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Expression of DNA Methyltransferase (Dnmt1) in Testicular Germ Cells during Development of Mouse Embryo

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ABSTRACT. **The DNA methylation pattern is reprogrammed in embryonic germ cells. In female germ cells, the short-form DNA methyltransferase Dnmt1, which is an alternative isoform specifically expressed in growing oocytes, plays a crucial role in maintaining imprinted genes. To evaluate the contribution of Dnmt1 to the DNA methylation in male germ cells, the expression profiles of Dnmt1 in embryonic gonocytes were investigated. We detected a significant expression of Dnmt1 in primordial germ cells in 12.5–14.5 day postcoitum (dpc) embryos. The expression of Dnmt1 was downregulated after 14.5 dpc after which almost no Dnmt1 was detected in gonocytes prepared from 18.5 dpc embryos. The short-form Dnmt1 also was not detected in the 16.5–18.5 dpc gonocytes. On the other hand, Dnmt1 was constantly detected in Sertoli cells at 12.5–18.5 dpc. The expression profiles of Dnmt1 were similar to that of proliferating cell nuclear antigen (PCNA), a marker for proliferating cells, suggesting that Dnmt1 was specifically expressed in the proliferating male germ cells. Inversely, genome-wide DNA methylation occurred after germ cell proliferation was arrested, when the Dnmt1 expression was downregulated. The present results indicate that not Dnmt1 but some other type of DNA methyltransferase contributes to the creation of DNA methylation patterns in male germ cells.**

Key words: DNA methyltransferase/DNA methylation/gonocytes/cell proliferation

In mammals, genomic DNA is often methylated at the 5th position of the cytosine residues in CpG sequences (Antequera and Bird, 1993). Dynamic regulation of DNA methylation is known to contribute to various biological phenomena such as tissue-specific gene expression (Shen and Maniatis, 1980; Razin and Cedar, 1991; Tajima and Suetake, 1998), genomic imprinting (Jaenisch, 1997), X chromosome inactivation (Riggs and Porter, 1996), and carcinogenesis (Laird and Jaenisch, 1996). In mammals, two types of DNA methyltransferase activity have been reported: *de novo*- and maintenance-type DNA methyltransferase activities. In mouse, *de novo*-type DNA methyltransferase activity contributes to the creation of DNA methylation patterns at the implantation stage of embryogenesis (Monk, 1990) and during gametogenesis (Kafri *et al*., 1992; Jaenisch, 1997; Tada *et al*., 1998: Coffigny *et al*., 1999). To date, three DNA methyltransferases, Dnmt1, Dnmt3a, and Dnmt3b, have been reported (Tajima and Suetake, 1998; Okano *et al*., 1998). Although Dnmt3a and Dnmt3b are the strong candidates for *de novo*-type DNA methyltransferases (Okano *et al*., 1998; Okano *et al*., 1999), Dnmt1, which contributes to the maintenance of methylation patterns during replication and maintains cell lineage-specific methylation patterns in somatic cells, also catalyzes *de novo* DNA methylation activity *in vivo* (Tajima *et al*., 1995; Takagi *et al*., 1995; Pradhan *et al*., 1999).

Reprogramming of DNA methylation patterns occurs in mouse embryo after implantation in both somatic and germ cells (Kafri *et al*., 1992; Jaenisch, 1997; Tada *et al*., 1998: Coffigny *et al*., 1999). A subset of mammalian genes is marked differently in male and female gametes by an epige-

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Abbreviations: dpc, day postcoitum; Dnmt, DNA methyltransferase; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride.

netic process, possibly DNA methylation, which is called genomic imprinting. The marking for genomic imprinting in female germ cells starts at the stage of growing oocytes (Kono *et al*., 1996; Obata *et al*., 1998), and at that stage, oocyte-specific Dnmt1, which lacks amino-terminal 118 amino acids starts to accumulate (Carlson *et al*., 1992; Mertineit *et al*., 1998). Unlike somatic cells, in which the expression of Dnmt1 is under the control of cell cycle (Szyf *et al*., 1991; Leonhardt *et al*., 1992; Liu *et al*., 1996; Suetake *et al*., 1998), Dnmt1 is highly accumulated in the cytoplasm of growing oocytes, where the cells are arrested (Mertineit *et al*., 1998). Recently, it has been shown that the depletion of this oocyte-type Dnmt1 does not contribute to the methylation pattern formation in oocytes, but is crucial for maintaining the methylation state of imprinted genes during embryogenesis (Howell *et al*., 2001). On the other hand, in male germ cells, the DNA methylation patterns of imprinted genes are formed earlier, around 16.5 days postcoitum (dpc), when the cells are in diploid (Ueda *et al*., 2000). However, it is not known which DNA methylating enzymes play crucial roles in the genome-wide DNA methylation including imprinting genes in primordial germ cells in gonad, which were designated as gonocytes (Roosen-Runge, 1977).

In the present study, we examined whether or not the expression of Dnmt1 in testis prepared from 12.5 to 18.5 dpc and newborn mouse coincides with the stage of creation of DNA methylation pattern. Dnmt1 was highly expressed in germ cells at 12.5 and 14.5 dpc, downregulated after 15.5 dpc, and increased in newborn mouse. The expression of Dnmt1 was positively correlated with the proliferative state of germ cells as it was in somatic cells, and inversely correlated to the DNA methylation level in gonocytes. It was therefore suggested that not Dnmt1 but some other DNA methyltransferase plays a primary role in creating DNA methylation patterns in mouse male germ cells.

Materials and Methods

Antibodies

Anti-mouse Dnmt1 antibodies were raised against amino-terminal 118 amino acid sequence (anti-amino-terminal antibodies) and carboxyl-terminal sequence (anti-carboxyl-terminal antibodies) of Dnmt1 as described elsewhere (Takagi *et al*., 1995; Suetake *et al*., 2001). Monoclonal antibody reactive with 5-methyl-2'-deoxycytidine, FMC9, was used to detect methylated DNA (Mizugaki *et al*., 1996). Rat monoclonal antibody, TRA104, which reacts with germ-cell-specific nuclear antigen (GENA), was kindly provided by "Dr. H. Tanaka" at the Research Institute of Microbial Diseases, Osaka University. GENA starts its expression in testis at 12 dpc (Tanaka *et al*., 1997). Mouse monoclonal antibody reactive with PCNA was purchased from Oncogene, Cambridge, NY.

Immunostaining

Testis was dissected from embryos of each stage or newborn mouse, and fixed with 4% paraformaldehyde for 12 hr. After overnight washing with sucrose-PBS (10 mM phosphate buffer (pH 7.2), 0.9% NaCl, 7% sucrose), the tissues were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo), and were cryosectioned at 10μm in cryostat microtome. The sections were pretreated with 1% BSA, 0.3% SDS, 2% Triton X-100 in PBS for the anti-carboxyl-terminal Dnmt1 antibodies or 1% BSA, 0.1% Triton X-100 in PBS for the anti-amino-terminal Dnmt1 antibodies. For the staining with anti-Dnmt1 antibodies reactive with the amino- and carboxyl-terminal region, sections were incubated for 1 hr at room temperature with the antibodies. After the incubation, the specimens were incubated with anti-rabbit IgG conjugated to ALEXA 488 (Molecular Probe, Eugene, OR) as secondary antibodies.

For the staining of GENA, monoclonal antibody TRA104 was detected with rhodamine-conjugated anti-rat IgG. For the detection of PCNA, cryostat sections were irradiated with microwave for 2 min, blocked with 1% BSA, 1% skimmed milk for 1 hr and incubated overnight at 4°C with anti-PCNA antibody. PCNA was detected with Cy5-conjugated anti-mouse IgG.

For the staining of methylated DNA, testes were fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 50 mM phosphate buffer (pH 7.4) for 12 hr at 4°C. After washing with sucrose-PBS, the tissues were embedded in paraffin. The paraffin sections were sequentially treated with pepsin (4 mg/ml in 10 mM HCl) for 20 min at 37°C, 50 mM glycine for 10 min, 4 M HCl for 5 min, 50 mM glycine for 10 min, and 1% BSA in PBS for 1 hr. The samples were incubated with anti-5-methyl-2'-deoxycytidine monoclonal antibody overnight at 4°C. After the washing, specimens were incubated with Cy5-conjugated anti-mouse IgG for 1 hr. All samples were observed with Confocal Laser scaning microscope (Biorad MRC1024, Nippon Bio-Rad Lab., Tokyo, Japan).

Immunoprecipitation

Excised testis was washed with PBS added with 1 mM PMSF, homogenized with 8 volumes of 1% SDS, and then boiled for 15 min. Dnmt1 was immunoprecipitated, electrophoresed, Western blotted, and detected as described elsewhere (Suetake *et al*., 2001).

Results

DNA methylation in embryonic testis

In mammalian germ cells, DNA methylation pattern is erased and then created thereafter (Kafri *et al*., 1992; Jaenisch, 1997; Tada *et al*., 1998; Coffigny *et al*., 1999). This reprogramming of the DNA methylation pattern in male germ cells, both genome-wide and imprinted gene-specific ones, occurs during the differentiation of gonocytes to spermatogonia (Coffigny *et al*., 1999; Ueda *et al*., 2000). The genome-wide DNA methylation level in testis cells was

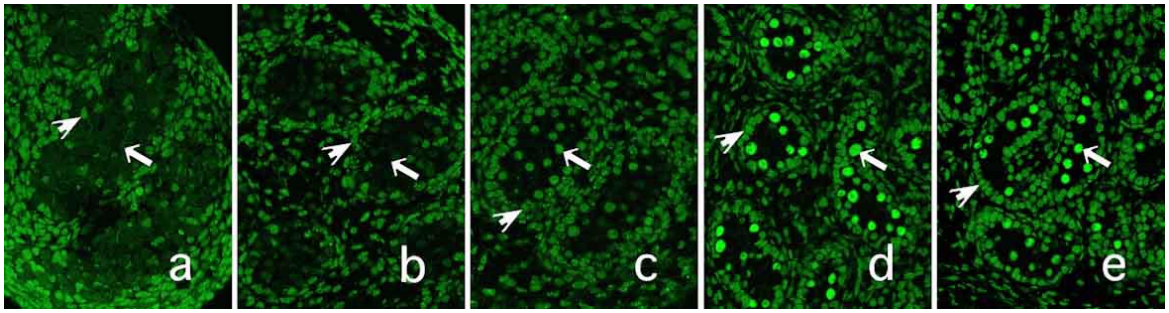

Fig. 1. Methylated DNA in germ cells in mouse testis was stained with methylated DNA-specific antibody. Samples prepared from 12.5 (a), 14.5 (b), 16.5 (c), 18.5 dpc (d), and newborn (e) testis were stained with anti-5-methyl-2'-deoxycytidine, FMC9 (a2–e2, green). Arrows indicate gonocytes and arrowheads indicate typical Sertoli cells.

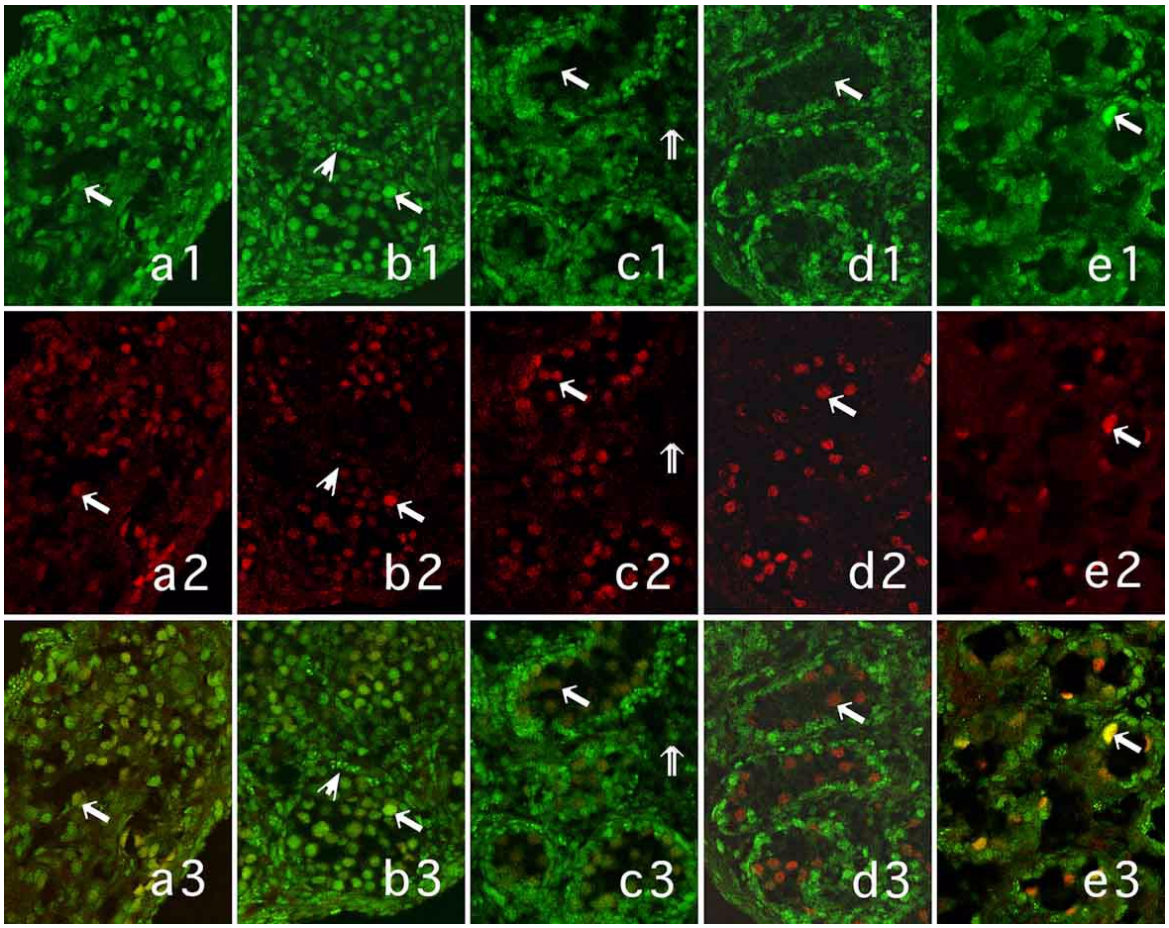

Fig. 2. Expression of Dnmt1 in germ cells in mouse testis. Samples prepared from 12.5 (a). 14.5 (b), 16.5 (c), 18.5 dpc (d), and newborn (e) testis were stained with anti-amino-terminal Dnmt1 antibodies (green, 1) and TRA104 (red, 2), and images of 1 and 2 were merged (3). Arrows indicate typical gonocytes and those expressing GENA reactive to TRA104. Arrowheads in b indicate typical Sertoli cells that are GENA-negative cells. Double arrows in c indicate typical stroma cells.

monitored by anti-5-methyl-2'-deoxycytidine monoclonal antibody that reacts with methylated DNA (Fig. 1). Large gonocytes in seminiferous tubules were weakly stained in 12.5 (Fig. 1a) and 14.5 dpc (Fig. 1b), but became positive in 16.5 dpc (Fig. 1c) and strongly stained in 18.5 dpc and newborn mice (Figs. 1d and 1e). As the exposing conditions were identical in each specimen, the intensities of the staining positively correlated to DNA methylation level. As

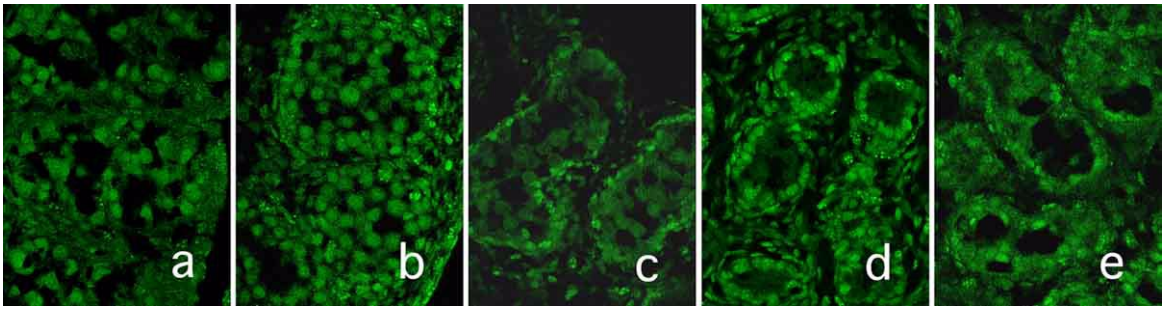

Fig. 3. Expression of Dnmt1 in germ cells in mouse testis. Samples prepared as in Fig. 2, 12.5 (a), 14.5 (b), 16.5 (c), 18.5 dpc (d), and newborn (e) testis, were stained with anti-carboxyl-terminal antibodies (green).

gonocytes are arrested the cell division after 15.5–16.5 dpc (Coffigny *et al*., 1999), genome-wide methylation increased in the non-proliferating cells.

Dnmt1 expression was downregulated after 14.5 dpc

To see if Dnmt1 contributed to the formation of DNA methylation observed in late stage of gonocytes, Dnmt1 protein was immuno-detected in testis obtained from 12.5 to 18.5 dpc and newborn mice. In testis at 12.5 dpc embryos, round-shaped gonocytes, which were positively stained with the monoclonal antibody TRA 104 reactive with the antigen expressed in germline cells (Tanaka *et al*., 1997) (Fig. 2a2), expressed a significant amount of Dnmt1 in their nuclei (Fig. 2a1). Sertoli and stroma cells also expressed Dnmt1 at this stage (Fig. 2a1 and 2a3 merge). Similar expression profiles were observed at 14.5 dpc (Fig. 2b). The gonocytes at this stage expressed a significant amount of Dnmt1 (Fig. 2b1 and 3). At stages 12.5 and 14.5 dpc, sporadic localization of Dnmt1 was observed in some Sertoli cells, but was not found in gonocytes (Fig. 2b1 and 3, arrowheads).

At 16.5 dpc, almost all of the gonocytes were faintly or negative stained with anti-Dnmt1 antibodies (Fig. 2c1 and 3). By contrast, the nuclei of Sertoli cells at the periphery of the seminiferous tubules and stroma cells were strongly stained with anti-Dnmt1 antibodies (Fig. 2c1 and 3). At 18.5 dpc, Dnmt1 was completely negative in gonocytes but strongly expressed in Sertoli cells and stroma cells at this stage (Fig. 2d1 and 3).

After birth, germ cells are differentiated into spermatogonia to enter the proliferation stage. As shown with arrows in Fig. 2e1 and 3, Dnmt1 was highly expressed in some gonocytes as reported previously (Trasler *et al*., 1992).

Gonocytes and Sertoli cells mainly expressed somatic-type Dnmt1

It was reported that growing oocytes, female germ cells, express a large amount of short-form Dnmt1 lacking amino-terminal 118 amino acid residues and play a crucial role in maintaining the methylation state of imprinted genes (Carlson *et al*., 1992; Mertineit *et al*., 1998; Howell *et al*., 2001). Since we used anti-Dnmt1 antibodies raised against the amino-terminal 118 amino acid sequences, the staining did not detect the short-form Dnmt1. It might be possible that the short-form Dnmt1 was expressed in the gonocytes after 14.5 dpc and contributed to the formation or maintenance of methylation pattern. To see if the gonocytes, male germ cells, expressed the short-form Dnmt1 after 14.5 dpc, we next detected both the full-length and short-form Dnmt 1 molecules expressed in testis using the anti-Dnmt1 antibodies raised against the carboxyl-terminal region of the molecule. As shown in Fig. 3, the staining profiles of Dnmt1 were basically identical to those in Fig. 2. The result indicates that no specific expression of the short-form Dnmt1 occurred in male germ cells after 16.5 dpc at least at

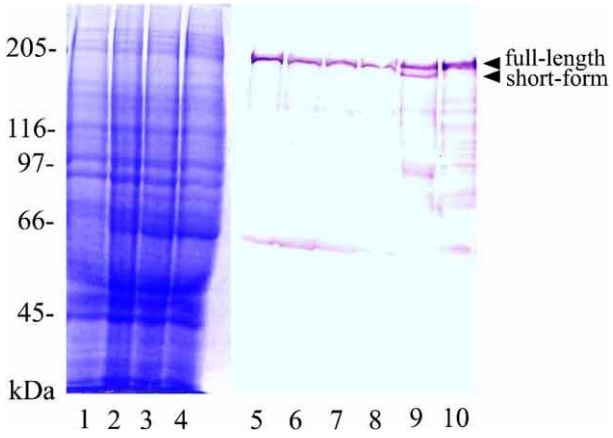

Fig. 4. Detection of Dnmt1 by Western blotting in germ cells in mouse testis. Samples (40 μg protein per lane) prepared from 14.5 (lanes 1 and 5), 16.5 (lanes 2 and 6), 18.5 dpc (lanes 3 and 7), and newborn (lanes 4 and 8) testes were separated by SDS-PAGE. The protein bands were Coomassie stained (lanes 1–4), and Dnmt1 bands were immunodetected with the anti-carboxyl-terminal antibodies (lanes 5–10). Lanes 9 and 10 indicate the sizes of short-form and full-length Dnmt1 exogenously expressed in HEK 293T cells, respectively. The full-length-sized band in lane 9 is an endogenous full-length Dnmt1 expressed in HEK 293T cells. Molecular weight markers are also indicated (kDa).

high level. In addition, no short-form Dnmt1 was detected throughout the stages of 14.5–18.5 dpc and newborn testis by Western blotting (Fig. 4). A significant amount of full-length Dnmt1 was detected in 16.5 and 18.5 dpc testes by Western blotting (Fig. 4, lanes 7 and 8). The major contribution of the full-length Dnmt1 at these stages could be due to non-gonocytes, since the gonocytes expressed negligible amounts of Dnmt1 by immunofluorescence detection under microscope (Figs. 2c1 and 2d1).

Proliferation stage-dependent expression of Dnmt1 in testis

Although there are a few exceptions to Dnmt1 being highly expressed in non-proliferating cells (Mertineit *et al*., 1998; Inano *et al*., 2000), the expression level of Dnmt1 is basically under the control of the cell cycle (Szyf *et al*., 1991; Liu *et al*., 1996; Suetake *et al*., 1998; Suetake *et al*., 2001), that is, it is high under proliferative conditions and low at post-mitotic stage. Since it is reported that Dnmt1 interacts with PCNA (Chuang *et al*., 1997), a marker for proliferating cells, we next detected the expression of PCNA in embryonic testis. As expected, stage-specific expression profiles of PCNA in the cells in seminiferous tubules were identical to that for Dnmt1 (Fig. 5a–e). PCNA was expressed in 12.5–14.5 dpc gonocytes and downregulated in 16.5–18.5 dpc gonocytes (Fig. 5a2–e2). By contrast, PCNA was expressed in 12.5–18.5 dpc Sertoli cells.

Discussion

In the present study, we demonstrated that in male germ cells, full-length, somatic-type Dnmt1 was highly expressed in gonocytes in 12.5–14.5 dpc, and downregulated in 16.5–18.5 dpc. It is reported that in mouse non-proliferating growing oocytes, a short-form Dnmt1 lacking amino-terminal 118 amino acid residues is expressed, and plays a crucial role in early embryogenesis, especially in the maintenance of the methylated state of imprinted genes (Mertineit *et al*.,

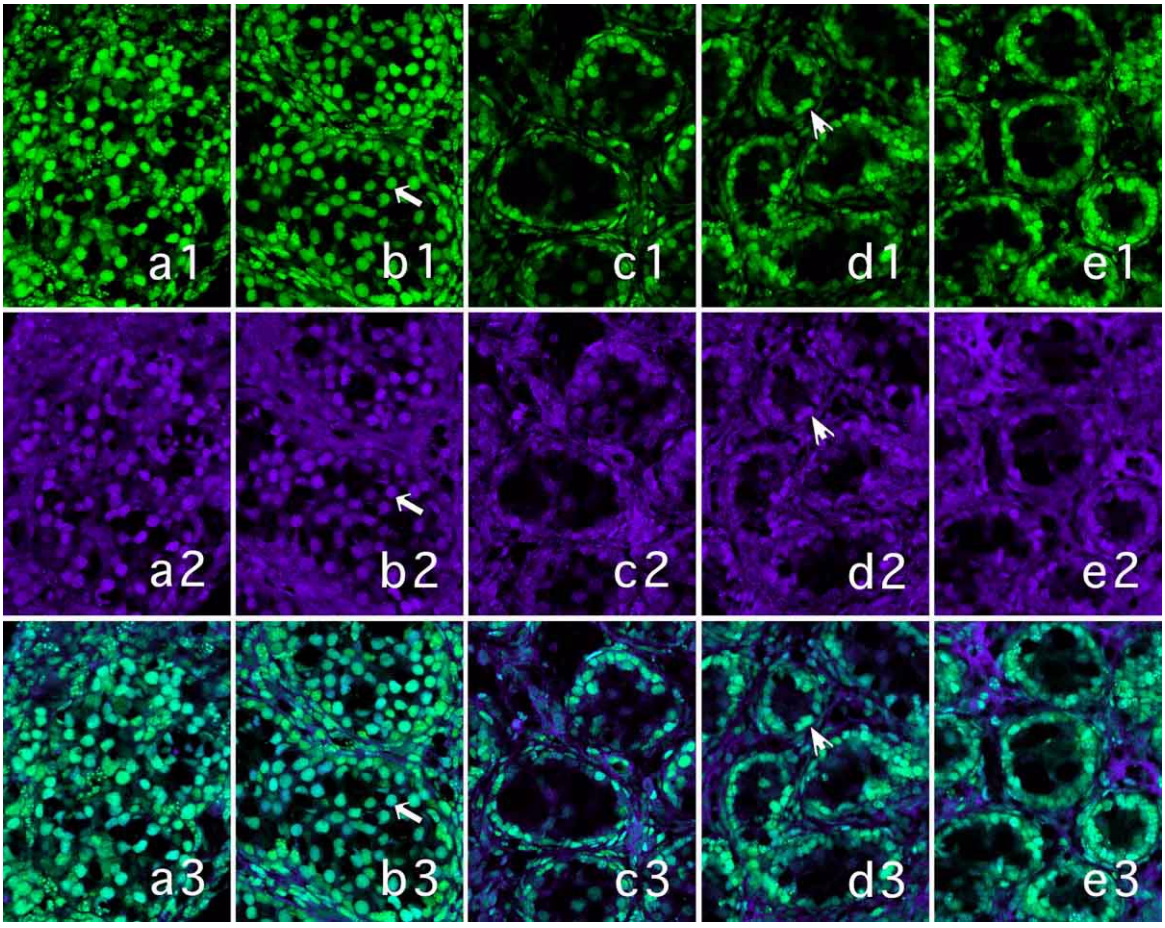

Fig. 5. Expression of Dnmt1 and PCNA in germ cells in mouse testis. Samples prepared from 12.5 (a). 14.5 (b), 16.5 (c), 18.5 dpc (d), and newborn (e) testis were stained with anti-amino-terminal Dnmt1 antibodies (green, 1) and anti-PCNA antibodies (purple, 2), and the images were merged (3). Arrows in b and arrowheads in d indicate typical gonocytes and Sertoli cells, respectively.

1998; Howell *et al*., 2001). Thus there exists a possibility that this short-form Dnmt1 might be expressed in male germ cells, gonocytes, even after the arrest of cell division after 15.5 dpc till birth (Coffigny *et al*., 1999) to affect the DNA methylation state. However, this short-form Dnmt1 was not detected in 16.5–18.5 dpc and 14.5–18.5 dpc gonocytes, by immunofluorescent detection under microscope and by Western blotting, respectively.

In male germ cells, DNA methylation imprinting in *H19* gene is established in the arrested gonocytes around 16.5 dpc (Tada *et al*., 1998; Ueda *et al*., 2000), and the methylation of specific genes (Kafri *et al*., 1992) as well as a genome-wide DNA methylation occurs at 16.5–18.5 dpc (Coffigny *et al*., 1999; Fig. 1). Since the expression levels of Dnmt1 and DNA methylation were in inverse correlation (Fig. 6), it strongly suggested that not Dnmt1 but some other DNA methyltransferase(s) such as Dnmt3a or Dnmt3b contributes to the creation of DNA methylation patterns including the imprinted genes. This conclusion, however, does not mean that once the DNA methylation pattern is formed in the arrested gonocytes, it is not maintained by Dnmt1 in spermatogonia when the cells reenter the proliferation stage and Dnmt1 is strongly expressed in those cells (Jue *et al*., 1995).

The expression profiles of Dnmt1 in gonocytes and Sertoli cells, in which Dnmt1 was expressed throughout 12.5–18.5 dpc, were similar to that of PCNA, a prerequisite factor for replication and repair and a marker for proliferating cells. It is reported that Dnmt1 is highly expressed in proliferating cells (Szyf *et al*., 1991), and interacts directly with PCNA with its amino-terminal region (Chuang *et al*., 1997). Therefore, it is reasonable that the expression stage of Dnmt1 was quite similar to that of PCNA in testicular cells. Recently it was reported that gonocytes incorporate bromodeoxyuridine until 15.5 dpc (Coffigny *et al*., 1999), suggesting that in *in vivo* state gonocytes still proliferate at 12.5–15.5 dpc. At stages 12.5 and 14.5 dpc, sporadic localization of Dnmt1 was observed in some Sertoli cells and stroma cells, but was not found in gonocytes (Fig. 2b1 and 3, arrowheads). In mouse somatic cells, replication foci combine to form large dots, and Dnmt1 accumulates in these dots (Leonhardt *et al*., 1992). The replication foci may not combine in gonocytes at these stages, perhaps due to low DNA methylation levels or differences in chromatin state. The present results confirm that Dnmt1 in gonocytes are also under the control of the cell cycle as is true for PCNA. When Dnmt1 in somatic cells is eliminated, apoptosis is induced in the cells (Jackson-Grusby *et al*., 2001), suggesting that the expression of Dnmt1 in proliferating somatic cells is indispensable for cell survival. The Dnmt1 in gonocytes during the proliferative state may contribute basically to the survival of the cells.

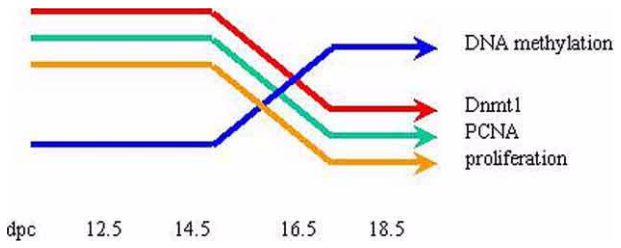

Fig. 6. Schematic illustration of the expression profiles of Dnmt1 and PCNA, DNA methylation levels, and proliferation state in gonocytes.

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