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Boyd Orr Lecture
Epigenetics and maternal nutrition: nature *v*. nurture

Rebecca Simmons

Department of Pediatrics, Children's Hospital Philadelphia and University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

Under- and over-nutrition during pregnancy has been linked to the later development of diseases such as diabetes and obesity. Epigenetic modifications may be one mechanism by which exposure to an altered intrauterine milieu or metabolic perturbation may influence the phenotype of the organism much later in life. Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene expression from one generation of cells to the next. This review highlights our current knowledge of epigenetic gene regulation and the evidence that chromatin remodelling and histone modifications play key roles in adipogenesis and the development of obesity. Epigenetic modifications affecting processes important to glucose regulation and insulin secretion have been described in the pancreatic β-cells and muscle of the intrauterine growth-retarded offspring, characteristics essential to the pathophysiology of type-2 diabetes. Epigenetic regulation of gene expression contributes to both adipocyte determination and differentiation in *in vitro* models. The contributions of histone acetylation, histone methylation and DNA methylation to the process of adipogenesis *in vivo* remain to be evaluated.

Diabetes: β-cell: Fetus: Programming: Obesity

Link of low birth weight to later development of disease

It is becoming increasingly apparent that the *in utero* environment in which a fetus develops may have long-term effects on subsequent health and survival[1,2]. The landmark cohort study of 300 000 men by Ravelli and colleagues showed that exposure to the Dutch famine of 1944–45 during the first one-half of pregnancy resulted in significantly higher obesity rates at 19 years of age[3]. Subsequent studies of English men demonstrated a relationship between low birth weight and the later development of CVD[4] and impaired glucose tolerance[5–8]. Other studies of populations in the USA[9–11], Sweden[12], France[13,14], Norway[15] and Finland[16], have demonstrated a significant correlation between low birth weight and the later development of adult diseases. The associations with low birth weight and increased risk of CHD, stroke and type-2 diabetes (T2D) remain strong, even after adjusting for lifestyle factors (e.g. smoking, physical activity, occupation, income, dietary habits and childhood socio-economic status) and occur independent of the current level of obesity or exercise[17,18].

Low birth weight and insulin secretion

It remains controversial as to whether the adverse effects of intrauterine growth retardation on glucose homeostasis are mediated through programming of the fetal endocrine pancreas[1]. Growth-retarded fetuses and newborns have been reported to have both a reduced population of pancreatic β-cells[19] or a normal percentage of pancreatic area occupied by β-cells[20]. Both of these studies were observational, morphometric analyses were not optimal, and only a small number of fetuses/newborns were examined. It is likely that a significant proportion, but not all growth-retarded fetuses will have reduced β-cell numbers. A more

Abbreviations: IUGR, intrauterine growth retarded; Pdx1, pancreatic duodenal homeobox-1; SGA, small for gestational age; T2D, type-2 diabetes.

Corresponding author: Dr R. Simmons, fax +1 215 573 7627, email rsimmons@mail.med.upenn.edu

clinically relevant consideration is the impact of fetal growth retardation upon β-cell function.

Intrauterine growth-retarded (IUGR) fetuses have been found to exhibit lower insulin and glucose levels and higher glucose : insulin ratio in the third trimester as measured by cordocentesis[21]. Two recent studies showed that IUGR infants display decreased pancreatic β-cell function, but increased insulin sensitivity at birth[22,23]. Low birth weight has been associated with reduced insulin response after glucose ingestion in young non-diabetic men, whereas other studies have found no impact of low birth weight upon insulin secretion[17,18]. However, none of these earlier studies adjusted for the corresponding insulin sensitivity, which has a profound impact upon insulin secretion. Therefore, Jensen *et al.*[24] measured insulin secretion and insulin sensitivity in a well-matched Caucasian population of 19-year-old glucose-tolerant men with birth weights either below the 10th percentile (small for gestational age (SGA)) or between the 50th and 75th percentile (controls). To eliminate the major confounders such as 'diabetes genes', none of the participants had a family history of diabetes, hypertension, or IHD. There was no difference between the groups with regard to current weight, BMI, body composition and lipid profile. When controlled for insulin sensitivity, insulin secretion was reduced by 30%. Insulin sensitivity, however, was normal in the SGA subjects. The investigators hypothesised that defects in insulin secretion may precede defects in insulin action and that once SGA individuals accumulate body fat, they will develop insulin resistance[24].

Genetics *v.* environment

Several epidemiological and metabolic studies of twins and first-degree relatives of patients with T2D have demonstrated an important genetic component of diabetes[25–28]. The association between low birth weight and risk of T2D in some studies could theoretically be explained by a genetically determined reduced fetal growth rate. In other words, the genotype responsible for T2D may itself cause retarded fetal growth *in utero*. This forms the basis for the fetal insulin hypothesis, which suggests that genetically determined insulin resistance could result in low insulin-mediated fetal growth *in utero* as well as insulin resistance in childhood and adulthood[29]. Insulin is one of the major growth factors in fetal life, and monogenic disorders that affect fetal insulin secretion or fetal insulin resistance also affect fetal growth. Mutations in the gene encoding glucokinase have been identified that result in low birth weight and maturity onset diabetes of the young[30,31].

The recently described T2D susceptibility gene transcription factor 7-like 2 confers a risk-allele frequency of approximately 30%[32]. Studies of non-diabetic subjects show that transcription factor 7-like 2 diabetes-risk genotypes alter insulin secretion[33–35]. A large study of 24 053 subjects combined from six studies demonstrated that transcription factor 7-like 2 is the first T2D gene to be reproducibly associated with altered birth weight. Each maternal copy of the T allele at *re7903146* increased offspring birth weight by 30 g, and the investigators suggest that the most likely mechanism is through reduced maternal-insulin secretion resulting in maternal hyperglycemia and increased insulin-mediated fetal growth[36].

Recent genetic studies suggest that the increased susceptibility to T2D of subjects who are born SGA also results from the combination of both genetic factors and an unfavourable fetal environment. Polymorphisms of PPAR-γ 2, a gene involved in the development and in the metabolic function of adipose tissue, have been shown to modulate the susceptibility of subjects who are born SGA to develop insulin resistance later in life[37,38]. The polymorphism is only associated with a higher risk of T2D if birth weight is reduced[37,38]. There is obviously a close relationship between genes and the environment. Not only can maternal gene expression alter the fetal environment, but the maternal intrauterine environment also affects fetal gene expression and both influence birth weight.

Association between obesity in pregnancy and later obesity in the offspring

Obesity is one of the most pervasive and burdensome public health problems in modern times. The steady increase in overweight reproductive-age women is correlated with increases in rates of childhood and infant obesity. A possible link between the abnormal intrauterine environment and abnormal growth and development of offspring must be considered. Maternal obesity significantly increases fetal and neonatal adiposity in the human subjects; thus, enhanced adipocyte development *per se* must play an important role in the genesis of obesity in the offspring[39].

A number of epidemiological studies have shown that there is a direct relationship between birth weight and BMI in childhood and in adult life[40,41]. In the US Growing Up Today Study, a cohort study of over 14 000 adolescents, a 1-kg increment in birth weight in full-term infants was associated with an approximately 50% increase in the risk of overweight at ages 9–14 years[42]. When adjusted for maternal BMI, the increase in risk remained significantly elevated at 30%. A study of Danish military conscripts showed that even after controlling for birth length and maternal factors, BMI at ages 18–26 strongly correlated with birth weight[40].

What animal models can tell us

Animal models have a normal genetic background upon which environmental effects during gestation or early postnatal life can be tested for their role in inducing disease later in life. Ontogeny of β-cell development in the rodent approximates what has been observed in human subjects[43,44]. The most commonly used animal models for IUGR are energetic or protein restriction, glucocorticoid administration, or induction of uteroplacental insufficiency in the pregnant rodent. In rats, maternal dietary protein restriction (approximately 40–50% of normal intake, termed LP) throughout gestation and lactation has been reported to alter insulin secretory capacity and reduce β-cell mass through a reduction in the β-cell proliferation

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rate and an increase in apoptosis[45–53]. Expression of pancreatic duodenal homeobox-1 (Pdx1), a homeodomain-containing transcription factor that regulates early development of both endocrine and exocrine pancreas, and later differentiation and function of β-cells[54], is also reduced in islets from pups of LP mothers[55]. In adulthood, rats born from LP mothers still have reductions in β-cell mass and insulin secretion and show glucose intolerance, but usually not overt diabetes[45,46,53]. In old age, LP offspring develop fasting hyperglycemia associated with insulin resistance[56–60].

Total energetic restriction during the last week of pregnancy and throughout lactation also reduces β-cell mass and impairs insulin secretion in the offspring[61,62]. When maternal undernutrition is prolonged until weaning and normal nutrition is given to the offspring from weaning onwards, growth retardation and β-cell mass reduction persists in adulthood[62].

Treatment of pregnant rats with dexamethasone during the last week of gestation retards fetal growth[63]. Insulin content of fetal β-cells is reduced and is associated with a reduction in Pdx1[63].

An ovine model of IUGR induced by placental insufficiency (heat induced) results in a significant reduction in β-cell mass in fetuses near term (0·9 of gestation) from decreased rates of β-cell proliferation and neogenesis[64]. Plasma insulin concentrations in the IUGR fetuses are lower at baseline and glucose-stimulated insulin secretion is impaired. Similar deficits occur with arginine-stimulated insulin secretion. A deficiency in islet glucose metabolism also occurs in the rate of islet glucose oxidation at maximal stimulatory glucose concentrations. Thus, pancreatic islets from nutritionally deprived IUGR fetuses caused by chronic placental insufficiency have impaired insulin secretion caused by reduced glucose-stimulated glucose oxidation rates, insulin biosynthesis and insulin content. This impaired glucose-stimulated insulin secretion occurs despite an increased fractional rate of insulin release from a greater proportion of releasable insulin as a result of diminished insulin stores[65].

To extend these experimental studies of growth retardation, we developed a model of IUGR in the rat that restricts fetal growth[66–68]. Growth-retarded fetal rats have critical features of a metabolic profile characteristic of growth-retarded human fetuses: decreased levels of glucose, insulin, insulin-like growth factor-I, amino acids and oxygen[66–70]. Birth weights of IUGR animals are significantly lower than those of controls until approximately 7 weeks of age, when IUGR rats catch up to controls. Between 7 and 10 weeks of age, the growth of IUGR rats accelerates and surpasses that of controls, and by 26 weeks of age, IUGR rats are obese[67]. No significant differences are observed in blood glucose and plasma insulin levels at 1 week of age. Between 7 and 10 weeks of age, however, IUGR rats develop mild fasting hyperglycemia and hyperinsulinemia. IUGR animals are glucose intolerant and insulin resistant at an early age. First-phase insulin secretion in response to glucose is also impaired early in life in IUGR rats, before the onset of hyperglycemia. There are no significant differences in β-cell mass, islet size or pancreatic weight between IUGR and control animals at 1 and 7 weeks of age. In 15-week-old IUGR rats, however, the relative β-cell mass is 50% that of controls, and by 26 weeks of age, β-cell mass is less than one-third that of controls. This loss of β-cell mass is accompanied by a reduction in Pdx1 expression that is greater than that in β-cell mass[71]. By 6 months of age, IUGR rats develop diabetes with a phenotype remarkably similar to that observed in the human subject with T2D: progressive dysfunction in insulin secretion and insulin action[67]. Thus, despite different animal models of IUGR, these studies support the hypothesis that an abnormal intrauterine milieu can induce permanent changes in β-cell function after birth and lead to T2D in adulthood.

Animal model of obesity in pregnancy induces obesity in the offspring

Several investigators have used animal models of high-fat or western style-diet-induced obesity (a diet that has increased fat and carbohydrate content) and have shown that maternal over-nutrition induces increased adiposity and induces permanent changes in metabolism in the offspring[72–84]. Using a similar Western diet, female Sprague–Dawley rats were started on the designated diet at 4 weeks of age. The rats were mated at 12–14 weeks of age, and all pups were weaned onto a control diet[85]. Offspring from dams fed the Western diet had significantly increased adiposity as early as 2 weeks as well as impaired glucose tolerance compared to offspring of dams fed a control diet. Inflammation and oxidative stress were increased in pre-implantation embryos, fetuses and newborns of obese dams. Oxidative stress was correlated with increased expression of pro-adipogenic and lipogenic genes in fat tissue and in pre-implantation embryos. These results suggest that obesity is programmed as early as the pre-implantation stage of development.

Chromatin structure, DNA methylation and gene expression

Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene expression from one generation of cells to the next. Epigenetic states can be modified by environmental factors, which may contribute to the development of abnormal phenotypes. There are at least two distinct mechanisms through which epigenetic information can be inherited: histone modifications and DNA methylation[86,87].

In eukaryotes, the nucleosome is formed when DNA is wrapped around an octameric complex of two molecules of each of the four histones: H2A, H2B, H3 and H4. The N-termini of histones can be modified by acetylation, methylation, sumoylation, phosphorylation, glycosylation and ADP ribosylation. The most common histone modifications involve acetylation and methylation of lysine residues in the N-termini of H3 and H4. Increased acetylation induces transcription activation, whereas decreased acetylation usually induces transcription repression. Methylation of histones, on the other hand, is associated with both transcription repression and activation[86,87].

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Moreover, lysine residues can be mono-, di-, or trimethylated *in vivo*, providing an additional mechanism of regulation[86,87].

The second class of epigenetic regulation is DNA methylation, in which a cytosine base is modified by a DNA methyltransferase at the C5 position of cytosine, a reaction that is carried out by various members of a single family of enzymes[87]. Approximately 70% of CpG dinucleotides in human DNA are constitutively methylated, whereas most of the unmethylated CpG are located in CpG islands. CpG islands are CG-rich sequences located near coding sequences, and serve as promoters for their associated genes. Approximately half of mammalian genes have CpG islands[87]. The methylation status of CpG islands within promoter sequences works as an essential regulatory element by modifying the binding affinity of transcription factors to DNA-binding sites. In normal cells, most CpG islands remain unmethylated; however, under circumstances such as cancer[88–90] and oxidative stress, they can become methylated *de novo*. This aberrant methylation is accompanied by local changes in histone modification and chromatin structure, such that the CpG island and its embedded promoter take on a repressed conformation that is incompatible with gene transcription. It is not known why particular CpG islands are susceptible to aberrant methylation.

DNA methylation is commonly associated with gene silencing and contributes to X-chromosomal inactivation, genomic imprinting as well as transcriptional regulation of tissue-specific genes during cellular differentiation (reviewed in[91–93]). It is not known why some genes are able to undergo aberrant DNA methylation; however, a study by Feltus *et al.*[94] suggests that there is a 'DNA sequence signature associated with aberrant methylation'. Of major significance to T2D is their finding that *Pdx1*, a pancreatic homeobox transcription factor, was one of only fifteen genes (of 1749 examined) with CpG islands within the promoter that were methylation-susceptible (which was induced by over-expression of a DNA methyltransferase). This study demonstrates that genes essential to pancreatic development, like *Pdx1*, are susceptible to epigenetic modifications, which could ultimately affect gene expression.

Histone methylation can influence DNA methylation patterns and vice versa[91]. For example, methylation of lysine 9 on histone 3 (H3) promotes DNA methylation, while CpG methylation stimulates methylation of lysine 9 on H3[92]. Recent evidence indicates that this dual relationship between histone methylation and DNA methylation might be accomplished by direct interactions between histone and DNA methyltransferases[91]. Thus, chromatin modifications induced by adverse stimuli are self-reinforcing and can propagate.

Maternal nutritional supplementation and epigenetic modifications in the offspring

The role of environmental regulation of epigenetic phenomena in the offspring has been established by experiments performed in agouti mice (reviewed in[95]). Wild-type expression of the agouti protein results in a phenotypic brown coat colour in the mouse. In this mouse model, an endogenous retrovirus-like transposon sequence is inserted close to the gene coding for the agouti protein. An unmethylated retrotransposon promoter overrides the wild-type agouti promoter, resulting in ectopic agouti expression and a yellow coat colour. A methylated retrotransposon is silenced and results in a wild-type agouti (brown) coat. Wolff *et al.* have investigated whether maternal diet can alter the phenotype of the agouti mouse[96] and found that when pregnant females are fed a diet supplemented with methyl donors, a larger proportion of offspring have a wild-type agouti coat colour as compared to the offspring of mothers fed a standard diet. These studies indicate that the maternal methyl donor diet leads to increased methylation of the offspring's retrotransposon. Methylation silences the offspring's retrotransposon allowing the wild-type agouti promoter to be expressed, thus resulting in a mouse with a wild-type (brown) coat colour. These results suggest that a maternal nutritional environmental exposure can change the stable expression of genes in the offspring through an epigenetic modification that takes place *in utero*.

Epigenetic regulation of gene expression in fetal growth retardation

A number of studies suggest that uteroplacental insufficiency, a common cause of IUGR, induces epigenetic modifications in offspring[97–100]. Epigenetic modifications affecting processes important to glucose regulation and insulin secretion, characteristics essential to the pathophysiology of T2D have been described in the IUGR liver, pancreatic β-cells and muscle[97–100].

Chromatin remodelling in the β-cell of intrauterine growth retarded rats

*Pdx*1 is a homeodomain-containing transcription factor that plays a critical role in the early development of both the endocrine and exocrine pancreas, and in the later differentiation and function of the β-cell. As early as 24 h after the onset of growth retardation, *Pdx1* mRNA levels are reduced by more than 50% in IUGR fetal rats. Suppression of *Pdx1* expression persists after birth and progressively declines in the IUGR animal, implicating an epigenetic mechanism.

Changes in histone acetylation are the first epigenetic modification found in β-cells of IUGR animals. Islets isolated from IUGR fetuses show a significant decrease in H3 and H4 acetylation at the proximal promoter of *Pdx1*[99]. These changes in H3 and H4 acetylation are associated with a loss of binding of upstream stimulatory factor-1 to the proximal promoter of *Pdx1*[99]. Upstream stimulatory factor-1 is a critical activator of *Pdx1* transcription, and its decreased binding markedly decreases *Pdx1* transcription[101,102]. After birth, histone deacetylation progresses and is followed by a marked decrease in H3K4 trimethylation and a significant increase in dimethylation of H3K9 in IUGR islets[99]. H3K4 trimethylation is usually associated with active gene transcription, whereas H3K9

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dimethylation is usually a repressive chromatin mark. Progression of these histone modifications parallels the progressive decrease in *Pdx1* expression that manifests as a deterioration in glucose homeostasis and increased oxidative stress in the aging IUGR animals[99]. Nevertheless, at 2 weeks of age, the silencing histone modifications in the IUGR pup are responsible for the suppression of Pdx1 expression since there is no appreciable methylation of CpG islands in mice at this age[99]. Reversal of histone deacetylation in IUGR islets at 2 weeks of age, is sufficient to nearly normalize Pdx1 mRNA levels permanently, perhaps due to active β-cell replication present in the neonatal rodent[99].

In IUGR, *Pdx1* is first silenced due to the recruitment of co-repressors, including histone deacetylase 1 and mSin3A (mammalian Sin 3) [99]. These repressors catalyse histone deacetylation. Binding of these deacetylases facilitates loss of trimethylation of H3K4, further repressing *Pdx1* expression[99]. We found that inhibition of histone deacetylase activity by trichostatin A treatment normalises H3K4me3 levels at *Pdx1* in IUGR islets[99]. These data suggest that the association of histone deacetylase 1 at *Pdx1* in IUGR islets likely serves as a platform for the recruitment of a demethylase, which catalyses demethylation of H3K4.

The molecular mechanism responsible for DNA methylation in IUGR islets is likely dependent on the methylation status of lysine 9 on H3 (H3K9). Previous studies have shown that changes in methylation of H3K9 precede changes in DNA methylation[103,104]. It has also been suggested that DNA methyltransferases may act only on chromatin that is methylated at H3K9[105]. Histone methyltransferases specifically DNA methyltransferase 3A and DNA methyltransferase 3B, bind to DNA methylases, thereby initiating DNA methylation[105].

These results demonstrate that IUGR induces a self-propagating epigenetic cycle in which the mSin3A–histone deacetylase complex is first recruited to the *Pdx1* promoter, histone tails are subjected to deacetylation and *Pdx1* transcription is repressed. At the neonatal stage, this epigenetic process is reversible and may define an important developmental window for therapeutic approaches. However, as dimethylated H3K9 accumulates, DNA methyltransferase 3A is recruited to the promoter and initiates *de novo* DNA methylation, which locks in the silenced state in the IUGR adult pancreas resulting in diabetes.

How do these epigenetic events lead to diabetes? Targeted homozygous disruption of *Pdx1* in mice results in pancreatic agenesis, and homozygous mutations yield a similar phenotype in human subjects[106]. Milder reductions in *Pdx1* protein levels, as occurs in the *Pdx*±mice, allow for the development of a normal mass of β-cells[106], but result in the impairment of several events in glucose-stimulated insulin secretion[106]. These results indicate that *Pdx1* plays a critical role in the normal function of β-cells[106] in addition to its role in β-cell lineage development. This may be the reason that human subjects with heterozygous missense mutations in *Pdx1* exhibit early- and late-onset forms of T2D[106].

The discovery of a critical developmental stage during which aberrant epigenetic modifications may be reversed represents a therapeutic window for the use of novel agents that could prevent common diseases with late-onset phenotypes. T2D is one such disease, where predisposed individuals could be treated with agents that normalize the epigenetic programming of key genes, thus providing protection against development of the adult diabetic phenotype.

Genome-wide DNA methylation is disrupted in intrauterine growth-retarded islets

Epigenetic modifications are not confined to the *Pdx1* locus in the IUGR rat. We mapped DNA methylation across approximately 1 000 000 loci using the HELP assay[107]. Comparison of IUGR with normal rats at 7 weeks of age prior to the onset of diabetes, revealed changes in DNA methylation at a number of novel loci, not limited to canonical CpG islands or promoters. We found that IUGR in the rat causes consistent and non-random changes in cytosine methylation, affecting $<1\%$ of HpaII sites in the genome in the islet. The majority of these changes take place not at promoters but at intergenic sequences, many of which are evolutionarily conserved. Furthermore, some of these loci are in proximity to genes manifesting concordant changes in gene expression and are enriched near genes that regulate processes that are markedly impaired in IUGR islets (e.g. vascularization, proliferation, insulin secretion and cell death).

Summary

The studies described above clearly show that environmental effects can induce epigenetic alterations, ultimately effecting expression of key genes linked to the development of T2D including genes critical for pancreatic development and β-cell function, peripheral glucose uptake and insulin resistance and atherosclerosis. Recent progress in understanding the epigenetic programming of gene function has led to the development of novel therapeutic agents with epigenetic targets in diseases such as cancer. Understanding the role of developmental programming of genes crucial to the development of T2D may unveil a critical window during which epigenetic therapeutic agents could be used as a means to prevent the later development of a disease. Prior to the use of such therapeutic agents there remains much to be learned about the programming of the epigenetic code, especially on a genome-wide scale. Much of the recent progress in understanding epigenetic phenomena is directly attributable to technologies that allow researchers to pinpoint the genomic location of proteins that package and regulate access to the DNA. The advent of DNA microarrays and inexpensive DNA sequencing has allowed many of those technologies to be applied to the whole genome. It is now possible that epigenetic profiling of CpG islands in the human genome can be used as a tool to identify genomic loci that are susceptible to DNA methylation. Aberrant methylation may then be used as a biomarker for disease. The genome-wide mapping of histone modifications by ChIP (chromatin immunoprecipitation)-chip and ChIP-seq has led to

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important insights regarding the mechanism of transcriptional and epigenetic memory, and how different chromatin states are propagated through the genome in yeast and in mammalian cells[108,109]. Although CHIP-seq experiments are currently being performed in human tissue, obstacles such as intrinsic human epigenetic variability (including age-related changes), and tissue-specific epigenetic variability must be characterized and mapped in the healthy, non-diseased state before this information can be applied to diseases such as T2D. Eventually genome-wide epigenetic characterization will lead to specific therapies with epigenetic targets and also will allow monitoring of genome-wide epigenetic consequences of these therapies once they are applied.

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