

Transgenerational Glucose Intolerance With *Igf2/H19* Epigenetic Alterations in Mouse Islet Induced by Intrauterine Hyperglycemia

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Gestational diabetes mellitus (GDM) has been shown to be associated with high risk of diabetes in offspring. However, the mechanisms involved and the possibilities of transgenerational transmission are still unclear. We intercrossed male and female adult control and first-generation offspring of GDM (F1-GDM) mice to obtain the second-generation (F2) offspring in four groups: C σ -C ϕ , C σ -GDM ϕ , GDM σ -C ϕ , and GDM σ -GDM ϕ . We found that birth weight significantly increased in F2 offspring through the paternal line with impaired glucose tolerance (IGT). Regardless of birth from F1-GDM with or without IGT, high risk of IGT appeared as early as 3 weeks in F2 offspring and progressed through both parental lineages, especial the paternal line. IGT in male offspring was more obvious than that in females, with parental characteristics and sex-specific transmission. In both F1 and F2 offspring of GDM, the expression of imprinted genes *Igf2* and *H19* was downregulated in pancreatic islets, caused by abnormal methylation status of the differentially methylated region, which may be one of the mechanisms for impaired islet ultrastructure and function. Furthermore, altered *Igf2* and *H19* gene expression was found in sperm of adult F1-GDM, regardless of the presence of IGT, indicating that changes of epigenetics in germ cells contributed to transgenerational transmission. *Diabetes* 61:1133–1142, 2012

A growing body of research suggests that exposure to an abnormal environment in the uterus can lead to chronic health problems later in life (1,2). Intrauterine hyperglycemia is a major characteristic of gestational diabetes mellitus (GDM) and has been suggested to be an important determinative factor for the risk of diabetes in adulthood, in addition to the effects of genetic factors (3–6). Although many studies have investigated intrauterine growth retardation and later diabetes, the mechanism involved in the association between intrauterine hyperglycemia and a high risk of diabetes in offspring remains unclear (7,8).

In mammals, epigenetic reprogramming is involved in germ cells and early embryonic development (9,10). Evolutionarily and environmentally acquired genomic susceptibilities can

induce epigenomic modulations early in life, which impact on the later development of human disease (11). Moreover, frequent tissue-specific and disease-specific de novo methylation events occur during somatic cell development and differentiation (12). Because erasure and establishment of the genomic imprints for some imprinted genes begin when migratory primordial germ cells (PGCs) enter the embryonic genital ridge through gametogenesis, epigenetic abnormalities that occur during this phase may be involved in transgenerational transmission (13,14). We hypothesized that the hyperglycemic intrauterine environment of GDM could result in a high risk of diabetes in offspring by altering epigenetic modification. In addition to intergenerational transmission (F1 offspring), intrauterine hyperglycemia may also have effects on the second generation (F2 offspring).

Since it is difficult to analyze the comparative parental contributions and the underlying mechanisms that result in F2 offspring outcomes in humans, in the current study we established a GDM mouse model of intrauterine hyperglycemia. The female (ϕ) and male (σ) F1 adults of control and GDM mice were intercrossed to obtain F2 offspring of four groups: 1) C σ -C ϕ , 2) C σ -GDM ϕ , 3) GDM σ -C ϕ , and 4) GDM σ -GDM ϕ . Besides the phenotype, we examined the expression of imprinted genes *Igf2*, *H19*, and *Plagl1* in islets. Abnormal *Igf2* production has been shown to develop β -cell dysfunction and result in diabetes and appears to be an early landmark in the pathological sequence leading to apoptosis of β -cells in the fetal Goto-Kakizaki (GK) rat (15–17). *H19* is an imprinted gene at 90 kb 3' of *Igf2* and is reciprocally imprinted with respect to *Igf2*, regulating its imprinting and expression (18,19). *Plagl1*, known as transcription factor zinc finger protein, is involved in the pathogenesis of transient neonatal diabetes mellitus (20–22). We further analyzed the methylation status of differentially methylated regions (DMRs) in *Igf2/H19* in mouse islets to investigate whether intrauterine hyperglycemia affected imprinted gene expression by regulating epigenetic modification. In addition, we examined imprinted genes in sperm of F1-GDM to explore the underlying mechanism of transgenerational transmission by germ cells.

RESEARCH DESIGN AND METHODS

All animal protocols were reviewed and approved by the Zhejiang University Animal Care and Use Committee. At the age of 8 weeks, virgin female ICR mice ($n = 60$) were mated with normal males. Onset of pregnancy was determined by the presence of a copulation plug after overnight mating (designated as day 0 [D0] of pregnancy). After a 12-h fast, the females were randomly divided into a control group and an intrauterine hyperglycemia group with GDM (GDM group). Mice in the GDM group were injected with a single intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO) in 0.1 mmol/L citrate buffer (pH 4.5) at a dose of 150 mg/kg body wt. Control pregnant females received an equal volume of citrate buffer. On D3 of pregnancy, diabetes was confirmed by measurement of blood glucose concentration via the tail vein

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and defined as a glucose level between 14 and 19 mmol/L (252–342 mg/dL), which was also measured on D7 and D20 of pregnancy to confirm the diabetes condition as previously described (23–26).

The pregnant mice were allowed to deliver spontaneously. The litter size was randomly reduced to 10 at birth to assure uniformity. The pups from the GDM group were fostered by normoglycemic females until they were weaned at the age of 3 weeks.

The ♀ and ♂ F1 adults of control (F1-C) and GDM (F1-GDM) mice were intercrossed, and the phenotypes of their F2 offspring were characterized. F2 offspring were obtained from 4 groups: 1) C♂-C♀, 2) C♂-GDM♀, 3) GDM♂-C♀, and 4) GDM♂-GDM♀ (Fig. 1A).

In vivo glucose and insulin analysis. Intraperitoneal glucose injection (2 g/kg body wt) was performed in unrestrained conscious mice after a 12-h overnight fast. The area under the curve (AUC) of glucose against time was calculated for analysis of glucose tolerance as previously described (27). Blood samples of fasted or fed condition were taken for evaluation of serum insulin concentrations by solid-phase enzyme-linked immunosorbent assay using commercially available kits (Linco Research, St. Charles, MI).

Pancreatic islet isolation and insulin secretion. In anesthetized 8-week-old mice, 2 mg/mL collagenase (Worthington Biochemical, Lakewood, NJ) was injected into the bile duct as previously described (28). The pancreas was dissected out, with static incubation at 38°C for 20 min followed by shaking incubation at 38°C for 8 min. After ficoll gradient separation, freshly isolated islets of similar size were handpicked under a stereomicroscope. Islets (20 per well) were preincubated in RPMI-1640 (Sigma-Aldrich, St. Louis, MO), washed, and incubated in 1 mL fresh RPMI-1640 that contained indicated glucose for 30 min (37°C). Fifty microliters of the medium were removed for insulin analysis.

Islets were extracted in acid ethanol (4°C) and stored (–20°C) for insulin content assay. In mice at embryonic day 17 (E17), the pancreas was directly dissected out and incubated in 2 mg/mL collagenase under the same conditions as described above. Isolated islets were incubated in RPMI-1640 containing different glucose concentrations (5 mmol/L or 25 mmol/L) for 24 h (37°C).

Sperm collection. Sperm were obtained from the caudal epididymis of 8-week-old adult male mice. Then, the sperm were washed in phosphate-buffered saline and centrifuged to pellet.

Gene expression (quantitative PCR). Total RNA was isolated from islets or sperm using RNeasy (Qiagen, Valencia, CA). The cDNA was synthesized using oligo-dT and random primers (TaKaRa, Dalian, China) for real-time quantitative PCR (ABI Prism 7900HT; Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase served as an internal control.

DNA methylation (bisulfite genomic sequencing PCR). Genomic DNA was extracted from islets of 8-week-old mice in the control, F1-GDM, and GDM♂-GDM♀ groups. Bisulfite was converted using the EpiTect bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer's instructions to deaminate cytosine to uracil; 5-methyl-cytosine was protected from deamination. Analysis of the methylation status of the *Igf2* and *H19* DMR was determined by cloning and sequencing of bisulfite-treated DNA. The purified PCR products were cloned using the pMD19-T vector system (TaKaRa, Dalian, China). The sequence obtained by cloning was analyzed with 3730 DNA Analyzer polymers (Applied Biosystems, Carlsbad, CA).

Electron microscopy. Samples were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide and then stained with aqueous 2% uranyl acetate for 30 min, dehydrated, and embedded in epoxy resin. Sections of 120 nm were stained with uranyl acetate and lead citrate. Samples were examined at 80 kV

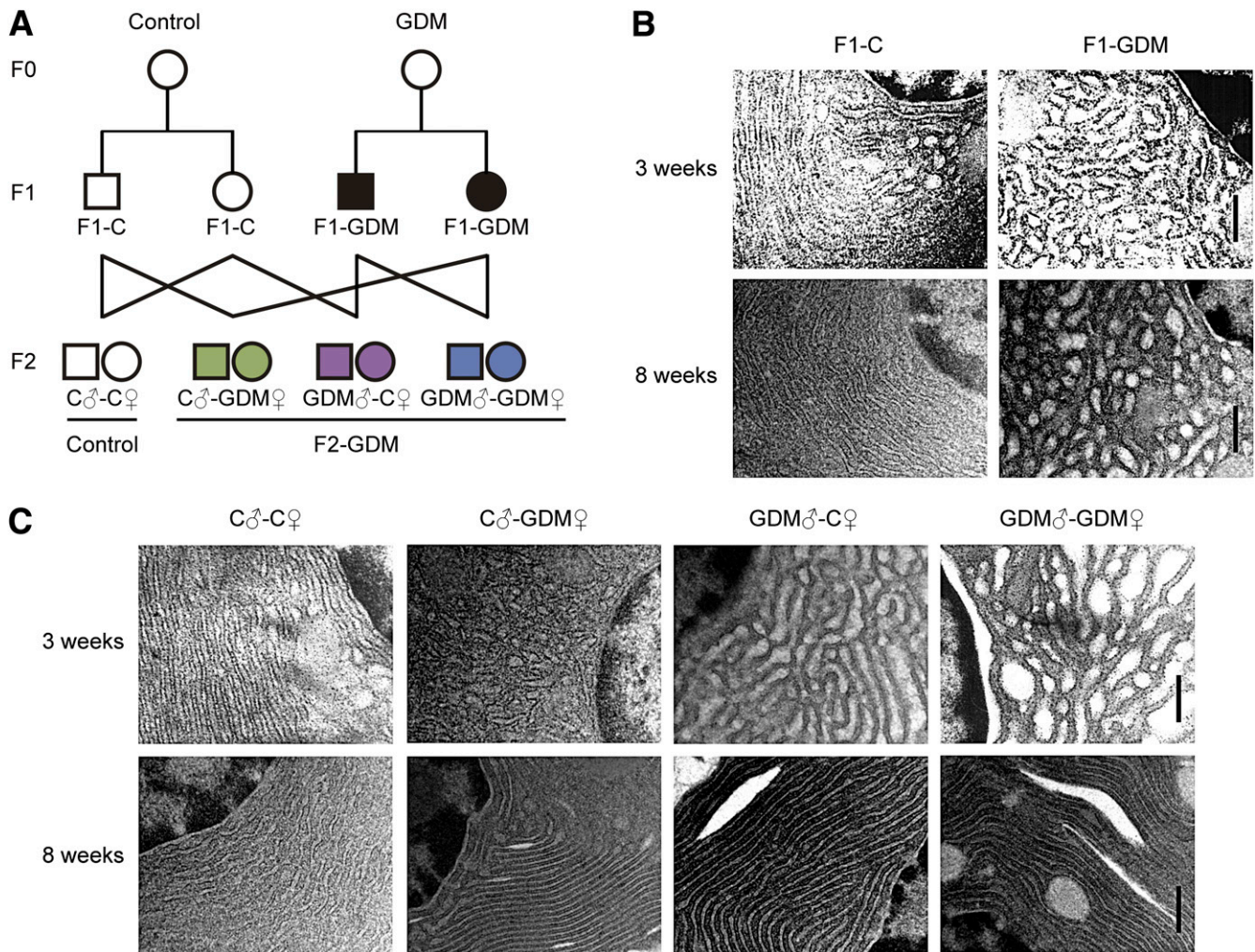


FIG. 1. Experimental design and islet ultrastructure of mice. A: Experimental design. Circles designate females and squares designate males. Note that mating pairs were nonsiblings. The pups from the GDM group were fostered by normoglycemic females until weaned. **B: Islet ultrastructure of F1 offspring under transmission electron microscopy.** Scale bar, 500 nm. **C: Islet ultrastructure of F2 offspring under transmission electron microscopy.** Scale bar, 500 nm.

under a TECNAI 10 transmission electron microscope (Philips, Amsterdam, the Netherlands).

Statistical analysis. Data are presented as means \pm SE. Statistical analysis was performed by unpaired two-tailed Student *t* test, one-way ANOVA, or χ^2 test as described in the Table and Figure legends (version 16.0; SPSS). *P* < 0.05 was considered statistically significant.

RESULTS

Intrauterine hyperglycemia induced transgenerational effect on birth weight and islet structure. We established a GDM mouse model by inducing moderate hyperglycemia after pregnancy. Female and male F1 adults of control and GDM mice were intercrossed to obtain F2 offspring (Fig. 1A). We found that the birth weight of F1-GDM offspring was not changed significantly (Table 1). After fostering by the normoglycemic mice, the body weight of both F1-GDM males and females at 3 or 8 weeks of age was similar to that of F1-C offspring (Table 1). There was no significant difference of birth weight between control and F2-GDM mice born from F1-GDM with relative normal glucose tolerance (data not shown). After birth from F1-GDM with impaired glucose tolerance (IGT), the birth weight in the GDM σ -C φ and GDM σ -GDM φ groups significantly increased compared with that in C σ -C φ (Table 1). However, the birth weight in the C σ -GDM φ group did not increase obviously. There was also no significant difference in birth weight between GDM σ -C φ and GDM σ -GDM φ groups (Table 1). The ratio of pancreas weight to body weight in F1-GDM offspring at 3 weeks was significantly higher than that in F1-C, although there was no difference at 8 weeks (Table 1). With the same tendency of F1-GDM mice, the pancreas weight-to-body weight ratio in F2-GDM offspring whose one and/or two parents experienced intrauterine hyperglycemia was significantly higher than that in the C σ -C φ group at 3 weeks (Table 1). There was no significant difference of pancreatic islet morphology, insulin distribution, or glucagon distribution between F1-C and F1-GDM (data not shown). However, analyzed by transmission electron microscopy, the structure of the endoplasmic reticulum in islet cells was obviously swollen and disordered

in F1-GDM offspring at 3 weeks of age compared with that of F1-C and progressed at 8 weeks of age (Fig. 1B). In F2 offspring, compared with the C σ -C φ group, the structure of the endoplasmic reticulum in islet cells was obviously swollen and disordered in F2-GDM groups at 3 weeks of age, especially in GDM σ -C φ and GDM σ -GDM φ groups (Fig. 1C). At 8 weeks of age, the structure of endoplasmic reticulum in all the F2-GDM groups was almost recovered, with the exception of some fissures (Fig. 1C).

Intrauterine hyperglycemia induced transgenerational transmission of glucose intolerance and abnormal insulin level. Blood glucose levels of all the 3- and 8-week-old F1-GDM and F2-GDM offspring did not differ from those in the corresponding controls in either the fasting or random-fed condition (Table 2). We further performed glucose tolerance test (GTT) by intraperitoneal injection of glucose (2 g/kg body wt). At 3 weeks, in both males and females, there were no differences in the GTT AUC between F1-GDM and F1-C, although the blood glucose level significantly increased in the males at 30 min after injection (Fig. 2A). However, at 8 weeks, IGT was found in both the male and female mice of the F1-GDM group, whose blood glucose level significantly increased at 30 min after injection (Fig. 2B). F1-GDM males showed more evident IGT than females (Fig. 2B). After GTT was performed by intraperitoneal injection of glucose (2 g/kg body wt), IGT appeared in both 3- and 8-week-old male and female F2-GDM offspring through both parental lineages. The male F2-GDM offspring showed much more IGT than females (Fig. 2C and D). At 3 and 8 weeks of age, in both males and females, although fasting glucose levels were normal, fasting insulin levels were much lower in F1-GDM than in F1-C (Table 2). In contrast, in the random-fed condition, the insulin levels of F1-GDM mice significantly increased. The abnormal insulin level of the F1-GDM males was more obvious than that of the F1-GDM females (Table 2). In F2 offspring, the fasting insulin levels of the F2-GDM males at 3 and 8 weeks were significantly lower than those of the control group, and the fasting insulin levels in 8-week-old F2-GDM females were also significantly decreased compared with the control

TABLE 1
Body weight and the ratio of pancreas weight to body weight in F1 and F2 offspring

	F1 offspring		F2 offspring			
	F1-C	F1-GDM	C σ -C φ	C σ -GDM φ	GDM σ -C φ	GDM σ -GDM φ
<i>n</i>	30	20	30	20	20	20
Body weight (g)						
At birth	1.65 \pm 0.02	1.62 \pm 0.01	1.68 \pm 0.02	1.69 \pm 0.03	1.75 \pm 0.02 ^{†‡}	1.78 \pm 0.03 ^{†‡}
At 3 weeks						
σ	13.1 \pm 0.13	12.2 \pm 0.52	13.2 \pm 0.29	13.3 \pm 0.29	13.4 \pm 0.85	13.8 \pm 0.49
φ	13.1 \pm 0.22	11.5 \pm 0.57	13.1 \pm 0.37	12.3 \pm 0.34	12.9 \pm 0.55	13.4 \pm 0.81
At 8 weeks						
σ	37.9 \pm 0.20	38.7 \pm 0.68	38.2 \pm 0.31	37.6 \pm 1.15	37.0 \pm 0.57	39.5 \pm 0.73
φ	30.7 \pm 0.88	30.5 \pm 0.52	31.0 \pm 0.71	29.7 \pm 0.84	32.3 \pm 0.33	28.8 \pm 0.66
Pancreas-to-body weight ratio (%)						
At 3 weeks						
σ	0.26 \pm 0.01	0.48 \pm 0.01*	0.27 \pm 0.02	0.41 \pm 0.03 [†]	0.38 \pm 0.02 [†]	0.41 \pm 0.02 [†]
φ	0.28 \pm 0.03	0.57 \pm 0.03*	0.30 \pm 0.02	0.46 \pm 0.02 [†]	0.41 \pm 0.02 [†]	0.44 \pm 0.02 [†]
At 8 weeks						
σ	0.49 \pm 0.02	0.53 \pm 0.02	0.50 \pm 0.01	0.53 \pm 0.04	0.51 \pm 0.08	0.53 \pm 0.03
φ	0.48 \pm 0.04	0.54 \pm 0.02	0.49 \pm 0.04	0.54 \pm 0.04	0.53 \pm 0.05	0.52 \pm 0.03

Data are means \pm SE. Significance was determined by Student *t* test in F1 offspring and ANOVA in F2 offspring. **P* < 0.05 vs. F1-C. [†]*P* < 0.05 vs. C σ -C φ . [‡]*P* < 0.05 vs. C σ -GDM φ .

TABLE 2
Blood glucose and insulin levels in F1 and F2 offspring

	F1 offspring		F2 offspring			
	F1-C	F1-GDM	C♂-C♀	C♂-GDM♀	GDM♂-C♀	GDM♂-GDM♀
<i>n</i>	8	6	8	6	6	6
Fasting glucose (mg/dL)						
At 3 weeks	82.2 ± 1.5	79.2 ± 3.7	81.0 ± 2.0	82.2 ± 3.3	81.6 ± 2.6	80.4 ± 1.5
♂						
♀	76.8 ± 4.2	79.2 ± 9.0	78.0 ± 3.6	87.0 ± 3.6	80.4 ± 2.1	80.4 ± 6.6
At 8 weeks						
♂	83.4 ± 1.5	82.8 ± 2.0	81.6 ± 1.5	83.4 ± 3.1	85.2 ± 4.2	84.6 ± 8.1
♀	93.0 ± 3.6	97.8 ± 2.4	90.0 ± 3.7	85.8 ± 3.3	91.8 ± 3.6	88.2 ± 4.5
Fed glucose (mg/dL)						
At 3 weeks						
♂	134.4 ± 2.1	139.8 ± 4.6	133.8 ± 2.1	139.2 ± 4.3	142.8 ± 3.1	146.4 ± 5.9
♀	129.0 ± 1.5	127.2 ± 2.6	130.8 ± 1.5	129.0 ± 2.4	132.0 ± 2.1	142.2 ± 5.4
At 8 weeks						
♂	143.4 ± 2.6	151.2 ± 4.7	145.8 ± 2.0	147.6 ± 7.8	147.0 ± 3.6	160.8 ± 13
♀	132.6 ± 1.5	133.8 ± 4.3	132.6 ± 3.1	126.6 ± 2.1	128.4 ± 2.1	130.2 ± 3.9
Fasting insulin (pg/mL)						
At 3 weeks						
♂	552 ± 24	188 ± 12*	508 ± 19	208 ± 26†	250 ± 15†	188 ± 36†
♀	496 ± 26	135 ± 11*	477 ± 13	485 ± 23	346 ± 28†	182 ± 26†
At 8 weeks						
♂	628 ± 36	207 ± 20*‡	653 ± 17	320 ± 29†	344 ± 12†	153 ± 13†
♀	605 ± 13	303 ± 19*	572 ± 21	386 ± 49†	300 ± 14†	129 ± 11†
Fed insulin (pg/mL)						
At 3 weeks						
♂	585 ± 20	1,157 ± 22*‡	535 ± 37	752 ± 18†	847 ± 35†	1,142 ± 79†
♀	597 ± 49	822 ± 11*	539 ± 20	821 ± 73†	1,015 ± 92†	1,137 ± 73†
At 8 weeks						
♂	923 ± 49	1,296 ± 72*	1,169 ± 39	1,036 ± 74	2,539 ± 115†‡	2,839 ± 156†‡
♀	939 ± 13	1,187 ± 68*	941 ± 27	1,063 ± 58	1,316 ± 45†	1,875 ± 55†

Data are means ± SE. **P* < 0.05 vs. F1-C. †*P* < 0.05 vs. C♂-C♀. Significance was determined by Student *t* test in F1 offspring and ANOVA in F2 offspring. ‡*P* < 0.05 vs. female. Significance was determined by Student *t* test.

group (Table 2). In contrast, in the random-fed condition, the insulin levels in all 3-week-old F2-GDM offspring were significantly higher than in controls (Table 2). At 8 weeks, the insulin levels of GDM♂-C♀ and GDM♂-GDM♀ offspring were significantly higher than in C♂-C♀ (Table 2).

Intrauterine hyperglycemia impaired insulin secretion.

To define further the potential secretory defects that could contribute to glucose intolerance in offspring exposed to intrauterine hyperglycemia, we assessed glucose-stimulated insulin secretion of islets isolated from 8-week-old male mice. In the F1-C group, insulin secretion significantly increased with glucose exposure in a dose-dependent manner. By contrast, islets of the F1-GDM group showed higher insulin secretion in response to moderate glucose concentration but lower insulin secretion in response to high glucose concentration compared with F1-C (Fig. 3A). Likewise, regardless of birth from F1-GDM with or without IGT, in F2 offspring in vitro glucose-stimulated insulin secretion was also impaired in the F2-GDM group (Fig. 3B). Impaired insulin secretion in female mice was similar to that of males, despite not being very significant (data not shown).

Intrauterine hyperglycemia downregulated *Igf2* and *H19* in islets.

We collected islets from 8-week-old mice and identified the purity and specificity of islets by incubating with dithizone or detecting insulin with immunofluorescence (Fig. 4A). We found that the relative mRNA levels of *Igf2* and *H19* in islets from 8-week-old males and females were both significantly lower in F1-GDM mice. The abnormal expression of both *Igf2* and *H19* in F1-GDM

males was more obvious than that in females. There were no significant differences in *Plagl1* expression levels between F1-GDM and F1-C (Fig. 4B).

In the F2-GDM offspring, regardless of birth from F1-GDM with or without IGT, the relative mRNA levels of *Igf2* and *H19* in islets from 8-week-old males and females were all significantly lower than those in controls. There were no significant differences in *Plagl1* expression levels between F2-GDM offspring and controls. It is interesting that in F2-GDM offspring, *Igf2* decreased most significantly in the C♂-GDM♀ group, whereas *H19* decreased most significantly in the GDM♂-C♀ group. Showing the same tendency as that in F1-GDM offspring, in F2-GDM offspring, the abnormal expression of *Igf2* and *H19* in males was also more obvious than that in females (Fig. 4C).

In addition, we collected islets of fetal mice at E17 (Fig. 4D). In order to verify the direct effect of high glucose on islet development, fetal islets were cultured in a medium containing different concentrations of glucose for 24 h. We found that the *Igf2* and *H19* mRNA levels were significantly lower in fetal mouse islets exposed to a high glucose concentration (25 mmol/L) than to a physiological concentration (5 mmol/L) (Fig. 4E).

Intrauterine hyperglycemia induced hypermethylation of *Igf2*/*H19* DMRs in islets.

As imprinted genes, *Igf2* and *H19* allelic expression in mice is regulated by allele-specific methylation at four DMRs (21). We collected islets from 8-week-old male mice of the control, F1-GDM, and GDM♂-GDM♀ groups. We analyzed the methylation levels of 12

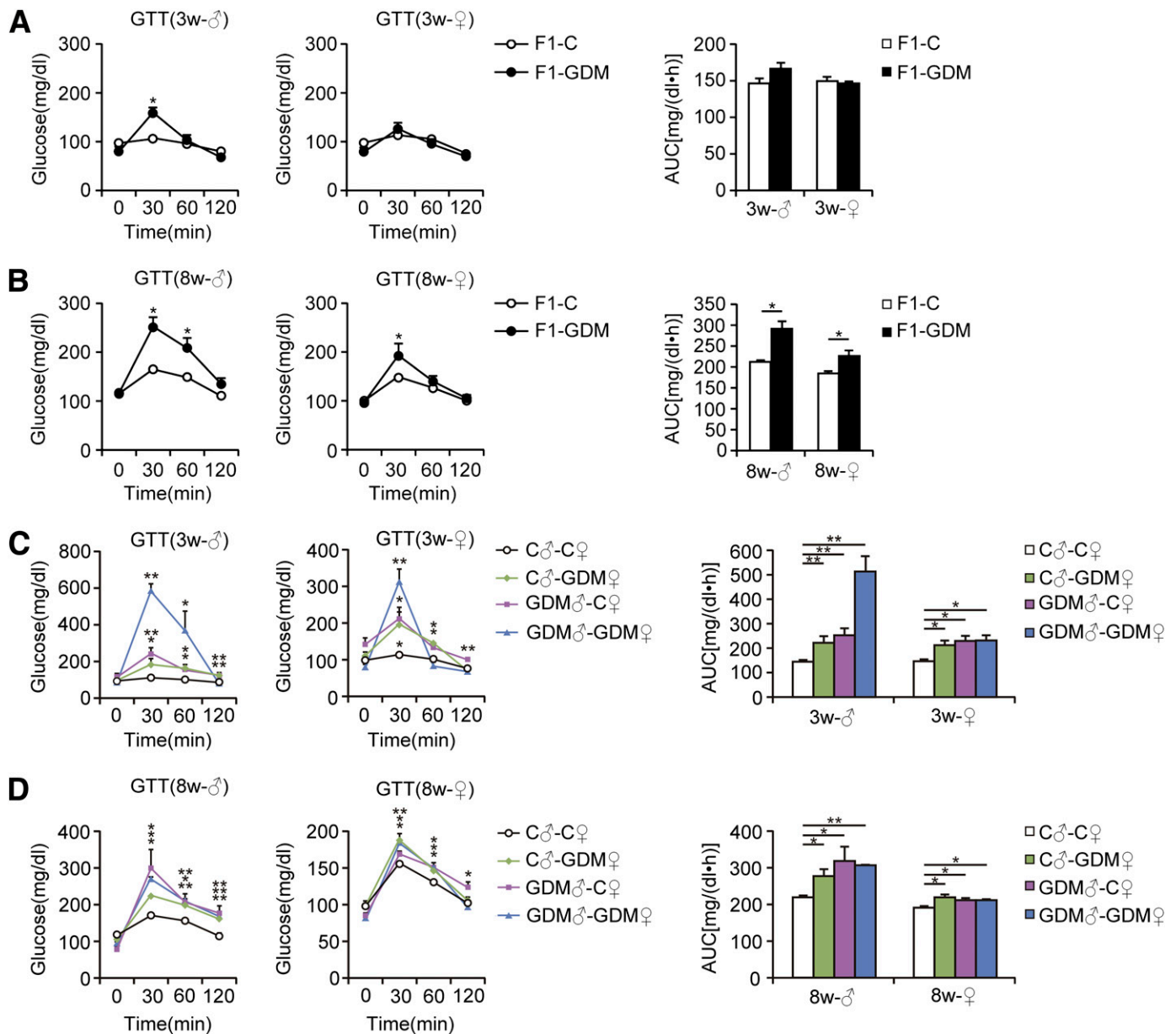


FIG. 2. GTT of F1 and F2 offspring. **A:** GTT (2 g/kg body wt) and AUC of 3-week-old F1 offspring. **B:** GTT and AUC of 8-week-old F1 offspring. **C:** GTT and AUC of 3-week-old F2 offspring. **D:** GTT and AUC of 8-week-old F2 offspring. Data are presented as means \pm SE ($n = 8$ in F1-C and C♂-C♀ groups and $n = 6$ in all other groups). F1 offspring, * $P < 0.05$, ** $P < 0.01$ vs. F1-C (Student t test); F2 offspring, * $P < 0.05$, ** $P < 0.01$ vs. C♂-C♀ (ANOVA). w, week.

cytosine phosphate guanine (CpGs) of the *Igf2* DMR0, 4 CpGs of the *Igf2* DMR1, and 13 CpGs of the *Igf2* DMR2 by bisulfite genomic sequencing PCR. Furthermore, we analyzed the methylation levels of the 12 CpGs located in the CCCTC-binding factor binding sites of the *H19* DMR (Fig. 5A).

In *Igf2* DMR0 and *Igf2* DMR1, the methylation status was all hypermethylated in control, F1-GDM, and GDM♂-GDM♀ groups with no significant differences (Fig. 5B and C). In *Igf2* DMR2, we found that the CpGs were moderately methylated in the control group, while the F1-GDM and GDM♂-GDM♀ groups showed significantly higher methylation levels (Fig. 5D). The *H19* DMR upstream of the *H19* gene (which is 90 kb 3' of *Igf2*) acts as a methylation-sensitive boundary element. Analyzed by bisulfite genomic sequencing PCR, *H19* DMR in both the F1-GDM and GDM♂-GDM♀ groups was much more highly methylated than that in the control group (Fig. 5E).

Intrauterine hyperglycemia affected *Igf2* and *H19* expression in sperm of F1 offspring.

In the GDM♂-C♀ group, without an abnormal intrauterine environment, sperm of F1-GDM was the only factor to affect the offspring. To investigate the underlying mechanism of epigenetic alteration of F2 offspring, we collected sperm of F1-GDM and examined *Igf2* and *H19* expression. F1-GDM male mice were further divided into the obvious IGT group and relative normal glucose tolerance group. We found that, regardless of whether F1-GDM male mice were with or without IGT, *Igf2* and *H19* were both downregulated in their sperm. Moreover, the change of *H19* was more obvious than that of *Igf2* (Fig. 6A and B).

DISCUSSION

Previous studies have demonstrated that low birth weight (LBW) is associated with adult diabetes (29–32). However,

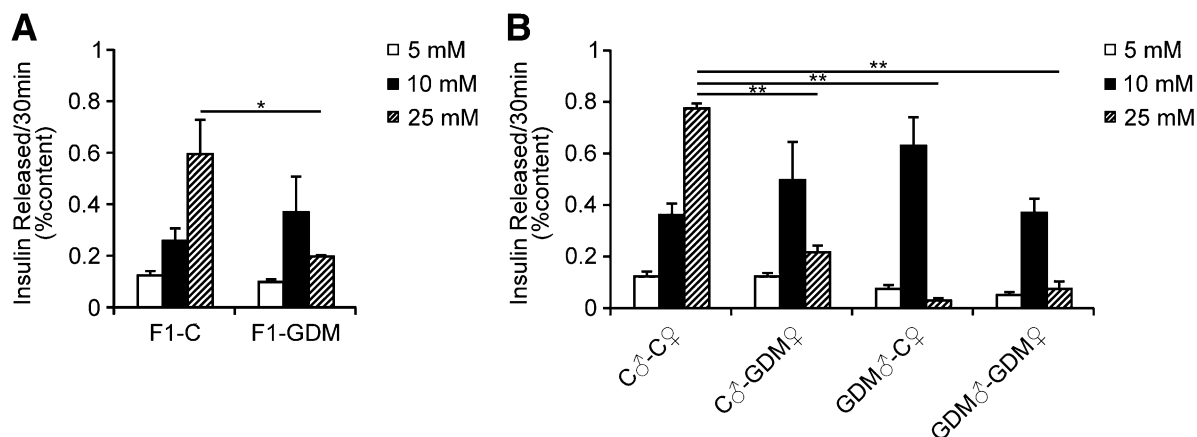


FIG. 3. Glucose-stimulated insulin secretion in vitro. **A:** Glucose-stimulated insulin secretion in islets isolated from 8-week-old mice of F1 offspring ($n = 5$ replicates/group in at least two independent isolations). **B:** Glucose-stimulated insulin secretion in islets isolated from 8-week-old mice of F2 offspring ($n = 4$ replicates/group in at least two independent isolations). Isolated islets were incubated with the indicated glucose concentration for 30 min. Results are expressed as means \pm SE. * $P < 0.05$; ** $P < 0.01$. Significance was determined by Student t test in F1 offspring and ANOVA in F2 offspring.

in clinical practice, newborns of mothers with GDM often have normal or higher birth weight, suggesting that the mechanism of high risk of diabetes in GDM offspring may be different from that of LBW-related adult diabetes.

In our study, moderate intrauterine hyperglycemia during pregnancy did not obviously affect birth weight in F1 offspring. Although the body weight was similar, the pancreas weight in 3-week-old F1-GDM was significantly higher than that of controls. Endoplasmic reticulum in pancreatic islets of F1-GDM was swollen and disordered, which suggests that hyperglycemia of intrauterine environment may lead to disorganization of fetal islet cells. The abnormal islet ultrastructure may be related to impaired β -cell activity and the development of IGT, which is primarily caused by insulin deficiency. Actually, although there was no difference in the fasting and fed blood glucose levels, the fasting insulin levels were decreased while random-fed insulin levels were increased in both the male and female F2-GDM offspring. IGT occurred in adults of the F1-GDM group. In vitro experiment also verified reduced glucose-stimulated insulin secretion in F1-GDM islets. Moreover, glucose intolerance of male GDM offspring was more obvious than that of female offspring, suggesting that male fetus might be more sensitive to the intrauterine environment than female.

Human epidemiological investigation indicated a link between mothers exposed to famine in utero with LBW in their offspring (33). Animal models have shown that maternal undernutrition during pregnancy could induce LBW, IGT, and obesity in both first- and second-generation offspring (34,35). In our study, after exposure to intrauterine hyperglycemia, transgenerational transmission was found in F2 offspring with parental characteristics. In F2 offspring born from F1-GDM with IGT, birth weight increased through the paternal line, but not maternal line, indicating that abnormal intrauterine environment had transgenerational effects on the birth weight in a parental-specific manner. For F2 offspring, compared with maternal line, impact factors of the paternal line are much more prone to be transmitted. The pancreas weights in 3-week-old F2 offspring after intrauterine hyperglycemia were all significantly higher than in controls. IGT and abnormal insulin levels appeared as early as 3 weeks and progressed until

adulthood in both males and females, through both parental lineages, although the endoplasmic reticulum in adult islets was recovered. Impaired glucose-stimulated insulin secretion in vitro showed in islets of F2-GDM offspring. The intrauterine hyperglycemia induced transgenerational transmission of glucose intolerance and abnormal insulin levels. In all F2-GDM offspring groups, IGT of males was more obvious than in females, suggesting that the effect of intrauterine hyperglycemia on F2 males was more obvious than in F2 females. A number of diabetes-related animal models show sex differences, with males having a more profound phenotype (36). In the Nagoya-Shibata-Yasuda (NSY) mice, there is a marked sex difference, with almost all males developing hyperglycemia but less than one-third of females being affected (37). Many genetically induced forms of insulin resistance have a more severe phenotype in males than in females (38,39). The sex differences in transmission of phenotypes indicate that epigenetic mechanisms may be involved in transgenerational effect.

Reduced glucose-stimulated insulin secretion may contribute to impaired glucose tolerance in both F1 and F2 offspring from intrauterine hyperglycemia. Many factors are related to β -cell dysfunction. In the current study, we focused on the imprinted genes involved in islet development and the pathogenesis of diabetes: *Igf2*, *H19*, and *Plagl1*. Downregulated expression of *Igf2* and *H19* exhibited not only in F1-GDM but also in F2-GDM offspring through both paternal and maternal lines, confirming that dysregulation of *Igf2* leads to inappropriate insulin production and secretion and induces diabetes (15–17). Moreover, in F2-GDM offspring, decreased level of *Igf2* and *H19* expression was associated with parental characteristics. *Igf2* reduced the most through the maternal line, while *H19* was reduced the most through the paternal line. In accordance with the tendency of phenotype changes, the change of imprinted gene expression was also more obvious in male offspring than in female.

In vitro culture confirmed the effect of high glucose on *Igf2* and *H19* expression in fetal islets, providing a probable explanation for intrauterine hyperglycemia directly influencing imprinted genes and inducing β -cell dysfunction. However, during pregnancy period of adult

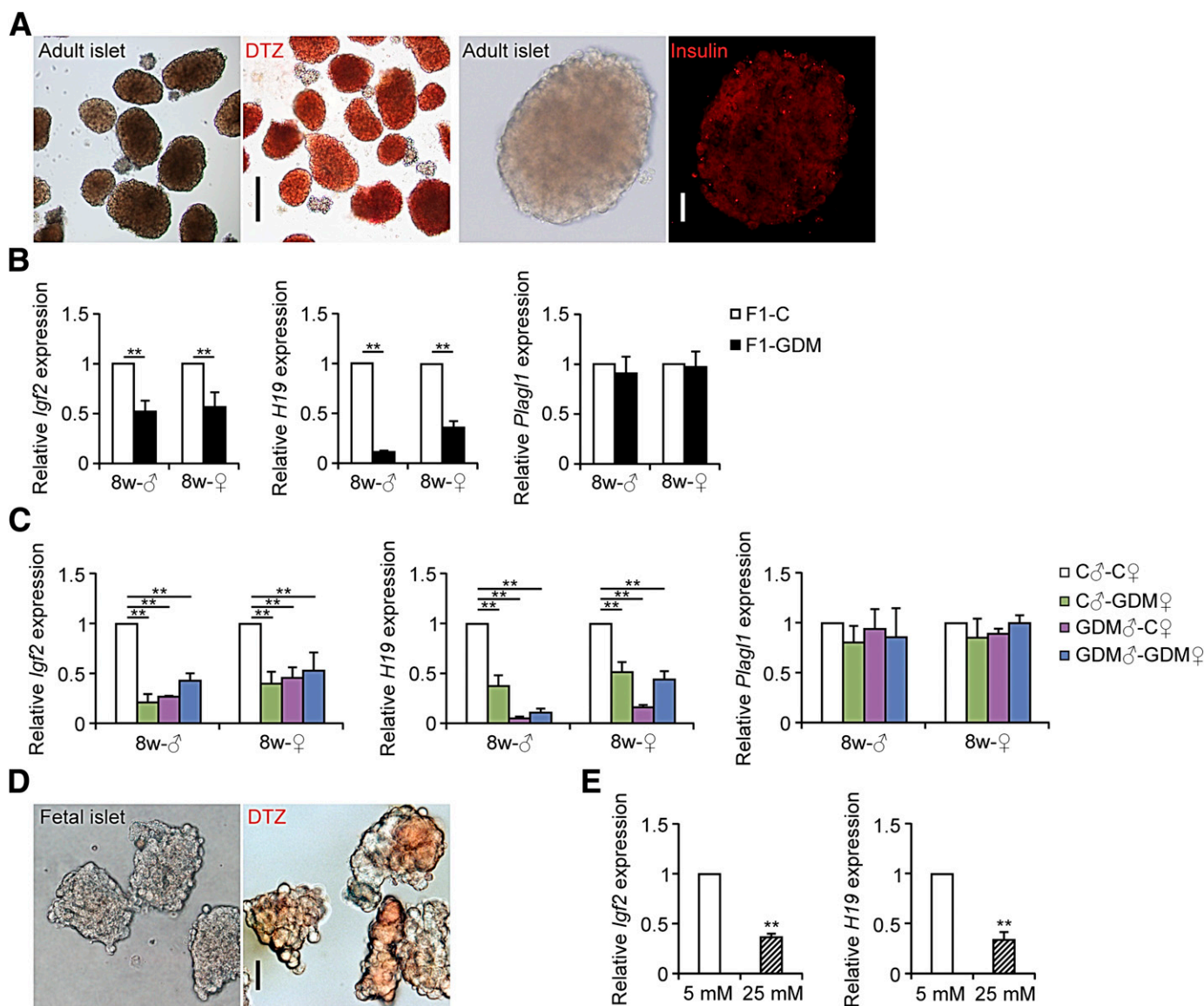


FIG. 4. *Igf2*, *H19*, and *Plagl1* gene expression assessed by real-time quantitative PCR. **A:** Adult islets were identified by incubating with dithizone (DTZ) and detecting insulin with immunofluorescence. Black scale bar, 200 μ m; white scale bar, 50 μ m. **B:** *Igf2*, *H19*, and *Plagl1* gene expression in islets of 8-week-old F1 offspring ($n = 6$ mice in F1-C group; $n = 4$ mice in F1-GDM group). **C:** *Igf2*, *H19*, and *Plagl1* gene expression in islets of 8-week-old F2 offspring ($n = 6$ mice in C σ -C ϕ group; in F2-GDM groups, $n = 4$ mice per group). **D:** Fetal islets were identified by incubating with dithizone. Scale bar, 50 μ m. **E:** *Igf2* and *H19* gene expression in fetal islets cultured in medium containing indicated concentrations of glucose for 24 h ($n = 5$ replicates/group in at least two independent isolations). Data were analyzed with the Eq. $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ (treatment group) - ΔCT (control group), and $\Delta CT = CT$ (sample) - CT (internal control). The values were presented as relative expression levels of mRNA. In all panels, results are expressed as means \pm SE. ** $P < 0.01$. Significance was determined by Student *t* test in F1 offspring and ANOVA in F2 offspring. (A high-quality digital representation of this figure is available in the online issue.)

female F1-GDM, the random glucose level was normal, which means both fetus of C σ -GDM ϕ and GDM σ -GDM ϕ groups developed in a relatively normoglycemic intrauterine environment. Especially in the GDM σ -C ϕ group, there was a completely normal maternal metabolic environment during development in utero. The paternal factor of F1 offspring played a more important role in growth and glycometabolism of F2 offspring, suggesting that epigenetic changes occurring in the germ line and inherited through meiosis (13,14,40) may be an explanation for transgenerational transmission. Thus, we further investigated the expression of *Igf2* and *H19* in sperm of adult F1-GDM male mice. It is interesting that, regardless of whether F1-GDM male mice were with or without IGT, *Igf2* and *H19* were both downregulated in their sperm.

Moreover, the change of *H19* was more obvious than that of *Igf2*. Although relevant epigenetic modification needed further exploration, the results indicated that intrauterine hyperglycemia could alter imprinted gene expression of germ cells and transmitted the risk of disease to next generation.

Parental imprinting is an important genetic mechanism by which some genes are repressed or expressed depending exclusively on their inheritance from mother or father (41). DNA methylation can control gene expression by modulating enhancer access to the gene promoter through regulation of an enhancer boundary. *Igf2*, an imprinted gene, shares an enhancer with its neighboring imprinted gene *H19* (42–45). *Igf2* and *H19* allelic expression in mice is regulated by allele-specific methylation at four *Igf2/H19* DMRs: *Igf2*

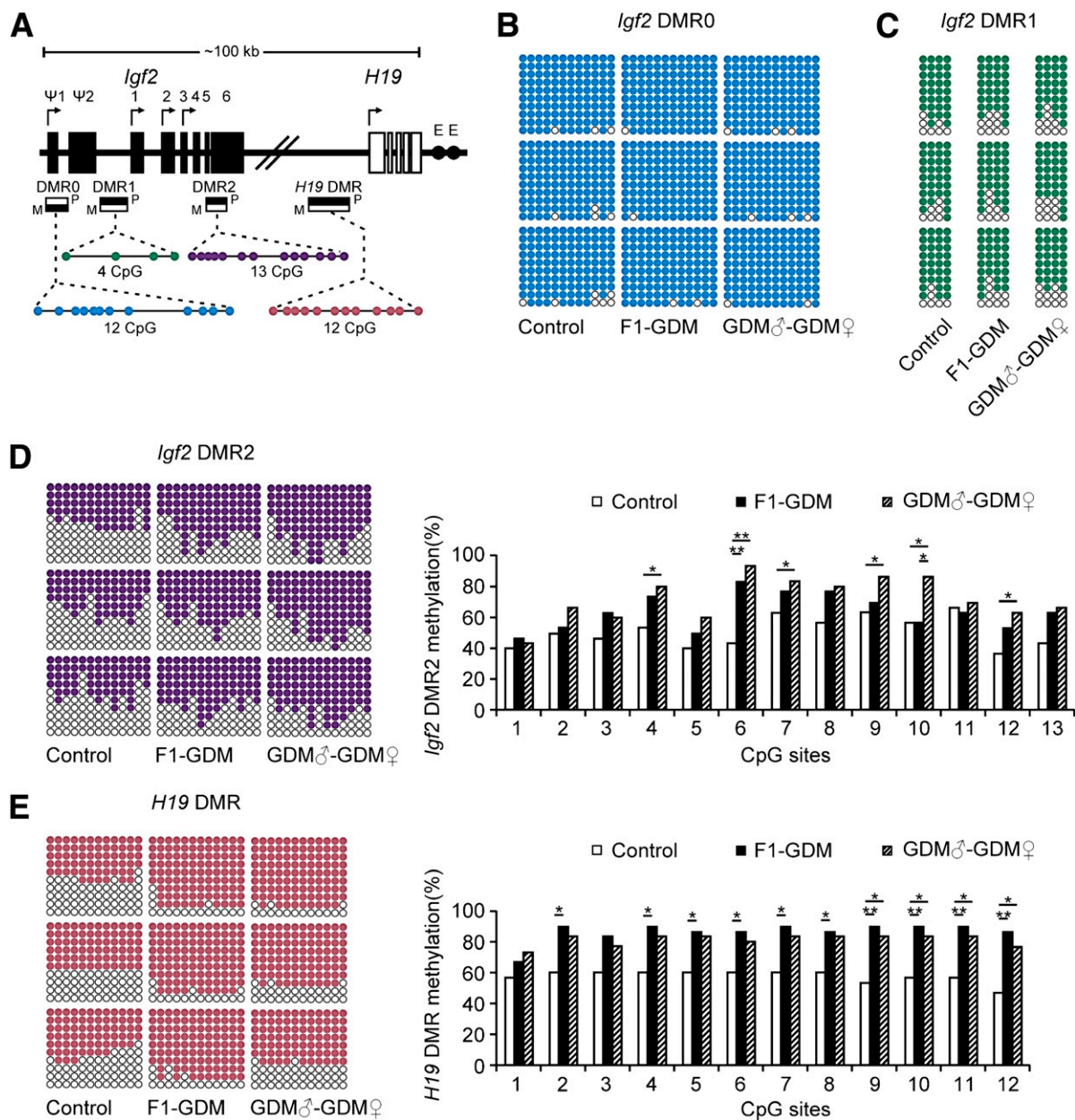


FIG. 5. Methylation analysis of *Igf2*/*H19* DMRs by bisulfite genomic sequencing PCR. **A:** Schematic representation of mouse imprinted locus, showing the relative positions of the *Igf2* and *H19* genes and indicating the location of the four DMRs known to contribute to *Igf2* imprinting. The number and position of upstream exons ($\Psi 1$ and $\Psi 2$ for *Igf2*) and exons are shown as black (*Igf2* gene) and white (*H19* gene) rectangles with arrows for transcription start sites. Enhancers (E) are indicated as black circles. The locations of the four DMRs within the *Igf2*/*H19* imprinted locus represented by boxes are shaded to indicate preferential methylation of the maternal (M) or paternal (P) allele in each region. **B:** Methylation status of individual DNA strands of *Igf2* DMR0 containing 12 CpG sites. **C:** Methylation status of individual DNA strands of *Igf2* DMR1 containing 4 CpG sites. **D:** Methylation status of individual DNA strands of *Igf2* DMR2 containing 13 CpG sites and the average methylation ratio in each CpG site. **E:** Methylation status of individual DNA strands of *H19* DMR containing 12 CpG sites and the average methylation ratio in each CpG site. Ten clones per mouse; a total of 30 clones per group were sequenced. Each line represents the sequence of a single clone. CpG sites are shown as blank (unmethylated) or filled (methylated) circles. In the histograms, results are expressed as methylation percentage of each CpG site ($n = 3$ mice per group). * $P < 0.05$, ** $P < 0.01$ vs. control (χ^2 test).

DMR0, *Igf2* DMR1, *Igf2* DMR2, and *H19* DMR (18,46,47). Because IGT and gene alteration were both more obvious in male offspring, we examined all of the *Igf2*/*H19* DMRs in islets of F1-GDM and GDM♂-GDM♀ male mice. Although there were no differences in methylation of *Igf2* DMR0 and *Igf2* DMR1 compared with controls, in islet of F1-GDM and GDM♂-GDM♀ the methylation levels were significantly higher in *Igf2* DMR2, which is a methylation-sensitive activator within the last exon of *Igf2* (18). The methylation levels of *H19* DMR were also significantly much higher than in

controls. These results indicate that intrauterine hyperglycemia could cause hypermethylation at *Igf2* DMR2 and *H19* DMR in both F1 and F2 offspring, suggesting that altered expression of *Igf2* and *H19* in islets is associated with the altered modification of *Igf2* DMR2 and *H19* DMR. Previous studies have also supported that the methylation state at *Igf2* DMR2 is especially labile. Maintenance of the methylation status at DMR2 depends on interactions with other *Igf2*/*H19* DMRs; mice carrying a deletion of either the *H19* DMR or *Igf2* DMR1 show aberrant methylation at *Igf2* DMR2, whereas

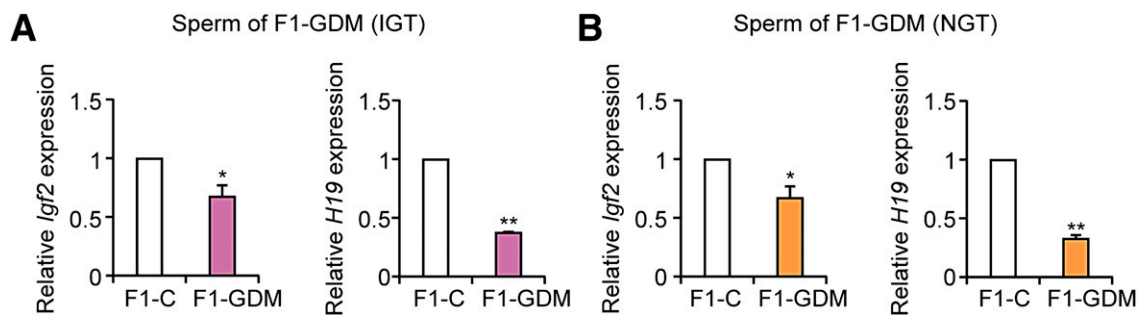


FIG. 6. *Igf2* and *H19* gene expression in sperm. **A:** *Igf2* and *H19* gene expression in sperm of F1-GDM with obvious IGT. **B:** *Igf2* and *H19* gene expression in sperm of F1-GDM with relative normal glucose tolerance (NGT). Data are presented as means \pm SE ($n = 6$ mice per F1-C group, $n = 5$ mice per F1-GDM group). * $P < 0.05$, ** $P < 0.01$ vs. F1-C (Student *t* test).

deletion of *Igf2* DMR2 does not reciprocally affect the other DMRs (47). Importantly, human genomic regions corresponding to mouse *H19* DMR, *Igf2* DMR0, and *Igf2* DMR2 have been identified and exhibit allele-specific methylation (48–50).

In conclusion, using a GDM mouse model we found that during pregnancy, intrauterine environment with moderate hyperglycemia did not affect birth weight of F1-GDM but increased birth weight of F2 offspring born from F1-GDM with IGT, which showed sex-specific characteristics. Furthermore, intrauterine hyperglycemia induced IGT and abnormal insulin levels in both F1 and F2 offspring regardless of birth from F1-GDM with or without IGT, which are partly due to the deficient islet ultrastructure. Abnormal *Igf2* expression related to dysregulation of *Igf2/H19* methylation of islet is one of the mechanisms involved in the effects of intrauterine hyperglycemia on F1 and F2 offspring. Altered *Igf2* and *H19* gene expression was also found in sperm of adult F1-GDM with or without IGT, indicating that changes of epigenetics in germ cells contributed to transgenerational transmission.

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G.-L.D. and F.-F.W. designed and performed experiments, analyzed data, and wrote and edited the manuscript. J.S., S.T., Y.J., D.Z., and N.W. researched data and contributed to discussion. Q.L., Y.Z., F.J., and P.C.K.L. contributed to discussion and edited the manuscript. J.-Z.S. and H.-F.H. designed and supervised the research, contributed to discussion, and edited the manuscript. H.-F.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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