The novel role of epigenetics in primary prevention of cardiovascular diseases

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Abstract

A great deal of evidences indicate that impaired fetal growth and *in utero* exposure to risk factors, especially maternal hypercholesterolemia, may be relevant for human pathophysiological signs of atherosclerosis and subsequent development of cardiovascular disease (CVD) during different life stages. Despite the underlying mechanisms of fetal programming are still unknown, epigenetics has been suggested as one of the possible explanations for the associations between intrauterine risk factors and CVD development. Indeed, a lot of translational studies support the hypothesis that epigenetic changes are related to increased CVD risk although it is still not possible to establish a direct causality in humans. Notably, epigenetic modifications can be reversible through therapeutic approaches employing histone deacetylase inhibitors, histone acetyltransferase inhibitors and commonly used drugs like statins. Thus, the whole comprehension of these mechanisms will provide in the next future the rationale for the development of novel tools to be used in the primary prevention and therapy of CVD.

Introduction

Cardiovascular disease (CVD) encompasses a range of conditions extending from congenital heart disease to myocardial infarction, unstable *in crescendo* angina, coronary heart disease, peripheral arterial disease, and ischemic stroke, all of which recognize a different degree of heritability. The genetics of most types of CVD is still poorly defined.[1] Indeed, since CVD refers mainly to complex multifactorial disorders, together with genetic predisposition (often related to polymorphisms of critical genomic loci), we must consider the environmental factors and lifestyle (diet, physical exercise, smoke, alcohol consumption, psychosocial factors) to which the individual is exposed, either acutely or chronically. Although candidate gene or genome-wide association studies led to the identification of more than thirty alleles in association with various forms of CVD, common alleles account for a relatively small fraction of the total heritability of these traits.[1] Indeed, ischemic heart disease and/or coronary artery disease are due to atherosclerosis, which derives from the harmful synergy between genetic, environmental, local and systemic risk factors.

The earliest lesion of atherosclerosis is the fatty streak, an intimal thickening due to the focal accumulation of serum lipids underneath the endothelial surface of the involved arterial segment. Clinical studies, like Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study and the Bogalusa cohort study, established the pathogenetic role of hypercholesterolemia in atherosclerotic lesion progression in youth.[2-4] Furthermore, we reported the early appearance of fatty streaks also in human fetal arteries;[5,6] the formation of these lesions was greatly increased in fetuses from hypercholesterolemic mothers.[5] Furthermore, we also observed the atherogenic influence of maternal hypercholesterolemia in the Fate of Early Lesions in Children study.[7] Remarkably, the development of atherosclerotic lesions is dependent on the arterial district.[8-10]

Studies of identical twins have clearly indicated that the early environment, including maternal malnutrition, plays an important role in programming the healthy/diseased status in later life.[11] Maternal cholesterol levels increase during the third trimester physiologically, even in normocholesterolemic mothers, and this phenomenon may be even more relevant in hypercholesterolemic mothers due to the transplacental passage of normal and oxidized fatty acids, which may promote their detrimental effect on atherosclerosis.[12] Moreover, several studies indicate that also low birth-weight is associated with increased hypertension, diabetes, and CVD.[13-15]

Thus, the hypothesis of early life programming is widely accepted. However, the mechanisms through which early life events exert long-term effects on the metabolism in the adult life are not fully elucidated. However, according to a general view, such programming should be the result of the fetus trying to adapt to unfavorable *in utero* conditions; this programming has been suggested to be generally independent of genomic DNA sequence, but rather mediated by epigenetics-related mechanisms.[14-17] Other mechanisms include the permanent structural changes occurring in an organ due to suboptimal concentrations of an important factor during a critical period of development and permanent effects on the regulation of cellular aging.[18]

The term *epigenetics* is referred to heritable changes in gene expression that do not involve

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changes in the genetic code.[19] Epigenetic control is one of the central regulatory systems within the cell contributing to lots of phenotypic differences between cell types in multicellular organisms. Indeed, epigenetics involves changes in gene function, which can be inheritable for several cell divisions and sometimes transgenerationally via the gametes, although this property is still debated, especially in humans.[20-23] Regarding CVD it may explain why subjects having similar genetic settings and risk factors for these diseases display a very different outcome in their clinical manifestation.

Interestingly, epigenetics could affect the development and outcome of CVD by regulating the regenerative potential of damaged tissues. Indeed, several studies on stem cells have revealed that among mechanisms contributing to the maintenance of pluripotency and self-renewal, there are also epigenetic modifications.[24] For instance, recent study also showed that stem cell DNA is subjected to different methylation mechanisms.[24]

Basic epigenetic mechanisms

The essential mechanisms of epigenetic modifications in mammals include DNA methylation, histone modifications and microRNA alterations.

Figure 1 provides a rapid outline of epigenetic mechanisms involved in the early pathogenic events linked to CVD development. DNA is packaged into chromatin, a protein-DNA complex consisting of nucleosomes (where DNA is wound around histone proteins) and non-histone proteins.[16,17] Epigenetic mechanisms alter the accessibility of chromatin to transcription factors by modifying DNA and nucleosomes, in response to environmental factors.[25,26]

DNA methylation

An essential role in epigenetics inheritance is played by DNA methylation that is generally associated with low gene activity.[27,28] DNA methylation is detected at the C5 position of cytosine residues in a CpG dinucleotide as result of DNA methyltransferases (DNMTs) which are capable both of methylation and demethylation rendering the modification reversible.[27,28]

Histone modifications

Covalent post-translational modifications of histone tail residues can also alter chromatin structure; these modifications include phosphorylation, methylation, acetylation, SUMOylation and ubiquitination. Over 70 modifications are currently acknowledged, mostly represented by acetylation and methylation of lysine residues. Acetylation/deacetylation of lysine residues is correlated with chromatin accessibility and gene activation whereas the role of histone methylation depends on the precise methylated residue and the number of added methyl groups.[16] Yet, arginine residues can also be specifically methylated and acetylated as well as SUMOylation and ubiquitination of histones have also been observed.[16] Overall, this chromatin plasticity is essential to keep DNA in an open or a closed state in order to switch genes on or off according to the cellular needs.[16] Modifications of histones and DNA methylation are functionally linked activities.[16,27,28] Throughout semi-conservative DNA replication, the methylation of the daughter strand and recruitment of histone-modifying proteins maintain the epigenome in the next cell generation.[29] Epigenetic modifications are naturally reversible, mainly due to the counterbalancing actions of several enzymes taking part to the maintenance of epigenome (Figure 1).[30-32]

Other epigenetic mechanisms may involve acetyltransferases/deacetylases and methyltransferases/demethylases targeting non-histone proteins, like NF- B.[33-36]

MicroRNA related epigenetic mechanisms

More recently, also non-coding RNAs, such as microRNAs (miRNAs) expressed from genes and intergenic regions, have been shown to influence epigenetic changes of DNA methylation and histone code,[37-38] in diseases like CVD.[37] Synthesized as a larger precursor in the nucleus, miRNAs are processed in the cytoplasm into mature miRNAs, where they target specific mRNAs thereby inducing degradation or translational inhibition (Figure 1).[39] A huge number of miRNAs have already been identified and some of them are also involved in inflammation and atherosclerosis.[37,40-42]

For instance, it is well known that heavy ethanol consumption during pregnancy can lead to several defects including cardiovascular ones. In this regard, specific ethanol-sensitive miRNAs control the ethanol addiction[43] and seem to confer mammalian-specific patterns of sensitivity to teratogens like

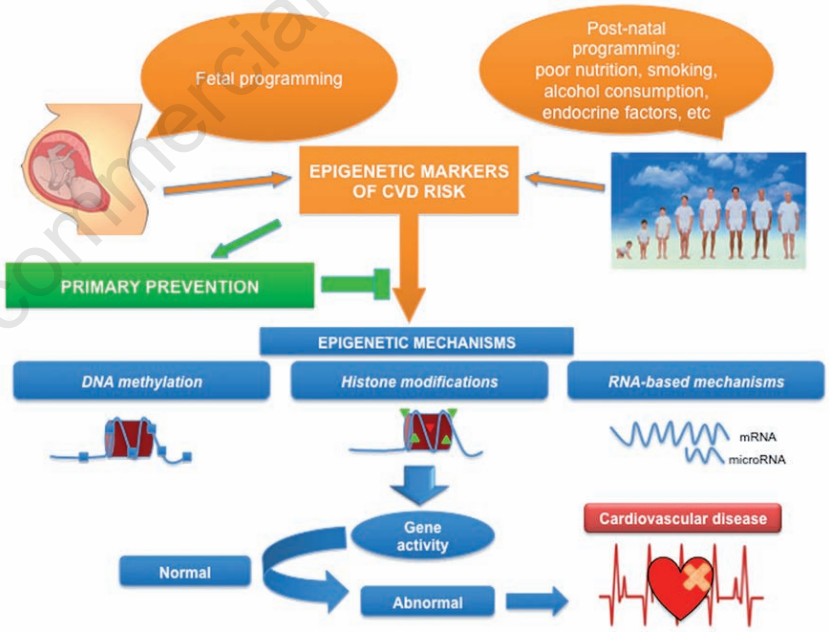

Figure 1. Epigenetic modifications during fetal or postnatal life can influence the mature phenotype and determine sensitivity to later environmental factors and subsequent risk of cardiovascular diseases (CVD). Primary prevention of CVD can benefit from early epigenetic markers and can eventually inhibit or reverse epigenetic mechanisms thus reducing the incidence of CVD. Epigenetic modifications of DNA and histones control the access of transcription machinery -RNA polymerase (RNA-pol), Mediator complex (MED) and transcription factors (TFs)- thus modulating the mRNA synthesis and protein expression. The active chromatin is characterized by the presence of acetyl groups (Ac) on specific lysine residues of core histones. CpG sequences in the promoter regions of active genes are usually unmethylated, allowing the binding of TFs. Inactive chromatin is characterized by histone deacetylation, promoter CpG methylation and decreased binding of TFs. Post-transcription control can also be provided by miRNA molecules that bind to complementary sequences of mRNA reducing the rate of protein synthesis.

ethanol.[44] The role of miRNAs in drug addiction is now starting to be investigated. Recent studies indicate that miRNAs play important roles in the actions of ethanol and the emerging picture is very complex. For example, ethanol can cause simultaneous upregulation of some miRNAs and downregulation of others. Moreover, the effect of ethanol on a particular miRNAs depends on both dose and cell context.[43,44] A further example is miR-33, an intronic miRNA located within the gene encoding a transcriptional regulator of cholesterol synthesis, the sterol-regulatory element-binding protein-2, which modulates the expression of genes involved in cellular cholesterol metabolism.[45]

Transcription factors activity

Epigenetic mechanisms can also affect CVD by influencing the expression of atherosclerosis-related genes via modulation of transcription factors. These proteins can be divided into four classes (I-IV) classified by structural elements, like basic leucine zipper or basic helix-loop-helix, which mediate their DNA binding activity, but also determine the classes of drugs that can affect their activity.[16,46] In fact, the potent cholesterol-lowering statins have been demonstrated to modulate the activation of the class-I transcription factor sterol responsive element-binding protein, whose target genes are involved in cholesterol and fatty acid metabolism.[46] Similarly, insulin-like drugs target the nuclear receptor peroxisome proliferator-activated-receptor-gamma (class-II transcription factor), several anti-inflammatory drugs inhibit activation of nuclear factor kappa B (class-IV transcription factor), while others (*e.g.* flavopiridol, rapamycin, and paclitaxel) target cell cycle regulating proteins.[46]

Recently, the Mediator complex has been involved in epigenetic mechanisms since it connects gene expression and chromatin architecture.[16,47,48] This is a ubiquitous conserved complex of approximately 30 subunits that regulates transcription by coordinating RNA polymerase II binding to target promoters through gene-specific activators and repressors.[49]

Evidence of the epigenetic role in fetal programming

Maternal programming

The PDAY and the Bogalusa studies, together with the observations that the early appearance of fatty streak lesions in human fetal arteries and the development of early atherosclerotic lesions in infants and adolescents are linked to the hypercholesterolemia of mothers during pregnancy, have strongly advocated the role of epigenetic mechanisms in fetal programming, which is now further supported by several translational studies.[2,3,5-7]

A link between atherosclerosis/neointima formation and histone modifications was established in a study where the administration of trichostatin A, a lysine deacetylase inhibitor, exacerbated atherosclerosis in low density lipoprotein (LDL) receptor-deficient mice.[50] More recently, in a murine model of ApoE deficiency, both *in utero* programming and diet-induced hypercholesterolemia were associated with histone methylation modifications and altered lysine methyltransferases in vascular endothelial cells and smooth muscle cells.[51] These studies strongly suggest that *in utero* environment can lead to epigenetic (re)programming and contribute to atherosclerosis development during the adult life.

Our previous studies addressed the issue of prenatal and postnatal modulation of risk factors linked to atherosclerosis and CVD. Indeed, we found that the maternal hypercholesterolemia was associated with enhanced formation of oxidized (ox) LDL and atherosclerosis in offspring when compared to that of normocholesterolemic mothers in both a rabbit[52] and a murine model.[53] More recently, pretreatment of oxLDL-exposed cells with statins has been shown to reduce the histone modification, as well as recruitment of the genes involved.[54] Moreover, oxLDL reduced histone deacetylase (HDAC) 1 and 2 expression, and statins partially restored global HDAC-activity.[54] Overall, these findings suggest that maternal hypercholesterolemia may affect atherosclerosis in offspring via epigenetic-related mechanisms. However, within the fetal programming theory, the exact epigenetic mechanisms involved in the promotion of CVD are still largely unclear. Nevertheless, studies on pregnant rats showed that a protein-restricted diet can lead to a reduced DNMT1 expression, with a consequent hypomethylation of specific promoters.[55] Analogously, a preclinical mouse model of prenatal protein restriction exhibited a hypermethylation of the liver X-receptor gene promoter, suggesting that prenatal nutrition may influence adult lipid metabolism by DNA methylation.[56] Restriction of vitamin B12, folate and methionine supply in a sheep model during the maternal periconceptional period, instead, elicited an altered immune response, insulin resistance and elevated blood pressure accompanied by alteration of DNA methylation in offspring.[57] In addition, a perturbation of the fetal environment, for example, through poor nutrition,[55] inappropriate energy metabolism,[58] exposure to ethanol,[59] methyl donors,[60] glucocorticoids,[61,62] endocrine disruptors,[63] and tobacco smoke,[64] can all lead to disease.

Paternal programming

Despite most studies of epigenetic programming have been studied on maternal behavior, a recent study, performed on a rat model, demonstrated that also a father diet can affect his daughters' health. Particularly, high-fat diets administered to fathers altered the development of their sperm, which then promoted an adult-onset disease, such as impaired glucose-insulin homeostasis, in their female offspring.[65] Generally, the mechanism of this transgenerational transfer is not still clear, although DNA methylation, histone modification and microRNAs may all contribute to inheritance by altering post-transcriptional processing of factors affecting early embryonic development.

It is also important to distinguish between epigenetic inheritance and direct epigenetic changes in the fetus' genes. Indeed, in the first case, the epigenetic changes in maternal DNA are transmitted to the fetus. In the second case, epigenetic changes in the fetus genome occur independently of maternal nuclear DNA changes. The first scenario is intriguing and less established whereas the second scenario is rather ordinary and common.[16] In this context, a group of genes, called *metastable epialleles*, have been suggested to establish the epigenetic state in the embryo. These alleles, not yet identified in humans, are variably expressed in genetically identical individuals due to epigenetic modifications that are established during early development. Moreover, they are probably inherited transgenerationally and can be modulated by environmental agents thus providing an explanation for some transgenerational effects, including transmission of CVD risk.[16] Several associated epimutations have also been reported.[16] In the attempt to estimate the relative contribution of environmental and genetic factors to the overall neonatal epigenome asset, a recent study has analyzed DNA methylation in multiple tissues from newborn twin pairs.[66] Interestingly, this study revealed that both genetic and intrauterine components contributed to variation in the human neonatal epigenome. Indeed, DNA methylation was different between tissues and between unrelated individuals, as well as within twin pairs, even though it was greater in dizygotic pairs than monozygotic ones.[66]

Clinical perspectives

Primary prevention of cardiovascular disease: can epigenetics help?

Coronary artery disease, heart failure, and stroke, the most disabling expressions of CVD, recognize multiple genetic and environmental determinants, and epigenetic changes are emerging as closely related factors. Primary prevention is the most promising strategy to reduce the health and economic societal bur-

Table 1. Epigenetics and cardiovascular disease development: principal ongoing observational trials.

Clinical trial	Clinical Trials.gov Identifier	Status	Conditions
Possible epigenetic changes in offspring of women with pregestational and gestational diabetes	NCT01255384	Not yet recruiting	Gestational and pregestational diabetes, IDM, epigenetic changes
Developmental pathways to metabolic disease - growing up in Singapore towards healthy outcomes (GUSTO)	NCT01174875	Recruiting	Metabolic diseases, diabetes mellitus
The early origins of cardiovascular disease	NCT00923039	Unknown	Cardiovascular disease
To investigate the influence of ethnicity in metabolic disease in healthy, overweight and obese subjects (SAMS-1)	NCT00988819	Unknown	Overweight, obesity
Personalized medicine for morbid obesity	NCT01365416	Not yet recruiting	Obesity surgery and diabetes
Testing the developmental origins hypothesis (CHIPS-Child)	NCT01545492	Recruiting	Diabetes, stroke, obesity
Study of offspring of women with type 1 diabetes (EPICOM)	NCT01559181	Recruiting	Type 1 diabetes
Dietary, physiological, genetic, and behavioural predictors of health in a young, ethnically-mixed population (InSight)	NCT00945633	Active, not recruiting	Obesity
Progression of early subclinical atherosclerosis (PESA)	NCT01410318	Recruiting	Atherosclerosis
Metabolic effects of birth weight on overweight and obese Chinese adults and their responses to weight loss (SAMS-2)	NCT01080378	Unknown	Overweight, obesity
Observational study of early metabolic and vascular changes in obesity (STYJOBS/EDECTA)	NCT00482924	Recruiting	Vascular burden in obesity, brown/white adipose tissue, adipokines, fatty liver disease, insulin resistance

IDM, infant of diabetic mothers.

den of chronic degenerative diseases like CVD. Therefore, a major aim in the field has always been to define the risk profile both at the population and the individual level, particularly in apparently healthy people. The latter level can be more challenging due to the interindividual variations. In this regard, epigenetics could be particularly promising because individual variability is generated by the different environmental stimuli interacting with different genetic backgrounds at different ages. Epigenetic signatures may, therefore, help to better define individual susceptibility to CVD, thus leading to more efficient and tailored preventive strategies. In order to reach this target you need to establish novel biomarkers that allow identifying as at-risk individuals also those that have a very low level of risk according to the traditional predisposing factors.

In this scenario, epigenetics can significantly contribute to improve primary prevention of CVD. Indeed, it is possible that future studies will analyze the cardiovascular risk according to atherogenic epigenetic modifications; since these markers can derive from fetal programming or also can become evident in the first years of life, there is the possibility of an early classification of at-risk individuals. This novel possibility is particularly critical because the earlier you start primary prevention, the better it is in terms of disease prevention or, at least, delaying the onset. Of course, the cost/benefit parameter is always to be considered at the population level.

Novel therapies

The reversible nature of epigenetic alterations has encouraged the development of therapeutic strategies targeting various epigenetic components, like DNA methylation, histone code and miRNAs. Those strategies should take the atherogenic epigenome back to its physiological status.

Indeed, several DNMT and HDAC inhibitors have been studied in clinical trials and some of these agents have been also FDA approved for treatment of several malignancies and other diseases. Newly, also histone methylation and microRNA expression are under study as therapeutic targets.[67,68]

However, despite the accumulated knowledge, no epigenetically active agents have entered in clinical trials for CVD. Indeed, as regard to atherosclerosis, there is only one study where administration of curcumin (a lysine acetyltransferase inhibitor) caused significantly lowered LDL levels and increased high-density lipoprotein levels in healthy volunteers.[69] In a rat model of heart failure, consumption of curcumin led to p300 histone acetyltransferase (HAT) activity inhibition, prevented ventricular hypertrophy, and preserved systolic function.[70] Furthermore, after interleukin 1-b exposure, early growth response factor-1 transcription in human vascular smooth muscle cells was stimulated via the acetylation of histone H3 and prevented by garcinol reflecting the efficacy of HAT inhibition during thrombus formation.[71] However, research on the potential therapeutic use of epigenetically active compounds is still very preliminary and prospective clinical trials should address selectively this question.

Currently, only observational studies are ongoing to definitely establish the link between epigenetics and CVD development. Table 1 is a list of the principal observational trials in this context. These studies will be also helpful to translate into humans the concepts that have been already acquired both in *in vitro* models[72] and animal studies.[73-76]

Furthermore, future studies will also provide more detailed information at molecular level such as the identification of the specific genes that are directly affected by DNA methylation and/or histone modifications. Nowadays, innovative technologies, such as next-generation sequencing (NGS), have given the opportunity to analyze the entire genome and epigenome thereby providing new opportunities for epigenetic research.[1,77] Moreover, NGSs allow the simultaneous genome-wide measurement of multiple epigenetic modifications together with the transcriptome analysis of the same biological sample.[77] This research should be of great importance since it will help clarifying the mechanism of action of available small molecules that can inhibit the function of DNA and histone modifying enzymes, thus altering the expression of target genes and providing new pharmacological tools for therapeutic intervention.

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