DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages

Danielle M. Maatouk1, Lori D. Kellam1, Mellissa R. W. Mann2, Hong Lei3, En Li3, Marisa S. Bartolomei2 and James L. Resnick1,*

DNA methylation is necessary for the silencing of endogenous retrotransposons and the maintenance of monoallelic gene expression at imprinted loci and on the X chromosome. Dynamic changes in DNA methylation occur during the initial stages of primordial germ cell development; however, all consequences of this epigenetic reprogramming are not understood. DNA demethylation in postmigratory primordial germ cells coincides with erasure of genomic imprints and reactivation of the inactive X chromosome, as well as ongoing germ cell differentiation events. To investigate a possible role for DNA methylation changes in germ cell differentiation, we have studied several marker genes that initiate expression at this time. Here, we show that the postmigratory germ cell-specific genes Mvh, Dazl and Scp3 are demethylated in germ cells, but not in somatic cells. Premature loss of genomic methylation in Dnmt1 mutant embryos leads to early expression of these genes as well as GCNA1, a widely used germ cell marker. In addition, GCNA1 is ectopically expressed by somatic cells in Dnmt1 mutants. These results provide in vivo evidence that postmigratory germ cell-specific genes are silenced by DNA methylation in both premigratory germ cells and somatic cells. This is the first example of ectopic gene activation in Dnmt1 mutant mice and suggests that dynamic changes in DNA methylation regulate tissue-specific gene expression of a set of primordial germ cell-specific genes.

Key words: Mouse, Primordial germ cells

INTRODUCTION
Primordial germ cells (PGCs) are the founding population of cells that will ultimately give rise to the mature gametes. Unlike organisms that have a mosaic-determined germ line, PGCs in the mouse embryo are specified by an inductive mechanism that requires the presence of several bone morphogenetic proteins (BMPs) emanating from the surrounding somatic cells (Fujiiwara et al., 2001; Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). PGCs can first be detected in the extra-embryonic mesoderm at 7.25 days post-coitus (dpc) (Ginsburg et al., 1990). By 8.5 dpc, PGCs enter the embryo proper and actively migrate through the hindgut endoderm, colonizing the developing gonads by 10.5 and 11.5 dpc. During this time, PGCs proliferate from an initial population of 45 cells at 7.5 dpc to 25,000 cells at 13.5 dpc when proliferation ceases (Tam and Snow, 1981). Sexual differentiation of the germline begins at 13.5 dpc with female germ cells entering prophase I of meiosis. Within the male gonad, a signal thought to originate from the testis cords prevents entry into meiosis and male PGCs enter a mitotic arrest by 14.5 dpc (McLaren, 1983). Prior to these changes, male and female PGCs are sexually indifferent, capable of following either the male or female pathway (McLaren and Southoe, 1997).

Shortly after PGCs enter the urogenital ridges, both male and female germ cells undergo a common set of changes independent of sexual differentiation. Changes in cell morphology and cell-adhesion properties occur as the germ cells transition to a non-migratory state (De Felici et al., 1992; Donovan et al., 1986; Garcia-Castro et al., 1997). Male and female PGCs also cease proliferating, have decreased potential to form pluripotent stem cell lines (Matsui et al., 1992; McLaren, 1984; Resnick et al., 1992), and undergo a wave of apoptosis (Coucouvanis et al., 1993; Wang et al., 1998). These differentiation events are accompanied by changes in gene expression as some germ cell marker genes, such as Tnap (Akp2 – Mouse Genome Informatics) and Zfp148, are downregulated (Donovan et al., 1986; Hahnel et al., 1990; Takeuchi et al., 2003). Other genes, including Mvh (Ddx4 – Mouse Genome Informatics), Scp3 (Scp3 – Mouse Genome Informatics), Dazl, Mageb4 and Genal are upregulated during this time (Cooke et al., 1996; Di Carlo et al., 2000; Fujiwara et al., 1994; Osterlund et al., 2000).

In addition to the differentiation events mentioned above, PGCs mediate two essential epigenetic processes. First, female PGCs reactivate their silenced X chromosome, thereby ensuring that each oocyte carries an active X chromosome (Monk and McLaren, 1981; Tam et al., 1994). Interestingly, the ability to reactivate the inactive X chromosome is not confined to female germ cells, as XXX male germ cells also possess this reactivation capability (Mroz et al., 1999). Second, migratory germ cells carry parent-of-origin-specific imprinting marks and high levels of allele-specific methylation that contribute to monoallelic expression in migratory PGCs. These differentially methylated regions become hypomethylated as PGCs colonize the gonads, leading to a loss of imprinting and biallelic gene expression (Hajkova et al., 2002; Lee et al., 2002; Szabo et al., 2002). However, this wave of demethylation is not restricted to imprinted loci and genes of the X chromosome, as several non-imprinted genes and repetitive sequences also show decreased methylation at this time (Hajkova et al., 2002; Lane et al., 2003; Lees-Murdoch et al., 2003).

1Department of Molecular Genetics and Microbiology, PO Box 100266, University of Florida, Gainesville, FL 32610-0266, USA. 2Howard Hughes Medical Institute and Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA. 3Epigenetics Program, Models of Disease Center, Novartis Institute for Biomedical Research, 250 Massachusetts Avenue, Cambridge, MA 02139, USA.

*Author for correspondence (e-mail: resnick@mgm.ufl.edu)

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We have been investigating regulatory mechanisms underlying postmigratory germ cell differentiation. Several studies suggest that continuing PGC development is regulated by a cell intrinsic program rather than by inductive signals from the gonads. PGCs located in ectopic locations enter meiosis and initiate expression of the postmitotic marker GCNA1 on schedule without exposure to the urogenital ridges (Wang et al., 1997). Embryonic stem cells have been shown to differentiate to form PGC-like cells that can go on to form cells resembling both oocytes and spermatocytes, further demonstrating that PGC differentiation can occur independently of the gonadal environment (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003). Last, cessation of germ cell proliferation has also been suggested to be cell intrinsic (Ohkubo et al., 1996).

We previously tested the potential of premigratory germ cells to differentiate in culture and reported that 8.5 dpc premigratory PGCs in feeder culture can differentiate to express GCNA1 on the correct temporal schedule (Richards et al., 1999). Surprisingly, the rate of differentiation in culture increased when PGCs were exposed to the DNA demethylating agent 5-azacytidine or the histone deacetylase inhibitor trichostatin A (Maatouk and Resnick, 2003). This suggests that epigenetic mechanisms may contribute to the regulation of germ cell differentiation.

Here, we further investigate the role of DNA methylation in the process of PGC differentiation. We present evidence that several postmitotically germ cell-specific genes are demethylated in germ cells as they colonize the genital ridges and that DNA demethylation controls the temporal expression of these genes in vivo. In addition, we show that these postmitotically germ cell-specific genes are ectopically expressed in DNA methyltransferase mutant embryos, suggesting that DNA methylation is a mechanism of silencing germ cell-specific genes in somatic tissues. These results provide the first in vivo evidence of tissue-specific embryonic gene regulation mediated by dynamic changes in DNA methylation.

MATERIALS AND METHODS

Mouse strains

Primordial germ cells were purified from embryos obtained from timed matings of B6C3F1 mice (Jackson Laboratories, Bar Harbor, ME). Noon of the day on which a mating plug was first visible was taken to be 0.5 dpc. For RNA analysis, mice carrying either the Dnmt1<sup>–/–</sup> (Li et al., 1992) or Dnmt1<sup>–/–</sup> allele (Lei et al., 1996) were maintained on the B6(CAST7) mixed background (Mann et al., 2003). Dnmt1<sup>–/–</sup> embryos used for immunostaining were also maintained on a mixed background (129/SvJae × C57BL/6).

Primordial germ cell isolation and purification

Gonads were collected from 10.5 dpc and 13.5 dpc embryos. At 13.5 dpc, embryos were sex segregated based on the presence of testis cords in the male gonad. PGCs were immunomagnetically purified using the TG-1 antibody as described (Pesce and De Felici, 1995). Purified fractions were greater than 85% (10.5 dpc) and 90% (13.5 dpc) germ cells, as judged by alkaline phosphatase staining. Immunodepleted fractions contained less than 1% PGCs and primarily contained somatic cells from the gonad and mesonephros.

Bisulfite conversion and DNA sequencing

Genomic DNA isolated from both purified and immunodepleted fractions was subjected to bisulfite conversion as described (Clark et al., 1994). Bisulfite primers were designed against the converted DNA sequences and are listed in Table 1. PCR amplification was performed on two independent germ cell purifications to avoid inconsistencies that might arise from conducting PCR on small amounts of DNA. PCR products were gel purified using Wizard DNA Clean-up System (Promega) and cloned using the pGEM-T Easy Vector System (Promega). Plasmid sequencing was carried out using ABI Prism BigDye terminator (PerkinElmer) by the Center for Mammalian Genetics DNA Sequence Core.

RNA analysis

RNA was isolated from 9.5 dpc embryos using the HighPure RNA Tissue Kit (Roche Molecular Biochemicals) with minor modifications of the manufacturer’s recommendations. Random-primed cDNA was prepared with Superscript II as recommended (Invitrogen). For RT-PCR, forward and reverse primers were located in separate exons to exclude any bands that might arise from genomic DNA contamination. Primer sequences are listed in Table 1. RT-PCR was performed using Trimplastmer Taq (Eppendorf) and PCR products were run on 2% agarose gels, Southern blotted and hybridized with α-[<sup>32</sup>P]dCTP labeled probes. Probe fragments were generated by gel purification of the RT-PCR products obtained from testis cDNA.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
<th>Region analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mvh-F</td>
<td>TTTGGCTCATATATGATGCCGG</td>
<td>210 bp</td>
</tr>
<tr>
<td>Mvh-R</td>
<td>ACACCTTGTGATCTTGAGAATGACC</td>
<td></td>
</tr>
<tr>
<td>Mage-b4-F</td>
<td>ACGCGAGGTATCTCCGGG</td>
<td>180 bp</td>
</tr>
<tr>
<td>Mage-b4-R</td>
<td>GGCGCTAAGGGGCAACC</td>
<td>335 bp</td>
</tr>
<tr>
<td>Dazl-F</td>
<td>TTCTGCTCCACCTTGGGGT</td>
<td>450 bp</td>
</tr>
<tr>
<td>Dazl-R</td>
<td>CATCTTCTGCACATCCCCAATGTA</td>
<td></td>
</tr>
<tr>
<td>Scp3-F</td>
<td>CCAATCAGCAGAGAGGTTG</td>
<td>250 bp</td>
</tr>
<tr>
<td>Scp3-R</td>
<td>AGCTGGCGCTGTCACACAC</td>
<td>360 bp (-540 to –180)</td>
</tr>
<tr>
<td>Hprt-F</td>
<td>GCTGTTGAAAAGACCTCT</td>
<td>248 bp (-378 to –77)</td>
</tr>
<tr>
<td>Hprt-R</td>
<td>CACAGGACTAGAAAACCTGC</td>
<td>381 bp (-241 to +141)</td>
</tr>
<tr>
<td>B-Daz-F</td>
<td>GAAGAGAGAGAGAACTAGCTGTAGGC</td>
<td></td>
</tr>
<tr>
<td>B-Daz-R</td>
<td>AAAAAACAAAAAAATCTCCCAC</td>
<td></td>
</tr>
<tr>
<td>B-Mvh-F</td>
<td>TGAATGAAATATAATGATGATGATGTT</td>
<td></td>
</tr>
<tr>
<td>B-Mvh-R</td>
<td>AAAATACAAAAAAATATACAAAAAA</td>
<td></td>
</tr>
<tr>
<td>B-Scp3-F</td>
<td>GAATGAGGATTAGTGAAGAAGATGTT</td>
<td></td>
</tr>
<tr>
<td>B-Scp3-R</td>
<td>CCCCCATCTCATACCAACCCAC</td>
<td></td>
</tr>
<tr>
<td>B-Tnap-F</td>
<td>GAAGAGGAAAAACTAGCTGTAGGC</td>
<td>128 bp (-213 to –85)</td>
</tr>
<tr>
<td>B-Tnap-B</td>
<td>AAAAAACAAAAAAATCTCCCAC</td>
<td></td>
</tr>
</tbody>
</table>
Immunohistochemical methods

Embryos were collected at 8.5 and 9.5 dpc and fixed overnight in methanol:dimethyl sulfoxide (4:1) at 4°C. Endogenous peroxidase activity was inactivated by a 2-hour incubation in methanol:dimethyl sulfoxide: 30% hydrogen peroxide (4:1:1) at room temperature. Embryos were stored in 100% methanol at –20°C.

Antibody to GCNA1 was generously provided by George Enders. For GCNA1 staining of paraffin sections, fixed embryos were dehydrated through an ethanol series, cleared in Citrisolv (Fisher) and embedded in paraffin. Cross-sections were cut at 7 μm. Immunostaining for GCNA1 in paraffin sections was performed using the 10D9G11 monoclonal antibody as previously described (Richards et al., 1999). Whole-mount embryos were immunostained using the same procedure with several adjustments. Blocking was performed in PBSMT (PBS, 2% w:v nonfat dry milk, 0.1% Triton X-100) and a horseradish peroxidase (HRP)-conjugated mouse anti-rat IgM (Zymed) secondary antibody was used. Color development was performed using the Liquid DAB (3,3′-diaminobenzidine) Substrate Kit following the manufacturer’s instructions (Zymed). Paraffin sections were stained for SSEA1 immediately after GCNA1 staining as previously described (Richards et al., 1999).

RESULTS

Postmigratory germ cell-specific genes are demethylated as PGCs colonize the urogenital ridges

We have previously shown that initial expression of the postmigratory germ cell marker GCNA1 is controlled by a cell-intrinsic timing mechanism. PGCs removed from 8.5 dpc embryos and plated in feeder culture initiate GCNA1 expression after a 2- to 3-day delay, consistent with their in vivo temporal expression pattern (Richards et al., 1999). Furthermore, we found that the DNA demethylating agent 5-azacytidine accelerates the rate and extent of germ cell differentiation in culture (Maatouk and Resnick, 2003). Because the expression of GCNA1 in cultured PGCs is sensitive to changes in DNA methylation, we explored whether induction of other postmigratory germ cell genes could be regulated by a demethylation event. As the gene encoding GCNA1 remains unknown, we investigated the methylation status of several postmigratory germ cell-specific genes that share a similar expression pattern with GCNA1. Mvh, Scp3 and Dazl are exclusively expressed by both male and female germ cells as they enter the urogenital ridges between 10.5-11.5 dpc (Cooke et al., 1996; Di Carlo et al., 2000; Fujiwara et al., 1994). Fig. 1 shows that the first exon of each of these genes is contained within a CpG island.

To examine the methylation status of these postmigratory germ cell genes, genomic DNA obtained from immunomagnetically purified 10.5 and 13.5 dpc PGCs was subjected to bisulfite sequence conversion. Approximately 20 CpG residues were analyzed for the presence of methylation. For each gene, 10.5 dpc PGCs showed high levels of methylation, but by 13.5 dpc all three genes showed a significant loss of methylation with most clones being completely unmethylated (Fig. 2). No significant differences in methylation were observed between male and female PGCs, consistent with the idea that PGCs are indifferent prior to 12.5 dpc. These results suggest that DNA demethylation of the germline between 10.5 and 12.5 dpc functions not only to reprogram imprinted loci and reactivate the X chromosome, but may contribute to additional postmigratory differentiation events.

Although most clones isolated from the PGC fraction were significantly hypomethylated at 13.5 dpc, some clones retained high levels of methylation. Some of these clones may be explained by the presence of contaminating somatic cells in the purified germ cell preparations, as cells of the immunodepleted fraction remained highly methylated at 13.5 dpc (Fig. 2). However, because 13.5 dpc PGC preparations are routinely more than 90% pure, we favor the explanation that some PGC genomes have not undergone demethylation by 13.5 dpc. Consistent with this interpretation, some germ cells initiate GCNA1 expression by 11.5 dpc, but many germ cells do not express the marker until 14.5 dpc (Enders and May, 1994).

Fig. 2 demonstrates that the germ cell-specific genes Mvh, Dazl and Scp3 exhibit loss of methylation as they first become expressed. In contrast to this pattern of expression, several genes are negatively regulated in PGCs as they differentiate into gonocytes. Tnap is expressed as germ cells are initially allocated in the extra-embryonic mesoderm at 7.25 dpc, but expression is lost between 13.5 and 14.5 dpc (Donovan et al., 1986; Ginsburg et al., 1990). We examined the Tnap locus to explore any potential role of dynamic DNA methylation changes on a gene that is negatively regulated in gonocytes. Bisulfite analysis of eight CpG dinucleotides upstream of exon 1 shows that this region of Tnap is unmethylated at 10.5, 13.5 and 14.5 dpc in both germ cells and somatic cells. This result is consistent with the notion that DNA demethylation occurs in genes that are positively regulated as germ cells transition into gonocytes, rather than a characteristic of all genes expressed in germ cells.

Expression of GCNA1 is sensitive to DNA methylation in primordial germ cells

Primordial germ cells in culture express the postmigratory germ cell marker GCNA1 on an accelerated schedule when exposed to the DNA demethylating agent 5-azacytidine (Maatouk and Resnick, 2003). Although many genes respond to this agent in culture, Walsh and Bestor (Walsh and Bestor, 1999) found that few genes are subject to dynamic DNA methylation changes in vivo. To determine whether GCNA1 expression is regulated by DNA methylation in vivo, we next tested whether GCNA1 is prematurely expressed by PGCs in DNA methyltransferase 1 (Dnmt1) mutant embryos. Dnmt1 maintains methylation patterns during DNA replication and...
the null Dnmt1c mutation results in a 98% loss of genomic methylation (Lei et al., 1996). Dnmt1c/c mutant embryos were cross-sectioned and immunostained for the PGC marker SSEA1 and for the postmigratory germ cell marker GCNA1. GCNA1 is normally not detectable prior to 10.5 dpc (Enders and May, 1994). In 8.5 dpc Dnmt1c/c embryos, PGCs located in the yolk sac endoderm simultaneously expressed GCNA1 and SSEA1, with GCNA1 being detected 2-3 days earlier than expected (Fig. 4B). PGCs in the posterior region of 9.5 dpc embryos also prematurely expressed GCNA1 (Fig. 4C). These results indicate that in vivo expression of GCNA1 is temporally controlled by DNA methylation.

**DNA methylation is a primary silencing mechanism for the postmigratory germ cell marker GCNA1 in somatic cells**

In addition to demonstrating that DNA methylation regulates GCNA1 expression in germ cells, Fig. 4 suggested that some somatic cells also express this germ cell-specific marker when DNA methylation is reduced by mutation of Dnmt1. To further investigate the expression pattern of GCNA1 in Dnmt1 embryos, 9.5 dpc Dnmt1n/n embryos were subjected to whole-mount immunostaining. This hypomorphic mutation leads to a 70% reduction in DNA methylation, compared with the 98% reduction observed in Dnmt1c/c embryos (Lei et al., 1996). Surprisingly, not only was GCNA1 prematurely expressed in PGCs, but ectopic expression was observed in somatic cells scattered throughout the entire embryo (Fig. 5C,D). Because these cells do not express the PGC markers SSEA1 (Fig. 4) or OCT4 (Hattori et al., 2004), we suggest that they are not PGCs that have migrated to aberrant locations.

As only a small number of cells ectopically expressed GCNA1, it seemed likely that the low levels of functional Dnmt1 enzyme present in the hypomorphic Dnmt1n/n mutants might attenuate promiscuous gene activation. To test this idea GCNA1 immunostaining was also performed on 9.5 dpc Dnmt1c/c embryos. The more severe mutation consistently caused much higher levels of ectopic expression than observed in the Dnmt1n/n embryos (Fig. 5F,G). As expected, wild-type and heterozygous embryos at these stages exhibited no GCNA1 expression (Fig. 5A,E). Development of Dnmt1-deficient mutants is frequently retarded such that 9.5 dpc Dnmt1c/c embryos more closely resemble wild-type 8.5 dpc
embryos. Fig. 5H demonstrates that GCNA1 expression is not readily detected in a more closely stage-matched 8.5 dpc Dnmt1+/c embryo. Together, these results indicate that the postmigratory germ cell marker GCNA1 is ectopically expressed both temporally and spatially in embryos lacking a functional Dnmt1 enzyme.

**Premature expression of postmigratory primordial germ cell genes in Dnmt1 mutant embryos**

Several postmigratory germ cell-specific genes are demethylated as the germ cells colonize the developing gonads (Fig. 2). Additionally, GCNA1 is ectopically expressed under conditions of reduced methylation. To determine if additional postmigratory germ cell-specific genes are prematurely expressed in Dnmt1 mutant embryos, RT-PCR analysis was performed to examine the expression profiles of Mvh, Scp3, Dazl and another PGC-specific gene that shares a similar expression pattern, Mageb4 (Osterlund et al., 2000) (Fig. 6). As these genes are expressed only after PGCs enter the developing gonads, little or no expression was detected in wild-type and heterozygous 9.5 dpc embryos, as expected. However, embryos homozygous for either the Dnmt1<sup>-/-</sup> or Dnmt1<sup>-/-</sup> mutation precociously expressed each of the germ cell genes analyzed. In addition, expression seemed to be greater in the more severe Dnmt1<sup>-/-</sup> mutant. These results support the notion that postmigratory PGC gene expression is dependent upon the genome-wide demethylation event that occurs during colonization of the gonads.

**DISCUSSION**

DNA methylation regulates germ cell-specific gene expression

Recent results from several laboratories demonstrate that between 10.5 and 12.5 dpc the germ cell genome undergoes a wave of genomic demethylation that affects genes on the inactive X chromosome, imprinted loci and some repetitive elements (Hajkova et al., 2002; Lane et al., 2003; Lee et al., 2002; Lees-Murdock et al., 2003). Although most CpG islands are unmethylated regardless of tissue or expression status (Ioshikhes and Zhang, 2000; Rollins et al., 2006), we found that several germ cell-specific genes are highly methylated at 10.5 dpc and are included in this wave of germ cell demethylation as they are first expressed (Fig. 2). These genes remain methylated and silent in somatic cells. Loss of methylation, as observed in Dnmt1 mutants, correlates with their premature expression each of the germ cell genes analyzed. In addition, expression seemed to be greater in the more severe Dnmt1<sup>-/-</sup> mutant. These results support the notion that postmigratory PGC gene expression is dependent upon the genome-wide demethylation event that occurs during colonization of the gonads.
expression and ectopic expression of somatic cells. Together, these results strongly suggest that the naturally occurring demethylation of these genes in germ cells is rate limiting for their expression, and that DNA methylation is necessary to maintain silencing of these genes in somatic cells.

The rapid rate of demethylation and the presence of nuclear Dnmt1 protein led Hajkova et al. to propose that germ cell demethylation results from an active mechanism, rather than the passive process of replication without further methylation (Hajkova et al., 2002). Our results are consistent with this proposal as we observed individual PGC genomes having intermediate levels of methylation, probably representing PGCs in the process of being actively demethylated (Fig. 2). Precocious expression of germ cell-specific genes, presumably owing to passive demethylation in Dnmt1-deficient cells in culture, was found to be ectopically expressed in placentas of Dnmt1 mutant embryos (Hattori et al., 2004). Here, we report extensive ectopic expression of germ line-specific genes resulting from loss of DNA methylation.

Expression of postmigratory germ cell genes is attenuated in DNA-deficient mutants

If DNA methylation is necessary to silence germ cell genes in somatic cells, why are only some cells positive for GCNA1 in the Dnmt1-deficient embryos? Although Dnmt1c/c embryos lack detectable Dnmt1 activity, Dnmt1n/n embryos produce low levels of functional Dnmt1 enzyme and retain about 30% of genomic methylation (Lei et al., 1996). The experiments reported here were not performed under directly comparable conditions; however, Mvh, Dazl, Scp3 and Mageb4 all show greater expression in the Dnmt1c/c compared with the Dnmt1n/n mutants relative to the Hprt control (Fig. 6). This was also observed for the expression of GCNA1 in Dnmt1n/n compared with Dnmt1c/c embryos (Fig. 5). Repressive chromatin structure or compensation by de novo DNA methyltransferases may maintain silencing in non-expressing cells. Alternatively, DNA demethylation in mutant embryos, which occurs by a passive replication-dependent mechanism, may occur more slowly in some cells as loss of Dnmt1 may decrease the rate of cell proliferation (Jackson-Grusby et al., 2001; Milutinovic et al., 2003). Slower cell cycles could lengthen the time it takes to passively demethylate, causing delayed gene activation. This may account for the large number of cells observed in the Dnmt1c/c mutant that do not initiate GCNA1 expression.

How could DNA methylation silence germ cell-specific genes in both germ and somatic lineages? Several mechanisms, including restricted expression of positive acting transcription factors, steric interference with transcription factor binding sites, attraction of methyl DNA binding proteins and DNA methylation induced...
changes in histone modifications have been proposed (Jaenisch and Bird, 2003). Interestingly, recent reports suggest that the repressive transcription factor E2F6 is necessary to silence several spermatogenic genes in somatic cells, and that promoters of these genes are hypomethylated in E2F6-deficient cells (Pohlers et al., 2005; Storre et al., 2005). We are currently investigating whether E2F6 and DNA methylation share a common pathway to repress germ cell-specific genes.

**DNA methylation mediated regulation of germ cell development**

DNA methylation has previously been proposed to regulate the expression of tissue-specific genes; however, the lack of substantial in vivo evidence has narrowed the proposed role of methylation to silencing of endogenous retrotransposons and maintaining monoallelic gene expression within imprinted loci and on the inactive X chromosome in females (Jaenisch, 1997; Walsh and Bestor, 1999). Our data provide strong evidence that methylation may indeed control tissue-specific gene expression for a set of germ cell-specific genes that are coordinately activated upon germ cell entry into the gonads.

Seki et al. (Seki et al., 2005) recently investigated genome-wide changes in chromatin modifications during primordial germ cell development. Using antibodies to 5-methylcytosine, they observed that PGCs at the base of the allantois at 8.0 dpc have similar methylation levels as somatic cells; however, migrating PGCs in the hindgut displayed lower methylation levels. This first wave of germ cell demethylation may signify the transition from a somatic cell fate to a more pluripotent state, as germ cells at this stage resemble cells of the inner cell mass in their expression profiles and their ability to give rise to pluripotent stem cell lines (Donovan and de Miguel, 2003; Matsui et al., 1992; Resnick et al., 1992).

Our data suggest that the second wave of demethylation, which temporally coincides with entry into the gonads, controls the expression of several genes required for gametogenesis, as well as contributing to imprint erasure, reactivation of the inactive X chromosome and expression of IAP retrotransposons. Other aspects of PGC differentiation may also be linked to DNA demethylation; however, the lethality of Dnmt1 mutant embryos prior to 10.5 dpc prevents the examination postmitotic germ cell demethylation events. Conditional deletion of Dnmt1 in PGCs might allow for further analysis of other changes that temporally overlap this wave of demethylation.

**Cancer testis antigens**

Efforts to identify cancer-derived gene products as targets for immunotherapy have revealed an association between genes normally expressed only in germ cells, but ectopically activated in tumors. Currently, 89 transcripts grouped into 44 families are recognized as cancer testis (CT) antigens (Scanlan et al., 2004). Boon and colleagues (De Smet et al., 1996; De Smet et al., 2004) have demonstrated lower levels of promoter methylation in tumors expressing the MAGEA1 cancer testis antigen compared with non-expressing cells. Furthermore, MAGEA1 expression could be induced in response to demethylating agents. This led to the suggestion that the loss of DNA methylation that accompanies tumor progression may be responsible for MAGE gene expression.

Kosowski et al. (Kosowski et al., 2004) reported that more than half of CT genes are expressed in premeiotic germ cells and that several could be induced in peripheral blood leukocytes by 5-azacytidine treatment. Similarly we found that several premeiotic germ cell-specific genes are following loss of DNA methylation, including Mageb4, the murine homolog of a human CT antigen. Our data provide direct in vivo evidence that premeiotic gene expression is linked to hypomethylation, and provides a likely explanation for the frequent appearance of germ cell-specific genes in certain tumors.

**Evolution of the germ cell lineage**

Boule and Vasa, the Drosophila homologs of Dazl and Mvh, were originally identified as components of Drosophila germ plasm, and are highly conserved in germ cell development. While organisms with a mosaically determined germ line inherit these gene products as maternal factors, Dazl and Mvh are expressed in postmitotically germ cells in the mouse, 3-4 days after the germ line is specified. Interestingly, divergent mechanisms of germ cell specification operate within the amphibian class. The Xenopus germ line is mosaically determined, while salamanders (Axolotl) specify germ cells by an inductive mechanism similar to mammals (Johnson et al., 2003). As salamanders delay expression of axdazl and axvh until germ cells arrive at the gonad (Bachvarova et al., 2004), it would be interesting to investigate the potential role of methylation in the expression of axdazl and axvh. The observation that methylation were to regulate expression of these genes in salamanders would suggest that control of germ cell differentiation by DNA methylation may be a widely conserved mechanism among species that use inductive signals to specify the germ cell lineage. Additionally, this would suggest that DNA methylation in the germ line initially arose to regulate the timing of germ cell differentiation rather than in epigenetic processes such as genomic imprinting.

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