



HHS Public Access

Author manuscript

Nat Rev Nephrol. Author manuscript; available in PMC 2019 December 03.

Published in final edited form as:

Nat Rev Nephrol. 2019 June ; 15(6): 327–345. doi:10.1038/s41581-019-0135-6.

Epigenetics and epigenomics in diabetic kidney disease and metabolic memory

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Abstract

The development and progression of diabetic kidney disease (DKD), a highly prevalent complication of diabetes mellitus, is influenced by both genetic and environmental factors. DKD is an important contributor to the morbidity of patients with diabetes mellitus, indicating a clear need for an improved understanding of disease aetiology to inform the development of more efficacious treatments. DKD is characterized by an accumulation of extracellular matrix, hypertrophy, and fibrosis in kidney glomerular and tubular cells. Increasing evidence shows that genes associated with these features of DKD are regulated not only by classical signalling pathways, but also by epigenetic mechanisms, involving chromatin histone modifications, DNA methylation, and non-coding RNAs. These mechanisms can respond to changes in the environment and importantly, might mediate the persistent long-term expression of DKD-related genes and phenotypes induced by prior glycaemic exposure, despite subsequent glycaemic control, a phenomenon called metabolic memory. Detection of epigenetic events during the early stages of DKD could be valuable for timely diagnosis and prompt treatment to prevent progression to end-stage renal disease. Identification of epigenetic signatures of DKD via epigenome-wide association studies might also inform precision medicine approaches. Here, we highlight the emerging role of epigenetics and epigenomics in DKD, and the translational potential of candidate epigenetic factors and non-coding RNAs as biomarkers and drug targets for DKD.

Introduction

The worldwide prevalence of both type 1 mellitus (T1DM) and type 2 diabetes mellitus (T2DM) has rapidly increased over the past 30 years^{1,2}, with consequential increases in the prevalence of microvascular and macrovascular diabetes complications³⁻⁷. Epidemiological studies show that improvements in diabetes management have led to reductions in diabetes-

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Author Contributions

Both authors contributed to researching data for the article, discussing the article's content, writing the article and reviewing and editing the manuscript before submission.

Competing interests

The authors declare no competing interests.

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Databases

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associated cardiovascular morbidity over recent decades; however, the impact of these strategies on rates of end-stage renal disease (ESRD) have been minimal⁸. Approximately 30–40% of patients with diabetes mellitus (T1DM and T2DM) develop DKD, and about 50% of them can progress to ESRD^{9–12}. Current therapies to manage DKD include control of blood pressure and glucose levels, and treatment with angiotensin-receptor blockers (ARBs) and angiotensin-converting-enzyme inhibitors; however, these modalities show limited efficacy in preventing the progression of DKD. Genetic factors and signal transduction pathways affect the expression of genes and phenotypes associated with DKD. However, the escalating rates of DKD indicate the need for a more in-depth understanding of the underlying molecular mechanisms to identify better therapies for this disease^{13–16}.

Epigenetic mechanisms can affect gene expression and function without changing the underlying DNA sequence and can mediate crosstalk between genes and the environment.^{17,18} As the development and progression of diabetes mellitus and DKD are strongly influenced by environmental factors, such as nutritional status, which in turn alters epigenetic states, it is likely that epigenetic mechanisms have a key role in the pathogenesis of DKD.^{14,19,20} In this Review we describe our current understanding of the role of epigenetics and epigenomics in DKD. We discuss how epigenetic factors and non-coding (nc)RNAs could be used as biomarkers and drug targets for DKD diagnosis, prognosis and treatment, and highlight how our rapidly expanding knowledge of epigenetic variations opens a window of opportunity for improved clinical management of DKD.

Pathogenesis of DKD

DKD is characterized by an accumulation of extracellular matrix (ECM) proteins such as collagen and fibronectin in renal compartments, resulting in tubular interstitial fibrosis, glomerular mesangial hypertrophy and expansion, thickening of the glomerular basement membrane, podocyte foot process effacement, and inflammation due to the infiltration of monocytes and macrophages. All of these factors contribute to renal dysfunction and can ultimately lead to ESRD.^{13,21–23} Diabetogenic stimuli, including high blood glucose levels (> 15 mM); advanced glycation end products (AGEs); growth factors such as transforming growth factor beta 1 (TGF- β 1), angiotensin II (AngII) and platelet-derived growth factor; and inflammatory cytokines have been implicated in the pathogenesis of DKD owing to their adverse effects on multiple renal cell types.^{24–29} Upregulation of the above-mentioned growth factors and cytokines, activates signal transduction pathways, including protein kinase C and Akt kinase cascades, leading to the activation of key effector transcription factors such as SMADs, nuclear factor kappa B (NF- κ B) and upstream stimulatory factors (USFs) (Fig. 1). Together, these mechanisms promote the expression of genes associated with fibrosis, hypertrophy, apoptosis, inflammation, oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and autophagy (Fig. 1).^{13–16,22,28–36} In particular, TGF- β 1, which is overexpressed in several renal cell types in diabetes mellitus, has been widely studied in DKD progression due to its pro-fibrotic effects.^{13,21,26,27} Identification of these pathways and the use of unbiased transcriptomic profiling approaches to uncover additional pathogenic factors have led to the initiation of clinical trials to assess new therapeutic targets in DKD.^{37–42} However, the escalating rates of DKD underscore the need to explore the contribution of other mechanisms, such as epigenetics, to DKD, particularly

for patients with long-standing diabetes mellitus who experience persistent complications even after glucose normalization, a phenomenon attributed to metabolic (or hyperglycaemic) memory.

Metabolic memory in DKD

Most complications in patients with diabetes mellitus are associated with hyperglycaemia. Although glucose levels can be controlled through diet, exercise, and medications including insulin, many patients continue to experience numerous life-threatening complications following glucose normalization, suggesting the existence of a ‘memory’ of the prior glucose exposure in target cells, which leads to persistence of its detrimental effects long after glycaemic control has been established.⁴³⁻⁴⁶ The phenomenon of hyperglycaemic memory has been observed in clinical trials and experimental models. The landmark Diabetes Control and Complications Trial (DCCT) showed that intensive glycaemic control delayed progression of microvascular complications, including nephropathy and neuropathy, compared to conventional therapy in patients with T1DM. After cessation of the DCCT in 1993, both groups of patients received intensive glycaemic control and were followed long-term (1994 to present) as part of the observational Epidemiology of Diabetes Intervention and Complications (EDIC) study⁴⁷. During EDIC, both groups achieved similar mean haemoglobin A1c (HbA1c) levels of approximately 8%.^{43,44} However, patients in the original DCCT intensive treatment group demonstrated a significantly lower risk of developing microvascular and macrovascular complications relative to those of patients in the original conventional treatment arm of DCCT.⁴³⁻⁴⁶ This difference in risk between the two groups was attributed to prolonged hyperglycaemia in the conventional therapy group during DCCT. The UK Prospective Diabetes Study reported similar long-term benefits of intensive glycaemic control in patients with T2DM. These benefits lasted well after cessation of the intervention, suggesting a ‘legacy effect’ of glycaemic control.⁴⁸

The persistent effects of high glucose through metabolic memory remain a major hurdle in the effective management of diabetic complications. An increasing body of evidence supports a role for epigenetic mechanisms in metabolic memory, suggesting that targeting these mechanisms might offer opportunities for the development of novel approaches to prevent and treat persistent diabetic complications, including DKD.

Epigenetics: new insights into DKD

Genome-wide association studies (GWAS) have identified only a few potential genes, loci, and single nucleotide polymorphisms (SNPs) associated with DKD, and few of these have achieved genome-wide significance in replication studies.⁴⁹⁻⁵² By contrast, a growing body of literature implicates a role for epigenetic modifications in DKD, particularly because they can induce changes in the expression of genes in response to environmental factors^{14,53-56}. Epigenetic modifications enable mitotically and/or meiotically heritable changes in gene function to occur without altering the underlying DNA sequence^{17,18}. Of note, most disease-associated SNPs are present in non-coding regions of the genome, including regulatory regions such as promoters and enhancers, and in ncRNAs,⁵⁷ which can affect gene expression by altering transcription factor binding, chromatin accessibility, and through

other epigenetic mechanisms. Thus, findings from GWAS and epigenome studies show convergence on shared pathways, suggesting that combined analyses of genetic and epigenetic susceptibility loci might provide a powerful approach to identify pathogenic pathways in DKD, as has been done in CKD⁵⁸. In order to understand the mechanisms and consequences of epigenetic changes, it is important to first understand the organizational structure of nuclear DNA and chromatin.

Nuclear DNA is packaged into a histone–protein complex called chromatin (Fig. 2). The basic subunit of chromatin is the nucleosome, which comprises an octamer of two copies of each of the core histone proteins H2A, H2B, H3 and H4, wrapped by 147 base pairs of chromosomal DNA.^{59,60} Epigenetic modifications affect chromatin structure and the expression of genes by either covalently modifying DNA or histone proteins to affect the binding of transcriptional regulators to DNA, or through actions of non-coding (nc)RNAs [G]. The most well-studied epigenetic marks include DNA methylation [G] of cytosines, histone post-translational modifications (PTMs), and (nc)RNAs. Whereas ‘epigenetics’ refers to epigenetic modifications that occur in single genes or sets of genes, ‘epigenomics’ refers to the broader, genome-wide profiles and effects of these modifications. The epigenome therefore collectively dictates cell identity and the gene expression and phenotypes of specific cells. Epigenetic control of gene expression is important in several important biological processes, including development, cellular identity, genomic imprinting [G] and cell differentiation. In the context of diabetes mellitus, epigenetic control of gene expression can mediate cellular responses to environmental factors and contributes to the differential susceptibility of monozygotic twins to diabetes mellitus and its complications.

Changes in the environment, including nutritional changes, can either transiently or chronically affect epigenetic states.^{54,61,62} Furthermore, as epigenetic marks are heritable, maternal behaviour and nutritional status (that is, undernutrition or overnutrition) can induce intrauterine and early postnatal epigenetic changes that might predispose offspring to metabolic diseases.^{54,62} A number of studies have also highlighted a connection between low birth weight, poor nutrition, low nephron endowment and the development of renal disease — a series of events most likely mediated by epigenetic factors.⁶³ Notably, the role of epigenetics in mediating the effects of maternal nutritional status on kidney development was elegantly demonstrated in a 2018 study⁶⁴, which showed that in a rat model of intrauterine growth restriction, kidneys of postnatal day 1 pups had reduced weight as well as pronounced DNA hypomethylation relative to controls. In mice, deletion of *Dnmt1*, which encodes the maintenance DNA methyltransferase 1, in nephron progenitor cells, phenocopied the effects of intrauterine growth restriction, with reduced nephron number at birth and reduced expression of genes critical for nephrogenesis,⁶⁴ supporting a role for DNA methylation in mediating the effects of altered nutritional status on renal programming.

Epigenetics has also been strongly associated with risk of both T1DM and T2DM,^{65–67} but this aspect is not discussed in this Review.

DNA methylation in DKD

DNA methylation is mediated by DNA methyltransferases DNMT1, DNMT2 and the DNMT3 family members DNMT3a, DNMT3b and DNMT3-like (DNMT3L). DNMT3a/3b mediate de novo DNA methylation (that is, the establishment of new DNA methylation patterns), whereas DNMT1 is a maintenance methyltransferase that copies the methylation pattern of parental DNA strands.⁶⁰ On the other hand, the function of DNMT2 is relatively less clear with data suggesting it serves more as a tRNA methyltransferase.⁶⁸ DNA methylation can be reversed by ten-eleven translocation (TET) proteins, which convert 5-methylcytosine to 5-hydroxymethylcytosine.⁶⁹ Methylation of DNA at gene promoter regions can repress gene expression through various mechanisms, including the recruitment of transcriptional repressors and by interfering with transcription factor binding, whereas methylation of DNA at gene bodies/transcribed regions can regulate the elongation [G] phase of transcription and alternative splicing [G].⁶⁰

Several experimental and clinical studies indicate the existence of links between CpG DNA methylation and DKD. For example, experimental models support the involvement of DNA methylation in glomerular and proximal tubular epithelial cell functions, including filtration, glucose and solute handling;⁷⁰ dysregulation of critical genes associated with these processes has a critical role in the pathogenesis of CKD and DKD. In models of TGF- β 1-induced renal fibrosis, upregulation of TGF- β 1 induced hypermethylation of *Ras1* promoter, leading to reduced expression of this Ras-GTP suppressor and increased Ras-GTP signalling, increased fibroblast activation, and fibrosis.⁷¹ Changes in *Ras1* promoter DNA methylation were reversed by TET3-mediated hydroxymethylation, with concomitant reduction in fibrosis.⁷² In another study, mice with streptozotocin-induced albuminuria showed decreased expression of the histone deacetylase [G] SIRT1. Interestingly, tubule-specific overexpression of SIRT1 prevented albuminuria by inducing hypermethylation of the *Cldn1* gene (as SIRT1 deacetylates and activates DNMT1), leading to downregulation of the tight junction protein Claudin-1, in podocytes, suggesting crosstalk between podocytes and proximal tubules through epigenetic mechanisms.⁷³ On the other hand, mice with proximal tubule-specific deletion of *Sirt1* showed aggravation of albuminuria in association with reduced *Cldn1* methylation, enhanced histone acetylation, and upregulation of Claudin-1.⁷³ These and other studies showing the protective effects of SIRT1 in DKD have prompted further evaluation of SIRT1 as a therapeutic target for DKD.⁷³ In the *db/db* mouse model of diabetic kidney disease, transcriptional repression of the transcription factor KLF4 was associated with increased DNA methylation at the promoter region of the gene that encodes nephrin, along with podocyte apoptosis and proteinuria.⁷⁴ Another in vivo study showed that the promoter region of *Agt*, which encodes angiotensinogen, and is associated with hypertension and CKD, exhibited DNA hypomethylation and acetylation of histone H3 at lysine 9 (H3K9) along with increased expression in early stages of DKD in *db/db* mice. These epigenetic marks were resistant to treatment with the antidiabetic drug pioglitazone, suggesting that conventional therapies might be unable to reverse epigenetic marks (as discussed later).⁷⁵ A 2017 study demonstrated that cultured proximal tubule cells exposed to high glucose, and kidneys from streptozotocin-treated mice exhibited DNA hypomethylation of *MIOX*, associated with enhanced expression of its encoded protein, myo-inositol oxygenase. Hypomethylation of

MIOX was associated with enhanced binding of the transcription factor, Sp1, to its promoter and the expression of genes involved in oxidative stress, hypoxia and fibrosis.⁷⁶ In a further study, levels of SP1, NFκB, and DNMT1 were markedly increased in podocytes from diabetic *db/db* mice; inhibition of DNMT1 with 5-azacytidine, 5-aza-2'-deoxycytidine, or Dnmt1 small interfering RNA, induced renal protection, suggesting that epigenetic regulation of the SP1–NFκB–DNMT1 signalling pathway might be involved in podocyte injury in DKD.⁷⁷ Increased expression of the DNA methylation eraser, TET2, increased expression of TGF-β1 by demethylating CpG islands in the TGF-β1 regulatory region.⁷⁸ Demethylation of USF1 binding sites in the *Tgfb1* promoter, associated with increased USF1 binding, decreased DNMT1 binding and increased expression of *Tgfb1* has also been demonstrated in primary mesangial cells from diabetic *db/db* mice.⁷⁹ In this study, treatment of mesangial cells with hydrogen peroxide also induced demethylation of the *Tgfb1* promoter, leading to upregulation of *Tgfb1* expression, suggesting that reactive oxygen species might mediate mesangial fibrosis in DKD through aberrant DNA methylation of the *Tgfb1* locus.

DNA methylation EWAS

An increased appreciation of the contribution of epigenetic mechanisms to human disease, including complications of diabetes, has led to a surge in epigenome-wide association studies (EWAS) of DNA methylation status in populations of patients with CKD and DKD (Table 1).

In a 2010 case-control study of 192 patients with T1DM,⁸⁰ researchers used the Illumina HumanMethylation27 BeadChip (which assesses ~27,000 CpGs across the genome) to compare DNA methylation levels from genomic DNA of patients with and without DKD. Nineteen CpGs correlated with time to DKD development, including one near *UNC13B*, which contains a SNP associated with DKD. In another early study, DNA from saliva was used to compare DNA methylation levels in >14,000 genes in African American and Hispanic patients with diabetes mellitus (3 T1DM and 21 T2DM) and ESRD, with those of patients with diabetes mellitus but no DKD.⁸¹ Interestingly, 21% of the 187 differentially methylated genes had been previously associated with CKD or DKD, suggesting potential functional relevance of the findings to disease pathogenesis. Another study that used the Illumina HumanMethylation27 BeadChip to assess epigenetic markers of renal function in 972 African Americans (including 298 with DKD) from the Genetic Epidemiology Network of Arteriopathy (GENOA) study⁸² found that 24 of the top 30 DNA methylation markers significantly associated with estimated glomerular filtration rate (eGFR) were related to aging, inflammation, or cholesterol pathways.

The development of improved DNA methylation arrays, high-throughput sequencing technologies, and powerful approaches for data analysis have prompted additional EWAS in DKD and CKD populations over the past ~ 10 years with the aim of improving disease prognosis and patient classification.⁸³ In the first EWAS study of kidney biopsy samples, DNA methylation profiling of microdissected tubuli from patients with DKD or nondiabetic CKD compared with those of control individuals without renal disease revealed significant differences between the two groups.⁸⁴ Notably, the differentially methylated regions

localized mostly to enhancers and were enriched in binding sites for important kidney specific transcription factors. Key genes associated with renal fibrosis, such as collagens, showed differential methylation patterns that correlated with gene expression. Another study, used the Illumina HumanMethylation450 BeadChip (which assesses ~450,000 CpG features across the genome) to profile DNA methylation in 255 T1DM patients with CKD (including 113 with DKD) and 152 controls (including 113 T1DM) without renal disease.⁸⁵ The researchers then evaluated the biological and functional relevance of the differentially methylated genes using in silico analyses based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database of pathways. These analyses led to the identification of 23 genes that had significant methylation changes associated with CKD, and suggested a potential functional role for DNA methylation changes in influencing CKD development.

Mitochondrial function has an important role in the pathogenesis of DKD. A study of DNA methylation alterations in genes related to mitochondrial function in patients with DKD compared the methylation of blood DNA from 196 patients with T1DM and DKD to that of 246 patients with T1DM but with no renal disease.⁸⁶ The researchers identified 54 CpG sites where methylation changes were significantly associated with DKD using both the HumanMethylation27 and HumanMethylation450 BeadChips; from a further sub-analysis, 46 of the top-ranked identified variants for DKD, including several genes related to mitochondrial function, were found to be differentially methylated in patients with ESRD relative to those without renal disease.

An analysis of DNA methylation levels in participants from the Chronic Renal Insufficiency Cohort (CRIC) with the lowest and highest rates of eGFR decline (that is, those with stable renal function versus rapid decliners) using the HumanMethylation450 BeadChip⁸⁷ identified associations between methylation at CpG islands in certain genes and renal fibrosis. Participants (20 patients including 10 with DKD) with stable renal function had greater DNA methylation of *NPHP4*, *IQSEC1*, and *TCF3* than rapid decliners (20 patients including 10 with DKD). Genes associated with oxidative stress and inflammation were also differentially methylated between the two groups. These data support a role for epigenetic changes in renal function decline in patients with CKD. Replication studies are needed to validate these findings and to determine whether these key epigenetic differences exist in patients with DKD, although these CKD cohorts had a significant number of subjects with DKD.

The role of DNA methylation in the trans-generational inheritance of metabolic diseases has been well studied because of the ability of diet and the environment to alter epigenetic marks at genes associated with metabolic functions.^{54,62,88} A study of the effects of foetal exposure to hyperglycaemia on kidney development and function⁸⁹ used the HumanMethylation27 BeadChip to examine leukocyte DNA methylation in 29 adult non-diabetic offspring of mothers with T1DM (the case group) and 28 non-diabetic offspring of fathers with T1DM (the control group). Differentially methylated CpGs were not located in any gene directly related to kidney development or function, but DNMT1, a key DNA methyltransferase involved in gene regulation in early development, showed reduced DNA methylation in the case group, prompting the researchers to conclude that T1DM and exposure to hyperglycaemia in utero might predispose offspring to changes in kidney function via

pathways related to imprinting. Additional studies in larger cohorts are needed to strengthen these conclusions and determine whether kidney-specific processes are also involved.

Many of the early EWAS were limited by small sample sizes, cross-sectional design, lack of replication samples, and/or the presence of comorbidities in the patient population under study. A 2017 EWAS⁹⁰ assessed whole blood DNA methylation profiles associated with kidney function in patients from the Atherosclerosis Risk in Communities (n=2,264 including 586 with diabetes) and Framingham Heart (n=2,595 including 394 with diabetes) studies, representing the largest EWAS of a CKD cohort to date. Using the HumanMethylation450 BeadChip, the researchers identified 19 CpGs associated with eGFR and CKD, five of which associated with renal fibrosis and showed concordant DNA methylation changes in renal cortical biopsy samples. The top CpGs mapped to enhancer regions; moreover, the researchers identified methylation at a CpG site cg19942083 at *PTPN6* that was associated with low renal *PTNP6* expression, higher eGFR, and reduced renal fibrosis, implying that *PTPN6* could be further evaluated as an epigenetic biomarker for fibrosis/CKD because locus-specific DNA methylation changes may cause CKD through alteration in the expression of the related functionally relevant genes. Notably, the data also shows the utility of non-invasive blood based DNA methylation profiling to screen for kidney disease specific targets. They also observed significant enrichment of eGFR-associated CpGs in regions that bind to the transcription factors *EBF1*, *EP300*, and *CEBPB*. In addition, they used epigenome data from ENCODE [G] to determine putative functions of these CpGs which showed the presence of active enhancers around them implying that they are associated with gene expression. This well-designed EWAS has several key strengths, including its large sample size, use of appropriate methodologies to correct for white blood cell subtype composition, the evaluation of important kidney phenotypes, use of an ethnically diverse population, its replication of findings in an independent cohort, and its rigorous analytical methods. Future studies are needed to stratify data obtained by this study according to diabetes status to identify methylation changes that are also specific to DKD. Nevertheless, many of the data analyses and covariate correction approaches used in this study will likely inform future EWAS efforts to establish epigenetic markers of renal function in patients with CKD and DKD.

The Pima Indians of Southwestern USA have among the highest incidence of T2DM of any population in the world, and also very high incidence of DKD. A 2018 study that examined leukocyte DNA methylation patterns at ~400,000 CpG sites in 181 Pima Indians with T2DM⁹¹ identified an association between methylation at 77 CpG sites and eGFR decline over a 6-year period. Furthermore, three of these 77 probes showed directionally consistent associations with fibrosis in matched kidney tissue, adding to the growing number of reports supporting a role of DNA methylation and epigenetic variations as potential biomarkers of DKD progression.

EWAS: challenges and opportunities

A limitation of EWAS of renal phenotypes is the paucity of available renal biopsy material. In one approach, to circumvent this limitation, a 2018 study used a network in silico analytic approach to identify renal compartment-specific gene expression and methylated sites

related to DKD through mining publicly available gene expression omnibus (GEO) database for glomerular and tubular datasets, enriched function analyses by gene ontology (GO), and KEGG analyses, and integrated the expression data with Illumina 27k methylation ChIP data. This endeavour enabled the authors to generate glomerular and tubular specific networks of genes associated with related tissue-specific functions in DKD pathogenesis, including mitochondrial function and acid-base regulation.⁹² With increasing availability of transcriptome, epigenome and other datasets from numerous clinical trials and consortia, as well as more powerful data integrative tools and reference datasets, it is likely that such in silico approaches will help accelerate new discoveries in DKD epigenome research.

Since epigenetic marks affect gene expression in a cell-specific manner, early EWAS were often confounded by the cellular heterogeneity of the samples. This challenge has been addressed to some extent by the adoption of computational deconvolution approaches that use published reference DNA methylation datasets to adjust for differences in the cell composition between samples from different participants, so that methylation changes can be corrected for any variation caused by cell type composition.⁹³ In addition, efforts have been made to compare EWAS findings from whole blood genomic DNA with candidate DNA methylation and gene expression changes in matched renal biopsy samples in the same patient to determine the extent to which changes in blood reflect those in the kidney.⁹⁰

Another challenge is the limited genome coverage provided by the HumanMethylation27 and HumanMethylation450 BeadChips. Although these BeadChips have been used extensively for population-based studies, they cover only a small fraction of the genome with little coverage of enhancer regions, and might therefore miss important epigenetic changes. The more recently developed Illumina EPIC arrays cover nearly 850,000 CpG sites, and have many improved features, including coverage at many enhancers.⁹⁴ Sequencing methods, including methylated DNA immunoprecipitation sequencing (Me-DIP-seq) [G], reduced representation bisulfite sequencing (RRBS-seq) [G] and whole genome bisulfite sequencing [G], are also increasingly being used to identify more numbers of differentially methylated sites across the genome, facilitated by steadily decreasing costs. These sequencing approaches, especially whole genome bisulfite, are powerful and can not only uncover differential changes genome-wide, but can also be combined with in silico bioinformatics and data analysis packages, as well as publicly available omics datasets for integrative analyses to obtain unprecedented new biological data. This information in turn can accelerate discovery of disease relevant loci and drug targets through associations with clinical phenotypes, biomarkers in biofluids (serum and urine) as well as subject-specific variations for personalized medicine.⁹⁵ As mentioned earlier, given that most disease-associated SNPs identified by GWAS are present in non-coding regions of the genome, identification of epigenotype [G] variations using EWAS can complement genotype variations uncovered by GWAS and provide powerful information about disease susceptibility and causality. In addition, advances in single-cell (transcriptome and epigenome) sequencing of multiple kidney cell types will greatly aid future EWAS efforts.⁹⁶ Exciting reports of single-cell and single-nucleus sequencing of mouse and human kidney cells have already uncovered cell types related to specific kidney diseases because epigenetic changes and their effects on gene expression and DKD can be renal cell type-specific , for

example, podocytes, endothelial cells, tubular cells and mesangial cells in the kidney.⁹⁷⁻¹⁰⁰ Thus, a combination of GWAS, EWAS, and single-cell or single-nucleus sequencing, along with integrative analyses with other omics datasets, has potential to uncover novel new causal genes or loci associated with DKD and determine the role of epigenetic changes in regulating the expression of these genes. Furthermore, controlling the epigenetic changes at these newly identified genes and loci might be a novel therapeutic approach for treating DKD and metabolic memory.

Histone modifications

PTMs of nucleosomal histone proteins in chromatin regulate gene expression and are an important component of epigenetic regulation⁵⁹ (Fig. 2). Genome-wide profiling of modified histones using chromatin immunoprecipitation (ChIP) sequencing [G] (ChIP-seq) has shown that specific different types of histone modifications are enriched in specific genomic locations such as regulatory regions (enhancers and promoters), transcriptionally-repressed regions, transcriptional start sites, and actively transcribed regions.¹⁰¹ The major histone PTMs are lysine acetylation, methylation, and ubiquitination; serine and threonine phosphorylation; and arginine methylation.^{59,102}

Histone acetylation can unwind and relax chromatin to enhance gene expression by various mechanisms which facilitate accession of transcription factors and cofactors, leading to transcription initiation and elongation through nucleosome remodelling⁵⁹ (Fig. 2, Fig. 3). In general, histone acetylation is enriched at promoters and enhancers of actively transcribed genes and reduced in repressed genomic regions. By contrast, the location of histone methylation varies. For example, monomethylation, dimethylation or trimethylation of H3 at lysine 4 (H3K4me1/2/3) and dimethylation or trimethylation of H3 at lysine 36 (H3K36me2/3) are enriched at transcriptionally active genome regions, whereas trimethylation of H3 at lysine 9 (H3K9me3), trimethylation of H3 at lysine 27 (H3K27me3), and trimethylation of H4 at lysine 20 (H4K20me3) are enriched in repressed regions,⁵⁹ and histone monomethylation of H3 at lysine 4 (H3K4me1) is enriched at enhancer regions.¹⁰³ Active enhancers are marked by enrichment of acetylated H3 at lysine 27 (H3K27ac) along with mediator complexes [G] and transcription factors. Super-enhancers, which are long stretches of enhancers enriched for binding motifs of transcription factors, are cell type-specific; hence, examination of their differential regulation by epigenetic marks could provide a wealth of information about cell-type function alterations in disease states.¹⁰⁴ Poised promoters [G] in developmental genes are enriched with bivalent chromatin modifications, for example, both active (H3K4me3) and repressive (H3K27me3) marks which allow dynamic changes in expression (silenced to highly expressed) of developmentally regulated genes in a stage-specific manner.^{101,102} Although the role of DNA methylation in epigenetic transmission and heredity is generally better accepted than that of histone modifications, some repressive histone PTMs can be epigenetically inherited.¹⁰⁵

The histone acetyltransferases p300 and CREB-binding protein (CBP) are transcriptional coactivators and catalyse histone acetylation; histone deacetylases (HDACs) and sirtuins can act as transcriptional corepressors and remove acetylation marks from histones.^{59,102}

Histone lysine methyltransferases (HMTs) catalyse lysine methylation whereas histone lysine demethylases (KDMs) remove methyl groups from histones.⁵⁹ HMTs and KDMs use specific lysine residues as substrates¹⁰⁶ and can also target non-histone proteins. Acetyltransferases and methylases are called epigenetic writers; deacetylases and demethylases are called epigenetic erasers; and proteins such as the bromodomain-containing proteins (for example, bromodomain-containing protein 4), which recognize acetylated proteins at promoters and enhancers and have an active role in gene transcription, are called epigenetic readers.¹⁰⁷⁻¹⁰⁹

Studies of histone PTMs at key metabolic genes suggest that their epigenetic regulation have a critical role in the pathogenesis of T1DM, T2DM, obesity, and the metabolic syndrome.^{65,110,111} These findings indicate that histone PTMs are also involved in diabetic complications — a proposal supported by preclinical studies of DKD. For example, induction of the pro-fibrotic genes *Pai1* (which encodes plasminogen activator inhibitor 1) and *p21* in cultured rat mesangial cells in response to TGF β or high glucose is associated with enrichment of H3K9ac and H3K14ac and the presence of p300 and CBP at Smad and SP1 binding sites in the promoters of these genes.¹¹² ChIP assays of glomeruli of diabetic mice showed that increases in *Pai1* and *p21* expression in vivo were associated with a similar enrichment of H3K9ac and H3K14ac at their promoters. Furthermore, increased expression of *Pai1*, *Coll1a1* (which encodes the collagen α -1(I) chain), and *Ctgf* (which encodes connective tissue growth factor) induced by TGF- β 1 was accompanied by enrichment of active histone methylation (H3K4me1/2/3) and decreased repressive marks (H3K9me2/3), at their promoters, together with increased occupancy of the HMT SET7.¹¹³ Consistent with these findings, exposure of cultured rat mesangial cells to 12(*S*)-hydroxyeicosatetraenoic acid, which is increased in DKD, induced similar effects to TGF- β 1 on these histone PTMs and pro-fibrotic genes, and the effects of this oxidised lipid on fibrosis were also mediated in part via SET7.¹¹⁴

Changes in the expression of key DKD-associated non-coding RNAs can also be regulated by histone PTMs. TGF- β 1 induced histone acetylation (H3K9ac, H3K14ac and H3K27ac) of the promoter region of micro RNA (miR)-192 in renal cells is induces the expression of Smads and the proto-oncogene *Ets-1*.^{30,115,116} As discussed below, miR-192 is upregulated by TGF- β 1 and has an important role in renal fibrosis and DKD pathogenesis.

In vivo studies in the *db/db* model of T2DM have demonstrated that several chromatin histone PTMs, occur in the promoters of *Pai1* and *Ager* (which encodes the AGE-specific receptor, RAGE) in glomeruli, together with increased recruitment of the RNA polymerase II suggesting that both active and repressive epigenetic marks cooperate to promote chromatin unwinding and enhance the recruitment of transcription factors to these DKD-associated genes. Of note, administration of the ARB losartan ameliorated key indices of DKD and reduced H3K9ac enrichment at *Pai1* and *Ager* promoters, but did not reverse other diabetes-induced histone PTMs in these mice.¹¹⁷ This incomplete amelioration of epigenetic changes following treatment could be a reason why some patients do not respond well to routine DKD drugs such as ARBs. Combination therapy with conventional therapies and drugs that target epigenetic processes could be a viable option for such patients.²⁴ In another study, increased recruitment of RNA polymerase II and enriched H3K4me2, but diminished

H3K27me3, correlated with the expression of DKD-related genes in mouse and rat models of T1DM. However, there were some differences in the expression of a few genes (*Mcp-1* and *Lamc1*) and epigenetic regulators (*Ezh2* K27 methylase and the corresponding demethylase) between rats and mice, suggesting some epigenetic changes could be species-specific.¹¹⁸ This aspect should be taken into consideration while translating findings of epigenetic changes/mechanisms in animals to human DKD. Podocyte-specific deletion of the HMT EZH2 decreased H3K27me3 levels at the promoter region of *Jag1*, which encodes the Notch ligand Jagged1, to increase *Jag1* expression, and sensitized mice to glomerular disease in three injury models, including diabetes (db/db T2DM mice) because *Jag1* can activate Notch signalling and glomerular disease.¹¹⁹ By contrast, inhibition of the KDMs *Jmjd3* (also known as *Kdm6b*) and *Utx* (also known as *Kdm6a*) increased H3K27me3 levels and attenuated glomerular disease in these models. Lower H3K27me3 levels and higher *UTX* levels were also detected in glomerular podocytes from humans with DKD, suggesting that *JAG1* induction by histone demethylation through UTX1 and to activate Notch signalling in podocytes may also be involved in the development of glomerular dysfunction in human DKD.¹¹⁹

Increased renal inflammation as a result of macrophage infiltration contributes to the pathogenesis of DKD. Histone modifications have been implicated in mediating the activation of NF- κ B and other inflammatory factors in vascular cells and monocytes in response to high glucose.^{120,121} Epigenome profiling studies have demonstrated differences in H3K4me2 and H3K9me2 marks at genes associated with diabetes and inflammation in high glucose-treated monocytes, and in blood monocytes and lymphocytes from patients with T1DM diabetes compared to those in normoglycaemic controls.¹²¹⁻¹²³ The HMT SET7 promotes methylation of H3K4 and coactivates inflammatory genes downstream of NF κ B in monocytes.¹²⁴ Given the above-described role of SET7 in promoting the expression of pro-fibrotic genes in mesangial cells exposed to TGF β , these findings suggest that this HMT might have a general role in regulating the expression of key pathological genes in various cell types including monocytes, endothelial and mesangial cells associated with diabetic complications, and supports efforts to explore small molecule inhibitors of SET7.¹²⁵

Together, these data from cell, animal, and clinical models clearly suggest that epigenetic regulation of DKD-related genes through histone PTMs in addition to DNA methylation has a critical role in the pathogenesis and progression of DKD (Fig. 1). Population-based EWAS of histone PTM profiles are less common than those of DNA methylation status because they require relatively large amounts of high quality ChIP-grade antibodies, fresh tissues or blood samples, and larger sample sizes. However, the increased availability of online datasets of enhancers with histone PTM profiles and of the chromatin states of multiple cell types should prove useful for integration with data from RNA sequencing and DNA methylation. Having such integrative and imputed data can significantly aid in interpreting the functional relevance of epigenome changes to the etiology and pathogenesis of DKD for future development of biomarkers and drug targets.

Epigenetics in metabolic memory

Epigenetic mechanisms, including DNA methylation and histone PTMs, have been implicated in the metabolic memory of diabetic complications, including DKD (Fig. 3). Analysis of chromatin and DNA in blood monocytes and lymphocytes of patients with T1DM who participated in the DCCT and EDICs trials^{126,127} showed significant enrichment of H3K9ac at key inflammatory genes in monocytes from patients who received conventional therapy during DCCT and experienced progression of nephropathy or retinopathy (cases) during subsequent EDIC follow-up, compared with levels in patients who received intensive glycaemic control during DCCT and who did not experience progression of nephropathy and retinopathy (controls) during EDIC.¹²⁶ Furthermore, this study identified a significant association between HbA1c level and H3K9ac, suggesting that H3K9ac might contribute to metabolic memory in patients with T1DM¹²⁶ owing to hyperglycemia induced sustained chromatin relaxation at susceptible genomic regions via histone hyperacetylation.

Another study of the DCCT and EDIC cohort¹²⁷ used the HumanMethylation450 BeadChip to profile DNA methylation in genomic DNA from whole blood taken at DCCT baseline and from blood monocytes obtained ~17 years later during the EDIC phase from the same set of T1DM patients. After adjustment for multiple co-variates, including age, gender, and cell-type composition, the researchers identified a set of 150–250 regions that were differentially hypomethylated or hypermethylated in cases (patients who received conventional therapy during DCCT and experienced progression of nephropathy or retinopathy by EDIC year 10) and controls (patients who received intensive glycaemic control during DCCT and did not experience progression of nephropathy or retinopathy during EDIC). Notably, 12 differentially methylated loci were common in both whole blood and monocytes, including hypomethylation in cases at the 3' untranslated region (UTR) of *TXNIP*, which encodes thioredoxin-interacting protein, and is associated with hyperglycaemia and related complications including DKD.^{127,128} Similarly, exposure of cultured THP1 human monocytes to high glucose induced persistent hypomethylation at the 3'-UTR of *TXNIP*. Furthermore, association analyses were performed between DNAm in whole blood and monocytes from each DCCT patient (samples collected ~ 17 years apart) and their individual mean HbA1c levels measured at multiple time points from the start of DCCT to the time of monocyte collection during EDIC years 16/17. This led to the identification of a set of differentially methylated loci (including *TXNIP* 3'UTR) that depicted similar association with prior HbA1c in both whole blood and monocyte DNA, i.e the association persisted for ~17 years in the same patient. These findings of a persistence of memory in an epigenetic signature and association with prior history of hyperglycemia strongly support a role for epigenetic mechanisms in metabolic memory. Interestingly, hypomethylation of *TXNIP* at the same site (cg19693031) has also been observed in other cohorts, in association with higher fasting blood glucose levels and lipidaemia¹²⁹, indicating that metabolic abnormalities and diabetes might promote similar DNA methylation changes at specific genomic loci that in turn lead to sustained complications.

Studies of experimental models also support a role for epigenetic mechanisms in metabolic memory. One study showed that, compared to VSMCs from aortas of non-diabetic

heterozygous *db/+* mice, VSMCs from diabetic *db/db* mice showed high expression of inflammatory genes, associated with lower levels of the HMT SUV39H1 and lower levels of promoter H3K9me3 — a repressive mark.¹³⁰ This difference in histone methylation persisted even after the VSMCs had been cultured for several passages. VSMCs from diabetic *db/db* mice also showed upregulation of miR-125b, which targets SUV39H1, demonstrating that miRNAs are key regulators of epigenetic factors in metabolic memory.^{130,131} In another study, endothelial cells cultured for short periods under conditions of high glucose exhibited persistent induction of the NF- κ B subunit p65, indicative of inflammation, accompanied by sustained enrichment of H3K4me1 and the corresponding HMT SET7 at the p65 promoter, long after normalization of glucose levels.^{132,133} In parallel, the researchers observed sustained and persistent increases in the expression of NF- κ B-dependent inflammatory genes *Ccl2* (also known as *Mcp-1*) and *Vcam-1*. These cell culture data demonstrate the existence of metabolic memory mediated by epigenetic mechanisms in cultured cells, and are supported by in vivo findings from studies of glucose-infused normal mice and in diabetic mice prone to atherosclerosis.¹³²⁻¹³⁵ Similar evidence of metabolic memory have also been reported in experimental models of diabetic retinopathy and cardiovascular disease,¹³⁶ suggesting that metabolic memory is an important component of the epigenetic changes that occur in diabetes, and that it has consequences for disease outcomes and response to treatment.

RNA modifications in DKD

In addition to DNA, RNA can also be epigenetically modified (Fig. 2). RNA methylation regulates gene expression by altering RNA stability and degradation. The methyltransferase complex METTL3–METTL14–WTAP converts adenosine to N6-methyladenosine (m6A) on RNA.¹³⁷ In human stem cells, activation of SMAD2/3 by TGF- β signalling promotes binding of the m6A methyltransferase complex to transcripts involved in early cell-fate decisions: the rapid downregulation (that is, degradation) of these transcripts enables the timely exit of cells from pluripotency.¹³⁸ Given the major role of TGF- β 1 in DKD, similar epigenetic crosstalk between TGF- β , SMAD2/3, and m6A methyltransferase could be involved in DKD. Indeed, according to the Nephroseq database (<https://www.nephroseq.org>), the expression of *METTL3*, *METTL14*, and *WTAP* is lower in glomeruli from diabetic mice, patients with CKD, and patients with focal segmental glomerulosclerosis than in healthy control samples, suggesting that negative feedback of m6A methylation could be disrupted resulting in sustained expression of TGF- β and SMAD2/3. This mechanism might also contribute to the persistent expression of genes associated with DKD.

Non-coding RNAs

Data from ENCODE and other consortia show that the majority of the genome is transcribed into RNA, much of which is non-coding.^{101,139,140} ncRNA includes not only small ncRNAs, such as miRNAs, but also long ncRNAs (lncRNAs), both of which are part of the epigenome and have regulatory functions.

MicroRNAs in DKD—miRNAs are well established epigenetic regulators and modulators of gene expression. These endogenous small ncRNAs are 20–25 nucleotides in length, and typically silence expression of target genes by hybridizing to the 3'UTR of target mRNAs, leading to translational repression and/or mRNA degradation.¹⁴¹ miRNAs are thought to regulate up to 60% of human protein-coding genes. They are generated as relatively long primary transcripts (pri-miRNAs) following transcription by RNA polymerase II in the nucleus, which are then processed to ~70 nucleotide stem-loop structures called precursor miRNAs (pre-miRNAs) by the Drosha–DGCR8 complex. Pre-miRNAs are transported to the cytoplasm and processed to mature miRNAs by Dicer. Genetic deletion of Dicer or Drosha in mice results in severe renal phenotypes, including proteinuria, underscoring the functional relevance of miRNAs in the kidney.^{142–146} The first miRNA shown to have a functional role in DKD was miR-192, which acts by targeting key repressors to promote the expression of ECM and collagen, and to augment the pro-fibrotic effects of TGF- β 1.^{30,116,147,148} Numerous miRNAs are now thought to regulate key features of DKD, such as podocyte apoptosis, the accumulation of ECM, glomerular and tubular hypertrophy, and fibrosis. The roles of miRNAs in DKD have been reviewed elsewhere.^{14,149–151} Of note, MeCP2 (methyl CpG binding protein 2) suppresses miRNA processing through Drosha–DGCR8 complex¹⁵² and modulates miR-25-NOX4 expression related to oxidant stress in diabetic kidney,¹⁵³ suggesting an interesting connection from DNA methylation to miRNA regulation.

Long non-coding RNAs in DKD—LncRNAs are long transcripts (>200 nucleotides and up to ~100kb in length) that have many similarities with mRNAs but lack protein-coding (translation) potential.^{139,154} Increasing evidence suggests that lncRNAs have distinct cellular roles that affect various biological mechanisms and processes, including gene transcription, splicing, mRNA stability, epigenetic regulation, cell cycle control, differentiation, and the immune response.^{155,156} lncRNAs regulate the expression of local and distal genes through various mechanisms, which include binding and targeting histone modifying complexes to various loci to affect chromatin states and modulate transcription.^{155–157} lncRNAs also serve as host RNAs for miRNAs, and act as modular molecules with individual domains that enable them to specifically associate with DNA, RNA, and/or proteins to affect gene expression.^{28,30,31,156–158} Thus, lncRNAs form an integral part of the epigenome. Interest in lncRNAs increased following the discovery that many lncRNAs are dysregulated in human diseases; furthermore, many GWAS studies have observed SNPs in lncRNA loci,^{155,159} underscoring the potential connection of lncRNAs to disease states, including diabetes and its complications.

Reports showing the involvement of lncRNAs in DKD have emerged only in the past few years.¹⁶⁰ A 2009 report showed that key miRNAs (miR-216a and miR-217) with DKD-related functions were induced by TGF- β 1 together with their host lncRNA RP23–298H6.1–001 in mesangial cells.²⁸ Another study in mouse mesangial cells treated with TGF- β 1 (mentioned earlier) demonstrated that miR-192 is co-regulated with its host lncRNA CJ241444 through histone acetylation of the lncRNA promoter and the proto-oncogene Ets-1, leading to the enrichment of Smads and up-regulation of both the lncRNA and miR-192.³⁰ Administration of AngII — a peptide hormone that is associated with renal

dysfunction, hypertension, and DKD — to cultured VSMCs induced several lncRNAs; including *Inc-Ang362*, which serves as a host for miR-221 and miR-222, known to promote VSMC proliferation. Findings from this study showed that *Inc-Ang362* might drive VSMC proliferation through these miRNAs.¹⁶¹ Another AngII-induced novel lncRNA called *Giver* is regulated by the nuclear receptor NR4A3 and promotes oxidant stress and inflammation in VSMCs.¹⁶² Expression of *Giver* was increased in AngII infused mice with hypertension, and expression of a human orthologue *GIVER* was elevated in arteries from patients with hypertension. A further study showed that key lncRNAs induced by AngII overlap with enhancers regulated by AngII in VSMCs; CRISPR–Cas9-mediated deletion of candidate lncRNA-associated enhancers reduced the expression of nearby AngII-regulated genes, demonstrating crosstalk between these two epigenetic systems.¹⁶³

The lncRNA plasmacytoma variant translocation 1 (*PVT1*) is also involved in fibrosis and DKD pathogenesis.¹⁶⁴ The *PVT1* locus was first implicated in diabetes-associated ESRD in a GWAS in 2007.¹⁶⁵ Several miRNAs that map to the *PVT1* locus, including miRs1204–1208, were subsequently shown to be upregulated in human mesangial cells in response to high glucose conditions, inducing the expression of ECM-related genes.¹⁶⁶ Another study found that 21 lncRNAs were upregulated in two models of renal fibrosis but downregulated in *Smad3*-knockout mice, indicating a role for TGF β –SMAD signalling in the regulation of these lncRNAs.¹⁶⁷ A 2016 study identified *Inc-megacluster (Inc-MGC)*, a lncRNA host for nearly 40 miRNAs in the miR-379 cluster, to promote mesangial cell ECM accumulation and hypertrophy in mouse models of early DKD.³¹ Expression of *Inc-MGC* and several of its resident miRNAs were increased in glomeruli of mouse models of T1DM and T2DM, and in cultured mouse mesangial cells exposed to high glucose and TGF- β 1 through mechanisms involving the endoplasmic reticulum (ER) stress-related transcription factor, CHOP (C/EBP homologous protein)). miR-379 targets (and therefore represses) *Edem3*, which encodes an inhibitor of ER stress, whereas miR-494, another miRNA in the miR-379 cluster, targets *Atf3*, a repressor of CHOP. Thus, upregulation of *Inc-MGC* and this miR-379 cluster can augment ER stress in DKD, which in turn might lead to persistent expression of *Inc-MGC* and miR-379 cluster miRNAs, continuing the circuits and potentially contributing to metabolic memory (Fig. 4).³¹ Notably, a human orthologue of *Inc-MGC* is also upregulated in human mesangial cells following exposure to high glucose and TGF- β 1.³¹ This finding is important because unlike miRNAs, lncRNAs are generally not well conserved across species, so it suggests that this lncRNA cluster could also be relevant to human DKD.

Podocyte foot process effacement, apoptosis, and dysfunction are key features of proteinuria and DKD. In mouse podocytes, the lncRNA *Tug1* was shown to regulate changes in mitochondrial bioenergetics associated with DKD via interactions with PPAR gamma coactivator α (PGC-1 α), and subsequent effects on key PGC-1 α targets that are important for mitochondrial biogenesis. Mice with diabetes showed downregulation of *Tug1*; however, overexpression of *Tug1* in mice was sufficient to reverse the negative impact of diabetes on mitochondrial function and ameliorate DKD¹⁶⁸. These data suggest that decrease of *Tug1* in podocytes in diabetes reduces mitochondrial function and biogenesis due to down-regulation of PGC-1 α target genes.

Several reports have described crosstalk between lncRNAs and miRNAs in the pathogenesis of DKD.^{169,170} For example, the lncRNA *ErbB4-IR* promotes diabetic kidney injury by hybridizing and inhibiting miR-29b, which targets genes involved in collagen production and ECM accumulation.¹⁶⁹ Another lncRNA, downregulation of *Lin01619* promotes oxidative stress and podocyte injury in DKD through reduced sequestering of miR-27a, which inhibits forkhead transcriptional factor Foxo1 and induces ER stress.¹⁷⁰ The lncRNA *1700020I14Rik* reduces cell proliferation and fibrosis in DKD through interactions with the miR-34a-5p–Sirt1–HIF-1 α signaling pathway.¹⁷¹ Finally, the lncRNA *MALAT1* controls renal tubular epithelial pyroptosis [G] in experimental models of DKD by inhibiting the expression of miR-23c, leading to increased expression of its gene target *Elav11* which induce pro-inflammatory programmed cell death in tubular epithelial cells related to tubular injury,¹⁷² further highlighting the numerous mechanisms by which lncRNAs induce tissue injury in DKD.

As mentioned earlier, infiltration of inflammatory cells and inflammation have important roles in DKD. Transcriptional profiling of blood cell samples from patients with T2DM has revealed several dysregulated lncRNAs with potential roles in inflammation and insulin resistance.¹⁷³ RNA sequencing of bone marrow macrophages from obese, diabetic *db/db* mice showed differential expression of several lncRNAs compared to those of control non-diabetic *db/+* mice, including increased expression of *E330013P06*, a pro-inflammatory lncRNA that enhances the formation of foam cells [G].¹⁷⁴ Levels of *E330013P06* were also higher in macrophages from mice fed a high-fat diet and in monocytes from humans with T2DM than in chow-fed and non-diabetic counterparts, respectively. A 2018 study showed that another pro-inflammatory lncRNA (*Dnm3os*) was similarly upregulated in macrophages of obese and diabetic mice, and in monocytes of humans with T2DM.¹⁷⁵ *Dnm3os* binds to nucleolin, a major nucleolar protein of eukaryotic cells; the available evidence indicates this interaction is disrupted under diabetic conditions.¹⁷⁵ Disruption of *Dnm3os*–nucleolin binding enables *Dnm3os* to enhance histone acetylation (H3K9ac) at promoters of key inflammatory genes, increasing their transcription and promoting macrophage dysfunction.¹⁷⁵ Human macrophage lncRNAs have also been associated with cardiometabolic disorders,¹⁷⁶ indicating that macrophages in the circulation might represent a non-invasive source for the identification of lncRNAs associated with inflammation and DKD.

Together, these reports highlight not only the involvement of lncRNAs in the pathogenesis of DKD, but also illustrate crosstalk between epigenetic layers (that is, between lncRNAs, miRNAs and chromatin), and provide insights into the signalling circuits these factors trigger to mediate fibrotic events, inflammation, and changes in mitochondrial bioenergetics associated with DKD.^{15,28,31,151,168,177} We expect that future studies will uncover additional lncRNAs related to DKD, including causal SNPs in lncRNA loci.

Epigenetic biomarkers

Early detection of DKD is important for preventing progression to renal failure; thus, predictive biomarkers could have a major clinical impact in the diagnosis and treatment of DKD. Several biomarkers of DKD progression have been reported¹⁷⁸ with most being proteins, peptides, growth factors, and cytokines. As discussed earlier, EWAS have also

identified epigenetic signatures of specific DNA methylation sites and histone PTMs that are associated with key DKD clinical phenotypes, including eGFR, albuminuria, and aberrant renal morphology. Such signatures could be developed as predictive biomarkers of DKD development as has been done in other fields. For example, the methylation status of the gene that encodes insulin has been exploited as a biomarker to examine pancreatic beta cell loss,¹⁷⁹ and tracking methylation patterns in circulating DNA can be used to identify tissue-specific cell death.¹⁸⁰ As podocytes are observed in the urine of patients with DKD, methylation of podocyte-specific genes in urine could serve as a biomarker of podocyte dysfunction and death. Combining traditional biomarkers (such as urinary albumin, serum cystatin C, serum and urine neutrophil gelatinase associated lipocalin (NGAL), urine kidney injury molecule 1 (KIM-1), and urine transferrin) with novel biomarkers, including circulating proteins, DNA and ncRNAs, could greatly enhance the early detection of renal function decline in patients with diabetes mellitus.

miRNAs in biofluids (that is, urine, serum and plasma) are already being investigated as non-invasive epigenetic biomarkers of various diseases including DKD, and remain a very active area of translational research.^{14,151} These ncRNAs are of particular interest as diagnostic biomarkers of DKD owing to their inherent stability in exosomes [G] and biofluids and availability of methods for their detection and quantification. A number of reports have characterized profiles of key miRNAs in urine, urinary sediment, and plasma of patients with various kidney diseases, showing varying levels of correlation with nephropathy or fibrosis.¹⁸¹⁻¹⁸⁷ Urinary exosomes, which originate from most renal cells, can be extremely valuable for miRNA profiling in renal disorders.^{186,188} Urinary exosomes from patients with T1DM and microalbuminuria, and mesangial cell-derived exosomes from cultured mesangial cells exposed to high glucose levels showed enrichment for miR-145 which is associated with inflammatory pathways and thus might be a potential biomarker for DKD.¹⁸⁹ Of note, however, the heterogeneity of extracellular vesicles, including exosomes, and the diversity of circulating extracellular RNAs are poorly understood, as are the mechanisms by which they elicit cellular signalling and communication. These limitations have hampered progress in this field and caution must therefore be taken when evaluating the potential of exosomes and their contents as biomarkers of disease.¹⁹⁰

A study that profiled over 700 miRNAs in the urine of 40 patients with T1DM and various stages of DKD provided strong support for the use of miRNA profiles as molecular predictors of DKD.¹⁹¹ Other studies have profiled miRNAs in urine, urinary sediment, and serum from patients with differing degrees of DKD, fibrosis, renal function decline and albuminuria,^{181,184,187,189,192-194}. For example, one report suggested that TGF- β 1-regulated circulating miRNAs can be used as biomarkers of renal function decline and rapid progression to ESRD in patients with T1DM.¹⁹³ Another study reported tissue-specific miRNA expression patterns for four types of kidney disease —namely, DKD, focal segmental glomerulosclerosis, IgA nephropathy, and membranoproliferative glomerulonephritis, suggesting such patterns could be used as biomarkers for these diseases and provide clues as to the underlying pathological mechanisms.¹⁹⁵

Of note, as miRNAs and DNA are relatively stable in paraffin-embedded sections, archived pathology sections represent a rich resource for the future development of epigenetic

biomarkers. Methods to assess circulating lncRNAs in biofluids are also under development and the utility of lncRNAs as biomarkers of disease is expected to be another active area of research. For example, one study demonstrated the expression of the lncRNA *NR_033515* to be significantly higher in- serum of patients with DKD than in controls and to correlate with the severity of DKD and other diagnostic markers of kidney injury such as KIM-1.¹⁹⁶

Translating epigenetic findings in DKD

Targeting epigenetic factors

Currently available therapies for DKD, such as ARBs, show limited efficacy in preventing the progression of renal disease in many patients²⁴. Studies in animal models show that these agents are limited in their ability to inhibit or reverse epigenetic changes that occur in the diabetic kidney^{14,117}, suggesting that future therapeutic approaches might require a combination of conventional therapy plus epigenetic agents. The potential reversibility of epigenetic marks provides exciting opportunities for therapeutic intervention.

Some epigenetic drugs are already being used in oncology, neuronal diseases, inflammation, and fibrosis¹⁹⁷, including HDAC inhibitors and DNA methylation inhibitors¹⁹⁸, suggesting their efficacy could be assessed in DKD. A 2017 computational systems-level network analysis of microarray data from 33 independent HDAC inhibitor studies, revealed enrichment of pathways involved in the metabolic syndrome and diabetes mellitus.¹⁹⁹ Integration of these data with ENCODE CHIP-seq datasets suggested that these HDAC inhibitors suppressed gene targets of the transcription factor EP300, which had previously been implicated in the pathogenesis of diabetes. These in silico data were tested experimentally by treating vascular endothelial cells from a diabetic patient with HDAC inhibitors or EP300 inhibitors which blocked diabetes associated EP300 target genes.¹⁹⁹ This type of in silico computational method could be useful to identify new drugs for diabetes and DKD, and also repurpose drugs that are already in use for other diseases. Although early generation HDAC inhibitors have been tested in animal models of DKD with some showing beneficial effects,²⁰⁰ these agents were generally non-specific; thus, it may be of value to study the efficacy of newer, more selective inhibitors or activators of HDACs. The class III HDAC protein, SIRT1, suppresses AGE-induced expression of pro-fibrotic genes via upregulation of antioxidant gene in glomerular mesangial cells.²⁰¹ Administration of the SIRT1 agonist BF175 to diabetic OVE26 mice attenuated kidney injury, suggesting that targeting (upregulating) this HDAC might be of therapeutic value.²⁰² Inhibitors of the HMT EZH2 have also been studied, mostly in models of renal fibrosis^{200,203,204}.

As described earlier, AngII-regulated enhancers overlap with key AngII-regulated genes in VSMCs. This study also showed that an inhibitor of the epigenetic reader bromodomain-containing protein 4, called JQ1, which also inhibits enhancer function, inhibited AngII-regulated genes in vitro, as well as AngII-induced hypertension in mice,¹⁶³ again highlighting the translational potential of targeting epigenetic marks for diabetic vascular complications.

Targeting non-coding RNAs

The finding that several miRNAs are dysregulated in DKD suggests they could represent valuable targets for epigenetic therapies.¹⁸⁶ miRNA levels have traditionally been manipulated using antisense miRNAs or miRNA mimics; however, technical advances have led to the development of stable modified nuclease-resistant oligonucleotides that can act as miRNA inhibitors and mimics. These chemical modifications to the oligonucleotides enhance target affinity, nuclease resistance in biofluids and bioavailability, with reduced toxicity. miRNAs that are upregulated can be inactivated using miRNA sponges, antisense oligonucleotides, or through gene editing. One widely used modification is LNA (locked nucleic acid), a high-affinity RNA analogue in which the ribose ring is chemically locked in a relatively rigid conformation by a 2'-O,4'-C methylene bridge. LNA-modified oligonucleotides possess high thermal stability when hybridized with their complementary mRNA targets, as well as superior sensitivity and specificity in detecting corresponding miRNAs.²⁰⁵ Locked nucleic acid (LNA) [G]-modified anti-miRNAs (antagomirs [G]) (antisense oligonucleotides that hybridize with target miRNAs can inhibit specific miRNAs.^{28,147,206} One such antagomir is being evaluated in clinical trials for the treatment of hepatitis C virus infection.²⁰⁵

In the context of diabetes, administration of a LNA-modified inhibitor of miR-192 to mice with streptozotocin-induced diabetes inhibited both miR-192 and its downstream miRNAs (miR-216a, miR-217, and the miR-200 family), as well as p53 expression in the renal cortex, and reduced key parameters of DKD.^{28,147,148,207} Several studies have also reported efficacy of miR-21 inhibitors in mouse models of DKD,²⁰⁸⁻²¹¹ while another study showed that injection of 2'-O-methyl antisense oligonucleotides, which target miR-29c, reduced rates of DKD in diabetic *db/db* mice.²¹² The upstream mechanisms that control the expression and/or transcription of miRNAs could also be targeted to prevent or treat DKD. For example, the induction of miR-192 by TGF- β 1 is mediated by Smad2/3 and acetylation of Ets-1 and histones by the histone acetyltransferase p300 (which is activated by Akt)³⁰, suggesting that miR-192 expression could be therapeutically targeted by inhibiting Akt or histone acetyltransferases to prevent DKD.

Despite the potential of miRNA-targeted therapies, it will be a challenge to avoid undesired off-target effects, especially in a heterogeneous organ such as the kidney. Stable, synthetic, and biodegradable miRNA carriers, as well as optimal delivery methods, are being assessed to ensure efficiency without toxicity.²¹³⁻²¹⁶

Beyond miRNAs, a number of studies have demonstrated the value of targeting lncRNAs in DKD. Subcutaneous injection of an LNA-modified chimeric antisense oligonucleotide (GapmeR) that targets the DKD-associated lncRNA megacluster, *lnc-MGC*, led to accumulation of the GapmeR in the kidneys of diabetic mice, led to reduced expression not only of *lnc-MGC*, but also key cluster miRNAs hosted within it, and attenuated key features of early DKD.³¹ A similar GapmeR that targets human *lnc-MGC* also reduced fibrotic gene expression in human mesangial cells cultured with high glucose or TGF- β mimicking diabetic conditions, suggesting translational potential of this agent for human DKD.³¹ As described above, miR-379–Edem3 and miR-494–Atf3 signalling can activate circuits that

augment ER stress to induce persistent expression of *Inc-MGC* and miR-379 cluster miRNAs as a potential mechanism of metabolic memory; thus, GapmeR-mediated inhibition of *Inc-MGC* might confer additional benefits by erasing metabolic memory (Fig. 4).

Epigenetic editing

Genome editing via CRISPR–Cas9 and other methods²¹⁷⁻²²⁰ is a powerful approach to modify genetic and epigenetic changes. The CRISPR–Cas9 system is relatively easier and faster than other genome editing methods. It can be used to introduce site-specific mutations, large deletions and replacements, even in mammalian cells, and to create animal models harbouring such mutations (Fig. 5)²²¹⁻²²³. By fusing Cas9 proteins with transcriptional activators or repressors, this system can also be used for the site-specific control of gene activation or repression.^{224,225} Similarly, fusion of Cas9 with DNA methyltransferases, TETs, or histone modifying enzymes, enables locus-specific alteration of epigenetic marks, DNA methylation, or histone acetylation or methylation, thus enabling erasure or reversal of epigenetic memory (Figs. 3 & 6).²²⁶⁻²³¹ In the future, such site-specific epigenetic modifications could be used improve efficacy of pharmacological treatments, and overcome the broad off-target, non-specific genomic effects of currently available epigenetic-modifying drugs.

Conclusions

DKD pathogenesis is driven by complex interactions between metabolic and haemodynamic factors. Epigenetic mechanisms that might integrate these interactions include DNA and chromatin modifications, miRNAs and lncRNAs. Epigenetics therefore offers new insights into DKD that will complement GWAS data. Long-term persistence of epigenetic modifications and ncRNAs triggered by diabetic stimuli could be key mechanisms underlying metabolic memory. Several HMTs and corresponding histone PTMs, DNA methylation as well as multiple miRNAs and lncRNAs, have been implicated in the expression of fibrotic and inflammatory genes associated with DKD. Approaches for controlling these molecules using small molecules or genetic interventions, including CRISPR-Cas9 editing, might lead to the development of new therapeutics for DKD and could potentially even erase metabolic memory. On-going efforts to evaluate epigenetic changes and ncRNA profiles in archived or freshly obtained biological samples, blood cells, and renal tissues from clinical cohorts of patients with DKD and CKD will lead to the discovery of non-invasive biomarker signatures of renal function decline and DKD. Integrating epigenetic datasets with available genetic patient data using experimental and in silico methods is expected to accelerate the identification of new mediators of DKD progression.

As epigenetics mostly affects gene expression in a cell type-specific manner, challenges exist to the interpretation of epigenome data from heterogenous white blood cell samples and renal biopsy samples. Computational deconvolution approaches to correct for cell-type composition have partially overcome these hurdles,^{127,232} but additional reference datasets are needed. Research in the area of EWAS in DKD is highly active and expected to further escalate, as better computational deconvolution approaches, multi-omics reference datasets,

and human kidney single-cell sequencing datasets become available. This rapidly expanding field is aided by unprecedented technological and scientific advances in the fields of high-throughput sequencing, sophisticated bioinformatics, and gene editing, as well as the wealth of publicly available datasets from the NIH Roadmap Epigenetics Project, ENCODE, and other international consortia. EWAS coupled with GWAS and other omics profiles, including data from enhancer and chromatin accessibility studies,⁸³ will help determine the functional relevance or causality of genetic variants, many of which occur in non-coding regulatory regions, including lncRNAs and enhancers.

Unlike genetics, epigenetic changes are mostly reversible generating exciting opportunities for therapeutic intervention through the use of emerging approaches that enable locus-specific control of epigenetic marks. In addition, epigenetic biomarkers could support precision medicine approaches to optimally stratify patients for effective treatment (Fig. 7). Thus, evaluation of epigenetic and epigenomic alterations is expected to yield new insights into the pathogenesis of DKD and metabolic memory that, in turn, can be translated into novel biomarkers and therapeutic modalities.

Acknowledgements

The authors gratefully acknowledge funding from the National Institutes of Health (NIDDK and NHLBI), the Wanek Family Project to Cure Type 1 Diabetes at City of Hope, and the Juvenile Diabetes Research Foundation. The authors thank Sarah T. Wilkinson and Kerin Higa (Office of Faculty and Institutional Support, Beckman Research Institute of City of Hope) for help editing the manuscript before submission.

Glossary

Non-coding RNA (ncRNA)

An RNA molecule that is not translated into protein; ncRNAs can regulate gene expression at the transcriptional and post-transcriptional levels

DNA methylation

An epigenetic modification that adds a methyl (CH₃) group to DNA, typically at the 5'-cytosine (C) of CpG dinucleotides

Histone post-translational modifications (PTMs)

Covalent modifications (e.g. methylation, phosphorylation, and acetylation) on histone residues that facilitate epigenetic regulation of gene expression by altering chromatin structure or recruiting chromatin modifiers

Genomic imprinting

A phenomenon that maternal and paternal chromosomes (or genes) are differentially marked and either maternal or paternal genes is selectively suppressed. DNA methylation is a key mark for genomic imprinting

Genome-wide association study (GWAS)

A cataloguing of genetic variants across the genomes of multiple individuals that are associated with a specific phenotype

Epigenome-wide association study (EWAS)

A cataloguing of epigenetics marks (typically focused on DNA methylation) across the genomes of multiple individuals that are associated with a specific phenotype

Enhancers

Short (50–1500 bp) regions of DNA that can be bound by transcriptional activators to increase the transcription of a particular gene. An enhancer may be located upstream or downstream of the gene it regulates, and does not have to be close to the transcription initiation site

Epigenotype

Encompasses a set of epigenetic marks (DNA methylation and histone modifications) unique to specific cells or individuals which determines their cell fate or phenotypes (disease susceptibility) respectively

Expression quantitative trait loci

(eQTLs) are genomic loci that explain the variation in expression levels of mRNAs

Elongation

A process of copying DNA into the growing RNA chain by RNA polymerase during the elongation phase of transcription

Alternative splicing

A process which allows multiple mRNAs to be transcribed from a single gene. Usually certain exons of a gene are included or excluded from the final processed mRNA

Histone deacetylase

Enzymes which remove the acetyl group from histones

ENCODE (Encyclopedia of DNA Elements)

A public research consortium funded by the National Human Genome Research Institute (NHGRI) to systematically identify all functional elements in the human and mouse genomes

Methylated DNA immunoprecipitation sequencing (Me-DIP-seq)

A method to identify the profile of genome wide DNA methylation, by sequencing DNA immunoprecipitated with antibody to 5-methylcytosine (5mC)

Reduced representation bisulfite sequencing (RRBS-seq)

A technique for analyzing the genome-wide DNA methylation profiles with single nucleotide resolution at relatively low cost. It combines restriction enzyme and bisulfite sequencing to enrich (~ 1% of genome) for areas of the genome with a high CpG content

Whole genome bisulfite sequencing

A gold standard for analyses of DNA methylation of single cytosines at base resolution in the whole genome. It involves next-generation sequencing of bisulfite converted DNA. Sodium bisulfite converts unmethylated cytosine into uracil which eventually will appear as thymine after sequencing. Methylated cytosines appear as cytosine

Chromatin immunoprecipitation (ChIP) sequencing

A method to analyze protein interactions with DNA. ChIP-seq combines chromatin immunoprecipitation (ChIP) using specific antibodies to target protein followed high throughput DNA sequencing to identify the binding sites of DNA-associated proteins in a genome-wide fashion.

Mediator complex

A multiprotein complex that is required for gene transcription by RNA polymerase II and functions as a transcriptional coactivator in eukaryotes. Subunits of the complex relay information from signals and transcription factors to the RNA polymerase II machinery to control the expression of specific genes

Poised promoters

Promoters which are defined by the simultaneous presence of histone modifications associated with both gene activation and repression (bivalent). They are frequently found at the promoters and enhancers of developmental and lineage specific genes

Pyroptosis

A process of pro-inflammatory programmed cell death which is also a form of regulated necrosis, a lytic type of cell death inherently associated with inflammation

Foam cells

Lipid-laden macrophages that serve as the hallmark of early stage atherosclerotic lesion formation

Exosomes

Cell-derived vesicles that are present in biofluids including blood, urine, cerebrospinal fluid and conditioned medium of cultured cells. The diameter of exosomes is between 30 and 100 nm

Locked nucleic acid (LNA)

A modified RNA nucleotide in which the ribose moiety is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation, which is often found in the A-form duplexes. LNA nucleotides are very stable and have higher affinity to the target DNA or RNA residues, hybridizing with them according to Watson-Crick base-pairing rules

Antagomirs

A small synthetic RNA that is designed to silence endogenous miRNAs

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Key Points

- Diabetic kidney disease (DKD), involves the accumulation of extracellular matrix, hypertrophy, and fibrosis in kidney glomerular and tubular cells, can lead to end-stage renal disease (ESRD).
- Genes associated with DKD are regulated not only by classical signalling pathways and transcription factors, but also by epigenetic mechanisms, including chromatin histone modifications, DNA methylation, and non-coding RNAs.
- Metabolic memory is a phenomenon characterized by the persistent expression of DKD-related genes and phenotypes induced by glycaemic exposure, despite subsequent glycaemic control.
- Persistent epigenetic changes (DNA methylation and histone modifications) and signalling circuitry mediated by non-coding RNAs may be involved in metabolic memory.
- Targeting epigenetic factors (using small molecules or locus-specific epigenetic editing via the CRISPR–Cas9 system) or non-coding RNAs (using chemically-modified antisense oligonucleotides) are potential approaches to prevent or treat DKD and erase metabolic memory.
- Epigenetic profiling through epigenome-wide association studies (EWAS) and RNA (transcriptome) profiling of patient samples (blood, urine, and biopsy samples) might facilitate a precision (personalized) medicine approach for the treatment of DKD.

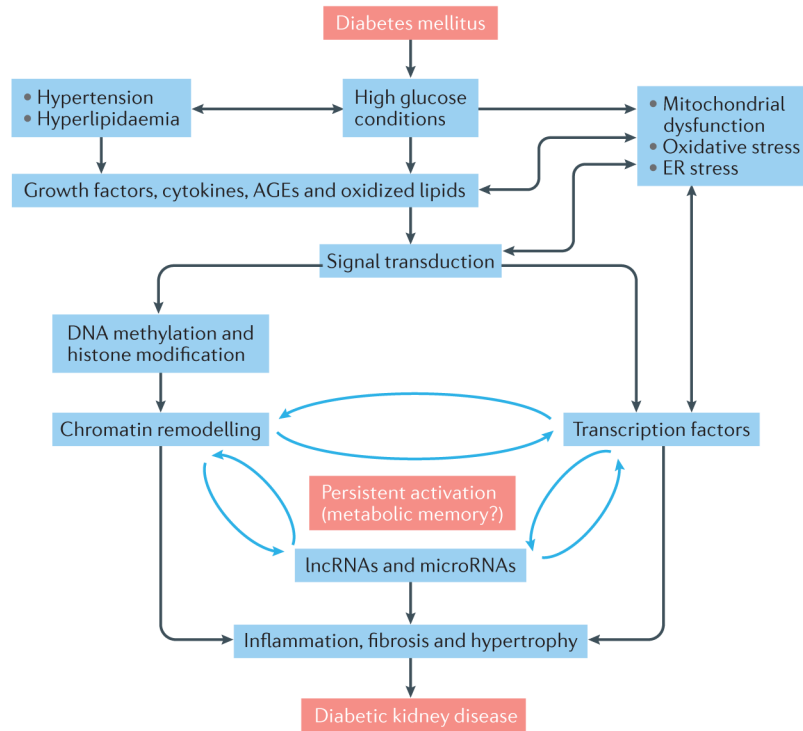


Fig. 1. Molecular mechanisms of diabetic kidney disease.

The pathophysiology of diabetic kidney disease (DKD) is complex and involves interactions between genetic factors, epigenetic factors, and the environment. High glucose levels in the context of diabetes mellitus, together with hypertension, and hyperlipidaemia induce the expression of growth factors, angiotensin II (AngII), cytokines, advanced glycation end products (AGEs), and produce oxidized lipids, mitochondrial dysfunction, and oxidative and endoplasmic reticulum (ER) stress. Growth factors, such as transforming growth factor β 1 (TGF- β 1), stimulate signal transduction pathways, including those involving MAPK and AKT kinases that activate downstream transcription factors, such as upstream stimulatory factors, SMADs, AP1 and the ER stress-related transcription factor, CHOP. They also affect epigenetic processes, including DNA methylation, histone modifications, and the expression of non-coding RNAs (microRNAs and long non-coding RNAs), resulting in altered chromatin accessibility and/or altered expression of target genes. Interactions between transcription and epigenetic factors lead to the persistent activation of signalling pathways, which might contribute to the phenomenon of metabolic memory, whereby exposure to high glucose levels leads to continued detrimental effects, even after glycaemic control has been established. Together, these conditions promote inflammation, fibrosis, and glomerular hypertrophy, which are hallmarks of DKD.

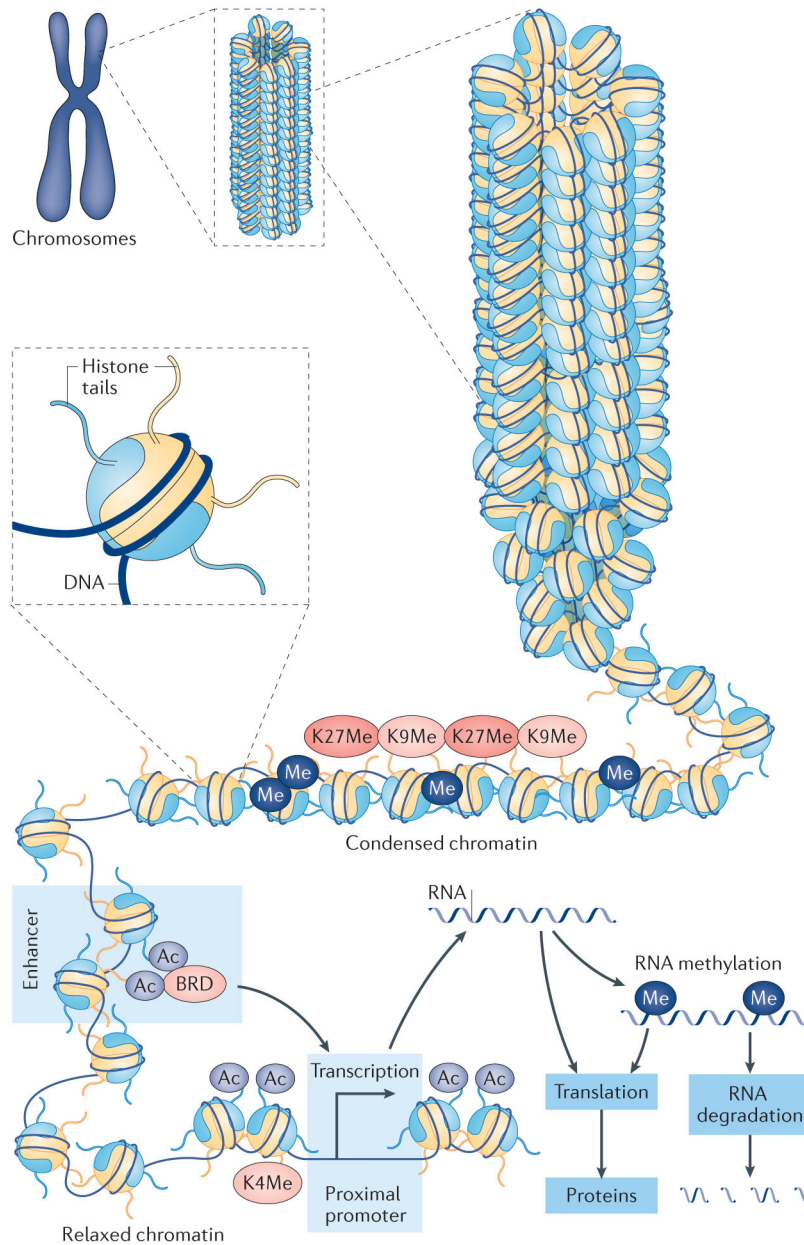


Fig. 2. The effects of epigenetic modifications on chromatin structure.

Chromosomes are composed of DNA–protein complexes called chromatin. The basic subunit of chromatin is the nucleosome, which comprises an octamer of two copies of each of the core histone proteins H2A, H2B, H3 and H4, wrapped by 147 base pairs of chromosomal DNA. Condensed chromatin is generally characterized by promoter DNA methylation (Me) and histone Me depending on the site of modification on histone (for example, lysine 9 methylation (K9Me) and lysine 27 methylation (K27Me) on histone H3), resulting in repressed gene expression. Relaxed chromatin is marked by histone lysine acetylation (Ac) and methylation of lysine 4 on histone 3 (K4Me) in the promoter regions, which allows efficient gene expression. Histone lysine acetylation at the enhancer region also enhances gene expression by interacting with the promoter region through

bromodomain-containing proteins (BRD) and other proteins. RNA methylation (Me) is a form of epigenetic regulation related to the translation and degradation of RNAs.

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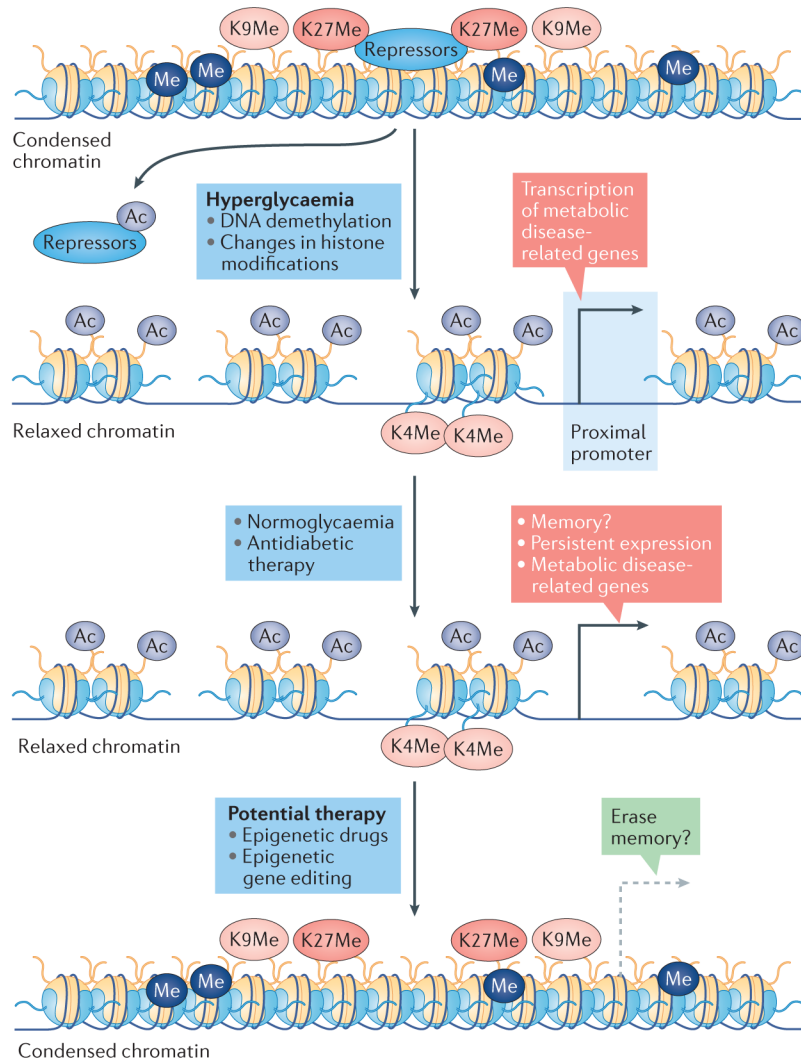


Fig. 3. Epigenetic mechanisms of metabolic memory.

Hyperglycaemia alters histone modifications and DNA methylation status, resulting in a change in chromatin structure from condensed (marked by DNA methylation (Me), methylation of lysine 9 on histone 3 (K9Me), and methylation of lysine 27 on histone 3 (K27Me)), which is associated with repressed gene expression, to relaxed (marked by histone lysine acetylation (Ac) and methylation of lysine 4 on histone 3 (K4Me)) to induce the expression of pathogenic genes. Acetylation relaxes chromatin and enhances gene expression through various mechanisms, including by facilitating the release of transcriptional repressors from condensed chromatin. Once established, the chromatin status (including DNA methylation and histone modification status) is maintained, even after normoglycaemia has been established by administration of insulin or antidiabetic drugs, with persistent expression of pathogenic genes, in a phenomenon called 'metabolic memory'. This epigenetic memory could potentially be erased using epigenetic drugs or by inducing locus-specific changes in histone modifications or DNA methylation through approaches such as gene editing directed by guide RNAs complementary to a specific locus.

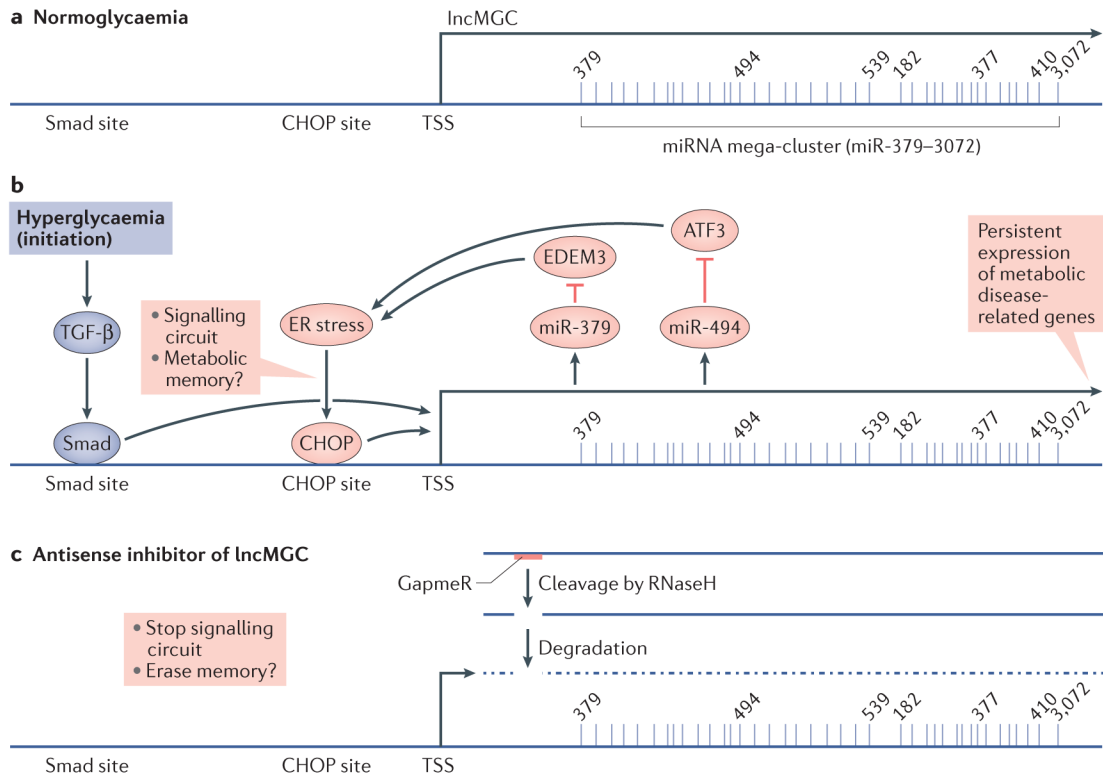


Fig. 4. Contribution of signalling circuits to persistent long non-coding RNA expression.
 a | The long noncoding RNA megacluster, *Inc-MGC* hosts nearly 40 miRNAs within a microRNA cluster called the miR-379 cluster. Smad site and CHOP site are binding sites for SMAD and CHOP transcription factors in the promoter region of *Inc-MGC*. b | Expression of *Inc-MGC*, is induced by hyperglycaemia through the actions of TGF- β , which induces the binding of transcription factors such as SMADs. The resultant induction of miR-379 and miR-494 (which are located in the miR-379 cluster) targets and reduces the expression of negative regulators of endoplasmic reticulum (ER) stress, such as EDEM3 and ATF3, which in turn induces activation of the transcription factor CHOP, which in turn increases the expression of *Inc-MGC* and the miR-379 cluster. This signalling circuit might facilitate the persistent expression of *Inc-MGC* and the miR-379 cluster even after blood glucose levels have been controlled by antidiabetic agents, providing a potential mechanism for metabolic memory. c | Erasure of this mechanism of metabolic memory might be achieved with use of a locked nucleic acid (LNA)-modified chimeric antisense oligonucleotide (a GapmeR) that targets *Inc-MGC*. Such an approach would result in cleavage of the *Inc-MGC* RNA by RNaseH and stop the persistent expression of *Inc-MGC* and the miR-379 cluster induced by the signalling circuit. TSS, Transcription start site.

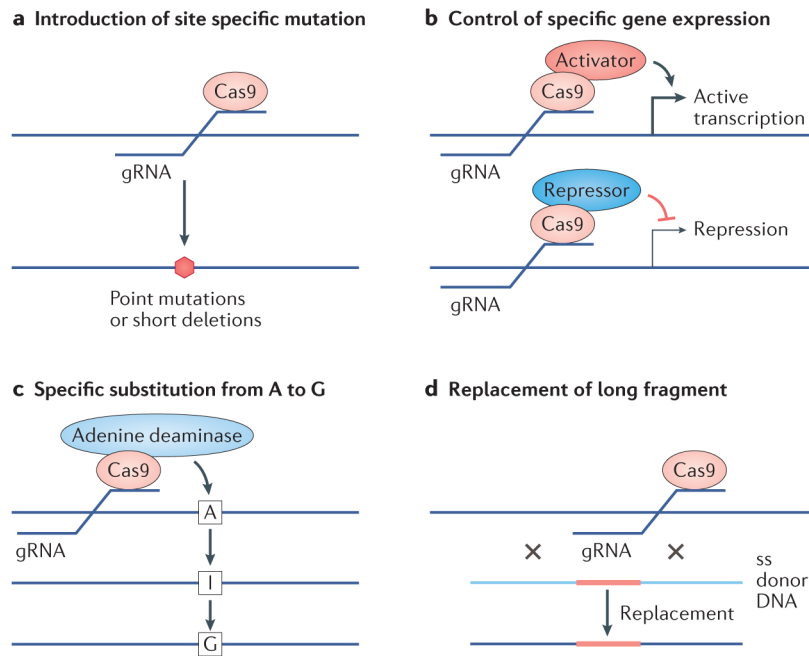


Fig. 5. Approaches to generate locus-specific genetic changes as a therapy for diabetic kidney disease.

CRISPR-Cas9 is a tool that enables targeted editing of the genome. Briefly, the Cas9 endonuclease is delivered into cells with a guide RNA (gRNA) that is designed to bind Cas9 and the target gene of interest. Following targeted Cas9 cleavage, the DNA is repaired by the cellular repair machinery (through non-homologous end joining or homology-directed repair). The system can be modified to achieve various outcomes, including (a) locus-specific induction of point mutations or short deletions, (b) the control of expression of specific genes (by fusing Cas9 with transcriptional activators or transcriptional repressors), (c) specific substitutions (for example by fusing Cas9 to adenine deaminase, which converts adenine (A) to inosine (I), which pairs with cytosine (C) and will be eventually converted to guanine (G) during replication), and (d) replacement of a long fragment of the genome by homology-directed repair. DNA damage (double-strand breaks) induced by CRISPR-Cas9 system can be repaired by homology-directed repair if the homologous template is close to the site. In this process, if an extra sequence is inserted between left arm and right arm of the homologous template (including extra sequences), the targeted sites can be replaced with the desired extra sequence.

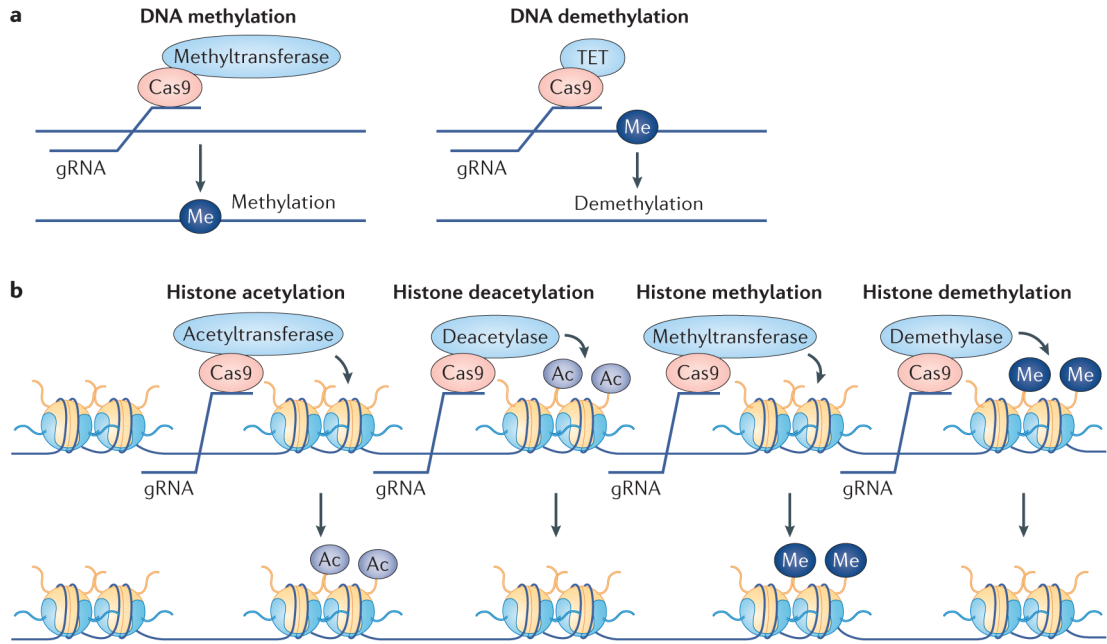


Fig. 6. Locus-specific epigenetic changes as potential therapy for diabetic kidney disease. In addition to introducing locus-specific genetic changes, the CRISPR-Cas9 system can also be used to introduce locus-specific epigenetic changes, including (a) the induction of DNA methylation (by fusing Cas9 with DNA methyltransferase) or demethylation at specific sites (by fusing Cas9 with ten-eleven translocation (TET) proteins that convert 5-methylcytosine into 5-hydroxymethylcytosine) and (b) the induction of histone modifications, including histone acetylation (by fusing Cas9 with acetyltransferase), histone deacetylation (by fusing Cas9 with histone deacetylase), histone methylation (by fusing Cas9 with histone methyltransferase), and histone demethylation (by fusing Cas9 with histone demethylase). Such changes of epigenetic marks might facilitate the erasure of epigenetic memory and improve long-term outcomes for patients with diabetic kidney disease. gRNA, guide RNA.

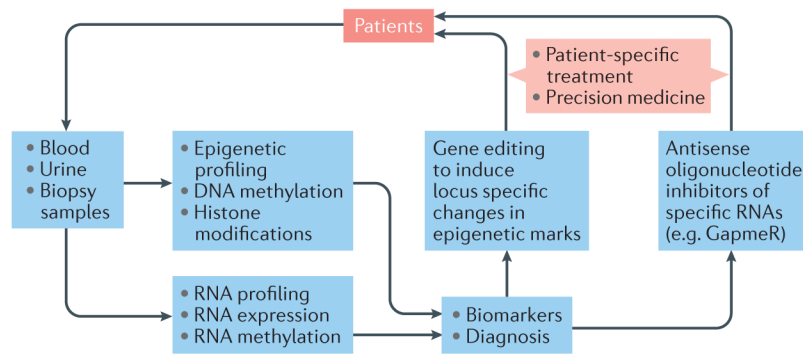


Fig. 7. Epigenetic and RNA profiling to support precision medicine.

Epigenetic changes such as DNA methylation and histone modifications in patients represent potential biomarkers for particular renal diseases, including diabetic kidney disease (DKD). Locus-specific correction of such epigenetic changes using the CRISPR-Cas9 system could be a future avenue for patient-specific treatment. Circulating RNAs (including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)) in biological fluids, such as blood and urine, or biopsy tissue are also potential diagnostic biomarkers for DKD. Synthetic chemical inhibitors of specific RNAs (for example, antisense oligonucleotides (for example, GapmeRs) could also facilitate patient-specific treatment.

Table 1.

Summary of EWAS studies in patients with diabetic kidney disease

Study design	Population	Tissue	Method	Gene (s) modified	Main findings	Ref
DKD in T1DM	192 patients of Irish descent with T1DM (with and without DKD)	Whole blood	Illumina HumanMethylation27 BeadChip (~27,000 CpGs)	<i>UNC13B</i>	19 CpGs correlated with time to DKD development, including one near <i>UNC13B</i> , which contains a SNP associated with DKD.	80
Microdissected tubuli from subjects with DKD and CKD and controls without	87 microdissected human kidney tubule samples: 21 from patients with DKD (cases) and 66 from controls for 450K arrays; 12 CKD cases and 14 controls for the HELP assay	Microdissected tubuli	Illumina 450K Bead Chip; and HpaII fragment enrichment by ligation mediated PCR (HELP) assay and hybridization to oligonucleotide arrays	<i>COL4A1/A2</i> , <i>SIX2</i> , <i>HNF</i> , and <i>TCFAP</i> <i>EZR</i> <i>TGFBR3</i> , <i>SMAD3</i> , <i>SMAD6</i> <i>SMAD3</i> <i>RUNX1</i> and others	Differentially methylated regions localized to enhancers and were enriched in binding sites for key renal TFs. Key genes associated with renal fibrosis, such as collagens, exhibited differential methylation that correlated with gene expression.	84
CKD versus controls including DKD	255 cases with CKD including 113 with DKD; 152 controls without renal disease including 113 T1DM	Whole blood	Illumina HumanMethylation450 BeadChip (~450,000 CpG features)	<i>CUX1</i> , <i>ELMO1</i> , <i>FKBP5</i> , <i>INHBA-AS1</i> , <i>PTPRN2</i> , <i>PRKAG2</i> <i>ELMO1</i> and <i>PRKAG2</i>	23 genes with significant methylation changes associated with CKD.	85
Mitochondrial function in T1DM with versus without DKD	196 patients with T1DM and DKD ; 246 patients with T1DM without renal disease	Whole blood	A combination of Illumina HumanMethylation 27K and 450K BeadChips	<i>TAMM41</i> , <i>PMPCB</i> , <i>TSEFM</i> , and <i>AUH</i> at multiple CpGs	54 probes were associated with DKD; 46 of the top-ranked variants for DKD, including genes related to mitochondrial function, showed differential methylation in subjects with ESRD.	86
eGFR decline in CKD including DKD	Chronic Renal Insufficiency Cohort (CRIC): 20 (including 10 with DKD) with the lowest and 20 (including 10 DKD) with the highest rates of eGFR decline	Whole blood	Illumina HumanMethylation450 BeadChip (~450,000 CpG features)	<i>NPHP4</i> , <i>IQSEC1</i> and <i>TCF3 NOS3</i> , <i>NFKBIL2</i> , <i>CLU</i> , <i>NFKBIB</i> , <i>TGFB3</i> and <i>TGFB1</i>	CpG islands in certain genes associated with renal fibrosis (<i>NPHP4</i> , <i>IQSEC1</i> , and <i>TCF3</i>) showed greater DNAm in participants with stable renal function.	87
Offspring exposed in utero to T1DM	29 adult non-diabetic offspring of mothers with T1DM (cases) and 28 non-diabetic offspring of fathers with T1DM (controls)	Blood leukocytes	Illumina HumanMethylation27 BeadChip (~27,000 CpGs)	<i>DNMT1</i>	DNMT1, a DNA methyltransferase involved in gene regulation in development, showed reduced DNAm in cases.	89
eGFR in CKD including DKD	Patients from the Atherosclerosis Risk in Communities (n=2,264 including 586 DM) and Framingham Heart (n=2,595 including 394 DM) studies	Whole blood	Illumina HumanMethylation450 BeadChip (~450,000 CpG features)	<i>PTPN6</i> <i>PHB2</i> , <i>ANKRD11</i> , and <i>TNRC18</i>	Of 19 CpG sites significantly associated with eGFR and/or CKD five also associated with renal fibrosis in biopsies from CKD patients and showed concordant DNAm changes in kidney cortex.	90

Study design	Population	Tissue	Method	Gene (s) modified	Main findings	Ref
Pima Indians with T2DM and DKD	181 diabetic Pima Indians	Blood leukocytes	Illumina HumanMethylation450 BeadChip (~450,000 CpG features)	<i>CDGAP</i> , <i>FKBPL</i> and <i>ATF6B</i>	Methylation at 77 sites were associated with eGFR decline; three of these 77 probes showed directionally consistent and significant associations with fibrosis in matched kidney tissue.	⁹¹
DCCT / and EDIC cohort of T1DM	Patients who received conventional therapy in DCCT and showed retinopathy or albuminuria progression by EDIC year 10 (cases) versus patients who received intensive therapy in DCCT who did not develop retinopathy or DKD progression by EDIC year 10 (controls).	Whole blood (sampled in ~1993), Monocytes (sampled in ~2010)	Illumina HumanMethylation450 BeadChip (~450,000 CpG features)	<i>GATADI</i> , <i>BSN</i> , <i>PRKCE</i> , <i>TXNIP</i> , <i>LHX6</i> , <i>MAP7</i> , <i>CDH3</i> , <i>SMYD1</i> , <i>CLCN7</i> , <i>ZNF167</i> , <i>CUED1</i>	12 differentially methylated loci were common in both whole blood and monocytes, i.e., sustained over 16 years. Loci included <i>TXNIP</i> known to be associated with hyperglycaemia and related complications, including DKD. Demonstrate a connection between DNAm and human metabolic memory.	¹²⁷

ChIP, chromatin immunoprecipitation; CKD, chronic kidney disease; DCCT, Diabetes Control and Complications Trial; DKD, diabetic kidney disease; DNAm, DNA methylation; EDIC, Epidemiology of Diabetes Intervention and Complications; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HELP, HpaII fragment enrichment by ligation mediated PCR; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TFs, transcription factors