Transforming growth factor-β1 (TGF-β1)–induced fibrotic and inflammatory genes in renal mesangial cells (MCs) play important roles in glomerular dysfunction associated with diabetic nephropathy (DN). TGF-β regulates gene expression in MCs by altering key chromatin histone modifications at target gene promoters. However, the role of the repressive histone H3 lysine 27 trimethylation (H3K27me3) modification is unclear. Here we show that TGF-β reduces H3K27me3 at the Ctgf, Serpine1, and Ccl2 gene promoters in rat MCs (RMCs) and reciprocally up-regulates the expression of these pro-fibrotic and inflammatory genes. In parallel, TGF-β down-regulates Enhancer of Zeste homolog 2 (Ezh2), an H3K27me3 methyltransferase, and decreases its recruitment at Ctgf and Ccl2 but not Serpine1 promoters. Ezh2 knockdown with siRNAs enhances TGF-β–induced expression of these genes, supporting its repressive function. Mechanistically, Ezh2 down-regulation is mediated by TGF-β–induced microRNA, miR-101b, which targets Ezh2 3′-UTR. TGF-β also up-regulates Jmdj3 and Utx in RMCs, suggesting a key role for these H3K27me3 demethylases in H3K27me3 inhibition. In RMCs, Utx knockdown inhibits hypertrophy, a key event in glomerular dysfunction. The H3K27me3 regulators are similarly altered in human and mouse MCs. High glucose inhibits Ezh2 and increases miR-101b in a TGF-β–dependent manner. Furthermore, in kidneys from rodent models of DN, fibrotic genes, miR-101b, and H3K27me3 demethylases are up-regulated, whereas Ezh2 protein levels as well as enrichment of Ezh2 and H3K27me3 at target genes are decreased, demonstrating in vivo relevance. These results suggest that H3K27me3 inhibition by TGF-β via dysregulation of related histone-modifying enzymes and miRNAs augments pathological genes mediating glomerular mesangial dysfunction and DN.

Diabetes, metabolic disorders, and activation of the renin-angiotensin-aldosterone system can all promote renal fibrosis and inflammation, which play key roles in the progression of diabetic kidney disease, also known as diabetic nephropathy (DN), a chronic condition that can lead to end-stage renal disease (1). The accumulation of extracellular matrix proteins in renal glomerular mesangial cells (MCs), podocytes, and the tubulointerstitium enhances fibrosis, a hallmark of chronic kidney disease and the major driving force for renal dysfunction in diabetes (1, 2). Transforming growth factor-β1 (TGF-β), a major mediator of kidney dysfunction, is induced by metabolic insults such as high glucose (HG), advanced glycation end products, growth factors, and peptide hormones such as angiotensin II in renal glomerular and tubular cells involved in the pathogenesis of DN. TGF-β induces fibrotic, inflammatory, and cell cycle genes in MCs to promote glomerular dysfunction (1–5). Inhibition of TGF-β confers renoprotection in animal models of DN, further supporting TGF-β as a major regulator of inflammation and fibrosis in DN (6). Despite substantial efforts to understand the molecular mechanisms involved in the pathological actions of TGF-β in the kidney, efficient renoprotective and anti-fibrotic strategies are still not available for DN, suggesting a critical need for the identification of new and novel effectors of TGF-β to design better therapeutic approaches (6–8).
Dysregulation of epigenetic mechanisms, including chromatin histone modifications and DNA methylation, plays a critical role in the expression of genes involved in the pathogenesis of renal disease (7–9). Changes in histone modifications at pathological gene promoters have been demonstrated in renal cells under diabetic conditions and in glomeruli and kidneys from animal models of diabetes (9). These studies show that key permissive histone modifications (e.g. histone H3 lysine 9/14 acetylation (H3K9/14Ac), and H3 lysine 4 methylation (H3K4me1–3) were enriched, whereas repressive histone modifications, such as H3K9me3, were decreased at fibrotic and inflammatory gene promoters. These changes were accompanied by altered expression and activity of relevant histone acetyl transferases (HATs), histone deacetylases (HDACs), and histone lysine methyl transferases (10–18). Overexpression of HATs could augment the expression of candidate fibrotic genes (15), and HDAC inhibitors, such as valproic acid, reduced fibrosis and proteinuria in diabetic rodents (19, 20). In type 2 diabetic db/db mice, increases in key permissive histone modifications in renal glomeruli were inhibited by treatment with the anti-hypertensive drug losartan, along with amelioration of proteinuria and glomerular hypertrophy (10). Further, siRNAs targeting the H3K4me transferase Set7/9 and pharmacological inhibitors of endoplasmic reticulum stress blocked increases in promoter H3K4me1 levels and expression of pro-inflammatory Ccl2 and also ameliorated renal glomerular hypertrophy in db/db mice (18). However, the role of the repressive histone modification, H3K7me3, in TGF-β actions in MCs is not clear.

In the current study, we evaluated the mechanisms by which TGF-β and diabetic conditions can promote the expression of pathological genes due to loss of repressive epigenetic histone marks and cross-talk with key microRNAs (miRNAs). Polycomb repressive complex 1 (PRC1) and PRC2 mediate epigenetic mechanisms that lead to gene repression and are involved in normal tissue homeostasis (21–23). PRC2 has four core subunits, including the histone methyltransferase Enhancer of Zeste homologue 2 (Ezh2, also known as KMT6A), which catalyzes H3K27me3. Increase in H3K27me3 at target gene promoters/enhancer leads to chromatin compaction and inhibition of transcription (21–23). Ezh2 function is associated with several pathophysiological processes, including stem cell differentiation and development, cancer, and diabetes (23, 24). In addition, H3K27me3 is erased by H3K27me3 demethylases UTX (KDM6A) and JMJD3 (KDM6B), which play key roles in fine-tuning the repressive functions of H3K27me3 (25). Reports show that Ezh2 can have both protective and pathological roles in animal models of different renal diseases (26–29). Notably, podocyte-specific deletion of Ezh2 decreased H3K27me3 levels and sensitized mice to glomerular disease. On the other hand, inhibition of the Jumonji C domain–containing H3K27me3 demethylases Jmjd3 and UTX increased podocyte H3K27me3 levels and attenuated glomerular disease in models of nephrotoxicity, nephrectomy, and diabetes (29). Furthermore, podocytes in glomeruli from humans with DN had reduced H3K27me3 and elevated UTX levels, altogether supporting a protective role of H3K27me3 in glomerular podocyte functions (29). However, the roles of Ezh2 or related demethylases and H3K27me3 in TGF-β–induced gene expression in MC dysfunction and DN are unclear.

In addition to histone modifications, noncoding RNAs like miRNAs are also part of the epigenetic machinery. miRNA-dependent mechanisms are involved in diverse biological processes. Mature miRNAs are short noncoding RNAs (~22 nucleotides in length) that down-regulate target mRNA levels or inhibit translation via binding to the 3’-UTRs of target genes (30). TGF-β and diabetes dysregulate several miRNAs in MCs, which promote glomerular dysfunction associated with DN (8, 31, 32). However, the function of TGF-β–induced miRNAs targeting epigenetic regulators in MC dysfunction is not well-known. Notably, dysregulated expression of Ezh2, as well as miR-101 that targets Ezh2, has been implicated in several cancers (23, 27, 33). However, the role of miR-101 in TGF-β–induced gene regulation in MCs, particularly via modulating Ezh2, is unclear.

In this study, we found that TGF-β–induced expression of key MC genes implicated in DN was associated with reduction in H3K27me3 levels at the promoters/enhancers of these target genes. This occurred, at least in part, via miR-101b–mediated down-regulation of Ezh2 as well as increased expression of H3K27me3 demethylases Jmjd3 and Utx. HG also down-regulated Ezh2 protein and increased miR-101b levels in a TGF-β–dependent manner. Furthermore, increased fibrotic gene expression in vivo was associated with similar changes in Ezh2, miR-101b, and H3K27me3 demethylases in MCs and kidneys from rodent models of streptozotocin-induced diabetes. These findings support a protective (repressive) function of H3K27me3 in MCs. Disruption of such epigenetic mechanisms by TGF-β, HG, and diabetes, at least in part via misregulation of the related methylases, demethylases, and miR-101b, can contribute to glomerular dysfunction in DN.

Results

**TGF-β down-regulates Ezh2 levels in RMCs**

To evaluate whether Ezh2 modulates TGF-β–induced fibrotic and inflammatory genes related to glomerular MC dysfunction, we first examined the expression and function of Ezh2 in rat MCs (RMCs). Serum-starved RMCs were treated without (control) or with TGF-β (5 ng/ml) for 24 and 48 h. Ezh2 mRNA and protein levels were determined by RT-qPCR and Western blotting, respectively. Results showed that Ezh2 mRNA (Fig. 1A) and protein levels (Fig. 1B and C), representative blots and bar graph quantification (31) were significantly down-regulated after 24 and 48 h of TGF-β treatment, compared with control untreated RMCs. In addition, immunofluorescence staining with Ezh2 antibodies also showed that nuclear Ezh2 levels were reduced by TGF-β treatment versus control (Fig. 1, D and E). We also performed RT-qPCR to determine mRNA expression of the fibrotic genes connective tissue growth factor (Ctgf) and serpin family E member 1 (Serpine1) and of inflammatory chemokine C-C motif chemokine ligand 2 (Ccl2). All three genes were significantly increased at 24 and 48 h after TGF-β treatment relative to controls (Fig. 1, F and G). These results
demonstrate that TGF-β down-regulates Ezh2 at the same time it increases expression of fibrotic and inflammatory genes in RMCs.

Reduced enrichment/occupancy of H3K27me3 and Ezh2 at the promoters of TGF-β-induced genes

Ezh2 inhibits gene expression by depositing the repressive histone modification H3K27me3 at target gene promoters and enhancers in mammalian cells (22). Because TGF-β down-regulated Ezh2, we next examined whether it also reduced H3K27me3 levels at target gene promoters using ChIP assays with H3K27me3 and Ezh2 antibodies. Because TGF-β significantly inhibited Ezh2 levels by 24 h, we used the 24-h time point in these and all subsequent experiments, unless indicated otherwise. ChIP-enriched DNA was analyzed by qPCR using primers specific to the Ctgf and Serpine1 promoters near Smad-binding elements (SBE) and to the Ccl2 promoter and enhancer near NF-κB–binding sites (Fig. 2A and Table 1), because TGF-β is known to regulate these genes via Smad and NF-κB TFs (34). TGF-β treatment significantly suppressed H3K27me3 levels at the Ctgf, Serpine1, and Ccl2 promoters, as well as the Ccl2 enhancer, compared with control (Fig. 2, B–E). However, no significant differences were observed at the promoter of the housekeeping gene β-actin (Actb; Fig. 2F) or at a control region ∼2 kb upstream of the Serpine1 promoter (Cp4; Fig. 2G), confirming specificity of the ChIP assay results. Furthermore, ChIP assays with Ezh2 antibody showed that, in parallel, Ezh2 occupancy was also significantly reduced by TGF-β at the Ctgf (Fig. 2H) and Ccl2 (Fig. 2I) promoters and the Ccl2 enhancer (Fig. 2K), but not at the Actb promoter (Fig. 2L), compared with controls. TGF-β did not inhibit Ezh2 occupancy at Serpine1 promoter (Fig. 2J), suggesting an Ezh2-independent mechanisms for the observed parallel reduction in H3K27me3.
As the H3K27me3 mark is known to be erased by the histone lysine demethylases Utx and Jmjd3 (25), increased expression of these demethylases may also inhibit H3K27me3. Accordingly, our RT-qPCR analysis revealed that Utx and Jmjd3 mRNA levels were indeed significantly increased by TGF-β/H9252 compared with controls (Fig. 2M), supporting a role for these histone demethylases in TGF-β–induced gene regulation in RMCs. Taken together, these results suggest that H3K27me3 inhibition plays a key role in TGF-β–induced regulation of key genes in RMCs.

**Ezh2 gene silencing enhances TGF-β–induced gene expression in RMCs**

We next used siRNA-mediated gene silencing approaches to evaluate the functional relevance of TGF-β–induced Ezh2

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**Table 1**

| Gene ID | Forward | Reverse |
|---------|---------|---------|
| cDNA primers | | |
| Ezh2 (r) | CCGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Serpine1 (r) | CGGGGATCCGCCGTCTGAGGGAGA | GGGGATCCGCCGTCTGAGGGAGA |
| Ccl2 (r) | GCCGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Tgfβ (r/m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ctgf (r/m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Actb (r/m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Pdia (r) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Serpine1 (m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ezh2 (m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| EZH2 (m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| CTGF (h) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| SERPINE1 (h) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Jmjd3 (r) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ltx (r) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Jmjd3 (m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ltx (m) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |

**ChIP primers (r)**

| Serpine1 (CP1) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Serpine1 (CP2) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ctgf (CP1) | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |
| Ccl2 promoter | CGGTCCCTCTCTGGTTACCTGACATGAA | CGGAGGGAGGAGAGGAGGAGA |

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As the H3K27me3 mark is known to be erased by the histone lysine demethylases Utx and Jmjd3 (25), increased expression of these demethylases may also inhibit H3K27me3. Accordingly, our RT-qPCR analysis revealed that Utx and Jmjd3 mRNA levels were indeed significantly increased by TGF-β compared with controls (Fig. 2M), supporting a role for these histone demethylases in TGF-β–induced gene regulation in RMCs. Taken together, these results suggest that H3K27me3 inhibition plays a key role in TGF-β–induced regulation of key genes in RMCs.

**Ezh2 gene silencing enhances TGF-β–induced gene expression in RMCs**

We next used siRNA-mediated gene silencing approaches to evaluate the functional relevance of TGF-β–induced Ezh2
down-regulation in RMCs. RMCs were transiently transfected with SMART-pool siRNA oligonucleotides targeting Ezh2 (siEzh2) or nontargeting control oligonucleotides (siNTC). Examination of expression of Ezh2 and other TGF-β target genes 72 h post-transfection showed that siEzh2 treatment resulted in significant inhibition of Ezh2 mRNA (Fig. 3A) and protein levels (Fig. 3, B and C) relative to siNTC in both control and TGF-β-treated RMCs. Fig. 3, D–F shows that, in the absence of TGF-β, Ezh2 knockdown was associated with up-regulation of Ccl2 mRNA, whereas Ctgf and Serpine1 mRNAs were not altered. However, Ezh2 knockdown significantly enhanced TGF-β–induced expression of all three genes, Ctgf, Serpine1, and Ccl2, compared with siNTC–transfected RMCs (Fig. 3, D–F). Of note, Ezh2 knockdown also up-regulated Serpine1 (Fig. 3E), even though Ezh2 enrichment at its promoter was not affected (Fig. 2I), suggesting indirect regulation by Ezh2 or cooperation with the demethylases. These results suggest that Ezh2 functions to limit the expression of key fibrotic and inflammatory genes, and its down-regulation can lead to derepression and contribute to TGF-β–induced increased expression of these genes in RMCs.

**TGF-β induces miRNAs that target Ezh2**

Increasing evidence implicates a key role for several miRNAs in regulating the expression of genes relevant to TGF-β actions and kidney dysfunction (8, 32). Therefore, we hypothesized that TGF-β might down-regulate Ezh2 via miRNA-dependent mechanisms. Bioinformatics analysis revealed the presence of binding sites for a number of miRNAs in the rat Ezh2 3′-UTR (Fig. 4A). RT-qPCR analysis showed that the levels of three of these miRNAs, miR-138, miR-26a, and miR-101b (Fig. 4, B–D), but not miR-101a (Fig. 4E), were significantly increased after TGF-β treatment versus control. To determine which of these miRNAs might contribute to TGF-β–induced Ezh2 down-regulation in RMCs, we constructed a reporter plasmid in which rat Ezh2 3′-UTR was fused to firefly luciferase reporter gene. RMCs were co-transfected with this Ezh2 3′-UTR reporter plasmid along with negative control mimic (NC-M) or miR-138 (138-M), miR-26a (26a-M), or miR-101b mimic (101b-M) oligonucleotides, and luciferase activity in cell lysates was expressed as fold over control cells, after normalization with internal control U6 (Fig. 4B). Data are expressed as mean ± S.D. (error bars); *, p < 0.05; **, p < 0.01; ***, p < 0.001. TGF-β (B, numbers on the left indicate protein molecular weight markers (kDa).

**Increased levels of miR-101b down-regulate Ezh2 and enhance TGF-β–induced gene expression in RMCs**

We further investigated the functional role of miR-101b–mediated Ezh2 down-regulation. RMCs were transfected with NC-M or 101b-M oligonucleotides, and 48 h post-transfection, cells were treated without or with TGF-β. Ezh2 protein (Fig. 5, A and B) and mRNA (Fig. 5C) levels were significantly decreased in cells transfected with 101b-M compared with NC-M in both untreated and TGF-β–treated cells. Transfection with 101b-M had no effect on basal levels of Ctgf, Serpine1, or Ccl2 in RMCs (Fig. 5, D–F) but significantly enhanced TGF-β–induced expression of Ctgf and Serpine1 relative to NC-M control cells (Fig. 5, D and E). In contrast, transfection of 101b-M inhibited TGF-β–induced Ccl2 expression (Fig. 5F), suggesting Ezh2– or miR-101b–independent effects. Together, these results demonstrate that down-regulation of Ezh2 via
H3K27me3 regulation by TGF-β in renal mesangial cells

miR-101b plays a key/mechanistic role in TGF-β–induced expression of candidate genes in RMCs.

**TGF-β also alters H3K27me3 regulators and miR-101b in human and mouse MCs**

To further investigate whether the TGF-β–mediated Ezh2 down-regulation observed in RMC is conserved in human and mouse MCs, we examined the effect of TGF-β on mRNA and protein levels of Ezh2 in these cells. TGF-β treatment (24 h) significantly inhibited expression of Ezh2 mRNA (Fig. 6A) and protein (Fig. 6B) and increased expression of Ctgf mRNA and SERPIN1 mRNA (Fig. 6D) as well as miR-101b mRNA (Fig. 6F) in human MCs. In mouse MCs, TGF-β–treatment reduced Ezh2 mRNA expression (Fig. 6G), but Ezh2 protein levels were not significantly inhibited (Fig. 6H and J). On the other hand, similar to RMCs, TGF-β–treatment increased protein levels of H3K27me3 demethylases Utx and Jmjd3 in mouse MCs (Fig. 6, panels H and I and panels K and L, respectively). In addition, Ctgf mRNA expression was also induced by TGF-β as expected (Fig. 6M). These results demonstrate that TGF-β alters the expression of key epigenetic enzymes and miRNAs that can contribute to the reduction of H3K27me3 levels at pathological genes not only in RMC, but also in mouse and human MCs.

**TGF-β mediates HG-induced Ezh2 down-regulation in RMCs**

Diabetes is associated with hyperglycemia, and experimental evidence shows that HG (25 mM glucose) induces TGF-β in MCs, which then subsequently mediates HG-induced epigenetic changes and gene expression involved in MC dysfunction (2, 9). We therefore tested our hypothesis that HG may inhibit Ezh2 expression in RMCs, and this might occur via TGF-β. We cultured RMCs in normal glucose (NG; 5.5 mM) or HG (25 mM) or osmotic control mannitol (5.5 mM glucose + 19.5 mM manitol) for 48 and 72 h and measured Ezh2 mRNA and protein expression. Ezh2 protein levels were reduced at 48 h (Fig. 7A) and 72 h (Fig. 7B) after HG treatment relative to NG, but mannitol had no significant effect. However, Ezh2 mRNA levels were not affected at these time points (data not shown), suggesting the role of post-transcriptional mechanisms, such as inhibition of protein levels by miR-101b. This was supported by the observation that HG also increased miR-101b levels at both 48 and 72 h post-HG treatment versus NG in RMCs. In contrast, mannitol did not significantly affect miR-101b levels (Fig. 7, C and D).

To examine whether TGF-β plays a mediatory role in HG–induced Ezh2 down-regulation, we pretreated RMCs with TGF-β–neutralizing antibodies or control IgG before HG treatment. HG (72 h) significantly reduced Ezh2 protein in RMCs pretreated with IgG, but this effect of HG was ameliorated in RMCs pretreated with TGF-β–neutralizing antibodies compared with control IgG (Fig. 7, E and F). These results demonstrate that HG down-regulates Ezh2 in a TGF-β–dependent manner in RMCs.

**Role of H3K27me3-modifying enzymes in mesangial cell hypertrophy**

Next, we examined the role of Ezh2 and Utx on mesangial cell hypertrophy, a key event in renal dysfunction in DN. RMCs were transfected with siEzh2 or control siNTC oligonucleotides, and total protein content was determined as a marker for hypertrophy. Protein concentration normalized with cell numbers showed that Ezh2 knockdown, mimicking diabetic conditions, did not have a significant effect on RMC hypertrophy relative to siNTC-transfected cells (Fig. 7G). Similarly, transfection of RMCs with miR-101b mimic (101b-M) that targets Ezh2 also did not significantly affect hypertrophy versus negative control NC-M (Fig. 7H). We next examined the role of Utx in RMC hypertrophy. Because our data show that Utx expression is increased by TGF-β, we examined whether its knockdown might inhibit TGF-β–induced hypertrophy. RMCs were transfected with siUtx or siNTC, and total protein content in control and TGF-β–induced cells was determined. siUtx significantly inhibited both basal and TGF-β–induced hypertrophy relative to siNTC (Fig. 7I). These results demonstrate that Utx plays key role in TGF-β–induced hypertrophy in RMCs.

**Dysregulation of H3K27me3-modifying enzymes in kidneys of diabetic rats and mice**

Next, to determine in vivo relevance, we used a streptozotocin–induced rat model of type 1 diabetes and DN. Sprague–Dawley rats were rendered diabetic by injecting a single dose of streptozotocin (STZ) and then euthanized at 4 and 16 weeks post-diabetes to analyze diabetes-induced renal changes relative to citrate buffer–injected control (NS) rats. Diabetic rats (STZ) exhibited significant renal hypertrophy at 4 weeks post-diabetes as evidenced by increased kidney/body ratio and glomerular expansion using PAS staining of renal sections (Fig. S1, A–D). RT-qPCR showed that expression of glomerular Tgfβ and other key DN-related genes Serpine1 and Ccl2 were significantly up-regulated in STZ rats versus control NS rats (Fig. 8, A–C). Furthermore, ChIP assays revealed that
H3K27me3 and Ezh2 enrichment were both reduced at the Serpine1 promoter and Ccl2 enhancer (Fig. 8, D–G), confirming that these key epigenetic changes are also induced by diabetes in vivo.

We also isolated and cultured RMCs from the glomeruli of control (NS) and diabetic (STZ) rats at 4 weeks post-diabetes and examined Ezh2 levels. Ezh2 protein levels were significantly decreased in RMCs derived from diabetic rats (STZ) relative to control (NS) rats (Fig. 8, H and I). Immunofluorescence further confirmed Ezh2 down-regulation in RMCs from diabetic (STZ) rats versus controls (NS) (Fig. 8, J and K). Conversely, Tgf and downstream pro-fibrotic genes Ctgf and Serpine1 were up-regulated in diabetic RMCs (Fig. 8, L–N). Furthermore, Ezh2 protein levels were also significantly lower in kidney cortex of diabetic rats at 16 weeks post-diabetes versus control rats (Fig. 8, O and P). Expression of miR-101b was also significantly increased in RMCs and kidney cortex at 4 and 16 weeks post-diabetes, respectively (Fig. 8, Q and R). In addition, mRNAs of the H3K27me3 demethylases Utx and Jmjd3 were significantly up-regulated in kidney cortex at 16 weeks post-diabetes (Fig. 8, S and T). We also examined changes in Utx and Jmjd3 in glomeruli derived from STZ-induced diabetic mice (STZ) and controls (NS) at 6 weeks post-diabetes. RT-qPCR showed that Utx and Jmjd3 mRNAs were increased in glomeruli from STZ mice versus NS (Fig. 8, U and V). In addition, Utx protein levels were also significantly increased in these glomeruli from diabetic mice (Fig. 8, W and X). Jmjd3 protein levels could not be determined in these mouse samples due to lack of a specific antibody that could detect Jmjd3 in mouse glomerular lysates. Overall, these results from rodent models of diabetes support in vivo relevance of our findings to DN pathogenesis.

Discussion

In this study, we observed that TGF-β can induce pathologic genes in MCs via reducing enrichment of the epigenetic repressive mark H3K27me3 at gene promoters. These epigenetic effects of TGF-β appear to be mediated via concomitant down-regulation of Ezh2 due to increases in miR-101b as well as up-regulation of H3K27me3 demethylases. In addition, HG treatment also induced miR-101b and inhibited Ezh2 in a TGF-β-dependent manner in RMC. Similar mechanisms were conserved in human and mouse MCs. Furthermore, in vivo relevance of these findings was noted in RMC and renal tissues obtained from rodent models of DN. These data suggest that the inhibition of repressive epigenetic mechanisms contribute to TGF-β-induced glomerular dysfunction.

Gene silencing mediated by PRC1 and PRC2 is a well-studied epigenetic mechanism in mammalian cells. The PRC2 complex recruits HDACs to remove H3K27ac and deposit H3K27me3 at target gene promoters and enhancers (22, 23). In contrast, H3K27me3 is erased by the demethylases Utx and Jmjd3, which can result in de-repression and increased transcription (25). Disruption of this fine balance in the actions of these H3K27me3-modifying enzymes can be a key epigenetic mechanism by which pathological genes are up-regulated in the diabetic kidney. We observed that H3K27me3 is decreased by TGF-β at fibrotic and inflammatory gene promoters at least in part via inhibition of Ezh2 expression and its promoter occupancy. Our results also identified miR-101b as a key player in Ezh2 down-regulation in RMCs because Ezh2 is a bona fide target of miR-101b. On the other hand, we did not evaluate the specific mechanisms involved in the loss of Ezh2 occupancy. TGF-β signaling through type I and type II receptors leads to activation of Smad TFs, which mediate the expression of down-stream genes, including Ctgf and Serpine1 (4, 5, 34). Other TFs as well as co-activator HATs, such as CBP/p300 and the H4Kme1-methyltransferase Set7, have also been implicated in TGF-β-mediated regulation of fibrotic and inflammatory genes (8, 9, 34). Studies in other cell types suggest that Smad2...
and Smad4 can displace Ezh2 from target gene promoters (35). Therefore, it is likely that in MCs, TGF-β activated Smad TFs can displace Ezh2 to reduce repressive H3K27me3. However, Ezh2 occupancy was not affected by TGF-β at the Serpine1 promoter, suggesting that H3K27me3 demethylases may be involved in reducing H3K27me3 in this case. This was supported by our data showing up-regulation of Imjmd3 and Utx after TGF-β treatment. Thus, TGF-β may remove H3K27me3 via histone demethylases such as UTX and Jmjd3 at the Serpine1 promoter, whereas miR-101b–mediated Ezh2 down-regulation may be required to prevent deposition of H3K27me3 and promote optimal induction of Serpine1 expression. Interestingly, TGF-β did not reduce Ezh2 protein levels in mouse MCs but up-regulated H3K27me3 demethylases in the mouse cells, suggesting species-specific differences in H3K27me3 inhibition. Notably, studies in podocytes showed that up-regulation of Utx can lead to increases in pathological genes in mice (29). Further examination using knockdown or overexpression approaches could help determine the exact role of H3K27me3 demethylases in TGF-β–induced gene expression in rat and mouse MCs.

Interestingly, several miRNAs predicted to target Ezh2 were induced by TGF-β, but only miR-101b appeared to inhibit Ezh2 in RMCs. In rats, miR-101 is expressed from two genomic locations, chromosome 5 (miR-101a) and chromosome 12 (miR-101b). We found that TGF-β increased only miR-101b levels, and overexpression of miR-101b with mimic oligonucleotides down-regulated Ezh2 via targeting its 3’-UTR and also enhanced TGF-β–induced fibrotic gene expression. However, miR-101b did not enhance Ccl2 expression, likely due to the effects of miR-101b on other targets. Overall, these findings highlight the involvement of miRNAs targeting histone-modifying enzymes in TGF-β–induced gene regulation in RMCs.

We also found that HG, the major upstream effector in DN, reduced Ezh2 expression and up-regulated miR-101b in a TGF-β–dependent manner in RMCs. Similar Ezh2 down-regulation as well as up-regulation of miR-101b and H3K27me3 demethylases were observed in RMCs and kidneys of type 1 diabetic rats. Furthermore, diabetes-induced renal hypertrophy was associated with up-regulation of pro-fibrotic and pro-inflammatory genes as well as reduced enrichment of H3K27me3 and Ezh2 at these gene promoters. We also found up-regulation of Utx and Jmjd3 in glomeruli from diabetic mice. These results further support the in vivo relevance of HG and TGF-β–mediated epigenetic effects.

Evidence shows that prior episodes of hyperglycemic exposure in diabetic patients and in experimental animal models can lead to long-term persistence of renal and other complications even long after glycemic normalization, a phenomenon termed “metabolic memory” (36, 37). Persistence of epigenetic histone modifications and DNA methylation have been suggested to be key mechanisms associated with metabolic memory, which can result in a persistent increase in pathological genes or decrease of protective genes despite glucose normalization (36-38). Interestingly, we found that down-regulation of Ezh2 and up-regulation of miR-101b as well as H3K27me3 demethylases and fibrotic genes persisted in RMCs that were isolated from diabetic rat glomeruli and cultured for several passages in vitro under nondiabetic conditions (Fig. 8), indicating a memory of the in vivo hyperglycemic exposure. These results suggest the involvement of a novel cross-talk between two epigenetic layers, chromatin and noncoding RNAs (miRNAs), in the metabolic memory of sustained DN progression despite glycemic control.

Recent studies showed a protective role for H3K27me3 and Ezh2 in podocytes (26, 29). These studies demonstrated that Ezh2 inhibition and increases in H3K27me3 demethylases led to decreases in H3K27me3 at key oxidative stress genes and promoted podocyte apoptosis and glomerular dysfunction (26, 29). Furthermore, epigenetic inhibitors, such as DZNep or...
miR-101b targeting Ezh2, or genetic deletion of Ezh2 enhanced glomerular dysfunction \(^\text{(26, 29)}\), whereas Utx inhibitors ameliorated these effects in mice with renal injury and DN \(^\text{(29, 39)}\). Our current studies demonstrate that Ezh2 is down-regulated in RMCs, in human and mouse MCs treated with TGF-β/H9252, and in glomeruli from diabetic rats, suggesting a conserved protective function in renal cells, although more studies are needed in different cell types. Overall, these reports in podocytes and our new results in MCs support a protective role for H3K27me3 in glomeruli. Furthermore, our data also reveal new roles for the Ezh2/miR-101 axis and H3K27me3 demethylases in the regulation of H3K27me3 by TGF-β and diabetes in glomerular MCs.

In contrast, another study reported that Ezh2 protein levels are increased by TGF-β/H9252 in renal fibroblasts, and DZNep treatment ameliorated interstitial fibrosis associated with unilateral ureteral obstruction (UUO), suggesting a pathological role for Ezh2 in fibrosis \(^\text{(28)}\). However, only changes in fibrotic proteins were examined and not transcriptional regulation or changes in H3K27me3 at specific gene promoters. Furthermore, unlike DN, UUO is primarily associated with tubular fibrosis \(^\text{(40)}\). Notably, evidence shows that, whereas DzNep is effective against fibrosis, it is not specific to Ezh2, as it targets not only H3K27me3, but also other active and repressive histone modifications \(^\text{(41)}\). These discordant results also indicate cell-, disease-, and even species-specific differences.
in Ezh2-mediated gene regulation and effects in kidney disease.

Interestingly, our findings demonstrated that Utx knockdown inhibited basal and TGF-β-induced hypertrophy in RMC. These results suggest that Utx up-regulation by TGF-β and diabetes may play a key role in mesangial expansion in DN. Previous studies showed that H3K27me3 was decreased in podocytes from humans with glomerular diseases such as focal segmental glomerulosclerosis and DN. This was associated with increased expression of Utx. Furthermore, treatment of mice with Utx inhibitor ameliorated diabetes-induced or adriamycin-induced glomerular dysfunction (29). Our current findings implicate a role for Utx in mesangial cell hypertrophy, further supporting the importance of Utx in renal disease. In podocytes, Utx dysregulates the Notch pathway (29), but further studies are needed to determine Utx target genes that mediate MC dysfunction.

In summary, our results suggest that H3K27me3 and Ezh2 play a role in the regulation of gene expression in MCs under diabetic conditions. Disruption of this epigenetic mechanism by TGF-β and diabetes via key miRNAs and histone demethylases can lead to de-repression and up-regulation of genes involved in glomerular MC as well as podocyte dysfunction (Fig. 9). A clear understanding of the cross-talk between TGF-β–induced miRNAs and chromatin modifications (two key epigenetic layers) can provide important insights into epigenetic mechanisms underlying chronic diabetic complications and metabolic memory that, in turn, could aid the development of novel therapeutics for DN.

**Experimental procedures**

**Materials**

Recombinant human TGF-β1 was purchased from R&D Systems. Primary antibody against Ezh2 (catalogue no. 612667) for Western blotting was from BD Transduction Laboratories (San Diego, CA). Primary antibodies against H3K27me3 (catalogue no. 39155) and Ezh2 (catalogue no. 39901) for ChIP assays were from Active Motif (Carlsbad, CA). In experiments with rat glomeruli, H3K27me3 antibody from Cell Signaling Technology (catalogue no. 9733, Beverly, MA) was used. Primary antibody against β-actin (catalogue no. A5441) was from Sigma-Aldrich (St. Louis, MO). Normal anti-mouse IgG (catalogue no. 12-371) and anti-rabbit IgG (catalogue no. PA64B) horseradish peroxidase-conjugated secondary antibodies were from Millipore (Billerica, MA). Reverse transcriptase kits for cDNA preparation, SYBR Green kits for real-time PCR, and magnetic Protein A/G Dynabeads for ChIP assays were from Thermo Fisher Scientific. Nucleofection reagents were from Lonza, and RNAiMax was from Thermo Fisher Scientific (Carlsbad, CA). Dual-Luciferase assay reagents and Renilla luciferase reporter vector (pRL-TK) were from Promega (Madison, WI). RNAeasy kits were from Qiagen (Valencia, CA). miRIDIAN miRNA mimic oligonucleotides representing miR-101b, miR-26a, and miR-138 and a negative control were purchased from Thermo Fisher Scientific (Carlsbad, CA), Inc. ON-TARGETplus SMARTpool siRNAs targeting Ezh2, Utx, and control oligonucleotides were purchased from Horizon-Dharmacon (Lafayette, CO). The Script miRNA cDNA synthesis kit and perfeCTa SYBR Green SuperMix were from Quanta Biosciences.

**Isolation and treatment of rat mesangial cell cultures**

All animal studies were performed in accordance with protocols approved by the City of Hope Institutional Animal Care and Use Committee. Primary RMCs were isolated from renal glomeruli of Sprague–Dawley rats (9 weeks), and mouse MCs were isolated from 8-week-old C57BL/6 mice as described (11, 14). MCs were cultured in complete medium (RPMI supplemented with 5.5 mM glucose, 10% FBS, penicillin/streptomycin, and 5 μg/ml plasmocin). MCs were used between passages 5 and 10. Human MCs were obtained from Lonza and cultured in manufacturer-supplied medium. In all the experiments, MCs were transferred to serum depletion medium (SD; RPMI containing 5.5 mM glucose, penicillin/streptomycin, plasmocin, and 0.1% BSA) for 24–48 h and treated with SD alone (control) or SD containing TGF-β (5 ng/ml) or high glucose (HG; 25 mM) for the indicated time periods.

**Induction of diabetes in rats and mice with streptozotocin injections**

Type 1 diabetes was induced in 16-week-old male Sprague–Dawley rats by intraperitoneal injection with a single dose of streptozotocin dissolved in citrate buffer (65 mg/kg). Control rats were injected with citrate buffer alone. Diabetic (STZ) and
control (NS) rats were sacrificed at 4 and 16 weeks post-diabetes (blood glucose levels were >300 mg/dl in STZ rats versus 145 mg/dl in control rats). Glomerular fractions were purified and processed for gene expression analysis and ChIP assays. Paraffin-embedded sections of renal cortex from STZ and NS groups were also analyzed by PAS staining to evaluate glomerular histopathology. RMCs were isolated from glomeruli of control and STZ rats at 4 weeks post-diabetes as described (11). These RMCs were cultured in NG and used to analyze gene expression (RT-qPCR) and protein levels (Western blotting and immunofluorescence) as indicated. Kidney cortex samples were used to analyze changes in gene expression at 16 weeks post-diabetes as indicated. Mice (C57BL/6) were made diabetic as described before by injecting streptozotocin dissolved in citrate buffer (50 mg/kg) intraperitoneally for 5 consecutive days (42). Control mice were injected with citrate buffer. After 1 week, blood glucose levels were >350 mg/dl in STZ-injected mice versus 163.5–171.75 mg/dl in control mice. Kidneys were collected from control (NS) and diabetic (STZ) mice at 6 weeks post-diabetes, and glomeruli were isolated by sieving as described before (42) and used for RNA extraction and protein assays.

RNA isolation and RT-qPCR
Total RNA was isolated from RMCs and glomerular fractions using RNeasy columns. Total RNA (500–1000 ng) was used for complementary DNA (cDNA) synthesis by RT using Gene Amp RNA PCR kits with random hexamer primers. Gene expression was analyzed by qPCR using a SYBR Green PCR Master Mix kit with gene-specific primers on ABI 7500 real-time PCR thermal cyclers as described (14). Reactions were performed in triplicate in a final volume of 20 μl. To determine the levels of miRNAs, cDNA was synthesized using the qScript miRNA cDNA synthesis kit, and expression was analyzed by qPCR with perfeCTa SYBR Green SuperMix. Reactions were performed in triplicate in a final volume of 15 μl using miRNA-specific and universal primers. RT-qPCR data were analyzed using the comparative CT method (2−ΔΔCt) using internal controls Ppia or Actb for mRNAs and U6 for miRNAs (11, 14, 43). Results are expressed as -fold over control. Sequences of the PCR primers used in this study are listed in Table 1.

ChIP assays
ChIP assays were performed as described, with some modifications (11, 14, 44). Serum-starved RMCs were treated with TGF-β (5 ng/ml) for the indicated time periods, fixed with 1% formaldehyde for 10 min, and quenched with 125 mM glycine for 5 min at room temperature. The cross-linked RMCs were washed twice with cold PBS containing protease inhibitors and lysed in ChIP lysis buffer. Lysates were sonicated using Bioruptor (Diagenode). Sheared chromatin (0.2–0.6-kb fragments) was immunoprecipitated with 2–5 μg of H3K27me3 or Ezh2 antibodies or control IgG overnight at 4°C. We followed the manufacturer’s recommendation for the concentration of antibodies used in ChIP assays with glomeruli. Immune complexes were captured on Protein A/G magnetic beads (25–30 μl), washed to remove nonspecific proteins, and bound DNA–proteins were eluted. Eluted DNA was reverse cross-linked for 4 h at 65°C, and ChIP-enriched DNA was isolated by phenol/chloroform extraction, followed by ethanol precipitation. Input DNA samples and antibody-enriched ChIP DNA samples were analyzed by qPCR using primers to amplify promoter and enhancer regions of the indicated genes (Fig. 2A and Table 1). Data were analyzed using the 2−ΔΔCt method and normalized with input samples as described (11, 14, 44). Results are expressed as -fold over control cells.

Construction of rat Ezh2 3′-UTR reporters
The Ezh2 3′-UTR was amplified by PCR using rat genomic DNA and cloned into the pZeol-luc vector (45) downstream of firefly luciferase at XbaI/ApaI cloning sites using standard molecular cloning techniques. Insertion in the reporter construct was verified by DNA sequencing.

Transfection of RMCs with siRNAs, Ezh2 3′-UTR reporters, and luciferase assays
RMCs were transiently transfected with ON-TARGETplus SMARTpool siRNAs (Dharmacon) targeting Ezh2 (siEzh2) or nontargeting control oligonucleotides (siNTC) (0.3–0.5 μg) using the NucleofectorTM device (Lonza) as described (43) and plated in 6-well plates. Transfected cells were serum-depleted for 24 h, stimulated with TGF-β (5 ng/ml) for 24 or 48 h, and processed for the extraction of RNA and protein. To analyze Ezh2 3′-UTR activity, RMCs were co-transfected with rat Ezh2 3′-UTR reporter plasmids and internal control plasmid pRL-TK, along with miRNA mimics or control mimic oligonucleotides, using the Nucleofector™ device and plated in 24-well plates. 48 h later, luciferase activity was determined in cell lysates using Dual-Luciferase assays in a 96-well plate reader according to the manufacturer’s instructions (Turner Biosystems, Promega). Firefly luciferase activity was normalized with internal control Renilla luciferase activity expressed from pRL-TK, and results are expressed as -fold over control. In some experiments, RMCs were transfected with siRNAs and miRNA mimics using RNAiMax (Thermo Scientific) using the manufacturer’s recommendations.

Immunofluorescence with Ezh2 antibodies
Immunofluorescence was performed as described, with some modifications (14, 43). Briefly, cells were grown on coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.02% Triton X-100, and blocked with 1% BSA in PBS. Then cells were immunostained using primary antibody against Ezh2 (1:50) followed by FITC-conjugated secondary antibody (1:250). To detect nuclei, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst dye. Cells were visualized through a ×40 objective using appropriate filters on an Olympus BX51 fluorescence microscope (Olympus America). Separate images for each color were captured with a Pixera 600 CL camera using Viewfinder version 3.0 software (Olympus). Images were merged, and fluorescence intensity (green) was quantified using Image-Pro Premier software (Media Cybernetics).

Determination of MC hypertrophy
RMCs were plated in 24-well plates and transfected with the indicated siRNA or miRNA mimic oligonucleotides. The fol-
lowing day, they were transferred to serum depletion medium and 24 h later treated with or without TGF-β. Two days later, cells were detached using trypsin and counted using a Z2 Coulter Counter (Beckman Coulter, Indianapolis, IN). Cells were pelleted by centrifugation, washed with PBS, and lysed in 0.5% SDS. Total protein content in cell lysates was estimated using the DC Protein assay kit (Bio-Rad). Protein content was normalized to total cell number, and results are expressed as -fold over control.

**Western blotting**

Protein lysates were prepared, and Western blotting with the indicated antibodies was performed as described (14). Equal amounts of cell lysates (20–30 μg) were run on 4–15% denaturing polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer containing 5% milk, incubated with primary antibody (1:1000 dilution in blocking buffer), washed, and incubated with appropriate horseradish peroxidase–conjugated secondary antibody (1:3000). Subsequently, membranes were washed, and protein bands were visualized using an enhanced chemiluminescence kit (PerkinElmer Life Sciences). Autoradiograms were scanned using a GS-800 calibrated densitometer, and contrast was adjusted uniformly across the entire autoradiogram to better visualize the protein bands. Intensity of protein bands was quantified using Quantity One or Image Lab software (Bio-Rad). Results were normalized to internal control β-actin or cyclophilin A and expressed as -fold over control.

**Statistical analyses**

Data from multiple experiments are expressed as mean ± S.D. Unpaired Student’s t tests were used to compare two groups, or one-way analysis of variance followed by post hoc tests were used for multiple-group comparisons, using PRISM software (GraphPad Software, La Jolla, CA). Statistical significance was detected at the p < 0.05 level.

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