Maternal Diabetes Causes Alterations of DNA Methylation Statuses of Some Imprinted Genes in Murine Oocytes 1

Authors: Ge, Zhao-Jia, Liang, Xing-Wei, Guo, Lei, Liang, Qiu-Xia, Luo, Shi-Ming, et al.

Source: Biology of Reproduction, 88(5)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.112.105981

BIOLOGY OF REPRODUCTION (2013) **88**(5):117, 1–9
Published online before print 20 March 2013.
DOI 10.1095/biolreprod.112.105981

Maternal Diabetes Causes Alterations of DNA Methylation Statuses of Some Imprinted Genes in Murine Oocytes[1]

Zhao-Jia Ge,[3,4] Xing-Wei Liang,[3] Lei Guo,[3,4] Qiu-Xia Liang,[3,4] Shi-Ming Luo,[3] Ya-Peng Wang,[3,4] Yan-Chang Wei,[3] Zhi-Ming Han,[3] Heide Schatten,[5] and Qing-Yuan Sun[2,3]

[3]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*
[4]*University of Chinese Academy of Sciences, Beijing, China*
[5]*Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri*

ABSTRACT

Maternal diabetes has adverse effects not only on oocyte quality but also on embryo development. However, it is still unknown whether the DNA imprinting in oocytes is altered by diabetes. By using streptozotocin (STZ)-induced and nonobese diabetic (NOD) mouse models we investigated the effect of maternal diabetes on DNA methylation of imprinted genes in oocytes. Mice which were judged as being diabetic 4 days after STZ injection were used for experiments. In superovulated oocytes of diabetic mice, the methylation pattern of *Peg3* differential methylation regions (DMR) was affected in a time-dependent manner, and evident demethylation was observed on Day 35 after STZ injection. The expression level of DNA methyltransferases (DNMTs) was also decreased in a time-dependent manner in diabetic oocytes. However, the methylation patterns of *H19* and *Snrpn* DMRs were not significantly altered by maternal diabetes, although there were some changes in *Snrpn*. In NOD mice, the methylation pattern of *Peg3* was similar to that of STZ-induced mice. Embryo development was adversely affected by maternal diabetes; however, no evident imprinting abnormality was observed in oocytes from female offspring derived from a diabetic mother. These results indicate that maternal diabetes has adverse effects on DNA methylation of maternally imprinted gene *Peg3* in oocytes of a diabetic female in a time-dependent manner, but methylation in offspring's oocytes is normal.

imprinted gene, maternal diabetes mellitus, oocyte

INTRODUCTION

Previous studies suggest that fetuses of poorly controlled diabetic mothers display a higher incidence of malformations, primarily neural tube defects and skeletal/cardiovascular abnormalities and fetal death than those of nondiabetic pregnant mothers [1–5]. Furthermore, their offspring are susceptible to obesity, glucose intolerance, and type 2 diabetes [6, 7]. These adverse effects may be the result of lower oocyte quality and a disturbed uterine environment. Diabetic mice have been shown to exhibit uterine atrophy [8, 9], reduced mating ability [10], and alterations of the hypothalamic-pituitary-ovarian axis [11]. The ovarian function is impaired, and also the ovulation rate is lower in type 1 diabetic mice than in nondiabetic mice [9, 12].

Numerous studies have shown that if oocytes are exposed to diabetic conditions during folliculogenesis and meiotic maturation, meiotic maturation and subsequent developmental potential are negatively affected [12, 13]. Evidence suggests that the mitochondrial function, glucose metabolism pathways, and communications between cumulus cells and the oocyte are all changed in follicles of maternal diabetic mice [14–17].

The maternal diabetic state is also detrimental to pre- and postimplantation embryo development in rodents and in humans. Preimplantation embryo development is significantly delayed in both chemically induced and spontaneous diabetic models, and the embryo has a high incidence of degeneration and fragmentation [18–20]. Even one-cell embryos isolated from diabetic mice and transferred to nondiabetic mice still experience malformations and growth retardation in the process of development [21].

In order to know the mechanisms underlying these abnormal phenomena, genetic factors are of interest. Several studies have shown that gene expression in embryos is affected by maternal diabetes. [22–26]. However, we do not know whether the methylation status of imprinted genes in oocytes is influenced by maternal diabetes.

Epigenetics presents a link between gene and environment, and it has an important role in embryo development and diseases [27]. A series of experiments showed that diploidy alone is not sufficient for normal embryo development, but proper regulation of both parental genomes is required as well [28, 29]. Maternal or paternal expression is regulated by genomic imprinting being dependent on whether a parental allele is inherited from the spermatozoon or oocyte [30, 31]. Monoallelic expression is the result of DNA methylation at the cytosine residue of the CpG site in the regulatory domain of the imprinted gene [30, 32]. Modification of DNA methylation in imprinted genes in the oocyte is established during embryo development and oocyte growth [33]. Many studies show that establishing improper DNA methylation in imprinted gene is important for embryo development and disease [34–39]. Moreover, the DNA methylation re-establishment process of imprinted gene is susceptible to being affected by environment. Thus, we hypothesized that maternal diabetes might disturb the DNA remethylation process during oogenesis and oocyte maturation in diabetic mice. In the present study, streptozotocin (STZ)-induced and nonobese diabetic (NOD) diabetic models were selected to analyze methylation patterns of differential methylation regions (DMR) of the maternally methylated genes

[1]Supported by the National Basic Research Program of China (2012CB944404 and 2011CB944501).
[2]Correspondence: Qing-Yuan Sun, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, #1 Beichen West Road, Chaoyang District, Beijing 100101, China.
E-mail: sunqy@ioz.ac.cn

Received: 1 November 2012.
First decision: 20 December 2012.
Accepted: 5 March 2013.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Peg3 and *Snrpn* and paternally the methylated gene *H19* in oocytes.

MATERIALS AND METHODS

All CD-1 mice were provided by the Beijing Vital River Experimental Animals Centre and maintained at 12D:12L in a temperature-controlled room. NOD mice (NOD/ShiLtJ) were supplied by Model Animal Research Centre of Nanjing University. All procedures described were reviewed and approved by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences.

Production of Diabetic Mice

To generate a diabetic model, female CD-1 mice (age 6–7 weeks) received a single intraperitoneal injection of STZ at a dose of 230 mg/kg [40, 41] (Fig. 1A). Four days after injection, the glucose concentration was checked after cutting the tip of the tail, using a glucometer (Accu-CHEK active;Roche Diagnostic). If glucose level was higher than 17.0 mmol/L, the mouse was used for further study. Mice of similar age injected with buffer were selected as control animals.

NOD Mice

NOD mice were nonobese and spontaneous diabetic individuals and exhibited polyuria and glucosuria accompanied by rapid weight loss [42]. All NOD mice (NOD/ShiLtJ, Nanjing, China) had glucose checked at approximately 13–14 weeks by using a tail blood sample. If glucose level was more than 14.0 mmol/L, the mouse was considered to have the mutation. Age-matched controls were NOD females with glucose levels ~9.0 mmol/L.

Oocyte Collection

Oocytes were collected on Days 15, 25, and 35 after injection of STZ or buffer. Female mice were superovulated (Fig. 1A) by intraperitoneal injection of 8 IU of equine chorionic gonadotropin, followed by injection with 8 IU of human chorionic gonadotropin (hCG; Tianjin Animal Hormone Factory) 46–48 h later. At 13–14 h after injection of hCG, female mice were killed, and oocytes were recovered from oviductal ampullae; cumulus cells were removed by brief incubation in 1 mg/ml hyaluronidase. The oocytes were then washed with M2 medium (Sigma) until no cumulus cells were observed. Oocytes from offspring of diabetic and nondiabetic mice were also collected for analysis as described above.

Bisulfite Treatment and PCR Amplification

Bisulfite treatment of oocyte DNA was conducted according to previous procedures described by our laboratory [23]. Briefly, oocytes collected from diabetic and nondiabetic mice and offspring from diabetic and nondiabetic mice were divided into PCR tubes, each containing 5 oocytes. Samples were then digested in lysis solution (0.5 M EDTA, pH 8.0, 2 mg/ml proteinase K; Amresco) at 37°C for 37 min and denatured with 0.3 M NaOH at 37°C for 15 min. A 15-μl portion of melted 2% low-melting point agarose (Sigma) was added into the tube and mixed (total volume, <25 μl). The mixtures were placed in chilled mineral oil and incubated for 15 min on ice allowing formation of beads. The beads were carefully removed to another new 2-ml Eppendorf (EP) tube and then 500 μl of fresh bisulfite solution (2.5 M sodium metabisulfite, Merck; 125 mM hydroquinone, Sigma; pH 5) was added. The tube was covered with 200 μl of mineral oil followed by incubation at 50°C for 4 h in the dark. The reaction was stopped by transferring the beads to a new 2-ml tube, adding 1 ml of TE (Tris-HCl and EDTA) and incubation at room temperature for 15 min, and this treatment was repeated three times. Following desulfonation twice with 0.5 ml 0.3M NaOH for 15 min each, washing twice for 15 min each with TE and water, respectively, the beads were used for amplification by PCR.

Single oocytes isolated from diabetic and nondiabetic mice were placed into individual PCR tubes with a small amount of M2 medium, and bisulfite treatment was performed as described above with only slight modifications. The time of digestion with lysis solution was 30 min, and the final concentration of NaOH during the denaturation stage did not exceed 0.3 M. All manipulations were carried out with the necessary care.

The bisulfite-modified DNA was used as template for PCR according to previous reports [23]. Briefly, nested PCR was carried out using one bead or 0.5-μl solution with modified DNA in the first-round PCR and 2 μl of product of first-round PCR was added into the second reaction mixture system as template. All reaction mixtures contained 0.4 mM primers, 0.2 mM of all dNTPs, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 1.25 units of *Taq* Hotstart polymerase (TaKaRa). The reaction conditions for the first-round PCR were 6 min at 94°C, followed by 35 cycles of PCR for 1 min at 94°C, 2 min at annealing temperature, and 2 min at 72°C and then extended 7 min at 72°C. The conditions for the second-round PCR were 4 min at 94°C, followed by 35 cycles of PCR for 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C and then extended 7 min at 72°C. The annealing temperature and primers [33] are shown in Table 1. To verify the specification of PCR amplification, products of each second-round PCR were checked by 1.5% agarose gel electrophoresis.

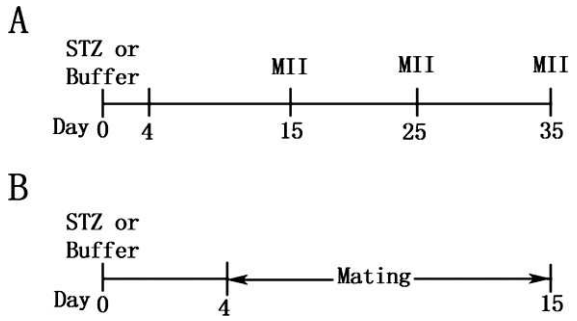


FIG. 1. Schematic diagram showing production of diabetic mice, collection of oocytes, and mating. **A**) Female mice received an injection of 230 mg/kg streptozotocin (STZ) on Day 0. Four days later, blood glucose level was evaluated with a commercial glucometer. Mice exhibiting glucose levels ≥17.0 mmol/ml were determined to be diabetic and were then superovulated, and oocytes were collected on Days 15, 25, and 35. **B**) After we determined the mice were diabetic, the estrous diabetic and nondiabetic mice were mated with normal male mice within 15 days after STZ was injected.

Determination of Methylation Status by Combined Bisulfite Restriction Analysis (COBRA) and Sequencing

To analyze the methylation patterns of DMR, the products of second-round PCR were digested by one or two restriction endogenous enzymes. These enzymes had different recognition sites at CpG loci located in DMRs of imprinted genes: *Taq*$^{\alpha}$I (T/CGA), *Rsa*I (GTAC/), and *Bst*UI (CG/CG). The digested products were analyzed by 2.5% agarose gel electrophoresis. According to COBRA analysis, we cloned the second round PCR products to T vector and sequenced (Invitrogen, Beijing, China) to further investigate the methylation status.

Generation of Embryos and Offspring

Four days after injection with STZ or buffer (Fig. 1B), the estrous diabetic/nondiabetic mice were mated with normal male mice within 15 days. On the next morning, if a vaginal plug was observed after mating, it was recognized as gestational 0.5 day. A portion of the pregnant mice was killed by cervical dislocation at 10.5 days postcoitum (dpc), and the embryos were collected. Others were fed until the offspring were born.

RNA Purification and Quantitative Real-Time PCR

RNA was extracted from oocytes by using RNeasy micro kit (Qiagen) according the manufacturer's instructions. The first cDNA strand was synthesized using Superscript II (Invitrogen). Quantitative real-time PCR (qRT-PCR) was carried out using fast real-time PCR systems (ABI). Triple samples were analyzed for each gene, and the housekeeping gene of peptidylprolyl isomerise A (*Ppia*) was used as control gene. The expression level was evaluated by $2^{-\triangle\triangle Ct}$. Primers are shown in Table 1.

Statistical Analysis

Data are presented as means ± SD, and the significance between groups was compared by one-way ANOVA. Pregnancy rate was analyzed by chi-square test. A probability level of <0.05 was considered significant.

TABLE 1. Oligonucleotides and annealing temperatures used for nested PCR.

Primer name and/or amplification	Primer sequence		Annealing temperature (°C)
	Forward	Reverse	
H19			
Out	5′-GAGTATTTAGGAGGTATAAGAATT-3′	5′-ATCAAAAACTAACATAAACCCCT-3′	55
In	5′-GTAAGGAGATTATGTTTATTTTTGG-3′	5′-CCTCATTAATCCCATAACTAT-3′	55
Snrpn			
Out	5′-TATGTAATATGATATAGTTTAGAAATTAG-3′	5′-AATAAACCCAAATCTAAAATATTTTAATC-3′	55
In	5′-AATTTGTGTGATGTTTGTAATTATTTGG-3′	5′-ATAAAATACACTTTCACTACTAAAATCC-3′	55
Peg3			
Out	5′-TGATAATAGTAGTTTGATTGGTAGGG-3′	5′-TAATTCACACCTAAAACCCTAAAACC-3′	45
In	5′-TTTTGTAGAGGATTTTGATAAGGAGG-3′	5′-AAATACCACTTTAAATCCCTATCACC-3′	45
qRT-PCR			
DNMT1	5′-CCTAGTTCCGTGGCTACGAGGAGAA-3′	5′-TCTCTCTCCTCTGCAGCCGACTCA-3′	
DNMT3a	5′-GCCGAATTGTGTCTTGGTGGATGACA-3′	5′-CCTGGTGGAATGCACTGCAGAAGGA-3′	
DNMT3b	5′-TTCAGTGACCAGTCCTCAGACACGAA-3′	5′-TCAGAAGGCTGGAGACCTCCCTCTT-3′	
DNMT3l	5′-GTGCGGGTACTGAGCCTTTTTAGA-3′	5′-CGACATTTGTGACATCTTCCACGTA-3′	

RESULTS

Ovulation Rate Is Lower in Diabetic Mice

The average number of MII oocytes was similar at different time points in the diabetic or nondiabetic group, but the ovulation rate was significantly lower in the diabetic group than in nondiabetic group (diabetes vs. nondiabetes, 9.2 vs. 23.5, 15 days; 12.6 vs. 27.2, 25 days; 10.1 vs. 28.0, 35 days; $P < 0.01$) (Fig. 2A).

Maternal Diabetes Affects DNA Methylation Re-establishment in DMRs of Maternally Imprinted Genes in Oocytes in a Time-Dependent Manner

To investigate whether the methylation re-establishment process was affected, we observed the methylation patterns of DMRs of the maternally imprinted genes *Peg3* and *Snrpn* and the paternally imprinted gene *H19* in oocytes by using COBRA and sequencing. A total of 90–120 MII oocytes per imprinted gene were analyzed in each group. The products of *H19*, amplified by nested PCR, were digested using nucleic acid restriction endonuclease *Taq*$^{\alpha}$I and *Rsa*I (Fig. 3A). Data showed that all of the products were undigested by both enzymes in all groups. This indicates that the methylation pattern of DMR of the paternally imprinted gene *H19* is not affected by maternal diabetes.

Products of the maternally imprinted genes *Peg3* and *Snrpn* were digested by *Taq*$^{\alpha}$I, *Bstu*I, or *Bstu*I, respectively. For *Peg3* (Fig. 3C), the COBRA results showed that most samples were completely digested by both enzymes except for a few samples (Fig. 3C, red arrow) for 15-day and 25-day groups. However, some samples were partially digested by both enzymes (Fig. 3C) for 35 days, and these uncut bands (Fig. 3C, red arrows) indicated that some CpG loci of *Peg3* DMR were unmethylated. For *Snrpn*, some samples were also partially digested by *Bstu*I (Fig. 3C, red arrows) for 25 days and 35 days, but similar results were not observed for 15 days (Fig. 3B). Because there was only one enzyme which could be used to digest the products of *Snrpn*, the unmethylated CpG loci may only locate at the recognition sites of the enzyme.

To further reveal the detailed methylation of *Peg3* and *Snrpn* at 15 days, 25 days, and 35 days after STZ injection, we pooled the uncut and/or cut samples together according to the COBRA results and sequenced the samples (Fig. 3, D and E). Because there were no samples of *Peg3* being completely undigested by both enzymes for 25 days and 15 days, we pooled all of the samples together at each time point and sequenced them, respectively. For the samples of *Peg3* on Day 35, the uncut and cut samples were separately pooled and sequenced. As to *Snrpn*, because only one enzyme was used to digest products of PCR, all the samples of 15 days, 25 days, and 35 days were separately pooled and sequenced, respectively. Sequencing results showed that the methylation patterns were properly acquired on Days 15 and 25 for *Peg3* in oocytes from diabetic mice, but on Day 35, the methylation status in DMR of *Peg3* was significantly affected by maternal diabetes (Fig. 3D). For *Snrpn*, the methylated status was similar between the two groups (Fig. 3E).

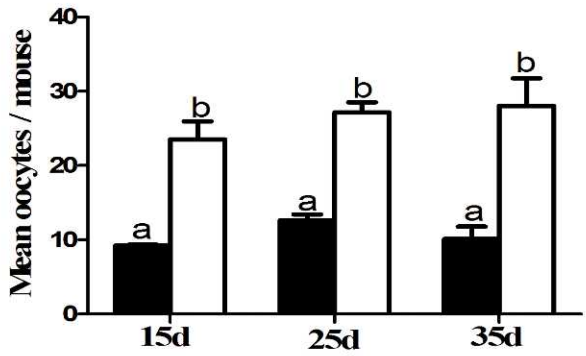
Proportion of Oocytes in Which the Methylation Status of Maternal Imprinting Genes Is Changed as Evaluated by COBRA

To further determine the rate of oocytes in which the DMRs' methylation status was changed on Day 35 after STZ injection, the sample was analyzed by COBRA. Part of the COBRA results are shown in Figure 2C. For the maternally methylated gene *Peg3*, the rate (total undigested samples/[total undigested + total digested]) of unmethylated oocytes was significantly higher ($P = 0.001 < 0.01$; 21.98%, n = 91) in the diabetic group than in the nondiabetic group (6.2%, n = 113). For *snrpn*, the rate of unmethylated oocytes was slightly higher ($P = 0.352 > 0.05$; 7.5%, n = 80) in the diabetic group than in the nondiabetic group (4.0%, n = 75) at 35 days after STZ injection (Fig. 2B).

Expression Levels of DNMTs Are Decreased in a Time-Dependent Manner in Oocytes from Diabetic Mice

To investigate how maternal diabetes affects the methylation patterns of some imprinted genes in a time-dependent manner, we analyzed the expression levels of DNMTs in oocytes by using qRT-PCR. As shown in Figure 4, their expression levels were significantly lower in diabetic group than in the control ($P < 0.05$). On Days 15 and 25, the expression levels of DNMTs in oocytes were similar in diabetic mice but were significantly decreased on Day 35 compared to Days 15 and 25 ($P < 0.05$).

A



B

Rate of unmethylated oocytes on day 35

Genes	Unmethylated oocytes (%)	
	Nondiabetic	Diabetic
Peg3	6.2	22**
Snrpn	4	7.5

Asterisks represent significant differences between nondiabetic and diabetic groups (**P<0.01)

C

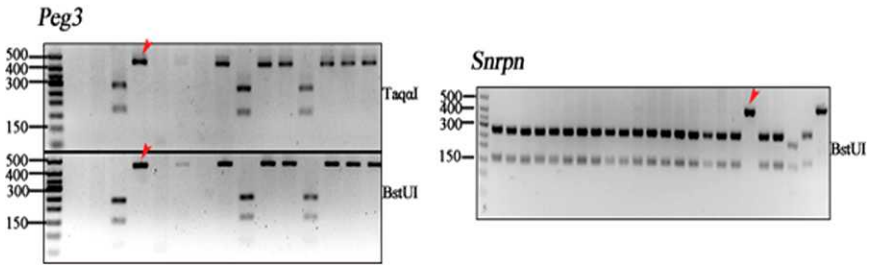


FIG. 2. Average oocyte number per mouse and percentage of unmethylated oocytes. **A**) Oocytes from diabetic and nondiabetic mice at different time points were counted and presented as means ± SD. Different letters show significant differences: same letter indicates a *P* value >0.05; different letters indicate a *P* value <0.01. Black bar, diabetic; white bar, nondiabetic. **B**) Percentage of *Peg3* (total oocytes, n = 91 diabetic and 113 nondiabetic) and *Snrpn* (total oocytes, n = 80 diabetic and 75 nondiabetic) unmethylated oocytes were evaluated by COBRA using single-oocyte samples. n = 36 diabetic and 17 nondiabetic females were used in the assay. Data are percentages. $^{**}P < 0.01$. **C**) Representative COBRA results of the methylation patterns in DMRs of *Peg3* and *Snrpn* in a single oocyte on Day 35 after STZ injection. Blank column, sample lost.

Methylation Patterns of Imprinted Genes Are Not Significantly Influenced in Oocytes from Offspring of Diabetic Mice

To determine whether maternal diabetes disturbs the DNA methylation reprogramming process in oocytes of offspring of diabetic mice (OD), DNA methylation patterns of DMRs in MII oocytes were assessed by COBRA and sequencing. A total of 90–120 MII oocytes per imprinted gene were analyzed in each group. We observed that some samples were not completely cut by *Bstu*I for *Snrpn* (Fig. 5B, red arrow). This indicates that some CpG sites were not remethylated in oocytes of OD. Because only one enzyme was used, the unmethylated CpG locus may be located at the recognition sites of the enzyme. To investigate in more detail, we pooled all the samples together and sequenced them. The sequencing results showed that only a few CpG sites were unmethylated (Fig. 5E), and they were located at the enzyme recognition loci, and the total methylation rate was similar between the two groups. However, at the loci (Fig. 5E, red arrows), the methylation rate was significantly lower than that of ON (offspring from nondiabetic mice). For the paternally imprinted gene *H19*, the COBRA result showed that several samples were partially digested by Taq^{α}I (Fig. 5A), but the sequencing results showed that there was only a slight ($P > 0.05$) increase in the total methylation rate of *H19* (Fig. 5D).

Pregnancy Rate and Embryo Development Are Affected by Maternal Diabetes

As shown in Figure 6, we observed that the pregnancy rate was only 73.68% (14 of 19) in diabetic mice in which vaginal plugs were observed, and the death rate of embryos at 10.5 dpc was 16.56% (26 of 157) in diabetic females, while in nondiabetic females, the pregnancy rate and embryo death rate were 100% and 0, respectively. The average number of live embryos per litter in diabetic group was significantly ($P < 0.05$) lower (9.36, 131 of 14) than that of the nondiabetic mouse (16.0, 192 of 12) at 10.5 dpc. We generated 86 pups that were born by 11 diabetic females, and the birth rate per mouse

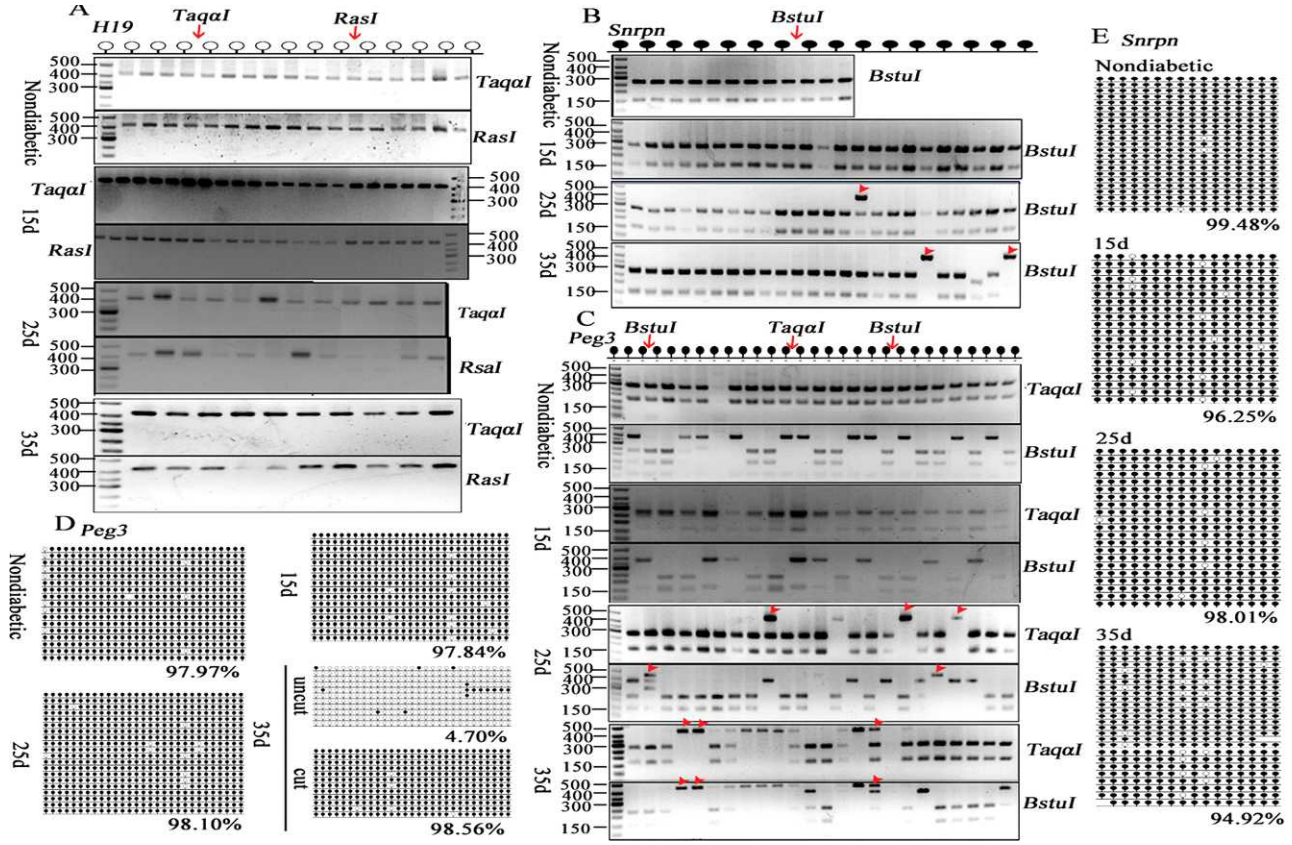


FIG. 3. DNA methylation status of DMRs of *Peg3*, *Snrpn*, and *H19* in nondiabetic and diabetic groups. Data represent the nondiabetic group (Nondiabetic) and the diabetic group oocytes collected on Days 15 (15d), 25 (25d), and 35 (35d) after STZ injection. **A**–**C**) Methylation patterns of *H19*, *Snrpn*, and *Peg3* were analyzed by COBRA in oocytes collected at different time points from diabetic and nondiabetic mice, respectively. Oocytes were treated with bisulfite and amplified by nested PCR and then digested by enzymes. Each sample included 5 oocytes, and the number column on the left of the pictures showed the bands' size of the marker. The line of cycle showed the digested loci of the enzymes in DMRs of the imprinted genes. **A**) For *H19*, in the diabetic and nondiabetic groups, all of the samples were undigested by enzymes. **B**) For *Snrpn*, the digested result was similar to that of the nondiabetic group on Day 15, but a few samples were not completely digested by enzyme on Days 25 and 35 (red arrowheads). **C**) For *Peg3*, in the diabetic group on Day 15, all of the samples were digested by both enzymes as nondiabetic group, and a few samples were not completely digested by only one enzyme on Day 25 (red arrowheads), but on Day 35, many samples were not digested by both enzymes (red arrowheads). **D** and **E**) The methylation rates of *Peg3* and *Snrpn* on Days 15, 25, and 35 were analyzed by sequencing. **D**) For *Peg3*, all of the samples were pooled together and sequenced for nondiabetic group, and 15-day (15d) and 25-day (25d) oocytes in diabetic group; methylation rates were similar among these groups, but on Day 35, the samples with uncut bands and without uncut bands were pooled and sequenced, respectively. Methylation rates of the samples with uncut bands were 4.70%, and the methylation rate of samples without uncut bands was 98.56%. **E**) For *Snrpn*, the samples were pooled together and sequenced for all groups, and the methylation rates were counted as shown. Black cycle, methylated; white cycle, unmethylated; blank loci, CpG lost.

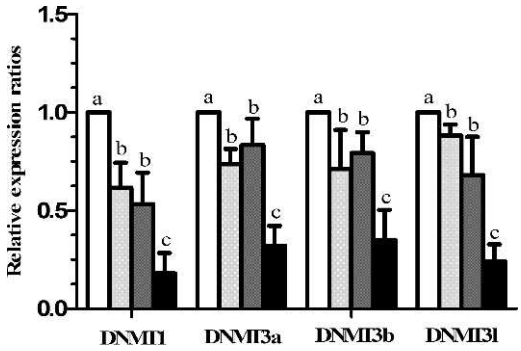


FIG. 4. Expressions of DNMTs in oocytes. Oocytes were collected at different time points for both groups, and RNA was reverse-transcribed to cDNA. It was used as a template to evaluate the expression of DNMTs by qRT-PCR. White bar, control; light gray bar, 15 days; dark gray bar, 25 days; black bar, 35 days. Same letter, $P > 0.05$; different letters, $P < 0.05$.

was 7.82 (86 of 11), which was significantly ($P < 0.001$) lower than that in nondiabetic groups (13.7, 164 of 12). A total of 43.02% (37 of 86) of pups died in 10 days of birth in diabetic groups, significantly ($P < 0.01$) higher than that of non-diabetetic groups (13.41%, 22 of 164). These results suggest that embryo development was adversely affected by maternal diabetes and that spontaneous abortion occurred at an earlier than the mid-gestation period.

NOD Genetic Diabetic Mice Show Altered Methylation Pattern of Peg3 *DMR in a Time-Dependent Manner*

To test whether methylation alteration in *Peg3* DMR in oocytes from STZ-induced diabetic mice was caused by STZ itself rather than maternal diabetes, we examined the methylation pattern of *Peg3* DMR in NOD mouse model. Approximately 80–110 ovulated MII oocytes (not including failed samples) were analyzed by COBRA at each time point and similar results were obtained. On Days 15 and 25, only a few samples were undigested by one or two enzymes (Fig. 7, B and C) like in controls (Fig. 7A). On Day 35, the undigested

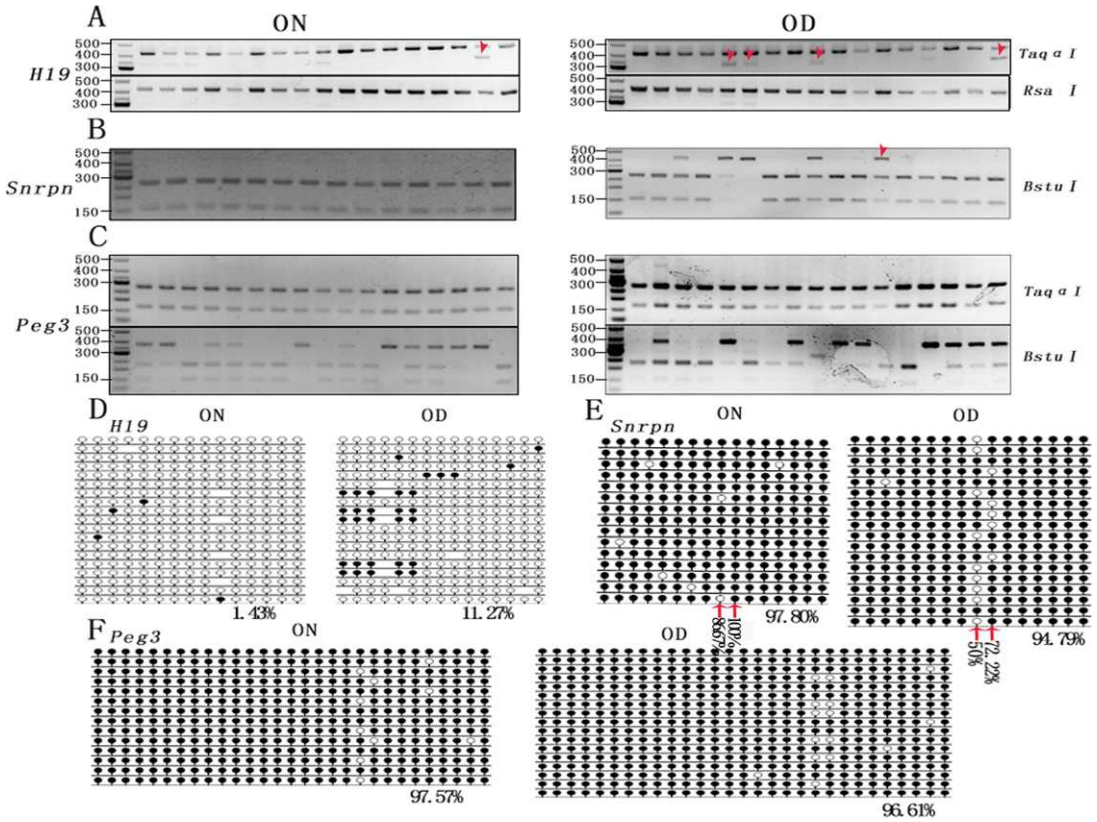
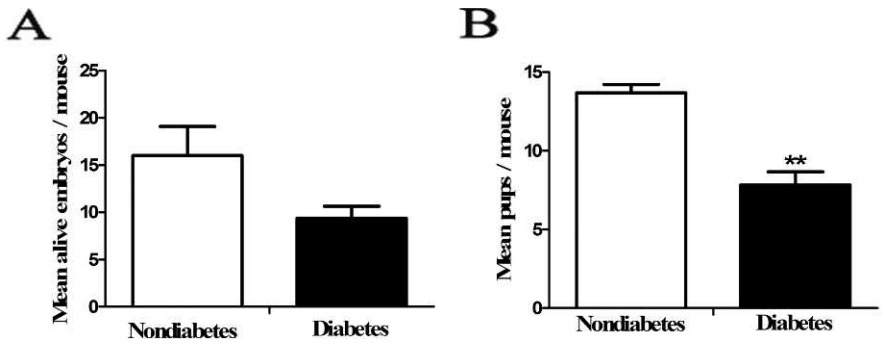


FIG. 5. Methylation patterns in DMRs of imprinted genes in oocytes from offspring of nondiabetic (ON) and diabetic (OD) females. ON and OD mice (7–8 weeks old) were superovulated, and oocytes were collected and treated with bisulfite and then amplified by nested PCR. Each sample included 5 oocytes and the number column on the left of the pictures showed the bands' size of the marker. **A**–**C**) The products of PCR were digested by enzymes and analyzed with 2.5% agarose gel electrophoresis. Red arrowheads indicate samples that were not completely digested by enzymes. **D** and **E**) Methylation rates in DMRs of *Peg3*, *Snrpn*, and *H19* were evaluated by sequencing. **E)** Red arrows point to the CpG loci methylation rates in *Snrpn*. Black cycle, methylated; white cycle, unmethylated; blank loci, CpG lost.



C

Pregnancy rate and death rate of pups in 10 days after birth

	% Pregnancy	% Death after birth
Nondiabetic (n=6 litters)	100	13.41
Diabetic (n=19 litters)	73.68	43.02**

Asterisks represent significant differences between nondiabetic and diabetic groups (P<0.01)**

FIG. 6. Effects of maternal diabetes on embryonic development and pregnancy parameters. **A**) Diabetic and nondiabetic mice were mated with normal males within 15 days of STZ injection and the embryos were collected at 10.5 dpc of gestation. Data are presented as means ± SD (n = 14 diabetic, 6 nondiabetic). **B**) Diabetic and nondiabetic females were mated with normal males and pups were born. Data are means ± SD (n = 11 diabetic, 6 nondiabetic). **C**) Pregnancy rate was evaluated as the percentage of pregnant animals within the total number of animals with vaginal plugs. Death rate after birth was evaluated as percentage of dead pups of the total number of pups within 10 days. $^{**}P < 0.01$.

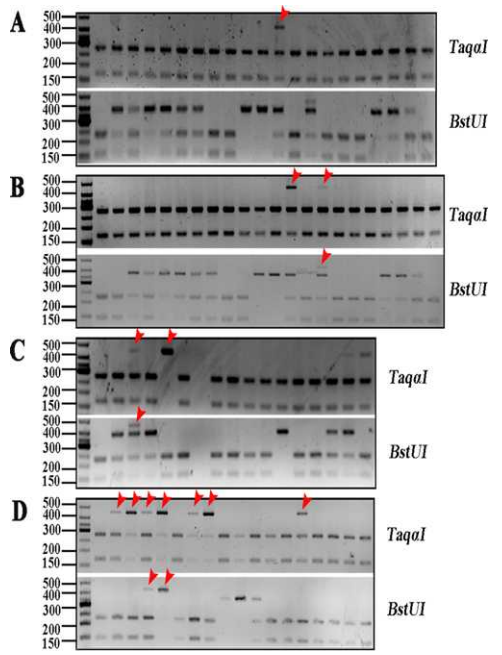


FIG. 7. DMR methylation status of *Peg3* analyzed by COBRA in NOD mouse model. DNA from ovulated MII oocytes from NOD mice was modified by bisulfite and amplified using nested PCR. The products were digested by enzymes. Control (**A**), Day 15 (**B**), Day 25 (**C**), Day 35 (**D**). Red arrows show undigested samples. Blank column, sample lost.

samples (Fig. 7D) were apparently more than the other time points and controls.

DISCUSSION

The genomic DNA methylation is subjected to two demethylation and remethylation processes during gametogenesis and embryo development [43]. If imprinting is not properly acquired in oocytes and/or not maintained during preimplantation development, fetal development may be abnormal [44, 45]. It is well known that germ cell quality and fertility are adversely affected by diabetes mellitus, however, whether imprinting methylation is affected in germ cells is not known. In this study, by using a STZ-induced mouse model, which was used by many reports where the authors obtained similar results compared to those of a mutational mouse model [14, 46], we for the first time investigated whether DNA methylation of imprinted loci are disturbed by maternal diabetes in oocytes of diabetic females and their offspring. We showed that maternal diabetes mellitus caused adverse effects on DNA methylation imprinting in oocytes of diabetic females (Fig. 3) but did not evidently affect imprinting pattern in oocytes of their offspring (Fig. 5).

Previous studies have demonstrated that oocyte quality is adversely affected by maternal diabetes [14–17]. During oogenesis, a crucial event is to establish proper genome-wide DNA methylation for later embryo development and survival. In mice, parthenogenetic and androgenetic embryos die before Day 10 of gestation and have distinctive phenotypes: 25-somite embryos with poor extraembryonic tissue and retarded embryos with proliferated trophoblasts, respectively [29]. This may be related to improper genomic imprinting in oocytes. In diabetic females, the oocyte quality is compromised, indicated by reduced glucose metabolism, compromised communication between cumulus cells and oocytes, mitochondrial malfunction, and decreased ovulation rate [14–17]. In our study, we also demonstrated the ovulation rate was decreased (Fig. 2). We found that methylation patterns of the imprinted genes' DMRs were not evidently changed at Days 15 and 25 of STZ injection but methylation was apparently affected on Day 35 of STZ injection. However, if the samples were contaminated by somatic cells, it might lead to improper evaluation of methylation patterns in DMRs of imprinted genes in oocytes. However, the paternally methylated imprinted gene *H19* showed hypomethylation in all oocytes, so it could be used as evidence to exclude somatic cells contamination of samples. The results analyzed by COBRA showed that the DMR methylation status of *H19* was not affected by maternal diabetes, also indicating that the samples were not contaminated by somatic cells (Fig. 3A). Additionally, we obtained similar results in NOD mouse model (Fig. 7). This demonstrates that the DNA imprinting abnormality is caused by diabetic conditions, and this will occur only when the adverse factors are accumulating to a level in vivo. In female germ line, methylation establishment of imprinted genes begins at the postnatal oocyte growing stage and finishes at MII stage [33], and this process is catalyzed by DNA methyltransferase (DNMT) 3A, 3B, and 3L [47, 48], which are coordinately regulated, and expression peaks during the stage of postnatal oocyte development when maternal methylation imprints are established [49]. Therefore, we evaluated the expression of DNMTs at different time points in oocytes from diabetic and control groups. We found that the DNMTs expression was decreased in a time dependent manner (Fig. 4), and this was coincident with the methylation status of imprinted genes. Approximately 24–28 days are required from primordial germinal cell to mature oocyte. During oogenesis, dramatic environment changes and/or stimulation may disturb remethylation of imprinted genes. Market-Velker et al. [24] showed that superovulation induced loss of maternal and paternal imprinted methylation in a dose-dependent manner. Lenzen [40] demonstrated that mouse injected with STZ showed hyperglycemia, and the Animal Models of Diabetic Complications Consortium Protocols suggests testing the levels of hyperglycemia 2 days later, after STZ injection too. Therefore, we suggest that only the adverse factors are accumulated to a threshold level in vivo, the remethylation process may be significantly affected, and mosaic methylation in DMRs of imprinted genes be caused.

We did not identify significantly abnormal methylation patterns in DMRs of *Peg3*, *Snrpn*, and *H19* in oocytes isolated from diabetic mice before Day 25 of STZ injection, but there are some samples partially undigested by only one enzyme in the results of COBRA, and the sequencing results showed that these were caused by a few unmethylated CpG loci which were located at the recognition loci of the enzyme (Fig. 3). From the results of COBRA and sequencing on Day 35, we found that because some oocytes were properly methylated in DMRs of imprinted genes, some samples were partially undigested by two enzymes. On Day 15, we did not find improper methylation status in DMRs of imprinted genes, but the oocyte quality, embryo development, and offspring of diabetic female were still affected [14, 50].

The methylation patterns of *Igf2*/*H19* in embryos and offspring are adversely affected by diabetes mellitus [51, 52], but the status of imprinting in oocytes of diabetic offspring is not known. We next investigated the methylation of imprinted genes in offspring derived from diabetic females. According to our investigation and the report by Van Belle [53], it was too difficult to get pups from diabetic females mating at 35 days after STZ injection, so diabetic female mice were mated with

normal male mice at 15 days after injection of STZ, and pups were produced. Our data showed that the methylation patterns of DMRs of imprinted genes were not significantly altered in offspring oocytes. Thus, diabetic intrauterine environment may not affect DNA imprinting re-establishment during oogenesis in offspring of diabetic mice.

In summary, maternal diabetes causes adverse effects on imprinting patterns in oocytes, but no evident imprinting abnormality is observed in oocytes from female offspring derived from a diabetic mother.

ACKNOWLEDGMENT

We thank Yi Hou (Institute of Zoology, Chinese Academy of Sciences), Ying-Chun Ouyang (Institute of Zoology, Chinese Academy of Sciences), En-Kui Duan (Institute of Zoology, Chinese Academy of Sciences), and Qi Chen (Institute of Zoology, Chinese Academy of Sciences) for technical assistance.

REFERENCES

1. Sadler TW, Hunter ES III, Balkan W, Horton WE Jr. Effects of maternal diabetes on embryogenesis. Am J Perinatol 1988; 5:319–326.
2. Eriksson UJ. Congenital malformations in diabetic animal models–a review. Diabetes Res 1984; 1:57–66.
3. Mills JL, Simpson JL, Driscoll SG, Jovanovicpeterson L, Vanallen M, Aarons JH, Metzger B, Bieber FR, Knopp RH, Holmes LB, Peterson CM, Withiamwilson M, et al. Incidence of spontaneous-abortion among normal women and insulin-dependent diabetic women whose pregnancies were identified within 21 days of conception. N Engl J Med 1988; 319: 1617–1623.
4. Suhonen L, Hiilesmaa V, Teramo K. Glycaemic control during early pregnancy and fetal malformations in women with type I diabetes mellitus. Diabetologia 2000; 43:79–82.
5. Moley KH, Chi MM, Manchester JK, McDougal DB, Lowry OH. Alterations of intraembryonic metabolites in preimplantation mouse embryos exposed to elevated concentrations of glucose: a metabolic explanation for the developmental retardation seen in preimplantation embryos from diabetic animals. Biol Reprod 1996; 54:1209–1216.
6. Cox NJ. Maternal component in NIDDM transmission—how large an effect. Diabetes 1994; 43:166–168.
7. Yessoufou A, Moutairou K. Maternal diabetes in pregnancy: early and long-term outcomes on the offspring and the concept of ''metabolic memory''. Exp Diabetes Res 2011; 2011:218598.
8. Tatewaki R, Otani H, Tanaka O, Kitada J. A morphological study on the reproductive organs as a possible cause of developmental abnormalities in diabetic NOD mice. Histol Histopathol 1989; 4:343–358.
9. Garris DR. Effects of diabetes on uterine condition, decidualization, vascularization, and corpus luteum function in the pseudopregnant rat. Endocrinology 1988; 122:665–672.
10. Hassan AA, Hassouna MM, Taketo T, Gagnon C, Elhilali MM. The effect of diabetes on sexual behavior and reproductive tract function in male rats. J Urol 1993; 149:148–154.
11. Katayama S, Brownscheidle CM, Wootten V, Lee JB, Shimaoka K. Absent or delayed preovulatory luteinizing hormone surge in experimental diabetes mellitus. Diabetes 1984; 33:324–327.
12. Colton SA, Pieper GM, Downs SM. Altered meiotic regulation in oocytes from diabetic mice. Biol Reprod 2002; 67:220–231.
13. Diamond MP, Lavy G, Polan ML. Progesterone production from granulosa cells of individual human follicles derived from diabetic and nondiabetic subjects. Int J Fertil 1989; 34:204–208.
14. Wang Q, Ratchford AM, Chi MMY, Schoeller E, Frolova A, Schedl T, Moley KH. Maternal diabetes causes mitochondrial dysfunction and meiotic defects in murine oocytes. Mol Endocrinol 2009; 23:1603–1612.
15. Ratchford AM, Esguerra CR, Moley KH. Decreased oocyte-granulosa cell gap junction communication and connexin expression in a type 1 diabetic mouse model. Mol Endocrinol 2008; 22:2643–2654.
16. Ratchford AM, Chang AS, Chi MMY, Sheridan R, Moley KH. Maternal diabetes adversely affects AMP-activated protein kinase activity and cellular metabolism in murine oocytes. Am J Physiol Endocrinol Metab 2007; 293:E1198–1206.
17. Colton SA, Downs SM. Potential role for the sorbitol pathway in the meiotic dysfunction exhibited by oocytes from diabetic mice. J Exp Zool a Comp Exp Biol 2004; 301:439–448.
18. Diamond MP, Moley KH, Pellicer A, Vaughn WK, DeCherney AH. Effects of streptozotocin- and alloxan-induced diabetes mellitus on mouse follicular and early embryo development. J Reprod Fertil 1989; 86:1–10.
19. Beebe LF, Kaye PL. Maternal diabetes and retarded preimplantation development of mice. Diabetes 1991; 40:457–461.
20. Moley KH, Vaughn WK, DeCherney AH, Diamond MP. Effect of diabetes mellitus on mouse pre-implantation embryo development. J Reprod Fertil 1991; 93:325–332.
21. Wyman A, Pinto AB, Sheridan R, Moley KH. One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring. Endocrinology 2008; 149:466–469.
22. Scholler M, Wadsack C, Lang I, Etschmaier K, Schweinzer C, Marsche G, Dieber-Rotheneder M, Desoye G, Panzenboeck U. Phospholipid transfer protein in the placental endothelium is affected by gestational diabetes mellitus. J Clin Endocrinol Metab 2012; 97:437–445.
23. Liang X-W, Ge Z-J, Guo L, Luo S-M, Han Z-M, Schatten H, Sun Q-Y. Effect of postovulatory oocyte aging on DNA methylation imprinting acquisition in offspring oocytes. Fertil Steril 2011; 96:1479–1484.
24. Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MRW. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. Hum Mol Gen 2010; 19:36–51.
25. Stouder C, Deutsch S, Paoloni-Giacobino A. Superovulation in mice alters the methylation pattern of imprinted genes in the sperm of the offspring. Reprod Toxicol 2009; 28:536–541.
26. Wang Z, Xu L, He F. Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. Fertil Steril 2010; 93:2729–2733.
27. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Gen 2011; 13:97–109.
28. McGrath J, Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 1984; 37:179–183.
29. Barton SC, Surani MA, Norris ML. Role of paternal and maternal genomes in mouse development. Nature 1984; 311:374–376.
30. Bartolomei MS, Webber AL, Brunkow ME, Tilghman SM. Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. Genes Dev 1993; 7:1663–1673.
31. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell 1991; 64:849–859.
32. Zemel S, Bartolomei MS, Tilghman SM. Physical linkage of two mammalian imprinted genes, H19 and insulin-like growth factor 2. Nat Genet 1992; 2:61–65.
33. Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. Genomics 2002; 79: 530–538.
34. Kaneko-Ishino T, Kuroiwa Y, Miyoshi N, Kohda T, Suzuki R, Yokoyama M, Viville S, Barton SC, Ishino F, Surani MA. Peg1/Mest imprinted gene on chromosome 6 identified by cDNA subtraction hybridization. Nat Genet 1995; 11:52–59.
35. Sasaki H, Jones PA, Chaillet JR, Ferguson-Smith AC, Barton SC, Reik W, Surani MA. Parental imprinting: potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II (Igf2) gene. Gen Dev 1992; 6:1843–1856.
36. Kuroiwa Y, Kaneko-Ishino T, Kagitani F, Kohda T, Li LL, Tada M, Suzuki R, Yokoyama M, Shiroishi T, Wakana S, Barton SC, Ishino F, et al. Peg3 imprinted gene on proximal chromosome 7 encodes for a zinc finger protein. Nat Genet 1996; 12:186–190.
37. Barr JA, Jones J, Glenister PH, Cattanach BM. Ubiquitous expression and imprinting of Snrpn in the mouse. Mamm Genome 1995; 6:405–407.
38. Walsh C, Glaser A, Fundele R, Ferguson-Smith A, Barton S, Surani MA, Ohlsson R. The non-viability of uniparental mouse conceptuses correlates with the loss of the products of imprinted genes. Mech Dev 1994; 46: 55–62.
39. Buiting K. Prader-Willi syndrome and Angelman syndrome. Am J Med Genet C Semin Med Genet 2010; 154C:365–376.
40. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 2008; 51:216–226.
41. Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM, Schedl T, Moley KH. Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice. PLoS One 2010; 5:e15901.
42. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 1980; 29:1–13.
43. Surani MA, Hayashi K, Hajkova P. Genetic and epigenetic regulators of pluripotency. Cell 2007; 128:747–762.
44. Howell CY, Bestor TH, Ding F, Latham KE, Mertineit C, Trasler JM, Chaillet JR. Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. Cell 2001; 104:829–838.

45. Cirio MC, Martel J, Mann M, Toppings M, Bartolomei M, Trasler J, Chaillet JR. DNA methyltransferase 1o functions during preimplantation development to preclude a profound level of epigenetic variation. Dev Biol 2008; 324:139–150.
46. Chang AS, Dale AN, Moley KH. Maternal diabetes adversely affects preovulatory oocyte maturation, development, and granulosa cell apoptosis. Endocrinology 2005; 146:2445–2453.
47. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 1998; 19: 219–220.
48. Lees-Murdock DJ, Shovlin TC, Gardiner T, De Felici M, Walsh CP. DNA methyltransferase expression in the mouse germ line during periods of de novo methylation. Dev Dyn 2005; 232:992–1002.
49. Lucifero D, La Salle S, Bourc'his D, Martel J, Bestor TH, Trasler JM. Coordinate regulation of DNA methyltransferase expression during oogenesis. BMC Dev Biol 2007; 7:36.
50. Vambergue A, Fajardy I. Consequences of gestational and pregestational diabetes on placental function and birth weight. World J Diabetes 2011; 2: 196–203.
51. Ding G-L, Wang F-F, Shu J, Tian S, Jiang Y, Zhang D, Wang N, Luo Q, Zhang Y, Jin F, Leung PCK, Sheng J-Z, et al. Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. Diabetes 2012; 61:1133–1142.
52. Shao W-J, Tao L-Y, Gao C, Xie J-Y, Zhao R-Q. Alterations in methylation and expression levels of imprinted genes H19 and Igf2 in the fetuses of diabetic mice. Comp Med 2008; 58:341–346.
53. Van Belle TL, Taylor P, von Herrath MG. Mouse Models for Type 1 Diabetes. Drug Discov Today Dis Models 2009; 6:41–45.