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Histone modifications, not DNA methylation, cause transcriptional repression of p16 (CDKN2A) in the mammary glands of offspring of protein-restricted rats☆

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Abstract

Maternal nutrition during pregnancy is an important intrauterine factor that results in persistent alteration of the offspring epigenome and that associates with health outcome in later life. This study examined the effect of maternal low-protein diet on the regulation of the p16 cell-cycle gene expression in the mammary gland of offspring rats. Timed-pregnant Sprague-Dawley rats were fed during gestation one of two isocaloric diets, control (18% casein) or low protein (LP, 9% casein). The expression of p16 mRNA in the mammary gland of the LP offspring was decreased by 75% vs. control. We also detected decreased p16 protein content in the mammary glands of pups gestated under the LP diet. Analysis of transcriptional and epigenetic regulation in offspring rats with maternal LP diet revealed the regulatory mechanisms underlying decreased p16 expression. Chromatin immunoprecipitation (ChIP) assay demonstrated that the altered p16 mRNA level and transcription rate in LP offspring resulted from histone code changes, including the reduced acetylation of histone H4 and the dimethylation of histone H3 at lysine 4 residues within the p16 promoter region. These results supported the hypothesis that maternal protein restriction during pregnancy programs p16 expression through histone code alterations in offspring mammary gland.
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1. Introduction

Recently documented developmental origins of adult human diseases have emphasized the effects of maternal nutrition during fetal development on long-term health outcome in offspring. Epigenetic regulations mediated by macro- or micronutrients are increasingly recognized as prenatal mechanisms that program offspring susceptibility to disease development [1]. Offspring gene expression alterations, stemming from changes in either DNA methylation or histone modifications, have been reported in the rat liver, kidney, skeletal muscle and adrenal gland following a restricted diet during gestation [2–4]. These epigenetic events have been shown to affect tumor suppressor loci in mammary gland cancer cells, including p16 (CDKN2A), E-cadherin and BRCA1, [5–7]. However, it is not known how these tumor-suppressive genes are regulated by epigenetic changes in the mammary glands *in vivo* under maternal low-protein (LP) diet.

The prenatal period is critical in the development of the mammary gland. Findings with experimental rodent models revealed that exposures to dietary factors during the *in utero* and pubertal periods, when the mammary gland is undergoing extensive modeling and remodeling, alter susceptibility to mammary tumor development [8]. p16 (CDKN2A), a cyclin-dependent kinase inhibitor encoded by CDKN2A (INK4a/ARF), binds to cyclin/cdk4 or cyclin/cdk6 kinase complexes, thereby blocking their kinase activity and inhibiting cell-cycle progression at the G1/S boundary [9–11]. Although recent studies demonstrated that regulation of p16 (CDKN2A) and cyclin D1 is critical for growth arrest during mammary involution [12], the role of p16 (CDKN2A) in mammary gland growth before puberty is poorly understood. p16 (CDKN2A) and p53 are two examples of genes that are frequently inactivated in many tumor types, including mammary carcinomas [13]. The inactivation of p16 (CDKN2A) and p53 occurs through a variety of mechanisms, including point mutation, deletion and epigenetic silencing [14]. Recently, the Ozanne group [15] reported that compensatory mammary growth in the offspring following protein restriction during both pregnancy and lactation increased the number of early-mammary tumors. The increased incidence of mammary tumors was accompanied by elevated expression of receptors to insulin, IGF-1, epidermal growth factor and estrogen. This report is the first study on the effect of maternal LP diet on mammary cancer development and provides an extremely relevant model for further study of such processes and, ultimately, the development of potential interventions.

In the present study, we employed a well-established model [2,15] to measure the effect of maternal protein restriction during

Abbreviations: ChIP, chromatin immunoprecipitation; LP, low protein; p16 (CDKN2A), cyclin-dependent kinase inhibitor 2A; Pol II, RNA polymerase II.

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pregnancy on the promoter chromatin status and expression of p16 (CDKN2A) in the mammary gland of the offspring after weaning. Our present study took advantage of the clinical features of the rat maternal LP model and addressed the mechanistic question by uncovering changes in epigenetic regulation related to p16 (CDKN2A). The results obtained from our analysis support that maternal LP diet programs p16 (CDKN2A) gene expression in offspring mammary glands through histone modifications and transcriptional repression.

2. Methods and materials

2.1. Animal and diet

Timed-pregnant Sprague-Dawley rats (Charles River Laboratories) were obtained at Day 2 of gestation and fed one of two isocaloric diets, control (180 g/kg casein) or low protein (LP, 90 g/kg casein) as described by Lillycrop et al. [2], throughout gestation. Both groups had *ad libitum* access to food and drinking water. Animals were individually housed in standard polycarbonate cages and maintained in a humidity- and temperature-controlled room on a 12-h light–dark cycle. Pups were weighed on a scale within 12 h of birth and returned to their mother. Twenty-four hours after birth, the litters were limited to six pups to minimize variation in pups' nutritional status during suckling. Dams were all fed a standard diet through lactation [2]. At Day 24, in both experimental groups, the pups were weaned from the dams and maintained with the same standard diet. When the pups were 38 days old, female pups were sacrificed, and the fourth abdominal mammary gland was collected, snap-frozen in liquid nitrogen and stored at −70°C.

2.2. RNA isolation and one-step real-time quantitative PCR

Frozen mammary gland samples were ground with a mortar and pestle in liquid nitrogen before total RNA was isolated with TRI reagent (Sigma, St. Louis, MO, USA). All purified RNA samples exhibited A260/A280 ratios ≥1.6. For quantification, 25 ng template RNA per well was mixed with iTag SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA), which is a dye that can bind to dsDNA generated during amplification, in a 96-well plate on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The samples were first incubated at 48°C for 30 min for reverse transcription to take place. To determine the exponential phase of amplification, kinetic analysis was conducted for the different primers. Total cDNA was denatured, and hot start RNA polymerase was activated at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. To ensure that a unique product was amplified in the reaction, dissociation curves were generated after PCR by stepwise increase of the temperature from 55°C to 95°C. Primers used for PCR are shown in Table 2.

2.3. Protein isolation and Western blotting

Twenty-five milligrams of frozen mammary gland sample was ground in liquid nitrogen and put into 500 μl of protein sample buffer [0.125 M Tris-HCl (pH 6.8), 5% 2-mecaptoethanol, 1% SDS, 20% glycerol, 0.4% bromphenol blue, protease inhibitor]. Protein was sonicated (Fisher Scientific Model 100 Sonic Dismembrator, Pittsburgh, PA, USA) on ice with 25 pulses at power setting 2. Lowry assay was used to determine protein content, and samples containing 20 μg of protein were resolved by SDS-PAGE. After electrotransfer to a polyvinylidene fluoride membrane (0.2 μm, Bio-Rad) using a wet transfer protocol, incubation in 5% milk in TBS/T [30 mM Tris base (pH 7.6), 200 mM NaCl and 0.1% Tween 20] was performed for 1 h at room temperature to block the membrane. The membrane was then incubated with mouse monoclonal antibody against p16 (CDKN2A) (sc-81156, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% BSA at a 1:200 dilution at 4°C overnight. The next day, the membrane was washed five times for 5 min with TBS/T on a shaker and then incubated with peroxidase-conjugated goat anti-mouse secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in 1% milk at a 1:5000 dilution for 1 h at room temperature. The membrane was then washed five times for 5 min. The bound secondary antibody was detected using a SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL, USA), and Western blot images were captured and analyzed by a Chemi Doc system (Bio-Rad).

2.4. Methylation-sensitive PCR

Methylation analysis was performed using a modified methylation-sensitive PCR (MSP) protocol, based on digesting DNA with methylation-sensitive enzymes and subsequently performing quantitative PCR (qPCR) to detect DNA methylation. Following cleavage with methylation-specific endonucleases, promoter-specific PCR primers amplify intact genomic DNA, while genomic DNA that had been cleaved by one of the two restriction endonucleases remained unamplified. Briefly, DNA was isolated from the mammary gland samples and quantified using a Spectrophotometer (Bio-Rad, SmartSpec Plus). DNA was digested with either *Aci*I or *Hha*I restriction enzymes, which are unable to cut at methylated sites (New England BioLabs, Ipswich, MA, USA), using Buffer 3 and 4, respectively. Both "cut" and "uncut" samples were incubated in the appropriate buffer for 1 h at 37°C and then incubated at 65°C to halt enzyme activity. Digestion longer than 1 h has been employed without any deleterious consequence (data not shown). Following enzyme digestion, samples were analyzed by qPCR using a 7300 Real-Time PCR System (Applied Biosystems). Detection of amplified products was enabled by SYBR Green fluorescent dye. To ensure specificity, a no-template control was included. The PCR amplification was performed in a 96-well optical plate with a 20-μl reaction volume. PCR was performed at the following conditions: 95°C for 10 min, followed by 45 cycles at 95°C for 1 min; 60°C for 10 s. To ensure amplification of desired products, a dissociation curve was generated following the real-time reaction with a temperature range of 55°C–95°C. The amount of methylation is shown as the % ratio of cut/uncut DNA normalized to control in each dietary treatment.

2.5. Chromatin immunoprecipitation

In order to determine whether a given protein binds to a specific DNA sequence *in vivo*, chromatin immunoprecipitation (ChIP) analysis was performed according to a modified protocol [16]. Briefly, 200 mg of frozen mammary gland was ground in liquid nitrogen, resuspended in PBS and cross-linked in 1% formaldehyde for 10 min at room temperature. The tissue pellet was resuspended in nuclei swelling buffer [5 mM Pipes (NaOH) (pH 8.0), 85 mM KCl, 0.5% NP40] containing protease inhibitor. The separated nuclei were lysed in SDS lysis buffer [50 M Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS] containing protease inhibitors. The resultant chromatin was sonicated (Fisher Scientific, model 100 Sonic Dismembrator, Pittsburgh, PA, USA) on ice with 10 bursts for 40 s at power setting 5, with 2-min cooling interval between each burst. The average length of sonicated chromatin was determined to be around 500 bp by resolving on a 1.6% agarose gel. The sample was then centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris from the crude chromatin lysate. Sheared chromatin (1 ml) was diluted in ChIP dilution buffer (total 10 ml). Ten percent of the diluted lysate was subsequently incubated overnight at 4°C on a hematology mixer (Model 346, Fisher Scientific) with 2 μg of primary antibodies of interest. Preblocked salmon sperm DNA/Protein A agarose beads (60 μl, 50% slurry; Upstate Biotechnology, Lake Placid, NY, USA) were then added to the chromatin for 2 h, followed by centrifugation at 2000 rpm for 1 min at 4°C. Supernatant of normal rabbit IgG was saved as the input control for PCR after cleanup. The pellets containing immunoprecipitated complexes were washed sequentially with 1 ml of low-salt solution [0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl], high-salt solution [0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl] and LiCl solution [0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)], and twice with TE (pH 8.0). Antibody/protein/DNA complexes were eluted from Protein A agarose beads by adding 250 μl of the elution buffer (50 mM $NaHCO_3$ and 1% SDS) followed by shaking at 37°C at 300 rpm for 15 min and a flash spin down at room temperature. The combined supernatants were incubated at 65°C for 4–5 h after addition of 20 μl 5 M NaCl and 1 μg of RNase A, to reverse the formaldehyde cross-linking and release the DNA fragments. Samples were then treated with proteinase K at 37°C for 1 h to remove protein. DNA was purified with a Wizard SV Gel and a PCR Clean up System (Promega, Madison, WI, USA). Five percent of immunoprecipitated DNA was used for each real-time PCR reaction. The standards and the samples were simultaneously amplified using the same reaction master mixture at the 25-μl scale. Primers were used to amplify genomic sequences at the promoter region of p16 (CDKN2A) gene (Table 2).

2.6. Transcriptional activity assay

To measure the transcriptional activity from the p16 (CDKN2A) gene, the method of Sandoval et al. [17] was used, which relies on ChIP analysis to examine RNA polymerase II (Pol II) binding at a coding region distal to the promoter. For this purpose, primers were designed within the protein coding region of the p16 (CDKN2A) gene (Table 2).

2.7. Statistical analysis

Results are expressed as mean±S.E.M. Comparison of mRNA expression or histone modification between either control and treated groups was performed by *t* test. Significance testing was set at the $P<.05$ level (two-sided).

3. Results

3.1. Birth and body weight of offspring

On the 21st day of gestation, the control dams delivered all pups after noon, while the LP dams delivered 43% of the pups before noon and 57% of the pups after noon (Table 1). At birth, the control pups weighed 6.32±0.19 g, while the LP pups weighed slightly less, 5.92±0.09 g. As the pups grew to 15 days, average body weight was

Table 1
Offspring vital results

A.

	Control (4)[a]	Low protein (4)[a]
Pups delivered before noon (%)[b]	0	43
Pups delivered after noon (%)[b]	100	57
Litter size	11.5±0.65	10.3±0.86
Litter sex distribution, M/F	1.11	1.25

B.

Age (days)	Body weight (g)[c]		
	Control (4)	Low protein (4)	P
Birth (Day 0)	6.32±0.19	5.92±0.09	.11
7	16.55±0.69	14.89±0.46	.10
15	35.06±1.14	30.73±0.70	.02
22	59.37±1.40	49.98±1.63	.006
35	138.56±2.58	125.21±3.54	.006

[a] Number of litters shown in parentheses.
[b] Timing of delivery by the dams.
[c] Body weight is shown as the average value at the specific day. Data are means±S.E.M. Significance testing was set at $P<.05$ derived from two-tailed *t* test.

significantly lower in the LP pups than in the control (at a *P* value of .02). Table 1 compares the average of body weight at birth and at Days 7, 15, 22 and 35 of postnatal life, showing that the average weights of the LP offspring were reduced by 12% at Day 15, 16% at Day 22 and 13% at Day 35 ($P<.05$) as compared to the control pups. There were no differences in the daily food intake, body weight of the dams during pregnancy (data not shown), litter sizes or litter sex distribution (Table 1) between control and LP mothers.

3.2. Maternal LP diet reduced p16 mRNA level in offspring mammary gland

The effect of maternal LP diet on offspring gene expression was examined through real-time qPCR. The p16 (CDKN2A) expression in mammary gland of the LP pups notably decreased by 75.8% at Day 38 relative to the control pups (Fig. 1B; $P<.001$). The p16 (CDKN2A) mRNA level did not differ between the two groups of dams (Fig. 1A). Because tumor protein 53 (p53) and p16 (CDKN2A) function following a compensatory mechanism in cell cycle arrest [18], the expression of p53 mRNA was examined. However, there was no significant change in p53 expression between the LP and control pups (Fig. 1C).

3.3. p16 protein content was decreased by maternal LP regime

To examine whether the altered p16 (CDKN2A) mRNA produces a change in protein content in offspring mammary gland, we performed Western blot analysis using an anti-p16 (CDKN2A) antibody. p16

Table 2
Primer sequence used in qPCR and ChIP

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Analysis of mRNA expression		
p16 (CDKN2A)	TCCGAGAGGAAGGCGAACTC	GCTGCCCTGGCTAGTCTATCTG
p53	CTTACCATCATCACGCTGGAAGAC	GCACAAACACGAACCTCAAAGC
Analysis of CpG island		
p16 (CDKN2A)	ACTGGGCGGGCACTGAATCTC	TTCGGGGCGTTTGGTGAAG
Analysis of histone modification		
p16 (CDKN2A)	AGCATGGAGTCCTCTGCAGATAGAC	GAAAGTGTTCGGGGCGTTTG
Analysis of transcription rate		
p16 (CDKN2A)	GGCAACGCGAGACTAGCATA	CTCCCTCCCTCTGCTAACCT

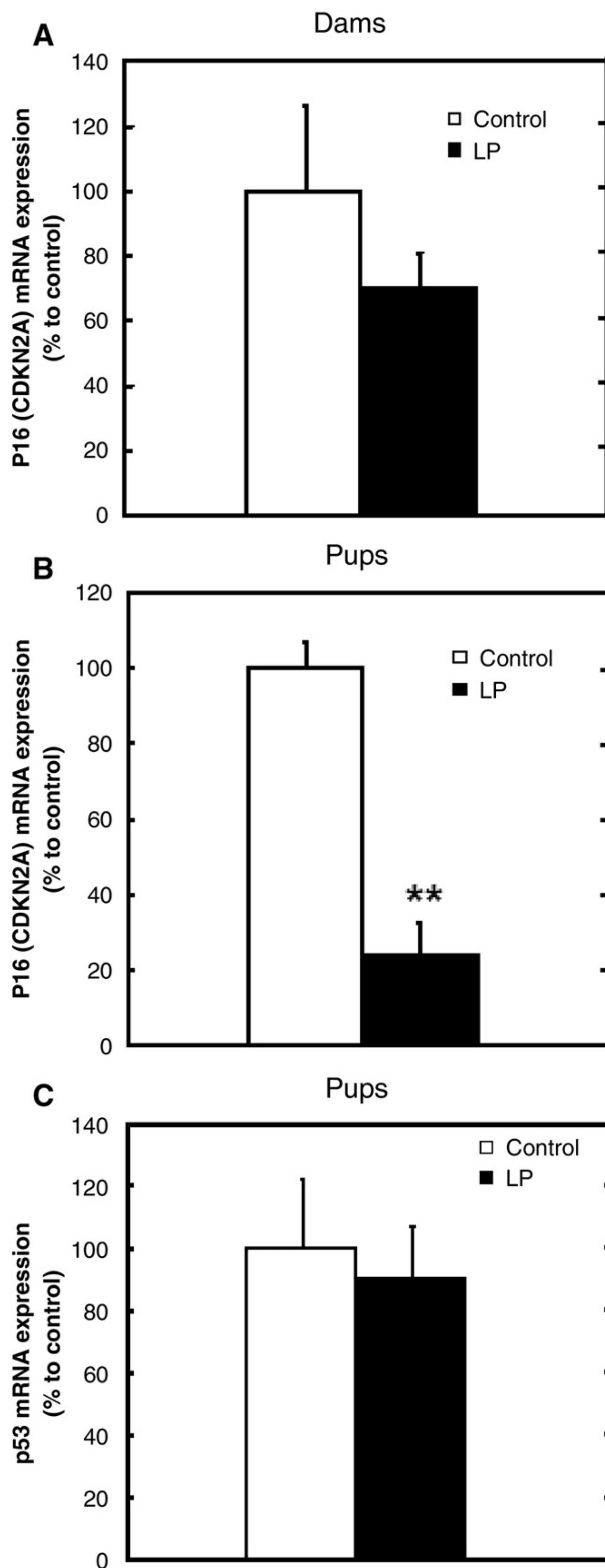

Fig. 1. Expression of p16 (CDKN2A) and p53 mRNA in rat mammary gland. Expression of p16 (CDKN2A) mRNA levels in rat mammary gland by real-time qPCR. (A) p16 (CDKN2A) mRNA in dams ($n=4$) who were fed control or LP diet; (B) p16 (CDKN2A) mRNA in female pups ($n=6$) of LP mothers vs. control; (C) p53 mRNA level in female pups ($n=6$) of LP mothers vs. control. LP mRNA levels are expressed as mean percentage of controls. Values are means±S.E.M., $^{**}P<.001$.

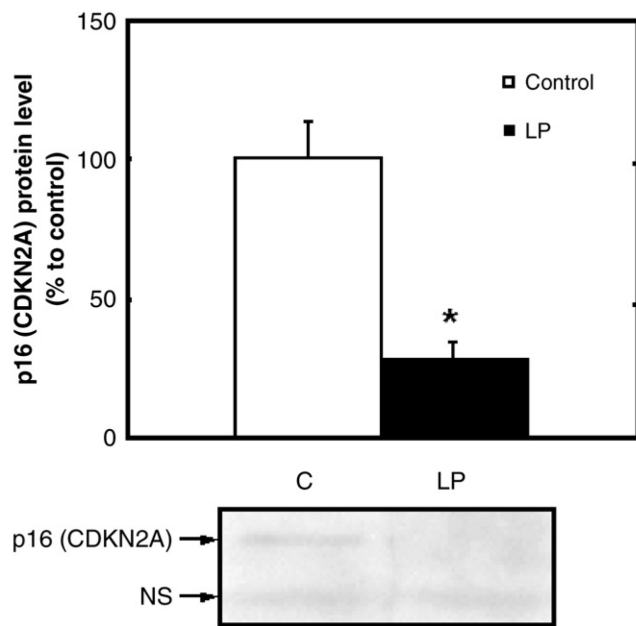

Fig. 2. p16 (CDKN2A) protein content in offspring mammary gland. Expression of p16 (CDKN2A) protein content in female offspring mammary gland by Western blot (n=4). The bands present the average level of p16 (CDKN2A) protein in control and LP groups. NS: Nonspecific binding. LP protein levels were expressed as mean percentage of control. The values are mean±S.E.M., *P<.05.

(CDKN2A) protein was reduced by maternal LP diet in female offspring mammary gland (Fig. 2) by 71% (P=.004), normalized with internal control. Significantly lower p16 (CDKN2A) protein content in female LP pups' mammary gland was consistent with the trend observed in its mRNA expression.

3.4. Restricted maternal LP diet did not change p16 gene methylation

DNA methylation has been reported as a mechanism to regulate p16 (CDKN2A) gene expression in mammary cells [19]. Fig. 3A represents the rat p16 gene structure with the promoter, exons and a CpG island located at the promoter region. In this study, the p16 methylation status was assessed by MSP, presented in Fig. 3B and C. A methylation-specific endonuclease, *Aci*I, was used to digest genomic DNA. The MSP primers we designed covered the whole CpG island as shown in Table 2. Fig. 3B shows that a PCR product was amplified, indicating that the p16 promoter was methylated. However, CpG methylation levels were consistent between the offspring of both control and LP dams. *Hha*I endonuclease, which has no cutting site within the p16 promoter, was used as a positive control (Fig. 3C). The expression levels of the three DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, were analyzed utilizing qPCR (Fig. 3D–F). The expression levels of the methyltransferases were not altered in LP offspring mammary glands. Therefore, the reduced p16 expression in offspring was not due to the DNA methylation state difference in offspring.

3.5. Programming chromatin structure at the p16 promoter through maternal diet

Changes in chromatin structure caused by chemical modifications of histone proteins, such as methylation and acetylation, regulate gene transcription by affecting the ability of eukaryotic RNA polymerase II (Pol II) to transcribe DNA in order to synthesize precursors of mRNA and most snRNA and microRNA [20,21]. ChIP assays were performed to investigate Pol II binding status at the p16 (CDKN2A) promoter. Recruitment of Pol II to the p16 (CDKN2A) promoter was decreased by 82.8% in LP pups compared to control (Fig. 4A; P=0.04). Data was well associated with the decreased p16 (CDKN2A) mRNA expression. Normal rabbit IgG antibody was used as negative control, indicating nonspecific binding. Other antibodies were classified as 'no binding' if the resulting value was equal to or less than rabbit IgG value (≤0.003 ratio to input).

To further determine whether the altered transcriptional level of p16 (CDKN2A) was regulated by the changes in chromatin structure at the p16 (CDKN2A) promoter, antibodies to either methylated histone or acetylated histone were employed in the ChIP assay. Histone acetylation is associated with increased gene transcription and, hence, with transcriptionally active chromatin domains. Although histone methylation is generally associated with transcriptional repression, methylation of different lysine or arginine residues of histone also results in transcriptional activation. For example, methylation of lysine residue 4 of histone H3 (H3K4) is an activator, while methylation of lysine 9 of histone H3 (H3K9) is a repressor of gene transcription [22]. Fig. 4B shows the histone modifications at the p16 (CDKN2A) promoter in the mammary gland of offspring rats. An 84.6% decrease in acetylated H4 (P=.01) and a 92.5% decrease in methylation at H3K4 (P=0.02) were detected in the LP pups, correlating to the 75.8% transcript repression (Fig. 1). No difference in histone H3 acetylation or methylation at lysine 9 residues was detected. These data suggest that the altered p16 (CDKN2A) gene transcription might be regulated by the changes in histone modification at its promoter region, specifically histone H4 acetylation as well as histone H3 methylation at lysine 4.

3.6. Maternal LP diet reduced the transcription rate of p16 (CDKN2A)

To confirm the transcriptional activity from the p16 (CDKN2A) gene, ChIP DNA samples were used to monitor RNA polymerase II translocation to a region distal to the promoter, as demonstrated by Sandoval et al. [17]. The measurement of Pol II binding within the coding region of a gene reflects the transcriptional activity at that gene, which usually has a short half-life. It has been reported that the p16 (CDKN2A) gene has a short half-life [23]. Our results showed that the transcription rate decreased by approximately 85% in LP pup mammary gland relative to control pups (P=.02; Fig. 4C). Therefore, decreased transcription appears to account for most of the reduction in p16 (CDKN2A) mRNA following maternal LP regime.

4. Discussion

The nutritional environment at the fetal and neonatal stages has been suggested to be a critical factor in breast cancer susceptibility. Our data showed for the first time that maternal protein restriction during gestation affected not only the growth of the pups but also p16 (CDKN2A) gene expression through histone code modifications. These observations were consistent with a previous report [15] showing that a LP maternal diet resulted in offspring with not only low birth weight and a slow growth rate during lactation, but also retarded mammary gland development at 3 weeks of age. A rapid compensatory mammary epithelial growth in offspring pups after weaning with normal diet was observed and led us to hypothesize that maternal protein restriction resulted in the repression of some cell-cycle regulators, which could contribute to cancer risk.

The protein p16 (CDKN2A) is known to negatively control cell cycle and to retain pRb in a hypo-phosphorylated form in order to inhibit cellular growth [9]. Down-regulated p16 (CDKN2A) expression has been related to aggressive cell growth, leading to many types of tumors in humans [24]. In the epithelial cells from female mice, the p16 (CDKN2A) expression level has been found to be differentially expressed in a DNA methylation-independent manner during normal

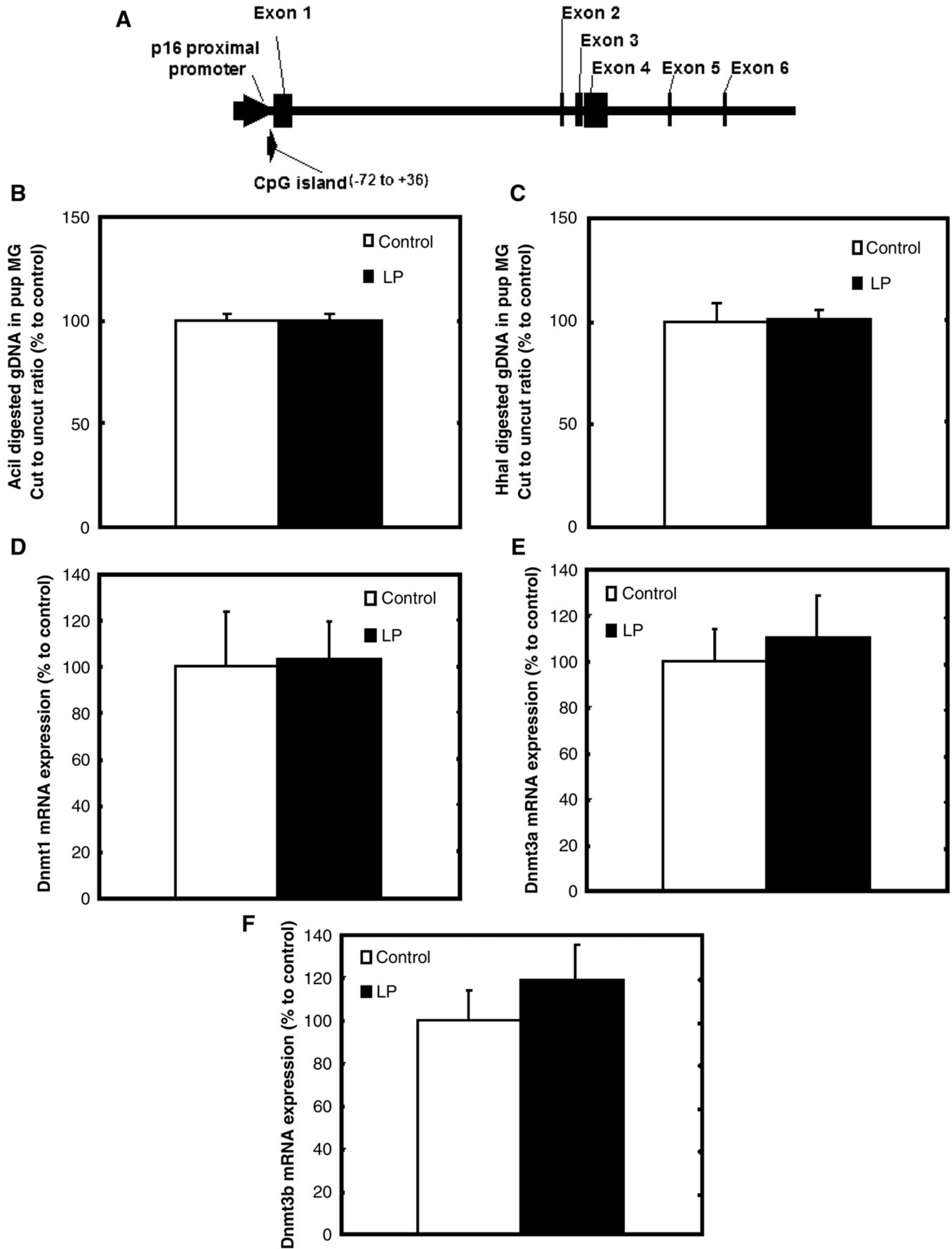

Fig. 3. Expression of DNMT mRNA in rat mammary gland and CpG island methylation status. (A) The rat p16 gene structure. A CpG island located between −72 and +36 bp. (B, C) MSP analysis was performed at the CpG island of p16 (CDKN2A) genes. Amount of methylation of all genes is presented as the % ratio of cut/uncut normalized to control. (D–F) Expression of DNMT1, DNMT3a and DNMT3b mRNA levels in rat mammary gland was measured by real-time qPCR. LP mRNA levels are expressed as mean percentage of controls. Values are means ±S.E.M., n=5.

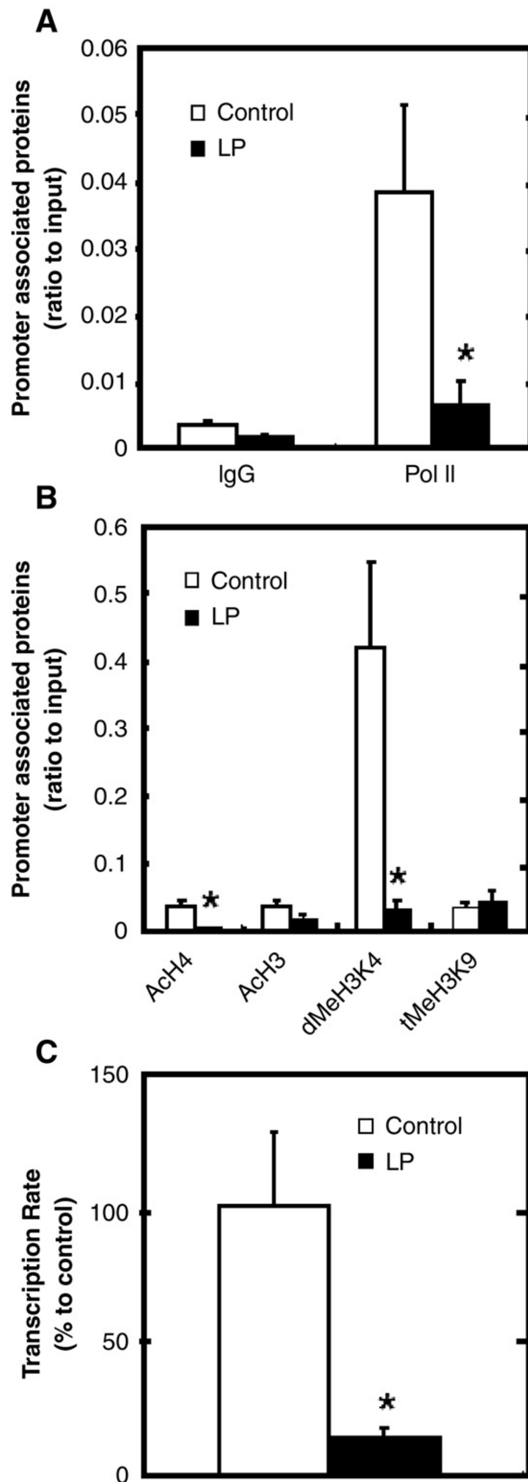

Fig. 4. Chromatin structure changes at the p16 (CDKN2A) promoter region. (A) ChIP assay to demonstrate binding of RNA polymerase II (Pol II) at the p16 (CDKN2A) promoter region in offspring mammary gland (n=5). Normal rabbit IgG antibody was used as negative control to show nonspecific binding. Data are shown as a ratio to the input DNA, which serves as an internal control to eliminate DNA quantity variation. (B) ChIP assay demonstrating the changes in histone modifications at p16 (CDKN2A) promoter region in offspring mammary gland (n=5). Data are shown as a ratio to the input DNA. AcH4: Acetylated histone H4; AcH3: acetylated histone H3; dMeH3K4: dimethylated histone H3 at lysine 4 residues; tMeH3K9: trimethylated histone H3 at lysine 9 residues. (C) Transcription rate of the p16 (CDKN2A) gene. The real transcription rate for p16 (CDKN2A) gene was analyzed by testing Pol II binding at the coding region. Data are plotted as the percentage decrease relative to control (n=5). Values are means±S.E.M., *P<.05.

mammary gland development [25]. The regulatory mechanism of p16 (CDKN2A) remains to be elucidated. As a comparison, in this study, the expression of p16 (CDKN2A) in the mammary gland of offspring with maternal protein restriction is substantially reduced to 24% of the control group. Potentially, down-regulation of p16 (CDKN2A) resulting from maternal LP diet may increase the offspring's breast cancer risk later in life.

Tumor protein 53 (p53, also known as protein 53) is a transcription factor encoded by the human *TP53* gene. p53 can promote cell-cycle arrest or apoptosis in response to a variety of cellular stresses, including DNA damage and hypoxia [26]. p16 gene activity inversely modulates p53 status and function in primary human mammary epithelial cells. p53 and p16 (CDKN2A) are mutated at high frequency in many tumor types, implying that their action is central to limiting tumor development. In fact, oncogenic Ras activates p53 and p16 (CDKN2A) to provoke premature cell senescence. Constitutive activation of the MEK/MAPK cascade induces p53 and p16 (CDKN2A) simultaneously [27]. p53 and p16 (CDKN2A) can also act in a compensatory mechanism that suppresses Ras-induced transformation [18]. Disruption of either p53 or p16 (CDKN2A) prevents cell-cycle arrest and is sufficient for Ras-induced transformation. In our study, although p16 (CDKN2A) expression is significantly suppressed, there is no detectable alteration in the regulation of p53 between control and maternal LP groups. This implies the presence of a nutritional signaling pathway that regulates p16 (CDKN2A) and p53 differently in the maternal protein restriction scenario.

There is growing evidence that maternal nutritional status can alter the epigenetic state of the fetal genome through histone modifications [4]. Histone modifications, such as acetylation or methylation of specific amino acid residues in the N-terminal tails, exhibit diversified effects on gene transcriptional regulation [28]. Histone acetylation or methylation can alter the histone–DNA interactions and the affinity of histone binding to DNA, thereby affecting gene expression [29]. Deacetylation at specific lysine residues in the histone tails is generally associated with transcriptional activity [30]. In contrast, methylation of lysine has been linked to either activation or repression, depending on which lysine residue is modified. Methylation of lysine 4 of histone H3 (H3K4) promotes transcriptional activity [31], while di- and trimethylation of lysine 9 on histone H3 (H3K9) have been shown to inhibit gene transcription [32]. However, the histone epigenetic response to environmental stimuli is much less studied. Our results indicated that, at the p16 (CDKN2A) promoter region, maternal LP diet resulted in deacetylation of histone H4, but not of histone H3, and in dimethylation of H3K4. A number of proteins have been identified that are recruited to histone modifications and bind *via* specific domains [33]. The difference we observed between acetylation of histone H4 and of histone H3 might be due to the varied binding proteins that recognize either the acetylated histone H4 or the acetylated histone H3 to tether different enzymatic activities onto chromatin. The communication is mechanically regulated by a variety of factors, which are much under investigation, although some proteins have been reported to potentially bind with modified histones, for example, Rsc4 binding to acetylated histone H3 and Brd2 binding to acetylated histone H4. However, a related research is undergoing and the functions of the binding proteins have not been clearly revealed [33]. In addition to the changes in histone modification, RNA polymerase II recruitment to the promoter is significantly reduced. We also observed a slight increase in methylation of H3K9, although the result is not statistically significant.

In summary, maternal protein restriction causes a down-regulation of p16 (CDKN2A) transcription and protein levels in offspring mammary glands. It demonstrates that a histone code modification of p16 (CDKN2A) is an epigenetic outcome of maternal dietary LP intake.

Although DNA methylation was also reported to regulate p16 (CDKN2A) gene expression in mammary cells of women with high risk of breast cancer incidence [19], other literature of *in vivo* studies showed DNA methylation-independent regulation of p16 [25]. Similarly, we did not observe any changes to DNA methyltransferase (Dnmt1 or Dnmt3a) expression or CpG island methylation status (Fig. 3) in our animal model, suggesting that it is not a DNA methylation-dependent regulation. A further elucidation of the signaling pathway leading to histone modification of p16 (CDKN2A) and other tumor suppressor genes will contribute to an improved understanding of molecular markers to promote breast cancer prevention through maternal–fetal nutrition.

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