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Timing of Prenatal Exposure to Trauma and Altered Placental Expressions of HPA-Axis Genes and Genes Driving Neurodevelopment

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Abstract

Prenatal maternal stress increases the risk for negative developmental outcomes in offspring, however the underlying biological mechanisms remain largely unexplored. In this study, alterations in placental gene expression associated with maternal stress were examined to elucidate potential underlying epi/genetic mechanisms. Expression levels of 40 selected genes involved in regulating fetal HPA-axis and neurodevelopment were profiled in placental tissues collected from a birth cohort established around the time of Superstorm Sandy. Objective prenatal traumatic stress was defined as whether mothers were exposed to Superstorm Sandy during pregnancy. Among the 275 mother-infant dyads, 181 dyads were delivered before Superstorm Sandy (i.e., Control), 66 dyads were exposed to Superstorm Sandy during the first trimester (i.e., Early Exposure) and 28 were exposed to Superstorm Sandy during the second or third trimester (i.e., Mid-Late Exposure). Across all trimesters, expression of *HSD11B2*, *MAOA*, *ZNF507,* and *DYRK1A* was downregulated among those exposed to Superstorm Sandy during pregnancy. Furthermore, trimester specific differences were also observed: exposure during early gestation was associated with downregulation of *HSD11B1* and *MAOB,* and upregulation of *CRHBP*; exposure during mid-late gestation was associated with upregulation of *SRD5A3*. Our findings suggest that placental gene expression may be altered in response to traumatic stress exposure during pregnancy, and the susceptibility of these genes is dependent on the time of the exposure during pregnancy. Further studies can elucidate the biological mechanisms that underlie trimester-specific exposure by evaluating the differential impact on offspring neurodevelopment later in childhood.

Key words: pregnancy; prenatal stress; mRNA; placenta

Introduction

During critical periods in pregnancy, the fetus has a heightened susceptibility to prenatal maternal stress (PNMS) [1,2]. Animal research shows that PNMS is associated with altered development of fetal neurobiological systems, particularly the central nervous system (CNS) and the hypothalamic-pituitary-adrenal (HPA) axis, which subsequently leads to impairment of health, cognition, affect, and behavior [3]. Human population studies also have demonstrated associations between PNMS, fetal CNS, HPA-axis development [4,5] and offspring's long-term neurobehavioral and neurodevelopmental aberrations [6]. However, these human studies are not yet able to causally link PNMS and CNS/HPA development due to possible confounds in recalling stress during pregnancy. While random assignment to reduce the impact of confounds is possible in animal research, a controlled experiment with random assignment of stress is not ethically feasible using a human population. Alternatively, a stressor with substantial negative valence, such as war or natural disaster, where individuals in the same region are independently and randomly exposed regardless of their demographic, genetic and psychosocial characteristics can be leveraged to study its effects on subsequent changes and health consequences. Indeed, studies have already shown that PNMS as a result of acute trauma, natural and man-made disasters increased risks for emotional, cognitive, behavioral, and physical problems in offspring [7–9].

In an attempt to understand the underlying biological mechanisms that influence sub/optimal development of the fetus due to PNMS, changes in placental gene expression have attracted interest in the research community in recent years [10–13]. The human placenta is the major interface between the mother and fetus, and is the critical organ that regulates fetal homeostasis, growth, and development. The disruption of the maternal milieu by stress can program vital aspects of placental functioning in response, including the expression of placental genes. In addition, since the placenta is derived from the extraembryonic layer of the blastocyst, the placenta shares genetic and epigenetic characteristics of the developing embryo/fetus [14]. PNMS has been linked to fetal growth and development, which may be partially explained by changes in placental functioning and its underlying genomics. These

programming processes have been studied in greater detail for the genes encoding 11-beta hydroxysteroid dehydrogenase enzymes (*HSD11B2*) [11,12,15], glucocorticoid and mineralocorticoid receptors (*NR3C1* and *NR3C2*) [11–13,16], and monoamine oxidase A (*MAOA*)[10]. However, the types of PNMS investigated also vary, from prenatal depression and prenatal anxiety, to prenatal perceived stress. For example, prenatal depression was associated with upregulation of *NR3C1* and/or *NR3C2* [13,16]. Prenatal depression was also associated with downregulation of *MAOA* [10]. In addition, decreased *HSD11B2* expression was found to be associated with prenatal anxiety but not with prenatal depression [15]; while increased *HSD11B2* expression was associated with perceived prenatal stress and negative health related stress [11]. The types and degree of PNMS may affect distinct molecular pathways/placental processes, and the role of placental gene expression in relaying the effects of PNMS from disaster or trauma exposure remains elusive.

Furthermore, examinations aimed at elucidating the role of PNMS exposure timing on placental functioning are underexplored. Animal studies (e.g., mice, rats, guinea pigs) suggest that the programming effects of PNMS on offspring outcomes are subject to the time of exposure [17–20]. For example, one study has found that only rats exposed to PNMS during the first trimester suffered behavioral and physiological deficits [20]. Male mice exposed to stress in early or mid-gestation showed increased stress reactivity (e.g., elevated levels of corticotrophin releasing factors, reduced hippocampal glucocorticoid receptor expression), cognitive deficits in learning and memory and anxiety-related behaviors [18]. Stress early in pregnancy was also associated with upregulation of placental peroxisome proliferator-activated receptor alpha (*PPARa*), insulin-like growth factor binding protein 1 (*IGFBP-1*), hypoxia-inducible factor 3a *(HIF3a*), and glucose transporter 4 (*GLUT4*) gene expression in male mice [18]. These studies broadly suggest that early pregnancy is a sensitive period for development. Stress exposure, especially during the first trimester, may disrupt developmental programming and potentially increase the risk of long-term neurodevelopmental disorders in offspring. Similarly, human studies provide evidence suggesting that PNMS exposure during early pregnancy may bring about the most

devastating consequences [17]. One study reported that women exposed to an earthquake in their first trimester experienced the highest level of stress and had infants with lower gestational ages at birth than women exposed during later trimesters [21]. King and Laplante (2005) found that exposure to a natural disaster in early and mid-pregnancy was associated with lower mental development scores [8]. In addition, many prior studies show that increased risk for schizophrenia is associated with extreme stress in early pregnancy [22–25]. For example, Khashan et al. (2008) found that pregnant women who experienced a familial death during the first trimester of pregnancy had children who were at a higher risk for schizophrenia and related disorders later in life [22]. Another study also linked higher risk for schizophrenia to first trimester exposure to the Dutch famine of 1944-1945 [24]. However, there is also evidence that the risks for other outcomes, such as autism, are associated with stress experienced in mid- or late- pregnancy. For instance, Beversdorf et al. (2005) reported the PNMS during the second and third trimesters, but not the first trimester, was associated with greater risk for autism [26]. Similarly, a study that investigated the effect of PNMS from a tropical storm or hurricane found that storm exposure during mid (5-6 months) and late (9-10 months) pregnancy predicted an increased risk for autism [27]. Taken together, it is of importance to examine whether early trimester has specific noxious influences on developing organisms and if so through what molecular mechanisms.

Studies specifically investigating stress exposure on placental changes also observe differences based on the timing of exposure. For example, Reynolds et al. (2015) found that higher prenatal depression throughout pregnancy was associated with upregulated placental *NR3C1* and *NR3C2* expression, and these effects were particularly significant for symptoms experienced in the third trimester for *NR3C1* and in the second trimester for *NR3C2* [16]. However, this study focused on a small subset of genes.

To date, differences in the timing of the exposure to PNMS on gene expression in the placenta have not yet been systematically investigated. Uncovering the biological mechanisms that are associated with earlier or later stress exposure and its subsequent

influence on developmental and mental health outcomes could further explain the somewhat inconsistent findings and move our understanding forward.

In this study, we aimed to evaluate acute PNMS experienced earlier and later in pregnancy by virtue of a devastating natural disaster, Superstorm Sandy. Superstorm Sandy was one of the worst natural disasters on record in the United States and was the second costliest cyclone to hit the U.S. since 1900. The New York metropolitan area was severely affected by the storm in October 2012 [28]. Superstorm Sandy drove extensive storm surge, waves, rainfall and flooding into the New York coastlines, where residences, businesses, cars and other property was heavily damaged. In New York, over 300,000 homes were severely destroyed primarily due to the storm. Significant damage also occurred to public transportation, particularly the subway system, resulting in suspensions of services, which ranged from a few hours to as long as several weeks. Other significant effects included widespread and prolonged power outages and a gasoline shortage. There were 117 deaths total (53 deaths in New York) attributed to Superstorm Sandy [29]. Because of its magnitude in size and impact, Superstorm Sandy brought to the population residing in the affected area both economical and psychological damages as a result of the destruction, providing us with a unique opportunity to conduct a quasi-experimental study. The quasi-experiment allows us to understand whether PNMS as a result of a natural disaster and its gestational timing may lead to dysregulation of the placental genome, particularly for 40 candidate genes known to be associated with HPA-axis functioning and neurodevelopment (Supplementary Table 1).

Materials and Methods

Study Population

The Stress in Pregnancy (SIP) Study is an on-going longitudinal study that enrolls and follows mothers throughout pregnancy and their offspring after their birth. All women were recruited as part of the SIP Study from the prenatal obstetrics and gynecological clinics at Mount Sinai Medical Center and New York Presbyterian Queens in New York City. The unexposed participants are comprised mainly of women who reside in Manhattan and

received obstetric care at Mount Sinai Hospital, while the Sandy exposed participants are comprised of women who reside in regions of Queens and Long Island devastated by the storm. Participants were excluded if positive for HIV infection, maternal psychosis, maternal age < 15 years, life-threatening medical complications related to the mother, and congenital or chromosomal abnormalities in the fetus. A detailed description of the study population can be found elsewhere [14,30]. Demographic information, such as mother's race, marital status, education, age, smoking behavior during pregnancy and prenatal normative psychosocial stress measures were collected during the second trimester. Data on mode of delivery, gestational age (in weeks) at birth, infant sex, and birth weight (grams) were recorded at birth.

A total of 328 placental tissues collected from mothers who were pregnant before or during Superstorm Sandy were included in the current study. Preterm infants born before 34 weeks (n = 10) were not included due to higher risks of developing severe health and developmental problems [31,32]. An additional 43 cases were excluded due to missing normative psychosocial stress measures, resulting in a final sample of 275 in this current study. Table 1 shows the demographic characteristics of the sample used in the current study. Included (N = 275) and excluded participants (N = 43) did not differ on major demographic characteristics, such as infant sex, gestational age at birth, birthweight, maternal age, race, or education. Missing education (n = 1), marital status (n = 1) and mode of delivery data (n = 7) have been imputed.

All participants gave written informed consent before any assessment or data collection. All procedures involving human subjects in this study were approved by the Institutional Review Boards at the City University of New York, New York Presbyterian/Queens, and the Icahn School of Medicine at Mount Sinai.

Timing of Trauma Exposure during Pregnancy

Among the 275 mother-infant dyads, 181 mothers included in this study gave birth before Superstorm Sandy (Control) and 94 mothers were pregnant during Superstorm Sandy. Among these 94, 66 were exposed to Superstorm Sandy during the first trimester

(Early Exposure), and 28 were exposed to Superstorm Sandy during the second or the third trimester (Mid-Late Exposure).

Selected genes known to modulate HPA-axis and neurodevelopment

The 40 candidate genes were identified *a priori* for their involvement in HPA-axis functioning and neurodevelopment, as based on extensive literature search and the Ingenuity ® Knowledge Data. Among the 20 HPA-axis functioning genes, 14 genes were expressed in the placenta and 6 genes were not sufficiently expressed. Among the remaining 20 genes associated with neurodevelopment, 13 genes were sufficiently expressed in the placenta and 7 genes were not sufficiently expressed. Details regarding candidate genes can be found in Supplementary Table 1.

Placenta Collection and Gene Expression Profiling

Biopsies, free of maternal decidua, were collected from each placenta quadrant midway between the cord insertion and the placenta rim, within one hour of delivery to prevent RNA degradation. The collected tissues were first snap-frozen in liquid nitrogen and then stored at -80°C. RNA was extracted with the Maxwell 16 automated DNA/RNA extraction equipment (Promega – Madison, WI, USA) using the proprietary extraction kits following the manufacturer's protocol. RNA was quantified with Nanodrop spectrophotometer (Thermo Electron North America – Madison, WI, USA).

Placental RNA expression was profiled using the nCounter platform (nanoString Technologies, Seattle, WA) as previously described [33]. The nanoString Norm package [34] was used to normalize data. Differences in sample content were accounted for by normalizing the data against the geometric mean of the housekeeping genes Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Ribosomal Protein L19 (*RPL19*), and Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*). Genes where more than 50% of the samples fell below the limit of detection were considered unexpressed. After filtering out unexpressed genes, a total of 27 genes remained in the final analysis.

Covariates

Demographic variables. Various maternal and child demographic and health characteristics were included as covariates. Maternal characteristics included: maternal age, race (white, non-white), education, marital status (married/common law, single, divorced/separated/widowed), and smoking behavior during pregnancy (smoking, non-smoking). Infant characteristics included infant sex (male, female), gestational age, and mode of delivery (C-section, vaginal).

Normative psychosocial stress measures. Normative psychosocial stress during pregnancy was defined as a composite of prenatal depression, pregnancy related anxiety, perceived stress, state and trait anxiety, and negative stressful events. The co-experience of multiple types of normative psychosocial stress during pregnancy is relatively commonplace, capturing various domains of stress that mothers experience during pregnancy and using an aggregate measure of stress would increase the validity and reliability of the normative prenatal stress measure, as opposed to relying on only a single stressor [35–37]. These variables were measured using maternal self-report scales completed during the second trimester of the pregnancy and were used as a covariate when investigating the relationship between effects of prenatal trauma exposure and gene expression in the placenta. *Prenatal depression* was measured by the Edinburgh Postnatal Depression Scale (EPDS) [38]. Mothers were asked to report how they felt during the past seven days on a 4-point Likert scale based on severity. This inventory is well-validated in several languages and has acceptable reliability ranging from 0.79 to 0.86 [39–42]. *Pregnancy related anxiety* was measured by the Pregnancy Related Anxieties Questionnaire-revised (PRAQ-R) [43], which measures pregnancy related fears and worries. *Perceived stress during pregnancy* was measured by the Perceived Stress Scale (PSS-14) [44], which assessed the degree to which the rater appraises situations as stressful. The PSS-14 has good reliability and validity [44]. *State and trait anxiety during pregnancy* was measured by the State-Trait Anxiety Inventory (STAI) [45], which assessed temporary “state anxiety” and long-standing, characterological “trait anxiety.” Each of the two subscales consists of 20 items rated on a 4-point Likert scale. A meta-analysis of 45 articles reporting Cronbach’s alpha for internal consistency for this

inventory determined the mean to be 0.92 [46]. *Negative stressful events during pregnancy* was measured by the Psychiatric Epidemiology Research Interview Life Events Scale (LES) [47], assessed the occurrence of stressful events in five major areas of life: relationships, health, legal matters, work and financials, and friendships. This measure is widely used, has been shown to have good validity with narrative reports of life events, and has low intra-category variability [48].

The measures of normative psychosocial stress above were categorized to create a composite latent measure created by latent profile analysis (LPA). Model fits were assessed by Bayesian Information Criteria (BIC) [49], adjusted BIC (ABIC) [50], Lo-Mendell-Rubin (L-M-R) test [51] *p* values, and the entropy values for the two to four class models. LPA was performed using the full maximum likelihood estimation using Mplus version 6 [52]. Methodological details on the extraction of the latent confounding variable are provided in the Supplementary Methods. Overall, all stress variables were significantly correlated (Supplementary Table 2). LPA indicated that the three-class solution provided the best solution (Supplementary Table 3). The composite latent measure was categorized into three values from (0) low normative stress, (1) medium normative stress, to (2) high normative stress. 104 individuals were labeled as “low normative stress”, 127 individuals as “medium normative stress”, and 44 as “high normative stress” (Supplementary Table 4).

Main Statistical Analysis

Analyses of variance (ANOVAs) for continuous variables and Chi-square/Fisher’s exact tests for categorical variables were conducted to examine the differences among groups (Control, Early Exposure, and Mid-Late Exposure) across demographic and psychosocial factors. Generalized linear model (GLM) was used to evaluate the effects of acute PNMS on gene expression by comparing group differences on the placental expression of each gene, adjusting for covariates determined *a priori*. Significance of main effects (significance $p < 0.05$) was further examined using the sequential Bonferroni (Holm)

multiple comparison tests. All main statistics were conducted using SPSS version 19; while LPA was done using Mplus version 6.

Results

Characteristics of Study Population

The distribution of the demographic characteristics of the 275 dyads included in the present study is shown in Table 1. The population consisted of infants (mean age at gestation = 39.31 weeks), with roughly equivalent numbers of males and females (females = 45.5%). The SIP study consists of an urban, ethnically diverse cohort, with over half of the population reported to be of Hispanic/Latino descent (52.7%). Enrolled mothers were largely single (57.8%) and of mixed educational background ranging from no high school degree (19.3%) to post/college degree (18.5%).

Except for significant differences in maternal education ($p = 0.003$) and marital status ($p < 0.001$), with relatively more educated and married women in the exposed groups as opposed to the control; no significant group differences were observed for other demographic or psychosocial factors (Table 1).

Timing of Superstorm Sandy Exposure and Gene Expression in Placental HPA-axis Genes

Table 2 and Figure 1 show results for the overall group differences and follow-up pairwise comparisons with Holm correction for multiple testing. There are significant overall group differences in *CRHBP*, *DYRK1A*, *HSD11B1*, and *HSD11B2.* When adjusted for multiple comparisons, *CRHBP* gene expression level was upregulated in those exposed in early gestation as compared to the unexposed controls ($p = 0.030$). *DYRK1A* gene expression level was downregulated in those exposed in mid-late gestation as compared to the unexposed controls ($p = 0.005$). *HSD11B1* gene expression level was downregulated in those exposed in early gestation when compared with the unexposed controls ($p = 0.038$) and those exposed in mid-late gestation ($p = 0.038$). *HSD11B2* gene expression level was

downregulated in those exposed in early gestation (p = 0.043) and mid-late gestation (p < 0.001) as compared to the unexposed controls.

Timing of Superstorm Sandy Exposure and Gene Expression in Placental Neurodevelopment Genes

The bottom half of Table 2 shows significant group differences in neurodevelopment genes, including *MAOA*, *MAOB*, *MECP2*, *SRD5A3*, and *ZNF507*. As indicated in Figure 1, when adjusted for multiple comparisons, *MAOA* gene expression level was downregulated in those exposed in early (p = 0.039) and mid-late gestation (p = 0.011) compared to the unexposed controls. *MAOB* gene expression level was downregulated in those exposed in early gestation compared to the unexposed controls (p < 0.001). *SRD5A3* gene expression level was upregulated in those exposed in mid-late gestation when compared with the unexposed controls (p = 0.019) and those exposed in early gestation (p = 0.019). *ZNF507* gene expression level was downregulated in those exposed in early (p = 0.005) and mid-late gestations (p = 0.001) when compared to the unexposed controls.

Discussion

Accumulating evidence from animal and human research suggests that PNMS exposure exerts long-term impacts on fetal programming by altering placental function, which may be reflected in the gene expression profile in placenta. Given the predominant fetal origin of the placenta, our findings offer interesting insights into the impacts of acute PNMS on offspring.

Our results showed that PNMS, as a result of exposure to a natural disaster, at different stages of pregnancy was associated with downregulation of *HSD11B2*, *MAOA* and *ZNF507* genes. The trend of downregulation of *DYRK1A* across pregnancy was also observed, while the effect was significant for mid-late gestation, it was marginally significant for early gestation (p = 0.084). Overall, many of these downregulated genes across trimesters are vital for placental function and fetal development.

The placental 11*β*-hydroxysteroid dehydrogenase type 2 (11*β*-HSD2) enzyme (encoded by the *HSD11B2* gene), which converts active cortisol into inactive cortisone, acts as a barrier regulating the transfer of the maternal cortisol to the fetus [53]. Cortisol is essential to fetal growth but, may be harmful to the fetus when in high concentrations [54]. Under normal circumstances, 11*β*-HSD2 largely converts cortisol into cortisone, thereby protecting the fetus from excessive glucocorticoid exposure [53]. This is supported by the finding that fetal blood has 13-fold lower cortisol concentrations than maternal blood [55]. Studies demonstrate that stressed mothers commonly secrete greater amounts of glucocorticoids [56,57], despite contradictory evidence [58,59]. As a consequence, elevated levels of glucocorticoids may enter the fetal circulation and influence fetal HPA-axis development. Our results suggest that when exposed to acute PNMS, the protective effect of placental *HSD11B2* can be overwhelmed. Indeed, lower placental *HSD11B2* has been associated with poor infant outcomes, including decreased infant movement quality and lower muscle tone [60,61]. Our findings are inconsistent with some of the previous literature regarding stress-related effects on *HSD11B2* gene expression. For example, prenatal anxiety, but not depression, has been associated with lower *HSD11B2* expression [15]. The distinction may be explained by a lack of evolutionary benefit for prenatal depression as compared to prenatal anxiety, such that a depressed mother may not perceive danger, and therefore it may not play a role in affecting fetal and child development in future dangerous and stressful situations [62]. Furthermore, prenatal perceived stress and health related stress were reported to be positively associated with *HSD11B2* [11]. It has also been suggested that mild to moderate levels of PNMS may not decrease, but in fact may enhance development. For example, mid-level PNMS, such as nonspecific stress and prenatal depressive symptoms, were found to be positively associated with mental and motor development in younger children [63]. Our study is the first to have associated acute PNMS due to a natural disaster and decreased *HSD11B2* expression and therefore requires further replication. Nevertheless, this finding advocates that different types of stressors may exert differential impacts on gene expression that in turn program distinct fetal and child outcomes [64].

In addition to maternal glucocorticoids, serotonin is an important stress related neurotransmitter [65] which is synthesized in the placental and fetal compartments and is vital for fetal brain development. *MAOA* metabolizes serotonin, dopamine and norepinephrine. Maternal blood serotonin can cross the placenta and enter the fetal circulation; overexposure to serotonin disrupts fetal brain development [66]. Recent research suggests that PNMS is associated with elevated levels of serotonin [67] and a reduction in *MAOA* gene expression [10,67]. Mutations in *MAOA* have been linked to disordered neurodevelopment and behaviors, including autism-like disorders and antisocial behaviors [68,69].

Little is known about how the expression levels of placental genes, such as *DYRK1A* and *ZNF507,* influence the development of brain function and behavior in typically developing children. *DYRK1A* is involved in cell proliferation and has been implicated in Down syndrome [70]. *ZNF507* modulates transcriptional regulation; reduced expression of *ZNF507* has been related to schizophrenia [71]. Stress induced downregulation of these genes may also have an impact on placental function, intrauterine homeostasis and fetal growth.

Furthermore, these downregulated genes are more markedly altered among women exposed during mid-late gestation, which suggests that the impact of PNMS may be exaggerated as women advance throughout pregnancy, yet this warrants further investigation. Our group comparison results reflect no significant statistical differences between early and mid-late gestation (*HSD11B2*, $p = 0.053$; *MAOA*, $p = 0.270$, *ZNF507*, $p = 0.168$ and *DYRK1A*, $p = 0.124$), which may be attributed to the relatively small sample of exposed participants during mid-late gestation.

Prior animal and human research suggests the timing of exposure appears to be crucial when considering the effect of PNMS on offspring outcomes [67]. PNMS is believed to be associated with adverse outcomes, particularly in cases of early gestation exposure. Our findings are partially consistent with this line of research. Specifically, our results show that upregulation of *CRHBP* and downregulation of *HSD11B1* and *MAOB* were observed among those exposed to Superstorm Sandy in early pregnancy. Due to a relatively small group of

mid-late gestation, we did not observe significant differences between early and mid-late gestation exposure for *CRHBP* (p = 0.697) and *MAOB* (p = 0.055) expression.

CRHBP encodes the corticotrophin-releasing hormone (CRH)-binding protein, which inactivates CRH that stimulates the production of adrenocorticotropic hormone (ACTH) and cortisol throughout pregnancy in the maternal and the fetal compartments [72,73]. Increased circulating maternal CRH concentrations have been associated with lower concentrations of CRHBP [73]. In a normal human pregnancy, maternal CRH, derived from the placenta, provides information on the length of gestation [74,75]. Circulating maternal CRH concentrations rise over the course of gestation, correlating with increased placental *CRH* mRNA expression [73]. While an elevation in circulating maternal CRH concentrations increases risks for fetal growth restriction during early gestation [76]; an increase in these concentrations during the last few weeks of pregnancy accompanied by a fall in the concentrations of CRH-binding proteins allows for the preparation of events leading to parturition [77]. It has been suggested that exposure to stress, especially during early gestation, is associated with an increase in placental CRH concentrations in plasma [78]. Our results suggest that for individuals exposed to Superstorm Sandy in early pregnancy, a rise in CRH may lead to upregulation of *CRHBP,* which can produce prolong excessive CRH-binding proteins that prevent inappropriate pituitary-adrenal stimulation but disrupts the developmental increase of maternal CRH concentrations. In adults, *CRHBP* dysfunctionality is associated with posttraumatic stress and depression symptoms [79,80].

Comparably, the expression and activity of *HSD11B1,* which is primarily involved in reactivation of cortisol from cortisone*,* increases during normal pregnancy. Decreased *HSD11B1* has been associated with reduced cortisol regeneration and increased risks for newborns with intrauterine growth restriction, i.e., small-for-gestational-age [81]. Offspring exposed to the traumatic event in early gestation may be more vulnerable to these disruptions as the consequences of deficient *HSD11B1* expression.

Similar to *MAOA*, *MAOB* plays a critical role in regulating dopamine metabolism and dietary amines including phenylethylamine [82]. The placental tissue contains a small amount

of *MAOB* [83,84]. *MAOB* activity increases with aging in humans and is associated with neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [85,86]. The role of *MAOB* gene expression in the placenta has not been well described. Lower *MAOB* platelet activity has been linked to mood disorders, alcoholism, sensation seeking and impulsivity [87]. Downregulation of placental *MAOB* may increase risks for neuropsychiatric and behavior disorders in offspring exposed to PNMS in early gestation.

Finally, upregulation of *SRD5A3* was observed among those exposed to Superstorm Sandy in mid-late pregnancy. *SRD5A3* plays an important role in protein glycosylation [88], is widely expressed in the human brain tissues and body organs (e.g., retina, skin, kidney), and plays a crucial role in brain development [89]. Mutations in *SRD5A3* have been linked to a congenital defect in dolichol metabolism [89,90]. Animal research has found that placental *SRD5A3* is altered by Triclosan (an antimicrobial agent often used in personal care products) exposure [91]. The three human 5α-reductases are encoded by the *SRD5A1*, *SRD5A2*, and *SRD5A3* genes. During pregnancy, the 5α-reductases in the placenta provide precursors for the synthesis of allopregnanolone, a neurosteroid that may exert neuroprotective effects on fetal brain development [92,93]. Therefore, it is essential to further investigate the prenatal risk factors such as maternal stress that may influence the allopregnanolone synthesis pathway. One recent investigation found that maternal plasma allopregnanolone concentrations were not related to the genotypes of *SRD5A1* and *SRD5A2* and maternal depressive symptoms during pregnancy [94]. However, little is known about the role of *SRD5A3* gene in the human placenta and its relation to the prenatal stress influences.

Our findings suggest that trauma exposure may uniquely impact developmental processes through changes in expression of genes which foster distinct developmental processes. Furthermore, it is likely that some of the genes we identified are fully developed and begin functioning during early pregnancy, whereas others only begin functioning during mid-late pregnancy. Although we were able to identify changes in gene expression as a result of placental development, the underlying molecular mechanisms by which this occurs requires further exploration, thus replication is needed. Expression of placental genes likely

varies across gestation to accommodate the dynamically changing needs of the developing fetus, although the molecular basis of placental development has yet to be fully uncovered [95]. Our findings suggest that several genes may be more vulnerable to maternal trauma exposure depending on the timing of exposure during gestation.

We acknowledge several limitations of this study. Although we observed associations between PNMS and differences in gene expression, implications of these findings on neurodevelopmental outcomes in childhood and adulthood remain unknown. As we see significant observations between CRH binding proteins and maternal stress in the current study, follow up studies will include further characterizing the response of the corticotrophin signaling pathway, HPA-axis functioning, and maternal stress, by evaluating additional components of the pathway, including placental levels of *CRH*, *ACTH* and *CORT*. Furthermore, the RNA integrity and quality were not assessed in the present study, while they should have been evaluated for each extraction especially since placental tissues contain high levels of RNase. Our opportunistic sample was relatively small especially once divided it into groups by windows of exposure, requiring that our conclusions be corroborated by future studies with larger sample sizes. The small size of our groups also supported the combining of mid and late trimester groups into one. This grouping is justifiable given that results of prior animal studies show that the first trimester is when the fetus might be most vulnerable to PNMS; however, it may have been more informative to have kept each trimester as a separate group. Furthermore, while prior research has shown that sex is likely a significant moderator of the effect of PNMS, our small sample size did not provide us with sufficient power to evaluate potential sex-specific effects [96]. Readers should also be reminded that the control and exposed groups were different with regards to marital status and education. Prior research has associated socio-economic status (i.e., education level) and altered placental gene expression levels [97]. While these differences could have happened by chance alone, the control group was composed of mainly women residing in Manhattan who received obstetric care at Mount Sinai Hospital, while the exposed group was mainly composed of women residing in storm devastated regions, Queens and Long

Island due to the study design. As such, while our findings were independent of a range of covariates; statistical control may not have been fully adequate in addressing group differences. Additionally, investigating the associations between gene expression and the covariates was outside the scope of the current study, and exploring such relationships may be worth pursuing in future studies. Finally, stress is a subjective experience and we did not include measures of how subjectively stressful each mother's experience of Superstorm Sandy was.

Despite these shortcomings, this is the first study, to our knowledge, to present an analysis of a list of candidate genes in HPA-axis regulation and neurodevelopment in a functional organ (placenta) by exposure to a traumatic event during pregnancy. In comparison with previous research, we were able to study how the timing of trauma exposure impacts placental gene expression. Our observations suggest that PNMS from trauma across trimesters downregulates placental expression of *DYRK1A*, *HSD11B2, MAOA,* and *ZNF507.* However, traumatic stress exposure in early gestation is associated with upregulation of *CRHBP* and downregulation of *HSD11B1* and *MAOB*, while exposure in mid-late gestation is associated with upregulation of *SRD5A3*. Our findings also demonstrated the importance of corroborating and extending the results of animal research in human populations. Longitudinal follow-up studies are needed to investigate how the alterations in the expression of these genes affect the neurobehavioral and neurodevelopmental outcomes in the offspring.

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Figure 1: Timing of trauma exposure and gene expression in placenta. Only significant overall differences in gene expression related to trauma exposure are reported. The bar represents the average expression level of each gene using housekeeping genes for normalization and the whisker the standard error. The pairwise comparisons were adjusted for multiple comparisons using sequential Bonferroni procedure. ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$.

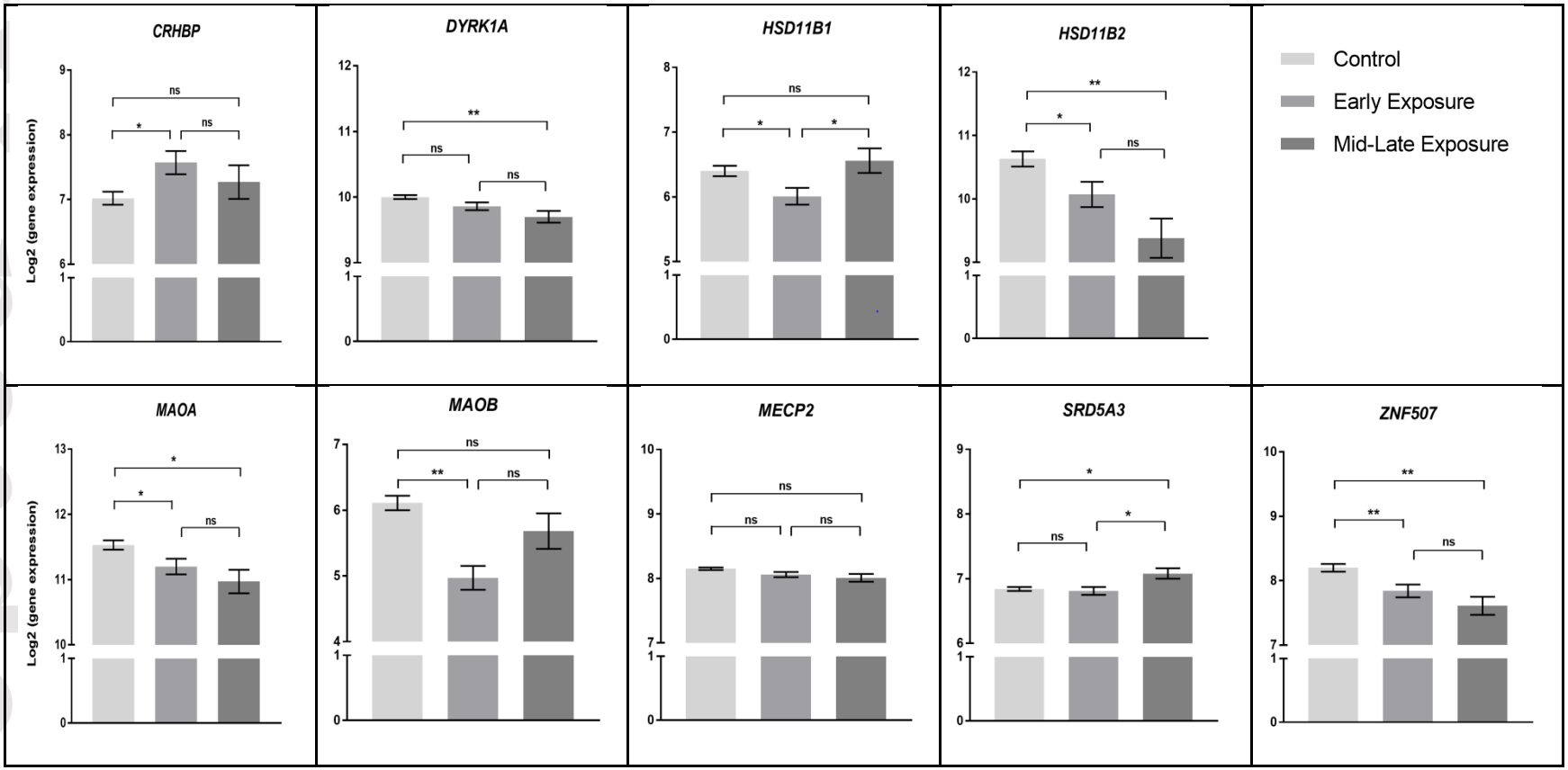

Table 1. Characteristics of the study population in total and by stress groups (Control, Early Exposure, and Mid-Late Exposure).

		Total (N = 275)	**Control (n = 181)**	**Early Exposure (n = 66)**	**Mid-Late Exposure (n = 28)**	***p* value** [a]
Infant sex						.282
Males	N (%)	150 (54.5)	98 (54.1)	40 (60.6)	12 (42.9)	
Females	N (%)	125 (45.5)	83 (45.9)	26 (39.4)	16 (57.1)	
Gestational age, weeks	Mean (SD)	39.31 (1.47)	39.30 (1.49)	39.31 (1.47)	39.33 (1.38)	.997
Birthweight						.196
< 2500 g	N (%)	15 (5.5)	13 (7.2)	1 (1.5)	1 (3.6)	
≥ 2500 g	N (%)	258 (93.8)	166 (91.7)	65 (98.5)	27 (96.4)	
Missing	N (%)	2 (.7)	2 (1.1)			
Mode of delivery						.389
C-section	N (%)	93 (33.8)	65 (36)	19 (28.7)	9 (32.1)	
Vaginal	N (%)	175 (63.6)	114 (63)	45 (68.2)	16 (57.1)	
Missing	N (%)	7 (2.5)	2 (1.1)	2 (3)	3 (10.7)	
Maternal age, years	Mean (SD)	27.76 (5.90)	27.65 (6.18)	27.70 (5.34)	28.68 (5.35)	.688
Mother's race						.219
White	N (%)	27 (9.8)	13 (7.2)	10 (15.2)	3 (10.7)	
Non-White	N (%)	248 (90.2)	168 (92.8)	56 (84.8)	25 (89.3)	
Black	N (%)	68 (24.7)	53 (29.3)	12 (18.2)	3 (10.7)	
Hispanic/Latino	N (%)	145 (52.7)	98 (54.1)	29 (43.9)	19 (67.9)	
Asian	N (%)	21 (7.76)	7 (3.9)	12 (18.2)	2 (7.1)	
Others	N (%)	14 (5.1)	10 (5.5)	3 (4.5)	1 (3.6)	
Maternal education						.003
Less than high school	N (%)	53 (19.3)	46 (25.4)	5 (7.6)	2 (7.1)	
High school graduate	N (%)	62 (22.5)	44 (24.3)	13 (19.7)	5 (17.9)	
Some college	N (%)	108 (39.2)	69 (38.1)	27 (40.9)	12 (42.9)	
College graduate	N (%)	30 (10.9)	13 (7.2)	11 (16.7)	6 (21.4)	
Graduate degree	N (%)	21 (7.6)	9 (5)	9 (13.6)	3 (10.7)	
Missing	N (%)	1 (.4)		1 (1.5)		

Mother's marital status						< .001
Married	N (%)	90 (32.7)	39 (21.5)	35 (53)	16 (60.7)	
Common law	N (%)	20 (7.3)	13 (7.2)	6 (9.1)	1 (3.6)	
Single	N (%)	159 (57.8)	126 (69.6)	22 (33.3)	11 (39.3)	
Divorced/separated/widowed	N (%)	5 (1.8)	3 (1.7)	2 (3.0)		
Missing	N (%)	1 (.4)		1 (1.5)		
Smoking during pregnancy						.246
No	N (%)	238 (86.5)	154 (85.1)	57 (86.4)	27 (96.4)	
Yes	N (%)	35 (12.7)	27 (14.9)	7 (10.6)	1 (3.6)	
Missing	N (%)	2 (.7)		2 (3)		
Prenatal depression	Mean (SD)	7.35 (5.4)	7.26 (5.36)	7.62 (5.37)	7.36 (5.88)	.902
Prenatal related anxiety	Mean (SD)	5.87 (2.3)	5.89 (2.28)	6.04 (2.34)	5.36 (2.32)	.423
Perceived stress during pregnancy	Mean (SD)	36.20 (7.43)	36.37 (7.47)	35.58 (7.58)	36.49 (6.96)	.748
State anxiety	Mean (SD)	38.00 (11.50)	37.87 (11.35)	38.58 (12.62)	37.57 (10.11)	.896
Trait anxiety	Mean (SD)	38.43 (10.75)	38.46 (10.83)	38.35 (10.31)	38.50 (11.61)	.997
Negative stressful events	Mean (SD)	1.57 (1.97)	1.55 (1.93)	1.62 (2.04)	1.57 (2.10)	.974
Normative psychosocial stress group						.744
Low	N (%)	104 (37.8)	69 (38.1)	24 (36.4)	11 (39.3)	
Moderate	N (%)	127 (46.18)	82 (45.3)	34 (51.5)	11 (39.3)	
High	N (%)	44 (16)	30 (16.6)	8 (12.1)	6 (21.4)	

NB. [a] *p* values for the test for the differences among the 3 groups: ANOVA for continuous variables and Chi-square/Fisher's exact tests for categorical variables.

Table 2. Gene expression differences among stress groups (Control, Early Exposure, and Mid-Late Exposure). There are significant overall group differences in HPA-axis genes (*CRHBP*, *DYRK1A*, *HSD11B1*, and *HSD11B2*) and genes driving neurodevelopment (*MAOA*, *MAOB*, *MECP2*, *SRD5A3*, and *ZNF507*). Mean value denotes the average expression level of each gene, using housekeeping genes for normalization. SE represents standard error.

Group	**Control (n = 181)**	**Early Exposure (n = 66)**	**Mid-Late Exposure (n = 28)**	
	Mean (*SE*)	**Mean (*SE*)**	**Mean (*SE*)**	**Overall *p* value**
HPA-Axis				
AVPR1B	3.80 (.06)	3.85 (.09)	4.00 (.14)	.453
CFL1	12.13 (.02)	12.06 (.03)	12.07 (.05)	.109
CREB1	9.23 (.03)	9.32 (.05)	9.25 (.07)	.282
CREBBP	4.33 (.05)	4.42 (.08)	4.38 (.12)	.599
CRHBP	7.02 (.10)	7.57 (.18)	7.27 (.26)	.035
DYRK1A	10.00 (.03)	9.86 (.06)	9.70 (.09)	.003
HSD11B1	6.40 (.08)	6.01 (.13)	6.56 (.19)	.016
HSD11B2	10.63 (.12)	10.07 (.20)	9.38 (.31)	<.001
NCOR1	10.39 (.03)	10.33 (.05)	10.25 (.07)	.121
NCOR2	9.35 (.04)	9.38 (.06)	9.45 (.10)	.664
NR3C1	10.85 (.03)	10.94 (.05)	10.76 (.08)	.107
NR3C2	7.22 (.05)	7.33 (.08)	7.00 (.12)	.078
NR4A1	7.53 (.07)	7.19 (.13)	7.60 (.19)	.053
POMC	3.62 (.06)	3.59 (.10)	3.68 (.14)	.869
Neurodevelopment				
ADRA2A	5.95 (.06)	5.82 (.10)	5.86 (.16)	.538
CDKL5	7.43 (.04)	7.43 (.06)	7.28 (.09)	.285

DBH	3.80 (.07)	3.96 (.11)	4.11 (.17)	.176
FOXP1	6.49 (.04)	6.42 (.07)	6.51 (.10)	.644
HTR1B	5.35 (.06)	5.30 (.10)	5.29 (.16)	.886
MAOA	11.53 (.07)	11.20 (.12)	10.97 (.18)	.004
MAOB	6.11 (.11)	4.97 (.18)	5.68 (.27)	<.001
MECP2	8.15 (.02)	8.06 (.04)	8.01 (.06)	.044
PON3	4.68 (.06)	4.89 (.09)	4.89 (.14)	.107
SNAP25	3.61 (.06)	3.66 (.10)	3.74 (.14)	.701
SRD5A3	6.84 (.03)	6.81 (.06)	7.08 (.08)	.017
ZNF507	8.20 (.06)	7.84 (.10)	7.61 (.14)	<.001
ZNHIT6	7.91 (.03)	7.89 (.05)	7.85 (.08)	.792

NB: *p* values are calculated based on generalized linear models controlling for maternal age, race, education, marital status, smoking behavior during pregnancy, infant sex, gestational age, mode of delivery, and normative psychosocial stress.

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Brief Report

Prenatal exposure to 1-bromopropane causes delayed adverse effects on hippocampal neuronal excitability in the CA1 subfield of rat offspring

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Abstract: Objectives: Neurotoxicity of 1-bromopropane (1-BP) has been reported in occupational exposure, but whether the chemical exerts developmental neurotoxicity is unknown. We studied the effects of prenatal 1-BP exposure on neuronal excitability in rat offspring. **Methods:** We exposed dams to 1-BP (700 ppm, 6 h a day for 20 days) and examined hippocampal slices obtained from the male offspring at 2, 5, 8, and 13 weeks of age. We measured the stimulation/response (S/R) relationship and paired-pulse ratios (PPRs) of the population spike (PS) at the interpulse intervals (IPIs) of 5 and 10 ms in the CA1 subfield. **Results:** Prenatal 1-BP exposure enhanced S/R relationships of PS at 2 weeks of age; however, the enhancement diminished at 5 weeks of age until it reached control levels. Prenatal 1-BP exposure decreased PPRs of PS at 2 weeks of age. After sexual maturation, however, the PPRs of PS increased at 5-ms IPI in rats aged 8 and 13 weeks. **Conclusions:** Our findings indicate that prenatal 1-BP exposure in dams can cause delayed adverse effects on excitability of pyramidal cells in the hippocampal CA1 subfield of offspring.
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Key words: 1-Bromopropane, Delayed adverse effect, Electrophysiology, Excitability, Prenatal exposure, Rat hippocampal slices

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Introduction

Social concerns have been raised regarding the developmental neurotoxicity of prenatally absorbed environmental chemicals, which may exert delayed adverse effects on brain function after birth. It is now recognized that some industrial chemicals (e.g., lead, methylmercury, polychlorinated biphenyls, arsenic, and toluene) can exert developmental neurotoxicity, which results in clinical or subclinical brain dysfunction in humans and in laboratory animals[1)]. Many neurotoxic chemicals are present in industrial work settings, and it is not known whether prenatal exposure to industrial chemicals leads to developmental neurotoxicity.

1-Bromopropane (CH_3-CH_2-CH_2Br; 1-BP), one substitute for specific chlorofluorocarbons, is currently used as a solvent in a variety of industrial and commercial applications. Products containing 1-BP include degreasers and cleaners, spray adhesives, spot removers, coin cleaners, paintable mold release agents, automotive refrigerant flushes, and lubricants. Adverse effects on the central and peripheral nervous system have been found in industrial workers who used 1-BP[2-4)]. Adult rats exposed to 1-BP have also exhibited central neurotoxicity, alteration of mRNA levels of brain neurotransmitter receptors[5)], and hippocampal disinhibition caused by a decrease in γ-aminobutyric acid (GABA)-mediated function[6)]. In *in vitro* studies using rat hippocampal slices, 1-BP directly suppressed the synaptic plasticity, referred to as a long-term potentiation, in the granule cells of the dentate

gyrus[7].

Developmental toxicity is one reason for the threshold limit value set by the American Conference of Governmental Industrial Hygienists for 1-BP[8]. We recently reported that prenatal exposure to 1-BP suppressed the occurrence of kainate (KA)-induced "wet dog shake" behavior in 2-week-old rat pups[9]. However, whether or not prenatal 1-BP exposure changes neuronal function at the cellular level in the brain of the offspring remains unknown. Therefore, we studied the effects of prenatal 1-BP exposure on neuronal excitability after birth. In studying neuronal excitability, population spikes (PSs) were recorded in the CA1 subfield of hippocampal slices. We analyzed stimulation-dependent responses, stimulation/response (S/R) relationships, and the ratio of responses to double-pulse stimulations (paired-pulse ratios or PPRs). PPRs have been used as a simple method for assessing excitability in neuronal networks[6,10] In the present study, we evaluated rats at 2, 5, 8, and 13 weeks of age, to determine whether prenatal 1-BP exposure exerts delayed effects after birth.

Materials and Methods

Animals and exposure protocol

Preparation of rats and 1-BP inhalation were made according to our previous study[9]. Briefly, adult male and female Wistar rats were purchased from Kyudo Co., Ltd. (Tosu, Japan). The rats were housed in plastic cages with paper-made chips (ALPHA-dri, Shepherd Specialty Papers, Richland, MI, USA) on a 12-h light/dark cycle (light period: 7 AM-7 PM). The temperature was controlled at 22°C-23°C. The relative humidity was approximately 50%-70%. The animals were allowed to consume food and water ad libitum. Female rats at the proestrus stage were mated with male rats. On the morning of the following day, the existence of sperm in the vaginal plug or vaginal smear was verified as gestation day (GD) 0. 1-BP was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Dams were exposed to 1-BP vapor at a concentration of 700 ppm (6 h/day) for 20 days from GD 1 to GD 20 in an exposure chamber, whereas the other dams were provided fresh air in the same type of chamber. The exposure concentration was designed to be 700 ppm, which was higher than the LOAEL (400 ppm) for decrease in inhibition, so-called disinhibition[11]. Rats were not allowed access to food and water during the inhalation period. At postnatal day (PND) 2, the litter size was counted. Until the experimental days, male and female rat pups were housed separately after weaning. Some pups in the control and prenatally 1-BP-exposed groups were sourced from pups that were not injected with KA in our previous study[9]. The prenatally 1-BP-exposed groups are abbreviated as the 1-BP group. The number of dams in the control group and 1-BP group was 15 and 12, respectively. The total number of pups in the control group and 1-BP group was 29 and 20, respectively.

The experiments were conducted under the guidance of the Ethics Committee of Animal Care and Experimentation in accordance with the Guiding Principle for Animal Care Experimentation, University of Occupational and Environmental Health, Japan, which conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Japanese Law for Animal Welfare and Care.

Hippocampal slice preparation

Electrophysiological tests were conducted in male rats at 2 (PND 14), 5, 8, and 13 weeks of age. The total number of tested slices in the control group and 1-BP group was 119 and 86, respectively. The slices were prepared following previously reported methods[6]. Briefly, the rats were deeply anesthetized using a diethyl ether vapor. After decapitation, the brain was removed and dipped in an ice-cooled artificial cerebrospinal fluid (ACSF) (3°C-4°C) saturated with an O_2/CO_2 mixture (95%:5%). The ACSF was composed of 124 mM NaCl, 2 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 26 mM $NaHCO_3$, and 10 mM glucose. The bilateral hippocampi were separated from the other brain regions. Further, transverse slices were obtained from the middle third region of the hippocampus using a McIlwain tissue chopper (Mickle Laboratory Engineering, Co., Ltd., Guildford, UK). The thickness of the slice was 600 μm for 2-week-old rats and 450 μm for 5-, 8-, and 13-week-old rats. The slices were transferred to an interface-type recording chamber, which was controlled at 32 ± 0.2°C, and perfused with ACSF saturated with a mixture of O_2/CO_2 (95%:5%) at a flow rate of 1 ml/min.

All the chemicals used in this study were of reagent grade and purchased from commercial sources.

Stimulation and recordings

After a stabilizing period of 1-2 h, bipolar stimulation electrodes made with stainless steel wires (50 μm in diameter) were placed on the stratum radiatum, where the Schaffer collateral and commissural fibers run up in the CA1 subfield (Fig. 1A). PS was recorded from the pyramidal cell layer in the CA1 subfield using glass microelectrodes (1-2 MΩ). Stimulations consisted of square-wave pulses (200 μs) from a stimulator (SEN7203, Nihon Koden Co., Tokyo, Japan) via an isolator (SS202J, Nihon Koden Co.). Stimulation intensities were 10 μA and 50 μA and increased by 100 μA every 2 min from 100 μA to a current of 600 μA in the slices from the 2-week-old rats. In the slices from the 5-, 8-, and 13-week-old rats, the stimulation was delivered every 30 sec with intensities of 20, 40, 60, 80, 100, 140, 200, and 300 μA. The S/R relationship in the extracellular recording configuration represents basic excitability of the local area responding to

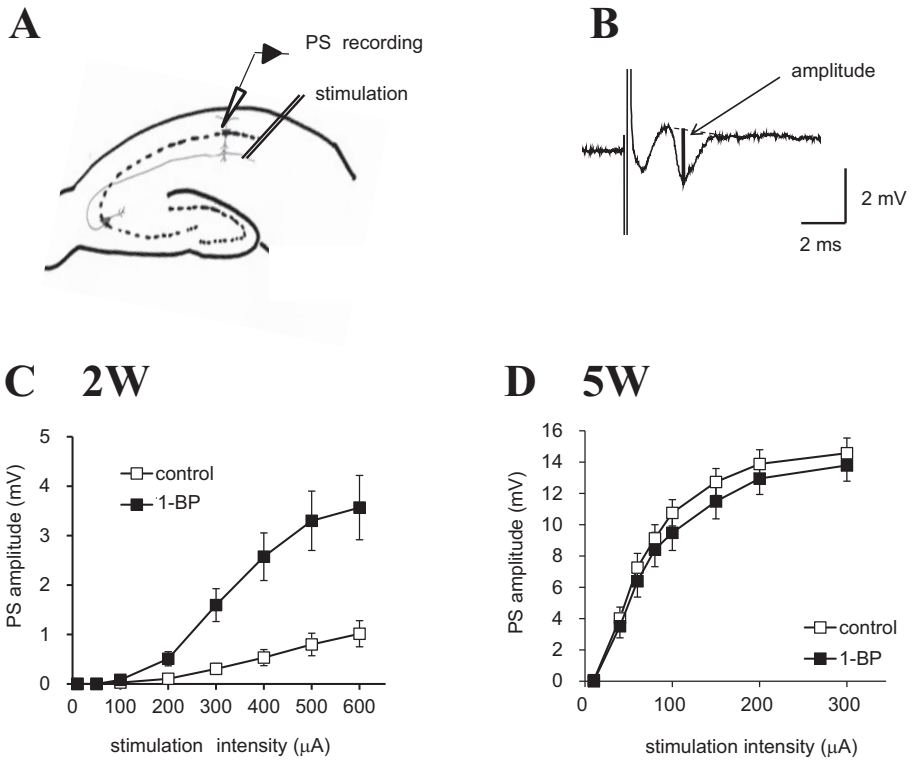

Fig. 1. Recording of population spikes (PSs) from the hippocampal CA1 field and PS stimulus/response (S/R) relationships
(A) Stimulation electrode and recording electrode set on the CA1 subfield of the hippocampal slice. The stimulation electrode was set in the stratum radiatum, supplying stimulation to Schaffer collateral and commissural fibers. The PS recording electrode was set in the pyramidal cell layer. (B) Typical PS recorded in the CA1 field of the hippocampal slice obtained from a 2-week-old control rat. The thick line represents the PS amplitude measurement; stimulation intensity was 600 μA. (C) At 2 weeks of age, S/R relationships of the PS amplitude obtained from the 1-BP-exposed rats were significantly enhanced compared to S/R relationships in control rats (p <0.001 by repeated-measure ANOVA). (D) At 5 weeks, the enhancement observed in the 1-BP-exposed rats disappeared, and the S/R relationship decreased to control levels (PS amplitude: p=0.5 by repeated-measure ANOVA). The horizontal axis represents stimulation intensity; the vertical axis represents PS amplitude. Data of 16-19 slices were averaged.

electrical stimulation, and the responses are prefigured to increase as the stimulation strengthens. For the paired-pulse configuration, after the S/R relationship experiment, the current amplitude was adjusted to result in an almost-maximum PS, 600 μA for slices from the 2-week-old rats, and 300 μA for slices from the 5-, 8-, and 13-week-old rats. Interpulse intervals (IPIs) of the paired-pulse stimulation were 5 and 10 ms and delivered every 2 min for slices from the 2-week-old rats and every 1 min for slices from older rats. Electrophysiological signals were amplified using a high-impedance amplifier (Axoclamp 2B, Molecular Devices, Sunnyvale, CA, USA). The signals were then digitized with an AD converter (Digidata 1200, Molecular Devices) and stored on a computer using pCLAMP software (Molecular Devices).

Electrophysiological analysis

PS amplitude was measured as described in our previous study[6] (Fig. 1B). PPRs were calculated as follows:

PPR of PS = second PS amplitude/first PS amplitude

In our previous inhalation studies using adult rats[6,11], PPRs of PS evoked with paired-pulse stimulation at IPIs of 5 and 10 ms in the CA1 subfield were <1 in the hippocampal CA1 of control adult rats, representing the presence of feedback inhibition. Compared to those of adult rats, PPRs of PS in immature rats can be 1 or higher[12]. Thus, in either case of inhibition or facilitation, paired-pulse configuration in extracellular recordings in the slices is useful to examine the excitability of the local area responding to double-pulse stimulations.

Statistical analysis

Statistical significance was evaluated by repeated-measure analysis of variance (ANOVA) for the S/R relationship. For PPRs, unpaired Welch's *t*-test was used to determine a difference between the 1-BP and control groups, when the data were normally distributed. Otherwise, the Mann-Whitney U test was applied, and p values <0.05 (two-tailed) were considered statistically significant. Electrophysiological data are expressed as means ± standard errors of the mean (SEMs). Litter size represents mean ± standard deviation. Statistical tests were performed in Ekuseru-Toukei 2010 for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

There was no difference between the litter sizes of the control group and 1-BP group (control: 14 ± 2 pups, 15 dams; 1-BP: 14 ± 3 pups, 12 dams).

As shown in Fig. 1C, the PS amplitude was 4 times greater in the 1-BP group than in the control group at 600 μA of stimulation intensity in 2-week-old rats. In 5-week-old rats, the enhancement disappeared, and the levels decreased to the control level of the S/R relationship of the PS amplitude (Fig. 1D). No difference was observed between the 1-BP and control groups at 8 and 13 weeks of age (data not shown). Increased excitability of pyramidal neurons was a transient change.

The left column of Fig. 2 shows examples of paired-pulse responses recorded from the hippocampal CA1 subfield of the control and 1-BP groups. As shown in Fig. 2A, at 2 weeks of age, the averaged PPR was approximately 2 in the control group, suggesting a facilitatory effect. In contrast, inhibition rather than facilitation was observed at the 5-ms IPI in the 1-BP group. At the 10-ms IPI, PPRs showed a slight facilitation but were significantly decreased compared to PPRs in the control group. At 5 weeks of age, PPRs were lower than 0.2, displaying an apparent feedback inhibition in both groups (data not shown), and the effects of prenatal 1-BP exposure on PS PPRs disappeared. At 8 and 13 weeks of age, PPRs were still lower than 1 but increased significantly at 5 ms of IPI in the 1-BP group compared with that of the control group (Fig. 2B and 2C). There was no significant difference in the disinhibitory effects between 8- and 13-week-old rats.

Discussion

In general, the effects of prenatal chemicals on the brains of offspring with more littermates may be different from those with fewer littermates. In addition, litter size may be affected by inhalation of 1-BP by the dams. However, because there was no difference between the litter sizes of the control and 1-BP groups in this study, the delayed developmental toxicity observed was not likely to be associated with changes in litter size.

The present study revealed that prenatal exposure to 1-BP enhanced the excitability of CA1 pyramidal neurons and caused a decrease in PPRs of PS amplitude in hippocampal slices from 2-week-old rats. The lactation period after birth is considered to be the period of synaptogenesis in rat brains[13]; thus, neuronal development during the lactation period may be sensitive to prenatal chemical exposures. In a previous study[9], we reported that prenatal exposure to 1-BP suppressed KA-induced "wet dog shake" behaviors in 2-week-old rats. In this study, prenatal 1-BP exposure rendered the hippocampal CA1 subfield highly responsive to a single stimulation but suppressive to double stimulations. The decrease in PPRs in the 1-BP group shown in Fig. 2A may have been caused by the higher sensitivity of PS1. In the control group, PS1 may have been developmentally suppressed for some unknown reason (as shown in Fig. 1C), and the suppression may have been lifted following the second stimulation, as shown in PS2. Prenatal 1-BP exposure may weaken or lift the suppression in the PS1. The PS2 amplitude in the control group was similar to that seen in the 1-BP group. This indicates that prenatal 1-BP exposure may make CA1 neurons hyperexcitable at the developmental stage, which is quite different from normal brain development. Because 1-BP is metabolized in the womb of pregnant rats and biotransformed into metabolites, it is unclear whether those effects were caused by 1-BP itself.

Although a decrease in PPRs of PS amplitude was observed in the 1-BP group at 2 weeks of age, the difference diminished at 5 weeks of age, as did the S/R relationship. In contrast to that of the 2-week-old pups, the 8- and 13-week-old groups displayed an increase in PPRs of the PS, also known as disinhibition. In the 13-week-old rats, we observed that the disinhibitory effect induced by the prenatal 1-BP exposure was greater in female than in male rats (data not shown). It is unclear, however, whether sex-specific effects would be observed at other ages, and this should be investigated in the future. Thus, prenatal exposure to 1-BP can exert developmental effects linked to the excitatory function of neurons and network excitability. Disinhibition has been reported in relation to subclinical and clinical changes in brain excitability in epileptic patients and animals[14], as well as in anxiety disorders[15]. We did not observe any spontaneous abnormal behaviors in the 1-BP group during breeding. To date, developmental neurotoxic effects caused by 1-BP exposure have not been reported in children whose mothers were exposed occupationally during pregnancy. However, because disinhibition can be associated with the hyperexcitable brain and epilepsy, it should not be concluded that disinhibition is merely a phenomenon restricted to rats. Because disinhibition is interpreted as a disturbance of the excitation/inhibition balance in the hippocampal CA1 area, disin-

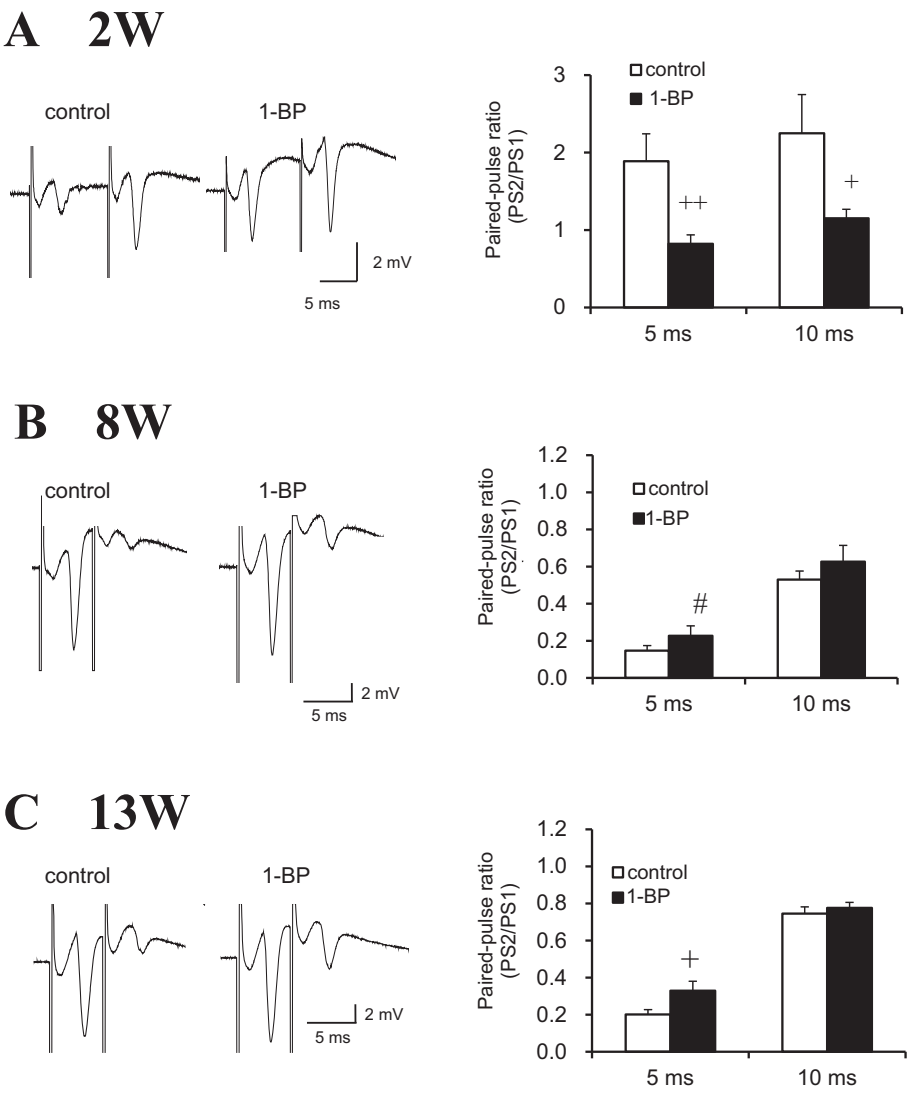

Fig. 2. Paired-pulse ratios (PPRs) of the population spikes (PSs) evoked with a double stimulation of 5 and 10 ms interpulse intervals (IPIs) in the CA1 subfield of hippocampal slices obtained from 2-, 8-, and 13-week-old male rats and 13-week-old female rats

Left column: Representative examples of paired-pulse responses recorded from the hippocampal CA1 subfield of the control and 1-BP groups. Right column: (A) At 2 weeks of age, PPRs decreased substantially in the 1-BP group (++p <0.01 vs. the control group at the 5-ms IPI, +p<0.05 vs. the control group at the 10-ms IPI by Welch's t-test). (B) At 8 weeks of age, PPRs were lower than 1 in both groups, indicating an apparent inhibition. At the 5-ms IPI, the PPR of the 1-BP group increased compared with that of the control group (#p <0.05 by Mann-Whitney U test). (C) Similar to the 5-ms IPI at 8 weeks of age, PPR of male rats in the 1-BP group increased compared with that of the control male rats at 13 weeks of age (+p <0.05 by Welch's t-test). The horizontal axis represents the IPIs; the vertical axis represents the PPRs of PS amplitude. Data of 16-25 slices were averaged.

hibitory effects can be classified as adverse effects.

The enhancement of excitability induced by prenatal 1-BP exposure was observed only in the 2-week-old group, and may therefore have been only a transient effect. Alternately, one could argue that the excess basal excitability during synaptogenesis is not coincidental with disinhibition after maturation. If so, the PS S/R relationship can be useful as a new index marker for developmental neurotoxicity of chemicals before the appearance of neurophysiological changes in the brain after maturation. To validate this method for assessing the developmental neurotoxicity of industrial chemicals, we should test chemi-

cals that are already known to exert developmental neurotoxicity. To this end, we are currently investigating valproic acid, an antiepileptic drug used in an established animal model of the developmental disorder, autism. Synaptic transmission generates action potentials; we are also studying field excitatory postsynaptic potentials.

In conclusion, we demonstrated that prenatal 1-BP exposure can cause delayed neurotoxicity, although the underlying mechanism is not known yet, and requires further investigation.

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Conflicts of interest: There are no conflicts of interest.

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SCIENTIFIC REPORTS

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Microbiome profile of the amniotic fluid as a predictive biomarker of perinatal outcome

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Chorioamnionitis (CAM), an inflammation of the foetal membranes due to infection, is associated with preterm birth and poor perinatal prognosis. The present study aimed to determine whether CAM can be diagnosed prior to delivery based on the bacterial composition of the amniotic fluid (AF). AF samples from 79 patients were classified according to placental inflammation: Stage III (n = 32), CAM; Stage II (n = 27), chorionitis; Stage 0-I (n = 20), sub-chorionitis or no neutrophil infiltration; and normal AF in early pregnancy (n = 18). Absolute quantification and sequencing of 16S rDNA showed that in Stage III, the 16S rDNA copy number was significantly higher and the α-diversity index lower than those in the other groups. In principal coordinate analysis, Stage III formed a separate cluster from Stage 0-I, normal AF, and blank. Forty samples were classified as positive for microbiomic CAM (miCAM) defined by the presence of 11 bacterial species that were found to be significantly associated with CAM and some parameters of perinatal prognosis. The diagnostic accuracy for CAM according to miCAM was: sensitivity, approximately 94%, and specificity, 79–87%. Our findings indicate the possibility of predicting CAM prior to delivery based on the AF microbiome profile.

Preterm birth, which occurs in 5–18% of all pregnancies, is caused by multiple pathological conditions[1,2] and is the leading factor in perinatal mortality and morbidity, and childhood neurological problems[3–5]. Intrauterine infection is linked to spontaneous preterm labour, which accounts for about two-thirds of all preterm births[1,6,7], and a minimum of 25–40% of premature infants are born to mothers with intrauterine infection[7–9]. In 30% of intrauterine infections, bacteria are identified in the foetal circulation[1,10], and it is known that foetal infections induce a systemic inflammatory response[11], which is suggested to cause abnormalities in the central nervous system, especially the white matter, by epidemiologic studies and animal experiments[12–14].

Chorioamnionitis is an inflammation of the foetal membranes (amnion and chorion) histologically diagnosed by the presence of acute inflammatory cells, such as neutrophils. In addition to being a gold standard for corroborating intrauterine infection, chorioamnionitis is associated with preterm birth and poor infant prognosis and is recognized as a risk factor for cerebral palsy and chronic lung disease[15–19]. The proposed diagnostic criteria prior to delivery[19,20] have low prediction accuracy for chorioamnionitis and intrauterine infection, and do not help prevent prematurity and neonatal sepsis[19,21].

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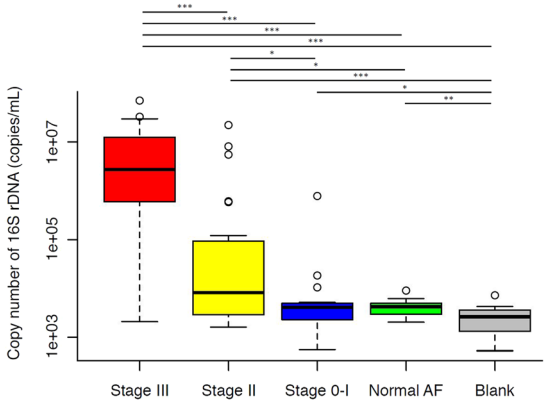

Figure 1. Microbial abundance in amniotic fluid samples. Microbial load was assessed based on 16S rDNA copy numbers per 1 mL AF using dPCR with universal primers 27Fmod and 338R and EvaGreen dye. The copy numbers in Stage III and Stage II were significantly higher than those in Stage 0-I/Normal AF/Blank; no differences were detected, only between Stage 0-I and Normal AF. Two-tailed probabilities were calculated by the Mann–Whitney test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

The amniotic fluid (AF) is considered to be sterile; however, this is frequently not the case in preterm birth, and a low gestational age at delivery is shown to be associated with bacterial infection in the AF[8]. Various bacterial species are detected in the AF in cases of preterm birth[22–26]. However, *Ureaplasma* spp. are also observed at 16–20 weeks in cases of normal delivery[27], and the detection rates of aerobic and anaerobic bacteria in the AF in full-term births are similar to those in preterm ones[28,29]; in addition, bacteria have been detected in umbilical cord blood and meconium[30,31]. These findings indicate that the AF is not necessarily sterile, even in normal pregnancies, and that the association between preterm deliveries and infection should be further clarified.

Molecular biology techniques have been proved effective for detecting *Ureaplasma* spp. and other bacteria difficult to identify using conventional culture methods[32]. However, to the best of our knowledge, metagenomic analysis with next-generation sequencing of the AF to verify the relationship between bacterial diversity and chorioamnionitis has not been conducted. Here, we performed absolute quantification of 16S ribosomal DNA (rDNA) copy numbers and sequencing of 16S rDNA amplified from the AF obtained by aseptic methods for comprehensive, quantitative analysis of AF microbiome. By examining the association between inflammation in the placenta and the bacterial composition of the AF, we demonstrated, for the first time, that microbial profiling of the AF can be used to diagnose chorioamnionitis with a high degree of accuracy prior to delivery, and to predict perinatal complications.

Results

Assessment of study subjects. Seventy-nine patients selected for the study were divided based on the stage of placental inflammation (Blanc's classification)[33]: Stage III (n = 32), chorioamnionitis; Stage II (n = 27), chorionitis; and Stage 0-I (n = 20), sub-chorionitis or no neutrophil infiltration. AF samples collected in the early second trimester (mean ± SD: 16.1 ± 0.6 weeks of pregnancy) during the same period were used as the AF control (Normal AF; n = 18), while laboratory-grade water was used as blank control (Blank; n = 24) for DNA extraction and library preparation (Table S1). Demographic and clinical characteristics for Stage III, Stage II, and Stage 0-I patients were extracted from medical records (Table S2).

Stage III was significantly different from Stage 0-I regarding multigravida, preterm premature rupture of membranes, antibiotic use before amniocentesis, number of caesarean sections, gestational age at amniocentesis, maternal inflammation (white blood cell [WBC] count, C-reactive protein [CRP] value), WBC count in the AF, funisitis of umbilical cord, extended days of hospital stay from admission to birth, neonatal inflammation (WBC count, CRP value, IgM level, funisitis of the umbilical cord), and antibiotic use for newborns (Table S2). Between Stage II and Stage 0-I, significant differences were observed only in caesarean sections, maternal CRP value, extended days of hospital stay, and neonatal IgM value (Table S2).

Quantification of microbial colonization. To assess bacterial load, we determined 16S rDNA copy numbers per 1 mL of AF using digital (d)PCR with a universal primer set[34,35] and EvaGreen dye. The median 16S rDNA copy number in Stage III was 328 and 656 times higher than that in Stage II and Stage 0-I, respectively (2.70×10^6 vs. 8.25×10^3 and 4.12×10^3, respectively; $P < 0.001$), and that in Stage II was 2.0 times higher than that in Stage 0-I ($P = 0.019$) (Figs 1, S1).

While Stage 0-I and Normal AF demonstrated significantly higher copy numbers than Blank ($P = 0.035$, 0.003, respectively), no difference was observed between Stage 0-I and Normal AF ($P = 0.696$), indicating that the AF in the early second trimester of a normal pregnancy is not necessarily as sterile as laboratory-grade water.

Comparison of bacterial diversity. Using the same universal primer set, we amplified and performed parallel sequencing of 16S rDNA in 97 AF samples and 24 blank controls. Only one sample (N11) did not yield sufficient reads for metagenomic analysis. Operational taxonomic units (OTUs) were created and within-community

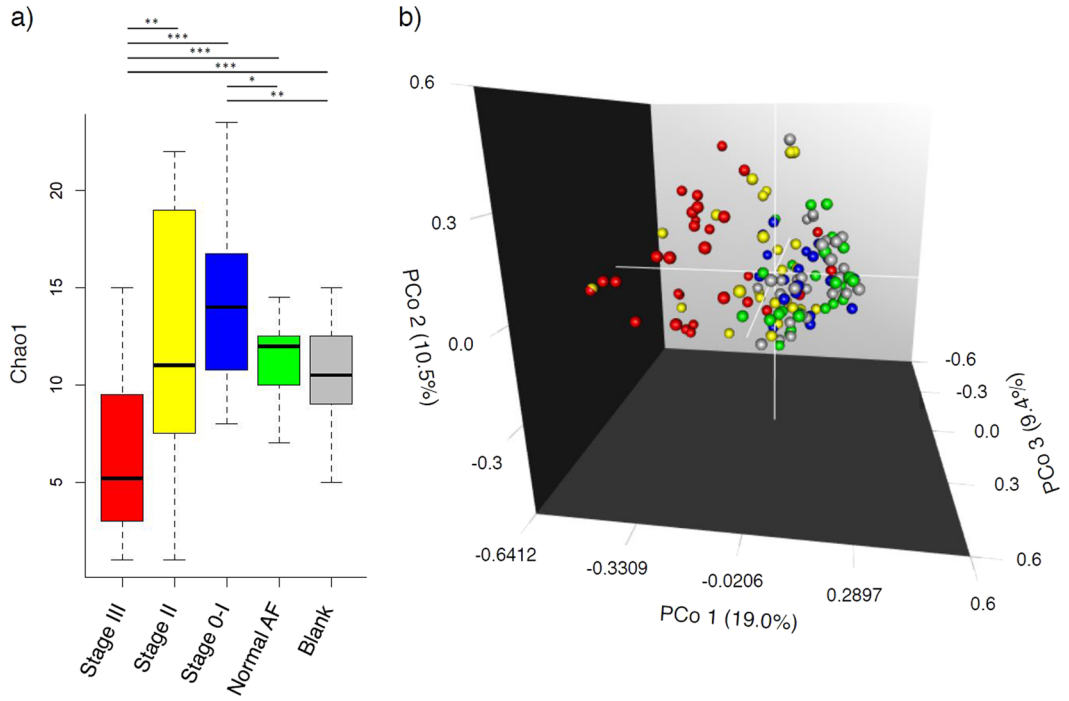

Figure 2. Numbers of OTUs (Chao1 index) and 3D-PCoA based on un-weighted UniFrac distances. Amplicons of 16S rDNA were sequenced using 27Fmod and 338R primers. (**a**) Sequences were clustered into OTUs with a 97% identity threshold and the α-diversity index (Chao1) was calculated for each sample. In Stage III, Chao1 was significantly lower than in the other groups. (**b**) Multidimensional composition of each group was determined based on matrix data for un-weighted UniFrac distance. Clustering of Stage III (red) samples differed from that of Stage 0-I (blue)/Normal AF (green)/Blank (grey); Stage II (yellow) was scattered between the two clusters. Three-D PCoA was performed with R; $*P<0.05$, $**P<0.01$, $***P<0.001$ by Mann–Whitney test.

(alpha) diversity was assessed by comparing the α-diversity index (Chao1) between the groups (Fig. 2a). Interestingly, Stage III demonstrated a significantly lower Chao1 than Stage II, Stage 0-I, Normal AF, and Blank ($P=0.001$, $P<0.001$, $P<0.001$, and $P<0.001$, respectively) and Stage 0-I demonstrated a significantly higher Chao1 than Normal AF and Blank ($P=0.038$, 0.008, respectively), while no other between-group differences were detected.

To compare phylogenetic relatedness of the microbial communities, we determined UniFrac distances between samples according to OTU data[34–36]. In principal coordinate analysis (PCoA) based on un-weighted UniFrac distances, Stage 0-I, Normal AF, and Blank clustered together, while Stage III formed a separate cluster, and Stage II was scattered between these two clusters (Fig. 2b). Analysis of un-weighted UniFrac distances with PERMANOVA revealed that Stage III and Stage II were significantly different from the other groups (Stage 0-I/Normal AF/Blank), and Stage 0-I was significantly different from Blank, but there were no differences among the other groups (Table S3). In analysis of weighted UniFrac distances with PCoA and PERMANOVA, similar to the results of un-weighted UniFrac distances, Stage III was significantly different from the other groups (Stage 0-I/Normal AF/Blank), and Stage II was scattered (Fig. S2, Table S3).

Analysis of bacterial composition in individual samples. Phylum-, genus-, and species-level OTUs were created with identity thresholds of 70%, 94%, and 97%, respectively, and taxonomic structure in each OTU was assessed by similarity searching against the standard database. Sample rearrangement by hierarchical cluster analysis using Ward's method based on un-weighted UniFrac distances (Figs 3a, S3a) mostly showed phylum-level distribution (Fig. 3). Consistent with the PCoA results, Stage III and Stage 0-I/Normal AF/Blank formed roughly separate clusters, while Stage II was scattered between the two clusters (Figs 3, S3). Consistent with the results shown in Fig. 1a, genus-level analysis indicated that multiple samples of Stage 0-I/Normal AF/Blank demonstrated a relatively high species richness, indicating complex compositions (Fig. S3). In contrast, the numbers of species in Stage III and in some samples of Stage II were extremely low (Fig. S3). Particularly noteworthy is the relative abundance of *Ureaplasma* spp. (minimum 35.5%) in fifteen samples (A2, 3, 6–8, 11, 13–15, 18, 20, 22, 24, 25, 31) of Stage III (64.3%) and six samples (B3, 4, 12, 16, 18, 19) of Stage II (22.2%) compared to all other samples (maximum 8.8%).

Selection of bacterial species as candidate diagnostic markers. To assess the association of particular bacterial species with chorioamnionitis, the data on relative abundance of the 28 most representative species were re-clustered according to the 79 samples in Stage III, Stage II, and Stage 0-I (Fig. 4).

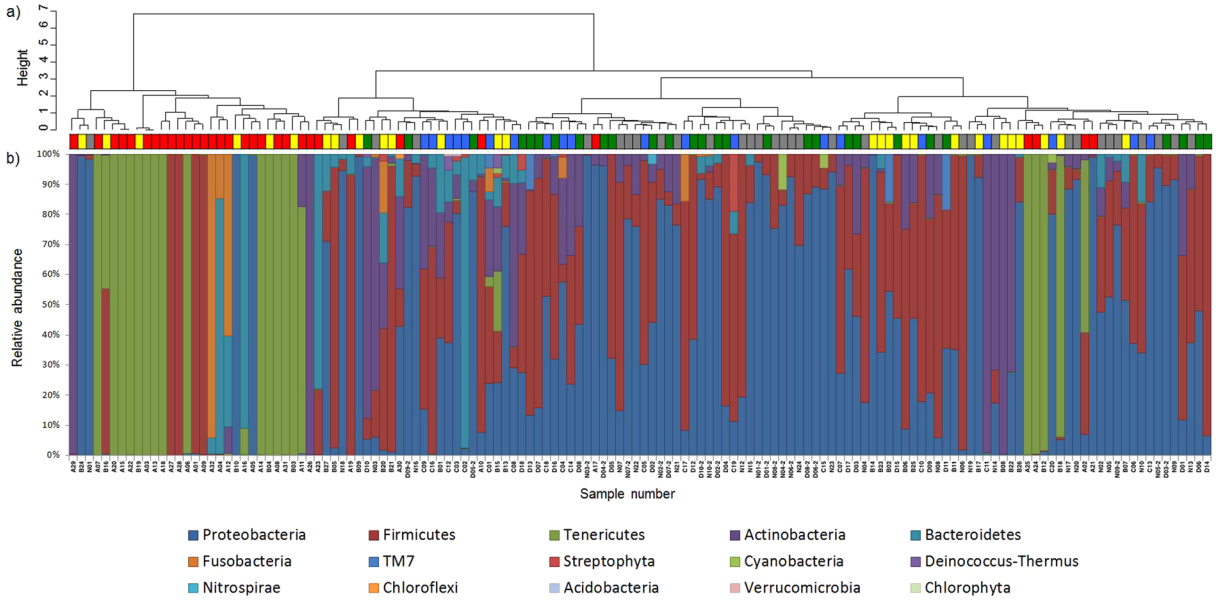

Figure 3. Relative abundances of different bacterial phyla in each sample. Sequences were clustered into OTUs with a 70% identity threshold and taxonomic assignments were performed by similarity searching against the standard database. The samples were rearranged by hierarchical cluster analysis using Ward's method based on un-weighted UniFrac distances. Stage III and Stage 0-I/Normal AF/Blank formed separate clusters, while Stage II was scattered between the two clusters.

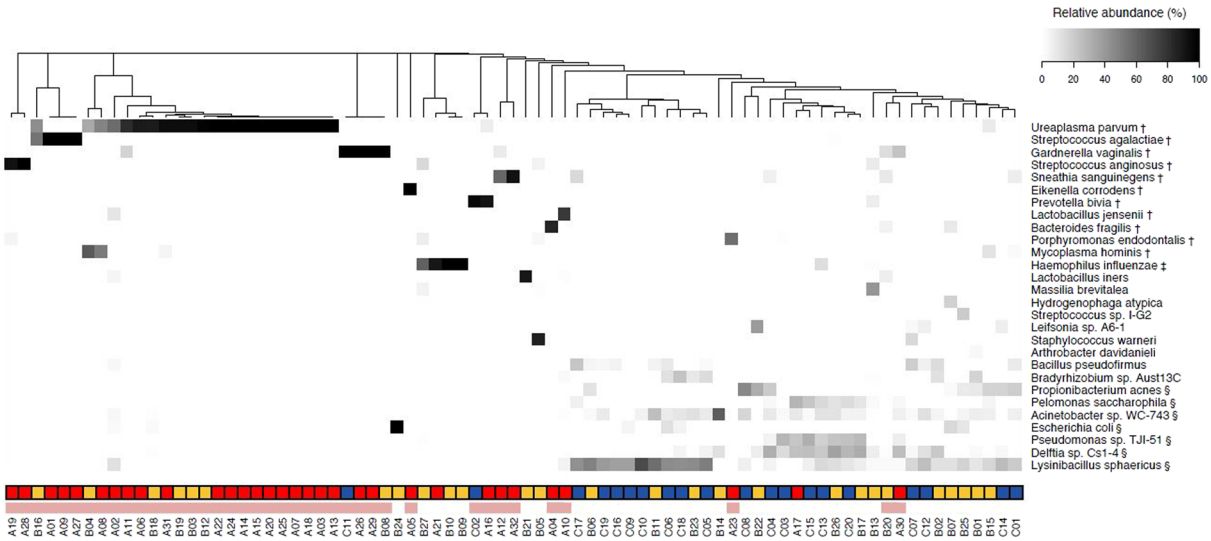

Figure 4. Relative abundances of the 28 most dominant species. Data on the relative abundances of the 28 most representative species were re-clustered according to the 79 samples in Stage III, Stage II and Stage 0-I. Stage III and Stage 0-I roughly formed separate clusters, while Stage II was scattered between the two clusters. The 11 most dominant species in Stage III (†) were almost non-existent in Stage0-I, while the seven most dominant species in Blank (§) were not dominant in Stage III and 40 miCAM samples (indicated by a pink bar). *H. influenza* (‡), which was dominant in one sample in Blank (N16), was dominant in some samples in Stage III and Stage II.

Stage III and Stage 0-I roughly formed separate clusters, while Stage II was scattered between the two clusters. In Stage III samples, the 12 most dominant species were *Ureaplasma parvum*, *Streptococcus agalactiae*, *Gardnerella vaginalis*, *Streptococcus anginosus*, *Sneathia sanguinegens*, *Eikenella corrodens*, *Prevotella bivia*, *Lactobacillus jensenii*, *Bacteroides fragilis*, *Porphyromonas endodontalis*, *Mycoplasma hominis*, and *Haemophilus influenzae*; however, except for *H. influenzae*, these species were nearly absent in Normal AF and Blank (maximum: 3.46%). In Blank samples, the most dominant species were *Lysinibacillus sphaericus*, *Delftia* sp. Cs1-4, *Pseudomonas* sp. TJI-51, *Escherichia coli*, *Acinetobacter* sp. WC-743, *Pelomonas saccharophila*, and *Propionibacterium acnes*, which can

be attributed to inevitable very low amounts of contamination during sample preparation. *H. influenzae*, which was dominant in some samples of Stage III and Stage II (A21; B9, 10, 27), was also dominant in one Blank sample (N16); indicating that contamination is nearly unavoidable.

The 11 species (*U. parvum, S. agalactiae, G. vaginalis, S. anginosus, S. sanguinegens, E. corrodens, P. bivia, L. jensenii, B. fragilis, P. endodontalis, and M. hominis*) that were remarkably dominant in Stage III but not in Blank were considered as candidate markers for prenatal diagnosis of chorioamnionitis; therefore, the samples in which any of these species demonstrated the highest abundance were considered positive for microbiomic chrioamnionitis (miCAM). According to this criterion, 30 out of 32 samples in Stage III (94%), 8 out of 27 samples in Stage II (30%), and 2 out of 20 samples in Stage 0-I (10%) were miCAM-positive (Table S4). The accuracy of chorioamnionitis diagnosis (Blanc's stage III) based on miCAM was as follows: sensitivity, 93.8%; specificity, 78.7%; positive predictive value, 75.0%; negative predictive value, 94.9%.

Comparison of clinical characteristics between miCAM and non-miCAM samples. We compared continuous variables related to maternal and infant perinatal outcomes between miCAM and non-miCAM samples (Fig. 5, Table S5). Although no significant difference was observed in gestational age and neonatal body weight at birth, Apgar score, or umbilical arterial pH; the miCAM subgroup demonstrated significantly shorter duration of maternal hospital stay from admission to birth, and higher WBC count, CRP value, and IgM levels in neonatal peripheral blood immediately after birth (Fig. 5). These results indicated a significant correlation between miCAM and adverse prognostic parameters in both mother and infant.

Diagnostic accuracy for miCAM and chorioamnionitis assessed by clinical and metagenomic sequence data. Next, we used clinical and laboratory data, DNA concentrations, and 16S rDNA copy numbers to calculate the area under the curve (AUC), the Youden index for the receiver operating characteristic (ROC) curve, cut-off value, and diagnostic sensitivity and specificity (Table S6, Fig. S4). Compared to body temperature, heart rate, WBC count, and CRP level in maternal peripheral blood, DNA amount and especially, 16S rDNA copy number demonstrated higher diagnostic accuracy for both miCAM and chorioamnionitis. Regarding miCAM, the diagnostic accuracy according to 16S rDNA copy number with an AUC of 0.909 (asymptotic 95% confidence interval [CI]: 0.838–0.980) and a cut-off value of 1.19×10^4 was: sensitivity, 94.9%, and specificity, 78.9%. For chorioamnionitis (Blanc's stage III), the diagnostic accuracy with an AUC of 0.926 (asymptotic 95% CI: 0.868–0.985) and a cut-off value of 1.73×10^4 was: sensitivity, 93.5%, and specificity, 87.0% (Table S6, Fig. S1).

Discussion

A close relationship between intrauterine infection and preterm labour have been confirmed[22,23,26,36], and recent studies have shown the association of placental microbiome composition with preterm birth and chorioamnionitis[37,38]. However, to the best of our knowledge, there was no report on metagenomic analysis of the AF to verify the relationship between bacterial community structure and placental inflammation. We conducted a metagenomic analysis of the AF obtained by aseptic methods from patients with or without chorioamnionitis and defined miCAM, which showed predictive utility in the identification of patients with poor prognosis regarding preterm delivery and neonatal status.

Of the 28 most dominant species identified (Fig. 4), the 12 most dominant species in Stage III (*U. parvum, S. agalactiae, G. vaginalis, S. anginosus, S. sanguinegens, E. corrodens, P. bivia, L. jensenii, B. fragilis, P. endodontalis, M. hominis, and H. influenzae*) primarily colonize the urogenital system (except *B. fragilis and P. endodontalis*, which are indigenous to the intestinal tract and oral environment, respectively); many of these may cause foetal infections such as meningitis and/or pulmonary disease, which can lead to brain dysfunction, epilepsy, hearing loss, and developmental disorders[19,39–45].

Eleven of the 12 bacterial species dominant in Stage III (except *P. endodontalis*) have been previously identified qualitatively in AF collected under sterile conditions[22–24,26,36]; among these, *Ureaplasma* spp. is frequently detected in spontaneous preterm birth[36,39]. Moreover, lipoprotein multiple-banded antigen from *U. parvum* has been shown to cause preterm birth in experimental animals[46], supporting the correlation between *Ureaplasma* presence and pregnancy outcome.

In the present study, we used samples from two distant hospitals, which were sequenced twice, to avoid regional and experimental bias. For the five most dominant species (*U. parvum*, *S. agalactiae*, *G. vaginalis*, *S. anginosus* and *S. sanguinegens*) in Stage III, the data for the two institutions corresponded well. In our previous study, we had confirmed a relationship between placental inflammation (Blanc's classification) and the 11 species defining miCAM[36]. In seven out of 10 cases (70%) with chorioamnionitis (Stage III), at least one of these species were dominantly detected; in two cases, different species, but from the same genera as those of the 11 dominant species, were detected; and in one case, no bacteria were detected[36]. These results were remarkably consistent with our current results. Moreover, in five major reports on the AF in cases of preterm birth[22–24,26,36], in at least 70% of all cases, at least one of the 10 most dominant genera in Stage III (*Ureaplasma*, *Streptococcus*, *Gardnerella*, *Sneathia*, *Eikenella*, *Prevotella*, *Lactobacillus*, *Bacteroides*, *Porphyromonas*, *Mycoplasma*) was qualitatively detected. Thus, our results can be regarded reliable.

The seven most dominant species in Blank (*L. sphaericus*, *Delftia* sp. Cs1–4, *Pseudomonas* sp. TJI-51, *E. coli*, *Acinetobacter* sp. WC-743, *P. saccharophila*, and *P. acnes*) were estimated to originate mainly from contamination during library preparation. Of the seven most dominant species in Blank, six genera (*Delftia*, *Pseudomonas*, *Escherichia*, *Acinetobacter*, *Pelomonas*, and *Propionibacterium*) have been previously reported as common contaminants[47]. *L. sphaericus* is primarily observed in mosquito larvae; it is a spore-forming bacterium resistant to heat and ultraviolet radiation, and common in aquatic environments[48–51]; therefore, trace amounts of DNA may have contaminated reagents, tubes, or instrumentation. The low abundance and high α-diversity (more complex

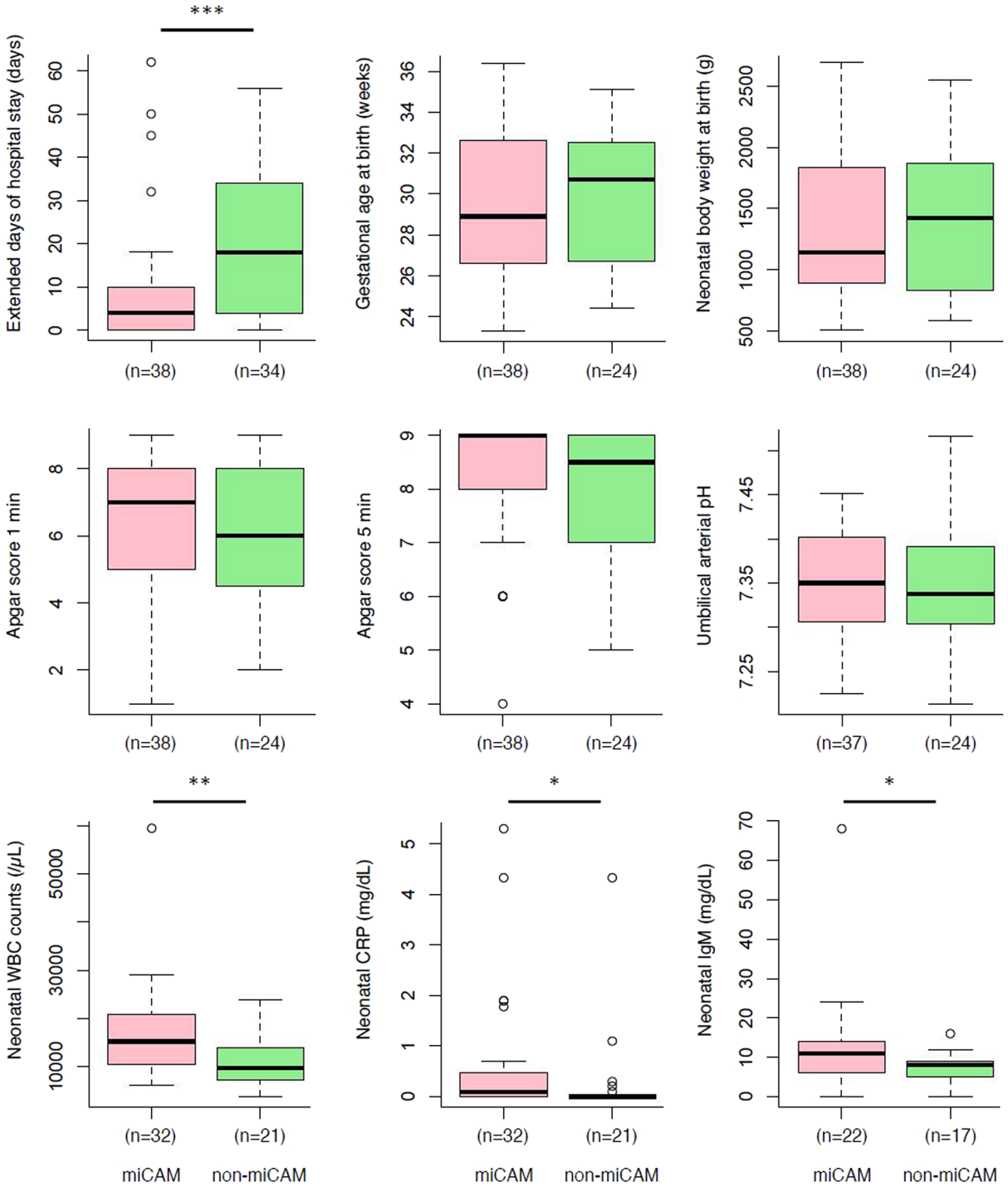

Figure 5. Comparison of perinatal outcomes between miCAM and non-miCAM subgroups. Comparison of continuous variables related to maternal and perinatal outcomes between miCAM and non-miCAM samples revealed that miCAM was significantly associated with many prognostic parameters of perinatal outcome. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Mann–Whitney test.

composition) of the seven species were consistently demonstrated in all samples dominated by these species, which is in agreement with previous reports.

For a long time, attempts have been made to diagnose chorioamnionitis and intrauterine infection prior to delivery[19,20,52–56]. Although high-accuracy diagnostic biomarkers have been reported[53], no diagnostic standards have been established[19,55] because of significantly overlapping confidence intervals and inconsistent associations between preterm birth and placental microbiome[1,37,38,55]. In the present study, we demonstrated that it is possible to diagnose chorioamnionitis with a high level of accuracy according to miCAM defined by metagenomic sequence profiles and 16S rDNA copy numbers in the AF. Therefore, miCAM can be used to assess the state of

intrauterine infection during pregnancy, which would help in the management of cases with high risk of preterm birth.

The quantification of 16S rDNA by dPCR has been recently reported useful for evaluating the prevalence of low-abundance bacteria[57,58]. We discovered, with high reproducibility, that when placental inflammation was mild (Stage $\leq$ I), microbial abundance in the AF was as low as in the early second trimester, even in preterm birth (Fig. 1).

Contamination of laboratory-grade water, PCR reagents, and DNA extraction kits can potentially significantly affect structural analysis of microbiome with low abundance[47,59]. Therefore, in this study, we made every effort to operate under strictly sterile conditions. However, complete prevention of contamination is considered impossible[47]. Therefore, we used a blank control (laboratory-grade water) in DNA extraction and library preparation, which, consistent with previous data[58], showed an extremely low presence of 16S rDNA (1–10 copies/μL). The blank samples were used to document bacterial sequences introduced during sample processing, which were excluded from the miCAM-defining species.

This study had some limitations. First, sample selection had a bias. The analysed AF specimens were obtained at caesarean section or were left-overs from clinical testing by amniocentesis[22], which is an invasive procedure resulting in miscarriage or preterm birth in approximately 0.1–2% of cases[60–63], and is not performed for all pregnancies. Therefore, the retrospective case-control study design made it difficult to achieve ideal control of patient characteristics, as evidenced by significant differences between groups in multigravida, preterm premature rupture of membranes, antibiotic administration before amniocentesis, caesarean section, and gestational age at amniocentesis. While there is a possibility that maternal use of antibiotics before amniocentesis influenced the results of metagenomic analysis, we considered it to be negligible; data of all of the 21 samples (A2, 10, 15, 18, 27; B6, 7, 14, 21–26; C6, 10, 12, 16–19) from patients that had not used antibiotics before amniocentesis, were in line with the findings based on the other samples. Second, 16S rDNA sequencing is inferior to whole-genome shotgun sequencing in terms of bacterial quantification potential, because 16S rDNA sequencing has a PCR bias. However, samples with low microbial abundance are contaminated with human DNA in typical DNA extraction methods, leading to enormous whole-genome shotgun sequencing costs; therefore, a method not susceptible to the effects of human DNA should be developed. Third, metagenomic analysis cannot distinguish between live and dead bacteria, and microbial profiles in antibiotic-treated patients may not be objective.

In the present study, we comprehensively and quantitatively analysed the microbiome of the AF and, by examining its association with the degree of placental inflammation, identified bacteria significantly associated with chorioamnionitis. Further studies should focus on achieving higher diagnostic sensitivity and specificity and on developing non-invasive testing methods, which would contribute to timely diagnosis and improve perinatal outcome.

Materials and Methods

Study design. In total, 8,172 births occurred at the Center for Maternal, Fetal, and Neonatal Medicine, Fukuoka University Hospital and at the National Hospital Organization Saga Hospital, between August 2009 and April 2017. Placental pathology examination was conducted in 4,373 cases; among them, amniocentesis was performed for 183 patients who provided informed consent for study participation, and left-over AF samples were cryopreserved. Moreover, 10 samples were added which were obtained at caesarean section within the same period under absolutely sterile condition. To minimize bias, we established the following exclusion criteria: multiple pregnancies and amniocentesis in the early second trimester. As a result, 79 patients who passed the exclusion criteria and for whom $\geq$ 3 mL AF was available by amniocentesis or caesarean section were included in the study.

Patients were divided into three groups based on Blanc's classification of placental inflammation severity[33]: Stage III (n = 32), Stage II (n = 27), and Stage 0-I (n = 20). We also established two control groups to ensure accuracy of data assessment considering the extremely low amounts of microbial DNA that can contaminate samples at any point from sample collection to sequencing. In the early second trimester (16.1 ± 0.6 weeks; mean ± S.D.), amniocentesis was performed for foetal genetic testing, and AF samples from normal pregnancies were considered as a control group (Normal AF; n = 18). Additionally, we used blank controls consisting of laboratory-grade water (Blank; n = 24) during DNA extraction and sequencing library preparation.

All methods were performed in accordance with the STARD guidelines and regulations for reporting diagnostic accuracy studies. The study was approved by the review boards of the Fukuoka University Hospital, National Hospital Organization Saga Hospital and the National Research Institute for Child Health and Development (protocol numbers 15-2-08, 23–4, and 699, respectively). Informed consent was obtained from all participants, who were explained the potential risks, including accidental leaks of personal information and project data, prior to the study. For patients who wished to withdraw content, we were able to dispose of the remaining samples, extracted DNA, and all project data at any time; however, we could not delete metagenomic sequence data which had been made publicly available through an open-access database.

Diagnostic criteria. Histological chorioamnionitis was defined as the presence of acute inflammatory lesions of the chorion or amnion according to Blanc's criteria[33]: stage I (sub-chorionitis): patchy or diffused accumulation of neutrophils within the sub-chorionic plate or decidua; stage II (chorionitis): more than a few scattered neutrophilic infiltrations in the chorionic plate or membranous chorionic connective tissue; and stage III (chorioamnionitis): neutrophilic infiltrates reaching sub-amniotic connective tissue and the amniotic epithelium. Funisitis was defined as neutrophilic infiltration in the umbilical vein wall or Wharton's jelly.

Sample collection and DNA extraction. AF samples were obtained by transabdominal ultrasound-guided amniocentesis performed percutaneously or at caesarean section under sterile conditions. The AF samples were collected in sterile tubes and preserved at 4 °C; leftover samples not needed for testing were

transported on ice within 24 h to a neighbouring laboratory. To minimize changes in bacterial composition, all laboratory procedures were performed rapidly on ice. Samples were centrifuged at low speed (1,450 × *g* at 4 °C for 10 min) as previously described[22,24]; the supernatant was rapidly frozen in liquid nitrogen and preserved at −80 °C until DNA extraction.

Samples were thawed and lysed using Pathogen Lysis Tubes L (Qiagen, Hilden, Germany), and DNA was extracted using the QIAamp UCP Pathogen Mini Kit (Qiagen) according to the manufacturer's protocol, at the National Research Institute for Child Health and Development and Fukuoka University.

Absolute quantification of 16S rDNA. dPCR was conducted with EvaGreen dye, using universal primers (27Fmod and 338R) for 16S rDNA sequencing, as previously described[34,35,58]. DNA (1 μL) was diluted in 19 μL of Bio-Rad QX200 reagents (Bio-Rad, Hercules, CA, USA), and each sample was then partitioned into approximately 20,000 droplets using the QX200 Droplet Generator (Bio-Rad). PCR was performed in a 96-well plate using the following cycling conditions according to the manufacturer's protocol: 5 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 60 °C, then 5 min at 4 °C and 5 min at 90 °C; the temperature was then decreased to 4 °C at a ramp rate of 2 °C/s. Fluorescence was detected using the QX200 Droplet Reader (Bio-Rad) and analysed using the Bio-Rad QuantaSoft software. Copy number of 16S rDNA per 1 mL of the sample was then calculated.

Sequencing of 16S rDNA amplicons. The same universal primers (27Fmod and 338R) were used for PCR amplification of the variable region (V1–2) of the 16S rRNA gene. A 16S Metagenomic Sequencing Library was prepared according to the Illumina protocol (16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. A; Illumina, San Diego, CA, USA). PCR was performed using the KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Boston, MA, USA) for 25 amplification cycles at 60 °C as an annealing temperature according to the manufacturer's recommendation. The amplified products were purified using the Agencourt AMPure XP Kit (Beckman Coulter, Tokyo, Japan) and tagged with indexes in eight cycles using the Nextera XT Index Kit (Illumina). Amplicons were quantified using the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) or the Agilent 2100 Bioanalyzer (Agilent Technologies) and thoroughly mixed to achieve homogeneity. Then, size selection for next-generation sequencing was performed using Pippin Prep (Sage Science, Beverly, MA, USA), with approximately 300–600 bp of the mixed library as a target. MiSeq sequencing (paired-end, 300 bp) was conducted using MiSeq Reagent Kit v3 (600-cycle format; Illumina) mixed with 20% of PhiX Control Kit v3 (Illumina) according to the manufacturer's protocol.

Analysis of sequencing data. Sequencing data was analysed as previously described[34–36]. Two paired-end reads were merged using the fastq-join program based on overlapping sequences. Low-quality sequence reads (quality value < 25) and suspected chimeric reads (BLAST match length of <90% with reference sequences in the databases [Ribosomal Database Project v. 10.27 and/or in-house 16S sequenced database in Tokyo University]) were filtered out (Table S7). Following adapter sequence trimming, 1,300 reads were randomly selected. Using a 97% pairwise-identity cut-off in the UCLUST program[64] version 5.2.32 (http://www.drive5.com/), the selected reads were clustered into OTUs.

Taxonomic assignment for each OTU was done by similarity searching against the above-mentioned databases using the GLSEARCH program (data provided in Supplementary Dataset 1). For the assignment at the phylum, genus, and species levels, sequence similarity thresholds of 70%, 94% and 97%, respectively, were applied.

UniFrac distance was used to assess dissimilarity (distance) between each sample pair[65], and a 3D PCoA model was plotted according to UniFrac distances using the R package.

Statistical analysis. Because of the relatively low sample number, we calculated exact significance probabilities (two-tailed) as *P*-values using the Mann–Whitney test for continuous variables and Fisher's exact test for categorical variables. To assess diagnostic accuracy, we constructed ROC curves and calculated the AUC. These analyses were performed using SPSS version 16.0J for Windows Base System SC (SPSS Japan, Tokyo, Japan). For comparison of bacterial composition between groups, we used vegan package in R to calculate R^2 and *P*-values in PERMANOVA. Differences at $P < 0.05$ were considered statistically significant.

Data and material availability. The 16S sequencing data generated in the present study have been deposited in DDBJ Sequence Read Archive (DRA) (accession number DRA005144).

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Author Contributions

D.U. designed the study, enrolled subjects, developed the extraction and amplification protocols, conducted data acquisition; analysed the data, and wrote and edited the manuscript. S.M. and K.H. designed the study, obtained funding, enrolled subjects, developed the extraction and amplification protocols, and wrote and edited the manuscript. W.S., M.H. and E.O. conducted data acquisition, analysed the data, and wrote and edited the manuscript. M.N. designed the study, enrolled subjects, and conducted data acquisition. R.A., C.K., M.K., A.S., F.Y., M.M., and K.N. conducted data acquisition. S.Y. and S.S. interpreted the analysed data, and wrote and edited the manuscript.

Additional Information

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総　説

ミニ特集　発達期環境に起因する疾患素因の形成―DOHaD の視点から―

生殖と発生異常にかかわるエピゲノム変化と環境の影響

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Reproductive/Developmental Abnormalities Induced by Epigenetic Aberrations and Possible Environmental Causes

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Abstract　Foetal environmental factors, including maternal nutrition, hormonal disturbance, and chemical exposure, affect foetal growth and can cause birth defects. Recent studies have shown the link of poor foetal growth with increased risks of coronary heart disease, type II diabetes, kidney disease, and brain disorders in adulthood. Epigenetic modifications, such as DNA methylation and histone modifications, are involved in tissue- and developmental stage-specific gene expression and silencing, and they can be transmitted stably through mitotic cell division, thereby inducing long-term changes in gene regulation. Developmental programming during the foetal period, therefore, could affect adult health through epigenetic mechanisms. In fact, many studies using animal models have demonstrated that nutrient manipulation during pregnancy induces epigenetic alterations at specific loci or globally in the offspring. In this review, we summarize our findings that elucidate the effects of *in utero* environments on the human placental epigenome.

Key words: epigenetics（エピジェネティクス），Developmental Origin of Health and Disease（DOHaD），infertility（不妊・不育症），Small for Gestational Age（SGA 児）

1. はじめに

生殖や発生異常では，染色体検査を行っても明らかな異常（ゲノムの異常）が見つからないことが稀ではない。その理由として，これらが多因子性疾患であること，あるいは従来技術では同定できないような微細な染色体構造異常や点変異が関与していること，等が推測される。一方，近年エピジェネティックな因子の生殖・発生への関与が明らかにされつつあり，これらの遺伝子機能欠失モデルマウスでは多くの場合，生殖や発生の重篤な表現型が観察される。加えて，一部のヒト先天奇形症候群でも，モデルマウスと同様の症状や分子生物学的変化が観察される。これらの状況証拠から，ヒトの生殖細胞，胎児，胎盤の発生・分化・発育には，エピジェネティックな機構による遺伝子発現制御が必須であること，そして，前述の「ゲノムの異常が見つからないヒト生殖・発生異常症例」の一部には，未知のエピジェネティックな異常を伴う症例が存在すること，が推測される。

さらに，環境の影響を受けて起こり得る胎児や生殖細胞のエピゲノム変化もわかってきた。少なくともモデル生物では，胚培養などの人為的操作や妊娠母獣の食餌などの影響により，初期胚や胎児にエピゲノム変化が生じ，出生後も遺残して遺伝子発現に影響する事が示されている。また一部のモデル系では，生殖細胞を介した（世代間の）エピゲノム変化の伝達も報告されている。これらのメカニズムは，「受精時，胎芽期，胎児期の子宮内及び乳幼児期の望ましくない環境がエピゲノム変化を起こ

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し，それが疾病素因となり，出生後の環境との相互作用によって疾病が発症する。生活習慣病等の多因子疾患はこの 2 段階を経て発症する。」（日本 DOHaD 研究会ホームページより引用）という DOHaD（Developmental Origin of Health and Disease）学説の有力な分子病態の一つと考えられる。本総説では，生殖や発生にかかわるエピゲノム変化と環境の影響に関する最近の知見を概説すると共に，ヒト胎盤で観察されるエピゲノム変化を，我々の知見を交えてお示しする。

2. 生殖と発生にかかわるエピジェネティックな遺伝子調節の基盤

哺乳類の正常な発生には卵子と精子が必要で，決して単為発生しない。卵子と精子の有するジェネティックな情報（遺伝子の配列）は同一であっても，エピジェネティックな情報（インプリンティング遺伝子領域の DNA メチル化状態など）は異なるため，卵子と精子の遺伝情報が等価ではない。一方で，大部分のゲノム領域では，受精直後に精子由来のメチル化は速やかに消去され（能動的脱メチル化），卵子由来のメチル化はそれに遅れ，DNA 複製依存的に維持されずに失われていく（受動的脱メチル化）。親由来の DNA メチル化修飾情報はこのように大部分が一度消去され，その後胚盤胞期まで低メチル化状態にあり，着床後，複数の DNA メチル化酵素（DNMTs）によってメチル化状態が再構築され，発生段階特異的かつ組織特異的なメチル化修飾（エピゲノム）が確立されていく。雄性生殖細胞系列では，胎児期にメチル化修飾が行われ，出生前の精原細胞ですでにゲノム全体が高度にメチル化されているのに対し，雌性生殖細胞系列では，出生後の卵母細胞成長期にメチル化修飾が入る（図 1）。特にヒトでは，卵子の解析は倫理的・手技的に困難であり，精子の解析が進んでいるが，精子（配偶子）形成に必要な遺伝子のプロモーターはバイバレントな修飾（正と負の調節修飾が同時に起こっている状態）を受け，かつ，高メチル化修飾（>60% methylation）されている特徴を持つ。成熟精子ゲノムにはこのような，発現活性型の高メチル化プロモーターがプロタミン遺伝子や piRNA クラスターなどの，成熟精子形成過程に必須な遺伝子に見出された。これらの領域はプロタミンに置換されることなく，成熟精子でもヒストンヌクレオソームに位置する（1）。少なくとも精子において

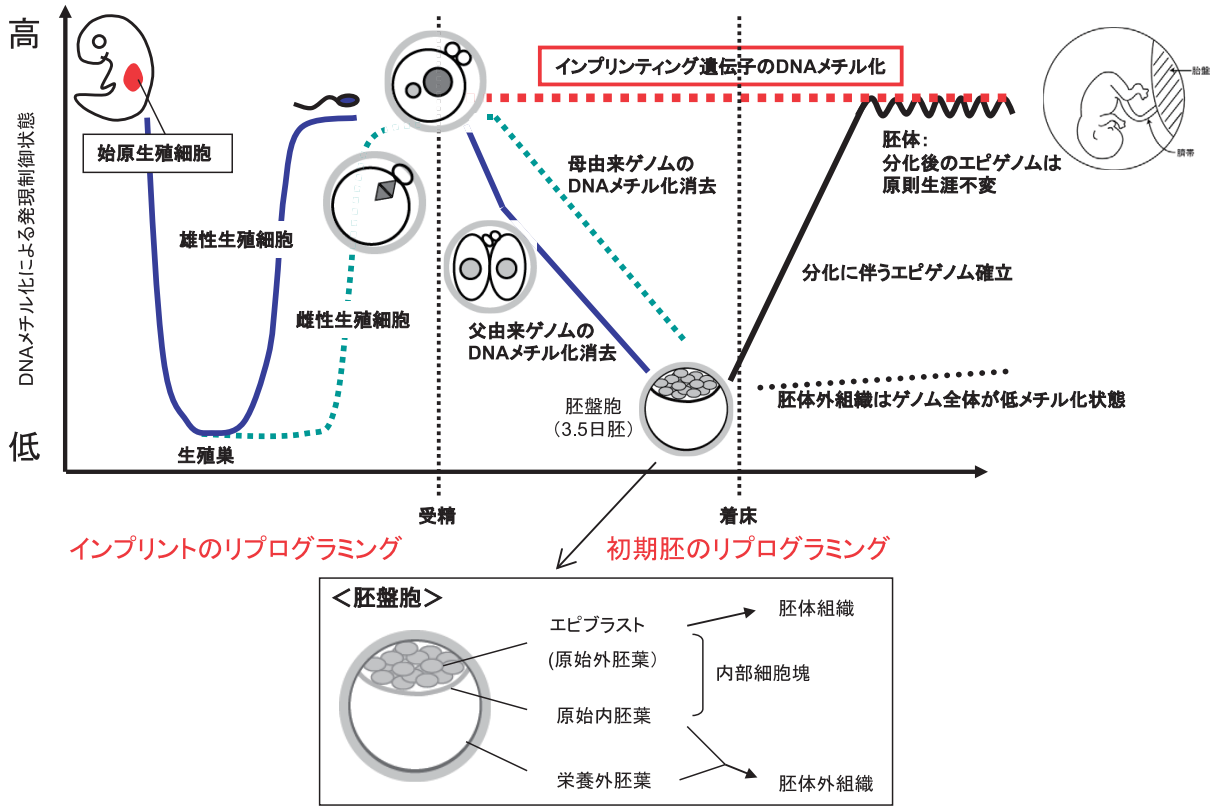


図 1　発生過程の組織特異的遺伝子のエピジェネティックな制御。受精後，着床までの間に，精子由来・卵子由来の両親のエピゲノム情報は消去され，着床後時期特異的ならびに組織特異的なメチル化修飾が再び胚ゲノムに入る。精子由来，卵子由来のインプリント領域のメチル化は脱メチル化から逃れる機構を備えており，両親のインプリント領域のメチル化修飾は胎児に遺残する。この精子由来・卵子由来のインプリント領域のメチル化は，両親の生殖細胞の分化の過程で確立されている。両親の生殖細胞は，胎児期の始原生殖細胞において自身の両親からのメチル化修飾が消去され，インプリント領域を含む精子ゲノム，卵子ゲノム特異的なメチル化修飾が生殖細胞の分化に伴い書き直される。

は，胎児期のエピゲノム修飾獲得が鍵となり，その修飾状態が生涯にわたり維持され，妊孕能，胚発生分化能に関与している可能性が示唆される。

3. 不妊症や不育症で報告されているエピゲノム異常

これまでに，原因不明と診断された不妊を呈す様々な疾患の精子ゲノムにおいてインプリント領域の DNA メチル化異常が報告されている。インプリンティング遺伝子は，DNA メチル化により制御され，厳密な片親アレル発現を保持する。これらのエピジェネティックな制御は，前述の発生初期のゲノム全体の DNA メチル化消去と再構築から免れているため，配偶子のエピゲノム（DNA メチル化）異常が胚発生を左右し，不妊や発育異常の直接の原因となりうる。

特に，精子ゲノムの *H19* インプリント領域低メチル化は自然流産，不育症，乏精子症，精子無力症の患者で認められている（2–5）。他に，乏精子症，精子無力症の精子では *PEG1/MEST* の高メチル化も報告されている（4–7）。これら乏精子症や精子無力症のメチル化異常は，全ての精子で生じているのではなく，正常なメチル化パターンを持った精子と異常パターンの精子がモザイク状に存在することも示された。さらに，乏無力精子症の精子ゲノム *H19* 低メチル化は，母由来アレルの低メチル化に起因することも認められている（5）。

非インプリント領域においても，不妊を呈するヒト精子ゲノムにおいて，葉酸代謝関連酵素遺伝子のメチル化異常や（8），生殖細胞特異的な piRNA 機構に関与する遺伝子のメチル化異常が検出されている（9）。特定の遺伝子に対するエピゲノム異常にとどまらず，不妊症例では本来精子特異的に高メチル化が認められる領域が広く DNA メチル化異常を起こしていることも認められている（10）。

動物実験では，これらのメチル化異常誘導因子の一つとして，父親の葉酸摂取不足が指摘されている。雄マウスの葉酸不足は，精子形成や精子の数・受精能・胚形成には変化を及ぼさないものの，着床後の流産率を高め，解剖学的異常を呈する胎仔の出生率を高めた。この雄マウスの精子では，ヒストンのメチル化修飾が全体的に減少しており，かつ，発生にかかわる遺伝子近傍の DNA メチル化修飾に変化が認められた（11）。このモデル系では，胎仔期から離乳まで葉酸欠乏状態に曝しており，胎仔期の葉酸不足がこの雄マウスの精子エピゲノム異常にクリティカルである可能性も示唆される。

4. 精子エピゲノム確立に寄与する環境

胎児期環境の攪乱が，生殖細胞のインプリンティング遺伝子インプリント機構の異常を介して生殖機能や次世代の発生に影響を及ぼすことが動物実験で確認されている。高分子樹脂として汎用され，広く環境中に存在する合成エストロゲンのビスフェノール A（BPA）に新生仔期に曝露すると，ラットの精子ゲノムあるいはその精子が受精した胚で，*H19* インプリント領域の低メチル化と遺伝子発現異常が認められ，最終的に流産する（12）。防カビ剤成分のビンクロゾリンに胎仔期に曝露すると，精子エピジェネティック異常と精子形成細胞のアポトーシスが 3 世代後まで遺残することが認められている（13, 14）。ビンクロゾリンによる世代を超えたエピジェネティクス異常はいくつかのインプリント領域でも生じている一方で，世代を超えるごとに徐々に正常化しているとの報告もある（15）。

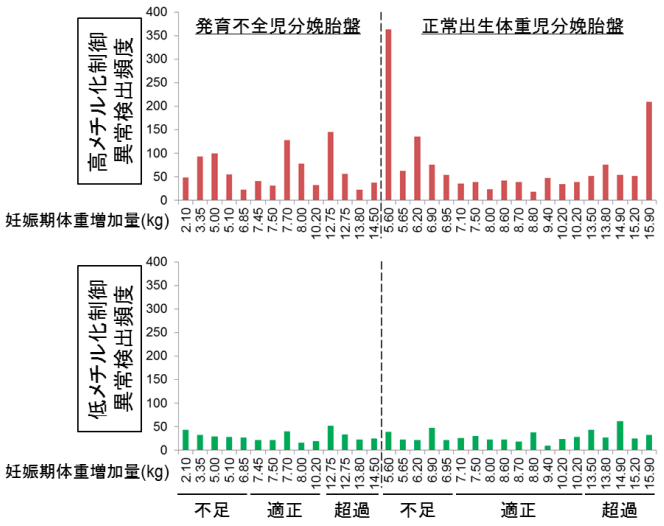
胎児期ではなく成人期の環境が，精子エピゲノミクス異常を介して次世代に影響する現象もモデル生物において分子機構の解明とともに報告されている。雄の食事内容が精子エピゲノム調節異常を介して次世代の肥満を呈するメカニズムがショウジョウバエで明らかにされ，標的となるのは代謝にかかわる遺伝子のヒストンの抑制マーク（H3K27me3, H3K9me3）の修飾異常であることが解明された（16）。

5. 子宮内環境に影響を受けるエピゲノム

我々は，胎生期の環境がエピゲノミクス制御に深く関与する例として，胎盤エピゲノムが母体の妊娠中の体重増加量によって左右される現象を見出した（17）。SGA 児（Small for Gestational Age）では予想通り，AGA 児（Appropriate for Gestational Age）と比較して胎盤の DNA メチル化状態の乱れが観察された。驚いたことに，AGA 児であっても，妊娠期体重増加量が不適切なほど（痩せすぎていたり太り過ぎていたりすると），個々の症例で DNA メチル化の外れ値を示す領域の数が増えた（DNA メチル化が乱れていた）（図 2）。共通の異常を示す遺伝子は見つからなかったが，これらのメチル化外れ値領域は，転写因子プロモーターに多く認められることから，実際に遺伝子発現を乱している可能性が示唆された。これらの転写因子の中には，HOX genes や SOX genes，FOX genes がいくつか認められた。これらの遺伝子群は，発生時期・組織特異的に発現し，それ以外ではエピジェネティックに抑制されていることが知られている（18）。ヒトの発生過程においても，ヒストンの抑制マークとそこにある DNA 領域のメチル化制御が，エピゲノム異常感受性の強い場所として，修飾変化を受ける可能性が示唆された。

6. おわりに

以上のように，受精前や胎児期の環境が，胎児ゲノムの様々な領域にエピジェネティック変化を引き起こし，出生後も長期にわたり遺残して成人期の疾患素因となる可能性は，様々なモデルで示されている。ヒトでも，不



有意と考えられるDNAメチル化変化が観察された遺伝子の例
胚性幹細胞においてH3K27me3修飾を受けている遺伝子（赤字）
胚性幹細胞においてH3K9me3修飾を受けている遺伝子（青字）

category	Subject	Gene Symbol	# of hyper-methylated outliers in pTSS	average Δβ of outliers
児:正常_適正増加	N_adequate_5	ZNF649	6	0.19
児:正常_超過増加	N_excessive_1	GBX2	3	0.25
	N_excessive_1	ZNF350	8	0.30
	N_excessive_2	ZFP37	4	0.44
	N_excessive_5	ZHX2	8	0.15
	N_excessive_5	CDKN1C	4	0.28
	N_excessive_5	PAX6	2	0.23
児:正常_不足増加	N_insufficient_1	KCNH8	4	0.24
	N_insufficient_1	FOXL2	10	0.21
	N_insufficient_1	F2R	3	0.20
	N_insufficient_1	SOX7	3	0.18
	N_insufficient_1	NFIB	2	0.37
	N_insufficient_1	HMX2	2	0.23
	N_insufficient_2	TFCP2	5	0.19
	N_insufficient_3	PROX1	3	0.15
	N_insufficient_3	FOXC1	8	0.19
	N_insufficient_3	FOXB1	2	0.17
	N_insufficient_5	ETV1	6	0.11
	N_insufficient_5	ZNF426	2	0.17
児:発育不全	FGR_adequate_1	PER1	6	0.16
	FGR_adequate_3	ZNF619	4	0.27
	FGR_adequate_3	ZKSCAN4	3	0.25
	FGR_adequate_3	PGBD1	7	0.21
	FGR_adequate_3	HOXB7	4	0.23
	FGR_adequate_4	ESR1	3	0.17
	FGR_adequate_4	MGA	3	0.17
	FGR_excessive_1	RFX8	3	0.33
	FGR_excessive_1	ZNF483	2	0.20
	FGR_excessive_1	ZNF254	3	0.21
	FGR_excessive_2	ZNF577	6	0.43
	FGR_excessive_3	ZNF655	2	0.35
	FGR_insufficient_1	ZNF562	8	0.36
	FGR_insufficient_1	ZNF805	2	0.30
	FGR_insufficient_2	ZNF583	6	0.27
	FGR_insufficient_3	ZNF354C	7	0.27
	FGR_insufficient_3	ETV1	6	0.16
	FGR_insufficient_4	ZIK1	2	0.42

図 2　妊娠中の母体体重増加量に影響を受ける胎盤の DNA メチル化。胚発生と同時期に形成される胚体外組織である胎盤も，子宮内環境の要因の一つである妊娠期母体体重変化量の違いによって，抑制修飾を受けるヒストンヌクレオソーム領域に特徴的に DNA メチル化変化を受ける可能性が示唆された。

妊症・不育症・胎児発育異常等の疾患には，明らかに様々なエピゲノム異常が関与していることが推測され，一部の疾患では実際に証明されている。更に，一見正常であっても，隠れたエピゲノム変化が存在する可能性が我々のデータからは示唆された。短期的には，配偶子や初期胚のエピゲノム診断法を確立すれば，エピゲノム異常の観点から配偶子や胚の品質評価が可能となり，不妊症や不育症のリスク因子の一部を軽減できると期待できる。今後はさらに，これらのエピゲノム異常と長期予後との分子疫学的な検証が待たれる。

利益相反なし

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Novel Nonsense Mutation in the *NLRP7* Gene Associated with Recurrent Hydatidiform Mole

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Key Words
Recurrent hydatidiform mole · DNA methylation · *NLRP7* · Genomic imprinting

Abstract
Aim: This study aimed to clarify the genetic and epigenetic features of recurrent hydatidiform mole (RHM) in Japanese patients. ***Methods:*** Four Japanese isolated RHM cases were analyzed using whole-exome sequencing. Villi from RHMs were collected by laser microdissection for genotyping and DNA methylation assay of differentially methylated regions (DMRs). Single nucleotide polymorphisms of *PEG3* and *H19* DMRs were used to confirm the parental origin of the variants. ***Results:*** A novel homozygous nonsense mutation in *NLRP7* (c.584G>A; p.W195X) was identified in 1 patient. Genotyping of one of her molar tissue revealed that it was biparental but not androgenetic in origin. Despite the fact that the RHM is biparental, maternally methylated DMRs of *PEG3*, *SNRPN* and *PEG10* showed complete loss of DNA methylation. A paternally methylated DMR of *H19* retained normal methylation. ***Conclusions:*** This is the first Japanese case of RHM with a novel homozygous nonsense *NLRP7* mutation and a specific loss of maternal DNA methylation of DMRs. Notably, the mutation was identified in an isolated case of an ethnic background that has not previously been studied in this context. Our data underscore the involvement of *NLRP7* in RHM pathophysiology and confirm that DNA methylation of specific regions is critical.

Y. Ito and K. Maehara contributed equally to this work.

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Introduction

Complete hydatidiform mole (CHM) is an abnormal pregnancy that typically arises from an androgenote (diploid conception without maternal chromosomes) and predominantly gives rise to the development of only trophoblastic tissues [1, 2]. Recent studies of rare familial cases with recurrent molar pregnancies have shown that mutations of *NLRP7* and *KHDC3L (C6orf221)* are associated with recurrent hydatidiform moles (RHMs) [3, 4]. However, more than 20% of RHM cases have no mutations in these genes [5], and the mechanism underlying the occurrence of molar pregnancy associated with *NLRP7* and *KHDC3L (C6orf221)* mutation remains unknown. To clarify the genetic etiology of RHMs, further comprehensive genetic screening in various ethnic backgrounds is important.

In Japan, the incidence of CHM has become as low as that in western countries. The incidence of CHM per 1,000 live births was 0.49 in 2,000, with a recurrence rate of 1.3% [6, 7]. Nonetheless, a genetic and epigenetic analysis of RHM in Japan has not been performed previously.

CHM conceptions exhibit abnormal genomic imprinting. Although some villi of RHM possess normal biparental alleles, they exhibit loss of maternal DNA methylation of differentially methylated regions (DMRs) within imprinted loci, and thus have aberrant genomic imprinting [8, 9]. Because the histopathological findings of diploid biparental RHM are identical to those of diploid androgenetic CHM, diploid biparental RHM cannot be distinguished from typical CHM without a genetic and epigenetic diagnosis. To clarify the molecular mechanisms of RHM, we performed comprehensive genetic and epigenetic analyses of isolated Japanese RHM cases and in doing so, we identified a novel nonsense *NLRP7* mutation. The RHM tissue contained biparental alleles and showed a loss of maternal DNA methylation of imprinted genes. This evidence strongly supports the role for *NLRP7* in maternal DNA methylation of imprinted genes and the involvement of *NLRP7* in RHM.

Materials and Methods

Study Participants and Samples

Patients with RHM (n = 5) and CHM (n = 9) were included in this study. The profiles of the 5 RHM patients are shown in table 1. Each sample was histopathologically diagnosed as CHM or partial hydatidiform mole and previously genetically diagnosed as an androgenote or a triploid, with the exception of Patients 1, 2, 4 and 5 [10]. All the villi of the 9 CHM conceptions were androgenote. None of the CHM patients had a family history of the condition. Genomic DNA was extracted from peripheral blood cells and molar tissues. This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development, Tokyo, Japan (approval number 234), and written informed consent was obtained from all patients.

Table 1. Profiles of 5 patients with RHM

Patient ID	Pathological diagnosis	Genetic diagnosis	Familial history
1	5 CHM	This study	No
2	2 CHM	No samples	No
3	PHM, NP, SA, CHM	Triploid[1]	No
4	CHM, PHM	No samples	No
5	SA, PHM, NP, PHM	No samples	No

PHM = Partial hydatidiform mole; NP = normal pregnancy; SA = spontaneous abortion.

[1] Genetic diagnosis of the first molar pregnancy (PHM).

Whole-Exome Sequencing

The whole-exome library was prepared from the peripheral blood cells of 4 RHM patients (Patients 1–4 in table 1) by using Agilent SureSelect Human All Exon V3 capture reagent (Agilent Technologies, Inc., Santa Clara, Calif., USA), and sequenced using the Illumina HiSeq1000 platform. Data analysis procedures are described in the online supplementary materials and methods (for all online suppl. material, see www.karger.com/doi/10.1159/000441780).

NLRP7 Mutation Analysis

The mutations were confirmed by direct sequencing [11]. Ten coding exons and 1 non-coding exon of *NLRP7* were amplified using primers and PCR conditions that were previously described [12].

Genotyping of Molar Tissue

The molar tissue of Patient 1 was genotyped. The villi were selectively laser microdissected using an LMD7000 (Leica Microsystems GmbH, Wetzlar, Germany), and genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The refSNP(rs) numbers of the 8 genotyped loci are shown in online supplementary table S1.

DNA Methylation Assay

The villi of a CHM from a patient with RHM (Patient 1), an androgenetic CHM and normal placenta were examined for *PEG3*, *SNRPN*, *PEG10* and *H19* gene methylation by bisulfite sequencing [13]. Bisulfite conversion was performed using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) with primers listed in online supplementary table S2.

Results

RHM is most likely caused by genetic factors, and 2 candidate genes have been previously reported [3, 4]. To search for additional mutations in genes that have not

Table 2. SNVs detected by whole-exome sequencing

Chromosome[1]	Position	Gene	Location	SNV	Protein alteration	RefSNP	Allele frequency in HGVB	Patient 1	Patient 2	Patient 3	Patient 4
1	52828383	*CC2D1B*[1]	Exon 3	c.G105A	p.M35I	rs183845075	0.02	G/G	G/G	G/G	A/A
3	10452493	*ATP2B2*[1]	Exon 3	c.C206T	p.P69L	N/A	N/A	T/T	C/C	C/C	C/C
3	16254129	*GALNT15*[1]	Exon 6	c.C1251A	p.H417Q	rs185944497	0.008	A/A	C/C	C/C	C/C
9	140007466	*DPP7*[1]	Exon 7	c.C809T	p.A270V	rs181036640	0.013	T/T	C/C	C/C	C/C
11	69063476	*MYEOV*[1]	Exon 3	c.C559T	p.R187W	rs116926312	0.044	C/C	C/C	C/C	T/T
13	41835028	*MTRF1*[1]	Exon 2	c.T16C	p.C6R	N/A	0.009	T/T	C/C	T/T	T/T
16	30735148	*SRCAP*[1]	Exon 25	c.C4403T	p.S1468L	rs75035256	0.019	T/T	C/T	C/C	C/C
17	27030713	*PROCA1*[1]	Exon 4	c.G874A	p.E292K	rs3744637	0.043	C/C	C/C	C/C	A/A
19	55451603	*NLRP7*	Exon 4	c.G584A	p.W195X	N/A	N/A	A/A	G/G	G/G	G/G
21	38439597	*PIGP*[1]	Exon 4	c.G239A	p.S80N	rs114319840	0.004	A/A	G/G	G/G	G/G

HGVB = Human Genetic Variation Browser; N/A = not available.
[1] Except for *NLRP7*, the 10 SNVs detected have no particular reported/predicted pathogenic features in the reproduction system.

Table 3. Summary of the SNVs found in *ZFP57*, *NLRP7* and *NLRP2* in 4 patients with RHM by whole-exome sequencing

Chromosome	Position	Gene	Location	SNV	Protein	RefSNP	Patient 1	Patient 2	Patient 3	Patient 4
6	29644668	*ZFP57*	Exon 14	c.G113>A	p.R38Q	rs142917604	G/G	G/G	G/A	A/A
19	55441902	*NLRP7*	Exon 9	c.A2775>G	p.A925A	rs269950	G/G	G/G	G/G	A/G
19	55441995	*NLRP7*	Exon 9	c.T2682>C	p.Y894Y	rs269951	G/G	G/G	G/G	A/G
19	55451050	*NLRP7*	Exon 4	c.G1137>A	p.K379K	rs10418277	A/A	G/G	G/G	G/G
19	55451232	*NLRP7*	Exon 4	c.G955>A	p.V319I	rs775882	A/A	G/G	G/G	G/G
19	55451603	*NLRP7*	Exon 4	c.G584>A	p.W195X	N/A	A/A	G/G	G/G	G/G
19	55451797	*NLRP7*	Exon 4	c.G390>A	p.Q130Q	rs775883	A/A	G/G	G/G	G/G
19	55485899	*NLRP2*	Exon 4	c.G312>A	p.K104K	rs2217659	G/G	A/A	G/G	A/A
19	55494881	*NLRP2*	Exon 7	c.C1815>G	p.L605L	rs11672113	G/G	C/C	G/G	C/C
19	55512137	*NLRP2*	Exon 14	c.C3060>A	p.I1020I	rs12768	A/A	C/A	A/A	A/A
19	55512232	*NLRP2*	Exon 14	c.C3155>A	p.A1052E	rs1043673	C/C	C/C	A/A	C/C

N/A = Not available.
[1] A novel homozygous nonsense mutation of *NLRP7* (c.584G>A; p.W195X) was found in Patient 1. Other SNVs are all commonly known SNPs with RefSNP(rs) numbers.

been previously associated with RHM, we performed whole-exome sequencing using peripheral blood cells from 4 RHM patients (Patients 1–4 in table 1). Quality filtering in the patients resulted in a set of 176,663 single nucleotide variations (SNVs) in coding regions. We excluded all non-coding SNVs and SNVs with more than 5% frequency in the 1000 Genomes database (http://www.1000genomes.org/, May 5, 2013) or the Human Genetic Variation Browser (http://www.genome.med.kyoto-u.ac.jp/SnpDB/). The remaining 10 SNVs (table 2) contained no particular potential pathogenic genes involved in the reproduction process except for an *NLRP7* mutation, as described later.

Next, we focused on *NLRP7*, *NLRP2*, *ZFP57* and *KHDC3L (C6orf221)*, which were previously reported as candidate genes for RHM or were associated with DNA methylation defects [3, 4, 14, 15]. Based on the minor allele frequency information of the 1000 Genomes database and other reports [3, 5, 9, 12, 16], the homozygous nonsense mutation of *NLRP7* (c.584G>A; p.W195X) identified in Patient 1 is considered a mutation, which was not previously reported (table 3). Other SNVs found in *ZFP57*, *NLRP7* and *NLRP2* are common variants. Sanger sequencing of genomic DNA from 5 RHM patients (Patients 1–5 in table 1), 9 patients with typical androgenetic CHM and 86 controls confirmed that the *NLRP7* muta-

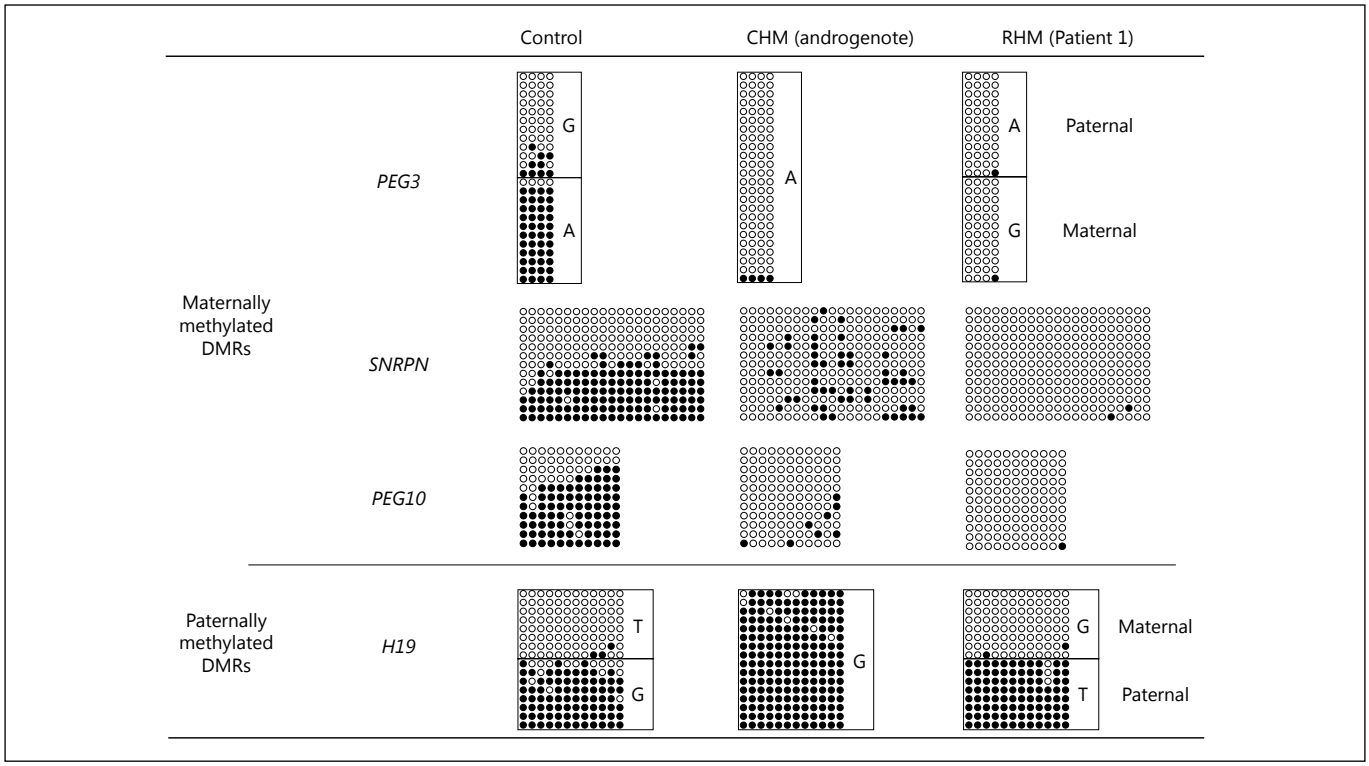

Fig. 1. DNA methylation assay of DMRs. Bisulfite sequencing was performed for 3 maternal DMRs (*PEG3*, *SNRPN* and *PEG10*) and 1 paternal DMR *(H19)*. Each line indicates a single clone, and each circle denotes a CpG; filled and open circles represent methylated and unmethylated cytosine residues, respectively. Allele-specific methylation was definitively diagnosed by SNPs in *PEG3* and *H19* DMRs. All maternally methylated DMRs (*PEG3*, *SNRPN* and *PEG10*) showed loss of DNA methylation in the villi of RHM (Patient 1). In contrast, paternal methylation of the *H19* DMR was maintained normally, even in the villi of RHM. Methylation defects were observed only in maternally methylated DMRs.

tion (c.584G>A; p.W195X) was observed only in 1 RHM (Patient 1; online suppl. table S3, fig. S1).

Previous reports have shown that the origin of a subset of RHM is not androgenic but biparental [12–14]. To precisely confirm the genotype of the villi of RHM Patient 1, the villi were selectively collected by laser microdissection, and a small amount of genomic DNA was obtained. Since the extracted DNA was fragmented and of low quality, analysis of polymorphic DNA markers was inconclusive. We then performed a comprehensive SNP analysis of the biological parents to confirm the origin of the villi of RHM Patient 1. Eight loci in which each parent (Patient 1 and her husband) possesses the opposite major or minor homozygous allele were genotyped, and all showed heterozygous genotypes in the villi (online suppl. table S1). These results clearly show that the villi of RHM Patient 1 contain both parental alleles and thus it is not androgenic, but biparental.

To clarify epigenetic abnormalities in the villi of Patient 1 RHM, DNA methylation analysis of DMRs of imprinted genes was performed using bisulfite sequencing (fig. 1). Three maternally methylated DMRs in *PEG3*, *SNRPN* and *PEG10* and a paternally methylated DMR in *H19* were analyzed. SNPs of *PEG3* and *H19* were used to distinguish between paternal and maternal alleles, enabling specific estimations of allele-specific DNA methylation defects of DMRs. The villi of RHM Patient 1 showed a loss of methylation in all the analyzed maternal DMRs and retained completely normal methylation in the paternal DMR.

Discussion

It is currently not possible to distinguish diploid biparental RHM from typical diploid androgenetic CHM with conventional pathological criteria. In fact, the RHM of Patient 1 and control CHM are histopathologically identical and were both p57KIP2-negative upon immunohistochemical analysis (online suppl. fig. S2). Though striking features of RHM are being revealed with genetic and epi-

genetic analyses [3, 8, 17–19], there is no clear mechanistic understanding as to why certain candidate genes are associated with the condition. Furthermore, no candidate genes have been identified in approximately 20% of all cases [5]. Thus, there is a clear need to explore genetic mutations and epigenetic aberrations in different genetic backgrounds. Additionally, whole-exome sequencing is a potentially promising approach to identifying unknown genetic factors [20]; however, to our knowledge, this is the first report of whole-exome sequencing in RHM patients.

To clarify the features of these Japanese isolated RHM cases, we performed comprehensive genetic and epigenetic analyses. We successfully detected a novel pathogenic homozygous nonsense *NLRP7* mutation (c.584G>A; p.W195X). Since we could not obtain genomic DNA to confirm the mutation in the parents of Patient 1, the parental origin of the mutation is unknown. The approximate 1-Mb region around the *NLRP7* mutation (c.584G>A) shows copy neutral loss of heterozygosity (data not shown). Although the parents of the Patient 1 do not appear to be consanguineous, we speculate that the *NLRP7* region of Patient 1 most likely has alleles identical by descent.

The examination of DNA methylation abnormalities is indispensable for a definitive diagnosis of epigenetic mutations specifically observed in diploid biparental RHMs. In this study, the parental origin of the *PEG3* and *H19* DMRs were definitively confirmed by SNP analysis. The villi of RHM Patient 1 clearly showed complete loss of DNA methylation of maternally methylated DMRs (*PEG3*, *SNRPN* and *PEG10*) but retained methylation of a paternally methylated DMR *(H19)*. Although the villi of the RHM were genetically normal, they showed abnormal DNA methylation and their DNA methylation profile was quite similar to that in a typical androgenetic CHM. This is in contrast to previous studies, in which patterns including hypermethylation of *H19* DMR [21] and retention of methylation at *PEG10* DMR were observed [12]. These differences are likely due to the multifactorial nature in which the DNA methylation machinery is regulated; this would account for the variable methylation changes within DMRs, as we previously reported in our study of *Dnmt3L* knockout mice [22].

RHM patients with *NLRP7* mutations are most likely to have failed in the establishment of methylation of maternal DMRs during oogenesis. The p.W195X mutation in Patient 1 is located upstream of the known nonsense mutations in RHM patients [5]. It is, therefore, expected that RHM from Patient 1 would exhibit the typical phenotype, and indeed this patient had 5 molar pregnancies. Some reported missense mutations could be hypomorphic and show stochastic effects on the methylation of DMRs [9, 12]. If DMR methylation defects are partial, they may cause ordinary abortion or may be sufficiently mild so as to allow normal pregnancy rather than cause molar pregnancy [21]. In fact, patients with *NLRP7* missense mutations have a mixed reproductive history of molar pregnancy and abortion or normal pregnancy [23]. Thus, some unexplained cases of infertility might be attributed to such stochastic epigenetic aberrations.

The NLR family proteins have roles in inflammation and apoptosis [24]. *NLRP7* is involved in the secretion of IL-1β [25–27], but there is no direct evidence that perturbations to the NLRP7-IL-1β axis cause loss of DNA methylation and molar pregnancy. Since *NLRP7* is present in oocytes [16] and preimplantation embryos [28], it could be involved in the hypomethylation of DMRs observed in the villi of RHMs. However, because *NLRP7* has no ortholog in mice [24], evaluation of its function in oogenesis and early embryogenesis is challenging. Therefore, identification of genetic and/or epigenetic mutations in isolated cases and in different genetic backgrounds remains an important aspect of studies designed to unravel the mechanisms underlying RHM pathogenesis.

In conclusion, we have, for the first time, identified a novel nonsense homozygous mutation of *NLRP7* in a Japanese RHM patient. Our study is the first to report an isolated RHM case in a previously unreported ethnic background. This comprehensive genetic and epigenetic analysis approach can facilitate the definitive molecular diagnosis of diploid biparental RHM in isolated cases and can be performed using fragmented DNA extracted from formaldehyde-fixed and paraffin-embedded tissue samples. *NLRP7* mutations cause abnormal DNA methylation in DMRs [21]. However, the mechanisms underlying region-specific DNA methylation remain unknown. Further analysis of RHM will shed light on the unknown etiology of infertility and the mechanisms that control region-specific DNA methylation.

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Disclosure Statement

None to declare.

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Augmenting effects of gestational arsenite exposure of C3H mice on the hepatic tumors of the F_2 male offspring via the F_1 male offspring

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ABSTRACT: Gestational exposure can affect the F_2 generation through exposure of F_1 germline cells. Previous studies reported that arsenite exposure of only F_0 females during their pregnancy increases hepatic tumors in the F_1 males in C3H mice, whose males are predisposed spontaneously to develop hepatic tumors later in life. The present study addressed the effects of gestational arsenite exposure on tumorigenesis of the F_2 males in C3H mice. Expression analysis of several genes in the normal livers at 53 and 80 weeks of age clearly showed significant changes in the F_2 males obtained by crossing gestational arsenite-exposed F_1 (arsenite-F_1) males and females compared to the control F_2 males. Some of the changes were shown to occur in a late-onset manner. Then the tumor incidence was assessed at 75–82 weeks of age in the F_2 males obtained by reciprocal crossing between the control and arsenite-F_1 males and females. The results demonstrated that the F_2 males born to arsenite-F_1 males developed tumors at a significantly higher rate than the F_2 males born to the control F_1 males, irrespective of exposure of F_1 females. Gene expressions of hepatocellular carcinoma markers β-catenin (CTNNB1) and interleukin-1 receptor antagonist in the tumors were significantly upregulated in the F_2 males born to arsenite-F_1 males compared to those born to the control F_1 males. These results show that arsenite exposure of only F_0 pregnant mice causes late-onset changes and augments tumors in the livers of the F_2 males by affecting the F_1 male offspring. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: arsenic; gestational exposure; hepatic tumor; transgenerational; gene expression

Introduction

Gestation is known to be vulnerable to a variety of environmental conditions, including chemical exposure and nutritional imbalances, and the adverse effects of environmental conditions in this period can lead to a number of adult-onset diseases in the F_1 offspring and in subsequent multiple generations (Aiken and Ozanne, 2014; Guerrero-Bosagna and Skinner, 2012; Perera and Herbstman, 2011). A possible causative route of the effects on the F_2 and subsequent generations by gestational chemical exposure is through direct exposure of germline cells in the F_1 fetuses and the responsible germ cells can be those of male, female, or both (Aiken and Ozanne, 2014; Guerrero-Bosagna and Skinner, 2012; Perera and Herbstman, 2011).

Naturally occurring inorganic arsenic, which is known as a human carcinogen, is causing serious health problems, including cancer, in many areas in the world (Hughes *et al*., 2011). Although animal models are pivotal to elucidate the mechanism of arsenic toxicity, it has been difficult to verify the carcinogenicity of arsenic in rodents (Rossman *et al*., 2002). Pioneering studies by Waalkes and colleagues showed that exposure of pregnant C3H mice (F_0) from gestational day (GD) 8–18 to inorganic arsenite results in an increase in tumors in the liver and adrenal gland of their F_1 male offspring at 74 weeks of age (Waalkes *et al*., 2003). As male C3H mice are predisposed to spontaneously develop hepatic tumors in adulthood (Köhle *et al*., 2008; Maronpot *et al*., 1995), the finding by Waalkes and colleagues supports the notion that arsenic acts in combination with other tumor promoting factors or dispositions (Klein *et al*., 2007). The results obtained by Waalkes *et al*. (2003) also indicate that gestation is a vulnerable period for arsenic carcinogenicity. Epidemiological studies have reported that gestational exposure to arsenite is associated with increased cancers in adulthood (Smith *et al*., 2006; Yuan *et al*., 2010). These findings on the sensitivity of gestational period against arsenic imply the possibility that gestational arsenic exposure may also affect F_1 germline cells and have an impact on late-onset tumorigenesis in the F_2 and subsequent generations, while those issues have not been addressed.

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We previously investigated the causal factors of hepatic tumor augmentation in the F_1 male offspring by gestational arsenite exposure in the experimental model reported by Waalkes *et al.* (2003) Nohara *et al.*, 2012). The results showed several characteristic changes, such as late-onset gene expression changes in the normal livers and an increase in hepatic tumors, particularly those having a C to A somatic mutation at codon 61 in oncogene Ha-*ras*, in the F_1 offspring by gestational arsenite exposure (Nohara *et al.*, 2012). In the present study, we addressed the effects of gestational arsenite exposure on the F_2 males of C3H mice by investigating gene expression changes in the normal livers, the hepatic tumor incidence, and the incidence of Ha-*ras* mutation at codon 61 in the tumors. In the assessment of tumor incidence, we performed a reciprocal crossing experiment between the control and arsenite-F_1 males and females to clarify the F_1 sex responsible for the tumor augmentation in the F_2 males. We also measured gene expression of several human hepatocellular carcinoma markers in the tumors of the F_2 males for confirmation of the tumor augmenting effects of gestational arsenite exposure.

The results of the present study made the novel findings on the tumor augmenting effects of gestational arsenite exposure on the F_2 generation.

Materials and methods

Design of Animal Experiments

Pregnant C3H/HeN mice (F_0) were purchased from CLEA Japan (Tokyo, Japan) and given free access to a standard diet (CA-1; CLEA Japan) and tap water (control mice) or tap water containing 85 ppm sodium arsenite (Sigma, St. Louis, MO, USA) from day 8 to 18 of gestation as described previously (Nohara *et al.*, 2012). Throughout the experiments, arsenite was only given to F_0 pregnant mice and not to F_1 or F_2 mice. To assess the tumor incidence and the F_1 sex responsible for the F_2 tumor augmentation, we did a reciprocal crossing experiment among the control F_1 males and females and arsenite-F_1 males and females, which originated from 22 control F_0 females and 29 arsenite-F_0 females. Male and female mice were mated at 10 weeks of age. The resulting F_2 males were reared until 75–82 weeks (17.5–19 months) of age and used for the assessment. Hepatic tumors were examined macroscopically (Nohara *et al.*, 2012) and some were subjected to histological analysis as described below.

The animals were handled in accordance with the National Institute for Environmental Studies guidelines for animal experiments.

Histological Analysis

Sections prepared from paraffin-embedded liver tissues were stained with hematoxylin and eosin as previously described (Nohara *et al.*, 2012). The histology of the liver neoplasms was classified as hepatocellular adenoma or hepatocellular carcinoma. Briefly, hepatocellular adenoma is characterized by a well-circumscribed lesion composed of well-differentiated hepatocytes, and hepatocellular carcinoma is characterized by an abnormal growth pattern and both cytological and nuclear atypia (Harada *et al.*, 1999).

cDNA Preparation and Real-Time Polymerase Chain Reaction

Total RNA of individual livers was prepared with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). After checking the quality of the RNA by electrophoresis, reverse transcription reactions were performed with an AMV Reverse Transcriptase XL (TaKaRa Bio, Shiga, Japan) using 100 ng of total RNA. Quantitative real-time polymerase chain reaction (PCR) analysis was performed on a LightCycler 480 instrument, version 1.5 (Roche Diagnostics, Basel, Switzerland) as described previously (Nohara *et al.*, 2006). The primer sequences and annealing temperatures used for real-time PCR are shown in Table 1.

Ha-*ras* Mutation

Ha-*ras* mutations at codon 61 were analyzed by the pyrosequencing method (Ogino *et al.*, 2005). The DNA region containing the sequence coding codon 61 in the Ha-*ras* gene was amplified by PCR using the biotinylated primers (5′-cggaaacaggtggtcattgat-3′ and biotin-5′-tgatggcaaatacacagaggaag-3′) and a PyroMark PCR kit (Qiagen). Amplification was achieved by heating at 95 °C for 5 min, cycling at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and was followed by extension at 72 °C for 10 min after the final cycle. The biotinylated PCR product was captured on streptavidin-coated beads (GE Healthcare Bio-Science, Little Chalfont, UK), denatured and washed. The sequence primer for codon 61 (5′-ggacatcttagacacagca-3′) was annealed to the biotinylated PCR product and pyrosequencing analysis was performed by using a

Table 1. Primers for real-time polymerase chain reaction analysis

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Annealing temp. (°C)
Creld2	gcagacagcagaaggcaaa	tgcccgtcacaaatcctc	60
Slc25a30	gaacgcccagaagatgaaac	ctgttctgtgcttgcattcg	60
Ell3	ccagaaacgcctggacaa	cttgaggctagaggcagagc	64
Fabp4	cagcctttctcacctggaag	ttgtggcaaagcccactc	60
Gpat-1	agcaagtcctgcgctatcat	ctcgtgtgggtgattgtgac	64
Afp	cccaaccttcctgtctcagt	tggctctcctcgatgtgttt	64
Il-1rn	tgcacaacactagaggctga	agtgatcaggcagttggtga	64
Ctnnb1	ccctgagacgctagatgagg	tgtcagctcaggaattgcac	64
Cpb	agactgttccaaaaacagtgga	gatgctctttcctcctgtgc	64
rRNA	tgcgaatggctcattaaatcagtt	ccgtcggcatgtattagctctag	64

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PyroMark Q96 ID system (Qiagen) and PyroMark Q96 ID Software 2.5 (Qiagen) according to the manufacturer's instructions.

Western Blotting

Tissues were homogenized in ice-cold lysis buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholic acid, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris–HCl [pH 7.5] and 150 mM NaCl) with a pellet mixer and then with a Bioruptor UCD-200TM (Cosmo Bio, Tokyo, Japan). The supernatant was boiled with the same volume of × 2 SDS sample buffer (100 mM Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM DTT, 0.002% bromophenol blue), and subjected to SDS-polyacrylamide gel electrophoresis. The first antibodies used were anti-p44/42 MAPK (Cell Signaling Technology, Danvers, MA, USA; 9102), anti-phospho-p44/42 MAPK (Cell Signaling Technology; 9101) and anti-β-actin (Sigma; A5441). The second antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and antimouse IgG (Sigma). The membranes were developed using the ECL Prime Western Blotting Detection System (GE Healthcare) and the images were captured using a VersaDoc imaging system (Bio Rad, Hercules, CA, USA).

Statistical Analysis

The difference in the tumor incidence between the two groups was analyzed by a chi-squared test. The difference in gene expression between the two groups was analyzed by Student's *t*-test. Those analyses were conducted with Stata11 (Stata Corporation, College Station, TX, USA).

Results

Late-Onset Gene Expression Changes Detected in the Normal Liver of the F_2 Generation

Analyses of mouse livers with Affymetrix GeneChips in our previous study showed that expression of two genes, *Creld2* and *Slc25a30*, was upregulated more than twofold and expression of another two genes, *Fabp4* and *Ell3*, was downregulated more than twofold in the normal livers of arsenite-F_1 males in comparison with those of the control males at 74 weeks of age (Nohara *et al.*, 2012). The expression changes of these genes were found to occur in an adult-onset manner (Nohara *et al.*, 2012). To examine whether arsenite exposure of F_0 pregnant mice has any effect on the F_2 offspring, we measured the expression of these four genes in the normal livers of the control F_2 males and the F_2 males obtained by crossing arsenite-F_1 males and females (arsenite-F_2) at 53 weeks and 80 weeks of age.

The results of the measurements showed significant downregulation of *Creld2* from 53 weeks of age and late-onset downregulation of *Slc25a30* at 80 weeks of age in arsenite-F_2 males in comparison with the control F_2 males. On the other hand, those genes were upregulated in arsenite-F_1 males in comparison with the control F_1 males at 49 and/or 74 weeks of age. Thus, direction of the changes by gestational arsenite exposure in the arsenite-F_1 and arsenite-F_2 males was the opposite (Fig. 1). Expression of *Ell3* was significantly upregulated in arsenite-F_2 males in comparison

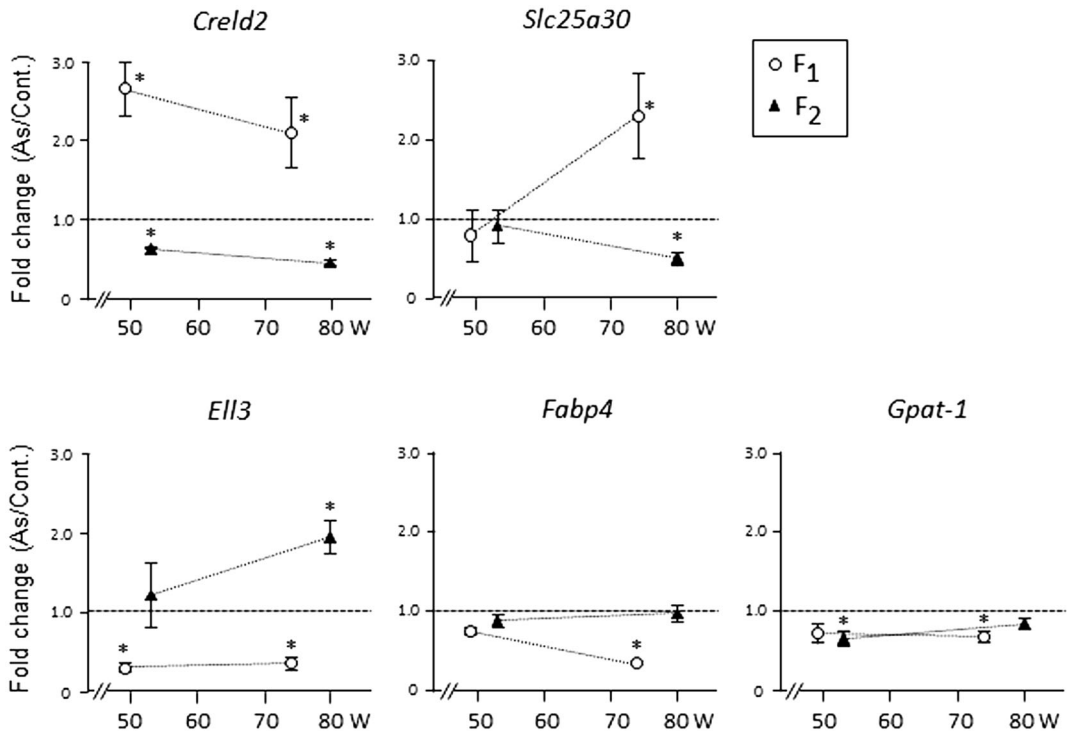


Figure 1. The changes in hepatic gene expression in the arsenite-F_1 males and arsenite-F_2 males in comparison with their control males. The expressions of five genes in the livers of the control F_2 males and arsenite-F_2 males were measured by real-time polymerase chain reaction at 53 weeks of age ($n = 4$ in each group) and 80 weeks of age ($n = 6$ in each group) and normalized to expression of *Cpb*. For the gene expressions in the F_1 generation, data obtained at 49 weeks of age ($n = 4$ in each group) and 74 weeks of age ($n = 8$ in each group) in our previous study (Nohara *et al.*, 2012) were used. The graphs show the ratios of the expression in the arsenite-exposed group normalized to the expression in the control group. The error bar shows the standard error. *Significant difference ($P < 0.05$) between the offspring of the control females and the offspring of the arsenite-exposed females.

with the control F_2 males also in a late-onset manner. The effect of gestational arsenite exposure on *Ell3* expression in the F_2 males was again the opposite of its effect in the F_1 males. Downregulation of *Fabp4*, which was detected in the arsenite-F_1 males, was not detected in the F_2 generation (Fig. 1).

In our previous study, we also observed downregulation of a lipid metabolism-related gene *Gpat-1* at 74 weeks of age in the normal livers of arsenite-F_1 males in comparison with the control males (Nohara *et al.*, 2012). This gene was downregulated in arsenite-F_2 males in comparison with the control F_2 males at 53 weeks of age (Fig. 1).

These changes in gene expression clearly showed significant effects of gestational arsenite exposure on the F_2 males, and the effects on the F_1 and the F_2 were varied.

Increased Hepatic Tumors in the F_2 Males Born to the Arsenite-F_1 Males

We performed the reciprocal crossing experiment with the control and arsenite-F_1 males and females as shown in Fig. 2 to assess the effects of gestational arsenite exposure on tumorigenesis in the F_2 males and determine the F_1 sex responsible for the F_2 tumor augmentation.

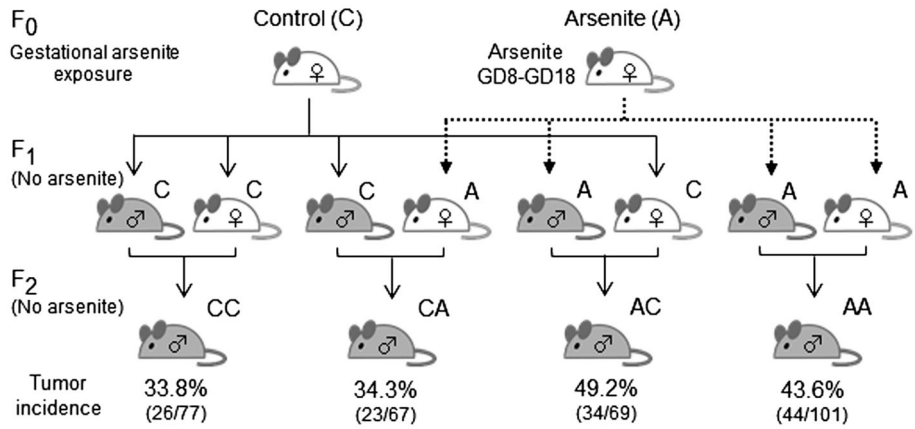
The tumor incidences in each F_2 group were 33.8% in CC, 34.3% in CA, 49.2% in AC and 43.6% in AA (Fig. 2A). Comparison of the tumor incidence between the F_2 male offspring of arsenite-F_1 males (AC and AA) and the control F_1 males (CC and CA) showed a significantly higher tumor incidence in the F_2 offspring of arsenite-F_1 males (45.9% in AC and AA vs 34.1% in CC and CA) (Fig. 2B). On the other hand, the tumor incidence of the F_2 male offspring of arsenite-F_1 females (39.9% in CA and AA) and the control F_1 females (41.1% in CC and AC) were not different (Fig. 2B). These results showed that the tumor-augmenting effect of gestational arsenite exposure is transmitted to the F_2 males via the F_1 male offspring, but not via the F_1 female offspring.

Pathological examination of tumor tissues from the control F_2 and arsenite-F_2 males (10 samples from each group) showed that hepatocellular adenoma was the predominant histological tumor type (Fig. 3). This finding was consistent with the observation of the hepatic tumors of the control mice and arsenite-F_1 mice in our previous study (Nohara *et al.*, 2012).

Ha-*ras* Mutation in the Tumors of the F_2 Generation

Our previous study showed that gestational arsenite exposure particularly increased the percentage of hepatic tumors containing

A)



B)

Comparison to assess the F_1 sex responsible for the F_2 tumor augmentation

	Tumor incidence		p value
F_2 group	Offspring of control F_1 male (CC, CA)	Offspring of arsenite-F_1 male (AC, AA)	
	34.1% (49/144)	45.9% (78/170)	0.033
F_2 group	Offspring of control F_1 female (CC, AC)	Offspring of arsenite-F_1 female (CA, AA)	
	41.1% (60/146)	39.9% (67/168)	0.827

Figure 2. Increase in the tumor incidence in the F_2 male offspring born to arsenite-F_1 males but not to arsenite-F_1 females. (A) The F_2 males were obtained by reciprocally crossing the control and arsenite-F_1 males and females as shown in the figure. The F_2 mice were macroscopically examined for hepatic tumors at 75–82 weeks of age I n an age-matched manner. Small lesions (≤1 mm in diameter) were omitted. The number in parenthesis is that of mice bearing hepatic tumors/the number of mice investigated. (B) The difference between the tumor incidences in the two groups was analyzed by chi-squared test. GD, gestational day.

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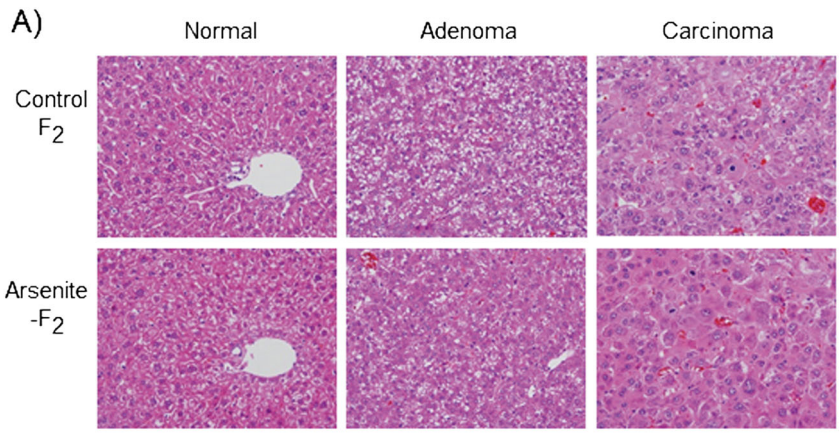

B)

Occurrence of hepatocellular adenoma and hepatocellular carcinoma

	Number of tumors	
	Adenoma	Carcinoma
Control F_2 (n=10)	8	2
Arsenite-F_2 (n=10)	7	3

Figure 3. Histological analysis of the hepatic tumors of the control and arsenite-F_2 males. (A) Representative sections of hepatocellular adenoma and hepatocellular carcinoma of the control F_2 males and arsenite-F_2 males. (B) Occurrence of hepatocellular adenoma and hepatocellular carcinoma.

the Ha-*ras* C61A somatic mutation in the F_1 males (Nohara *et al.*, 2012). Ha-*ras* mutation is thought to be involved in carcinogenesis by activating several signaling pathways, including the RAF/MEK/ERK cascade (Pylayeva-Gupta *et al.*, 2011). The investigation in the present study confirmed that the Ha-*ras* mutations increase the activating phosphorylated forms of ERKs, ERK1 (p44 MAPK) and ERK2 (p42 MAPK) in the tumor tissues harboring codon 61 Ha-*ras* mutations in both the control F_2 males and arsenite-F_2 males (Fig. 4A). p44/p42 MAPK was shown to be hypophosphorylated in the non-tumor tissues of tumor-bearing livers, which do not contain mutated Ha-*ras* (Fig. 4B).

On the other hand, Ha-*ras* C61A mutation or the total Ha-*ras* mutations in codon 61 in the tumor tissues was shown not to be increased in the F_2 males born to arsenite-F_1 males (AC and AA) in comparison with the F_2 males born to the control males (CC and CA) (Table 2). Thus, the existence of Ha-*ras* mutations seems not to be the major causation of the increased hepatic tumor incidence in the F_2 males born to arsenite-F_1 males.

Upregulation of Cancer Related Genes in the Tumor Tissues of F_2 Males Born to the Arsenite-F_1 Males

In an effort to establish early diagnostic markers for detecting human hepatocellular carcinoma, increases in several serum proteins, including α-fetoprotein, β-catenin (CTNNB1) and interleukin-1 receptor antagonist (IL1-RN), were shown to be closely associated with hepatocellular carcinoma (Sun *et al.*, 2008). The expression of those genes was shown to be higher in the tumor tissues compared to the normal tissues or the non-tumor tissues of tumor-bearing livers, while the expression of *Afp* is varied widely among samples (Fig. 5). The expressions of *Ctnnb1* and *Il1rn* genes were significantly higher in the F_2 males born to arsenite-F_1 males (AC and AA) than those born to the control males (CC and CA) (Fig. 5).

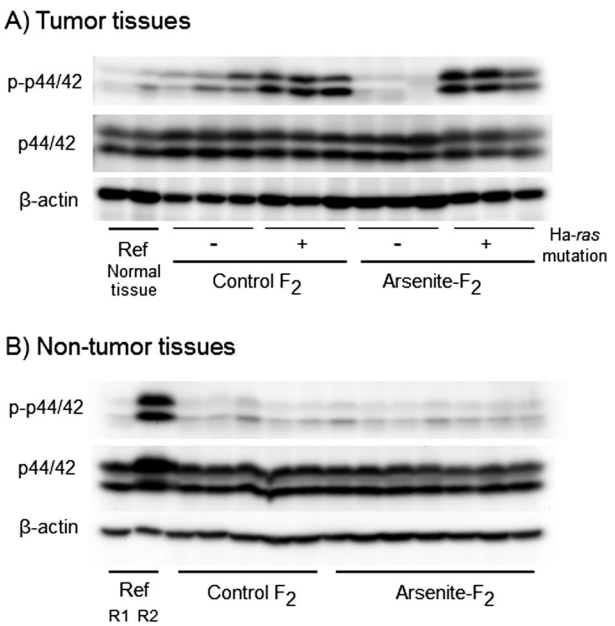

Figure 4. p44/42 MAP kinases activation as a result of Ha-*ras* mutation in the hepatic tumors of C3H males. Western blots of hepatic tumor tissues (A) and non-tumor tissues in the tumor-bearing livers (B) of the control F_2 and arsenite-F_2 males were prepared as described in the Materials and methods section. Labels in (B) are R1, normal tissue and R2, tumor tissue with a Ha-*ras* mutation.

Interestingly, when the gene expression was assessed separately in tumor tissues with and without Ha-*ras* mutation, the expression of *Il-1rn* seemed to be upregulated by Ha-*ras* mutation (Fig. 5).

Table 2. Spectra of Ha-*ras* codon 61 mutations in the hepatic tumors of the F_2 males

F_2 group (*n*)		Ha-*ras* codon 61 type (%)			
		CAA wild-type	AAA	CTA	CGA
CC	(29)	31	41	7	7
CA	(33)	61	18	3	3
AC	(34)	38	35	3	3
AA	(49)	39	45	6	6

The hepatic tumor tissues were obtained in the reciprocal crossing experiment (Fig. 2) and analyzed for mutations in Ha-*ras* codon 61 by a pyrosequencing method.

Discussion

The earliest studies carried out primarily in the middle 1900s reported that maternal exposure or germ cell exposure to radiation and some carcinogenic chemicals transgenerationally affect the susceptibility of the progeny to cancer (Tomatis, 1994). The transgenerational effects of radiation, chemicals and nutritional imbalances on cancers and other disorders have been reported to be transmitted paternally, maternally, or both paternally and maternally (Aiken and Ozanne, 2014; Anway *et al.*, 2005; Barber *et al.*, 2002; Mohamed *et al.*, 2010; Tomatis, 1994). The individual molecular mechanisms of the transmission are yet to be clarified.

The present study showed that gestational arsenite exposure of pregnant C3H mice from GD8 to GD18 increases the incidence of hepatic tumors in the F_2 males born to arsenite-F_1 males (AC and AA in Fig. 2) compared to the F_2 males born to the control males (CC and CA in Fig. 2), irrespective of exposure of the F_1 females. These results showed that tumor augmenting effects by gestational arsenite exposure is transmitted to the F_2 males through the F_1 males. We also detected significant late-onset changes in gene expression in the normal livers of arsenite-F_2 (AA F_2) males compared to those of the control F_2 males (Fig. 1), indicating that transient gestational arsenite exposure of pregnant females causes a significant impact on the F_2 generation. Furthermore, the cancer-related genes *Ctnnb1* and *Il1rn* were shown to be upregulated in the hepatic tumors of the F_2 males born to arsenite-F_1 males (AC and AA) compared to those born to the control males (CC and CA) (Fig. 5). These results show the augmenting effects of gestational arsenite exposure on the hepatic tumors of the F_2 male offspring.

The F_1 male fetuses in the present study were exposed from GD8 to GD18, when the primordial germ cells appear and differentiate into the sperm precursor cells (Sasaki and Matsui, 2008). The epigenetic profile of the primordial germ cells undergoes dynamic alterations, including imprint erasure, during the development stage, and disruption of the epigenetic profile during this stage has been implicated in transgenerational effects of F_0 gestational exposure (Aiken and Ozanne, 2014; Guerrero-Bosagna and Skinner, 2012; Perera and Herbstman, 2011). A previous study (Devesa *et al.*, 2006) reported the concentrations of inorganic arsenic and methylated arsenic at GD18 in the fetus organs, including

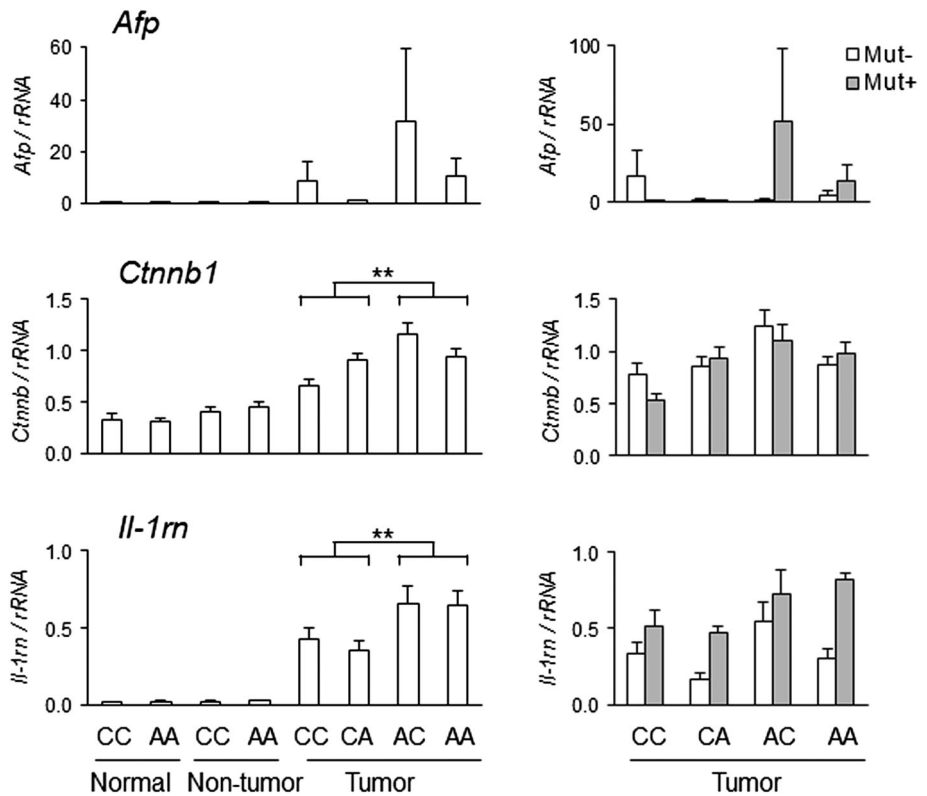

Figure 5. Expression of cancer-related genes in the hepatic tissues in the F_2 mice. The expressions of *Afp*, *Ctnnb* and *Il-1rn* were measured by real-time PCR for the normal livers ($n = 6$), non-tumor tissues from tumor-bearing livers ($n = 6$) and tumor tissues ($n = 9$–11) from the F_2 males and normalized to the expression of rRNA. The difference in the gene expressions in the tumor tissues between the F_2 males born to the control F_1 males (CC and CA) and those born to arsenite-F_1 males (CA and AA) was analyzed by Student's *t*-test. $^{**}P < 0.01$.

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the liver and blood in the same gestational arsenite exposure model of C3H mice we used. The study showed that fetus organs are directly exposed upon gestational arsenite exposure. As arsenic has been reported to induce epigenetic changes, particularly DNA methylation changes (Reichard and Puga, 2010; Ren *et al.*, 2011; Suzuki *et al.*, 2013), the gestational arsenite exposure may alter the phenotype of the F_2 generation by affecting the epigenetic profile of genes, possibly including imprinted genes, in the F_1 male germ cells.

Arsenic has been thought to be a weak mutagen or not a mutagen based on the results of assays using bacteria and mammalian cells *in vitro* (reviewed by Rossman, 2003). Our recent study using *gpt* delta transgenic mice clarified that arsenite exposure greatly increases the incidence of G:C to T:A transversion *in vivo* (Takumi *et al.*, 2014). Thus, mutation in the proliferating and differentiating primordial germ cells might be another possible causation of the F_2 effect by arsenite exposure of F_0 pregnant mice.

In the present study, we found that gestational arsenite exposure significantly affects hepatic expression of *Creld2*, *Slc25a30* and *Ell3* even in the F_2 generation, but the effect of the exposure was the opposite of its effect on the F_1 generation (Fig. 1). The difference seems to be attributable to the fact that exposure of the F_1 and F_2 mice occurred in a different manner, e.g., the fetal liver is directly exposed in the F_1 and F_2 is exposed at the germ cell stage. On the other hand, *Gpat-1* was significantly downregulated in the liver of the F_2 generation, as was also observed in the F_1 generation (Nohara *et al.*, 2012) (Fig. 1). *Gpat-1* is one of the target genes of sterol regulatory element-binding protein 1, a member of the central transcription factors that control intracellular cholesterol and fatty acid levels (Raghow *et al.*, 2008; Wendel *et al.*, 2010). Lipid accumulation in the liver has been implicated in hepatic carcinogenesis through an increase in oxidative stress (Ziech *et al.*, 2011). The change in *Gpat-1* expression in the liver of the F_1 and F_2 mice may indicate involvement of lipid metabolism changes in the increase in hepatic tumors.

We previously found a higher proportion of C61A Ha-*ras* mutation in the hepatic tumors of arsenite-F_1 males (Nohara *et al.*, 2012). In the present study, we confirmed that Ha-*ras* mutation increases activated forms of ERKs in the hepatic tumors of C3H mice (Fig. 4). However, the percentage of Ha-*ras* mutation in the tumors was not increased in the F_2 males born to arsenite-F_1 males (Table 2), which indicated that the tumor increase in the exposed F_2 males could not be attributed to the increase in Ha-*ras* codon 61 mutation 1.

In summary, we demonstrated the novel finding that gestational arsenite exposure of F_0 pregnant mice increases hepatic tumor incidence in the F_2 male offspring through the impact on the F_1 males. Further studies will be required to identify the factors that cause tumor augmentation in the liver of the F_2 generation by arsenite exposure of F_0 pregnant mice and to explore changes in the F_1 male germ cells that induce such tumor-augmenting factors in the liver of the F_2 generation.

Acknowledgments

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Increased epigenetic alterations at the promoters of transcriptional regulators following inadequate maternal gestational weight gain

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Epigenetic modifications are thought to serve as a memory of exposure to *in utero* environments. However, few human studies have investigated the associations between maternal nutritional conditions during pregnancy and epigenetic alterations in offspring. In this study, we report genome-wide methylation profiles for 33 postpartum placentas from pregnancies of normal and foetal growth restriction with various extents of maternal gestational weight gain. Epigenetic alterations accumulate in the placenta under adverse *in utero* environments, as shown by application of Smirnov-Grubbs' outlier test. Moreover, hypermethylation occurs frequently at the promoter regions of transcriptional regulator genes, including polycomb targets and zinc-finger genes, as shown by annotations of the genomic and functional features of loci with altered DNA methylation. Aberrant epigenetic modifications at such developmental regulator loci, if occurring in foetuses as well, will elevate the risk of developing various diseases, including metabolic and mental disorders, later in life.

Foetal environmental factors, including maternal nutrition, hormonal disturbance, and chemical exposure, affect foetal growth and can cause birth defects. Recent studies have linked poor foetal growth to increased risks of coronary heart disease[1], type II diabetes[2,3], kidney disease[1,4], and brain disorders[5] in adulthood. Barker first proposed that nutritional conditions *in utero* may be responsible, at least in part, for the developmental programming of the foetus and placenta, potentially predisposing the individual to adult metabolic disease[6]. The concept that the foetal environment affects health later in life has been defined as the developmental origins of health and disease (DOHaD)[7], in which epigenetic modifications are thought to serve as a memory of exposure to *in utero* environments[8,9]. Epigenetic modifications, such as DNA methylation and histone modifications, are involved in tissue- and developmental stage-specific gene expression and silencing, and they can be transmitted in a stable manner through mitotic cell division, thereby inducing long-term changes in gene regulation. Developmental programming during the foetal period, therefore, could affect adult health through epigenetic mechanisms. In fact, many studies using animal models have demonstrated that nutrient manipulation during pregnancy induces epigenetic alterations at specific loci or globally in the offspring[10]. On the other hand, human studies showing associations of *in utero* conditions and epigenetic alterations have been relatively limited[11].

The predominant form of DNA methylation is methylation of cytosine in the context of CpG. The diploid human genome contains more than 10^8 cytosines, of which more than 10^7 are present in the context

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of CpGs[12]. The genome-wide DNA methylation pattern in various types of cells in the body is bimodal, with the majority of CpG sites being highly methylated (>85%), while CpG islands (CGIs) are largely unmethylated (<10%). After fertilisation of the oocyte by sperm, the paternal genome becomes actively demethylated in the zygote, and the maternal genome undergoes passive demethylation until the early blastocyst stage[13]. DNA methylation patterns are re-established in a lineage-specific manner[14,15]. The genome in cells of the placenta remains relatively hypomethylated compared to that in somatic tissues. A genome-wide DNA methylation analysis of promoter regions in human placental tissues collected during the first, second, and third trimesters revealed that there is a significant increase in the average methylation level in autosomes from the second-trimester placenta to the full-term placenta[16]. Interindividual variations in DNA methylation levels have also been shown to increase during gestation[16]. Foetal environmental factors, such as maternal weight, maternal alcohol intake, maternal smoking, and maternal psychological stress, have recently been shown to affect DNA methylation in the human placenta[11,17,18]. However, compared to the number of studies that have examined the DNA methylation levels at repetitive sequences, such as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), and at certain imprinted loci[19–21], studies evaluating the alterations in DNA methylation on the genome-wide scale in relation to the foetal environment have been limited[18,21–24].

Maternal gestational weight gain (GWG) influences the foetal nutritional environment during gestation. The Japan Ministry of Health, Labour, and Welfare recommends that women with a prepregnancy body mass index (BMI) between 18.5 and 25 should gain about 7–12 kg body weight during the gestational period. Excessive GWG has been reported to be associated with increased neonatal obesity during infancy and adulthood[25–27]. On the other hand, insufficient GWG is related to increased risk of low birth weight[28,29], which is known to be associated with metabolic syndrome, including impaired glucose tolerance, insulin resistance, and coronary heart disease, during adulthood[1–4]. Foetal growth restriction (FGR), which may be caused by foetal, placental, and/or maternal factors[30], is defined as a foetus that has not reached its growth potential (below the 10th percentile for gestational age).

In this study, we elucidate the effects of *in utero* environments on the human placental epigenome. To this end, we examine a collection of postpartum placentas using array-based genome-wide DNA methylation analysis and evaluate DNA methylation levels in placental tissues in relation to GWG and birth weight. We demonstrate that inadequate GWG perturbs the placental epigenome variably among subjects, and that such epigenetic alterations occur preferentially at the CGI promoters of genes encoding transcriptional factors. Therefore, our results demonstrate that epigenetic alterations accumulate in the placenta under adverse *in utero* environments, supporting the importance of appropriate *in utero* conditions and maternal health in foetal development.

Results

Alterations in placental DNA methylation were associated with FGR and GWG. We subjected placentas from 14 births exhibiting FGR and 19 births within the normal range of birth weight (Table 1) to genome-wide DNA methylation analysis, and assessed whether the FGR placentas contained CpG sites that were differentially methylated compared with the placentas with a birth weight within normal range. Wilcoxon rank-sum tests[31] did not detect any CpG sites as significantly differentially methylated between two groups (significance level = Benjamini–Hochberg [BH] adjusted p-value of 0.05). Comparisons of FGR and normal placentas within subgroups depending on maternal GWG (insufficient, adequate, and excessive) also did not detect any differentially methylated CpG sites in the FGR placentas (Supplementary Fig. 1). Next, we assessed whether placentas from subjects with excessive or insufficient maternal GWG contained CpG sites that were differentially methylated compared with those in placentas from subjects with adequate GWG. Four comparisons (i.e., insufficient versus adequate and excessive versus adequate within FGR and normal categories) did not detect any significantly differentially methylated CpG sites between two subgroups (Supplementary Fig. 1). These results suggested that no specific CpG sites showed consistent changes in DNA methylation associated with the FGR phenotype or inadequate maternal GWG in this study.

Next, we considered the possibility that the FGR phenotype and/or inadequate GWG may affect the placental epigenome in different ways among individual subjects rather than showing similar effects for all individuals within a group. To evaluate this possibility, we searched for CpG sites whose methylation level differed significantly in one placenta (as compared with all of the other placenta samples) by performing Smirnov-Grubbs' outlier test with Bonferroni multiple test corrections (significant level = 0.1) for each placenta. We detected 2,983 and 1,416 CpG sites as hyper- and hypomethylated outliers, respectively, among the 33 subjects. To reduce the numbers of outliers that could have been detected spuriously due to SNPs at/near the target CpG sites, we excluded the CpG sites whose corresponding probes are annotated to contain known SNPs as described in the Methods. When 89,678 probes were regarded as potentially SNP-containing based on the Illumina probe annotation, 2,521 (85%) and 977 (69%) CpG sites remained as hyper- and hypomethylated outliers, respectively (Fig. 1 and Supplementary Tables 1 and 2).

We subjected these remaining outliers to further data analyses. Hypomethylated outliers coincided with SNP-containing probes more often than hypermethylated outliers (439/1,416 (31%) versus 462/2,983 (15%)). The mean (standard deviation [SD]) β values of the 2,521 hyper- and the 977 hypomethylated outliers were 0.24 (0.13) and 0.56 (0.19), respectively. The mean (SD) $\Delta\beta$ values ($\Delta\beta$ = the β value of the

	FGR			Normal		
	adequate	insufficient	excessive	adequate	insufficient	excessive
	(n = 5)	(n = 5)	(n = 4)	(n = 9)	(n = 5)	(n = 5)
BMI of pre-pregnancy (kg/m^2)	20.3 ± 2.0	18.7 ± 1.1	19.6 ± 1.0	20.1 ± 1.9	19.7 ± 2.2	19.8 ± 1.1
BMI at delivery (kg/m^2)	23.7 ± 2.5	20.4 ± 1.4[a]	25.2 ± 1.6	23.5 ± 1.7	22.3 ± 1.9	25.5 ± 1.2[d]
B.W. of pre-pregnancy (kg)	49.0 ± 5.3	49.4 ± 5.9	46.5 ± 1.7	51.0 ± 5.4	48.0 ± 7.0	50.6 ± 3.6
B.W. at delivery (kg)	57.2 ± 5.7	53.9 ± ± 6.5	60.0 ± 2.2	59.7 ± 4.8	54.3 ± 6.5	65.3 ± 3.9[d]
Gestational weight gain (kg)	8.2 ± 1.2	4.5 ± 1.8[a]	13.5 ± 0.9[b]	8.7 ± 1.1	6.3 ± 0.7[c]	14.7 ± 1.0[d]
B.W. of newborn (g)	1984 ± 296[e]	1702 ± 282[f]	1860 ± 535[g]	2937 ± 297	3010 ± 483	3452 ± 200
B.W./B.H. of newborn (cm/g)	45.4 ± 5.2[e]	40.9 ± 5.5[f]	43.8 ± 7.7[g]	61.3 ± 4.0	62.5 ± 7.9	68.3 ± 3.6[d]
Placental weight (P.W.) (g)	417.0 ± 47.9[e]	351.0 ± 83.2[f]	487.5 ± 24.0[b,g]	631.7 ± 189.2	639.0 ± 132.9	652.0 ± 97.8
B.W. of newborn/P.W.	4.8 ± 0.5	5.0 ± 1.1	3.8 ± 1.1[g]	4.9 ± 1.2	4.8 ± 1.0	5.4 ± 0.7
Gestational weeks	37.0 ± 1.4	35.8 ± 1.3[f]	36.3 ± 3.0	38.0 ± 1.7	39.6 ± 1.7	39.6 ± 1.1
Ratio of C. section to vaginal delivery	3 to 2	2 to 3	2 to 2	2 to 7	1 to 4	1 to 4
Ratio of male to female newborns	3 to 2	2 to 3	0 to 4	6 to 3	1 to 4	3 to 2
Age at delivery	31.4 ± 3.6	28.6 ± 2.4	26.8 ± 4.0	32.0 ± 7.1	31.4 ± 7.6	32.6 ± 5.9

Table 1. Characteritics of mothers, newborns, and placentas enrolled in this study. BMI, body mass index; B. W., body weight; B. H., body height. [a]$p < 0.05$ in the t-test between FGR_insufficient and FGR_adequate. [b]$p < 0.05$ in the t-test between FGR_excess and FGR_adequate. [c]$p < 0.05$ in the t-test between Normal_insufficient and Normal_adequate. [d]$p < 0.05$ in the t-test between Normal_excess and Normal_adequate. [e]$p < 0.05$ in the t-test between FGR_adequate and Normal_adequate. [f]$p < 0.05$ in the t-test between FGR_insufficient and Normal_insufficient. [g]$p < 0.05$ in the t-test between FGR_excess and Normal_excess.

outlier–the mean β value of the other samples) of hyper- and hypomethylated outliers were 0.18 (0.11) and –0.27 (0.12), respectively.

While the numbers of outliers in the "normal_adequate" category were low and relatively consistent among subjects (ranging from 46 to 74), those in the other five categories were higher, exhibiting statistical significance (Tukey's multiple comparison test p-value < 0.001; Fig. 1B) and diverse among the subjects (ranging from 44 to 421). In normal subjects, the greater the insufficiency or excessiveness of maternal GWG, the higher the number of methylation outliers, as represented by the U-shaped appearance of the bar plots for the number of outliers in normal subjects sorted according to weight gained during pregnancy (Fig. 1C). In FGR subjects, all three subcategories (FGR_insufficient, FGR_adequate, and FGR_excessive) contained significantly higher numbers of outliers than the "normal_adequate" category (Fig. 1B). The numbers of outliers in FGR_insufficient and FGR_excessive categories were also significantly higher than those in FGR_adequate (Fig. 1B). The numbers of outliers were neither associated with C-section nor correlated with gestational weeks (Supplementary Table 4). These results suggested that both FGR and inadequate GWG conditions affected the placental epigenome independently and additively.

Next, we examined the numbers of hyper- and hypomethylated outliers in each subject (Fig. 1E,G). While the numbers of hypomethylated outliers were not much different among subjects (Fig. 1F), the numbers of hypermethylated outliers were significantly higher in subjects in the other five categories compared to subjects in the "normal_adequate" category ($P < 0.001$; Fig. 1D). Therefore, only hypermethylated outliers occurred with FGR pregnancies and normal pregnancies with inadequate GWG. Because of the nature of the Smirnov-Grubbs' outlier test, the identified methylation outliers were all specific to individuals (deviated only in one sample among the cohort). Our results demonstrate that the adverse pregnancy conditions, FGR and inadequate GWG, affected the placental epigenome variably among individuals.

Contrasting genomic features of hyper- and hypomethylated outliers. We subsequently annotated the genomic features of 2,521 hyper- and 977 hypomethylated outliers (Fig. 2). Among these outliers, 2,107 (84%) and 758 (78%) CpG sites were located in genic regions (in 1,001 and 606 genes, respectively). Hypermethylated outliers were found to be predominantly located in CGIs or their shores/shelves (94% in total) and proximal to the transcriptional start sites (defined as "pTSS" hereafter; i.e., TSS1500, TSS200, the 5′ untranslated region [UTR], and the first exon categories; 77%). In contrast, hypomethylated outliers were most frequently located outside of CGIs, shores, and shelves (open sea,

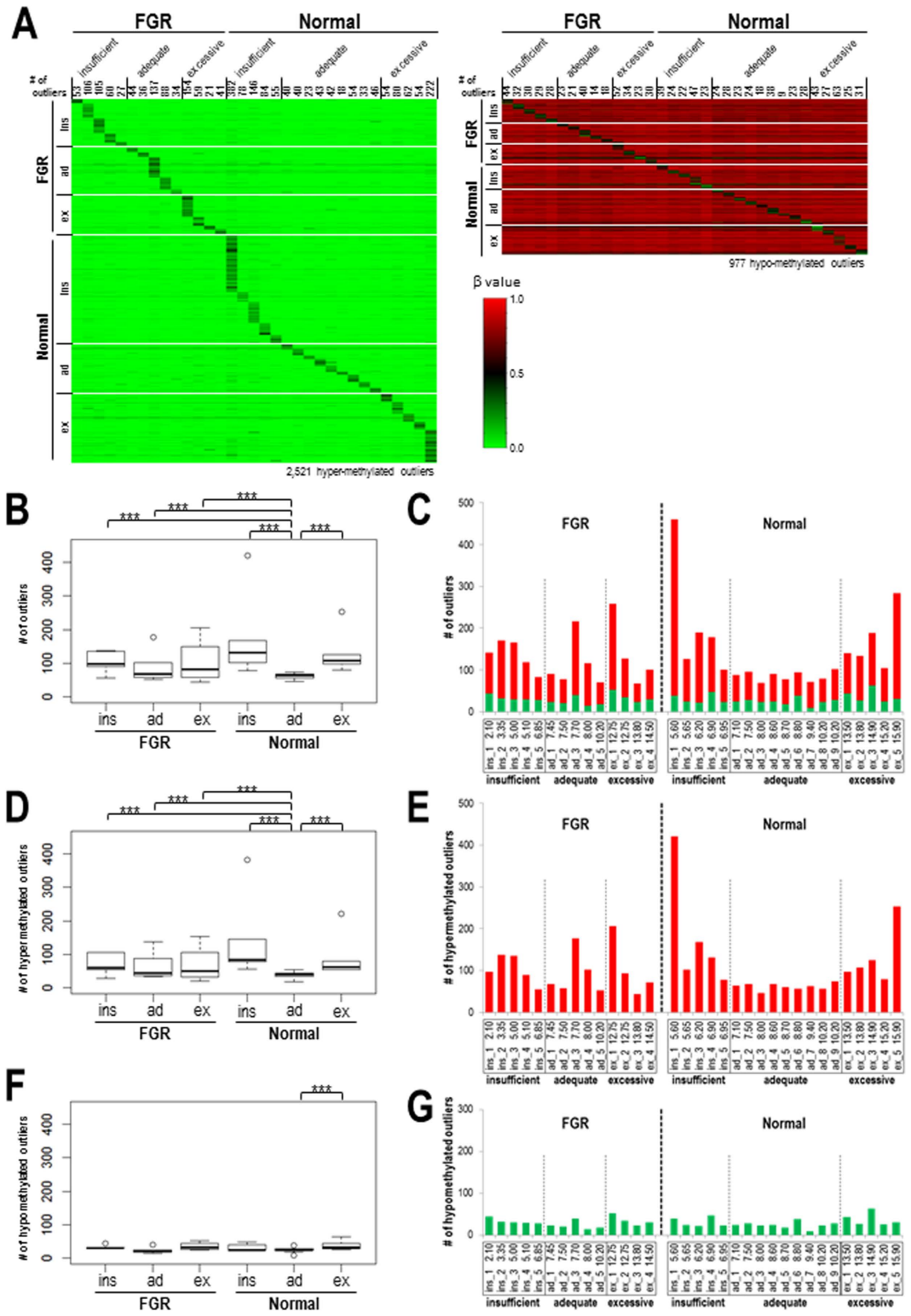

Figure 1. (**A**) Heatmap visualisation of the β value of methylation outliers detected by Smirnov-Grubbs' outlier tests. The numbers of outliers detected in each placenta are indicated above the heatmap. The colour scale represents the β value from 0 to 1. The left and right panels represent hypermethylated and hypomethylated outliers, respectively (**B,D,F**). Box plots showing the distribution of the numbers of outliers in each of six placental categories (***, Tukey's multiple comparison test P-value < 0.001). ins, insufficient; ad, adequate; ex, excessive (**C,E,F**). Bar plots for the numbers of outliers in FGR and normal subjects sorted according to weight gained during pregnancy. Plots for all outliers (**B,C**), hypermethylated outliers only (**D,E**), and hypomethylated outliers only (**F,G**) are shown. Red and green bars represent the numbers of hyper- and hypomethylated outliers, respectively (**C,E,F**).

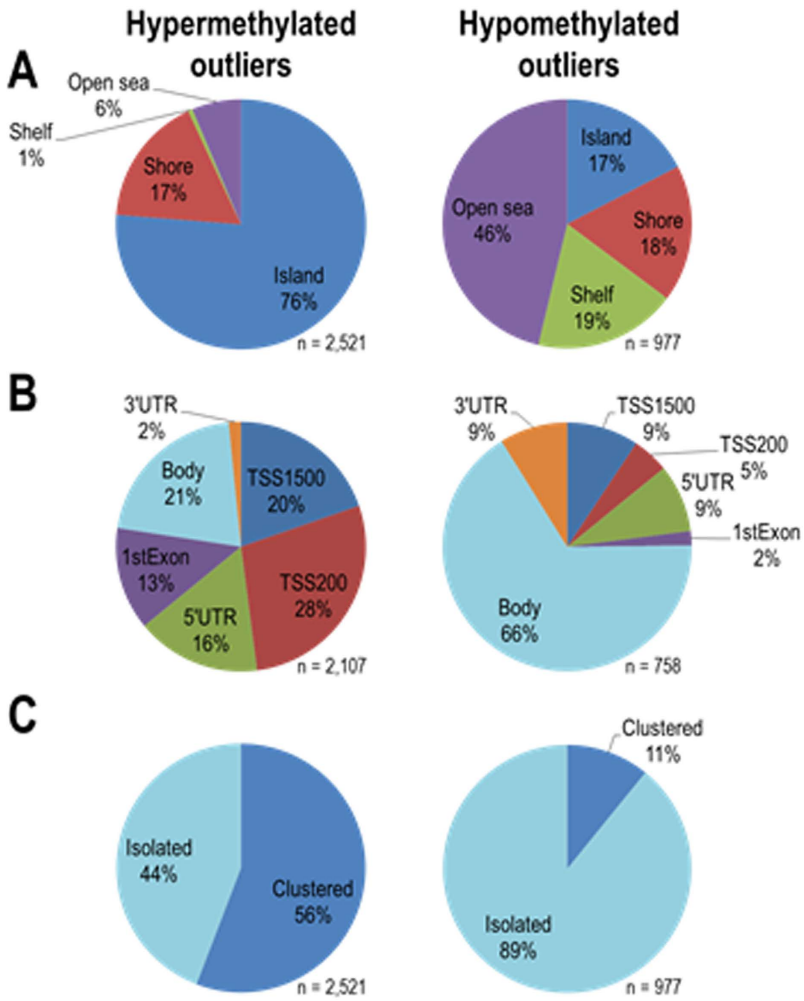

Figure 2. Genomic features of 2,521 hyper- and 977 hypomethylated outliers. Distribution of outliers in relation to CGIs and their shores and shelves (**A**) and to gene feature groups (**B**). Six gene feature categories, i.e., TSS1500, TSS200, the 5′UTR, the first exon, the gene body, and the 3′UTR, are regarded as genic regions, in which 2,107 (84%) and 758 (78%) CpG sites were located. The ratio of clustered and isolated outliers is shown (**C**).

46%) and in gene bodies (66%) (Fig. 2A,B). Hypermethylated outliers tended to be detected consecutively at two or more adjacent probes ("clustered"; 56%), while hypomethylated outliers did not (11%; Fig. 2C). These results implied that hypermethylated outliers tended to be clustered within CGI promoters.

We therefore scrutinised the extent of hypermethylation and the positional distribution relative to the TSS of hypermethylated outliers by visualising β and $\Delta\beta$ values on the Integrative Genomics Viewer (IGV, www.broadinstitute.org/igv/home). Indeed, we found that hypermethylated outliers were often distributed in a promoter-wide manner (i.e., located consecutively and clustered around the TSS) with relatively large methylation differences, as exemplified by *FOXC1*, *FOXL2*, and *HOXB7* loci (Fig. 3). The methylation statuses in the outlier sample and a control (Normal_adequate_7) at these promoter regions were validated to be hypermethylated and unmethylated, respectively, by targeted bisulfite sequencing analyses (Fig. 3). The appearance of both of heavily methylated and unmethylated clones in individual outlier samples may indicate the mosaic composition of normal and epimutated cells in these placentas.

The observation that hypermethylated outliers were often clustered at CGI promoters suggested that placental hypermethylation events do not occur in a purely random manner in terms of genomic location, but instead occur due to dysfunction of certain intrinsic mechanisms regulating the epigenetic status of CGI promoters under adverse *in utero* environments.

Hypermethylated outliers were frequently associated with genes encoding transcriptional regulators. In order to search for functional characteristics of genes containing hypermethylated outliers, we performed gene ontology (GO) analysis; 1,001 genes hosting hypermethylated outliers (as well as 606 genes hosting hypomethylated outliers for comparison) were analysed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7. The 606 genes hosting hypomethylated

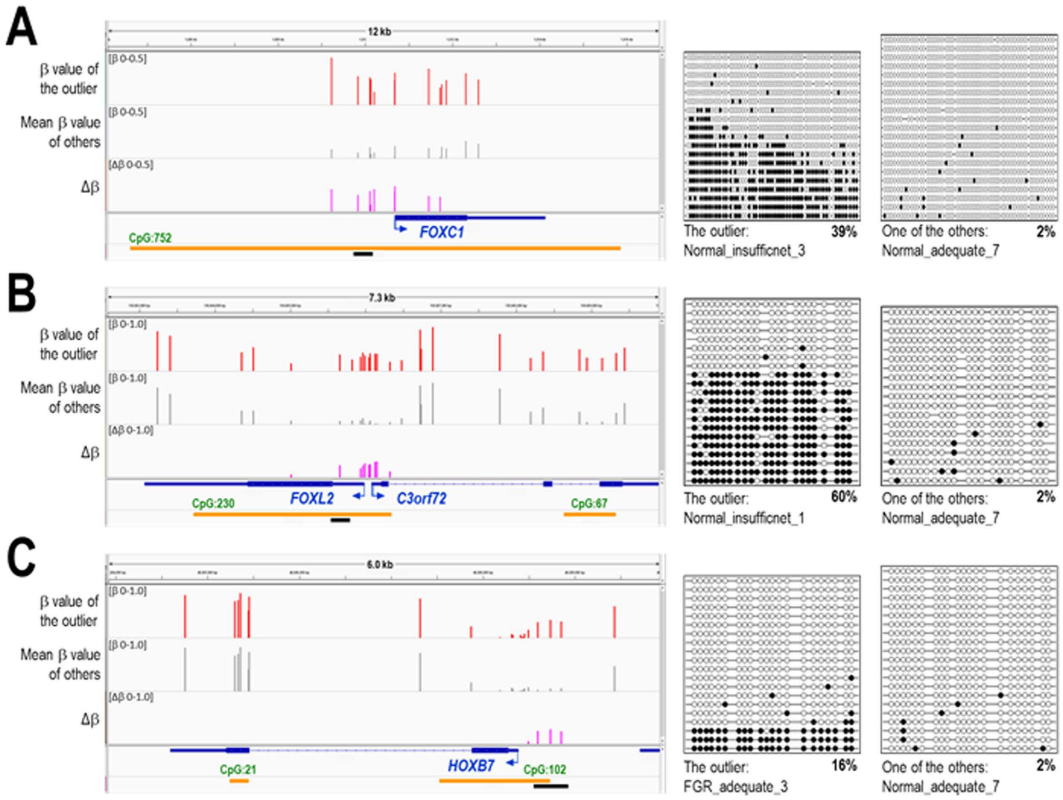

Figure 3. Examples of promoter-wide hypermethylation at *FOXC1* (**A**), *FOXL2* (**B**), and *HOXB7* (**C**) loci. The β value of the outlier, the mean of the β values of samples other than the outlier, and the Δβ are shown together with Refseq gene and UCSC-defined CGIs using IGV at the left side in each panel. The data range of 0 to 0.5 (or 0 to 1.0) is shown for β and Δβ values. The outlier samples for the three loci are Normal_insufficient_3 (**A**), Normal_insufficient_1 (**B**), and FGR_adequate_3 (**C**). DNA methylation status of these promoter regions were validated by targeted bisulfite sequencing (BS). The black horizontal bar at the bottom in each panel shows the interval of the bisulfite-PCR amplicon. The BS results for the outlier sample and a control (Normal_adequate_7) are shown at the right side in each panel. Open and closed circles represent unmethylated and methylated CpG sites, respectively. Each row of circles corresponds to an individual clone sequenced. The overall methylation rate (%) is shown underneath each panel of the BS results.

Gene ontology term (Biological Process)	Gene count	Fold enrichment	*p*-value	Benjamini's adjusted *p*-value
GO:0006355 regulation of transcription, DNA-dependent	157	1.70	6.73E-12	1.96E-08
GO:0030182 neuron differentiation	60	2.63	1.29E-11	1.88E-08
GO:0051252 regulation of RNA metabolic process	157	1.66	3.85E-11	3.74E-08
GO:0045449 regulation of transcription	199	1.47	2.39E-09	1.74E-06
GO:0007409 axonogenesis	31	3.08	6.61E-08	3.85E-05

Table 2. Top 5 gene ontology (GO) Biological Process terms significantly enriched among the 1,001 genes hosting 2,521 hypermethylated outliers.

outliers were found to be weakly enriched with only one term, "cytoskeletal protein binding", in the Molecular Function (MF) category (Benjamini's corrected $Pc = 0.0025$). However, the 1,001 genes hosting hypermethylated outliers were highly enriched with terms related to transcriptional regulators and neuronal differentiation in the Biological Process (BP) and MF categories (e.g., BP terms "regulation of transcription, DNA-dependent" [$Pc = 1.96 \times 10^{-8}$] and "neuronal differentiation" [$Pc = 3.16 \times 10^{-8}$]; Table 2 and Supplementary Table 3). We subsequently performed GO analysis for subgroups of genes:

409 genes hosting highly deviated ($\Delta\beta > 0.2$) hypermethylated outliers, 709 genes hosting hypermethylated outliers in the pTSS, and 317 genes hosting two or more clustered hypermethylated outliers. These subgroups of genes were also found to be significantly enriched with terms related to transcriptional regulators (Supplementary Table 3). These results supported our observation that hypermethylated outliers are often distributed in a promoter-wide manner and that the genes hosting such outliers are significantly enriched with genes encoding transcriptional regulators. We further performed GO analysis for the 163 genes hosting highly deviated ($\Delta\beta > 0.2$) and clustered hypermethylated outliers in the pTSS. Among those, 36 genes were assigned to the category "GO:0006355~regulation of transcription, DNA-dependent" with a statistical significance [$Pc = 0.0038$] and showed a higher fold enrichment value to the term than that of the entire (1,001) genes (2.25 versus 1.70, Supplementary Table 3). Importantly, in 35 out of the 36 genes encoding transcriptional regulators (97%), promoter hypermethylation was detected in the placentas from cases of inadequate GWG or FGR (Table 3).

Discussion

In this study, we demonstrated the possibility that inadequate maternal GWG enhances aberrant DNA methylation in the placenta. We initially failed to identify specific loci whose methylation was commonly altered across all subjects in each of the GWG categories. We subsequently used Smirnov-Grubbs' outlier tests, which detect the most significantly deviated outlier among subjects, for each of the CpG probes and found that hypermethylated loci accumulated in normal pregnancies with inadequate GWG and in FGR pregnancies. The results suggested that the epigenetically affected loci due to adverse *in utero* environments were variable among the subjects examined in this study. It should be noted that the relatively small number of the enrolled subjects (partly due to exclusion of the subjects with certain types of pregnancy complications) with various layers of heterogeneities (e.g., genetic, phenotypic, and environmental) may account for a primary cause of the absence of commonly epigenetically affected loci and the variation of affected loci among the individuals studied. While many animal studies have clearly demonstrated direct associations between *in utero* nutritional conditions during foetal development and epigenetic alterations (at certain loci or globally)[10], evidence from studies in human populations has been limited. Unlike the homogeneous genetic backgrounds of animal models and the well-controlled environmental and experimental conditions that can be easily achieved in animal studies, individuals in human studies are genetically heterogeneous and have not been exposed to identical environments throughout their lives. These unavoidable genetic and environmental heterogeneities in human subjects very likely give rise to individual variations in epigenetically affected loci, even when the subjects were exposed to similar nutritional environments for a certain period. Provided that epimutations could occur not only at common loci but at variable loci among subjects, Smirnov-Grubbs' outlier test is effective in evaluating the extent of the accumulation of the latter type of epimutations under certain disease and/or malnutrition conditions and may be applicable to a wide range of epigenetic studies in human populations.

FGR is idiopathic in most cases and is generally thought to be caused by foetal, placental, maternal, and/or environmental factors[30]. Therefore, the hypermethylation events observed with significantly high frequencies in placentas from FGR births in this study may also be explained by various factors. Unidentified genetic factors, such as foetal and/or placental chromosomal abnormalities and mutations at certain genes, if they exist, could affect the epigenomes of both the foetus and placenta, regardless of *in utero* conditions. Maternal and environmental factors deteriorating *in utero* conditions and contributing to the FGR phenotype may not have been identified in some subjects enrolled in this study. On the other hand, in normal pregnancy cases with inadequate GWG, since the body weights of the babies were within the normal range, the foetuses (and the placentas) were considered to be genetically normal. Under this assumption, promoter hypermethylation observed with higher frequencies in placentas with inadequate GWG than in those with adequate GWG can be regarded as environmentally induced epigenetic alterations.

Multiple independent studies have shown that genetic variants can cause variations in DNA methylation levels, defined as sequence-dependent allele-specific DNA methylation (ASM)[32]. A recent methylC-Seq study of the mouse genome revealed that sequence-dependent ASMs typically exist as isolated CpG sites in intergenic and intronic regions, but are relatively depleted from proximal promoters[33]. Moreover, sequence-dependent ASMs are influenced by defined sequences nearby and they appear to have little effect on gene expression. The genomic features of hypomethylated outliers in our study were similar to those of sequence-dependent ASMs. On the other hand, the characteristics of the hypermethylated outliers, being clustered (56%) in the pTSS (77%), were distinct from those of sequence-dependent ASMs. It is generally challenging to distinguish whether differentially methylated regions among genetically heterogeneous human populations are epimutations or sequence-dependent ASMs. However, considering the above-mentioned genomic features of the hypermethylated outliers as well as their enrichment in the promoter regions of transcriptional regulator genes (which will be discussed in detail in the next paragraph), at least a portion of these outliers likely represent genuine epigenetic alterations rather than sequence-dependent changes in DNA methylation.

We initially considered that placental epimutations may have occurred randomly under aberrant *in utero* environments; our data subsequently revealed that hypermethylated outliers were not found completely randomly in terms of genomic location, but tend to be frequent at the promoters of genes

Subject	Gene Symbol	Gene Name	# of hyper-methylated outliers in pTSS	average Δβ of outliers
N_adequate_5	*ZNF649***	zinc finger protein 649	6	0.19
N_excessive_1	*GBX2**	gastrulation brain homeobox 2	3	0.25
N_excessive_1	*ZNF350***	zinc finger protein 350	8	0.30
N_excessive_2	*ZFP37***	zinc finger protein 37 homolog	4	0.44
N_excessive_5	*ZHX2**	zinc fingers and homeoboxes 2	8	0.15
N_excessive_5	*CDKN1C**	cyclin-dependent kinase inhibitor 1C	4#	0.28
N_excessive_5	*PAX6**	paired box 6	2	0.23
N_insufficient_1	*KCNH8**	potassium voltage-gated channel, subfamily H, member 8	4	0.24
N_insufficient_1	*FOXL2**	forkhead box L2	10	0.21
N_insufficient_1	*F2R**	coagulation factor II (thrombin) receptor	3	0.20
N_insufficient_1	*SOX7**	SRY (sex determining region Y)-box 7	3	0.18
N_insufficient_1	*NFIB**	nuclear factor I/B	2	0.37
N_insufficient_1	*HMX2**	H6 family homeobox 2	2	0.23
N_insufficient_2	*TFCP2**	transcription factor CP2	5	0.19
N_insufficient_3	*PROX1**	prospero homeobox 1	3	0.15
N_insufficient_3	*FOXC1**	forkhead box C1	8	0.19
N_insufficient_3	*FOXB1**	forkhead box B1	2	0.17
N_insufficient_5	*ETV1*	ets variant 1	6	0.11
N_insufficient_5	*ZNF426***	zinc finger protein 426	2	0.17
FGR_adequate_1	*PER1*	period homolog 1	6	0.16
FGR_adequate_3	*ZNF619***	zinc finger protein 619	4	0.27
FGR_adequate_3	*ZKSCAN4***	zinc finger with KRAB and SCAN domains 4	3	0.25
FGR_adequate_3	*PGBD1*	piggyBac transposable element derived 1	7	0.21
FGR_adequate_3	*HOXB7**	homeobox B7	4	0.23
FGR_adequate_4	*ESR1**	estrogen receptor 1	3#	0.17
FGR_adequate_4	*MGA*	MAX gene associated	3	0.17
FGR_excessive_1	*RFX8**	hypothetical protein LOC731220	3	0.33
FGR_excessive_1	*ZNF483***	zinc finger protein 483	2	0.20
FGR_excessive_1	*ZNF254***	zinc finger protein 254	3#	0.21
FGR_excessive_2	*ZNF577***	zinc finger protein 577	6#	0.43
FGR_excessive_3	*ZNF655***	zinc finger protein 655	2	0.35
FGR_insufficient_1	*ZNF562***	zinc finger protein 562	8	0.36
FGR_insufficient_1	*ZNF805***	zinc finger protein 805	2	0.30
FGR_insufficient_2	*ZNF583***	zinc finger protein 583	6	0.27
FGR_insufficient_3	*ZNF354C***	zinc finger protein 354C	7	0.27
FGR_insufficient_3	*ETV1*	ets variant 1	6	0.16
FGR_insufficient_4	*ZIK1***	zinc finger protein interacting with K protein 1	2	0.42

Table 3. The list of 36 genes assigned with transcription factor-related gene ontology terms among the 163 genes hosting highly-deviated and clustered hypermethylated outliers in pTSS. *Polycomb repressive complex 2 (PRC2) target genes in mouse or human embryonic stem cell lines (16/36, 44%). The 653 mouse PRC2-targets identified in Ref.36 and the ChIP-seq dataset of the PRC2 components (EZH2 and SUZ12) for a human ES cell line (H1-hESC) produced by the Encyclopedia of DNA Elements (ENCODE) Consortium were refered to search for PRC2-targets among the 36 genes. **Zinc-finger genes (15/36, 42%). #indicates gene body probes nearby (<1 kb) the pTSS region.

encoding transcription factors. Considering that the promoter regions of genes encoding developmental regulators, such as homeobox proteins and other developmental transcription factors, have been reported to be mostly devoid of sequence-dependent ASMs[33], the hypermethylated outliers located at the promoter regions of such genes identified in this study (Table 3) most likely represent epigenetic alterations due to aberrant *in utero* environments. In a recent genome-wide DNA methylation study using reduced representation bisulphite sequencing (RRBS) in a murine model of FGR, genes hosting differentially methylated regions in the placenta upon maternal calorie restriction are significantly enriched ($P < 0.05$) with GO terms such as homeobox and transcription factor activity, among others[34]. Notably, our own annotations for the 131 genes hosting hypermethylated regions in the placenta upon maternal gestational calorie restriction[34] using DAVID revealed that these genes were moderately enriched with genes assigned with the GO Molecular Function term "DNA binding" (17 out of the 131 genes were assigned this term). Therefore, although the statistical method used for detecting differentially methylated regions is different from that in our study, some aspects of this murine study were consistent with our findings demonstrating the enrichment of placental epimutations in transcriptional regulator genes.

Our findings also suggested the possibility that certain epigenetic regulatory systems are susceptible to the disruptive effects of aberrant *in utero* environments. In fact, a careful analysis of the 36 genes assigned with GO terms related to transcriptional regulation (Table 3) revealed that polycomb group repressive complexes (PRCs)[35] represent a primary candidate of such regulatory mechanisms. We found that seven out of the 36 genes (i.e., *HOXB7*, *GBX2*, *HMX2*, *SOX7*, *F2R*, *FOXL2*, and *FOXC1*) were included in the 653 PRC2 targets in mouse embryonic stem cells, as identified by a ChIP-on-chip analysis[36]. Further annotations of the 36 genes using the ChIP-seq data for EZH2 and SUZ12, which are components of PRC2[35], from a human ES cell line (H1-hESC) produced by the Encyclopedia of DNA Elements (ENCODE) Consortium (http://genome.ucsc.edu/ENCODE/) identified additional eight PRC2 targets (Table 3). Consistent with our observations, epigenetic variation between twin-twin transfusion syndrome children, wherein twin foetuses occasionally exhibit striking growth differences, is most prominent at the CpG sites within the target regions of PRCs[37]. Furthermore, Wilhelm-Benartzi *et al.* reported significant associations of placental LINE-1 and AluYb8 methylation levels with birth weight percentile and significant differences in the methylation levels of these repetitive elements upon maternal alcohol or tobacco use during pregnancy[21]. Interestingly, the authors also revealed the positive association of increased placental AluYb8 methylation with the average methylation levels of CpG sites in polycomb group target genes. Therefore, evidence from these previous reports and our current findings suggest the possibility that PRCs occasionally fail to recognise their targets with a stochastic nature in the placenta under improper *in utero* environments, leading to epigenetic switching from PRC marks (H3K27me3) to DNA methylation. Another striking feature of these 36 genes was that 15 (42%) were zinc-finger genes (Table 3). Zinc-finger genes are often silenced through H3K9me3-mediated gene silencing coupled with promoter DNA methylation in toxicant-induced carcinogenesis, suggesting the existence of an unknown epigenetic mechanism through which many zinc-finger genes are coregulated[38]. This hypothetical regulatory mechanism may also be susceptible to the effects of adverse *in utero* environments.

In addition to the enrichment of GO terms related to transcriptional regulation, the genes hosting hypermethylated outliers were also found to be enriched with the GO term "neuron differentiation" (Supplementary Table 3).This seemingly unexpected observation is consistent with those of previous studies. In an array-based expression study that identified 7,519 genes exhibiting differential expression between human placentas sampled during the first and third trimesters, both up- and downregulated genes in the third trimester were found to be enriched with genes involved in human neurogenesis[39]. The authors of the study have suggested that the brain and placenta possibly share common developmental routes. In the above-mentioned RRBS study of the murine model of intrauterine malnutrition[34], GO terms found to be enriched in genes hosting altered placental DNA methylation upon maternal caloric restriction were shown to contain neuron-related terms[34]. Additionally, several neural factors, such as BDNF[40], NGF[41], and serotonin[42], have been shown to be secreted from the placenta. Among these factors, BDNF has also been shown to potentiate placental development and play an important role in cytotrophoblast differentiation[43,44]. Furthermore, placental BDNF expression has been reported to be significantly correlated with neonatal birth weight[40] and to be decreased upon maternal malnutrition in rats[45]. Because of the functional significance of a subset of genes in both the placenta and brain, it is tempting to speculate that the foetuses may have gained epigenetic alteration patterns that are similar to those observed in the placenta in pregnancies with inadequate GWG. Hypermethylation at the promoter regions of genes encoding developmental regulators (PRC2 targets) and neuronal regulators at early embryonic stages would reduce their expression levels when these genes are expressed in a spatio-temporal manner, and such aberrant expression of critical developmental regulators may elevate the risk of developing various diseases, including metabolic and mental disorders, later in life.

In this study, we demonstrated that loci with alterations in the placental DNA methylation under inadequate GWG were not common among subjects but were instead distributed in an individual-specific manner. Furthermore, such epigenetic alterations under the adverse pregnancy condition were found to occur preferentially at the CGI promoters of genes encoding transcriptional factors. Our novel findings support the necessity of large-scale epigenomic studies of placental tissues and samples (e.g., cord blood) from newborns for pregnancies under normal and malnutrition conditions, together with follow-up

studies when the newborns reach adulthood in order to elucidate the epigenetic mechanisms underlying developmental programming in humans and their roles in health and disease in later life.

Materials and Methods

Study design. The present study was approved by the Ethics Committee of the National Center of Child Health and Development (NCCHD), Japan and by the Human Study Committee of the Hokkaido University Hospital, Japan. Informed consent was obtained from all subjects. Pregnant Japanese women who did not have pregnancy complications of gestational diabetes, pre-eclampsia, or pregnancy-induced hypertension were enrolled. All enrolled subjects did not smoke or drink alcohol, and did not exhibit hypertension or proteinuria during pregnancy. Subjects ($n = 33$) were categorised into six categories according to GWG and newborn birth weight: FGR_adequate, FGR_insufficient, FGR_excessive, normal_adequate, normal_insufficient, and normal_excessive, consisting of 5, 5, 4, 9, 5, and 5 placentas, respectively. Prepregnancy BMIs were similar among all groups. The characteristics of each group are shown in Table 1. BMI, body weight, GWG, and additional clinical information (maternal complication, gestational week, delivery method, and newborn's gender) for each of the subjects are provided as Supplementary Table 4. Although the Institute of Medicine of the United States recommends that pregnant women whose prepregnancy BMI is in the normal range (18.5–24.9) should gain 11.3–15.9 kg during pregnancy, we defined adequate GWG as gaining 7–12 kg in this study in accordance with the recommendations of the Japan Ministry of Health, Labour, and Welfare[46]. This difference is also consistent with the different average BMIs of Japanese and US women (21.14 ± 3.28[47] versus 27.05 ± 0.35[48], respectively).

Genomic DNA extraction and DNA methylation profiling. Full-term placental samples were obtained from normal caesarean sections or vaginal deliveries. Chorionic villous tissue was obtained from the foetal side of the placenta. Genomic DNA was purified from the tissue using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Genomic DNA (1.5 μg) was bisulphite converted using an EpiTect Plus DNA Bisulfite Kit (Qiagen). After determining the concentration of bisulphited DNA, 300 ng of bisulphite DNA from each sample was subjected to Illumina Infinium HumanMethylation450 BeadChip analysis using the manufacturer's standard protocol.

Data processing. To calculate the DNA methylation levels of more than 480,000 CpG sites assayed on the HumanMethylation450 BeadChip (Illumina), the signal intensity data (.idat files), produced by the Illumina iSCAN system, were processed using Illumina GenomeStudio Methylation Analysis Module v1.9.0 with background subtraction and control normalisation options. The methylation levels were calculated as β values ranging from 0 (completely unmethylated) to 1 (completely methylated; β value = intensity of the methylated allele/[intensity of the unmethylated allele + intensity of the methylated allele + 100]). The obtained data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession number GSE62733. From 485,577 probes on the BeadChip array, the following probes were excluded: the probes on sex chromosomes, the probes for 65 random SNPs (which assay highly-polymorphic SNPs rather than DNA methylation), and the probes whose detection *p*-value was higher than 0.01 or whose β value was missing in one or more samples. The β values (methylation levels) of the remaining 449,848 probes were corrected by an Empirical Bayes method, ComBat[49], to remove the array-batch effect, and subjected to statistical tests.

To detect differentially methylated CpG sites between groups, the Illumina Methylation Analyzer (IMA)[31] was run using the Wilcoxon rank-sum test for inference of differences between categorical groups. The BH procedure was used for multiple testing corrections, and the cut-off for the adjusted *p*-values was set to 0.05. Smirnov-Grubbs' outlier test with Bonferroni multiple test corrections was performed using the R Package 'outliers' (http://cran.r-project.org/web/packages/outliers/outliers.pdf) and custom R scripts to detect outlying CpG sites, and the cut-off for the corrected *p*-values was set to 0.1.

The Illumina-provided probe annotation, HumanMethylation450_15017482_v.1.1.csv, was used to sort out the outlying CpG sites whose β value could possibly have been affected by sequence variation within the corresponding probe sequence. This table lists 89,678 probes as SNP-containing in its "probe_SNPs" and "probe_SNPs_10" columns based on the information of NCBI dbSNP Build 131. The refSNP information registered in dbSNP Build 142 was also tested for the same purpose of SNP filtering (Supplementary Fig. 2).

When a single CpG site was assigned to multiple gene symbols or gene features in the Illumina probe annotation, only the lead-off gene symbol or feature was used for gene ontology and genome feature annotations.

Targeted bisulfite sequencing. Bisulfite sequencing analysis was performed as described previously[50] using bisulfite-PCR primers designed by the MethPrimer website[51]. The forward and reverse primer sequences, and the genomic interval (hg19) of the amplicon are: 5′-GAGAGGTTGGGGTAATTTTAG-3′, 5′-AAAAACTTCTAAACTTTTAAACATCC-3′ and chr6:1609671-1610171 (501bp) for the *FOXC1* locus; 5′-GGGGTAGTTGGTTATTATGATAAAGT-3′, 5′-ACTCCCCATAACCAAAAACTAAACT-3′, and chr3:138665547-138665794 (248 bp) for the *FOXL2* locus; 5′-AGTTTTGTGGATTGGGGTTG-3′,

5′-ACACCTAAAAAAACTTACTCCATCTC-3′, and chr17:46688533-46688920 (388 bp) for the *HOXB7* locus. The obtained sequence data were analysed using the QUMA website[52].

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Author Contributions

T.K. designed the project. T.Y., R.A., H.M. and K.H. contributed materials. T.K. and H.K. obtained the experimental data. T.K., K.A., K.O. and K.N. analysed the data. T.K. and K.N. wrote the manuscript. K.N. and K.H. cosupervised the project.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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