Maternal Obesity Induces Epigenetic Modifications to Facilitate Zfp423 Expression and Enhance Adipogenic Differentiation in Fetal Mice

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ABSTRACT

Maternal obesity (MO) predisposes offspring to obesity and type 2 diabetes (T2D) despite poorly defined mechanisms. Zfp423 is the key transcription factor committing cells to the adipogenic lineage and with exceptionally dense CpG sites in its promoter. We hypothesized that MO enhances adipogenic differentiation during fetal development through inducing epigenetic changes in the Zfp423 promoter and elevating its expression. Female mice were subjected to a control (Con) or obesogenic (OB) diet for two months, then mated and maintained in their diets during pregnancy. Fetal tissue was harvested at E14.5, when the early adipogenic commitment is initiated. The Zfp423 expression was 3.6-fold higher and DNA methylation in the Zfp423 promoter was lower in OB compared to Con. Correspondingly, repressive histone methylation (H3K27me3) was lower in OB Zfp423 promoter, accompanied by reduced binding of Enhancer of Zeste 2 (EZH2). Gain and loss of function analysis showed that Zfp423 regulates early adipogenic differentiation in fetal progenitor cells. In summary, MO enhanced Zfp423 expression and adipogenic differentiation during fetal development, at least partially through reducing DNA methylation in the Zfp423 promoter, which is expected to durably elevate adipogenic differentiation of progenitor cells in adult tissue, programming adiposity and metabolic dysfunction later in life.

Key words: DNA methylation, EZH2, fetus, mother, obesity, pregnancy, Zfp423
INTRODUCTION

According to the latest NHANES survey (2009-2010), 31.9% of non-pregnant women in the U.S. 20-39 years of age are obese, and another one third are overweight (1). Maternal obesity (MO) represents a special problem that can result in poor fetal development, leading to harmful, persistent effects on offspring, including pre-disposition to obesity and Type 2 diabetes (T2D) (2-5). Indeed, T2D and obesity are increasing at alarming rates in teenagers and even children (6). Mechanisms linking MO to the increased incidence of obesity and T2D in their descendants remain poorly defined.

Obesity, especially central obesity, is well correlated with diabetes, hyperlipidemia and cardiovascular diseases (7). Visceral fat, especially epididymal fat, is the first fat depot to develop, mainly between birth up to 4 weeks of age in rodents (8). Adipocyte number in epididymal fat increases little after weaning (9) and further development is primary due to adipocyte hypertrophy (8). Similarly, in humans, morbid adiposity only slightly elevates visceral adipocyte number (10). Therefore, visceral fat is developed mainly during the late fetal and nursing stages, when the mother provides all nutrients. As a result, MO is expected to enhance adipogenesis and visceral fat development. Indeed, a recent study using MR imaging found that MO correlates with abdominal adiposity in human neonates (11), consistent with enhanced central adiposity in offspring of obese mice (12; 13).

Skeletal muscle composes of about 40 to 50% of the body mass of adults, and insulin resistance in skeletal muscle is indispensable for the incidence of T2D (14). Intramuscular fatty accumulation leads to muscle insulin resistance (15; 16). In addition, it also impairs muscle contractile function, resulting in muscle weakness and even immobility (17). MO enhanced intramuscular adipogenesis in fetal muscle, which is associated with higher intramuscular fat
content and also visceral adiposity in offspring, providing another explanation between MO and the increased incidence of obesity and T2D in offspring (5; 13; 18). Up to now, mechanisms leading to enhanced adipogenesis in progeny due to MO remain poorly defined.

Adipogenic differentiation has been studied for decades, and 3T3-L1 cells are the major model for studying adipogenesis. Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) and CCAAT-enhancer-binding proteins (C/EBPs) as critical regulators inducing adipogenic differentiation (19). Quite recently, zinc-finger protein Zfp423 was discovered to initiate the early preadipocyte commitment in mature adipose tissue, which induces PPAR$\gamma$ expression and adipogenic differentiation (20). It remains unclear whether Zfp423 regulates adipogenesis during fetal development and how Zfp423 expression is regulated. We speculated that during adipose tissue development Zfp423 expression is regulated primarily through epigenetic modifications.

Cell differentiation including adipogenesis is indispensable of epigenetic modifications. The Polycomb repressive complex 2 (PRC2) has histone methyltransferase activity due to the presence of Enhancer of Zeste 1 and 2 (EhZ1/2), which catalyzes histone 3 lysine 27 trimethylation (H3K27me3), a marker for gene silencing (21). Though no specific DNA binding element for PRC2 has been identified, PRC2 tends to bind promoters with rich GC sites, which attracts PRC1 binding (22). Trithorax group (trxG) catalyzes H3K4 trimethylation (H3K4me3), which activates gene transcription (23). Interestingly, H3K4me3 and H3K27me3 co-exist, forming a ‘bivalent state’ (24), which poise genes for activation or inhibition. During cell differentiation, non-induced bivalent genes loss active H3K4me3 but keep repressive H3K27me3 mark, leading to largely permanent inhibition of gene expression by inducing DNA methylation (25). De novo DNA methylation serves to convert plastic gene inhibition by PRCs to permanent silencing (25). Our bioinformatic analyses showed that the Zfp423 promoter presents
exceptionally rich CpG sites and islands, meeting the characteristics of “key developmental gene” with high CpG density promoters (24). Because Zfp423 is a critical transcription factor initiating adipogenic commitment, we hypothesized that MO enhances Zfp423 expression and adipogenic differentiation during fetal development via attenuating DNA methylation and inhibitory histone modifications in the Zfp423 promoter.

MATERIALS AND METHODS

Animals. Animal studies were conducted according to the protocol approved by the Animal Use and Care Committees of both the University of Wyoming and Washington State University. The diet induced obesity model was used. Female C57BL/6J mice (4 weeks of age) were fed ad libitum either a control diet (D12450B, Research Diets, New Brunswick, NJ) with 10% energy from fat (Con) or a high energy diet (D12451) with 45% energy (OB) from fat for 8 weeks at 12-h light/12-h dark cycles. Then, both Con and OB female mice were mated with C57BL/6J male mice (on the normal rodent diet) and mating was confirmed by the presence of vaginal plug. During pregnancy, each female was individually housed. At E14.5, mice were sacrificed and fetuses were collected and weighed. Then, under a dissecting microscope, fetal tissue was harvested after removing head, heart, lung and liver, surface gelatinous tissue, and spinal cord and primordial bones. Due to the small size, fetal tissue from the same litter was pooled and the litter was considered as an experimental unit.

Real-time Quantitative PCR (RT-PCR). RT-PCR was conducted as previously described. Primer sequences and the amplicon sizes are listed in Table 1. Relative expression of mRNA was determined after normalization to 18S reference using ∆∆-Ct method (26).
**Western blot analysis.** Western blotting was conducted as previously described (26). Polyclonal antibody against Zfp423 was purchased from Santa Cruz, against EZH2 from Cell signaling technology (Boston, MA), and against β-Actin from Earthox (San Francisco, CA).

**Genomic DNA isolation and sodium bisulfite sequencing (BSP).** Genomic DNA was extracted using the phenol-chloroform method. Genomic DNA was modified with sodium bisulfite using EZ DNA Methylation Kit (Zymo Research, Irvine, CA). The Methprimer software was used to search CpG islands and design primers (27). Primers specific to the bisulfite-modified sequences of the Zfp423 promoter region were listed in Table 1.

Silica Bead DNA Gel Extraction Kit was purchased from Fermentas (Thermo Fisher Scientific, Waltham, MA). pGEM®-T Easy Vector System (Promega, Madison, WI) was used to clone bisulfite-modified sequences via TA cloning. Plasmids were extracted using a GeneJET™ Plasmid Miniprep Kit (Thermo). DNA methylation status was evaluated by comparing the cloned sequence against the genomic sequence and methylation map was generated using Quantification Tool for Methylation analysis (QUMA, Kyoto, Japan) (28).

**Embryonic fibroblast preparation and adipogenic induction.** Mouse embryonic fibroblasts (MEF) were separated from E14.5 fetal mice according to a common procedure as previously described with slight modifications (29-32). Briefly, fresh tissue separated under a stereomicroscope was minced and then digested in medium containing 0.75 U/ml collagenase D (Roche, Pleasanton, CA) and 1.0 U/ml Dipase type II (Roche) for 20 min at 37 °C. The lysate was filtered sequentially through 70 µm and 40 µm cell strainers. The adipogenic differentiation was induced using a medium containing 0.5 mM isobutyl-1-methylxanthine, 1 µM dexamethasone, and 10 µg/ml insulin in DMEM with 15% fetal bovine serum for 8 days and
then changed to insulin (10 µg/ml) only DMEM with 10% fetal bovine serum for 4 more days (26).

Progenitor cells were further sorted using surface markers, Sca-1+/CD45-, by Magnetic Cell Sorting System (MACS). The antibody MicroBeads kit and MACS separation column were bought from Miltenyi Biotec (Germany). Sca-1+ marks a group of progenitor cells with high adipogenic potential (55,56), while CD45+ cells are of blood origins (57). Sorted cells were induced adipogenic differentiation as described above.

**Oil-red O staining of differentiated adipocytes.** Cells were fixed and stained with Oil-Red O (Sigma Chemical Co., Saint Louis, MO) as described previously (26). Following microscopic observation, Oil-Red O dye was solubilized with isopropanol and the light absorbance was measured at 510 nm.

**Chromatin immunoprecipitation assay.** Fetal tissue was homogenized in 1% formaldehyde solution and incubated for 10 min and centrifuged. The pellet was resuspended in PBS containing 125 mM glycine. After centrifuge, pellet was lysed in a cold lysis buffer (10 mM Tris-HCl, PH8.0, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40) containing 10 µl protease inhibitor cocktail (Thermo), sonicated and centrifuged. The supernatant was precleaned with ChIP grade protein G (Cell Signaling Technology) and then incubated with an antibody against H3K27me3, H3K4me3, EZH2, H3K9ac, or H3K27ac (Cell Signaling), or normal rabbit IgG overnight at 4 °C. Then, the antibody-chromatin complex was precipitated with protein G and further treated with RNaseA (fermentas) and then Proteinase K (sigma) for 2 h. DNA were purified with ChIP DNA Clean & Concentrator™ (Zymo research) and used as templates for PCR using primers listed in Table 1. Relative enrichment folds of detected proteins were determined after normalization to input DNA using ΔΔ-Ct method.
**Plasmid and transfection In Vitro.** Plasmid pMSCVFLAG-ZFP423 (Cat. 24764) and pMKO.1-Zfp423 (Cat. 35972) were bought from Addgene (Cambridge, MA). Plasmid transfection was performed using PolyJet in vitro DNA transfection reagent (SignaGen Laboratory, Ijamsville, MD) following the manufacturer’s instruction.

**Statistical analyses.** Each pregnancy was considered as an experimental unit. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). The differences in the mean values were compared by the Student’s t-test, and mean ± standard errors (SE) were reported. Statistical significance was considered as $P < 0.05$.

**RESULT**

**Maternal and fetal body weights.** After 2 month feeding of a high energy diet, OB mice were heavier than Con mice (Con vs. OB, 17.44 ± 0.21 vs. 23.18 ± 0.24 g, $P < 0.01$), which maintained until E14.5 when maternal mice were euthanized (Con vs. OB, 30.32 ± 1.08 vs. 35.40 ± 1.01 g, $P < 0.05$), consistent with our previous report (13). There was no difference in fetal weight between treatments (Con vs. OB, 0.50 ± 0.03 vs. 0.52 ± 0.03 g).

**MO enhanced Zfp423 expression and adipogenic differentiation in OB fetal tissue.** In this study, we chose E14.5 fetal tissue for analyzing adipogenesis and Zfp423 epigenetic modifications. Though white adipocytes are not detectable in fetal mice at this stage, molecular changes associated with the early stage of commitment to adipogenesis have initiated and thus fetal tissue at this stage is ideal for testing pre-adipocyte commitment and the early events of adipogenic differentiation. Indeed, the expression of Zfp423, the initiator of adipogenic commitment, was very high in E14.5 fetal tissue but was absent when assayed on E9.5 and had
only weak expression on P0 (Fig. 1A). On the other hand, the expression of PPARγ2, a marker of differentiated adipocytes, was undetectable in fetal tissue at E14.5, showing that there were no differentiated adipocytes (Fig. 1A).

Compared to Con pregnancy, the Zfp423 mRNA expression was more than 3.6 fold greater in OB fetal tissue (Fig. 1B). Similarly, Zfp423 protein content was also higher (Fig. 1CD), showing that the Zfp423 expression was enhanced in OB fetal tissue.

**MO enhanced the Zfp423 expression and adipogenic differentiation in OB progenitor cells.**

To analyze whether there was overall difference in adipogenic commitment and adipogenesis, we prepared mouse embryonic fibroblasts (MEFs) from fetal tissue; as demonstrated in previous studies, MEFs contain a large number of progenitor cells and have high adipogenic capacity (29-31), and primary MEFs do not express PPARγ (20). These cells were passaged once and adipogenesis was induced with an adipogenic cocktail. The Zfp423 expression was higher in OB compared to Con MEFs during the whole 12 days of adipogenic differentiation. Correspondingly, the expression of PPARγ2 was clearly detectable after 6 days of differentiation with OB higher than Con MEFs (Fig. 2A). After 12 days of adipogenic differentiation, much more adipocytes were detected in OB compared to Con MEFs (Fig. 2BC). Because these cells have been cultured *in vitro* for numerous cell generations, the difference in their adipogenic differentiation strongly indicates that MO had long-term promoting effects on the adipogenic differentiation of MEFs in OB fetal tissue.

Because MEFs contain cells at various stages of early commitment and differentiation, we further sorted MEFs using Sca-1+/CD45- as surface markers. Sca-1+ marks a group of progenitor cells with high adipogenic potential (33) while CD45+ cells are of blood origins (34). After 12 days of adipogenic differentiation, more adipocytes were detected in OB compared to
Con sorted progenitor cells (Fig. 2DE), consistent with data obtained from MEFs. Of note, the adipogenic capacity of sorted cells was lower than that of MEFs (Fig. 2BD), showing that there were additional populations of cells with high adipogenic capacity in fetal tissue. To be inclusive for all cells, for further studies, we used MEFs or fetal tissue, which represents the overall adipogenic commitment in E14.5 fetuses due to MO. This approach fits our objective to explore the impact of MO on Zfp423 expression, associated epigenetic modifications and the adipogenic commitment during fetal development.

**Zfp423 regulates adipogenic differentiation of MEFs.** We have demonstrated that MO enhanced Zfp423 expression in OB fetal tissue. The remaining question is whether high Zfp423 expression is critical for enhanced adipogenic differentiation during fetal development. To test this, we prepared MEFs from both Con and OB fetal tissue, which were then transfected with either a vector over-expressing Zfp423 or a shZfp423 plasmid to knockdown Zfp423 mRNA. After 48 hours, Zfp423 expression was dramatically increased in Zfp423-transfected cultures, accompanied by enhanced PPARγ expression. In contrast, knockdown of Zfp423 decreased PPARγ expression compared to MEFs transfected with EGFP (P < 0.05) (Fig. 3A). The same pattern of changes was observed after 6 days (Fig. 3B). After 12 days of adipogenic differentiation, much more adipocytes were visually detected in MEFs over-expressing Zfp423 while reduced by Zfp423 knockdown (Fig. 3C). Furthermore, comparing Con and OB MEFs, over-expression of Zfp423 was sufficient to enhance adipogenic differentiation of Con MEFs while knockdown Zfp423 reduced high adipogenic capacity of OB MEFs (Fig. 3D). These data showed that Zfp423 regulates adipogenesis of MSCs, and suggest that Zfp423 mediates effects of MO on adipogenic differentiation of fetal progenitor cells during development.
DNA methylation of Zfp423 promoter was lower in OB compared to Con fetal tissue. The next question is why Zfp423 expression was enhanced in OB compared to Con tissue. Through the analysis of ChIP-seq data, we found that the Zfp423 promoter and its 5’upstream region had abundant presence of H3K27me3 and H3K4me3, showing that the Zfp423 promoter is in a ‘bivalent state’ (Fig. 4A). The promoters of genes with bivalent histone modifications may lose active histone modifications and convert to stable DNA methylation when lacks of stimuli to activate gene expression (25). To analyze whether there was change in DNA methylation we further analyzed the abundance of CpG sites in the Zfp423 promoter. As expected, exceptionally rich CpG sites were detected in the Zfp423 promoter (Fig. 4B). The location of CpG islands overlays with the presence of H3K27me and H3K4me3. To elucidate whether there is difference in DNA methylation, we compared DNA methylation status using the sodium bisulfite sequencing. Interestingly, DNA methylation was largely absent in CpG sites approximate to the transcription start site (TSS) in both Con and OB tissue, and the higher density of CpG methylation was detected upstream of TSS. Overall, DNA methylation was more abundant in the Zfp423 promoter of the Con compared to OB tissue (Fig. 4C), consistent with the lower Zfp423 expression in Con fetal tissue.

Histone methylation and acetylation in the Zfp423 promoter of Con and OB fetal tissue.
PcG induces inhibitory histone modification, H3K27me3, and PcG protein EZH2 further acts as a recruitment platform for DNA methyltransferases (DNMTs) (35). To explore mechanisms leading to the reduction of Zfp423 DNA methylation in OB fetal tissue, we analyzed H3K27me3 and H3K4me3 in the Zfp423 promoter. OB decreased H3K27me3 modification in the GC-rich region of the Zfp423 promoter (2.6 folds, P = 0.01), showing that PRC2 was likely involved in the epigenetic modifications (Fig. 5AB). On the other hand, active H3K4me3 histone marker was
significantly higher in the OB fetal tissue (Fig. 5ACD), consistent with reduced Zfp423 methylation and its enhanced expression, as well as reduction of H3K27me3 in the OB tissue (Fig. 4). These data further confirm Zfp423 as a member of genes with bivalent histone modifications, which are known to be critical for early cell commitment and differentiation (36).

In addition, the binding of Suz12, EZH2 and RNG1B, components of PGC2, were highly enriched in the Zfp423 promoter (Fig. 6A). EZH2 catalyzes H3K27me3, and the mRNA expression and protein content of EZH2 were higher in OB compared to Con tissue (Fig. 6BC). This was unexpected but consistent with a previous report, where EZH2 expression was shown to be elevated during adipogenic differentiation (37). Despite of the increase in overall EZH2 content, MO reduced the binding of EZH2 to the Zfp423 promoter of OB compared to Con fetal tissue, corroborating H3K27me3 and DNA methylation data (Fig. 6D).

Active gene expression is associated with enhanced histone acetylations, which were further analyzed (H3K9ac and H3K27ac). There was no difference in H3K27ac, but H3K9ac was higher in the Zfp423 promoter of OB compared to Con tissue (Fig. 7AB), accentuating the role of these active epigenetic marks in the heightened Zfp423 expression in OB compared to Con fetal tissue.

Based on these data, we propose that MO reduced the binding of Polycomb group proteins to the Zfp423 promoter, which reduced inhibitory H3K27me3 histone modification and DNA methylation in the promoter, activating Zfp423 expression in fetal progenitor cells to predispose these and their derived cells to adipogenic differentiation (Fig. 7C).

**DISCUSSION**

Obesity is associated with excessive adipose tissue accumulation in three major depots in
the body, including visceral, subcutaneous, and intermuscular/intramuscular depots. However, fat accumulation is not evenly distributed to these depots, and the accumulation of fat in the visceral depot is correlated with insulin resistance and the risk for T2D (38; 39). In addition, fatty accumulation inside skeletal muscle leads to muscle insulin resistance which has not been appreciated until recently (40).

Adipose tissue, including both white and brown adipose tissue, is mainly developed during the early developmental stages. Enhancing adipogenic differentiation during fetal development forms more preadipocytes and white adipocytes, which lead to adiposity in specific depots in offspring. In this study, we observed that adipogenic differentiation was enhanced in OB compared to Con fetal tissue, which is consistent with previous studies. MO enhanced adipogenic differentiation in fetal tissue (41; 42), and increased intramuscular adipose tissue in MO offspring (43), as well as central adiposity (12; 13). Of note, we did not observe an increase in fetal body weight at E14.5, which could be due to the smallness and immaturity of fetuses at this stage. Using the same dietary-induced obesity model, the body weight of neonatal mice of OB was higher than that of Con mice (13), and MO leads long-term obesity in offspring (12; 13).

To define the mechanisms leading to the enhanced adipogenic differentiation in OB fetal tissue, we first analyzed whether Zfp423 regulates adipogenic commitment in fetal tissue. To our knowledge, our data, for the first time, suggest that Zfp423 regulates adipogenic commitment and adipogenesis in the developing fetal tissue. The remaining question becomes how MO regulates Zfp423 expression during fetal development. Zfp423 promoter presents exceptionally rich CpG sites and islands, meeting the characteristics of “key developmental gene” with high CpG density promoters (36) and previous ChIP-seq results showed that during embryonic development, these rich CpG sites recruit PRC2 which catalyzes H3K27me3 in MEFs (44).
These data strongly support the notion that MO regulates Zfp423 expression and adipogenesis through altering DNA methylation and other epigenetic modifications. To test this, we designed primers corresponding to the CpG islands overlaying H3K27me3 peak in the Zfp423 promoter. Indeed, we observed that MO reduced DNA methylation in the Zfp423 promoter. However, DNA methylation in these CpG rich regions was low and largely unmethylated especially for regions located on TSS, which was unexpected but in agreement with previous reports that the majority CpG islands of high CpG genes remains unmethylated or low methylation status during differentiation (36), and DNA methylation mainly occurs in the shore area of CpG islands (45). Indeed, we found that the methylation level was much higher in those regions located upstream of TSS. As expected, the DNA methylation was lower in OB compared to Con fetal tissue, negatively associated with the higher expression of Zfp423 in OB fetal tissue.

Stem cells maintain their pluripotency through reversible inhibition of lineage-specific genes while allowing genes needed for self-renewal to express. Conversely, during differentiation, lineage-specific genes are expressed while pluripotency genes are inhibited (25). Polycomb repressive complexes (PRCs) are mainly responsible for reversible inhibition of genes. There are two well characterized PRCs, which are PRC1 and PRC2. PRC2 tends to bind promoters with rich GC sites, which attracts PRC1 binding (22; 25). Because the Zfp423 promoter has exceptionally rich GC sites, PRC2 is positioned as a key mediator of Zfp423 expression and adipogenic commitment, which induces H3K27me3 (21). De novo DNA methylation serves to convert plastic gene inhibition by PRCs to permanent silencing (25). Our data show that the H3K27me3 and EZH2 levels in the Zfp423 promoter were lower in OB compared to Con fetal tissue, consistent with the lower DNA methylation and the high expression of Zfp423. However, it is surprising that total EZH2 expression in OB was higher.
than in Con tissue albeit its consistence with a previous report (37). These data suggest that the specific binding of EZH2 to the Zfp423 promoter, not the EZH2 content, is critical for the epigenetic modifications in the Zfp423 promoter, which warrants further studies.

TrxG catalyzes H3K4 trimethylation (H3K4me3), which activates gene transcription. It appears that H3K4me3 is transient, and only induced when gene expression is needed to counter the inhibitory effect of Polycomb group (23; 46). The level of H3K4me3 in the Zfp423 promoter was slightly higher in OB fetal tissue, which indicates that trxG was also involved in the control of Zfp423 expression due to MO but appeared minor compared to PRC2.

In summary, for the first time, we observed that MO reduced DNA methylation in the Zfp423 promoter, which was correlated with higher Zfp423 expression and enhanced adipogenesis of progenitor cells. We further show that histone modification, H3K27me3, a reaction catalyzed by PRC2, was lower in OB fetal tissue, which provides a mechanism for the reduced DNA methylation in the Zfp423 promoter, and enhanced Zfp423 expression and adipogenesis in OB fetal tissue. Because DNA methylation is stable, the reduced DNA methylation in the Zfp423 promoter and enhanced Zfp423 expression in fetal progenitor cells likely enhances the adipogenic capacity of their derived cells in offspring adipose tissue, predisposing offspring to adiposity and the accompanied metabolic dysfunction, which might partially explain the increasing rates of obesity and even T2D in teenagers and even children.

AUTHOR CONTRIBUTIONS

QY researched data, wrote manuscript. JL researched data. CR and JZ contributed to discussion. MZ contributed to discussion, reviewed/edited manuscript. MD wrote manuscript, designed experiments. Dr. Min Du is the guarantor of this work and, as such, had full access to
all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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ABBREVIATIONS

C/EBPs, CCAAT-enhancer-binding proteins; EED, Embryonic ectoderm development; EZH1/2, Enhancer of Zeste 1 and 2; H3K4me3, histone 3 lysine 4 trimethylation; H3K27me3, histone 3 lysine 27 trimethylation; MO, maternal obesity; PPARγ, Peroxisome proliferator-activated receptor γ; PRC2, Polycomb repressive complex 2; RbAp48, Retinoblastoma-associated protein 48; SUZ12, Suppressor of Zeste 12; T2D, Type 2 diabetes; trxG, Trithorax group; TSS, transcription start site; Zfp423, Zinc finger protein 423.
Table 1: Primers used for RT-PCR and bisulfate sequencing

| Name            | Forward                              | Reverse                                | Amplicon sizes (bp) |
|-----------------|--------------------------------------|----------------------------------------|---------------------|
| Zfp423          | GTCACCAGTGCCCAGGAAGAAGAC             | AACATCTGGTTGCACAGTTTACACCTCAT          | 144                 |
| Zfp423-ChIP1    | CCATCATAATTTCCAAACCAGGCAT            | GTCCGGAGCGCAGGAGCTTTAGTA               | 146                 |
| Zfp423-ChIP2    | TGTATTTCCAGCGCTGTCCATCG              | CAGGAGGATGAGGAGCGGAGGT               | 131                 |
| MethPrimer Sec1 | GGTGTATATGTGTGGGTGTCTCCAAATACACTCTAT| CTAACACTCCTCCATAAAACAAAAACT           | 265                 |
| MethPrimer Sec2 | GTGTGTGTGGGTGTGTGGGTGTACTCCCTACTTAA| ATATCCCTCAACTCAAACCTACTTAA            | 322                 |
| Methprimer Sec3 | TTGTATAAAAATTCGGTAAGGGG              | CCTCCCTATAAAAAACACAACTT              | 292                 |
| MethPrimer Sec4 | GGATGTGTAGAGGTGGGTGTATTGTTGCAACTCAT | ACAAAACAATAAACCTCAACGAAAAC            | 225                 |
| 18S             | GTAACCCGTTGACCCCTCTAGTGGAAC          | CCATCCAATCGTGTAGTGACAG              | 151                 |
| EZH2            | AGATGCTGGTAACACTGTGTCGACTCCACACAT   | AGATGCTGGTAACACTGTGTCGACTCCACACAT   | 112                 |
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Figure legends

FIG.1. Zfp423 expression in fetal tissue of Con (□) and OB (■) fetal tissue. A: Zfp423 and PPARγ protein contents in fetal and offspring mice (due to the absence of mature adipose tissue in E9.5, E14.5 and 0D animals, whole fetal tissue was used; for 1.5, 15 and 60 days, adipose tissue was used). B: Zfp423 mRNA expression was enhanced in OB mouse. C-D: OB fetal tissue contained more Zfp423 protein than Con tissue. (*P<0.05; **P < 0.01; Mean ± SE; n = 5).

FIG.2. Adipogenesis of mouse embryonic fibroblasts (MEFs) separated from Con (□) and OB (■) fetal tissue. A: Adipogenesis of Con and OB MEFs were induced using standard adipogenic cocktail, and the contents of Zfp423 and PPARγ were analyzed at 0, 3, 6, 9 and 12 days. B-C: After 12 days of differentiation, adipocytes derived from Con and OB MEFs were visualized by Oil-Red O staining (Upper panel at 40 x and lower panel at 100 x magnifications respectively) (B), and stained Oil Red-O dye in MEFs was extracted and measured by spectrometry at 510 nm (C). D-E: MEFs were sorted by Sca-1+/CD45−, and adipogenesis of sorted cells was induced for 12 days, and adipocytes were visualized by Oil-Red O staining (100 x magnification) (D), and stained Oil Red-O dye in sorted cells was extracted and measured by spectrometry at 510 nm (E). (***P < 0.001; Mean ± SE; n = 5)

FIG.3. ZFP423 regulated adipogenic differentiation of MEFs from fetal tissue. MEFs were separated from Con and OB E14.5 fetuses, and transfected with eGFP control vector, ZFP423 overexpression vector, or ZFP423 shRNA vector with target genes driven by the PCMV viral LTR, and then induced adipogenic differentiation. A-C: MEFs separated from Con E14.5 fetal tissue were induced adipogenic differentiation, and Zfp423 and PPARγ2 expression was
analyzed after 48 h (A), 6 days (B), and both Zfp423 and PPARγ2 protein contents after 14 days (C).  

D: Oil-Red O staining of both Con and OB MEFs after 14 days of adipogenic differentiation.  200 x magnification. (*P < 0.05; **P < 0.01; ***P < 0.001; Mean ± SE; n = 5)

FIG. 4. H3K4me3 and H3K27me3 abundance, CpG sites and DNA methylation in the Zfp423 promoter of Con (□) and OB (■) fetal tissue.  

A: ChIP-Seq data of the Zfp423 promoter region in MEFs (Bioinformatic analysis based on genome-wide epigenetic data in NCBI: http://www.ncbi.nlm.nih.gov/epigenomics. Data were accessed by choosing Browse Experiments and, then, choosing Filters including: Species, Mus musculus; Biological Source, fibroblasts; and Feature Type, H3K27me or H3K4me3. Finally, mouse embryonic fibroblast and MEF_ChIPSeq were chosen to view epigenetic modifications on Zfp423).  

B: The genomic structure and CpG sites of the Zfp423 promoter (Blue regions show CpG islands and short lines represents each CpG dinucleotides, and the regions analyzed by BSP data were shown) (47).  

C: Bisulfite genomic sequencing and methylation of the Zfp423 promoter, and percentages of methylated CpG sites (Sec1-Sec4 as shown in Panel B; white and black circles indicate unmethylated and methylated CpGs respectively).

FIG. 5. Histone modifications (H3K27me3 and H3K4me3) in the Zfp423 promoter of Con (□) and OB (■) fetal tissue. Amplicon levels obtained from ChIP-qPCR analysis was normalized by that of input genomic DNA content. Normal Rabbit IgG was used as the negative control.  

A: Amplicon levels of ChIP-qPCR normalized to the input genomic DNA.  

B: H3K27me3 modification (primers based on -1.3 to -1.5 kb kb of Zfp423 promoter).  

C: H3K4me3
modification (primers based on -1.3 to -1.5 kb of Zfp423 promoter. \( D \): H3K4me3 modification (primers based on -0.6 to -0.75 kb of Zfp423 promoter). \(*P < 0.05; \text{Mean} \pm \text{SE}; n = 5\)

FIG. 6. Abundance of EZH2 and other Polycomb group proteins in the Zfp423 promoter of Con (□) and OB (■) fetal tissue. \( A \): Enrichment of Polycomb group proteins, EZH2, Suz12, and RING1B in the Zfp423 promoter (Bioinformatic analysis based on the genome-wide epigenetic data in NCBI as described in FIG 4.legends). \( B-C \): EZH2 mRNA expression and protein content were higher in OB samples. \( D \): Less abundance of EZH2 in the Zfp423 promoter of OB samples (Two ChIP-Sep assays were conducted using primers based on -0.6 to -0.75 kb and -1.3 to -1.5 kb of the Zfp423 promoter). \(*P < 0.05; \text{Mean} \pm \text{SE}; n = 5\)

FIG. 7. Histone acetylation and the proposed model for epigenetic modifications in the Zfp423 promoter of Con (□) and OB (■) fetal tissue. \( A \): H3K27 acetylation (H3K27AC) was tended to be higher in OB samples demonstrated by two ChIP assays with primers based on -0.6 to -0.75 kb and -1.3 to -1.5 kb of the Zfp423 promoter). \( B \): H3K9 acetylation (H3K9AC) was higher in OB samples demonstrated by two ChIP assays with primers based on -0.6 to -0.75 kb and -1.3 to -1.5 kb of the Zfp423 promoter). \( C \): Proposed model for the effects of maternal obesity on epigenetic modifications in the Zfp423 promoter which enhances Zfp423 expression and adipogenic differentiation of progenitor cells. \(*P < 0.05; \text{Mean} \pm \text{SE}; n = 5\)
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