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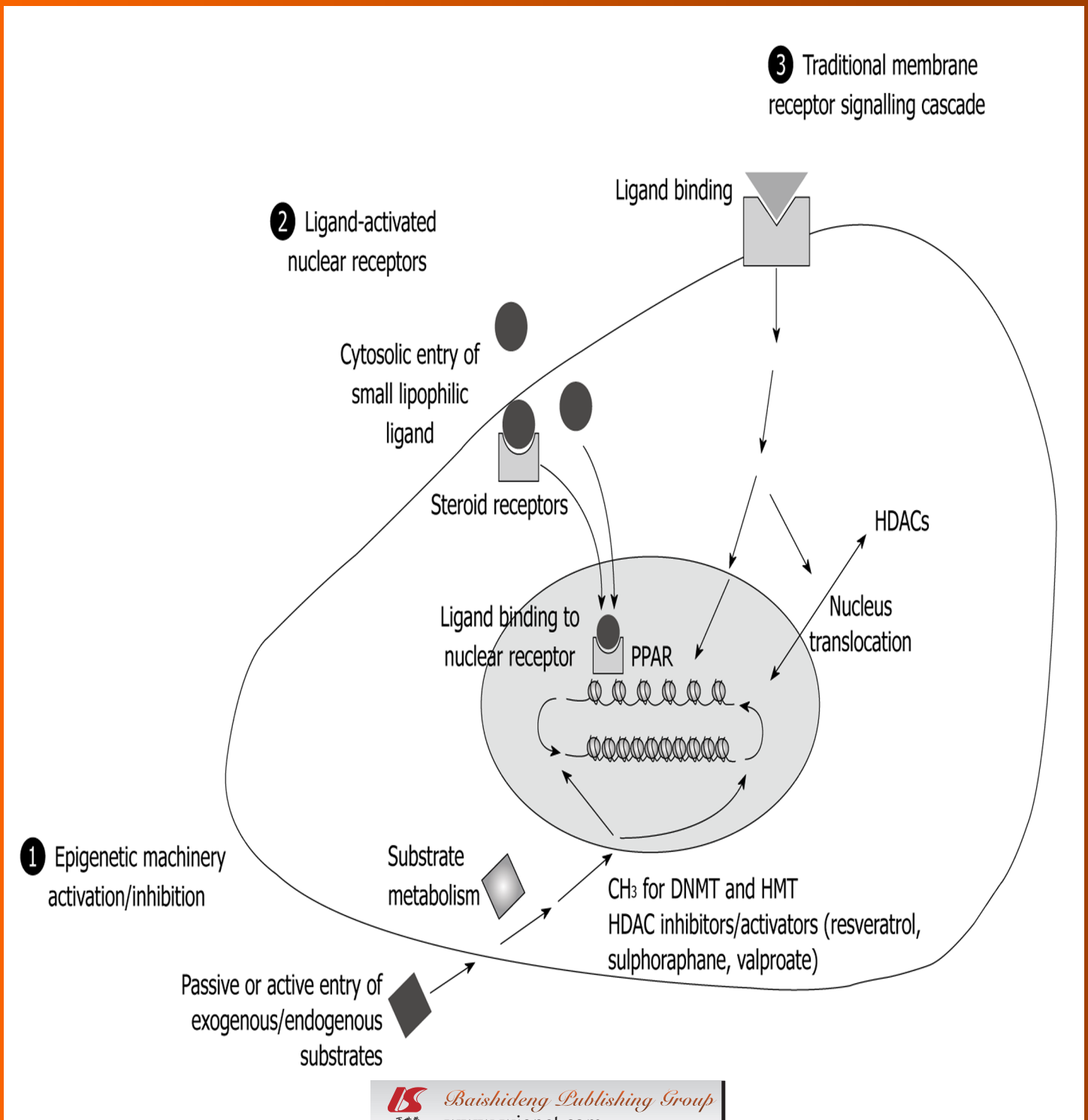

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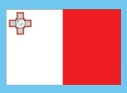

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EDITORIAL

MicroRNAs in hepatic pathophysiology in diabetes

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Abstract

MicroRNAs (miRNAs or miRs) are small approximately 22 nucleotide RNA species that are believed to regulate diverse metabolic and physiological processes. In the recent past, several reports have surfaced that demonstrate the role of miRNAs in various biological processes and numerous disease states. For a disease as complex as diabetes, the emergence of miRNAs as key regulators leading to the disease phenotype has added a novel dimension to the area of diabetes research. On the other hand, the liver, a metabolic hub, contributes in a major way towards maintaining normal glucose levels in the body as it can both stimulate and inhibit hepatic glucose output. This equilibrium is frequently disturbed in diabetes and hence, the liver assumes special significance considering the correlation between altered hepatic physiology and diabetes. While the understanding of the mechanisms behind this altered hepatic behavior is not yet completely understood, recent reports on the status and role of miRNAs in the diabetic liver have further added to the complexities of the knowledge of hepatic pathophysiology in diabetes. Here, we bring together the various miRNAs that play a role in the altered hepatic behavior during diabetes.

Key words: MicroRNA; Liver; Diabetes; Insulin; Non-alcoholic fatty liver disease

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INTRODUCTION

"In diabetes, the thirst is great; for the fluid running off dries the body…. For the thirst, there is need of a powerful remedy, for in kind it is the greatest of all sufferings; and when a fluid is drunk, it stimulates the discharge of urine…."[1].

These are the words of one of the most celebrated physicians of ancient Greece, Aretaeus of Cappadocia. He went on to say that "a person suffering from diabetes leads a life that is disgusting and painful, followed by a speedy death". The term diabetes also seems to have been introduced into medical nomenclature by Aretaeus himself. A notable Roman physician, Galen, regarded diabetes as a disease of the kidneys, or as he put it, "diarrhoea of the urine"[2]. Several hypotheses followed thereafter and it was only after the 1500s that some of the intricate explanations of this complex disorder became known to mankind. In 1674, Thomas Willis[3] first differentiated diabetes from other causes of polyuria by the sweet taste of diabetic urine and also suggested that this sweetness

first appeared in the blood. Later, Matthew Dobson established that the sweetness of urine was due to sugar and the sweet component was later on identified precisely as glucose by Eugene Chevreul[2]. Thus, it was due to the keen observational skills of these early researchers that we started gaining some understanding of diabetes.

In the present day, diabetes and its mechanisms are much more understood although several complexities still exist. A contributing factor to this has been the fact that, in recent times, there has been a tremendous increase in the number of individuals affected by diabetes together with varied mechanisms of the disease manifestation. Its exploding trend is evident from world-wide predictions that forecast a number as high as 366 million patients with diabetes in 2030[4]. Mainly classified as two types, diabetes mellitus can be due to either insulin deficiency (due to loss of insulin producing pancreatic β-cells) referred to as type 1 diabetes mellitus, or due to decreased responsiveness of the body tissues to insulin referred to as type 2 diabetes mellitus (T2DM). The skeletal muscle, adipose and liver mainly comprise the insulin target tissues that together contribute towards maintaining a circulating euglycemic status in the presence of insulin. While the adipose tissue and the skeletal muscle majorly participate in sequestering glucose into the cells, the liver critically regulates hepatic glucose output by controlling the pathways of gluconeogenesis, glycolysis and glycogenolysis. In addition, the liver also communicates with other tissues and thereby regulates metabolism in extra-hepatic tissues, such as adipose and muscle. Therefore, the liver is believed to be a central organ in the regulation of whole body glucose homeostasis and is central to the onset and progression of diabetes. In this article, we discuss the hepatic abnormalities during diabetes and role(s) of microRNAs (miRNAs or miRs), the recently discovered small RNA species.

LIVER IN DIABETES

The role played by liver in whole body metabolism and its implication in the pathogenesis of T2DM becomes even more significant as this tissue is sensitive to the two important hormones involved in glucose homeostasis, namely insulin and glucagon. Any disturbance in the delicate equilibrium maintained by the liver often leads to abnormal glucose levels within the body[5]. Moreover, owing to the hepatic portal system, the liver has an upper edge in encountering any changes in the nutritional status of an individual. Any manipulation in its physiology involving the intricate regulation of glucose homeostasis by alternating between cycles of glucose output and its inhibition contributes to the onset and progression of diabetes. Hence, it is not surprising that the liver is one of the affected and/or contributing tissues in diabetes mellitus.

One of the most prominent and evident symptoms of T2DM is a fatty liver. While adipocytes are normally believed to accumulate fats, under abnormal conditions (such as over nutrition), other organs like the liver and muscle become additional sites for fat deposition. This "ectopic" fat accumulation causes several derangements, not only in the normal functioning of the organ involved but also in the whole body metabolism. One such abnormality that is evident in the liver during T2DM is non-alcoholic fatty liver disease (NAFLD).

NAFLD is a broad term comprising of liver disorders which are usually related to insulin resistance, metabolic syndrome or type 2 diabetes. NAFLD starts with hepatic steatosis that is characterized by fat accumulation in the hepatocytes. This fat accumulation is thought to be caused by metabolic imbalances such as higher amounts of dietary lipids, increased trafficking of free fatty acids from adipose to liver and increased *de novo* lipogenesis. This may also result from reduced fatty acid oxidation or impaired triglyceride secretion from liver *via* VLDLs. Hepatic steatosis might progress to a more severe form of NAFLD i.e., non-alcoholic steatohepatitis (NASH). In some cases, this condition may worsen, progressing to fibrosis (activation of hepatic stellate cells resulting in collagen deposition) and cirrhosis and finally, might also end up in hepatocellular carcinoma[6].

The underlying molecular mechanisms that lead to such hepatic abnormalities during diabetes are far from being completely understood. The association among insulin resistance, accumulation of triglycerides in the liver and diabetes is quite well established[7] but the precise mechanistic events are still a matter of debate. The contribution of genes, urban lifestyle and intra-uterine environment in making us prone specifically to T2DM and its complications are understandable, but still are not completely sufficient to explain these mentioned phenomena. As researchers from diverse fields of genetics, epigenetics, molecular biology and evolutionary biology are still struggling to address these intriguing questions, the emergence and identification of microRNAs as new regulators in this disease have added a whole new layer of complexity. The next section focuses on the established role(s) of various miRNAs during the onset and progression of hepatic abnormalities.

MICRORNAS AND THEIR ROLE IN THE DIABETIC LIVER

MicroRNAs represent a new class of small RNA molecules with an ability to fine-tune gene expression. These are endogenous, single-stranded approximately 22 nucleotide RNA molecules that have been identified in over 80 species, including those encoded by viral genomes[8]. They predominantly regulate target gene expression by binding to the 3'UTRs of the respective mRNAs (messenger RNA) and inhibiting their translation into the respective proteins. It is believed that expression of 30% of human genes may be regulated by miRNAs[8].

In the last few years, several reports mentioned the association of miRNAs with diverse metabolic pathways, especially in the liver, that lead to the diabetic phenotype[9]. These studies not only underscore the fact that

these tiny RNA species are big players in hepatic pathophysiology during diabetes but also categorically emphasize that this field is growing quite rapidly. In one such study, a set of 18 distinct miRNAs were differentially expressed in the livers of hyperglycemic Goto-Kakizaki (GK) rats as compared to normal Brown Norway rats[10]. In particular, specific miRNAs namely miR-195 and miR-103 that exhibit highest levels of expression in the livers of the diabetic GK rats followed an almost linear co-relative expression pattern with the hyperglycemic phenotype. Also, miR-191 was significantly up regulated in GK rats as compared to other strains. Predicted targets to these altered miRNAs are involved in pathways pertaining to T2DM and therefore suggest the relevance of an altered miRNA status in the diabetic liver.

miRNAs were shown to play regulatory roles in the aberrant energy status during NAFLD. In a comparative study in ob/ob mice (a well established mouse model for diabetes with NAFLD) *vs* streptozotocin (STZ)-induced diabetic mice (this mouse model exhibits hyperglycemia without fatty liver), miR-107, miR-103, miR-126-3p, miR-100 and miR-29c were identified to be differentially expressed. On the other hand, miR-34a was specifically up regulated in STZ-induced diabetic mice in contrast to normal mice[11]. Such distinct patterns of miRNA expression identify an important role of miRNAs in the altered energy metabolism and pathophysiology of the liver during T2DM. Livers of ob/ob mice also exhibit elevated levels of miR-103/107 levels[12] and in an attempt towards reconfirming this in humans, patients with alcoholic liver disease, NAFLD and NASH also depicted increased levels of these miRNAs. Interestingly, antagonism of miR-103/107 in ob/ob mice resulted in increased insulin sensitivity and improved glucose homeostasis while gain of miR-103/107 function led to impaired glucose homeostasis. One of the mechanisms described for these miRNAs is *via* targeting Caveolin-1 that is involved in the activation of insulin signaling. Caveolins comprise a family of integral membrane proteins that are major components of membrane invaginations called caveolae. They are involved primarily in receptor-mediated endocytosis and caveolin-1, in particular, modulates insulin signaling by regulating glucose uptake[13,14]. miR-103/107, by targeting caveolin-1, therefore appears as a key regulator of insulin sensitivity and identifies a new target for the treatment of T2DM.

In addition to the above mentioned reports[9-12], there are an increasing number of studies focusing on miRNA patterns in various stages of fatty liver disease. One of the earliest reports that came along in this area was by Jin *et al*[15]. The authors identified the differential miRNA expression patterns in liver among different stages of NAFLD using fat rich-diet based rat models. By applying prediction analysis of Microarray, miRNA signatures were identified specific to the stages of simple steatosis and steatohepatitis as compared to the normal liver. This, the first of its kind of study, provided not only an evidence for miRNA deregulation in fatty liver disease but also suggested that miRNA signatures can be applied as diagnostic markers to differentiate among various pathological states of the liver. While miR-122, miR-27a, miR-31, miR-451, miR-145 were down-regulated, miR-200a, miR-200b were up-regulated in diet-induced NAFLD rat models[16]. Pputative targets of these altered miRNAs are involved in specific relevant pathways of lipid and carbohydrate metabolism, signal transduction and apoptosis. They also exhibited an inverse pattern of expression and this suggests a potential involvement of miRNAs and their target proteins in the pathogenesis of diet-induced NAFLD. Differential miRNA expression profiling in the hepatic tissues of patients of NASH identified a total of 46 miRNAs to be deregulated in these subjects[17]. Of these, silencing and over expression of miR-122 (one of the down regulated miRNAs) could regulate the expression of its predicted target genes mainly those involved in the lipogenic pathway (FASN, HMG-CoA reductase, SREBP-1c and SREBP-2), suggesting modulation of the hepatic fatty acid metabolism *in vivo*. Incidentally, an altered miRNA expression pattern in the livers of diet-induced mouse models for alcoholic as well as NASH was reported by Dolganiuc *et al*[18], suggesting specific miRNA signatures in these hepatic pathologies even though both these abnormalities share common phenotypes.

In a rat model of hepatic fibrosis, the miR-34 family that targets ACSL1 and modulates lipid metabolism is altered[19]. ACSL1 belongs to the long-chain fatty acid CoA ligase family that activates long chain fatty acids to acyl-CoAs and hence by modulating lipid and fatty acid metabolism also contributes to the development of hepatic fibrosis. In patients of chronic hepatitis, the hepatic levels of miR-199a, miR-199*, miR-200a and miR-200b are markedly elevated and also exhibit a positive correlation with the progression of the disease[20]. Fibrosis related genes, specifically TIMP1 and matrix metallo-proteinase 13, are increased in the presence of these miRNAs that lead to the development of hepatic fibrosis. All these suggest a diverse array of miRNAs getting altered during hepatic fibrosis and suggest their potential in therapeutic interventions.

Vinciguerra *et al*[21] described that unsaturated fatty acids increase the levels of miR-21 in hepatocytes, which in turn targets phosphatase and tensin homolog (PTEN), leading to steatosis. PTEN is a ubiquitously present protein that specifically dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate and inhibits the AKT signaling pathway. This makes it important for the insulin signaling pathway of which AKT is a major intermediate and therefore the role of decreased PTEN expression has been well correlated with insulin resistance and fatty liver[22] but its regulation through miR-21 has further added a new mechanistic layer of regulation. The insulin-AKT axis and glucose homeostasis is also targeted by elevated miR-143 levels in the obese liver[23]. Over-expression of miR-143 impairs insulin-stimulated AKT activation that confers insulin resistance in obese mice. miR-143 does so by targeting oxysterol-binding protein related protein 8

(ORP8) that regulates the ability of insulin to phosphorylate and activate AKT. ORP8 is a member of the oxysterol-binding-protein family that binds 25-OH cholesterol and regulates the activation of AKT[23]. In a steatosic LO2 cell line model, miR-10b is up regulated that lead to steatosis *via* regulating its target PPARα, a nuclear hormone receptor that is essential during lipid metabolism[24]. This interaction results in modulation of lipid metabolism by triggering the expression of genes required for fatty acid oxidation as well as by increasing the translocation of fatty acids into mitochondria. In another study, miR-27a and miR-27b were identified to be critical in the trans differentiation of normal quiescent HSCs (Hepatic Stellate Cells) into activated HSCs which play a significant role in liver fibrosis[25]. These miRNAs were able to modulate fat metabolism in HSCs by targeting RXRα, the expression of which is decreased in activated HSCs. As compared to normal quiescent HSCs, miR-335 is down-regulated in activated HSCs that lead to increased expression of matrix proteins, α-SMA and collagen type 1[26]. Tenascin C, an extracellular matrix glycoprotein involved in cell migration, is down-regulated by miR-335 and the miR-335-tenascin C pair is identified, at least in part, to regulate HSC migration during hepatic fibrosis. Inhibition of HSC activation and migration is considered an effective therapeutic strategy and here miR-335 offers a promising treatment scheme for hepatic fibrosis.

Altered miRNA levels, therefore, are believed to determine the deregulated hepatic physiology that is frequently associated with T2DM and promise to be significant targets for therapeutic intervention.

CONCLUSION

Current emerging studies indicate that miRNAs might be perceived as potential therapeutic targets. That this could be so is evident from reports where miRNA overexpression or antagonism led to regression of tumors in animal models[27,28]. These studies provide a proof of principle for the use of miRNAs in medicine. Since miRNAs exhibit partial complementarity with their target mRNAs, multiple mRNAs are targeted by each miRNA for example, a miRNA might target several components of a particular molecular pathway. miRNA based therapy might involve miRNAs, their mimics or their inhibitors known an antagomiRs to modulate the levels of the corresponding miRNA and their target(s) within a cell. So, miRNAs can be treated as multi-target therapeutic agents that might prove far more efficient than targeting a single gene or protein. However, a major obstacle envisaged is the targeted delivery and maintaining the stability of the miRNAs or their antagomiRs *in vivo*. Lipid conjugation and chemical modifications, especially additions of phosphorothioate, 2'-O-methyl, 2'-O-methoxyethyl moieties and locked nucleic acid motifs to the nucleotide backbone are being considered to overcome these obstacles[29]. These are believed to increase the target affinity and also enhance the serum stability of these small RNA species. Indeed, *in vivo* inhibition of liver specific miRNA, miR-122 led to improvement in hepatic steatosis by increasing fatty acid oxidation and down regulating lipogenesis in diet-induced obese mice[30]. It also resulted in decreased cholesterol levels. Their therapeutic potential is also apparent in a recent study by Trajkovski *et al*[12] where miR-103/107 antagonism led to improved insulin sensitivity and restored glucose homeostasis in obese mice.

In addition, miRNAs can be utilized as superior biomarkers for diagnosis of various diseases as well as distinguishing different forms of the disease. Diagnosis remains a major unresolved issue related with NAFLD[6]. To date, there is no gold standard technique to either identify NAFLD or to differentiate among different stages of this disease. Serological assays based on conventional liver tests for alanine aminotransferase and aspartate aminotransferase and imaging techniques such as hepatic ultrasonography, magnetic resonance imaging and proton magnetic resonance that are routinely used for the detection of NAFLD have their associated shortcomings. Some recent studies have shown that different types of cancers can be discriminated by their specific miRNAs expression signatures[27]. Also, presence of miRNAs in body fluids such as serum and increased stability as compared to mRNAs since they circulate within membrane vesicles which protect them from endogenous RNase activity[31], make them even more attractive agents as biomarker diagnostics. In fact, circulating miRNA profiles have been explored as potential non-invasive biomarkers in several diseases[32]. Plasma levels of cardiac-specific miR-208 and miR-499 are elevated in acute myocardial infarction[33,34] and in a mouse mole of drug-induced liver injury, miR-122 and miR-192 are specifically elevated[35]. Reduced levels of plasma miR-132 are associated with rheumatoid arthritis and osteoporosis[36]. Circulating miRNA levels have been most identified in various types of cancer; plasma miR-21 levels are elevated in B-cell lymphoma patients[37] and in leukemia patients, miR-92a levels are down-regulated[38]. Serum miR-141 levels are elevated in prostate cancer patients[39] and the miR-17-92 cluster is associated with colon cancer[40]. These reports suggest that circulating miRNAs are altered in diverse disease states and might be explored as potential biomarkers to identify the stage and progression of the disease.

Not much has been done as far as the circulatory miRNA profiles in T2DM or hepatic pathophysiologies are concerned. Identification of altered circulatory levels of miRNAs in T2DM is just at the beginning[41-43] but nevertheless suggest a promising aspect of their use as potential non-invasive biomarkers in T2DM. Although not in relation to diabetes yet, plasma levels of miR-122, mir-34a and miR-16 were markedly elevated in NAFLD subjects that also correlated with the disease severity[44]. Therefore, although at its nascent stage, an altered miRNA signature offers a strong possibility to be exploited to identify hepatic abnormalities and also the stage of the disease during T2DM. Such a therapeutic potential of miRNAs in targeting the challenging issues of obesity

and T2DM has recently been described by Czech *et al*[29] in 2011.

Considering the increasing body of evidence that reveal altered hepatic miRNA patterns in diabetes, a worthwhile thought would be to utilise these small RNA species in the diagnosis and prognosis of this abnormality that would put forth novel criteria for their clinical identity.

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TOPIC HIGHLIGHT

Didier Vieau, Professor, *Series Editor*

Epigenetic mechanisms involved in developmental nutritional programming

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Abstract

The ways in which epigenetic modifications fix the effects of early environmental events, ensuring sustained responses to transient stimuli, which result in modified gene expression patterns and phenotypes later in life, is a topic of considerable interest. This review focuses on recently discovered mechanisms and calls into question prevailing views about the dynamics, position and functions of epigenetic marks. Most epigenetic studies have addressed the long-term effects on a small number of epigenetic marks, at the global or individual gene level, of environmental stressors in humans and animal models. In parallel, increasing numbers of studies based on high-throughput technologies and focusing on humans and mice have revealed additional complexity in epigenetic processes, by highlighting the importance of crosstalk between the different epigenetic marks. A number of studies focusing on the developmental origin of health and disease and metabolic programming have identified links between early nutrition, epigenetic processes and long-term illness. The existence of a self-propagating epigenetic cycle has been demonstrated. Moreover, recent studies demonstrate an obvious sexual dimorphism both for programming trajectories and in response to the same environmental insult. Despite recent progress, we are still far from understanding how, when and where environmental stressors disturb key epigenetic mechanisms. Thus, identifying the original key marks and their changes throughout development during an individual's lifetime or over several generations remains a challenging issue.

Key words: DNA methylation; Developmental origin of health and disease; Epigenetics; Histone modifications; Nutrition

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INTRODUCTION

Epigenetic marks are candidate memories of early life events. All the cells in the body have identical genomes. However, each cell has one of many "epigenomes", unique sets of epigenetic instructions for establishing and maintaining lineage-specific expression profiles[1]. The genome is programmed to express appropriate sets

of genes, in particular tissues, at specific time points during the individual's life. Epigenetic events create a memory of cell identity, maintaining genomic functions such as the maintenance of cell identity after differentiation, the propagation of essential features of chromosomal architecture and dosage compensation[2].

Unlike genetic information, which is extremely stable, epigenetic events are reversible, responding to endogenous and exogenous (environmental) signals. There is convincing experimental evidence to suggest that epigenetic marks serve as a memory of exposure, in early life, to inappropriate environments. These marks induce long-term changes in gene expression, potentially leading to disease in later life, the "developmental origin of health and disease" (DOHaD) hypothesis[3,4].

We focus here on the most recently discovered mechanisms. Significant advances in analytical technologies have led to epigenome characterization becoming a key element in increasing numbers of investigations[5-10]. These recent data challenge prevailing views about the dynamics, relevant position and functions of many epigenetic marks and their complex patterns of crosstalk. We highlight improvements in our understanding of the relationships between epigenetic processes and environmental factors, such as maternal nutrition, and discuss the gaps in our knowledge that remain to be filled. The reversibility of the chromatin modification states determining gene expression status is essential for interaction between the environment and the dynamic epigenome. However, some epigenetic marks laid down early in development, under the influence of environmental factors, must remain stable, acting as a memory of the event long after exposure has ceased. The basis of this paradox, the need for both reversibility and stability, remains unclear.

RECONSIDERING DNA METHYLATION DOGMAS

Cytosine methylation is the only epigenetic modification directly affecting the DNA molecule. It is required for correct embryonic development in mammals. The DNA of most vertebrates is depleted in CpG dinucleotides, the main target for DNA methylation. Furthermore, the role of DNA methylation in genome regulation, other than in genomic imprinting and X inactivation, remains unclear. CpG islands (CGIs) and promoters have been studied in detail because they are easily accessible in terms of the techniques available and sequence specificity. However, other sequences should be taken into consideration (Figure 1).

Mammalian genomes are punctuated by CGIs, DNA sequences with an unusually high frequency of CpG sites[11]. There is considerable evidence for a functional role of CGI-promoter methylation in transcription, but the correlation between CGI methylation and transcription status is poor for many genes. Recently, "CGI shores" were defined as sequences up to 2 kb around CGI and their methylation are highly conserved, tissue-specific and strongly related to gene expression[12,13]. Several large-scale methylation studies have called into question some of the prevailing views about the dynamics and function of DNA methylation. Weber and co-workers investigated the function of DNA methylation in *cis*-regulatory regions and its impact on gene expression by mapping DNA methylation throughout the genome with a methylated DNA immuno precipitation-chip approach and defined three classes of promoters in terms of CpG frequency[14]. They showed that (1) the methylation of CpG-poor promoters did not prevent gene expression; (2) DNA methylation was not a general mechanism of gene repression, as most CGI promoters remain unmethylated even when inactive; and (3) DNA methylation was principally involved in regulating key developmental genes. Thus, promoter CpG density and gene function are the main predictors of promoter methylation state. Shen and co-workers reported that a subset of CGIs within the promoters of key developmental genes were subject to tissue-specific methylation during development. Such methylation had previously been reported only for imprinted and X-inactivated genes. This observation suggests the existence of a programmed mechanism of DNA methylation[15]. Unmethylated regions, recently identified as non-promoter CGIs, become methylated during development in a tissue-specific manner, potentially modifying gene expression[16]. Thus, the methylation of other regulatory elements may also be important for transcriptional regulation. Moreover, as first observed for the active X chromosome, gene-body methylation may be a hallmark of active genes in the whole genome[11,17,18].

Most studies have focused on the methylation of CpG nucleotides, but a potential role of non-CpG methylation has been demonstrated in embryonic cells and adult tissues. In non-CpG contexts, methylation is observed principally in gene bodies, being much rarer at protein-binding sites and enhancers and entirely absent after the induction of differentiation in embryonic stem (ES) cells[19-21]. In a pathological context, Barrès *et al*[20] showed that non-CpG methylation was readily detectable in the skeletal muscles of patients with type 2 diabetes (T2D). They found that the peroxisome proliferator-activated receptor g coactivator-1a (PGC-1a) gene displayed hypermethylation in diabetic subjects, which was negatively correlated with PGC-1a mRNA and mitochondrial DNA levels. Bisulfite sequencing revealed the proportion of non-CpG methylation to be highest. Exposure to tumor necrosis factor α or free fatty acids resulted in a short-term increase in non-CpG methylation in human myotubes. Thus, non-CpG methylation, previously reported almost exclusively in plants and ES cells, may have a physiological role in human skeletal muscle[21,22].

Finally, hydroxymethylation of cytosine was recently identified in mouse ES and neuronal cells[23,24]. Altogether, these new findings highlight the complexity of DNA methylation and the importance of not focusing solely on CGIs and promoters, which should be taken into account in future studies.

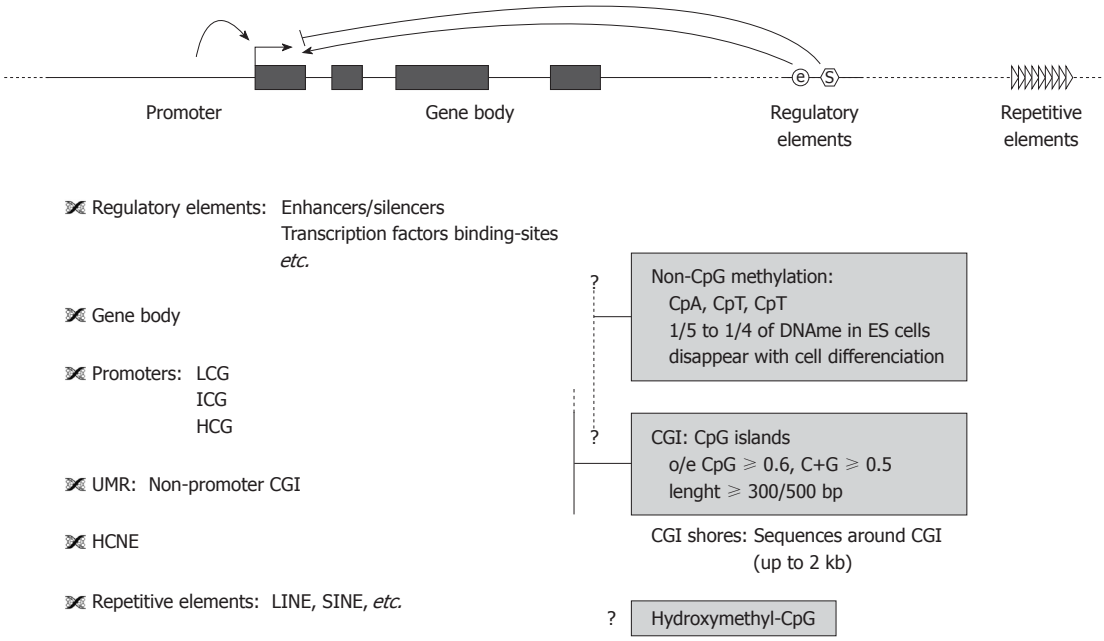

Figure 1 Target sequences for DNA methylation studies. The majority of DNA methylation concerns methylcytosine on CpG dinucleotides but recently hydroxymethylcytosine and methylation on non-CpG sites were identified. Non-CpG methylation was reported in gene body, promoters and repetitive elements; its expanse needs to be further investigated. CpG islands (CGI) and gene promoters are preferred targets in many studies as they correspond to a tractable fraction of the genome with obvious regulatory potential. CGIs are defined algorithmically, as sequences with an observed-to-expected ratio of CpG greater than 0.6, a G+C content greater than 0.5 and, in most cases, a length of more than 500 bp. Three classes of promoters were defined according to their CpG content: LCP have the highest probability to be methylated but methylation correlates poorly to transcription, HCP have low probability to be methylated but this correlates with gene expression. However, transcriptional regulation of genes depends also on distal regulatory elements such as enhancers, insulators, locus control regions and silencing elements. In addition, recent studies show that gene-bodies in active transcription sites are enriched in DNA methylation. Moreover, non-promoter CGIs unmethylated regions (UMR) were recently identified, initially unmethylated they become methylated during development in a tissue-specific manner. "CGI shores" sequences were described around CGI, their methylation in normal tissues is highly conserved, tissue-specific and strongly related to gene expression and were highly sensitive to DNA alterations in colon cancer, as opposed to promoters or CGIs. Highly methylated repetitive elements and highly conserved non-coding elements can also be interesting targets for DNA methylation studies. LCG: Low-CpG promoters; ICG: Intermediate-CpG promoters; HCG: High-CpG promoters; HCNE: Highly conserved non-coding elements.

EVOLVING WORLD OF HISTONE MODIFICATIONS

Recent studies have greatly modified our understanding of histone modifications. Histone modifications lead to the recruitment and binding of critical DNA-regulatory proteins controlling transcription, replication, recombination and repair. Each modification constitutes a signal that is read alone or in combination with other marks on the same or neighboring histones, constituting a "histone code". Histone protein tails display at least 9 different types of post-translational modifications (e.g., acetylation, methylation, ubiquitination, phosphorylation…) with many target sites and at least 50 different modifications having been identified[25]. Histone-modifying enzymes, such as histone methyltransferases or histone demethylases, histone acetyl transferases or histone deacetylases (HDACs) add or remove epigenetic marks on histone tails[26]. Their presence on histones may induce a higher-order chromatin structure and may co-ordinate the ordered recruitment of enzyme complexes for DNA manipulation. For example, acetylation is associated exclusively with active chromatin states, whereas lysine and arginine methylation may be associated with active transcription or repression. Histone modifications may thus influence many fundamental biological processes and may be epigenetically inherited[25].

A complex picture is emerging in which DNA methylation and histone modifications act in concert in an epigenetic program integrating gene-silencing networks within the cell[27,28]. Crosstalk occurs between DNA methylation and histone modifications and is mediated by methyl- or histone-binding proteins, which decipher the regulatory information encoded by the DNA methylation and histone marks[29].

Ambiguous chromatin structure of ES cells

After fertilization, the acquisition of pluripotency involves the epigenetic resetting of the gamete genome to allow the activation of essential genes, such as pluripotency-associated genes. ES cells have an open chromatin structure that is essential for pluripotency and allows the transcription of developmentally regulated genes. The gene expression program of ES cells keeps these cells in a pluripotent state, but also allows them to differentiate into more specialized cells in response to appropriate signals.

One particular group of transcription factors, "pioneer factors", is essential early in development. Pioneer factors binding to promoters and enhancers enable chromatin access for other tissue-specific transcription factors. Such proteins, including members of the fork head

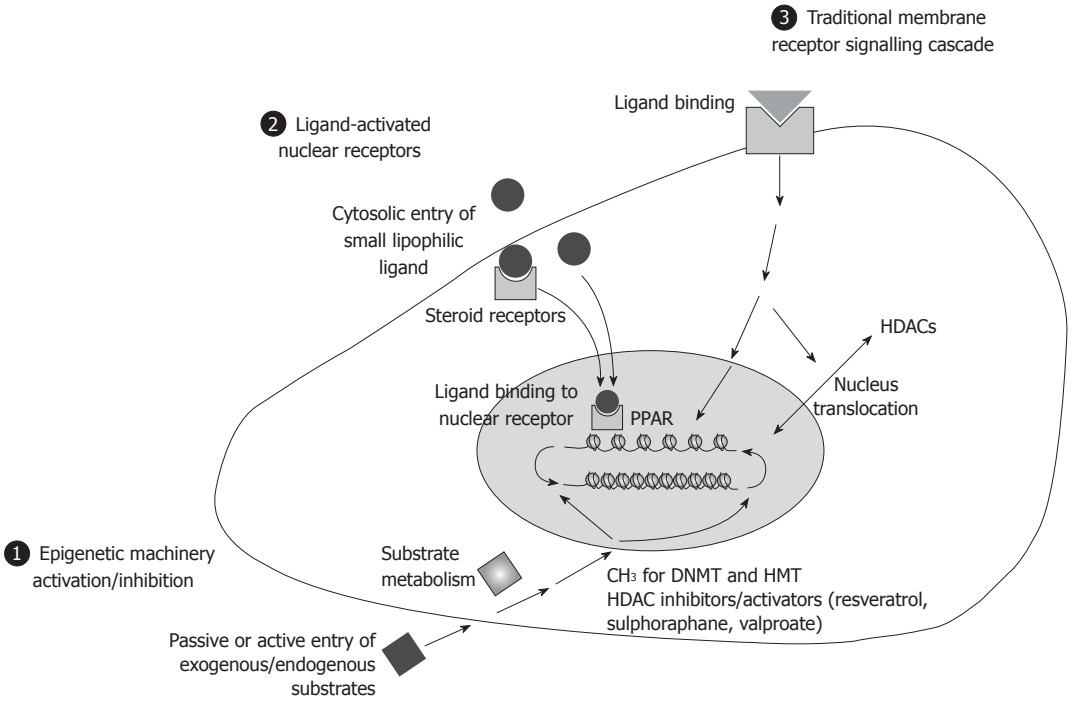

Figure 2 Mechanistic pathways for environmental factors involved in epigenetic reprogramming. There are three ways to link environmental factors such as nutrients or drugs from the cell membrane to the chromatin structure: (1) Some environmental factors, aging and gender may target chromatin modifying enzymes or their substrate availability. Exogenous/endogenous substrates [methyl donors such as folates, histone deacetylase (HDAC) inhibitors such as TSA, *etc.*], after passive or active entry through the cell membrane, undergo cell specific metabolism. Thus, endogenous or exogenous compounds may lead to the alteration of a critical balance of chromatin remodelling enzymes, at the whole genome level, or to specific regions targeted by specific enzymes, e.g. HDACs; (2) Some other compounds (like endocrine disruptors) specifically bind to nuclear receptors, like steroid receptors, may be present in the cytoplasm, bind to their ligand, undergo several modifications and be subsequently translocated to the nucleus where they bind to their responsive elements (RE). The binding of other nuclear receptors, like PPARs and retinoid X receptor (RXR), with their natural polyunsaturated fatty acids ligands or drugs like fibrates lead to the recruitment of co-activators and chromatin remodelling factors. The appropriate modifications of the epigenetic marks at PPAR/RXR RE in target gene promoters modulate the expression of a particular set of genes, in a tissue-specific manner depending on the presence of appropriate co-factors; and (3) Traditional membrane receptor-signalling cascades may be involved. It is possible, depending on the type of ligand, that different pathways could be used. The maintenance of epigenetic patterns is dependent on the preservation of the balance of factors such as DNMTs, Histone acetyl transferases/HDACs or histone methyltransferases/histone demethylases and on the translocation of these enzymes into the nucleus. Extra- or intracellular signalling pathways could trigger activation of one of these factors and result in loci-specific modifications. PPAR: Peroxisome proliferator-activated receptor; DNMT: DNA methyltransferase.

family Foxa, have been found to play important roles at many stages of mammalian life, in early development, organogenesis and, finally, metabolism and homeostasis in the adult[30].

Bivalent chromatin domains, with overlapping repressive H3K27me3 and activating H3K4me3 histone modifications, have recently been shown to mark the promoters of more than 2000 silent regulatory genes involved in developmental processes[7,28,31-36]. Although not unique to ES cells, these bivalent marks seem to play a special role in differentiating cells, keeping developmental genes poised for expression during differentiation. Moreover, inactive unmethylated CGI promoters have high H3K4me2 levels, which may protect DNA from methylation[14]. In addition, Guenther *et al*[37] found that nucleosomes with H3K4me3 and H3K9/K14ac modifications, together with RNA polymeraseII, occupied the promoters of most protein-coding genes in human ES cells. Only a subset of these genes produces detectable full-length transcripts and is occupied by nucleosomes with H3K36me3 modifications, a hallmark of elongation. The others display transcription initiation but not elongation, consistent with regulation principally at post-initiation steps. The genes encoding most developmental regulators fall into this group.

These data suggest a model in which epigenetic marks restrict and define differentiation potential during development[14,38,39]. Identification of the markers involved in establishing transcriptional competence in pluripotent cells should make it possible to explore potential disturbance due to environmental factors. If modulation by environmental stressors occurs at these early stages, the resulting epigenetic marks should escape the resetting process, allowing them to manifest during adulthood[40,41].

How early nutrition sculpts our epigenomes

Throughout evolution, organisms have been faced with the challenge of sensing changes in their environment, such as food depletion and stress, and adapting to them, to ensure their survival. These responses implicitly involve mechanisms, such as chromatin targeting, for adapting the expression of fundamental genes and ensuring genome integrity. Environmental factors, such as diet, nutrients, drugs or the social environment, can be linked

to chromatin structure in several ways (Figure 2)[42].

There is emerging evidence that the environmental changes, triggered at different stages of development, lead to the self-propagation of epigenetic marks associated with changes in gene expression and an adult-onset phenotype[43-55] (for review see[56]). Most studies have dealt with DNA methylation, histone modifications or chromatin occupancy by components of the epigenetic machinery, rarely with combinations of these factors. Such combined studies would provide (1) a better definition of the respective roles of the different epigenetic alterations; (2) an understanding of the consequences of crosstalk between different modifications; (3) identification of the initiating mark; or (4) the matrix for subsequent marks as the individual progresses from an asymptomatic, latent state towards full-blown disease.

Environmental conditions before conception and implantation

Preimplantation development in mammals has recently been shown to be sensitive to environmental conditions, both *in vivo* and *in vitro,* modifying blastocyst potential and leading to long-term changes in fetal and postnatal health and physiology. Similarly, the environment inhabited by a breeding female before conception and early in pregnancy has striking effects on the oocytes developing in the ovarian follicle and embryos in the early stages of development in the reproductive tract. Environmental conditions at these stages may also alter behavior, cardiovascular function and reproductive function throughout postnatal life[57-64]. Low maternal protein consumption or vitamin B and methionine status leads to behavioral and cardiovascular abnormalities in offspring, sex-specific changes in hepatic gene expression in rat fetuses and changes in imprinted gene expression in the rat embryo-fetal axis[65-68]. It has recently been shown that *in vitro* culture conditions, as found in assisted reproduction technology, may affect global patterns of DNA methylation and gene expression. Gametes or early embryos from couples undergoing treatment for infertility may therefore display epigenetic modifications. An association was indeed observed between *in vitro* conception and changes in DNA methylation, potentially affecting the long-term pattern of expression of genes involved in chronic metabolic disorders, such as obesity and T2D[45]. Thus, identifying the specific features and functions of the epigenetic build-up at these stages and determining the mechanisms by which environmental factors may affect them in the long term will be a major milestone in the domain of DOHaD investigation[69,70].

Post-translational histone modifications have been implicated in the complex changes in gene expression driving early mammalian development. Optimization of the chromatin immunoprecipitation technique enables analysis of histone modifications in mouse embryos in culture, from the 8-cells stage to blastocysts. An increase in H4ac and H3K4me in the promoters of *Hoxb1* and *Hoxb9* was observed after the exposure of embryos to the HDAC inhibitor valproic acid. These changes are heritable, even after removal of the inhibitor, at least until the blastocyst stage. These findings illustrate the way in which an environmental signal can generate an inherited epigenetic modification during early development with potential long-term consequences[40].

Ontogeny of chromatin remodeling: An ongoing process

Recent epigenomic profiling and functional studies have provided insights into the dynamics and regulatory complexity of the transcriptional repression mediated by histone-modifying enzymes and other chromatin-associated proteins. These machineries clearly function in a sequential manner. Furthermore, the repressed chromatin state is dynamic rather than static and reflects the balance between antagonistic enzyme activities[41]. A full understanding of the role of chromatin in transcriptional regulation will require knowledge of the relative levels of antagonistic histone modifications and their spatial distributions with respect to transcription factor binding sites and RNA polII[71].

Pinney *et al*[72] studied epigenetic events at the promoter of the gene encoding Pdx1, a critical transcription factor for β cell function and development, the expression of which is reduced in intrauterine growth retardation (IUGR), promoting the development of diabetes in adulthood. They demonstrated that IUGR induces a self-propagating epigenetic cycle, in which the mSin3A/HDAC complex is first recruited to the Pdx1 promoter at the fetal stage, leading to histone deacetylation and a loss of binding of major transcription factors to the Pdx1 promoter, resulting in transcriptional repression. In the postnatal period, as histone deacetylation progresses, active H3K4me3 levels decrease and repressive H3K9me2 accumulates. This epigenetic process is still reversible at this stage, which may be an important developmental window for therapeutic approaches. H3K9me2 accumulation promotes the recruitment of DNMT3A, initiating *de novo* DNA methylation and locking *pdx1* in a silent state in the pancreas of adults born with IUGR.

Similarly, Raychaudhuri *et al*[53] focused on the sequence of epigenetic mechanisms responsible for the weak expression of Glut4 in the skeletal muscle of subjects with IUGR[53]. Different DNMTs bound the Glut4 promoter at different ages: DNMT1 bound postnatally, whereas DNMT3a and 3b bound in adults. DNA methylation was unaffected in subjects with IUGR, but they displayed greater binding of DNMTs to the Glut4 promoter, resulting in higher levels of methyl CpG-binding protein (MeCP2). H3K14 deacetylation mediated by HDAC1 recruitment and enhanced HDAC4 binding were observed. This set the stage for Suv39H1 methylase-mediated dimethylation of H3K9 and an increase in the recruitment of heterochromatin protein 1, which partially inactivates postnatal and adult Glut4 gene transcription in subjects with IUGR. This study demonstrated that perinatal nutrient restriction resulting in IUGR leads to histone modifications in skeletal muscle that directly de-

crease Glut4 gene expression. This effectively creates a metabolic knockdown of this important regulator of peripheral glucose transport and insulin resistance, thereby contributing to the adult T2D phenotype.

Finally, two groups recently demonstrated that mice with disrupted H3K9 demethylase Jhdm2a gene develop adult-onset obesity, hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and hyperleptinemia, hallmarks of metabolic syndrome. Thus, this H3K9 demethylase is a crucial regulator of genes involved in energy expenditure and fat storage[73-75]. The disruption of epigenetic components may therefore play a key role in the progression of obesity and metabolic syndrome.

Thus, histone modifications can be stably inherited while giving rise to additional alterations, and the epigenetic landscapes established under the influence of an environmental factor at a given stage, in a specific chromatin context, may evolve with time. The epigenetic landscapes observed subsequently may therefore not fully reflect the mechanisms initially involved.

Physiological "hyperglycemic metabolic memory" is based on epigenetic modifications

Diabetic patients continue to develop inflammation and vascular complications, even when glycemia is controlled. This poorly understood metabolic memory phenomenon poses major challenges for diabetes treatment. Recent studies have highlighted the persistent and dramatic effects of short-term hyperglycemic spikes on vascular cells in animal models and humans. They have demonstrated a link between epigenetic changes (H3K9me and H3K4me) and the expression of transcription factors, such as NFkB, involved in modulating inflammatory gene expression[76-78]. Brasacchio *et al*[77] reported that hyperglycemia induced dynamic cooperativity between histone methylase and demethylases, associated with gene-activating epigenetic marks on the H3 lysine tail. Thus, an increase in NFkB gene expression is associated with the persistence of epigenetic marks after the removal of a cell from its hyperglycemic environment, providing evidence that epigenetic modifications contribute to changes in gene expression, potentially forming the basis of a physiological "hyperglycemic memory".

Malprogramming other than that associated with early nutrition is beyond the scope of this review. The long-term outcomes of epigenetics alterations in malprogramming to other later-onset diseases or to short-term outcomes of epigenetic changes are progressively declined. However the similitude of the mechanisms involved, whatever the environmental factor (i.e. circadian, nutritional, hormonal or exercise-induced changes) is striking and should help complete our understanding of the picture[55,70,79-84].

SEXUAL DIMORPHISM OF GENE EXPRESSION AND EPIGENETICS

The vast majority of common diseases, including atherosclerosis, diabetes, osteoporosis, asthma, neuropsychological and autoimmune diseases which often take root in early development, display sex bias. Moreover, the risk of developing complex disease in offspring often depends on the sex of the affected parent. The relevance of epigenetic mechanisms underlying the physiological differences between sexes, particularly in drug metabolism, fits well into the epigenetic theory of complex disease (reviewed in[85]).

This bias could be explained by the role of sex chromosomes, the different regulatory pathways underlying sexual development of most organs and finally, lifelong fluctuating impact of sex hormones. Many tissues exhibit sexual dimorphism for a substantial proportion of the genes that they express[42,86]. In fact, sex-specific expression appears to be under the control of sex-specific epigenetic marks. Environmental factors such as social behavior, nutrition or chemical compounds can influence, in a sex-related manner, these flexible epigenetic marks during particular temporal windows of life. For example, modifications of histone H3 are sexually dimorphic in the developing mouse brain and patterns of acetylation, but not methylation, are masculinized in females by testosterone in utero[87]. There are many examples of sex differences in the effects of prenatal and early postnatal life exposures on the risks of subsequent metabolic dysfunction[42,88-92].

It's not all hormones: Roles of sex chromosomes

Sexual dimorphism has been explained traditionally by the regulatory pathways that underlie sexual development of the gonads, brain and other organs, and the impact of lifelong fluctuations in the circulating level of sex hormones. Mammalian sexual differentiation was assumed to be initiated by the presence or absence of the testis-determining factor SRY, encoded on the Y chromosome, in a very narrow spatiotemporal window restricted to the Sertoli cells between 6 and 7 wk of gestation. However, recent findings propose that sexual dimorphism precedes gonadal development. Recently, it was found that the sexual dimorphism between male and female cells in their response to chemical exposure to either ethanol or camptothecin apparently occurred at fetal stages that preceded the production of sex hormones and, accordingly, could be directly attributed to a sex chromosome effect[93,94]. Sex-determining genes on sex-chromosomes can influence not only the development of non-gonadal secondary sexual organs but also of organs outside of the reproductive system, such as brain[95]. Indeed, at the level of the whole body, the sex-chromosomes are crucial for establishment of sex-dimorphism of cellular functions[42]. All male cells possess a single X chromosome of maternal origin and a Y chromosome of paternal origin. Female cells consist of two populations, both of which possess two X chromosomes: one population with inactive maternally inherited X and the second population with inactive paternally inherited X. As a consequence of this random female mosaicism, it is possible that certain

traits, such as cognitive traits, show a greater degree of variability amongst females than amongst males[96].

Extent of global sexual dimorphism

A substantial proportion of dimorphic gene expression might be under the control of sex-specific epigenetic marks. The regulatory pathways underlying sexual differentiation clearly result in extensive differences in gene expression in adults. The genetic and transcriptional mechanisms regulating differences between the sexes have intensively been investigated in the liver but dimorphic gene expression have also been reported in mouse kidney, blastocysts, lacrimal gland, placenta and brain[96-104], and more global differences[105-109]. A recent microarray analysis of 23 574 transcripts by Yang *et al*[86] revealed the extent of sexual dimorphism in gene expression to be much greater than previously recognized. The degree of sexual dimorphism ranged from 14% of active genes in the brain to 70% in the liver. These genes displayed highly tissue-specific patterns of expression, correlated with high levels of activity of distinct pathways. Differences in expression level of a factor of less than 1.2 between tissues were observed for 70% of the sexually dimorphic genes. Interestingly, these genes displayed evidence of clustering, not only on the sex chromosomes, but also on several autosomes.

Sexual dimorphism of gene expression in the liver

Gene expression in somatic cells and tissues can be influenced by external factors, such as the extracellular hormonal milieu. A good example of hormonal regulation is the effect of growth hormone (GH) on gene expression in the liver, which leads to sex-differences in many metabolic processes, such as steroid and fatty acid metabolism, cholesterol homeostasis and drug metabolism[110]. Important sex differences also characterize responses to various hepatic stresses in both rodent models and humans. For example, alcohol-induced liver fibrosis is more prevalent in women than in men, whereas sepsis and hepatitis virus-induced liver fibrosis, hepatic ischemia/reperfusion injury and hepatocellular carcinoma are more prevalent in men than in women; and these sex differences are at least in part due to hormonal factors[110,111]. Support for the involvement of chromatin features in the regulation of genes showing sex differences in liver comes from the discovery of short genomic regions that show sex-dependent and GH-regulated differences in chromatin accessibility ("hypersensitivity sites") in liver tissue, as probed using the enzyme DNase I. Thus, increased hypersensitivity to DNase I cleavage in the male liver tissue compared to that of female liver tissue is seen in the promoter regions of two male-specific genes, *Ca/Slp*, sex-limited protein, and *Cypc,* which catalyzes testosterone hydroxylation[110-113]. DNase hypersensitive chromosomal regions, such as these, have increased access to transcription factors and other DNA-binding proteins, and include promoters, enhancers, silencers and insulators. These findings of sex differences in DNase hypersensitivity are indicative of a sex-specific liver chromatin organization, which is presumably established and/or maintained by the sexually dimorphic patterns of pituitary GH secretion that emerge at puberty, and through their downstream signaling, which leads directly to the sex-dependent patterns of nuclear STAT5b activity[110,114-117].

Sexual dimorphism of placenta

The placenta has long been considered to be an asexual organ, with most placental studies consistently pooling data for male and female placentae into a single group[96]. However, predisposition to metabolic disease differs between the sexes, with women more likely to develop obesity and men cardiovascular disease. This sexual dimorphism may already exist during development. Indeed, there is mounting evidence to suggest that the sex of the embryo, through the embryo-derived tissues of the placenta, plays a significant role in determining fetal size, nutrition, morbidity and survival[96,118]. Only a handful of studies have reported differences between the sexes, in terms of the expression of individual genes or pathways in male and female human and rodent placentae. These studies also addressed the impact of differences in the quality of the maternal diet on placental gene expression, with a systematic investigation of the relationship between diet and the expression of sexually dimorphic genes, providing insight into the different sensitivities of male and female fetuses to what the mother eats[96,101-105,108,109].

We have data showing that gene expression and DNA methylation are sexually dimorphic in male and female placentae under control conditions. Surprisingly, in stressful conditions, say at high fat or low calorie diet, or maternal overweight/obesity, the placentae from male and female fetuses do not use the same gene pathways and networks to cope with the stress. Does that lead to different outcomes? Maybe this leads to sex-dependent differences in the outcome of programming with long lasting effects. Alternatively, males may "climb the mountain" taking the north face while females take the south face but they ultimately reach the same peak after using different paths.

CONCLUSION

The DOHaD science is still accumulating proof of evidence of fetal programming: a developmental insult (diet, drugs, lifestyle, social interventions, *etc.*) leading to long-term consequences (metabolic syndrome, psychiatric diseases, *etc.*). A new field is emerging, aiming to identify epigenetic targets to improve our understanding of the ontology of chronic diseases in response to environmental factors. Experiments in this area must be carefully designed.

How should such studies be carried out? Investigations should first be carried out in appropriate animal models exposed to specific environmental factors during critical developmental windows. Many analytical procedures are available; each with its own biases and limita-

tions and the choice depends on the question posed. We may need genome-wide or gene-specific approaches targeting regulatory regions (promoters, enhancers, gene body or elsewhere) and assessing functional significance[9]. New high-throughput tools are becoming available and may soon be applied more widely as DNA sequencing costs drop, to studies of the epigenomic changes associated with developmental shifts, environmental changes, and disease states[14,15,18,38,119-123].

Where should we look for epigenomic effects? Each environmental factor may target specific cell types, leading to a unique, specific epigenome identifiable only in the appropriate tissue[124], which will often contain mixed cell populations. However, the appropriate tissues are generally not available for study in humans. Fortunately, recent data suggest that the traces left by specific environmental factors can be visualized in leukocytes, at least for dietary factors[81]. However, the question remains as to whether surrogate tissues obtained by minimally invasive procedures, such as the placenta or cord blood, truly reflect early programming *in utero*, cataloguing intrauterine environmental events, or whether adult tissues and cells, such as lymphocytes, monocytes or buccal smears, reflect the lifelong metabolic memory[43,76,77,125].

When should epigenetic effects be studied? Circadian and seasonal rhythms are important components. Sampling at the right time may unmask pertinent marks important for determining both the nature of the challenge and the extent of the effect[126].

What are we actually studying? Are the marks observed the cause or just a consequence? It would be very interesting to carry out studies at several time points, to unravel the sequence of epigenetic events and to distinguish between causal changes and the resulting epigenetic landscape.

Who should be studied? It should be borne in mind that men and women have different programming trajectories[42]. Different recent studies show an obvious sexual dimorphism in response to the same environmental insult.

Why not? The inherent reversibility of epigenetic marks is promising for treatment approaches. However, one major potential problem is that epigenetic processes associated with the disturbance of programming by early environmental events may disappear during differentiation, may be leaky, leading to irreparable changes in the number of nephrons, β cells of the pancreas, or changes in the function of target tissues, or may remain dormant until the appropriate environmental stimulus comes along to activate them[127].

All epigenetic changes are, in theory, flexible, but can interventions really modify them? Without side effects[128]? A growing number of studies have demonstrated the potential reversibility or compensation of misprogramming with appropriate nutrients or epigenetic drugs[52,129-132].

This should make it possible to identify the specific epigenetic marks induced by specific environmental factors and to study their changes during the individual's life and potential reversibility, using appropriate epigenetic tools.

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London, United Kingdom

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9. Gastro Forum München
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Gastroenterology: New Zealand CME Cruise Conference
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March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology
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March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform
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March 25-27, 2011
MedicReS IC 2011 Good Medical Research
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March 28–30, 2011
The Second World Congress on Interventional Therapies for Type 2 Diabetes
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The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition
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May 7-10, 2011
Digestive Disease Week
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June 11-12, 2011
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The major task of *WJD* is to report rapidly the most recent results in basic and clinical research on diabetes including: metabolic syndrome, functions of α, β, δ and PP cells of the pancreatic islets, effect of insulin and insulin resistance, pancreatic islet transplantation, adipose cells and obesity, clinical trials, clinical diagnosis and treatment, rehabilitation, nursing and prevention. This covers epidemiology, etiology, immunology, pathology, genetics, genomics, proteomics, pharmacology, pharmacokinetics, pharmacogenetics, diagnosis and therapeutics. Reports on new techniques for treating diabetes are also welcome.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Books

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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