Expression of DNA methyltransferases in the mouse uterus during early pregnancy and susceptibility to dietary folate deficiency

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Abstract

We have characterized the uterine expression of DNA methyltransferases (DNMTs) during early pregnancy in mice and determined whether a folate-deficient diet (FDD) can affect DNMTs in this context. Within endometrial cells, expressions of DNMT (cytosine-5) 1 (*Dnmt1*), *Dnmt3a*, and *Dnmt3b* were significantly elevated during the prereceptive phase of pregnancy but generally returned to baseline levels during receptive and postimplantation periods. As such, the transcription of DNMT genes is temporally regulated during early pregnancy. When comparisons were made between implantation sites (IS) and inter-IS on day 5 of pregnancy, lower levels of *Dnmt3a* were detected at IS. Comparisons between IS and inter-IS did not reveal significant expression differences for other DNMT genes. When tissue sections were examined, DNMT3A was specifically lower in the stroma of IS. Reduced DNMT1 and DNMT3B levels were also observed in the luminal and glandular epithelia of IS, whereas no obvious differences in the stroma were detected. In pseudo-pregnant mice subjected to a FDD, levels of *Dnmt1* and *Dnmt3a* (but not *Dnmt3b*) were significantly upregulated in endometrial tissues, as compared with controls. When tissues from these folate-deficient mice were examined, DNMT1 levels were elevated in both the luminal and glandular epithelia, whereas DNMT3A was upregulated in the luminal epithelium and the stroma. A slight increase in DNMT3B levels was detected in the glandular epithelium. These results indicate that DNMTs may regulate the transcription of endometrial genes associated with embryo implantation and that levels of DNMTs are affected by dietary folate in mice.

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Introduction

The methylation of the cytosine guanine dinucleotide (CpG) is an epigenetic modification that silences gene transcription and plays a critical role in cancer development and progression (Issa *et al.* 1994, Laird & Jaenisch 1996). Three DNA methyltransferase (DNMT) enzymes catalyze DNA methylation. DNMT (cytosine-5) 1 (DNMT1) maintains methylation patterns during DNA replication, whereas DNMT3A and DNMT3B catalyze *de novo* DNA methylation (Okano *et al.* 1999, Robert *et al.* 2003). Altered levels of DNMTs can cause hyper- and hypo-methylation, which are associated with down-regulating and reactivating gene expression, respectively (Baylin *et al.* 1991, Kanai & Hirohashi 2007, Morey Kinney *et al.* 2008).

Changes to the pattern of DNA methylation may functionally alter endometrial cells (Munro *et al.* 2010). For example, DNA methylation of the estrogen receptor 2 (ESR2) promoter downregulates ESR2 levels (both mRNA and protein) in endometrial stromal cells (Xue *et al.* 2007). The CpG-rich region within the homeobox A10

promoter is hypermethylated in the endometria of women who have intrauterine devices (Lu *et al.* 2010). Both prolactin and insulin-like growth factor-binding protein 1 are markers for decidualization and both are upregulated in human endometrial stromal cells after treatment with a demethylation agent (5'-aza-2'-deoxycytidine) (Logan *et al.* 2010). E-cadherin is regulated by DNA methylation during the establishment of a receptive endometrium *in vitro* (Rahnama *et al.* 2009). Finally, expression levels of DNMT1, DNMT3A, and DNMT3B are altered during the female menstrual cycle and are regulated by both progesterone and estrogen (Yamagata *et al.* 2009, Vincent *et al.* 2011). Taken together, these results strongly suggest that DNA methylation affects the endometrium, but very little is known about the roles played by DNMTs during early stages of pregnancy.

Folate is an important dietary vitamin, and its deficiency in humans is associated with several diseases (Botez *et al.* 1978, Kim 2005, Burren *et al.* 2008). Folate deprivation has also been implicated in early pregnancy loss (Ronnenberg *et al.* 2007) and recurrent spontaneous abortions (Sutterlin *et al.* 1997, George *et al.* 2002).

Folate provides S-adenosylmethionine, the primary methyl donor for most methylation reactions, and thereby modulates DNA methylation in a wide range of biological contexts (Burdge & Lillycrop 2010). Moderate depletion of folate results in the site-specific hypomethylation of tumor protein TP53 (p53) in endometriotic stromal cells (Wasson *et al.* 2006) and cyclin-dependent kinase inhibitor 2a (*CDKN2A*) in head and neck squamous cell carcinoma (Kraunz *et al.* 2006). Reduced levels of folate, however, also lead to genomic DNA hypermethylation (Kotsopoulos *et al.* 2008) and *de novo* methylation of *Cdkn2a* in adult rat liver and early preneoplastic liver (Pogribny & James 2002). Despite these paradoxical results, folate clearly affects both DNA methylation and gene transcription within the associated genetic regions. Furthermore, levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* are altered in the liver and serum of nonpregnant mice subjected to a folate-deficient diet (FDD) and in serum samples from women who have taken supplemental folate (Ghoshal *et al.* 2006, Piyathilake *et al.* 2008). We have examined, therefore, the expression of DNMTs in the uteruses of early pregnant mice and in the uteruses of pseudo-pregnant mice that were subjected to a FDD. We sought to determine whether the expression of DNMTs in the mouse uterus varies with respect to the time of embryo implantation during early pregnancy and whether those expression levels are susceptible to dietary folate deficiency.

Results

DNMT gene expression levels in the mouse uterus during early pregnancy

RNA samples were isolated from mice uteruses during the earliest stages of pregnancy. qPCR analysis was then used to quantify the expression levels of genes that encode DNMTs. Expression of *Dnmt1* in endometria increased during the prereceptive phase of pregnancy from D1 to D3 (Fig. 1A). Expression gradually returned to baseline during the receptive phase and early post-implantation period (D4, D5, and D7). On D5 and D7, levels of *Dnmt1* were not significantly different between implantation site (IS) and inter-IS. The temporal pattern of expression of *Dnmt3a* was similar to that of *Dnmt1* (Fig. 1B). One major difference, however, is that on D5 significantly lower levels of *Dnmt3a* expression were detected at IS as compared with those at inter-IS. These values were still generally higher than those on D1 (Fig. 1B). *Dnmt3b* expression peaked on D3. From D4 to D7, *Dnmt3b* levels were dramatically reduced, dropping well below D1 levels. No differences in *Dnmt3b* expression were detected between IS and inter-IS on D5 and D7 (Fig. 1C).

Figure 1 qPCR analysis of mRNA levels of DNA methyltransferases. Levels within the mouse endometrium during early pregnancy are shown for *Dnmt1* (A), *Dnmt3a* (B), and *Dnmt3b* (C). *Dnmt* mRNA levels were normalized to *Gapdh*. Bars indicate the mean $\pm$ s.d. Bars labeled with different lowercase letters indicate that the data are significantly different between groups ($P < 0.05$; $n = 6$ per group).

Western blot analysis of DNMT protein in the mouse uterus during early pregnancy

On both D5 and D7, DNMT3A protein levels were significantly lower at IS compared with inter-IS ($P < 0.01$ and $P < 0.05$ respectively; Fig. 2B). Similar differences for DNMT1 and DNMT3B were not detected (Fig. 2A and C). At IS, protein levels for DNMT1 and DNMT3B were significantly lower on D7 than on D5 ($P < 0.01$; Fig. 2). Similar differences for DNMT3A were not detected. In general, DNMT protein levels were lower on D7 than on D5.

Figure 2 Western blot analysis of DNMT proteins during early pregnancy. Protein levels of DNMT1 (A), DNMT3A (B), and DNMT3B (C) at Inter-IS and IS within the endometrium at D5 and D7 of pregnancy in mice are shown ($n=6$ per group). Data are the mean $\pm$ s.d. from three independent experiments normalized to β -actin. ** $P<0.01$, and * $P<0.05$.

DNMT immunohistochemistry in the mouse uterus during early pregnancy

To determine whether DNMT protein levels are affected by implantation, we used immunohistochemistry to assess DNMT localization in the mouse uterus during early pregnancy. On D5, DNMT1-staining intensity at inter-IS was moderate in the stroma, the luminal epithelium, and the glandular epithelia. At IS, DNMT1 staining was moderate in the stroma, but no staining was detected in either the luminal or glandular epithelia. On D7, DNMT1 staining at inter-IS was weak in the stroma but was maintained at a moderate level in both the luminal and glandular epithelia. At IS, DNMT1 staining

was weak in the stroma but was absent from both the luminal and glandular epithelia (Fig. 3 and Table 1).

DNMT3A localized exclusively to stromal cells, as staining was absent from both the luminal and glandular epithelia, regardless of the time point. On D5, DNMT3A staining was strong in the stroma at inter-IS but was greatly reduced at IS (Fig. 3 and Table 1). On D7, DNMT3A staining was still elevated in stromal cells at inter-IS compared with IS, but this difference was reduced as compared with that on D5 (Fig. 3 and Table 1). Thus, DNMT3A protein levels in stroma are associated with the time of embryo implantation. DNMT3A protein was absent or weak in luminal and

Figure 3 DNMT localization within uteruses of pregnant mice. DNMT1, DNMT3A, and DNMT3B expression at IS and Inter-IS at D5 and D7 of pregnancy is shown. On D5, DNMT1 staining intensity at Inter-IS was moderate in the stroma, le, and ge. At IS, DNMT1 staining was moderate in the stroma, but no staining was detected in either the le or ge. DNMT3A staining was strong in the stroma at Inter-IS but was greatly reduced at IS. DNMT3B staining at both IS and Inter-IS was moderate in the stroma, weak in the le, and absent from the ge. On D7, DNMT1 staining at Inter-IS was weak in the stroma but was maintained at a moderate level in both the le and ge. At IS, DNMT1 staining was weak in the stroma but absent from both the le and ge. DNMT3A staining was still elevated in stromal cells at Inter-IS as compared with IS. DNMT3B staining was reduced in all cell types with the exception of the le of Inter-IS, where protein levels were moderately elevated. The binding of antibodies to DNMT was detected as a brown precipitate using the streptavidin-biotin-immunoperoxidase method. Blue indicates an absence of DNMT expression. Sections that were not exposed to the primary antibody were used as negative controls (NEG). For detailed comparisons of staining intensities between groups, see Table 1. em, embryo; le, luminal epithelium; ge, glandular epithelium; s, stroma. Scale bar, 50 µm.

glandular epithelia of decidualized endometrium, with strong expression in undecidualized endometrium (data not shown).

On D5, DNMT3B staining at both IS and inter-IS was moderate in the stroma, weak in the luminal epithelium, and absent from the glandular epithelium (Fig. 3 and Table 1). On D7, DNMT3B staining was reduced in all cell types with the exception of the luminal epithelium at inter-IS, where protein levels were moderately elevated (Fig. 3 and Table 1).

DNMT expression in uteruses of pseudo-pregnant mice fed with FDD

To confirm the effect of the FDD on folate levels, six mice that had been fed the control diet were randomly selected for the determination of residual folate levels before the experimental groups were established. Folate levels did not vary significantly among these mice, and the average folate level for these mice was not significantly different from that of the pseudo-pregnant mice that were fed the control diet (data not shown).

Table 1 DNA methyltransferase (DNMT) expression in mouse uterine cells at inter-IS and IS during early pregnancy.

Protein	Days of pregnancy	Region	Stroma	Luminal epithelium	Glandular epithelium
DNMT1	D5	Inter-IS	++	++	++
		IS	++	—	—
	D7	Inter-IS	+	++	++
		IS	+	—	—
DNMT3A	D5	Inter-IS	+++	—	—
		IS	+	—	—
	D7	Inter-IS	++	—	—
		IS	+	—	—
DNMT3B	D5	Inter-IS	++	+	—
		IS	++	+	—
	D7	Inter-IS	+	++	—
		IS	+	—	—

—, no staining; +, weak; ++, moderate staining; and +++, strong staining.

Figure 4 The effects of folate deficiency on *Dnmt* expression. qPCR analysis was used to determine mRNA levels of *Dnmt* genes in the endometrium of pseudo-pregnant mice subjected to a FDD. *Dnmt* mRNA levels were normalized to *Gapdh* ($n=6$ per group). Bars indicate the mean $\pm$ s.d. ** $P<0.01$ vs control (CTR; pseudo-pregnant mice that were fed a control diet).

Pseudo-pregnant mice subjected to a FDD, when compared with pseudo-pregnant mice that were fed the control diet, did, however, have significantly reduced levels of folate both in the liver (2.93 ± 0.38 vs 12.63 ± 1.27 μ g folate/g respectively; $n=6$, $P<0.01$) and serum (2.41 ± 0.42 vs 15.33 ± 2.14 ng folate/ml respectively; $n=6$, $P<0.01$). In pseudo-pregnant mice with folate deficiencies, expression of both *Dnmt1* ($P<0.01$) and *Dnmt3a* ($P<0.01$) was significantly elevated in the endometrium (D5 of pregnancy was analyzed). In contrast, no change was detected in *Dnmt3b* levels of expression ($P=0.373$; Fig. 4). Western blot analysis confirmed these results (Fig. 5).

We next examined the tissue sections to determine whether the FDD affected DNMT expression in a cell type-specific fashion within the uterus. Compared with controls, mice fed with FDD had elevated levels of DNMT1 protein in the luminal and glandular epithelia, with no change observed in the stroma (Fig. 6 and Table 2). DNMT3A expression was slightly increased in the luminal epithelium and was dramatically elevated in the stroma of FDD-fed mice (Fig. 6 and Table 2). Finally, a FDD resulted in a slight increase in DNMT3B protein levels in the glandular epithelium (Fig. 6 and Table 2).

Discussion

Within human endometrial tissues, mRNA levels of *DNMT1* and *DNMT3A* are lower during the secretory phase than the proliferative phase. In contrast, protein levels of DNMT1, DNMT3A, and DNMT3B do not change during the menstrual cycle (Yamagata *et al.* 2009). In agreement with this result, analyses of endometria during the early and late secretory menstrual phases detected lower levels of *DNMT3A* mRNA but no

changes in DNMT protein levels (Vincent *et al.* 2011). Despite these discrepancies between DNMT mRNA and protein levels, results such as these suggest that the expression of DNMT-encoding genes is associated with endometrial changes during menstruation in humans. Correlations between DNMT expression and the DNA methylation of specific genes in the uterus during reproductive cycles, however, have not been sufficiently characterized.

Significantly higher DNA methylation levels have been detected in the proliferative phase when compared with the secretory phase in women (Ghabreau *et al.* 2004). In addition, both mRNA and protein levels of ESR2 are downregulated in the endometrial stromal cells by DNA methylation of the promoter (Xue *et al.* 2007). Similarly, both prolactin and insulin-like growth factor-binding protein 1, which are vital for decidualization, are upregulated in human endometrial stromal cells that have been treated with a demethylation agent (5'-aza-2'-deoxycytidine) (Logan *et al.* 2010). However, it is unclear whether these genes are regulated by DNA methylation during the reproductive cycle or in association with embryo implantation.

Here we have examined mice uteruses during early stages of pregnancy to determine the levels of expression of genes that encode DNMTs. Transcript levels for all three DNMT genes peaked during the prereceptive phase of pregnancy (D3). Levels subsequently decreased back to baseline levels (or lower) during the receptive phase (D4 and D5) and the postimplantation period (D5 and D7). The maximum level of DNMT expression thus coincided with the period of steroid hormone dominance during early pregnancy in the mouse. As such, increased DNMT expression may be linked to active DNA methylation and to the downregulation of genes that generate a receptive endometrium. Decreased expression of DNMT genes during receptive and postimplantation phases could be associated with passive DNA demethylation, by increasing the expression of genes controlling endometrial cell

Figure 5 Western blot analysis of DNMT protein in the endometrium of pseudo-pregnant (PD5) mice fed a FDD. Data for DNMT1, DNMT3A, and DNMT3B are shown ($n=6$ per group). Bars indicate the mean $\pm$ s.d. from three independent experiments normalized to β -actin. ** $P<0.01$ vs control (CTR; pseudo-pregnant mice that were fed a control diet).

Figure 6 Protein localization patterns for DNMTs in endometria of pseudo-pregnant mice (PD5) fed either a control diet (CTR) or FDD. Immunostaining for DNMT1, DNMT3A, and DNMT3B is shown. DNMT was detected as a brown precipitate using the streptavidin-biotin-immunoperoxidase method. Blue indicates an absence of DNMT expression. Sections that were not exposed to the primary antibody were used as negative controls (NEG). For detailed comparisons of staining intensities between the groups, see Table 2. em, embryo; le, luminal epithelium; ge, glandular epithelium; s, stroma. Scale bar, 50 μ m.

proliferation and differentiation (decidualization). Passive mechanisms of demethylation rely on DNA replication and the incorporation of unmethylated cytosines into new DNA strands, a process that does not require DNMT activity (Teperek-Tkacz *et al.* 2011). DNMTs play a dual role in the transcriptional cycling of ESR- α target genes by facilitating both the methylation and demethylation of these targets (Kangaspeska *et al.* 2008, Metivier *et al.* 2008). This supports the hypothesis that the DNA methylation status of the genome, or of a specific set of target genes, in the endometrium may vary during the reproductive cycle in mouse and human. In addition, growth arrest and DNA damage-inducible protein α (GADD45A), which is thought to be involved in active DNA demethylation (Barreto *et al.* 2007), is upregulated in human endometrium during the mid-secretory phase (Tseng *et al.* 2010). In fact, many genes are downregulated in the mouse uterus during the

prereceptive phase of pregnancy and are subsequently upregulated during receptive and implantation phases (Wang & Dey 2006).

Here we have shown that both mRNA and protein levels of DNMT3A are significantly lower at IS than at Inter-IS on D5. As such, DNMT downregulation may be associated with embryo implantation, and *Dnmt3a* downregulation may play an important role in regulating the DNA methylation of genes that control implantation. To the best of our knowledge, no specific gene expression changes in the mammalian endometrium have been attributed to DNA methylation during implantation. Further experimentation is required to clarify this claim.

Previous studies have used immunohistochemistry to characterize the localization pattern of DNMT proteins within the human uterus. These proteins have been detected in endometrial stroma and epithelia during the proliferative phase but not the secretory phase (Liao *et al.* 2008). However, high levels of DNMT1, DNMT3A, and DNMT3B have also been detected during all menstrual phases (Yamagata *et al.* 2009). This discrepancy may have resulted from the isolation of different cell populations in the examined uterine tissue. Here we have evaluated the cell type-specific expression of DNMTs. At IS, DNMT staining was absent from the luminal epithelium on D5 and D7 of pregnancy (except for DNMT3B, which showed weak staining). In contrast, DNMTs were present (except for DNMT3A) in cells of the luminal epithelium at inter-IS. This suggests that DNA methylation may regulate, at least in part, genes that generate a receptive endometrium. Moreover, DNMT staining was mainly localized to the stroma at IS. Of note, DNMT3A levels were lower in stromal cells at IS than in stroma at inter-IS on both D5 and D7. Low levels of DNA methylation and subsequent transcriptional upregulation of genes at IS, therefore, may be associated with stromal cell differentiation during embryo implantation.

Recent evidence indicates that the expression of *DNMT3A* and *DNMT3B* is regulated by estrogen and progesterone in the human endometrium, suggesting that female sex steroid hormones may affect DNA methylation. These steroids, which act in part through

Table 2 DNMT expression in uterus cells on D5 from pseudo-pregnant control mice (CTR) and mice fed with FDD.

Protein	Treatment	Stroma	Luminal epithelium	Glandular epithelium
DNMT1	CTR	+	++	+
	FDD	+	+++	++
DNMT3A	CTR	+	++	+++
	FDD	+++	+++	+++
DNMT3B	CTR	+	+	—
	FDD	+	+	+

—, no staining; +, weak; ++, moderate staining; and +++, strong staining.

nuclear hormone receptors, are influenced by DNA methylation in the endometrium, myometrium, ovary, and breast (Piva *et al.* 1989, Lapidus *et al.* 1996, Hori *et al.* 2000, Ghabreau *et al.* 2004, Mehrotra *et al.* 2004, Ren *et al.* 2007, Suzuki *et al.* 2008, Li *et al.* 2010, Wei *et al.* 2012). These findings further suggest that the levels of DNMTs in endometrial tissues are regulated by estrogen and progesterone during menstruation.

Folate can also alter DNA methylation status (Pogribny & James 2002, Kim 2005, Oommen *et al.* 2005, Kraunz *et al.* 2006, Wasson *et al.* 2006, Kotsopoulos *et al.* 2008). A folate- and methyl-deficient diet increases the expression of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in the livers of rats (Ghoshal *et al.* 2006), and folate fortification increases *DNMT1* expression in the human cervix (Piyathilake *et al.* 2008). Interestingly, our study has shown that a FDD significantly upregulated DNMT1 and DNMT3A (both mRNA and protein) in the uteruses of pseudo-pregnant mice, suggesting that folate regulates DNMT expression. Furthermore, the DNA methylation status of the genes that control endometrial changes may be affected by folate levels. Further studies are required, however, to elucidate the molecular mechanisms by which DNMT levels are regulated during pregnancy and to determine which genes are specifically affected by folate deficiency.

Materials and Methods

Animals and treatment

This research plan was approved by the ethics committee of Chongqing Medical University (No. 20110016). Male and female white Kunming mice (8 weeks old, 20–24 g) were purchased from the Laboratory Animal Center of Chongqing Medical University (China). Before experiments were initiated, all mice were fed the same diet, which contained 2 mg folate/kg (Research Diets, New Brunswick, NJ, USA) for 2 weeks. Six female mice were randomly selected to measure the residual folate levels. Six groups ($n=8$ each) of randomly selected female mice were mated with fertile males and were used to measure the gene expression on days 1–7 of pregnancy; pregnant day 1 (D1) was the day when a vaginal plug was detected. Mice from an additional group ($n=8$) were fed the control diet, which contained 2 mg folate/kg, for 35 days

and then were mated with vasectomized males to induce pseudo-pregnancy; pseudo-pregnant day 1 (PD1) was the day when a vaginal plug was detected. The folate-deficient group ($n=16$) was fed a diet that lacked folate for 35 days (Research Diets; Walzem & Clifford 1988, Bills *et al.* 1992, Kim *et al.* 1996, Kotsopoulos *et al.* 2005). These 16 mice were then mated with infertile males to induce pseudo-pregnancy as described above. Male mice were fed the control diet. To ensure that the diets for the mice were similar, only mice that became pregnant within 5 days of mating were selected for further experimentation. Mice were subjected to the FDD, therefore, for 40–45 days. For all groups of mice, serum and liver folate levels were measured by HPLC (Patring *et al.* 2005). Mean levels of folate were calculated in groups ($n=6$ each) on D5 (or PD5). After the mice were killed, the uteruses were excised, and the endometrial tissues were finely separated. ISs were distinguished from inter-ISs using i.v. injection of Chicago Blue B solution (Paria *et al.* 1993). The left uteruses were embedded in paraffin or snap-frozen in liquid nitrogen.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from endometrial tissues using the TRIzol method (Invitrogen). DNase-treated total RNA was converted to cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). mRNA expression was evaluated using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) with SYBR Premix Ex Taq II (T TaKaRa). The primers (Table 3) were designed using Primer Premier v5.0 (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Sangon Biotechnology (Shanghai, China). qPCR was carried out at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, T_m (Table 3) for 30 s, and at 72 °C for 45 s. PCR products were processed in triplicate, subjected to a melting curve analysis, and examined using an agarose gel to confirm their size. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with *Gapdh* as the normalization control.

Western blot analysis

Protein was extracted from endometrial tissues using the Mouse/Rat Tissue Extract Protein Array Kit (Sigma-Aldrich). Samples were boiled in 5× SDS sample-loading buffer for 10 min. Equal amounts of protein (30 µg) were separated using 10% (w/v) SDS-PAGE and transferred to nitrocellulose

Table 3 Primer sequences for mouse DNMT genes and *Gapdh*.

Genes	Sequence (5' → 3')	Accession no. ^a	Size (bp)	T_m ^b (°C)
<i>Dnmt1</i>	F: GGGTCTCGTTCAGAGCTG R: GCAGGAATTCATGCAGTAAG	NM_010066.4	201	60
<i>Dnmt3a</i>	F: CCGCTCTCTTTGAGTTCTAC R: AGATGTCCCTCTTGCTCACTAACG	NM_007872.4	125	55
<i>Dnmt3b</i>	F: ATGGAGATCAGGAGGGTATGGA R: GTCGCTTGGAGGTGGCTTTC	NM_001122997.1	177	56
<i>Gapdh</i>	F: TGCCCCCATGTTTGTGATG R: TGTGGTCATGAGCCCTTCC	NM_00103792	249	60

^aAccession numbers correspond to GenBank entries. ^bThe melting temperature.

membranes (Bio-Rad Laboratories). Membranes were incubated with blocking buffer at 37 °C for 2 h and then with primary rabbit polyclonal antibodies against DNMT1, DNMT3A, DNMT3B, and β -actin at 4 °C overnight (each at 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the blots were incubated for 1 h with HRP-conjugated anti-goat or -rabbit secondary antibodies (Zhongshan Goldenbridge, Beijing, China) in the blocking buffer. After additional washes, the immunoreactive bands were visualized using ECL reagents (Beyotime, Shanghai, China). Protein expression was normalized to that of β -actin. Densitometry was performed using Quantity One v4.4.0 (Bio-Rad Laboratories).

Immunohistochemistry

Uteruses were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin wax. After deparaffinization and dehydration in a graded series of xylene and alcohol, sections were boiled for 15 min in 0.01 M sodium citrate buffer for antigen retrieval. After cooling in the buffer for 60 min at room temperature, sections were incubated with 0.3% (v/v) H_2O_2 /methanol for 30 min. After three washes in PBS with 0.1% (v/v) Tween 20 (PBST), sections were incubated with 5% (v/v) normal rabbit serum in PBST to block nonspecific staining at room temperature for 30 min. Sections were then incubated with primary rabbit polyclonal antibodies against DNMT1, DNMT3A, or DNMT3B (each at 1:200) at 4 °C overnight. Secondary antibodies were visualized using the avidin-biotin method (Zhongshan Goldenbridge) followed by washing. After incubation with tetrahydrochloride (Zhongshan Goldenbridge), sections were counterstained with hematoxylin. Negative control sections were processed with nonimmune serum (Zhongshan Goldenbridge) instead of the primary antibody and counterstained with hematoxylin and eosin. Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA) was used to quantify images. The intensity of DNMT staining was classified as no staining (–), weak staining (+), moderate staining (++), or strong staining (+++).

Statistical analysis

SPSS v 11.5 (SPSS, Inc., Chicago, IL, USA) was used for the analyses. Data are described as mean $\pm$ s.d. The uterine or endometrial samples from six pregnant (or pseudo-pregnant) mice in each group were analyzed. Western blot and qPCR analyses were independently repeated three times for each group. Differences between two groups were analyzed using the Student's *t*-test. Differences between three or more groups were analyzed using a one-way ANOVA. *Post hoc* comparisons between groups involved Fisher's protected least significant difference test. Results were considered statistically significant for $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y B Ding and Y X Wang conceived and designed the experiments; C L Long and Y B Ding performed the experiments; Y B Ding, C L Long, J L He, and X M Chen contributed reagents, materials, and analysis; Y B Ding wrote the paper.

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