Current Diabetes Reports (2019) 19:91
https://doi.org/10.1007/s11892-019-1214-6

PATHOGENESIS OF TYPE 2 DIABETES AND INSULIN RESISTANCE (M-E PATTI, SECTION EDITOR)

Cytosine Methylation Studies in Patients with Diabetic Kidney Disease

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Abstract

Purpose of the Review Kidney disease is the major cause of morbidity and mortality in patients with diabetes. Poor glycemic control shows the strongest correlation with diabetic kidney disease (DKD) development. A period of poor glycemia increases kidney disease risk even after an extended period of improved glucose control—a phenomenon called metabolic memory. Changes in the epigenome have been proposed to mediate the metabolic memory effect, as epigenome editing enzymes are regulated by substrates of intermediate metabolism and changes in the epigenome can be maintained after cell division.

Recent Findings Epigenome-wide association studies (EWAS) have reported differentially methylated cytosines in blood and kidney samples of DKD subjects when compared with controls. Differentially methylated cytosines were enriched on regulatory regions and some correlated with gene expression. Methylation changes predicted the speed of kidney function decline. Site-specific methylome editing tools now can be used to interrogate the functional role of differentially methylated regions.

Summary Methylome changes can be detected in blood and kidneys of patients with DKD. Methylation changes can predict future kidney function changes. Future studies shall determine their role in disease development.

Keywords Epigenetics · Cytosine methylation · Diabetic kidney disease · Epigenome editing · Epigenome-wide association analysis (EWAS) · Metabolic memory

Introduction

Kidney disease development explains most excess mortality associated with diabetes. The clinical definition of DKD is based on the presence of albuminuria and reduced kidney function (GFR) [1]. However, the gold standard disease definition requires a kidney biopsy and the identification of glomerular basement membrane thickening, mesangial expansion, and nodular sclerosis, indicating that multiple cell types are affected and a complex underlying mechanism [2]. Depending on the criteria used, roughly a third to half of patients with diabetes will eventually develop DKD. Therapeutic options for patients with DKD remain limited, reflecting our limited understanding of disease pathogenesis [3].

Multiple molecular pathways have been proposed to play a role in DKD development. As kidney function is a highly heritable trait, large genome-wide association studies (GWAS) have been performed to identify genetic variations associated with DKD [4, 5]. Despite intense efforts, only a few genomic regions show associations that pass the genome-wide significance level. Early studies from Diabetes Control and Complications Trial (DCCT) and UK Prospective Diabetes Study (UKPDS) established the critical role of poor glycemic control in DKD. Strangely enough, new analyses from the DCCT and UKPDS studies indicate that the risk of DKD development is still increased after 25 years in individuals who had an initial period of poor glycemia, even though subsequent glucose control was improved. This phenomenon is called "metabolic memory" [6].

This article is part of the Topical Collection on *Pathogenesis of Type 2 Diabetes and Insulin Resistance*

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Published online: 30 August 2019

Metabolic Memory and Epigenetic Mechanisms

Metabolic programming, or lasting effects of a metabolic insult experienced during prenatal development or early postnatal life, has also been suggested to contribute to renal disease. Human epidemiologic studies of children exposed to undernutrition, such as during the Dutch famine during the winter of 1944–1945, or the famines in Ukraine around 1930 and in China at the end of the 1960s [7], indicate that in utero undernutrition is associated with an increased risk of diabetes, hypertension, cardiovascular, and kidney disease in adults. Rodent experiments further support the role of developmental programming [8–11]. In animal models, maternal exposure to high-fat diet or caloric restriction during gestation was associated with hypertension and metabolic dysregulation during adult life in the F1 generation offspring.

The metabolic memory phenomena suggest that the transcriptional activity of the genome is permanently changed even after the removal of the environmental insult [12•, 13, 14]. Changes in the epigenome can provide a possible explanation for such phenomenon as the epigenome controls gene expression, and these epigenetic changes are maintained even after cell division. The epigenome is under the environmental influence as epigenome editing enzymes use substrates of intermediate metabolism (Fig. 1) [15].

In the eukaryotic nucleus, the DNA forms a complex with histone proteins called chromatin (Fig. 1). Two of each of the H2A, H2B, H3, and H4 histones form the histone octamer and 147 bp of DNA is wrapped around the histone octamer to form the nucleosome. This basic chromatin structure is identical in each somatic cell. This first level of DNA compaction is followed by further cell type-specific genome compaction allowing the nuclear folding of the genomic DNA. Mechanisms underlying this cell-specific higher order chromatin organization are not fully understood, but are critical for appropriate regulation of gene expression. Covalent modifications of the chromatin result in the cell-specific separation of the genome into open and closed states. Silenced chromatin domains are called heterochromatin, while open chromatin is called euchromatin. Histones are highly conserved small globular proteins with protruding N- and C-terminal tails (Fig. 1). Most epigenetic modifications involve basic residues of the N-tail of the histones. Histone acetylation of the lysine residues is usually associated with active gene expression (H3K27 acetylation) [16]. Histone methylation can occur on active promoters, enhancers, and actively transcribed gene bodies (H3K4 methylation, H3K36 methylation) or silenced regions (H3K9 methylation, H3K27 methylation) [17]. The role of other histone modifications such as phosphorylation, ubiquitylation, arginine methylation, and citrullination is much less well understood.

Genomic DNA can also be covalently modified, such as by methylation of cytosines at the 5th position (Fig. 2). Most cytosines in the genome are methylated [18]. Cytosine methylation plays a key role in repression of transposable elements, the footprint of ancient integrated DNA, that represent more than half of the human genome. Cytosines are usually not methylated in cytosine and guanine (CpG)-rich regions, called CpG islands. Methylation of promoter and enhancer regions can alter the strength of transcription factor binding, either directly or via the recruitment of methyl-binding domain proteins [19]. In the most simplistic view, methylation of promoters and enhancers is associated with gene silencing, while unmethylated promoters and enhancers allow the binding of transcription factors and gene expression to occur (Fig. 2). The exact role of methylation in gene expression regulation is not fully understood and is highly context-specific as gene body methylation can be associated with enhanced transcription. In mammals, methylation is essential for normal development, and dynamic methylation changes in promoters and

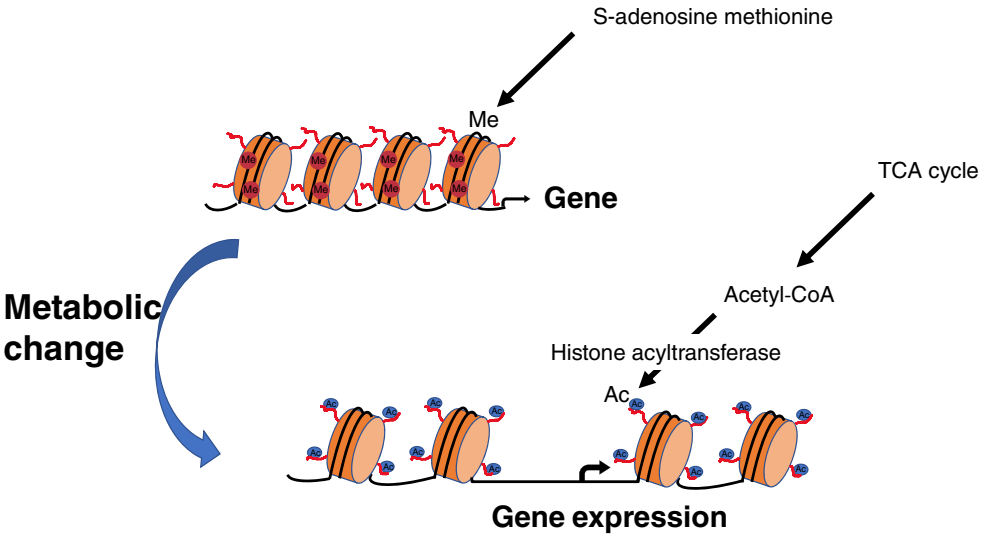

Fig. 1 Schematic representation of chromatin structure. Heterochromatin region the nucleosomes are tightly compacted and genes are not expressed. Metabolic stress can change the epigenome. Lower panel indicates an active euchromatin region where gene expression can occur. Epigenome editing enzymes use substrates of the intermediate metabolisms such as acetyl-CoA from the TCA cycle and methionine. Me: methyl group, Ac: acetylation

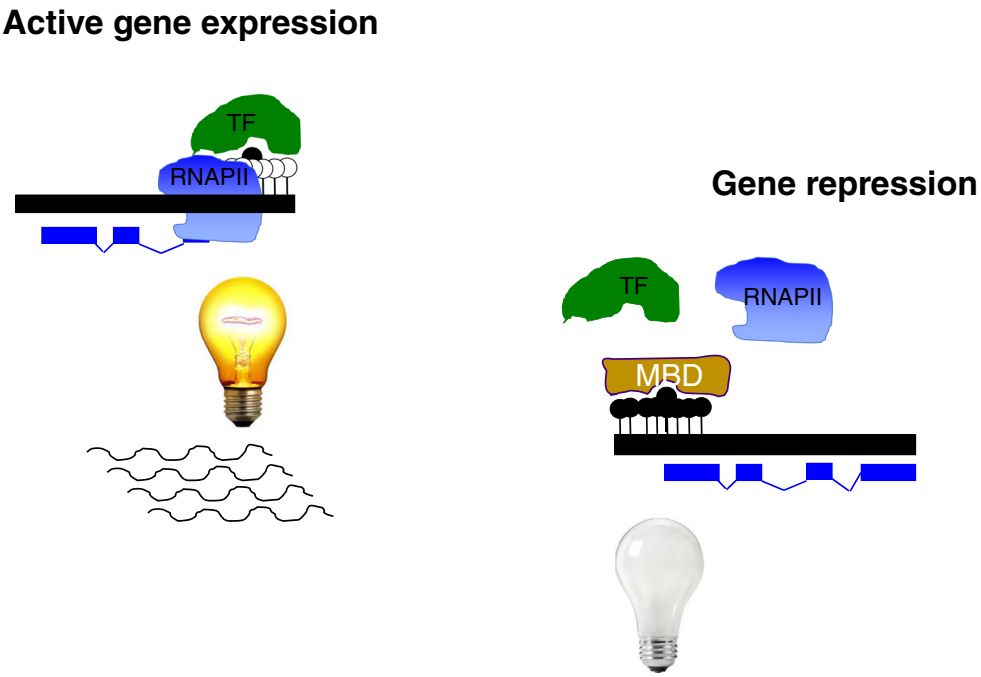

Fig. 2 Cytosine methylation Methylated cytosines on gene promoter and enhancer regions attract methyl binding proteins and associated with gene silencing. Transcription factors can bind to lowly methylated promoters and enhancers and allow gene transcription. TF: Transcription factor. RNAPII: RNA Polymerase II, MBD: methyl binding domain protein, Black circles represent methylated cytosines, while open circles represent unmethylated cytosines

enhancers are considered to be important for cell-type specification [20]. Several enzymes are essential for DNA methylation. Dnmt3a and 3b are considered de novo methyltransferases as they methylate unmethylated DNA and therefore are critical for cell differentiation. By contrast, Dnmt1 is regarded as a maintenance methyltransferase as it methylates the newly synthesized DNA during cell division based on the methylated DNA template [21]. The ten-eleven translocation (Tet1–3) enzymes can oxidize 5-methylcytosine (5mC) into 5-hydroxymethylation (5hmC), 5-formylmethylation (5fmC), and 5-carboxymethylation (5caC) eventually to non-modified cytosine [22]. In addition, cytosine methylation can decrease passively during cell division in the newly synthesized DNA strand.

This complex network of epigenetic modifications forms the epigenetic code [23], where the co-occurrence of some covalent modifications and the absence of others in the same region maintain an active or silenced gene expression patterns. The epigenome is cell type-specific, reflecting both cellular differentiation and previous environmental fluctuations. In addition, the epigenetic code essentially defines the cell type-specific gene regulatory logic.

Experimental Approaches to Probing the Epigenome

A plethora of techniques has been developed to investigate the epigenome [24, 25]. Given the key species-specific differences in the regulatory genome, model organism studies cannot replace studies performed on patients or human materials. Thus, epigenomic methods are particularly valuable in translational and clinical research and could potentially be used to understand the footprint of current and previous cellular environments and its impact on gene regulation.

Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) has been the most widely used method to characterize histone modifications on a genome-wide scale. ChIP-seq can be applied to describe the DNA binding of a transcription factor and also for mapping gene regulatory regions with the use of regulatory region-specific antibodies. ChIP-seq can provide a comprehensive genome-wide tissue- or cell-specific activity map of promoters, enhancers, active gene regions, and repressed heterochromatin. Limitations of the ChIP-seq studies are the need for a relatively large amount of starting material and the significant technical variability due to their antibody dependence. Open chromatin analysis using Tn5 transposases as established in the ATAC-seq method appears to generate data that are more robust and quantitative. However, this method is unable to differentiate promoter and enhancer regions [24]. New methods are aimed at to generate high quality data for low input and archived materials [26–28].

The majority of clinical studies, such as epigenome-wide association analyses (EWAS), are focused on analyzing DNA methylation as it can be performed on archived material (Fig. 3). Sodium bisulfite converts non-methylated cytosines to uracil, which can be distinguished from the modified cytosines, as they remain resistant to the chemical treatment [29]. Bisulfite conversion–based methylation detection approaches were developed 25 years ago [30]. The drawback of the method is that both methylated and hydroxymethylated cytosines are resistant to the treatment, thus cannot be distinguished. Alternative techniques are required to detect 5hmC. Recently,

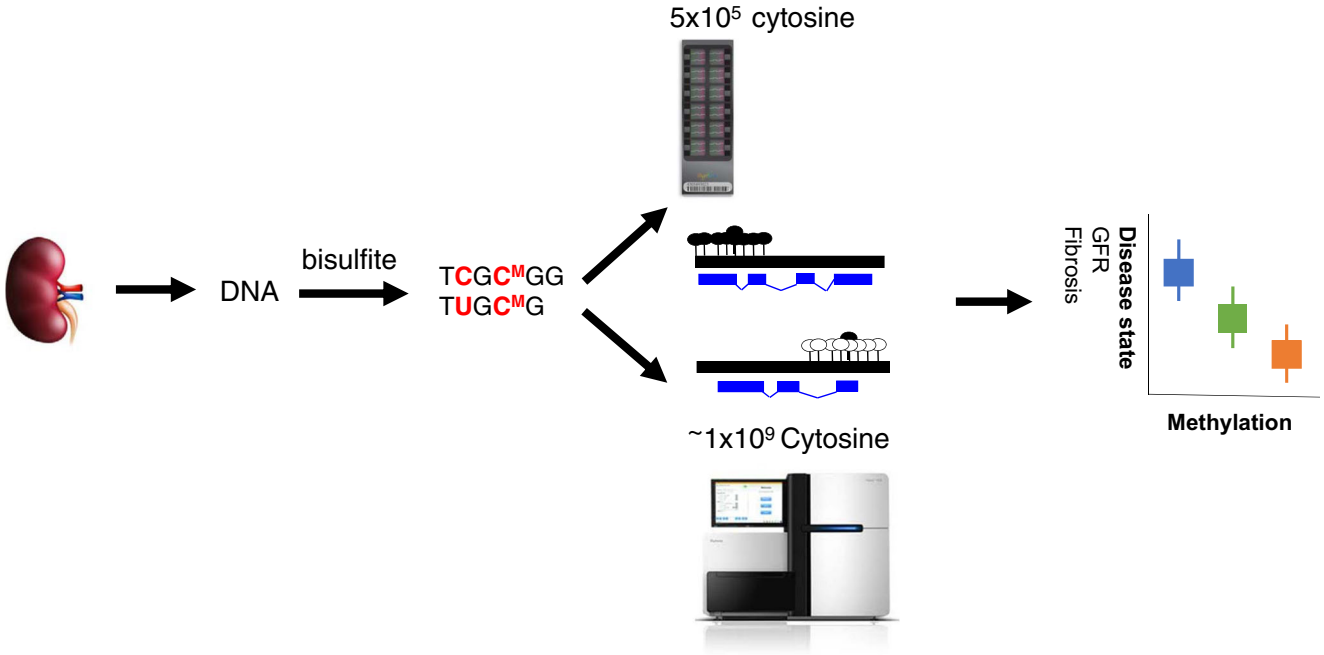

Fig. 3 Epigenome-wide association analysis. Blood or kidney tissue samples collected from cases and controls are used for methylation analysis. Bisulfate converts unmethylated cytosine to uracil, whole methylated cytosine is resistent to bisulfate. Array-based methylation analysis or whole genome bisulfite sequencing is used for methylome profiling, with subsequent analysis to identify regions related to disease state

an APOBEC-dependent method has also been developed (ACE-seq), which can specifically detect 5hmC [31]. Alternatively, mass spectrometry–based approaches can be used to separate all different DNA modifications simultaneously [32]. However, as more than 95% of cytosine modifications are methylation, detection of DNA methylation itself remains a potent tool for analysis.

Whole genome bisulfite sequencing (WGBS) provides genome-wide and base resolution methylation read-out and therefore the most comprehensive method for analysis of cytosine methylation. However, both the data acquisition and the analysis are cost-prohibitive. Instead, reduced representation bisulfite sequencing (RRBS) is widely used since it generates high-quality methylome datasets from restriction enzyme-digested genomic DNA [21]. The RRBS data is enriched for regulatory elements, which often overlap with CpG islands. For clinical materials, array-based capturing has been the favored technique, such as the Illumina Infinium arrays. While initial arrays analyzed methylation at only limited numbers of sites (27,000), subsequent releases contained 450,000 probes (450 K), with the current version probing 850,000 CpG sites [33, 34].

Methylation Studies in Surrogate Cell Types in Patients with Diabetic Kidney Disease

A key challenge in epigenetic analysis of diabetic kidney disease has been the identification of consistent, validated genome-wide significant methylation changes associated with disease phenotypes, such as albuminuria or decline in kidney function. Once such changes are described, they can be correlated with environmental factors to potentially understand whether the elusive metabolic memory effect is encoded by the epigenome.

As the human kidney is not an easily accessible tissue, most groups have focused on analyzing whole blood or isolated blood leukocyte samples as they have been stored for large cohorts. Immune cells present within these samples might play a role in DKD development, providing additional justification for this approach. Initial studies, performed using 27K Illumina arrays, analyzed whole blood samples of 192 subjects of European descent with T1DM [35]. The authors noted that age and experimental design significantly influenced the outcome, but 19 CpGs were identified using a statistical threshold of false discovery rate (FDR) less than 0.05, using the time of DKD development as an outcome.

In a follow-up study, methylation profiles in genes related to mitochondrial function were examined to assess association with DKD in T1DM. This early case-control study included 196 individuals with DKD and 246 individuals without renal disease. Fifty-four CpG probes across 51 unique genes were significantly associated ($p \leq 10^{-8}$) with DKD across both the 450K and 27K methylation arrays. A sub-analysis, employing the 450K array, identified 755 CpG probes in 374 genes that were significantly associated ($p \leq 10^{-8}$) with end-stage renal disease [36].

Qiu et al. performed a genome-wide methylation analysis of blood samples obtained from participants of Pima heritage [37]. This study utilized the Infinium 450K arrays to assess association between methylation levels and eGFR and to test whether methylation levels can predict future GFR decline. Methylation levels at 77 sites showed significant association with eGFR decline after correction for multiple comparisons. A model including methylation level at two probes (cg25799291 and cg22253401) improved prediction of eGFR decline compared with models that only included baseline eGFR and albuminuria. Furthermore, some probes that were associated with eGFR decline in blood samples also showed directionally consistent and significant association with fibrosis in microdissected human kidney tissue.

Chen et al. examined epigenetic changes in one of the best characterized cohorts, the DCCT and in its long-term follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) Study [12•]. Cytosine methylation profiles in genomic DNA of whole blood isolated at baseline of the EDIC study in 32 cases (DCCT conventional therapy group subjects showing retinopathy or albuminuria progression) were compared with 31 controls (DCCT intensive therapy group subjects without these complications). Furthermore, cytosine methylation was also profiled in blood monocytes of the same patients obtained during EDIC follow-up. Using a case-control design, hundreds of differentially methylated regions (fold change ≥ 1.3; $p < 0.005$) were identified. Twelve annotated differentially methylated loci were common to both white blood cells and monocytes, including Thioredoxin interacting protein (TXNIP). Cell culture studies confirmed the role of TXNIP in hyperglycemia-induced complications development [12•].

The largest epigenome-wide association studies for eGFR and CKD in whole blood DNA included 2264 ARIC Study and 2595 Framingham Heart Study participants [38]. The study identified 19 CpG sites significantly associated ($p < 1^{-07}$) with eGFR/CKD. In this study, the association observed in the blood samples was also replicated in microdissected human kidney tubule biopsies, and five CpG sites showed association with renal fibrosis in biopsies from CKD patients and showed concordant DNA methylation changes in the kidney cortex. The lead CpG at protein tyrosine phosphatase non-receptor type 6 and prohibitin 2 locus mapped to an active enhancer in the kidney cortex. Increases in methylation levels of this region in the kidney cortex were associated with lower renal protein tyrosine phosphatase non-receptor type 6 expression, higher eGFR, and lower renal fibrosis.

Unfortunately, many of these early studies analyzed limited numbers of samples and were thus underpowered to detect small differences. As of now, no clear consistent changes have been observed across multiple cohorts. However, a limiting factor is that different studies used slightly different outcome definitions and variable methods for covariate adjustment. As the majority of studies used the Illumina platforms, the data opens avenues for straightforward future meta-analyses.

Methylome Changes in Human Kidney Samples of Patients with Diabetic Kidney Disease

Ko et al. were the first to report methylation changes in 26 microdissected human kidney samples [39], using a methylation sensitive enzymatic DNA digestion method. Despite the modest sample size, a large number of differentially methylated sites were identified. Changes in methylation were rarely observed in promoters, but mostly overlapped with putative enhancer regions; differentially methylated loci were enriched in consensus binding sequences for important renal transcription factors. A core set of genes known to be related to kidney fibrosis, including genes encoding collagens, showed cytosine methylation changes. Methylation changes correlated with downstream transcript levels, indicating their potential importance.

In a follow-up study, Gluck et al. examined kidney cytosine methylation using Illumina Infinium 450K arrays from 91 participants with and without diabetes and varying degrees of kidney disease using a cross-sectional design [40••] and a similar sample cohort for validation studies. They first identified cytosine methylation changes associated with kidney structural damage (fibrosis) and kidney function (eGFR), further considering those 65 probes with genome-wide significance. Using machine learning approaches, they built models and identified both clinical variables and methylation changes that predicted kidney function. Methylation of 471 probes improved the model for kidney function decline, even though the base model that includes baseline age, GFR, and biochemical parameters already had a high predictive value [40••]. Methylation probes associated with kidney damage and functional decline were enriched for kidney regulatory regions and were associated with gene expression changes, including epidermal growth factor (EGF). Kidney and urinary EGF levels have been known to correlate with and predict kidney damage in multiple cohorts. Together, the work showed that kidney methylation differences could be detected in patients with diabetic kidney disease and can improve kidney function decline models indicating that they are potentially functionally important [40••] (Fig. 3).

A critical deficiency of the initial studies has been the lack of base resolution methylome information. In a recent study, Park et al. generated a base resolution methylome map by whole genome bisulfite sequencing (WGBS) of microdissected kidney tubule samples from patients with DKD and controls [28, 41••], identifying a large number of differentially methylated cytosines (DMC) and differentially methylated regions (DMR). Methylation changes identified by WGBS were

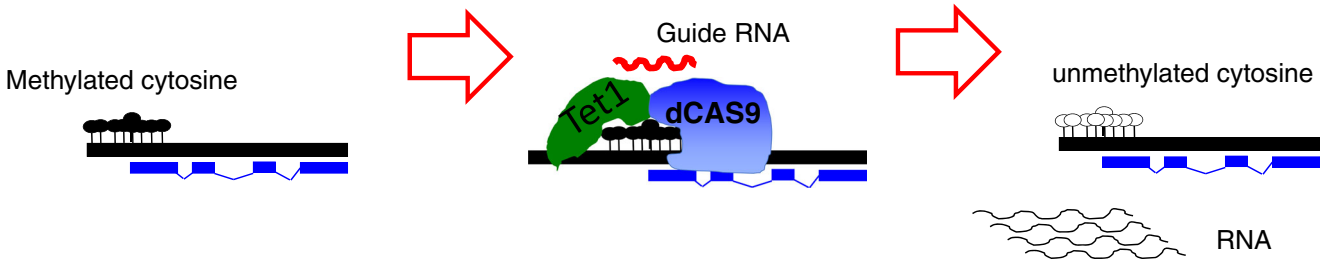

Fig. 4 Schematic representation of epigenetic editing. Methylated cytosine of CpG islands associated with silenced genes. Using a site-specific guide RNA, the Tet1-dCas9 can be targeted to the specific genomic region. Methylated cytosine is oxidized to hydroxymethyl by Tet ultimately reducing cytosine methylation, and a downstream increase in gene expression

consistent and could be validated by prior array-based studies. Parallel RNA sequencing of the same samples and subsequent pathway analysis highlighted coordinated changes of methylation and gene expression, including changes in immune signaling, such as the tumor necrosis factor alpha (TNF) cascade [41••]. Alterations in TNF methylation correlated with kidney function decline.

Kidney-specific studies are limited in number, and they have almost exclusively been performed by a single lab. Validation studies will be critical to understand consistency between additional cohorts and groups. However, the available data do support significant differential methylation in human kidney DKD samples and have highlighted changes in pathways encompassing known disease biomarkers such as EGF and TNF.

Understanding the Functional Role of Methylation Changes in Diabetic Kidney Disease

The next critical question in the field is whether methylation changes play a role in disease development. While methylation changes can influence gene expression, changes in gene expression can also alter the methylome. Several indirect approaches are used for causality establishment. These include focusing on changes that are established prior to disease development such as studies performed on the Pima Indian samples by Qiu et al., the DCCT cohort by Chen et al., or the kidney-specific analysis by Gluck et al. [12•, 37]. As the epigenome is species and cell-type specific, it is essential to focus on changes occurring in the cell type of interest [42]. Kidney cell-specific analysis will be essential to generate a hypothesis focused on the mechanistic role of methylation in DKD development.

The model of methylation-induced gene expression alterations invokes differences observed in transcription factor binding affinity to methylated vs. unmethylated DNA. Therefore, methylation changes that are observed in regulatory regions, such as enhancers and promoters, of disease relevant cell types are prioritized as functionally more relevant (Fig. 2). Changes identified in DKD kidneys show consistent enrichment on kidney-specific enhancer regions, suggesting that they are likely to be functionally important. Correlation between methylation and gene expression levels, as reported by the majority of studies, could be another important approach to prioritize functionally relevant sites. A caveat to these observations is there is no set location for enhancers in relation to the regulated gene; therefore, it is not trivial to match enhancers to their proper target genes [40••]. In addition, methylation and gene expression correlations might be condition-specific.

Recently, CRISPR/Cas9-based site-specific methylome editing methods have been developed [43]. The studies use a mutated Cas9 that binds DNA with high affinity, but does not cut the DNA (deadCas9; dCas9). Using a guide RNA, the complex can be directed to specific cytosines in the genome. Cas9 linked to a DNA methyltransferase or Tet enzyme can modify the cytosines in a site-specific manner with high precision (Fig. 4). Unfortunately, it is difficult to use array-based data for such site-specific functional studies, as arrays do not provide information at an individual cytosine resolution. Park et al. used the dCas9 method to show that cytosine methylation influences TNF levels and is likely functionally important for kidney disease development [41••].

In summary, in the future, a combination of computational and experimental methods will be needed to define methylation changes that drive gene expression alterations. As the epigenome is species-specific, it is difficult to use animal models to understand the functional role of cytosine methylation. Human kidney organoids could provide an important intermediate to test the role of regulatory elements that are specific for kidney cells; however, at present, they mostly resemble fetal rather than adult kidney tissue.

Conclusions

In conclusion, we are at the early stages in our understanding the role of epigenetics in diabetic kidney disease development. Current studies are mostly focused on analysis of patient samples to identify robust genome-wide significant differences

that could potentially explain the metabolic memory effect and DKD development. These initial results and studies should be synthesized and combined with further larger studies to address the critical need for translation of these discoveries into improved disease understanding or disease therapeutics. An important avenue is the identification of key epigenome editing enzymes. Most methylome and histone modifying enzymes can be targeted by small molecules for human studies, and some of them are already used in the clinics. Indeed, the Ishibe group had shown that valproic acid an important histone modifying compound (histone deacetylase inhibitor) had been associated with slower DKD progression [44]. As valproate is a widely used drug in the clinics, these results are highly attractive for future clinical studies.

In summary, initial studies indicate important changes in the methylome of blood and kidney samples of patients with DKD. These methylome changes can potentially serve an important disease biomarkers or therapeutic targets.

Compliance with Ethical Standards

Conflict of Interest Tamas Aranyi declares that he has no conflict of interest.

Katalin Susztak reports grant support from GSK, Regeneron, Boehringer Ingelheim, Merck, Bayer, Eli Lilly and Company, and Gilead; and consulting for Chemocentryx, Janssen, and Maze Bio. However, the work is not related to any of the studies supported by industry.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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