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Adverse events *in utero,* such as intrauterine growth restriction (IUGR), can permanently alter epigenetic mechanisms leading to the metabolic syndrome, which encompasses a variety of symptoms including augmented cholesterol. The major site for cholesterol homeostasis occurs via the actions of hepatic cholesterol 7α-hydroxylase (Cyp7a1), which catabolizes cholesterol to bile acids. To determine whether posttranslational histone modifications influence the long-term expression of Cyp7a1 in IUGR, we used a protein restriction model in rats. This diet during pregnancy and lactation led to IUGR offspring with decreased liver to body weight ratios, followed by increased circulating and hepatic cholesterol levels in both sexes at d 21 and exclusively in the male offspring at d 130. The augmented cholesterol was associated with decreases in the expression of Cyp7a1. Chromatin immunoprecipitation revealed that this was concomitant with diminished acetylation and enhanced methylation of histone H3 lysine 9 [K9,14], markers of chromatin silencing, surrounding the promoter region of *Cyp7a1*. These epigenetic modifications originate in part due to dietary-induced decreases in fetal hepatic Jmjd2a expression, a histone H3 [K9] demethylase. Collectively, these findings suggest that the augmented cholesterol observed in low-protein diet-derived offspring is due to permanent repressive posttranslational histone modifications at the promoter of *Cyp7a1*. Moreover, this is the first study to demonstrate that maternal undernutrition leads to long-term cholesterol dysregulation in the offspring via epigenetic mechanisms. **(*Molecular Endocrinology* 25: 785–798, 2011)**

High circulating cholesterol, in addition to hypertension, central obesity, and insulin resistance, characterizes the metabolic syndrome and remains an important cardiovascular risk factor in adults (1, 2). The major site for the regulation of cholesterol homeostasis occurs in the liver, mainly through the conversion of cholesterol to bile acids, via the rate-limiting enzyme cholesterol 7α-hydroxylase (Cyp7a1) (3). The expression of Cyp7a1 is under the regulation of the orphan nuclear receptor, the liver X receptor (LXR) (4). Male and female mice deficient in *Cyp7a1* have elevated circulating cholesterol and decreased low-density lipoprotein (LDL) receptors, collectively leading to a proatherogenic phenotype (5). Although current therapies using the statin class of lipid-lowering drugs have been successful in treating patients with high cholesterol (6), some patients experience adverse side effects such as rhabdomyolysis (7). Thus, additional novel strategies are warranted to prevent the onset of hypercholesterolemia and reduce the long-term use of these drugs.

Clinical studies in humans have demonstrated that adverse events *in utero* that result in placental insuffi-

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Abbreviations: ChIP, Chromatin immunoprecipitation; Cyp7a1, cholesterol 7α-hydroxylase; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; IUGR, intrauterine growth restriction; [K9], lysine 9; LDL, low-density lipoprotein; LP, low protein; LXR, liver X receptor; LXRE, LXR response element; SDS, sodium dodecyl sulfate.

ciency-induced intrauterine growth restriction (IUGR, representing 8% of newborns), can also alter physiological processes leading to the metabolic syndrome (8, 9). Moreover, 50% of metabolic syndrome-related diseases can be linked to adverse events *in utero* (10–12). With respect to cholesterol, David Barker and colleagues (13, 14) have demonstrated that impaired fetal growth is linked to elevated LDL cholesterol and hypertension in adult humans. Moreover, the association between birth weight and cholesterol was more apparent in males and independent of social class, current body weight, cigarette smoking, and alcohol consumption (13). Given that hypercholesterolemia in mothers is associated with increased incidence of aortic fatty streaks in fetal and adolescent life (15), it is conceivable that postnatal cholesterol concentrations may be altered by the perinatal environment, although the underlying molecular mechanisms are unknown.

Various animal models have broadened our understanding of how early nutrition in pregnancy may influence liver development (16–19). The majority of studies have demonstrated that maternal nutrient or protein restriction leads to decreased liver growth (16–19). Other studies have demonstrated that a low-protein (LP) diet during pregnancy and lactation leads to asymmetrical IUGR (20), associated with alterations in hepatic glucose output, decreased glucagon receptor and glucokinase expression, along with increases in insulin degradation and hepatic lobular volume (16, 17). However, very little is known about how maternal nutrition may alter cholesterol homeostasis in postnatal life. In one study, maternal dietary iron restriction led to a 21% decrease in fetal growth and an increase in fetal liver cholesterol (21). This was further associated with decreased expression of Cyp7a1 (21). More recently, a LP diet throughout pregnancy in mice was demonstrated to lead to reduced LXRα expression at embryonic d 19.5 (22); however the long-term effects of this *in utero* nutritional insult on cholesterol homeostasis in postnatal life remain elusive.

Although transcriptional changes may mediate the expression of target genes involved in fetal programming, there is limited knowledge on the links between epigenetic mechanisms *in utero* and the long-term expression of hepatic target genes (18, 19, 23, 24). Previous studies have demonstrated that diminished acetylation and/or an increased methylation of histone H3 lysine 9 [K9,14] has been associated with chromatin silencing and decreased RNA polymerase II binding (25, 26). Elegant studies by Park *et al.* (27) have demonstrated that uterine ligation-induced IUGR rat offspring developed type 2 diabetes as a result of epigenetic silencing of pancreatic and duodenal homeobox 1 (*Pdx1*), a critical transcription factor regulating β-cell differentiation. This included both deacetylation and hypermethylation of histone H3 [K9] (27). Recently it was demonstrated that in a model of a LP diet during pregnancy, LXRα expression was suppressed in the fetal liver as a result of DNA hypermethylation at the *LXRα* promoter (22); however the long-term effects of this diet on LXR-target genes (*e.g.* Cyp7a1) in postnatal life were not examined. In addition, the effects of a low-protein diet on posttranslational histone modifications during liver development have never been explored.

In the present study, we tested the hypothesis that a LP diet in pregnancy may impair cholesterol homeostasis long term via repressive changes to histone modifications throughout development in a promoter-specific manner. We observed whether the timing of the LP diet throughout pregnancy and lactation led to increases in circulating cholesterol in both early (3 wk) and late (18 wk) postnatal life. To accomplish this, four dietary regimes (Control; Low Protein, LP1–LP3) were employed to assess the effects of a LP diet throughout life (LP1), until the end of lactation (LP2), and exclusively during gestation (LP3). By examining all three LP dietary regimes, it is possible to assess the impact of the duration of the LP diet and the timing of protein restoration on liver development and circulating cholesterol. Given that Cyp7a1 plays a major role in regulating cholesterol catabolism in the liver, we also demonstrated whether changes in circulating cholesterol were linked to alterations in Cyp7a1 expression. Finally, we investigated whether any changes to Cyp7a1 expression in fetal and postnatal life were due to differences in the acetylation and trimethylation of histone H3 [K9] surrounding the *Cyp7a1* promoter.

Results

A LP diet during pregnancy and lactation leads to impaired body weight and liver growth

A LP diet during pregnancy has been previously demonstrated to decrease birth weight (16–19) and lead to increases in visceral fat, impaired glucose tolerance, and symptoms of type II diabetes in adulthood (28–31) in the rat offspring. Although others have demonstrated that offspring derived from this LP dietary regime were not hypertensive at 4 wk of age (32), LP females placed on a high-carbohydrate diet after lactation exhibited elevated systolic blood pressure at 1 yr of age (33). In this study, a LP diet resulted in a significant decrease in fetal to placental weight ratio and a marked decrease in fetal liver to body weight ratio at embryonic d 19 (Table 1). In agreement with previous reports (34, 35), a LP diet did not alter maternal food intake, and there was no observed change

TABLE 1. The effect of LP dietary regimes LP1, LP2, LP3 on liver growth and body weight in rat offspring at embryonic day 19 (e19), postnatal day 21 (d21), and postnatal day 130 (d130)

		d21		d130	
	e19	Male	Female	Male	Female
Body weight (g)					
Control	5.67 ± 0.30	50.30 ± 1.15	48.87 ± 1.49	565.50 ± 8.21	348.20 ± 9.64
LP1	4.87 ± 0.18[a]	25.63 ± 0.75[a]	25.63 ± 0.91[a]	469.10 ± 14.00[a]	281.40 ± 8.05[a]
LP2	N/A	N/A	N/A	511.1 ± 12.8[a]	298.0 ± 7.3[a]
LP3	N/A	48.00 ± 2.17	47.44 ± 1.38	579.00 ± 18.74	362.70 ± 13.53
Liver-body weight ratio					
Control	0.091 ± 0.004	0.0391 ± 0.001	0.0386 ± 0.001	0.0314 ± 0.001	0.0317 ± 0.001
LP1	0.056 ± 0.006[a]	0.033 ± 0.001[a]	0.034 ± 0.001[a]	0.033 ± 0.001	0.031 ± 0.001
LP2	N/A	N/A	N/A	0.0330 ± 0.001	0.0307 ± 0.001
LP3	N/A	0.0360 ± 0.001	0.0384 ± 0.001	0.0306 ± 0.001	0.0302 ± 0.001

Dietary regimes and the liver to body weight ratio of offspring. To assess body weight (g), the fetal to placental weight ratio was determined for offspring killed at e19, and the body weight was determined for offspring killed at d21 and d130. To assess liver growth, liver to body weight ratio was determined for all three developmental time points. All data was expressed as SEM. For e19, dietary effects were determined using Student's unpaired *t* test whereas for d21 and d130, a one-way ANOVA followed by a Bonferroni's Multiple Comparison *post hoc* test was performed.

[a] Significant difference ($P < 0.05$) *vs.* control (n = 10–14/group). N/A refers to when the LP dietary regimes were the same.

in litter size or sex ratio (data not shown). To further examine the effect of the timing and duration of the low-protein diet on postnatal growth and liver development, various dietary regimes (LP1, LP2, LP3) were implemented (Table 1). At d 21, there was a significant decrease in both body weight and liver to body weight ratio in the male and female offspring derived from the LP1/LP2 diet compared with control (Table 1). However, offspring of either sex derived from the LP3 dietary regime displayed no difference in body weight and liver to body weight ratios when compared with the offspring derived from the control diet. We next observed the effects of LP1–LP3 on liver and body growth at 130 d of age. We examined this age based on our previous study that demonstrated sex-specific differences in glucose homeostasis at this time point (35). Although the body weights of LP1 or LP2 male and female offspring regimes remained significantly lower at d 130, their liver to body weight ratio was restored compared with the control animals (Table 1). Furthermore, no change in the food intake of the offspring was observed in the four dietary regimes (Control, LP1–3; data not shown), suggesting that it was a difference in the protein content of the diet and not appetite that was a primary factor in inducing any of the observed developmental changes.

A LP diet during pregnancy and lactation leads to augmented circulating cholesterol in both sexes in early development and exclusively in the male offspring in adulthood

To determine whether LP1–LP3 led to postnatal changes in cholesterol levels, total circulating cholesterol was measured in the offspring at d 21 and d 130. Total circulating cholesterol levels was observed to be significantly increased in the male and female offspring derived from the LP1/LP2 dietary regime at d 21 (Fig. 1A) and exclusively in the male offspring derived from the LP2 dietary regime at d 130 (Fig. 1B). In addition, hepatic total cholesterol concentrations were similarly increased in male LP2 offspring at d 130 (Fig. 1C). In a small cohort of control animals given a LP diet from birth to d 21 (reverse LP3), circulating cholesterol was not significantly different from the control or LP3 offspring (Fig. 1A). The increase in hepatic cholesterol in LP2 males at d 130 was not attributed to alterations in 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase or the LDL receptor (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Furthermore, when plasma triglycerides were measured at d 21 and d 130, there was no statistical difference in triglyceride concentrations between any dietary groups at both ages (data not shown).

Decreases in Cyp7a1 protein levels coincide with the respective increases in total circulating cholesterol levels in offspring derived from the LP2 dietary regime at d 21 and d 130

To elucidate the underlying mechanisms for the augmented cholesterol in the LP2 offspring, immunoblot analysis was performed to determine the protein levels of hepatic Cyp7a1, the rate-limiting enzyme involved in the conversion of cholesterol to bile acids, during fetal and postnatal development. At embryonic d 19, a LP diet led to a significant decrease in hepatic Cyp7a1 protein levels (Fig. 2A). At d 21, both the male and female offspring derived from the LP1/LP2 dietary regime also had significantly lower Cyp7a1 protein levels (Fig. 2, B and C), which was associated with the augmented circulating cholesterol observed in both sexes at this age (Fig. 1A). Interestingly, when maternal proteins were restored to the

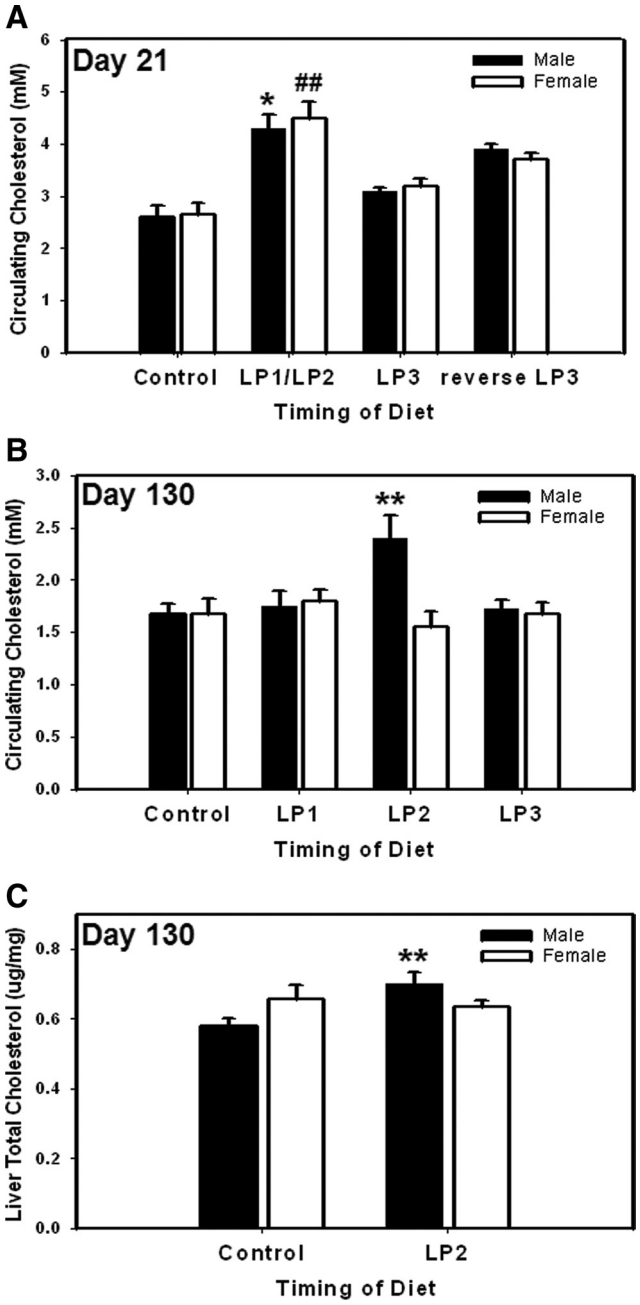

FIG. 1. The effect of different LP dietary regimes on circulating cholesterol concentrations (mM) in rat offspring at postnatal d 21 (n = 10–14/group) in A. Results were expressed as the mean ± SEM. Dietary effects were determined using a two-way ANOVA followed by a Bonferroni's Multiple Comparison *post hoc* teand postnatal d 130 (n = 10–14/group) in B. C, The effect of LP2 on median lobe liver total cholesterol at postnatal d 130 (n = 5–6/groupst. **, Significant difference ($P < 0.01$); *, significant difference ($P < 0.05$) *vs.* male control; ##, significant difference ($P < 0.01$) *vs.* female control.

LP offspring during lactaction (LP3), Cyp7a1 protein levels significantly increased compared with control (Fig. 2D). Finally, when the expression of hepatic Cyp7a1 was examined in both sexes from the LP2 dietary regime at d 130, only the male LP2 offspring exhibited significant decreases in Cyp7a1 protein levels (Fig. 2, E and F). This gender-specific decrease in Cyp7a1 coincided with changes in circulating and hepatic cholesterol at this age (Fig. 1, B and C).

The LP2-induced reduction in hepatic Cyp7a1 protein in the male offspring is due to a decreased recruitment of RNA polymerase II at the *Cyp7a1* initiation site throughout fetal and postnatal development

To explore the effects of LP2 on transcriptional mechanisms, chromatin immunoprecipitation (ChIP) was employed to examine the recruitment of RNA polymerase II to the *Cyp7a1* initiation site. Quantitative real-time PCR was used to amplify an approximate 100-bp genomic region surrounding the TATA box and the initiation site (Fig. 3, A, C, E, and G). This region was demonstrated to have maximal enrichment of RNA polymerase II compared with distal sites of the *Cyp7a1* promoter (Supplemental Fig. 2). At embryonic d 19, it was observed that there was a significant decrease in the recruitment of RNA polymerase II to the initiation site of hepatic *Cyp7a1* in the LP fetuses (Fig. 3A). Moreover, the decreased binding of RNA polymerase II to the *Cyp7a1* promoter persisted in the LP2-derived male offspring at both d 21 and d 130 (Fig. 3, C and E). The decrease in recruitment of RNA polymerase II was specific to the *Cyp7a1* promoter region given that no significant alterations in its recruitment were observed at the promoter of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), a housekeeping gene (Fig. 3, B, D, F, and H). ChIP performed using nonimmune IgG displayed negligible level of binding to either the *Cyp7a1* and *Gapdh* promoter region. The decrease in the recruitment of RNA polymerase to the promoter of *Cyp7a1* in LP2 offspring corresponded with both the decrease in Cyp7a1 protein levels (Fig. 2, A, B, and D) and augmented cholesterol (Fig. 1, A and B). Given that there were sex-specific differences in circulating cholesterol and Cyp7a1 expression between males and females at d 130, the recruitment of RNA polymerase II to the *Cyp7a1* promoter was examined at that age for both sexes. Unlike the males, ChIP revealed no corresponding differences in RNA polymerase II binding to the promoter of *Cyp7a1* in the females at this age (Fig. 3G).

Decreased recruitment of RNA polymerase II in the LP2 dietary regime is associated with chromatin silencing of *Cyp7a1* promoter region via posttranslational histone modifications

It is well established that decreased acetylation and/or an increased methylation of histone H3 [K9,14] is associated with chromatin silencing and decreased RNA polymerase II binding (25, 26). Therefore, to in-

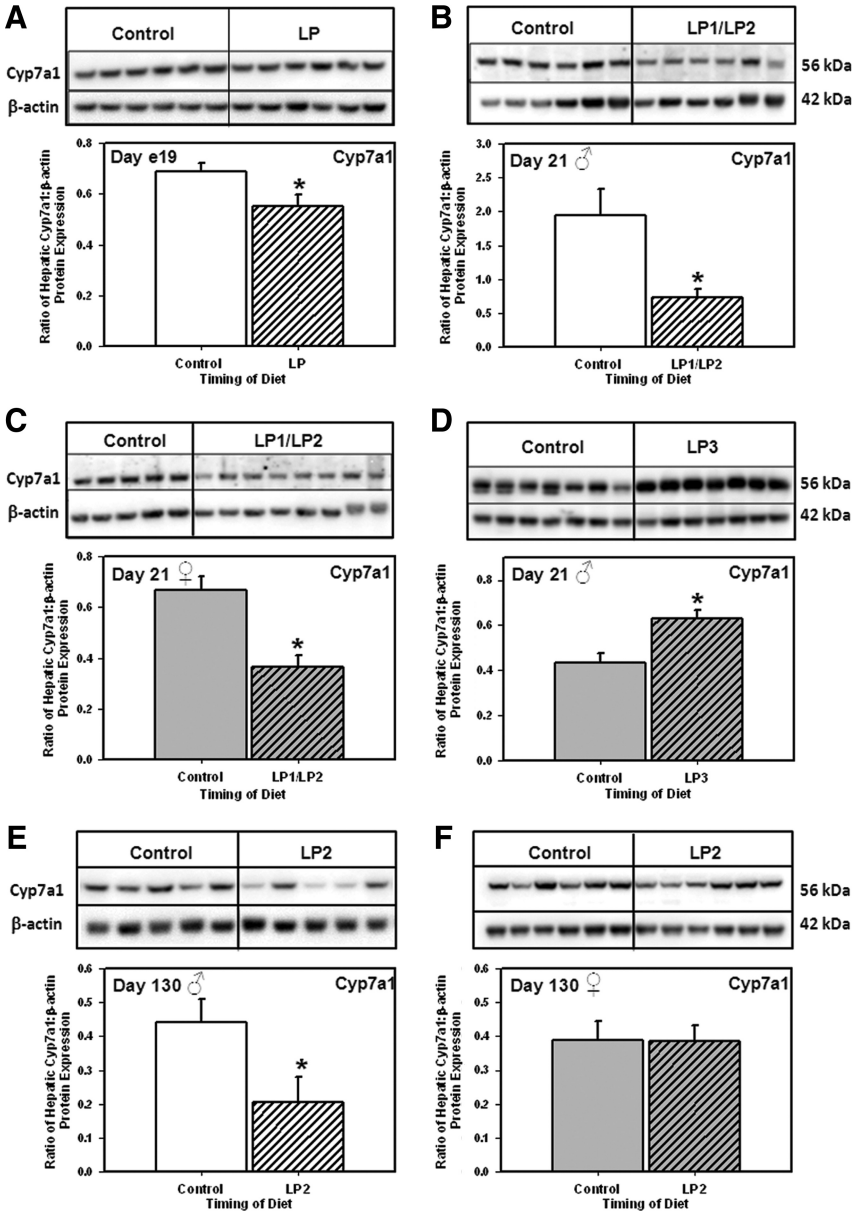

FIG. 2. The effect of the LP2 dietary regime on hepatic Cyp7a1 protein levels in rat offspring at embryonic d 19 (A), male (B), and female (C) rat offspring at postnatal d 21 and male (E) and female (F) rat offspring at postnatal d 130. D, The effect of LP3 dietary regime on hepatic Cyp7a1 protein levels in male rat offspring at postnatal d 21. Relative hepatic Cyp7a1 protein levels were determined using Western blot analysis. Total membrane protein was isolated and Cyp7a1 protein was detected on a Western blot using the Cyp7a1 primary antibody. The Cyp7a1 protein level was quantified using densitometry and normalized to that of β-actin protein levels. Results were expressed as the mean ± SEM. *, Significant difference ($P < 0.05$); n = 4–7/group, where each n represents a single offspring derived from a different mother.

vestigate whether posttranslational histone modifications influenced chromatin remodeling and RNA polymerase II recruitment to the *Cyp7a1* promoter during LP2, we employed ChIP utilizing antibodies directed against histone H3 methylation [K9] and/or acetylation [K9,14]. At embryonic d 19, an increase in the acetylation of histone H3 (Fig. 4A), but no change in trimethylation (Fig. 4B), was observed surrounding the active site of *Cyp7a1* promoter. This was despite the fact that the protein levels of Cyp7a1 were slightly reduced (Fig. 2A). However, a significant decrease in the acetylation, along with an increase in the methylation of histone H3 [K9], was observed at the hepatic *Cyp7a1* promoter derived from LP2 male offspring at both d 21 (Fig. 4, C and D) and d 130 (Fig. 4, E and F). At both ages in LP2 males, these epigenetic changes correspond exactly with the observed decreased recruitment of RNA polymerase II in the *Cyp7a1* promoter (Fig. 3, C and E), diminished expression of Cyp7a1 (Fig. 2, B and D), and the observed increases in circulating and hepatic cholesterol (Fig. 1, A–C). In contrast, no alterations in the acetylation [K9,14] or methylation [K9] of histone H3 were observed at the hepatic promoter of *Gapdh* at this age (Supplemental Fig. 3). Interestingly, in d 130 females, where no differences in circulating cholesterol or Cyp7a1 expression were observed (Figs. 1B and 2E), there was also no corresponding changes in acetylation (Fig. 4G) or methylation (Fig. 4H) surrounding the active site of *Cyp7a1*.

Given that the methylation of histone H3 [K9] can be influenced by specific [K9] demethylases (36), we next examined whether changes in their expression facilitated the increases in the trimethlyation of histone H3 [K9] associated in the LP2 males. We examined only the steady-state mRNA levels of those [K9] demethylases (Jmjd2a, Jmjd2b, Jmjd2c, Jmjd2d) that removed trimethyl groups from [K9] of histone H3 (36). Real-time quantitative PCR revealed that significant decreases in the hepatic Jmjd2a mRNA were observed only at embryonic d 19 in LP fetuses (Fig. 5A). No significant changes in the hepatic mRNA levels of Jmjd2b and Jmjd2c were observed between diets at this age, although there was a trend for a decrease in their expression due to the LP diet (Fig. 5, B and C). The steady-state mRNA level of all of these [K9] demethylases was the same at d 21 (Fig. 5, D–F) and undetect-

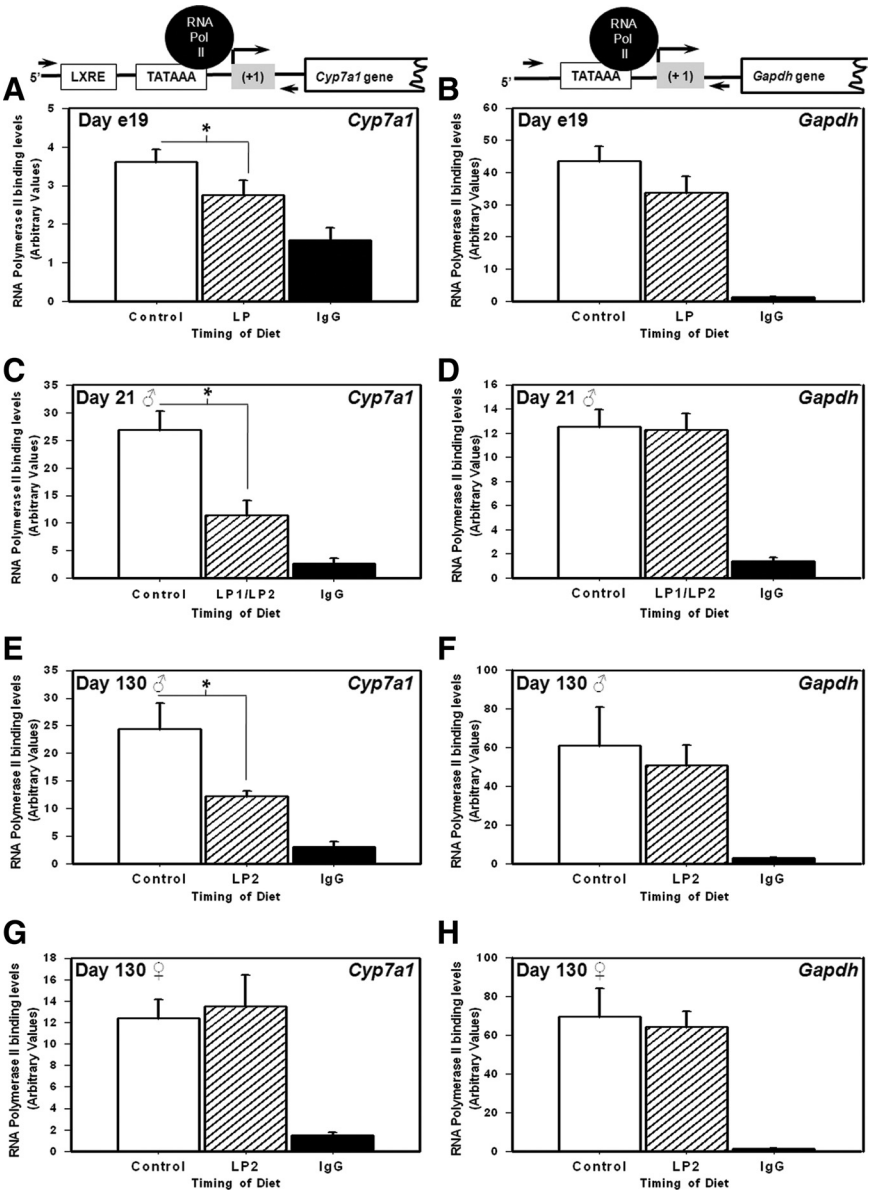

FIG. 3. The effect of the LP2 dietary regime on the *in vivo* RNA polymerase II (RNA Pol II) binding at the initiation site of hepatic *Cyp7a1* (A, C, E, and G) and the initiation site of *Gapdh* gene (B, D, F, and H) in rat offspring at embryonic d 19 (e19), male rat offspring at postnatal d 21, male and female rat offspring at postnatal d 130, respectively. ChIP was carried out on snap-frozen liver tissues by immunepreciptation with antibody specific for RNA polymerase II. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real-time PCR via use of primers specific for the initiation sites on the promoter regions of hepatic *Cyp7a1* and *Gapdh*. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.*, Significant difference ($P < 0.05$); n = 4–6/group, where each n represents a single offspring derived from a different mother.

Protein restoration during lactation (LP3 dietary regime) enhances the recruitment of LXRα to the promoter of *Cyp7a1* at postnatal d 21

Given the important role of LXRα in the transcriptional regulation of Cyp7a1, we next investigated whether hepatic LXRα levels were altered in the LP offspring by postnatal d 21, the earliest time point of cholesterol dysregulation and impaired Cyp7a1 expression. Intriguingly, in both LP2 and LP3 offspring, increases in total hepatic LXRα protein levels (Fig. 6, A and B) were observed. We next employed ChIP to investigate the *in vivo* binding of LXRα to the promoter of *Cyp7a1* containing a well-characterized LXR response element (LXRE) site. Despite an increase in total LXRα, LP2 offspring did not exhibit any alterations in the binding of LXRα to the promoter of *Cyp7a1* at d 21 (Fig. 6C). However, when the LP offspring received a 20% protein diet during lactation (LP3), LXRα binding was enhanced (Fig. 6D). This is in association with restored Cyp7a1 expression in this dietary cohort (Fig. 2D).

Discussion

In this study we present evidence for the first time that a LP diet during pregnancy and lactation led to hypercholesterolemia in both sexes at postnatal d 21, with augmented cholesterol persisting in the male offspring at d 130. This was found to coincide with transcriptional repression of Cyp7a1, governed in part due to changes in posttranslational histone modifications surrounding the *Cyp7a1* promoter during postnatal life. Moreover, LP diet-induced decreases in the steady-state levels of Jmjd2a mRNA *in utero* may mediate the increased trimethylation of histone H3 [K9] associated with this promoter at postnatal d 21 and d 130. These identified epigenetic and transcriptional mechanisms help in further understanding the link between IUGR and long-term cholesterol

able at d 130 in the control and LP2 dietary regimes (data not shown). The hepatic Jmjd2d mRNA was low to undetectable at all ages examined. This LP diet-induced decrease in the fetal hepatic expression of this [K9] demethylase may initiate the hypermethylation of histone H3 surrounding the *Cyp7a1* promoter observed in LP2 offspring.

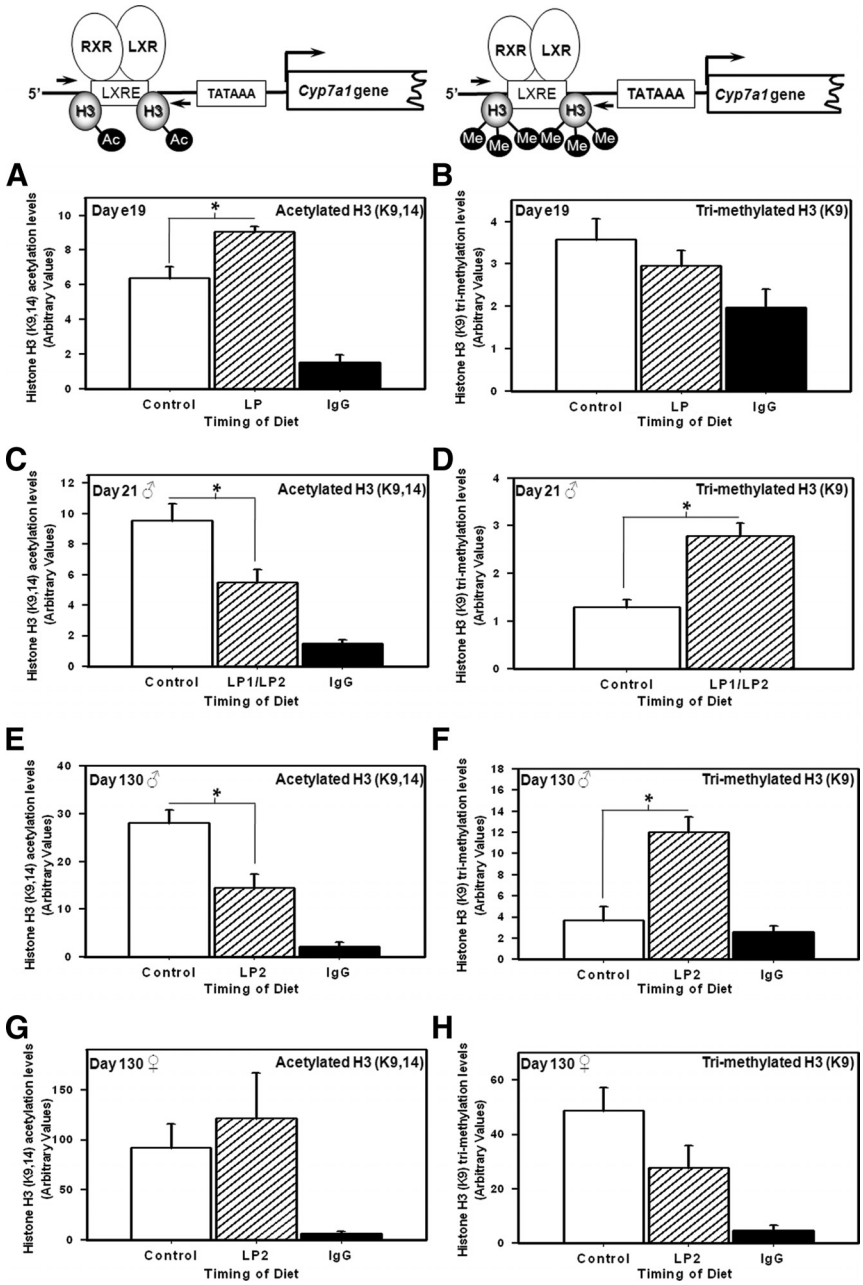

FIG. 4. The effect of LP2 dietary regime on the *in vivo* level of acetylated histone H3 [K9,14] (A, C, E, and G) and trimethylated histone H3 [K9] (B, D, F, and H) at the hepatic *Cyp7a1* LXRE site in rat offspring at embryonic d 19 (e19), male rat offspring at postnatal d 21, male and female rat offspring at postnatal d 130, respectively. ChIP was carried out on snap-frozen liver tissues by immunoprecipitation with antibodies for acetylated histone H3 [K9,14] and trimethylated histone H3 [K9]. Quantification analysis on the immunoprecipitated solubilzed DNA was carried out by real-time PCR via use of primers specific for the LXRE site on the promoter regions of hepatic *Cyp7a1*. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.*, Significant difference ($P < 0.05$); n =4–6/group, where each n represents an offspring derived from a different mother. RXR, Retinoid X receptor.

dysregulation. Uncovering these molecular mechanisms is critical given that 50% of metabolic syndrome-related diseases can be linked to adverse events *in utero* (10–12). In identifying some of the epigenetic mechanisms underlying the *in utero* origins of hypercholesterolemia, this study helps identify early life dietary and/or drug intervention strategies to lower cholesterol and reduce the incidence of the metabolic syndrome. Such strategies could reduce the long-term use of cholesterol-lowering drugs in adulthood.

As expected from previous studies by others (14–17) and in our laboratory (16–19), a LP diet resulted in reduced fetal to placental weight, neonatal growth, and liver to body weight ratio by postnatal d 21. Furthermore, this decrease in body weight persisted to adulthood (d 130) in offspring derived from a LP diet during pregnancy and lactation, with (*e.g.* LP2) or without (*e.g.* LP1) a restoration in maternal protein content after lactation. This compliments previous studies suggesting that a LP diet in early development has long-term implications on the overall growth of rats (31). The maternal protein restriction model of IUGR in rats is a relevant model to study the fetal origins of adult diseases given that amino acids play a critical role in fetal growth (37). Moreover, given that placental insufficiency in humans can produce protein deficiency in the fetus (38), this model share features in common with human placental insufficiency-IUGR. The impaired body weight is due to the LP content in the diet given that maternal food intake was similar in all dietary regimes. Interestingly, the liver to body weight ratio of offspring was not significantly different at postnatal d 130 in any dietary regime, suggesting that the impaired liver growth is ultimately recovered by adulthood, even though deficiencies in body weight may still be apparent.

As evident from their body weight at d 21, restoration of protein in the maternal diet at birth (*e.g.* LP3) resulted in complete catch-up growth in these offspring, with no changes in the liver to body weight ratio. Moreover, they also exhibited enhanced Cyp7a1 protein levels, contributing to the prevention of hypercholesterolemia. Given that during this period there is a high rate of replication, neogenesis, and

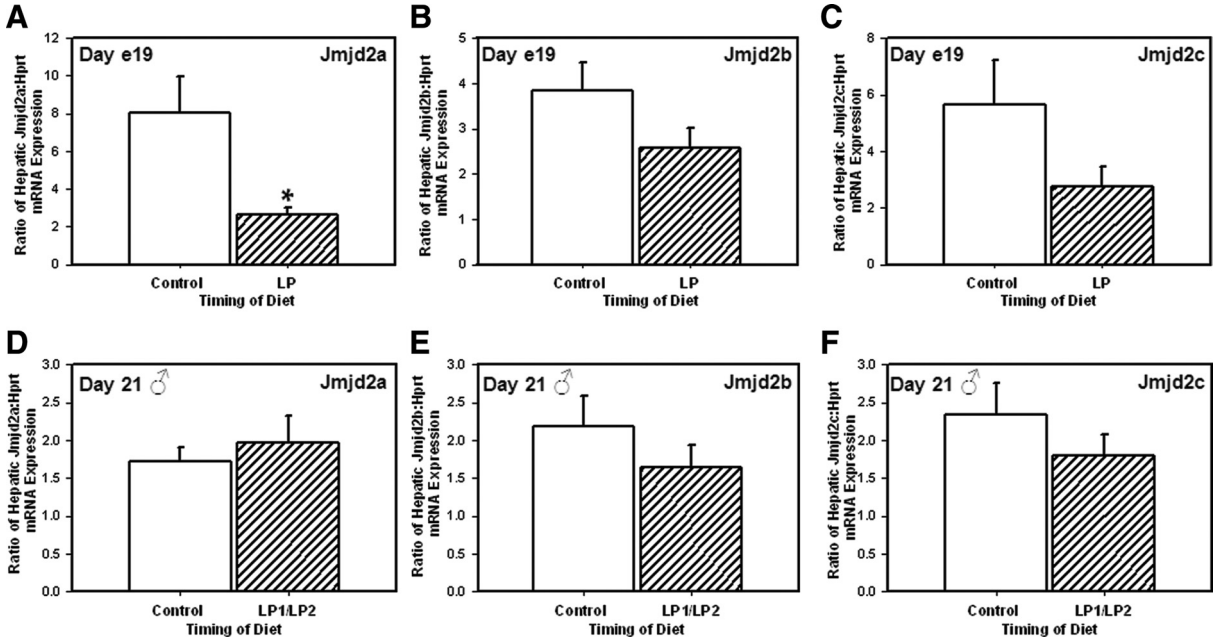

FIG. 5. Quantitative RT-PCR mRNA level analysis of Jmjd2a (A and D), Jmjd2b (B and E), and Jmjd2c (C and F) in the livers of rat offspring derived at embryonic d 19 (e19) and male rat offspring at postnatal d 21, respectively. RNA was extracted and reverse transcribed for quantitative RT-PCR. mRNA level expression was assessed via Q-RT-PCR using primers specific for Jmjd2a, Jmjd2b, Jmjd2c, and Hprt. The relative levels of each mRNA transcript were normalized to that of the levels of each Hprt mRNA transcript. Results were expressed as the mean ± SEM. *, Significant difference ($P < 0.05$); n = 4–9/group at embryonic d 19, where each n represents an offspring derived from two different mothers and n = 5–6/group at postnatal d 21, where each n represents an offspring derived from a different mother.

apoptosis leading to extensive liver remodeling in the newborn (39), it is not surprising that a restoration of protein in the maternal diet during this developmental window rescued hepatic growth and function. Other studies have implicated the neonatal period as a period of plasticity in liver development (23, 40). Raab *et al.* (23) have demonstrated that in IUGR rats derived from uterine-ligated dams, neonatal administration of Exendin-4 (a GLP-1 analog), prevents the development of hepatic oxidative stress and insulin resistance. Further understanding the molecular mechanisms underlying this plasticity could lead to improved therapies designed to enhance hepatic growth and function long term.

A LP diet during pregnancy and lactation (*e.g.* LP1/LP2) was associated with hypercholesterolemia (~4.5 mM) at postnatal d 21 in both sexes. At this age, hepatic Cyp7a1 expression in these offspring was reduced two thirds compared with control. This is intriguing given that Cyp7a1 has been previously demonstrated to display maximal expression at postnatal d 21 (41, 42). A LP1/LP2-induced increase in circulating cholesterol by 3 wk of age is of considerable interest with regards to the early development of cardiovascular disease. Interestingly, when a LP diet was fed to nursing mothers fostering control diet offspring, circulating cholesterol levels was not significantly different, highlighting the importance of the *in utero* environment in the long-term regulation of cholesterol levels. In LP2 offspring at d 130, circulating and hepatic cholesterol was significantly higher (~30%) in the male LP2 offspring. The decrease in Cyp7a1 is likely the major contributor to the observed 30% increase in hepatic cholesterol given that there was no difference in the protein levels of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (involved in cholesterol synthesis) and the LDL receptor (involved in cholesterol flux).

The augmented circulating and hepatic cholesterol observed in these LP2 offspring is similar to what is observed in *Cyp7a1*-deficient mice and in humans with a mutation in *CYP7A1* (5, 43). This elevation in baseline cholesterol in combination with other diet and lifestyle factors (*i.e.* high-fat diet) could make these animals more susceptible to hypertension and cardiovascular disease. For example, LP2 female offspring that were fed a control diet higher in carbohydrates after lactation exhibited elevated systolic blood pressure at 1 yr of age (33). Although males were not examined in that study, it remains possible from our study that the augmented cholesterol from as early as 3 wk could collectively contribute to a similar or worse phenotype. Moreover, the combination of impaired glucose tolerance (31), visceral obesity (29), and augmented cholesterol in these LP2 offspring would lead toward the development of metabolic syndrome.

It is interesting to note that whereas LP2 offspring had augmented cholesterol at d 130, neither LP1 nor LP3 offspring exhibited this phenotype. This is likely due to the fact that a LP environment during development results in adap-

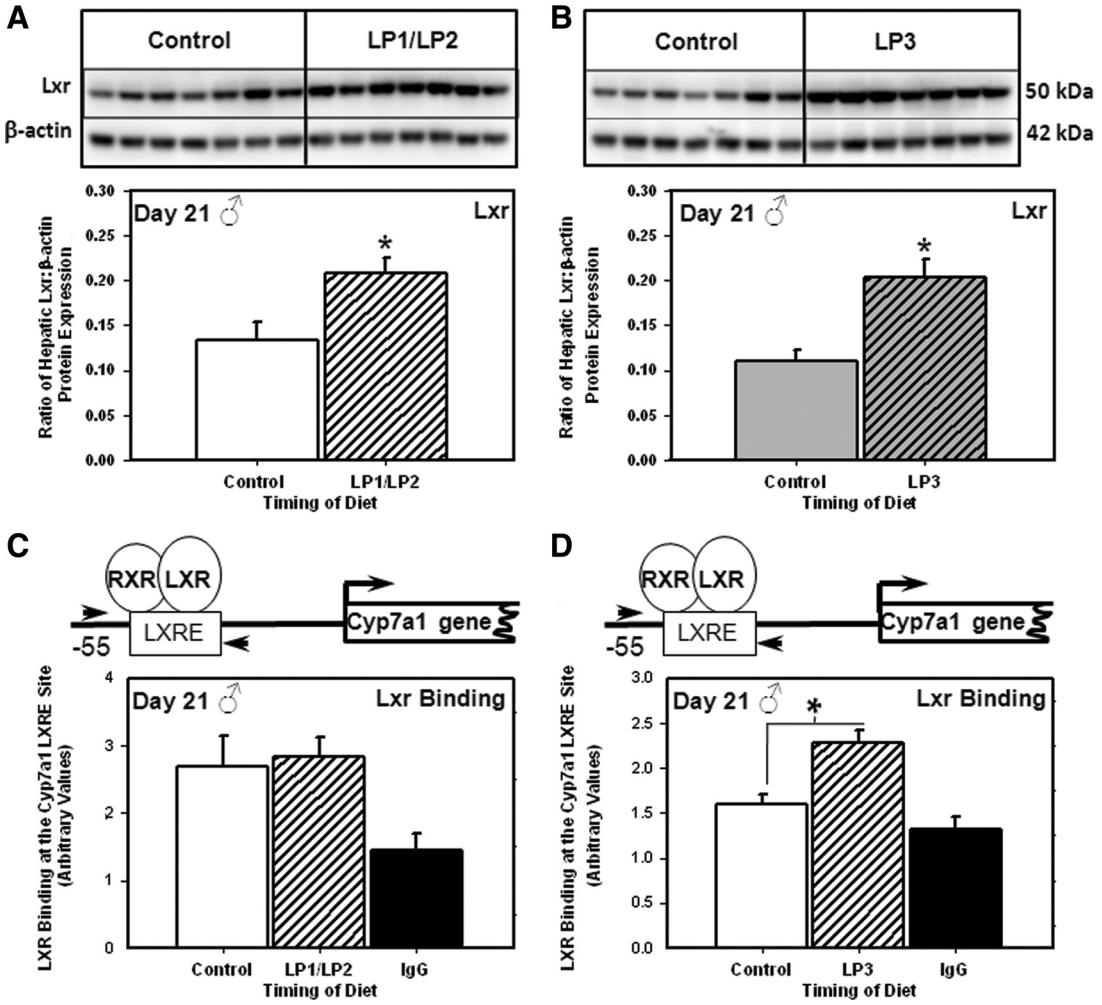

FIG. 6. The effect of LP1/LP2 (A and C) and LP3 (B and D) dietary regime on hepatic LXRα protein and binding levels to *Cyp7a1* LXRE site in rat offspring at postnatal d 21. Relative hepatic LXRα protein levels were determined using Western blot analysis. Total protein was isolated and LXRα protein was detected on a Western blot using the LXRα primary antibody. The LXRα protein level was quantified using densitometry and normalized to that of β-actin protein levels. Relative hepatic LXRα binding levels to *Cyp7a1* LXRE site were determined using ChIP analysis. ChIP was carried out on snap-frozen liver tissues by immunoprecipitation with antibody specific for LXRα. Quantification analysis on the immunoprecipitated solubilized DNA was carried out by real-time PCR via use of primers specific for the LXRE site on the promoter regions of hepatic *Cyp7a1*. The relative level of immunoprecipitated genomic DNA was normalized to the total genomic DNA. Results were expressed as the mean ± SEM.*, Significant difference ($P < 0.05$); n =4–7/group, where each n represents a single offspring derived from a different mother. RXR, Retinoid X receptor.

tations that are better suited to a similar environment later on in life (44). Therefore, when a switch is made to a 20% protein diet after liver development (45) (*e.g.* LP2), the offspring become more susceptible to physiological disorders. It was surprising to find that the LP3 animals had normal circulating cholesterol in our study, given previous work that has demonstrated that rapid catch-up growth in humans is more tightly associated with components of the metabolic syndrome (46). The answer may be due to the differences in liver development and the duration of the low-protein insult. Liver development in rodents and humans consists of embryonic cell specification, budding, and then differentiation (45). In rodents, liver differentiation develops later in gestation with a large portion of it occurring in postnatal life. In contrast, liver differentiation in humans is already occurring by early to midgestation (45). Therefore, at birth the rat liver is less mature and has greater plasticity than the human liver. This makes the rat liver more susceptible to further insult or recovery during that period of neonatal life. This plasticity in liver development is evident in the LP3 offspring whereby Cyp7a1 expression was restored at postnatal d 21 due to increases in LXRα activation. This is in contrast to the LP2 offspring, which did not exhibit alterations in LXRα binding to the *Cyp7a1* promoter. Collectively, this would suggest that it is the difference in the duration of the dietary LP insult (LP2 = 43 d *vs.* LP3 = 21 d) throughout the crucial stages of rat liver development that likely plays a greater role in long-term liver impairment than catch-up growth.

Epigenetic mechanisms have been previously demonstrated to underlie developmental programming of gene transcription. However, investigation of the epigenetic mechanisms linking an *in utero* insult to the development of disease in postnatal life is limited (18, 19, 23, 24). Because a conserved CpG island was not found in the promoter region of the human and rodent *Cyp7a1*, it is more likely that posttranslational histone modifications may influence its long-term expression compared with DNA methylation. In the present study, significant decreases in the acetylation and increases in methylation of histone H3 [K9], promoting a repressive chromatin environment, were observed surrounding the hepatic *Cyp7a1* promoter in LP2 male offspring at postnatal d 21. This persisted into adulthood (d 130), long after the switch to a control diet. The observed repressive chromatin environment correlated with decreased recruitment of RNA polymerase II, diminished Cyp7a1 protein levels, and the observed increases in circulating cholesterol. This is not surprising because trichostatin A, a histone deacetylase inhibitor, has been previously demonstrated to increase Cyp7a1 expression and cholesterol metabolism when administered in mice (47). It is interesting to note that the significant increase in histone H3 trimethylation at postnatal d 21 was preceded by a decrease in the expression of the [K9] demethylase Jmjd2a during fetal life. This suggests that an early loss of demethylation in the fetal liver, due to a LP diet, may initiate the enhanced trimethylation of histone H3 observed in LP2 offspring.

Whereas an increase in the acetylation of histone H3 [K9,14] was observed surrounding the *Cyp7a1* promoter at embryonic d 19, recruitment of RNA polymerase II to its promoter and Cyp7a1 protein levels were modestly reduced. Moreover, increases in the overall trimethylation of histone H3 [K9] were not observed at this time in development. Therefore in fetal life, the decreased expression of Cyp7a1 is likely also due to transcriptional mechanisms. Recently, it was demonstrated that a low-protein diet in mice led to decreased expression of LXRα and certain downstream target genes at embryonic d 19.5 as a result of increased DNA methylation at *LXRα* promoter (22). Therefore, the decrease in recruitment of RNA polymerase II to the *Cyp7a1* promoter in the fetuses derived from a LP diet could possibly be due to decreased recruitment of LXRα at the *Cyp7a1* LXRE site.

To date, very little is known about the links between malnutrition and epigenetic mechanisms during development, specifically with regards to the regulation of histone-modifying enzymes. One study looking at fetal surfactant protein A expression demonstrated that lower oxygen tension *in vitro* can promote alterations in these enzymes, leading to a repressive chromatin environment (48). Given that this LP model leads to asymmetric IUGR (20), and that this brain-sparing effect is associated with fetal hypoxia (49, 50), this could conceivably mediate the observed decrease in JmjD2a expression. As a result, this would lead to the observed hypermethylation and hypoacetylation surrounding the promoter of *Cyp7a1* during development.

Recent studies in animal models have suggested that perturbations to the maternal environment during pregnancy can lead to sex-specific, long-term consequences in postnatal life. For example, LP2 offspring exhibited insulin resistance (35) and visceral obesity (29) exclusively in the adult male offspring at postnatal d 130. In this study, circulating cholesterol increased whereas a concomitant decrease in Cyp7a1 protein levels and RNA polymerase II recruitment was observed only in the adult male offspring at postnatal d 130. Furthermore, the male-specific silencing of Cyp7a1 expression observed at d 130 was governed, in part, due to male-specific repressive changes in posttranslational histone modifications surrounding the active site of the *Cyp7a1* promoter. Given that cholesterol and Cyp7a1 expression are deregulated in both sexes at d 21, but only in males at d 130, it is unlikely that imprinting is involved. Instead, it is plausible that gender-specific hormonal differences after lactation may mediate these programmed effects. It has been postulated that sex steroids (*e.g.* estrogen) may offer the female some protective effects against the development of these disease processes, including elevated blood pressure (51). Evidence from this comes from the aromatase knockout (*ArKO*) mouse, which cannot synthesize endogenous estrogens due to disruption of the *Cyp19* gene (52). *ArKO* females, when challenged with a high-cholesterol diet, had higher circulating cholesterol and lower Cyp7a1 expression compared with wild-type females and males of either genotype (52), where estrogen replacement reversed the hepatic steatosis (53). However, the stimulatory effects of synthesizing estrogen may be only part of the reason for the sexual dimorphism observed in LP2 offspring. Given that LP2 males have suppressed levels of circulating testosterone compared with control males (35), it is conceivable that the loss of this male sex steroid may also underlie the male-specific impairment of Cyp7a1 and cholesterol catabolism. Aside from sex steroids, another important difference in LP2 males and females in that study was circulating basal insulin concentrations. LP2 male offspring at postnatal d 130 have 2-fold higher levels of circulating insulin (35), which has been previously demonstrated to inhibit Cyp7a1 in both the rat hepatocytes and streptozotocin-induced diabetic rats via decreases in the binding of the transcription factors FoxO1 and Smad3 to the promoter of *Cyp7a1* (54). Moreover, stud-

ies have now established that these hormones can influence posttranslational histone modifications. For example, the estrogen receptor has been demonstrated to bind to coregulators (*i.e.* CREB-binding protein/p300, steroid receptor coactivator-1, steroid receptor coactivator-2) that enhance promoter-specific histone H3 acetylation, leading to an active chromatin state surrounding the promoter regions of estrogen-responsive genes (55–57). Therefore in LP2 females, it is conceivable that their histone code was altered at the promoter of *Cyp7a1* via the actions of these hormones.

In conclusion, our results demonstrate that LP diet-induced IUGR leading to augmented cholesterol in postnatal life results from permanent epigenetic silencing of the hepatic *Cyp7a1* promoter, via posttranslational histone modifications. Moreover, our study identifies the *in utero* environment as a novel risk factor for cholesterol dysregulation and aids our understanding of why a prevalence of metabolic syndrome-related diseases can be linked to adverse events in fetal life. Given the plasticity of the liver in fetal and neonatal life, further studies will undoubtedly lead to early life dietary and/or drug intervention strategies to lower cholesterol and reduce the incidence of the metabolic syndrome in adulthood.

Materials and Methods

Animals and dietary regimes

All procedures were performed in accordance with the guidelines set by the Canadian Council of Animal Care and upon approval of the Animal Care Committee of the University of Western Ontario. Female and male Wistar rats at breeding age (250 g) were purchased from Charles River (La Salle, St-Constant, Quebec, Canada). These rats were housed in individual cages and maintained at room temperature on a 12-h light, 12-h dark cycle. For 3 wk, these rats were left to acclimatize to the animal care facility, and their reproductive cycles were followed. At the onset of proestrus, these rats were mated. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning.

Upon confirmation of impregnation (gestation d 1), the rats were fed either a control diet containing 20% protein or a LP diet containing 8% protein. The LP diet contained similar fat content and was made isocaloric by the addition of carbohydrates (Bio-Serv, Frenchtown, NJ). At birth, the litter size was reduced to eight animals (four females and four males), with weights closest to the litter mean. This ensured a standard litter size for all mothers. Four different dietary regimes were administered to these offspring (Table 1). Offspring derived from a maternal LP diet were either administered the LP diet throughout postnatal life (LP1), until the end of lactation (LP2) or until birth (LP3). Otherwise, they were given a control diet.

Food and water was provided *ad libitum*, and food intake was recorded during pregnancy and in the offspring. At embryonic d 19, a subset of pregnant rats were killed, the weights of the fetus, placenta, and liver were measured, and the fetal liver was excised and frozen. Another subset of rats was allowed to deliver spontaneously. The growth curves and food intake of these offspring were monitored by measuring their body weight and food consumption every third day. At both postnatal d 21, the selected time for weaning, and at postnatal d 130, the pups were also killed for blood and medial lobe liver tissue analysis.

Plasma lipid measurements

Measurements of total cholesterol and triglycerides from plasma of all blood samples were automatically detected using the COBAS analyzer (Roche, Mississauga, Ontario, Canada) at St. Joseph's Health Care (London, Ontario, Canada). For the measurement of circulating cholesterol, cholesterol esters were cleaved by cholesterol esterase and subsequently converted to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. A colorimetric assay resulting from breakdown of hydrogen peroxide via the Trinder reaction allowed for the quantification of cholesterol levels.

Hepatic cholesterol measurements

To determine the total amount of cholesterol present in hepatic tissue, a chloroform-methanol (1:1) extraction was performed to extract and separate total cholesterol and phospholipids from aqueous matter. After samples were dried, the total amount of cholesterol was determined by the Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA), which produces resorufin as a cholesterol byproduct. This was measured using a fluorescence spectrometer at 590 nm.

Real-time PCR analysis

Total RNA from Wistar rat medial lobe liver tissue was extracted at embryonic d 19, postnatal d 21, and postnatal d 130 by the one-step method of Chomczynski and Sacchi (58) (TRIzol, Invitrogen). RNA was treated with deoxyribonuclease to remove any contaminating DNA. Of the total RNA, 4 μg were reverse transcribed to cDNA using random primers and Superscript II RNase H-reverse transcriptase (Invitrogen). Primer sets directed against rat Jmjd2a, Jmjd2c, Jmjd2d, β-actin, and Hprt (rRNA) were generated via Primer Express software (PE Applied Biosystems, Boston, MA) based on published sequences (Table 2). The relative abundance of each transcript was determined by real-time quantitative PCR as previously published (59). For the quantitative analysis of mRNA expression, the Bio-Rad CFX384 Real Time System was employed using the DNA binding dye IQ

TABLE 2. Real-time PCR Primers

Gene	Primer (5′-3′)	Reference no.
Jmjd2a	Forward, GCG AGC AGG AGC TGG CAG AG	NM 001107966
	Reverse, AAC ACC AGC GGG TGA TGG CG	NM 001107966
Jmjd2b	Forward, GCG AGC TGG TGG AGC TGC GG	BC161813
	Reverse, GGG ACC GTA CCC TCT TGG GC	BC161813
Jmjd2c	Forward, ATG GAG GAG TTT CGG GAG TT	BC158850
	Reverse, CAT GGG CTT TTT CTG GAT GT	BC158850
Jmjd2d	Forward, GGG GCA GCC ACG AGC TTT CC	NM 001079712
	Reverse, GGG CAT CAG CTC AGT CAG GG	NM 001079712
Hmg-CoA	Forward, TAC ATC CGT CTC CAG TCC AAA A	NM 013134
Reductase	Reverse, CAG GTT TCT TGT CGG TGC AA	NM 013134
Ldlr	Forward, GGG TTC CAT AGG GTT TCT GCT	NM 175762
	Reverse, TGG TAT ACT CGC TGC GGT CC	NM 175762
Hprt	Forward, TTG CTC GAG ATG TCA TGA AGG A	NM 012583
	Reverse, AGC AGG TCA GCA AAG AAC TTA TAG	NM 012583
β-actin	Forward, ACG AGG CCC AGA GCA AGA	NM 031144
	Reverse, TTG GTT ACA ATG CCG TGT TCA	NM 031144

SYBER green supermix (Bio-Rad). The cycling conditions were 50 C for 2 min, 95 C for 10 min, followed by 45 cycles of 95 C for 15 sec, and 60 C for 1 min. The cycle threshold was set at a level where the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. We calculated the relative fold changes using the comparative cycle times (Ct) method with rRNA (Hprt) or β-actin as the reference guide. Over a wide range of known cDNA concentrations, all primer sets were demonstrated to have good linear correlation (slope = −3.4) and equal priming efficiency for the different dilutions compared with their Ct values (data not shown). Given that all primer sets had equal priming efficiency, the ΔCt values (primer internal control) for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared with calibrator was determined by the formula, $2^{\Delta\Delta Ct}$, in which ΔΔCt is the calibrated Ct value.

ChIP

ChIP was performed on snap-frozen medial lobe liver tissue excised at embryonic 19, postnatal d 21, and postnatal d 130 from male and female offspring derived from the control or LP2 dietary regime. ChIP was performed using a modification (60) of previously published methods (61). Briefly, a small piece of snap-frozen liver was homogenized and incubated with 1% formaldehyde for 10 min at room temperature to cross link proteins and DNA. Cross-linking was terminated by the addition of glycine (0.125 M, final concentration). The liver tissue was washed once with cold PBS and placed in 500 μl of sodium dodecyl sulfate (SDS) lysis buffer (Millipore, Etobicoke, Ontario, Canada) with protease inhibitor cocktail (Roche). The lysates were sonicated on ice to produce sheared, soluble chromatin. The lysates were diluted 10 times with the addition of ChIP dilution buffer (Millipore) and aliquoted to 400-μl amounts. Each of the aliquots was precleared with protein A/G Plus agarose beads (40 μl, Millipore) at 4 C for 30 min. The samples were microfuged at 12,500 rpm to pellet the beads, and the supernatant containing the sheared chromatin was placed in new tubes. The aliquots were incubated with 4 μg of antibodies against RNA polyermase II (catalog no. 05-623B), acetylated histone H3 (K9,14, catalog no. 05-399), trimethylated histone H3 (K9, catalog no. 07-442, all from Millipore), or LXRα (catalog no. sc-13068x, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 C overnight. Two aliquots were reserved as controls, one incubated without antibody and the other with nonimmune IgG (Millipore). Protein A/G Plus agarose beads (60 μl) were added to each tube, the mixtures incubated for 1 h at 4 C, and the immune complexes collected by centrifugation. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in wash buffer I (20 mM Tris-HCl, pH 8.1; 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), wash buffer II (same as I, except containing 500 mM NaCl), wash buffer III (10 mM Tris-HCl, pH 8.1; 1 mM EDTA; 1% Nonidet P-40; 1% deoxycholate; 0.25 M LiCl), and in 2× Tris-EDTA buffer. The beads were eluted with 250 μl elution buffer (1% SDS, 0.1 mM $NaHCO_3$ + 20 μg salmon sperm DNA) (Sigma-Aldrich, Oakville, Ontario, Canada) at room temperature. This was repeated once and eluates were combined. Cross-linking of the immunoprecipitated chromatin complexes and input controls (10% of the total soluble chromatin) was reversed by heating the samples at 65 C for 4 h. Proteinase K (15 μg, (Invitrogen) was added to each sample in buffer (50 mM Tris-HCl, pH 8.5; 1% SDS; 10 mM EDTA) and incubated for 1 h at 45 C. The DNA was purified by phenol-chloroform extraction and precipitated in EtOH overnight at 20 C. Samples and input controls were diluted in 10–100 μl Tris-EDTA buffer just before PCR. Real-time PCR was employed using forward (5′-TGCTTTGGTCACTCAAGTTCA-3′) and reverse (5′-GCAATTCCCCAAATCAAAGA-3′) primers that amplify a *−164*-bp to *+65*-bp region encompassing the TATA box and the *Cyp7a1* initiation site (62), forward (5′-CGT-AGCTCAGGCCTCTGCGCCCTT-3′) and reverse (5′-CTG-GCACTGCAC-AAGAAGATGCGGCTG-') primers that amplify a *−123*-bp to *+53*-bp region encompassing the TATA box and the rat *Gapdh* initiation site (63), and forward (5′-GGC-CGGGTAATGCTATTTTT-3′) and reverse (5′-CCGAAA-CAGTGGGTCTGACT-3′) primers that amplify a *−156*-bp to *+25*-bp region encompassing the rat *Cyp7a1* LXRE site (−128 bp to −81 bp) and the TATA box (PE Applied Biosystems, Boston, MA) (62). Using serial dilutions of rat liver chromosomal DNA, these primers were demonstrated to have equal efficiency in priming to their target sequences. Primers against distal regions of the *Cyp7a1* promoter were employed to ensure this region has the greatest recruitment of RNA polymerase II.

Preparation of tissue membrane extracts and immunoblot analysis

Wistar rat liver tissue membrane extracts were prepared using modifications of previously published methods (64). Briefly, a small piece of snap-frozen medial lobe liver tissue was homogenized in a 1.2 ml of buffer A solution (250 mM sucrose, 2 mM $MgCl_2$, 20 mM Tris-HCl at overall pH 7.5) with protease inhibitor cocktail (Roche). The homogenate was centrifuged at 2000 × *g* for 10 min at 4 C. The supernatant was retained and buffer A was added. This was centrifuged at 120,000 × *g* for 45 min at 4 C, and the pellet was retained as the membrane fraction, which was resuspended in 200 μl of buffer B (80 mM NaCl, 2 mM $CaCl_2$, 1% Triton X-100, 50 mM Tris-HCl at over-all pH 8) with protease inhibitor cocktail (Roche). Equal concentrations of membrane proteins normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI), were fractionated in gradient polyacrylamide gels (Invitrogen) and transferred onto polyvinylidenedifluoride membrane (Millipore). Blots were probed using Cyp7a1 rabbit polycolonal antibody (1:500, catalog no. sc-25536, Santa Cruz Biotechnology), LXRα (catalog no. sc-13068x, Santa Cruz Biotechnology), and monoclonal horseradish peroxidase-conjugated β-actin (1:50,000, catalog no. A3854, Sigma-Aldrich) diluted in 5% milk-1× Tris-buffered saline-Tween 20 buffer and with horseradish peroxidase-conjugated donkey antirabbit IgG (1:10,000, catalog no. 711-035-152, Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in 5% milk-1× Tris-buffered saline-Tween 20 buffer as the secondary antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA).

Statistics

All results were expressed as the mean of arbitrary values ± the SEM. The significance of differences ($P < 0.05$) between mean values was evaluated using the unpaired Student's *t* test for results from quantitative RT-PCR, ChIP, and immunoblot analysis. Two and one-way ANOVA followed by a Bonferroni's Multiple Com-

parison *post hoc* test, was used to evaluate significance of differences for results comparing the effect of all the dietary regimes.

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