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Epigenomes: the missing heritability in human cardiovascular disease?

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Abstract

Cardiovascular disease is a tremendous burden on human health and results from malfunction of various networks of biological molecules in the context of environmental stress. Despite strong evidence of heritability, many common forms of heart disease (heart failure in particular) have not yielded to genome-wide association studies to identify causative mutations acting via the disruption of individual molecules. Increasing evidence suggests, however, that genetic variation in non-coding regions is strongly linked to disease susceptibility. We hypothesize that epigenomic variation may engender different chromatin environments in the absence of (or in parallel with) changes in protein or mRNA sequence and abundance. In this manner, distinct—genetically encoded—chromatin environments can exhibit distinct responses to environmental stresses that cause heart failure, explaining a significant portion of the altered susceptibility that is observed in human disease.

Keywords

chromatin; genomics; heart failure; human studies; systems biology

Introduction

One of the most important problems in biology is how the genome is read in different cells. Somatic mutations aside, the sequence of the genome is invariant between cells in eukaryotes. Of course every other tier of biological information varies greatly—from the transcriptome, to the proteome and metabolome and obviously the phenotype—between cell type and amongst copies of the same cell. The developmental basis for this variation has been studied extensively, with detailed transcriptional pathways elucidated to underlie the various mammalian lineages. Indeed it was in this context that the hypothesis of an epigenetic landscape was put forth by Waddington, wherein gene interactions determine unidirectional cell fate progression [1]. A contemporary definition of epigenetics is more appropriately *heritable (through mitosis and, in rare cases, meiosis) changes that do not involve alteration of the DNA sequence*, although colloquial usage of the term has come to refer to *anything that involves the nucleus and gene expression*. For this paper, we consider

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epigenetics to refer to persistent changes in chromatin features that establish and/or maintain cellular phenotype.

Cardiovascular disease is the leading cause of death worldwide. The major forms of cardiovascular disease, including myocardial infarction, hypertension, atherosclerosis, heart failure and arrhythmias have all been shown to have a significant heritable component, yet genome-wide association studies (GWAS) have had only mixed success in identifying causative loci in genes [2–4]. One interpretation of this observation is that rather than a few rare variants having a large effect, the genetic component of common diseases like cardiovascular disease arises from multiple common sequence variants acting alone or in a combinatorial manner. Heart failure is the end result of many forms of cardiovascular disease and affects 5–6 million Americans [5]. Discerning the cellular mechanisms underlying the onset and progression of polygenic diseases like heart failure, and moreover how to diagnose and treat them, is a major challenge in clinical medicine. The purpose of this article is to discuss the increasingly recognized role of the epigenome as a point of integration between genetics and environment in the context of complex, common diseases. While the existence of epigenetic mechanisms has been known for decades, we are recently becoming aware of the central role that chromatin structure plays in shaping cellular phenotype and disease response.

Heritability of Cardiovascular Disease: Role of Common Genetic Variation

Cardiovascular disease accounts for 31.9% of deaths in the United States and is the leading cause of death in this country and worldwide [5]. Cardiovascular disease involves a spectrum of interrelated and coincident conditions affecting the heart and vasculature, including hypertension (high blood pressure), atherosclerosis (lipid deposition and inflammation of the vasculature), myocardial infarction (death of cardiac myocytes), arrhythmias (abnormal cardiac conduction) and heart failure (inability of the heart to supply the oxygen demands of the body). Metabolic syndrome and type II diabetes, which are prevalent in developed societies, are strongly linked with increased risk for cardiovascular diseases [6]. Current clinical management of heart failure involves fluid balance modulation, blood pressure control and regulation of cardiac inotropy, which have powerful effects to mitigate the symptoms of the disease but fail to prevent its progression and have been relatively unchanged for decades. Regenerative therapies remain on the horizon but the advent of low cost genomic sequencing makes personalized heart failure therapy a near term possibility. However, its success is dependent on a greater understanding of the cellular networks through which genetic variation impacts disease.

One of the best-characterized genetic causes of heart disease is hypertrophic cardiomyopathy, caused by mutations in a class of genes mostly encoding sarcomeric proteins. As the name implies, this disease involves increased cardiac myocyte size, increased muscle mass without chamber dilation and a predisposition to arrhythmias and sudden death. Although the effects on the individual patient are devastating, the incidence of hypertrophic cardiomyopathy due to these mutations is estimated at 1 in 500 [7], compared to the approximately 5–6 million Americans known to suffer from heart failure (about 1 in 55). In most cases described clinically, the mutations causing hypertrophic cardiomyopathy

occur in the coding regions of sarcomeric genes, disrupting the expression level and/or function of the protein [8]. This is one example where genetic screening is playing a key role in clinical treatment: families known to harbor hypertrophic cardiomyopathy mutations are frequently screened to identify at risk individuals [9]. The study of heart rhythm disorders (e.g. long QT syndrome) has also revealed these diseases to have strong genetic components, often due to mutations in cardiac ion channels [10, 11].

GWAS for coronary artery disease, a frequent precursor to heart failure, have yielded insights into the genetic contributors to this disease [12–15]. A more recent study in dilated cardiomyopathy—a condition not associated with ischemia or obvious vascular lesions—revealed two loci, near the genes *HSPB7* and *BAG3* (both of which encode heat shock/stress response proteins), associated with increased incidence of the disease [16]. Interestingly, when variants in the *BAG3* sequence were further explored it was found that nonsynonymous mutations, which were rare, were linked with monogenic familial forms of the disease (that is, the mutation in *BAG3* caused the disease). Meanwhile, more commonly occurring variation was also observed in the *BAG3* sequence, with these SNPs associated with sporadic (i.e. non-familial) incidence of dilated cardiomyopathy [16]. These findings demonstrate both the heritable and somatic genetic insults that cause disease can do so by converging on the same genomic regions. Dilated cardiomyopathy, like hypertrophic cardiomyopathy, has also been linked to a series of mutations in a small subset of genes, screening of which has made an impact clinically [17]. In a 2013 report, GWAS was used to identify a novel risk locus for dilated cardiomyopathy after which the investigators used expression quantitative trait loci (eQTL) analyses, which investigates how variation in a region of the genome affects the transcriptome, to demonstrate that the locus controls inflammatory gene expression [18].

For common forms of heart failure—as distinguished from familial forms and accounting for the major portion of the disease burden [5]—there is still strong evidence of heritability, although the mechanisms are unclear. In a Framingham cohort of ~1500 patients, longitudinal analyses revealed that having a parent with heart failure increased the risk of the disease to an incidence of 2.72% (versus 1.62% in patients without a parent with heart failure), an ~70% increase in risk [2]. The original study implicating *HSPB7* came from subgenomic analyses of heart failure patients (in a case-control format), showing that SNPs in this gene (as well as in the gene *FRMD4B*) indicate increased risk for heart failure. The association was not observed in an African American sub-cohort of patients [19], but was found to be equally associated with ischemic and non-ischemic disease, suggesting *HSPB7* is a bona fide risk indicator for heart failure, as opposed to acting only as a marker for one of its clinical precursors like coronary artery disease [20]. Subsequent discovery of *HSPB7* as a risk allele for dilated cardiomyopathy [16] also supports this interpretation.

Despite this progress, known genetic variation explains only a small portion of the observed heritability of heart failure in humans. There are several possible explanations for this (aside from the real possibility that the heritability of the disease is inflated in the first place [21]) including incomplete sampling of individual populations, differences in racial lineages and localization of casual variants near other variants with larger effects. The heritability can also come from variation other than SNPs, including copy number variation (although this

was shown not to be the case for early onset myocardial infarction [12]) and chromosomal translocations. An alternative explanation is that the genetic risk reveals itself only through a combination of SNPs, which is in agreement with the common interpretation of GWAS findings in cardiovascular disease as indicating many common SNPs each with small contribution to the phenotype. Studies from the non-cardiovascular arena have shown the utility of such multi-SNP approaches, including for body mass index [22] and type II diabetes [23]. But what are the cellular mechanisms through which these multiple SNPs, many of which reside in non-protein coding regions [24], interact to cause complex common disease? Recent evidence suggests that chromatin may be a regulatory hub through which common genetic variation controls phenotype.

Chromatin Regulation in the Heart

In considering how the epigenome may serve to integrate genetic variation and environmental stimuli, it can be instructive to reflect on what is known about the structural hierarchy of chromatin. DNA alone [25] or in complex with the nucleosome heteromultimer [26] is structurally resolved with atomic resolution. Also characterized *in vitro* are the fibers of DNA-protein that form when nucleosomes are reconstituted on DNA in a cell free system, which range in scale from 30 to 100s of nanometers and are affected by non-nucleosomal chromatin structural proteins [27, 28]. Beyond this scale, however, our understanding of structure-function relationships within eukaryotic genomes, outside of the context of mitosis, is lacking. This issue has been discussed in other recent papers [29, 30] and is not the focus of this work. It is critical to consider, however, the manner in which chromatin structural changes can affect phenotype and how these changes may come about due to variation in non-coding regions of the genome and/or epigenomic features that integrate genetics and environment.

It is increasingly recognized, that many SNPs associated with cardiovascular and other common diseases lie outside of coding regions, suggesting these variants are acting in trans to regulate gene expression. The commonly assumed mechanism is one in which the SNP falls in a distal enhancer, disrupting its ability to bind a protein responsible for transcriptional regulation and thereby exerting a biological effect. A second, and to our knowledge less explored mechanism involves the disruption of local chromatin structure, presumably by changing a consensus motif that alters the binding of chromatin structural proteins and/or lncRNAs, the function of which have been shown to exert chromatin regulatory effects in the heart [31, 32] and other organs [33] (Figure 1).

Many of these mechanisms have been understood for decades—what has changed is the ability to (a) interrogate them in a global manner with locus specificity based on advances in nucleotide sequencing [34]; (b) reveal the extent of the protein [35] and modification [36] landscape based on advances in mass spectrometry-based proteomics; and (c) to reveal combinatorial behavior that confers biological function (that is, groups of proteins/histone PTMs that when localized together affect DNA in a predictable manner [37]). The heart presents several interesting biological challenges to our understanding of how chromatin dynamics regulate phenotype. Genetic engineering in mice has revealed a role for multiple chromatin-regulating enzymes in cardiac and vascular development [38] and developmental

mechanisms may hold insights into novel approaches for cardiac regeneration [39]. How chromatin is regulated during mammalian heart development has been recently explored with next generation sequencing approaches combined with immunoprecipitation of well characterized chromatin marks (ChIP-seq) to reveal the genome-wide patterns of epigenetic modification during lineage commitment in mice [40] and humans [41]. These studies reveal that combinations of chromatin marks distinguish different regions of the genome throughout differentiation, predisposing genes to appropriate activation at the correct developmental stage. These combinations of chromatin marks also discriminate between genes that are drivers of cardiac differentiation and known to participate in disease. Chromatin states—that is, combinations of histone post-translational modifications at a given locus that correspond to a transcriptional behavior—are predictive of gene expression and are dynamically regulated developmentally. For example, proteins with powerful effects on gene expression (like transcription factors) have a unique chromatin signature contrasted with other genes, in which the transcription factors and signaling molecules controlling cardiac development exhibit high H3K27me3 signal in the pluripotent state (which is lost with commitment to the cardiac lineage, commensurate with acquisition of H3K4me3 and H3K36me3). In contrast, genes for structural proteins, which are equally cell type specific but which lack the ability to robustly alter phenotype that is possessed by transcription factors, acquire the activating marks with differentiation but lack a strong silencing mark in the primitive state [41]. Intriguingly, very recent studies explored chromatin modifications in the setting of cardiac hypertrophy [42] using an *in vivo* model of pressure overload, revealing that, similar to development, patterns of histone post-translational modifications appear to specify distinct gene expression regimes. These approaches have been powerful for the identification of new master regulators of differentiation and have revealed the general principles for how different chromatin features affect transcriptional outcomes.

Despite these important advances, much is still unknown about how chromatin is coordinated on a genome-wide scale in the adult myocyte. How genetic variability between individuals alters the genomic structure/function *in vivo* to alter the transcriptome is unknown in the cardiovascular system, as is at what scale do changes in chromatin structure matter for function (SNPs—histone modifications—chromatin binding proteins—higher order structure). In the realm of phenotypic plasticity, two recent observations suggest potentially therapeutic roles for chromatin remodeling in cardiac rejuvenation: fibroblasts can be directly reprogrammed to cardiac myocytes *in vivo* [43, 44], and the efficiency of reprogramming of fibroblasts to pluripotency is hindered by extensive deposition of silencing marks on chromatin [45]. Could strategies to selectively loosen regions of chromatin necessary for cardiac gene expression complement the current transcription factor-based reprogramming strategies, enhancing efficacy and repair?

Epigenomic Regulation of Adult Phenotypes: Heritability of Diseased Proteomes

It has recently become apparent that proteome diversity is a heritable trait, independent of transcript diversity. A study in humans using a cohort of individuals from the HapMap project demonstrated that proteome variation could be used to define protein quantitative

trait loci, or regions of genetic variation that explain proteome level differences between individuals [46]. This level of variation was not captured in the transcriptome, illustrating that these tiers of information vary independently. Previous studies in model organisms had demonstrated this same phenomenon. A study precisely monitoring the abundance of yeast ribosomal genes and proteins demonstrated that genetic variation of proteins was distinct from that of their cognate transcripts [47]. A study in mice also observed low correlation between the loci controlling transcript or protein levels in the liver [48]. Together, these studies support the now widespread observation of a small correlation between abundances of mRNAs and their respective proteins—modern methods of measuring protein abundance have put to rest the concerns that this lack of correlation between mRNA and protein arises from poor quantitation at the level of protein measurements. Transcript networks are related to protein networks through non-linear relationships and both of these networks can be independently controlled by genetics. An argument for the importance of these findings is that they prompt a need to explain the mechanisms for how these networks interact to transfer the genetic information residing in the genome...bringing the discussion back to chromatin.

The Encyclopedia of DNA Elements (ENCODE) Consortium systematically examined the human epigenome for histone post-translational modifications, measures of DNA accessibility and nucleosome occupancy, transcription factor association and production of RNA. The most recent reports from this study have been very high profile and purported to upend long held dogma regarding the portion of the genome that is functional. ENCODE investigators reported that the vast majority, ~80% according to some estimates, of the genome is 'functional', with large stretches of DNA once thought to be silent now shown to be transcribed at some level [49]. The implications of ENCODE findings have been discussed in detail in other venues and have not escaped vociferous criticism [50]—what these studies serve to highlight is a general appreciation for the importance of chromatin for interpreting genomic function (there are now countless papers demonstrating differences in chromatin marks as predictive of development and disease, as discussed above). Perhaps the best way to determine a causal role for chromatin features is in the context of inheritance. For example, GWAS studies from multiple organs and disease states have shown that 88% of trait associated SNPs reside in introns (45%) or intergenic DNA (43%), that is, outside of protein-coding genes [51]. Many of these disease-associated SNPs live in DNase I hypersensitive sites [24], supporting the concept that their effects may involve changing chromatin structure. Regardless of what these non-coding regions are doing (whether one chooses to call them 'junk DNA' or 'functional elements', for example, is inconsequential), the fact that genetic variation outside of a gene affects the expression of genes and proteins implicates the 3D structure of the genome in the mechanism of action, be it alteration in enhancer function, changes in nucleosome occupancy or disruption of other types of RNAs, like long noncoding RNAs, that may impact gene expression by modulating chromatin structure/function.

So far, we have discussed how SNPs in non-coding regions can mediate heritability to common diseases by regulating genomic structure to affect transcription. Because the majority of SNPs associated with these diseases are in fact in non-coding regions, this offers an important, and under-explored area of research into genetic predisposition. However,

there is also evidence for chromatin to regulate disease susceptibility in a heritable fashion without resulting from genetic mutation. There is a school of thought [52, 53] that various environmental factors can impinge on chromatin in a manner that is heritable, through modulation of DNA methylation or even protein-based chromatin marks such as histone modifications, with recent examples including the role of paternal diet in the offspring's metabolic make-up [54, 55]. But whether epigenetic changes are heritable independent of genetic change can be very hard to prove. As has been described adroitly elsewhere, epigenetic changes like histone modifications and DNA methylation can influence DNA sequence: for example by affecting mismatch repair, which can happen directly in the case of DNA methylation, or by altering the accessibility of DNA repair genes through nucleosome features, thereby affecting DNA sequence in trans. These epigenetic changes throughout life may be selectable, regardless of whether they affect DNA sequence directly, so long as they change the phenotype [56]: there can be a direct causal link between environmental stress, epigenetic change, phenotypic change and natural selection that need not involve changes in DNA sequence. In this manner, chromatin can integrate environmental stress independent from the genome, thereby serving as a substrate for affecting natural selection. To be clear, in this model heritability is still through a conventional, Darwinian genetic mechanism, even if selection (also Darwinian) occurs independent of genetic variation.

A recent multi-center study of genetic cardiomyopathies resulting from de novo mutations made the interesting observation that a preponderance of these disease-inducing new variants occurs in histone modifying enzymes[57]. While most of the mutations affect the gene product—and therefore the pathogenic mechanisms presumably result from altered regulation of genes regulated by histone modifications deposited by these enzymes—these human genetic studies provide additional evidence for chromatin as a point of convergence for cardiac disease mechanisms.

Epigenomics for Breakthroughs in Cardiovascular Medicine?

In closing, we reflect on how advances in our understanding of multi-genic diseases and chromatin biology together may influence the study and treatment of cardiovascular disease (Table 1). As we have discussed, much of the heritable component of cardiovascular disease has not been linked to single gene mutations through GWAS or other studies. How, then, do these multi-genic diseases 'work' at the molecular level? While recent evidence has implicated global chromatin remodeling in the response to heart disease [42], we suggest that transformative advances in the diagnosis and treatment of cardiovascular disease will come from systematic analysis of epigenomic features in the context of heritability. That is, how does genetic variation lead to altered chromatin structure, which in turn influences disease susceptibility?

Although many studies in cardiovascular medicine perform multivariable adjustments to correct for comorbid conditions (e.g. the Framingham Offspring study found an increased risk of heart failure in offspring of those with the disease even after correcting for age, sex, height, weight, systolic blood pressure, valve disease, diabetes, prior myocardial infarction and treatment for hypertension [2]), most of these studies do not account for other

environmental factors that are often shared within families, including dietary habits, use of recreational drugs, exposure to air pollution, and physical activity as examples. Future studies must take environmental factors into account when studying human populations, including how these environmental factors can impact chromatin to change cellular phenotype. Furthermore, this limitation highlights the importance of model organisms, wherein strict environmental control can be implemented in parallel with the study of genetic effects. A terrific example of this principle is seen with the hybrid mouse diversity panel: ~100 strains of densely genotyped mice allowing strong genetic differences in phenotype to be explored using modern 'omics techniques [58]. The HMDP has proven to be a powerful model for revealing the genetic basis of common human traits including bone density [59], blood cell parameters [60], inflammation [61] and obesity [62]. Because all the animals in a given strain are genetically identical and all mice in the experiments are exposed to the same environmental conditions, the underlying genetic contribution to complex disease, including the manner in which this manifests through gene, transcript, protein and metabolite networks, can be measured—ruling out a 'reverse causality' situation in which an environmental factor that selects for a given phenotype is incorrectly interpreted as a genetic predisposition.

It is likely that networks of genes, transcripts and proteins are all independently under genetic control and all independently contribute to disease susceptibility and progression. What remains unclear is at what level of resolution we need to understand these networks to target them in disease (Figure 2). Chromatin is a perfect example of the intersection between networks of different biological molecules. Determining at what scale of genomic structure chromatin modifications influence pathological transcription will be critical and will involve studies into how altered modification of individual bases (DNA methylation) or chromatin structural proteins (such as histone post-translational modifications) can affect global gene expression and phenotype. How are these marks integrated and coordinated across the genome? What gives rise to myocyte specific chromatin structure and how is this maintained in the terminally differentiated myocyte? Further complicating analysis of cardiovascular disease is that it is a multi-systems disease, in which heritability can come from multiple epigenomes. The same SNP may confer a positive benefit in the context of one organ's epigenome while at the same time being deleterious to cellular function in another organ's epigenome.

A solution moving forward is large human cohort studies that link complete genomic sequencing with deep phenotyping and extensive, quantitative multi-'omics profiling. What would arise from these studies is a molecular knowledgebase for human cardiovascular health. To accomplish this goal, one would collect multiple non-biased datasets from large human populations (on the scale of 50–100,000) with diverse ethnic background and with/without pre-existing cardiovascular disease, including genomics, proteomics, epigenomics, transcriptomics and metabolomics. This data would be matched with patient electronic medical records to reveal genetic features that control susceptibility to and progression of cardiovascular disease. As a longitudinal study, this project would follow these patients for at least the next decade but preferably for the rest of their lives. One could then investigate the relationship between molecular genetic features and cardiovascular disease traits, to reveal new diagnostic insights and therapeutic approaches. These studies would enable

development of new risk prediction algorithms that would enhance decision making for the physician caregiver and improve patient outcomes.

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Abbreviations

ChIP-seq	chromatin immunoprecipitation and next generation sequencing
ENCODE	Encyclopedia of DNA Elements
eQTL	expression quantitative trait loci
GWAS	genome wide association study
H3K27me3	trimethylation of lysine 27 on histone H3
SNP	single nucleotide polymorphism

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Figure 1. Chromatin variation and disease susceptibility

A. Two patients will manifest different phenotypes under the same stress due to differences in their epigenomic landscapes, which result in different proteomes. **B.** GWAS hits often reside in intergenic regions. **C.** The majority of validation experiments probe for simple eQTL relationships between a single SNP and a single gene. However, the relative contribution of these simple relationships to phenotype, as compared to more complex interactions between chromatin domains, is unknown (**D**). **E.** A challenge is to distinguish between chromatin packaging differences with functional consequences versus those that do not alter cellular phenotype. Shown is an example of three DAPI-stained neonatal rat cardiomyocyte nuclei all from a single isolation (note differences in relative DAPI intensity and distinct relative abundance of heterochromatin), representing variability in global chromatin structure within a range ostensibly tolerated by the cell. Scale bar is 5 μ m. Images acquired on a Nikon A1 confocal microscopy with a 63x oil immersion lens and converted to black and white in Photoshop.

Figure 2. Physiological differences in molecular networks affect patient response to therapy
Multiple molecular networks interact to control cell phenotype. In disease, two individuals can present with a similar phenotype (i.e. symptoms), such as impaired cardiac function, but the manner in which the cellular networks are altered to produce the phenotype is different. Here, node size represents molecular abundance. A drug targeting this cellular function may produce the same effect in both individuals at an individual node (in this example, increased expression/activity of the grey node) but the ability of the drug to alter clinical phenotype is dependent on the other components in the networks of the two individuals. Therefore, in only one patient does treatment correct the phenotype, due to the interaction of the drug-induced change with the rest of the network that controls inotropy. Furthermore, the abundance of a given network component can be influenced in non-intuitive ways by genetic variation that impacts chromatin structure directly and transcript/protein abundance through secondary, nonlinear relationships.

Table 1**Open Questions Regarding Epigenomics as a Regulator of Heart Disease Heritability**

- i. How do multi-genic diseases ‘work’ at the molecular level? What is the role of network level interactions and at what level of resolution do we need to understand these networks to target them therapeutically?
- ii. How does genetic variation lead to altered chromatin structure?
- iii. How are chromatin marks integrated and coordinated across the genome? Beyond observing correlations amongst marks, we must determine the logic for how they encode phenotype.
- iv. What gives rise to myocyte-specific chromatin structure and how is this maintained in the terminally differentiated myocyte?
- v. What are the contributions from non-CV epigenomes—that is, from the epigenomes in other organs (e.g. brain, adrenal gland, kidneys)—that together influence function of CV system?