Integrative Omics Analyses Reveal Epigenetic Memory in Diabetic Renal Cells Regulating Genes Associated With Kidney Dysfunction

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Diabetic kidney disease (DKD) is a major complication of diabetes and the leading cause of end-stage renal failure. Epigenetics has been associated with metabolic memory in which prior periods of hyperglycemia enhance the future risk of developing DKD despite subsequent glycemic control. To understand the mechanistic role of such epigenetic memory in human DKD and to identify new therapeutic targets, we profiled gene expression, DNA methylation, and chromatin accessibility in kidney proximal tubule epithelial cells (PTECs) derived from subjects with and without type 2 diabetes (T2D). T2D-PTECs displayed persistent gene expression and epigenetic changes with and without transforming growth factor- β 1 treatment, even after culturing in vitro under similar conditions as nondiabetic PTECs, signified by deregulation of fibrotic and transport-associated genes (TAGs). Motif analysis of differential DNA methylation and chromatin accessibility regions associated with genes differentially regulated in T2D revealed enrichment for SMAD3, HNF4A, and CTCF transcription factor binding sites. Furthermore, the downregulation of several TAGs in T2D (including *CLDN10*, *CLDN14*, *CLDN16*, *SLC16A2*, and *SLC16A5*) was associated with promoter hypermethylation, decreased chromatin accessibility, and reduced enrichment of HNF4A, histone H3-lysine-27-acetylation, and CTCF. Together, these integrative analyses reveal epigenetic memory underlying the deregulation of key target genes in T2D-PTECs that may contribute to sustained renal dysfunction in DKD.

Diabetic kidney disease (DKD) is a major vascular complication of diabetes and the leading cause of end-stage renal failure (1). Despite strict glucose and blood pressure

control, >25% of patients with diabetes develop DKD. The key features of DKD include kidney glomerular and tubular dysfunction associated with fibrosis as a result of increased deposition of extracellular matrix (ECM) proteins such as collagens (2,3). Many studies have emphasized the importance of early and intensive glycemic control for the prevention/delayed onset of diabetic complications, including DKD (4–6). These studies identified a memory of prior intensive or conventional glycemic control therapy, which affects the risk of developing future complications despite subsequent glucose normalization, referred to as metabolic memory or legacy effect. Studies have implicated epigenetic mechanisms in metabolic memory (7), but the molecular mechanisms for such epigenetic memory in renal cells/tissues from subjects with diabetes despite glucose normalization are not clear. A comprehensive study of the underlying epigenomic changes and related mechanisms leading to persistent gene expression may enable the development of new therapeutics to erase the memory and prevent DKD progression.

Epigenetic memory refers to dynamic, heritable epigenetic regulation of gene expression in response to previous stimuli (e.g., hyperglycemia in diabetes) (8,9). Epigenetic/transcriptional memory may also potentiate gene expression upon reexposure to the same stimulus. Although studies suggest that DNA methylation (DNAm), chromatin histone modifications, or chromatin states/accessibility play significant roles in regulating epigenetic memory (8), the integrative effects of these mechanisms in DKD need to be defined. Evidence from experimental models and epigenome-wide associations studies suggests associations between aberrant epigenetic changes, especially DNAm and histone modifications, in renal fibrosis, inflammation,

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and pathogenesis of DKD (9–17). A few systematic studies have used integrative analyses to characterize the functional consequences of the interplay between the genome and the epigenome and key kidney disease-related loci (18,19). However, the direct impact of epigenetic memory from prior hyperglycemic exposure on gene expression in renal cells of subjects with diabetes needs to be determined. Furthermore, one key consequence of hyperglycemia is the upregulation of transforming growth factor- β 1 (TGF- β 1), a master regulator of DKD-related fibrosis (3,20). TGF- β 1 can induce epigenetic changes associated with the expression of fibrotic and inflammatory genes in renal glomerular and tubular cells (21–23), suggesting that TGF- β 1 signaling may mediate and amplify hyperglycemic epigenetic memory in DKD.

In the current study, we focused on identifying persistent transcriptomic and epigenomic changes in primary human proximal tubular epithelial cells (PTECs) derived from the kidneys of donors with type 2 diabetes (T2D-PTECs) compared with donors without T2D (N-PTECs) to uncover potential new mechanisms and therapeutic targets for metabolic memory of DKD. We compared the transcriptome, DNA methylome, and chromatin accessibility of these N-PTECs and T2D-PTECs cultured under similar growth conditions with or without TGF- β 1. We performed integrative analyses of these genomic data sets to identify T2D, as well as TGF- β 1-dependent and -independent, changes. Our results provide strong evidence of epigenetic memory in the deregulated transcription of fibrotic and key transport-associated genes (TAGs) in T2D-PTECs and show that T2D-PTECs are more sensitive to TGF- β 1. These data demonstrate novel mechanisms for chronic tubular dysfunction in DKD, even after glycemic control.

RESEARCH DESIGN AND METHODS

Renal PTEC Culture

PTECs were from two donors each for N-PTECs (CC-2553; Lonza, Morristown, NJ) and T2D-PTECs (CC-2925; Lonza) (details in Supplementary Table 1). All PTECs (two independent replicates for each donor) were cultured in renal epithelial growth medium (REGM) (Lonza) for four passages before harvesting at ~80% confluency for downstream experiments. TGF- β 1 signaling was induced by adding TGF- β 1 (10 ng/mL; Invitrogen, Carlsbad, CA) to the REGM at ~70% cell confluency. To check for epigenetic memory of TGF- β 1 actions, N- and T2D-PTECs were first treated with TGF- β 1 (10 ng/mL) for 24 h and 96 h. Cells were then washed with PBS and cultured in REGM without TGF- β 1 for 96 h.

RNA Sequencing and Data Analysis and RT-PCR

RNA was harvested using an RNeasy Kit (74106; QIAGEN). Briefly, total RNA extracted from replicates of two independent cultures of each PTEC (8 samples total) was used to make RNA sequencing (RNA-seq) libraries (16 in total, with and without TGF- β 1 treatment) using the Ribo

Zero Gold (Illumina, San Diego, CA) protocol. Strand-specific, paired-end, 100-base pair sequencing for ~50 million reads/sample was performed on an Illumina HiSeq 2500 system and aligned to hg38 (GENCODE v25) using Spliced Transcripts Alignment to a Reference (STAR) software (24). Analysis for differentially expressed gene (DEG) was performed using DESeq2 (25), with an adjusted $P < 0.05$ as the threshold significance level. A gene was considered expressed if its transcripts per million (TPM) was >1 in at least one donor PTEC. DESeq2 analysis was performed for the indicated comparison groups (Fig. 1A); with gene ontology (GO) analysis and pathway analysis performed using Bioconductor, significant enriched terms had $P < 0.05$ and Bonferroni correction with $q\text{-value} < 0.01$. RNA-seq data were validated using quantitative RT-PCR (RT-qPCR) in triplicate with *GAPDH* as the control using an Applied Biosystems 7500 Fast Real-Time PCR system. Primers used are shown in Supplementary Table 2. To quantify expression, average threshold cycle (Ct) values from triplicates were normalized with average Ct values of *GAPDH*.

DNAme Profiling and Data Analysis

Genomic DNA was isolated from PTECs using a QIAamp DNA Micro Kit (QIAGEN) and 1 μ g DNA used for Infinium MethylationEPIC BeadChip arrays (Illumina). Independent replicates from each PTEC were used (14 samples in total, without the addition of TGF- β 1, and after 24, 72, or 96 h of TGF- β 1 treatment). Raw data were analyzed using minfi and ChAMP packages (26,27). Briefly, data were preprocessed for background and control normalization followed by Subset-quantile Within Array Normalization. Differentially methylated CpG sites (DMP) and regions (DMR) were identified per ChAMP package. β -Values (% of DNAme) were calculated as methylated / (methylated + unmethylated). Changes in β -values ($\Delta\beta$) were calculated as T2D: β – N: β . CpGs meeting any of these criteria were removed: detection $P > 0.01$, methylation $<30\%$ in at least one sample, general single nucleotide polymorphisms, and multihits. Enrichment analysis of genes within 50 kilobases (kb) of DMRs was performed. Locus-specific methylation was measured using EpiTect Methyl assays (59436; QIAGEN) for selected TAGs in triplicate. Primers were designed on the basis of genomic coordinates (hg38, GENCODE v25) for DMRs (primer sequences in Supplementary Table 2).

Assay for Transposase-Accessible Chromatin Sequencing and Data Analysis

Assay for transposase-accessible chromatin sequencing (ATAC-seq) library preparation was performed as described (28) with 50,000 PTECs, one from each donor, with or without TGF- β 1 treatment (Supplementary Table 3). DNA was purified using QIAGEN MinElute Reaction Cleanup Kit and amplified with NEBNext High-Fidelity 2X PCR Master Mix (NEB) followed by library purification using AMPure XP beads. One hundred-base pair paired-end libraries were

Figure 1—Transcriptome profiling of cultured N-PTECs and T2D-PTECs. **A**: Schematic of the experimental design showing PTEC samples and conditions used to study epigenetic memory. PTECs from two control donors without diabetes (N-PTECs) and donors with T2D (T2D-PTECs) were cultured under similar glucose conditions (7 mmol/L) and subsequently treated with or without TGF-β1 (10 ng/mL) for 24 h. Cells were processed for profiling as shown. **B**: Bar chart showing expression of kidney proximal tubule-specific genes in TPM. Each bar represents the average TPM for replicates, and the red line represents 1 TPM. Individual data points are shown as dots. Error bars = SD. **C**: Heat map of hierarchical clustering of normalized count data for DEGs in N and T2D; pseudocolor normalization ranges from light yellow (downregulation) to dark purple (upregulation) on a log₂ fold change (log₂FC) (T2D vs. N) scale. Donor 1 and donor 2 represent the two donors each for N and T2D. Two columns for each donor represent independent RNA-seq replicates. The heat map of scaled log₂FC is based on normalized read counts from RNA-seq for N- and T2D-PTECs and generated using DESeq2 (see RESEARCH DESIGN AND METHODS). Each row represents scaled values for a gene across all the samples on a pseudoscale to highlight the variations across the samples. **D**: Bar plot representing the top nine enriched KEGG pathways, GO terms, and TF motifs for N vs. T2D DEGs. Bar = -log₁₀(p). **E**: Venn diagram of number of DEGs common between the current study and a published DKD study by Woroniecka et al. (38). **F**: Pseudocolor heat map displaying expression of selected genes common to both the current study and by Woroniecka et al. The heat map of scaled log₂FC is based on normalized read counts from RNA-seq for N- and T2D-PTECs and generated using DESeq2 (see RESEARCH DESIGN AND METHODS). Each row represents scaled values for a gene across all the samples on a pseudoscale to highlight the variations across the samples. **G**: RT-qPCR validation of candidate TAGs. Bar = average FC

sequenced on an Illumina HiSeq 2500 system. Reads were aligned to hg38 (GENCODE v25) using Bowtie 2 (29) and peaks called using MACS2 (30). Differential peak analysis was performed using DESeq2, with adjusted $P < 0.05$ as the threshold for significance levels. Each differential peak (*d*-ATAC) was associated with the closest transcription start site (TSS) (± 50 kb). Motif analysis of *d*-ATACs was performed using Homer (31). GO analysis and pathway analysis were performed using Bioconductor with $P < 0.05$ and Bonferroni correction with $q\text{-value} < 0.01$.

Integrative Analysis

We used regression analysis and bedtools to quantify correlations among DNase-seq, ATAC-seq, DEGs, and hepatocyte nuclear factor 4 α (HNF4A) motifs. Script used is accessible upon request at <https://github.com/bansalanita/EM>.

Immunofluorescence Assays

We used CellROX Orange Reagent (C10443; Invitrogen) for detecting oxidative stress (OS) and Col-F green fluorescence kit (6346; ImmunoChemistry Technologies, Bloomington, MN) for detecting collagens per the manufacturer's protocols. Briefly, PTECs were cultured in triplicate in six-well plates (50,000 cells), washed twice, treated with CellROX or Col-F reagents, and measured using a plate reader.

5-Azacytidine Treatments

PTECs ($\sim 70\%$ confluence) were treated with 500 nmol/L 5-azacytidine (5-AZA) (A2385; Sigma, St. Louis, MO) for 72 h, followed by downstream experiments.

Global DNase-seq Assay and ELISA for HNF4A

Global methylation changes (%) after 5-AZA treatment were measured using MethylFlash Global DNA Methylation ELISA Easy Kit (P-1030, $n = 3$; Epigentek). HNF4A protein was measured using Human HNF-4 α ELISA Kit (ab210581, $n = 3$; Abcam).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed in triplicate using 5 million cells with antibodies for HNF4A (ab41898; Abcam), CCCTC binding factor (CTCF) (C15410210; Diagenode), RNA polymerase II (Pol II) (CTD4H8; Santa Cruz Biotechnology) at 1:1,000 dilution, and H3K27ac (ab4729, 1:2,000 dilution; Abcam) following ENCODE protocols (32). qPCRs were performed on 1 μ L of ChIP-enriched DNAs and input DNA (control) using an ABI system (primer sequences are shown in Supplementary Table 2). Percent enrichment was calculated as 1) $\Delta Ct = Ct_{\text{ChIP}} - Ct_{\text{input}}$ and 2) % enrichment = $100 / 2^{\Delta Ct}$.

Statistics

Unless otherwise specified, we used R software for statistical analyses and ANOVA to calculate the level of significance with Tukey post hoc testing.

Data and Resource Availability

The data sets generated in this study are deposited into Gene Expression Omnibus (series GSE145747). They are also available from the corresponding author upon reasonable request.

RESULTS

Experimental Design

We used deidentified human PTECs from two control subjects without diabetes (N-PTECs) and two subjects with T2D (T2D-PTECs) from commercial sources (Supplementary Table 1). We cultured the cells in vitro under similar growth conditions with near-normal glucose (7 mmol/L) in the medium for four passages and treated them without or with TGF- β 1 (T) (10 ng/mL) for 24 h. T2D-PTECs appeared larger and more elongated in culture than N-PTECs, and this (fibroblast-like) elongated phenotype was even more pronounced after TGF- β 1 treatment in both N-T and T2D-T cells (Supplementary Fig. 1). We performed RNA-seq, DNase-seq, and ATAC-seq profiling on PTECs to identify transcriptomic and epigenomic signatures characterizing epigenetic memory of diabetic renal tubular dysfunction (Fig. 1A). For differential analyses, we compared 1) T2D versus N to compare untreated T2D-PTECs and N-PTECs, 2) N-T versus N, and 3) T2D-T versus T2D to evaluate the TGF- β 1-induced changes in N- and T2D-PTECs. Furthermore, we cultured N- and T2D-PTECs from each donor with TGF- β 1 (10 ng/mL) for up to 96 h followed by culture without TGF- β 1 to examine potential epigenetic memory of TGF- β 1 exposure. Validations were performed using RT-qPCRs, DNase-seq, and ChIP assays for candidate genes.

Characterization of Persistent Gene Expression Signatures in T2D-PTECs

To first verify the integrity of cultured PTECs, we queried our RNA-seq data sets for the expression of known proximal tubule-specific genes (33,34). RNA-seq detected expression (TPM ≥ 1) of several candidate tubular-specific genes (e.g., *AGMAT*, *ANPEP*, *AQP1*, *BHMT*, and *CDH2*) in both N-PTECs and T2D-PTECs (Fig. 1B). We did not detect other tubule-specific genes *SLC5A2* and *SLC34A1* in either group, suggesting a potential impact of in vitro culture.

The hierarchical clustering of the RNA-seq data sets showed distinct DEG profiles for N-PTECs and T2D-PTECs (Fig. 1C). We identified 1,486 DEGs (adjusted $P < 0.05$), of

in T2D vs. N ($n = 3$); $P < 0.05$, ANOVA with Tukey post hoc testing. FC is calculated as $2^{-\Delta\Delta Ct}$, and individual data points are shown as dots. H: Interaction map displaying representative TAGs as predicted targets for HNF4A, ESR1, and FOXA1 TFs on the basis of the presence of respective TF binding sites in their promoters (± 2 kb). AGE-RAGE, advanced glycosylation end product-receptor of advanced glycosylation end product.

which 801 showed a more than twofold change (Supplementary Fig. 2A and B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs revealed enrichment for cell adhesion, Ras signaling, ECM-receptor interactions, and advanced glycation end products and receptor for advanced glycation end products signaling, all related to DKD (2,3,35) (Fig. 1D). GO for molecular functions showed enrichment for membrane and solute transport, ion channel, and peptidase activity (Fig. 1D), which are related to tubular functions. Analyses of promoter regions (5 kb $\pm$ TSS) identified binding sites for transcription factor (TF) families (e.g., HNF, forkhead box protein O [FOXO], nuclear factor- κ B1 [NF- κ B1], nuclear factor of activated T cells [NFAT]) (Fig. 1D), some of which have been implicated as potential targets for DKD (36,37). To assess the extent to which our cultured PTECs recapitulated gene expression signatures observed in vivo, we compared our data with published studies in tubuli isolated from patients with DKD (38). Approximately 32% of the DEGs (i.e., 253 genes identified in our study) overlapped with DKD-associated genes identified by Woroniecka et al. (38) (Fig. 1E and F), suggesting that our cultured T2D-PTECs maintain their tubular characteristics and express genes associated with DKD.

Considering that filtration and reabsorption are primary functions of proximal tubules, and GO analysis showed enrichment of TAGs, we further explored the deregulated TAGs. We found differential expression of 103 TAGs in T2D-PTECs, including solute carrier (*SLC*) and claudin (*CLDN*) genes (Fig. 1G and Supplementary Fig. 3A). Fifty-nine of these 103 TAGs are also differentially expressed in at least one of three published studies of tubular samples or whole kidney from subjects with DKD (38–40) (Supplementary Fig. 3B). Motif analysis predicted the enrichment of HNF4A, FOXA1, and estrogen receptor 1 (ESR1) binding sites in the deregulated TAGs, especially *SLCs* and *CLDNs* (Fig. 1H). *SLC* transporters are membrane-bound proteins mediating the transport of molecules across cellular membranes, while *CLDN* proteins are critical components of tight junctions regulating paracellular transport (41–43). Hence, the sustained deregulation of *SLC* and *CLDN* genes in T2D cells cultured in near-normal glucose conditions could have profound effects on metabolic memory, kidney function, and clinical outcomes; however, there is only limited information on their roles in diabetes and DKD (44). Together, these RNA-seq data demonstrate that T2D-PTECs maintain a distinct cellular context and transcriptional memory of the prior diabetic environment relative to N-PTECs, which are preserved even after in vitro culture under nondiabetic conditions.

Differential Gene Expression Profile of T2D-PTECs in Response to TGF- β 1 Exposure

We next examined whether T2D-PTECs display differential responses to TGF- β 1 compared with N-PTECs. Treatment of N-PTECs with TGF- β 1 resulted in >2,000 DEGs (N-T vs. N) but >3,000 DEGs in T2D-PTECs (T2D-T vs. T2D)

(Fig. 2A and Supplementary Fig. 4A). We identified 1,798 TGF- β 1-dependent genes, including those common between N-T and T2D-T (Fig. 2A). We found 142 deregulated genes in T2D-PTECs versus N-PTECs and responsive to TGF- β 1 in both N- and T2D-PTECs (i.e., common to all three groups) (Fig. 2A). These 142 could represent genes that are TGF- β 1 dependent and constitutively deregulated under diabetic conditions. Notably, 1,533 DEGs exhibited variable expression only in T2D and T2D-T cells, implying that the diabetic environment influences TGF- β 1 signaling.

To delineate the transcriptional outcome of TGF- β 1 signaling in the diabetes environment, we compared the fold change in DEGs common to three groups: T2D versus N, N-T versus N, and T2D-T versus T2D (Fig. 2B and C). The 142 overlapping DEGs common to all three comparisons showed the least variation in T2D versus N and the highest variation in T2D-T versus T2D (Fig. 2B). Similarly, when comparing the 1,798 TGF- β 1-responsive DEGs common between N-T and T2D-T, T2D-T versus T2D showed significantly higher TGF- β 1-induced response (Fig. 2C–E). These data demonstrate that the T2D cells are more sensitive to TGF- β 1, which may be due to epigenetic memory of the diabetic environment.

DEGs unique to T2D versus N (potentially TGF- β 1 independent) showed the highest enrichment for ECM-receptor interactions, while genes common to TGF- β 1 response in both PTECs showed enrichment for phosphoinositide-3-kinase-Akt (PI3K-Akt), TGF- β , Ras-proximate-1 (Rap1), and FOXO signaling, tight junction, and cell-cell junctions (Fig. 2F). Motif analyses of promoters of DEGs unique to T2D versus N showed enrichment of binding sites for NF- κ B, HNF, interferon regulatory factor (IRF), and forkhead factors, whereas TGF- β 1-responsive DEGs in T2D-T and N-T revealed enrichment for HNF, mothers against decapentaplegic homolog (SMAD), GATA binding proteins (GATA), and E26 transformation-specific (ETS) binding sites (Fig. 2G).

Interestingly, we observed deregulation of 320 TAGs in T2D-T and 246 TAGs in N-T. Nineteen TAGs showed differential expression exclusively in N-T (Supplementary Fig. 4B and C). Forty TAGs showed TGF- β 1-independent deregulation in T2D. Because only 19 TAGs were unique to N-T versus N, while the remaining TAGs were altered in T2D-PTECs, it suggests that numerous TAGs are uniquely deregulated by T2D-related memory (Supplementary Fig. 4B and C).

We next evaluated whether the observed transcriptional memory in T2D in the cultured PTECs was also associated with a memory of phenotypic traits for DKD, such as OS and fibrosis. Fluorescent staining revealed higher OS in T2D and T2D-T compared with N and N-T PTECs, respectively (Fig. 2H). Fluorescent staining for collagens showed that TGF- β 1 increases the expression of collagens in both N-T and T2D-T cells. Additionally, the magnitude of Col-F change was significantly higher in T2D-T versus N-T, suggesting that T2D cells are also more sensitive TGF- β 1-mediated cellular phenotypes related to DKD (Fig. 2I).

Figure 2—Transcriptome and phenotypic changes in PTECs after TGF-β1 treatment. **A**: Venn diagram showing overlapping DEGs observed in three groups: N-T vs. N, T2D-T vs. T2D, and T2D vs. N. **B** and **C**: Violin plot for distribution of the change in expression levels of 142 DEGs common to the three groups (**B**) and expression levels of DEGs common to N-T vs. N and T2D-T vs. T2D cells (**C**). **D** and **E**: Bar plots depicting differential response to TGF-β1 treatment in T2D and N for candidate upregulated (**D**) and downregulated (**E**) DEGs in N-T vs. N and T2D-T vs. T2D. **F** and **G**: Pseudocolor representation of KEGG pathways (**F**) and TF families (**G**) enriched for DEGs in Venn diagram shown in panel **A**. 1) T2D vs. N only, 2) T2D-T vs. T2D, 3) N-T vs. N. Color in each box for panels **F** and **G** represent $-\log_{10}(p)$. **H** and **I**: Measurement of OS in PTECs using CellROX Orange assay (**H**) and collagen using Col-F fluorescent assay (**I**). Bar = average relative fluorescent units (RFUs) ($n = 3$). **J–M**: Measurement of change in *Col6A2* (**J** and **K**), *SLC16A5*, *SCNN1A*, *SLC12A3*, *SLC47A1*, *CLDN10*, *CLDN16*, and *TUBB* (**L** and **M**) expression observed in N and T2D samples with TGF-β1 treatment and subsequent removal of TGF-β1 using RT-qPCR. Bars = average fold change (FC) ($n = 3$). Error bars = SD. Black, N; green, T2D; purple, N-T (24-h TGF-β1 treatment); dark purple, N-T (96-h TGF-β1 treatment); pink, T2D-T (24-h TGF-β1 treatment); dark pink, T2D-T (96-h TGF-β1 treatment). Shaded bars represent change in expression for indicated genes 96 h after removal of TGF-β1. Error bars = SD. Individual data points are shown as dots. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. ns, not significant.

To evaluate this further, we next examined whether the expression of TGF-β1-inducible collagen/TAGs shows persistent expression between N- and T2D-PTECs, even after the removal of TGF-β1. *COL6A2* was upregulated in T2D versus N and was also increased by TGF-β1 in both N-T and T2D-T PTECs, with higher induction in T2D-T (Fig. 2J and K). Interestingly, while *COL6A2* levels were reduced significantly in N-T after removing TGF-β1 from culture media for 96 h (Fig. 2J), it remained elevated in T2D-T cells (Fig. 2K). Expression of candidate TAGs *SCL16A5*, *SCNN1A*, *SLC12A3*, *SLC47A1*, *CLDN10*, and *CLDN16* also exhibited similar behavior, depicting reversal in N-T cells but not in T2D-T cells after removing TGF-β1 for 96 h (Fig. 2L and M). These data show that relative to N-T, T2D-T cells exhibit a more exaggerated response to TGF-β1, as well as a memory of TGF-β1 exposure, on genes and phenotypes associated with DKD.

Identification of Differential Methylation Signatures in T2D-PTECs

Next, to determine the role of epigenetics in the observed transcriptional memory, we profiled DNAm signatures in PTECs using EPIC arrays. We identified 6,772 hypermethylated and 4,383 hypomethylated probes (DMPs, false discovery rate <0.05) (Supplementary Fig. 5A and B) in T2D versus N-PTECs. More than 40% of DMPs were located within gene bodies, with no differences in the distribution of DMPs relative to CpG islands (Supplementary Fig. 5C). Further analysis identified 376 hypomethylated and 526 hypermethylated DMRs, showing at least 20% variation ($\Delta\beta$) in T2D versus N (Fig. 3A and B), and

another 405 DMRs showed $\Delta\beta < 20\%$. Genomic annotation revealed an abundance of T2D hypermethylated DMRs in the 3' un translated region (UTR) and intronic regions and more enrichment of hypo-DMRs in the promoters and 5'UTR (Fig. 3C). Notably, >200 TAGs had a DMR within 20 kb of TSS (Fig. 3D).

KEGG analysis of DMR-associated genes (within 50 kb of DMRs) showed enrichment for genes associated with membrane transport, such as glucose, amino acid, and ion transport and SLC-mediated transport (Fig. 3E), and disease ontology analysis identified diabetes, hypertensive disease, and liver cirrhosis as top terms (Fig. 3F). Furthermore, TF motif analysis of DMRs identified enrichment of binding sites for CTCF, HNF4A, ETS, FOXO3A, and so forth (Fig. 3G). We also identified 241 DMRs in T2D-T (96-h TGF-β1 treatment) versus T2D. However, we did not find any DMRs in N-T versus N (96-h TGF-β1) or either T2D- or N-PTECs after 24 h of TGF-β1 treatment (Supplementary Fig. 5D). Relatively small numbers of DMRs with TGF-β1 treatment only in T2D-T and only after 96 h suggest that methylation changes are less dynamic. Altogether, DNAm studies revealed persistent DNAm changes in the T2D-PTECs cultured in vitro and suggest a previously unknown role of diabetes-associated DNAm and epigenetic memory in the regulation of TAGs.

The Landscape of Chromatin Accessibility in PTECs and the Effect of TGF-β1

We next performed ATAC-seq to examine chromatin accessibility as another index of epigenetic memory. ATAC-seq yielded >30 million nucleosome-free reads in PTECs

Figure 3—DNAm profiles of PTECs showing differential patterns in N- and T2D-PTECs. **A**: Manhattan plot of DMRs in T2D-PTECs compared with N-PTECs. Top DMR-associated genes are highlighted. Each dot depicts a DMR; black and gray dots are for DMRs in even and odd chromosomes, and green dots highlight DMRs associated with TAGs. **B**: Pie chart showing number of DMRs on the basis of fold change (FC) (T2D vs. N) and that have an adjusted $P < 0.05$. Orange indicates hypomethylated DMRs and purple, hypermethylated DMRs. Shaded areas show DMRs with <20% FC, whereas solid colors represent DMRs with >20% FC. **C**: Percent genomic distribution of hyper- and hypomethylated DMRs. **D**: Manhattan plot highlighting candidate TAGs with a DMR within 20 kb of their TSS. **E–G**: Enriched GO pathways (E), disease ontology terms (F), and TF motifs (G) for genes within 50 kb of DMRs. Bar = $-\log_{10}(p)$. TTS, transcription termination site.

and displayed expected ATAC-seq fragment length profiles (Supplementary Fig. 6), with replicates showing ~100,000 overlapping peaks in N, T2D, N-T, and T2D-T (Supplementary Table 3). Hierarchical clustering indicated the involvement of TGF- β 1 signaling in modifying chromatin accessibility because we observed closer correlations among N-T, T2D, and T2D-T relative to N cells (Fig. 4A). Genomic annotation of the peaks showed differential enrichment in various genomic regions, with N cells having the lowest and T2D-T cells having the highest number of peaks annotated to promoters (± 1 kb of TSS), with opposite trends for intronic and distal intergenic regions (Fig. 4B).

To assess variations in chromatin accessibility around DEGs, we created a master list combining all DEGs for all three groups together (T2D versus N, T2D-T versus T2D, and N-T versus N) and plotted peak densities around TSS. We observed (Fig. 4C) significantly more nucleosome-free reads around TSS of the DEGs master list in T2D- versus

N-PTECs. The number of nucleosome-free reads around DEGs increased further in both T2D-T and N-T, with T2D-T showing the highest. Similar trends were observed for mononucleosome reads (Fig. 4D). Differential analysis for nucleosome-free reads identified 5,890 (T2D vs. N), 7,358 (N-T vs. N), and 8,897 (T2D-T vs. T2D) differential peaks. Of note, 825 differential peaks were present in all groups, while the majority were unique to each group (Fig. 4E). Differential ATAC-seq analysis showed that T2D cells have more changes than N, and the effects of TGF- β 1 are more pronounced in T2D.

Furthermore, GO analysis again identified top enrichment terms as transcriptional activators, membrane transport, and ECM proteins (Fig. 4F), as was also seen in the RNA-seq and DMR analyses. Motif analysis also showed enrichment of binding sites for HNF4A, SMAD3, ETS1, and GATA2 TFs (Fig. 4G). Interestingly, we identified *d*-ATACs associated with ~400 TAGs in T2D. Together, ATAC-seq yielded novel insights into the role of TGF- β 1

Figure 4—ATAC-seq profiles of PTECs show more chromatin accessibility in T2D and after TGF- β 1 treatment. **A**: Dendrogram showing hierarchical clustering of *d*-ATACs of various PTECs, two replicates each, one from each donor for both N and T2D. **B**: Bar plots showing genomic distribution of ATAC peaks in PTECs. **C** and **D**: Profile plots showing ATAC-seq peaks in PTECs, centered on TSS for nucleosome-free reads (**C**) and mononucleosome reads (**D**). **E**: Venn diagram showing overlapping and unique *d*-ATACs in PTECs. **F** and **G**: Pseudocolor representation of top enriched pathways (**F**) and TF motifs (**G**) for genes associated with *d*-ATACs (within 50 kb) in 1) T2D vs. N, 2) N-T vs. N, and 3) T2D-T vs. T2D. Color in each box of panels **F** and **G** represents $-\log_{10}(p)$.

and diabetes-induced alterations in chromatin accessibility in regulating the expression of associated genes like TAGs in PTECs and provided more evidence of epigenetic memory.

Integrative Genomic Analyses of DEGs, DMRs, and d-ATAC in T2D Versus N

We observed an inverse correlation ($r^2 = -0.42, P < 0.05$) between key hyper-DMRs and corresponding DEGs (Fig. 5A) in T2D versus N. Of note, several TAGs were hypermethylated and downregulated in T2D-PTECs (Fig. 5B). We identified >1,000 DEGs having d-ATACs within 50 kb of TSS and a positive correlation ($r^2 = 0.62, P < 0.05$)

implying altered chromatin accessibility in the regulation of DEGs (Fig. 5C). Moreover, 513 d-ATACs in T2D were within 1 kb of a DMR; of these, 179 hypo-DMRs overlapped with more accessible sites, and 234 hyper-DMRs overlapped with less open sites in T2D (Fig. 5D). Association of DMRs with d-ATACs implies that hypermethylation is associated with closed/inaccessible chromatin.

Accordingly, >1,800 DEGs in T2D versus N, including 59 TAGs (CLDNs, SLCs), collagen, and *TGFβ1* (all related to kidney function), showed association with DMRs and nearby d-ATACs, depicting negative correlations between DEGs and hyper-DNAme and positive correlations between DEGs and d-ATACs (Fig. 5E and F). Notably, Fig. 5F

Figure 5—Integrative analysis of RNA-seq, DNase profile, and ATAC-seq data sets. **A:** Scatter plot showing correlation between DEGs (log₂ fold change [FC]) and DMRs (Δβ) for T2D- vs. N-PTECs. Top genes associated (within 50 kb) with DEGs and DMRs are highlighted. **B:** Scatter plot showing correlation between candidate TAGs and associated DMRs within 50 kb. **C:** Quadrant plot showing correlation between DEGs and d-ATAC peaks in T2D- vs. N-PTECs. Top genes associated within 50 kb of d-ATAC are highlighted. **D:** Pie chart showing variation in ATAC-seq peaks (more closed or open chromatin) within hyper- or hypo-DMRs. **E:** Pseudocolor heat map showing T2D-associated key DEGs and corresponding DMRs and d-ATAC peaks. **F:** Pseudocolor heat map showing T2D-associated differential TAGs, DMRs, and d-ATAC. **E** and **F:** Heat maps are scaled from −1 to 1, where −1 indicates downregulation of gene expression, hypomethylation, and decreased ATAC peaks and 1 represents upregulation of gene expression, hypermethylation, and increased ATAC peaks. Orange indicates downregulation and purple, upregulation in panels C, E, and F. **G:** Enriched TF families for overlapping DMR and d-ATAC associated with DEGs. Color in each box represents −log₁₀(p). **H:** Line plot of in silico analysis showing DNase and HNF4A (TF binding site) motifs around DEGs in T2D vs. N.

depicts positive correlations between DEGs and *d*-ATACs and a negative correlation with hypermethylation for the 59 differential TAGs. In addition, our data show differential regulation of 36 mitochondrial function-associated genes along with associated *d*-ATAC peaks and DMP/DMRs within 50 kb of their TSS (Supplementary Fig. 7). This is in line with the observed variation in OS in TGF- β 1-treated T2D-PTECs, and the known role of mitochondrial dysfunction in DKD. Motif analysis of downregulated DEGs associated with DMRs and closed *d*-ATACs revealed associations with TF binding sites, such as HNF4A and CTCF, whereas upregulated DEGs and open *d*-ATACs displayed enrichment for SMAD3, PAX7, and forkhead box binding sites (Fig. 5G).

Reports have shown the potential role of HNF4A in the regulation of TAGs, especially SLCs and CLDNs (45,46). Binding sites for TFs like HNF4A in downregulated genes may become inaccessible in diabetes either because of hypermethylation or inaccessible chromatin. Our *in silico* analyses of overlapping *d*-ATACs and DMRs in T2D also revealed HNF4A motifs around DMRs (Fig. 5H). For example, *SLC16A5*, a downregulated TAG in T2D, showed hypermethylation and reduced chromatin accessibility in its promoter region (Supplementary Fig. 8A–C). Accessibility to HNF4A binding sites may be reduced in diabetes because of persistent epigenetic changes (DNAm and closed chromatin), leading to the downregulation of its target genes (e.g., *SLC16A5*). Conversely, *SLC7A7*, an upregulated TAG in T2D, showed persistent hypomethylation and enhanced chromatin accessibility in its promoter regions overlapping forkhead TFs (Supplementary Fig. 8A–C).

HNF4A as a Potential Regulator of Candidate TAGs

By motif analysis, we found enrichment of HNF4A binding sites in all three data sets (DEG, DMR, and *d*-ATAC), and evidence has shown that HNF4 regulates many TAGs (46) (Fig. 1H). Thus, we evaluated connections between epigenetic modulations and HNF4A in the deregulation of TAGs in T2D cells, using the demethylating agent 5-AZA for candidate TAGs *CLDN10*, *CLDN14*, *CLDN16*, *SLC16A2*, and *SLC16A5*. 5-AZA treatment (500 nmol/L for 72 h) led to an ~50% reduction in global DNAm in both N- and T2D-PTECs (Fig. 6A) without affecting cell survival (Supplementary Fig. 9A). The *HNF4A* gene was hypomethylated in the gene body (Supplementary Fig. 9B) but showed no significant change in expression in T2D versus N (Supplementary Fig. 9C), although it was downregulated by TGF- β 1 in N-T and T2D-T (Supplementary Fig. 9C). We did not observe any changes in HNF4A protein expression in T2D-versus N-PTECs, although slight decreases were observed in N-T and T2D-T (Supplementary Fig. 9D). 5-AZA treatment did not alter HNF4A protein expression in N- and T2D-PTECs (Supplementary Fig. 9D), implying that HNF4A binding, and not its expression, may regulate expression of TAGs. Locus-specific methylation PCR assays verified a significant reduction in T2D-induced promoter DNAm for

CLDN10 and *SLC16A5* after 5-AZA treatment (Fig. 6B). *CLDN10* and *SLC16A5* are representative TAGs depicting hypermethylation (Supplementary Fig. 9E).

The expression of candidate T2D-downregulated TAGs (Fig. 6C) increased after 5-AZA treatment (Fig. 6D), while control genes (hypomethylated [*LY75*, *SPARC*] or no DMR [*HNF4A*, *RIPK4*, *TUBB*] in T2D-PTECs) showed no changes. Next, ChIP-qPCRs for HNF4A at promoters of candidate SLCs and CLDNs (TAGs) showed decreased HNF4A binding in T2D versus N, which was reversed after 5-AZA treatment (Fig. 6E). Furthermore, ChIP-qPCR also showed a reversal of T2D-associated chromatin modulations, with increased enrichment for H3K27ac (a mark of active promoters, enhancers, and open chromatin) (Fig. 6F), Pol II (Fig. 6G), and CTCF (Fig. 6H) in the promoters of selected TAGs after 5-AZA treatment. The observed reversal in HNF4A binding and chromatin modifiers after 5-AZA treatment supports our hypothesis that in T2D, HNF4A binding upstream of TAGs like *CLDN16*, *CLDN10*, *CLDN14*, *SLC16A2*, and *SLC16A5* is disrupted as a result of persistent epigenetic changes, including DNAm and chromatin accessibility.

DISCUSSION

In this study, we used human T2D-PTECs and N-PTECs cultured *in vitro* under similar conditions to discern mechanisms underlying epigenetic memory of diabetes-induced renal tubular dysfunction. Using a multiomics approach, we identified the sustained deregulation of known and novel genes in T2D cells and related epigenetic changes. We found persistent gene expression and epigenetic (DNAm, chromatin accessibility) changes in T2D-PTECs versus N-PTECs, even when cultured long term *in vitro* under similar nondiabetic conditions. We also found that T2D-PTECs are more sensitive to diabetes/DKD-related secondary stimulus (TGF- β 1), depicted by enhanced TGF- β 1-regulated gene expression and epigenetic profiles in T2D-PTECs. These persistently deregulated signatures in T2D-PTECs might be associated with long-term renal dysfunction and chronic DKD, even after glycemic control.

Notably, our data revealed a previously unrecognized epigenetic memory and persistent deregulation of several TAGs in T2D. Although the role of the kidney and, particularly, proximal tubules in transport and absorption of nutrients (e.g., glucose, amino acids, cations) and filtration of wastes (e.g., creatinine, ammonia) are well known, little is known about the epigenetic regulation of various TAGs (47). Many SLCs are therapeutic targets for T2D, with *SLC5A1* and *SLC5A2* and the sodium-glucose cotransporter 2 inhibitors among the best studied (48). Further investigation of other TAGs identified here could provide a rationale for evaluating their therapeutic potential for DKD and metabolic memory in the future.

We found persistent deregulation (transcriptional and epigenetic) of several key TAGs, such as *CLDN16*, *CLDN10*, *CLDN14*, *SLC16A2*, and *SLC16A5*, in T2D-PTECs and their association with TFs, such as HNF4A and CTCF. Additionally,

Figure 6—HNF4A, a potential regulator of candidate SLCs and CLDNs. **A**: Bar plot showing percent reduction in global DNAME as measured using ELISA after 5-AZA treatment (500 nmol/L for 72 h) in N and T2D. **B**: Percent methylation measured in the promoters of *CLDN10* and *SLC16A5* in N, T2D, N-AZA, and T2D-AZA using QIAGEN EpiTect Methyl II PCR Assay. **C** and **D**: Fold change (FC) in expression of candidate TAGs (*SLC*, *CLDN*), *HNF4A*, and control genes as measured by RT-qPCR ($n = 3$). Bar = average FC compared with N (**C**) and T2D (**D**). **E–H**: ChIP-qPCR assays showing enrichment of HNF4A (**E**), H3K27ac (**F**), Pol II (**G**), and CTCF (**H**) in PTECs at gene promoters and overlapping DMRs. Error bars = SD. Individual data points are shown as dots. Bar = average FC ($n = 3$). Significance was calculated using ANOVA followed by Tukey post hoc test. * $P < 0.05$, ** $P < 0.005$ for T2D vs. N; # $P < 0.05$ for T2D-AZA vs. T2D. **I**: Proposed schematic model for epigenetic memory in the persistent deregulation of candidate TAGs in T2D and the role of HNF4A TF. Hyperglycemia associated with diabetic conditions induces persistent epigenetic changes (i.e., epigenetic memory) in PTECs, which is represented by changes in DNAME or chromatin states (obtained by ATAC-seq). Our study shows that these epigenetic changes persist even when diabetic PTECs are cultured under normal glucose conditions in vitro. In addition, the persistent epigenetic changes render the diabetic PTECs more sensitive to secondary DKD-related stimuli like TGF- β 1. Our data suggest that epigenetic changes associated with diabetes reduce the binding/enrichment of HNF4A upstream of key TAGs along with increased DNAME and reduced chromatin accessibility, hence reducing their transcription/expression in T2D. The purple line depicts the open or more accessible chromatin regions with DNA hypomethylation and greater chromatin accessibility in the promoters. The blue line depicts the closed regions with DNA hypermethylation and lower chromatin accessibility in the promoters.

our data suggest that the diabetic state induces hypermethylation and closed chromatin at candidate TAGs, leading to disrupted HNF4A binding and their consequent downregulation. Altered binding of TFs like HNF4A as a result of long-lasting epigenetic changes during T2D could contribute to misregulation of key HNF4 target genes, chronic renal dysfunction, and DKD progression, even after glucose normalization. Our new data show that the deregulation of TAGs in T2D-PTECs may occur, at least in part, through epigenetic mechanisms. In particular, downregulation in T2D of *CLDN16*, a gene almost exclusively expressed in the kidney cortex on the basis of Genotype-Tissue Expression (49), may serve as a biomarker of DKD progression.

Our study also underscores a potential role for nuclear TFs like HNF4A and CTCF as crucial players in epigenetic memory. Although reports have shown interactions between HNF4A and DNAm (12,50), the functional role of HNF4A in epigenetic memory of DKD has not been investigated. Here, we propose a schematic model (Fig. 6I) that links epigenetic memory (persistent DNAm and chromatin changes) to enduring, long-lasting basal and inducible gene expression signatures in T2D-PTECs, even after glucose reduction.

We are aware of limitations, which include potential artifacts of the in vitro culture of PTECs. We also needed a glucose concentration of 7 mmol/L to maintain PTECs in culture, which is slightly above nondiabetic fasting glucose (~5.5 mmol/L), although close to postprandial levels. Another limitation is our small sample size because of the limited availability of primary PTECs, especially from donors with T2D. However, the recapitulation of about one-third of DEGs (Fig. 1E) and DMRs (data not shown) from other studies with DKD tissues (12,14,17,38) provides assurance that despite the limitations, genomic changes and novel DEGs identified here can provide vital insights into epigenetic memory in DKD. Since donors of T2D-PTECs in our study have no known overt renal disease, it is possible that our observed changes are primarily driven by diabetes. However, correlation analysis with published DKD transcriptome studies using tubular or whole-kidney tissues showed that DEGs in our T2D-PTECs depict significant correlation ($r^2 = 0.33$, $P = 0.0005$) (39) with DKD but not with hypertensive kidney disease unrelated to diabetes ($r^2 = 0.18$, $P = 0.21$) (39) and with early DKD ($r^2 = 0.28$, $P = 0.0031$) but not late DKD ($r^2 = 0.09$, $P = 0.1$) (51). These data, along with our multiomics data showing that the T2D-PTECs retain DKD-like behavior with increased OS, and altered expression, chromatin accessibility, and DNAm at genes related to tubular function, even after culture under nondiabetic conditions, suggest that the T2D-PTECs have phenotypes reminiscent of early DKD.

In conclusion, our integrative study shows the existence of epigenetic memory in PTECs from subjects with T2D in regulating key genes, including previously unexplored TAGs, that might be associated with DKD. Exploration of mechanisms underlying sustained aberrant binding of TFs

like HNF4-regulating genes in tubular cells could yield a deeper understanding of epigenetic memory and identify novel therapeutics for DKD.

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