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Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells

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Abstract

The development of germ cells is a highly ordered process that begins during fetal growth and is completed in the adult. Epigenetic modifications that occur in germ cells are important for germ cell function and for post-fertilization embryonic development. We have previously shown that male germ cells in the adult mouse have a highly distinct epigenetic state, as revealed by a unique genome-wide pattern of DNA methylation. Although it is known that these patterns begin to be established during fetal life, it is not known to what extent DNA methylation is modified during spermatogenesis. We have used restriction landmark genomic scanning (RLGS) and other techniques to examine DNA methylation at multiple sites across the genome during postnatal germ cell development in the mouse. Although a significant proportion of the distinct germ cell pattern is acquired prior to the type A spermatogonial stage, we find that both *de novo* methylation and demethylation occur during spermatogenesis, mainly in spermatogonia and spermatocytes in early meiotic prophase I. Alterations include predominantly non-CpG island sequences from both unique loci and repetitive elements. These modifications are progressive and are almost exclusively completed by the end of the pachytene spermatocyte stage. These studies better define the developmental timing of genome-wide DNA methylation pattern acquisition during male germ cell development.

Keywords: Spermatogenesis; DNA methylation; Epigenetics; RLGS; Germ cell; Mouse

Introduction

Epigenetic marks in the form of DNA methylation are involved in the development of germ cells and are important in the maintenance of fertility. Catalyzed by a family of DNA methyltransferase (DNMT) enzymes, mammalian DNA is commonly modified by the addition of a methyl group to the 5th position of the cytosine ring in CpG dinucleotides. DNA methylation is thought to act by promoting heterochromatin formation that can lead to gene repression when present in regulatory regions of genes (Klose and Bird, 2006). Several studies have highlighted the importance of DNA methylation to male germ cells. DNMTs are expressed in male germ cells in a developmentally regulated fashion, and some are expressed as germ cell-specific alternative transcripts (Mertineit et al., 1998; La Salle et al., 2004; Shovlin et al., 2007; La Salle and Trasler, 2006). The *Dnmt3L* gene, encoding a DNMT lacking catalytic activity, is expressed at especially high levels in the gonocytes in fetal testes beginning at 15.5–18.5 days *post coitum* (dpc) (Bourc'his et al., 2001; La Salle et al., 2004). Males lacking *Dnmt3L* are infertile due to a complete lack of mature germ cells (Bourc'his et al., 2001; Hata et al., 2002) and display abnormal meiotic chromosome structures (Bourc'his and Bestor, 2004; Webster et al., 2005); in these mice, male germ

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cell DNA methylation is not fully acquired at several repetitive and non-repetitive sequences, including imprinted and non-imprinted loci (Bourc'his and Bestor, 2004; Kaneda et al., 2004; Webster et al., 2005; Hata et al., 2006; Oakes et al., 2007). Germ cell-specific disruption of *Dnmt3a* results in infertility and a loss of methylation at imprinted genes but not at repeat sequences (Kaneda et al., 2004).

The importance of DNA methylation in male germ cells is also inferred by the presence of distinct patterns. We have shown that genome-wide DNA methylation patterns involving an array of sequence types are highly unique in spermatozoa compared to somatic tissues in mouse (Oakes et al., 2007). Recently, a study in humans concluded that DNA methylation in sperm is highly distinct from cells and tissues of somatic origin (Eckhardt et al., 2006). Other studies have found distinct DNA methylation states in repetitive sequences (Sanford et al., 1984) and some testis-specific genes in male germ cells (MacLean and Wilkinson, 2005). This unique state of DNA methylation arises from a genome-wide reprogramming event that occurs specifically in the primordial germ cells (PGCs) of the developing embryo (Reik et al., 2001). Between 10.5 and 12.5 dpc, patterns of DNA methylation in imprinted and testis-specific genes are erased in PGCs (Hajkova et al., 2002; Maatouk et al., 2006). Repetitive elements, such as the intracisternal A particle (IAP), LINE-1 (L1) and minor satellites undergo a similar demethylation although not to the same extent as is seen for single-copy genes (Walsh et al., 1998; Hajkova et al., 2002; Lees-Murdock et al., 2003). In the male, DNA methylation begins to be reestablished around 15.5 dpc for imprinted genes (Davis et al., 2000; Ueda et al., 2000; Li et al., 2004). The repeat sequences IAP and L1 are remethylated by 17.5 dpc (Lees-Murdock et al., 2003). This also coincides with the developmental time point where germ cells stain strongly using an antibody directed against 5-methylcytosine (Coffigny et al., 1999).

Although several studies have addressed the acquisition of DNA methylation during fetal germ cell development, few have investigated the behavior of DNA methylation patterns during spermatogenesis. Spermatogenesis is a well defined, complex developmental process whereby morphologically distinct, haploid spermatozoa that are capable of fertilization are produced from diploid germ cell precursors (Russell et al., 1990). In mammals, this process continually produces a supply of spermatozoa for the duration of the life of the adult animal. Spermatogonial stem cells occupy the seminiferous tubules of the testis and continually provide a pool of undifferentiated diploid cells called type A spermatogonia. These cells undergo several rounds of mitosis before entering meiotic prophase I, where tetraploid spermatocytes pair and recombine homologous chromosomes. After recombination, spermatocytes are reduced to haploid spermatids that undergo morphological changes from round spermatids to elongating spermatids and finally to spermatozoa. These spermatozoa exit the testis and complete their maturation process during epididymal transit.

Studies of the paternally methylated imprinted gene, *H19*, show that although initial acquisition occurs before birth, complete levels of DNA methylation are not achieved until the pachytene spermatocyte phase of spermatogenesis (Davis et al., 1999). The two other known paternally methylated imprinted genes, *Rasgrf1* and *Gtl2*, similarly acquire most of their DNA methylation in the prenatal window, but have yet to acquire the levels found in spermatozoa (Li et al., 2004). Other data show that some sequences have fully acquired their DNA methylation status before the beginning of spermatogenesis, including some repetitive elements, such as IAP, L1 and satellite sequences (Walsh et al., 1998; Lees-Murdock et al., 2003; Bourc'his and Bestor, 2004). The hypomethylated state of *Pgk-2*, a testis-specific gene expressed in spermatocytes, is also established prior to spermatogenesis (Geyer et al., 2004). Although limited data point to the acquisition of DNA methylation patterns beyond the fetal development window, a comprehensive study of the timing and the range of sequences involved has not been done.

Restriction landmark genomic scanning (RLGS) is a highly reproducible technique that is used to investigate genome-wide patterns of DNA methylation in a variety of sequences. In combination with a recently developed second generation virtual RLGS resource that uses genomic sequence to produce simulated RLGS profiles (Smiraglia et al., unpublished), individual genomic loci that display alterations of DNA methylation can be identified. In this study, we produce a non-biased, detailed view of the patterns of DNA methylation in a variety of sequences as male germ cells progress through spermatogenesis. We find that both *de novo* methylation and demethylation occur during spermatogenesis in a sequence-specific manner. Most importantly, we establish that, in addition to prenatal acquisition, patterns of DNA methylation at multiple sites across the genome are acquired postnatally and are complete prior to meiosis in male germ cells.

Materials and methods

Isolation of purified spermatogenic cells

Adult male C57BL/6NCrl mice were obtained from Charles River Laboratories (St-Constant, Quebec). All animal studies were conducted in accordance with the principles and procedures outlined in the *Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care. Purified populations of type A spermatogonia, early pachytene and pachytene spermatocytes, and round and elongated spermatids were obtained from the testes of mice using the sedimentation velocity method (Bellvé et al., 1977). Type A spermatogonia were obtained from 8-day *post partum* (dpp) mice with an average purity of 86% ($n=2$, 100 mice pooled per cell separation). Early pachytene spermatocytes were obtained from 17 dpp mice with a purity of 75% ($n=1$ cell separation, 100 mice pooled). Pachytene spermatocytes (average purity=85%), round spermatids (average purity=95%) and elongated spermatids (average purity of nucleated cells=97%) were obtained from 70 dpp mice ($n=3$, 12 mice pooled per cell separation). Spermatozoa were isolated from the cauda epididymidis of 70 dpp mice ($n=4$, 12 mice pooled per purification, average purity=99%) as described previously (Alcivar et al., 1989). Primitive type A spermatogonia were isolated from the testes of 6 dpp GOF18deltaPE-Oct4/GFP (Yoshimizu et al., 1999) mice that have been bred into C57BL/6 background for 3+ generations. Germ cells were isolated using fluorescence-assisted cell sorting (FACS) as described in Oakes et al. (2007) ($n=3$, 3–4 mice pooled per purification). The qAMP method was used (see below) to determine the level of inter-strain variability in DNA methylation levels. Primitive type A spermatogonia were isolated from the testes of 6 dpp GOF18deltaPE-Oct4/GFP bred to CD1 mice and methylation levels for approximately 20 randomly chosen genomic loci were found to be similar (<15% variation) to GOF18deltaPE-Oct4/GFP mice bred into C57BL/6. Liver, intestine and brain tissues were isolated

from adult C57BL/6 mice. Genomic DNA was isolated using proteinase K and phenol followed by dialysis for the RLGS and Southern blotting experiments (Okazaki et al., 1995) or the DNeasy Tissue Kit from Qiagen (Germantown, MD, USA) for qAMP analysis.

RLGS and spot identification

RLGS was done as described previously (Okazaki et al., 1995). Densitometry of RLGS spots was done by exposing the RLGS gel to a phosphorimager screen from Kodak (Rochester, NY, USA). Images were analyzed using the ImageQuant v5.1 software from GE Healthcare (Piscataway, NJ, USA). Spot density values were obtained by comparing a spot of interest to approximately 10–15 surrounding spots of unchanged intensity. In order for a spot to be identified as having altered DNA methylation, the alteration had to be consistent in all RLGS profiles of the same cell type and spot densitometry had to reveal a difference of greater than 25%. The genomic location of spots was identified using a virtual RLGS resource (Smiraglia et al., unpublished). All identified spots were confirmed by the BAC mixing gel method (Oakes et al., 2007). CpG islands were defined as done previously (Gardiner-Garden and Frommer, 1987) and 5′ regions were defined as being within 1 kb of the transcriptional start site or within 200 bp of a CpG island that was found within the first exon or up to 5 kb from the transcriptional start site. Spermatogenic cell-type-specific gene expression data were obtained from the Mammalian Reproductive Genetics database (http://mrg.genetics.washington.edu/).

DNA methylation analysis using qAMP

The qAMP method was done as described previously (Oakes et al., 2006). Briefly, genomic DNA is digested in separate reactions with either no enzyme (sham digest), methylation sensitive restriction enzymes (MSREs) and the methylation-dependent restriction enzyme, McrBC. Primers are designed to flank restriction sites of interest and individually digested DNA samples are amplified using real-time PCR. Shifts in Ct value (ΔCt) between the sham- and enzyme-digested samples are used to calculate the percentage of methylation at the various CpG sites within the amplified region MSREs: % methylation = 100 ($2^{-\Delta Ct}$); McrBC: % methylation = 100($1-2^{-\Delta Ct}$). All ΔCt values are the means of triplicate reactions. Due to the curved relationship between ΔCt and percent methylation, MSREs are more accurate in the low % methylation range (<50%) and McrBC is more accurate in high % methylation ranges (>50%). Primers used to analyze genomic regions identified by RLGS are as follows: *AK137601*, 5′-CTCCCCCATTCTCCCTCAC 5′-CCTAACTTCTTGCCGTGCTC; *Polg*, 5′-CAGACCTCCACGTCGAACA 5′-CAGAGCCTGCCTTACTTGGA; *Abt1*, 5′-CCATGGGCGTGTTATGTAGA 5′-TGCTTGATGGGATGTTCATT; *Ibtk*, 5′-ACTCTCCTGCCTTGCACCT 5′-GCTGTCCACTCGGTGTCAT; *Tcf3*, 5′-GCAAGGGCCTGGATAGGA 5′-GCTACCCACTCCGAGCAA. Primers used to analyze differentially methylated regions (DMRs) of imprinted genes are as follows: *H19*, 5′-AAAAGCAGAAGGCAGGACAC 5′-ATGTTCCAGAGACAGCCAAAG; *H19* (McrBC), 5′-AGCCGTTGTGAGTGGAAAGA 5′-CATAGCGGCTTCGGACATT; *Rasgrf1*, 5′-CTGCACTTCGCTACCGTTTC 5′-CAGCAGCAGCAGTAGCAGTC; *Gtl2*, 5′-CCGTGAACTAGCGAGGAGGT 5′-ATAATGCAGCCCTTCCCTCA. In the chromosome-wide survey, a region was considered to be different if at least one of the enzyme digests detected a reproducible difference of 15% or greater in each replicate ($n=2$ for both primitive type A spermatogonia and spermatozoa) and that the enzyme used to detect the difference was within its accurate percent range. The difference in both enzyme digests had to be in the same direction or unchanged. Primers used to analyze regions on chromosome 7 are listed in Supplementary Table 1; primers for chromosomes 4, 10, 17 and X were described previously (Oakes et al., 2007). Primers were designed using the Primer3 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Genomic sequence data were obtained from the University of California at Santa Cruz Genome Browser, version mm7 (http://www.genome.ucsc.edu).

Bisulfite sequencing and Southern blotting

Bisulfite sequencing was done as described previously (Warnecke et al., 1998). Primers used to amplify the *Abt1* gene were 5′-GGTGTTTGGATTAGAGTTGGAG and 5′-AACCTACAAACCACTTTATAAAAC. Primers used to amplify the *Tcf3* gene were 5′-GGAAAGAGGTTGGGTTTGTAGTA and 5′-TTAACCTCACCAACTACCCCTAC. Southern blots were performed as described (Trasler et al., 1990) and visualized by autoradiography. Minor satellite probes were constructed by PCR amplification of mouse genomic DNA using primers 5′-CATGGAAAATGATAAAAACC and 5′-CATCTAATATGTTCTACAGTGTGG (Lehnertz et al., 2003). The ribosomal DNA (rDNA) repeat probe was constructed using primers 5′-CGTTATGGGGTCATTTTTGG and 5′-CAGACCCAAGCCAGTAAAAAG to analyze *Hpa*II sites located in the proximal promoter of the rDNA repeat. The IAP probe has been used previously (Michaud et al., 1994; Walsh et al., 1998). DNA was digested completely with either *Msp*I or its methylation-sensitive isoschizomer *Hpa*II. The membrane was stripped and reprobed according to the manufacturer's recommended conditions (Hybond, GE Healthcare).

Results

Detection of alterations of DNA methylation during spermatogenesis using RLGS

RLGS investigates genome-wide patterns of DNA methylation by separating genomic DNA that has been digested with the methylation-sensitive restriction enzyme, *Not*I, by two-dimensional gel electrophoresis. In the mouse, *Not*I sites occur in a variety of sequence types. To determine if the pattern of genome-wide DNA methylation in spermatozoa is acquired during spermatogenesis, RLGS profiles of purified populations of type A spermatogonia, pachytene spermatocytes from two developmental time points (early and mid–late pachytene) as well as post-meiotic round and elongating spermatids were generated (Fig. 1a). The intensity of a total of 19 RLGS spots was observed to be different between these cell types; 11 demonstrated increased methylation (*de novo* methylation) and 8 demonstrated decreased methylation (demethylation) during spermatogenesis, as indicated by a loss or a gain of spot intensity, respectively (Fig. 1b). The majority of the changes in individual spot intensities, in *de novo* methylation and demethylated directions, occurred between type A spermatogonia and early pachytene spermatocytes. The intensity of some spots continued to change between early and mid–late pachytene and always occurred in the same direction. With the exception of one spot, all spots did not gain or lose measurable amounts of methylation after the pachytene stage. Other than the progressive changes that occur between type A spermatogonia and spermatocytes, no *de novo* or demethylation events were observed in any of the cell types tested. Virtual RLGS analysis reveals that, in the analyzable window of the RLGS gel, there are 2954 potential RLGS spots that originate from approximately 2600 *Not*I sites (Table 1). This indicates that only a small fraction (<0.7%) of the assayable *Not*I sites displayed modified DNA methylation during spermatogenesis, leaving greater than 99% unchanged.

Germ cell-specificity of spots that show altered methylation during spermatogenesis

To determine if the spots that display altered methylation during spermatogenesis have a methylation status that is

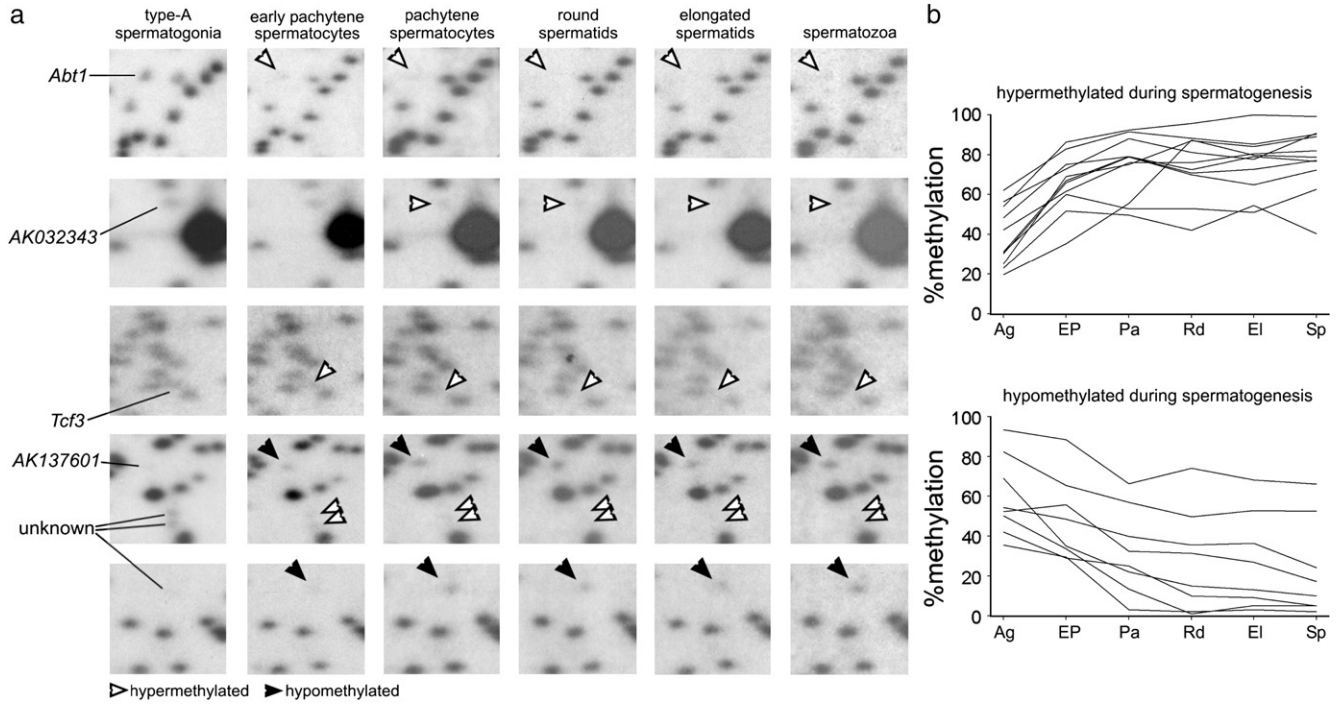


Fig. 1. Examination of RLGS profiles from purified spermatogenic cell types. (a) RLGS profiles are produced by digestion of genomic DNA with the methylation-sensitive enzyme, *Not*I; these cleavage sites are radiolabeled and DNA fragments are separated by two-dimensional gel electrophoresis. Visible spots reveal hypomethylated sites, absent spots are hypermethylated. Enlargements of RLGS profiles produced from type A spermatogonia, early and mid–late pachytene spermatocytes, round and elongated spermatids and spermatozoa are shown. Selected enlargements are representative of areas throughout the two-dimensional RLGS profile. Spots that are *de novo* methylated and demethylated relative to type A spermatogonia are indicated by open and black arrows, respectively. The identified genes that contain the differentially methylated spots are shown. (b) RLGS densitometry of spots that are *de novo* methylated and demethylated during spermatogenesis. Cell-type-specific spot intensity of spots was determined by comparing the intensity of spots of interest with unchanged, surrounding spots. Percent methylation values are determined by the inverse of the relative spot density.

unique to male germ cells, the intensity of changed spots was examined in three somatic tissues: liver, intestine and brain (Fig. 2a). Thirteen of the nineteen spots were hypermethylated (absent) in all three somatic tissues studied, and none of them was hypomethylated in all three tissues. A minority of spots were hypomethylated in a tissue-specific manner (2–4 of 19) (Fig. 2b). Spots that were *de novo* or demethylated during spermatogenesis demonstrated equal levels of germ cell specificity, indicating that the unique hypomethylated state of these loci is not related to the methylation states in a particular phase of spermatogenesis. The dissimilarity between spermatozoa and somatic profiles (Oakes et al., 2007), versus the relative similarity between type A spermatogonia and spermatozoa, indicates that the bulk of germ cell-specific methylation pattern is acquired prior to the type A spermatogonia stage.

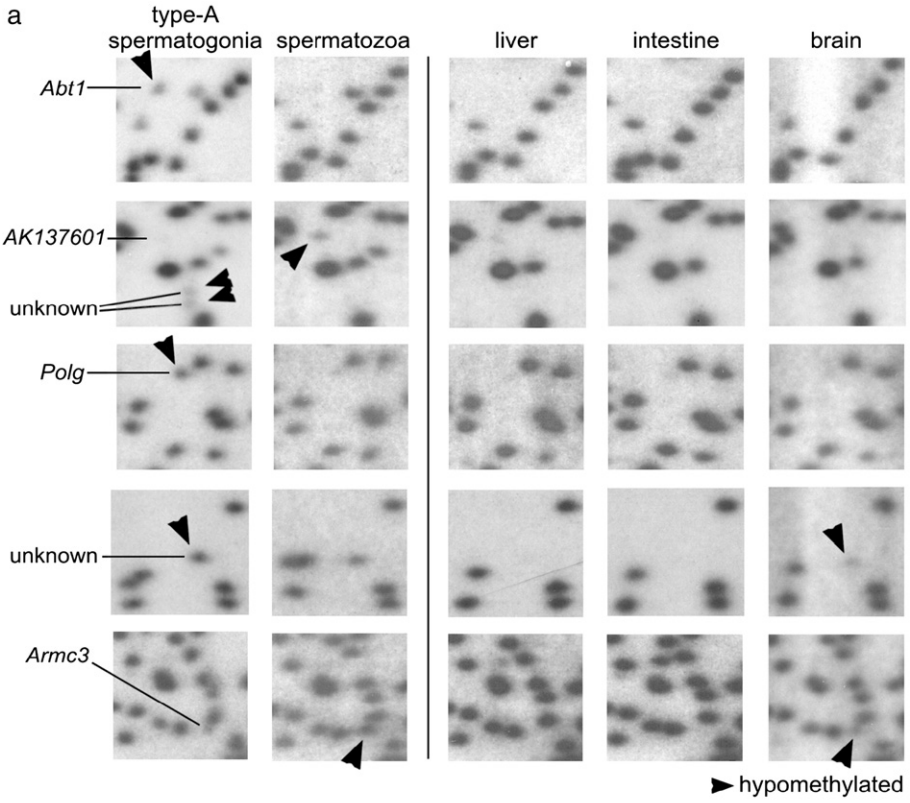
Table 1
RLGS spot summary

Methylation during spermatogenesis	# of spots	% of total
Hypermethylated during spermatogenesis	11	0.37
Hypomethylated during spermatogenesis	8	0.27
Unchanged	2935	99.4
Total[a]	2954	100

[a] Derived from virtual RLGS profile.

Identification of spots that show alterations during spermatogenesis

Identification of the genomic location of spots of interest was accomplished by using a second-generation virtual RLGS resource to identify candidate loci (Smiraglia et al., unpublished); confirmation of the identity of each spot was done using the BAC mixing gel method (data not shown). Using these methods, we identified 5/11 spots that were *de novo* methylated and 3/8 spots that were demethylated during spermatogenesis (Table 2). Spots were found on several chromosomes, were located within a variety of positions relative to known genes and within various sequence types (i.e. CpG islands (CGIs), repeats, etc.). All sites were in the vicinity of expressed sequences; either in the 5′ region, body or 3′ end of genes. Only 2/8 identified spots were within CGIs. Interestingly, these two sites were found only 42 kb apart on chromosome 7, one within the 5′ CpG island of the *Polg* (mitochondrial DNA-directed polymerase) gene and an mRNA, *Ak032343*, located upstream. Due to the GC-rich nature of the *Not*I recognition site, 75% of the approximate 8000 *Not*I sites found throughout the mouse genome are found within CGIs; 2/8 (25%) are less than the proportion that is expected, suggesting that altered DNA methylation occurs more commonly in non-CGI sequences. Most interestingly, all three identified sites that were demethylated during



b

The somatic methylation status of spots that are differentially methylated during spermatogenesis (n=19)

	liver	intestine	brain
hypermethylated	16	17	15
hypomethylated	3	2	4
% hypomethylated	16%	11%	21%

Fig. 2. Determination of the somatic methylation state of spots that are differentially methylated during spermatogenesis. (a) Enlargements of selected portions of RLGS profiles produced from type A spermatogonia, spermatozoa, liver, intestine and brain are shown. Hypomethylated spots that are differentially methylated during spermatogenesis are indicated by black arrows. The known genes associated with the differentially methylated spots are shown. (b) The proportion of spots that are differentially methylated during spermatogenesis that are hypomethylated in liver, intestine or brain. Spots that are differentially methylated during spermatogenesis are largely germ cell-specific.

spermatogenesis were found in small solitary long terminal repeats (LTRs) that belong to the mammalian retroposon-like (MaLR) and endogenous retroviral-K (ERVK) families of the LTR class of repetitive sequences, whereas all identified *de novo* methylated sites were in unique sequences.

Male germ cells possess a global gene expression profile that is highly unique from somatic tissues (Su et al., 2002; Shima et al., 2004). These transcripts are found to be highly regulated during spermatogenesis. DNA methylation has been proposed to function as a transcriptional regulator by causing gene repression

Table 2
Characteristics of identified RLGS spots

Methylation during spermatogenesis	RLGS spot	Gene	*Not*I site position	CpG island	Repeat (family)	Genome position
Hypermethylated	5ax2	*Tcf3*	3′	N	Non-repetitive	chr6:72645454
Hypermethylated	1gx8	*Polg*	5′	Y	Non-repetitive	chr7:75333081
Hypermethylated	4C13	*AK032343*	Body	Y	Non-repetitive	chr7:75377378
Hypermethylated	3ex8	*Ibtk*	Body	N	Non-repetitive	chr9:85800773
Hypermethylated	2F36	*Abt1*	Body	N	Non-repetitive	chr13:22791131
Hypomethylated	3ex3	*AK137601*	5′	N	LTR (MaLR)	chr1:36371367
Hypomethylated	2G75	*Armc3*	Body	N	LTR (ERVK)	chr2:19315058
Hypomethylated	4dx1	*AK035353*	Body	N	LTR (ERVK)	chr4:9701990

when present in 5′ regulatory sequences (Klose and Bird, 2006). To investigate if the status of DNA methylation in specific spermatogenic cell types correlates with transcriptional activity, the DNA methylation status of all identified RLGS loci located in 5′ regions of known genes was compared to known levels of gene expression in these same cell types. Gene expression data were obtained from the Mouse Reproductive Genetics Database. Approximately 400 spots have been identified on mouse RLGS profiles that are located within the 5′ regions of transcribed sequences (Smiraglia et al., unpublished). Of these, expression levels have been determined for 166 known genes in type A spermatogonia, pachytene spermatocytes and round spermatids (Shima et al., 2004). Despite greater than 90% (140) of these genes demonstrating a greater than 1.5-fold difference in expression between spermatogenic cell types (66% show greater than 2-fold expression differences), greater than 99% (165/166) showed no detectable change in methylation status (Supplementary Table 2). In addition, for the only spot originating from a 5′ region that demonstrated a change in methylation, *Polg*, increased methylation was correlated with an increase in expression, the opposite of what would be expected in this instance.

Quantitative DNA methylation analysis of selected identified loci

To determine whether changes that are observed by RLGS at *Not*I sites are representative of the DNA methylation status of neighboring CpGs, sites within small regions (~200 bp) flanking the *Not*I sites were chosen for analysis by the qAMP method. In addition, to confirm and expand upon the results found in type A spermatogonia, a further cell type, primitive type A spermatogonia, was analyzed. These cells are obtained from a time point 2 days earlier in spermatogonial development (6 dpp). *Not*I sites that are found in various positions relative to genes were chosen for analysis: 3′ end (*Tcf3*), body region (*Abt1*), 5′ upstream region (*AK137601*) and 5′ CGI (*Polg*). DNA methylation is gained (*Abt1*, *Tcf3* and *Polg*) or lost (*AK137601*) at multiple CpGs surrounding the RLGS *Not*I sites (Fig. 3). The percent methylation values of *Not*I sites determined using densitometry of RLGS spots was similar to the percent value determined by qAMP in each of the cell types investigated. Neighboring CpG sites generally showed similar levels of methylation to the *Not*I site and gained or lost methylation in a similarly progressive manner. Changes were found to be virtually complete by the pachytene spermatocyte stage, supporting the RLGS findings.

Although neighboring CpG sites generally showed similar levels of methylation, differences in the percent methylation between some adjacent CpGs were observed. This suggests that a mosaic, heterogeneous state of methylation may exist between neighboring CpG sites in various spermatogenic cell types. To determine the level of heterogeneity between CpGs on individual DNA strands, bisulfite sequencing was used to analyze *Abt1* and *Tcf3* in primitive type A spermatogonia, pachytene spermatocytes and spermatozoa (Fig. 4). This analysis revealed that heterogeneous methylation exists between neighboring CpG sites on individual DNA strands in all samples

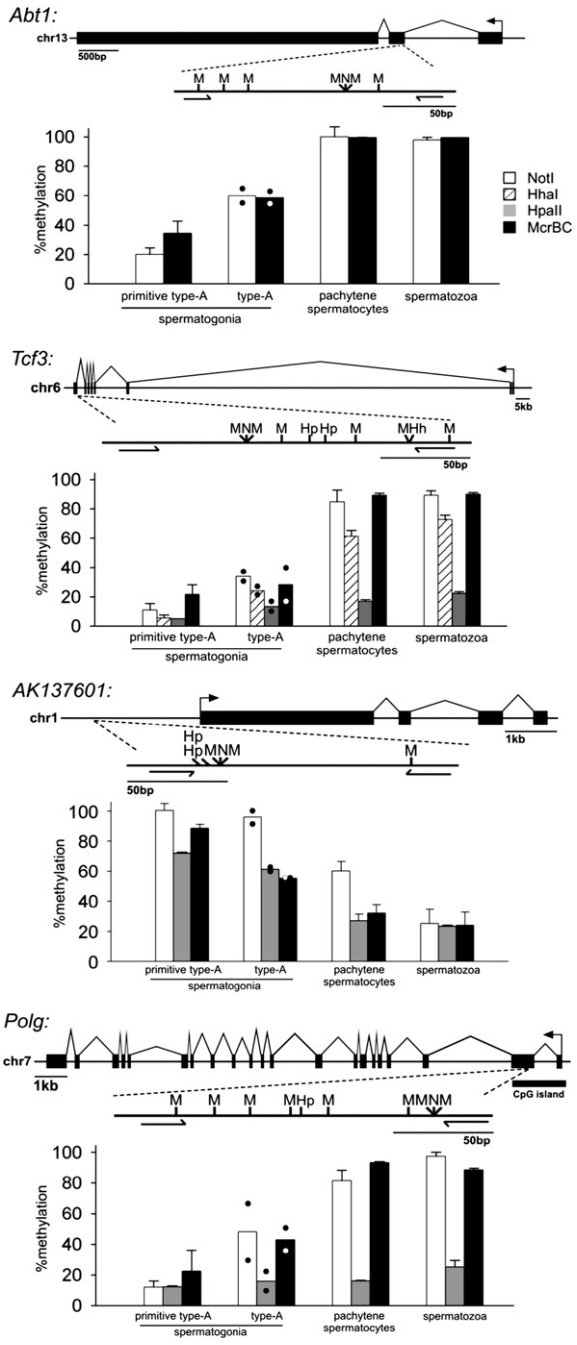


Fig. 3. Detailed examination of differentially methylated loci using the qAMP method. DNA is digested using methylation-sensitive restriction enzymes and the methylation-dependent enzyme, McrBC. Primers are designed to flank the *Not*I site along with neighboring restriction sites (assayable CpGs) and are amplified using real-time PCR. The positions of the assayed regions relative to known genes are shown. All genomic sequences are orientated from centromere to telomere in gene diagrams. The percent methylation at different CpG sites (or groups of sites) determined by independent enzyme digests in primitive type A and type A spermatogonia, pachytene spermatocytes and spermatozoa are shown. Results are represented as a mean±SEM of $n=3$–4 independent samples of pooled animals except for type A spermatogonia where individual values obtained for two independent pools (from $n=100$ mice/pool) of animals are represented by dots. N, *Not*I; Hh, *Hha*I; Hp, *Hpa*II; M, McrBC.

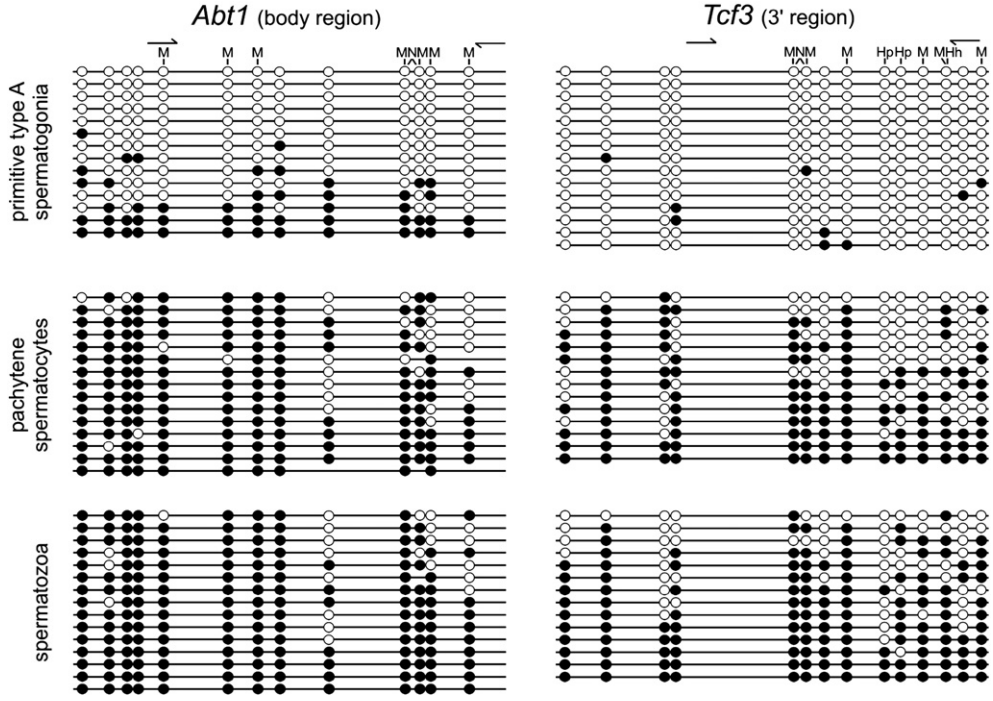


Fig. 4. Bisulfite sequencing analysis of *Abt1* and *Tcf3*. Bisulfite-treated DNA from primitive type A spermatogonia, pachytene spermatocytes and spermatozoa was amplified with primers that flank the regions of *Abt1* and *Tcf3* that were analyzed by the qAMP method; 14–15 individual DNA strands were cloned and sequenced per cell type. Open and closed circles represent unmethylated and methylated CpG sites, respectively. The CpGs analyzed by various qAMP digests as well as the qAMP primer positions are indicated. N, *Not*I; Hh, *Hha*I; Hp, *Hpa*II; M, McrBC.

analyzed. The percent methylation determined by qAMP closely matched the bisulfite sequencing results, demonstrating that differences in the percent methylation between adjacent restriction sites determined by qAMP are due to heterogeneous methylation between neighboring CpGs. For example, the percent methylation of the CpGs analyzed by *Not*I, *Hha*I, *Hpa*II and McrBC in the *Tcf3* gene in pachytene spermatocytes was determined by qAMP to be 85±8.2%, 61±4.2%, 17±1.2% and 89±1.3% (mean±SEM) respectively. Bisulfite sequencing of predicts percent values of 79%, 64%, 29% and 93% for each respective enzyme digest, closely matching the qAMP values. Due to the mosaic methylation states, the use of MSREs with multiple recognition sites will yield lower percentage values because all sites are required to be methylated simultaneously in order for amplification to occur. These results also confirm that, despite the heterogeneous methylation found in individual cell types, the pachytene spermatocyte and spermatozoa stages have similar overall levels of methylation, while developmental acquisition of DNA methylation clearly occurs in between the type A spermatogonia and pachytene spermatocyte stages. Furthermore, these results show that the methylation status of CpGs that are not analyzed by qAMP has a methylation status that is generally representative of the analyzed CpGs in each individual cell type.

Acquisition of DNA methylation at paternally methylated imprinted DMRs

Imprinted genes acquire a parent-specific pattern of methylation during germ cell development. Previously, the *H19* gene was shown to possess an incomplete level of methylation in spermatogonia, specifically on the maternal allele, which was later completed by the pachytene stage (Davis et al., 1999). There are two other well described regions that possess paternally methylated DMRs. Unfortunately, none of the paternally methylated DMRs are present on our RLGS profiles. To determine if DNA methylation is being acquired during spermatogenesis, the qAMP method was used to investigate DMRs in the *Dlk1-Gtl2* region (Takada et al., 2002) and the *Rasgrf1* gene (Yoon et al., 2002). The previously defined *H19*-Igf2 region (Tremblay et al., 1995) was used as control. This analysis revealed that the majority of CpGs investigated have acquired their full methylation status by the primitive type A spermatogonial stage; however, a small amount of methylation was acquired up to the pachytene stage (Fig. 5). The *Hha*I enzyme digest of the *Rasgrf1* DMR displayed the largest percentage increase of DNA methylation during spermatogenesis. There are three *Hha*I sites in the amplified region of *Rasgrf1*; if only one of the three is unmethylated, the strand will not amplify and contribute to the percentage of unmethylated strands. This particular digest revealed that a small proportion of CpGs were unmethylated in the *Rasgrf1* DMR in type A spermatogonia, and by the pachytene stage, DNA strands gained their fully methylated status. Changes in DNA methylation at other sites were minor.

Chromosome-wide survey of non-CpG island unique sequences

RLGS analysis has a strong bias towards CGIs; however, a higher proportion of non-CGI sites were shown to display

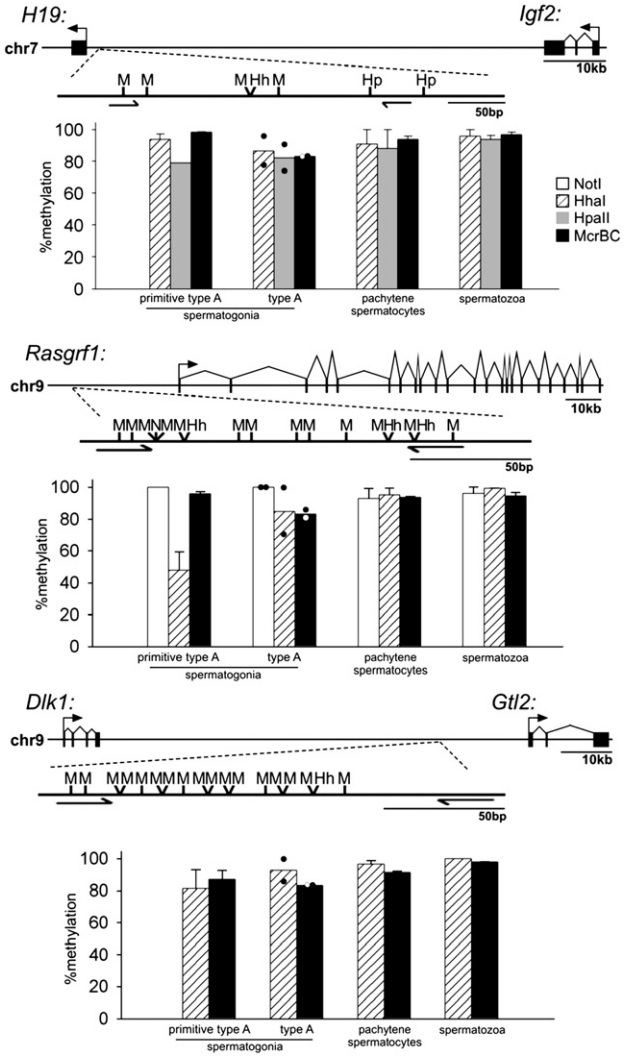


Fig. 5. Examination of paternally methylated imprinted DMRs. Primers were designed to flank restriction enzyme sites within the DMRs of *H19*-Igf2, *Rasgrf1* and *Dlk1-Gtl2*. The location of the primers used to assay the regions and the restriction enzyme sites examined are shown for each DMR. All genomic sequences are orientated from centromere to telomere in gene diagrams. The percent methylation at different CpG sites (or groups of sites) determined by independent enzyme digests is shown in primitive type A and type A spermatogonia, pachytene spermatocytes and spermatozoa. Data are represented as described in the legend of Fig. 3. N, *Not*I; Hh, *Hha*I; Hp, *Hpa*II; M, McrBC.

altered patterns of DNA methylation during spermatogenesis. To determine the prevalence of DNA methylation changes occurring at non-CGI sites, small groups of CpGs (regions) were chosen for quantitative analysis by the qAMP method at approximately 5 Mb intervals across chromosomes 4, 7, 10, 17 and X. These regions were chosen at random other than not being proximal (>10 kb) to a CGI or the transcriptional start site of a known gene. Regions were also chosen to be solely within non-repetitive sequences. Analysis of 125 total regions in primitive type A spermatogonia and spermatozoa revealed differences in DNA methylation in a region-specific manner (Fig. 6). Regions displaying high, intermediate (partial) and low levels of DNA methylation were detected, revealing that a full range of methylation levels can be found in germ cells in a site-specific manner. Differences were observed in 12 regions on 4 of 5 chromosomes. The number and/or extent of methylation differences were observed to be similar between the autosomes and the X chromosome, chromosomal position (telomeric versus centromeric), G- and R-banding patterns and flanking GC content (data not shown). Interestingly, all 12 regions that showed a difference were gaining methylation during spermatogenesis. Differences were in the same range (<60%) as detected at RLGS sites.

A closer examination of the changes to 6 of the 12 sites revealed that, like other changes observed, methylation acquisition during spermatogenesis is completed by the pachytene stage (Fig. 7). Several of the changes were specific to the CpGs investigated by a particular restriction enzyme, indicating that heterogeneous methylation exists at these sites. Further examination of the methylation of these regions in primitive type A spermatogonia showed that most CpGs investigated have methylation states that are similar to type A spermatogonia; however, substantial increases were observed at sites in two of the six regions.

Examination of repetitive sequences

The observation that all demethylated sequences identified by RLGS were of repetitive origin suggests that changes might be occurring in repetitive sequences during spermatogenesis. We chose to analyze three different types of repeat sequences that have been previously determined to have different levels of methylation: the minor satellite repeat, the ribosomal DNA repeat and IAP, an interspersed LTR-containing endogenous retroviral sequence (Fig. 8). Equal amounts of DNA isolated from type A spermatogonia to spermatozoa were digested with *Hpa*II, along with somatic tissues as control. Differences were observed between somatic and germ cells for both the minor satellite repeat and IAP; however, no changes were observed to occur during spermatogenesis for these classes of repeat sequences. Furthermore, several hundred IAP and early transposon (ETn) repeats of the LTR class of repeat sequences are distinctly visible on virtual RLGS profiles (Oakes et al., 2007). No change in the methylation status of full-length IAP repeats was found by a comparison of real and virtual RLGS profiles. The spots corresponding to these repeat sequences were not observed in any spermatogenic cell type, indicating invariable hypermethylation during spermatogenesis (data not shown).

Discussion

We have examined a wide variety of sequence types to determine the development of DNA methylation patterns during spermatogenesis. Our findings demonstrate that *de novo* and demethylation events occur in a sequence-specific manner. Through the use of several methods, we have shown that sequences which undergo changes in methylation during spermatogenesis include CGI and non-CGI sequences that are found within various positions within known genes or in

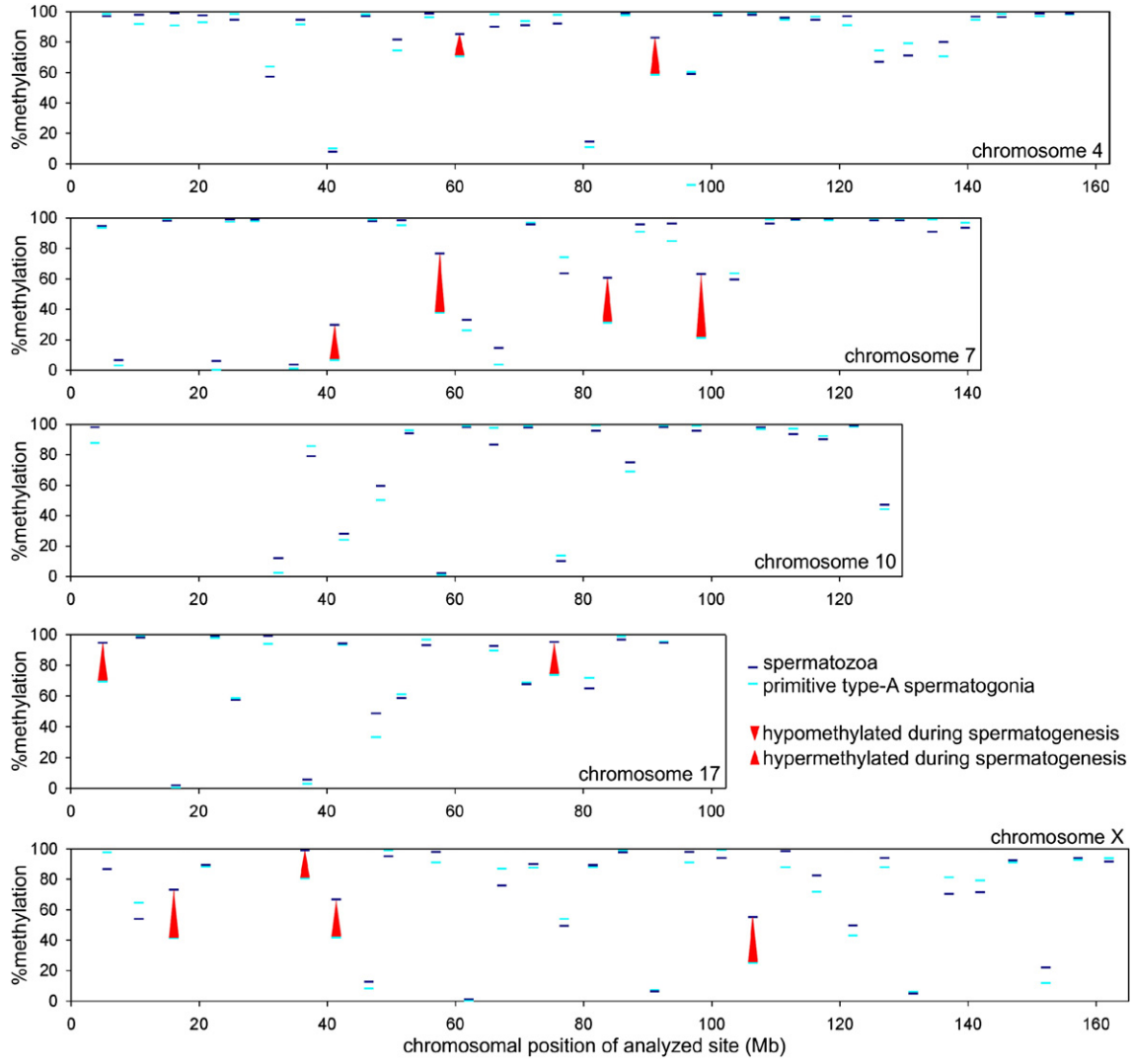


Fig. 6. Chromosome-wide analysis of non-CGI, non-repetitive sequences. Using the qAMP method, *Hha*I and McrBC sites were randomly chosen for examination at approximately 5 Mb intervals across chromosomes 4, 7, 10, 17 and X. Chosen sites were within non-repetitive sequences and >10 kb from a CpG island or the transcriptional start site of a known gene. Percent methylation values for the two digests were averaged to give a single value for primitive type A spermatogonia (light blue dash) or spermatozoa (dark blue dash). Regions that demonstrated a change in methylation during spermatogenesis are indicated (red arrows).

intergenic sequences. These modifications occur in a specific developmental window during spermatogenesis. Both *de novo* and demethylation events occur in the early phases of spermatogenesis, and, regardless of the direction of the change or the sequence type, are complete by the end of the pachytene stage. During spermatogenesis, the reported *de novo* DNA methyltransferase enzymes, DNMT3a and DNMT3b, display their highest levels of expression in spermatogonia (Shima et al., 2004; La Salle and Trasler, 2006) and are probable candidates to facilitate *de novo* methylation events in early germ cell types. Germ cells in the early phases of spermatogenesis undergo frequent DNA replication, thus, demethylation may occur passively. Demethylation does not occur in spermatogenic cell types that are not replicating DNA. Sequences that acquire *de novo* methylation during spermatogenesis are generally non-repetitive. Demethylated sequences are observed in solitary LTR fragments, a category of small, divergent interspersed repeat sequences that are the remnants of transposition events involving full-length LTR repeats. The specific fragments identified are from MaLR and ERVK families of repeats, sequences that have previously been found to be expressed in oocytes and early embryos (Peaston et al., 2004). Although the numbers of identified demethylated sequences are low, it is interesting to observe this dichotomy between the behavior of small repetitive and non-repetitive sequences. This difference is probably confined to a subset of repeat sequence types as no other types of repeats tested demonstrated this behavior.

We also find that DNA methylation changes are only present in a minor proportion of the sequences investigated. Using RLGS, where 75% of all sites examined are within CGIs, only 0.7% (19/2954) of *Not*I sites were observed to change during spermatogenesis. The survey of non-CGI, non-repetitive sequences across five chromosomes revealed a higher 9.6% (12/125) proportion of sequences that were changing during spermatogenesis. A few possibilities exist to explain this

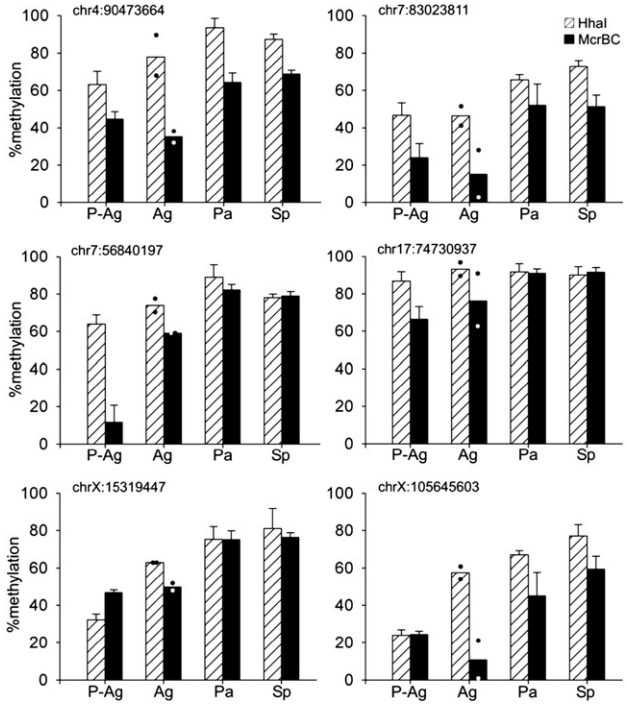


Fig. 7. Detailed analysis of differentially methylated CpGs identified in the chromosome-wide analysis. Percent methylation determined by *Hha*I and McrBC individual restriction enzyme digests is shown for primitive type A (P-Ag) and type A (Ag) spermatogonia, pachytene spermatocytes (Pa) and spermatozoa (Sp). Data are represented as described in the legend of Fig. 3.

discrepancy. Firstly, non-CGI sequences demonstrate a higher level of variability between tissues (Oakes et al., 2007) and a similar phenomenon may be present between developing spermatogenic cell types. Because only a fraction of *Not*I sites in the mouse genome are in non-CGI, non-repetitive sequences (Fazzari and Greally, 2004), fewer changes are expected to be observed using RLGS. Secondly, due to the random nature of spot positions on two-dimensional RLGS profiles, spots displaying altered intensity can be overlapped or obscured by others and would be missed. Thirdly, the qAMP method is more sensitive to small-scale changes (error range ± 5%) (Oakes et al., 2006) than is RLGS. For these reasons, the fraction of loci found to be changed using RLGS would be considered to be an underestimate. However, even with these caveats considered, it remains clear that changes occur only in a limited proportion of the sequences examined.

Some small differences are observed between primitive type A and type A spermatogonia. One explanation is that these differences are developmental, although we cannot exclude the possibility that some of these small differences are representative of the differences in purity levels of these cells. Differences in percent methylation in excess of 15–20% (*Abt1*, *AK137601* in Fig. 3; *Rasgrf1* in Fig. 5; and chr7:56840197 and chrX:105645603 in Fig. 7) support a developmental difference between these cell types because it is more than the maximum amount that could be attributed to somatic contamination. Type A spermatogonia have an average purity of 85%, whereas primitive type A spermatogonia isolated by flow cytometry are more highly purified. Contamination in cell fractions isolated by sedimentation velocity is likely to be Sertoli cells.

Acquisition of DNA methylation during spermatogonial development has been previously demonstrated at the DMRs of paternally methylated imprinted genes (Davis et al., 1999; Li et al., 2004). To compare our approach with previous findings, we investigated the developmental acquisition of methylation at the *H19* DMR. Our results indicate that a low amount of DNA methylation is acquired during the phases up to the pachytene stage in paternally methylated DMRs. Because the *Hha*I restriction enzyme in the *Rasgrf1* amplified region has the most restriction sites of all of the MSREs used, this region is the most sensitive for detecting DNA strands that are incompletely methylated. This particular measurement reveals that complete methylation is achieved by the pachytene stage. Based on the allele-specific differential acquisition of DNA methylation at *H19* (Davis et al., 1999; Li et al., 2004), we predict that unmethylated CpGs are likely to be found on alleles of maternal origin.

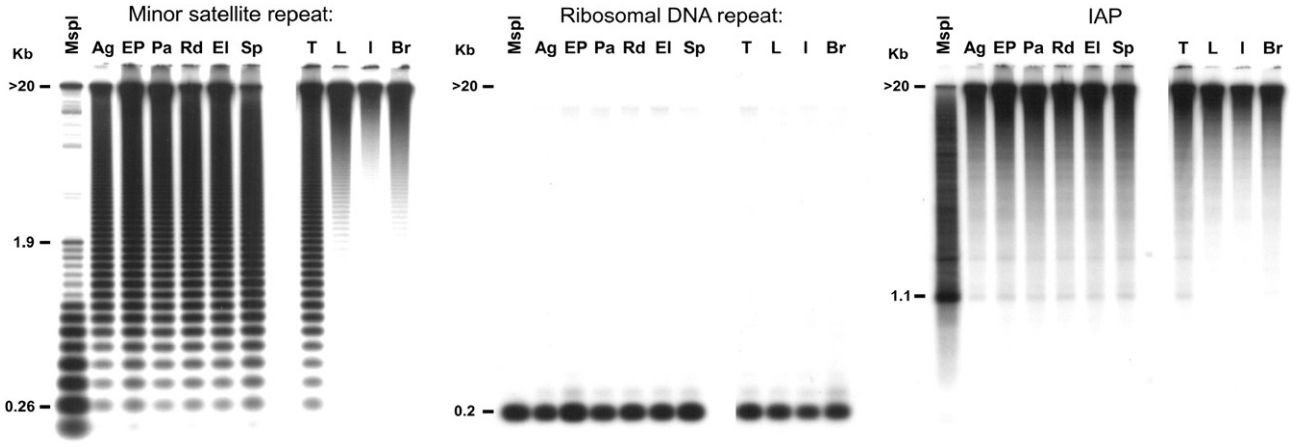


Fig. 8. DNA methylation of repetitive elements during spermatogenesis and in somatic tissue. Genomic DNA was digested with *Msp*I (lane 1) or *Hpa*II (all other lanes) and hybridized to probes specific for the minor satellite, ribosomal DNA and IAP repeats. Each blot was produced by stripping and hybridizing the same membrane to each respective probe. Ag, type A spermatogonia; EP, early pachytene spermatocytes; Pa, pachytene spermatocytes; Rd, round spermatids; El, elongated spermatids; Sp, spermatozoa; T, testis; L, liver; I, intestine; B, brain.

The prevailing view of the primary biological role of DNA methylation involves the promotion of heterochromatin formation in gene promoter regions leading to a transcriptional repression. The global transcriptional profile of individual spermatogenic cell types is highly distinct, especially in the pachytene spermatocyte and round spermatid cell types where a burst of unique transcripts is produced presumably to facilitate meiotic and spermiogenic processes (Shima et al., 2004). Although the expression of a limited number of testis-specific genes has been shown to be correlated with testis-specific hypomethylation of 5′ regions (MacLean and Wilkinson, 2005), a primary role for DNA methylation in the global direct control of spermatogenic cell-type-specific levels of gene expression is not supported by our data. Of 166 genes examined, no 5′ region was shown to be hyper- or hypomethylated in a cell type where expression was repressed or increased, respectively. Furthermore, changes in DNA methylation during spermatogenesis were more commonly found away from the regulatory (5′) regions of genes. These results do not challenge the prevailing view that DNA methylation and gene expression are mechanistically linked, rather they point to potential alternative functions for DNA methylation in germ cells. In addition, there are several explanations that could contribute to the lack of correlation, including RNA stabilization. There is evidence to suggest that changes in DNA methylation play a role in establishing an epigenetic state in the early stages of germ cell development that is permissive for transcription to occur at a later stage (Geyer et al., 2004). It is likely that other regulatory mechanisms, such as transcription factor regulation, are responsible for the variability observed in transcript levels between spermatogenic cell types.

An alternate role for these modifications of DNA methylation is their involvement in the organization of a germ cell-specific chromatin configuration. Alternate roles for DNA methylation have been described and include silencing of repetitive elements and chromatin stability/organization (Bestor and Tycko, 1996). The results of the present study indicate that the bulk of the unique germ cell-specific pattern that is achieved by meiosis has already been established in primitive type A spermatogonia. Thus, one possible explanation is that these changes represent the final modifications that are important for the organization of a specialized, genome-wide chromatin configuration necessary for passage through meiosis. There are a few observations that support this hypothesis: firstly, the majority of modifications are non-5′. The involvement of non-5′ methylation in meiotic chromosomal organization is suggested by the abnormal chromosomal structures in *Dnmt3L*-null spermatocytes (Bourc'his and Bestor, 2004). These germ cells fail to gain normal methylation patterns at interspersed repetitive and intergenic/intronic loci (Bourc'his and Bestor, 2004; Oakes et al., 2007). Secondly, very few changes were observed to occur after meiosis, despite highly dynamic chromatin modulations in spermatid stages. Thirdly, most changes that occur are partial (20–60%) changes, indicating that some methylation has been acquired in prior stages at these sites. Finally, changes are generally restricted to sites with germ cell-specific (non-somatic) methylation states, supporting a connection to the distinct, post-meiotic patterns. An example of this connection is that the three identified loci that are demethylated during spermatogenesis are of the same family of repetitive sequences as 21 other identified RLGS spots, 19 of which are already hypomethylated in germ cells despite being hypermethylated in somatic tissues (Oakes et al., 2007). It is reasonable to believe that the selective demethylation of these repeat sequences during spermatogenesis reflects a requirement for male germ cells to have sequences of this type hypomethylated.

In summary, we find that in addition to the acquisition of DNA methylation that occurs in prenatal male gonocytes, patterns continue to be acquired during spermatogenesis in a sequence-specific manner. These studies raise the possibility that male germ cells may be especially sensitive to potential 'epimutations'; further studies will be required to test if these processes render male germ cells particularly sensitive to environmental influences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.05.002.

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