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Article

In Utero Undernutrition in Male Mice Programs Liver Lipid Metabolism in the Second-Generation Offspring Involving Altered *Lxra* DNA Methylation

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SUMMARY

Obesity and type 2 diabetes have a heritable component that is not attributable to genetic factors. Instead, epigenetic mechanisms may play a role. We have developed a mouse model of intrauterine growth restriction (IUGR) by in utero malnutrition. IUGR mice developed obesity and glucose intolerance with aging. Strikingly, offspring of IUGR male mice also developed glucose intolerance. Here, we show that in utero malnutrition of F1 males influenced the expression of lipogenic genes in livers of F2 mice, partly due to altered expression of *Lxra*. In turn, *Lxra* expression is attributed to altered DNA methylation of its 5′ UTR region. We found the same epigenetic signature in the sperm of their progenitors, F1 males. Our data indicate that in utero malnutrition results in epigenetic modifications in germ cells (F1) that are subsequently transmitted and maintained in somatic cells of the F2, thereby influencing health and disease risk of the offspring.

INTRODUCTION

Complex noncommunicable diseases, such as obesity, type 2 diabetes, or cardiovascular disease, have a heritable component that is not entirely attributable to genetic variation (Gluckman et al., 2007). Instead, nongenomic mechanisms, including epigenetics and mechanisms related to parental physiology or behavior, may play an additional role in mediating inheritance of disease risk (Youngson and Whitelaw, 2008). Increasing evidence suggests that environmental factors are relevant in influencing the inheritance of disease risk, especially when occurring during early stages of development, such as the fetal and/or neonatal periods. Among early environmental exposures, nutrition plays a key role predisposing to type 2 diabetes, not only to exposed individuals, but also to their offspring and, in a few striking examples, their grand-offspring (Patti, 2013). Offspring of people exposed to malnutrition in utero have increased prevalence of cardiovascular disease and type 2 diabetes risk (Painter et al., 2008; Veena et al., 2007; Veenendaal et al., 2013; Walker et al., 1998). Likewise, increased grand-paternal food supply during the prepubertal growth period augmented the risk of cardiovascular and diabetes-related death in their grandchildren (Bygren et al., 2001; Kaati et al., 2002; Pembrey et al., 2006). In agreement, in rodent models, nutritional imbalance during fetal and/or early neonatal development also increased the risk of obesity, glucose intolerance, insulin resistance, and type 2 diabetes in the following generation (Benyshek et al., 2006; Burdge et al., 2007, 2011; Dunn and Bale, 2009, 2011; Jimenez-Chillaron et al., 2009; King et al., 2013; Pentinat et al., 2010; Zambrano et al., 2006).

It has been proposed that transmission of such environmentally acquired phenotypes may be mediated by epigenetic mechanisms, which is conceptually termed as transgenerational epigenetic inheritance (Gluckman et al., 2007). This refers to the transmission of a specific phenotype to the next generation offspring via epigenetic modifications in the germline (Daxinger and Whitelaw, 2012). Yet, to really ascertain transgenerational epigenetic inheritance, the phenotypic changes should be studied up to the third-generation offspring (F3) (Figure S1 available online) (Jirtle and Skinner, 2007; Skinner, 2008). The reason is that when an F0 gestating mother is exposed to an environmental challenge, her embryos/fetuses (F1) and the already developing germline (that will give rise to the F2) are also directly exposed. This is what some authors define as a multigenerational effect, rather than a transgenerational effect. Hence, for accuracy purposes, we will maintain this conceptual distinction in this article.

In addition, to further evaluate whether epigenetic mechanisms mediate a specific transgenerational (or multigenerational)

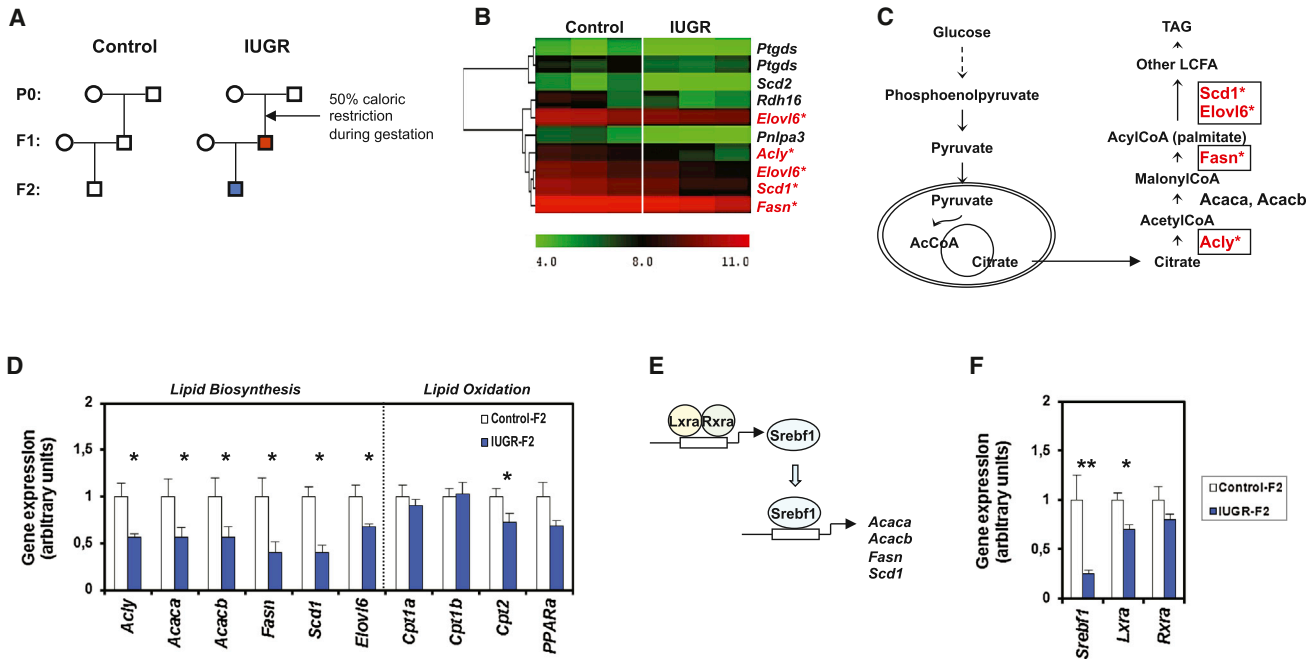

Figure 1. The Expression of Lipogenic Genes Is Altered in Livers from IUGR-F2 Male Mice

(A) Breeding strategy to generate the first- and second-generation offspring.

(B) Heat map including the statistically significantly expressed genes that belong to the lipid biosynthetic process Gene Ontology. The genes that regulate TAG synthesis from citrate are highlighted in red.

(C) Lipid biosynthetic pathway. The genes that appeared differentially expressed in the microarray are highlighted in red.

(D) Gene expression (qPCR) of genes from the lipid biosynthesis and free fatty acid oxidation pathways. Values in are mean ± SEM. N, C-F2 $\geq$ 8; IUGR-F2 $\geq$ 8. *p < 0.05; **p < 0.01, Student's t test.

(E) Hierarchical structure of the transcription factors that regulate the lipid biosynthesis: Srebf1, Lxra, and Rxra.

(F) Gene expression (qPCR) analysis of the transcription factors that regulate the lipid biosynthetic process.

Values are mean ± SEM. n, C-F2 $\geq$ 8; IUGR-F2 $\geq$ 8. *p < 0.05; **p < 0.01, Student's t test. See also Table S1 and Figures S1 and S2.

effect, it is important to distinguish whether the inheritance of an environmentally acquired trait is transmitted through the maternal or the paternal lineage. Maternal effects comprise a plethora of conditions where, in addition to epigenetics, the female physiology and/or behavior influence the physiology of her own offspring, thereby perpetuating a phenotype across generations (Ferguson-Smith and Patti, 2011). Paternal effects, on the other hand, strongly implicate epigenetic mechanisms via the germline. This is particularly clear in animal models where males are removed from the cage after fertilization and do not contribute to rearing their offspring.

We have previously developed a mouse model of fetal malnutrition-associated diabetes. Briefly, a 50% global caloric restriction was imposed to pregnant females (F0) during the last week of gestation (Jimenez-Chillaron et al., 2005). Offspring from caloric-restricted females showed intrauterine growth restriction (IUGR) and low birth weight. In analogy to observations in humans, IUGR-F1 male mice developed many features reminiscent of the metabolic syndrome, including obesity, mild hyperglycemia, and glucose intolerance by age 4–6 months. Strikingly, deregulation of metabolism persisted in the next generation offspring (IUGR-F2) through the paternal lineage (Jimenez-Chillaron et al., 2009).

In the present study, we tested the hypothesis that epigenetic modifications, namely DNA methylation, contribute to the development of metabolic dysfunction in the second-generation offspring through the paternal lineage. To evaluate this, we analyzed global gene expression profile and specific DNA methylation signatures in livers from IUGR-F2 mice. Second, we determined whether the epigenetic signatures identified in the liver were already present in the sperm of their progenitors (IUGR-F1). Here, we show that in utero malnutrition alters the patterns of DNA methylation of the *Lxra* locus in sperm samples of IUGR-F1 males and liver samples of their offspring (IUGR-F2). These data suggest that in utero undernutrition may reprogram the epigenome of cells from the germline/mature gametes that can be inherited into the next generation offspring, thereby influencing health and disease risk.

RESULTS

In Utero Undernutrition Influences the Expression of Lipogenic Genes in the Second-Generation Offspring

We determined the gene expression profile in liver samples from second-generation offspring (GeneChip Affymetrix microarrays) (Figure 1A). Despite the fact that IUGR-F2 mice were not exposed to nutritional stress in utero, 172 genes were significantly differentially expressed between IUGR-F2 and control-F2 mice at q value $\leq$ 0.05 (Table S1). The gene ontology term with the highest enrichment score was the lipid biosynthetic

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Table 1. Gene Ontology Terms with the Highest Significance of the Functional Clusters

Gene Ontology Term	Category	Cluster Enrichment Score	Count	%	p value	% FDR
0008610	Lipid biosynthetic process	2.979	8	8.421	0.00044	0.660
0006954	Inflammatory response	2.188	7	7.368	0.00076	1.124
0006085	Acetyl-CoA biosynthetic process	1.791	3	3.157	0.00034	0.507
0006959	Humoral immune response	1.457	4	4.210	0.00227	3.309

Up- and downregulated significant gene data sets were considered separately. FDR, false discovery rate.

process (DAVID Bioinformatics Resources; Gene Ontology Tool) (Table 1). Many genes included in this cluster are key players in regulating triglyceride (TAG) biosynthesis from citrate, including ATP citrate lyase (*Acly*), fatty acid synthase (*Fasn*), stearoyl CoA desaturase 1 (*Scd1*), and elongation of very-long-chain fatty acids protein 6 (*Elovl6*) (Figures 1B and 1C). Acetyl-CoA carboxylase 1 and 2 (*Acaca*, *Acacb*), two other important players of the lipogenic pathway (Figure 1C), showed a tendency to be reduced in the microarray, although they did not reach statistical significance (not shown). Next, we confirmed the microarray data by qPCR in an extended set of samples (eight livers/group) that included additional livers from sibling and nonsibling mice (Figure 1D). Coordinated downregulation of lipogenic targets was highly specific because expression of genes that regulate free fatty acid oxidation was not globally altered (Figure 1D).

Lxra and *Srebf1* Contribute to Regulate Expression of Lipogenic Genes in IUGR-F2 Mice

De novo lipogenesis is regulated through a cascade of upstream transcription factors, including the sterol regulatory element binding transcription factor 1 (*Srebf1*), the liver X receptor-alpha (official gene name, *Nr1h3*; alternate gene name, *Lxra*), and the retinoid X receptor-alpha (*Rxra*) (Figure 1E). *Srebf1* expression was reduced by 70% in livers from IUGR-F2 male mice (Figure 1F). Likewise, *Lxra* was statistically downregulated, whereas *Rxra* remained unaltered in IUGR-F2 livers (Figure 1F). Finally, expression of transcription factors upstream of *Lxra*, including the peroxisome proliferator-activated receptor alpha (*Ppara*), the peroxisome proliferator-activated receptor gamma (*Pparg*), and the hepatic nuclear factor 4 alpha (*Hnf4a*), was unaltered (Figure S2). Collectively, these data support that reduced lipogenic gene expression can be explained, at least partly, by altered expression of *Lxra* and *Srebf1*.

DNA Methylation of *Lxra* Is Altered in Liver Samples from IUGR-F2 Mice

We hypothesized that the deregulated expression of *Lxra* might, at least in part, be explained by altered epigenetic modifications. We therefore measured DNA methylation patterns in the 5′ regulatory regions of the gene (Chr2: 91,194,917–91,195,232; mm10). Two CpG islands (CGI) were identified in the 5′ proximal region of *Lxra* (EMBOSS Cpgplot software; http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) (Figures 2A and S3). The first one (CGI-A) spans from −63 to +43 based on the transcription start site (TSS), and the second one (CGI-B) spans from +68 to +138, within the 5′ UTR.

Quantitative DNA methylation analysis by pyrosequencing showed that the methylation profile of the CGI-A was similar among groups (Figure 2A). In contrast, the methylation levels of the CpG sites 1, 2, 3, 5, and 6 of the CGI-B were statistically reduced in liver samples from IUGR-F2 mice (Figure 2A). Likewise, overall methylation of the CGI-B was reduced as assessed by the median DNA methylation level (Figure 2A). We additionally measured the DNA methylation profile of *Srebf1*, the immediate LXR downstream target, and *Fasn*, the rate-limiting enzyme of the lipogenic pathway. We confirmed that both genes contain a canonical CGI around the transcription start site (Figure S3). The pattern of methylation of both genes was unaltered in livers from IUGR-F2 mice (Figures 2B and 2C).

Lxra Methylation Is Already Altered in Sperm Samples from IUGR-F1 Mice

The key question was to determine whether the altered methylation patterns of the *Lxra* locus were inherited from IUGR-F1 male mice through the gametes, or emerged secondarily as IUGR-F2 mice developed metabolic dysfunction. To address this, we determined CGI-B methylation in (1) sperm samples from IUGR-F1 male mice, and (2) fetal liver (ED14.5) from IUGR-F2 mice (Figure 3A). Strikingly, the epigenetic signature that we detected in livers from adult IUGR-F2 mice was already present in both tissues (Figures 3C and 3D). Even more, the CpG sites 1, 3, and 5 were significantly hypomethylated in sperm-F1, fetal liver-F2, and adult liver-F2 (Figure 3C). Again, this effect was highly specific because the CGI-A remained unaltered in sperm-F1 and fetal liver-F2 (Figures S4A–S4C). Likewise, DNA methylation in the promoter region of *Fasn* also remained unaltered in sperm-F1 and fetal liver-F2 (data not shown).

Given that altered patterns of methylation were already present in sperm samples, we analyzed whether they were also detectable in other tissues of IUGR-F2 mice, including skeletal muscle, white adipose tissue, and islet cells (Figure 3B). DNA methylation patterns were altered in skeletal muscle but not in white adipose tissue and in islet cells of IUGR-F2 mice (Figures 3E and 3F). These data suggest that there is a tissue-specific postzygotic epigenetic reprogramming of the transmitted marks in islets and adipose tissue.

Epigenetic Regulation of Lxra Expression

Next, we addressed whether the *Lxra* gene expression is truly regulated by changes in DNA methylation or they just show a positive association in our model. Therefore, we combined (1) treatment of the mouse hepatocyte cell line (Hepa1c) with 5-azacytidine (5-AZA), and (2) chromatin immunoprecipitation assays (ChIP) in vivo. 5-AZA depletes DNA methyltransferases in replicating cells leading to DNA demethylation. We confirmed that 5-AZA reduced moderately *Lxra* DNA methylation (Figure 4A).

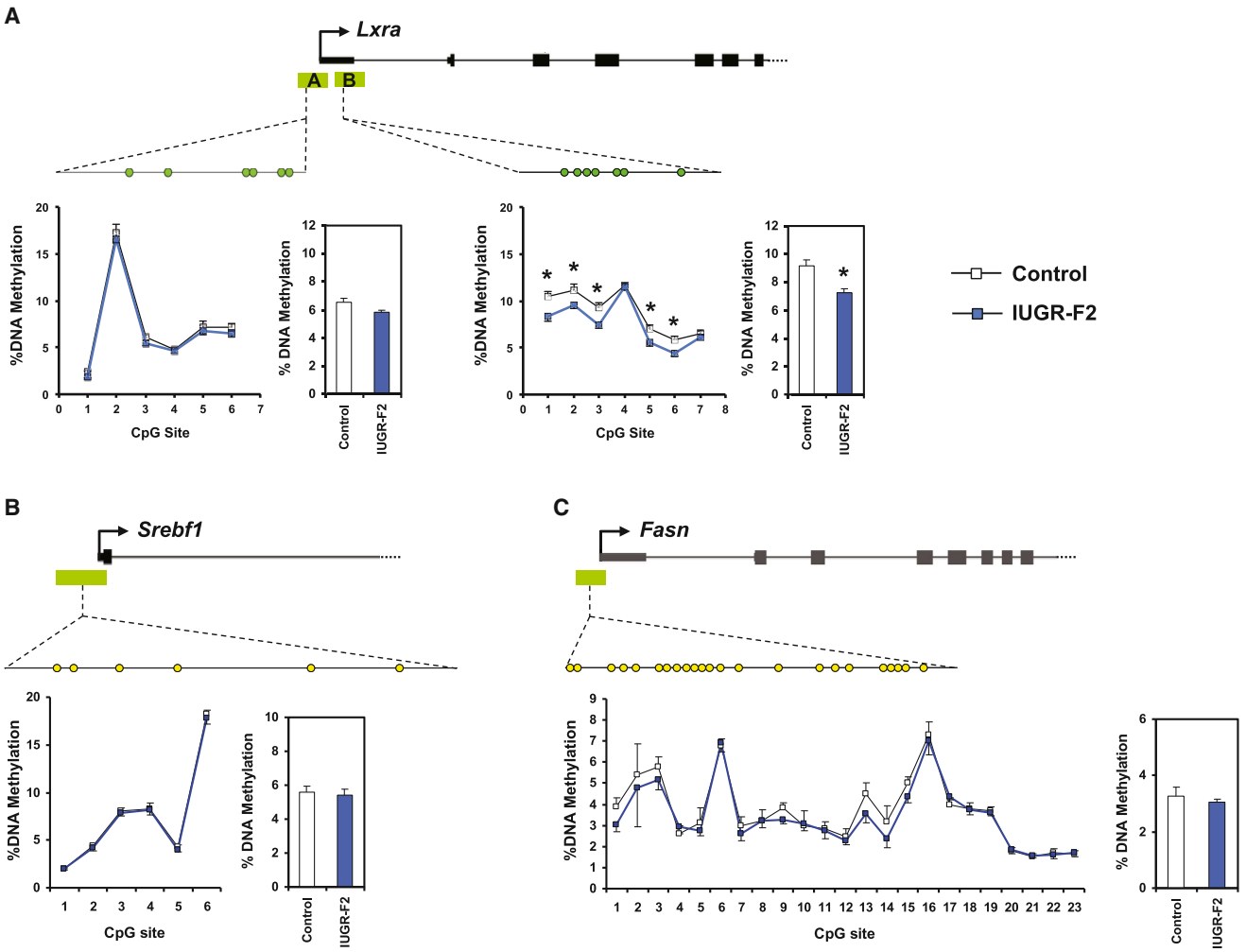

Figure 2. Lxra DNA Methylation Is Altered in Liver Samples from IUGR-F2 Mice

(A) Lxra structure, detailing the CGIs in green. The line graphs represent the percentage of DNA methylation of individual CpG sites within the island (pyrosequencing). The bar graph is the median of %DNA methylation for each region.

(B) Transcription start site and CGI for *Srebf1*. Line graph and bar graph represent the same conceptual information as in (A).

(C) Transcription Start Site and CGI for *Fasn*. Line graph and bar graph represent the same type of information as in (A) and (B).

Values are the mean ± SEM. n = 12 C-F2 and 12 IUGR-F2 mice. *p < 0.05, Student's t test. See also Figures S1 and S3.

In agreement with our in vivo data, 5-AZA also reduced *Lxra* gene expression in a dose-dependent manner (Figure 4B). This effect appeared to be specific for *Lxra* because 5-AZA reduced the pattern of DNA methylation for *Fasn* (Figure 4C), but increased its expression (Figure 4D).

The positive correlation between *Lxra* DNA methylation and expression suggested that reduced methylation might be associated to a combination of histone marks and/or transcription factors that result in transcriptional repression of the gene. ChIP-qPCR assays confirmed that indeed there was an enrichment of repressive histone marks (H3K9me2, H3K27me3) at the CGI-B in liver samples from IUGR-F2 mice (Figure 4E). Conversely, levels of the active transcription mark histone H3K4me1 were reduced in livers from IUGR-F2 mice (Figure 4E). Furthermore, promoter analysis of the CGI-B (http://rvista.dcode.org/) demonstrated consensus-binding sites for known activators (PPARα, PPARγ, HNF4α) and potential repressors of *Lxra* (Stat1, Stat3) (Figure 4F). As stated previously, expression of *Ppara*, *Pparg*, and *Hnf4a* was similar in control-F2 and IUGR-F2 mice (Figure S2). Likewise, expression of *Stat1* and *Stat3* was comparable between groups (Figure S5). However, Stat3 bound to the promoter region of *Lxra* was enriched in IUGR-F2 mice (Figure 4E). In contrast, the percentage of Stat1 and PPARγ bound to the island was similar in control-F2 and IUGR-F2 livers.

We further confirmed that DNA methylation of *Lxra* influences its regulatory properties. Vehicle- and AZA-treated Hepa1c cells were incubated with insulin (that activates *Lxra* transcription), rosiglitazone (a PPARγ agonist), and TNF-α (that represses *Lxra* through the activation of the Jak-Stat pathway) (Figure 5A). Insulin moderately increased *Lxra* transcription in vehicle-treated Hepa1c cells (Figure 5B). This effect was lost in AZA-treated cells. This outcome was specific to *Lxra*, because insulin increased transcription of *Fasn* in both vehicle- and AZA-treated

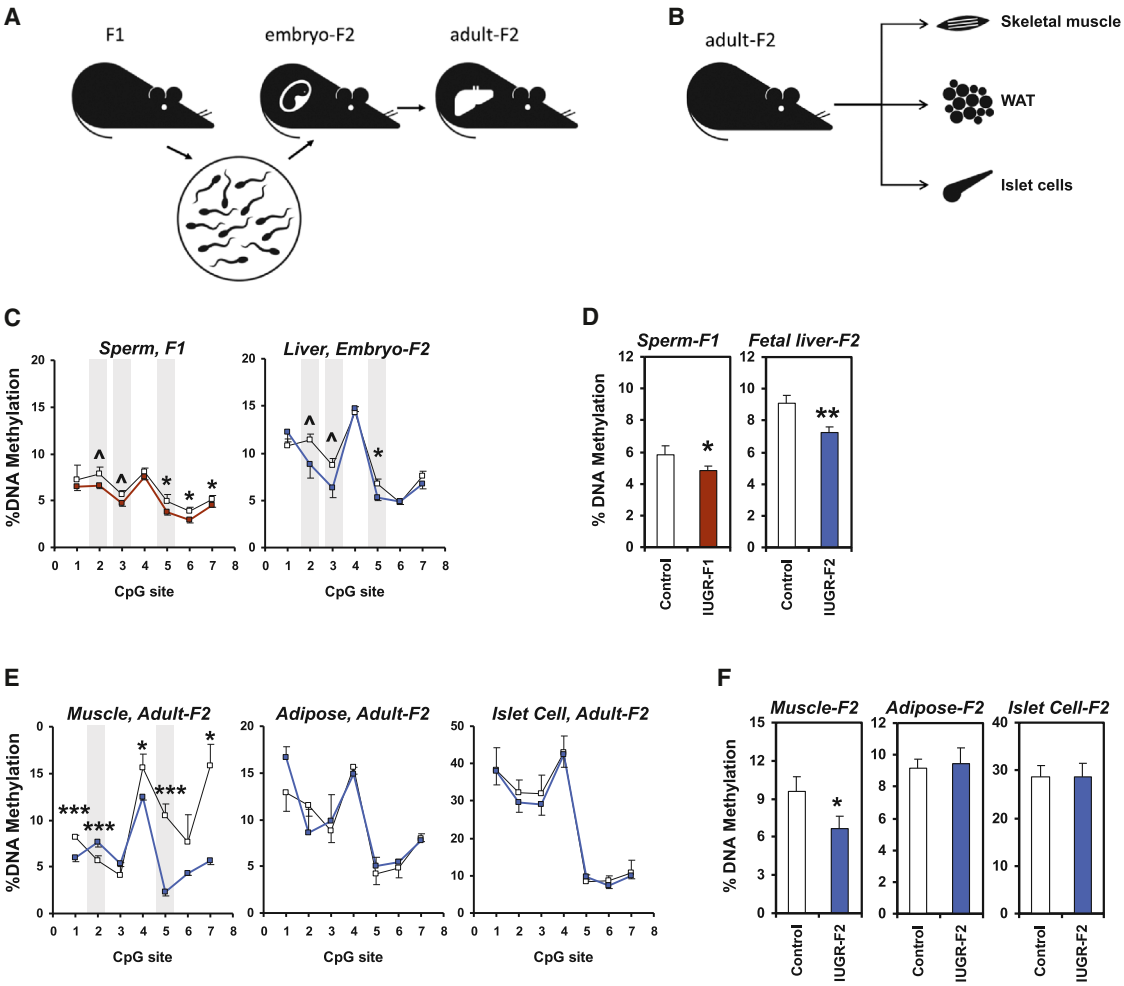

Figure 3. Altered Lxra Methylation Is Already Present in Sperm Samples from the Progenitors, IUGR-F1 Male Mice

(A) Multigenerational exposure to maternal undernutrition.

(B) Multigenerational transmission of epigenetic marks into somatic tissues: skeletal muscle, white adipose tissue, and islet cells.

(C) Percentage of DNA methylation (pyrosequencing) of Lxra in sperm samples from IUGR-F1 mice (C-F2 = 9 mice; IUGR-F2 = 14 mice) and liver samples of IUGR-F2 fetuses from embryonic day ED14 (C-F2 = 8 mice; IUGR-F2 = 8 mice).

(D) Median DNA methylation for sperm-F1 and fetal liver-F2.

(E) Percentage of DNA methylation (pyrosequencing) of Lxra in skeletal muscle, adipose tissue, and islet cells from adult control-F2 and IUGR-F2 mice.

(F) Median DNA methylation level for skeletal muscle (C-F2 = 6 mice; IUGR-F2 = 8 mice), adipose tissue (C-F2 = 8 mice; IUGR-F2 = 6 mice), and islet cells (C-F2 = 3 mice; IUGR-F2 = 9 mice).

Values are the mean ± SEM. ^$p < 0.1$, *$p < 0.05$, ***$p < 0.001$. Student's t test. See also Figures S1 and S4.

cells (Figure 5B). Likewise, rosiglitazone induced *Lxra* and *Fasn* gene expression by more than 2-fold in the vehicle-treated cells (Figure 5C). Strikingly, this effect was abolished for *Lxra* and mildly impaired for *Fasn* in 5-AZA-treated cells. Finally, TNF-α reduced *Lxra* expression more potently in AZA-treated cells than in vehicle-treated hepatocytes (Figure 5D). Together, these data suggest that changes in methylation of the CGI-B fine-tune the normal physiologic regulation of the gene.

Impact of Lxra on Metabolism of IUGR-F2 Mice

Finally, we addressed whether deregulated *Lxra* expression contributed to the development of metabolic dysfunction in the mouse model. Lxra regulates both fatty acid and cholesterol metabolism (Figure 6A). As previously shown, Lxra regulates lipogenesis through transcriptional control of *Srebf1* (Figures 1D and 1F). In agreement, hepatic de novo lipogenesis was impaired in IUGR-F2 mice during a fasting refeeding test in vivo (Figure 6B). Likewise, production of VLDL, as assessed by progressive accumulation of TAG after tyloxapol treatment, was also impaired in IUGR-F2 mice (Figure 6C). Lxra also contributes to the regulation of lipoprotein metabolism (Figure 6A). Accordingly, expression of hepatic *Apoa5* and *Lpl* was reduced in livers from IUGR-F2 mice (Figure 6D). This can partly explain the slight increase in serum TAG observed in adult IUGR-F2 mice (Figure 6E).

Finally, Lxra regulates the transcriptional program of cholesterol export from hepatocytes to HDL (*Abca1*, *Abcg1*) and to bile (as cholesterol, *Abcg5*, *Abcg8*; conversion to bile acids,

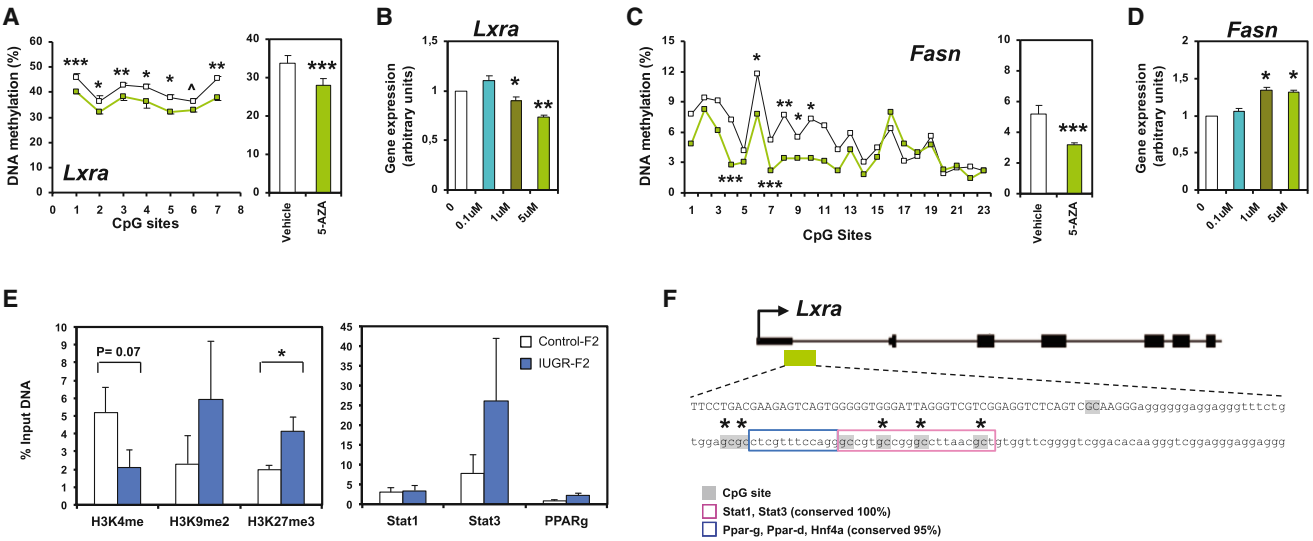

Figure 4. Promoter Lxra DNA Methylation Influences *Lxra* Gene Expression

(A) Quantification of Lxra DNA methylation (pyrosequencing) after 48 hr treatment with 5-azacytidine (5-AZA). The line graph represents the average DNA methylation for each CpG site. The bar graph integrates the median DNA methylation of the entire island.

(B) *Lxra* gene expression (qPCR) after 48 hr treatment with 5-AZA or vehicle.

(C) Quantification of Fasn DNA methylation (pyrosequencing) after 48 hr treatment with 5-AZA. The line graph and bar graph display the same information as in (A).

(D) *Fasn* expression (qPCR) after 48 hr treatment with 5-AZA or vehicle.

(E) ChIP-qPCR in vivo of liver samples from 4-month-old adult mice. The left panel includes ChIP from three histone marks (H3K4me, H3K9me2, H3K27me3) and the right panel includes three relevant transcription factors (Stat1, Stat3, Pparg) (n, C-F2 = 4; IUGR-F2 = 4).

(F) Sequence analysis of the CGI-B. The blue and pink boxes indicate the consensus-binding sites for Stat1, Stat3, Pparg, and Hnf4a. The degree of conservation with the human sequence is denoted in the figure. CpG dinucleotides are labeled in gray. Differentially methylated CpG sites are labeled with an asterisk.

See also Figure S5.

Cyp7a1) (Figure 6A). We show that expression of *Abca1* and *Abcg1* was slightly decreased although not significantly in IUGR-F2 livers (Figure 6D). This change may contribute at least to reduce cholesterol transport to HDL, which correlated with reduced serum HDL cholesterol levels in IUGR-F2 mice (Figure 6F). Finally, *Cyp7a1* and *Abcg5* were also reduced in livers from IUGR-F2 mice (Figure 6D), potentially explaining the slight accumulation of hepatic free cholesterol (Figure 6G). In summary, we demonstrate that Lxra-dependent pathways are moderately altered in IUGR-F2 mice and can potentially contribute to deregulated metabolic function with aging.

DISCUSSION

Here, we show that in utero undernutrition in male mice influences the expression of lipogenic genes in the following-generation offspring (F2), thereby increasing their risk for metabolic syndrome. Deregulated lipogenic gene expression in the liver can be explained, in part, by reduced expression of the key transcription factor *Lxra*. In turn, altered *Lxra* expression can be partially attributed to changes in DNA methylation within its 5′ UTR region. This epigenetic signature was already present in sperm samples from their progenitors (F1). Hence, our data strongly suggest that in utero malnutrition alters patterns of DNA methylation in germ cells and/or mature sperm that are subsequently transmitted and maintained in somatic cells, thereby influencing health and disease risk in the offspring.

Other investigators have previously shown that nutritional stress during early development, including low-protein diet, high-fat feeding, and hyperglycemia, can alter DNA methylation patterns in the following-generation offspring (Burdge et al., 2007, 2011; Carone et al., 2010; Ng et al., 2010; Ding et al., 2012; Fullston et al., 2013). It is arguable that these alterations might be the product of epigenetic inheritance via the gametes (Daxinger and Whitelaw, 2012). Yet, evidence of epigenetic alterations in germ cells and/or mature gametes is so far not very strong (Ferguson-Smith and Patti, 2011; Skinner, 2010). In addition, it could be possible that germline-mediated transmission of phenotypic traits may be due to the combination of developmental abnormalities and aberrant DNA alterations in germ cells, rather than to the continuity of epigenetic marks between F1 and F2 (Burdge et al., 2011).

In this context, our work strongly suggests transmission of epigenetic marks through the gametes: the epigenetic signature identified in the liver of IUGR-F2 adult mice is already present in sperm samples from their fathers, IUGR-F1. These data constitute, per se, evidence of the continuity of an epigenetic mark between generations. Furthermore, we confirmed that the methylation signature of the Lxra locus was also present in liver samples of IUGR-F2 fetuses, well before they develop metabolic alterations that may secondarily lead to epigenetic de novo modifications (Jiménez-Chillarón et al., 2012). Therefore, the presence of the same signature in sperm of IUGR-F1 mice, liver of IUGR-F2 fetuses, and liver of IUGR-F2 adults strongly support transmission of the epigenetic mark through the male gametes

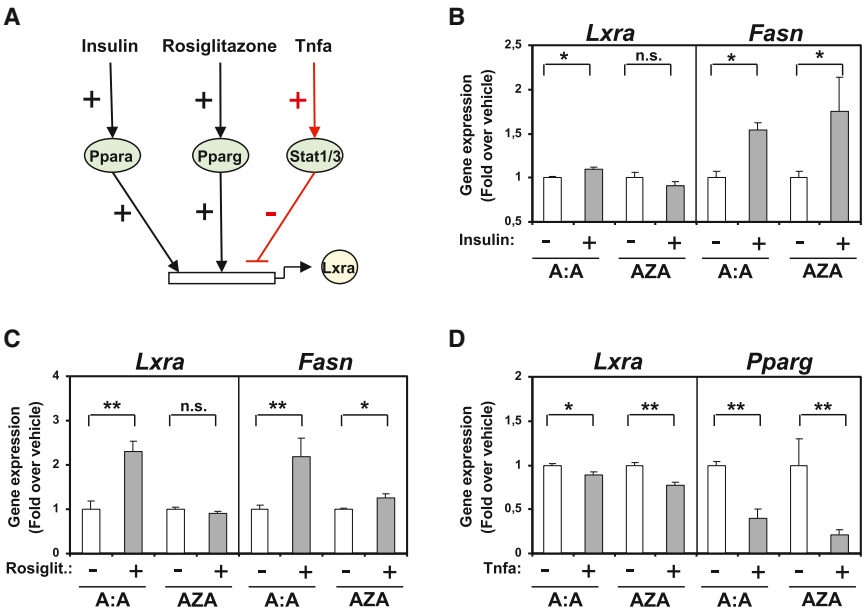

Figure 5. Lxra Promoter Methylation Modulates Gene Expression by Specific Transcription Factors

(A) Transcriptional regulation of *Lxra* by insulin, rosiglitazone, and TNF-α, through their specific transcription factors (Ppara, Pparg, Stat1/3).

(B) Insulin-stimulated *Lxra* and *Fasn* expression (qPCR) after treatment of Hepa1c cells with 5-AZA or vehicle (A:A).

(C) Rosiglitazone-stimulated *Lxra* and *Fasn* expression (qPCR) after treatment of Hepa1c cells with 5-AZA or vehicle (A:A).

(D) Tnfa-stimulated *Lxra* and *Pparg* repression (qPCR) after treatment of Hepa1c cells with 5-AZA or vehicle (A:A).

Values are the mean ± SEM. n = 8 AZA and 8 vehicle in (B)–(D). *p < 0.05, **p < 0.01. n.s., not statistically significant. Student's t test.

rather than a de novo event (Burdge et al., 2011). In addition, if the epigenetic marks in the sperm are truly inherited, they should be present in other tissues as well. In accord, similar patterns of DNA methylation were observed in skeletal muscle from adult IUGR-F2 mice. Nevertheless, this pattern was not visible in two other tissues of metabolic relevance, such as fat and islet cells. This suggests that tissue-specific postnatal remodeling of DNA methylation may occur. Indeed, this effect has been described previously, suggesting that this process might be a common event (Zeybel et al., 2012).

Evidence of epigenetic inheritance in mammals is very limited (Jablonka and Raz, 2009). Indeed, this scarcity has generated an open debate about whether this is a relevant biological process or just a rare event (Grossniklaus et al., 2013) restricted to a particular set of conditions, including (1) intracisternal A particles (IAP) (Blewitt et al., 2006; Morgan et al., 1999; Rakyan et al., 2003; Vasicek et al., 1997), (2) perinatal exposure to endocrine disruptors (Anway et al., 2005; Guerrero-Bosagna et al., 2010; Guerrero-Bosagna and Skinner, 2012), and more recently, (3) hypomorphic mutations of the enzyme methionine synthase reductase, which maintains folate and methionine cycles and, hence, provision of methyl groups for DNA methylation (Padmanabhan et al., 2013). In this context, two recent reports (Zeybel et al., 2012; Vassoler et al., 2013), together with ours, widen above-mentioned panorama and show nongenomic transmission of complex phenotypes in rodents, likely through epigenetic modifications. First, liver damage in male mice induced epigenetic signatures that resulted in improved wound healing in the offspring and the grand-offspring (Zeybel et al., 2012). Second, self-administration of cocaine to male rats induced resistance to cocaine in the following-generation offspring through epigenetic reprogramming of the germline (Vassoler et al., 2013). It has to be noted that in the previous examples, including ours, nongenomic transmission of traits occurred in the context of multigenerational setting (Figure S1) (Jirtle and Skinner, 2007; Skinner, 2008): the F0 gestating mother, the F1 embryo/fetus, and its germline, which will give rise to the F2, are exposed to the same environmental cues during the same time. This is conceptually relevant because in the multigenerational framework inheritance of phenotypic traits via the gametes might be partly mediated by mechanisms other than epigenetics. However, it has to be emphasized that the recognition that environmental cues may lead to the transmission of disease risk, either through epigenetic mechanisms or not, is still extremely important because it has profound implications for human health at large.

Finally, epigenetic inheritance in mammals has not been extensively studied until recently. The reason is that from a mechanistic point of view it is well-established that genomes undergo a massive epigenetic reprogramming during the gametogenesis and the first postzygotic divisions. Thus, the epigenetic modifications existing in germ cells are erased. The goal of this process is precisely to avoid inheritance of environmentally acquired epigenetic marks. However, recent systematic genome-wide mapping of epigenetic events occurring in germ cells has shown that there are many regions that remain substantially methylated in all stages of germ cell development until mature oocytes and sperm (Hackett and Surani, 2013; Hackett et al., 2013; Seisenberger et al., 2012). These regions included primarily IAPs and, importantly, a group of CGIs with variable degrees of stable methylation. It is proposed that these specific islands could mediate epigenetic inheritance across generations.

We show that the 5′ UTR locus of *Lxra* is hypomethylated in liver samples from IUGR-F2 mice. In agreement, altered DNA methylation of Lxra has previously been described in another model of prenatal nutritional stress (van Straten et al., 2010). Low protein intake during gestation resulted in hypermethylation of the promoter region of *Lxra* in the fetal liver from the offspring. Together, both studies suggest that the 5′ regulatory region of *Lxra* is especially vulnerable to early nutritional stress. Alternatively, it might be possible that changes in Lxra DNA methylation are accounted for by a bias in liver cell population. The liver is a heterogeneous tissue formed by different cell types including hepatocytes (60%), Kupffer cells (20%), lymphocytes (15%–20%), stellate cells and oval cells (<1%), etc. It might, therefore, be

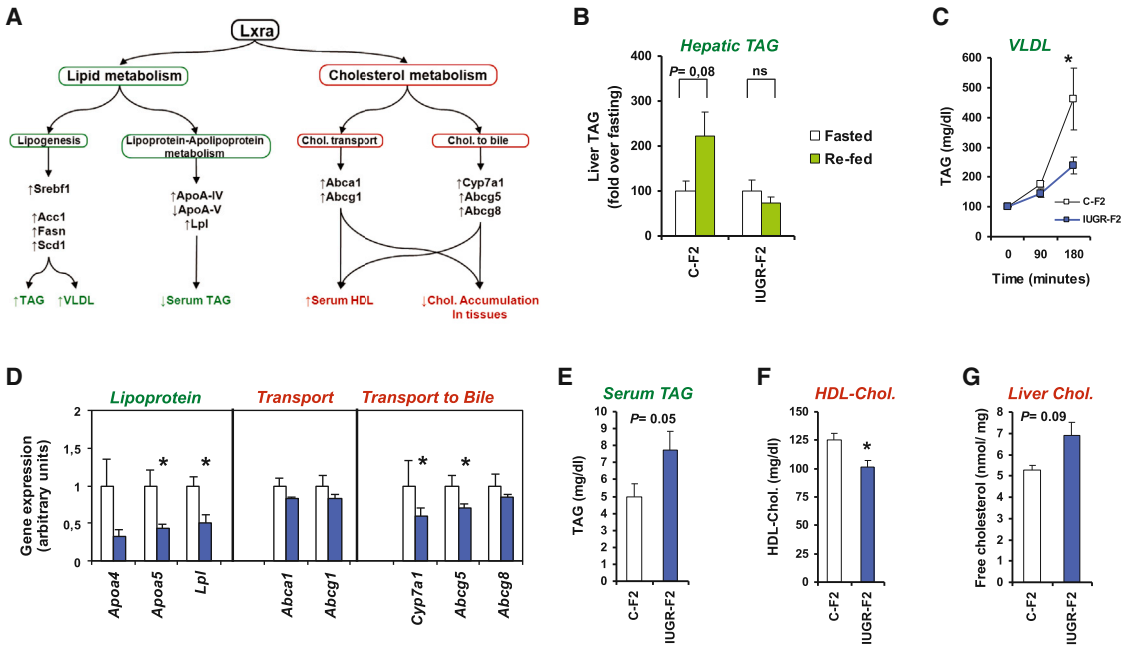

Figure 6. Altered *Lxra* Expression Influences FFA-Cholesterol Metabolism in IUGR-F2 Mice

(A) Targets of Lxra and their role on fatty acid and cholesterol metabolic pathways.

(B) Hepatic TAG content during the fast-to-refeeding transition (C-F2 $\geq$ 3; IUGR-F2 $\geq$ 4).

(C) VLDL production after intraperitoneal treatment with tyloxapol (C-F2 = 12; IUGR-F2 = 12).

(D) Hepatic gene expression analysis (qPCR) of genes that regulate lipoprotein synthesis, cholesterol transport, and transport to bile acids (C-F2 = 8; IUGR-F2 = 8).

(E) Serum triglyceride content in 4-month-old mice (C-F2 = 8; IUGR-F2 = 8).

(F) Serum HDL-cholesterol in 4-month-old mice (C-F2 = 15; IUGR-F2 = 16).

(G) Free cholesterol content in liver samples from 4-month-old mice (C-F2 = 6; IUGR-F2 = 12).

Values are the mean ± SEM. *p < 0.05. Student's t test.

possible that reported changes in methylation reflect, in part, a shift in the abundance of these cell types. Although this possibility deserves further investigation, it has to be noted that in our model the change in methylation is similar in magnitude and direction in at least three independent tissues (sperm, liver, skeletal muscle). Hence, at this point, the possibility that cell type composition accounts for differences in DNA methylation across several tissues is very unlikely.

In our model, *Lxra* expression and DNA methylation are positively correlated. Yet general consensus, primarily from the field of cancer, points out that promoter methylation tends to correlate negatively with gene expression. Here, we show that treating Hepa1c cells with 5-AZA resulted in progressive DNA demethylation and concomitantly reduced *Lxra* gene expression. In addition, the methylation status of the CGI-B locus influenced the physiological regulation of *Lxra* in response to insulin, Pparg, and Tnfa. Together, these data confirm (1) that the methylation state of the CGI-B contributes to the normal physiologic regulation of the gene, (2) that small changes in methylation are enough to influence gene expression, and (3) that methylation of the 5′ UTR and expression of *Lxra* correlate positively. In agreement with the last issue, many transcription factors are effectively able to bind to methylated DNA and induce gene expression in a methylation-dependent manner (Hu et al., 2013; Rönn et al., 2013; Spruijt et al., 2013).

Furthermore, ChIP-qPCR data suggest that reduced DNA methylation results in a combination of histone marks and transcription factors that are associated to repression of *Lxra*. Indeed, repressive histone marks were enriched (H3K9me2, H3K27me3), whereas active histone marks (H3K4me) were reduced at the CGI-B of IUGR-F2 mice. Moreover, promoter analysis of *Lxra* demonstrated consensus-binding sites for known activators (PPARγ, PPARγ, HNF4α) and potential repressors (STAT1, STAT3). We confirmed that, in livers from IUGR-F2 mice, there is a preferential although nonstatistical enrichment of Stat3 bound to the CGI-B. Lack of significance could be due to combined low N value and high interindividual variation. In sum, we provide evidence that *Lxra* expression is influenced by the DNA methylation patterns in the proximal promoter region, which allows the association of specific transcription factors and histones that ultimately determine the transcriptional activity of the gene.

Lxra is involved in the control of cholesterol and fatty acid metabolism (Tontonoz and Mangelsdorf, 2003). In hepatocytes, *Lxra* regulates lipogenesis primarily by its binding and direct activation of the transcription factor *Srebf1* (Repa et al., 2000), which, in turn, activates the transcription of its downstream targets (*Fasn*, *Scd1*, and *Acaca/b*). On the other hand, *Lxra* also controls several genes involved in cholesterol homeostasis, including *Cyp7a1*, *Abca1*, *Abcg1*, *Abcg5*, and *Abcg8* (Peet et al., 1998; Repa and Mangelsdorf, 2000). In agreement, in our model, reduced *Lxra* expression correlates with the expression of its downstream FFA and cholesterol target genes. The effects of *Lxra* on FFA metabolism appeared more potent than

those on cholesterol metabolism, because cholesterol target genes were moderately reduced (between 5%–20%) and only *Abcg5*, *ApoA5*, and *Lpl* reached significance.

The small reduction in *Lxra* expression is sufficient to alter liver metabolism: moderated hypertriglyceridemia, increased VLDL production, reduced HDL cholesterol, and augmented hepatic free cholesterol levels. In agreement, heterozygous mice for *Lxra* (*Lxra*$^{+/-}$) also did show a mild effect in the expression of its downstream targets and very little effect on hepatic TAG and cholesterol homeostasis on a regular chow diet (Kalaany et al., 2005; van der Veen et al., 2007). Notably, the metabolic effects in our model (which shows a 20%–30% reduction in *Lxra* expression) appear as striking as those in *Lxra*$^{+/-}$ mice (where *Lxra* is reduced by 50%). This might be attributed to the fact that (1) in our model additional alterations, other than *Lxra*, might contribute to the whole phenotype, and (2) we are using different strains that can have different sensitivity to *Lxra* deregulation. In summary, we provide evidence that deregulated expression of *Lxra* contributes to alter FFA and cholesterol metabolism in IUGR-F2 mice. This alteration can partially contribute to the development of glucose intolerance and whole-body metabolic dysfunction observed in the IUGR-F2 mice.

To conclude, we show evidence of the transmission of an epigenetic modification, via the sperm, that is stably maintained in somatic tissues (liver) of the offspring and that contributes, in part, to the development of metabolic dysfunction in the second-generation offspring. Our data suggest that transmission of environmentally acquired epigenetic modifications may occur in nature more frequently than previously expected. This is particularly relevant in humans where nongenomic transmission of ancestral disease risk has been demonstrated in many populations. Therefore, better understanding of mechanisms mediating such non-Mendelian forms of inheritance is clearly relevant to design nutritional interventions aimed to prevent such effects and improve health of the upcoming generations.

EXPERIMENTAL PROCEDURES

Animal Care and Experimental Design

Protocols were approved by the Universitat de Barcelona Animal Care and Use Committee. Eight-week-old ICR(CD-1) mice were purchased from Harlan Laboratories. A single virgin female was mated with one nonsibling male. After confirmation of pregnancy by vaginal plug (day 0.5), the male was removed from the cage and the female was maintained individually throughout gestation. On pregnancy day 12.5, the females (F0) were randomly assigned to either the control (C; 10 females) or the intrauterine growth restriction (IUGR; 13 females) groups (Figure 1A). Food intake of IUGR-F0 dams was restricted to 50% compared with that consumed by C-F0 from day 12.5 until delivery. After delivery, litter size was adjusted to eight pups to avoid metabolic drifts due to nutrient availability during lactation. Offspring from C-F0 and IUGR-F0 dams were designated as the first-generation offspring (C-F1 and IUGR-F1) (Figure 1A). F1 pups were nursed freely and weaned at 3 weeks onto standard chow (2014 Tekland Global, Harlan Iberica), provided ad libitum. To generate the second-generation offspring (F2), eight C-F1 and seven IUGR-F1 unrelated nonsibling males from two independent breedings were mated at age 2 months with external control virgin females. Pregnant females were not subjected to food restriction. At birth, F2 litters were adjusted to eight pups per dam, and all mice had free access to standard chow at weaning.

In Vivo VLDL Production

Production of very-low-density lipoproteins in vivo was assessed by measuring the progressive accumulation of TAG in serum of mice treated with Tyloxapol (Sigma). Intraperitoneal injection of Tyloxapol (500 mg/kg) was performed on conscious mice after 4 hr fast. Blood samples were obtained from the tail vein at 0, 90, and 180 min after Tyloxapol administration.

Serum and Tissue TAG Metabolites

Triglycerides were measured using colorimetric methods in 2 μl serum samples (Biosystems). Hepatic lipids were extracted as described (Bligh and Dyer, 1959). Commercially available kits for total and free cholesterol (DiaSys Diagnostic Systems) and TAG (Roche Diagnostics) were used to determine the lipid profiles in the liver.

Tissue Culture and In Vitro Assays

Hepa1c cells were maintained under standard growth conditions (DMEM, 10% fetal bovine serum). Confluent (60%–70%) cells were treated with 5 μM 5-AZA (Sigma) or vehicle for 48 hr. After treatment, cells were additionally incubated with insulin (400 nM) for 6 hr, rosiglitazone (10 μM) for 24 hr, or Tnfa (100 ng/μL) for 24 hr. All products were obtained from Miltenyi Biotech.

Sperm and Islet Cell Isolation

Sperm was isolated from 2- to 3-month-old mice. The reproductive tract was retrieved, the epididymal conduct of both sides was punctured with a needle, and sperm was isolated by gently shaking the epididymis. The sperm was collected in a culture dish containing warmed PBS solution. Purity of the sperm was initially assessed by microscopy and further evaluated by determining the level of DNA methylation at two Ctcf binding sites in the imprinting control region upstream of *H19* (Figure S6). As expected, the level of methylation was >85% in sperm samples, which is very reminiscent of what was observed in healthy human men at the corresponding locus (Boissonnas et al., 2010), confirming that the isolation yielded a high purity in spermatozoa with minimal contamination of accompanying somatic cells.

Islet cells were isolated from 2-month-old mice as previously described (Jimenez-Chillaron et al., 2005).

DNA and RNA Extraction

Genomic DNA from tissues was extracted using the Wizard Genomic DNA Purification Systems Kit (Promega Biotech Ibérica S.L.). Sperm DNA was isolated by using the DNeasy Blood & Tissue Kit (Izasa-QIAGEN). Total RNA was isolated by using TriReagent (Sigma-Aldrich).

Microarray Experiments and Differential Expression Analysis

Total liver RNA was amplified by MEGAScript T7, retrotranscribed to cDNA, and labeled with the IVT kit. Labeled cRNA (15 μg) was fragmented and hybridized to oligonucleotide GeneChip Affymetrix Mouse 430 2.0 whole-genome arrays. Three microarrays were hybridized for each group (C-F2, IUGR-F2). Each array contained the pooled RNA from three independent mice. Expression values were summarized after background correction and normalization steps using the robust multiarray average (RMA) methodology (Irizarry et al., 2003).

Differential expression analysis was performed by the nonparametric approach Rank Prod (Breitling et al., 2004). Oligonucleotides presenting changes between groups with q values lower than 0.05 were considered significant. The tool DAVID (Huang et al., 2009) was used for the calculation of the functional clustering enrichment statistics of the Gene Ontology Biological Process database considering the list of significant genes.

Assessment of DNA Methylation by Pyrosequencing

Quantitative DNA methylation analysis was performed by pyrosequencing of bisulfite-treated DNA (Tost and Gut, 2007). One microgram of DNA was bisulphite converted using the EpiTect 96 Bisulfite kit (QIAGEN). Regions of interest for validation were amplified using 30 ng of bisulfite-treated genomic DNA and 5–7.5 pmol of forward and reverse primer, one of them being biotinylated (Table S2) (Tost and Gut, 2007). Quantitative DNA methylation analysis was carried out on a PSQ 96MD system with the PyroGold SQA Reagent Kit (QIAGEN), and results were analyzed using the PyroMark CpG software (V.1.0.11.14, QIAGEN).

RT-qPCR Analysis

cDNA was synthesized from 1 μg of total RNA using Random Primers (Promega Biotech Ibérica S.L.). The amplification of the genes of interest

was performed using real-time quantitative PCR on a 7500 Real-Time PCR System and 7500 Software v.2.0.4 (Applied Biosystems) using SYBR (Promega Biotech Ibérica S.L.) and TaqMan 2x Universal PCR Master Mix (Applied Biosystems). The list of primers is available in Table S2.

Western Blot and Chromatin Immunoprecipitation

Western blots were performed using 50 μg of protein extract as previously described (Jimenez-Chillaron et al., 2005). The list of antibodies is detailed in Table S3.

ChIP analyses were performed as follows. Liver tissue (100 mg) of was sliced in PBS. Formaldehyde (1.5%) and glycine (0.125 M) were added for 5 min to quench the crosslinking reaction. Chromatin was extracted with lysis buffer (50 mM Tris, 10 mM EDTA, 1% SDS, 1× protease inhibitor, and 2 mM phenylmethylsulfonyl fluoride [PMSF]). Lysates were sonicated and cleared by centrifugation at 10,000 × *g* for 25 min at 4°C. Samples were diluted 1/10 in dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl). Fifty microliters of diluted samples were saved as Input DNA. The remaining sample (450 μl) was immunoprecipitated overnight at 4°C with 5 μg of the corresponding antibody (Table S3). Samples were washed and boiled to revert the crosslink, and DNA was recovered for RT-qPCR analysis.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using a two-tailed t test or a one-way ANOVA as indicated (IBM SPSS Statistics 19). A p value < 0.05 was considered significant.

ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE55304.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.03.026.

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