ORIGINAL RESEARCH

DNA methylation of germ-cell-specific basic helix-loop-helix (HLH) transcription factors, Sohlh2 and Figla during gametogenesis

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ABSTRACT: Regulation of germ-cell-specific transcription is essential for the differentiation and other physiological processes of germ cells. Germ-cell-specific transcription factors, Sohlh2 and Figla, play key roles in gametogenesis. To elucidate whether an epigenetic mechanism is involved in the controlled expression of Sohlh2 and Figla, we examined the dynamics of DNA methylation at two gene loci. Results showed changes in methylation patterns in the promoter regions of both genes during the period of germ cell differentiation, while the methylation patterns in first exons and first introns (near the transcription initiation sites) remained constant. The methylation reprogramming at the cytosine-phosphate-guanine (CpG) locus in the Figla promoters (P1: –812 to –568 bp and P2: –692 to –438 bp) and in the Sohlh2 promoter (sohlh2-P: –202 to 173 bp) was found to correlate with the expression of Figla and Sohlh2 transcripts, respectively. Data therefore suggested that a dynamic DNA CpG methylation in the Sohlh2 and Figla promoters, but not in the intron and exon sequences, is linked to the regulation of gene expressions, even though CpG islands are also present in their introns or exons.

Key words: gametogenesis / DNA methylation / transcription factors / Sohlh2 / Figla

Introduction

In mice, the formation of the primordial germ cells (PGCs) first appears at ~6.25 days post coitum (dpc), with a group of six cells in the proximal epiblast (Saitou et al., 2002; Ohinata et al., 2005). Once the founder number reaches 40 PGCs, cells migrate to reside in extra-embryonic tissues until gastrulation is complete. Subsequently, the cells migrate to the nascent gonads and proliferate at around 10.5–11.5 dpc when they begin the process of erasure of genomic imprints (McLaren, 1983, 2003). Following differentiation of the gonads to testes or ovaries, germ cell sex is determined to be male or female and diagnostic sex-specific imprint gene methylation patterns are established (Saffman and Lasko, 1999). The oogonia stop dividing and enter meiosis at ~13.5 dpc to become oocytes, which are eventually arrested in prophase I. Shortly after 13.5 dpc, female germ cells undergo a series of incomplete cell divisions resulting in clusters called cysts or nests (Pepling and Spradling, 1998). Close to birth, mouse germ cell cysts break down into individual oocytes that become surrounded by somatic pre-granulosa cells to form primordial follicles (Pepling and Spradling, 1998). During the process of cyst formation and subsequent break down, a subset of oocyte-specific genes begin to sequentially express, including factor in the germ line a (Figla), bone morphogenetic protein 15 (Bmp15), growth and differentiation factor 9 (Gdf9), newborn ovary homebox (Nobox), zona pellucida 1–3 (Zp1–3), octamer-binding transcription factor 4 (Oct4, also known as POU5F1) and other genes (Dong et al., 1996; Elvin et al., 2000; Dean, 2002; Varani et al., 2002; Hattori et al., 2004; Rajkovic et al., 2004).

Previous studies have identified many tissue-specific basic helix-loop-helix (bHLH) transcription factors that play critical roles in cell differentiation (Davis et al., 1987; Villares and Cabrera, 1987); however, the full complement of oocyte-specific transcription factors is not yet to known. Mammalian oogenesis and spermatogenesis require germ-specific transcriptional regulators. For example, Tcf/5 is a testis-specific HLH transcription factor that binds calmodin gene promoter, although its function in spermatogenesis is unknown (Siep et al., 2004). Sohlh1 (spermatogenesis and oogenesis-specific bHLH transcription factor 1) is an essential regulator for oogenesis
Table I  The primers used for real-time RT–PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences of primers</th>
<th>Productions (bp)</th>
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<tr>
<td></td>
<td>R: 5′-GGGGAGGAGGAGGTCCTTATAC-3′</td>
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<tr>
<td>Figla</td>
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<td>225 bp</td>
<td>NC_00072</td>
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<td></td>
<td>R: 5′-TGTTAGCTTATCTCGAAAG-3′</td>
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<tr>
<td>Ube1</td>
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<td>Female: 217 bp;</td>
<td>NC_00086</td>
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<td></td>
<td>R: 5′-GGGCTTAAGGTTTGCGGGA-3′</td>
<td>male: 217 and 198 bp</td>
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<tr>
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<td>255 bp</td>
<td>NM_007393</td>
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<tr>
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<td>R: 5′-TGCCCTTAGGTTTGCGGGG-3′</td>
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DNA methylation of Sohlh2 and Figla during gametogenesis

(Ballow et al., 2006b; Pangas et al., 2006; Pangas and Rajkovic, 2006). Sohlh2 (spermatogenesis and oogenesis-specific HLH transcription factor) shares 84 and 50% identity with the rat and human ortholog, respectively (Ballow et al., 2006a; b; Choi et al., 2008). In a previous study, the mRNA of Sohlh2 was detected as early as 13.5 dpc, and the SOHLH2 protein was first detected at 17.5 dpc in the ovaries and abundantly expressed in germ cell clusters and primordial follicles of the newborn ovaries (Ballow et al., 2006b). Figla encodes a 219 amino acid protein that contains a bHLH domain. Figla binds as a heterodimer with E12 to the E-box in the promoter region of all three zona pellucida genes (ZP1, ZP2, ZP3) and has the ability to transactivate reporter gene constructs in vitro.

Previous studies suggested that methylation occurs almost exclusively on the cytosine-phosphate-guanine (CpG) dinucleotide in DNA and shows no preference for sequence context (Lees-Murdock and Walsh, 2008). Methylation of CpGs is associated with repression of transcription. In addition to direct transcriptional silencing, DNA methylation is important for suppression of chromosome recombination and histone activity (Csankovszki et al., 2001; Stewart et al., 2005), and resetting this information is therefore necessary for the maintenance of genomic stability. In the mouse germ line, as part of the normal course of development, embryonic germ cells are dynamically reprogrammed during germ cell migration and differentiation, and it is a characteristic of the PGCs to show monoallelic expression of imprinted genes throughout the lifetime of an organism (Sapienza et al., 1987; Panning and Jaenisch, 1996; Kass et al., 1997; Razin, 1998). Since the imprinting control region of imprinted genes is demethylated in post-migratory germ cells and imprinted expression patterns must be maintained for normal mammalian development (McGrath and Solter, 1984; Surani et al., 1984), de novo methylation subsequently occurs in the maturing gametes to reset the imprint, which ensures correct dosage compensation in the next generation. Although the full complement of oocyte-specific transcription factors is unknown, more and more studies indicated that transcription of those genes involved in gametogenesis and maturity is not independent of methylation; that those genes become passively methylated and demethylated during the gamete differentiation processes (for example, the postmigratory germ-cell-specific genes Mvh, Dazl and Syce3 are demethylated in germ cells as they colonize the genital ridges); and that DNA demethylation controls the temporal expression of these genes in vivo (Maatouk et al., 2006). Furthermore, H1FOO is the predominant linker histone found in mouse oocytes, expressed beyond the germinal vesicle stage in polar bodies as well as in the early zygote. Analysis on the dynamics of DNA methylation at the H1FOO locus in germ and somatic cells revealed a tissue-dependent and differentially methylated region (T-DMR) upstream of the H1FOO gene; this region was hypermethylated in sperm, somatic cells and stem cell lines, but specifically unmethylated in the ovulated oocytes (Maeda et al., 2008); moreover, DNA methylation may play a role in the permanent shutdown of promoter 2-directed CYP19A1 expression in large lutein cells (Vanselow et al., 2010).

Data from previous studies and our own observations led us to hypothesize that methylation reprogramming may be involved in the up and down-regulations of Sohlh2 and Figla, and the complete shutdown of their expressions after female germ cell maturation (Newell-Price et al., 2000; Nojima et al., 2004). In this study, we attempt to elucidate the involvement of DNA methylation in the oocyte-specific expression of Figla and Sohlh2. We analyzed germ cells of different developmental stages and found that the Figla and Sohlh2 loci display distinct methylation patterns specific to the female germ line.

Materials and Methods

Animal breeding and isolation of germ cells

All procedures described in the study were reviewed and approved by the Ethical Committee of Qingdao Agricultural University. CD1 mice, a widely used experimental strain, were used for all experiments. Gonads were isolated from 11.5 to 13.5 dpc mouse embryos and PGCs were immunomagnetically purified by using the monoclonal antibody SSEA-1 (MILLIPORE Cat#MAB4301 Temecula, CA, USA) according to the manufacturer’s instructions. Techniques for dissecting PGC-containing regions from the embryonic gonads according to embryonic age and have been described previously by De Felic group (Pesce and De Felici, 1995; De Felici, 2000). Briefly, urogenital ridges (11.5 dpc) or gonads (12.5–13.5 dpc or until birth) were dissected from mouse embryos, the sex of 11.5 dpc gonads were distinguished by using Ube1 PCR (primers of Ube1 in Table I) (Imai et al., 1992; Chuma and Nakatsuji, 2001) and 12.5 dpc embryos could be distinguished by sex segregated based on the presence of testis cords in the male gonad. The ovaries were collected from 3, 5, 7, 14 to 21 dpp mice, respectively and oocytes were obtained by digesting the ovaries with parenzyme and collagenase mixture in a 37°C incubator for 8 min. The cells were collected and stored at −80°C for the following experiments.

Germ cells from 15.5 to 17.5 dpc gonad ridges were purified by the adherent culture method: the gonads were first dispersed as single cells, briefly, pairs of 15.5 or 17.5 dpc gonads were dispersed with 0.25% trypsin (Gibco-BRL), 0.2% collagenase IV (Gibco-BRL) plus 0.02%...
DNA isolation, bisulfite modification, amplification and sequencing

DNA was isolated from oocytes by using a micro DNA isolation kit (Tiangen) according to the manufacturer’s instructions. Briefly, nucleic acid released from oocytes was adsorbed on a Pellecisil column, and cell lysate was passed through the absorption column at 13 400g for 10 min supplied by a high speed centrifuge. After several washing cycles, DNA was dissolved into nuclease-free water and collected into a collecting duct by centrifugation at 13 400g for 5 min. In each sample, at least 300–500 oocytes were prepared for handling. The isolated DNA was treated with sodium bisulfite by use a MethylampTM DNA modification kit (Epigentek, USA) according to the manufacturer’s instructions. The bisulfite-treated DNA was amplified by nested (semi-nested) PCR for different regions of Sohlh2 and Figla genes with primers and PCR conditions described (Table II). The PCR products were separated by electrophoresis in 1% agarose gel, and correct bands were isolated from the gel and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Then the purified DNA was cloned into a PMD18-T Vector (Takara) according to the manufacturer’s instructions. The positive clones were obtained by antibiotic selection and the insert was sequenced at GeneScript (Nanjing, China). Generally, about 10 clones were chosen for each sample.

Statistical analysis

The percentage of methylation for each sample was calculated from the number of methylated CpG (black circles in figures) divided by total CpG loci (all circles in figures). Relative gene expression level for each sample was normalized with that of β-actin and plotted. Following arcsine transformation, percentage of DNA methylation in oocytes was analyzed by analysis of variance, and differences were located with the Tukey’s test. All analyses were carried out with the Statistical Analysis System.

Table II The primers used for amplification of gene by PCR.

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<td>R: 5′-CCCAACCTTTAATAAACACCT-3′</td>
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<td>Figla-P2</td>
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<td>375 bp</td>
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<td>Figla-MLH</td>
<td>F: 5′-TTGATTAGTTAGTATGTTGG-3′</td>
<td>255 bp</td>
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<td>R: 5′-AAATAAAACACAAAAACTAACAC-3′</td>
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<td>Sohlh2-P</td>
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<td>R: 5′-CCCAACCTTTAATAAACACCT-3′</td>
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In silico screening for transcription factors binding sites and CpG islands

The genes were screened for the presence of CpG islands using the EMBOSSCpGPlot/CpGReport/Isochore online tool (http://www.ebi.ac.uk/Tools/emboss/cpgraphplot/index.html) with default settings (window: 100; step: 1; observed to expected ratio of C plus G: 0.6; minimum average percentage of G plus C:50; minimum length of reported CpG island: 200 bp) and online tool MethPrimer (http://www.urogene.org/methprimer/index1.html) with default settings (window: 100; shift:1; Obs/Exp: 0.6; GC%: 50%).
Results

Dissociated germ cells from 11.5 to 17.5 dpc gonad ridges

Mouse germ cells dissociated from 11.5 to 13.5 dpc gonads were collected and purified following the miniMACS separation protocol (Pesce and De Felici, 1995). Alkaline phosphatase staining revealed that >95% of the germ cells were present in the eluted fraction from disaggregation of germ-cell-containing tissues (Supplementary data, Fig. S1A and C). STAT3 immune fluorescent staining detected dissociated germ cells from 15.5 to 17.5 dpc gonad ridges, indicating an embryonic age dependency. The recovery rates of positive cells purified by the adherent culture method were >92% (Supplementary data, Fig. S1B and C).

Cell-type-dependent DNA methylation of the Figl2 gene

With the online tools of Methprimer (http://www.urogene.org/methprimer/), TFSearch (http://mbs.cbcrc.jp/research/db/TFSEARCH.html) and p-match (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi), the promoter region of Figl2 was characterized. The first exon, first intron, GC-rich sites and putative transcription factor binding sites were identified. Results showed that more CpG loci and transcription factor binding sites were present next to the transcription initiation point. Three regions of Figl2 sequence—Figl2-P1 (−812 to −568 bp), Figl2-P2 (−692 to −438 bp) and Figl2-HLH (+120 to +461 bp)—were then selected for cloning (Figs. 1A, 2A, Supplementary data, Fig. S2A). Meanwhile, genomic DNAs from germ cell were treated with bisulfite and subjected to sequence analysis.

Results showed that both Figl2-P1 and Figl2-P2 are located in the promoter region of Figl2, and they encompass a total of 5 CpG dinucleotides and 8 CpG dinucleotides, respectively. And there is a duplicate sequence (−692 to 568 bp) between the Figl2-P1 (−812 to −568 bp) and Figl2-P2 (−692 to −438 bp), which encompass two CpGs dinucleotides, through the identification of transcription factor binding sites and CpG regions of Figl2-P1 and Figl2-P2 sequences. Sequencing of individual bisulfite-converted genomic DNAs revealed that the CpG loci of Figl2-P1 and Figl2-P2 were mostly highly methylated from 11.5 to 17.5 dpc (Figs. 1B and 2B). In contrast, the rate of methylation decreased significantly (down to 22.8% for Figl2-P1 and 25.92% for Figl2-P2) during the period when germ cell cyst began to breakdown and primordial follicle began to form (0 dpp). This hypomethylation status remained low at 7 days post birth, and it reached as high as 70% for Figl2-P1 and 65.625% for Figl2-P2 at 14 dpp, the methylation rate was 80% for Figl2-P1 and 79.68% for Figl2-P2; at 21 days after birth, it remained high (78.13%) for Figl2-P2 (Figs. 1B and 2B).

A CpG island was found during the screening of the Figl2-HLH sequence, a 341 bp fragment downstream of the transcription initiation site of the Figl2 gene. Several transcription factors, such as NRE-2, GATA-1 and ADR-1, are contained in the Figl2-HLH sequences. Data further showed that CpGs of Figl2-HLH maintained the hypermethylation status from 11.5 dpc until 5 days after birth regardless of the differentiation status of female germ cells. Similar hypermethylation status was observed in male germ lines (Supplementary data, Fig. S2).

Cell-type-dependent DNA methylation of the Sohlh2 gene

A 600 bp fragment (from −202 to +173) upstream of the putative transcription initiation codon of Sohlh2 and the first intron of Sohlh2 (180–534 bp) were chosen for methylation analysis. These two fragments were named Sohlh2-P and Sohlh2-I, respectively (Figs. 3A and 4A). Analysis of putative transcription factor binding sites and GC rich regions containing the CpG pattern indicated that the Sohlh2-P fragment is highly concentrated with transcription factor binding sites (such as HSF, ADR-1, SP1, Nkx-2 and others). The data also revealed a CpG island in the core promoter region (Fig. 3A). Overall, data suggested that epigenetic changes in CpG sites of the Sohlh2-P sequence may have a strong correlation with the regulation of Sohlh2 gene expression.

To examine the changes of Sohlh2-P DNA methylation status during female germ cell differentiation, genomic DNAs from germ cells were collected at different stages and treated with bisulfite before subjecting to sequence analysis. Sohlh2-P encompasses a total of 18 CpG dinucleotides, from −202 bp upstream of the translation initiation site to the +173 bp. Sequencing of individual bisulfite-converted genomic DNAs revealed that CpG loci of Sohlh2-P were mostly hypermethylated in undifferentiated PGCs (11.5 dpc, 83.33%, Fig. 3B). At 13.5 dpc, the rate of methylation showed a significant decrease, down to 47.22% (Fig. 3B). In contrast, the CpG loci were almost completely demethylated after birth. This hypomethylation status continued at 5 dpp (4.17%, Fig. 3B) and at 7 dpp (0%, Fig. 3B). However, at 12 days after birth, the rate of methylation came back to reach a high level of 52.08%, indicating that the Sohlh2 promoter has undergone a de novo DNA methylation after the differentiation of germ cells (Fig. 3B).

When the same method was used to analyze the other CpG rich regions in the first intron, we counted 19 CpG dinucleotides in Sohlh2-I. In addition, our study indicated that there was a 144 bp CpG island (259–402 bp) in this region, as well as many transcription factor binding sites (such as HSF, SRY, HOXA3 etc.). Surprisingly, results also showed that the CpGs of Sohlh2-I remained hypermethylated regardless of the differentiation status of female germ cells, while interestingly and in contrast, the CpGs in male germ cells were maintained with low methylation (Fig. 4B).

Regulation of gene expression via DNA methylation in Sohlh2 and Figl2 genes

To elucidate the timing and specificity of Figl2 and Sohlh2 transcription, expression analyses of germ cells were performed. Using β-actin mRNA as a load control and for assessment of RNA integrity, the mRNA expression of Figl2 and Sohlh2 was detected in germ cells. Figl2 and Sohlh2 were not detected in liver tissues or other somatic tissues (data not shown), thereby confirming germ-cell-specific expression (Liang et al., 1997; Soyal et al., 2000). The Figl2 transcripts in females were preferentially expressed in ovaries (Fig. 5A). Figl2 transcripts were barely detectable in the female urogenital ridges at 11.5, 13.5 and 15.5 dpc. Low quantities of Figl2 mRNA were detected at ~17.5 dpc, and it continued to rise until peaked shortly after birth. At 3 days after birth, there was still considerable transcript expression.
although the level decreased slightly compared with at birth. Then Figlα mRNA level showed a marked decrease at 5 dpp, and at 7 days after birth its level was barely detectable. The expression of Sohlh2 transcripts in female urogenital ridges was barely detected at 11.5 dpc. Low levels of Sohlh2 transcripts were first observed between 13.5 and 17.5 dpc. The transcript abundance markedly increased at birth and peaked approximately at 3 dpp (Fig. 5B). However, the Sohlh2 mRNA level was noticeably decreased at 5 dpp, and at 7 days after birth it was at the baseline level (Fig. 5B). The methylation reprogramming of CpG loci in the Figlα promoter (−812 to −438 bp) was associated with the expression of Figlα transcripts. In Sohlh2, a similar CpG locus is present at −202 to +173 bp. In conclusion, dynamic changes of CpG methylation status in the promoter regions of Sohlh2 and Figlα, but not in the intron or exon sequences are linked to the regulation of gene expression.

**Discussion**

The bHLH transcriptional factors, especially those that are tissue-specific, play important roles in cellular differentiation during various
developmental stages of organogenesis (Murre et al., 1989; Lee et al., 1995; Massari and Murre, 2000). However, few bHLH transcription factors are known to be involved in germ cell development. Recently, a spermatogenesis- and oogenesis-specific bHLH transcription factor, Sohlh2, was reported to be essential for both spermatogenesis (Hao et al., 2008; Toyoda et al., 2009) and oogenesis (Choi et al., 2008).

The expression pattern of Sohlh2 mimics that of Sohlh1, which is preferentially expressed in oocytes and required for oogenesis, and Sohlh1 disruption perturbs follicular formation in part by causing down regulation of three critical regulators of oogenesis: Nobox, Figla and Lhx8 (Lim Homeobox Gene) (Suzumori et al., 2002; Rajkovic et al., 2004; Pangas et al., 2006). SOHLH2 deficiency accelerates post-natal oocyte loss in the ovary and causes infertility in female mice. In addition, SOHLH2 deficiency affects the expression of numerous oocyte-specific genes in the ovary, including Sohlh1, Nobox, Figla, Gdf9, Pou5f1, Zpf1, Zpf3, Kit, Ospx1, Nlpl1, H1fao and Stra8. Furthermore, Sohlh1 and Sohlh2 are independently required for successful oocyte differentiation (Choi et al., 2008). However, later research did not support this view, at least in the male reproductive system, because the expression pattern of Sohlh2 in the testis is also similar.

Figure 2 Methylation dynamics of Figla-P2 during the period of germ cell differentiation in CD1 mice. (Aa). Transcription factor binding sites and CpG-pattern rich regions. The red horizontal line represents the input sequence. Blue vertical bars stand for putative transcription factors that bind to specific loci. The red vertical lines represent the positions of the 8 CpG sites within the 255 bp fragment. (b) Detailed Figla-P2 sequence from the −692 to −335 bp locus. The top and bottom sequences correspond to the bisulfite sequencing result and the original sequence, respectively. The black horizontal lines indicate the location of primers. Black boxes indicate the distribution of the CpG site in the sequence. (B) Comparison of Figla-P2 methylation levels between germ cells and somatic cells. Each line represents an individually sequenced clone and each circle represents a CpG residue. White and black circles stand for unmethylated and methylated cytosines, respectively (F, female; M, male; L, livers).
to that of Sohlh1, which is expressed in spermatogonia (Ballow et al., 2006a, b). Moreover, by providing a crucial checkpoint to optimize the numbers of spermatocytes entering meiosis during each cycle of spermatogenesis, Sohlh2 is required for progression of differentiating type A spermatogonia into type B spermatogonia (Hao et al., 2008). A subsequent study indicated that Sohlh2 gene may coordinate with the Sohlh1 gene to promote the differentiation of KIT+ germ cells in vivo. By over-expressing both Sohlh2 and Sohlh1 in HEK293 cells and subsequently examining their association, it was found that FLAG-SOHLH1 co-immunoprecipitated with SOHLH2, while the anti-SOHLH2 antibody did not cross-react with the SOHLH1 protein (Toyoda et al., 2009). Further, bHLH proteins with similar amino acid sequences, such as HANDs (Firulli et al., 2003), can form heterodimers. All these studies suggested that Sohlh2 and Sohlh1 may dimerize in vivo to form a network that regulates early spermatogenesis and oogenesis. Figl1a is germ-cell-specific, with RNA expression detectable in both male and female gonads (Liang et al., 1997), and it is critical in the early development of oocyte, but no phenotype was observed in

Figure 3  Methylation dynamics of Sohlh2-P during the period of germ cell differentiation in CD1 mice. (Aa) Transcription factor binding sites and CpG-pattern rich regions. The red horizontal line stands for the input sequence. Blue vertical bars represent transcription factors that probably bind to specific gene loci. The red vertical lines indicate the positions of the 18 CpG sites within a 375 bp fragment. (Ab) Detailed Sohlh2-P sequence from the −202 to +173 bp locus. The top and bottom sequences correspond to bisulfite sequencing and original sequence, respectively. Black horizontal lines indicate the location of primers. Black boxes indicate the distribution of the CpG sites in the sequence. (B) Comparison of Sohlh2-P methylation levels between germ cells and somatic cells. Each line represents an individually sequenced clone and each circle represents a CpG residue. White and black circles stand for unmethylated and methylated cytosines, respectively (F, female; M, male; L, livers).
Figure 4 Methylation dynamics of Sohlh2-I during the period of germ cell differentiation in CD1 mice. (A) Transcription factor binding sites and CpG-pattern rich regions. The red horizontal line represents the input sequence. Blue vertical bars represent putative transcription factors that bind to specific gene loci. The red vertical lines indicate the positions of the 19 CpG sites within the 354 bp fragment. The pink arrows represent the actual amplification sequence. (b) Detailed sohlh2-I sequence from the +180 to +534 bp locus. The top and bottom sequences correspond to bisulfite sequencing and the original sequence respectively. The black horizontal lines represent the location of primers. The black box indicates the distribution of CpG sites in the sequence. (B) Comparison of Sohlh2-I methylation levels between germ cells and somatic cells. Each line represents an individually sequenced clone and circles represent CpG residues. White and black circles stand for unmethylated and methylated cytosines, respectively (F, female; M, male; L, livers).
male mice that lack Figlα (Soyal et al., 2000). Although the promoter regions of all three mouse zona genes (Zp1–3) have little sequence homology, a canonical E-box (CANNTG) has been identified ~200 bases upstream of their transcription start sites (Millar et al., 1991; Epifano et al., 1995). E-box is a known binding site for bHLH transcription factors (Murre et al., 1989), and Figlα may co-operate with E12 to bind the E-box upstream of Zp genes to regulate their expressions (Liang et al., 1997).

DNA methylation is a principal epigenetic mechanism underlying gene regulation and normal development of mammals, which is often associated with the stable repression of certain genes (Panning and Jaenisch, 1996; Razin, 1998; Ng and Bird, 1999; Csankovszki et al., 2001; Jackson et al., 2002). During germ cell migration and differentiation, embryonic germ cells are dynamically reprogrammed. DNA methylation is believed to be of particular importance in maintaining the genetic integrity of germ cells due to its unique role in bridging successive generations (Walsh et al., 1998; La Salle et al., 2004; van der Heijden and Bortvin, 2009). Previous studies found that CpG islands are discrete CpG rich regions present in the promoters of 50–70% of human genes (Bird, 1986), and there may be an association between the widespread CpG loci in promoters and the regulation of gene expression. Previous reports from several laboratories demonstrated that between 10.5 and 12.5 dpc, the germ cell genome undergoes a wave of methylation that affects genes on the inactive X chromosome, imprinted loci and some repetitive elements (Hajkova et al., 2002; Lee et al., 2002; Lees-Murdock et al., 2003). Our study indicated that demethylation reprogramming does occur in genes of Figlα and Sohlh2 at their specific promoter loci simultaneously during gametogenesis. Using the online tool, we screened the promoter regions of Sohlh2 and Figlα for the presence of CpG rich regions or CpG islands.

We found no typical CpG island in the Figlα promoter but one is present in its first exon, and typical CpG islands are present in both the promoter region and the first intron of Sohlh2. In our study, Figlα transcription was barely detectable in the female urogenital ridges at 11.5 and 13.5 dpc, just when shortly after the onset of sexual dimorphism of the gonads and following female germ cells begin to enter into the prophase of meiosis I, then low levels of Figlα transcripts were first detected at 15.5 dpc, the mRNA level began to increase and peaked approximately at birth, and the expression level was kept high at 3 dpp. These findings match the results from several previous laboratory studies, which also indicated that Figlα transcripts were clearly restricted to oocytes within the ovary and they are absent in male gonads. Previous studies and our own finding also demonstrated that the mRNA of Figlα began to increase at 17.5 dpc and subsequently peaked around birth (Liang et al., 1997; Soyal et al., 2000), and a rapid demethylation of Figlα-P1 and Figlα-P2 was accompanied with increase of Figlα gene expression, suggesting that DNA methylation and gene silencing of Figlα is at least not a mutually independent event (Herman et al., 1994; Gonzalez-Zulueta et al., 1995). The Figlα mRNA levels gradually decreased from 3 dpp but still maintain at a relatively high level, and at 7 days after birth, the level of transcripts became very low; meanwhile the epigenetic reprogramming of Figlα-P1 and Figlα-P2 at 3 dpp (P1: 20%; P2: 34.42%) and 5 dpp (P1: 17.5%) were still kept at the low methylation status. It is possible that low methylation of promoter-specific regions (Figlα-P1 and Figlα-P2) is necessary to keep the Figlα expression in moderation. These results strongly suggest that the naturally occurring demethylation at specific loci of the Figlα promoter is rate limiting for its expression in germ cells, and that DNA methylation is necessary to maintain the gene silencing of Figlα expression, while demethylation is required to release the gene.

**Figure 5** Correlations between gene expression and promoter methylation status of Figlα (A) and Sohlh2 (B) in female cells. The methylation levels (%) against relative gene expression of female germ cells and replicates were determined by regression analysis and plotted using SigmaPlot8.0.
expression. In our study, the Sohlh2 transcription was initiated before the primordial follicle stage, although Sohlh2 transcripts were barely detectable in female urogenital ridges at 11.5 dpc and at low levels at 13.5 dpc shortly after the onset of sexual dimorphism of the gonads, when female germ cells began to enter the prophase of meiosis I, the mRNA level started to increase until it peaked dramatically at birth and at ~3 dpp. Previous studies and our finding also showed that the mRNA emergence of Sohlh2 and demethylation of Sohlh2-P occurred simultaneously, with the demethylation Sohlh2-P being almost complete at ~3 dpp (Ballow et al., 2006; Toyoda et al., 2009). Interestingly, the demethylation of Sohlh2-P continued to occur rapidly, while the gene transcription of Sohlh2 also decreased markedly at 3 dpp. At 7 days after birth, the level of Sohlh2 transcripts was already very low. Nevertheless, the epigenetic reprogramming pattern of Sohlh2-P strongly suggested that the naturally occurring demethylation step is the rate-limiting factor for the gene expression of Sohlh2, and that DNA methylation is necessary to maintain gene silencing of Sohlh2, while demethylation is required to release the gene expression in germ cells.

Figla-HLH is located at the first exon (and partially in the first intron) region of Figla. Figla-HLH encompasses a total of 16 CpG dinucleotides with a classic CpG island. Although in female germ cells, the rate of CpG methylation progressively increases as mouse ages, DNA methylation analyses of fetal germ cells revealed that Figla-HLH kept highly methylated in females from 11.5 dpc until 5 dpp, and the same high methylation were found in male germ cells at both 0 and 3 dpp; Figla-HLH is also highly methylated in somatic cells, suggesting that Figla-HLH is not correlated with gene expression in comparison with Figla-P1 and Figla-P2.

Sohlh2-I is located at the first intron region of Sohlh2 and it encompasses a total of 19 CpG dinucleotides. Our study indicated that there is a 144 bp length CpG island (from 259 to 402 bp) in this region. Intriguingly, we found that Sohlh2-I is a T-DMR. The DNA methylation analyses of fetal germ cells revealed the hypermethylation of Sohlh2-I in females of 11.5 dpc–7 dpp (Ballow et al., 2006b; Toyoda et al., 2009). Interestingly, we found that Sohlh2-I is not correlated with gene expression in comparison with Figla-P1 and Figla-P2.

However, further studies are still needed to illuminate the relationship between gene silencing and methylation, and other potential epigenetic modification also need to be analyzed to establish the full extent of epigenetic regulation.

### Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

### Authors’ roles
B.P., H.C., B.C. and L.Z. acquisition of data; L.L. and X.S. played a role in analysis of data; W.S. was involved in conception and design.

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### References


Maatouk DM, Kellam LD, Mann MR, Lei H, Li E, Bartolomei MS, Resnick JL. DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages. Development 2006;133:3411–3418.


van der Heijden GW, Bortvin A. Transient relaxation of transposon silencing at the onset of mammalian meiosis. Epigenetics 2009;4:76–79.

Vanselow J, Spitschak M, Nimz M, Furbass R. DNA methylation is not involved in preovulatory down-regulation of CYP11A1, HSD3B1, and CYP19A1 in bovine follicles but may have a role in permanent