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An *Arthrobacter* spp. Bacteremia Leading to Fetal Death and Maternal Disseminated Intravascular Coagulation

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A 34-year-old parous woman developed high fever and threatened preterm labor after a 1-day trip, for which she was receiving prenatal care at a hospital. Three days after onset, at 24 4/7 weeks of gestation, she was transferred to our hospital in an emergency. Soon after the woman's arrival at our hospital, the infant was spontaneously stillborn via a transvaginal delivery. Laboratory tests revealed severe maternal disseminated intravascular coagulation with renal and liver insufficiency. Histopathologic examination of the placenta revealed vast fibrin deposition and remarkable neutrophilic infiltration in the intervillous space, suggesting a rare bacterial infection caused by *Arthrobacter* spp. The bacteria were predominantly detected in the placenta and maternal blood serum by common bacterial 16S rRNA sequencing after polymerase chain reaction amplification. We report the first case, to our knowledge, of bacteremia with *Arthrobacter* spp., which may lead to maternal disseminated intravascular coagulation and intrauterine fetal death.

Keywords arthrobacter, disseminated intravascular coagulation, fetal death, sepsis

INTRODUCTION

Maternal sepsis is sometimes related to disseminated intravascular coagulation (DIC) during pregnancy. A hypercoagulable state during pregnancy may favor the ensuing formation of intravascular fibrin during severe sepsis and possibly contribute to DIC pathogenesis [1]. *Escherichia coli* is frequently responsible for severe sepsis during pregnancy [2]. To our knowledge, severe sepsis during pregnancy caused by *Arthrobacter* spp. is not yet described in the literature. During maternal sepsis, pathogens in the maternal blood, which reach the placental intervillous space of the placenta, can spread to the fetus through the villous capillaries [3, 4]. Intervillositis causes formation of a placental abscess [5]. We describe the case of a 34-year-old woman who experienced an acute onset of severe sepsis caused by *Arthrobacter* spp., which was followed by placental abscess formation and eventually intrauterine fetal death (IUFD).

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CASE REPORT

A 34-year-old parous woman had been receiving prenatal care at a maternity hospital since early gestation and was in a healthy condition. At 24 1/7 weeks of gestation, she went on a 1-day trip to the seashore, following which she developed a mild fever (37°C) and malaise. On the following day, she visited the hospital where she was given routine prenatal care. On examination, her temperature was 39°C. Urinalysis revealed 1+ protein content with 10^4 white blood cells per high power field. She was diagnosed as having a urinary tract infection, and was prescribed oral antibiotics (cefcapene pivoxil hydrochloride hydrate, 100 mg thrice a day for 2 days) for the same. However, the high fever persisted till the following day, and she also experienced vaginal bleeding and lower abdominal pain. She was admitted to the hospital and administered beta-adrenergic drugs in addition to the oral antibiotics for the treatment of threatened preterm labor. At 24 4/7 weeks of gestation, IUFD was detected, and she was transferred to our hospital in an emergency.

On admission, the patient had atopic dermatitis, although she had refrained from taking any medication during this pregnancy. Her previous delivery was through caesarean section because of pregnancy-induced hypertension. She did not consume alcohol, smoke, or use illicit drugs. Her mother had a history of breast cancer. On examination, her temperature was 36.6°C, blood pressure 102/66 mm Hg, pulse 120 beats/min, and oxygen saturation 100% in room air. Her abdomen was hard. A pelvic examination revealed vaginal bleeding and continuous uterine contractions. The cervix was dilated to 4 cm and was 80% effaced. Ultrasonography of the uterus revealed absence of the fetal heart beat. The test results for coagulation as well as those for renal and liver function were abnormal. The platelet count and hemoglobin level were low, whereas the C-reactive protein level was high; laboratory test results are shown in Table 1.

Upon arrival, delivery was in progress. Approximately 1 h later, she gave birth to a stillborn infant weighing 635 g via transvaginal delivery. Fetal appearance was normal. The amniotic fluid was clear and did not have a foul odor.

On the first day in our hospital, she received fresh frozen plasma (1080 mL) and platelet (600 mL) transfusions to treat DIC. She also received red blood cell (560 mL) transfusion for anemia. At midnight, she developed a high fever (39.5°C) with chills. Blood and urine bacterial cultures were taken, following which she was administered intravenous antibiotics (ceftoriaxon 1 g twice a day and clindamycin 600 mg twice a day). Several hours after onset of the high fever, the oxygen saturation decreased. A chest X-ray revealed pulmonary edema. Results of post-transfusion tests revealed prolonged insufficiency of coagulation and renal and liver insufficiency. Laboratory test results are shown in Table 1. On the second hospital day, she was admitted to the intensive care unit (ICU), where she received flosemide and gabexate mesilate in addition to the above-mentioned intravenous antibiotics. She was discharged from the ICU in good condition on the fifth day. At a 3-month follow-up visit, she was healthy.

The patient's placenta was subjected to a complete histopathologic examination to determine the cause of IUFD. Gross examination had revealed severe fibrin deposition on the maternal surface of the placenta. Histologic examination revealed remarkable neutrophilic infiltration in the intervillous space, diffuse severe intervillous abscess (acute intervillositis covering almost the entire placenta), thrombosis, massive intervillous fibrin deposition, villitis, and mild chorioamnionitis (Figure 1). These findings suggested that the bacterial infection had spread to the placenta hematogenously. We obtained placental specimens and examined them using polymerase chain reaction (PCR) to identify pathogenic microorganisms. 16S rRNA gene

TABLE 1 Laboratory Data

	Reference range	On admission,	4 h after	2nd Day, 20 h after	3rd Day	5th Day
	Adults	This Hospital	Admission	Admission		
Hemoglobin (g/dl)	11.5–15	11.1	6.9	10.4	9.7	9.5
White-cell count (per mm^3)	4000–9000	7600	7200	11,000	10,300	6900
Platelet Count (per mm^3)	150,000–350,000	10,000	42,000	19,000	28,000	70,000
Fibrinogen (mg/dl)	200–400	142	152	212		339
Fibrinogen degradation product (μg/ml)	0–5	294.9	113.3	47.8		42.7
Aspartate aminotransferases (U/L)	12–35	110	74	85	51	37
Alanine aminotransferases (U/L)	5–30	37	28	34	30	28
Total birilubin (mg/dl)	0.2–1.2	3.6	2.6	3.2	3.5	1.6
Creatinine (mg/dl)	0.4–0.8	4.6	4.61	4.71	4.77	4.22
C-reactive protein (mg/dl)	0–0.3	22.1	12.3	16.7	23.8	5.4

The ranges used at Kansai Rosai Hospital are for adults who are not pregnant and do not have medical conditions that could affect the results.

was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, California) under the following cycling conditions: initial denaturation at 94°C for 10 min followed by 32 cycles at 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplicon was obtained using a primer set, 16S rRNA-357f 5'-ctcctacgggaggcagcag-3', and 16S rRNA-1100r 5'-gggttgcgctcgttg-3'[6]. These sequences were compared to sequence data deposited in GenBank using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We identified *Staphylococcus* spp. and *Arthrobacter* spp. (Table 2). The latter was not identified in the control placenta sample (data not shown). The blood and urine bacterial cultures that were taken on the second hospital day were both negative. We used PCR to examine the maternal serum that was taken on the first hospital day to identify pathogenic microorganisms and identified dominance of *Arthrobacter* spp. (Table 3). These results indicated that severe sepsis due to *Arthrobacter* spp. may have contributed to DIC and multiple organ insufficiencies, and the infections may have spread to the placenta hematogenously leading to placental abscess formation and IUFD.

Autopsy permission was declined, so we were unable to determine the exact cause of fetal death. Massive intervillous fibrin deposition also might be one of the causes of IUFD. However, massive intervillous fibrin might be related to the infection. So, we think the presence of diffuse severe placental abscess suggests fetal systemic infection that may have caused death.

DISCUSSION

The clinical course and histopathologic examination results revealed maternal DIC and IUFD due to severe maternal sepsis. Despite administration of oral antibiotics,

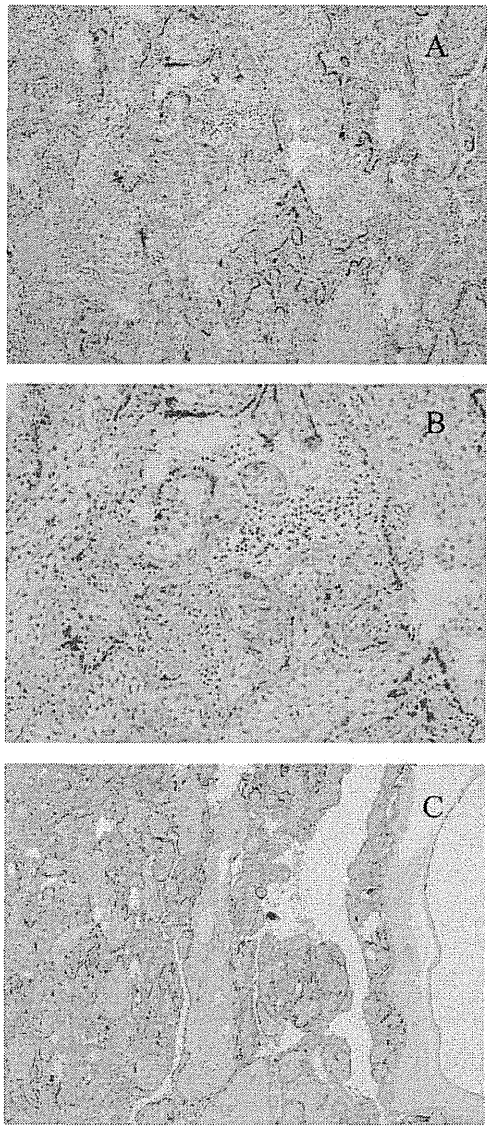

FIGURE 1 Histopathologic findings of the placenta. Photomicrograph demonstrating severe intervillous abscess formation and mild Leukocyte infiltration of subchorionic area (H & E, original magnification: ×10 [A], ×40 [B], ×4 [C]).

her infection rapidly progressed to sepsis and IUFD in only three days. *Arthrobacter* spp. was the causative pathogen in this case.

Arthrobacter spp. are often found in the environment and are recognized as opportunistic pathogens [7]. Human *Arthrobacter* spp. infections are very rare; therefore, we decided to conduct further examinations to confirm the same. Vaginal culture taken at 9 weeks post-partum revealed *K. pneumoniae* and *E. faecalis* infection. *Lactobacillus* spp., major bacteria in the normal vaginal flora, were not detected. *Ureaplasma parvum* (serotype 1) was detected in the vaginal smear by PCR amplification of 16S rRNA gene and MBA gene specific to the *Ureaplasma* spp. genome (data not shown). This abnormal composition of vaginal flora may have adversely affected *Arthrobacter* spp. bacteremia and the in-utero infection.

TABLE 2 Identification of bacterial DNA detected in the placental tissue

PCR results	
Staphylococcus spp.	31
Staphylococcus warnei	8
Staphylococcus epidermidis	9
Staphylococcus pasteuri	5
Staphylococcus lugdnensis	1
Staphylococcus undifferentiated	9
Lactococcus lactis	23
Arthrobacter spp.	17
Corynebacterium tuberculostearicum	3
Sphingomonas spp.	3
Lactobacillus casei	2
Propionibacterium acnes	2
Citrobacter freundii	1
Dietzia cinnamea	1
Streptococcus spp.	0
Streptococcus mitis	0
Streptococcus pneumonia	0
Bradyrhizobium spp.	0
Total	84

PCR amplification of 16S rRNA genes was used. The amplified 84 products were sequenced and determined. *Arthrobacter* spp. was dominantly identified.

Certain pathologic conditions in humans, such as scleroderma, bacteremia, vaginitis, and urine infections, are related to *Arthrobacter* spp. [8]. We have little knowledge of the pathogenic potential and clinical significance of this bacterial species [7, 9]. Our case indicates that *Arthrobacter* spp. are potential causative bacteria for severe sepsis during pregnancy.

TABLE 3 Identification of bacterial DNA detected in maternal serum.

PCR results	
Arthrobacter spp.	14
Staphylococcus epidemidis strain	2
Acinetobacter spp.	3
Streptococcus spp.	1
Brevundimonas spp.	2
Methylobacterium spp.	1
Staphylococcus warmeri strain	1
Propionibacterium acnes	1
Stenotrophomonas maltophilia strain	2
total	27

Sample was obtained on the first hospital day. PCR amplification of 16S rRNA gene was used. The amplified 27 products were sequenced and determined. Arthrobacter spp. was dominantly identified.

In this case, urinalysis performed at a previous hospital revealed bacteriuria. Considering these results and the clinical course, pyelonephritis was most likely the primary cause of maternal sepsis.

Previously, we have reported the characteristic pathologic features of the human placenta during *Ureaplasma* spp. Infections [10] and placental dysfunctions after systemic exposure of nanoparticles to maternal circulation in mice [11]. In this case, however, pathologic features of diffuse severe placental abscess were quite unique. To detect the possible etiologies, we retrospectively performed common bacterial 16S rRNA gene sequencing. *Arthrobacter* spp. were dominantly detected in the patient's placental tissue, but not in that of the control case. However, *Arthrobacter* spp. abundantly exist in the environmental soil. Another evidence of maternal systemic infection by *Arthrobacter* spp. was therefore needed to determine the etiology. Unfortunately, we did not have any blood samples at the onset, so we tried to detect bacterial DNA from the residual serum, which was not a routine strategy. As mentioned above, because *Arthrobacter* spp. were detected in both the placenta and residual blood sample, we report the first case, to our knowledge, of *Arthrobacter* spp. causing maternal DIC and IUFD.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Comparison of risk factors for gestational hypertension and preeclampsia in Japanese singleton pregnancies

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Abstract

Aim: To demonstrate the difference between risk factors for gestational hypertension (GH) and preeclampsia (PE).

Material and Methods: Using data from women with no essential hypertension and with singleton births between 2001 and 2005 delivering after 22 weeks of gestation at 125 centers in Japan (Japan Perinatal Registry Network) ($n = 241\,292$), we compared risk factors for GH and PE. Odds ratios were calculated using multivariate logistic regression analyses.

Results: Of 241 292 women, 2808 (1.2%) developed GH and 6423 (2.7%) developed PE. Thirty-five years or older, primiparity, diabetes mellitus, and renal disease increased the risk of both hypertensive conditions. Forty years or older was a risk factor only for GH, while primiparity, female baby, and renal disease were risk factors only for PE. Early-onset was a common risk factor for small-for-gestational-age (SGA) in GH and PE, but in late-onset only PE was a risk factor for SGA. The main population of SGA infants was composed of PE cases because PE accounted for 83.3% of early-onset type before 32 weeks. Girl preponderance in the PE women was observed (sex ratio: boys/girls = 0.904), while slight boy preponderance was seen in normotensive women (1.06) and GH (1.02).

Conclusion: Preeclampsia is associated with lower fetal sex ratio (girl preponderance) compared to GH or normotensive. Presence of hypertension is a risk factor for SGA in early-onset GH and PE, and hypertension and proteinuria are risk factors for SGA in late-onset group.

Key words: gestational hypertension, hypertension in pregnancy, preeclampsia, risk factor, sex ratio, small-for-gestational-age infant.

Introduction

Hypertensive disorders of pregnancy, consisting of gestational hypertension (GH) and preeclampsia (PE), approximately 7–12% of all singleton pregnancies worldwide, are the most important complications for maternal and perinatal morbidity and mortality.[1] GH and PE share many risk factors, while the complications associated with GH are less frequent and less severe. For example, Ros *et al.* showed the similarities in risk factors for these hypertensive conditions[2] and more recently, Villar *et al.* demonstrated that these disorders shared many risk factors.[3] However, although these reports showed the similarities in risk factor patterns, little attention has been paid to the selection of mothers. Because those studies have been limited to nulliparas aged 34 years or less, or contained about one percent of multiple pregnancies, there was a possibility that parity and twin pregnancy might mask subtle differences between GH and PE. In addition, it is well known that the frequency of small-for-gestational-age (SGA) infants in preeclamptic women is higher

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compared to that in GH cases, and yet the reason is not fully clarified.

The paper focused on the differences in risk factors and in perinatal outcomes between GH and PE in Japanese women. Using the data from the Perinatal Database of Japan Society for Obstetrics and Gynecology from 2001 through 2005, we clarified the clinical features characteristic of these two disorders (maternal backgrounds and perinatal outcomes) and we compared the frequencies of SGA in each gestational age between GH and PE.

Material and Methods

The Tokyo Women's Medical University Ethics Committee approved this study. Since 2001 the perinatal database (DB) has been assembled by the perinatal committee in the Japan Society of Obstetrics and Gynecology under a cooperative agreement with secondary or tertiary hospitals in Japan. This DB protects patients' anonymity because of unlinked information. We analyzed maternal background and perinatal outcomes in singleton pregnancies without essential hypertension ($n = 241\,292$) after 22 weeks of gestation at 125 centers of the perinatal network in Japan from 2001 through 2005, as previously described.[4] The Japan Perinatal Registry Network was started in 1974 and is managed by the Japan Society of Obstetrics and Gynecology. This network comprised of 125 perinatal medical centers in Japan, including 76 university hospitals, 14 national hospitals, 10 Red Cross hospitals, and 25 other hospitals. The database we used was converted to its present database structure in 2001. It included all live births and stillbirths after the 22nd week at those 125 perinatal medical centers of perinatal research network in Japan from 2001 to 2005 and covered 5.2% (56 671 registered births) of the total 1 094 434 births in Japan in 2005.

Women were classified to have pregnancy-induced hypertension when they had hypertension (systolic blood pressure 140 mmHg or diastolic 90 mmHg or more) on two occasions. Patients were diagnosed with GH if blood pressure was 140 mmHg systolic or 90 mmHg diastolic or more on at least two occasions without proteinuria; PE if blood pressure was 140 mmHg systolic or 90 mmHg diastolic or more on at least two occasions and if proteinuria was 1+ (30 mg/dL) or more. Patients were diagnosed with severe hypertension if blood pressure was 160 mmHg systolic or 110 mmHg diastolic or more on at least two occasions. Patients were diagnosed with severe proteinuria if proteinuria was 2 g/day or more or 3+ (300 mg/dL) or more on at least two occasions. Pregnancy-induced hypertension with the onset earlier than the 32nd week of gestation was defined as an early onset type and after the 32nd week was defined as a late onset type.

We compared GH with PE, with respect to background factors (maternal age, parity, smoking during pregnancy, alcohol intake during pregnancy, ovulation induction, artificial insemination by husband (AIH), and *in vitro* fertilization and embryo transfer (IVF-ET), underlying disorders (diabetes mellitus, uterine disease such as uterine leiomyoma and uterine anomaly, renal disease), perinatal outcomes (fetal death, admission at neonatal intensive care unit [NICU]), and sex ratio of her baby. Severity of SGA for gestational age and fetal gender was calculated using the formula, adjusted for gestational age based on Japanese population.[5]

Data analysis

The data were analyzed statistically by chi-square using a statistical software package (SAS version 9.1; SAS Institute, Cary, NC, USA). To adjust for the effects of potential confounders, we used logistic regression models to estimate risk ratios (RRs) and 95% confidence interval (CI). The criterion for statistical significance was a level of 0.05.

Results

Incidence of pregnancy-induced hypertension at delivery

Among 241 292 mothers without essential hypertension, the incidence of pregnancy-induced hypertension (PIH) [h for mild hypertension, H for severe hypertension, hp for mild hypertension with mild proteinuria, hP for mild hypertension with severe proteinuria, Hp for severe hypertension with mild proteinuria, HP for severe hypertension with severe proteinuria, S for superimposed type] is shown in Figure 1. Severe hypertension with severe proteinuria (HP) was the most common type of PIH less than 35 weeks of gestation in Japan and HP patients delivered their babies earlier.

Incidence of GH and PE at delivery

Among 241 292 mothers without essential hypertension, the incidence of GH was 1.2% ($n = 2810$) and that of PE was 2.7% ($n = 6426$) (Fig. 2). GH had its peak incidence at 39 weeks of gestation (median: 38.0 weeks) and

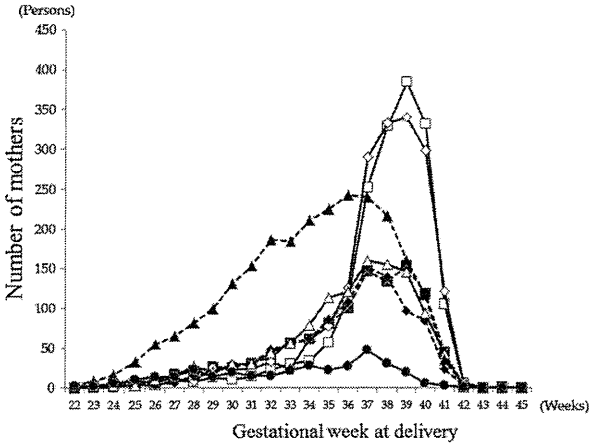

Figure 1 Incidence of pregnancy-induced hypertension in each gestational week at delivery. Markers represent the subtype of pregnancy-induced hypertension as follows: Open squares for mild hypertension (h), closed squares for severe hypertension (H), open diamonds for mild hypertension with mild proteinuria (hp), closed diamonds for mild hypertension with severe proteinuria (hP), open triangles for severe hypertension with mild proteinuria (Hp), closed triangles for severe hypertension with severe proteinuria (HP), and closed circles for superimposed patients (S). -□-, h (n = 1729); -▪-, H (n = 1081); -◇-, hp (n = 1826); -◆-, hP (n = 972); -△-, Hp (n = 1157); -▲-, HP (n = 2471); -●-, S (n = 346).

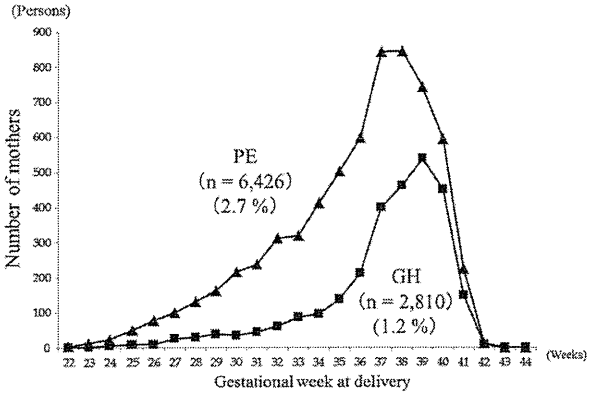

Figure 2 Incidence of gestational hypertension (GH) and preeclampsia (PE) in each gestational week at delivery. Closed squares represent the number of GH mothers and closed triangles represent the number of PE mothers. -▪-, GH; -▲-, PE.

PE at 38 weeks of gestation (median: 37.0 weeks). The incidences of GH at delivery before 28, 32, 34 weeks of gestation among total GH cases were 1.9%, 7.3%, 12.5%, respectively. Compared with GH, those incidences of PE among total PE cases were about two times (4.2%, 15.8%, 25.6%, respectively), showing that women with PE accounted for 83.3% of early-onset before 32 weeks. Rate of primiparity was substantially increased for women with PE compared with GH (66.5% and 59.2%, respectively).

Risk factors for GH and PE

Risk factors for GH and PE in Japan are summarized in Table 1. GH shared common risk factors with PE when compared with the normotensive (NBP) group. Maternal ages (35–39 years, and 40 years or older), primiparity, diabetes mellitus, and renal disease were common risk factors for GH and PE. Maternal ages (younger than 20 years, 35–39 years, and 40 years or older), primiparity, diabetes mellitus, and renal disease were similarly distributed in these groups.

Smoking during pregnancy increased the risk of GH only (RR = 1.36, 95% CI 1.15–1.60), while the influence of smoking during pregnancy on the risk of PE was statistically not significant (RR = 1.09, 95% CI 0.97–1.23). These findings were different from the previous reports in Western countries.[6,7]

Table 2 shows the differences in risk factors for GE and PE by univariate and multivariate analyses. As can be seen, advanced age (40 years or older) was the sole risk factor for GH, while primiparity and renal disease were identified as independent risk factors for PE.

Perinatal outcomes

Table 3a summarizes the perinatal outcomes of three study groups. Women with PE had a lower mean birth weight (2192 g) and a shorter mean gestational age (35.6 weeks of gestation) than those with GH (2571 g, 37.1 weeks of gestation) and the NBP women (2854 g, 38.1 weeks of gestation). The rate of NICU admission in babies from PE was significantly higher than in those from women with GH (38.8% and 25.7%, respectively; P = 0.001). Fetal and neonatal death was higher in the PE group (2.7%), compared with the GH group (1.0%), but there was no statistically significant difference (P = 0.064).

Fetal sex ratio in GH and PE

Table 3b shows the fetal sex ratio (boys/girls) in the NBP mothers, GH mothers, and PE mothers. Strikingly, distinct girl preponderance in the PE women (sex ratio: boys/girls = 0.904) was detected, while slight boy preponderance was seen in the NBP women (1.06) and the GH women (1.02).

Table 1 Risk factors for normotensive, gestational hypertension and preeclampsia (logistic regression analysis)

Risk factor	Odds ratio (95% CI)		
	NBP	GH	PE
Maternal age (years)			
<20	1	0.72 (0.49–1.08)	0.78 (0.61–1.01)
35–39	1	1.59 (1.44–1.77)	1.59 (1.48–1.71)
>40	1	2.62 (2.21–3.11)	1.96 (1.72–2.24)
Primiparity	1	1.49 (1.36–1.62)	1.91 (1.80–2.03)
Smoking during pregnancy	1	1.36 (1.15–1.60)	1.09 (0.97–1.23)
Diabetes mellitus	1	2.37 (1.91–2.94)	2.50 (2.17–2.90)
Renal disease	1	2.29 (1.67–3.13)	3.90 (3.30–4.62)

CI, confidence interval; GH, gestational hypertension; NBP, normotensive; PE, preeclampsia.

Table 2 Differences in risk factors between gestational hypertension and preeclampsia (univariate and multivariate analyses)

Risk factor	GH (%)	PE (%)	Univariate analysis			Multivariate analysis
			Risk difference		Risk ratio (95% CI)	Risk ratio (95% CI)
			χ^2	Fisher's		
Maternal age (years)						
<20	1.2	1.5	$P = 0.34$	$P = 0.34$	1.23 (0.84–1.82)	1.09 (0.68–1.74)
35–39	24.1	22.8	$P = 0.18$	$P = 0.18$	1.00 (0.89–1.14)	0.98 (0.88–1.12)
>40	7.2	5.3	$P < 0.001$	$P < 0.001$	0.74 (0.62–0.88)	0.76 (0.62–0.95)
Primiparity	59.2	66.5	$P < 0.001$	$P < 0.001$	1.12 (1.08–1.16)	1.28 (1.15–1.42)
Smoking during pregnancy	7.4	6.0	$P = 0.03$	$P = 0.03$	0.81 (0.67–0.98)	0.83 (0.67–1.01)
Diabetes mellitus	4.3	4.2	$P = 0.79$	$P = 0.78$	0.97 (0.78–1.19)	1.06 (0.82–1.36)
Renal disease	2.0	3.2	$P < 0.001$	$P < 0.001$	1.65 (1.23–2.22)	1.72 (1.21–2.43)

Risk ratio: an exact estimate of the cumulative incidence ratio. CI, confidence interval; GH, gestational hypertension; PE, preeclampsia.

Table 3 Differences in perinatal outcomes and fetal sex among normotensive, gestational hypertension, and preeclampsia

a. Perinatal outcomes according to hypertensive status

Outcomes	NBP ($n = 232\,015$)	GH ($n = 2820$)	PE ($n = 6457$)	P value
Infant's body weight (g) mean ± SD	2875.9 ± 588.7	2570.7 ± 757.1	2192.8 ± 847.9	$P < 0.001$*
				$P < 0.001$**
				$P < 0.001$***
Gestational age at delivery (weeks) mean ± SD	38.2 ± 2.8	37.1 ± 3.2	35.6 ± 3.8	$P < 0.001$*
				$P < 0.001$**
				$P < 0.001$***
Fetal and neonatal death (%)	1.5	1.0	2.7	$P = 0.003$*
				$P < 0.001$**
				$P = 0.064$***
NICU admission (%)	13.2	25.7	38.8	$P < 0.001$*
				$P < 0.001$**
				$P = 0.001$***

*NBP versus GH; **NBP versus PE; ***GH versus PE [Turkey type multiple comparison].

b. Fetal sex according to hypertensive status

Infant's sex	NBP ($n = 232\,015$)	GH ($n = 2820$)	PE ($n = 6457$)	P value
Boy	51.5%	50.6%	47.5%	$P = 0.914$*
				$P < 0.001$**
				$P = 0.022$***
Girl	48.5%	49.4%	52.5%	

*NBP versus GH; **NBP versus PE; ***GH versus PE [Bonferroni type multiple comparison]. GH, gestational hypertension; NBP, normotensive; PE, preeclampsia; SD, standard deviation.

Figure 3 displays the fetal sex ratios among these mothers stratified by maternal age. The fetal sex ratios in PE mothers were lower than one in each maternal age groups and the ratio was lowest in PE mothers under 20 years old. By contrast, the fetal sex ratios in NBP mothers were not influenced by maternal age and the ratios were higher than one. Moreover, the fetal sex ratio in GH mothers was inconstant in each maternal age groups.

Maternal body weight and body mass index

Information about prepregnancy body weight (BW), BW at delivery, prepregnancy body mass index (BMI), and BMI at delivery was available only for a small number of participants because there was discrepancy in the format of database; thus, $n = 29\,504$ for NBP; $n = 410$ for GH; $n = 870$ for PE. When the analyses were restricted to these women, prepregnancy BW, BW at delivery, and prepregnancy BMI in GH mothers were significantly higher than those in PE and normotensive mothers, while BMI at delivery in GH mothers tended to be higher than that in PE mothers (Table 4). Moreover, prepregnancy BMI and BMI at delivery were found to be independent risk factors for both GH and PE after logistic regression analysis ($P = 0.019$, $P < 0.001$, respectively).

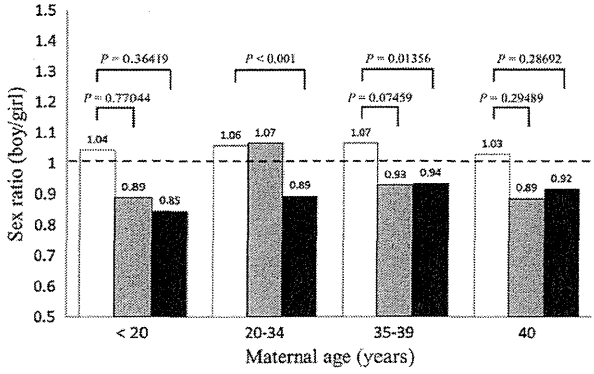

Figure 3 Maternal age at delivery and fetal sex ratio (boy/girl) in mothers with normotensive (NBP), gestational hypertension (GH), and preeclampsia (PE). □, NBP; ▨, GH; ■, PE.

Small-for-gestational-age (SGA) baby

Figure 4 illustrates the proportion of SGA babies delivered at or after 22 weeks of gestation in three groups. As can be expected, the percentage of SGA babies less than −1.5 SD was highest (27.9%) for the PE group, followed by the GH group (17.2%), and the NBP group (6.3%). Comparable results were obtained in cases of SGA babies less than −2.0 SD (17.0 %, 10.8 %, and 3.2 %, respectively) ($P < 0.001$).

To clarify the difference between the early-onset type and late-onset type, we compared the prevalence of SGA babies born before 32 weeks of gestation (early-onset type) with that born at or after 32 weeks of gestation (late-onset type). Figure 5 presents the proportion of SGA babies (SGA < −1.5 SD or <−2.0 SD)

Table 4 Maternal pre-pregnancy body weight, maternal body weight at delivery, maternal body mass index and maternal body mass index

a. Maternal pre-pregnancy body weight and body weight at delivery

	NBP ($n = 104\,481$)	GH ($n = 1371$)	PE ($n = 2930$)	Adjusted P-value (Kruskal-Wallis)	
Pre-pregnancy body weight (kg) mean ± SD	52.5 ± 8.9	58.7 ± 13.2	56.7 ± 12.1	No HT vs GH	$P < 0.001$
				No HT vs PE	$P < 0.001$
				GH vs PE	$P < 0.001$
Body weight at delivery (kg) mean ± SD	62.2 ± 9.3	67.3 ± 12.1	65.6 ± 11.5	No HT vs GH	$P < 0.001$
				No HT vs PE	$P < 0.001$
				GH vs PE	$P = 0.037$

b. Maternal pre-pregnancy body mass index (BMI) and BMI at delivery

	NBP ($n = 33\,297$)	GH ($n = 496$)	PE ($n = 948$)	Adjusted P-value (Kruskal-Wallis)	
Pre-pregnancy BMI (kg/m^2) mean ± SD	21.1 ± 3.4	23.6 ± 5.0	22.7 ± 4.5	No HT vs GH	$P < 0.001$
				No HT vs PE	$P < 0.001$
				GH vs PE	$P = 0.029$
BMI at delivery (kg/m^2) mean ± SD	24.9 ± 3.4	27.2 ± 4.8	26.4 ± 4.3	No HT vs GH	$P < 0.001$
				No HT vs PE	$P < 0.001$
				GH vs PE	$P = 0.121$

GH, gestational hypertension; NBP, normotensive; PE, preeclampsia; SD, standard deviation.

among the three mother groups. Before 32 weeks of gestation, the incidence of SGA babies delivered from PE women was surprisingly similar to that from GH women (54.3% versus 55.4%, not significant, for SGA babies <–1.5 SD; 37.4 % versus 35.3%, not significant, for SGA babies <–2.0 SD), indicating that the high frequency of SGA babies from PE mothers may be caused not by a factor/factors related to the presence of proteinuria but by a factor/factors related to the presence of early-onset type hypertension. In analysis at or after 32 weeks of gestation, the proportion of SGA babies in PE mothers was higher than that in GH mothers (22.9% versus 15.7%, $P < 0.001$, for SGA infants <–1.5 SD; 13.2% versus 8.9%, $P < 0.001$, for SGA infants <–2.0 SD), suggesting that hypertension and proteinuria are the risk factors for SGA in late-onset groups.

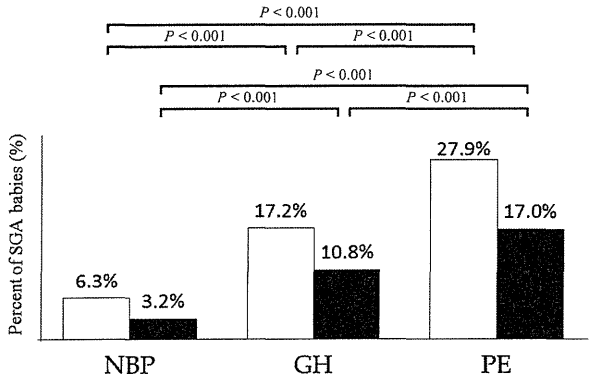

Figure 4 Proportion of small-for-gestational age (SGA) babies in mothers with normotensive (NBP), gestational hypertension (GH), and preeclampsia (PE). Open squares illustrate the percentage of SGA babies less than –1.5 standard deviation (SD). Closed squares represent the percentages of SGA babies less than –2.0 SD. □, SGA, <–1.5 SD; ■, SGA, <–2.0 SD.

Discussion

This study with sufficient numbers of cases revealed that GH and PE shared some risk factors, while the dissimilarities between them in maternal backgrounds and neonatal outcomes were seen. Older maternal ages, primiparity, renal disease, and diabetes mellitus were common risk factors for GH and PE. Prior works have documented the Ros *et al.*[1] found that high BMI (>29.0) was common risk factor. Villar *et al.*[3] reported that diabetes mellitus, renal disease, heart disease, past history of PE, age 40 years or older, high BMI (>30) were common risk factors. However, both reports contained a small amount (about 1%) of twin pregnancies. To remove the effect of multiple pregnancies on risk factors for GH and PE, we excluded multiple pregnancies from our study. We could, therefore, find the risk factors in singleton pregnancies and differentiate the risk factors only for GH from those only for PE. Our findings that age 40 years or older was a risk factor only for GH and that primiparity and renal disease were risk factors only for PE extend the previous findings from the data containing twin pregnancies. The discrepancy in risk factors between Western countries and Japan may be explained by racial differences in study population, such as physical frame. The strength of this investigation is its large sample size, which confers the sufficient power to evaluate the relation between maternal characteristics and GH or PE.

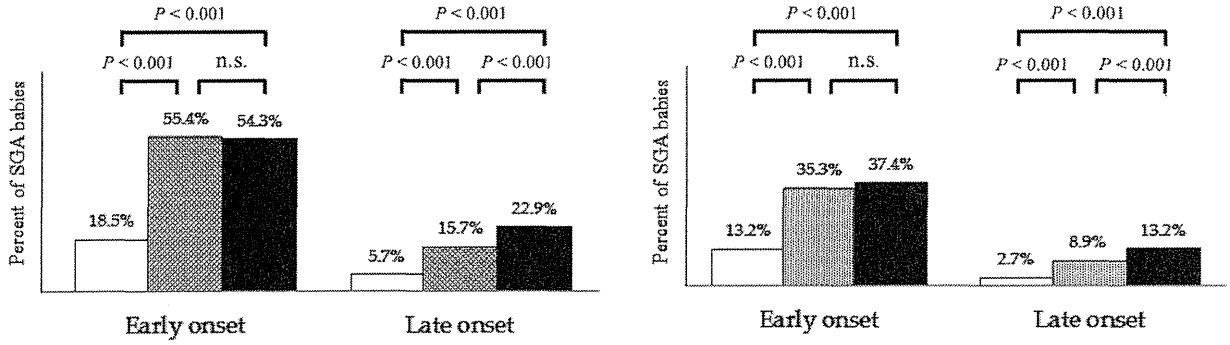

Figure 5 Proportion of small-for-gestational age (SGA) babies among normotensive (NBP), gestational hypertension (GH), preeclampsia (PE). (A) Proportion of SGA less than –1.5 standard deviation (SD). (B) Proportion of SGA less than –2.0 SD. Open bars show the percentage of SGA babies in NBP, hatched bars that in GH, closed bars that in PE. □, NBP; ▨, GH; ■, PE.

In addition, we found that the incidence of SGA babies in PE cases was significantly higher than that in GH, which is consistent with Lau's report.[8] Most notably, this is the first study to our knowledge to investigate the difference in the preponderance of SGA babies before 32 weeks of gestation between GH mothers and PE mothers. This study showed that mothers with early-onset type might have more SGA babies in the GH group as well as the PE group. Moreover we demonstrated that the number of SGA babies from the PE group was higher than that from GH, because PE mothers accounted for 83.3% of early-onset type (<32 weeks). Our study therefore indicates that we should carefully practice GH mothers as well as PE mothers in case of early-onset type.

Our data showed causative mechanism in the growth of babies in mothers with GH and PE; that is, mid-pregnancy hypertension itself was a risk factor for SGA and proteinuria might not be a direct cause of SGA. By contrast, hypertension with proteinuria was a risk factor for SGA in late-onset group.

We found that in virtually all cases, maternal hypertension after mid-pregnancy was associated with maternal BW and BMI. These findings are in agreement with those of Caruso *et al.*[9] and Catalano,[10] confirming that women with GH tend to exhibit metabolic features similar to those of patients with insulin resistance syndrome. The differences in BW and BMI between GH and PE are also consistent with the report by Caruso *et al.*[9] showing both elevated glucose intolerance and increased insulin resistance were frequently seen among GH women but not among PE women. These subtle differences may be due to maternal lipid profile (body fat mass, triglyceride, non-esterified fatty acid, or lipokine).

The literature in Western countries suggests that fetal male gender is associated with an increased risk of preeclampsia.[11–13] By contrast, our results showed that fetal female gender was associated with the risk factor of PE in singleton pregnancy. We also reported that fetal female gender was a risk factor for PE in Japanese twin pregnancy cases.[14] Therefore, our finding that mothers with a female fetus tend to suffer from PE, irrespective of gestational age and parity, points to the probability that pregnancy with a female fetus itself may be a risk factor for PE. In addition, the differences in sex ratio among PE mothers in our study were unchanged with maternal age. This study therefore suggests that there may be fetal gender-specific difference between GH and PE in the pathogenesis. We should clarify this mechanism in the future.

Several limitations and potential biases of this study also must be considered. First, we were unable to evaluate the risk of PE in relation to some factors previously reported to influence this disorder, such as race, maternal physical activity during pregnancy, change in paternity, length of sexual cohabitation before conceptions, and familial history of PE because these data were not available from our database. Second, our database (2001–2005) contributed about 5% of birth registered in Japan. Future work should therefore include a comprehensive analysis to clarify factors that affect maternal hypertension (for example, mother's physical, psychosocial, and financial status).

In summary, the risk factor patterns for GH and PE, evaluated in Japanese population, are similar, but there is a clear difference in the magnitude of the associations (that is, 40 years or older for GH, primiparity and renal disease for PE). The fact that PE mothers have more SGA babies is associated with our finding that there are more early-onset PE mothers.

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Disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Contribution of Intragenic DNA Methylation in Mouse Gametic DNA Methylomes to Establish Oocyte-Specific Heritable Marks

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Abstract

Genome-wide dynamic changes in DNA methylation are indispensable for germline development and genomic imprinting in mammals. Here, we report single-base resolution DNA methylome and transcriptome maps of mouse germ cells, generated using whole-genome shotgun bisulfite sequencing and cDNA sequencing (mRNA-seq). Oocyte genomes showed a significant positive correlation between mRNA transcript levels and methylation of the transcribed region. Sperm genomes had nearly complete coverage of methylation, except in the CpG-rich regions, and showed a significant negative correlation between gene expression and promoter methylation. Thus, these methylome maps revealed that oocytes and sperms are widely different in the extent and distribution of DNA methylation. Furthermore, a comparison of oocyte and sperm methylomes identified more than 1,600 CpG islands differentially methylated in oocytes and sperm (germline differentially methylated regions, gDMRs), in addition to the known imprinting control regions (ICRs). About half of these differentially methylated DNA sequences appear to be at least partially resistant to the global DNA demethylation that occurs during preimplantation development. In the absence of *Dnmt3L*, neither methylation of most oocyte-methylated gDMRs nor intragenic methylation was observed. There was also genome-wide hypomethylation, and partial methylation at particular retrotransposons, while maintaining global gene expression, in oocytes. Along with the identification of the many *Dnmt3L*-dependent gDMRs at intragenic regions, the present results suggest that oocyte methylation can be divided into 2 types: *Dnmt3L*-dependent methylation, which is required for maternal methylation imprinting, and *Dnmt3L*-independent methylation, which might be essential for endogenous retroviral DNA silencing. The present data provide entirely new perspectives on the evaluation of epigenetic markers in germline cells.

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Introduction

Throughout mammalian gametogenesis, dynamic DNA methylation changes occur in a sex- and sequence-specific manner. These changes result in the establishment of oocyte- and sperm-specific genomic imprints and unique methylation patterns of repetitive elements via DNA methyltransferase activity [1–4]. This process is indispensable for functional gamete and embryo development. For example, sex-specific methylation imprints are maintained throughout cell division after fertilization, despite genome-wide demethylation and *de novo* methylation during embryogenesis. These imprints control parent-of-origin specific monoallelic expression of a subset of genes, which are known as imprinted genes [5–9]. In addition, DNA methylation during spermatogenesis plays a crucial role in meiotic progression and retrotransposon silencing [10–14]. However, little is known about the profile and functional role of DNA methylation during oogenesis, except for the establishment of genomic imprints.

Recently, the epigenetic modifications which are responsible for regulating cell differentiation and embryo development have been studied in detail by using high-throughput sequencing: bisulfite sequencing ("BS-seq"); "Methyl-seq" with a methyl-sensitive restriction enzyme; "MeDIP-seq" with methylated DNA immunoprecipitation; and "MBD-seq" with a methyl-DNA binding domain protein antibody [15–26]. However, a major limitation of epigenomic studies is the lack of a standard methodology for DNA methylome analysis. Ideally, the gold standard is high resolution and genome-wide methylome analysis of germ cells. However, genome-wide methylome analysis of female germ cells has almost never been performed due to the limited availability of samples.

Author Summary

In mammals, germ-cell–specific methylation patterns and genomic imprints are established throughout large-scale *de novo* DNA methylation in oogenesis and spermatogenesis. These steps are required for normal germline differentiation and embryonic development; however, current DNA methylation analyses only provide us a partial picture of germ cell methylome. To the best of our knowledge, this is the first study to generate comprehensive maps of DNA methylomes and transcriptomes at single base resolution for mouse germ cells. These methylome maps revealed genome-wide opposing DNA methylation patterns and differential correlation between methylation and gene expression levels in oocyte and sperm genomes. In addition, our results indicate the presence of 2 types of methylation patterns in the oocytes: (i) methylation across the transcribed regions, which might be required for the establishment of maternal methylation imprints and normal embryogenesis, and (ii) retroviral methylation, which might be essential for silencing of retrotransposons and normal oogenesis. We believe that an extension of this work would lead to a better understanding of the epigenetic reprogramming in germline cells and of the role for gene regulations.

Shotgun bisulfite sequencing (SBS) may be able to overcome this limitation and enable the determination of the cytosine methylation status of individual CpG sites at a whole-genome level without a bias toward CpG-rich regions [22,23,26] and with only relatively small-scale DNA samples [24,27]. As a result, in this study, an improved SBS method for small-scale DNA samples was used to analyze the DNA methylome of mouse germ cells. In addition, the mouse germ cell transcriptome was investigated using high-throughput cDNA sequencing (mRNA-seq) to reveal relationships between DNA methylation and gene transcription in both male and female germ cells.

Results

Genome sequencing

We performed SBS analysis by using MethylC-seq [22] and a new SBS method called "whole bisulfitome-amplified DNA sequencing" (WBA-seq). The MethylC-seq and WBA-seq libraries were generated as shown in Figure S1. The MethylC-seq method generated 1010 and 1085 million tags (reads) from germinal vesicle (GV) stage oocytes and epididymal sperm, respectively. Oocyte DNA libraries generated by MethylC-seq showed higher redundancies than sperm DNA libraries. For example, 33.0% and 81.7% of the 21 million cytosines of CpGs in the mouse genome were covered by at least 1 sequence read from GV oocytes and sperm, respectively; whereas the average read depth (*i.e.*, the number of hits of reads that were mapped to a given position) was over 10× for both germ cells (Figure S2). The WBA-seq method generated 307 and 397 million tags from GV oocytes obtained from wild-type and *Dnmt3L*-deficient ($Dnmt3L^{-/-}$) mice, respectively. WBA-seq libraries for GV oocytes showed higher genome coverage (60% of genomic CpGs were covered by at least 1 read) but with smaller average read depth (7.4×) than MethylC-seq library. Some reads from the oocyte libraries strongly matched mitochondrial DNA (mtDNA), satellite, low complexity, or simple repeat sequences (Figure S3), which might have been due to a distinct genomic copy number bias in the mitochondria of germ cells or an over-amplification bias. Thus, SBS results were simplified by removing the redundancy information (only mtDNA was separately examined for DNA methylation) and combining MethylC-seq and WBA-seq results for wild-type oocytes. Consequently, the average read depth was 18.8×, 4.4×, and 12.5× for wild-type and $Dnmt3L^{-/-}$ oocytes, and sperm, respectively, and 70.8%, 45.6%, and 79.9% of genomic CpGs were covered by at least 1 sequence read from each cell type (Table 1 and Figure S3). Furthermore, the average read depths of MethylC-seq of mouse blastocysts and embryonic stem cells (ESCs), which served as zygote and stem cell controls, were 12.8× and 6.1×, respectively (Table 1).

Methylome of mouse germ cells

The average methylation level of wild-type oocytes (40.0%) was less than half that of sperm (89.4%) (Figure S4). This difference in global DNA methylation between male and female germ cells was consistent with results from the previous studies [28,29]. The $Dnmt3L^{-/-}$ oocyte genome was observed to be hypomethylated, exhibiting a methylation level of only 5.5%. Furthermore, blastocysts showed a lesser extent of methylation (21.3%) than did wild-type oocytes; ESCs, on the other hand, showed relatively high levels of methylation (70.6%). To elucidate the distribution of methylation levels on CpG sites, on regional and genome-wide scales, we created dot plots of CpG methylation for individual chromosomes and histograms of the methylation levels for all CpGs. These graphs revealed that hypermethylated CpGs in oocytes tended to cluster in transcribed regions of particular genes (*e.g.*, *Kcnq1* or *Rlim* genes, known to be expressed in oocytes [30,31]); the sperm genome was almost entirely hypermethylated, except at most CpG-rich regions (Figure 1 and Figure S5). Specifically, 55.7% of the CpGs in the oocyte genome exhibited <10% methylation, whereas another 32.0% of CpGs exhibited ≥90% methylation (Figure 2A). The $Dnmt3L^{-/-}$ oocyte genome was also hypomethylated in almost all chromosomal regions (Figure S6). The methylation level of the mtDNA genome in

Table 1. Summary of shotgun bisulfite sequencing data.

Sample	Method	Aligned tags (base)	Genome covarage		Read depth
			(>x1)	(>x5)	
Wild-type oocyte	MethylC-seq & WBA-seq	51,166,451,066	70.8%	39.4%	18.8
$Dnmt3L^{-/-}$ oocyte	WBA-seq	11,872,662,647	45.6%	19.6%	4.4
Sperm	MethylC-seq	34,153,237,944	79.9%	63.4%	12.5
Blastocyst	MethylC-seq	34,857,014,339	86.2%	79.4%	12.8
ESC	MethylC-seq	16,691,289,063	73.0%	38.9%	6.1

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