

Cardiovascular Epigenetics

Basic Concepts and Results From Animal and Human Studies

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Epigenetics is the study of heritable alterations in phenotypes and gene expression that occur without changes in the DNA sequence. Epigenetic mechanisms are flexible genomic factors that not only can change genome function under exogenous influence, but also can provide a molecular substrate that allows for the stable propagation of gene expression states from one generation of cells to the next.

The best understood of the epigenetic mechanisms is DNA methylation,¹ a covalent modification that, in mammals, occurs predominantly at cytosines followed by guanines (ie, CpG dinucleotides) to form 5-methylcytosines. CpG methylation generally is associated with gene silencing. DNA methylation states may vary over an individual's lifetime, and have been shown to regulate biological processes underlying cardiovascular disease, such as atherosclerosis, inflammation, hypertension, and diabetes.^{6,12,13} Methylation within gene promoters and CpG-dense sequences (CpG islands, usually unmethylated) has long been held to have the highest functional relevance to gene expression control (Figure 1). However, recent work has identified regions up to 2 kb from the islands (ie, on the island edges), termed *CpG island shores*, that have variable methylation and are the most enriched with functional CpG sites.² DNA methylation also is believed to play important roles in the maintenance of genome integrity by transcriptional silencing of retrotransposons, that is, retrovirus-like DNA sequences that can duplicate and transpose themselves across the genome. In particular, some retrotransposons, such as Alu and long-interspersed nucleotide elements-1 (LINE-1) have up to 1 million copies in the human genome, are heavily methylated, and have been extensively investigated in human epigenetics studies. DNA methylation is mitotically stable and can be transmitted through cell division and, in some loci identified in animal experiments, transgenerationally.

Histones are globular proteins that can be posttranslationally modified at specific amino acid residues. Histone 3 and histone 4 can be covalently modified by acetylation, methylation, ubiquitination, phosphorylation, sumoylation, citrullina-

tion, and ADP-ribosylation. These modifications alter histone interactions with DNA and other nuclear proteins. Consequently, histone modifications induce either repression or activation of transcription, depending on the type of modification and on the position of the modified amino acid residue.

The Growth of Epigenetics

The conceptual foundations of epigenetics were laid out in the early 1940s by Sir Conrad Waddington who first defined epigenetics as the "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being." Just a few years later, DNA methylation was first described in bacterial genomes (Figure 2), but only in 1975, did 2 groups independently hypothesize that 5-methylcytosines were duplicated through cell division and regulated mRNA expression. At that time, Allfrey et al had already demonstrated that histone acetylation and methylation contributed to gene expression control.²³

The past 20 years have seen the development and diffusion of easily accessible techniques for DNA methylation and histone modification analysis. Qualitative methods such as bisulfite sequencing and methylation-specific polymerase chain reaction have been developed and eventually adapted to produce semiquantitative calls. Quantitative methods such as pyrosequencing and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) recently have allowed for precise measurements of the copies of methylated genes/loci in a DNA sample.

Techniques for genome-scale analysis of both DNA methylation and histone modifications have become available that provide increasing degrees of genomic coverage. Currently, relatively inexpensive chip arrays are available that allow quantitative analysis of methylation at approximately 486 000 individual CpG sites. At a higher cost, whole-genome tiling arrays featuring 2.1 million probes can be used to provide qualitative calls on the methylation of genes or loci but without achieving individual methylation measures on single CpGs.

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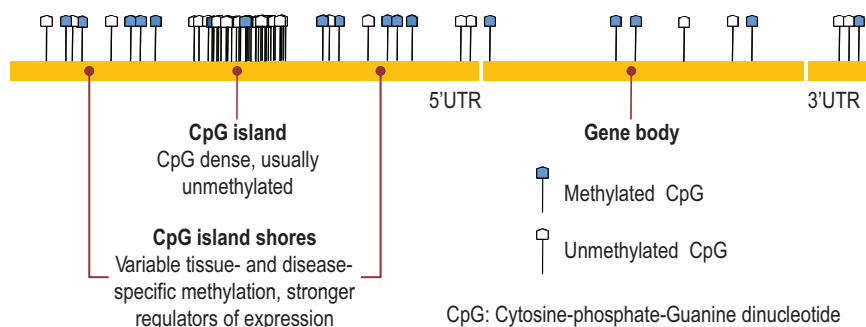


Figure 1. CpG islands, shores, and methylation sites in relation to a hypothetical gene. UTR indicates untranslated region.

Next-generation sequencing is increasingly used for histone modification analysis, coupled with chromatin immunoprecipitation, and is being developed further for DNA methylation analysis. Current genome-wide scale methods for epigenomic analyses are limited in that they are prone to analytic artifacts and measurement errors. Technical validation through a more-accurate technology, such as pyrosequencing or MALDI-TOF, is widely regarded as a necessary step to validate genome-scale findings.

Starting from the mid-1990s, there has been a steady growth in the number of laboratories and investigators involved in epigenetic research. In the words of Andrew Feinberg,¹ epigenetics has been moving at the very “epicenter of modern medicine because it can help to explain the relationship between an individual’s genetic background, the environment, aging, and disease.” All those questions that can be addressed in epigenetic studies are critical to cardiovascular research. Cardiovascular epigenetic research has been growing quasi-exponentially in recent years, albeit the increase in the number of cardiovascular publications has lagged a few years behind the general production in epigenetics (Figure 3). Only 12 epigenetic publications that involved cardiovascular disease or related issues appeared in PubMed in 1995, and that number was just 35 in 2002. The projected number for 2010 is 525, amounting to a 43-fold

increase since 1995. As a benchmark, the total number of PubMed publications on cardiovascular disease or related issues increased from 50 320 to 95 953 from 1995 to 2010 (91% increase). In 2010, the proportion of epigenetic publications on cardiovascular disease or related issues will be 5.4% compared to 1.6% in 1995.

Cardiovascular Epigenomics: A Conceptual Model

Epigenomics represents a critical link between genomic coding and phenotype expression that is influenced by both underlying genetic and environmental factors (Figure 4). Available data indicate that cardiovascular risk factors might influence and remodel epigenomic patterns and that cardiovascular biomarkers are associated with epigenetic modifications. Epigenetic modifications may contribute to subclinical and clinical cardiovascular disease. Epigenomics is inherently interconnected with genetics because epigenetic modifications can alter the expression of genetic variations, and genetic variation is one of the determinants of DNA methylation and histone modifications. In online-only Data Supplement Table S1, we provide an extended list of epigenetic publications on cardiovascular risk factors, biomarkers, and subclinical and clinical cardiovascular diseases.

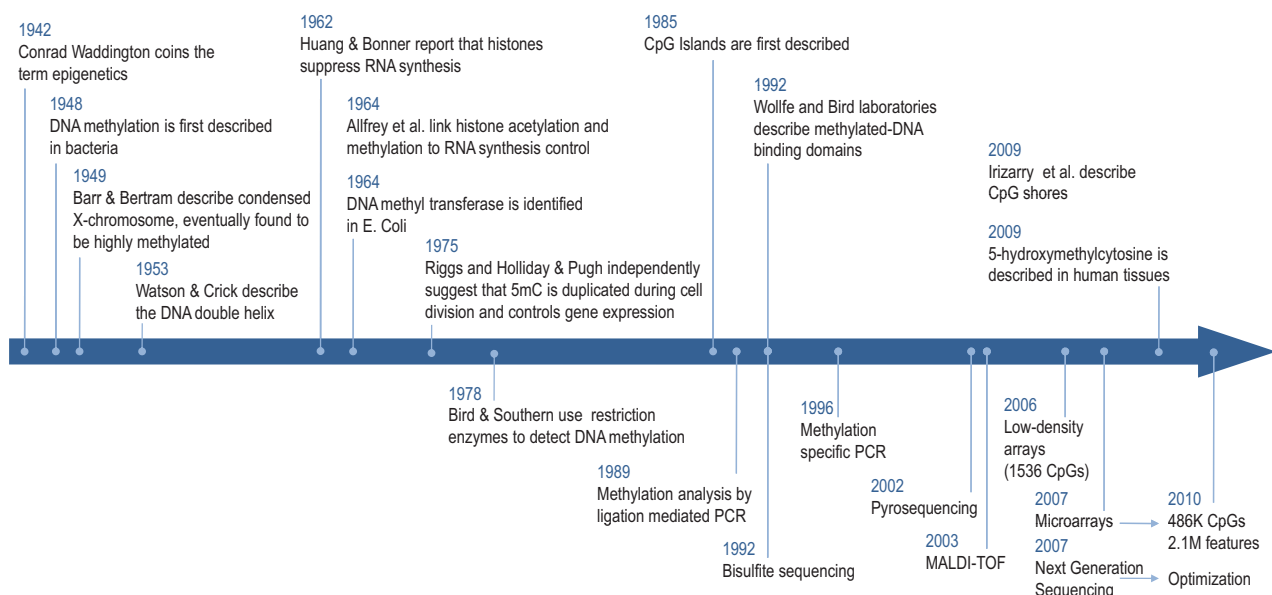


Figure 2. Timeline of milestones in epigenetics.

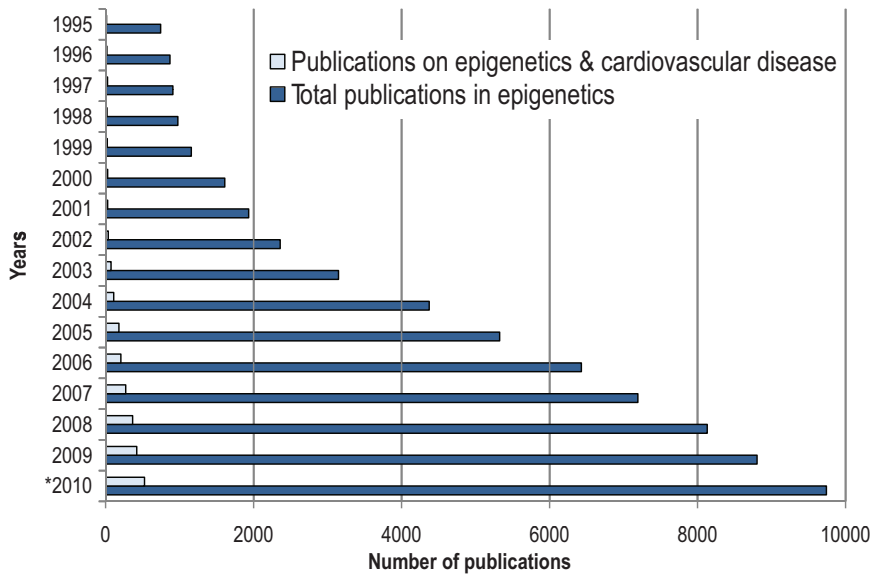


Figure 3. Epigenetic publications: total number and number related to cardiovascular disease from a PubMed Search performed on October 30, 2010, using the search terms (*epigenetics OR DNA methylation OR histone modifications*) for epigenetics; and (*cardiovascular OR cardiovascular disease*) for cardiovascular disease.

*Data from 2010 were projected by multiplying the 2009 publication numbers by the 2007 to 2009 yearly relative increment. As of October 30, 2010, PubMed listed 6586 publications on epigenetics and 280 on epigenetics and cardiovascular disease.

Animal Models Link DNA Methylation to Cardiovascular Disease

Experimental animal models have demonstrated that DNA methylation plays a critical role in the development of atherosclerosis and cardiovascular disease. Mice deficient in genes coding for methylation enzymes, such as DNA methyltransferases (*DNMTs*) (that establish or replicate DNA methylation) or *MTHFR* (related to methyl donor generation), show hypomethylation of their DNA. Increased expression of inflammatory mediators is found in leukocytes from *DNMT*-deficient mice.³ In *MTHFR*-deficient mice, DNA hypomethylation has been shown to precede the formation of aortic fatty streaks.⁴ Additionally, atherosclerosis-prone *apoE*^{-/-} mice develop specific changes in DNA methylation of transcribed gene sequences as well as in interdispersed elements both in peripheral blood leukocytes and in the aorta before developing vascular lesions.⁵ In *apoE*^{-/-} mice, changes in DNA methylation in peripheral blood leukocytes have been shown to contribute to the dysregulation of inflammation and promotion of atherosclerosis.⁵

Animal experiments also show how environmental influences during critical periods of pregnancy and early development may cause epigenetic modifications in the fetus, resulting in permanent offspring health effects. In Agouti viable yellow (*A^{vy}*) mice, the *A^{vy}* allele results from insertion of a retrotransposon (metastable epiallele) into the 5' end of the wild-type *A* allele. Unlike *A/A* wild type (brown) and *a/a* (black), *A^{vy}/a* mice have variable coat color, ranging from yellow to mottled to brown. Providing female mice around the time of conception with dietary supplements, such as folic acid, vitamin B₁₂, choline, and betaine, increases the availability of methyl groups and has been shown to cause higher CpG methylation at the *A^{vy}* locus in the offspring.⁴ *A^{vy}* methylation results in a phenotype characterized by brown coat color, reduced susceptibility to obesity, insulin resistance, cancers, and lengthened life span.

Atherosclerotic Lesions in Epigenetic Studies on Animal Models and Humans

Hypomethylation is characteristic of areas of smooth muscle cell proliferation that has been found in advanced atheroscle-

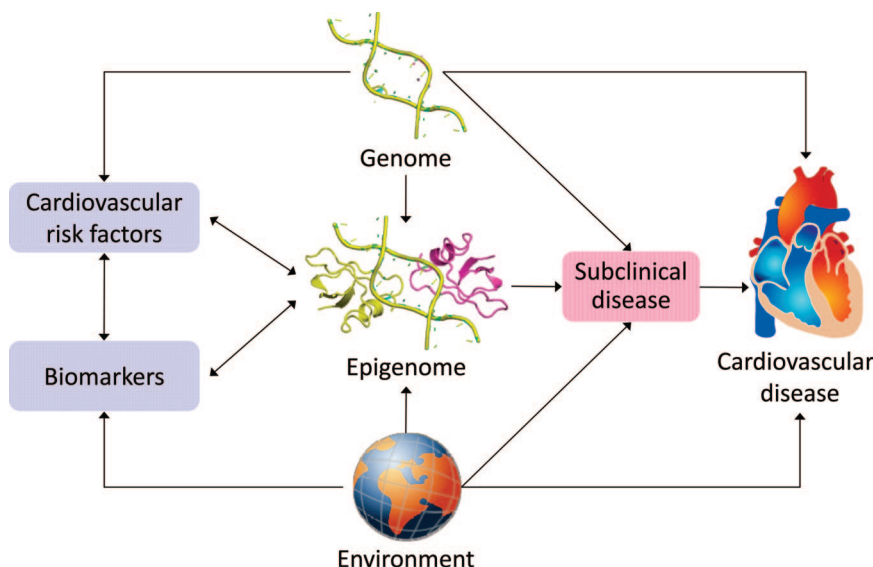


Figure 4. A conceptual model linking epigenomics to cardiovascular disease and cardiovascular risk factors.

rotic plaques in human pathology specimens as well as in atherosclerosis of apoE knockout mice and in neointimal thickenings of New Zealand White rabbit aortas. *ApoE* knockout mouse aortas exhibit a decrease in DNA methylation that can be detected as early as age 4 weeks, thus anticipating any histological changes associated with atherosclerosis.⁶ In human atherosclerotic tissues, the estrogen receptor α and β promoters show increased methylation. Estrogen receptor promoter methylation has been well demonstrated to increase with age even in normal tissues and reach near complete methylation level in the elderly.

Animal models have linked alterations in histone modifications with the development of atherosclerosis and cardiovascular disease. Trichostatin A, a specific histone deacetylase inhibitor, accelerates macrophage infiltration and development of fatty streak lesions in *LDLR*^{-/-} mice.⁶ In mouse models of type 2 diabetes, smooth muscle cells show increased expression of proatherogenic inflammatory genes. Turunen et al⁶ suggested that aberrant changes in histone modifications may represent a primary substrate for metabolic memory and proinflammatory phenotypes in smooth muscle cells in diabetic vascular disease.

Epigenetics and Cardiovascular Risk Factors

The epigenome, unlike the genome, undergoes dynamic changes throughout the life course that can contribute to and maintain adaptive and deviant gene-expression states. DNA methylation is established during embryonic stages (de novo DNA methylation) by DNMT3a and DNMT3b. In somatic cells, DNMT1 is responsible for mitotic replication (maintenance) of DNA methylation during mitosis. In mammalian cells, the fidelity of maintenance of methylation is 97% to 99.9% per mitosis. In addition, de novo methylation occurs in adult somatic cells in as much as 3% to 5% of mitoses, thus generating additional epigenetic changes.

Age, sex, and cardiovascular risk factors have been associated with specific patterns of DNA methylation and histone modifications. Loss of genomic DNA methylation has been found cross-sectionally in a variety of common age-related diseases. In work with the Normative Aging Study of men who receive care in Veterans Administration hospitals, Bollati et al⁷ showed a longitudinal decline in the average blood genomic DNA methylation of repetitive sequences, such as Alu and LINE-1, over 8 years of follow-up. Genome-wide profiling of DNA methylation in blood DNA samples taken 11 to 16 years apart in recent studies from 2 cohorts from Iceland and Utah demonstrated both losses and gains in methylation over time, depending on the loci.⁸ The dynamic changes in DNA methylation appear to be influenced by additional factors related to cardiovascular risk. Three independent studies consistently demonstrated that exposure to air pollution, an established risk factor for ischemic heart disease and stroke, is associated with reduced blood methylation of LINE-1. (For a review of epigenetic effects of environmental factors, see Baccarelli and Bollati.⁹) Using a candidate gene approach, hyper- and hypomethylation of specific genes was shown to be related to air pollutant exposures, including increased *CDKN2B* methylation and decreased *NOS2* and *MAGEA1* methylation.⁹ Recently, Breton et al¹⁰ showed that

second-hand smoke induced lower Alu and LINE-1 DNA methylation in child buccal cell DNA as well as changes in methylation of specific genes identified through methylation profiling. In peripheral blood leukocytes of patients with hypertension, recent studies have shown a loss of global genomic methylation content¹¹ as well as hypermethylation of the *HSD11B2* gene, linking epigenetics to blood pressure control.¹²

Several genes that are critical to glucose and lipid metabolism have been shown to be under epigenetic control, as reviewed by Ling and Groop.¹³ Participants in the Dutch Hunger Winter Families Study who were exposed in utero to the 1944 to 1945 famine, a condition that has been associated with overweight, impaired glucose homeostasis, and increased cardiovascular risk in adulthood, exhibited hypomethylation of the imprinted *IGF2* and *INSIGF* genes and hypermethylation of the *GNASAS*, *MEG3*, *IL10*, *ABCA1*, and *LEP* genes compared to unexposed siblings.¹⁴

Epigenetics and Cardiovascular Biomarkers

Because of its dynamic nature, the epigenome may show signatures associated with cardiovascular risk biomarkers. Additionally, the individual epigenomic background may determine the levels of these biomarkers or their responses to acquired risk factors. In particular, DNA methylation has been linked to several cardiovascular-related biomarkers, including homocysteine¹⁵ and C-reactive protein.¹⁶ DNA methylation is emerging as a primary regulator of inflammation. Methylation has been shown to control leukocyte functions related to cardiovascular risk, including the expression of soluble mediators and surface molecules that direct margination, adhesion, and migration of blood leukocytes in vascular tissues. Recent work in the Normative Aging Study showed that mean serum vascular cell adhesion molecule-1 was associated with decreasing blood methylation levels measured in LINE-1 repetitive elements, adjusting for potential confounders.¹⁷

Epigenetics and Subclinical Cardiovascular Disease

Cardiovascular disease often develops after a prolonged asymptomatic phase. Identifying epigenomic biomarkers that parallel the development of subclinical disease might open new paths for risk stratification and prevention. Based on results from animal models showing atherosclerosis-related DNA methylation alterations in peripheral blood leukocytes, DNA methylation has been suggested to reflect hyperproliferation and altered functions of cell types involved in immune or inflammatory responses during atherosclerosis.¹⁸ Peripheral blood leukocytes can be easily obtained in the community and have high potentials for the development of novel epigenomic biomarkers because of their roles in inflammation, atherosclerosis, and cardiovascular disease etiology. A study of 93 patients with chronic kidney disease failed to reveal significant associations between global DNA methylation content in peripheral blood DNA and intima media thickness, taken as a measure of subclinical atherosclerosis.¹⁹ Larger studies that include other measures of subclinical disease are needed.

Epigenetics and Clinical Cardiovascular Disease

Conversely, recent data associated DNA methylation profiles, as measured in peripheral blood leukocytes, with clinical cardiovascular disease. Castro et al²⁰ found lower DNA methylation content in peripheral blood leukocytes from patients with atherosclerotic cardiovascular disease. Recent findings from the Normative Aging Study showed that lower LINE-1 methylation in peripheral blood leukocytes is a predictor of incidence and mortality from ischemic heart disease and stroke.²¹ Elevated, not decreased, Alu methylation in peripheral blood leukocytes recently was related to prevalence of cardiovascular disease and obesity in Chinese individuals.²² Global methylation measures provide average estimates of methylation across the entire genome or in wide portions of the DNA, such as repetitive elements, and as such, do not have the resolution necessary to pinpoint individual genes or sequences responsible for cardiovascular disease risks. Decreased LINE-1 and Alu methylation may be accompanied by reactivation of different sets of silenced genes, which may be responsible for the opposite associations with cardiovascular risk.

Future Directions

DNA methylation and histone modifications represent attractive disease mechanisms because in principle, they might help to explain how environmental and lifestyle factors can impose aberrant gene expression patterns in an individual's lifetime that can result in increased cardiovascular risk. At the current stage, several questions are open in cardiovascular epigenomics.

How Many Epigenomes?

Previous and ongoing human studies often have relied on easily obtainable biospecimens, such as peripheral blood leukocytes. Because of the established roles of inflammation and leukocytes in atherosclerosis and cardiovascular disease, peripheral blood leukocytes represent a biologically relevant cell type for cardiovascular studies, which has unparalleled potential for development of biomarkers for clinical use. Animal experiments and, if possible, human studies will need to describe epigenomic signatures in multiple tissues involved in the etiology of cardiovascular disease, including but not limited to endothelial, smooth muscle, ventricular and atrial, and adipose tissues. Epigenomic markers show both tissue specificity and correlations across different tissues, depending on the loci. The extent, if any, to which easily obtainable tissues, such as peripheral blood leukocytes, reflect epigenomic signatures in cardiovascular tissues needs to be established in future research.

Going Genome-Wide: How To Replicate Epigenomic Findings?

Current technologies for DNA methylation analysis and histone modifications allow for the conduction of genome-scale studies that will produce high-dimensional data. We expect that stand-alone studies, even in large cohorts, will fall short of providing conclusive evidence on epigenomic signatures associated with cardiovascular disease. Collaborations across studies will be necessary for independent replication of findings.

How To Analyze Epigenomic Data?

Because adjacent epigenomic marks often are highly correlated, a pressing question for data analysis is whether epigenetic profiles can be analyzed in blocks and whether informative "tag epigenetic marks" can be identified and used in epigenomic investigations. Related questions include determining to what extent epigenomic patterns vary by age, sex, and race/ethnicity and how rapidly and how much they are influenced by environmental factors. Because epigenomic variations associated with ethnicity already have been described, the possibility that population stratification might produce bias at specific loci should be thoroughly considered.

How Do All the Omics Fit Together?

Because genomics, epigenomics, and transcriptomics are functionally interrelated, the understanding of epigenomic mechanisms cannot be complete without evaluating their relations with genomic data of genome-wide association studies and their relations with transcriptional profiles in human cardiovascular disease. Integrated omics analysis will help to clarify the mechanisms to determine familial clustering of epigenomic patterns (ie, whether epigenomic marks are heritable or regenerated at each generation on inherited genomic templates). Integrated analyses of epigenomics and transcriptomic data are necessary to identify the epigenetic marks that are functional in regulating gene expression.

Can We Keep Our Epigenome Healthy? How Can We Get It Back in Shape?

The growing evidence that cardiovascular risk factors and biomarkers are associated with epigenomic signatures in multiple tissues opens up several questions. In the first place, several studies just reported associations of epigenetic profiles with cardiovascular risk factors, biomarkers, or disease. Whether the observed epigenomic signatures are epiphenomena or part of the causal pathways leading to cardiovascular disease is still largely to be determined. If causally related to cardiovascular disease, primordial prevention would be expected to avoid or limit the development of these epigenomic signatures. Additionally, future work will need to address whether the signatures associated with cardiovascular risk factors or biomarkers can be reverted by removing or reducing the individual risk factor burden. A growing sector of pharmacological research has focused on the development of drugs that can modify the epigenome. For instance, animal experiments have identified histone deacetylase inhibitors that could be developed further to treat several cardiovascular conditions, including atrial fibrillation, cardiac hypertrophy and heart failure, and stroke outcomes. A major issue in cardiovascular epigenomics is how rapidly and how effectively these epigenomic drugs can be translated to humans and introduced in standard clinical practice.

Conclusion

This review is only a brief and necessarily partial introduction to cardiovascular epigenomics that we hope will help to motivate future research. A list of selected online resources that may help with retrieving additional information and facilitate further work in epigenetics is presented in the Table.

Table. Selected Online Resources for Epigenetics

Resource	Description	URL
Consortia and initiatives		
The NIH Roadmap Epigenomics Program	An NIH initiative to foster epigenomic research, develop comprehensive reference epigenome maps, and generate new technologies for comprehensive epigenomic analyses	http://nihroadmap.nih.gov/epigenomics/
The Epigenome Network of Excellence	A European Union-funded network of institutions and research groups	http://www.epigenome-noe.net/WWW/index.php
The Human Epigenome Projects	A public/private collaboration to catalog methylation-variable positions in the human genome	http://www.epigenome.org/
NAME21	A German national initiative to analyze DNA methylation patterns of genes on chromosome 21	http://biochem.jacobs-university.de/name21/
Databases		
The Human Epigenome Atlas	An atlas of human reference epigenomes and the results of their integrative and comparative analyses	http://www.genboree.org/epigenomeatlas/index.rhtml
MethDB	A searchable database for DNA methylation and environmental epigenetic effects	http://www.methdb.de/
Human Histone Modification Database (HHMD)	A searchable database of information from experimental data to facilitate understanding of histone modifications at a systematic level, with the current release incorporating 43 location-specific histone modifications in human	http://bioinfo.hrbmu.edu.cn/hhmd
NCBI Epigenomics	An online repository of epigenetic data sets	http://www.ncbi.nlm.nih.gov/epigenomics/browse
GeneImprint	A catalog of imprinted genes	http://www.geneimprint.com/site/genes-by-species
Catalogue of Parent of Origin Effects	Searchable database of imprinted genes and related effects	http://igc.otago.ac.nz/home.html
Tools and other resources		
MethPrimer	Primer design for methylation polymerase chain reaction	http://www.urogene.org/methprimer/index1.html
MethBlast	A sequence similarity program that checks primers for bisulfite-converted DNA by blasting them against unmethylated and methylated genomic sequences of man, mouse, and rat	http://medgen.ugent.be/methBLAST/
Methylator	A tool that attempts to predict whether CpGs in a DNA sequence are likely to be methylated	http://bio.dfci.harvard.edu/Methylator/
RMAP	A tool to map reads from the next-generation sequencing technology that supports bisulfite-treated reads mapping	http://rulai.cshl.edu/rmap/
Chromatin Structure and Function	Information on chromatin biology, histones, and epigenetics	http://www.chromatin.us/chrom.html
Epigenetic Station	A source for information, protocols, methods, techniques, products, vendors, kits, assays, analysis, bioinformatics, and databases on epigenetics	http://epigeneticstation.com/

NAME21, indicates National Methylation Project: Analysis of DNA Methylation Patterns of Genes on Chromosome 21; NCBI, National Center for Biotechnology Information; NIH, National Institutes of Health.

We look forward to future epigenetic investigations that will produce fundamental knowledge about the pathophysiology of cardiovascular disease and lead to improved prevention, risk stratification, and disease management.

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Disclosures

None.

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