Epigenetic Gene Promoter Methylation at Birth Is Associated With Child's Later Adiposity

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OBJECTIVE—Fixed genomic variation explains only a small proportion of the risk of adiposity. In animal models, maternal diet alters offspring body composition, accompanied by epigenetic changes in metabolic control genes. Little is known about whether such processes operate in humans.

RESEARCH DESIGN AND METHODS—Using Sequenom MassARRAY we measured the methylation status of 68 CpGs 5′ from five candidate genes in umbilical cord tissue DNA from healthy neonates. Methylation varied greatly at particular CpGs: for 31 CpGs with median methylation ≥5% and a 5–95% range ≥10%, we related methylation status to maternal pregnancy diet and to child's adiposity at age 9 years. Replication was sought in a second independent cohort.

RESULTS—In cohort 1, retinoid X receptor-α (RXRA) chr9: 136355885+ and endothelial nitric oxide synthase (eNOS) chr7: 150315553+ methylation had independent associations with sex-adjusted childhood fat mass (exponentiated regression coefficient [β] 17% per SD change in methylation [95% CI 4–31], P = 0.009, n = 64, and β = 20% [9–32], $P < 0.001$, n = 66, respectively) and %fat mass (β = 10% [1–19], P = 0.023, n = 64 and β =12% [4–20], P = 0.002, n = 66, respectively). Regression analyses including sex and neonatal epigenetic marks explained >25% of the variance in childhood adiposity. Higher methylation of RXRA chr9: 136355885+, but not of eNOS chr7:150315553+, was associated with lower maternal carbohydrate intake in early pregnancy, previously linked with higher neonatal adiposity in this population. In cohort 2, cord eNOS chr7:150315553+ methylation showed no association with adiposity, but RXRA chr9:136355885+ methylation showed similar associations with fat mass and %fat mass (β = 6% [2–10] and β = 4% [1–7], respectively, both P = 0.002, n = 239).

CONCLUSIONS—Our findings suggest a substantial component of metabolic disease risk has a prenatal developmental basis. Perinatal epigenetic analysis may have utility in identifying individual vulnerability to later obesity and metabolic disease.
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Fixed genomic variations explain only a fraction of the risk of human obesity and metabolic disease (1,2). However, there is increasing epidemiological evidence linking perinatal factors to later adiposity and metabolic disease risk (3,4). For example, famine during pregnancy is associated with obesity in the adult offspring (5) and normal variations in maternal body composition relate to child's later adiposity (6). Although understanding of the underlying mechanisms is limited, data from animal models suggest that epigenetic processes are an important link between the early life environment, for example maternal diet, and altered metabolism and body composition in the adult offspring (7–9).

Epigenetic processes such as DNA methylation and histone modifications allow the developmental environment to modulate gene transcription; many of these changes are then stable throughout the lifecourse (10,11). Regulated DNA methylation mostly occurs at a cytosine immediately 5′ to a guanine (CpG sites). Such processes are involved not only in cell differentiation and parental genomic imprinting but also in developmental plasticity through which the environment in early life can affect the developmental trajectory, with long-term effects on gene expression and phenotypic outcome (12–14). For example, in the rat unbalanced maternal diet during pregnancy induces changes in DNA methylation and covalent histone modifications in the 5′ regulatory regions of specific non-imprinted genes (15–17) and affects the offspring's later body composition and metabolic phenotype. Induced changes in phenotype can be prevented by nutritional interventions during pregnancy (18), or altered by nutritional interventions during the juvenile-pubertal period (19) or by hormonal interventions during suckling (20).

Although epigenetic processes operating in early development have been implicated in the origins of obesity (3,11), there is as yet no direct evidence for this proposition in humans. Furthermore, there has been considerable debate as to the relative importance of such developmental pathways in determining phenotypic outcomes. Any association between epigenetic state at birth and later phenotype would allow an estimate of the possible developmental contribution, irrespective of whether such an association was causal or simply reflected the developmental state.

We reasoned that the targeted measurement of DNA methylation in human fetal tissues at birth might not only provide evidence that environmental influences have affected prenatal development but, if they then correlated with later phenotype, would provide an approach to

demonstrating the role of the prenatal environment in predisposition to adiposity.

Here we first measured the methylation status of CpGs in the promoters of candidate genes in DNA extracted from umbilical cord tissue obtained at birth in children who were later assessed for adiposity at age 9 years (the Princess Anne Hospital [PAH] study) (21). Measurements of perinatal DNA methylation were related to adiposity in later childhood and to information on mother's diet during pregnancy. Because of the strong associations found, we then sought to replicate associations between umbilical cord CpG methylation and child's adiposity in a second independent group of children from the Southampton Women's Survey (SWS) (22).

RESEARCH DESIGN AND METHODS

We studied two prospective cohorts recruited antenatally in Southampton, U.K. In the PAH study, Caucasian women ≥16 years old with singleton pregnancies <17 weeks' gestation were recruited and a validated food frequency questionnaire (23) was administered at 15 weeks' gestation; patients with diabetes and hormonally induced conceptions were excluded. When the children approached 9 years, we wrote to 461 still living locally. Two hundred and sixteen (47%) attended a clinic, and adiposity was measured using dual energy X-ray absorptiometry (DEXA); 78 had DNA available from an umbilical cord sample collected at birth and stored at −80°C. None of the mothers were administered glucocorticoids during pregnancy.

In the SWS, women aged 20–34 years were recruited when not pregnant; those who subsequently conceived were followed through pregnancy and the offspring followed up (22). To replicate associations with adiposity found in the PAH cohort, we studied 239 children selected as having both umbilical cord DNA available and childhood adiposity measurements.

Both groups underwent adiposity measurement by DEXA (PAH study, age 9 years: Lunar DPX-L, pediatric software, version 4.7c; GE Corporation, Madison, WI [6]; SWS, age 6 years: Hologic Discovery, pediatric scan mode; Hologic, Bedford, MA). Instruments were calibrated daily; coefficients of variation were <1%. Follow-up of the children and sample collection/analysis was carried out under Institutional Review Board approval (Southampton and SW Hampshire Research Ethics Committee) with written informed consent. Investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Selection of candidate genes. DNA from 15 randomly selected PAH study umbilical cords underwent methylation-specific chromatin precipitation followed by hybridization to a commercial tiled oligomer microarray (NimbleGen Systems HG17_min_promoter array [24]), which uses 50-mer oligonucleotides positioned on average every 100 bp from −3,750 bp to +500 bp relative to the transcription start site of 24,134 human genes. We focused our analysis initially on a panel of 78 candidate genes, 28 selected on the basis of animal data from our and other laboratories (25,26) and 50 selected from the array as having the highest SD between subjects relative to the SD of within-subject replicates. For this panel we identified those with correlations between overall gene methylation status and DEXA measurements of adiposity at age 9 years and from these genes chose five genes for further study based on individual oligomers showing evidence of correlation with DEXA measurements at age 9 years, biological plausibility and feasibility of designing amplicons suitable for Sequenom analysis; genes selected were retinoid X receptor-α (*RXRA*), endothelial nitric oxide synthase (*eNOS*), superoxide dismutase-1 (*SOD1*), interleukin-8 (*IL8*), and phosphoinositide-3-kinase, catalytic, δ-polypeptide (*PI3KCD*). These genes all had associations of comparable strength between childhood body composition and both overall gene promoter methylation and the methylation of individual oligomers. All chromosomal coordinates are based on University of California Santa Cruz, human genome March 2006 assembly (hg18).

Sequenom and single nucleotide polymorphism analysis. Genomic DNA was purified from frozen cords by proteinase K digestion and phenol:chloroform extraction. DNA methylation was measured with the Sequenom MassARRAY Compact System (www.sequenom.com/). Briefly, gene-specific amplification of bisulfite-treated DNA was followed by in vitro transcription and analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (27,28). Sequenom assay design and methods were as per the manufacturer's protocol. DNA (1 μg) was bisulfite converted using EZ DNA Methylation kit (Zymo Research). PCR primers specific for bisulfite converted DNA were designed (Methprimer). Each reverse primer contained a T7-promoter tag for in vitro transcription (5′-cagtaatacgactcactatagggagaaggct-3′), and the forward primer was tagged with a 10mer to balance Tm (5′-aggaagagag-3′). Supplementary Table 1 lists amplicons, primer sequences, PCR annealing temperature, and genomic coordinates for the extended promoter regions measured. Bisulfite-treated DNA was PCR amplified (Qiagen HotStar Taq Polymerase) in 5 μL reactions and treated with Shrimp Alkaline Phosphatase (Sequenom) (20 min at 37°C), heat inactivated (85°C for 5 min), and simultaneous in vitro transcription/uracil-cleavage reaction was carried out (7 μL reactions) using Sequenom T-cleavage reagent mix. Transcription cleavage products were desalted with 6 mg of Clean-Resin and 20 nL spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung). Mass spectra were acquired using a MassARRAY MALDI-TOF MS (Bruker-Sequenom) and peak detection, signal-to-noise calculations, and quantitative CpG site methylation performed using proprietary EpiTyper software v1.0 (Sequenom). Consistent with the manufacturer's specification (www.sequenom.com) agreement between technical replicates was good, duplicate measures differing by <10% methylation in almost all cases. We excluded from analysis samples that failed to give a reliable PCR product or produced spectra with low confidence scores (<2.9 in EpiTyper). For fragments containing a single CpG site, DNA methylation was calculated by the ratio of methylated to unmethylated fragments. Limitations imposed by Sequenom analysis treat cleavage products containing multiple CpG sites as single units, and the methylation values reported are weighted averages across the unit (referred to as a CpG group). DNA quality and no-template controls, 0%, and 100% methylated DNA were included in all assays. Supplementary Table 2 shows the distributions of methylation values for CpGs and CpG groups and numbers of subjects with measurements available.

Pyrosequencing analysis (PyroMark Q96MD; Qiagen) was undertaken to exclude single nucleotide polymorphisms (SNPs) involving CpG dinucleotides, which would result in loss of potential for methylation. Briefly, DNA was PCR amplified, and the product annealed to streptavidin-coated sepharose beads and denatured (0.2M NaOH) to a single-stranded product. Nucleotides were incorporated to the open 3′ DNA strand in which pyrophosphate is released and used in a sulfurylase reaction emitting ATP, measured in a luciferase reaction, and analyzed using PyroMark MD v1.0 software (Qiagen) (29).

Statistical analysis. Using Stata 11 (StataCorp) study variables were transformed using Fisher-Yates (30) (for methylation measurements) or logarithmic transformations where necessary to satisfy statistical assumptions of normality. A priori we restricted the analysis to CpGs with median methylation ≥5% and a 5–95% range ≥10%. In PAH subjects we first used Pearson correlation (r_p) and linear regression to examine CpG methylation in relation to child's adiposity. Mutually adjusted regression models were built including CpG sites significantly associated with each outcome in univariate analyses ($P < 0.05$). Because the measurements of childhood adiposity are on the log scale and the CpG measurements are z scores, the exponentiated results are interpreted as percent change in childhood adiposity measurements per SD change in methylation. To allow assessment of the influence of taking account of the child's sex, exponentiated regression coefficients are first presented excluding and including adjustment for child's sex. We then preadjusted all adiposity measurements for sex and took account of the mother's age, adiposity (as continuous variables), and smoking during pregnancy (as a binary variable, yes/no) by including them as covariates in the regression model; we next related mother's diet to CpG methylation at birth. Finally, we sought replication of the associations between CpG methylation and child's adiposity in the SWS cohort. SWS measurements, taken over a wider age range than those in PAH subjects, were preadjusted for age and sex.

RESULTS

Median (interquartile range) birth weight, age, fat mass, and %body fat at follow-up values for the PAH subjects were 3,330 g (3,010–3,790), 8.66 years (8.52–8.73), 5.41 kg (3.97–8.41), and 18.3% (14.4–27.0), respectively. Comparable values for the 239 SWS children were 3,485 g (3,205–3,773), 6.59 years (6.41–6.78), 4.60 kg (3.66–5.69), and 23.4% (19.7–27.6), respectively. Similar percentages of mothers smoked in the PAH and SWS cohorts (21 vs. 24%, respectively); median maternal age and prepregnancy BMI were lower in the PAH mothers (28 vs. 31 years and 22.3 vs. 24.3 kg/m^2, respectively). The above characteristics are similar to the overall values for participants in the two cohorts.

PAH cohort methylation studies. Supplementary Table 2 shows the distribution of percent methylation of the 68 CpGs and CpG groups among the 78 PAH subjects; 31 CpGs/CpG groups met a priori criteria for further analysis.

Particularly marked interindividual variation in percent methylation was seen at some sites (for example, RXRA chr9:136355885+ had a median of 59% with 5–95th centiles 4–99%; eNOS chr7:150315553+ had a median of 93%, with 5–95th centiles 64–100%). Correlations between methylation of different CpGs were generally low (for example $r_p = -0.03$, $P = 0.81$, $n = 55$ between the above two CpGs).

Of the 31 CpGs with variable methylation above the a priori threshold, seven had significant associations with the child's adiposity at age 9 years (Supplementary Table 3). Table 1 shows the results of uni- and multivariate analyses of CpG sites with significant independent associations with the child's adiposity. Comparison of the regression coefficients for the univariate and multivariate associations of RXRA chr9:136355885+ and eNOS chr7:150315553+ methylation with child's fat mass, %fat, and ratio of trunk to limb fat shows that mutual adjustment for each other and sex has little effect on the coefficients; adiposity measurements were therefore preadjusted for sex in further analyses. RXRA chr9:136355885+ methylation had positive associations with childhood fat mass, %fat mass (scatterplots shown in Supplementary Fig. 1), and ratio of trunk to limb fat, with similar positive associations for eNOS chr7:150315553+: exponentiated regression coefficients (β) (95% CI) per SD change in methylation were 17% (4–31), $P = 0.009$; 10% (1–19), $P = 0.023$; and 6% (0–12), $P = 0.039$, respectively ($n = 64$). Comparable values for eNOS chr7:150315553+ methylation were 20% (9–32), $P < 0.001$; 12% (4–20), $P = 0.002$; and 7% (2–13), $P = 0.007$, respectively ($n = 66$). Methylation of RXRA chr9:136355885+ and eNOS chr7:150315553+ had similar positive associations with child's BMI (β = 3% [0–6], $P = 0.037$; and 4% [2–7], $P = 0.001$, respectively). Independently of RXRA chr9:136355885+ and eNOS chr7:150315553+ methylation, SOD1 chr21:31853660/63+ methylation was also inversely related to child's trunk-to-limb fat ratio ($P = 0.037$). The associations reflect clinically important shifts in adiposity such that the mean fat mass rose from 4.8 kg (17.3% body fat) in the lowest quarter of RXRA chr9:136355885+ methylation to 6.6 kg (21.3% body fat) in the highest quarter of the distribution. Taking account of RXRA chr9:136355885+ methylation and sex explained 26% of the variance in fat mass in PAH children, and simultaneous analyses showed that the association between RXRA chr9:136355885+ methylation and child's fat mass was not attenuated after adjustment for birth weight and mother's age, smoking, and BMI.

In this population we have previously linked lower maternal carbohydrate intake in early pregnancy with higher neonatal adiposity (21). Relating umbilical cord CpG methylation to maternal carbohydrate intake in early pregnancy, higher methylation of RXRA chr9:136355885+, but not of eNOS chr7:150315553+, was associated with a lower maternal carbohydrate intake (Fig. 1). The mother's early pregnancy fat and protein intakes in early pregnancy were not associated with cord RXRA chr9:136355885+ methylation ($P = 0.7$ and $P = 0.6$, respectively), and no additional variance in RXRA chr9:136355885+ methylation was explained by simultaneously taking account of maternal fat and protein intakes in early pregnancy or macronutrient intakes in late pregnancy. Relating umbilical cord CpG methylation to the infant's size at birth, only three CpGs showed a significant correlation with birth weight adjusted for sex and gestational age (PIK3CD chr1:9609980+ [$r_p = -0.32$, $P = 0.013$, $n = 58$], PIK3CD chr1:9635676/79+ [$r_p = -0.36$, $P = 0.028$, $n = 37$], and SOD1 chr 21:31853827+ [$r_p = -0.27$, $P = 0.029$, n = 65]). RXRA chr9:136355885+ methylation showed a weak association with the subjects' sex and age-adjusted height ($r_p = 0.24$, $P = 0.054$) but was not associated with the infant's weight or ponderal index at birth.

Methylation analysis in the SWS cohort. We next sought to replicate the stronger associations found in the first cohort (eNOS chr7:150315553+ and RXRA chr9:136355885+ CpG methylation and child's adiposity) in the SWS cohort (20). In these 239 SWS children, eNOS chr7:150315553+ showed no association with adiposity, but RXRA chr9:136355885+ showed remarkably similar and statistically significant associations (fat mass, β = 6% [2–10], $P = 0.002$; %fat mass, β = 4% [1–7], $P = 0.002$, both $n = 239$) to those in PAH children. As in the PAH cohort, these associations were little changed after taking account of birth weight and maternal age, smoking, and BMI. Figures 2 and 3 show the graded associations between cord RXRA chr9:136355885+ methylation and child's fat mass and %fat mass in the two cohorts.

Pyrosequencing across RXRA chr9:136355885+ in SWS subjects showed no C allele SNPs that could account for differences in methylation observed between individuals.

TABLE 1
Univariate and multivariate analyses of umbilical cord CpG methylation in relation to child's adiposity measured by DEXA at age 9 years

	eNOS chr7:150315553+ (%)	*P*	RXRA chr9:136355885+ (%)	*P*	Sex (%)	*P*	Variance explained (%)
Total fat mass (ln kg)							
Univariate analysis	21	0.001	21	0.003	39	0.001	
Multivariate analysis	19	0.002	23	0.001	47	0.001	44
Percentage fat (ln %)							
Univariate analysis	13	0.006	14	0.006	42	<0.001	
Multivariate analysis	11	0.009	14	0.005	48	<0.001	47
Ratio trunk to limb fat (ln)							
Univariate analysis	7	0.008	7	0.016	12	0.009	
Multivariate analysis	8	0.004	7	0.021	11	0.059	28

Values are the percent change in outcome variable per SD change in CpG methylation and *P* value. Univariate analysis rows show the regression coefficients and *P* values relating each of eNOS chr7:150315553+, RXRA chr9:136355885+, and sex to outcomes individually, whereas multivariate analysis rows show regression coefficients and *P* values for a combined analysis of eNOS chr7:150315553+, RXRA chr9:136355885+, and sex in relation to outcomes, together with the variance explained by the multivariate model. For variances explained, $n = 55$.

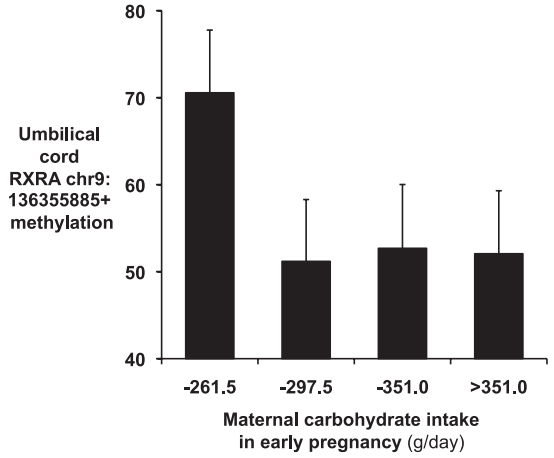

FIG. 1. Lower maternal carbohydrate in early pregnancy is associated with higher umbilical cord RXRA chr9:136355885+ methylation in the PAH cohort. Values are means + SEM.

DISCUSSION

This study provides novel evidence for the importance of the developmental contribution to later adiposity. We found that greater methylation of RXRA chr9:136355885+ measured at birth was strongly correlated with greater adiposity in later childhood in two independent cohorts. Although we studied a subset of children in both cohorts these were selected on the basis of subject and specimen availability, so it is unlikely that selection bias could explain the relationships observed unless the association between RXRA methylation and adiposity was different in the remainder of the cohort. The data build on animal experiments suggesting that the developmental environment acts through epigenetic processes to exert a strong influence on postnatal body composition and metabolic function (7–11).

Our study shows that specific components of the epigenetic state at birth predict later childhood adiposity. The associations with adiposity were linked to specific CpGs 5′ to the start site of the selected candidate genes. Although some of the CpGs studied were either within the proximal promoter or close to it, others were more distal and may be exerting effects through the regulation of other genes. Nevertheless the data indicate possible mechanistic pathways, suggesting avenues for future study. Our observation that adjacent or nearby CpGs within the same promoter showed differences in the strength of association with child's adiposity suggests highly specific changes in the transcriptional regulation of these genes induced by the developmental environment, rather than generalized changes in promoter methylation. Both CpG hyper- (eNOS chr7: 150315553+ and RXRA chr9:136355885+) and hypomethylation (SOD1 chr21:31853660/63+) at different sites were associated with body fat distribution, again indicating complexity in transcriptional control. The specificity of the associations between methylation of an individual CpG and both maternal diet and child's phenotype endorses the concept of a fine control of development by environmental factors via epigenetic processes.

Our observation indicates one potential mechanistic pathway involved, because induction of transcription by RXRA is dependent on its binding to ligands including the peroxisome proliferator–activated receptors, involved in insulin sensitivity, adipogenesis, and fat metabolism (31,32). Moreover, RXRA chr9:136355885+ is located in a region considered to contain positive regulatory elements of transcription (33). Figure 4 shows the proximity of RXRA chr9:136355885+ to proposed binding sites for RXR, MAF, NF-κB, and AP1. Retinoid receptor biology is complex, and increased RXRA methylation might be acting through a variety of pathways (34); however, an association between increased RXRA methylation and adiposity is consistent with the observation of strongly diminished RXRA expression in visceral white adipose tissue from obese mice (35). Moreover, a role for retinoid receptor methylation in developmental influences on later metabolic risk is supported by recent experimental data showing an influence of maternal diet during pregnancy on methylation of LXRA, a heterodimeric partner of RXRA (36).

Genome-wide association studies suggest that fixed genetic variation makes a relatively small contribution to risk of obesity, heart disease, and diabetes (1,2); our findings raise the possibility that the developmental environment component may be equally or more important. We excluded

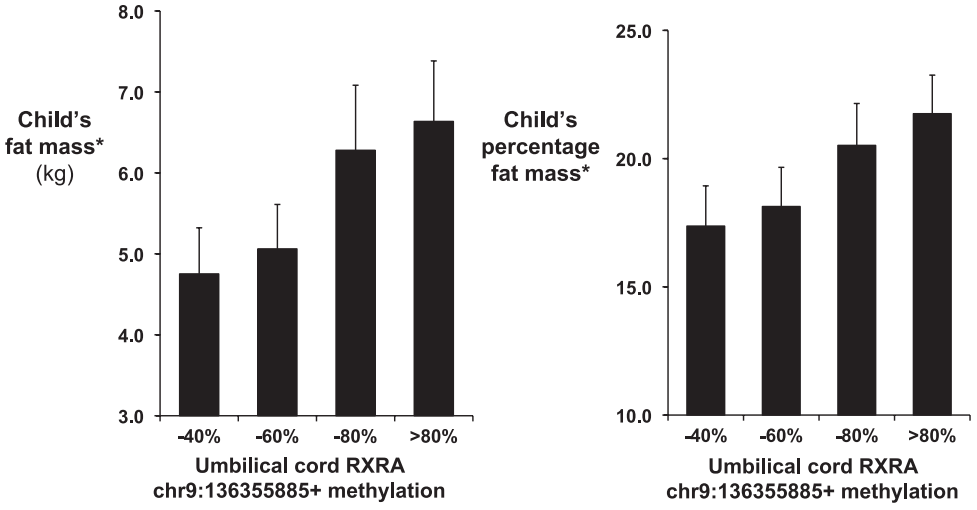

FIG. 2. Child's %fat mass and fat mass at age 9 years increase with higher umbilical cord RXRA chr9:136355885+ methylation in the PAH cohort. Values are means + SEM. *Fat mass and percentage fat mass are preadjusted for sex.

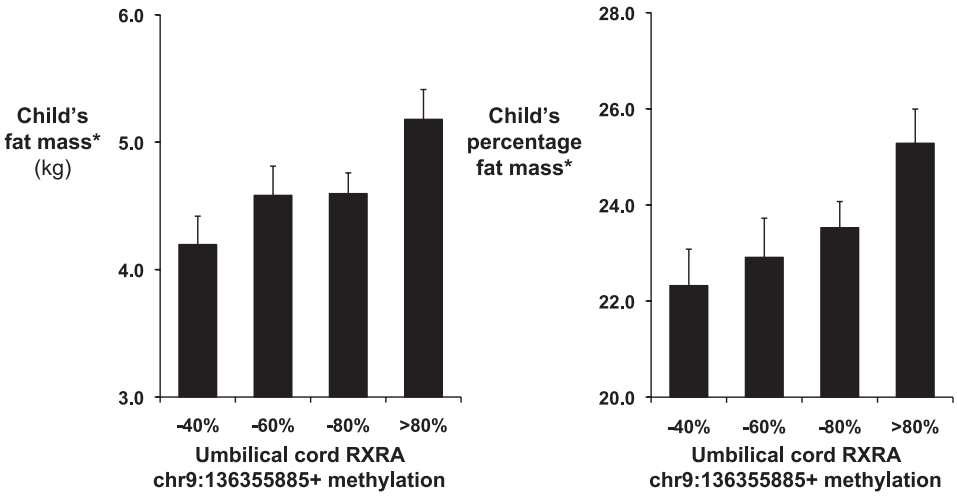

FIG. 3. In a second independent cohort, child's %fat mass and fat mass at age 6 years increase with higher umbilical cord RXRA chr9:136355885+ methylation in SWS subjects. Values are means + SEM. *Fat mass and percentage fat mass are preadjusted for age and sex.

the presence of a SNP at RXRA chr9:136355885+ by sequencing, but without genome-wide analysis it is not possible to exclude a genetic effect of distant SNPs, which could influence both DNA methylation of a particular sequence and child's phenotype. However, even if this were the case, our data clearly indicate that epigenetic measures at birth may have prognostic value. Although epigenetic changes can be dynamic, experimental studies have shown that environmental factors acting on the genotype during development relate to epigenetic profile in adulthood (7,15), and there are longitudinal human studies showing that DNA methylation is often stable over time (37). Such changes can be tissue specific, and in this respect the umbilical cord may be advantageous because it contains a high proportion of fetal vascular tissue and mesenchymal cells, which may be relevant to later adiposity. Furthermore, unlike the placenta it is a tissue in which consistency of sampling between individuals is more likely. Although experimental work in the rat suggests that methylation changes induced by maternal diet can be similar in the umbilical cord and liver (38), further work is needed to determine the relevance of epigenetic changes in human umbilical cord tissue. Recent data show that for some genomic regions methylation appears largely independent of tissue of origin, whereas for others there is a clear tissue-specific dependence (39).

Many epidemiological studies have shown associations between fetal development, through the proxy measure of birth size, and later adiposity and metabolic function (3,4), but the developmental contribution to such phenotypic characteristics has remained uncertain and controversial. This study provides the first estimate of the developmental contribution to phenotype associated with human disease risk based on measures of the underpinning biology: our data for RXRA chr9:136355885+ suggest that a substantial proportion of the variation in adiposity in prepubertal children can be explained by epigenetic measurements made at birth. Although our data are correlative and thus can only imply an association between DNA methylation at birth and later phenotype, the importance of the observation stands irrespective of whether the RXRA methylation is causally related to the development of adiposity. Even if it is simply a noncausal association, the changed epigenetic

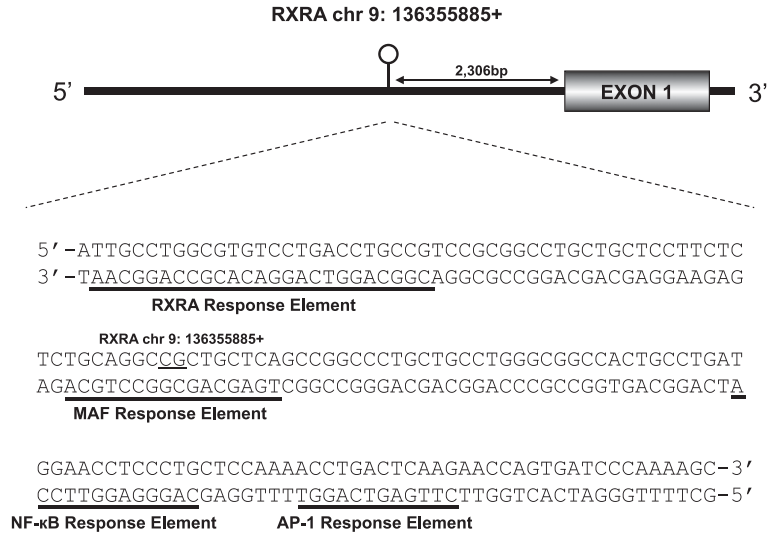

FIG. 4. Schematic diagram of the RXRA promoter region, showing the position of the CpG group at RXRA chr9:136355885+ (underlined) and of neighboring transcription factor binding sites.

status provides an objective marker of altered developmental trajectory by the time of birth. Despite the limitations of dietary intake assessment tools, the instrument we used is both validated and provides information that can be used to rank the nutrient intakes of individuals (23).

Methylation of eNOS chr7:150315553+ was associated with later adiposity in the initial cohort only. This may reflect a chance finding, or different maternal characteristics in the two cohorts, such as the greater maternal adiposity and maternal folate supplementation in the SWS cohort (22,40,41). Alternatively, adipocyte proliferation is high during the first year of life but then remains low until a second proliferative phase from age 9–14 years (42). Thus the processes that determine adiposity at age 9 years might differ from those at age 6 years. Nitric oxide synthesis by eNOS promotes preadipocyte differentiation (43). Therefore, a further possible explanation is that eNOS methylation in umbilical cord marks capacity for adipogenesis which has a greater net contribution to adiposity at age 9 years than age 6 years.

Our findings show strong associations between epigenetic markers and childhood total and central body fat. Beyond these simple associations, multivariate analysis indicates that the associations explain substantial proportions of the variances in outcomes, emphasizing an important developmental contribution to phenotypes associated with metabolic dysfunction and disease risk. It is noteworthy that the genes for which we report effects are not imprinted. In vitro fertilization increases risk of imprinting disorders (44), and methylation effects on imprinted genes have been reported in offspring of mothers exposed to famine during various periods of pregnancy (45,46), but with no associations with phenotype reported. The current study implicates the human prenatal environment with epigenetic changes in nonimprinted genes and is the first to link epigenetic status at birth with clinically relevant later phenotypic variation.

Variation in the degree of methylation of nonimprinted genes and in later cardiovascular and metabolic physiology can be induced experimentally by manipulation of the developmental environment, for example by altering maternal nutrition or administering glucocorticoids during pregnancy (8,9), often without necessarily affecting the birth size of the offspring. Although we found associations between some epigenetic markers and birth weight, these were weaker associations than those with later phenotype and were for different markers; moreover, previous reports on the same cohort showed only modest associations between birth weight and later body composition (6). Because birth weight in humans is influenced by multiple factors including the mother's own birth weight and height and by gestational length (40), epigenetic changes may provide a more sensitive index than birth weight of environmentally induced effects on fetal development.

There are potentially important implications of the strong and replicated association between RXRA chr9:136355885+ methylation and later adiposity. First, the effect is considerably greater than that of factors such as birth weight or maternal body composition, suggesting that epigenetic measurements made in the neonate may be useful predictors of later obesity and other phenotypic outcomes. Second, the association between CpG methylation and child's adiposity operates within the normal ranges of maternal nutritional state and birth size; this supports the argument that developmental programming is the consequence of an evolved and potentially adaptive process involving the mechanisms of developmental plasticity (10). Indeed the data provide strong evidence supporting a role for developmental plasticity in determining individual risk of metabolic disease. Third, the data suggest that developmental factors may be more significant in contributing to phenotypic variation and disease risk than generally considered. Fourth, the association between RXRA chr9:136355885+ methylation and mother's carbohydrate intake raises the possibility that conditions in early pregnancy could affect child's adiposity through this pathway. This provides additional support for the argument that all women of reproductive age should have appropriate nutritional, education, and lifestyle support to improve the health of the next generation. Finally, our data suggest that epigenetic measures at birth may have prognostic value and potential utility for monitoring programs to optimize maternal health and nutrition for long-term benefits to the offspring; however, evaluation of this possibility will require further research correlating methylation measurements in early life with those in later life.

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