Maternal regulation of SATB2 in osteo-progenitors impairs skeletal development in offspring

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Abstract
Nutritional status during intrauterine and/or early postnatal life has substantial influence on adult offspring health. Along these lines, there is a growing body of evidence illustrating that high fat diet (HFD)-induced maternal obesity can regulate fetal bone development. Thus, we investigated the effects of maternal obesity on both fetal skeletal development and mechanisms linking maternal obesity to osteoblast differentiation in offspring. Embryonic osteogenic calvarial cells (EOCCs) were isolated from fetuses at gestational day 18.5 (E18.5) of HFD-induced obese rat dams. We observed impaired differentiation of EOCCs to mature osteoblasts from HFD obese dams. ChIP-seq-based genome-wide localization of the repressive histone mark H3K27me3 (mediated via the polycomb histone methyltransferase, enhancer of zeste homolog 2 [Ezh2]) showed that this phenotype was associated with increased enrichment of H3K27me3 on the gene of SATB2, a critical transcription factor required for osteoblast differentiation. Knockdown of Ezh2 in EOCCs and ST2 cells increased SATB2 expression; while Ezh2 overexpression in EOCCs and ST2 cells decreased SATB2 expression. These data were consistent with experimental results showing strong association between H3K27me3, Ezh2, and SATB2 in cells from rats and humans. We have further presented that SATB2 mRNA and protein expression were increased in bones, and increased trabecular bone mass from pre-osteoblast specific Ezh2 deletion (Ezh2^{floxed}/lox Osx-Cre cko) mice compared with those from control Cre^{+} mice. These findings indicate that maternal HFD-induced obesity may be associated with decreasing fetal pre-osteoblastic cell differentiation, under epigenetic control of SATB2 expression via Ezh2-dependent mechanisms.

KEYWORDS
bone, epigenetic, high fat diet, osteoblast

Abbreviations: ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BMP4, bone morphogenetic protein 4; BMSCs, bone marrow-derived mesenchymal stem cells; ChIP-seq, chromatin immunoprecipitation with massively parallel DNA sequencing; cko, conditional knockout; E18.5, embryonic day 18.5; EOCCs, embryonic osteogenic calvarial cells; Ezh2, enhancer of zeste homolog 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3K27me3, tri-methylation of lysine 27 on histone H3; HFD, high fat diet; IGF1, insulin-like growth factor 1; Osx, osterix; PND, postnatal day; pQCT, peripheral quantitative computerized tomography; SATB2, special AT-rich sequence-binding protein 2; Tcfap2a, transcription factor AP2 alpha.

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1 | INTRODUCTION

The developmental origins of health and disease paradigm provide a framework linking gestational and early life experiences to adult chronic disease risk. Along these lines, evidence from experimental animal models suggest that maternal obesity (or obesogenic high fat diet, HFD), independent of birth weight, leads to developmental programming of adiposity and metabolic diseases in the offspring. Although bone mineralization within the skeletal envelope is strongly influenced by genetics and lifestyle factors such as diet and physical activity, intrauterine programming of osteopenia has also been suggested. In this context, several studies have shown that maternal obesity can negatively impact fetal and offspring bone development.

The links between maternal obesity and offspring phenotype alterations are complex and poorly understood. One widely hypothesized mechanism relates to the maternal obesity-associated signals altering epigenetic and epigenomic pathways in development, thus leading to altered bone formation. Consistent with this hypothesis, previous studies have shown that maternal obesity alters DNA methylation of genes including HoxA10. Another important aspect of epigenomic control of gene expression involves regulation of chromatin structure via post-transcriptional modifications of histones. The polycomb group protein enhancer of zeste homologue 2 (Ezh2), a histone lysine methyltransferase associated with transcriptional repression, catalyzes the addition of methyl groups to histone H3 at Lys 27 (H3K27me3) in target genes involved in numerous cellular processes, predominantly leading to gene repression. There is evidence that loss or inhibition of Ezh2 leads to enhanced osteogenic but inhibited adipogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) both from rodents and humans, as well as in murine pre-osteoblastic cell line MC3T3. Expression of Ezh2 suppresses the osteogenic genes and ligand-dependent signaling pathways (eg, WNT, PTH, and BMP2) to favor adipogenic differentiation. More interestingly, several studies have shown that inhibition of Ezh2 prevents estrogen deficiency-induced bone loss in animal models. Despite evidence for a role of Ezh2 in bone cell differentiation, it is not known if this system is involved in maternal obesity-HFD-associated offspring programming of bone.

Special AT-rich sequence-binding protein 2 (SATB2) is a critical transcription factor known to be involved in osteoblast differentiation via its regulation of the osterix (Osx) gene which is an osteoblast-specific transcription factor essential for osteoblast differentiation and bone formation. Recent evidence showed that SATB2 also functions as a key regulator of gene expression and chromatin remodeling. It can be epigenetically regulated by HIV, leading to decreased bone formation. Gene mutations of SATB2 result in SATB2 haploinsufficiency and SATB2-associated syndrome, which is characterized by behavioral problems, mild to severe intellectual disability, developmental delay, and abnormal craniofacial features. In the present report, we examine if maternal obesity-associated defects in skeletal development and in fetal pre-osteoblasts involve epigenetic alterations regulated through the Ezh2-SATB2 axis.

2 | EXPERIMENTAL PROCEDURES

2.1 | Animals and diets

Female Sprague-Dawley rats at weaning (postnatal day 25) were purchased from Harlan Industries (Indianapolis, IN). Subsequently, these rats were divided into two groups, one group of rats received control AIN-93G diet (17% fat) and the other group of rats received a HFD (25% protein, 45% fat corn oil, and 30% carbohydrate). After 12 weeks of diets, rats were time-impregnated (n = 6 per group) by control diet male rats, and this HFD-induced maternal obesity rat model was described previously. Pregnant rats were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility at the Arkansas Children’s Research Institute with constant humidity and lights on from 06:00-18:00 hours at 22°C. Pregnant HFD and control diet rats were sacrificed, and embryos were taken at gestational day 18.5 for analysis. Day 18 postcoitum with 0 considered the day of the positive vaginal smear indicates embryonic day 18.5, E18.5.

We purchased the Osterix-Cre (OsxCre) mice (stock no. 003771) and Ezh2floxfloxflox mice (stock no. 15499) strain from the Jackson Laboratory (Bar Harbor, ME). Two-month-old OsxCre mice were crossed with Ezh2floxfloxflox mice. The offspring were intercrossed to generate the OsxCre+ (Cre+ positive); Ezh2floxfloxflox OsxCre+ mice (cko) and others Ezh2floxfloxflox OsxCre−, Ezh2floxfloxflox OsxCre−, Ezh2floxfloxflox OsxCre−, and wild-type (Wt) mice. All mice were fed standard rodent chow diet, at 47 days old, a pilot study (n = 4) was conducted to analyze gene expression and bone phenotype of cko mice compare to their controls.

2.2 | Isolation of human umbilical cord mesenchymal stem cells at term

Umbilical cord-mesenchymal stem cells (UC-MSC) samples from six subjects obtained as part of a larger longitudinal study (Clinical Study: NCT0061639) were utilized for cell differentiation studies. Briefly, mothers in the parent study were recruited prior to 10 week of pregnancy and were either of normal BMI (19-24.9) or overweight/obese (early pregnancy BMI ≥ 25 kg/m²). We utilized UC-MSCs from n = 3
subjects from each of these groups, chosen randomly from the samples available. As described previously, 22 cells from the UC matrix (UC MSCs) were isolated from the cord-tissue collected at term, pooled, and expanded. Passage two UC MSCs were counted and plated in growth media in a single well of a six-well plate.

2.3  Fetal Alizarin red/alcian Blue staining and isolation of calvarial cells

Alizarin red/alcian blue staining for observation of fetal skeletal development and mineralization was used according to a method published previously. For the isolation of fetal calvarial cells, sequential collagenase digestion of calvarial tissue was performed, and cells from second and third digestion were collected and pooled. The procedure was similar to isolation of neonatal mouse or rat calvarial osteoblastic cells as previously published.

2.4  Bone peripheral quantitative computerized tomography

Peripheral quantitative computerized tomography (pQCT) was performed on formalin fixed mouse left tibia for bone mineral density (BMD) measurement using a STRATEC XCT 960 M unit (Resolution 90-500 µm, XCT Research SA, Norland Medical Systems, Fort Atkins, WI) specifically configured for small bone specimens. Software version 5.4 was used with thresholds of 470 mg/cm³ to distinguish cortical bone and 107 mg/cm³ to distinguish trabecular from cortical and sub-cortical bone. Tibial BMD and bone mineral content (BMC) were automatically calculated. The position for pQCT scanning was defined at a distance from proximal tibia growth plate (1 mm below) corresponding to 7% of the total length of the tibia. Distance between each scan was 0.5 mm, and a total of five scans (five slices) were carried out. Data were expressed as the mean of three contiguous slices with the greatest trabecular bone density.

2.5  Cell cultures

Isolated fetal/embryonic rat osteogenic calvarial cells (EOCCs) or human UC MSCs were cultured in α-MEM (# 41061-029, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), penicillin (100 Units/mL), streptomycin (100 µg/mL), and glutamine (4 mM). EOCCs were treated in the presence or absence of non-esterified fatty acids (NEFA) for 3 days followed by collection of RNA for real-time PCR and proteins for Western blotting. NEFA was individually purchased from Sigma-Aldrich, and they were dissolved in 95% ethanol at 60°C and then mixed with pre-warmed BSA (10%) to yield a stock concentration of 8 mM. Cells were treated with a mixture of NEFA at 400 µM (palmitate and oleic 1:2 mixture) for 48 hours. Ezh2 overexpression plasmid (Ezh2, #28060, Addgene) and shRNA Ezh2 (Ezh2-sh, #TG509722, Origene) were used. According to cell transfection method provided by manufacturer, we have used Lipofectamine 2000 DNA transfection reagent 10 µL/6-well plate, 0.1x Ezh2: cells transfected with 0.04 pmol/µL of DNA; 1X Ezh2: cells transfected with 0.4 pmol/µL of DNA; 0.1x Ezh2 shRNA: cells transfected with 1.25ng/µL of RNA; 1x Ezh2 shRNA, cells transfected with 12.5 ng/µL of RNA. #TG509722, pGFP-V-RS and #MC221218, PCMV6-entry empty vectors were used as controls for Ezh2 shRNA and overexpression respectively.

2.6  Rat embryo histology and immunostaining

Immunohistochemical staining for H3K27me3 (Active Motif) staining was performed on cryosections of whole-mount rat embryos either from HFD obese rats or control rats. Anti-SATB2 antibody from Abcam (#ab51502) and anti-Ezh2 antibody from Cell Signaling (#5346) were used for immune-staining for SATB2 or Ezh2 in human UC MSCs.

2.7  Chromatin-immunoprecipitation and ChIP-seq

The procedure for standard Chromatin-immunoprecipitation (ChIP) assay using H3K27me antibody (ChIP grade from Cell Signaling) has been described previously, and information for all primers used for ChIP assay were designed using Primer Express software 2.0.0 (Applied Biosystems). The genome-wide localizations of histone modifications, spanning varying transcriptional states, were analyzed via ChIP-seq in EOCCs from HFD obese dams and EOCCs from control diet dams. ChIP-grade antibody for H3K27me3 (Active Motif) was incubated with ChIP-grade protein A/G magnetic beads. Detailed ChIP-seq procedures are described in our previous report. The procedure for standard ChIP assay using H3K27me3 and Ezh2 (Cell Signaling) antibodies was described previously.

2.8  Real-time reverse transcription-polymerase chain reaction, western blot

Cell RNA from isolated cultured cells was extracted using TRI Reagent (MRC Inc, Cincinnati, OH) according to the manufacturer’s recommendation, followed by DNase digestion and
column cleanup using QIAGEN mini columns. Reverse transcription was carried out using an iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). All primers for real-time PCR analysis used in this report were designed using Primer Express software 2.0.0 (Applied Biosystems). Standard Western blots were performed using total protein isolated from cultured cells and bone. The following antibodies were used: H3k27me3 (#07-449, Millipore), Ezh2 (#5346, Cell Signaling), p-Ezh2 (#ab109398, Abcam), CDK1 (#ab18, Abcam), β-actin (#A1978, Sigma), SATB2 (#AB19016, Millipore), Col 1 (#sc-135650, Santa Cruz), SuperSignal West Pico chemiluminescent substrate (Pierce) was used for developing blots.

2.9 | Statistics

Numerical variables were expressed as means ± SEM (Standard Error of Mean), and n represents the number of samples/group. For in vitro and ex vivo experiments, differences within groups were evaluated using t test or one-way ANOVA followed by Tukey’s post hoc test comparing each genotype group to the control or cko group. P < .05 was considered significant. Cell culture experiments were conducted at least three independent times, and representative images are displayed. Dose or time response was assessed using Cruick’s non-parametric test for trend. The non-parametric Wilcoxon rank-sum test was used to compare cko to individual Osterix positive or Ezh2 flox control groups. Values were considered statistically significant at P < .05.

2.10 | Study approval

All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences (UAMS, Little Rock, AR). Written informed consent was obtained from mothers during the first trimester of pregnancy after approval by the Institutional Review Board at the UAMS.

3 | RESULTS

3.1 | Epigenetic regulation of fetal skeletal development in rats fed control diet or HFD

At gestational day 18 (E18.5), embryos were taken and fetal calvarial cells were isolated for experimental analysis (see experiment design in Figure S1); dam body composition at gestational day 0 was presented previously. Alizarin red/alcian blue staining for observation of fetal skeletal development and mineralization from two represented embryos (one from control diet lean dam and one from HFD obese dam) are shown in Figure 1A.

Proteins and genomic DNA were isolated from calvarial cells of 12 fetuses from 12 different dams, 6 from control, and 6 from HFD. Western blots showed increased tri-methylation of lysine 27 on histone H3 (H3K27me3) and expression of Ezh2 in embryos from HFD-fed dams; however, phosphorylation of Ezh2 and expression of CDK1 were decreased in EOCCs of HFD obese dams compared to EOCCs from control diet dams (Figure 1B). Higher expression of Ezh2 in EOCCs from HFD embryos was confirmed by real-time PCR (Figure 1C). Expression of H3K27me3 in EOCCs from HFD obese dams was further determined and compared using antibody immunostaining in three different tissue systems (calvaria, gut and vertebrae) of embryos (Figure S2A-C).

Since it is known that modification of histone tails plays an important role in regulating gene transcription, we performed H3K27me3 chromatin immunoprecipitation (ChIP) followed by Illumina sequencing (ChIP-seq), using chromatin isolated from EOCCs from either control or HFD obese rat dams. The solubilized chromatin fragments were immunoprecipitated with ChIP-seq grade antibodies against H3K27me3. Quality control of ChIP-seq data set and ChIP enriched regions of chromatin from EOCCs from either control or HFD obese dams were identified using MACS (Model-based Analysis of ChIP-Seq) (Figure S2D-F). A significant number of genes (including gene bodies and their promoters) from EOCCs of HFD obese dams had enriched (Figure 1D) levels of H3K27me3 compared to gene bodies and promoters of DNA from EOCCs of control diet dams. There are also a significant number of genes and gene promoters with decreased (Figure 1E) levels of H3K27me3. A more detailed gene list is presented in Figure S3.

3.2 | SATB2 is a target gene for maternal obesity regulation on osteoblast differentiation

There were many genes from EOCCs of HFD obese dams that had significantly enriched levels of H3K27me3, among those, SATB2, a well-known transcription factor involved in bone development, is one of the top 25 genes with significantly enriched levels of H3K27me3 shown in the heat map of Figure 1D. ChIP-seq analysis showed that SATB2 had a significantly enriched level of H3K27me3 within the gene promoter region (Figure 1D) and in gene body (Figure 2A). mRNA expression of SATB2 and osteoblast differentiation marker alkaline phosphatase (ALP) were confirmed to be significantly lower in EOCCs from obese dams (Figure 2B). To further detect whether HFD-associated maternal obesity enhances binding of H3K27me3 or Ezh2 to SATB2 gene, H3K27me3 and Ezh2 ChIP assays were carried out. Results showed significant associations between H3K27me3...
and SATB2, and between Ezh2 and SATB2 (Figure 2C,D). Compared to SATB2, we also noticed that transcription factor AP2 alpha (Tcfap2a) and insulin-like growth factor 1 (IGF1) showed significantly increased levels of H3K27me3 within their gene bodies (Figure S4A), and bone morphogenetic protein 4 (BMP4) had significantly decreased levels of H3K27me3 in its promoter region (Figure S4A) based on detection of peaks of enrichment. TCFAP2A and IGF1 are genes known to be involved in osteoblast differentiation, and BMP4 has been shown to be involved in a variety of functions involving commitment of adipocyte differentiation and osteoblast differentiation, and recently cellular senescence transduction.27 Similarly, using antibodies against H3K27me3 and Ezh2, and subsequent PCR amplification, H3K27me3 and Ezh2 ChIP assays were carried out for those osteoblast non-specific target genes (TCFAP2A, IGF1 and BMP4) (Figure S4B). H3K27me3 and Ezh2 recruitment to the BMP4 gene was lower, along with increased recruitment of Ezh2 and presence of H3K27me3 on the TCFAP2A and IGF1 genes in EOCCs from HFD obese dams compared to EOCCs from

FIGURE 1 Epigenetic regulation of skeletal development of fetuses from HFD (high fat diet) rat dams. A, Representative images from Alizarin red/Alcian blue staining of E18.5 embryos from dams fed either control diet or HFD. Cartilage and calcification stained dark blue; arrows indicate differences of skeletal ossification in the head. B, Western blots for H3K27me3, Ezh2, p-Ezh2 and CDK1 in proteins from EOCCs (embryonic rat osteogenic calvarial cells) either from six HFD obese dams or six control diet dams. M, male; F, female. C, Real-time PCR for Ezh2 mRNA expression in total RNA from EOCCs either from six HFD obese dams or six control diet dams. *P < .05 by t-test. D, and E, representing top genes and significantly H3K27me3 enriched or decreased gene body and gene promoter using Heat Map analysis of ChIP-seq data from EOCCs either from six HFD obese dams or six control diet dams (pooled).
control diet dams (Figure S4B,C). Their gene expression are presented in Figure S4D.

We previously hypothesized that such effects of HFD-induced maternal obesity on embryonic osteoblastic cells are, at least in part, due to increased levels of NEFAs (non-esterified fatty acids) in dams’ circulation or derived from lipolysis of HFD dam-derived blood triglycerides. Therefore, we next treated control EOCCs with either a mixture of NEFAs (a mixture of two major NEFAs with concentrations and ratios similar to their appearance in serum from HFD-fed rodents), or individual NEFAs palmitic acid or oleic acid for 48 hours. Increased H3K27 trimethylation and Ezh2 protein expression were found after NEFA treatments (Figure 2E). NEFA upregulated BMP4, but downregulated TCFAP2A and IGF1 mRNA expression (Figure S4E) consistent with their gene expression in vivo in EOCCs from HFD-induced obese dams compared to EOCCs from control diet dams (Figure S4D). These data indicated that HFD-induced maternal obesity decreases osteogenic transcriptional activity, at least in part, through epigenetic mechanisms, and that these processes might involve NEFA.

3.3 | Ezh2 controls SATB2 expression in osteoblastic cell progenitors

SATB2 is a critical transcription factor known to be involved in osteoblast differentiation. To confirm Ezh2 controls
SATB2 gene and protein expression in osteoblastic cell progenitors, we performed Ezh2 knock-in and knock-down experiments in EOCCs from HFD obese or control diet dams and mesenchymal stem cell line ST2 cells. EOCCs from HFD obese or control diet dams were transfected with Ezh2 overexpression plasmid (Figure 3A): over-expression of Ezh2 significantly downregulated SATB2 mRNA expression (Figure 3B). However, knock-down of Ezh2 expression (Figure 3A) using Ezh2 shRNA significantly increased SATB2 gene expression in EOCCs from HFD obese dams (Figure 3B). These relationships between Ezh2 and SATB2 gene expression were confirmed in a stem cell line, over-expression of Ezh2 in ST2 cells (Figure 3C) significantly decreased SATB2 (Figure 3D). Interestingly, when we transfected 10-fold lower concentrations of Ezh2 expression plasmid (0.1x) in ST2 cells (Figure 3C), SATB2 expression was down to half of control treated cells (Figure 3D). However, shRNA-mediated knockdown of Ezh2 expression in ST2 cells (Figure 3E) increased SATB2 mRNA expression (Figure 3F). When 10-fold lower Ezh2 shRNA was used (Figure 3E), SATB2 was significantly upregulated (Figure 3F). These data indicate that SATB2 expression is sensitively regulated by Ezh2 expression status.

### 3.4 Increased Ezh2 but decreased SATB2 expression in human UC MSCs from obese mothers

Human UC MSC cells either from overweight/obese or lean mothers were cultured in 8-well chamber slides, and increased expression of Ezh2 (stained red) in cells from overweight/obese mothers was clearly observed (Figure 4A). Relative to cells from non-overweight/obese mothers, there was lower expression of SATB2 (stained green, especially in the nucleus) in cells from overweight/obese mothers (Figure 4A). Consistent with those observations, real-time PCR and Western blot analysis showed increased Ezh2 expression (Figure 4B), but decreased SATB2 expression (Figure 4C) in human UC MSCs from overweight/obese mothers compared to those cells from lean mothers. To further detect whether

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**FIGURE 3** Ezh2 controls SATB2 expression in osteo-progenitors. A, Real-time PCR Ezh2 mRNA expression in EOCCs either from obese dams or control diet dams was transfected with either control vector, Ezh2 overexpression plasmids or Ezh2 shRNA. B, Real-time PCR SATB2 mRNA expression in EOCCs either from obese dams or control diet dams after cells was transfected either control vector, Ezh2 overexpression plasmids or Ezh2 shRNA. *P < .05 vs cells from control diet dams; #P < .05 vs vector transfected with controls. C, and D, Ezh2 and SATB2 protein and mRNA expression after ST2 cells were transfected with two different concentrations of Ezh2 overexpression plasmid. *P < .05 vs vector transfected. E, and F, Ezh2 and SATB2 protein and mRNA expression after ST2 cells were transfected with two different concentrations of Ezh2 shRNA. *P < .05 vs vector transfected. n = 3/treatment, Western blots represent duplicates.
maternal overweight/obese enhances binding of H3K27me3 or Ezh2 to SATB2 gene, H3K27me3 ChIP assays were carried out. Results showed significant associations between H3K27me3 and Ezh2, and between H3K27me3 and SATB2 (Figure 4D). We found that there were pronounced increase in the bindings of H3K27me3 to Ezh2 and SATB2 genes, but not to housekeeping gene GAPDH (Figure 4E).

3.5 | Deletion of Ezh2 in osterix+ cells increases SATB2 expression in bone

We examined if Ezh2 deletion in osterix+ (Osx+) cells causes aberrations of SATB2 expression in bone during development. Two Ezh2floxflox female mice and two OsxCre positive male mice were bred, and the offspring were intercrossed to generate six genotypes of mice including Ezh2floxfloxfloxflox OsxCre+ cko mice (Figure S5A). The frequencies of all individual genotypes were not as we expected, therefore, offspring samples were pooled from three intercrossed breeding. Due to difficulties in obtaining matched sample size for all genotype groups, we conducted a pilot study with four animals per group at age of day 47 pooled from three separate breedings to determine protein and gene expression in bone. Surprisingly, the SATB2 protein expression in bone from cko mice was higher than those from control groups of any genotyped mice [Wt OsxCre+ (Cre), Ezh2floxflox OsxCre− (flox), Wt OsxCre− (Wt)] (Figure 5A). Consistent with SATB2 protein expression in bone, SATB2 mRNA expression was significantly higher in cko mice compared to those from any other genotypic groups in males and females (Figure 5B data in males). Furthermore, osteocalcin and β-catenin mRNA expression in bone from male cko mice were also significantly higher than those from any other genotypic groups (Figure 5B). No differences in mRNA expressions of osteoclastic cell differentiation markers such as RANK and Cathepsin K were observed among all groups (Figure 5B). No differences in mRNA expressions of osteoclastic cell differentiation markers such as RANK and Cathepsin K were observed among all groups (Figure 5B).}

![FIGURE 4](image_url) Increased Ezh2/H3K27me3 but decreased SATB2 expression in human UC MSCs from obese mothers. A, UC MSCs from either lean or obese mothers were cultured, passage 2 cells were immune-stained with anti-Ezh2 antibody (red, white arrows) or anti-SATB2 antibody (green, yellow arrows). B, and C, Real-time PCR (Box & Whiskers graphs) and Western blots (under Box & Whiskers graphs) of Ezh2 and SATB2 mRNA and protein expression in UC MSCs either from lean or obese mothers. *P < .05 vs lean, t-test. D, ChIP of human Ezh2, SATB2 and GAPDH enhancer elements by specific anti H3K27me3 antibody, and E, ChIP of enrichment of human Ezh2, SATB2 and GAPDH after IP with H3K27me3 antibody, fold enrichment relative to IgG. *P < .01 by t-test vs lean.
**FIGURE 5** SATB2 expression is increased in bone from Ezh2^flox/flox Osterix Cre^+ osteoblastic cell specific cko mice. A, Western blot shows increased SATB2 protein expression in bone (total protein isolated from L1 vertebra after cleaning up bone marrow cells and surround connective tissues) from male Ezh2^flox/flox Osterix Cre^+ cko mice compared to other control genotypic animal groups. B, Real-time PCR shows significantly increased SATB2, beta-catenin and osteocalcin mRNA expression in bone in males from Ezh2^flox/flox Osterix Cre^+ cko mice compared to other control genotypic groups. n = 4 per group.

3.6 | Deletion of Ezh2 in Osx^+ cells increases trabecular bone density and content

BMD and BMC in Ezh2 osteoblastic cell-specific cko mice and other genotypic mice were analyzed using peripheral quantitative CT-scan (pQCT). As shown in representative pictures of slice 4 from five consecutive slices of a pQCT scan of male mouse tibia ex vivo in Figure 6A, total BMC and trabecular BMD in males of cko mice were higher than those from OsxCre^+ mice, but statistically did not reach significant differences in total BMD (Figure 6B,C). In trabecular, we found that the trabecular BMD was significantly higher in cko group compared to Cre mice (Figure 6D). However, in the cortical bone site, we found that there were no statistical differences on total cortical BMD among all groups (Figure 6E). Other parameters were found not significantly changed among groups (Table S1). In females, total BMD in cko mice were significantly higher than those from Cre mice (Figure S6A), there were no differences among groups: cko, OsxCre^+, Ezh2^flox/+OsxCre^−, Ezh2^flox/flox OsxCre^−, and Wt group (Figure S6A). There are no differences in total BMC (Figure S6B). It was surprising that trabecular BMD and BMC in female cko mice were significantly higher than those from Ezh2^flox/flox OsxCre^+ and OsxCre^+ mice (Table S2), trabecular BMD and BMC in female Ezh2^flox/flox OsxCre^+ and OsxCre^+ mice were significantly lower than those from groups of mice of Ezh2^flox/+ OsxCre^−, Ezh2^flox/flox OsxCre^−, and Wt (Table S2). In the cortical bone site, we found that there were no statistical differences on total cortical BMD among all groups in females (Table S2); however, total cortical BMC in all OsxCre^+ mouse groups were lower than OsxCre^− mouse groups, Ezh2^flox/+OsxCre^+ group had the lowest cortical BMC than any other groups (Table S2). These data indicate that loss of Ezh2 in osterix positive cells resulted in increased low limb trabecular bone density and content in both male and female mice compared with their OsxCre^+ control mice.
Maternal diet and obesity status prior to conception are now recognized as important determinants of risks for chronic diseases such as obesity, cardiovascular disease, diabetes and cancer in offspring. Although both experimental and clinical studies indicate that maternal obesity or obesogenic HFD is associated with poor skeletal development and bone health in offspring, maternal obesity-associated epigenetic regulation of the fetal skeletal and postnatal offspring bone development are still understudied. We have previously demonstrated that maternal HFD consumption prior to and during pregnancy in mice and rats is associated with impaired embryonic/neonatal skeletal development. Our results suggested that maternal HFD suppresses fetal bone ossification and osteoblast differentiation. Osteoblast differentiation requires an orchestrated series of events to activate osteoblastogenic transcription factors including Runx2, osterix, SATB2, ATF-4, and β-catenin. SATB2 is a special AT-rich binding transcription factor, and is a downstream regulator of osterix (Osx), while Osx may be a more specific transcription factor essential for osteoblast differentiation and bone formation. Adult offspring from obese dams showed a sustained decrease in SATB2 expression associated with reductions in bone quality, suggesting involvement of epigenetic and/or other persistent mechanisms. We identified a key regulation of SATB2 via repressive histone methylation. Our data suggest that maternal HFD increases Ezh2 expression in fetal osteoprogenitors, while Ezh2 also suppressed expression of osteoblast SATB2. We have suggested a potential model that removal of Ezh2 gene in pre-osteoblasts resulted in increased SATB2 expression in bone and increased trabecular bone mass in mice.

We hypothesized that the fetal skeleton may represent another target for developmental programming and might lead to changes in ability to attain peak bone mass and thus alter the risk of osteoporosis in later life. In the current study, we provide evidence that maternal obesity regulates polycomb-associated genes in rodent embryonic osteogenic calvarial cells (EOCCs) from obese dams. Such regulation of polycomb-associated genes might be through increased NEFA levels in dam-fetal circulation. Increased both total protein and mRNA expression of Ezh2 in EOCCs from obese dams were associated with increased H3K27me3. Ezh2 is known the catalytic subunit of polycomb repressive complex 2 (PRC2) and catalyzes the H3K27me3, which represses gene transcription. In this report, SATB2 was described one of such repressed gene associated with Ezh2/H3K27me3. This supports our hypotheses that the fetal skeleton is a target for developmental programming by maternal HFD. However, as in other tissues, intrauterine fetal skeletal programming is complex, and may undergo

4 | DISCUSSION

Maternal diet and obesity status prior to conception are now recognized as important determinants of risks for chronic diseases such as obesity, cardiovascular disease, diabetes and cancer in offspring. Although both experimental and clinical studies indicate that maternal obesity or obesogenic HFD is associated with poor skeletal development and bone health in offspring, maternal obesity-associated epigenetic regulation of the fetal skeletal and postnatal offspring bone development are still understudied. We have previously demonstrated that maternal HFD consumption prior to and during pregnancy in mice and rats is associated with impaired embryonic/neonatal skeletal development. Our results suggested that maternal HFD suppresses fetal bone ossification and osteoblast differentiation. Osteoblast differentiation requires an orchestrated series of events to activate osteoblastogenic transcription factors including Runx2, osterix, SATB2, ATF-4, and β-catenin. SATB2 is a special AT-rich binding transcription factor, and is a downstream regulator of osterix (Osx), while Osx may be a more specific transcription factor essential for osteoblast differentiation and bone formation. Adult offspring from obese dams showed a sustained decrease in SATB2 expression associated with reductions in bone quality, suggesting involvement of epigenetic and/or other persistent mechanisms. We identified a key regulation of SATB2 via repressive histone methylation. Our data suggest that maternal HFD increases Ezh2 expression in fetal osteoprogenitors, while Ezh2 also suppressed expression of osteoblast SATB2. We have suggested a potential model that removal of Ezh2 gene in pre-osteoblasts resulted in increased SATB2 expression in bone and increased trabecular bone mass in mice.

We hypothesized that the fetal skeleton may represent another target for developmental programming and might lead to changes in ability to attain peak bone mass and thus alter the risk of osteoporosis in later life. In the current study, we provide evidence that maternal obesity regulates polycomb-associated genes in rodent embryonic osteogenic calvarial cells (EOCCs) from obese dams. Such regulation of polycomb-associated genes might be through increased NEFA levels in dam-fetal circulation. Increased both total protein and mRNA expression of Ezh2 in EOCCs from obese dams were associated with increased H3K27me3. Ezh2 is known the catalytic subunit of polycomb repressive complex 2 (PRC2) and catalyzes the H3K27me3, which represses gene transcription. In this report, SATB2 was described one of such repressed gene associated with Ezh2/H3K27me3. This supports our hypotheses that the fetal skeleton is a target for developmental programming by maternal HFD. However, as in other tissues, intrauterine fetal skeletal programming is complex, and may undergo
postnatal reprogramming. Indeed, many components of food have the potential to decrease DNA methylation on certain genes.\textsuperscript{35} Consistent with increased expression of Ezh2 mRNA and protein in other fetal tissues from HFD obese dams,\textsuperscript{36} decreased phosphorylation of Ezh2 at threonine 487 in EOCCs from HFD dams leads to an increase in its activity, and therefore increased trimethylation of lysine 27 of histone H3 (Figure 1). It has been reported that CDK1-dependent phosphorylation of Ezh2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells.\textsuperscript{37} The evidence however support our results, de-phosphorylation of Ezh2 suppresses osteogenic differentiation, therefore, suggested that de-phosphorylation of Ezh2 and increased methylation of H3K27 are also CDK1-dependent. In the future, using cell type-specific CDK-1 or phosphorylated Ezh2 gene mutation models, detailed mechanistic insight could be determined.

Evidence has been shown that histone H3 lysine (H3K) methylation may be a barrier of phenotypic cell reprogramming.\textsuperscript{12} H3K-associated fetal tissue programming or even single gene methylation may affect several generations\textsuperscript{38} making it a difficult phenomenon to target for disease intervention. Hyper-H3K27me3 may lead to long-term, heritable modifications that are linked with active gene expression such as SATB2, TFAP2A, and IGF1. Although signaling molecules TFAP2A and IGF1 are not bone cell specific, aberrant IGF1 expression in EOCCs may contradict its expression in other tissues,\textsuperscript{39,40} such contradictory data may result from specific gene and nutritional interactions. Hypo-H3K27me3 leads to gene overexpression such as we presented with BMP4. As SATB2 was one of the top 25 H3K27me3 enriched genes in EOCCs from HFD dams and is a well-known transcription factor involved in bone development, it was focused on as a key molecule in our current study, though, these maternal HFD obesity-associated aberrant gene expressions may synergistically control osteoblast and adipocyte differentiation to favor a more adipogenic pathway.\textsuperscript{10} Nonetheless, specific blood-borne bioactive compounds, for example, increased NEFA levels in both maternal and fetal circulation, may be partially explained as causative mechanism of effects of maternal HFD obesity on bone in both dams and fetus. NEFAs can cross the maternal-fetal barrier into the fetal circulation, and we demonstrated that in vitro cell treatments with NEFAs resulted in similar gene expression patterns as those seen in vivo. Previous studies from us and others also supported that NEFAs may modify the epigenetic landscape of chromatin in the fetus from an obese mother.\textsuperscript{11} These evidence also supported our results in the current report that NEFA mixture and their individuals, palmitic acid and oleic acid were able to change gene methylation.

Most importantly, we believe that such maternal obesity-induced Ezh2-SATB2 events in rodents may occur in human cells. Considering that embryonic calvarial osteo-progenitor cells are not feasible to study in humans, we studied Ezh2 and SATB2 epigenetic signals in isolated MSCs from human UC upon delivery. These human MSCs are the most accessible fetal mesenchymal osteoprogenitor cells in infants during delivery. It is acknowledged that despite maternal obesity-induced Ezh2-SATB2 signaling matched up with outcomes in rodents; our findings in human cells are limited to a small sample size. Moreover, longitudinal clinical investigations of bone phenotype, epigenetic bone developmental signaling in offspring from obese mothers compare to those from lean mothers will be of interest. However, it is recognized that human studies, especially in postnatal offspring bone developmental signaling studies, are important to the field, it is difficult to control for confounding factors over a lengthy and expensive study period, and genetic variability adds a high level of complexity.

Ezh2 knock in or knock down significantly changed SATB2 expression in osteogenic cells. We described that Ezh2 gene deletion in osteoblastic specific cell lineage increased bone density, especially trabecular bone density and BMC, and increased SATB2 expression in bone (cko vs cre\textsuperscript{+} control). Consistent with previous reports, Osx cre mice showed that basic bone phenotype that is long bone (tibia) length is shorter than those control mice (Osx cre negative mice). This may be concerned, but it should not be problem for us to use this epigenetic animal model to study embryonic bone development by HFD feeding in dams in our future studies. It is recognized that the epigenetic status of specific genes between repressive H3K27me3 and active H3K27ac is essential in controlling osteoblast differentiation\textsuperscript{41}; however, there are only a few studies offering little and inconsistent insight on how epigenetic remodeling of bone-specific chromatin maintains bone cell development and bone mass in vivo. It has been shown that genetic inactivation of Ezh2 in calvarial bones using Ezh2\textsuperscript{lox/lox} and Prx1-Cre model enhances expression of osteogenic genes.\textsuperscript{17} Several studies have also previously presented evidence in support of the role of Ezh2 that inhibition of Ezh2 has bone stimulatory effects.\textsuperscript{13-15} Moreover, it has been shown that loss or inhibition of Ezh2 not only resulted in enhanced osteogenic, but, however, inhibited adipogenic differentiation of mesenchymal stem cells from both human and rodents.\textsuperscript{15} Ezh2 suppresses the expression of osteogenic genes and ligand-dependent signaling pathways (eg, WNT, PTH, and BMP2).\textsuperscript{42} These are all consistent to our current findings, ie, Ezh2 deletion enhanced bone-specific SATB2 gene expression, and other genes such as β-catenin, Runx2, and even mature osteoblastic marker osteocalcin. However, conflicting results on Ezh2 expression and function in pre-osteoblasts were also reported recently, Dudakovic et al published data showing that Ezh2 cko expressing the Osx-Cre driver have no changes on bone volume phenotype in young adult mice.\textsuperscript{43} Possible explanation for that may be as follows: (i) age, animal age presented...
in our study was younger, deletion of Ezh2 in pre-osteoblast leads to aberrant skeletal growth in different age of mice; (ii) sample size, we only presented four animals per group as a pilot study, our original purpose was to check if SATB2 gene expression changes in bone in pre-osteoblast specific Ezh2 deletion; (iii) different methods, we used pQCT to measure bone density, although we used micro-CT method, we had some failed and did not present in the current report; and (iv) different Cre mouse model used and different control mouse groups were compared. Nonetheless, from results of our current study, it was indicated once again that bone development or growth is epigenetically regulated.

In summary, we have presented evidence suggesting epigenetic regulation of embryonic osteogenic cell differentiation from obese mothers. We demonstrated that regulation of polycomb-regulated gene Ezh2 in embryonic rat from HFD obese dams occurred, and Ezh2 controls SATB2 expression. Increased enrichment of repressive histone mark H3K27me3 in the gene of SATB2 is associated with aberrant differentiation of EOCCs to mature osteoblasts. We found significantly increased SATB2 mRNA and protein expression in bone from Ezh2<sup>fl<sub>ox</sub>fl<sub>ox</sub></sup> Osterix-Cre<sup>+</sup> cko mice compared with those from control wild-type mice. Most importantly, our findings provided evidence that maternal HFD-induced obesity-associated decreasing of fetal pre-osteoblastic cell differentiation is under epigenetic control through SATB2 expression, and implicated new target for potential maternal obesity-associated chronic disease prevention.

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CONFLICT OF INTEREST
The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS
J.-R. Chen designed and performed the study, and wrote the paper; H. Zhao and O.P. Lazarenko performed cell, biochemical and molecular experiments, and in vivo sample analysis; M. Blackburn helped to perform ChIP analysis; K. Shankar contributed in the performance of experiments and study designs.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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