Introduction

The incidence of non-communicable diseases (NCDs), which already account for 60% of deaths worldwide, is expected to increase by 17% in the next decade (WHO). However the fundamental misconceptions associated with the current focus of action on obesity and NCDs call for a paradigm shift, incorporating a new dimension: the developmental origins of health and diseases (DOHaD). Indeed, early nutritional events can have lasting effects on cell fate, organogenesis, metabolic pathways and physiology, thereby influencing life-long physical degrees of diet-induced obesity (DIO) and/or type 2 diabetes (T2D), with different patterns of metabolic adaptation, even if maintained in seemingly identical environmental conditions. The reasons for this remain unclear. Likewise, despite the worldwide increase in obesity and related diseases, most individuals are neither overweight nor obese, and are obviously able to maintain a balance between dietary intake and energy expenditure, leaving them better “armed” than others to deal with the plethora of food on offer. According to the DOHaD concept, environmental conditions during specific windows of mammalian development can have lasting effects on cell fate, organogenesis, metabolic pathways and physiology, thereby influencing life-long physical
health and the susceptibility to lifestyle-induced diseases in adulthood [1,2]. There is evidence to suggest that maternal overnutrition, gestational diabetes and obesity are deleterious to the health of offspring, inducing the same range of defects as maternal mal- or undernutrition and leading to the development of metabolic syndrome [3,4,5,6,7] in the offspring, with a striking sex-specificity [8,9,10]. The number of overweight or obese women of child-bearing age is growing, and has reached 25% in Europe and 50% in the US (WHO). This could trigger a vicious cycle, with transmission to subsequent generations and an increasing prevalence of these lifestyle-induced disorders. Interestingly, the adverse metabolic consequences of dietary manipulations can be improved or prevented by applying mild food restriction or a normal control diet to the mother [11,12], by reducing maternal obesity by bariatric surgery [13,14] or by the addition to the maternal diet of specific nutrients involved in various levels of carbon metabolism essential for DNA methylation [15,16,17,18,19,20].

We previously showed that 83% of F1 females (F1LM), born to F0 CD-fed lean mothers develop hyperphagia, obesity and T2D in response to a post-weaning high-fat diet (HFD) for 20 weeks, but with 17% remaining lean, with normal insulin sensitivity, despite the HFD. When F1 females with diet-induced obesity (DIO) and T2D (F2OM) were fed a CD with an appropriate dietary fatty-acid profile during the periconceptional/gestation/lactation period, we observed a strong shift toward resistance (increasing from 17% to 43%) in their offspring fed an obesogenic diet after weaning. However, this shift in sensitivity was restricted to females [21]. This sex-specificity is frequently found in studies of developmental programming [8,9,10,22]. Thus, a CD can alleviate the malprogramming effects of maternal obesity and type 2 diabetes (T2D) in mice, in a sex-specific manner, probably through adaptation to adverse intrauterine conditions.

In this study, focusing on the female offspring born to either lean mothers (F1LM or F1) or to obese mothers (F2OM or F2) fed a CD during the periconceptional/gestation/lactation period, we investigated phenotypic and metabolic features, the transcriptional and epigenetic mechanisms underlying the metabolic response and adaptation to HFD, and the trait of resistance/susceptibility to the obesogenic effects of a HFD. We used a candidate gene approach to study categories of genes likely to be affected by lipid overload and to contribute to the hepatic response to HFD and long-term adaptation. A custom-built microarray [23] was used for the transcriptional analysis of 1) classical candidate genes controlling metabolism, 2) genes regulated by genomic imprinting potentially susceptible to nutritional disturbances [24,25,26], and, 3) genes involved in neurotransmission of potential importance for the control of hepatic function and energy status in the liver [27,28,29]. Quantitative RT-PCR (RT-qPCR) experiments were also carried out in the adipose tissue, which plays a key role in lipid storage. Global epigenetic modifications were assessed, by LUMA for DNA methylation and western blotting for pertinent histone marks, to identify changes in response to diet as a function of resistance status. We then investigated the relationship between these modifications and potential changes in enzymatic activities, by analyzing the expression of 15 genes encoding chromatin-modifying enzymes in tissues presenting global epigenetic changes, by RT-qPCR.

Results and Discussion

Phenotypic and Metabolic Data

As previously described, the offspring of lean control or obese, diabetic female mice fed a CD during the periconceptional/gestation/lactation period were fed a HFD for five months after weaning [21]. Figure 1 summarizes all the metabolic data. Mice with body weights more than two standard deviations above the mean weight of the controls were classified as obesity-prone (OP), whereas mice with body weights below this threshold were classified as obesity-resistant (OR). This highlights the step-by-step development of the metabolic phenotype, from mice prone to obesity [full phenotype] when challenged with an obesogenic diet after weaning (OP1), to obesity-resistant mice (OR2 mice) with an almost normal phenotype despite HFD-associated mild dyslipidemia. OP mice accounted for 83% (OP1) of the F1 daughters of the lean F0 mothers (F1LM) and for 57% (OP2) of the F2 daughters of the F1 obese and diabetic mothers (F2OM) and weighed, on average, 75% and 62% more than their respective controls (CD1, CD2), fed the control diet (CD) (Table 1). Obesity was generally associated with hyperphagia and high calorice intake in OP mice. However, 17% of the F1LM female offspring and 43% of the F2OM female offspring remained lean, with body weights similar to those of CD1 and CD2 mice (Figure 1), despite being fed the HFD. These mice were classified as obesity-resistant (OR1 and OR2 for the F1LM and F2OM, respectively; Figure 1). OR1 mice had a calorice intake slightly higher than that of CD1, but much lower than that of OP1 and OP2 mice. OR2 mice had a calorice intake similar to that for CD2 mice. Piximus technology and the dissection of adipose tissue from various locations showed that body fat content was much higher in OP1 and OP2 obese mice, reaching about 40% of body weight. OR1 and OR2 mice also had a slightly higher fat content than their respective controls, but this difference was much smaller than that for OP1 and OP2 mice (Table 1). OP1 mice had heavier livers than control mice, whereas OP2, OR1 and OR2 mice had liver weights similar to that in control mice (Figure 1). Severe pancreatic hypertrophy (tripling of organ weight) was also observed in OP1, OP2 and OR1 mice, but not in OR2 mice (Figure 1).

Determinations of several metabolites and hormone parameters (Table 1) showed that obesity was associated with hyperleptinemia and that the development of T2D was associated with higher plasma glucose and insulin concentrations in OP mice. In a previous study [21], we performed oral glucose tolerance tests (OGTT) and intraperitoneal insulin tolerance tests (ITT): OR2 females and CD-fed normal control mice displayed similar responses to insulin. Glucose homeostasis was impaired by high-fat feeding in OP1 and OR1 mice, and in OP2 mice. OP1 mice were hyperglycemic and hyperinsulinemic, whereas OP2 mice were hyperinsulinemic but not hyperglycemic, and OR2 mice were normoglycemic and normoinsulinemic at 24 weeks. Glycemia and insulinemia were thus similar in lean OR2 females and control mice (CD1 and CD2). OR1 and OR2 mice remained normoglycemic and normoinsulinemic. HOMA index values indicated abnormal fasting glucose concentrations in OP1 and, to a lesser extent, OP2 mice (table 1). All mice fed the HFD presented dyslipidemia, with high cholesterol and HDL-C levels, although this pattern was less pronounced in OR1 and OR2 mice.

The higher total circulating fatty acid concentration observed in the F1LM offspring (in both OP1 and OR1 mice) was not observed in the F2OM offspring (Table 1 and Figure 1). Detailed plasma fatty acid profiling showed that OP1 and OP2 mice had lower levels of C16:1 and higher levels of polyunsaturated n-6 C18:2 and C20:4 than their respective controls. OP1 mice displayed specific modifications, with higher levels of C18:0 and C22:6, which were not found in the F2OM offspring (OP2 mice). Moreover, the changes in C18:2 and C20:4 FA levels were more marked in OP1 than in OP2 mice. OR1 and OR2 mice had lower levels of C16:1 and higher levels of C18:2 than CD1 and CD2.
mice. These changes were of a similar magnitude to those observed in OP1 and OP2 mice. OR1 and OR2 mice had lower C16:0 levels than control mice, this effect being particularly pronounced in OR2 mice. Higher levels of C20:4 were found specifically in OR1 mice; but not in OR2 mice, which had C20:4 levels similar to those of CD2 mice. C18:1 levels were similar in all groups (Table 1).

Several studies have shown that maternal fat intake during the periconceptional/gestation/lactation period contributes to progression to non alcoholic fatty liver disease (NAFLD) in the offspring during adulthood [30,31]. We show here that feeding a CD to obese, diabetic females during the periconceptional/gestation/lactation period limits the progression of HFD-induced NAFLD in the offspring. This conclusion is supported by the normalization of liver weight in the offspring born to obese mothers (F2OM), in both OP2 and OR2 females, and by the lower levels of steatosis revealed by hematoxylin and red oil staining (Wu et al. unpublished data), associated with clear changes in hepatic lipid metabolism, as reflected by adaptive changes in circulating fatty acid levels.

Transcriptomic Data

The microarray used contained 992 gene-specific 50-mer oligonucleotides generated from 63 imprinted genes (See Table S1), 256 genes encoding proteins involved in neurotransmission and 157 genes encoding proteins related to energy homeostasis [23]. A direct, dye-swapping comparison strategy was used to compare conditions, and dye-swaps were replicated several times, with at least four animals/group. Heat maps were constructed by compiling the differentially expressed genes (DEG) in the various comparisons (OP1/CD1, OP2/CD2, OR2/CD2, OR2/OP2, OP2/OP1), facilitating further indirect comparison between groups. The heat maps shown in Figures 2 and 3 highlight the dysregulated expression of three categories of genes: genes relating to metabolism, neurotransmission and imprinted genes.

The microarray results were confirmed by RT-qPCR, by determining the expression levels of 15 genes (listed in the legend to Figure S1) that appeared to be differentially expressed in at least one comparison between the various mouse groups (CD1, OP1, CD2, OP2, OR2; n = 5 per group). Expression values were normalized with the Genorm factor, and RT-qPCR expression ratios (OP1/CD1, OP2/N2, OR2/CD2, OR2/OP2 and OP2/OP1) were calculated and plotted against the corresponding microarray data (Figure S1). The RT-qPCR and microarray values were consistent for most of the 75 ratios, as indicated by the statistical significance of the linear regression equation (r² = 0.718, p < 10⁻⁵), highlighting the robustness of the microarray data.

As summarized in Table 2, we identified four different patterns of gene expression for the metabolic response to HFD, susceptibility/resistance and adaptation to the obesogenic HFD (Figures 2 and 3 and Table 2): 1) Resistance to the obesogenic diet: 260 genes were either specifically associated with the maintenance of a lean phenotype under a HFD in the OR2/CD2 comparison, distinguishing OR mice from controls (CD), or showed a striking
difference in the OR2/OP2 comparison, distinguishing OR from OP mice. These genes were considered to be potentially involved in peripheral resistance to the obesogenic effects of the HFD; 2) Susceptibility to the obesogenic diet, with proneness to obesity: 287 genes associated with weight gain were either up- or downregulated in OP mice (comparisons OP1/CD1 and OP2/CD2). However, only 53 (19%) of these genes were dysregulated in a strictly similar manner by the HFD in both OP1 and OP2 mice, demonstrating differences in obesity phenotype despite similar weight gain; 3) Maternal effect: resistance or susceptibility to the obesogenic diet differed according to the leanness/obesity of the mother: 92 genes were differentially expressed between OP mice from the F1LM and F2OM groups. Thirty-five of these genes were identified by direct comparisons of OP2 and OP1 mice. Most of these genes (63%) were expressed less strongly in OP2 mice than in OP1 mice. In total, 57 genes were identified as differentially regulated between OP1 and OP2 mice (OP1/CD1 and OP2/CD2 comparisons) but similarly regulated between OP2 and OR2 mice (OP2/CD2 and OR2/CD2 comparisons). The pattern of gene expression in OP2 mice therefore prefigures the acquisition of resistance in OR2 mice. 4) Diet effect: 8 genes were either up- or downregulated by diet in both resistant and sensitive mice born to both lean and obese mothers (F1LM and F2OM mice).

Expression of liver metabolism genes: As shown in table 2, in OR2 mice, despite the higher lipid content of the HFD than of the CD, whole-lipid metabolism in the liver appeared to be restricted, with the strong repression of many genes involved in lipid storage and utilization, consistent with the normalization of liver weight and caloric intake. Genes regulated by Ppara and genes regulating lipid uptake, storage and utilization were restricted, with the strong repression of many genes involved in lipid storage and utilization, consistent with the normalization of liver weight and caloric intake. Genes regulated by Ppara and genes regulating lipid uptake, storage and utilization were restricted, with the strong repression of many genes involved in lipid storage and utilization, consistent with the normalization of liver weight and caloric intake.

Table 1. Phenotypic and metabolic characteristics of the mice after 24 weeks on the CD and HFD, for the F1LM (CD1, OP1 and OR1) and F2OM (CD2, OP2 and OR2) mice.

| Parameters | CD1 | OP1 | OR1 | CD2 | OP2 | OR2 |
|------------|-----|-----|-----|-----|-----|-----|
| Weight (g) | 21.35±0.12 | 37.28ab±0.55 | 22.33±0.33 | 21.14±0.14 | 34.37ab±1.09 | 21.5±0.28 |
| Caloric intake (kcal/5mo) | 1300±8 | 1856ab±83 | 1451±30 | 1283±19 | 1848ab±58 | 1344±30 |
| Fat mass (%) | 18.42±1.12 | 42.34ab±2.07 | 29.00±1.32 | 15.61±0.63 | 40.46ab±3.96 | 27.97±2.2 |

Organ weights

| Organ | CD1 | OP1 | OR1 | CD2 | OP2 | OR2 |
|-------|-----|-----|-----|-----|-----|-----|
| Heart (g) | 0.123±0.013 | 0.142±0.009 | 0.142±0.019 | 0.155±0.016 | 0.142±0.014 | 0.109±0.009 |
| Liver (g) | 0.826±0.110 | 1.165ab±0.068 | 0.902±0.063 | 0.785±0.120 | 0.902±0.050 | 0.723±0.090 |
| Spleen (g) | 0.100±0.015 | 0.107±0.010 | 0.112±0.018 | 0.120±0.020 | 0.112±0.019 | 0.106±0.035 |
| Pancreas (g) | 0.343±0.023 | 1.195±0.145 | 1.172±0.164 | 0.387±0.011 | 1.172ab±0.164 | 0.53±0.122 |
| Epididymal AT (g) | 0.176±0.014 | 1.851ab±0.260 | 2.080±0.588 | 0.256±0.045 | 2.552ab±0.453 | 0.686±0.301 |
| Retroperitoneal AT (g) | 0.123±0.016 | 1.602ab±0.554 | 1.294±0.397 | 0.112±0.043 | 1.583ab±0.356 | 0.436±0.173 |
| Subcutaneous AT (g) | 0.73±0.111 | 5.748±1.103 | 3.968±0.990 | 0.663±0.069 | 4.808ab±0.679 | 1.223±0.789 |

Plasma hormones and metabolites

| Parameter | CD1 | OP1 | OR1 | CD2 | OP2 | OR2 |
|-----------|-----|-----|-----|-----|-----|-----|
| Insulin (ng/ml) | 0.89±0.08 | 2.38ab±0.31 | 0.77±0.10 | 0.99±0.08 | 1.48ab±0.38 | 0.96±0.12 |
| Leptin (ng/ml) | 4.6±0.7 | 40.6ab±4.1 | 8.2±2.4 | – | 34.0±5.0 | 9.5±3.4 |
| Glucose (mmol/l) | 6.89±0.19 | 8.6ab±0.26 | 7.1±0.3 | 6.54±0.21 | 8.1ab±0.2 | 7.2±0.4 |
| Cholesterol (mmol/l) | 1.59±0.07 | 2.58ab±0.15 | 2.17±0.14 | 1.36±0.12 | 2.37ab±0.26 | 2.00±0.14 |
| HDL (mmol/l) | 1.03±0.03 | 1.61±0.08 | 1.53±0.09 | 0.89±0.09 | 1.28±0.16 | 1.20±0.06 |

Circulating fatty acids

| FA | CD1 | OP1 | OR1 | CD2 | OP2 | OR2 |
|----|-----|-----|-----|-----|-----|-----|
| Total FA (μg/ml) | 186.4±6.6 | 334.0±22.1 | 256.7±30.0 | 198.4±6.0 | 202.2±14.8 | 174.0±16.9 |
| C16:0 (μg/ml) | 45.2±3.4 | 36.8±3.0 | 39.7±7.3 | 34.5±4.8 | 26.1±2.3 | 18.8±3.6 |
| C18:0 (μg/ml) | 23.1±0.4 | 49.5±6.2 | 38.8±6.4 | 28.2±1.3 | 24.2±2.3 | 25.1±0.9 |
| C16:1 (μg/ml) | 7.0±0.5 | 1.8±0.3 | 3.8±0.2 | 5.3±0.4 | 3.3±0.05 | 2.0±0.06 |
| C18:1 (μg/ml) | 50.0±2.7 | 65.3±6.7 | 60.3±6.9 | 66.7±3.0 | 53.3±5.7 | 53.8±4.9 |
| C18:2 (μg/ml) | 22.7±1.3 | 63.4±5.7 | 52.2±5.2 | 26.3±1.8 | 46.8±3.5 | 38.7±4.1 |
| C20:4 (μg/ml) | 17.7±0.5 | 82.4ab±6.1 | 35.5±4.7 | 20.6±3.4 | 36.7±2.3 | 22.6±4.1 |
| C22:6 (μg/ml) | 4.7±0.2 | 26.9ab±1.6 | 6.8±6.1 | 8.0±1.5 | 6.3±0.75 | 5.4±0.4 |

The values presented are the means ±SEM. For weight and caloric intake, n = 50 to 70, for plasma hormone and metabolite determinations, n = 10 to 15, for FA level determination, n = 7, and for Piximus analysis and organ weights, n = 5.

*P < 0.05 for comparison with control.

*P < 0.05 for comparison between OP and OR mice.

*P < 0.05 for comparison between the F1LM and F2OM mice, assessed by Kruskal-Wallis tests followed by Dunn’s post hoc tests.

doi:10.1371/journal.pone.0066816.t001
Ppara were repressed and Thrβ signaling was upregulated in obesity-resistant, but not in obesity-prone mice (Table 2).

Similar transcriptional responses, resulting in the well known abnormalities of T2D, such as changes to the expression of insulin signaling-related genes and lipid metabolism-related genes and features of hypothyroidism were observed in OP1 and OP2 mice born to both lean and obese mothers (Figure 2).

However, OP mice also displayed specific expression patterns potentially responsible for the differences in metabolic phenotype observed between mice born to either lean or obese mothers. Specifically in OP1 mice, genes linked to de novo lipogenesis were activated, despite the lipid overload induced by HFD. This may have led to the deposition of larger amounts of lipids and to the development of steatosis in these mice. The expression of several genes involved in lipid regulation and hepatic glucose production

Figure 2. Heat map construction representing the differential expression of genes involved in metabolic function and neurotransmission. The heat map shows changes in the hepatic expression of genes encoding proteins involved in neurotransmission and genes encoding energy homeostasis-related proteins. Red, green and white squares represent upregulated, downregulated and unmodified genes, respectively.

doi:10.1371/journal.pone.0066816.g002
was also considerably modified in OP1 mice, whereas no such changes were observed in OP2 and OR2 mice. Genes involved in lipid storage and accumulation were more strongly repressed in OP2 mice than in OP1 mice. In addition, OP2 mice presented a striking upregulation of genes involved in fatty acid oxidation that was not observed in OP1 mice, based on the OP1/CD1 comparison. This may reflect an increase in the capacity to process excess fatty acids, preventing OP2 mice from developing hepatic steatosis (Table 2). Thus, in F1LM and F2OM mice, specific metabolic pathways were progressively recruited to spare the liver from lipid accumulation and hypertrophy and to maintain its capacity to respond to insulin.

Diet sensitivity was noted for genes or marks dysregulated in the same way in all groups fed the HFD. Transcriptomic analysis of the liver showed that eight lipid metabolism genes were similarly upregulated or downregulated in all groups fed the HFD, with OP mice generally more strongly affected than OR mice (Table 2).

Expression of liver neurotransmission genes: The traits of proneness or resistance to the obesogenic effects of a HFD were associated with major changes in the transcriptional regulation of neurotransmission genes in the liver. The central modulation of the expression of these genes to control food intake and energy expenditure is well documented, but it is intriguing that some of these “neurotransmission” genes appear to be modulated in the same way in the liver and the central nervous system.

Many genes encoding GABAergic receptors were dysregulated in OR2 mice, suggesting a potential GABAergic control of metabolism, as reported in studies on hepatic glucose production [32] (Table 2). Nicotinic acid receptors were downregulated in OR mice, whereas Chrm3 displayed the opposite pattern, being upregulated in OR2 mice. This is of particular interest, because acetylcholine (Ach) signaling has been implicated in the regulation of hepatic glucose metabolism and insulin responsiveness [33,34] and the effects of Ach on glycogen synthesis and neoglucogenesis seem to be mediated by this receptor [35] (Table 2).

In addition, OR2 mice had lower levels of orexigenic gene expression than OP2 mice, and displayed an upregulation of genes encoding anorexigenic factors in response to positive energy balance (OP/OR). A similar pattern has been reported for the hypothalamus, but this is the first report of such a pattern for the liver. In the context of Npy signaling, the specific downregulation of both Npy5r and Npy1r in OR2 mice is important, because these two genes underlie the potent effect of Npy on both feeding regulation and the control of energy expenditure [36,37] (Table 2).

The development of obesity and T2D in mice born to both lean and obese mothers, whereas others including Gnas-Goa and Itpr were repressed (Table 2).

Several imprinted genes were found to be upregulated in OP mice born to both lean and obese mothers, whereas others including Gnas-Goa and Itpr were repressed (Table 2).

Thus, a large proportion of imprinted genes were dysregulated in mice fed a HFD, clearly following the adaptive response to the obesogenic diet. Several genes, such as Igf2/Igf2as and Igf2r, play a role in embryonic and placental growth, by governing nutrient availability. In addition to Cdkal1, Kcnq1 and Kcnq1ot, these genes are involved in the overgrowth observed in BWS [43]. The Magn/
Table 2. Major functions involved in diet effect, proneness to obesity, resistance and adaptation to the obesogenic effects of a HFD.

|                             | Obesity resistance specific                                                                 | Obesity proneness specific                                                                 | Maternal effect : lean versus obese                                                                 | Diet effect                                                                 |
|-----------------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| **Circulating fatty acids** | (-) C16:0, in OR2 only                                                                     | (-) C16:1 n-7, in all; (+) C18:2 n-6, C20:4 n-6 in OP1, OP2, with OP1 > OP2; (+) C20:4 n-6, OP1 > OR1 < OR2 not OR2; (+) C18:0, C22:6 n-3, in OP1 only | (+) Total fatty acid, in OP1, OR1 only; (-) C16:0, in OR2 only; (+) C18:2 n-6, C20:4 n-6, OP1 > OP2; (+) C20:4 n-6, OP1 > OR1 < OP2 not OR2 | (-) C16:1 n-7, in all; = C18:1 n-3, in all                                                                 |
| **Liver transcriptome Metabolism** | (--) Acaca, Scd1, Apoa4, Cpt1, Ucp3, Pik3r4, Fabp5, (by Ppara) in OR2; (--) Angpt1, Fabp4, Fabp3, Acdcl, Cav1, Acdas, Ucp2, Rora, Fads2, in OR2 | (--) Plin3, Timp3, Lpin1, Lpin2, in OP with OP2 > OP1                                    | (-) Apoa5, Fabp5, Hnf4a, Lepr, Scd1, Slic1a3; (+) Accn1, Fabp2 with OP > OR                                  |
| Insulin signaling and sensitivity | (+/-) Pten, Ptp4n1, Inpp51, Pik3r1, Bdnf, Slc20a4 (Glut4), in OR2 compared to OP2         | (--) Irs2, Pik3r, Pikkca, Hnf4a, in OP1, OP2                                              |                                                                                  |                                                                                  |
| **Liver transcriptome Neurotransmission** | (--) Rora, Apoa4, Angpt4, Scd1, Lpin1, in OP1, OP2                                           | (+/-) Fads2, Insig1, Ppara, in OP1                                                     |                                                                                  |                                                                                  |
| Lipogenesis                  |                                                                                           | (+/-) Fads2, Insig1, Ppara, in OP1                                                     |                                                                                  |                                                                                  |
| Lipid regulation and hepatic glucose production | (--) Irs, Celbp, G6pc, Fasn, Pde3b, Scarb1, Eno2, in OP1 not in OP2, OR2 | (-) Angpt1, Fabp4, Timp3, Cave1, in OP2 and OR2                                        |                                                                                  |                                                                                  |
| Fatty acid oxidation         | (+/-) Acaca, Acdas, Adhr1, Adhr3, Fabp2, Cpt1, Ppara, Ucp3, in OP2 but not OP1            | (-) Acaca, Acdas, Chrnm, Chrna1, Chrna2, Chrmb2, in OP2                                  |                                                                                  |                                                                                  |
| Diverse                     | (--) Rora, Apoa4, Angpt4, Scd1, Lpin1, in OP1, OP2                                           | (+/-) Fads2, Insig1, Ppara, in OP1                                                     |                                                                                  |                                                                                  |
| Calcium signaling            | (-) Adora2b, Cacala1, Chrnm, Camlk4, CckA, CckBr, in OP1 to OP2 transition                  | (+/--) (+) Thrh1, Ucp1, Pax8, Ucp3, Adhr3, in OR2                                       |                                                                                  |                                                                                  |
| Thyroid signaling            | (++) (-) Thrh1, in OP                                                                       | (++) (-) Thrh1, in OP                                                                  |                                                                                  |                                                                                  |
| K+ channels                  | (++) (-) Kcna2, Kcna3, Kcnb2, Kcnb3, Kcnf1, in OR2                                           | (++) (-) Kcna2, Kcnb3, Kcnf1, Kcnf2, Kcnf3, Kcnf14, in OP                                | (-) Kcnb7, Kcnb3, Kcnf1, Kcnf4, Kcn1, Kcn1, Hcn1, in OP1 to OP2 transition; (+) Kcnb7, Kcnb9, Kcnf4, Kcn1, Kcn1 in OP and (-) or (=) in OP2 and OR2 | (-) Kcnb7, Kcnb3, Kcnf1, Kcnf2, Kcnf3, Kcnf14, in OP                                |
| Serotonin receptors          | (++) (-) Htr2a, Htr2b, Htr3a, Htr7, in OR2                                                  | (++) (-) Htr2a, Htr2b, Htr7, Htr1b, in OP1 and (-) or (=) in OP2 and OR2               | (+/-) (+) Htr2a, Htr2b, Htr7, Htr1b, in OP1 and (-) or (=) in OP2 and OR2               | (+/-) (+) Htr2a, Htr2b, Htr7, Htr1b, in OP1 and (-) or (=) in OP2 and OR2               |
| Cholinergic/Nicotinic Receptors | (--) Chmb2, Chme, Chmea6, in OR2, (--) Chmb3, (++) in OR compared with OP; (--) (+) Chmb3, Chmeb2, Chmrnd | (++) (-) Chmb3, Chmb2, Chmrnd, in OP1, OP2; (+) Chmba1, Chmb4, Chme, in OP1 and (+) or (=) in OP2, OR2 | (+/--) (+) Chmba1, Chmb4, Chme, in OP1 and (-) or (=) in OP2, OR2 | (+/--) (+) Chmba1, Chmb4, Chme, in OP1 and (-) or (=) in OP2, OR2 |
| Orexigenic                   | (+/-) (-) Npy, Agrip, Hcrt, Npy5r, Npy1r in OR                                             | (+/-) (+) Npy, Agrip, Hcrt, Npy5r, Npy1r in OR                                          |                                                                                  |                                                                                  |
| Anorexigenic                 | (+) Mc4r, Mc5r, Sst, in OR2 compared to OP2                                                  | (+/-) (-) Mc4r, Mc5r, Sst, in OP2 compared to OP2                                       |                                                                                  |                                                                                  |
| GABA receptors :            | (+) Gabra2, Gabrb3, Gabrd, Gabrg2, Gabrg3, Gabrq, Gabrb1, in OR2                            |                                                                                         |                                                                                  |                                                                                  |
| Liver transcriptome imprinted genes | (++) Gnas, Gld2, Mas1, Nnat, between OP2, OR2; (-) Igf2r, Gld2 in OR2                         | (+/-) Gnas, Gld2, Mas1, Nnat between OP2, OR2; (+) Slc22a18, Xist, Mf1, Gld2, (--) Gnas-Gln, in OP1, OP2 | (-) Ins2, Kcnf1a2, Gabrg2 in OP2, OR2 not in OP1                                             | (-) Ins2, Kcnf1a2, Gabrg2 in OP2, OR2 not in OP1                                             |

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Alleviation of Malprogramming
Table 2. Cont.

| Obesity resistance specific | Obesity proneness specific | Maternal effect: lean versus obese | Diet effect |
|-----------------------------|---------------------------|---------------------------------|-------------|
| BWS cluster                 | (+) Ascl2, Igf2-α, Stk22a18, Kcnq1, Kcnq10t, Cdkn1c, Igf2, Ins2, Cdkn1 in OR2 | (+) Kcnq1 in OP1, OP2, OR1, OR2 | (−) Ins2, Kcnq10t, in OP2, OR2 not in OP1 |

Adipose tissue Gene expression

| Adipogenesis, lipid storage Adipocyte size hypertrophy/hyperplasia | (+) Lep, Peg1, in OP2, OR2 | (+) Lep, Peg1, in OP1 | (−) normalization of adipocyte size, in OP2 compared to OP1 |

Liver epigenetic machinery

| Global DNA methylation | (−) in OP1 only | (−) in OP1 only |
|------------------------|----------------|----------------|
| Histone K-acetylation  | (+) Dnmt2, in OP2, OR2 only | (+) Dnmt2, in OP2 |
| Histone K-deacetylation | (+) Hdac5, in OR1 only | (+) Hdac5, in OR1 |

Liver epigenetic machinery

| Global DNA methylation | (−) in OP2 only | (−) in OP2 only |
|------------------------|----------------|----------------|
| Histone K-acetylation  | (+) Hat1, in OP2 only | (+) Hat1, in OP2 only |

Muscle epigenetic marks

| Global DNA methylation | (−) Jhdm2a, in OP2, OR2 | (−) Jhdm2a, in OP2, OR2 |

Muscle epigenetic machinery

| Global DNA methylation | (−) Jhdm2a, in OP2, OR2, OP2−OP2 |

Gtl2 gene plays important roles in postnatal growth, adaptation and adiposity. The Nfat gene is involved in regulating insulin secretion in pancreatic beta cells and is dysregulated in models of litter size-associated overnutrition [44,45]. Other genes, such as Smpn, Bsep, Pecr1, Gabrb3 and Gabrg3, have been identified as contributing to obesity and other symptoms of PWS. Of particular interest, Gnas-Gsa, which displayed opposite patterns of regulation in OP and OR mice, is one of the multiple products of the Gnas locus, which modulates susceptibility to obesity through various effects on insulin sensitivity, glucose and lipid metabolism [46,47,48]. (Table 2).

Several dysregulated imprinted genes (Gnas, Igf2, Igf2r, Gtl2, Cdkn1 and Sge1) constitute important nodes in the recently described network of coregulated imprinted genes (IGN) [49,50]. With the exception of Sge1, all are modulated under a HFD in OR2 mice and associated with the maintenance of leanness (Figure 3). The mRNA levels of most of the genes of the IGN have been shown to be significantly altered, in a coordinated fashion, in placentas obtained after in vitro fertilization and undergoing apparently normal development. This global modulation of imprinted genes is thought to act as a compensatory process, correcting the potential dysfunction of the placenta [51]. We recently demonstrated that a maternal HFD during pregnancy also results in the deregulation, in the placenta, of clusters of imprinted genes controlling many cellular, metabolic and physiological functions potentially involved in adaptation and/or
evolution [26]. The global dysregulation of genes from the IGN in the livers of OR mice could therefore also be interpreted as an adaptive process triggered to preserve liver function and necessary, under deleterious conditions of lipid overload, to maintain a fairly normal phenotype (Table 2).

**Adipose tissue gene expression:** As shown in figure 4, levels of gene expression in the adipose tissue were determined as a ratio between OP1 or OR1 and CD1 and between OP2 or OR2 and CD2 mice. The levels of expression (as assessed by RT-qPCR) of key genes relating to adipogenesis and adipocyte size (Pparg, Lep, Peg1), energy expenditure (Ucp1, Adhrβ3), glucose utilization (Ski2a4/Glut4), Pdk4, lipogenesis (Sdc1, Aqp7, Ppck) and lipolysis (Hsl, Atgl, Acc1, Ppara and Peg1a) were modified by resistance or susceptibility to obesity, or by maternal effects (lean versus obese mother) or by diet (Figure 4 and Table 2).

Adhrβ3, Lep and Peg1 were dysregulated in OP1 and OP2 mice only, and were therefore strictly associated with proneness to obesity. Ucp1, Acc1 and Sdc1 were similarly downregulated in response to the HFD in OR and OP mice born to both lean and obese mothers, suggesting an effect of diet (Table 2). Finally, the expression of Ppck, Aqp7, Atgl, Hsl, Ppara and Peg1a did not differ between conditions.

Changes in the adipose tissue also supported the metabolic adaptation of the mice according to maternal leanness or obesity status. Pparg, Glut4 and Pdk4 were differentially expressed in OP mice, with levels in OP2 mice similar to those in OR1 and OR2 mice. Levels of Pparg and Glut4 expression were lower and levels of Pdk4 expression were higher in OR1, OR2 and OP2 mice than in CD mice, whereas the expression of these genes was unaffected in OP1 mice. This response switches the energy source from glucose to fatty acids, to maintain blood glucose levels (Figure 5) [52]. In OP1 mice, lipid storage was associated with increases in the expression of both Lep and Peg1, reflecting adipocyte hypertrophy, which could lead to a worsening of the metabolic phenotype by promoting the development of a pro-inflammatory state and insulin resistance [37]. By contrast, OP2 mice, despite displaying a weight gain similar to that of OP1 mice, had much lower levels of Lep and Peg1 expression, suggesting a normalization of adipocyte size and an expansion of fat mass through hyperplasia, a major feature of the adaptive response [53,54]. Thus, adipose tissue displayed a pronounced dysregulation of gene expression, with an upregulation of genes involved in lipid storage and associated with adipocyte hypertrophy in OP1 mice, and hyperplasia in OP2 mice, according to maternal leanness or obesity status (Table 2).

**Major Functions and Networks of Interactions in Proneness, Resistance and Maternal Effects**

Genes either up- or downregulated in OR1 and OR2 mice reflect proactive mechanisms, leading to resistance to the deleterious effects of a HFD, combining both peripheral mechanisms conferring metabolic adaptation to the relative dispropor- tion of lipids and a probable central mechanism for preventing hyperphagia. We did not address the issue of hyperphagia directly in this study, but the dysregulation of neurotransmission genes in the liver strongly suggests that cross-talk occurs between the two mechanisms. However the mechanisms involved in OR1 and OR2 mice are strikingly different, suggesting that the stochastic resistance in the female offspring of lean mothers does not involve the same pathways. By contrast, the genes dysregulated in OP1 and OP2 obese mice probably reflect the pathological condition developed after 20 weeks on a HFD, leading, in different ways in OP1 and OP2 mice, to massive obesity and T2D. We investigated the functions and pathways most clearly related to the observed phenotypic characteristics, by carrying out Ingenuity pathway analysis (IPA) on the liver.

The response to a HFD in OR2 mice (OR2/CD2 comparison) was associated with changes in the expression of genes that appeared to be clustered into several networks (Figure 6A). The two main networks, “Genetic Disorders, Neurological Diseases” and “Nutritional Diseases, Lipid Metabolism and Small-Molecule Biochemistry”, are presented in Figure 6B and 6C. Network 1 highlights the global repression of serotonin receptors and an upregulation of Ach nicotinic acid receptors (Figure 6B). Network 2 highlights changes in the expression of genes directly involved in lipid metabolism (Figure 6C), with the repression of genes regulated by Ppara and the upregulation of Thrb signaling. Many genes encoding GABAergic receptors and potassium channels involved in neurotransmission were also dysregulated, and some

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**Figure 4. Analysis of mRNA levels for key adipogenic genes by RT-qPCR on the adipose tissue of mice fed either the CD or the HFD, born to either lean or obese/diabetic mothers (F1LM and F2OM).** The values shown are the ratios between OP1 or OR1 and CD1 and between OP2 or OR2 and CD2 (Figures 9 and 10). They are expressed as the mean±SEM, n = 6 per group. *p<0.05 for comparison with control CD, **p<0.01 for comparison between OP and OR mice, ***p<0.005 for comparison between F1LM and F2OM, assessed by Kruskal-Wallis tests followed by post hoc Dunn’s tests.
doi:10.1371/journal.pone.0066816.g004
Normal DNA methylation was associated with an upregulation of genes involved in fatty acid oxidation were globally upregulated. Changes to hepatic metabolism, together with the probable redirection of lipids to activated in OP1 mice and that insulin signaling was greatly disturbed. In OP2 mice, genes related to insulin signaling were less affected, whereas genes involved in fatty acid oxidation were globally upregulated. Changes to hepatic metabolism, together with the probable redirection of lipids to muscle thus spared the liver from lipid accumulation. Finally, in OR2 mice, lipid metabolism as a whole was downregulated, whereas thyroid hormone signaling was upregulated. The HFD response was also associated, in OP1 mice, with an upregulation of potassium channels and serotonin receptors, subsequently reversed in both OP2 and OR2 mice. Changes in DNA methylation were observed in the livers of OP1 mice and the muscle of OP2 mice. In the liver, Set7/9 expression was decreased by the HFD in mice born to either lean or obese/diabetic mothers (F1LM and F2OM), whether OP or OR. In the livers of OP1 mice, DNA hypomethylation was associated with an upregulation of Suv39h1 and Suv39h2 expression, whereas, in both OR2 and OP2 mice, normal DNA methylation was associated with a decrease in Jhdm2a expression and an increase in the level of Dnmt2 mRNA. In muscle, normal DNA methylation was associated with an upregulation of Suv39h1, Set7/9 and Dnmt2 in OP1 and OR2 mice, contrasting with the lower level of expression of the Set7/9 and Dnmt2 genes in the muscle of OP2 mice presenting DNA hypomethylation.

doi:10.1371/journal.pone.0066816.g005

Figure 5. Schematic diagram summarizing the transcriptional data obtained for the liver, muscle and adipose tissue and the results of epigenetic studies in OP1, OP2 and OR2 mice. Blue arrows represent potential lipid fluxes. In the hyperphagic OP1 and OP2 mice, lipid ingestion was much greater than in OR2 mice, in which caloric intake was normalized. In OP mice, excess lipids were initially stored in the adipose tissue, leading to adipocyte hypertrophy in OP1 mice and hyperplasia in OP2 mice, as a function of the level of expression of Lep and Peg1. In OR2 mice, lipids were stored in the adipose tissue without adipocyte abnormalities or ectopic storage, as in mice supplied with limited amounts of lipid. In OP1 mice, the excess lipids were stored in the liver, contributing to hepatic hypertrophy. Transcriptomic data indicated that de novo lipogenesis was activated in OP1 mice and that insulin signaling was greatly disturbed. In OP2 mice, genes related to insulin signaling were less affected, whereas genes involved in fatty acid oxidation were globally upregulated. Changes to hepatic metabolism, together with the probable redirection of lipids to muscle thus spared the liver from lipid accumulation. Finally, in OR2 mice, lipid metabolism as a whole was downregulated, whereas thyroid hormone signaling was upregulated. The HFD response was also associated, in OP1 mice, with an upregulation of potassium channels and serotonin receptors, subsequently reversed in both OP2 and OR2 mice. Changes in DNA methylation were observed in the livers of OP1 mice and the muscle of OP2 mice. In the liver, Set7/9 expression was decreased by the HFD in mice born to either lean or obese/diabetic mothers (F1LM and F2OM), whether OP or OR. In the livers of OP1 mice, DNA hypomethylation was associated with an upregulation of Suv39h1 and Suv39h2 expression, whereas, in both OR2 and OP2 mice, normal DNA methylation was associated with a decrease in Jhdm2a expression and an increase in the level of Dnmt2 mRNA. In muscle, normal DNA methylation was associated with an upregulation of Suv39h1, Set7/9 and Dnmt2 in OP1 and OR2 mice, contrasting with the lower level of expression of the Set7/9 and Dnmt2 genes in the muscle of OP2 mice presenting DNA hypomethylation.

doi:10.1371/journal.pone.0066816.g005

clustered into a third network, “Developmental Disorders” (not shown).

The 128 genes differentially expressed between OR2 and OP2 mice were associated, in particular, with “nutritional and metabolic disease”. They included genes related to adiposity, dyslipidemia, T2D and organ damage, together with genes linked to steatobesity and inflammation (Table S2). The main biological function altered in obesity resistance was “Cell-to-Cell Signaling”, due to the dysregulation of many genes encoding receptors modulating G-protein signaling (adrenergic, glutamatergic and serotonergic receptors), proteins modulating intracellular calcium levels and potassium channels. OR2 mice displayed lower levels of orexigenic gene expression than OP2 mice, and an upregulation of genes encoding anorexigenic factors (Table 2). Moreover, strict mirror-image patterns of expression were observed for 42 genes in OR2 and OP2 mice, in the OR2/CD2 and OP2/CD2 comparisons. These genes included 25 neurotransmission-related genes, many encoding potassium channels, six imprinted genes and several other genes involved in thyroid hormone signaling and insulin sensitivity.

The 53 genes dysregulated in both OP1 and OP2 mice had functions in pain; metabolic and nutritional disorders, with the altered genes related to adiposity, diabetes and hypothyroidism; obesity and hepatic system disorder, with genes associated with liver cancer and hematological disorders (Table S3A). Forty-five of these genes clustered into three main networks relating to “Lipid Metabolism, Molecular Transport, Small-Molecule Biochemistry”, “Endocrine System Development and Function” and “Organismal Injury and Abnormalities” (Table S3B). In total, 57 genes involved in the response to HFD were differentially regulated between OP1 and OP2 mice (comparisons between OP1/CD1 and OP2/CD2) but similarly regulated in OP2 and OR2 mice (comparisons between OP2/CD2 and OR2/CD2). Many of the genes upregulated in response to a HFD in OP1 mice and expressed or unmodified by a HFD in both OP2 and OR2 mice were related to neurotransmission, encoding potassium channels, cholinergic and serotonergic receptors, or involved in lipogenesis. In the OP2/OP1 comparison, most (65%) of the 35 genes displaying differential regulation between F1LM and F2OM obese mice were repressed and related to the following functions and diseases: “Nutritional Disorder”, “Synaptic Transmission”, “Genetic Disorder” and “Inflammatory Disorder” (Figure 7). In OP2 mice, the genes globally repressed with respect to OP1 mice were involved in lipid storage and accumulation, related to the calcium signaling pathway, or encoded potassium channels. These genes clustered together in a network related to “Nutritional Disease and Organismal Function” (Figure 7). This maternal effect (obese versus lean mother) was characterized, in adipose tissue, by a mirror-image for the expression of Pdk4. There were also specific changes in the expression of several genes of the epigenetic machinery in both the liver and muscle (Table 2).
Epigenetic Analyses

Global DNA methylation and histone modifications: Chromatin is regulated by modifications of about 80 known histones and DNA modifications, generating docking sites for enzymes that modify chromatin or remodel nucleosomes. DNA methylation is often associated with gene repression, whereas histone methylation may be associated with gene activation or gene repression, depending on the residue methylated, the degree of methylation and the position of the methylated residue with respect to the gene sequence. Histone acetylation in the promoter and coding regions of genes is essentially correlated with transcriptional activation [55]. Providing an additional level of complexity, recent studies have highlighted the crosstalk between marks [56]. However, given the large number of epigenetic marks...
| Relevant functions and diseases       | p-value  | Numbers of genes |
|--------------------------------------|----------|------------------|
| Nutritional disorder                 | 3.12E-12 | 14               |
| Ahr, Cckbr, Chrm1, Chrm4, drd4, Ins, Irs2, Kona3, Lpin1, Lpin2, Plin1, Pten, Sstr3, Ucp3 |
| Synaptic transmission                | 1.32E-09 | 10               |
| Camk4, Cckbr, Chrm1, Chra4, Chra6, Drd4, Grin2d, Grm1, Grm8, Kona1 |
| Genetic disorder                     | 2.24E-07 | 34               |
| Adora2b, Ahr, Cacna1dt, Cacna1s, Camk4, Cckar, Cckbr, Chrm1, Chrm4, Chra6, Drd4, Grin2d, Grm1, Grm8, Hcn1, Ins, Irs2, Kcna1, Kcna3, Kcnj4, Kcnj1, Lpin1, Lpin2, Mycn, Otx2, Plin1, Pten, Snrpn, Sstr3, Timp3, Ucp3, Vipr2 |
| Inflammatory disorder                | 4.02E-04 | 18               |
| Adora2b, Ahr, Cacna1d, Camk4, Cckar, Chrm1, Chrm4, Chra4, Grin2d, Grm8, Hcn1, Ins, Lpin1, Pten, Snrpn, Sstr3, Timp3, |

“Nutritional Disease and Organismal Function”
and modifiers in existence, we still know very little about the ways in which the diverse environmental factors that interfere with different chromatin landscapes and players induce global chromatin modifications and control the expression of individual genes [57].

We first evaluated the global level of DNA methylation with the LUMA technique. DNA methylation levels were found to be lower than normal in the livers of OP1 mice only (Figure 8A), and in the muscles of OP2 mice only (Figure 8A). No change in DNA methylation was observed in the adipose tissue or kidneys (data not shown). The DNA hypomethylation observed in mice on a HFD was not due to a lower intake of methyl donors, as the amounts of these compounds (choline, betaine, biotin, folic acid, methionine) provided by the diet were adjusted according to caloric content. However, efficiency of use of these substrates and of the cellular pathways involved may differ between OP and OR mice, thus contributing to differences in DNA methylation levels [58,59,60](Table 2).

Similarly, global changes to histone acetylation and methylation may also be observed under diabetic stimuli, such as exposure to high glucose concentrations, with effects on key genes related to diabetes, glycemic memory and inflammation [61,62,63,64]. The experiments described above used up large amounts of tissue. As a result, the remaining samples were sufficiently large for testing only for the OP2 and OR2 groups born to obese mothers (F2OM). Four posttranslational histone modifications, H3K9ac, H3K9me1, H3K9me3, and H3K4me3, were analyzed by western blotting in the livers of OP2, OR2 and CD2 mice (Figure 8B). Obesity resistance was characterized by a specific decrease in two histone marks – H3K9ac and H3K9me1 – in the livers of OR2 mice. Both H3K9 acetylation (H3K9ac) and H3K9me1 levels were significantly lower in OR2 mice than in CD2 mice, but did not differ significantly between OR2 and OP2 mice, suggesting an effect of diet. Levels of H3K4me3, a mark often associated with transcriptionally active chromatin, were significantly higher in both OP2 and OR2 mice, than in control mice, suggesting a diet effect. No differences were found in the levels of H3K9me3, a

Figure 7. Maternal effect (lean versus obese mother): Relevant functions and diseases, and representation of the major network of interaction identified by IPA analysis of the genes differentially expressed between OP1 and OP2 obese mice. The 35 genes differentially expressed between the obese mice born to lean and obese/diabetic mothers (F1LM and F2OM) obtained in the direct OP2/OP1 comparison were subjected to IPA analysis. The network shown contains 20 focal genes with a score of 50. The node color indicates the expression levels of genes: red, upregulated; green, downregulated.

doi:10.1371/journal.pone.0066816.g007

Figure 8. Analysis of global epigenetic modifications in several organs of mice fed either the CD or the HFD, born to either lean or obese/diabetic mothers (F1LM and F2OM). (A) Global DNA methylation analysis by LUMA in the liver and muscle of CD, OR and OP females. *HpoI/MspI levels indicate the degree of CCGG unmethylation. (B) Global analysis of posttranslational histone modifications by western blotting, for the liver of F2OM females. The values shown are the mean±SEM. *p<0.05 for comparison with control CD, **p<0.05 for comparison between OP and OR mice, ***p<0.05 for comparison between the F1LM and F2OM mice born to lean and obese mothers, respectively, assessed by Kruskal-Wallis tests and post hoc Dunn’s tests.

doi:10.1371/journal.pone.0066816.g008
In both the liver and muscle, Dnmt1, Dnmt3a and Dnmt3b expression levels were similar in OP and OR mice born to both lean and obese mothers and their corresponding controls, CD1 and CD2. Dnmt3b expression was not detectable in muscle and Dnmt3a mRNA was barely detectable in either tissue. DNA hypomethylation was therefore not correlated with lower levels of Dnmt1 or Dnmt3a and Dnmt3b expression after five months on a HFD. However, we cannot exclude the possibility that dysregulation of the production, activity or turnover of these DNMTs occurred earlier in the establishment of the response to the HFD and was subsequently normalized (Table 2).

Only Dnmt2 (Trdmt1) expression appeared to be modulated by the HFD in both liver and muscle. In the liver, Dnmt2 expression was modulated only in the offspring of obese mothers (F2OM), being upregulated in both OP2 and OR2 mice (Figure 9A). In muscle, mirror-image patterns were observed for the expression of the DNA methyltransferase gene Dnmt2, which was found to have increased in OP1, OR1 and OR2 mice, but to have decreased in OP2 mice (Figure 9B). The role of Dnmt2 remains a matter of debate, but a recent study highlighted its importance in the maintenance of genome stability, organ development, metabolic processes and resistance to oxidative stress [65]. Interestingly, Dnmt2 was upregulated in tissues with normal levels of DNA methylation, but downregulated in the muscles of OP2 mice, which displayed DNA hypomethylation (Figure 5, Table 2).

In the liver, the expression of the two H3K9 methyltransferase genes, Suv39h1 and Suv39h2, was upregulated only in OP1 mice (Figure 10), potentially inducing potential global changes in the level of H3K9 methylation, which is known to promote heterochromatin formation and gene silencing. However, as reported above, no difference in H3K9me3 level was found between the OP2, OR2, and CD2 groups. Nevertheless, we cannot exclude possible effects in the offspring of lean mothers (F1LM), in which it was not possible to study histone marks (Table 2).

The H3K4 methyltransferase gene Set7/9 was downregulated in the livers of both F1LM and F2OM mice. Despite this, F2OM mice had higher levels of H3K4me3 than controls, suggesting either a potent compensatory mechanism operating at the transcriptional level or the involvement of other molecules due to crosstalk between marks and enzymes. Suv39h1 and Set7/9 were upregulated in the muscles of both the OP1 and OR1 groups (Figure 10). In the offspring of obese mothers, Suv39h1 was upregulated in OR2 mice only and Set7/9 was regulated in opposite ways in OP2 and OR2 mice, tending to be upregulated in OR2 mice and significantly downregulated in OP2 mice. Suv39h2 expression levels were high specifically in OR1 and OR2 mice. The Set7/9 gene has been implicated in inflammation and diabetes, and its downregulation in monocytes has been shown to decrease the expression of key genes induced by inflammatory stimuli [66]. The expression of Jhdm2a, encoding an H3K9 demethylase, was weak only in OP2 mice. Jhdm2a is closely linked to several metabolic processes, as its product regulates the expression levels of genes encoding proteins involved in energy homeostasis, fat storage and glucose transport [67,68] (Table 2).

Expression of the histone acetyl transferase gene HAT was upregulated in the liver of OP2 mice only. Expression of the histone deacetylase gene Hdac3 was significantly stronger in OR1 mice than in CD1 and OP1 mice, whereas no differences between groups were found in the offspring of obese mothers F2OM (Table 2). Interestingly, the Hdac3 expression profile in muscle was similar to that in the liver, with specific upregulation in OR1 mice only, suggesting a role in obesity resistance specific to the offspring of lean mothers. Finally, the levels of expression of the Gen3 histone
acetyl transferase gene (Kat2a) in the liver and of Hat1 (Kat1) in muscle were similar between groups. Expression of the histone deacetylase genes Lsd1, Hdac3 and Hdac7a was not affected by the HFD or by susceptibility/resistance, in either the liver or muscle (data not shown).

Some tissue-specific expression patterns seemed to depend on the level of DNA methylation. In the liver, the hepatic expression of the H3K9 demethylase gene Jhdm2a (Kdm3a) was lower in both groups (OP2 and OR2) of offspring born to obese mothers F2OM than in the control. In muscle, normal DNA methylation was associated with the upregulation of Suv39h1 (Kdm1a), Dnmt2 (Trdmt1) and Set7/9 (Kmt7) in OP1 and OR2 mice. This upregulation contrasted with the lower levels of Set7/9 and Dnmt2 gene expression observed in the muscles of OP2 mice presenting DNA hypomethylation (Table 2).

In the liver, DNA methylation levels were low only in the OP1 group. By contrast, in muscle, DNA hypomethylation was observed in OP2 mice only. As far as the epigenetic machinery was concerned, histone K-methylation was affected in OP1 mice only, and histone K-acetylation was increased in OP2 muscle only (Table 2).

The lower levels of histone demethylation in the F2OM groups and the higher levels of histone K-deacetylation in OR1 mice probably reflect group-specific compensation mechanisms. It is therefore tempting to link DNA hypomethylation, lipotoxicity and tissue dysfunction. (Table 2).

The expression of genes encoding epigenetic machinery proteins involved in DNA methylation and histone K-deacetylation was altered in the livers of OR1 mice only. In OR mouse muscles, the only DNA methyltransferase gene displaying differential expression, Dnmt2, displayed opposite patterns of regulation in OR2 and OP2. The stronger expression of genes encoding proteins involved in histone K-methylation and histone K-deacetylation in OR1 and OR2 mice also suggests that specific epigenetic changes associated with either maternal leanness or obesity are involved in these processes of peripheral resistance. (Table 2).

Diet sensitivity was noted for genes or marks dysregulated in the same way in all groups fed the HFD. In the liver, the expression of

Figure 10. Analysis, by RT-qPCR, of the expression of genes encoding histone-modifying enzymes in the liver and muscle of mice fed the HFD, for both lean and obese mothers. The values shown are the ratios between OP1 or OR1 and CD1 and between OP2 or OR2 and CD2. They are expressed as the mean ± SEM, n = 8 per group. *p < 0.05 for comparison with control CD, †p < 0.05 for comparison between OP and OR mice, ‡p < 0.05 for comparison between the F1LM and F2OM (maternal effect: lean versus obese mother), assessed by Kruskal-Wallis tests and post hoc Dunn’s tests.
doi:10.1371/journal.pone.0066816.g010
Set7/9 was clearly affected by diet, in both groups, as was histone H3K4me3 level.

The response to HFD and resistance/susceptibility to the obesogenic effects of the HFD were thus associated with tissue-specific changes in the expression of chromatin-modifying enzymes. It is difficult to determine the functional significance of these global changes, but these marks could potentially serve as markers for an effect of diet or for a susceptibility/resistance trait. Genome-wide analyses would make it possible to identify the sequences or genes on which these epigenetic modifiers act to affect susceptibility/resistance to the obesogenic effects of HFD.

DNA hypomethylation is a major feature of cells undergoing tumor progression and is a hallmark of many types of cancer, including hepatocellular carcinoma (HCC) in particular [69,70]. It remains unclear whether HCC can develop from fatty liver, but it is striking that the risk of developing HCC is 4.5 times higher in obese individuals than in normal-weight subjects [71]. The DNA hypomethylation induced in DIO, with the development of hepatic steatosis, may therefore be a primary defect, as suggested by the dysregulation in the livers of OP mice of genes encoding proteins involved in liver cancer, with the potential to progress to more extensive cell damage and, potentially, carcinogenesis [72]. Thus, if this hypothesis can also be demonstrated in humans, DNA hypomethylation could be considered a marker of steatosis and, hence, of the risk of developing HCC. The association between DNA hypomethylation and NAFLD may provide new insight into the risk of HCC.

Conclusion

Despite the well-known deleterious effects of maternal obesity and T2D, the female offspring of obese mothers display greater resistance to the obesogenic effects of a HFD after weaning. Our results clearly demonstrate that, in a context of maternal obesity and diabetes, an appropriate dietary fatty-acid profile and intake during the periconceptional/gestation/lactation period helps the female offspring to cope with subsequent obesogenic conditions. These findings are reminiscent of those for other environmental factors, such as carbon tetrachloride or bile duct ligation triggering liver damage, and cocaine-self administration in rats [73,74]. Fathers exposed to these chemicals display deleterious effects of exposure, whereas their offspring are better adapted to cope with future fibrogenic challenges to the liver or display a cocaine-resistant phenotype respectively. Exposure in the parent may therefore lead to greater resistance in the offspring. Exposure to a HFD leads to complex adaptation processes in several peripheral organs of the offspring, involving changes in the expression of imprinted genes and genes involved in metabolism and neurotransmission, together with more general epigenetic changes, such as marked changes in global DNA methylation, posttranslational histone modifications and the tissue-specific expression of genes encoding several chromatin-modifying enzymes. These findings highlight the complexity of the epigenetic mechanisms linked to overall adaptation to and attenuation of the deleterious programming effects of maternal obesity and T2D.

The networks identified highlight important connections between metabolism, neurotransmission and the imprinted gene network. The role of relevant metabolic pathways showing mirror-image patterns of expression in obesity-prone and obesity-resistant mice, or recruited for maternal effects specifically associated with either leanness or obesity/T2D and the unpredicted involvement of classes of liver genes related to neurotransmission reveal the importance of neural liver-brain crosstalk and provide new peripheral targets for prevention and treatment. We suggest that these gene networks may be considered as new environmental sensors and, hence, as novel, peripheral targets for the management of obesity and T2D. The different transcriptional and epigenetic modifications, according to maternal leanness or obesity/T2D status, underlying resistance or susceptibility to an obesogenic diet and associated with either the OP or OR phenotype therefore represent potential markers worthy of further study, with the aim of increasing our understanding of the etiology of T2D.

Methods

Animals and Diet

All experiments on animals were conducted after ethical review, under French government license, for three generations [21]. F0 generation: Male and female C57BL/6j mice were obtained from Harlan at four weeks of age. After one week of adaptation, the animals were housed in individual cages in a controlled-temperature room with a 12 h/12 h light/dark cycle and free access to water and a chow diet. F1LM generation: At six months of age, F0 males and F0 females were mated. The males were removed once the females were pregnant. The day after delivery, all F1LM litterers were randomly reduced to four or five pups. After weaning, all mice, of both sexes, were transferred to individual cages and randomly assigned to the control (CD, 10% of calories from fat, 20% from protein and 70% from carbohydrates) or high-fat (HFD, 60% from fat, 20% from protein and 20% from carbohydrates) diets for 20 weeks. Diets were supplied in pellet form by Research Diets (CD: D12450B, HFD: D12492). F2 generation: At six months of age, F1LM female mice fed the HFD that became obese (OP1) were crossed with F1LM control male mice on the CD. The pregnant F1LM females were maintained on the CD from mating until the end of lactation and the F2OM offspring (male and female) was switched onto the HFD for 20 weeks after weaning. Mice were classified on the basis of their weight as sensitive (obesity-prone, OP1, OP2) or resistant (obesity-resistant, OR1, OR2) to the HFD [21]. In parallel, F1LM female mice on the CD (CD1) were crossed with F1LM control male mice to generate F2OM control mice fed the control diet after weaning (CD2). Reproducibility was ensured by carrying out three independent experiments with contemporary controls [21]. In these experiments, we were unable to obtain a sufficiently large number of pregnancies when females were maintained on a HFD, thus precluding comparisons with such a group.

Caloric Intake

Food consumption was estimated by subtracting the amount of feed left on the grid and the amount of spilled food from the initial weight of food supplied. Energy intake was then calculated, assuming 3.8 kcal/g for the CD and 5.2 kcal/g for the HFD.

Body Composition

Body fat and lean mass were determined in vivo by dual-energy X-ray absorptiometry (DEXA) after 20 weeks on the CD or HFD. DEXA investigations in anesthetized animals (70 mg/kg ketamine/7 mg/kg xylazine) were performed on the whole body, excluding the head, with a Piximus apparatus® (Lunar Corporation, Madison, Wis., USA).

Plasma Assays

Blood was collected after 20 weeks on the assigned diet. The animals were fasted for 6 h, and blood samples were then collected by retro-orbital sinus puncture. Glycemia, triglyceride concentration and total cholesterol and HDL cholesterol (HDL-C)
concentrations were determined with a Behring RXL. Plasma insulin and leptin concentrations were determined with the Ultra-Sensitive Rat Insulin and Leptin ELISA kits, using the mouse standard, reference 90090 (Crystal Chem Inc). The HOMA index was calculated as the product of the fasting plasma insulin level (μU/ml) and the fasting plasma glucose level (mmol/l), divided by 22.5.

**Determination of the Fatty-acid Composition of LDL**

Total fatty acids (FA) in LDL were analyzed by capillary gas chromatography on a Hewlett-Packard 5890 gas chromatograph attached to a 5971A mass detector (Hewlett-Packard), as previously described [75]. Total lipids were extracted from LDL as described by Folch et al. [76]. The extract was saponified by incubation at 60°C for 60 min with potassium hydroxide (13.2 g/l) and then esterified at 60°C for 60 min with boron trifluoride (BF3)-methanol, to generate fatty acid methyl esters. The chromatographic analysis was as described above for oxidized cholesterol derivatives, except for the oven temperature, which was increased from 220°C to 280°C at a rate of 10°C/min. Heptadecanoic acid (17:0) was added to each sample as an internal standard before extraction, and the fatty-acid contents for saturated FA: palmitic acid (C16:0), stearic acid (C18:0); mono-unsaturated FA: palmitoleic acid (C16:1), oleic acid (C18:1); polyunsaturated n-6: linoleic acid (C18:2), arachidonic acid (C20:4); and for polyunsaturated n-3: docosahexaenoic acid (C22:6), were determined from the ratio of the peak area of the sample to the peak area of the internal standard.

**Extraction of RNA and DNA**

Total RNA was extracted from liver and adipose tissue with the RNeasy Mini kit® (Qiagen S.A.). DNA was extracted from muscle, liver, kidneys, adipose tissue and testis with the DNeasy Blood and Tissue Kit® (Qiagen S.A.). DNA and RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and quality was assessed by agarose gel electrophoresis.

**Custom-built Liver Microarray**

The custom-built microarray design and array prehybridization and hybridization procedures have been described in detail elsewhere [23]. The microarray used contained 992 gene-specific 50-mer oligonucleotides generated from 63 imprinted genes (See Table S1), 256 genes encoding proteins involved in neurotransmission and 157 genes encoding proteins related to energy homeostasis. RNA quality was assessed by electrophoresis, in RNA 6000 assays on an Agilent 2100 bioanalyzer (Agilent Technologies). For cDNA synthesis, oligo-dT and RPO were added to 15 μg of total RNA and the mixture was incubated for 10 min at 70°C and then cooled on ice. We added 15 μl of 25× prexiprim buffer (500 μM dCTP, 500 μM dATP, 500 μM dGTP, 100 μM dTTP, 100 μM Cy3-dUTP/Cy 5-dUTP, 600 U Superscript II Reverse Transcriptase, 10 mM DTT and 1×reverse transcriptase buffer), 1 μl of Cy3 dUTP or Cy5 dUTP and 2 μl of Superscript II reverse transcriptase to each sample. Reverse transcription was carried out overnight at 42°C. We then added 15 μl of 0.1 N NaOH and incubated the mixture for 10 min at 70°C to degrade the RNA template, and then with 15 μl of 0.1 N HCl to neutralize the reaction. The product was purified with the QiAquick PCR Purification Kit® from Qiagen. A direct comparison strategy with dye-swapping was used to compare two conditions, and dye-swaps were replicated several times, with at least four animals/group. Comparisons between conditions were made according to an open-loop design, including the direct comparisons of major interest; OP1 vs CD1 (OP1/CD1); OP2 vs CD2 (OP2/CD2); OR2 vs CD2 (OR2/CD2); OR2 vs OP2 (OR2/OP2) and OP2 vs OP1 (OP2/OP1), such that secondary comparisons could be estimated [77]. Direct comparison between OR2 and OR1 mice was not possible due to the limited number of samples used for array hybridization. Arrays were normalized by LOESS regression, using the LIMMA library from the R/BIOCONDUCTOR packages to assess differential expression between conditions. Microarray experiments, described according to MIAME guidelines, have been deposited in NCBI’s Gene expression Omnibus repository under accession number GSE30085 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30085).

**Microarray Analysis**

Heat maps were constructed by compiling the differentially expressed genes (DEG) in the various comparisons (OP1/CD1, OP2/CD2, OR2/CD2, OR2/OP2, OP2/OP1), thereby allowing further indirect comparison between groups. Biologically relevant pathways were identified after importing Entrez Gene IDs into IPA software (version 8.7 Ingenuity® Systems, http://www.ingenuity.com). Several analyses were carried out with the lists of DEG obtained for the OR2/CD2, OR2/OP2 and OP2/OP1 comparisons and the subset of genes displaying dysregulation in both OP1/CD1 and OP2/CD2 comparisons. All the spot entries were recognized by the program and included in network and function/pathway analyses. The DEG were mapped to the genetic networks available in the Ingenuity database and were then ranked on the basis of their score. Only networks with scores of at least 20 were considered for interpretation, and functions and diseases were considered relevant if they had a p value<10^-5.

**Reverse Transcription and Quantitative PCR (RT-qPCR)**

First-strand cDNAs were synthesized from 2 μg of total RNA from adipose tissue samples, in the presence of 50 ng random hexamers (GE Healthcare), 400 nM dNTP and 200 U of SuperScript™ II RNase H Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. qPCR analyses were carried out with the Absolute Blue QPCR SYBR Green Rox Mix (Thermo Scientific), with a 7300 Real-Time PCR System (Applied Biosystems), according to the manufacturer’s instructions. Amplification was carried out in a final volume of 25 μl, with 2 x SYBR, 7.5 μM of each primer and 10 ng of the cDNA sample. Each reaction was run in duplicate. Eight-point standard curves were constructed from serial 10- fold dilutions, with cDNA pooled equally from all animals. 18S, Actb, Eif4a2, Silha and Mpi32 were tested as reference genes in the Genorm Software [78]. The qPCR were normalized with the Genorm normalization factor obtained with Actb, Eif4a2 and Silha. The differences in mRNA levels between CD and either OP or OR mice for each maternal effect (lean versus obese mother) were calculated and are expressed relative to CD levels. The complete list of primers used is provided in Table S4.

**Luminometric Methylation Assay (LUMA):** Global DNA methylation was analyzed as previously described by Karimi et al. [79]. Restriction enzymes (HpaII, MspI, and EcoRI) were purchased from New England Biolabs. Briefly, 500 ng of genomic DNA was subjected to double digestion with either HpaII + EcoRI in buffer 1 (10 mM bis-Tris propane-HCl, 10 mM MgCl2, 1mM DTT) or MspI + EcoRI in buffer 4 (50 mM potassium acetate, 20 nM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT) for four hours at 37°C. The digested DNA was subjected to polymerase extension assays on the pyrosequencing platform. For this purpose, pyrosequencing annealing buffer (Biotage) was added to each reaction, and the reaction mixture was transferred
to 24-well pyrosequencing plates and analyzed with PyroGold Q24 Reagents and the Pyromark Q24 Instrument, with an assay sequence defined as AC/TCGA. The level of cytosine methylation was finally determined by comparing the ratio of $\text{H}^\text{m}\text{C}2\text{G}$ to $\text{H}\text{m}\text{C}2\text{A}$ in liver DNA from each group of F2OM mice, as previously described [80]. Protein concentration was determined with a Pierce BCA assay kit (ThermoScientific). Histones (2 μg) were separated by electrophoresis in a 15% polyacrylamide SDS-PAGE gel and transferred onto PVDF membranes (0.2 μm pore, Millipore). The membranes were blocked by incubation with 5% nonfat milk in PBS-Tween. After washing in PBS-Tween, membranes were incubated overnight with the primary antibody in 2% nonfat milk (anti-H3K9me3 1:1000, anti-H3K4me3 1:1000, anti-H3K9ac 1:10000, anti-H3K9me1 1:1000; Millipore, 07-442, 07-473, 07-450, 07-352, respectively) and then for 1 hour with secondary antibody conjugated with horseradish peroxidase (anti-rabbit IgG 1:10000, Sigma A6667). Signals were detected with ECL Plus (Amersham) and LAS-1000 (Fujifilm). The membranes were stripped and incubated for one hour with anti-pan H3 antibody (Abcam ab1791, 1:5000). The secondary antibody and signal detection were as previously described. Signals were quantified with AIDA software and the signal for each specific modification was normalized to the amount of total H3. The whole western blotting procedure for each histone mark and total H3 was carried out twice. We then averaged the signals for the two experiments.

**Statistical analyses:** All statistical analyses were carried out with GraphPad Prism 2.0 Software. Phenotypic parameters (body weight, caloric intake, hormone levels) with a normal distribution were subjected to anova followed by Dunnett’s post hoc tests, and differences were considered to be significant if $p<0.05$.

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