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Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells ☆

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Abstract

The acquisition of genomic methylation in the male germ line is initiated prenatally in diploid gonocytes, while DNA methylation in the female germ line is initiated postnatally in growing oocytes. We compared the temporal expression patterns of the DNA methyltransferases, DNMT1, DNMT3a, DNMT3b, and DNMT3l in the male and female germ lines. DNMT1 expression was examined by immunocytochemistry and Northerns with an emphasis on the prenatal period. In the female, there is a gradual down-regulation of DNMT1 protein in prenatal meiotic prophase I oocytes that is not associated with the production of an untranslated transcript, as it is in the male; these results suggest that the mechanism of meiotic down-regulation differs between the sexes. In the male, DNMT1 is unlikely to play a role in the prenatal acquisition of germ line methylation patterns since it is down-regulated in gonocytes between 14.5 and 18.5 days of gestation and is absent at the time of initiation of DNA methylation. To search for candidate DNMTs that could be involved in establishing methylation patterns in both germ lines, real-time RT-PCR was used to simultaneously study the expression profiles of the three DNMT3 enzymes in developing testes and ovaries; DNMT1 expression was included as a control. Expression profiles of DNMT3a and DNMT3l provide support for an interaction of the two enzymes during prenatal germ cell development and de novo methylation in the male. DNMT3l is the predominant DNMT3 enzyme expressed at high levels in the postnatal female germ line at the time of acquisition of DNA methylation patterns. DNMT1 and DNMT3b expression levels peak concomitantly, shortly after birth in the male, consistent with a role in the maintenance of methylation patterns in proliferating spermatogonia. Together, the results provide clues to specific roles for the different DNMT family members in de novo and maintenance methylation in the developing testis and ovary.

Keywords: DNA methyltransferases; DNMT1; DNMT3a; DNMT3b; DNMT3l; Germ line; DNA methylation; Mouse; Real-time RT-PCR

Introduction

Genomic methylation patterns are established and maintained by DNA (cytosine-5)-methyltransferases (DNMTs). In mammals, five DNMTs have been characterized and classified according to similarities found in their C-terminal catalytic domain: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3l (reviewed by Bestor, 2000). Of these, only DNMT1, DNMT3a, and DNMT3b are known to be catalytically active in vivo. DNMT1, the major methyltransferase in somatic tissues, has a preference for hemimethylated DNA and is critical for the maintenance of methylation patterns during replication of DNA (Bestor, 1992; Lei et al., 1996; Li et al., 1992; Yoder et al., 1997). DNMT3a and DNMT3b are encoded by essential genes (Okano et al., 1999) that are expressed at high levels in mouse embryonic stem cells and during embryonic development and have

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been postulated to function predominantly in de novo methylation of DNA (Chen et al., 2003; Okano et al., 1998, 1999). Homozygous *Dnmt1*-deficient embryos have < 5% of the DNA methylation levels found in normal embryos and such embryos show bialleleic expression of most imprinted genes, inactivation of all X chromosomes, activation of retroposons, and apoptotic death before midgestation (Lei et al., 1996; Li et al., 1992; Panning and Jaenisch, 1996; Walsh et al., 1998). Gene-targeting studies in mice have also established the importance of DNMT3l. Male mice deficient in DNMT3l are infertile while females produce non-viable offspring due to aberrant acquisition of genomic methylation during oogenesis (Bourc'his et al., 2001). Since DNMT3l has not been shown to possess DNA methyltransferase activity, it may be involved in the acquisition of germ cell methylation through interactions with other factors. An oocyte-specific isoform of DNMT1 (DNMT1o), which lacks 118 amino acids of the N-terminal domain of the somatic isoform of DNMT1, has also been described (Carlson et al., 1992; Mertineit et al., 1998). However, depletion of DNMT1o shows that while it contributes to maintaining the methylation state of imprinted genes during embryogenesis, it does not play a role in the methylation of oocyte DNA (Howell et al., 2001).

Genomic methylation patterns are acquired in the germ line and differ markedly for male and female gametes (Driscoll and Migeon, 1990; Monk et al., 1987; Sanford et al., 1987; reviewed by Reik et al., 2001). These methylation differences are especially striking at imprinted loci where they have important implications for allele-specific gene expression in the offspring (reviewed by Reik and Walter, 2001). Although further modification of methylation patterns occurs after fertilization (Howlett and Reik, 1991; Kafri et al., 1992; Monk et al., 1987), some sequences, such as imprinted loci and IAP retroposons, retain their gamete-derived marking (Lane et al., 2003; Olek and Walter, 1997; Shemer et al., 1997; Tremblay et al., 1997). Accurate reprogramming is therefore required with every reproductive cycle to ensure proper erasure, acquisition, and maintenance of methylation marks. In both germ lines, DNA methylation patterns on most sequences appear to be erased around the time when primordial germ cells enter the gonad, at approximately days 10.5–12.5 of gestation in the mouse (Hajkova et al., 2002; Kato et al., 1999; Lee et al., 2002; Szabo and Mann, 1995; Szabo et al., 2002). In female germ cells, methylation patterns are acquired postnatally, after the pachytene phase of meiosis is complete, during the oocyte growth phase (Brandeis et al., 1993; Kono et al., 1996; Lucifero et al., 2002; Stoger et al., 1993; Walsh et al., 1998). In contrast, in the male, genomic methylation begins to be acquired before birth in prospermatogonia and is complete after birth and before the end of pachytene (Davis et al., 1999, 2000; Kafri et al., 1992; Lees-Murdock et al., 2003; Ueda et al., 2000; Walsh et al., 1998). No male germ cell-specific DNMT protein has been identified yet and, although DNMT3a and DNMT3l are thought to play important roles during spermatogenesis, their exact contributions are still unknown (Bourc'his et al., 2001; Okano et al., 1999). The role each DNA methylating enzyme plays in the genome-wide and sex-specific methylation events that take place in germ cells, as well as the mechanisms governing these events, still remain to be elucidated.

We report the expression patterns of DNMT1, DNMT3a, DNMT3b, and DNMT3l during the times when genomic methylation patterns are acquired in the male and female germ lines. We demonstrate that there are sex-specific differences in DNMT1 expression both before and shortly after birth, use anti-synaptonemal complex markers to determine the precise timing of events leading to DNMT1 down-regulation during prophase I in oocytes, and show the absence of DNMT1 expression at the time of acquisition of methylation in the prenatal male germ line. We identify candidate methyltransferases that are likely to be involved in establishing genomic methylation patterns in both germ lines as well as the dynamic relationship that exists between the enzymes during the putative windows of acquisition and maintenance of genomic methylation patterns.

Materials and methods

Isolation of Gonads

CD-1 mice were purchased from Charles River Canada Inc. (St-Constant, QC, Canada). Noon of the day on which the vaginal plug was found was designated as embryonic day (E) 0.5 and the day of delivery as postnatal day (dpp) 0. Genital ridges were isolated from E11.5 mouse embryos; genetic sexing using primers specific for *Zfy1* and *Zfy2* was used to determine the sex of the embryo (Nagamine et al., 1989). Ovaries and testes were distinguished in embryos collected at daily intervals from E12.5 to E18.5 by the presence of seminiferous cords in the testes. Pairs of urogenital complexes were dissected in Eagle Modified Essential Medium (MEM) containing Hank salts and 25 mM Hepes buffer, pH 7.3 (Gibco BRL, Grand Island, NY) and gonads were separated from adjacent mesonephric tissues. Fetal and postnatal testes and ovaries were decapsulated and rinsed in sterile DEPC-treated saline, pooled, and frozen in liquid nitrogen or between slabs of dry ice unless otherwise specified. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Preparation of germ cells and gonads for immunocytochemistry

Intact E11.5 genital ridges were fixed by immersion in Ste Marie's fixative (Trasler et al., 1996). Alternatively, genital ridges were pooled, washed briefly in Ca^{2+}–Mg^{2+}-free PBS, incubated in 0.2% EDTA-PBS solution for 20 min, transferred to MEM, and mechanically disrupted by

pipetting to release primordial germ cells; cells were fixed for 15 min in 3.7% formaldehyde in PBS. Fetuses from E13.5 to 18.5 and postnatal mice were perfused through the heart with physiological saline followed by twice diluted (50%) Ste Marie's fixative; ovary and testis sections were prepared as previously described (Mertineit et al., 1998). Oocytes in meiotic prophase were obtained from E15.5 to 17.5 ovaries as described previously (Amleh et al., 2000). Briefly, ovaries were pooled, digested with collagenase, washed in MEM, and further digested with trypsin in Rinaldini solution (Rinaldini, 1959). Ovaries were suspended in 10% fetal bovine serum in MEM, washed, transferred to Ca^{2+}–Mg^{2+}-free PBS, dissociated by pipetting, centrifuged, and resuspended in MEM. The cell suspension was applied to 0.5% NaCl droplets on glass multi-spot slides (Shandon Inc., Pittsburg, PA) and allowed to settle for 15 min. The slides were fixed in 2% paraformaldehyde, pH 8.2, rinsed in 0.4% Kodak Photo-Flo wetting agent (Eastman Kodak Co, Rochester, NY), pH 8.0, air-dried, and stored at −20°C.

Immunocytochemistry

All solutions for immunocytochemistry were prepared in PBS, pH 7.2 and procedures were carried out at room temperature, unless otherwise specified. The rabbit polyclonal anti-DNMT1 antibody PATH52, which recognizes epitopes in both the somatic (DNMT1s) and the oocyte-specific (DNMT1o) forms of DNMT1, has been described previously (Bestor, 1992; Li et al., 1992). The mouse monoclonal anti-stage-specific embryonic antigen-1 antibody (anti-SSEA-1; Solter and Knowles, 1978), which recognizes a cell surface glycoprotein on primordial germ cells, was a kind gift of Dr. T. Feizi (Northwick Park Institute for Medical Research, Harrow, UK). The mouse monoclonal anti-synaptonemal complex antibody (anti-SC), which labels the chromosomal components of germ cells in meiotic prophase, has been described previously (Dobson et al., 1994; Moens et al., 1987). Sections were processed as previously described in Mertineit et al. (1998) until incubation with the secondary antibody; slides were incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA), rinsed and incubated with the Vectastain ABC reagent (Vector Laboratories). Next, they were incubated with H_2O_2, diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and imidazole in 25 mM Tris-buffered saline, pH 7.6. Sections were counterstained with methylene blue and mounted with Permount (Sigma). Immunofluorescence was performed on E11.5 germ cell preparations and meiotic prophase oocytes, which were incubated with 1:500 PATH52 and either 1:1000 anti-SSEA-1 or 1:1000 anti-SC overnight, washed and then incubated with FITC and Texas Red secondary antibodies (Vector Laboratories), as Moens et al. (1987) described. Slides were mounted with Prolong antifade reagent containing 0.4 μg/ml DAPI (Molecular Probes, Eugene, OR) and examined with a Zeiss Axiophot or a Zeiss LSM410 (Carl Zeiss Canada Ltd, Toronto, ON) confocal microscope as described (Laird et al., 1995).

Northern blotting

Total RNA was extracted from the mesonephros, ovaries, and testes from prenatal and postnatal mice using TRIzol (Gibco BRL) as directed by the manufacturer and Northern Blotting was performed using 10 μg of RNA as previously described (Mertineit et al., 1998).

Real-time RT-PCR

Total RNA was extracted from three separate collections of pooled prenatal and postnatal gonads of mice coming from different litters using the RNeasy extraction kit with DNaseI treatment (Qiagen Inc., Mississauga, ON, Canada) as described by the manufacturer. Real-time/Quantitative RT-PCR (QRT-PCR) was performed on the Mx4000 QPCR system from Stratagene (Stratagene, La Jolla, CA) using the Quantitect SYBR Green RT-PCR kit (Qiagen). Gene-specific primers were used to determine the relative expression levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* according to the standard curve method (reviewed by Bustin, 2002). Primers (Table 1) were designed to span introns and pick up

Table 1
Details of primers used for Real-time RT-PCR

Gene	GenBank accession no.	Primers	Product size (bp)	Annealing temperature (°C)
Dnmt1	X14805	F: 5′-CCTAGTTCCGTGGCTACGAGGAGAA-3′ R: 5′-TCTCTCTCCTCTGCAGCCGACTCA-3′	137	58
Dnmt3a	AF068625	F: 5′-GCCGAATTGTGTCTTGGTGGATGACA-3′ R: 5′-CCTGGTGGAATGCACTGCAGAAGGA-3′	147	59
Dnmt3b	AF068626	F: 5′-TTCAGTGACCAGTCCTCAGACACGAA-3′ R: 5′-TCAGAAGGCTGGAGACCTCCCTCTT-3′	145	59
Dnmt3l	AJ404467	F: 5′-GTGCGGGTACTGAGCCTTTTTAGA-3′ R: 5′-CGACATTTGTGACATCTTCCACGTA-3′	120	63
18S	X00686	F: 5′-GCCCTGTAATTGGAATGAGTCCACTT-3′ R: 5′-GTCCCCAAGATCCAACTACGAGCTTT-3′	149	59–63

Note. F, forward primer; R, reverse primer.

all known isoforms of the various DNMTs, resulting in the detection of a single band on gels (data not shown). SYBR Green was used to detect the double-stranded DNA produced during the amplification reaction and 18S rRNA content to normalize for the input of RNA. Reactions were performed using approximately 10 ng or 100 pg of total RNA for the DNMTs and 18S, respectively. One-step RT-PCR reactions were performed in a 25-μl volume as directed by the manufacturer for 40 cycles. For each gene studied, a specific standard curve was established using single-use aliquots of the same stock of RNA (total RNA extracted simultaneously from multiple 6 dpp testes). In all cases, reactions were performed in triplicate on the same three independent samples (i.e., same three separate pools) of ovary or testis RNA. PCR products were cloned and sequenced to confirm their identity before undertaking the study; specificity was assessed with the melting curve analysis and confirmed on a 3% agarose gel after each QRT-PCR experiment (data not shown). QRT-PCR results were normalized to their corresponding 18S rRNA content and calibrated accordingly to the lowest-expressing time point. Data are presented as mean ± SEM.

Results

DNMT1 localization in fetal germ cells

We first examined the expression and localization of DNMT1 in primordial germ cells (PGCs) at E11.5 and then following sexual differentiation, from E12.5 to E18.5. The cell surface marker SSEA-1 was used to identify PGCs in the genital ridge of E11.5 embryos (Solter and Knowles, 1978). Genital ridges of both XX and XY embryos were double-labeled with anti-DNMT1 and anti-SSEA-1 antibodies; many cells, including SSEA-1-positive PGCs, showed strong expression of DNMT1 (Fig. 1A). SSEA-1 clearly marked the cell surface of isolated PGCs (Figs. 1C, D, F, and G), whereas DNMT1 was strongly expressed throughout these cells (Figs. 1B, D, E, and G). Z-sectioning through the entire depth of PGCs confirmed the presence of DNMT1 in the nucleus with some staining in the cytoplasm; the staining surrounded condensed mitotic chromosomes of germ (Fig. 1E) and somatic (Fig. 1H) cells.

Following sexual differentiation, marked changes in DNMT1 expression were observed in the developing fetal testis. Two ages, E13.5 and E18.5, representing times before and after the DNA of male gonocytes initially becomes methylated, are shown in Fig. 2. In contrast to the preimmune control (Fig. 2A), proliferating germ and somatic cells (Fig. 2B) showed strong expression of DNMT1. Mitotic gonocytes within the testicular cords revealed both intense DNMT1 nuclear staining, as well as staining around mitotic chromosomes (Fig. 2B). DNMT1 levels in E14.5 and E15.5 gonocytes gradually decreased (data not shown) and by E18.5, the enzyme was no longer detected although it was still abundant in the supporting cells along the base of the tubules (Fig. 2C). Noticeable changes were also detected in the fetal ovary. In the female mouse, germ cells actively proliferate by mitosis until E13.5–14.5 and then enter and reach the diplotene stage of meiotic prophase between E14.5 and birth. At E13.5, fetal ovaries incubated with rabbit preimmune serum remained negative (Fig. 2D), whereas sections incubated with PATH52 showed strong expression of the DNMT1 enzyme in germ cells (Fig. 2E).

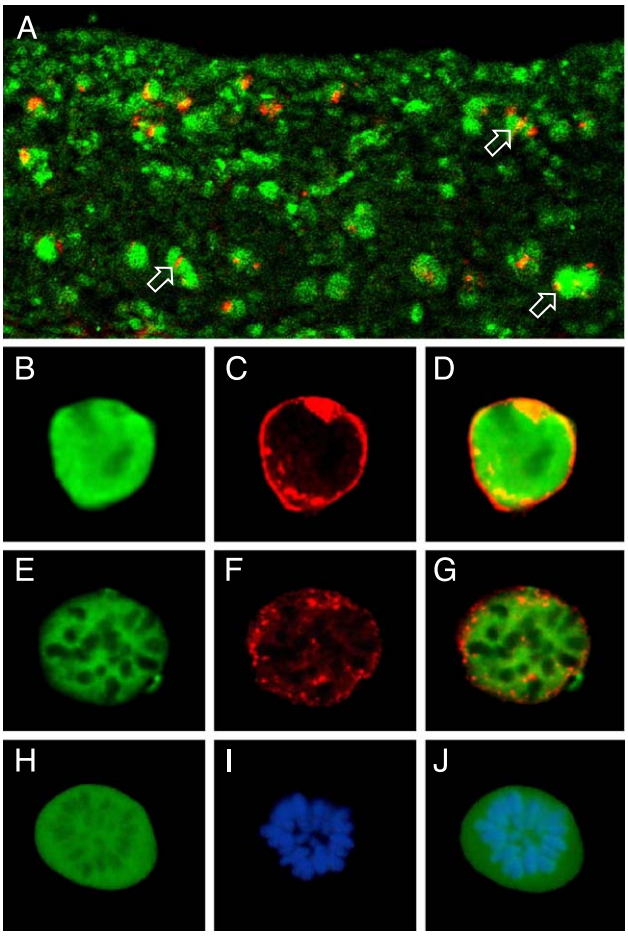

Fig. 1. Immunocytological localization of DNMT1 in the genital ridge. E11.5 genital ridge preparations were double-labeled with PATH52 to detect DNMT1 (green) and anti-SSEA-1 to identify primordial germ cells (PGCs; red), in addition to being counterstained with DAPI to visualize the nuclei (blue). (A) Longitudinal section of a male gonad. Examples of DNMT1-positive PGCs are indicated by open arrows. (B–D) High magnification of an isolated PGC imaged in the same z-plane via confocal microscopy. (B) DNMT1 immunoreactivity. (C) SSEA-1 immunoreactivity. (D) Superimposed confocal images of DNMT1 and SSEA-1 in the same germ cell. (E–G) High magnification of a PGC during the M-phase of the cell cycle imaged by confocal microscopy. (E) DNMT1 immunoreactivity surrounding condensed chromosomes. (F) SSEA-1 immunoreactivity. (G) Superimposed confocal images of DNMT1 and SSEA-1 staining. (H–J) High magnification of a somatic cell at metaphase photographed by conventional epifluorescence microscopy. (H) DNMT1 immunoreactivity. (I) DAPI staining of condensed chromosomes. (J) Double exposure of DNMT1 and DAPI.

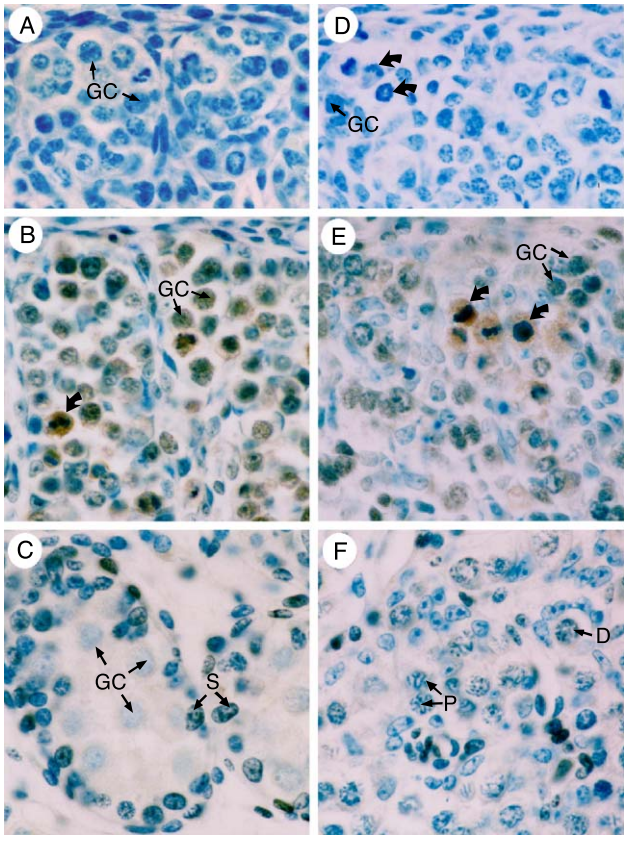

Fig. 2. Immunohistological localization of DNMT1 in the fetal mouse testis and ovary. (A–B) E13.5 testis; (C) E18.5 testis; (D–E) E13.5 ovary; (F) E18.5 ovary. Paraffin-embedded fetal gonads were stained with immunoperoxidase (brown) using PATH52 to detect DNMT1 (B, C, E, F) or rabbit preimmune serum (A and D). Examples of dividing cells are indicated by thick, curved arrows. Representative cell types are indicated as follows: D, diplotene stage oocyte; GC, germ cell; P, pachytene-stage oocyte; S, Sertoli cell.

DNMT1 staining of germ cells gradually decreased between E14.5 and E17.5 (data not shown) and by E18.5, when the majority of oocytes were at the pachytene or diplotene stages of meiotic prophase, they were devoid of DNMT1 while various supporting cells were positively stained (Fig. 2F).

DNMT1 down-regulation in prenatal meiotic prophase oocytes: timing and mechanisms

DNMT1 is expressed in preleptotene, leptotene, and zygotene spermatocytes before being down-regulated at pachytene (Jue et al., 1995). Evidence from whole female prenatal ovary staining, shown in Fig. 2, suggested that down-regulation of DNMT1 expression was also occurring during female meiosis, but did not allow precise timing to be determined. To assess the meiotic cell type-specific timing of down-regulation during female meiosis, dissociated fetal ovary preparations were double-labeled with anti-DNMT1 and anti-synaptonemal complex antibodies; the latter served as a marker to identify oocytes in the various phases of meiotic prophase I according to the state of synapsis between homologous chromosomes (Dobson et al., 1994; Moens et al., 1987; Fig. 3). At leptotene, the

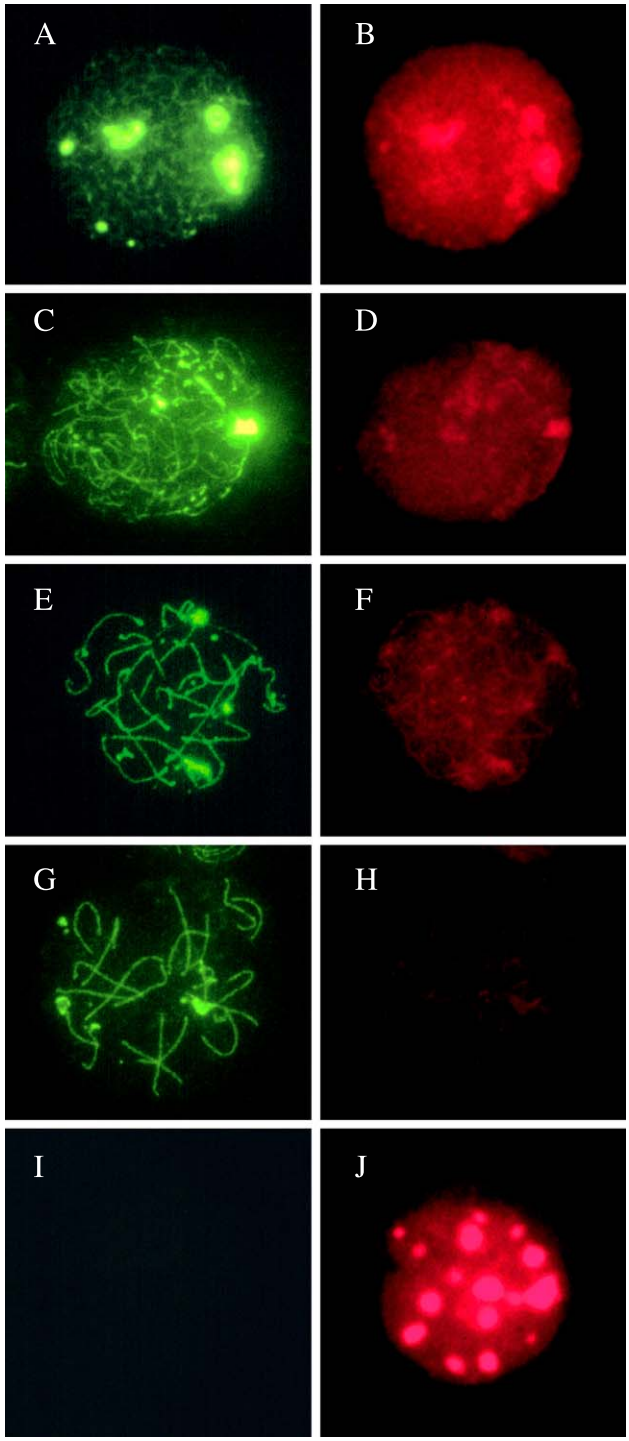

Fig. 3. DNMT1 expression in meiotic prophase oocytes. Fetal ovary preparations were double-labeled with PATH52 to detect DNMT1 (red) and anti-SC (green) to identify the various stages of meiotic prophase based on the state of synapsis between homologous chromosomes. (A–B) Leptotene; (C–D) zygotene; (E–F) early pachytene and (G–H) late pachytene oocytes. (I–J) Somatic cell. Note that DNMT1 staining is gradually down-regulated during meiotic prophase in the female mouse.

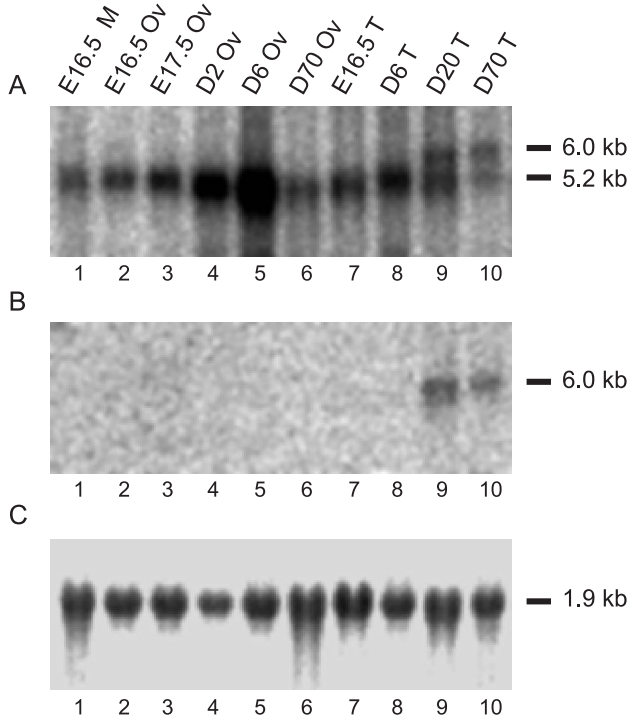

Fig. 4. Northern Blot analysis of Dnmt1 mRNA in the developing mouse ovary and testis. Total RNA was extracted from E16.5 mesonephros (lane 1), E16.5 ovary (lane 2), E17.5 ovary (lane 3), 2 dpp ovary (lane 4), 6 dpp ovary (lane 5), 70 dpp ovary (lane 6), E16.5 testis (lane 7), 6 dpp testis (lane 8), 20 dpp testis (lane 9), and 70 dpp testis (lane 10). (A) Membrane probed with a ^{32}P-labeled Dnmt1 cDNA probe (pR5; Bestor et al., 1988). (B) Membrane shown in (A) stripped and rehybridized with a ^{32}P-labeled cDNA probe to pachytene 1p 5' exon (Mertineit et al., 1998). (C) Loading validated using a ^{32}P-labeled oligonucleotide complementary to 18S rRNA (Benoit and Trasler, 1994).

chromosome cores appeared as fine threads (Fig. 3A) and DNMT1 staining was intense and largely diffuse throughout the nucleus (Fig. 3B). Zygotene oocytes, characterized by sites of chromosome pairing (Fig. 3C), stained moderately for DNMT1 throughout the nucleoplasm (Fig. 3D). Pachytene oocytes were identified by complete formation of the synaptonemal complex between pairs of homologous chromosomes (Figs. 3E and 3G). In early pachytene oocytes, DNMT1 staining was weak (Fig. 3F), while no staining was observed in late pachytene oocytes (Fig. 3H). In contrast to germ cells, somatic cells at all stages of fetal ovary development were identified by their lack of staining for the synaptonemal complex (Fig. 3I) and many exhibited bright punctuate foci with diffuse nucleoplasmic staining for DNMT1 (Fig. 3J). Taken together, these data show a gradual down-regulation of DNMT1 during meiotic prophase, to reach complete disappearance of the protein at the pachytene stage.

In previous studies, we identified an untranslated 6.0 kb transcript of *Dnmt1* that is expressed during male meiosis and is associated with down-regulation of DNMT1 in postnatal pachytene spermatocytes (Mertineit et al., 1998; Trasler et al., 1992). Northern Blot analysis was used here to determine if the same phenomenon was occurring during female pachynema. The ubiquitous 5.2-kb transcript, which gives rise to the full-length somatic form of DNMT1, was present at all times during development in both the ovary and the testis (Fig. 4A). In the postnatal ovary, the signal became especially strong compared to fetal mesonephros and ovaries (Fig. 4A, lanes 4 and 5) due to the expression of the 5.1 kb oocyte-specific transcript, undistinguishable on gel from the 5.2-kb transcript; the 5.1 kb transcript is absent before birth (Ratnam et al., 2002). A third transcript of 6.0 kb is also detected in the testis; it results from the use of a different first exon (exon 1p) and remains untranslated, causing a loss of DNMT1 protein during male meiosis. To determine if ovaries containing pachytene oocytes also

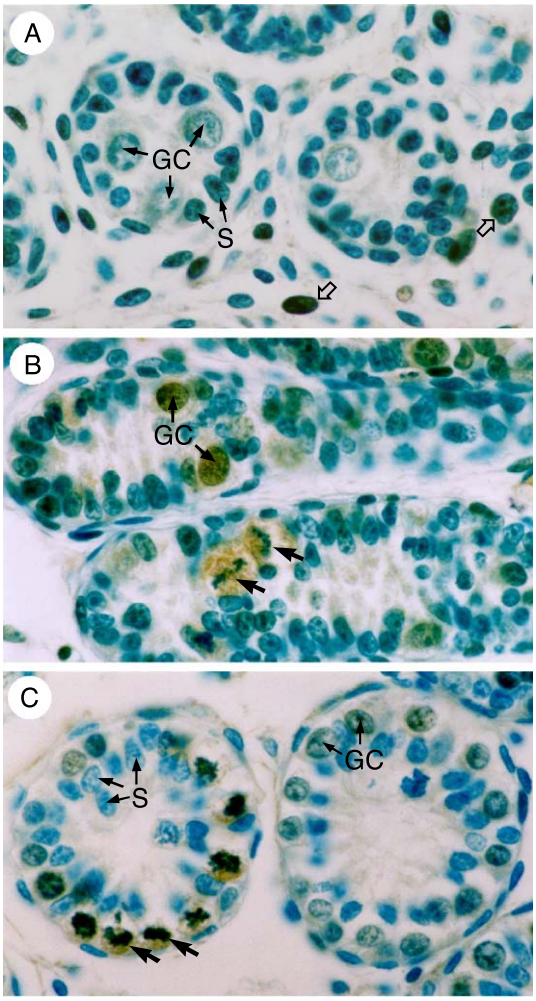

Fig. 5. Immunohistological localization of DNMT1 in the postnatal mouse testis. Immunoperoxidase staining (brown) using the PATH52 antibody was used to detect the presence of DNMT1 in (A) 0 dpp testis, (B) 3 dpp testis and (C) 6 dpp testis. Examples of dividing germ cells intensely stained for DNMT1 are indicated by large arrows and positively stained interstitial cells by open arrows. Representative cell types are indicated as follows: GC, germ cell; S, Sertoli cell. Note that germ cells in the center of the tubules are unreactive at 0 dpp but become highly reactive as they descend towards the basement membrane and resume mitosis.

express the 6.0-kb transcript, we stripped and rehybridized the membrane with a probe to exon 1p (Mertineit et al., 1998). Despite the fact that more than 30–50% of oocytes in E16.5–17.5 ovaries are at the pachytene stage of meiosis (McClellan et al., 2003; O'Keeffe et al., 1997; Speed, 1982), we failed to detect the 6.0-kb transcript in these samples (Fig. 4B). In contrast, the transcript was present in testes that contain pachytene germ cells (20 and 70 dpp), but not in testes that are devoid of pachytene cells (E16.5 and 6 dpp).

DNMT1 and the maintenance of methylation patterns in neonatal spermatogonia

We assessed DNMT1 re-expression in the neonatal period shortly after birth, at the boundary of mitosis resumption, by immunostaining testes of newborn mice of zero, 3 and 6 dpp. The prospermatogonia arrested at the G1 phase of the cell cycle remained negative for DNMT1 in comparison to neighboring Sertoli and interstitial cells, in the testis of 0 dpp mice (Fig. 5A). Between 3 and 4 dpp, the germ cells resumed their mitotic activity and relocated to the basal compartment of the seminiferous epithelium; consistently, they became highly reactive to PATH52 in 3 dpp testes (Fig. 5B). DNMT1 expression remained high in dividing spermatogonia in the 6 dpp testis, (Fig. 5C).

Expression of the DNMT3 enzymes in the developing testis and ovary

Previous studies (Howell et al., 2001; Sakai et al., 2001) as well as the data presented here make it unlikely that DNMT1 is responsible for the genome-wide de novo methylation events that take place in the male germ line prenatally and in the female germ line postnatally. We examined the expression dynamics of the other DNA methyltransferase genes, *Dnmt3a* and *Dnmt3b*, as well as the DNA methyltransferase 3-like gene *Dnmt3l*, using QRT-PCR in an effort to find candidate DNA methyltransferases responsible for the initial acquisition of DNA methylation patterns. Developmental stages before, during, and after genomic methylation patterns are first acquired in the male germ line were assayed using E13.5, E15.5, and E18.5 testes, respectively; the various populations of maturing germ cells in the postnatal testis were represented with 6, 16, 22, and 70 dpp testes (Bellvé et al., 1977). In the female, periods before and during, as well as the arrest in prophase I of meiosis were assayed with identical prenatal time points; the oocyte growth phase, the period during which methylation patterns are acquired in the female, was covered with the same postnatal time points as for the testis. Relative quantification using the standard curve method was used to

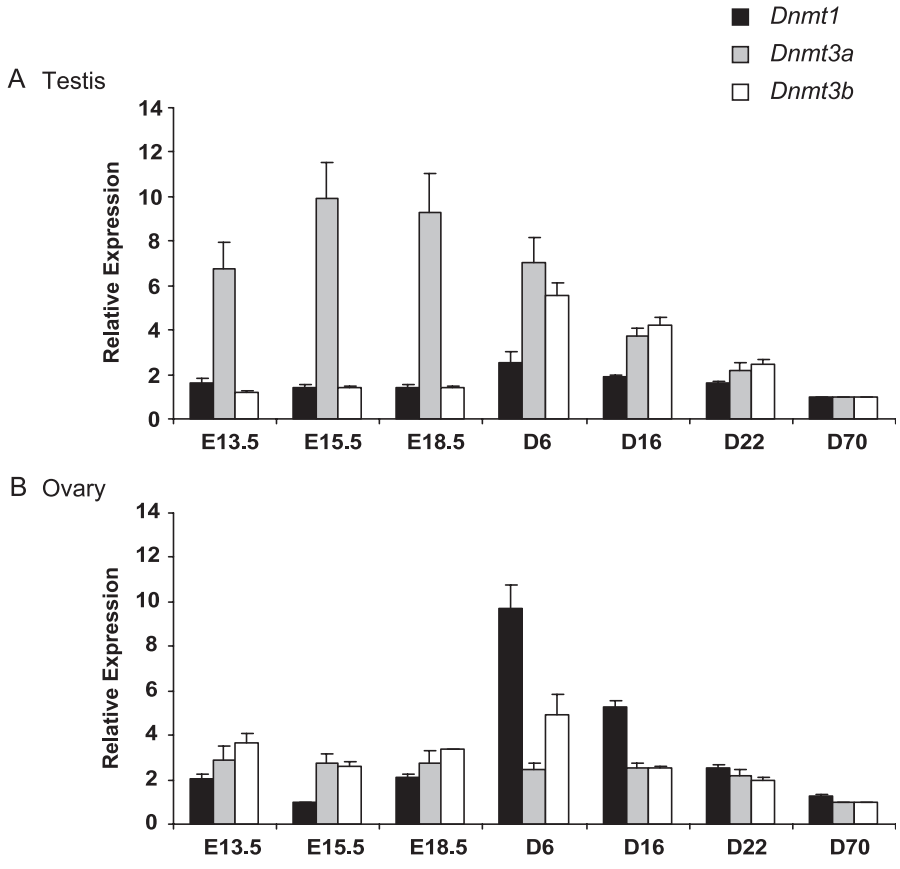

Fig. 6. Expression dynamics of DNA methyltransferases in the developing testis and ovary. Relative quantification of *Dnmt1* (black bar), *Dnmt3a* (gray bar), and *Dnmt3b* (white bar) in (A) testis and (B) ovary mRNA populations were done in triplicate on the same three individual samples of RNA extracted from pooled testes or ovaries at the indicated developmental stages via real-time RT-PCR. D = days. Mean ± SEM.

determine the fold changes in expression according to the lowest expressing time point (calibrator) for a given gene in whole testes and ovaries; all other quantities were expressed as an *n*-fold difference relative to the calibrator. Because the same stock of RNA was used to prepare all standard curves, the relative quantities determined using this method could be compared across individual experiments.

We first compared the expression profiles of the enzymes known to be capable of methylating DNA in both the testis (Fig. 6A) and the ovary (Fig. 6B). Validation of the assay was assessed by determining the pattern of expression of DNMT1 in the male germ line, which is shown in Fig. 4A and is consistent with previous reports (Benoit and Trasler, 1994; Trasler et al., 1992). Consistent with the Northern results in Fig. 4, DNMT1 showed higher levels of variation in the ovary (up to 9-fold) than in the testis (up to 3-fold). In the ovary, the highest DNMT1 levels were found early after birth, a time when folliculogenesis is active and DNMT1 may be required predominantly to maintain genomic methylation in dividing granulosa cells. DNMT3a and DNMT3b displayed unique developmental profiles that showed marked sex-specific differences. In the testis, DNMT3a expression was prominent before and early after birth and gradually returned to basal levels in the postnatal testis (Fig. 6A). In contrast, in the female, DNMT3a expression remained relatively constant throughout ovary development (Fig. 6B). In the male, DNMT3b was present at low levels before birth, only increasing above day E18.5 levels after birth, most notably on days 6 and 16 (Fig. 6A). The developmental pattern of expression of DNMT3b in the female (Fig. 3B) was similar to that in the male; however, in the ovary while expression peaked at 6 dpp, it decreased again by 16 dpp.

We subsequently analyzed the expression pattern of *Dnmt3l*, a member of the DNMT3 family known to lack DNA methyltransferase activity, but to nevertheless be essential for DNA methylation to occur. *Dnmt3l* transcripts were particularly abundant in both the testis and the ovary, varying up to 500- to 600-fold; however, peaks of expression were seen at very different developmental times in the two germ lines (Fig. 7). In the male, *Dnmt3l* was abundantly expressed before birth and was present at the highest levels at E15.5, just preceding the time methylation is known to be initiated in the male germ line. *Dnmt3l* expression in the testis dropped dramatically after birth; expression was approximately 470× lower at 22 dpp than at E15.5. A different pattern was seen in the ovary where DNMT3l was present at low levels before birth with an up-regulation shortly after birth. In the female germ line, expression peaked at 16 dpp with more than 600-fold the levels at the lowest expressing time point (E18.5).

Discussion

Although DNA methylation occurs at about 30 million sites in the mammalian genome (Bestor and Tycko, 1996), in a sex- and sequence-specific manner and is initiated in the germ line, little is known about the specific DNA methyltransferase enzymes involved, when they act developmentally and if and how the process differs between the sexes. Here, we provide evidence that the predominant DNA methylating enzyme, DNMT1, is present in prenatal gonocytes but is then down-regulated before and is absent at the time of acquisition of methylation in the male germ line, implicating other enzymes in the de novo methylation of DNA that is initiated in the prenatal period. Expression profiles showing concomitant peaks of DNMT3a and DNMT3l expression in the prenatal testis suggest that these two enzymes may interact to help establish DNA methylation patterns in the male germ line. DNMT1 and DNMT3b expression levels peak in the early postnatal

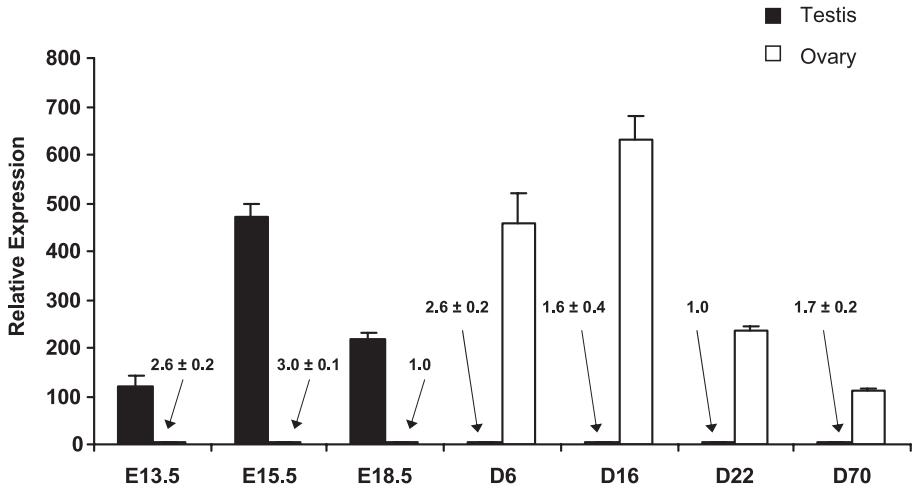

Fig. 7. Expression dynamics of a DNA methyltransferase-like gene, *Dnmt3l*, in the developing testis (black bar) and ovary (white bar). Relative quantification of Dnmt3l mRNA population was done in triplicate on the same RNA samples via real-time RT-PCR. D = days. Mean ± SEM.

period in the male suggesting a role for these enzymes in the maintenance of methylation patterns in rapidly proliferating spermatogonia. In the female, DNMT1 is expressed in prenatal oogonia but is then down-regulated during meiotic prophase; a similar process occurs in the male in the postnatal period. Expression profiles identified DNMT3l as the predominant DNMT3 enzyme present in the postnatal ovary coincident with the timing of acquisition of methylation patterns in the female germ line.

DNMT1 expression in the nuclei of male and female proliferating primordial germ cells

Previous studies of DNMT1 expression in the germ line focused primarily on the postnatal events taking place during gametogenesis (Benoit and Trasler, 1994; Jue et al., 1995; Mertineit et al., 1998; Numata et al., 1994; Ratnam et al., 2002). Here, we compared the DNMT1 cellular expression patterns in the male and female prenatal gonad, using the same well-characterized anti-DNMT1 antibody (PATH52) that we had previously used to localize DNMT1 in the postnatal testis and ovary. We first showed that mitotically dividing E11.5 PGCs of both sexes contain high levels of DNMT1 and that the protein is present in the nucleus where DNA methylation takes place; DNMT1 continues to be expressed in dividing male and female germ cells following sexual differentiation. Recent studies have examined the timing of methylation reprogramming events that take place between E10.5 and E12.5 in the PGCs colonizing the embryonic gonads. While the methylation at imprinted and single-copy loci is erased in E10.5 to E12.5 germ cells within about a 1-day window, it appears that some repetitive sequence elements, in particular the LTR of Intracisternal A Particle (IAP) elements, retain their methylation (Hajkova et al., 2002; Lane et al., 2003; Lees-Murdock et al., 2003). Consistent with our results, although sexing of the gonads was not carried out, Hajkova et al. (2002) showed that DNMT1 was expressed in E12.5 genital ridge germ cells. Thus, it is possible that DNMT1 is required in mitotic PGCs of both sexes to maintain the methylation of some repetitive elements, perhaps to prevent them from being expressed at a critical time when other sequences are losing their methylation.

Down-regulation of DNMT1 in the male and female fetal gonads following sexual differentiation and re-expression postnatally

Our data also show that, following sexual differentiation, as germ cells of both sexes gradually entered a non-proliferative state, DNMT1 staining was lost. In the male, mitotic arrest is associated with maturation of germ cells into prospermatogonia at about E14.5 followed by genome-wide acquisition of methylation between E15.5 and E18.5 (Davis et al., 1999, 2000; Kafri et al., 1992; Lees-Murdock et al., 2003; Ueda et al., 2000; Walsh et al., 1998). The current study shows that DNMT1 is not present in male germ cells during this critical period, although it is clearly present in surrounding somatic cells. Using a different anti-DNMT1 antibody, Sakai et al. (2001) found expression of DNMT1 in the prenatal testis to follow that of PCNA (a marker for proliferating cells), a finding consistent with our results. Together, the results indicate that DNMTs other that DNMT1 are responsible for the de novo acquisition of methylation patterns in male prenatal germ cells.

In the female germ line, DNMT1 was initially expressed in oogonia; expression then decreased between E13.5 and E18.5. However, unlike male germ cells that enter mitotic arrest during this time, female germ cells enter meiotic prophase I shortly after sexual differentiation in the embryo. Precise staging of meiotic prophase oocytes using a synaptonemal complex marker revealed a gradual decrease in nucleoplasmic DNMT1 staining as germ cells passed through meiotic prophase, such that by late pachytene, DNMT1 was undetectable in oocytes. We previously reported that DNMT1 protein levels were down-regulated at pachytene in the male mouse; this down-regulation is due to the production of a non-translated pachytene spermatocyte-specific 6.0 kb transcript derived from exon 1p of the *Dnmt1* gene (Jue et al., 1995; Mertineit et al., 1998; Trasler et al., 1992). We now report another example of DNMT1 down-regulation at pachytene, this time in the female germ line, in the absence of an alternative transcript, suggesting that the mechanism of meiotic down-regulation differs between the sexes. Several features of meiotic chromosomes render them vulnerable to de novo methylation during crossing over and DNMT1 preferentially targets DNA structures associated with recombination (Bestor and Tycko, 1996). Based on the results in the male, we proposed earlier (Bestor and Tycko, 1996; Jue et al., 1995) that germ cells may protect their meiotic DNA from inappropriate methylation by inactivating the expression of DNMT1. Our current results provide further evidence in support of this hypothesis.

Experiments in mouse 3T3 fibroblasts at S phase showed that DNMT1 localizes to discrete foci that are sites of DNA replication (Leonhardt et al., 1992). We previously observed nuclear localization of DNMT1 foci in isolated type A spermatogonia from 8-day-old mice; as this cell type is mitotically active, we thought the foci most likely represented sites of DNA replication as observed in S-phase 3T3 fibroblasts (Jue et al., 1995). However, we had not linked this observation to mitosis resumption early after birth, as germ cells exit their dormant state, relocate to the basement membrane, and recommence dividing. Here, we confirm that DNMT1 re-expression is synchronized with the cell cycle and, consistent with its role as a replication factor, associates with actively dividing germ cells at 3 dpp but not with G1 phase gonocytes at 0 dpp.

Differential expression of the DNMT3 family members and the timing of methylation in the male and female germ lines

De novo methylation begins in the male germ line before birth and is further consolidated after birth; once established, methylation patterns must be maintained during DNA replication that takes place in spermatogonia and preleptotene spermatocytes. In the female germ line, DNA methylation patterns are acquired during the oocyte growth phase after birth, but since there is no further DNA replication, there appears to be no need for maintenance methylation later in oogenesis. Recently, DNMT3a, DNMT3b, and DNMT3l have been characterized and were shown to be expressed in multiple tissues, including the testis and the ovary; DNMT3a and DNMT3b are postulated to be involved in de novo methylation events (Aapola et al., 2001; Chen et al., 2002; Okano et al., 1998). Results of gene-targeting experiments indicate that DNMT3l is dispensable for normal somatic development but is an important regulator of maternal imprint establishment as well as being essential for normal postnatal spermatogenesis (Bourc'his et al., 2001).

We determined the time windows during which the DNMT3 family members are expressed (summarized in Fig. 8). In the male, DNMT3a and DNMT3l levels peaked at the same time before birth, during the critical time window of DNA methylation pattern initiation (E15.5–E18.5); the results support an interaction of the two enzymes in the de novo methylation process taking place during prenatal male germ cell development. DNMT3l was recently shown to stimulate de novo methylation through DNMT3a, but not DNMT3b, at some

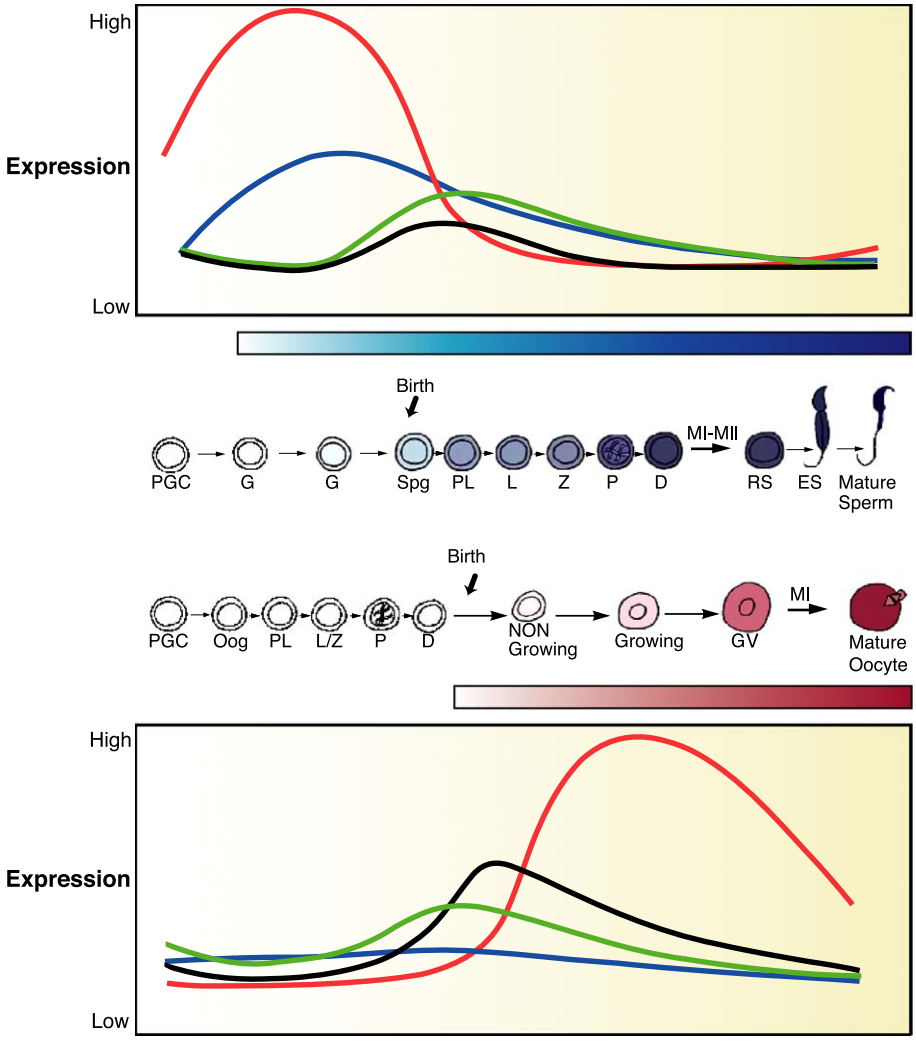

Fig. 8. Schematic representation of DNA methyltransferase dynamics during gametogenesis. Progression of the establishment of methylation marks at imprinted and non-imprinted loci and relative levels of Dnmt1 (black), Dnmt3a (blue), Dnmt3b (green), and Dnmt3l (red) are presented as functions of spermatogenesis and oogenesis. Intensity of the shading in the boxes reflects the methylation status of the paternal (blue) and maternal (red) genomes. Dnmt3l levels are not to scale. Representative cell types are indicated as follows: PGC, primordial germ cell; G, gonocyte; Spg, spermatogonia; Oog, oogonia; PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene; GV, germinal vesicle; RS, round spermatid; ES, elongating spermatid. MI–MII, meiosis I–II. Modified from Lucifero et al., 2002.

imprinted gene loci in vitro (Chedin et al., 2002) and we hypothesize it might be carrying out a similar function in the germ line in vivo.

In the female, particularly high levels of DNMT3l coincided with the timing of oocyte growth in the postnatal ovary, further supporting the important role it is thought to play in regulating the establishment of maternal imprints. In the current experiments, DNMT3a expression did not vary greatly during development of the ovary. Nevertheless, Hata et al. (2002) demonstrated that embryos derived from transplanted *Dnmt3a*$^{-/-}$/*Dnmt3b*$^{+/-}$ ovaries have perturbed maternal imprints, suggesting a role for DNMT3a in methylation events occurring during oocyte growth.

There is growing evidence that DNMT family members interact (Hata et al., 2002; Kim et al., 2002; Margot et al., 2003). For instance, biochemical fractionation experiments have revealed an association between DNMT1 and DNMT3b (Datta et al., 2003). Minor satellite repeats, found in the pericentromeric region of chromosomes, are thought to be specifically methylated by DNMT3b (Okano et al., 1999) and ICF syndrome (immunodeficiency, centromere instability, and facial anomalies), a rare immune disease causing hypomethylation of centromeric repeats in humans, is caused by mutations in the *Dnmt3B* gene (Xu et al., 1999). We show here that, in the male, DNMT3b is expressed at the highest levels postnatally, at times when the predominant cells are either mitotic spermatogonia (6 dpp) or spermatocytes entering meiotic differentiation (16 dpp). It is possible that DNMT3b plays a role at these early times during spermatogenesis in actively methylating centromeric regions to ensure proper pairing and recombination between homologous chromosomes. In addition, we suggest that DNMT1 and DNMT3b may cooperate in maintaining the integrity of the genome, since they show a similar pattern of expression in both germ lines.

Experiments over the last decade on DNMT1 in the germ line have uncovered several interesting features including highly regulated expression during male and female gametogenesis, the use of sex-specific first exons to produce translated and non-translated RNAs, as well as an important role in reproductive function due to an oocyte DNMT1 isoform essential for the maintenance of methylation in preimplantation embryos. The data presented here on the DNMT3 family members show striking gene-specific and germ line-specific patterns of expression, evidence that DNMT3a and DNMT3l may interact in de novo methylation in the male germ line, a potential role of DNMT3b in maintenance methylation in spermatogonia and high levels of DNMT3l expression at the time of methylation acquisition in the female germ line. Our findings suggest that in-depth studies to understand the roles of each of the DNMT3 enzymes in male and female germ cell development and the acquisition of sequence-specific methylation patterns are warranted. As for DNMT1, such studies will likely require the identification and characterization of germ cell specific isoforms, detailed cell localization studies in isolated populations of germ cells, as well as the creation of germ cell-specific knockout models.

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