Post-transcriptional Up-regulation of Tsc-22 by Ybx1, a Target of miR-216a, Mediates TGF-β-induced Collagen Expression in Kidney Cells†§

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Increased accumulation of extracellular matrix proteins and hypertrophy induced by transforming growth factor-β1 (TGF-β) in renal mesangial cells (MC) are hallmark features of diabetic nephropathy. Although the post-transcriptional regulation of key genes has been implicated in these events, details are not fully understood. Here we show that TGF-β increased microRNA-216a (miR-216a) levels in mouse MC, with parallel down-regulation of Ybx1, a miR-216a target and RNA-binding protein. TGF-β also increased protein levels of Tsc-22 (TGF-β-stimulated clone 22) and collagen type I and II (Col1a2) expression in MC through far upstream enhancer E-boxes by interaction of Tsc-22 with an E-box regulator, Tfe3. Ybx1 colocalized with processing bodies in MC and formed a ribonucleoprotein complex with Tsc-22 mRNA, and this complex formation was reduced by TGF-β, miR-216a mimics, or Ybx1 shRNA to increase Tsc-22 protein levels but enhanced by miR-216a inhibitor oligonucleotides. Chromatin immunoprecipitation (ChIP) assays revealed that TGF-β could increase the occupancies of Tsc-22 and Tfe3 on enhancer E-boxes of Col1a2. Co-immuno-precipitation assays revealed that TGF-β promoted the interaction of Tsc-22 with Tfe3. These results demonstrate that post-transcriptional regulation of Tsc-22 mediated through Ybx1, a miR-216a target, plays a key role in TGF-β-induced Col1a2 in MC related to the pathogenesis of diabetic nephropathy.

Diabetic nephropathy (DN)² is one of the major complications of diabetes. The main features of DN include renal glomerular and tubular hypertrophy and fibrosis due to progressive accumulation of extracellular matrix (ECM) proteins in component kidney cells (1). Transforming growth factor-β1 (TGF-β) levels are increased in renal cells including mesangial (MC) and other cells under diabetic conditions and up-regulate ECM proteins such as collagens. TGF-β promotes ECM gene expression via both Smad transcription factors and E-box-dependent mechanisms (2, 3). E-box elements are located in the far upstream enhancer region of the collagen gene (4–6). E-box repressors, Zeb1 (also known as ESE1) and Zeb2 (also known as Smad-interacting protein1, SIP1), repress target genes by binding to E-boxes in their promoters (7, 8). They are also repressors of collagen type I and type II genes (4, 5). TGF-β increases Col1a2 expression in MC by down-regulating Zeb1 and Zeb2 (9). E-boxes are positively regulated by transcription factor E3 (TFE3) (3, 10–12) as well as by upstream stimulatory factors (USFs) (13–17). TGF-β is also reported to promote MC hypertrophy by activating the phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway (10, 18–20), as well as other cell types (21, 22). PI3K phosphorylates membrane phosphatidylinositol-diphosphate to generate the second messenger lipid phosphatidylinositol-triphosphate, which in turn activates Akt. Activated Akt phosphorylates several downstream proteins to control cell survival and protein synthesis. PTEN (phosphatase and tensin homologue), a tumor suppressor that is frequently mutated in various human cancers, inhibits Akt activation by dephosphorylating phosphatidylinositol-triphosphate (23). Decreased Pten expression levels and enhanced Akt activation are associated with hypertrophy in MC treated with high glucose or TGF-β (10, 19). PI3K/Akt signal transduction is also involved in ECM gene up-regulation (20, 24).

MicroRNAs (miRNAs) are short non-coding RNAs of ~22 nucleotides that induce post-transcriptional gene repression by blocking protein translation (by binding to the 3’ UTR of their target genes) or by inducing mRNA degradation (25, 26). miRNAs also repress gene expression by recruiting target miRNAs to cytoplasmic RNA processing bodies (P-bodies) for RNA storage, decapping, deadenylation, or degradation (27). At least 60% of human protein-coding genes expressed in the genome are down-regulated by miRNAs (25). Recent evidence suggests that miRNAs regulate the expression of key genes relevant to cancer and kidney diseases (28–30). miR-192 is up-regulated in the renal glomeruli of diabetic mice and in glomerular MC treated with TGF-β and induces Col1a2 gene by inhibiting Zeb1 and Zeb2 (E-box repressors) (9). miR-192 is also up-regulated in renal fibrosis (mouse kidneys after unilateral ureteral obstruction and a rat model of remnant kidney

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**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary mouse MC (MMC) were isolated and cultured as described (9). Recombinant human TGF-β1 was from R&D Systems, Inc. Mouse kidney cell line TCMK-1 was maintained as reported (9).

**Mouse Models**—All animal studies were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Induction of diabetes by streptozotocin (STZ) injections in C57BL/6 mice was carried out as described (9, 10). Mice were used 7 weeks after the onset of STZ injections in C57BL/6 mice was carried out as described (9). Mice were used 7 weeks after the onset of TGF-β1-induced activation of Akt in mouse MC (10). miR-216a and miR-217 are both located in an intron of a non-coding RNA, RP23-298H6.1-001 (RP23). Interestingly, this non-coding RNA (as well as miR-216a and miR-217) is regulated by TGF-β and miR-192 through E-boxes present in the RP23 promoter region. These TGF-β-triggered miRNA circuits and downstream signaling cascades result in enhanced ECM accumulation, hypertrophy, cell survival, and oxidant stress in MC related to the pathogenesis of DN (10).

Several mechanisms of enhanced mRNA translation of ECM and other proteins related to diabetic kidney diseases have been proposed (1, 36). A recent report showed that heterogeneous nuclear ribonucleoprotein E1 phosphorylation by Akt activated by TGF-β up-regulates Dab2 and interleukin-like epithelial-to-mesenchymal transition inducer translation (37). However, a potential role for miRNA-mediated regulation of mRNA translation in response to TGF-β in renal cells has not been fully studied. Here we show that TGF-β induces miR-216a in MC and down-regulates its target Ybx1, which regulates the translation of a binding partner RNA, Tsc-22. Furthermore, we uncovered a new mechanism by which Ybx1, Tsc-22, and Tfe3 coordinately mediate TGF-β-induced Col1a2 expression in MC.

**Chromatin Immunoprecipitation (ChIP) Assays**—ChIP assays were performed as reported (9). Briefly, MMC were serum-depleted and treated with TGF-β for 24 h and then formaldehyde-fixed. The cross-linked chromatin was sheared and immunoprecipitated using antibodies against Tfe3, Tsc-22, or IgG (negative control). ChIP-enriched DNA was purified and used as template for real-time qPCRs using primers spanning E-box regions as described (9). 

**Plasmids and Ybx1 3′-UTR Reporters**—Mouse Ybx1 3′-UTR region was amplified by PCR using primers below and high
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fidelity polymerase Pfu Ultra polymerase, digested with Xhol and NotI, and cloned into the NotI-Xhol site of psiCheck2 (Promega). Plasmid containing mouse Ybx1-3′-UTR sequence was used as a reporter (psiCheck2mYbx1-3′-UTR), whereas the plasmid containing the 3′-UTR sequence harboring a mutation in the miR-216a site was a negative control (psiCheck2mYbx1-3′-UTR-Mut). For mutagenesis of the miR-216a target site, two primers below were used. Tfe3 and its dominant negative mutant and shRNA expression vectors were described (10).

Primers for PCR of Ybx1 3′-UTR region are: 5′-gtactcgag-TGCCGGCTTACCATCTCTAC-3′, 5′-actgctgggacgct-CTT-TATTAACAGGTGTTGCAGT-3′. Lower case denotes sequences added for cloning. miR-216a site mutagenesis primers into Ybx1 3′-UTR are: 5′-CATATCTGTCGA GTTCTCGAGTTTTAAGAA-A-3′, 5′-TTCTTAAAACTCGAGAA CATGACCAGATAG-3′. The mutated sites (and new Xhol site introduced) are underlined.

Luciferase Assays—MMC were transfected with plasmids and/or small RNAs using Nucleofector as described (10). After 24 h, luciferase (Luc) activities were measured as described (9). For miRNA and shRNA experiments, cells were treated for 48 h.

shRNA against Mouse Ybx1—U6 promoter-driven shRNA expression vector targeting Ybx1 was constructed as described (9). Briefly, U6 promoter region was amplified using 5′-U6 primer and Ybx1 target reverse primer below. The amplified fragment was cloned into pcR3.1EGFP, whereas plasmid expressing scrambled shRNA (pcR3.1EGFP-Scr) was used as control. The Ybx1 shRNA reverse primer is: 5′-GGACTCGAGGA- AAAAAAAGAAACCTCGTTGCGATGACCCTACACA- AAGGTCTACGCAACGAGGTTTCCGTGTTCGTC- TTCC-3′. The target sequence is underlined.

miR-216a Inhibits Ybx1 in Mouse Kidney Cells—To test whether exogenous miR-216a addition inhibits endogenous levels of Ybx1, miR-216a mimic oligonucleotides were transfected into MMC using nucleofection (10), and protein levels of Ybx1 were examined. Significant decrease of Ybx1 was confirmed as described (9). Protein Domain Structure Prediction—For prediction of domain structures of TFE3 and TSC-22, on-line database SWISS-MODEL (42) was used. Downloaded results were further analyzed using the 3D Molecular Viewer (Vector NTI, Invitrogen) to depict interaction of two proteins through the leucine zipper (LZ) structure.

RESULTS AND DISCUSSION

Decreased Ybx1 Levels in mouse MC (MMC) Treated with TGF-β and in Diabetic Kidney Glomeruli—TGF-β treatment led to a significant decrease in Ybx1 mRNA (from 6 to 24 h) and protein levels at 24 h in MMC (Fig. 1, A and B). Ybx1 mRNA levels were also significantly lower in kidney glomeruli from type 1 diabetic mice (STZ-injected) (Fig. 1C) as well as type 2 diabetic mice (db/db) (Fig. 1D) when compared with their respective controls (control C57BL/6 and db/db/+). Parallel decrease in Ybx1 expression in glomeruli of these diabetic mice by immunostaining was observed when compared with their non-diabetic controls (Fig. 1, E–J). Ybx1 was suggested to be a potential target of miR-216a (43), and according to the databases miRBase, TargetScan, and microRNA.org (Fig. 1A). We confirmed a significant increase in miR-216a levels in glomeruli of type 1 and type 2 diabetic mice when compared with corresponding control mice (Fig. 1, L and M) as reported previously (10). TGF-β and Colla2 gene expression levels were also increased in glomeruli from these diabetic mice (9). miR-216a levels were increased from 6 to 24 h (2–3.5-fold) in MMC treated with TGF-β (Fig. 1N). Under both conditions (diabetic glomeruli and MMC treated with TGF-β), increased miR-216a and decreased Ybx1 levels were observed. These results suggest that increased miR-216a under diabetic conditions may inhibit the expression of its target, Ybx1.

Because TGF-β activates Akt (10, 18, 19) and Akt phosphorylates Ybx1 (40, 44), phosphorylation of Ybx1 was examined (Fig. 1B). Increased Ybx1 phosphorylation was observed at 6 h after TGF-β treatment, but this disappeared by 24 h, whereas Akt activation (P-Akt) was sustained through 6–24 h. Ybx1 is phosphorylated by TGF-β-activated Akt (at 6 h); however, by 24 h, increased miR-216a subsequently reduces its target, Ybx1 protein. Therefore, Ybx1 is regulated by at least two different mechanisms by TGF-β.

miR-216a Inhibits Ybx1 in Mouse Kidney Cells—To test whether exogenous miR-216a addition inhibits endogenous levels of Ybx1, miR-216a mimic oligonucleotides were transfected into MMC using nucleofection (10), and protein levels of Ybx1 were examined. Significant decrease of Ybx1 was
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control (NC) and scrambled control (SCR) (Fig. 2, C and D). We confirmed these results using a mouse kidney cell line, TCMK-1 cells. Ybx1 protein levels were decreased by over 80% by the miR-216a mimic, whereas Ybx1 mRNA levels were not altered (supplemental Fig. S1, A and B). In parallel, Col1a2 mRNA levels were increased by miR-216a mimic (supplemental Fig. S1C). mRNA and protein levels of Ybx1 were significantly reduced by Ybx1 shRNA (supplemental Fig. S1, D and E), whereas Col1a2 mRNA levels were increased (supplemental Fig. S1F). These results strongly suggest that miR-216a targets endogenous Ybx1 and that Ybx1 is a key negative regulator of Col1a2 in mouse kidney MC.

Ybx1 3'-UTR Is a Direct Target of miR-216a—To verify whether the Ybx1 3’-UTR is a direct target of miR-216a, Luc reporter vectors containing the Ybx1 3’-UTR were constructed (Fig. 2E). miR-216a mimic significantly inhibited Luc activity of Ybx1 3’-UTR vector by almost 70% relative to a negative control mimic (NC Mimic) but not in the two control constructs, namely control without 3’-UTR (first pair of bars) or that with Ybx1 3’-UTR that has base substitutions in the miR-216a site (third pair of bars) (Fig. 2F). Because TGF-β increased miR-216a expression (Fig. 1N), we tested the effects of TGF-β on the Ybx1 3’-UTR reporter activity. TGF-β significantly inhibited Ybx1 3’-UTR vector Luc activity in MMC (by almost 80% relative to untreated cells) but had no significant effect in control or miR-216a site mutant vector-transfected cells (Fig. 2G). These results suggest that miR-216a increased by TGF-β can inhibit Ybx1 3’-UTR Luc activity. Then, we examined whether a miR-216a inhibitor reverses the effects in this system. miR-216a inhibitor demonstrated a dose-dependent increase in Luc activity from 5 to 20

FIGURE 1. Expression of Ybx1 and miR-216a. A, Ybx1 mRNA levels were significantly decreased in MMC treated with TGF-β (10 ng/ml). Bars represent the mean ± S.E. (n = 3). B, Ybx1 protein levels were decreased by 24 h in MMC treated with TGF-β. Phosphorylation of Ybx1 (P-Ybx1) was detected at 6 h but disappeared at 24 h. Increased Akt phosphorylation was observed at 6–24 h. CTR, control. C and D, mRNA expression levels of Ybx1 in mouse glomeruli. A significant decrease of Ybx1 mRNA levels in glomeruli from both type 1 and type 2 diabetic mice was seen when compared with control mice. Mean ± S.E. (n = 4). E-H, immunohistochemical staining of Ybx1 in glomeruli from normal and diabetic mice. A significant decrease in Ybx1 staining in glomeruli of type 1 (STZ) diabetic (F) when compared with control mice (E) (scale bar, 20 μm) and in db/db type 2 diabetic (H) was seen when compared with control db/+ mice (G). I and J, significant decrease of Ybx1 staining in diabetic mouse kidney glomeruli (STZ or db/db) when compared with control (CTR or db/+). Mean ± S.E. (n = 3). K, alignments of human and mouse miR-216a and Ybx1 3’-UTR sequences. L–N, miR-216a expression levels are increased in glomeruli from type 1 (STZ) diabetic mice (L) and from type 2 (db/db) diabetic mice (M) and in MMC treated with TGF-β (N) when compared with respective controls. Mean ± S.E. (n = 3). *** and * indicate p < 0.001, p < 0.01 and p < 0.05, respectively.

observed in MMC transfected with miR-216a mimic in a dose-dependent manner (Fig. 2, A and B). Reciprocally, Col1a2 mRNA levels were increased by miR-216a mimic or by Ybx1 shRNA when compared with their respective controls (negative nm (Fig. 2H), further confirming Ybx1 as a true target of miR-216a.

Mouse Col1a2 Proximal Promoter—Ybx1 was initially identified as a transcriptional factor. A potential Ybx1-binding
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A sequence is present in the proximal promoter of human COL1A2 gene along with a Smad-binding element (CAGA repeats). To evaluate a potential role in the mouse, we cloned this sequence along with a Smad-binding element (CAGA repeat) and a potential Y-box binding element (CAGCac) (Fig. 3, A). This human E-box sequence is present in the proximal promoter of human COL1A2 gene. Consensus Smad-binding element (CAGA repeat) and a potential Y-box were found in this promoter region (Fig. 3, A and B). However, this mouse proximal promoter did not respond to TGF-β (Fig. 3C, lowest set of bars), although the human COL1A2 proximal promoter was sufficient to respond to TGF-β (45, 46). Because our previous data showed that E-boxes in the far upstream region of the mouse Col1a2 gene respond to TGF-β (9), we cloned these E-boxes into the mouse proximal promoter. This reporter responded to TGF-β (Fig. 3C, middle set of bars), demonstrating that E-boxes are essential for TGF-β response in mouse cells. To further investigate this discrepancy, we compared the sequences of human and mouse proximal promoters and found that the human promoter has a putative E-box (CAGCTG), whereas the mouse Col1a2 promoter has a two-base substitution (CAGCac) (Fig. 3A). This human E-box sequence was conserved in chimpanzee, monkey, and pig but not in mouse or rat (Fig. 3A). Using the QuikChange site-directed mutagenesis method, we altered the sequence to introduce the E-box in the mouse proximal promoter. As expected, this humanized (+E-box) mouse proximal promoter of Col1a2 responded to TGF-β (Fig. 3C, top set of bars). These results demonstrate that the E-box is essential for TGF-β response and that the mouse proximal promoter cannot respond to TGF-β because of the sequence substitution in the potential E-box. Collaboration of far upstream enhancer E-boxes with the mouse Col1a2 proximal promoter is necessary for TGF-β response in mouse kidney cells.

E-boxes are essential for TGF-β response in the promoters of PAI-1, Smad7, and non-coding RNA RP23-298H6.1-001 (RP23) hosting miR-216a (3, 10, 47). These data are consistent with previous observations that the far upstream enhancer region (including E-boxes) is necessary for strong expression of Col1a2 reporter gene in transgenic mice (48) and that far upstream E-boxes are major regulators of the mouse Col1a2 gene (4, 5, 9, 10). This sequence variation in the Col1a2 gene between human and mouse or rat is intriguing and might explain the difference in their susceptibility to diabetic kidney diseases.

Tfe3 Is a Positive Regulator of E-boxes in the Mouse Col1a2 Promoter in Response to TGF-β—Our data show that E-boxes play key roles in Col1a2 promoter activity in response to TGF-β in MMC. E-box repressors, such as Zeb1 and Zeb2, act as neg-
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![Diagram of Col1a2 proximal promoters](image)

**FIGURE 3. Col1a2 proximal promoter activity.** A, comparison of the proximal promoter sequence of the Col1a2 gene in several species (human, chimpanzee, monkey, pig, rat, and mouse). Conserved consensus sequences of potential Smad site (CAGA repeats), Y-box, and TATA box (TA) were found in all the listed species. A potential E-box (CAGCTG) was found in the promoter of human, chimpanzee, monkey, and pig. However, two base substitutions that disrupted the E-box consensus were in rat and mouse (CAGCac). B, structure of the promoter region of mouse Col1a2 gene. A cluster of three E-boxes is located in the 16-kb far upstream region of mouse Col1a2 gene. C, response of the mouse proximal promoter to TGF-β. The cloned proximal promoter of mouse Col1a2 gene did not respond to TGF-β (bottom construct). The addition of far upstream E-boxes conferred TGF-β response to this mouse proximal promoter (third construct from the bottom). Deletion of the Y-box and Smad sites led to loss of basal promoter activity (second construct from the bottom) even if the upstream E-boxes were added (fourth construct from the bottom). Base substitutions, which created a putative E-box (the same sequence as the human promoter, humanized), restored the TGF-β response (top construct). Mean ± S.E. (n = 4). *** and * indicate p < 0.001 and p < 0.05, respectively.

active regulators of the Col1a2 gene (4, 9, 10). However, positive regulators of E-boxes in the Col1a2 gene promoter have not yet been described. In TGF-β signaling, Tfe3 is a major transcription factor associated with the expression of the PAI-1, Smad7, and RP23 (host gene for miR-216a) genes (3, 10, 47). Tfe3 is also a positive regulator of E-boxes in the IRS2 promoter (11). Therefore, we next tested whether Tfe3 is a candidate positive regulator of Col1a2 gene expression. Tfe3 protein expression was increased by TGF-β in a dose-dependent (6–24 h) manner in MMC (Fig. 4A). ChIP assays showed that the occupancy of Tfe3 at E-boxes in the far upstream enhancer region of the Col1a2 gene was significantly increased at 6 h after TGF-β treatment with 10-fold enrichment at 24 h (Fig. 4D), as noted earlier (9). These results demonstrate that the E-box repressor Zeb1 is replaced by the activator Tfe3 in response to TGF-β.

To further verify the positive role of Tfe3 on the Col1a2 promoter, a Tfe3 expression vector was co-transfected into MMC with two types of Col1a2 promoter reporter constructs. Tfe3 overexpression had no effect on the Col1a2 promoter without E-box (Col1a2P-luc) (Fig. 4E) (left set of bars), even after TGF-β treatment. However, Tfe3 increased the basal reporter activity of a Col1a2 promoter containing E-boxes (E-box-Col1a2P-luc) and its induction by TGF-β significantly (Fig. 4E) (right set of bars). On the other hand, a dominant negative Tfe3 mutant or Tfe3 siRNA clearly inhibited basal and TGF-β-induced Luc activity of the same construct (E-box-Col1a2P-luc) (Fig. 4F). These results further demonstrate that Tfe3 is a major positive regulator of the Col1a2 promoter at E-boxes and mediates TGF-β responses in MC.

In our current studies, we could not demonstrate any role for the potential Y-box in mouse proximal promoter in the TGF-β response. No change in promoter activity or TGF-β response was detected when the Y-box was deleted from the proximal promoter constructs, even when the far upstream E-boxes were present (Fig. 3C). Therefore, this potential Y-box might play a role in basal Col1a2 expression. We therefore next pursued other potential mechanisms by which Ybx1 might regulate Col1a2 gene under our experimental conditions.

Colocalization of Ybx1 with P-bodies in MMC—Ybx1 is also known as an RNA-binding protein that regulates mRNA stability and translation (40, 49, 50). Ybx1 was reported to be a component of cytoplasmic processing-bodies (P-bodies) and stress...
Tfe3 occupancy at the Col1a2 enhancer (E-box region) at 6–24 h after TGF-β treatment (n = 3). D, combined results of occupancies of Zeb1 and Tfe3 on the proximal promoter. The proximal promoter without upstream E-boxes (Col1a2P-luc) did not respond to TGF-β or to Tfe3 overexpression. However, the promoter containing three E-boxes (E-box-Col1a2P-luc) responded to TGF-β and Tfe3, suggesting that E-boxes located in far upstream of Col1a2 gene are essential for TGF-β response. F, effects of dominant negative Tfe3 (Tfe3dn) and Tfe3 siRNA on the mouse Col1a2 promoter (with E-boxes). Dominant negative Tfe3 or Tfe3 siRNA inhibited basal and TGF-β-induced reporter activity of E-box-Col1a2P-luc. Mean ± S.E. (n = 4). ** and * indicate p < 0.01 and p < 0.05, respectively.

granules (51, 52). Ybx1 was also found in the Ago-binding protein complex (53). Therefore, we examined the intracellular localization of Ybx1 in MMC. MMC were transfected with pcNEGFP-DCP1a, a well known P-body marker (38), and Ybx1 localization was detected by immunofluorescence. DCP1aGFP signals (green spots) were observed in the cytoplasmic region of MMC (Fig. 5A), indicating P-bodies. Ybx1 (Texas Red staining) was located in both the nucleus and the cytoplasmic regions, and many of the Ybx1-stained spots clearly overlapped with the green DCP1a spots (Fig. 5, B and C, arrows), suggesting that Ybx1 and P-bodies were clearly, but partially, colocalized. Interestingly, 24 h after TGF-β treatment, Ybx1 spots disappeared, and cytoplasmic Ybx1 staining was greatly attenuated...
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FIGURE 6. Ybx1 functions as an RNA-binding protein. A, Tsc-22 mRNA precipitated with Ybx1 but not Col1a2 mRNA. TGF-β decreased Ybx1-Tsc-22 mRNA binding. M, molecular weight markers. Input, RNA extracted from RNA-protein complex without IP. B, real-time qPCR of RNA-IP samples. TGF-β decreased Ybx1-Tsc-22 mRNA interaction significantly. Ybx1 RNP, Ybx1-containing ribonucleoprotein. Mean ± S.E. (n = 3). C, Tsc-22 protein levels were increased at 24 h after TGF-β treatment. D and E, Ybx1 shRNA (D) and miR216a mimic (E) significantly decreased Tsc-22 mRNA levels in Ybx1 ribonucleoprotein. SCR, scrambled control; Mimic NC, negative control inhibitor. F, miR-216a inhibitor significantly increased Tsc-22 mRNA levels in Ybx1 ribonucleoprotein. Inhibitor NC, negative control inhibitor. G, schematic model of the up-regulation of Tsc-22 translation by TGF-β or diabetic conditions. Ybx1 decrease by miR-216a releases Tsc-22 mRNA from the P-body, leading to enhancement of Tsc-22 translation. H–J, Immunostaining of Tsc-22 in mouse renal glomeruli. A significant increase of Tsc-22 in glomerul a from db/db type 2 mice was seen when compared with negative control mimic treatment (Fig. 5, N and O). Notably, Ybx1 signals in P-body (GFP)-positive cells were clearly weaker than in those without P-body signals (Fig. 5, P–R), whereas no difference was observed between GFP-positive and negative cells transfected with negative control (NC) (Fig. 5, M–O), indicating that cells transfected with miR-216a (GFP-positive) have lower levels of Ybx1 protein. These results are consistent with the data in Figs. 1 and 2 showing that TGF-β or miR-216a inhibits Ybx1 expression even in MMC. No clear change in P-bodies themselves was detected after treatment with TGF-β or miR-216a mimic relative to corresponding controls (Fig. 5, A, D, G, J, M, and P), suggesting that TGF-β and miR-216a do not directly affect P-bodies and that Ybx1 may function as an RNA-binding protein in cytoplasmic P-bodies in response to TGF-β or miR-216a.

Ybx1 Plays a Role as an RNA-binding Protein in Response to TGF-β—A series of transcripts has been reported to be Ybx1-binding RNAs (40). We first tested whether Col1a2 mRNA binds to Ybx1 in MMC using RNA immunoprecipitation (RNA-IP). However, Col1a2 mRNA was not precipitated with Ybx1 (Fig. 6A), although we did detect Tsc-22 (TGF-β-stimulated clone 22 or TGFβi4) transcripts, a reported positive control for this RNA-IP experiment (40). However, interestingly, Ybx1-Tsc-22 mRNA binding was greatly decreased after TGF-β treatment (Fig. 6A). qPCR further confirmed that the relative amount of Tsc-22 RNA bound to Ybx1 was significantly decreased by TGF-β (Fig. 6B). Conversely, Tsc-22 protein levels were increased by TGF-β in MMC (Fig. 6C), suggesting an enhanced translation of Tsc-22 after release of the Tsc-22 mRNA from Ybx1. Ybx1 shRNA or miR-216a mimic reduced

(Fig. 5, K and L, TGF-β 24 h), whereas no change was seen in control untreated cells (Fig. 5, compare E or B with F or C, respectively) or to 6 h TGF-β treatment (Fig. 5, H and I). miR-216a mimic also induced a decrease in cytoplasmic Ybx1 signals (Fig. 5, Q and R), similar to TGF-β actions, when compared

the interaction of Tsc-22 mRNA with Ybx1, whereas the miR-216a inhibitor increased the interaction (Fig. 6, D–F). These results suggest that TGF-β may enhance Tsc-22 translation through up-regulation of miR-216a, which targets and reduces Ybx1 (Fig. 6G). Tsc-22 levels were also increased in the glomer-
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A

| Relative occupancy | 0h | 6h | 24h | 0h | 6h | 24h |
|-------------------|----|----|-----|----|----|-----|
| Smad4             |    |    |     |    |    |     |
| Tsc-22            |    |    |     |    |    |     |

B

| Relative occupancy | 0h | 6h | 24h | 0h | 6h | 24h |
|-------------------|----|----|-----|----|----|-----|
| Smad4             |    |    |     |    |    |     |
| Tsc-22            |    |    |     |    |    |     |

C

| Relative occupancy | 0h | 6h | 24h |
|-------------------|----|----|-----|
| Smad4             |    |    |     |
| Tsc-22            |    |    |     |

D

Far-upstream enhancer region

E-box E-box E-box ~16kb Smad Y-box TATA Col1a2 gene

Proximal promoter

E

Mouse Tsc-22

Far-upstream enhancer region

F

Tsc-22 LZ (992-1047)

Tfe3 HLH LZ (205-289)

Leucine

G

IP IgG Tfe3 Tsc-22 Tsc-22 (2.5%)

MMC (TGF-β)

H

IP IgG Tfe3 Tsc-22 Tsc-22 -Tfe3

MMC

SD TGF-β

I

IP IgG Tfe3 Tsc-22 Tsc-22 -Tsc-22 WB

MMC

SD TGF-β

J

Relative luc activity

| Relative luc activity | 0h | 6h | 24h |
|-----------------------|----|----|-----|
| None                  |    |    |     |
| TGF-β                 |    |    |     |

K

Zeb1

Diabetic conditions TGF-β

Tsc-22

Tfe3

E-box

Col1a2

Col1a2
It should be noted that upstream stimulatory factors (USF1 and USF2) have been reported to be involved in TGF-β up-regulation by high glucose and diabetic conditions and associated with the pathogenesis of DN (13–15, 56). USFs are also E-box-regulators and have a LZ motif, which is critical for homo- or heterodimerization (16). USFs also mediate TGF-β-induced plasminogen activator inhibitor-1 (PAI-1), collaborating with EGF receptor signaling (17). Therefore, it is possible USFs may also be involved in TGF-β-induced Col1a2 in MMC by interacting with Tsc-22 through their LZ motifs. This is intriguing and remains to be elucidated in the future.

Collagen Induction by Diabetic Conditions and TGF-β Mediated by miR-216a, Ybx1, Tsc-22, and Tfe3—Our results demonstrate that miR-216a up-regulated by diabetic conditions or TGF-β targets Ybx1, an RNA-binding protein. Ybx1 reduction releases Tsc-22 mRNA (probably from P-bodies) to enhance Tsc-22 protein levels in MMC treated with TGF-β. Tsc-22 in turn interacts with Tfe3 and enhances Col1a2 expression through far upstream E-box region (supplemental Fig. S2). Tsc-22 was cloned as a gene induced by TGF-β (57) and regulated post-transcriptionally by TGF-β (58). Interestingly, a specific allele in the promoter region of the TSC-22 gene is associated with an increased risk of human DN (59), suggesting that TSC-22 plays a role in the development of human DN.

On the other hand, Ybx1 is also phosphorylated by Akt, and this promotes the translation of mRNAs bound to Ybx1 (40, 44, 60). In the current study, we also observed increased Akt activation and Ybx1 phosphorylation in TGF-β-treated MMC (Fig. 1B). Therefore, Ybx1 may be regulated by different miRNA-regulated mechanisms in response to TGF-β. One is via phosphorylation and inactivation of Ybx1 by TGF-β-induced Akt activation (via inhibition of Pten targeted by miR-216a) (10, 19). The other is via down-regulation of Ybx1 targeted by miR-216a. Both steps are mediated by miR-216a induced by TGF-β.

The promoter of the non-coding RNA RP23 (the host gene of miR-216a and miR-217) also contains E-boxes (10), suggesting that, apart from the Col1a2 promoter, increased miR-216a may also activate its own promoter through Tsc-22 and Tfe3. Therefore, the miR-216a/Ybx1 pathway can amplify the signaling initiated by TGF-β (supplemental Fig. S2). These positive feedback signaling loops can further accelerate and augment signaling through miR-216a and miR-217 and their targets Ybx1 and Pten. Therefore, once the signal is

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FIGURE 7. Tsc-22 interacts with Tfe3 and enhances Col1a2 expression through E-boxes. Tsc-22 interacts with Tfe3 through E-boxes and stimulates Col1a2 expression. A. ChIP analysis of Tsc-22 and Tfe3 on Col1a2 E-box region in response to TGF-β. B. ChiP analysis of Tsc-22 and Tfe3 on Col1a2 E-box region in response to TGF-β. C. Combined results of occupancy of Zeb1, Tfe3, Tsc-22, and Smad4 on the Col1a2 E-box region. Zeb1 occupancy was significantly decreased by TGF-β, whereas Tfe3 and Tsc-22 occupancies were increased. D. Tsc-22 siRNA inhibited the TGF-β-induced increase in Col1a2 E-box Luc reporter activity. E. The results suggest a novel function of Tsc-22 in response to TGF-β in enhancing Col1a2 expression by collaborating with Tfe3 at the far upstream E-box region (Fig. 7K).
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initiated by TGF-β, these downstream signaling pathways (including miRNAs, Ybx1 and Pten, and Akt kinase) are amplified. This could be a major mechanism for chronic renal diseases such as DN.

In summary, the current results demonstrate that post-transcriptional regulation by Ybx1, a target of miR-216a, which is induced by TGF-β in MCC, is a key mechanism for enhanced miRNA translation under diabetic conditions. This novel regulation linked with enhanced accumulation of ECM proteins such as collagen may be involved in pathological sclerosis associated with not only diabetic kidney disease but also other fibrotic disorders and wound healing.

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