Multigenerational programming in the glucocorticoid programmed rat is associated with generation-specific and parent of origin effects

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Key words: programming, glucocorticoids, multigenerational, epigenetics, methylation

Abstract: Exposure to an adverse early life environment is associated with increased cardio-metabolic disease risk, a phenomenon termed “programming.” The effects of this are not limited to the exposed first (F1) generation but can be transmissible to a second generation (F2) through male and female lines. Using a three generation animal model of programming by initial prenatal glucocorticoid overexposure we have identified effects on fetal and placental weight in both the F1 and F2 offspring. However, the expression of candidate imprinted genes in the fetus and placenta differed between the F1 and F2, with marked parent-of-origin effects in F2. Since DNA methylation at imprinted genes is maintained at fertilization, they are potential templates for the transmission of programming effects across generations. Although we detected alterations in DNA methylation at differentially methylated regions (DMRs) of the key prenatal growth factor Igf2 in F1 and F2 fetal liver, the changes in DNA methylation at these DMRs do not appear to underlie the transmission of effects on Igf2 expression through sperm. Thus, multigenerational programming effects on birth weight and disease risk is associated with different processes in F1 and F2. These findings have implications for the pathogenesis and future attempts to stratify therapies for the “developmental component” of cardiometabolic disease.

Introduction

An adverse prenatal environment, as marked by low birth weight, permanently alters offspring structure and function and increases the risk of cardiovascular, metabolic and neuropsychiatric disorders in adulthood, a phenomenon called fetal “programming”. Epidemiological studies in different populations suggest that the effects of early life exposure to environmental influences, including nutritional and psychosocial stressors, may not be limited to the directly exposed first (F1) generation but may be transmissible to subsequent generations. Such effects have been proposed as an explanation for the persistence of secular trends in health, including cardiovascular disease and its risk factors, in humans. The mechanisms underlying the non-genetic transmission of disease risk across generations remain incompletely understood. While exposure to persisting adverse environmental conditions or fetal exposure to programmed maternal physiology and structure (the “uterine effect”) may play a role, these factors do not adequately explain the transmission of programmed effects through the male line in both human studies and in animal experiments in which environmental conditions can be closely controlled. There is increasing evidence for a role for epigenetic mechanisms including DNA methylation, histone modifications and small non-coding RNAs as mediators of programming effects and growing interest in the potential for the inheritance of epigenetic marks through the germline as a mechanism for the transmission of disease risk across generations.

Epigenetic modifications are established during early development and maintained throughout life (i.e., are mitotically stable). During fetal development, the germ cells that will give rise to the F2 generation are present from early gestation and undergo extensive epigenetic reprogramming prenatally. Disruption of germ cell reprogramming by the same environmental insult causing the phenotype in the F1 offspring could therefore result in effects in the F2, if changes were preserved during the period of epigenetic reprogramming that occurs following fertilization. Genomic imprinting refers to the phenomenon by which a subset of genes is monoallelically expressed according to the parent
Imprinted genes play a key role in fetal and placental growth and in modulating placental nutrient supply and, in humans, abnormalities of imprinting have major effects on fetal and placental growth. The allele-specific epigenetic marks maintaining the expression of imprinted genes, which include DNA methylation, undergo epigenetic reprogramming in the germline but are preserved in the zygote following fertilization, making imprinted genes in the fetus and placenta plausible candidate targets for the transmission of programming effects to a second generation.

One of the major hypotheses to explain the relationship between exposure to an adverse early life environment and later disease risk is fetal overexposure to glucocorticoids. Glucocorticoids are essential for fetal maturation in late gestation, but excessive exposure reduces fetal growth in animal models and humans. Although glucocorticoids are highly lipophilic molecules, and should readily cross the placenta, fetal glucocorticoid levels are normally much lower than the levels in the maternal circulation. While glucocorticoids readily cross the placenta, fetal glucocorticoid levels are normally much lower than the levels in the maternal circulation.

This is thought to be mediated by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which catalyzes the conversion of active glucocorticoids to their inactive 11-keto metabolites; however, this barrier is not complete so that 10–20% of maternal cortisol can cross the placenta. We and others have modeled programming by prenatal overexposure to glucocorticoids in rodents and non-human primates, using a number of methods including maternal treatment with dexamethasone (Dex; a poor substrate for 11β-HSD2) or maternal physiologic stress, which increases the levels of maternal glucocorticoids to levels that may overcome the placental 11β-HSD2 barrier and additionally reduce placental 11β-HSD2. Such manipulations reduce birth weight and produce hypertension, insulin resistance/hyperglycemia and anxiety-related behaviors in the offspring. Prenatal glucocorticoid overexposure is associated with the transmission of programming effects across generations; birth weight is also reduced in the second (F2) generation following either maternal or paternal prenatal Dex exposure. DNA demethylation can mediate hormone-induced alterations in gene transcription and glucocorticoid-induced DNA demethylation may provide memory of a regulatory event during development. Thus, alterations in DNA methylation may be one mechanism underpinning glucocorticoid-induced programming effects and the transmission of these effects to a second generation.

We have explored the effects of prenatal Dex exposure on the expression of candidate imprinted genes in F1 and F2 offspring in both embryonic and extra-embryonic tissues in late gestation and determined whether any alterations in gene expression associated with changes in DNA methylation. We hypothesized that given the similar programmed phenotypes observed in this model, the effects on growth and imprinted gene expression would be similar in both generations and that these effects may be mediated by alterations in DNA methylation.

Results

The experimental design and major findings are summarized in Figure 1 and Table 1.

Placental and fetal growth. Birth weight was reduced in the F1 offspring of Dex-treated pregnancies (Fig. 2A) and at E20 both placental and fetal weights were reduced in F1 Dex pregnancies (Fig. 2B and C). In the F2 generation birth weight was reduced in Dex/Dex, Dex/Veh and Veh/Dex offspring compared to Veh/Veh controls (Fig. 2D), replicating our previous study in reference 11. Intriguingly, there was a paternal effect to reduce birth weight [F(1, 408) = 23.2, p < 0.001] and, additionally, an interaction between parental exposure (F(1, 408) = 14.93, p < 0.001) so that F2 Veh/Dex (paternally exposed) offspring were significantly smaller than all other groups.

At E20, fetal weight was significantly reduced only in F2 Veh/Dex offspring (Fig. 2E), suggesting that the reduction in fetal weight in the F2 generation occurs late in gestation, but further emphasizing the paternal Dex effect on fetal growth. There was, again, an effect of paternal Dex exposure at this time point to decrease both fetal and placental weight (Fig. 2E and placenta F(1, 397) = 12.25, p < 0.001; Fig. 2F, fetus F(1, 397) = 4.78, p = 0.029) and an interaction between parental exposures to reduce fetal weight (F(1, 397) = 6.57, p = 0.011). Unexpectedly, there was also an effect of maternal Dex exposure to increase F2 placental [F(1, 397) = 10.15, p < 0.01] and fetal (F(1, 397) = 17.69, p < 0.001) weights at E20.

Gene expression in fetal liver and placental labyrinth. Fetal liver. There were changes in the expression of a number of imprinted genes in fetal liver in the F1 offspring (Fig. 3A). The level of mRNA encoding the paternally expressed gene Igf2 was increased in liver of F1 Dex fetuses. Although increased expression of Igf2 would predict increased fetal growth, Dex exposure was also associated with increased expression of the maternally expressed genes Cdkn1c and Grb10, which act to reduce fetal growth, and with increased expression of H19. These Dex-induced changes in maternally expressed imprinted genes were specific, since there were no changes in Phlda2 or Igf2r.

In F2 fetal liver all mRNAs that showed altered expression were discordant with changes seen in the F1 liver. Thus, 2-way
ANOVA revealed an interaction between parental prenatal treatment to reduce Igf2 [F(1, 27) = 5.66, p = 0.025] and Phlda2 [F(1, 27) = 6.62, p = 0.016] (Fig. 3B) and a maternal effect to increase the expression of H19 [F(1, 27) = 4.93, p = 0.035]. No effects of Dex were found on Cdkn1c expression in F2 liver though this was strikingly increased in F1 liver.

Placental labyrinth. In F1 placenta, Dex exposure increased expression of the imprinted, paternally expressed system A amino acid transporter slc38a4 (Fig. 4A); however, contrary to the findings in the liver, placental expression of Igf2 was reduced by Dex exposure. Also, in contrast to the findings in fetal liver, there were no changes in the expression of a number of other imprinted genes in placenta (Fig. 4A).

In F2 placenta, the expression of a number of imprinted genes was altered, with parent of origin specific effects. Paternal prenatal Dex exposure increased expression of the maternally expressed genes Cdkn1c [F(1, 28) = 6.41, p = 0.017], Phlda2 [F(1, 28) = 6.078, p = 0.02] and H19 [F(1, 28) = 7.03, p = 0.013], whereas maternal prenatal Dex exposure increased the expression of slc38a4 [F(1, 28) = 7.78, p = 0.009] and the paternally expressed genes slc38a4 [F(1, 28) = 5.34, p = 0.028] and Mest [F(1, 28) = 6.33, p = 0.018] (Fig. 4B).

DNA methylation at Igf2 in F1 and F2 generations. In order to explore the mechanisms underpinning altered gene expression we examined DNA methylation at Igf2 DMRs in fetal liver and placenta (Fig. 5). Igf2 is expressed exclusively from the paternal allele during prenatal development\(^{32}\) and is methylated on the paternal allele at a number of DMRs including the intragenic DMR2 and the H19 ICR situated 5’ of the H19 gene.\(^{32,33}\) Methylation at DMR2 (including within the “core DMR2,” where DNA methylation is required for high level gene transcription)\(^ {33,34}\) was decreased in F1 Dex fetal liver (Fig. 6A). There were no differences in methylation across four CTCF binding sites within the ICR, the dominant transcriptional control region (Fig. 6B). In the F2 generation, liver DNA showed no changes in methylation at the Igf2 DMR2 (Fig. 6C), but there was an interaction between maternal and paternal prenatal Dex exposure to reduce DNA methylation at the fourth CTCF binding site within the ICR in F2 fetal liver when either parent was exposed to Dex in utero [F(1, 25) = 4.425, p = 0.046] (Fig. 6D). There were no differences in DNA methylation at Igf2 DMR2 or the ICR in F1 or F2 placenta (data not shown).

DNA methylation in F1 sperm. Since Igf2 is a paternally methylated gene, we studied DNA methylation at Igf2 DMR2 and the H19 ICR in sperm from F1 males to determine whether the effects on Igf2 DNA methylation following Dex exposure were associated with altered DNA methylation in sperm. Analysis of sperm DNA revealed no difference in DNA methylation at the Igf2 DMRs using pyrosequencing at the Igf2 DMR2 (Fig. 6E) and Sequenom MassArray at the ICR (Fig. 6F).

**Discussion**

Prenatal Dex reduced F1 fetal weight at E20 and altered liver mRNA levels of imprinted genes. The induction of hepatic

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**Table 1. Summary of main findings**

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<tr>
<th>Generation</th>
<th>F1</th>
<th>F2</th>
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<tr>
<td>E20 fetal weight</td>
<td>↓ in F1 dex</td>
<td>Effect of paternal dex to ↓ and effect of maternal dex to ↑ fetal weight at E20</td>
</tr>
<tr>
<td>E20 placental weight</td>
<td>↓ in F1 dex</td>
<td>Effect of paternal dex to ↓ and effect of maternal dex to ↑ placenta weight at E20</td>
</tr>
<tr>
<td>Birth weight</td>
<td>↓ in F1 dex</td>
<td>Birth weight ↓ in all groups compared to control</td>
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| Gene expression |
|-----------------|-----------------|
| Fetal liver | Effects of prenatal dex to increase expression of both maternally and paternally expressed imprinted genes: Igf2, cdkn1c, grb10, H19 |
| Placenta | ↓ expression Igf2 and ↑ expression slc38a4 in F1dex |

<table>
<thead>
<tr>
<th>DNA methylation</th>
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<tr>
<td>Igf2 DMR2</td>
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<td>H19 ICR</td>
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\(\text{placenta}^{(1)}\)
The effects on fetoplacental growth and gene expression in F2 differed from F1 and were dependent on the transmitting parent. There was a predominant paternal effect to reduce F2 placental and fetal weights as well as subsequent birth weight, as previously reported in this model; an effect also reported in a mouse model of undernutrition. Unexpectedly, there appeared to be maternal "compensation" with Dex via the maternal line increasing both placental and fetal weight at E20. Unlike our findings in the F1 offspring, in F2 fetal liver the expression of both *Igf2* and *Phlda2* mRNA was lowest in the smallest offspring, those of Dex-exposed fathers and control mothers. This reduction in hepatic Igf2 expression may be of major importance in the observed growth restriction in F2 offspring, since Phlda2 is thought to act primarily in the placenta. Intriguingly, Grb10 has been identified as a candidate mediator of early life programming effects because of its dual role in fetal growth and the regulation of body composition and later glucose-insulin homeostasis. While Dex exposure also reduced F1 placental size, the effects on gene expression were discordant with fetal liver. Recent evidence suggests that fetal Igf2 may link placental nutrient transport and fetal demand so that the increased expression of the nutrient transporter slc38a4 in Dex-exposed F1 placentas may reflect increased fetal demand mediated through increased fetal Igf2 production. Although it is unclear whether the primary effects of Dex exposure act through the maternal epigenome, with subsequent “compensation” by the paternal epigenome, or vice versa (or both), the ultimate effects of direct Dex exposure on fetal growth plausibly depend on the balance of growth factor expression and the interaction between maternal and paternal effects in both the placenta and fetus in late gestation.
glucocorticoid exposure, are presumably acting to drive this increase in expression rather than altered methylation. In contrast, in F2 fetal liver, DMR2 methylation was unchanged but there was an interaction between maternal and paternal prenatal Dex exposure to decrease methylation in the H19 ICR, the dominant epigenetic regulator of Igf2 expression and consistent with both the reduction in Igf2 gene expression and the reduction in fetal size in Veh/Dex offspring. Although the significance of relatively small changes in DNA methylation (~5–6%) are unclear, such modest differences in methylation have been documented in human studies in IUGR neonates, and differences in DNA methylation of around 20% at the Igf2 DMRs cause the marked abnormalities in prenatal growth seen in Beckwith-Wiedemann and Silver-Russell syndromes; consequently, small changes in these regions may have consequences for fetal growth.

In placenta, despite the marked alterations in Igf2 expression in the F1 generation, there were no alterations in Igf2 DMR2 or ICR methylation, a finding consistent with a study in mice. The effects of prenatal Dex exposure on the expression of imprinted genes in the placenta may be mediated by other epigenetic mechanisms, including histone modifications, altered transcription factor binding or post-transcriptional modification, or by the recently described epigenetic modification 5-hydroxymethylcytosine.
Figure 4. Gene expression in placental labyrinth. (A) Prenatal Dex exposure is associated with altered gene expression in F1 placental labyrinth at E20 (n = 12 Veh and 12 Dex). (B) Gene expression in F2 placental labyrinth. Maternal prenatal Dex exposure increased the expression of slc38a4, Mest and Glut 1 (*a: 2-way ANOVA, p < 0.05) while paternal Dex increased the expression of Cdkn1c, Phlda2 and H19 (*b: 2-way ANOVA, p < 0.05). n=9 Veh/Veh, 7 Dex/Dex, 8 Dex/Veh and 8 Veh/Dex. Values are mean +/- SEM. *p<0.05 **p<0.01.

Figure 5. Schematic diagram of the Igf2 and H19 genes showing location of DMRs including DMR2 and the imprinting control region ICR upstream of H19. DNA methylation was analyzed at DMR2 including at four CpGs within the “core DMR” region by pyrosequencing and across four CTCF sites within the H19 ICR by Sequenom MassArray. The sites of promoters P0, P1, P2 and P3 are also shown. Exons are marked by shaded boxes.
In conclusion, late gestation Dex exposure exerts tissue-specific effects on the expression of imprinted genes in fetal liver and placenta in the F1 generation. In the F2 generation, although birth weight is also reduced, effects on feto-placental growth and gene expression differed from those in the F1, with marked parent of origin effects, suggesting that the mechanisms underlying glucocorticoid programmed effects in F1 and F2 generations differ. Early life programming effects may represent an adaptive response made by an organism in order to prepare it for an expected future environment, and the transmission of such effects to subsequent generations might be an attempt to maintain a “predictive shadow” of the programmed phenotype in a second generation to promote survival in the event that these environmental changes persist. Our observations of parent of origin effects on feto-placental growth and gene expression have implications for pathogenesis and future attempts to stratify therapies for the “developmental component” of cardiometabolic disease.

The mechanisms by which the effects of prenatal Dex exposure are transmitted to the second generation in this model remain unclear. Reports of transgenerational epigenetic inheritance at murine epialleles and the transmission of environmentally-induced epigenetic modifications to a third unexposed generation and beyond suggest that some epigenetic marks may resist reprogramming in germ cells and the zygote, although the mechanisms by which this occurs remain poorly understood. Although we found alterations in DNA methylation at Igf2 DMRs in fetal liver in both generations, the affected DMRs differed between the generations and perhaps, not surprisingly, altered DNA methylation at these DMRs is not responsible for the transmission of effects on Igf2 expression through sperm. Alternative explanations may include effects on DNA methylation or hydroxymethylation at other sperm DMRs, histone modifications (which are enriched at imprinted gene loci in sperm) or through non-coding RNA, which remain to be explored.

Figure 6. Methylation at the DMRs of Igf2 in F1 and F2 liver and F1 sperm. (A) Methylation at 9 CpG sites within Igf2 DMR2 in F1 *p < 0.05. CpGs 1–4 lie within the “core” DMR2 (n = 8 Veh and 8 Dex). (B) Methylation across four CTCF sites within the H19 ICR in the F1 generation (n = 8 Veh and 8 Dex). (C) Methylation at 9 CpG sites within Igf2 DMR2 in F2 and (D) methylation across four CTCF sites within the H19 ICR within F2 generation (2-way ANOVA shows interaction between parental exposures to decrease methylation p < 0.05) (n = 8 Veh/Veh, 7 Dex/Dex, 8 Dex/Veh and 8 Veh/Dex). (E) Methylation at 9 CpG sites within Igf2 DMR2 and (F) methylation across four CTCF sites within the H19 ICR in F1 sperm (n = 8 Veh and 8 Dex).
Methods

Animals. Female Wistar rats (200–250 g; Harlan UK Ltd.,) were maintained under conditions of controlled lighting (lights on 7:00 am to 7:00 pm) and temperature (22°C) and allowed free access to food (standard rat chow, Special Diets Services, Witham, Essex, UK) and water. All studies were conducted under licensed approval by the UK Home Office, under the aegis of the Animals (Scientific Procedures) Act, 1986, and with local ethical committee approval. After two weeks acclimatization, rats were timed-mated. A single virgin female was housed with a male in a breeding cage until an expelled vaginal plug was noted [designated embryonic (E) day 0]; females were then housed singly until term (E21–22). Pregnant females (F0) were injected subcutaneously with Dex 100 μg/kg in 0.9% saline containing 4% ethanol (Dex mothers) or with an equivalent volume of vehicle (Veh; 0.9% saline containing 4% ethanol (Veh mothers) at the same time each morning between E15–E21 inclusive. A subgroup of F0 females (n = 8) from each group was culled at E20 and fetal litters. Pups and placentas were individually weighed and fetal liver and placenta collected as before. The rest of the females (n = 10 Veh and 9 Dex females per group) were allowed to deliver and offspring (n = 145 Veh and 103 Dex) were weighed at birth and culled to 8/litter. Litters were termed F1 Dex and F1 Veh. At maturity, F1 females weighing around 230–250 g were timed-mated with F1 non-sibling males in all combinations as follows: F1 Veh females with F1 Veh males (Veh/Veh), F1 Dex females with F1 Dex males (Dex/Dex), F1 Dex females with F1 Veh males (Dex/Veh), and F1 Veh females with F1 Dex males (Veh/Dex). Females were caged separately during pregnancy and not manipulated in any way. A subset (n = 7–9/group) were culled at E20 and fetal and placenta collected as before. The rest of the females (n = 6 Veh/Veh, 6 Dex/Dex, 6 Dex/Veh and 5 Veh/Dex) were allowed to go to term and offspring were weighed at birth and culled to eight. Experimental cohorts of F2 fetuses/offspring included males and females selected randomly from multiple litters.

mRNA expression studies. Total RNA was extracted from snap-frozen tissue samples using the Qiagen RNeasy system (Qiagen Ltd., Crawley, UK) and reverse transcribed using the Promega Reverse Transcription kit (Promega UK Ltd., Southampton, UK). Real time PCR was performed either using the UPL system from Roche Diagnostics Ltd., Burgess Hill, UK or pre-designed assays from Applied Biosystems, Warrington, UK (Table 2), using a Roche LightCycler® 480 as previously described in reference 58. Results were corrected for the mean of expression of cyclophilin A and TATA-box binding protein (TBP). We chose to measure the expression of a number of imprinted genes that would be predicted to affect fetal and placental growth through direct growth effects or through effects on placental nutrient transport. These included the paternally expressed growth factors Igf2 and Mesoderm-specific transcript homolog (Mest), which promote fetal growth, the maternally expressed genes Cyclin dependent kinase 1c (Cdkn1c) and Growth factor receptor-bound protein 10 (Grb10), which act to reduce fetal growth, Pleckstrin homology-like domain family A member 2 (Phlda2), which affects placental growth, insulin like growth factor receptor-bound protein 1 (Igf2r), which sequesters Igf2, and the maternally expressed gene H19, which encodes a non-coding RNA. We also measured the expression of imprinted genes encoding placental nutrient transporters: the paternally expressed system A amino acid transporter slc38a4 and the maternally expressed slc22a3.

Sperm collection and DNA extraction. Testes were dissected from adult males (n = 8 Veh and 7 Dex aged 1 year) and epididymal sperm collected by puncturing the cauda epididymis and allowing sperm to swim out into warmed PBS. 200 ul of sample was spun at 4,000 rpm for 10 minutes. The pellet was resuspended in 500 ul of Somatic Cell Lysis Solution (2% Triton X-100, 20 mM Tris pH 8, 400 μg/ml Proteinase K) and incubated at 56°C for 3 hours. The sample was then incubated with 60 μl of DNase 1 (Promega, Southampton UK) made up to 300 μl with water, pipetted onto 500 μl of dimethyl glutarate.

### Table 2. List of primers

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<td>CCT ACT AAC TAA CAC CTC CTC TCC</td>
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Epigenetics

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spun at 14,000 g and the supernatant discarded. 500 μl of Sperm Lysis Buffer (20 mM Tris pH 8, 10 mM EDTA, 100 mM NaCl, 1% SDS 100 ug Proteinase K and 0.04 M DDT) was added and the sample incubated overnight at 56°C. DNA was isolated by phenol chloroform extraction, washed with ethanol and resuspended in 50 μl TE.

**DNA methylation.** Genomic DNA was extracted from liver and placenta by phenol-chloroform extraction and methylation at Igf2 DMR2 and the H19 imprinting control region (ICR) (Fig. 5) was analyzed using pyrosequencing or Sequenom MassArray.

**Pyrosequencing.** Pyrosequencing was performed to assess methylation of the Igf2 DMR2 (including four CpGs within the core DMR2).34 1 μg of DNA was subjected to bisulphite conversion using the Epitect Bisulphite Kit (Qiagen, Crawley, UK) and amplified using pyrosequencing primers designed using PyroMark Assay Design 2.0 software (Qiagen, Crawley, UK). Primers are detailed in Table 2. Data was analyzed using Pyro Q-CpG Software (Qiagen, Crawley, UK). All primers were purchased from Eurogentec (Southampton, UK).

**Sequenom massarray.** For analysis of methylation at the H19 ICR, quantitative methylation was conducted by Sequenom, Inc., (Hamburg, Germany). This included analysis of DNA methylation at four of the CCCTC-binding factor (CTCF) binding sites within the H19 ICR. Methylation ratios for each of the residues (Methyl CpG/Total CpG) were then determined using a MassARRAY™ mass spectrometer employing proprietary peak picking and spectra interpretation tools.

**Statistical analysis.** Differences between F1 weight variables and gene expression were analyzed by independent Student’s t testing. Dex readily crosses the placenta to directly induce effects in both male and female F1 offspring, therefore to dissect the effects of F1 male and/or paternal prenatal exposure on the F2 offspring, data were analyzed by 2-way ANOVA with maternal and paternal prenatal exposure status (Dex or Veh) as independent variables and the outcome measures, e.g., placental and fetal weight, gene expression or DNA methylation, as dependent variables. Post hoc LSD testing was used where the ANOVA gave a p value of < 0.05. Data are expressed as mean ± SEM and for 2-way ANOVA as F statistic and p value for maternal or paternal effect or interaction.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Acknowledgments**
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**References**