Epigenetic Mechanisms in Diabetic Vascular Complications and Metabolic Memory: The 2020 Edwin Bierman Award Lecture

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Macrovascular complications such as atherosclerosis, myocardial infarction and stroke, and microvascular complications such as nephropathy, retinopathy, and neuropathy are the major causes of increased morbidity and mortality in both type 1 and type 2 diabetes. Increased inflammation, oxidative stress, and fibrosis are common features in most diabetes complications. Although extensive studies have examined the biochemical pathways leading to the expression of inflammatory, profibrotic, and other pathological genes, as well as genetic factors related to diabetes and associated complications, much less is known about the contribution of epigenetic changes that occur without alterations in the DNA sequence. Environmental factors, lifestyles, and improper diet implicated in diabetes can affect epigenetic states. Epigenetic modifications, including DNA methylation and histone modifications, can alter gene transcription in response to environmental stimuli and cooperate with noncoding RNAs. These epigenetic modifications have been observed in various target cells under diabetic conditions. Moreover, epigenetics has also been implicated in the phenomenon of metabolic memory observed in clinic trials and animal studies, in which prior episodes of poor glycemic control can confer continued risk of complications despite subsequent glucose normalization. Epigenome-wide association studies in cohorts with diabetes are uncovering epigenotype variations that provide new insights into diabetic vascular complications. Here, I discuss the role of epigenetics and noncoding RNAs in diabetes complications and metabolic memory, and their translation potential to serve as biomarkers and drug targets to improve clinical management of diabetic vascular complications.

ADA AWARD LECTURE

It was truly an honor to deliver the 2020 Edwin Bierman Award Lecture at the 80th Scientific Sessions of the American Diabetes Association. I dedicate this award to the victims of the COVID-19 pandemic and to our health care workers, heroes of these challenging times. It was a unique experience prerecording this talk all alone in my office via a zoom portal and then watching it on June 13th while responding to text messages from other attendees. Our kudos to the organizers for bravely embarking on this very first virtual Scientific Sessions.

Dr. Edwin Bierman's pioneering work in the fields of diabetes, obesity, and atherosclerosis has inspired so many scientists, including me, and emphasized the role of aberrant lipid metabolism in diabetic vascular disease. Chronic elevation in blood glucose levels in both type 1 diabetes (T1D) and type 2 diabetes (T2D) can cause blood vessel damage. Damage to arteries increases macrovascular complications including cardiovascular diseases (CVDs) such as atherosclerosis, myocardial infarction, hypertension, and stroke, while damage to small blood vessels results in microvascular complications such as nephropathy, neuropathy, and retinopathy. These complications lead to significantly increased morbidity and mortality (1–4). Because of shared mechanisms leading to vessel damage, micro- and macrovascular complications frequently coexist, with diabetic nephropathy (diabetic kidney disease [DKD]) being a major risk factor for CVDs (4).

Genetic disposition and key single nucleotide polymorphisms have been implicated in both T1D and T2D complications. However, increasing evidence suggests that complex interactions between genes and the environment may also be involved (5–7). Notably, nuclear chromatin is

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a crucial interface between the effects of genetics and environment, and the epigenetic modifications in chromatin including DNA methylation (DNAme) and posttranscriptional modifications (PTMs) of histone tails, along with noncoding RNAs, regulate gene transcription. Although several studies have identified key biochemical pathways triggered by hyperglycemia and diabetes in target cells leading to expression of genes related to diabetes complications, the role of epigenetic mechanisms is becoming increasingly evident. Clinical trials have emphasized the benefits of intensive glycemic control for reducing the risk of progression of complications. Even though diabetes can be controlled through medications, insulin, and lifestyle modifications, in some subjects with diabetes, prior periods of hyperglycemia due to relaxed glycemic control can be associated with continued risk of complications long after hemoglobin A_{1c} (HbA_{1c}) levels have been normalized. This "metabolic memory" of prior glycemic control could be due to epigenetic changes in target cells. In the Edwin Bierman Award Lecture, I outlined contributions from our laboratory and others to our understanding of epigenetics and epigenomics related to diabetic vascular complications and metabolic memory and potential implications for much-needed new therapies.

Vascular Complications of Diabetes: Inflammation and Fibrosis Are Key Culprits

Key events in macrovascular complications such as atherosclerotic plaque formation include activation of monocytes and endothelial cells (ECs); transendothelial migration of monocytes and their differentiation into macrophages, which become lipid-laden foam cells; and proliferation and migration of vascular smooth muscle cells (VSMCs) (2). Several experimental models in animals have illustrated mechanisms associated with diabetes-induced accelerated CVDs (4,8,9). In early collaborations with Gerrity and colleagues (10), we observed markedly increased oxidative stress in monocytes obtained from a swine model of diabetes-induced accelerated atherosclerosis. In addition, we found that treatment of VSMCs and human monocytes with high glucose (HG) (15–25 mmol/L) or advanced glycation end products (AGEs) mimicking the diabetes state leads to the activation of NF-κB and AP-1 transcription factors (TFs) associated with the transcriptional regulation of inflammatory genes like cytokines and adhesion molecules (11,12). These and several subsequent studies have clearly highlighted inflammation as a major feature of diabetic vascular disease, causing accelerated dysfunction of vascular and inflammatory cells and the affected target organs. In diabetic CVDs, monocyte/macrophage infiltration, inflammation, fibrosis, and hypertrophy of component cells in the heart, kidney, and blood vessels are key features. In DKD, renal dysfunction (proteinuria, abnormal glomerular filtration) is associated with inflammation, fibrosis, marked hypertrophy of renal mesangial cells (MCs) and tubular epithelial cells, and effacement/apoptosis of podocytes (7,13).

Hyperglycemia, a common feature of both T1D and T2D and a major risk factor for diabetes complications, especially microvascular, can act via production of AGEs; oxidized lipids; inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β and interleukin-6; growth factors such as angiotensin II (AngII); and transforming growth factor-β1 (TGF-β1) in target cells (4,7,9,13). Several signal transduction pathways downstream of HG, AGEs, dyslipidemia, and growth factors activate various kinases and TFs such as NF-κB, CREB, and SMADs. These TFs regulate the expression of inflammatory cytokines and growth factors as well as fibrotic and growth-related genes associated with CVDs and other diabetes complications (3,4,7). These processes regulate coding genes as well as noncoding genes such as miRNAs and long noncoding RNAs (lncRNAs) that modulate diabetes complications (7). Moreover, diabetes also disrupts key endogenous protective processes such as antioxidant factors, autophagy, and mitophagy (3).

Our laboratory began investigating potential epigenetic mechanisms (defined in the next section) in the regulation of genes and processes associated with diabetic vascular complications and metabolic memory. This is because gene-environment interactions, viruses, improper diet, and sedentary lifestyles, all of which affect epigenetics, augment risk for diabetes, inflammation, and related complications (7,13).

Epigenetics and Epigenomics: New Insights Into Complications

Epigenetics is broadly defined as mitotically and/or meiotically heritable changes in gene expression and function that occur without changes in the underlying DNA sequence (14,15). Epigenetic changes can occur dynamically in response to developmental, environmental, and nutritional cues to regulate gene expression, phenotypes, and metabolic abnormalities, some of which are heritable. Epigenetic changes can lead to discordant phenotypes and disease susceptibility among genetically identical twins (5–7).

Nuclear chromosomal DNA is tightly packaged with histone proteins into chromatin, which consists of subunits called nucleosomes. Each nucleosome is composed of an octameric histone protein complex containing dimers of core histones H2A, H2B, H3, and H4 and wrapped with 147 base pairs of DNA. Apart from the binding of TFs to promoters and classic transcription mechanisms, gene regulation can also be affected by epigenetic changes altering chromatin structure via nucleosome remodeling. Chromatin can be either tightly packed/condensed (heterochromatin) and inaccessible to TFs and transcriptional machinery or in relaxed/open (euchromatin) states accessible to TFs and the transcription machinery. These chromatin changes are regulated by epigenetic marks like cytosine DNAme, which usually occurs at CpG nucleotides (5-methylcytosine), and histone PTMs (16,17) and also modified by noncoding RNAs, including miRNAs and

lncRNAs. Mechanistically, promoter DNAme or repressive histone PTMs repress gene expression by recruiting corepressors and condensing chromatin to form heterochromatin (Fig. 1). In contrast, permissive histone PTMs drive gene expression by recruiting coactivators and chromatin remodeling proteins that increase chromatin accessibility (16,17). Together, these modifications modulate chromatin structure/function and cell-type gene expression patterns (Fig. 1). Epigenomics refers to genome-wide profiles of these modifications and their alterations during physiological and pathological conditions.

Advances in high-throughput sequencing (seq) technologies, along with efforts of major consortia such as the ENCODE, NIH Roadmap Epigenomics Mapping Consortium,

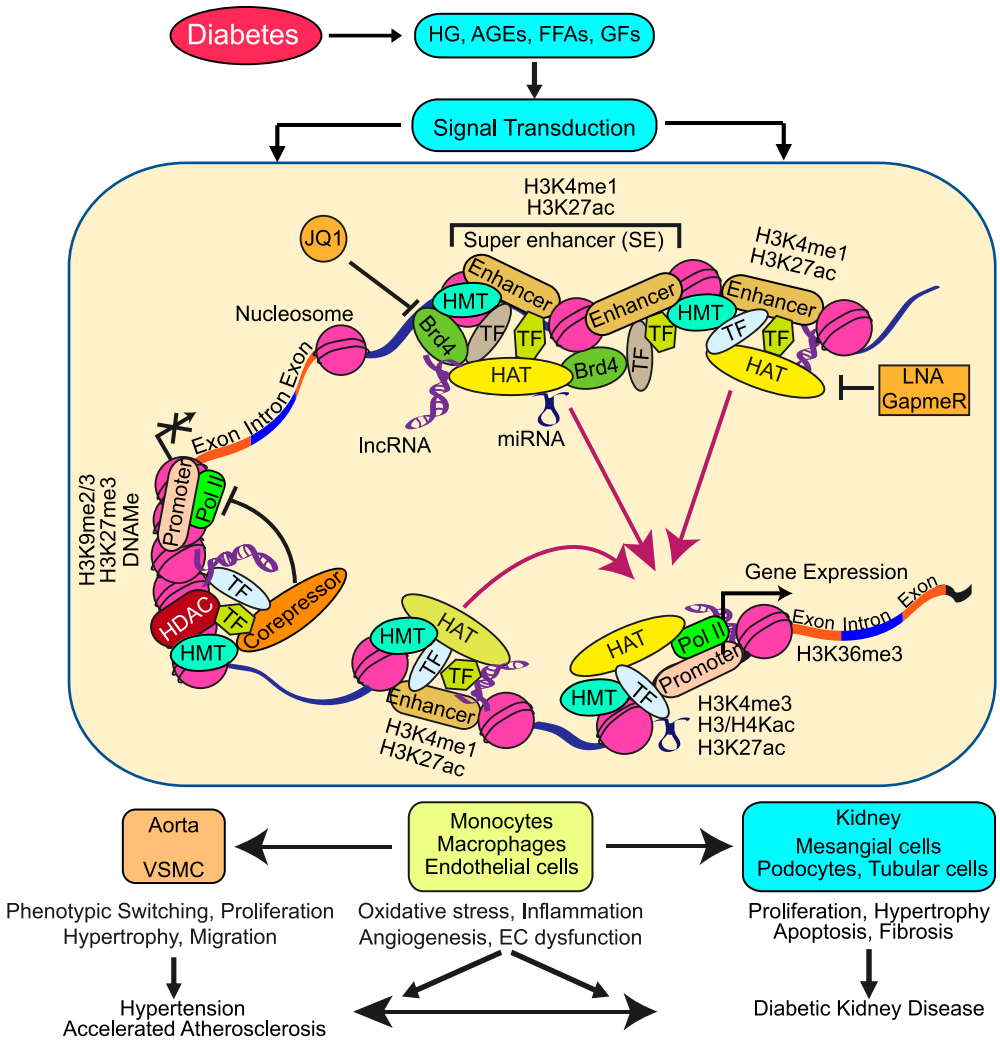

Figure 1—Schematic of epigenetic modifications at various *cis*-regulatory elements in chromatin that can be altered under diabetic conditions and impact the regulation of genes associated with diabetes complications. Chromatin consists of DNA packaged with histones in nucleosomes, which are the basic subunits of chromatin. Histone PTMs and DNAme at *cis*-regulatory elements such as promoters and enhancers regulate chromatin structure and function involved in gene regulation. Permissive histone PTMs like histone H3/H4Kac and H3K4me1/2/3 are associated with activate gene transcription, whereas repressive histone marks (H3K9me2/3 and H3K27me3) and DNAme at promoters are associated with suppressed gene expression. Active promoters are marked by H3K4me3 and H3K27ac, active enhancers are marked with H3K4me1 and H3K27ac, repressed promoters are marked by DNAme, H3K9me2/me3, and H3K27me3, and transcribed regions are marked by H3K36me3. Histone lysine acetylation and methylation are mediated by HATs and HMTs, respectively. Their actions are reversed by HDACs and HDMs (data not shown), respectively. DNAme is mediated by DNA methyl transferases and removed by the ten-eleven translocation group of enzymes (data not shown). Dynamic balance between various histone PTMs and DNAme as well as chromatin remodeling factors regulates chromatin access to TFs and determines the active or inactive states of chromatin. These epigenetic mechanisms are fine-tuned by lncRNAs that can interact with chromatin and chromatin-modifying proteins to alter gene expression. In addition, miRNAs repress target genes via posttranscriptional mechanisms. In diabetes, signal transduction pathways activated by the major pathological factors such as HG, AGEs, free fatty acids (FFAs), and growth factors (GFs) can dysregulate these epigenetic mechanisms and noncoding RNAs, leading to altered expression of genes and phenotypes associated with dysfunction of vascular, renal, and immune cells implicated in macrovascular complications (like hypertension and atherosclerosis) and microvascular complications (like DKD). LNA, locked nucleic acid.

and FANTOM (Functional Annotation of the Mammalian Genome), have enabled us to gain unprecedented details of the transcriptome, genome, and epigenome and chromatin states at high resolution under normal versus disease states (7). Some of these commonly used technologies include RNA-seq (to profile transcriptomes), assay for transposase-accessible chromatin-seq (for chromatin accessibility), chromatin immunoprecipitation-seq (for histone PTMs and TFs), and various platforms for DNAme (DNAme bead chips, methylated DNA immunoprecipitation-seq and whole-genome bisulfite-seq).

Alterations in epigenetic states have profound effects on gene regulation and phenotypes and thus mediate various human disorders, including cancer and diabetes (5–7). Along with the efforts of genome-wide association studies (GWAS) to identify complications-associated genotypes, evaluation of epigenotypes by epigenome-wide association studies (EWAS) is increasingly providing new insights into diabetes complications, metabolic memory, and related biomarkers/therapeutic targets. This is particularly significant because GWAS have revealed only few genetic variants related to diabetes complications, and most GWAS-associated signals are located in noncoding/regulatory regions whose functions and causality can be investigated by EWAS, along with assessment of chromatin states and related approaches (18,19).

Histone Modifications in Diabetes Complications

Histone PTMs can promote or repress transcription depending on their type and location. As shown in Fig. 1, promoters associated with active gene expression are enriched with histone H3 lysine 4 trimethylation (H3K4me3) and H3/H4 lysine acetylation (Kac), whereas repressed gene promoters are enriched with H3K9me2/3, H3K27me3, and/or DNAme. H3K4me1 is enriched at poised enhancers and H3K27ac at active enhancers (16). Superenhancers (clusters of multiple enhancers) are enriched with H3K27ac, the coactivator bromodomain-containing protein 4 (BRD4), and cell-specific TFs along with other proteins (20). Histone PTMs are mediated by specific "writers" or "erasers." At a specific histone tail position, lysine acetylation and methylation can be "written" by histone acetyltransferases (HATs) and methyltransferases (HMTs), respectively, and "erased" by histone deacetylates (HDACs) and demethylases (HDMs) respectively. PTMs at different locations can have important biological functions. Enhancers and superenhancers enhance expression of nearby genes (in *cis*) or genes located several hundred kilobases away (in *trans*) via chromatin looping that brings coactivators such as BRD4 and HATs, and TFs, in contact with target gene promoters (20) (Fig. 1).

We showed that histone PTMs can cooperate with TFs like NF-κB in regulating inflammatory and other genes under diabetic conditions in monocytes and vascular cells. Treatment of THP1 human monocytes with HG increased active histone marks H3Kac and H4Kac at the promoters of inflammatory genes (*TNF* and *PTGS2*) via increased recruitment of coactivator HATs (CBP/p300) and NF-κB (21). H3K4me1 and the corresponding HMT (SETD7) were involved in inflammatory gene expression in monocytes stimulated by ligands of RAGE (receptor for advanced glycation end products) and in macrophages from diabetic mice. SETD7 enhanced the transcriptional activation of NF-κB–regulated inflammatory genes *TNF* and *CCL2* (22). In addition, HG-induced (in vitro) and diabetes-induced (in vivo) genome-wide changes in repressive H3K9me2 at inflammatory and key autoimmune genes in monocytes and lymphocytes (23).

In ECs, HG induced sustained expression of NF-κB p65 subunit and inflammatory genes by increasing H3K4me1 via SETD7 activation and also decreased H3K9me3 (24,25). We found that diabetic conditions reduced enrichment of the repressive H3K9me3 mark at inflammatory gene promoters in VSMCs via inhibition of the repressive HMT Suv39h1 (26). In parallel, a key miRNA (miR-125b) that targets and downregulates Suv39h1 was upregulated, suggesting cross talk with the noncoding RNA layer (7). Diabetes also enhances production and actions of growth factors such as AngII to augment VSMC dysfunction associated with accelerated CVDs. We profiled AngII-regulated histone PTMs and enhancer and superenhancer repertoires genome wide in rat VSMCs and showed their close relations to AngII-induced gene expression (27). CRISPR-Cas9–mediated deletion of candidate enhancers/superenhancers revealed their target genes associated with VSMC dysfunction. Furthermore, the BRD4 inhibitor JQ1 attenuated AngII-induced inflammation, aortic medial hypertrophy, and hypertension in mice (27), illustrating the translational potential of epigenetic drugs like JQ1 for CVDs.

HG and TGF-β1 induce expression of fibrotic and inflammatory genes in renal cells via activation of TFs such as SMAD, AP1, and NF-κB, which play key roles in the pathogenesis of DKD (7). We and others have shown that histone PTMs participate in these processes in renal MCs, podocytes, and tubular epithelial cells (7). For example, in MCs, HG and TGF-β1 increased levels of permissive modifications H3K4me1/3 and H3K9ac and decreased repressive H3K9me2/3 at promoters of profibrotic genes. They also increased SETD7 expression and recruitment to enrich H3K4me1 at target gene promoters (28). Since then, several studies have demonstrated the involvement of various active and repressive histone PTMs, as well as HMTs and HDMs, in the regulation of genes associated with fibrosis, cell growth, and inflammation using cell and mouse models of DKD (7,19).

Thus, histone PTMs in vascular, inflammatory, and renal cells regulate the expression of genes involved in atheroprogression and renal dysfunction in diabetes and also interface with noncoding RNAs (Fig. 1). While these studies have focused on few candidate genes, histone modifiers, and PTMs, more genome-wide assessments of epigenetic changes using EWAS in relevant clinical and animal models can yield more comprehensive understanding of the association between epigenetics and various

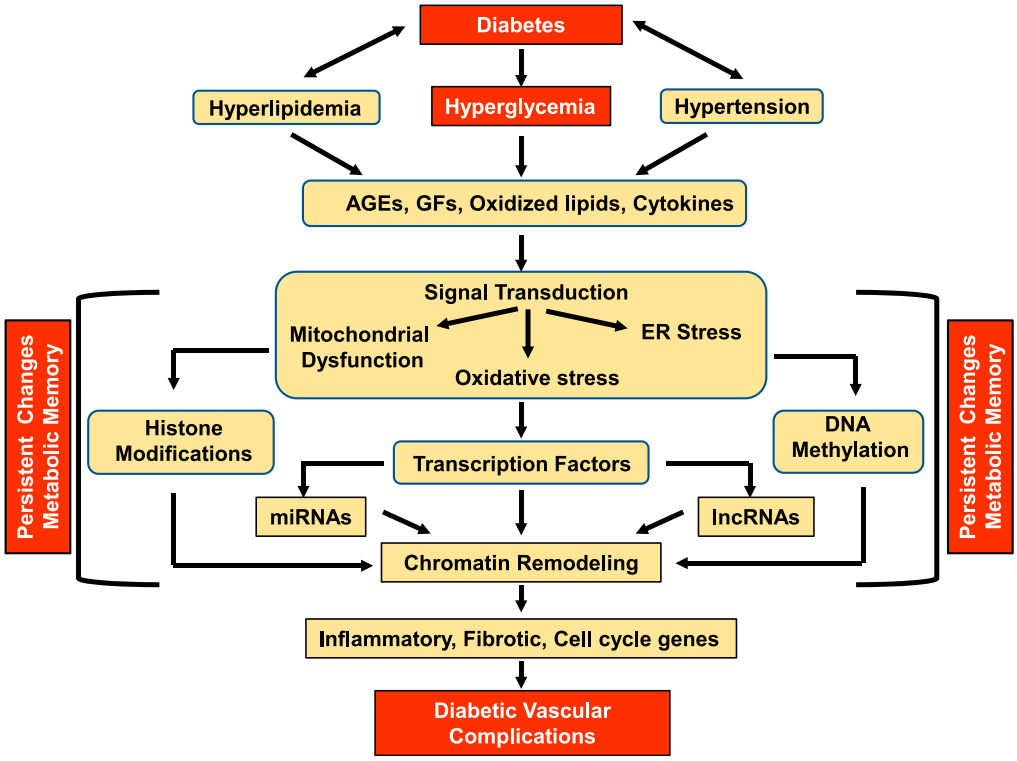

Figure 2—Schematic of epigenetic mechanisms involved in diabetes complications and metabolic memory. Diabetes and its complications are a result of complex interactions between various environmental, genetic, and epigenetic factors. In diabetes, hyperglycemia, hyperlipidemia and hypertension generate excessive levels of AGE, oxidized lipids, cytokines, and growth factors (GFs), which activate multiple signal transduction pathways, mitochondrial dysfunction, oxidative stress, and ER stress, leading to activation of TFs such as NF-κB, SMADs, AP1, and the ER stress–related TF CHOP. Furthermore, activation of these pathways dysregulates epigenetic processes, including histone modifications and DNAme, and alters the expression and function of noncoding RNAs like miRNAs and lncRNAs, resulting in changes in chromatin accessibility to TFs and expression of genes involved in inflammation, fibrosis, and cell cycle regulation that are implicated in diabetes complications. Some of these epigenetic modifications can be maintained through cell division. The persistence of such epigenetic changes over time might explain the phenomenon of metabolic memory in which good glycemic control has long-term beneficial outcomes, whereas a prior history of hyperglycemia leads to continued development of diabetes complications even after achievement of good glucose control.

diabetes complications (7,13). Most EWAS have focused on DNAme variations because DNAme is more stable than histone PTMs and can be studied in archived genomic DNA from clinical cohorts.

DNAme and EWAS in Diabetic Vascular Complications

DNAme at promoters usually leads to inhibition of gene expression, whereas the outcome of DNAme at gene bodies or introns is less predictable. DNA methyltransferase 3A (DNMT3A) and DNMT3B regulate de novo DNAme, whereas DNMT1 acts to maintain DNAme. Although DNAme is the most stable epigenetic mark, it can also be removed by the action of DNA demethylases such as ten-eleven translocation enzymes that catalyze the oxidation of 5-methylcytosine to 5′-hydroxymethylated cytosine (17). Unlike histone PTMs, DNAme does not depict much variation in cultured cells treated acutely with diabetic stimuli like HG and growth factors. However, some experimental models showed that such diabetic stimuli regulate DNAme in cell types related to diabetes complications at genes associated with vascular and renal inflammation and fibrosis (7,13).

In recent years, the availability of increasingly affordable DNAme arrays and high-throughput seq platforms, coupled with robust bioinformatics/data analyses tools to integrate multiomics data sets, has led to a remarkable increase in EWAS. Furthermore, exciting advances in single-cell seq approaches, including single-cell RNA-seq to obtain unbiased gene expression profiles and single-cell assay for transposase-accessible chromatin-seq for examining chromatin accessibility in all major cell types within heterogeneous samples (29), will most certainly enhance the outcomes of EWAS because of the cell type–specific nature of epigenetic changes.

Although DNAme has been implicated in atherosclerosis, much less is known under diabetic conditions. Analysis of multiple global methylation studies in CVD identified differential methylation of 84 genes involved in obesity, inflammation, and lipid and carbohydrate metabolism (30). Hypermethylation of multiple loci was observed in

atherosclerotic versus healthy regions of human aortas, which correlated with expression of genes involved in VSMC and EC functions (31). However, more EWAS are needed with blood and artery samples from subjects with both diabetes and atherosclerosis to find robust associations. For diabetic retinopathy and neuropathy, their associations with histone PTMs and DNAme have been found in experimental models (7,13). Moreover, in a recent report, key genes were identified as associated with diabetic peripheral neuropathy by examination of transcriptomic and DNAme profiles of sural nerves from patients with T2D (32).

On the other hand, several EWAS have associated aberrant DNAme with DKD or renal function alterations relevant to kidney and vascular disease (7). Many of these used Illumina Infinium 450K or EPIC 850K methylation-sensitive bead arrays that provide robust data at base resolution useful for big clinical cohorts. For example, with genome-wide DNAme profiles using blood DNA from participants in both the Atherosclerosis Risk in Communities study and Framingham Heart Study, links were discovered between renal function and DNAme at specific loci related to metabolism, apoptosis, and fibrosis (33). EWAS in kidney samples showed that DNAme at identified CpGs could improve renal function decline prediction in patients with DKD (34). These data highlight the value of epigenetics for early detection of complications. Because of the cell type–specific nature of epigenetics, it is advantageous to perform EWAS in samples from target organs adversely affected by diabetes such as kidney and arteries. However peripheral blood cells obtained noninvasively are more accessible than affected organs and often reflect the inflammation phenotypes seen in target tissues/cells.

Epigenetic Modifications in the Metabolic Memory Phenomenon and as Mediators of HbA_{1c}-Associated Complications

The landmark Diabetes Control and Complications Trial (DCCT) (1983–1993) showed that intensive glycemic control in subjects with T1D significantly delays/prevents microvascular complications compared with conventional therapy (CONV) (35). The profound benefits of intensive therapy (INT) led to the early termination of the DCCT, when all participants were advised to practice INT and followed long-term under the subsequent Epidemiology of Diabetes Interventions and Complications (EDIC) study. Remarkably, those participants originally in the CONV group during the DCCT, to this day, continue to have significantly slower progression of key microvascular complications such as nephropathy, retinopathy, and neuropathy, as well as macrovascular diseases (stroke, atherosclerosis, heart attacks, coronary artery calcification), relative to participants who were in the INT group during DCCT, despite between-group convergence to nearly similar HbA_{1c} levels during EDIC (36). This persistent and enduring benefit of early INT has been termed "metabolic memory" (37). Similar benefits have also been observed in the Pittsburgh Epidemiology of Diabetes Complications Study of T1D patients (38) and the UK Prospective Diabetes Study (UKPDS) of T2D patients (termed "legacy effect") (39).

Oxidant stress may be one of the factors perpetuating metabolic memory of sustained complications by damaging essential lipids, proteins, and DNA (4,7,13). In addition, AGEs that accumulate in diabetes act via their receptors to promote sustained end-organ damage (4,9). We and others examined nuclear epigenetic transcriptional mechanisms to explain sustained "memory/legacy" effects and aberrant phenotypes through multiple cell divisions over time under diabetic conditions (7,13,40).

Experimental models in cell culture and animals were initially used to demonstrate metabolic memory of retinopathy, nephropathy, vascular inflammation, and CVDs and the involvement of epigenetic mechanisms (7,13,40). For example, we found that VSMCs derived from T2D, insulin-resistant, obese *db/db* mice exhibited a preactivated phenotype and metabolic memory of the prior diabetes environment. Relative to VSMCs from nondiabetic *db/+* control littermates, even after culturing in vitro under similar conditions for several passages, VSMCs from diabetic *db/db* mice continued to exhibit increased inflammatory gene expression, migration, monocyte adhesion, oxidant stress, activation of NF-κB and CREB TFs, and signaling pathways associated with growth and migration. In parallel, there was persistent reduction in levels of the repressive HMT SUV39H1and related H3K9me3 at inflammatory gene promoters in VSMCs from diabetic *db/db* mice relative to *db/+* mice, suggesting a loss of chromatin repression at pathological genes in diabetes (26). El-Osta et al. (25) showed short-term exposure of cultured ECs to HG resulted in sustained increases in oxidant stress, and expression of NF-κB p65, inflammatory genes, and SETD7-mediated H3K4me1 at NF-κB, that persisted even after glucose normalization. This was supported by in vivo findings in diabetic mice prone to atherosclerosis (40), suggesting that SET7 could be a useful therapeutic target. Recently, we performed multiomics analyses of renal tubular cells from subjects with T2D versus control subjects, which revealed epigenetic memory of dysregulation of several key genes associated with DKD and renal function (41).

To determine connections between epigenetics and metabolic memory directly in humans, we performed epigenomic profiling in white blood cells from a subset of DCCT/EDIC participants, including 32 case subjects (from the DCCT CONV group, who had high mean DCCT HbA_{1c} and experienced metabolic memory/microvascular complications during EDIC follow-up up to year 10) and 31 control subjects (from the DCCT INT group, without complications up to EDIC year 10) (42). Results showed significant increases in enrichment of the active histone PTM H3K9ac at several genes associated with inflammation and vascular complications in case versus control subjects. Moreover, H3K9ac enrichment in monocytes was

positively associated with glycemic history (HbA_{1c}), suggesting that PTMs like H3K9ac might contribute to metabolic memory in T1D because histone hyperacetylation can promote chromatin relaxation and gene expression (42). However, histone PTMs are not stable, and the persistence of epigenetic marks in the same subject was not assessed in the study.

To address this, we next evaluated the role of DNAme, a more stable epigenetic mark, in metabolic memory and long-term persistence (43). We profiled DNAme using HumanMethylation450K BeadChips in whole blood DNAs archived at DCCT closeout (EDIC baseline, ~1993) from the same case and control DCCT/EDIC participants mentioned above, as well as in blood monocytes of the same participants collected 16–17 years later during EDIC (2010–2011). The results (after corrections for multiple covariates) showed that key DNAme differences identified between the case and control subjects at multiple loci persisted in the same participant 16–17 years apart. Among the 12 loci depicting most significant differential methylation in case versus control subjects that were common in both time periods, we found hypomethylation at cg19693031 in the 3′-untranslated region (3′-UTR) of *TXNIP*. Additionally, treatment of cultured THP1 monocytes with HG in vitro induced persistent hypomethylation at the 3′-UTR cg19693031 of *TXNIP* along with upregulation of *TXNIP* (43). Together, the study strongly supported epigenetic mechanisms in metabolic memory in humans. The significance is further underscored by the fact that TXNIP is a prooxidant associated with hyperglycemia and pancreatic islet dysfunction, as well as several micro- and macrovascular complications in diabetes (44).

However, the limitations and unanswered questions of this second study included the following: *1*) the small sample size, *2*) the case and control subjects differed in HbA_{1c} levels, hence, the association between DNAme at the end of DCCT and mean DCCT HbA_{1c} (i.e., preceding glycemic history) could not be assessed, and *3*) whether DNAme plays a mediatory role in the known association between HbA_{1c} and vascular complications was not determined. These important aspects were addressed in our recent study where we established the involvement of DNAme in metabolic memory using a larger DCCT cohort rigorously phenotyped for multiple complications (45). In this EWAS (using EPIC 850K arrays), we examined associations of DNAme with previous glycemic (HbA_{1c}) history and with subsequent development of complications over an 18-year period in blood DNAs of 499 DCCT/EDIC participants. We found significant associations between DNAme at DCCT closeout and mean HbA_{1c} during DCCT at 186 CpGs, many located in genes related to complications. Validations were performed with internal and external cohorts. Interestingly, we again found that cg19693031 in the *TXNIP* 3′-UTR was the most significant HbA_{1c}-associated CpG ($P = 5.16 \times 10^{-37}$). Moreover, we found multiple other HbA_{1c}-associated CpGs at the *TXNIP* locus as well as at other loci related to diabetes complications. In silico examination of biological functions and chromatin states/accessibility showed that the HbA_{1c}-associated CpGs were enriched in binding sites for C/EBP TF (which is related to myelopoiesis), as well as enhancer/transcription regions in both white blood cells and hematopoietic stem cells, and in open chromatin states in myeloid cells. Treatment of human primary bone marrow $CD34^+$ progenitor cells with HG lead to hypomethylation of cg19693031 and two other CpGs in the *TXNIP* 3′-UTR and increased TXNIP expression. Notably, mediation analyses showed that several CpGs in combination explain 68–97% of the association of mean DCCT HbA_{1c} with retinopathy and nephropathy development during EDIC (up to year 18).

These noteworthy data provide evidence in humans that DNAme changes due to history of different blood glucose control strategies could be maintained 16–17 years later when between-group HbA_{1c} differences converged. Further, DNAme at some of these key "persistent" CpGs mediate the association between prior history of hyperglycemia (HbA_{1c}) and future complications development (retinopathy, DKD) during 18-year follow-up, including metabolic memory. Importantly, since these DNAme changes can modify enhancer activity at stem cells, myeloid, and other cells (45), our data suggest that metabolic memory in humans may arise due to establishment of hyperglycemia-induced epigenetic changes in stem cells that are maintained for long periods in differentiated cells even after removal of the initial stimulus and contribute to chronic diabetes complications resistant to conventional therapies.

Taken together, results from experimental models and human studies support the notion that hyperglycemia-induced persistent epigenetic changes are a major driving force underlying metabolic memory (Fig. 2). Notably, there are now reports showing associations between hypomethylation of *TXNIP* at the same site (cg19693031) and T2D as well as T2D-related phenotypes (7,45,46), indicating that metabolic disorders might alter DNAme at key loci such as *TXNIP* related to sustained complications.

However, despite these important results, some questions remain unanswered, including the issue of causality. Our analyses could not differentiate as to whether DNAme at specific CpGs have causal effects on complication development. Nevertheless, multiple lines of evidence support the involvement of *TXNIP* in complications development. Moreover, using Mendelian randomization by integrating DNAme data with GWAS meta-analysis results, we detected a causal effect of DNAme at one CpG on DKD development (45). Future studies with larger EWAS cohorts and greater disease incidence (longer follow-up) to increase power can help validate additional loci with causal mediation functions and test their molecular mechanisms experimentally. Secondly, our study and findings were mainly related to hyperglycemia-induced (HbA_{1c}) complications (which are mediated in part by DNAme). We focused on hyperglycemia because it is the major risk factor for retinopathy and DKDs (the two complications

analyzed in our study). We therefore examined DNAme associated with mean DCCT HbA_{1c} by adjusting for several covariates including age, sex, diabetes duration, and baseline HbA_{1c} at DCCT entry. Sensitivity analyses ruled out a significant impact from other risk factors including blood pressure, BMI, and lipids. Interestingly, one report showed that as DCCT/EDIC participants age, the association between HbA_{1c} and subsequent CVD development can also be mediated by standard risk factors including blood pressure and lipids (47). Because factors like age, sex, blood pressure, and lipids can alter DNAme, additional studies are needed to investigate the role of DNAme at CpGs associated with such (risk) factors in complications. Thirdly, because our EWAS was from two time points (DCCT baseline and EDIC year 16–17), we cannot specify how long it takes for hyperglycemia to induce epigenetic changes (DNAme) and how long they persist. It is possible epigenetic changes are triggered early on during hyperglycemia, and while most of these can be reversed by glycemic control interventions, some may last longer through cell divisions. Future EWAS at multiple time points can help provide more insights into temporal changes. Lastly, the EDIC participants developed significant CVDs only beyond year 18. So, future EWAS in larger cohorts with higher rates of disease incidence (longer follow-up) can determine the direct connections of DNAme with HbA_{1c} and macrovascular and other complications and can identify methylation quantitative trait loci, as well as causality.

Noncoding RNAs and Diabetes Complications

Evidence shows that much of the genome is transcribed into RNA, the majority of which does not code for protein. These noncoding RNAs include miRNAs and lncRNAs (7). MiRNAs are short (~22-nucleotide) noncoding RNAs that bind to the 3′-UTRs of their target mRNAs, leading to posttranscriptional silencing and translational repression or RNA degradation. lncRNAs are long transcripts (>200 nucleotides) that exert cellular effects via more complex, diverse, cell- and location-specific mechanisms. They can regulate epigenetic mechanisms to fine-tune the expression of key target genes in *cis* and *trans* via interaction with key RNA binding proteins, epigenetic factors, enhancers, and chromatin and also modulate the actions of miRNAs, which in turn can affect biological and disease processes (Fig. 1). We and others have demonstrated the involvement of miRNAs and lncRNAs in the regulation of inflammatory, fibrotic, and other genes associated with various diabetes complications and also assessed their therapeutic potential (7).

Given the relatively poor conservation and cell type–specific expressions/functions of lncRNAs, characterization of lncRNAs has been quite challenging. However, advances in high-throughput sequencing and bioinformatics and availability of publicly available genomic data sets have significantly accelerated lncRNA research and led to identification of lncRNA dysregulation in most cell types implicated in diabetes complications. Work from our laboratory and others identified lncRNAs that regulate EC and VSMC dysfunction and VSMC phenotypic switching (48,49). We found that an AngII-induced lncRNA, *Giver*, promotes inflammatory gene expression, oxidative stress, and proliferation in rat VSMCs (50). Notably, a human ortholog *GIVER* was upregulated in hypertensive patients and attenuated in patients taking antihypertensive agents that block AngII signaling (50), thus suggesting a role for *GIVER* in hypertension, a major risk factor in diabetes complications.

Macrophage inflammation and foam cell formation play important roles in CVDs. We identified three separate macrophage lncRNAs that regulate inflammation under diabetes and obesity conditions in mice and humans. By transcriptome profiling, we found that several lncRNAs are upregulated in macrophages from T2D *db/db* mice, including *E330013P06* and *Dnm3os*, that were also increased in monocytes from T2D patients (49). Both lncRNAs were induced by HG and palmitic acid in cultured macrophages, and they upregulated inflammatory genes. *E330013P06* induced foam cell formation, whereas *Dnm3os* promoted phagocytosis in macrophages (49). We also showed the involvement of altered promoter histone PTMs and key lncRNA binding proteins in the mechanisms by which these macrophage lncRNAs regulate their target genes. The third lncRNA, *Mist*, was downregulated in macrophages from obese mice fed a high-fat diet. Based on several lines of evidence, we found that *Mist* has anti-inflammatory roles and is positively associated with insulin sensitivity and good cardiometabolic profiles. Hence, downregulation of "protective" lncRNAs like *Mist* and upregulation of other "harmful" lncRNAs in monocytes/macrophages might be associated with accelerated inflammation in obesity and diabetes (51). LncRNAs can therefore be valuable biomarkers of inflammation in cardiometabolic disease (52).

Dysregulated expression of several lncRNAs has been observed in renal cells cultured under diabetic conditions (like TGF-β1 treatment) and in kidneys from animals with DKD (7). Notably, we found that *lncMGC* was highly expressed in diabetic mice glomeruli and MCs (53). This lncRNA is a host gene for numerous miRNAs (the miR-379 cluster) and is induced by CHOP, an endoplasmic reticulum (ER) stress-responsive TF. In diabetic mice, administration of chemically modified antisense oligonucleotides (locked nucleic acid-modified gapmers) targeting *lncMGC* could inhibit the expression of *lncMGC* and key component miRNAs and ameliorate features of early DKD (e.g., glomerular extracellular matrix production and hypertrophy). These data suggest that lncRNA-dependent mechanisms are linked to oxidative/ER stress in DKD, and highlight the translational potential of targeting them for amelioration of DKD (53). Moreover, persistent upregulation of *lncMGC* via signaling circuits and ER stress may contribute to metabolic memory (7).

The interaction of lncRNAs and enhancers provides another epigenetic layer of trans-gene regulation that is gaining interest (49). Furthermore, GWAS have identified

single nucleotide polymorphisms in lncRNAs that can alter functions associated with CVD and metabolic disease (52). Thus, lncRNAs regulate key genes/functions in multiple cells and their dysregulation in diabetes can significantly contribute to vascular complications (7,48) (Fig. 1).

Translational Potential of Epigenetic Changes: Future Perspectives

The reversible nature of epigenetics presents key opportunities for therapeutic intervention. Currently, many inhibitors targeting epigenetic modifiers are being tested in cancer. Some of these inhibitors have been tested in experimental models of diabetes complications, including JQ1, and inhibitors of key HDACs and HMTs, but with mixed results (7), emphasizing the importance of optimal selectivity and limited off-target effects. Nonspecific genomic effects of currently available epigenetic drugs could be partly offset by taking advantage of epigenetic engineering and CRISPR-Cas9 editing for locus-specific alteration of epigenetic marks (DNAme or histone PTMs) (7). RNA-based therapies such as siRNAs or antisense oligonucleotides with nuclease-resistant modifications targeting epigenetic modifiers and lncRNAs/miRNAs show some promise (7), but further studies are needed to improve delivery, efficacy, and specificity. The rapidly expanding field of epigenomics is spurred by sophisticated technological and computational approaches, coupled with publicly available reference data sets. Moreover, single-cell-based transcriptomic and epigenomic profiling (29) have already yielded unprecedented insights into identities of specific cells and genes affected in diabetes complications. In this era of precision medicine, inclusion of epigenotypes, epigenetic drugs, and biomarkers along with genotypes and conventional therapies can help improve the clinical management of diabetic vascular complications and metabolic memory.

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References

1. Nathan DM. Long-term complications of diabetes mellitus. N Engl J Med 1993;328:1676–1685

2. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA 2002;287:2570–2581

3. Rask-Madsen C, King GL. Vascular complications of diabetes: mechanisms of injury and protective factors. Cell Metab 2013;17:20–33

4. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev 2013;93:137–188

5. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet 2007;8:253–262

6. Nilsson E, Ling C. DNA methylation links genetics, fetal environment, and an unhealthy lifestyle to the development of type 2 diabetes. Clin Epigenetics 2017; 9:105

7. Kato M, Natarajan R. Epigenetics and epigenomics in diabetic kidney disease and metabolic memory. Nat Rev Nephrol 2019;15:327–345

8. Kanter JE, Averill MM, Leboeuf RC, Bornfeldt KE. Diabetes-accelerated atherosclerosis and inflammation. Circ Res 2008;103:e116–e117

9. Naka Y, Bucciarelli LG, Wendt T, et al. RAGE axis: animal models and novel insights into the vascular complications of diabetes. Arterioscler Thromb Vasc Biol 2004;24:1342–1349

10. Natarajan R, Gerrity RG, Gu JL, Lanting L, Thomas L, Nadler JL. Role of 12-lipoxygenase and oxidant stress in hyperglycaemia-induced acceleration of atherosclerosis in a diabetic pig model. Diabetologia 2002;45:125–133

11. Shanmugam N, Reddy MA, Guha M, Natarajan R. High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. Diabetes 2003;52:1256–1264

12. Yerneni KK, Bai W, Khan BV, Medford RM, Natarajan R. Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. Diabetes 1999;48:855–864

13. Reddy MA, Zhang E, Natarajan R. Epigenetic mechanisms in diabetic complications and metabolic memory. Diabetologia 2015;58:443–455

14. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes Dev 2009;23:781–783

15. Bernstein BE, Stamatoyannopoulos JA, Costello JF, et al. The NIH Roadmap Epigenomics Mapping Consortium. Nat Biotechnol 2010;28:1045–1048

16. Zhou VW, Goren A, Bernstein BE. Charting histone modifications and the functional organization of mammalian genomes. Nat Rev Genet 2011;12:7–18

17. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484–492

18. Ahlqvist E, van Zuydam NR, Groop LC, McCarthy MI. The genetics of diabetic complications. Nat Rev Nephrol 2015;11:277–287

19. Sullivan KM, Susztak K. Unravelling the complex genetics of common kidney diseases: from variants to mechanisms. Nat Rev Nephrol 2020;16:628–640

20. Whyte WA, Orlando DA, Hnisz D, et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013;153: 307–319

21. Miao F, Gonzalo IG, Lanting L, Natarajan R. In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. J Biol Chem 2004;279:18091–18097

22. Li Y, Reddy MA, Miao F, et al. Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation. J Biol Chem 2008;283:26771–26781

23. Miao F, Smith DD, Zhang L, Min A, Feng W, Natarajan R. Lymphocytes from patients with type 1 diabetes display a distinct profile of chromatin histone H3 lysine 9 dimethylation: an epigenetic study in diabetes. Diabetes 2008;57:3189–3198

24. Brasacchio D, Okabe J, Tikellis C, et al. Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. Diabetes 2009;58: 1229–1236

25. El-Osta A, Brasacchio D, Yao D, et al. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. J Exp Med 2008;205:2409–2417

26. Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory

phenotype of vascular smooth muscle cells in diabetes. Proc Natl Acad Sci U S A 2008;105:9047–9052
27. Das S, Senapati P, Chen Z, et al. Regulation of angiotensin II actions by enhancers and super-enhancers in vascular smooth muscle cells. Nat Commun 2017;8:1467
28. Sun G, Reddy MA, Yuan H, Lanting L, Kato M, Natarajan R. Epigenetic histone methylation modulates fibrotic gene expression. J Am Soc Nephrol 2010;21: 2069–2080
29. Gawad C, Koh W, Quake SR. Single-cell genome sequencing: current state of the science. Nat Rev Genet 2016;17:175–188
30. Fernández-Sanlés A, Sayols-Baixeras S, Subirana I, Degano IR, Elosua R. Association between DNA methylation and coronary heart disease or other atherosclerotic events: a systematic review. Atherosclerosis 2017;263:325–333
31. Zaina S, Heyn H, Carmona FJ, et al. DNA methylation map of human atherosclerosis. Circ Cardiovasc Genet 2014;7:692–700
32. Guo K, Elzinga S, Eid S, et al. Genome-wide DNA methylation profiling of human diabetic peripheral neuropathy in subjects with type 2 diabetes mellitus. Epigenetics 2019;14:766–779
33. Chu AY, Tin A, Schlosser P, et al. Epigenome-wide association studies identify DNA methylation associated with kidney function. Nat Commun 2017;8: 1286
34. Gluck C, Qiu C, Han SY, et al. Kidney cytosine methylation changes improve renal function decline estimation in patients with diabetic kidney disease. Nat Commun 2019;10:2461
35. Nathan DM, Genuth S, Lachin J, et al.; Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–986
36. Nathan DM; DCCT/EDIC Research Group. The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications study at 30 years: overview. Diabetes Care 2014;37:9–16
37. Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study. JAMA 2003;290:2159–2167
38. Orchard TJ, Dorman JS, Maser RE, et al. Factors associated with avoidance of severe complications after 25 yr of IDDM. Pittsburgh Epidemiology of Diabetes Complications Study I. Diabetes Care 1990;13:741–747
39. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med 2008;359:1577–1589
40. Keating ST, Plutzky J, El-Osta A. Epigenetic changes in diabetes and cardiovascular risk. Circ Res 2016;118:1706–1722
41. Bansal A, Balasubramanian S, Dhawan S, Leung A, Chen Z, Natarajan R. Integrative omics analyses reveal epigenetic memory in diabetic renal cells regulating genes associated with kidney dysfunction. Diabetes 2020;69: 2490–2502
42. Miao F, Chen Z, Genuth S, et al.; DCCT/EDIC Research Group. Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. Diabetes 2014;63:1748–1762
43. Chen Z, Miao F, Paterson AD, et al.; DCCT/EDIC Research Group. Epigenomic profiling reveals an association between persistence of DNA methylation and metabolic memory in the DCCT/EDIC type 1 diabetes cohort. Proc Natl Acad Sci U S A 2016;113:E3002–E3011
44. Thielen L, Shalev A. Diabetes pathogenic mechanisms and potential new therapies based upon a novel target called TXNIP. Curr Opin Endocrinol Diabetes Obes 2018;25:75–80
45. Chen Z, Miao F, Braffett BH, et al.; DCCT/EDIC Study Group. DNA methylation mediates development of HbA1c-associated complications in type 1 diabetes. Nat Metab 2020;2:744–762
46. Soriano-Tárraga C, Jiménez-Conde J, Giralt-Steinhauer E, et al.; GENESTROKE Consortium. Epigenome-wide association study identifies TXNIP gene associated with type 2 diabetes mellitus and sustained hyperglycemia. Hum Mol Genet 2016;25:609–619
47. Bebu I, Braffett BH, Pop-Busui R, Orchard TJ, Nathan DM, Lachin JM; DCCT/EDIC Research Group. The relationship of blood glucose with cardiovascular disease is mediated over time by traditional risk factors in type 1 diabetes: the DCCT/EDIC study. Diabetologia 2017;60:2084–2091
48. Leung A, Natarajan R. Long noncoding RNAs in diabetes and diabetic complications. Antioxid Redox Signal 2018;29:1064–1073
49. Das S, Reddy MA, Natarajan R. Role of epigenetic mechanisms regulated by enhancers and long noncoding RNAs in cardiovascular disease. Curr Opin Cardiol 2020;35:234–241
50. Das S, Zhang E, Senapati P, et al. A novel angiotensin II-induced long noncoding RNA giver regulates oxidative stress, inflammation, and proliferation in vascular smooth muscle cells. Circ Res 2018;123:1298–1312
51. Stapleton K, Das S, Reddy MA, et al. Novel long noncoding RNA, macrophage inflammation-suppressing transcript (*MIST*), regulates macrophage activation during obesity. Arterioscler Thromb Vasc Biol 2020;40:914–928
52. Zhang X, Li DY, Reilly MP. Long intergenic noncoding RNAs in cardiovascular diseases: challenges and strategies for physiological studies and translation. Atherosclerosis 2019;281:180–188
53. Kato M, Wang M, Chen Z, et al. An endoplasmic reticulum stress-regulated lncRNA hosting a microRNA megacluster induces early features of diabetic nephropathy. Nat Commun 2016;7:12864