Legacy of Glucose Management

As arguably the single most important breakthrough in diabetes mellitus therapy, the discovery of insulin in 1921 was instrumental in prolonging life expectancy in patients with type 1 diabetes mellitus. Indeed, before insulin, 50% of individuals were dead within 20 months of their diagnosis. Although the advent of insulin changed this, extending the lifespan of such patients uncovered new long-term clinical challenges. Cardiovascular complications emerged as the leading causes of morbidity and premature mortality among those with diabetes mellitus.

Circulation Research Compendium on Obesity, Diabetes, and Cardiovascular Diseases

Obesity, Diabetes, and Cardiovascular Diseases: A Compendium
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Epidemiology of Obesity and Diabetes and Their Cardiovascular Complications
Lipid Use and Misuse by the Heart
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Cardiac Dysfunction and Vulnerability in Obesity and Diabetes

Philipp E. Scherer and Joseph A. Hill, Editors

Epigenetic Changes in Diabetes and Cardiovascular Risk
Samuel T. Keating, Jorge Plutzky, Assam El-Osta

Abstract: Cardiovascular complications remain the leading causes of morbidity and premature mortality in patients with diabetes mellitus. Studies in humans and preclinical models demonstrate lasting gene expression changes in the vasculopathies initiated by previous exposure to high glucose concentrations and the associated overproduction of reactive oxygen species. The molecular signatures of chromatin architectures that sensitize the genome to these and other cardiometabolic risk factors of the diabetic milieu are increasingly implicated in the biological memory underlying cardiovascular complications and now widely considered as promising therapeutic targets. Atherosclerosis is a complex heterocellular disease where the contributing cell types possess distinct epigenomes shaping diverse gene expression. Although the extent that pathological chromatin changes can be manipulated in human cardiovascular disease remains to be established, the clinical applicability of epigenetic interventions will be greatly advanced by a deeper understanding of the cell type-specific roles played by writers, erasers, and readers of chromatin modifications in the diabetic vasculature. This review details a current perspective of epigenetic mechanisms of macrovascular disease in diabetes mellitus and highlights recent key descriptions of chromatinized changes associated with persistent gene expression in endothelial, smooth muscle, and circulating immune cells relevant to atherosclerosis. Furthermore, we discuss the challenges associated with pharmacological targeting of epigenetic networks to correct abnormal or deregulated gene expression as a strategy to alleviate the clinical burden of diabetic cardiovascular disease. (Circ Res. 2016;118:1706-1722. DOI: 10.1161/CIRCRESAHA.116.306819.)

Key Words: atherosclerosis ■ cardiovascular diseases ■ chromatin ■ diabetes mellitus ■ epigenomics

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As arguably the single most important breakthrough in diabetes mellitus therapy, the discovery of insulin in 1921 was instrumental in prolonging life expectancy in patients with type 1 diabetes mellitus. Indeed, before insulin, 50% of individuals were dead within 20 months of their diagnosis. Although the advent of insulin changed this, extending the lifespan of such patients uncovered new long-term clinical challenges. Cardiovascular complications emerged as the leading causes of morbidity and premature mortality among those with...
diabetes mellitus. Because early regimens of insulin therapy did not anticipate the importance of glycemic control, much debate surrounded the extent that hyperglycemia represented the primary driver of chronic vascular complications.

It was 60 years after the first clinical use of insulin that the blood glucose hypothesis was tested in the landmark Diabetes Control and Complications Trial (DCCT). This randomized, controlled clinical trial of 1441 patients with recently diagnosed type 1 diabetes mellitus compared the conventional therapy of 1 or 2 daily insulin injections to an intensive regimen of multiple daily injections combined with close blood glucose monitoring. Distinct differences in glycemic control were detected between the treatment regimens, with median HbA1c levels of 7% versus 9% observed for the patients receiving intensive and standard therapy, respectively. The unequivocal clinical benefits of intensive glycemic control prompted early DCCT termination. In light of the significant reduction of incidence and progression of microvascular complications conferred by intensive insulin treatment, in 1993, those originally assigned conventional therapy were switched to the more beneficial intensive regimen along with the rest of the DCCT cohort under the Epidemiology of Diabetic Interventions and Complications (EDIC) observational study. In addition to microvascular complications, EDIC sought to examine the incidence of macrovascular events that were not observed in the relatively short DCCT. Despite the early convergence of HbA1c levels across the 2 arms formerly assigned intensive and conventional therapies, EDIC demonstrated further divergence in vascular outcomes between the 2 groups, suggesting some fundamental impact of the earlier more intensive insulin treatment.

The benefits of early intensive insulin therapy on the initiation and progression of diabetic vascular complications remain evident as DCCT/EDIC enters its fourth decade. Comparable findings were reported for type 2 diabetes in the United Kingdom Prospective Diabetes Study, where despite equalization of HbA1c, 10-year follow-up demonstrated a persistent reduction in vascular complications in those initially assigned intensive glycemic control when compared with conventional therapy. Similarly, 10-year follow-up of Veterans Affairs Diabetes Trial revealed long-term cardiovascular benefit of more intensive glucose control despite showing no difference in cardiovascular outcome in the initial study. The consistency across these and other clinical studies is noteworthy, especially in contrast to the almost uniformly negative initial results of cardiovascular outcome studies testing different novel glucose-lowering therapies. Thus, the deleterious influence of antecedent hyperglycemia stands out as a unique and important pathogenic factor. However, what is particularly provocative about these results is their indication that molecular mechanisms exist in humans through which previous hyperglycemic exposure was remembered and impacted outcomes, completely consistent with previous pioneering reports of metabolic memory in animal models. As early as 1987, the enduring development of diabetic retinopathy was described in dogs despite 2.5 years of insulin therapy and effective glycemic control. Similarly persistent microvascular complications were reported in diabetic rodents. More recent studies of diabetic ApoE−/− mice revealed persistent aortic atherosclerotic plaque development despite normalization of blood glucose levels for at least 8 weeks. Intensified research now implicates lasting gene expression changes in the vasculopathies instigated by prior glucose exposure. Together, these findings point to the presence of biological memory linked to hyperglycemia and the broader diabetic state as driving the molecular changes and clinical outcomes seen in diabetic vascular disease. Increasing evidence advocates for epigenetics as a robust mechanism that can help account for biological memory underlying cardiometabolic complications.

Epigenetic Mechanisms of Gene Regulation

Architectural compartmentalization and spatiotemporal regulation are fundamental features of genome organization in the differentiating and replicating mammalian cell. The tight coiling of DNA around chromatin—the dynamic polymer of chromosomal DNA and histone proteins organized into nucleosome subunits—solves the physical challenge of storing long lengths of DNA, while also generating the need for a mechanism by which previous hyperglycemic exposure was remembered and impacted outcomes, completely consistent with previous pioneering reports of metabolic memory in animal models. As early as 1987, the enduring development of diabetic retinopathy was described in dogs despite 2.5 years of insulin therapy and effective glycemic control. Similarly persistent microvascular complications were reported in diabetic rodents. More recent studies of diabetic ApoE−/− mice revealed persistent aortic atherosclerotic plaque development despite normalization of blood glucose levels for at least 8 weeks. Intensified research now implicates lasting gene expression changes in the vasculopathies instigated by prior glucose exposure. Together, these findings point to the presence of biological memory linked to hyperglycemia and the broader diabetic state as driving the molecular changes and clinical outcomes seen in diabetic vascular disease. Increasing evidence advocates for epigenetics as a robust mechanism that can help account for biological memory underlying cardiometabolic complications.
Chromatin marks generally consist of 2 types of modifications. The best characterized is the classically epigenetic postreplicative methylation of DNA at the 5-carbon ring of cytosine nucleotides (5-methylcytosine [5mC]) immediately adjacent to guanine nucleotides (CpG). Stably propagated through cell division by the sequence symmetry of CpGs, 5mC is commonly associated with gene suppression when enriched at or near gene regulatory regions by modulating transcription factor binding to directly interfere with gene activation or interacting with specific regulatory proteins. In the latter example, specific methylation-recognition factors such as MeCP1 and MeCP2 and other methyl-CpG binding domain proteins (1–4) translate the DNA methylation signature to functional states of chromatin by cooperating with chromatin-modifying corepressor complexes. Such complexes contain enzymes that remove transcriptionally permissive histone acetylation marks and enzymes that write transcriptionally repressive histone methylation marks, linking DNA methylation with post-translational histone modifications (discussed further below). In contrast, mechanistically distinct DNA methylation at gene bodies and intergenic regions is associated with transcriptional elongation, gene splicing, and regulation of enhancer activities. The heritability and gene regulating function coupled with alteration of DNA methylation patterns observed during development and disease underscores the enthusiasm surrounding this modification in sustaining cellular memories. Recent characterization of the dioxygenase activities of ten-eleven-translocation (TET) proteins conferring their ability to generate 5-hydroxymethylcytosine (5hmC) from existing 5mC, which can be further processed to 5-formylcytosine and 5-carboxylcytosine, has inspired strong interest in demethylating pathways. In addition, these variants of the methyl modification represent newly characterized stable epigenetic marks recently associated with activated, poised, and silenced distal regulatory elements. Advances in epigenomics using high-throughput sequencing technologies to profile differential DNA methylation at single nucleotide resolution have identified previously unrecognized roles for this modification in numerous human diseases as well as physiological changes in ageing.

Also important for gene regulation are the myriad post-translational modifications written to and erased from predominantly arginine and lysine amino residues on the protruding N-terminal tails of chromatinized histones by specialized enzymes and modifying complexes (Figure 1). Methyl groups also feature strongly on histone tails and are associated with both active and silent genes depending on the

Figure 1. Interpreting chromatin and constituent residues subject to modification. Chromatin regulates gene structure and function. DNA is packaged into a 30-nm macromolecular structure consisting of the chromatin fiber. Together with post-translational modifications (PTM) of histone residues, chromatin regulates the functions involving transcription, repair, replication, and condensation. The nucleosome at 11 nm is comprised of ≈147 bp of DNA comprising an octamer of 2 copies of each of the 4 core histone proteins: H2A, H2B, H3, and H4. The histone tail is subject to immense interpretation by writing enzymes that add PTM shown such as histone methyltransferase, Set7. Histone tails are also subject to erasing enzymes that specifically remove these modifications and interpreted by reader proteins that identify histone tail modifications. Cytosine residues of the DNA duplex are subject to 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) modifications interpreted by methyl-CpG binding domain (MBD) and ten-eleven translocation (TET) enzyme. Methylation-mediated gene suppression events involve targeted MBD recruitment whereas TET proteins are implicated in active DNA demethylation and regulation of gene expression.
specific location and degree of modification. Contrasting this dual role of histone methylation, histone lysine acetylation is exclusively associated with transcriptional competency. The influence of histone post-translational modifications on higher order chromatin structure and transcriptional modulation is largely attributed to (1) charge disruption of histone tails in the case of acetylation/deacetylation which alters affinity for adjacent histones and DNA, and (2) establishment of high-affinity binding sites for recruitment of complexes that actively remodel the chromatin.107

In conjunction with transcription factor networks and remodeling complexes, these covalent and post-translational modifications collaboratively drive the functional exchange between repressed and active states of chromatin to contextualize gene activity.16,108,109 The compact configuration of heterochromatic promoters precluding gene transcription is patterned by methylation of and specific histone N-terminal tail amino acids such as H3-lysine-9 (H3K9) and H3-lysine-27 (H3K27). On the contrary, the transcriptionally permissive arrangement of euchromatin is marked by methylation of sites that include H3 histones at lysines 4 (H3K4) and 36 (H3K36), as well as acetylation of H3K9, H3K14, and H3K27.14,110

Although key enzymes such as methyltransferases, demethylases, acetyltransferases, and deacetylases have been described, characterization of the specific factors and signaling pathways directing chromatin changes, for instance, the substrate cofactor roles of metabolic pathway intermediates,111,112 is a topic of active research. In addition, much recent interest surrounds the importance of genetic determinants of epigenetic modifications.98

Most chromatin-modifying enzymes function in large multisubunit macromolecular complexes that determine their genetic and biochemical specificity.113 Such context specificity is most clearly demonstrated when recombinant enzymes exhibit distinct properties to their native complexes.114 This is exemplified by MLL1 that functions as an H3K4 methyltransferase in a COMPASS-like complex. However, this methyltransferase activity is severely compromised in the absence of core complex components Ash2L, RbBP5, or Wdr5.115,116 Similarly, EZH2 can methylate H3K27 only when incorporated in Polycomb Repressive Complexes.59 Therefore, associated factors found within multisubunit chromatin-modifying complexes are critical for the specific activity of enzymes on chromatin. This is also observed in cases where a recombinant enzyme preferentially targets free core histones but requires associated factors in native complexes to efficiently target chromatin.114 Moreover, many complexes have enzymatic activities with distinct functions, allowing them to combine important

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gene-regulating functions. For example, the NuRD (nucleosome remodeling deacetylase) complex couples the regulation of histone acetylation with chromatin remodeling. In addition to the localization to specific histone tail residues, the precise genomic localization of chromatin modifications must also be tightly regulated. Many chromatin-modifying complex subunits have distinct recognition modules that can bind chromatin on particular histone marks, directing the complex to bind and target specific residues on chromatin. Here, a role in localization of chromatin signatures has recently emerged for noncoding RNA molecules. For example, primary microRNA-208b can direct Ezh2-mediated histone methylation to key cardiac genes in the hypertrophic hearts of mice.

Into this interplay between the placement and the removal of epigenetic marks are reader proteins, which bind to specific chromatin and DNA modifications, thus facilitating the assembly of key transcriptional players such as RNA polymerase, elongation factors, and transcription factors. One group of reader proteins that have received attention as determinants of transcriptional responses are the bromodomain and extraterminal (BET) domain-containing family of proteins BRD2, BRD3, BRD4, and BRD7. Bromodomains are a structural motif found in a group of proteins that bind to acetylated lysines; BETs bind to specific acetylated lysines located on histone tails associated with defined groups of genes. BETs facilitate the assembly of transcriptional machinery, including Mediator and RNA Polymerase II (RNA PolII), a process that demands the opening of chromatin so as to allow transcription to occur. BETs can interact with both promoter regions and enhancer elements. Characterization of BET involvement in cancers like multiple myeloma using chromatin immuno-precipitation suggest BETs, like BRD4, predominately occupy enhancer, rather than promoter sites, where they drive the expression of a key set of multiple myeloma-associated target genes. This concentrated activity at specific sites and in the control of discrete subsets of genes is reflected in the conceptual if not also biological term super enhancer or stretch enhancers. In addition to extensive data implicating BETs as regulators and therapeutic targets in different cancers, recent data also implicate these epigenetic reader proteins in inflammatory and cardiovascular settings, as discussed further below.

Epigenetics and the Diabetic Vasculature: From Sites to Cyes
The life-threatening macrovascular complications of diabetes mellitus derive from atherosclerosis, a chronic inflammatory disease defined by the formation of fibrofatty atherosclerotic plaques. Chronic damage to the endothelium elicits a state of endothelial dysfunction characterized by increased vascular permeability and induction of proinflammatory adhesion and chemotactic molecules that promote immune cell infiltration and reduced responsiveness to vasoactive stimuli. Macrophage recruitment and accumulation in the subendothelial space is followed by foam cell formation and cytokine secretion that intensifies the inflammatory state. As the disease progresses, activated vascular smooth muscle cells (VSMCs) migrate from the arterial media to the intima where they also actively secrete various proliferative, fibrotic, osteogenic and inflammatory factors. Although still incompletely defined, diabetes mellitus has been suggested to promote atherosclerosis and plaque rupture through (1) increased expression of adhesion and chemotactic factors by endothelial cells (ECs) exposed to the diabetic milieu, (2) hyperglycemia induced VSMC migration and proliferation, and (3) increased macrophage accumulation. Relevant to the discussion here, evidence now establishes that all these elements seem to be modulated by epigenetic mechanisms. Although genetic predisposition is a significant factor in atherosclerosis, genome-wide association studies indicate a heritable component in only a relatively small fraction of cases of cardiovascular disease. Hence, epigenetic changes are increasingly implicated in molecular events underlying the aforementioned atherogenic changes. Comparison of donor-matched healthy and atherosclerotic human aorta samples revealed an atherosclerosis-specific epigenetic signature characterized by genome-wide CpG hypermethylation in the diseased vessel, with specific changes mapped to genes functionally associated with vascular homeostasis. Consistently, recent follow-up uncovered many CpG loci in atherosclerotic plaques that increase in methylation with lesion progression.

As described above, atherogenesis is a complex heterocellular process. And despite sharing a common genetic sequence, the component cells have distinct epigenomes regulating gene expression and cell type–specific pathologies. Whereas loci-specific chromatin modifications are informative of individual gene regulation, an important challenge is to understand their cell-specific function. Accumulation of publicly available deep-sequencing datasets underscored by the ongoing Encyclopedia of DNA Elements project permit the generation of genome-wide maps to highlight the importance of cell type–specific chromatin signatures (Figure 2). Further understanding the multitude of discrete epigenomes that constitute the atherosclerotic lesion can reveal new insights in to the development and progression of the disease that can be translated to novel therapies.

Hyperglycemia and Persistent Endothelial Dysfunction
As the major regulator of vascular homeostasis, the endothelium intimately engages the challenges of circulating factors of the diabetic milieu. Profoundly impacted by metabolic and inflammatory signaling, the endothelial monolayer is particularly vulnerable to injury and is the site of atherogenesis, initiating an inflammatory and oxidative cascade. The proinflammatory effects of hyperglycemia on endothelial function are well established and the activated EC and endothelial progenitor cells have accordingly emerged as therapeutic targets in diabetes mellitus.

Hyperglycemic conditions mediate a variety of chromatinized changes underlying altered gene expression patterns in ECs. Epigenome-wide analysis of aortic ECs stimulated with high glucose identified distinguishable and predominately inverse acetylation of H3K9/K14 and DNA methylation patterns correlating with transcriptional activation of various gene pathways closely associated with metabolic and cardiovascular disease. Of specific importance to the long-term clinical implications of glucose control, transient hyperglycemia induces various histone lysine modifications including...
monomethylation of H3 histones at lysine 4 (H3K4m1), which persists at the promoter of the \textit{RELA} gene (encoding the nuclear factor [NF]-κB-p65 subunit) for ≤6 days after return of vascular ECs to normoglycemia.\textsuperscript{13,135} This key proinflammatory transcription factor is integral to the regulation of genes central to vascular inflammation and atherosclerosis, including adhesion molecules, cytokines, and chemokines. Accordingly, VCAM-1, a key promoter of monocyte adhesion to arterial ECs, and the monocyte chemoattractant protein-1 (MCP-1) chemokine that directs macrophage infiltration were increased by transient hyperglycemia, remaining elevated during subsequent incubation at physiological glucose concentrations. Further studies determined that the specific H3K4m1 enrichment is written by the Set7 (also Set9 or Set7/9) lysine methyltransferase,\textsuperscript{13,135} which is mobilized to enter the nucleus in response to changes in ambient glucose concentration.\textsuperscript{136} Persistent depletion of the repressive methylation of H3K9 and enrichment of the lysine-specific demethylase 1 at the \textit{RELA} promoter identified a cooperative program of methyl writing and erasure, further supporting the chromatinization of sustained regulatory changes induced by transient hyperglycemia.\textsuperscript{13} Indeed, the expression of genes encoding NF-κB-p65 and NF-κB–dependent MCP-1 and VCAM-1 were elevated in aortas of previously hyperglycemic ApoE\textsuperscript{-/-} mice. Recent genome-wide analysis of Set7 function in human vascular ECs not only confirmed the role of Set7 in regulating NF-κB dependent pathways but also characterized in detail its involvement in the regulation of a large number of other genes associated with vascular function including the effects of lysine methylation of nonhistone substrates\textsuperscript{107} such as transcription factors.\textsuperscript{137} Indeed, the induction of Set7 mediated upregulation of \textit{HMOX1} in response to high glucose occurs independently of histone methyl changes.\textsuperscript{136}

**Epigenetic Reader Proteins as Determinants of EC State**

In strong synergism with these findings, recent studies reveal involvement of the BET epigenetic reader proteins in dictating large scale, inflammatory transcriptional programs in ECs under both resting and proinflammatory conditions, as seen in vitro and in vivo.\textsuperscript{138} Knockdown of all 3 BETs present in ECs (BRD2, 3, and 4) as well as BET inhibition using highly specific chemical agents that bind selectively within
the bromodomain, blocking BET association with acetylated lysines, represses key proinflammatory and proatherosclerotic targets like VCAM-1, e-selectin, and intracellular adhesion molecule 1 (ICAM-1) in ECs stimulated with tumor necrosis factor-α (TNFα). Moreover, genome-wide chromatin immunoprecipitation studies reveal that BRD4 is associated with a group of specific enhancers associated with endothelial genes under basal conditions, driving RNA polymerase II (Pol II) activity; in response to TNFα, BRD4 undergoes a massive redeployment to a new, discrete set of enhancers associated with genes involved in the inflammatory program. These targets include genes involved in leukocyte recruitment (chemokines), cell adhesion (VCAM-1, ICAM-1), and thrombosis, suggesting BET involvement in integrated pathological programs underlying atherosclerosis. BRD4 localization is highly correlated with NF-kB-p65. The clustering of BRD4 in a relatively limited number of promoter elements is in keeping with the concept of subsets of enhancers at which transcriptional activity may be concentrated—the so-called super enhancers139 or stretch enhancers.140 In cancer, this smaller, more defined group of factors suggests that complex disease states might be more tractable than previously thought.141 Given the evidence linking BETs with canonical regulators of both cancer and atherosclerosis, these data also suggest that analyzing BET localization may offer a means of identifying novel players underlying complex disease states. BETs are also implicated in macrophage biology and sepsis, where their inhibition increases survival142 and also adipogenesis.143 BETs have been reported to take part in directing transcriptional programs involved in pulmonary arterial hypertension.144 In terms of atherosclerosis, BET inhibitors repress leukocyte adhesion and transendothelial migration in vitro and ex vivo and decrease atherosclerotic lesions in LDL receptor–deficient mice in vivo.138,145 BETs have also been found to be involved in VSMCs and transcriptional responses to carotid artery injury.146 BET inhibitors are in clinical trials for various disease states, including cancer and cardiovascular disease.121,147 Of note, BETs use a different set of targets to direct transcriptional programs in cardiomyocytes, where BET inhibition blocks cardiac remodeling in response to transaortic constriction and pressure overload.148

Sensory Cues Implicate Epigenetic Mechanisms
How does the epigenetic machinery sense and respond to different stimuli? Especially relevant to diabetes mellitus, how do cells couple sensed changes in cellular glucose concentration to epigenetic responses? Mitochondrial overproduction of reactive oxygen species (ROS) as a result of hyperglycemia—long implicated in development of diabetic vascular injury—activates 4 major pathways involved in the pathogenesis of cardiovascular complications including protein kinase C activation, increased production of advanced glycation end products, polypol pathway flux, and the hexosamine pathway.149 Several lines of evidence indicate hyperglycemia-mediated ROS as the major driver of glycemic memory in the vasculature. Forced expression of genes encoding proteins that prevent ROS accumulation, either uncoupling protein-1 or manganese superoxide dismutase effectively abolished the transcriptional activation of hyperglycemic markers in vascular ECs, including RELA. Similarly, overexpression of the glyoxalase 1 enzyme, a major detoxifier of the methylglyoxal byproduct of glycolysis that accumulates in hyperglycemic cells as a consequence of increased mitochondrial superoxide production140 and a precursor to advanced glycation end product formation, also prevented the increased RELA expression induced by transient hyperglycemia.145 Persistent activation of ROS-generating mechanisms has been shown to perpetuate the proinflammatory memory of hyperglycemia. For instance, increased levels of pro-oxidant enzymes protein kinase C (PKC)-β and NAD(P)H oxidase subunit p47phox persist despite glucose normalization in human ECs.150 In fact, recent findings describe a complex sequence of epigenetic modifications modulating the expression of pro-oxidant and proinflammatory genes that underpin a vicious cycle of persistent vascular stress.151 In human aortic ECs exposed to hyperglycemia and aortas of diabetic mice, Paneni et al152 demonstrated persistent activation of the mitochondrial adapter protein and critical mediator of oxidative stress p66Shc by elevated PKCβII despite normoglycemia restoration. This was paralleled by sustained PKCβII-dependent inhibitory phosphorylation of endothelial nitric oxide synthase (eNOS) at Thr-495 and persistent reduction of nitric oxide bioavailability. Importantly, in vivo siRNA-mediated p66Shc silencing at the time of glucose normalization blunted mitochondrial ROS production, restored basal PKCβII expression, attenuated eNOS inhibition, and restored nitric oxide availability, while suppressing the persistent increase of methylglyoxyl. Consistent with epigenetic changes underlying vascular hyperglycemic memory, elevated p66Shc expression was not only associated with sustained promoter CpG hypomethylation but was also driven by GCN5-mediated enrichment of H3 histone acetylation. In agreement with the latter finding, class III histone deacetylase inhibitors significantly increased p66Shc mRNA and protein expression in human umbilical vein ECs.153 Further implicating changes in histone acetylation, transcriptional upregulation of p66Shc during hyperglycemia was exacerbated by specific knockdown of the SIRT1 class III histone deacetylase and reversed by overexpression of the same enzyme. Furthermore, the elevated p66Shc expression observed in wild-type diabetic mice was significantly decreased in endothelial-specific SIRT1 transgenic diabetic animals, which also manifest reduced oxidative stress and less endothelial dysfunction compared with wild-type littermates. This study demonstrated that SIRT1 deacetylates H3 histones at the human endothelial p66Shc promoter to suppress transcription, as corroborated by significantly decreased H3 acetylation in the aortas of SIRT1 transgenic mice.153 These findings have significant implications for diabetic cardiovascular complications. Hyperglycemia initiates epigenetic upregulation of p66Shc expression and ROS production to activate PKCβII, which in turn maintains elevated p66Shc levels, thus promoting sustained ROS-dependent epigenetic changes by enzymes such as Set7. In addition to being a major producer of mitochondrial ROS, p66Shc also seems to promote downregulation of the antioxidant manganese superoxide dismutase, commensurate with unchallenged ROS accumulation in the vascular endothelium.151,155 Although experiments demonstrating the importance of a feed-forward mechanism of p66Shc expression for hyperglycemic memory were performed...
in models of chronic high glucose exposure (3 weeks in diabetic mice and 3 days for cultured ECs), this construct suggests how a legacy of transient hyperglycemic spikes in human diabetes mellitus could have enduring pathological consequences. The opposing activities of GCN5 and SIRT1 on p66Shc gene expression therefore represent important epigenetic factors for ROS-mediated hyperglycemic memory in the vascular endothelium. Taken together, modulation of the critical histone modifications conferred by GCN5, SIRT1, and Set7 holds tremendous therapeutic potential to reduce or reverse diabetic cardiovascular complications.

**Diabetes Mellitus Epigenetically Modulates the Phenotypic Plasticity of VSMCs**

The response of VSMCs to metabolic and hemodynamic cues is a major contributor to changes associated with the development of cardiovascular complications in diabetes mellitus. Recent studies in rodent models have begun to elucidate the epigenetic component of these pathological changes. For instance, angiotensin II induced widespread enrichment of trimethylated H3K4 and H3K36 in VSMCs isolated from rats.\(^{154}\)

Epigenetically regulated changes in transcription included the modulation of hundreds of long noncoding RNAs, including the novel Lnc-Ang362 transcript, which was further characterized as the host transcript of 2 micro-RNAs, miR-221 and miR-222, and found to be important for VSMC proliferation. Similarly, histone methylation changes are also implicated in the novel methylated H3K4 and H3K27 in VSMCs previously exposed to the diabetic milieu.\(^ {155}\) VSMCs derived from the diabetic db/db mouse model exhibited increased expression of interleukin-6 and MCP-1 that persisted for ≥8 weeks when cultured ex vivo under normal glucose conditions. This memory of hyperglycemia is mediated by the loss of repressive H3K9 trimethylation, paralleling decreased expression of the H3K9 methyltransferase Suv39h1. The same research group identified that increased expression of miR-125b in response to high glucose stimulation could decrease H3K9 methylation at inflammatory gene promoters by targeting Suv39h1 mRNA, and correspondingly increasing IL-6 and MCP-1 gene expression.\(^ {156}\) Although it remains unclear whether miR-125b is persistently elevated after glucose normalization, the lasting effects of diabetes mellitus on VSMC phenotype illustrate that important epigenetic changes in hyperglycemic memory extend beyond the endothelium.

Indeed, the role of epigenetic modifications in determining VSMC phenotype is an emerging theme in atherosclerosis research. VSMCs are not terminally differentiated, but retain remarkable plasticity that underlies functions important for vascular health and disease. Under homeostatic conditions, VSMCs exhibit a contractile phenotype characterized by a low proliferative index and a unique repertoire of contractile proteins, ion channels, and receptors\(^ {157}\) that help maintain structural integrity, vascular tone, blood pressure, and flow. However in response to chronic vascular injury in diabetes mellitus, VSMCs can assume a dedifferentiated synthetic and migratory phenotype characterized by loss of contractile function, increased proliferation, and secretion of extracellular matrix proteins that promote atherosclerosis.\(^ {158,159}\) Many genes characteristic of VSMC phenotype switching are under transcriptional regulation,\(^ {160}\) whereas several studies demonstrate a role for histone modifications in VSMC differentiation.\(^ {161}\) For example, DNA binding of the serum response factor master regulator of VSMC-specific gene expression of contractile proteins is dependent on histone methylation and acetylation at CarG motifs in mice.\(^ {162}\) While H3K4 di-methylation persists after vascular injury, loss of serum response factor binding was coupled with H4 histone deacetylation during suppression of VSMC differentiation.

More recently, a novel role for DNA demethylation by TET2 and its 5hmC enzymatic product in VSMC phenotypic plasticity was reported.\(^ {163}\) High levels of TET2 and 5hmC were associated with the differentiated VSMC phenotype, whereas significant loss of TET2 and 5hmC occurred in experimental models of VSMC dedifferentiation and human atherosclerosis. Moreover, TET2 overexpression induced the expression of VSMC contractile genes while significantly increasing the expression of synthetic phenotype markers like KLF4, KLF5, and OPN, and increasing proliferation of human coronary artery VSMCs. In contrast, TET2 overexpression induced a contractile phenotype in the absence of differentiation stimuli, while simultaneously decreasing expression of dedifferentiation and antiproliferative markers. Mechanistically, TET2 promoter binding seems to coordinate VSMC phenotypic modulation, with opposing effects on chromatin accessibility of contractile and synthetic genes by altering DNA methylation and 5hmC modifications while also influencing the respective permissive and repressive methylation patterns of H3K4 and H3K27. Taken together, this study describes a commanding role for TET2-mediated epigenetic signatures in balancing the distinct molecular states of contractile and dedifferentiated VSMCs phenotypes that may represent a new therapeutic target for controlling phenotypic modulation in cardiovascular disease. A recent provocative description of the transdifferentiation of VSMCs to a macrophage phenotype in atherosclerosis\(^ {164}\) contrasts the traditional view that lesional macrophages primarily derive from circulating monocytes.\(^ {165}\) What regulatory roles might chromatin modifications play in this shifting paradigm of phenotypic transition?

**Epigenetic Macrophage Memory**

It is well established that both tissue resident macrophages and circulating monocytes contribute to the immunologic landscape of the atherosclerotic lesion. Recent genome-wide analysis revealing increased monocyte H3 acetylation associated with conventional treatment compared with intensive treatment group subjects of the DCCT indicates a possible mechanism of metabolic memory in humans.\(^ {166}\) Consistent with previous studies in ECs, increased H3K4m1 by Set7 leading to upregulation of RELA and inflammatory gene expression was observed in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus.\(^ {167}\) Indeed, ≈25% of NF-κB-dependent genes were attenuated by Set7 knockdown in TNF-α–stimulated monocytes.\(^ {168}\) Although Set7 depletion did not interfere with monocyte-to-macrophage differentiation, this experimental strategy significantly reduced monocyte adhesion to ECs, a critical step in the pathogenesis of atherosclerosis.
Studies have recently started to define the epigenetic signatures of monocyte/macrophage phenotype. Chromatin modifications distinguish the opposing functional programs of monocyte-to-macrophage differentiation as well as trained immunity and tolerance. Monocytes and macrophages can retain a memory of previous immunologic stimulation by the Gram-negative bacterial endotoxin lipopolysaccharide via Toll-like receptor 4 signaling, entering a refractory state with reduced capacity to respond to repeated or prolonged stimulation. Single exposure of cultured human peripheral blood mononuclear cells to lipopolysaccharide induced an initial inflammatory response characterized by increasing expression of genes encoding inflammatory mediators such as TNF-α, IFN-γ, and interleukin-12 associated with NF-kB-p65, IFN-1, and STAT1 transcription factors, broadly classified as classical M1 polarization. This contrasts with the induction of tolerance by a second dose of lipopolysaccharide redirecting the cells to an alternative M2 polarization state of attenuated inflammatory gene expression while activating other immunologic functions such as chemotaxis indicated by the upregulation of MCP-1, as well as wound healing characterized by increased expression of matrix metalloproteinase 7 and matrix metalloproteinase 9. A separate study demonstrated that the dampening of previously expressed inflammatory genes (tolerable genes) after lipopolysaccharide restimulation in macrophages and genes induced in tolerant cells (nontolerable) were distinguishable by histone modification patterns. Although promoters of both classes of genes were inducibly acetylated at H4 histones, only nontolerable genes were reacetylated on lipopolysaccharide restimulation. Similarly, trimethylated H3K4 induced by lipopolysaccharide in naïve macrophages was selectively diminished at transiently silenced tolerable promoters, but maintained at nontolerable promoters.

Such findings may be explained by the recent description of persistent epigenetic programs in macrophages that at least partly rely on the induction of latent enhancers. These distal regulatory elements that are unbound by transcription factors and lack the histone modifications characteristic of distal regulatory elements acquire signature features of enhancers on stimulation. Once activated in response to factors such as lipopolysaccharide, most of these regions remain epigenetically modified to mediate a faster and more robust response to restimulation. Enrichment of H3K4m1 and acetylation of H3 histones at lysine 27 (H3K27ac) are characteristic hallmarks of enhancer regions. After lipopolysaccharide stimulation, Ostuni et al demonstrated the persistence of H3K4m1 despite loss of H3K27 acetylation at decommissioned distal elements, supporting the notion that similar to the inflamed EC, H3K4 methylation provides a mechanism for epigenetic memory in macrophages.

An intriguing example of how enhancers can influence macrophage biology is through their action on specific macrophage programs as a function of anatomic location. Gosselin et al found that the unique properties that macrophages adopt as a function of physical context may derive from differential activation of an enhancer repertoire and selective induction of different transcription factors interacting with such elements. Although these studies were performed in brain microglia and peritoneal macrophages, they are likely relevant in other settings, like distinctions between atherosclerotic lesions that are active, plaque-rupture-prone versus more stable disease, or macrophages in a diabetic versus nondiabetic environment.

Epigenetic mechanisms like these may relate to recent evidence linking chronically elevated serum lipopolysaccharide levels in diabetes mellitus to the development of vascular complications. Epidemiological studies indicate that elevated levels of circulating endotoxin are predictive of an increased risk of atherosclerosis. Consistently, weekly lipopolysaccharide injections have been shown to accelerate the development of atherosclerotic lesions in ApoE-/- mice. That lipopolysaccharide tolerance balances the inflammatory response to limit tissue damage however seems to conflict with the prevailing view that chronic endotoxin exposure influences atherogenesis by increasing inflammation. Indeed, monocytes from diabetic patients display an inflammatory phenotype characterized by increased secretion of proinflammatory cytokines. However, the increased expression of MCP-1 and elevated matrix metalloproteinase expression in lipopolysaccharide tolerant cells, known to influence macrophage infiltration and associated with plaque instability respectively could be just as critical for the formation of complex, rupture-prone lesions in diabetes mellitus. Indeed, macrophage-rich regions of atherosclerotic plaques exhibit the greatest vulnerability. Certainly, these same mechanisms suggested for lipopolysaccharide may also be relevant to other pathogenic stimuli such as classic cardiometabolic risk factors.

Understanding the contributions of individual components of the protective and proatherogenic milieu to monocyte/macrophage chromatin patterns and phenotype remains an important challenge. Impelled by seminal descriptions of chromatin-dependent trained innate immunity, non-microbial factors relevant to atherosclerosis are now under investigation for their roles in the epigenetic remodeling of myeloid cells. Exposure of isolated human monocytes to a low concentration of oxidized low-density lipoprotein induced the formation of macrophages with a long-lasting proatherogenic phenotype characterized by changes in histone methylation. Specifically, brief incubation with oxidized low-density lipoprotein augmented production of not only proinflammatory factors but also matrix metalloproteinases by lipopolysaccharide restimulation after 6 days in culture. Furthermore, oxidized low-density lipoprotein priming up-regulated CD36 and SR-A scavenger receptor expression leading to enhanced foam-cell formation. These trained changes in gene expression were paralleled by enrichment of promoter H3K4 trimethylation. Accordingly, pretreatment with a pan-histone methyltransferase inhibitor abolished the training induced by oxidized low-density lipoprotein, indicating that the reversibility of myeloid epigenetic modifications could offer opportunities to develop novel treatment strategies for atherosclerosis.

Harnessing Epigenetic Insights to Target Gene Regulators

The identification and subsequent elucidation of DNA and the genetic code is uniformly recognized as a fundamental advance that transformed our understanding and approach
human health and disease. This context foreshadows the potential importance of the more recent recognition that an epigenetic code also exists that can determine phenotype independent of gene variations. The studies highlighted here provide examples selected from many in a rapidly burgeoning arena of how epigenetic mechanisms underlie and help define cardiometabolic health and dysfunction.

Much interest surrounds the clinical development of pharmacological compounds that modulate epigenetic players. Although extended furthest in the field of oncology, interest in this promising treatment strategy is now extending to cardiovascular disease. Distinct classes of histone deacetylase (HDAC) inhibitors have demonstrated therapeutic potential in preclinical models of heart failure and strong interest surrounds their use for the clinical treatment of atherosclerosis. Indeed, explicit targets are beginning to emerge. For example, macrophage expression of HDAC3 is elevated in ruptured human atherosclerotic lesions. Macrophage-specific Hdac3 deletion in mice enhanced Tgfβ1 gene expression by promoter hyperacetylation, driving increased collagen production by VSMCs and subsequently inducing a stable plaque phenotype. Consistently, specific targeting of HDAC3 in bone marrow–derived macrophages promotes an atheroprotective macrophage phenotype, further indicating the therapeutic potential for HDAC3 inhibition for treating cardiovascular disease. Likewise, pharmacological targeting of Set7 using a recently developed and highly selective small molecule inhibitor was able to counter NF-κB–dependent proinflammatory cytokine production in cultured human epithelial cells. BET bromodomain inhibitors, which are in active studies for treating various cancers, are also being pursued for their potential action on cardiovascular disease.

The extent to which pathological chromatin changes can be manipulated in human cardiovascular disease remains to be established. Several issues represent key hurdles for such opportunities. First, although numerous enzymes and specific modifications have been described, understanding their specific modes of enzymatic action remains an unresolved but critical issue for developing novel therapeutic epigenetic modulators. Our recent description of noncanonical effects on chromatin by pharmacological HDAC inhibition highlights the complexities of epigenome editing. Related classes of epigenetic enzymes can have distinct effects on atherogenesis. Increased plaque size and macrophage accumulation in the aortic sinus of LDLR−/− mice by the pan-selective HDAC inhibitor Trichostatin A contrasts the beneficial effects of myeloid HDAC3 deletion. The complexities of epigenetic modulation are further exacerbated by cell type–specific roles of the chromatin modifying machinery. Although macrophage HDAC3 deletion is atheroprotective, deletion of the same enzyme in ECs enhances plaque development. Approaches that enable plaque- and cell-specific pharmacological targeting of chromatin regulators, such as a nanomedicine delivery strategies using drug-loaded high-density lipoprotein nanoparticles may facilitate greater precision of epigenetic interventions. Finally, the fundamental roles that epigenetic readers, writers, and erasers play in cellular biology raises questions about the ability to selectively target relevant proteins without untoward effects. Although significant hurdles exist, it is apparent that epigenetic mechanisms offer significant and still largely untapped opportunities for understanding and treating diabetes mellitus and cardiovascular disease. Although the convergence of diabetes mellitus and atherosclerosis is a broad intersection, among the most fundamental characteristics common to these 2 conditions is their chronic nature, involving pathogenesis that arises over years if not decades of subclinical phases. As such, ample opportunities exist for biological memory to be a central contributor to disease, as cells remember the hyperglycemia, elevated LDL levels, hypertension, oxidative stress, and inflammation to which they have been exposed. Perhaps epigenetic-based therapies will provide a way in which such exposures can be forgotten or at least ignored, and complications avoided.

Current Perspectives and Future Directions

Intensified interest surrounds the ability to pursue chromatin modifiers and complexes as therapeutic targets for numerous diseases including vascular complications associated with diabetes mellitus. With current technological and scientific advances, an improved understanding of chromatin-dependent disease mechanisms is already emerging that will underpin future therapeutic advances. However, the expansion from research to the clinic of pharmacological strategies targeting chromatin modifiers and readers awaits a more complete description of the epigenomic machinery. To this end, recently emerged concepts could offer further insight into chromatin regulation. Several intermediates of cellular metabolism are critical substrates for chromatin-modifying enzymes and fluctuating levels of metabolites could therefore signal for continual adjustment of gene expression. In addition, recent reports describe a role for long noncoding RNA transcripts in localizing chromatin signatures. Furthermore, it is increasingly appreciated that the enzymes responsible for writing and removing chromatin modifications also interact with, modify, and modulate transcription factors, representing an important consideration for pharmacological specificity.

Although remarkable parallels are observed across tissues, prominent differences are clearly distinguishable between distinct cell types. The heterocellular nature of atherosclerosis demands a thorough understanding of cell type–specific chromatin signatures underlying their specific pathologies. Endothelial, smooth muscle, and circulating immune cells constitute the 3 major cell types that directly influence the development and progression of atherosclerotic lesions. Understanding their individual epigenetically determined contributions to macrovascular complications in context of the diabetic milieu, including mechanisms underlying persistent gene expression, offers unprecedented opportunities to target cell type-specific enzymes and complexes to reduce the clinical burden of diabetes mellitus. Epigenetic variation is observed across individual subjects, highlighted by distinct chromatin patterns between men and women (Figure 3).

The epigenomes of circulating cells can be profiled with relative ease, whereas most epigenetic studies of the vascular wall have relied on cultured endothelial and smooth muscle cells. As such, knowledge of the mechanisms controlling vascular phenotypes in vivo is far from complete. This is partly because the interpretation of traditional analytic approaches using
immunoprecipitated chromatin is confounded by the composite signal of heterogeneous cell populations found in atherosclerotic lesions. To circumvent these technical limitations, Gomez et al. recently described a novel method that combines in situ hybridization and proximity ligation assays for visualizing histone modifications at single genomic loci with single-cell resolution in formaldehyde-fixed paraffin-embedded tissue sections, allowing unprecedented studies of epigenetic changes in the pathogenesis of atherosclerosis and other human diseases using existing tissue banks. Although this represents a major step forward, there remains a need to balance design tradeoffs such as genome coverage and cell specificity.

With the emergence of massive parallel sequencing and a consensus in handling datasets, the clinical applicability of epigenetic interventions for cardiovascular disease will be much clearer in the near future. Combining multilayered
epigenome-wide analyses with strategies to interrogate specific cell populations important for atherosclerosis is key to this progress. However, the truly comprehensive understanding necessitated by the complexity of gene regulation will only be achieved after large-scale epigenomic sequencing efforts provide a systematic, cell-specific, description of the relations between differential chromatin modification in response to specific stimuli, genome sequence variation, and major clinical phenotypes.

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