Treatment with constitutive androstane receptor ligand during pregnancy prevents insulin resistance in offspring from high-fat diet-induced obese pregnant mice

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Masuyama H, Hiramatsu Y. Treatment with constitutive androstane receptor ligand during pregnancy prevents insulin resistance in offspring from high-fat diet-induced obese pregnant mice. Am J Physiol Endocrinol Metab 303: E293–E300, 2012. First published May 29, 2012; doi:10.1152/ajpendo.00167.2012.—The constitutive androstane receptor (CAR) ligands have been reported to decrease insulin resistance even during pregnancy, while exposure to a high-fat diet (HFD) in utero in mice can induce a type 2 diabetes phenotype that can be transmitted to the progeny. Therefore, we examined whether treatment with a CAR ligand during pregnancy could prevent hypertension, insulin resistance, and hyperlipidemia in the offspring from HFD-induced obese pregnant mice (OH mice). We employed four groups of offspring from HFD-fed and control diet-fed pregnant mice with or without treatment with a CAR ligand. Treatment with a CAR ligand during pregnancy improved glucose tolerance and the levels of triglyceride and adipocytokine and restored the changes induced by HFD with amelioration of hypertension in the adult OH mice. This treatment also increased adiponectin mRNA expression, suppressed leptin expression in adipose tissues of OH mice, and abolished the effect of HFD on the epigenetic modifications of the genes encoding adiponectin and leptin in the offspring during immaturity and adulthood. Our data suggest that CAR might be a potential therapeutic target to prevent metabolic syndrome in adulthood of offspring exposed to an HFD in utero.

diabetology; constitutive androstane receptor; epigenetic change; insulin resistance

MATERNAL OBESITY IN HUMAN pregnancy often results in fetal overgrowth (5, 15). This increases the risk of the offspring developing obesity and metabolic syndrome later in life, thereby contributing to the increased incidence of type 2 diabetes (1–3). Whereas obesity is associated with an increased risk of almost every common complication of pregnancy, obesity in the mother might play a direct role in the transmission of an obesogenic and diabetogenic trait from generation to generation. In addition, maternal hyperglycemia during pregnancy has been associated with a high risk of obesity in offspring, independent of birth weight (11, 21), and there is evidence that a high birth weight in itself increases the risk of obesity (29).

Epigenetics involves somatically heritable states of gene expression resulting from changes in chromatin structure without alterations to the DNA sequence per se, including DNA methylation, histone modifications, and chromatin remodeling (24). In recent years, epigenetics has become an emerging issue for understanding a broad range of human diseases, such as type 2 diabetes mellitus, obesity, inflammation, and neurocognitive disorders (4). Exposure to a high-fat diet (HFD) in utero in mice can induce the phenotype of type 2 diabetes and hypertension that can be transmitted to the progeny (10, 26) and might cause a metabolic syndrome-like phenomenon through epigenetic modifications of adipocytokine, adiponectin, and leptin gene expressions (18).

The constitutive androstane receptor (CAR) is an orphan nuclear receptor. It is highly expressed in the liver and small intestine and at low levels in heart, skeletal muscle, brain, kidney, and lung (6). Recent in vivo studies have demonstrated that the activation of CAR improves insulin sensitivity via glucose and lipid metabolic pathways; moreover, CAR null mice had spontaneous insulin insensitivity that could be relieved by CAR ligands (7, 8), suggesting that CAR plays some roles in insulin resistance. We have recently demonstrated that the activation of CAR-mediated signaling can ameliorate insulin resistance under relatively high concentrations of estradiol and progesterone, which are compatible with pregnancy via decreased activities of transcription factors in gluconeogenesis in combination with CAR (19). We also observed that HFD-induced obese pregnant mice had high blood pressure and proteinuria, whereas treatment with CAR ligands ameliorated these signs (20).

Exposure to HFD in utero might cause a metabolic syndrome-like phenomenon through epigenetic modifications of adipocytokine, adiponectin, and leptin gene expressions in their offspring (10, 18), whereas treatment with CAR ligands ameliorated high blood pressure and proteinuria in HFD-induced obese pregnant mice (20). Therefore, we examined whether treatment with CAR ligands during pregnancy could prevent the hypertension, insulin resistance, and hyperlipidemia in the offspring from HFD-induced obese pregnant mice via amelioration of epigenetic modification in adipocytokine genes. In this study, we used 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) as a CAR ligand, because this molecule has been shown to bind specifically to murine CAR and to activate CAR-mediated signaling (6).

MATERIALS AND METHODS

Materials and animal procedures. TCPOBOP, D-glucose, and human insulin were purchased from Sigma-Aldrich (St. Louis, MO). Female, 8-wk-old ICR strain mice were obtained from Charles River (Tokyo, Japan), and six female pregnant mice and 24 offspring were examined per group for all in vivo experiments. After 4 wk of feeding with the HFD (energy content 62% fat, 18% protein, and 20% carbohydrate) or a control diet (CD; 12% fat, 28% protein, and 60% carbohydrate) purchased from Oriental Yeast (Tokyo, Japan), the mice were weighed and mated. Females were checked daily for postcopulatory plugs, and the presence of a plug was taken to indicate day 0.5 of pregnancy. Eight-week-old male mice for mating were fed with the CD for 4 wk before experiments or mating. The pregnant...
mice had free access to food and water, and their food consumption was estimated by weighing the remaining food every day. Pregnant HFD- and CD-fed mice received once-weekly intraperitoneal injections of TCPOBOP (0.5 mg/kg) dissolved in corn oil, or with corn oil alone (control) during pregnancy. Injections were started at day 0.5 of pregnancy or at the indicated day for specific experiments and finished at day 0.5 after delivery. The maternal weight on day 20 of gestation and neonatal weight on day 0.5 after birth were measured, and the offspring weights were measured every 2 wk. All offspring were weaned on the CD at 3 wk of age. Body composition was analyzed in live mice using EchoMRI-100 (Echo Medical Systems, Houston, TX). The systolic blood pressure of offspring was measured at 12 and 24 wk after birth by the tail-cuff method using a Softron BP98A tail-cuff hemodynamometer (Softron, Tokyo, Japan) after the behavior and heart rate of the mice had stabilized. Blood pressure is reported as the mean of at least three measurements recorded during the same session, which had to vary by <5%. Most of the blood pressure values were within the required range once the mice had stabilized. At 2, 12, and 24 wk of age, the mice were anesthetized with ether, and the white mesenteric adipose tissue were removed, frozen immediately, and stored at −70°C until analysis (n = 6, three male and three female mice for each group). Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. The mice were kept in a temperature- and light-controlled room with free access to food and water except during tolerance tests for glucose (GTT) and insulin (ITT). All animal procedures were approved by the Institutional Animal Care and Use Committee of Okayama University.

### Table 1. Maternal characteristics and neonatal weights

<table>
<thead>
<tr>
<th>Group</th>
<th>Maternal Wt, g</th>
<th>HOMA-IR</th>
<th>Triglycerides, mg/dl</th>
<th>Leptin, ng/dl</th>
<th>Adiponectin, μg/dl</th>
<th>Neonatal Wt, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>38.2 ± 3.4</td>
<td>3.51 ± 0.44</td>
<td>244 ± 24</td>
<td>3.8 ± 0.5</td>
<td>10.4 ± 1.2</td>
<td>1.24 ± 0.15</td>
</tr>
<tr>
<td>HFD</td>
<td>48.1 ± 6.8*</td>
<td>4.95 ± 0.61*</td>
<td>335 ± 48*</td>
<td>6.6 ± 0.8*</td>
<td>5.2 ± 0.5*</td>
<td>1.61 ± 0.18*</td>
</tr>
<tr>
<td>CD + TCPOBOP</td>
<td>37.8 ± 3.5</td>
<td>3.33 ± 0.49</td>
<td>221 ± 33</td>
<td>3.5 ± 0.4</td>
<td>11.0 ± 0.8</td>
<td>1.22 ± 0.11</td>
</tr>
<tr>
<td>HFD + TCPOBOP</td>
<td>39.3 ± 4.0**</td>
<td>3.63 ± 0.55**</td>
<td>237 ± 28**</td>
<td>3.9 ± 0.5**</td>
<td>10.4 ± 1.0**</td>
<td>1.28 ± 0.14**</td>
</tr>
</tbody>
</table>

Data are means ± SE. CD, control diet; HFD, high-fat diet; HOMA-IR, homeostasis model assessment-insulin resistance; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene. P < 0.01 vs. CD-fed pregnant mice (*) and vs. HFD-fed pregnant mice with TCPOBOP treatment (**).
GTT, ITT, and measurements of insulin, total triglyceride, adiponectin, and leptin levels. Offspring at 12 or 24 wk of age were fasted for 16 h before receiving an intraperitoneal injection of d-glucose (2 g/kg body wt) for the GTT or for 4 h before receiving an intraperitoneal injection of human insulin (1.0 U/kg body wt) for the ITT (n = 6, three male and three female mice for each group). Blood samples from tails were taken before and at 30, 60, 90, and 120 min after the injection. Blood glucose levels were measured by the glucose oxidase method using a Medisafe automated analyzer (Termo, Tokyo, Japan). Fasting insulin, total triglyceride, adiponectin, and leptin levels were determined using enzyme-linked immune sorbent assay (ELISA) kits [insulin and triglycerides (Morinaga Institute of Biological Sciences, Yokohama, Japan); adiponectin and leptin (R&D Systems, Minneapolis, MN)]. Blood sample volumes for each measurement were 10–20 μl, and the total sample volume collected from each mouse was <200 μl, which was <5% of total blood volume. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as the fasting insulin concentration (μU/ml) × fasting glucose concentration (mg/dl)/405 (12).

Real-time quantitative PCR. Real-time quantitative PCR was performed to measure the mRNA levels of the leptin and adiponectin genes using a StepOne Real-time PCR System and a TaqMan RNA-to-CT Gene Kit (Applied Biosystems, Carlsbad, CA). Specific primers for the mouse leptin, adiponectin, and β-actin sequences were purchased from Applied Biosystems. Sequences of specific primers and accession numbers were as described (8, 17, 33). RNA samples (25 ng) were assayed in triplicate using 15 pmol of gene-specific primers and 5 pmol of gene-specific probes. Because we observed that there were no significant differences of β-actin expression under different diet conditions or TCPOBOP treatment using another housekeeping gene, GAPDH as a control (data not shown), mouse β-actin mRNA levels were measured as an internal control using a predeveloped TaqMan primer and a probe mixture (Applied Biosystems). The mRNA levels of the target genes were normalized by the β-actin mRNA levels.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s protocol. Adipose tissues from the HFD-induced obese (OH) and OC groups (n = 6, three male and three female mice for each group) at 2 and 24 wk of age were taken for sampling. Briefly, 20-mg aliquots of frozen samples were ground in liquid nitrogen using a mortar and pestle and then washed with PBS at room temperature. The samples were then resuspended in PBS and cross-linked in 1% formaldehyde for 10 min. After centrifugation, the pellet was resuspended in nucleus-swelling buffer containing protease and phosphorylation inhibitors. The nuclei were lysed in SDS lysis buffer containing protease and phosphorylation inhibitors. The chromatin was sonicated to reduce DNA fragment lengths to 0.3–1.0 kb. Chromatin was precleared in the presence of 20 μl of normal serum, 2 μg
of salmon sperm DNA, and 80 µl of 25% protein A-agarose slurry. Precleared chromatin samples were subjected to immunoprecipitation at 4°C overnight in the presence of 2 µg of rabbit polyclonal antibodies against acetyl-histone H3 at lysine 9 (acetyl H3K9; Millipore, Bedford, MA), dimethyl histone H3 at lysine 9 (dimethyl H3K9; Millipore), and monomethyl histone H4 at lysine 20 (monomethyl H4K20; Abcam, Cambridge, MA), or nonimmune rabbit IgG (Millipore). After the complex was collected by incubation with 60 µl of a 25% protein A-Sepharose slurry and centrifugation, the beads were washed five times, and the chromatin-immune complex was eluted. After the cross-linking was reversed, DNA was purified and used as a template for PCR. PCR was performed using primer sets specific for the promoter region of the mouse adiponectin gene (positions −549 to −481) (25) and the promoter region of the mouse leptin gene (−181 to +20) (35).

Statistical analysis. Statistical analyses were performed by two-way ANOVA for comparison among four offspring groups, HFD-fed or CD-fed with or without TCPOBOP treatment during pregnancy, and by repeated-measures ANOVA for GTT and ITT followed by Dunnett’s test. All statistical analyses were performed using StatView software, version 5.0 (Abacus Concepts, Berkeley, CA). Data are presented as means ± SD, and P < 0.05 was taken to indicate statistical significance.

RESULTS

The weight, caloric intake, fat mass-to-body weight ratio, and blood pressure of offspring. The offspring from CD-fed dams with TCPOBOP treatment are designated OCT mice, and the offspring from CD-fed dams without treatment are designated OC mice; the offspring of HFD-fed dams with TCPOBOP treatment are designated OHT mice, and the offspring of HFD-fed dams without treatment are designated OH mice. TCPOBOP treatment decreased the maternal weight of HFD-fed pregnant mice on day 20 of gestation and restored the increased maternal weight induced by HFD (Table 1). Neonatal weights from the HFD-fed pregnant mice on day 0.5 after birth were significantly greater than that from CD-fed pregnant mice (P < 0.01), whereas TCPOBOP treatment decreased the mean neonatal weight of the offspring from HFD-fed pregnant mice and restored the increased fetal weight induced by maternal HFD (Table 1). There was no significant difference in mean litter size among the four groups (overall 9.1 ± 0.6). The mean weight of the OHT mice was significantly lower than the OH mice from 14 wk of age, and there were no significant differences of weight between the OHT and OC mice (Fig. 1A). The caloric intake of OHT mice was lower than that of the OH mice and was not significantly different from the OC and OCT mice from 8 wk of age, before a significant increase in body weight (Fig. 1B). Also, the gain of fat mass in the OHT mice was decreased significantly compared with the OH mice and was not significantly different from the OC and OCT mice from 12 wk of age using MRI analysis (Fig. 1C). Systolic blood pressure in the OH mice was elevated significantly compared with the OC mice at 24 wk of age (P < 0.01, Fig. 1E), but not at 12 wk (Fig. 1D). TCPOBOP treatment during pregnancy prevented the elevation of blood pressure in OH mice at 24 wk of age (Fig. 1E).

Fig. 3. The effects of treatment with a CAR ligand during pregnancy on serum triglyceride, adiponectin, and leptin levels in offspring. Pregnant HFD- and CD-fed mice received once-weekly ip injections of TCPOBOP (0.5 mg/kg) dissolved in corn oil or corn oil alone (controls). Offspring at 12 and 24 wk of age were fasted for 16 h, and blood samples were taken. Serum total triglyceride (A and D), adiponectin (B and E), and leptin (C and F) levels were determined using ELISA kits. The results are shown as means ± SD (n = 6/group). *P < 0.01 vs. OH, OCT, and OC mice (see legend to Fig. 1).
CAR activation during pregnancy prevented glucose intolerance and insulin resistance in OH mice. We performed GTTs and ITTs, measured serum insulin level, and calculated the HOMA-IR in offspring from HFD-fed or CD-fed pregnant mice with or without treatment with TCPOBOP at 12 and 24 wk of age, to examine the effects of TCPOBOP treatment during pregnancy on glucose metabolism in offspring. There were no significant differences of GTT, ITT, and HOMA-IR between the OH and OC mice at 12 wk of age (Fig. 2, A–C). However, the OH mice exhibited significantly worse glucose tolerance and insulin sensitivity and significantly increased HOMA-IR values compared with the OC mice at 24 wk of age (P < 0.01 for all significant differences; Fig. 2, D–F). Next, we examined whether TCPOBOP treatment during pregnancy could improve the glucose tolerance in the offspring. OHT mice exhibited significantly improved glucose tolerance compared with the OH mice at 24 wk of age (P < 0.01, Fig. 2, D and E). HOMA-IR was also decreased in OHT mice compared with the control vehicle-treated OH mice at 24 wk of age, and the HFD-induced changes were restored (P < 0.01, Fig. 2F). OCT mice showed no significant changes compared with control OC mice at 12 and 24 wk of age.

Treatments with CAR ligand during pregnancy improved serum triglyceride and adipocytokine levels in OH mice. To clarify whether TCPOBOP treatment during pregnancy would affect lipid metabolism and adipocytokine levels in offspring, serum triglyceride, leptin, and adiponectin levels were examined. The total triglyceride and leptin levels were significantly higher, whereas the adiponectin level was significantly lower in OH mice compared with OC mice at 12 (Fig. 3, A–C) and 24 (Fig. 3, D–F) wk of age (P < 0.01 for all significant differences). The triglyceride and leptin levels were decreased and the adiponectin level was increased in OHT mice, and the changes in triglyceride, leptin, and adiponectin levels were restored by TCPOBOP treatment during pregnancy (Fig. 3, A–F). OCT mice showed no significant changes compared with vehicle-treated control OC mice at both 12 and 24 wk of age.

Effects of treatment with a CAR ligand during pregnancy on leptin and adiponectin gene expression levels in the adipose tissue of offspring. The leptin gene was upregulated significantly, whereas the adiponectin gene was downregulated in the white adipose tissue of OH mice compared with the OC mice at both 12 (Fig. 4, A and B) and 24 wk (Fig. 4, C and D) of age (P < 0.01 for all significant differences). TCPOBOP treatment significantly decreased the leptin mRNA expression and increased the adiponectin mRNA expression in the white adipose tissue of the OHT mice compared with the OH control mice (P < 0.01 for all significant differences), and the changes in both expression levels induced by the HFD during pregnancy were restored (Fig. 4, A–D). There were no significant differences in these gene expression levels between the OC and OCT mice.

Effects of treatment with a CAR ligand during pregnancy on modifications to H3K9 and H4K20 in the promoter regions of the adiponectin and leptin genes in the adipose tissue of offspring. To investigate whether treatment with a CAR ligand during pregnancy would affect histone modifications in the promoter regions of the adiponectin and leptin genes in the adipose tissue of offspring, we performed ChIP assays using antibodies for acetyl and dimethyl H3K9 and monomethyl H4K20 at 2 and 24 wk of age. The acetyl H3K9 level in the adipose tissue of offspring was remarkably higher than the adipose tissue of OH mice (P < 0.01 for all significant differences). HFD treatment signifi- cantly decreased the leptin and adiponectin gene expression in the white adipose tissue of the OHT mice compared with the OH control mice (P < 0.01 for all significant differences), and the changes in both expression levels induced by the HFD during pregnancy were restored (Fig. 4, A–D). There were no significant differences in histone modifications between the OC and OCT mice.

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monomethyl H4K20 level was increased significantly in the leptin promoter region of OH mice compared with OC mice, but treatment with the CAR ligand during pregnancy abolished the effect of HFD on the modification of H4K20 at 2 and 24 wk of age. There were only weak detections and no significant differences in acetyl and dimethyl H3K9 levels in both groups at 2 and 24 wk of age (Fig. 5B). There were no effects of maternal diet on the association of IgG binding with the promoter regions of leptin or adiponectin in adipose tissues and no significant gender differences in epigenetic changes by maternal HFD and the effect of TCPOBOP (data not shown).

Effect of different treatment timing with a CAR ligand on modifications to H3K9 and H4K20 in the promoter regions of the adiponectin and leptin genes in the adipose tissue of offspring. We used offspring from HFD-fed dams with different timings of TCPOBOP treatment to examine the effects on epigenetic changes in the adiponectin and leptin genes (Fig. 6A). Treatments started at days −7.5, 0.5, and 7.5 of gestation abolished the effect of HFD on the modification of H3K9 at 24 wk of age, but the treatments started at day 14.5 of gestation and 0.5 days after birth had no effect on this modification (Fig. 6B). Moreover, the treatment with a CAR ligand started at days −7.5, 0.5, and 7.5 of gestation abolished the effect of HFD on the modification of H4K20 at 2 and 24 wk of age, but the treatments started at 14.5 of gestation and 0.5 day after birth also had no effect on this modification (Fig. 6C).

DISCUSSION

Adipose tissue functions as a highly specialized endocrine and paracrine tissue, producing an array of adipocytokines such as leptin, tumor necrosis factor-α, and adiponectin, as well as eliciting cell-mediated effects via proinflammatory and anti-inflammatory cells, producing various cytokines and chemokines. Such factors have local and systemic biological effects and influence insulin sensitivity and the development of metabolic diseases (9). Leptin plays important roles not only in modulating satiety and energy homeostasis (16, 23) and adiponectin might maintain endothelial function and its deficiency can lead to endothelial dysfunction/hypertension (14, 27, 30). Previously, we demonstrated that HFD-fed pregnant mice exhibited signs of preeclampsia, including hypertension, pro-

Fig. 5. Effects of treatment with a CAR ligand during pregnancy on modifications to histone H3 at lysine (H3K9) and histone H4 at lysine 20 (H4K20) in the promoter regions of the adiponectin (A) and leptin (B) genes of offspring at 2 and 24 wk of age using anti-acetyl and anti-dimethyl H3K9 and anti-monomethyl H4K20 antibodies. The results are shown as means ± SD (n = 6/group). *P < 0.01 vs. OH, OCT, and OC mice (see legend to Fig. 1).
CAR LIGAND PREVENTS METABOLIC SYNDROME IN OFFSPRING

Fig. 6. Effects of different treatment timings with a CAR ligand on modifications to H3K9 and H4K20 in the promoter regions of the adiponectin and leptin genes in the adipose tissue of offspring. A: schedule of TCPOBOP administration for each group. ChIP assays were performed on the chromatin extracts from the white mesenteric adipose tissue of OH and OC mice to examine the effects of different treatment timings with a CAR ligand on modifications to H3K9 and H4K20 in the promoter regions of the adiponectin (B) and leptin (C) genes at 24 wk of age using anti-acetyl and anti-dimethyl H3K9 and anti-monomethyl H4K20 antibodies. The results are shown as means ± SD (n = 6/group). *P < 0.01 vs. OH, OCT, and OC mice (see legend to Fig. 1).

We also observed that the timing of TCPOBOP administration affected the epigenetic changes of adiponectin and leptin genes differently in adipose tissues of OH mice. Thus, administration of TCPOBOP in midpregnancy as well as in the preconception stage and early pregnancy abolished the effect of HFD on the modification of adiponectin and leptin genes, but administration at late pregnancy and after birth had no effect on this. Findings from the Dutch famine of 1944 showed that maternal undernutrition during gestation has important effects on health in later life, but the effects on health depend on its timing during gestation, and early gestation seems to be a particularly vulnerable period (24). Moreover, differences in DNA methylation patterns after exposure to prenatal undernutrition have been demonstrated to be timing-specific in the mouse (32). Although there has been no report examining the effects of different HFD exposure times in utero on metabolic syndrome-like phenomena, treatment with a CAR ligand at a preconception period or during early pregnancy might prevent the onset of metabolic syndrome in adulthood of offspring exposed to an HFD in utero. However, we observed that the adipocyte function in OH mice seemed to be improved after TCPOBOP treatment during pregnancy although CAR has not been detected in adipose tissues (20). It is possible that treatment with a CAR ligand in utero may indirectly affect the epigenetic modification of adipocytokines in adipose tissue of OH mice through combined changes to gluconeogenesis and lipogenesis. Further analysis will be required to resolve this issue.

Measures to alleviate fetal overgrowth in obese and diabetic women represent an early intervention strategy that could help decrease the prevalence of obesity and diabetes in future generations. Previously, we demonstrated that TCPOBOP treatment ameliorated fetal growth in addition to improving maternal glucose metabolism and adipocyte function in HFD-fed pregnant mice (20). In the present study, we observed that TCPOBOP treatment during pregnancy abolished metabolic-like phenomenon in OH mice, suggesting that CAR activation will decrease the risk of offspring developing metabolic syndrome and diabetes in adulthood.

Taken together, our data suggest that treatment with the CAR ligand TCPOBOP during pregnancy can improve insulin sensitivity and adipocytokine levels and that these improvements were associated with the amelioration of adult hypertension in OH mice. CAR might be a potential therapeutic
target to prevent metabolic syndrome in adulthood of offspring exposed to an HFD in utero.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: H.M. conception and design of research; H.M. performed experiments; H.M. analyzed data; H.M. interpreted results of experiments; H.M. prepared figures; H.M. drafted manuscript; H.M. and Y.H. edited and revised manuscript; H.M. and Y.H. approved final version of manuscript.

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