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Chronic Maternal Protein Deprivation in Mice Is Associated with Overexpression of the Cohesin-Mediator Complex in Liver of Their Offspring[1–3]

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Abstract

Epigenetic mechanisms may play an important role in the developmental programming of adult-onset chronic metabolic diseases resulting from suboptimal fetal nutrition, but the exact molecular mechanisms are incompletely understood. Given the central role of the liver in metabolic regulation, we investigated whether chronic maternal dietary protein restriction has long-term effects on liver gene expression in the offspring. We fed adult C57BL/6J dams ad libitum an 8% maternal low-protein (MLP) or 20% protein control diet (C) from 4 wk prior to mating until the end of lactation. Male pups were weaned to standard nonpurified diet and singly housed at 21 d of age (d 21). Body weights were followed to 1 y of age (1 y). At d 21 and 1 y, organs were quantitatively dissected and analyzed. MLP offspring had significantly lower body weights at all ages and significantly lower serum activity of alanine aminotransferase and lactate dehydrogenase at 1 y. Gene expression profiling of liver at 1 y showed 521 overexpressed and 236 underexpressed genes in MLP compared to C offspring. The most important novel finding was the overexpression of genes found in liver that participate in organization and maintenance of higher order chromatin architecture and regulation of transcriptional activation. These included members of the cohesin-mediator complex, which regulate gene expression by forming DNA loops between promoters and enhancers in a cell type-specific fashion. Thus, our findings of increased expression of these factors in liver of MLP offspring implicate a possible novel epigenetic mechanism in developmental programming. J. Nutr. 141: 2106–2112, 2011.

Introduction

It is becoming increasingly evident that epigenetic mechanisms may play an important role in the developmental programming of adult-onset chronic diseases (1,2). Nutrition is known to be particularly important in the development of these disorders (3,4), which was first proposed by Hales et al. (5) and Barker (6). This observation formed the basis for the Developmental Origins of Health and Disease hypothesis, which suggests that environmental factors at critical developmental stages can alter later disease susceptibility (7). The exact molecular mechanisms and alterations in gene expression are still incompletely understood.

There are several proposed models that seek to explain the molecular mechanisms underlying the relationship between maternal nutrition and fetal or developmental programming. Historical data from the Dutch famine during World War II suggested a direct link between maternal nutrition and disease susceptibility in later life (8). In animal models where low-protein diets were administered during gestation and lactation, the responses observed in the offspring included permanent growth restriction (9), impaired glucose tolerance (10), changes in hepatic enzymes (11,12), insulin sensitivity (13), and a reduction in skeletal muscle mass (9). It has also been reported that maternal protein deficiency may result in changes in methylation and expression of genes (12,14,15) in the liver of their offspring by epigenetic phenomena.

Although genome-wide alterations in DNA methylation or histone modifications are the best known and studied epigenetic mechanism, it is now known that other levels of epigenetic reprogramming, involving higher-order chromatin conformation, are

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[3] Supplemental Figures 1–7 and Supplemental Tables 1–4 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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layered upon these chromatin modifications. Recently, interest has grown in a novel role for the cohesin complex in the reorganization of chromatin architecture to influence gene expression. Besides its well-known functions in chromosome segregation (16–18), transcriptional termination (19), and DNA damage repair (20), the cohesin complex was also described to mediate transcriptional insulation by CTCF[9] (21) and regulate tissue-specific transcription in a CTCF-independent manner (22). It was also reported that mediator and cohesin complexes cooccupy different promoters in different cells, thus generating cell type-specific DNA loops linked to the gene expression program of each cell (23).

Materials and Methods

Animals and tissue collections. To mimic chronic maternal dietary protein restriction in humans, 8- to 12-week-old C57BL6/J virgin dams consumed ad libitum an 8% [TD93033, Harlan Teklad; maternal low-protein (MLP) group; n = 9] or 20% protein diet (TD91352, Harlan Teklad; control (C) group; n = 9) 4 wk prior to timed mating. The composition of both diets is described in **Supplemental Table 1**. Litters were culled to 6 pups/dam on d 3 and male pups were weaned to laboratory nonpurified diet (Picolab Rodent Diet 20–5053, Labdiet) and singly housed on d 21. Body weights were measured weekly until 3 mo of age, then monthly up to 1 y. Mice were killed at d 21 and 1 y by cervical dislocation after isoflurane anesthesia. Organs and hind leg muscles were dissected, weighed, and snap-frozen in liquid nitrogen and stored at −80°C; tibia and femurs were dissected and their length measured using Vernier calipers. The protocol was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. All applicable institutional and governmental regulations concerning the ethical use of animals were followed.

GTT. To examine the effect of chronic maternal protein deprivation on glucose homeostasis, we performed GTT following 16 h of feed deprivation at d 21, d 183, and 1 y. Blood glucose was measured in a conscious state from tail vein bleeds 15, 30, 60, 90 and 120 min. after an i.p. glucose (G8270, Sigma Aldrich) injection of 1 g dextrose/kg body weight as previously described (24).

Serum chemistry tests. Serum of mice at 1 y was collected, flash frozen, and stored at −80°C until further analysis. Chemistry tests (AST, ALT, alkaline phosphatase, LDH, albumin, globulin, total protein, albumin:globulin ratio, creatinine, blood urea nitrogen, creatinine kinase, calcium, phosphorus, direct bilirubin, indirect bilirubin, total bilirubin, LDL-, HDL-, VLDL-, and total cholesterol, TG, cholesterol: HDL) on 9 mice from each group were performed by the Comparative Pathology Laboratory at the Center for Comparative Medicine at Baylor College of Medicine.

DNA and RNA isolation and manipulation. RNA was extracted from the liver of the 1-y offspring using the RNeasy Mini kit (74104; QIAGEN) from the muscle with the miRNeasy Mini kit (217004; QIAGEN). DNA was isolated with the Gentra Puregene Tissue kit (158622; QIAGEN). RT was performed with the qScript cDNA Supermix (Quanta BioSciences).

Expression arrays. To study the transcriptome of the skeletal muscle and liver, genome-wide RNA expression profiling was performed on 3 samples from each group on the Affymetrix Mouse Gene 1.0 ST Arrays (Affymetrix). Probes were prepared and hybridized to the array according to the manufacturer's instructions at the Baylor College of Medicine Microarray Core Facility.

Data analysis and integration. Data from RNA expression array experiments were analyzed with the Partek Genomic Suites Software (version 6.5, Partek) as previously described (25,26). The significantly over- and underexpressed genes in MLP compared to C cohort in each muscle type (soleus at d 21 and 1 y and tibialis anterior at 1 y) were detected using PGS with 2-way ANOVA (muscle type and treatment group as factors; $P < 0.01$) and ± 2-fold in MLP compared to C groups in each muscle type were considered significant. Clustering analysis of the muscle samples was performed on the imported data across all probes in the individual arrays using the PGS hierarchical Euclidean clustering and principal component analysis tool with the default limits. The significantly over- and underexpressed genes in livers of 1-y-old offspring of dams fed the MLP diet compared to C were detected using 1-way ANOVA at $P < 0.01$ and 1.5-fold enrichment with an false discovery rate < 0.1.

Network identification and canonical pathway analysis. Genes demonstrating significantly altered expression in the MLP group were analyzed using Ingenuity Pathways Analysis software version 8.7 (Ingenuity Systems) as previously reported (27). Briefly, data were imported with the Core Analysis tool; the analysis was performed using the Ingenuity Knowledge Database selecting all species with stringent filter, all tissue and cell lines, and direct and indirect interactions.

RT-qPCR. We used RT-qPCR to confirm the microarray data. Sequence-specific custom primers designed with Primer Express 3.0 (Applied Biosystems) were used for amplification and detection using the Perfecta Sybr Green Fastmix (Quanta BioSciences) on the StepOnePlus Real-Time PCR system (Applied Biosystems). Expression changes were quantified relative to 18S RNA as an endogenous control, using the $2^{-\Delta\Delta Ct}$ method. No differences in 18S RNA expression were detected among the groups. Sequences for primers used can be found in **Supplemental Table 2**.

Methylation analysis. To validate the methylation status in a set of genes that were differentially expressed, we performed the quantitative analysis of CpG with the EpiTYPER system using the MassARRAY system (Sequenom) at the Analytical Genetics Technology Centre (Princess Margaret Hospital) per the manufacturer's instructions. The gene-specific bisulfite primers were designed using the MethPrimer Software (28). Each analysis was performed in triplicate.

Histological analysis. Samples were flash frozen and stored at −80°C until histological processing performed by the Department of Pathology Core Services laboratory at Baylor College of Medicine. Sections were stained with hematoxylin-eosin, periodic acid-Schiff, and Oil Red O according to standard histological procedures. Histological analysis of the coded samples was performed by a pathologist who was blinded to the identity of the samples.

Statistical analyses. Multilevel analysis (29) and mixed effect models were used to describe changes in primary outcomes of interest (e.g. weight gain, GTT) and to compare the 2 study groups (8 and 20% protein diet) for these factors. A separate model was produced and fitted to each outcome. Within each model, we examined significance of within-subject effect and between subjects (between the 2 study groups). Interaction effects were included and tested. Changes over time were compared between groups using a likelihood ratio statistic, which follows a chi-square distribution. Adjustments were made for multiple comparisons using the Tukey test; differences were considered significant at $P < 0.05$. All analyses were performed using SAS.

Results

Body weight, muscle weights, and glucose tolerance. Offspring exposed to the MLP diet during the fetal and suckling

[9] Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, pups of dams fed the control diet from 1 mo before mating through lactation; CTCF, CCCTC-binding factor; GTT, glucose tolerance test; IPA, Ingenuity Pathway Analysis; LDH, lactate dehydrogenase; MLP, pups of dams fed the 8% low-protein diet from 1 mo before mating through lactation; PGS, Partek Genomic Suites.

periods had a lower body weight at d 21 ($P < 0.0001$) as well as 1 y ($P < 0.019$). When weight-over-time patterns were analyzed, MLP offspring weighed less at all analyzed ages between d 21 and 1 y ($P < 0.0012$) (**Supplemental Fig. 1***A*) and differed in standardized weight gain (all weights normalized to the mean d 21 C group weights; $P = 0.0006$) (Supplemental Fig. 1*B*). However, the weight change compared to the initial weight of the mice at d 21 (Supplemental Fig. 1C) did not differ between the 2 groups ($P = 0.52$).

Because it was previously reported that maternal dietary protein restriction may lead to impaired glucose tolerance (10,30,31), we performed i.p. GTT at d 21, d 183, and 1 y. In contrast to some previous studies (32) and despite selecting a mouse strain predisposed to development of glucose intolerance (33), MLP and C offspring did not differ at d 21 ($P = 0.58$), d 183 ($P = 0.99$), and 1 y ($P = 0.53$) (**Supplemental Fig. 2**).

At d 21, MLP offspring had significantly lower organ weights for kidneys ($P < 0.003$), heart ($P < 0.002$), and liver ($P < 0.034$) than C offspring. When adjusted for total body weight, the differences between the 2 groups were not significant, indicating that lower organ weights were proportional to the reduced total body weight. There was also a significant difference in kidney weights at 1 y ($P < 0.014$), which was proportional to the difference in total body weight (**Supplemental Fig. 3**).

Because the liver is one of the most complex organs in the body and a central regulator of numerous metabolic tasks, including carbohydrate and nitrogen metabolism, we examined the consequences of chronic gestational protein deprivation on several indices of hepatic function in the adult. Serum concentrations of LDH ($P < 0.04$) and ALT ($P < 0.006$) were lower and AST tended to be lower ($P = 0.07$) in the 1-y MLP offspring than in the C offspring. Histological analysis of fresh-frozen tissues stained with hematoxylin-eosin, periodic acid-Schiff, and Oil Red O identified no differences in the morphology of samples between the MLP and C groups.

Tibia lengths at d 21 ($P < 0.0001$), tibia and femur lengths at 1 y (tibia: $P < 0.01$; femur: $P < 0.05$), and weights of hind leg muscles at d 21 ($P < 0.001$) and 1 y ($P < 0.05$) were less in the MLP offspring compared to the C offspring. These differences persisted after the muscle weights were standardized to correct for the smaller overall body size in the MLP diet-exposed group by adjusting weights to total tibia and femur lengths. At d 21, absolute weights and weights adjusted for tibia length of the gastrocnemius, soleus, plantaris, quadriceps, tibialis anterior, and extensor digitorum longus muscles ($P < 0.001$) were lower in the MLP diet-exposed offspring. Weight differences adjusted for tibia and femur lengths persisted at 1 y for soleus, gastrocnemius, tibialis anterior, and extensor digitorum longus ($P < 0.05$) (**Fig. 1**).

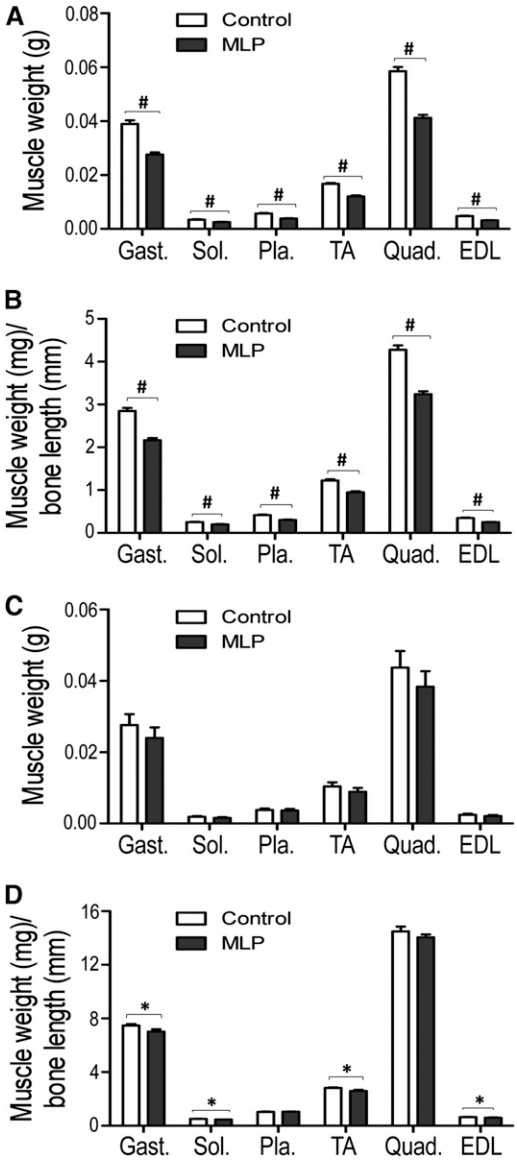

FIGURE 1 Absolute (*A,C*) and relative (*B,D*) hind leg muscle weights at d 21 and 1 y of male offspring of rat dams fed control or a low-protein diet from 1 mo before mating through lactation. In *B* and *D*, weights were adjusted to total tibia [gastrocnemius (Gast.), soleus (Sol.), plantaris (Pla.), tibialis anterior (TA), and extensor digitorum longus (EDL)] and femur quadriceps (Quad.) lengths. Values are mean ± SEM, $n = 6$ (C, 21 d), 19 (MLP, 21 d), or 15 (1 y). Symbols indicate that means differ, $*P < 0.05$; $\#P < 0.001$. MLP, pups of dams fed the 8% low-protein diet from 1 mo before mating through lactation.

Transcriptome of hind leg muscles. To study the possible gene expression differences in skeletal muscles of the offspring exposed to chronic maternal dietary protein deprivation in early development, we performed gene expression profiling at d 21 and 1 y on 2 hind leg muscles. Clustering analysis with the principal component analysis demonstrated an absence of clustering of the data from soleus at d 21 and 1 y and tibialis anterior at 1 y with regard to diet (**Supplemental Fig. 4***A*), which was also confirmed by Euclidean clustering (Supplemental Fig. 4*B*). There were very few differences in gene expression between the groups at the criterion used ($P < 0.01$, fold difference >2 by volcano plot analysis) (Supplemental Fig. 4*C–E*). Nevertheless, there were 6 significantly underexpressed genes at d 21 and 2 over- and 12 underexpressed genes at 1 y in soleus muscle of the MLP group relative to the C group. Confirmation of two hits with the highest fold difference could not be confirmed with RT-qPCR (data not shown).

Liver gene expression profiling. Total RNA from livers was hybridized to Affymetrix Mouse Gene 1.0 ST Arrays for the liver transcriptome study. The expression profile data confirmed distinct clustering of gene expression from the livers of the MLP diet-exposed offspring relative to livers of C diet-exposed offspring (**Supplemental Fig. 5**). There were 757 genes differentially expressed between the 2 groups using the following criteria: $P < 0.01$, false discovery rate < 0.1, and 1.5-fold change in expression. Of these, 521 genes were upregulated and

236 genes were downregulated in the MLP diet-exposed offspring. Next, we performed IPA of the 757 genes with significantly altered expression to identify the gene networks, pathways, and biological functions in the liver (27) that were preferentially affected by developmental exposure to the maternal low-protein diet. These 757 genes were significantly overrepresented within the following networks: embryonic and tissue development and antimicrobial response (network 1); gene expression, DNA replication, recombination and repair, and cell morphology (network 2); cell cycle, developmental disorder, and genetic disorder (network 3); cellular assembly and organization, genetic disorder, and metabolic disorder (network 4); and gene expression, tissue morphology, and cardiovascular disease (network 5) (**Supplemental Fig. 6**). IPA analysis also revealed the five biological functions that had the most genes with altered expression. These included cellular growth and proliferation, protein synthesis, posttranslational modification, protein folding, and RNA posttranscriptional modification (**Supplemental Table 3**).

The cohesin-mediator complex. Subsequently, to identify genes of particular interest for further characterization, we combined information about genes with the greatest differences in expression with the results of the IPA analysis to focus on those genes involved in the most significantly represented networks and pathways. We also considered known information on previously proposed or confirmed roles or associations with adult-onset disorders of interest: diabetes, obesity, and hypertension. The genes that were selected for validation and further analysis using these criteria are presented in **Table 1**. The genes that showed the most altered expression were involved in higher order chromatin structure and regulation (*Smarca5*, *Atrx*, *Arid4b*, *Smc1a*, *Smc3*, *Smc5*, *Smc6*, *Nipbl*, *Pcm1*) as well as transcriptional regulation (*Topi*, *Top2b*, *Smarca5*, *Med21*). We also confirmed altered expression of several genes already known to play a role in adult-onset disorders (*Rock1*, *Acp1*, and *Arid4b*).

RT-qPCR analysis was performed on genes exhibiting altered expression in livers of mice from the MLP diet-exposed group. We did not confirm altered expression for genes that were underexpressed in livers from MLP diet-exposed mice due to nearly undetectable expression levels for 9 of 11 studied genes in this category. For the 2 that had detectable expression by RT-qPCR, lower expression in liver of the MLP group was not confirmed (data not shown). In contrast, 12 of the 15 investigated overexpressed genes were validated (**Table 2**). Among these genes were the representatives of the core cohesin complex (*Smc1a*, *Smc3*, *Rad21*, *Stag2*; $P < 0.05$), members of the mediator complex (*Med4*, *Med8*) ($P < 0.05$), and *Nipbl* ($P < 0.05$), a cohesin-loading factor necessary for loading the cohesin complex onto chromatin. Recently, it was reported that mediator and cohesin protein complexes cooperate in the formation of enhancer-promoter loops, thus generating cell type-specific DNA loops linked to the regulation of gene expression (23). Other confirmed genes were involved in transcriptional regulation (*Acp1*, *Arid4b*, *Rock1*; $P < 0.05$) (**Supplemental Fig. 7***A*) and DNA repair (*Smc5*, *Smc6*; $P < 0.05$) (Supplemental Fig. 7*B*).

DNA methylation differences. Because DNA methylation has previously been shown to participate in gene expression changes induced by suboptimal nutrition (12), we investigated altered DNA methylation at CpG-rich sequences near a subset of the genes with altered expression in the livers of MLP diet-exposed mice. To perform this analysis, we used the EpiTYPER MassARRAY system to quantitate DNA methylation levels at CpG-rich sequences in known or predicted promoter regions upstream of 10 of the identified genes displaying the most significant differences in expression. This method uses base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight MS, previously used for SNP detection (34). Overall, we did not detect significant differences between the groups other than a few scattered CpG sites in 4 of the genes, with significantly lower methylation in the livers of the MLP diet-exposed mice compared to C (**Supplemental Table 4**).

Discussion

In this study, we examined the effects of chronic maternal protein deprivation on the offspring's phenotype and transcriptome. For this, we used a mouse model that mimics chronic dietary protein deprivation from before conception to the end of lactation in humans. This study allowed us to characterize the acute (d 21) and chronic phenotype (1 y) of the offspring. As we expected, the offspring of dams fed the MLP diet had lower body weights at d 21, which persisted until 1 y. The lower hind leg muscle masses when standardized to tibia and femur length likely contributed to the lower body weights, because the musculature comprises a large proportion of body mass. Our results indicate that even though all mice had free access to the same diet, MLP-exposed mice did not have "catch-up weight gain" in the first year, as has been previously described in other rodent models (9,10,35). We did not find significant weight differences among organs when adjusted for the difference in body weight. Although we utilized a strain of mice that is prone to developing diabetes (33,36), we did not detect any difference in the response to a glucose load in the MLP group compared to C. This finding was contrary to our expectations, because there are several publications describing altered glucose tolerance and insulin responsiveness in offspring born to mothers fed a protein-restricted diet during gestation (37,38), although characteristics such as sex, age, and the postweaning diet can influence the response (39,40). Conversely, other related studies report findings comparable to ours (41).

Decreased activity of LDH and ALT and the marginally significant reduction of AST suggest an effect of gestational dietary protein deprivation on the developmental programming of liver physiology. It is possible that gestational protein deprivation decreases the amino acid pool size, altering the catabolism of amino acids to pyruvate and oxaloacetate (42). Because these 2 molecules are directly connected and metabolized by LDH, ALT, and AST, we speculate that gestational protein deprivation may alter the developmental programming of the liver and thereby permanently alter the homeostasis of these enzymes.

Histological analysis of the liver did not reveal any morphologic differences between the 2 groups; this was not surprising, because maternal protein deprivation during gestation and lactation may be an environmental effect that is too subtle to cause permanent histological changes in the liver, even in the presence of altered gene expression.

To examine the acute and chronic effects of chronic maternal protein deprivation on the offspring's skeletal muscle, we performed gene expression analysis on soleus at d 21 and 1 y and on tibialis anterior at 1 y. Expansive data analysis revealed no significant differences in gene expression in the examined muscles between the MLP and C groups. These findings were also confirmed by RT-qPCR. We speculate that our approach may not be sensitive enough to detect the subtle gene expression

TABLE 1 Selected upregulated and downregulated genes in liver of 1-y-old mice exposed to chronic maternal protein deprivation[1]

Gene	CpG Island	Function	*P* value	Fold change
Hnmt	No	Histamine N-methyltransferase, DM, HTN, CAD	0.0096	2.248
Dlk1	Yes	delta-like 1 homolog (Drosophila) (imprinted gene)	0.0095	−1.548
Hrsp12	Yes	Heat-responsive protein 12 (DM)	0.0086	3.188
Tax1bp1	Yes	TRAF6-BP, apoptosis (HTN)	0.0085	2.193
Smc5	Yes	Structural maintenance of chromosomes, recruit cohesins to DSB	0.0083	2.209
Pcm1	Yes	Pericentriolar material 1, DM	0.0077	2.079
Dnajb6	Yes	DnaJ (Hsp40) homolog, subfamily B, member 6	0.0067	1.73
Ncl	Yes	Nucleolin	0.0062	3.048
Arid4b	Yes	RBP1L, subunit of the HDAC dependent SIN3A corepressor complex (HTN)	0.0060	2.491
Top1	Yes	DNA Topoisomerase 1	0.0058	2.965
Areg	Yes	Amphiregulin	0.0057	−1.548
Cntn2	Yes	Contactin 2 (axonal)	0.0049	−1.503
Top2b	Yes	Topoisomerase (DNA) II beta 180kDa	0.0048	2.177
Ube3a	Yes	Ubiquitin protein ligase E3A (imprinted gene)	0.0047	2.027
Tmf1	Yes	TATA element modulatory factor 1, transcriptional regulation (DM)	0.0047	1.941
Cox6c	Yes	Cytochrome c oxidase subunit VIc	0.0043	2.881
Tmod2	Yes	Tropomodulin 2	0.0043	−1.517
Nipbl	Yes	Nipped-B homolog (Drosophila)	0.0041	2.503
Chsy1	Yes	Chondroitin sulfate synthase 1	0.0040	−1.67
Mnx1	Yes	Motor neuron and pancreas homeobox 1	0.0039	−1.506
Phyhip	No	Phytanoyl-CoA 2-hydroxylase interacting protein	0.0038	−1.59
Med21	Yes	Mediator complex subunit 21	0.0037	2.219
Smc1a	Yes	Structural maintenance of chromosomes 1A	0.0035	1.743
Aire	Yes	Autoimmune regulator	0.0031	−1.611
Diras1	No	DIRAS family, GTP-binding RAS-like 1	0.0023	−1.562
Cdk5r2	Yes	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)	0.0023	−1.599
Il1r2	No	Interleukin 1 receptor, type II	0.0021	−1.582
Rock1	Yes	Rho assoc serine-threonine PK (HTN)	0.0020	3.437
Mxd3	Yes	MAX dimerization protein 3	0.0019	−1.518
Wnt7a	Yes	Wingless and int homolog 7A	0.0015	−1.505
B4galnt1	Yes	Beta-1,4-N-acetyl-galactosaminyl transferase	0.0014	−1.551
Acp1	Yes	Acid phosphatase 1 (obesity, T1DM[2], CVD[2])	0.0014	2.357
Rev3l	Yes	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	0.0010	1.543
Smc6	Yes	Structural maintenance of chromosomes, recruit cohesins to DSB	0.0008	2.225
Smc3	Yes	Component of cohesin complex	0.0007	2.803
Smarca5	Yes	SWI/SNF related regulator of chromatin	0.0004	2.505
Efna2	Yes	Ephrin A2	0.0003	−1.518
Atrx	Yes	Alpha thalassemia/mental retardation syndrome X-linked	0.0001	2.924
Col5a1	Yes	Collagen, type V, alpha 1	0.0001	−1.545
Hnrnpa3	Yes	Heterogeneous nuclear ribonucleoprotein A3	>0.0001	2.501

[1] Combined data analysis with PGS and IPA. CAD, coronary artery disease; CVD, cardiovascular disease; DM, diabetes mellitus; DSB, double-strand break; HDAC, histone deacetylase; HTN, hypertension; IPA, Ingenuity Pathway Analysis; PGS, Partek Genomic Suites; T1DM, type 1 diabetes mellitus.

changes responsible for the altered phenotypes; a more sensitive method, such as RNA sequencing, could address this issue. On the other hand, it is also possible that the gene expression changes that are initially responsible for the altered phenotype observed in the muscle are no longer present at the examined ages.

Expression profiling in the 1-y mouse liver revealed numerous genes with altered expression in the MLP group. Pathway analysis using IPA revealed that most of the significant hits were associated with chromatin regulation, higher order organization of chromatin architecture, chromosome integrity, transcriptional regulation, and metabolic disease. We acknowledge that results of our pathway analysis are influenced by the IPA software's Gene Ontology term assignment, depth of coverage of its associated mined database, and other possible factors that may influence pathway flux and the accuracy of literature mining. Nonetheless, it is one of the leading suites used for integrating biomolecular interaction networks with high-throughput expression data.

Further investigation of genes associated with higher order chromatin regulation revealed that there was increased expression of all members of the core cohesin complex in the MLP mice. Moreover, representatives of the mediator complex and an ancillary gene (*Nipbl*), which encodes a protein that is important for loading cohesins onto DNA, also showed higher expression. This altered expression of cohesins, mediators, and *Nipbl* is a novel observation that implicates a role for higher order chromatin regulation in the epigenetic modifications, which set up gene expression profiles in the liver; ultimately, this may

TABLE 2 Overexpression of genes of the cohesin-mediator complex in liver of 1-y-old mice following chronic MLP compared to C

Gene	Fold change	*P* value
Smc1a[1]	3.44	0.010
Smc3[1]	3.76	0.001
Rad21[1]	3.39	0.014
Stag2[1]	2.20	0.001
Med4[2]	2.72	0.027
Med8[1]	2.50	0.022
Nipbl[3]	3.84	0.037

[1] n = 5 (C, MLP). C, control group; MLP, maternal protein deprivation.
[2] n = 3 (C, MLP).
[3] n = 3 (C) or 4 (MLP).

contribute to the increased susceptibility to adult-onset metabolic disorders.

There are a growing number of reports highlighting the regulatory functions of the cohesin complex and their connections to physiological processes. One recent study revealed a relationship between NAD metabolism and the function of the DNA methylation-sensitive CTCF/cohesin complex in control of brain-derived neurotrophic factor expression in part by altering expression of cohesin components. This may have broad implications for metabolic effects on the regulation of gene expression (43). It has also been demonstrated that cohesin-mediator complexes form cell type-specific DNA loops with the contribution of the loading factor *Nipbl* to regulate gene expression in the cell (23). Other genes with confirmed altered expression are also implicated in chromatin and transcriptional regulation, chromosome integrity or previously have been implicated in adult-onset chronic disorders. We speculate that the altered expressions of these genes all participate in the developmental programming of adult-onset chronic disorders and that various epigenetic mechanisms cooperate to stabilize long-term global alterations in transcription.

One such mechanism that has been extensively studied is DNA methylation. We therefore investigated the methylation status of several selected genes with altered expression in livers of MLP offspring using the EpiTYPER MassARRAY system; this is a sensitive, accurate, and reliable technique that enables discrimination between methylated and nonmethylated samples and is informative in quantifying the methylation levels of DNA (44). Although various genes showed a small number of CpG sites with significantly different methylation in the MLP group, the analysis demonstrated an overall low methylation level at the CpG sites in a majority of the analyzed loci, without any significant difference between the groups. This finding is not surprising, because the majority of CpG islands in the genome are unmethylated. This observation may be due to the possibility that expression of these genes is regulated by other epigenetic mechanisms, such as histone modifications or the reorganization of higher order chromatin structure, as perhaps implicated from the gene expression profiling data in our current study.

We do not know if the changes in expression of these genes are specific to the dietary protocol used in the current study and if they are specific to the liver, or whether they highlight a more universal epigenetic mechanism that contributes to the developmental programming of health and disease by various forms of environmental exposures and stresses among different tissues. Our future aims are to examine this further by gene expression profiling in other tissues using the same animal model as well as different models. It will also be interesting to investigate the exact molecular mechanism by which the cohesin-mediator complex regulates gene expression in the liver of the MLP mice.

The altered expression of representatives of the cohesin-mediator complex and *Nipbl* in the offspring's liver resulting from chronic maternal dietary protein restriction is a novel finding. This discovery may contribute to a better understanding of the developmental genetic programming of adult-onset chronic disorders.

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