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Transforming Growth Factor β1 (TGF-β1) Enhances Expression of Profibrotic Genes through a Novel Signaling Cascade and MicroRNAs in Renal Mesangial Cells*

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Background: The mechanism by which TGF-β1 down-regulates miR-130b to promote diabetic nephropathy (DN) remains unclear.
Results: TGF-β1 initiates a signaling cascade, including NF-YC, RIK, miR-130b, and TGF-βR1, in mesangial cells to up-regulate profibrotic genes related to DN pathogenesis.
Conclusion: A novel pathway involving microRNAs and the host gene can augment mesangial fibrosis.
Significance: The reno-protective effects of miR-130b could be exploited for DN treatment.

Increased expression of transforming growth factor-β1 (TGF-β1) in glomerular mesangial cells (MC) augments extracellular matrix accumulation and hypertrophy during the progression of diabetic nephropathy (DN), a debilitating renal complication of diabetes. MicroRNAs (miRNAs) play key roles in the pathogenesis of DN by modulating the actions of TGF-β1 to enhance the expression of profibrotic genes like collagen. In this study, we found a significant decrease in the expression of miR-130b in mouse MC treated with TGF-β1. In parallel, there was a down-regulation in miR-130b host gene *2610318N02RIK* (*RIK*), suggesting host gene-dependent expression of this miRNA. TGF-β receptor 1 (TGF-βR1) was identified as a target of miR-130b. Interestingly, the RIK promoter contains three NF-Y binding sites and was regulated by NF-YC. Furthermore, NF-YC expression was inhibited by TGF-β1, suggesting that a signaling cascade, involving TGF-β1-induced decreases in NF-YC, RIK, and miR-130b, may up-regulate TGF-βR1 to augment expression of TGF-β1 target fibrotic genes. miR-130b was down-regulated, whereas TGF-βR1, as well as the profibrotic genes collagen type IV α 1 (*Col4a1*), *Col12a1*, *CTGF*, and *PAI-1* were up-regulated not only in mouse MC treated with TGF-β1 but also in the glomeruli of streptozotocin-injected diabetic mice, supporting *in vivo* relevance. Together, these results demonstrate a novel miRNA- and host gene-mediated amplifying cascade initiated by TGF-β1 that results in the up-regulation of profibrotic factors, such as TGF-βR1 and collagens associated with the progression of DN.

MicroRNAs (miRNAs)[2] are endogenously produced single-stranded, short (average 22 nucleotides), non-coding regulatory RNAs that can repress target gene expression (1, 2). Increasing evidence shows that miRNAs play important roles in gene regulation by binding to the 3′-untranslated regions (3′-UTRs) of their target mRNAs to block protein translation or to induce mRNA degradation. Thus, miRNAs have the potential to regulate a multitude of genes under specific physiological and pathological conditions. miRNAs are emerging as important players and modulators in the pathogenesis of many human diseases, including diabetic nephropathy (DN), a severe microvascular complication of diabetes.

Key structural features of DN include glomerular mesangial cell expansion and hypertrophy due to increased accumulation of extracellular matrix (ECM) proteins, such as collagens and fibronectin, as well as glomerular basement membrane thickening, tubulointerstitial fibrosis, and podocyte apoptosis, all of which contribute to renal dysfunction and end stage renal failure (3–5). Several biochemical pathways and profibrotic factors, such as transforming growth factor-β1 (TGF-β1) and angiotensin II, can enhance the expression of ECM proteins, including collagen, in renal cells and have been implicated in the pathogenesis of DN (3, 6–11). Increased TGF-β1 expression in glomerular mesangial cells (MC) has been shown to promote the accumulation of fibrotic factors, ECM proteins, and hypertrophy during the progression of DN (4, 5, 7–9, 12). Studies from our laboratory and others have recently demonstrated the mediatory role of several miRNAs and their key target genes in the actions of TGF-β1 and high glucose in MC and other renal cells related to the pathogenesis of DN as well as molecular mechanisms of regulation of key miRNAs by TGF-β1 and high glucose (13–21). However, it is likely that

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[2] The abbreviations used are: miRNA, microRNA; DN, diabetic nephropathy; ECM, extracellular matrix; MC, mesangial cell(s); MMC, mouse MC; STZ, streptozotocin; RIK, 2610318N02RIK; PAS, Periodic acid-Schiff; SD, serum-depleted; NC, negative control.

there are other as yet unidentified miRNAs that modulate TGF-β1-induced regulation of ECM genes in MC to promote DN.

We hypothesized that key miRNAs that target the same gene may exert synergistic and amplifying effects in the diabetic kidney. While examining miRNAs that are differentially regulated by TGF-β1 in renal MMC and in glomeruli of diabetic mice, we observed a significant down-regulation in the miRNAs, miR-130b and let-7b. Interestingly, through computational target predictions, we identified TGF-β receptor 1 (TGF-βR1), which mediates the fibrotic actions of TGF-β1, as a potential target for both miRNAs, miR-130b and let-7b. let-7b was originally identified in *Caenorhabditis elegans* as an important regulator for normal development (22). The let-7 family is highly conserved among various species. In mice and humans, it consists of 11 very closely related family members (23). let-7 family members have been extensively studied for their tumor-suppressive functions in lung, pancreatic, and breast cancer cells (24, 25). let-7b inhibits the expression of key oncogenes, such as *MYC*, *RAS*, and *HMGA2* (26–28). let-7 is regulated by the RNA-binding protein, Lin28, which inhibits the processing of let-7 family miRNAs and their expression levels. Thus, the expression levels of let-7 and Lin28 are inversely correlated (29). Recently, it was found that under normal conditions, let-7 maintains low levels of TGF-βR1 expression in human umbilical vein endothelial cells and in adult human liver cells (30, 31). In addition, in tubular epithelial cells, lipoxins were shown to down-regulate TGF-βR1-induced fibrosis by up-regulating let-7c (32). Another recent paper also showed that let-7b targets TGF-βR1 related to kidney fibrosis (33). On the other hand, very little is known about the function of miR-130b in any cellular system. A few reports indicate that miR-130b promotes the growth and self-renewal of $CD133^+$ (hepatocellular carcinoma marker) liver tumor-initiating cells via down-regulation of TP53INP1 (34, 35). However, the function of miR-130b in DN is unknown, and it is also not known if it cooperates with let-7b. Herein, we report the identification of miR-130b as a new endogenous anti-fibrotic protective miRNA in MC and also a novel miRNA-mediated cascade, where TGF-β1 is able to enhance the expression of TGF-βR1 and collagens via the regulation of a cascade of key interacting mediators (NF-YC, 2610318N02RIK, miR-130b, and let-7b) to promote the progression of DN. Together, these data provide further insights into the complex amplifying cross-talk between growth factors and miRNAs and the related post-transcriptional regulatory mechanisms that modulate the progression of DN and also identify additional miRNAs that can be evaluated as therapeutic targets.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary MMC were isolated and cultured in RPMI 1640 medium supplemented with 10% FBS as described previously (7). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN) and used at a final concentration of 10 ng/ml.

Mouse Models—All animal studies were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee. C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were injected with 50 mg/kg streptozotocin (STZ) intraperitoneally for 5 consecutive days. Blood glucose levels were measured 1 week after final STZ injection to confirm the onset of diabetes (fasting glucose >300 mg/dl). For this study, mice were sacrificed 4 weeks after the onset of diabetes. Sieved glomeruli from mouse renal cortical tissue were prepared as described (14, 15) and stored at −80 °C for further analysis.

Real-time Quantitative PCR—RNA was extracted from MMC using miRNeasy columns (Qiagen, Inc., Valencia, CA). The qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) or GeneAmp RNA PCR kit (Applied Biosystems, Carlsbad, CA) were utilized for cDNA synthesis. Harvested mature miRNAs were polyadenylated in a poly(A) polymerase reaction. The poly(A) tailed miRNAs were then converted to cDNAs using an oligo(dT) primer with universal tag. Forward-specific primer sequences were designed for amplification of mature miRNAs, and reverse primers were provided by the cDNA kit. PerfeCTa SYBR Green Supermix (Quanta Biosciences) or POWER SYBR Green mix (Applied Biosystems) was used for quantitative PCR amplification on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR primer sequences were designed as follows: *TGF-βR1*, 5′-CGGTTCTACTTTTCTCATTTCTTCAACCG-3′ and 5′- AAATTGCTCGACGCTGTTCTATTG-3′; *RIK*, 5′-AAAGAGCCACATGGTTGATCTGA-3′ and 5′-CGGAGTATGAGAAATGGAAGCCTCCG-3′; *Col4a1*, 5′-GCCTTCCGGGCTCCTCAG-3′ and 5′-TTATCACCAGTGGGTCCG-3′; *Col12a1*, 5′-TTTGCCTGGAGAGAAAGGTG-3′ and 5′-GATACCTGAGTTTCCTGGAC-3′; *NF-YC*, 5′-ACAGAGGATAACAAGCGTCG-3′ and 5′-GTCACAGACTGGCGTACC-3; *CTGF*, 5′-GCGAAGCTGACCTGGAGGA-3′ and 5′-CGCACGAGTGGTGGTTCTGTGCG-3′; *PAI-1*, 5′-GACGCCTTCATTTGGACGAA-3′ and 5′-CGGACCTTTTCCCTTCAAGAGTCCG-3′; *CypA*, 5′-ATGGTCAACCCCACCGTGT-3′ and 5′-TTCTTGCTGTCTTTGGAACTTTGTC-3′. Sequences of miRNA-specific forward primers were designed as follows: miR-130b, 5′-CAGTGCAATGATGAAAGGGCAT-3′; let-7b, 5′-CACATGAGGTAGTAGGTTGTGTG-3′; miR-30e*, 5′-CTTTCAGTCGGATGTTTACAGC-3′. CypA served as the internal control for mRNA, and 18SII RNA (Ambion) served as the internal control for miRNAs.

3′-UTR and Promoter Luciferase Reporters—The mouse TGF-βR1 3′-UTR was amplified using primers TGF-βR1–3′-UTR-F (5′-CAGCTCGAGTGAAACACCGTGGGAACTCTG-3′) and TGF-βR1–3′-UTR-R (5′-GTCGCGGCCGCATCCATAGTGCACAGAAAGGAC-3′). The PCR fragment of TGF-βR1 3′-UTR was digested with XhoI and NotI and cloned into psiCheck-2 (Promega, San Luis Obispo, CA). let-7b and miR-130b mutant constructs were made via site-directed mutagenesis using primers let-7bmut-F (5′-AGGCTGGTTGTTGGATCCCACTGAGAGAAC-3′) and let-7bmut-R (5′-GTTCTCTCAGTGGGATCCAACAACCAGCCT-3′) and primers miR-130bmut-F (5′-GTCCTTTCTGCTCGAGATGGATGCGGCCGC-3′) and miR-130bmut-R (5′-GCGGCCGCATCCATCTCGAGCAGAAAGGAC-3′). The promoter region of mouse 2610318NO2RIK (RIK) was amplified using primers RIKpromoter-F (5′-TCTCGGTACCTGGTCTCCTTGAATTCATAGAGA-3′) and RIKpromoter-R (5′-AGGGCT-

CGAGTCAGCACCTCGTGGCGCCCT-3′). The WT-RIK promoter (three NF-Y sites) was made by digesting the PCR fragment of the RIK promoter with KpnI and SacI and cloned into the pGL4.10 vector (Promega, San Luis Obispo, CA). To make the NF-Y site-deletion promoter construct (0 NF-Y sites), the PCR fragment was sequentially digested with KpnI and SmaI and cloned into pGL4.10 vector.

Luciferase Assays—MMC were transfected with plasmids and/or miRNA mimics or inhibitors using Nucleofector kit (Lonza, Walkersville, MD) and treated with 10 ng/ml TGF-β1. RIK promoter constructs were co-transfected with pRLTK as a control for *Renilla* luciferase (Promega). After 48 h, luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) and Veritas Microplate luminometer (Turner Designs, Sunnyvale, CA), in accordance with the manufacturers' instructions.

Western Blot Analysis—MMC were lysed using 1.5× SDS buffer. Lysates were then subjected to sonication for 2.5 min on high power (Diagenode-Bioruptor ultrasonicator). Protein concentrations were measured using Bio-Rad protein assay reagents. Protein lysates were then fractionated on a 4–15% gradient gel and transferred to a nitrocellulose membrane. Membranes were immunoblotted with the following antibodies: TGF-βR1 (1:1000 dilution; Santa Cruz Biotechnology, Inc., sc-398), NF-YC (1:500 dilution; Santa Cruz Biotechnology, sc-7715-R), and β-actin (1:3000 dilution; Cell Signaling, Beverly, MA). Protein quantitation was performed on GS-800 densitometer using Quantity One software (Bio-Rad).

miRNA Oligonucleotides and siRNAs—All oligonucleotides representing the miRNA mimics (M), negative control for mimics (NC-M), miRNA inhibitors (I), and negative control for the inhibitors (NC-I) were purchased from Thermo Fisher Scientific. Mimics and inhibitors were transfected into MMC at a final concentration of 20 nM. TGF-βR1 and NF-YC siGenome SMARTpool duplex and non-targeting siRNA control were purchased from Dharmacon (Lafayette, CO) and transfected into MMC at a final concentration of 20 nM.

MMC Transfection—MMC were trypsinized and spun down, and 1×10^6 cells were resuspended in 100 μl of Basic Nucleofection transfection reagent. miRNA mimic/inhibitor oligonucleotides, mutant constructs, SMARTpool siRNA, or negative control oligonucleotides were transfected using Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The transfected cells were serum-depleted the following day for 24 h and then treated with or without 10 ng/ml TGF-β1 for the indicated time periods. The cells were then harvested for luciferase reporter assays and RNA and protein analysis.

Immunohistochemistry—Formalin-fixed, paraffin-embedded sections were made from mouse kidneys collected 4 weeks after the onset of diabetes (14). These kidney sections were mounted onto positively charged slides and incubated with NF-YC antibody. Periodic acid-Schiff (PAS) staining was performed to determine extracellular matrix accumulation in the kidney sections. Images of control mice and diabetic mice kidney sections were taken at ×40 magnification using an Olympus BX51 microscope with In Studio (Pixera Corp., Santa Clara, CA) software. ImagePro software (Media Cybernetics Inc., Rockville, MD) was used to count and quantify staining.

RESULTS

Decreased Expression of miR-130b and let-7b in TGF-β1-treated MMC—We first sought to identify miRNAs that are differentially regulated under TGF-β1-induced (diabetic) conditions in MMC. In examining several candidate miRNAs, we found a consistent down-regulation of key members of the let-7 family, miR-30 family, and miR-130b. We observed a TGF-β1-induced decrease of miR-130b, let-7b, and miR-30e* at 6 and 24 h compared with the serum-depleted (SD) control condition (Fig. 1, *A*–*C*). Recent reports have identified TGF-βR1 as a target of let-7c and let-7b in renal cells (30, 32, 33). Interestingly, computational analysis using Target Scan software identified TGF-βR1 as a potential profibrotic target of not only let-7b but also the other two miRNAs, miR-130b and miR-30e*. Collagen type XII α1 (Col12a1) was also identified as a potential target of miR-30e* with the Col12a1 3′-UTR having three potential miR-30e* target sites (Fig. 1*D*). *TGF-βR1* and *Col12a1* mRNA expression levels were significantly increased at 24 h of TGF-β1 treatment, reaffirming the idea that miR-130b and let-7b may be targeting the TGF-βR1 3′-UTR, and miR30e* may be targeting the Co12a1 3′-UTR in a TGF-β1-induced manner in MMC (Fig. 1, *E* and *F*). The mRNA expression levels of profibrotic genes, *CTGF* (connective tissue growth factor) and *PAI-1* (plasminogen activator of inhibitor-1) were also evaluated in MMC as positive controls for TGF-β1 treatment because these genes have been previously shown to be enhanced by TGF-β1 in MC (36–38). As expected, we observed significant increases in *PAI-1* and *CTGF* mRNA expression, with peak expression occurring at 6 h after TGF-β treatment (Fig. 1, *G* and *H*).

TGF-β1 Induces TGF-βR1 Expression via Down-regulation of miR-130b and let-7b—It was recently reported that let-7c and let-7b directly bind and target the TGF-βR1 3′-UTR in renal epithelial cells, leading to an attenuation of downstream genes associated with renal fibrosis (32, 33). let-7 was also shown to target TGF-βR1 in adult human liver cells to regulate human liver development (30). However, miR-130b has not been examined previously in DN pathogenesis. We first determined whether miR-130b and let-7b directly target TGF-βR1 in MMC. We generated WT as well as mutant luciferase reporter constructs harboring a single mutation in either the miR-130b or let-7b binding site within the TGF-βR1 3′-UTR or a double mutation where both the miR-130b and let-7b binding sites were mutated (Fig. 2*A*). miR-130b sequence alignment on TGF-βR1 3′-UTR is at bp 180–186 (Fig. 2*B*). To determine the effects of miR-130b on TGF-βR1 3′-UTR luciferase activity, MMC were co-transfected with negative control oligonucleotides (NC) or miR-130b mimic or miR-130b inhibitor oligonucleotides. Luciferase activity was significantly reduced in the presence of exogenous miR-130b mimic (miR-130b-M). Conversely, MMC transfected with miR-130b inhibitor (miR-130-I) increased the TGF-βR1 3′-UTR activity (Fig. 2*C*), verifying that miR-130b targets TGF-βR1 3′-UTR.

Next, to verify the effects of let-7b on TGF-βR1 3′-UTR in MMC, luciferase reporter assays were performed where WT or Mut-let-7b (mutation in the let-7b binding site in the

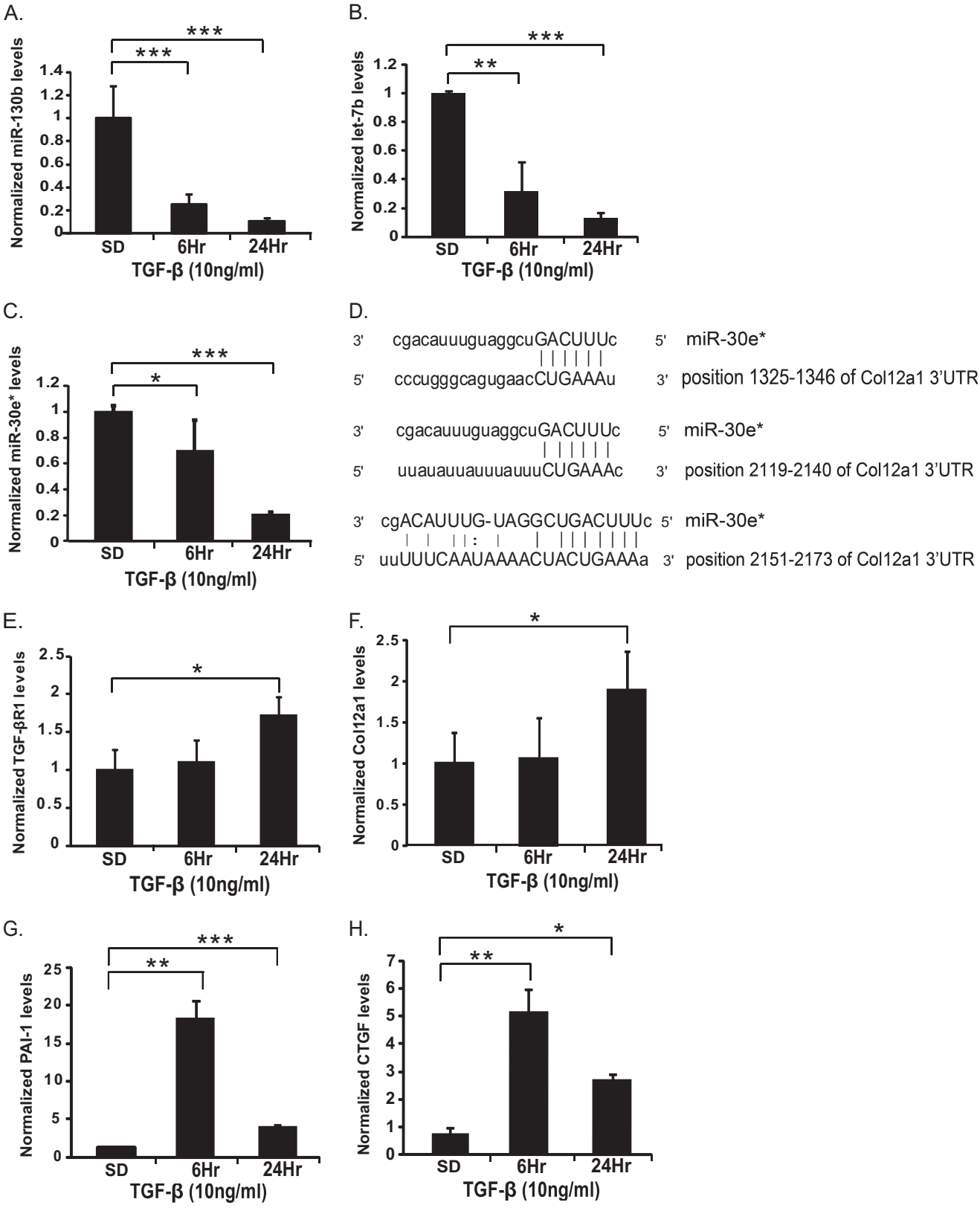


FIGURE 1. **Decreased expression of miR-130b and let-7b in TGF-β1-treated MMC.** *A–C*, quantitative PCR analysis of miR-130b, let-7b, and miR-30e* expression levels in SD MMC treated without or with 10 ng/ml TGF-β1 for 6 and 24 h. *D*, schematic representation of miR-30e* seed sequence alignment and Col12a1 3′-UTR. *E–H*, *TGF-βR1*, *Col12a1*, *PAI-1*, and *CTGF* mRNA levels were measured in MMC at 6 and 24 h of TGF-β1 treatment. *TGF-βR1* and *Col12a1* expression levels were significantly increased at 24 h of TGF-β1 treatment, whereas *PAI-1* and *CTGF* mRNA levels were increased at both time periods. Results for miRNAs were normalized with internal control 18 S. *, **, and ***, $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, *versus* SD control condition. Expression of other genes were normalized to *CypA* internal control. Results shown are mean ± S.E. (*error bars*) ($n = 3$). *, **, and ***, $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, *versus* SD control condition.

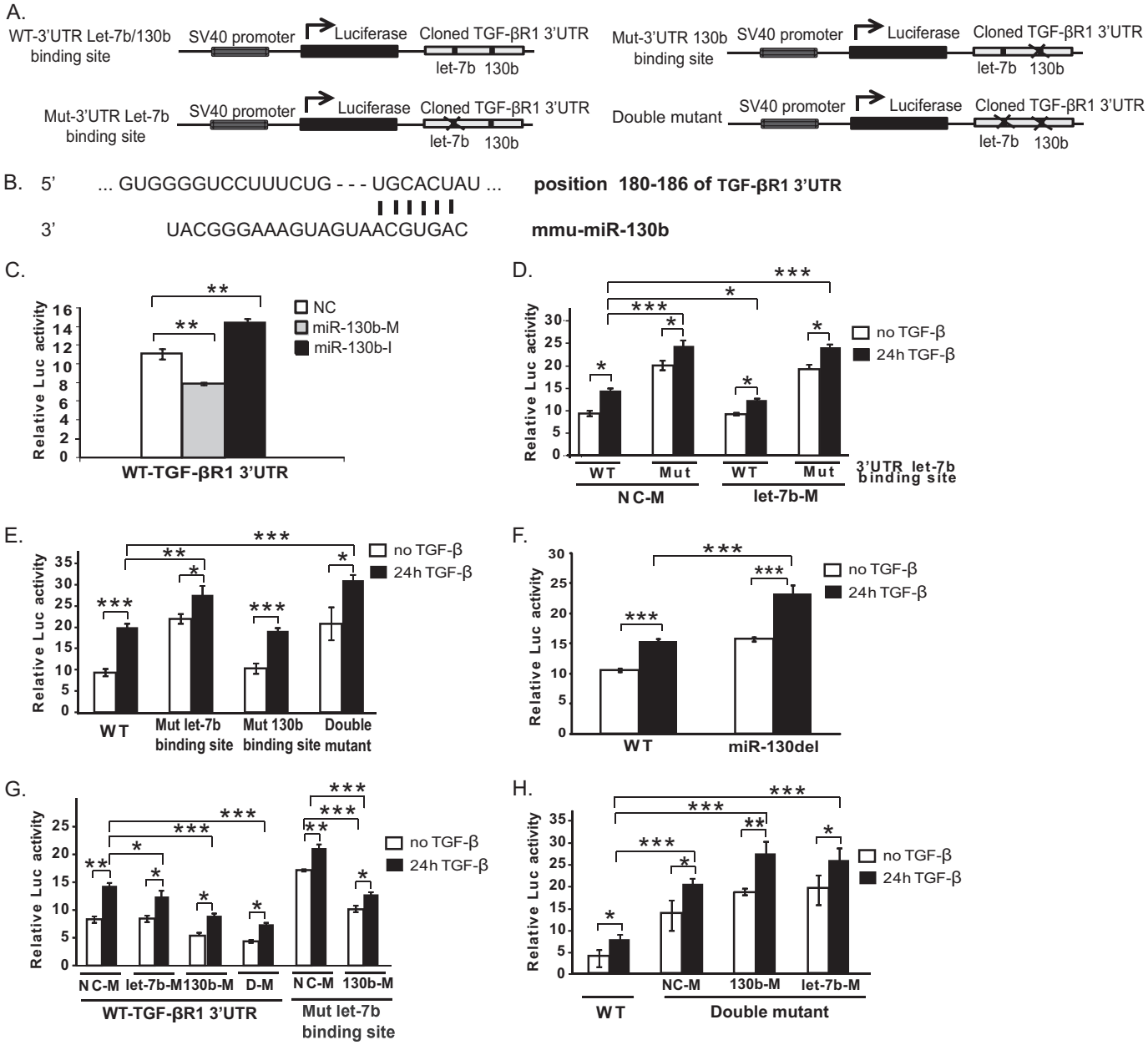


FIGURE 2. **TGF-β1 induces TGF-βR1 expression via down-regulation of miR-130b and let-7b.** *A*, schematic diagram showing several 3′-UTR luciferase reporter constructs, which include WT-TGF-βR1 3′-UTR (with WT intact let-7b and miR-130b target sites), Mut-miR-130b binding site, Mut-let-7b binding site, and double mutant (*DM*; where both miR-130b and let-7b binding sites have been mutated). *B*, schematic representation of miR-130b seed sequence alignment to the TGF-βR1 3′-UTR. *C*, luciferase reporter assays using WT-TGF-βR1 3′-UTR in MMC transfected with miR-130b mimic (*M*) or miR-130b inhibitor (*I*). Transfection with miR-130b-M decreased WT-TGF-βR1 3′-UTR activity; conversely, WT-TGF-βR1 3′-UTR reporter activity was increased with miR-130b-I relative to negative control (*NC*). *D*, luciferase reporter assay in cells co-transfected with WT-TGF-βR1 3′-UTR or Mut-let-7b with either negative control mimic (*NC-M*) or let-7b mimic (*M*) in the presence or absence of 10 ng/ml TGF-β1 for 24 h in MMC. *E*, luciferase reporter assays in MMC transfected with WT-TGF-βR1 3′-UTR, Mut-let7b, Mut-130b, or double mutant in the presence or absence of TGF-β1 for 24 h. *F*, MMC transfected with miR-130del mutant elicited higher basal activity and greater TGF-β1-induced activity compared with WT- TGF-βR1. *G*, luciferase reporter assay in cells co-transfected with WT-TGF-βR1 3′-UTR and either NC-M, let-7b-M, 130b-M, or double mimic (*D-M*; where both let-7b-M and 130b-M were transfected) and in cells with Mut-let-7b co-transfected with NC-M or 130b-M. *H*, luciferase reporter assays showing increased basal and TGF-β1-induced TGF-βR1 3′-UTR activity in MMC co-transfected with the double mutant construct and either NC-M, 130b-M, or let-7b-M compared with WT control. Results shown are mean ± S.E. (*error bars*) ($n = 3$). *, **, and ***, $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, *versus* WT or NC-M conditions.

TGF-βR1 3′-UTR) luciferase constructs were co-transfected with negative control mimic (NC-M) or let-7b mimic (let7b-M) and treated with or without TGF-β1 for 24 h. WT-TGF-βR1 3′-UTR co-transfected with NC-M showed a TGF-β1-induced increase in luciferase activity. However, cells transfected with Mut-let-7b showed an increase in basal activity and still responded to TGF-β1 compared with the WT control condition (Fig. 2*D*). Similar results were observed for cells transfected with let-7b-M, where the WT condition still responded to TGF- β1 and Mut-let-7b showed an increase in basal activity and also responded to TGF-β1, together demonstrating that loss of the functional let-7b site (Mut-let-7b-transfected condition) results in an increase in luciferase activity.

Next, we examined MMC transfected with WT-TGF-βR1, Mut-let-7b, Mut-130b, or double mutant constructs (where both miR-130b and let-7b binding sites are mutated) and treated with or without TGF-β1 for 24 h (Fig. 2*E*). Similar to previous results, Mut-let-7b resulted in an increase in basal activity but still responded to TGF-β1 treatment compared with WT control. Double mutation on the TGF-βR1 3′-UTR resulted in increased basal activity and a TGF-β1-induced increase in TGF-βR1 activity. However, no significant difference was observed with Mut-130b alone compared with WT control. Because this could be due to ineffectiveness of the miR-130b mutation, we designed another construct, namely a miR-130 deletion mutant in which several bases of the miR-130b target site in the TGF-βR1 3′-UTR were removed. This miR-130del construct now showed an increase in basal TGF-βR1 activity and an increase in TGF-β1 response compared with WT- TGF-βR1 (Fig. 2*F*).

To determine whether miR-130b and let-7b have additive or synergistic effects on the TGF-βR1 3′-UTR, MMC were co-transfected with WT-TGF-βR1 3′-UTR and either NC-M, let-7b-M, 130b-M, or double mimics (D-M; the addition of miR-130b mimic and let-7b mimic combined) with or without TGF-β1 for 24 h (Fig. 2*G*). TGF-β1-induced TGF-βR1 3′-UTR activity was decreased with let-7b-M or miR-130b-M alone. Interestingly, this decrease was even more pronounced in the D-M condition (Fig. 2*G*), suggesting an additive effect between let-7b and miR-130b actions on TGF-βR1 3′-UTR. In addition, Mut-let-7b constructs were co-transfected with NC-M or 130b-M in the presence or absence of TGF-β1 treatment for 24 h. The addition of exogenous miR-130b-M with the Mut-let-7b construct significantly attenuated basal and TGF-β1-induced increase in TGF-βR1 3′-UTR activity under these conditions (Fig. 2*G*, *last four bars*).

Finally, MMC were transfected with WT-TGF-βR1 3′-UTR alone or with double mutant constructs along with NC-M, 130b-M, or let-7b-M (Fig. 2*H*). Similar to previous experiments, whereas we observed a TGF-β1-induced increase in luciferase activity in WT, a further increase in basal activity was observed with the double mutant construct. The addition of exogenous mimics, 130b-M or let-7b-M, in combination with the double mutant construct had no further additional effect on the reporter activity relative to NC-M, suggesting, as expected, that the mimics did not recognize their target sites when mutated. Taken together, these results demonstrate that miR-130b and let-7b regulate TGF-βR1 3′-UTR through their own target sites in MMC.

In addition, constructs harboring a mutated miR-30e* binding site on TGF-βR1 3′-UTR were also tested. Results showed no significant change in TGF-βR1 3′-UTR activity with the miR-30e* binding site mutation (data not shown), suggesting that TGF-βR1 is not a *bona fide* target of miR-30e*.

miR-130b and let-7b Down-regulate Endogenous TGF-βR1 and Col4a1—Next, to determine the effects of miR-130b and let-7b on endogenous gene expression, we analyzed RNA samples from MMC transfected with NC-M, 130b-M, let-7b-M, or D-M (miR-130b-M and let-7b-M combined). *TGF-βR1* mRNA expression levels were significantly increased with TGF-β1 treatment in the presence of NC-M (Fig. 3*A*), but this was significantly reduced by the addition of exogenous 130b-M or let-7b-M. This effect was further abrogated in the TGF-β1-induced D-M condition, suggesting the additive actions of these two miRNAs on endogenous target gene expression. Similar to mRNA levels, TGF-β1-induced TGF-βR1 protein levels were also down-regulated with the addition of exogenous mimics compared with NC-M (Fig. 3*B*). Notably, there was a further decrease in the expression levels of TGF-βR1 protein in the D-M condition compared with the NC-M condition, again suggesting that miR-130b and let-7b can have additive effects on decreasing TGF-βR1 protein expression. β-actin was used as a loading control (Fig. 3*B*).

Identifying key players downstream of the miR-130b/let-7b/TGF-βR1 signaling pathway involved in the progression of DN is imperative to the understanding of this disease. Similar to *TGF-βR1*, *Col4a1* mRNA expression was increased with TGF-β1 treatment in the NC-M sample but significantly reduced in the presence of exogenous miR-130b and let-7b mimics, suggesting that *Col4a1* expression downstream of TGF-βR1 signaling is also functionally modulated by the actions of these miRNAs (Fig. 3*C*).

To further confirm that *Col4a1* expression is mediated via the TGF-βR1 pathway, we performed siRNA experiments. The successful knockdown of TGF-β1-induced *TGF-βR1* expression led to a decrease in TGF-β1-induced *Col4a1* mRNA expression levels compared with the NC (Fig. 3*D*), indicating that Col4a1 is a downstream target of the TGF-βR1 signaling pathway and can contribute to fibrosis.

To further test whether inhibition of miR-130b and let-7b affects TGF-βR1 expression, MMC were transfected with NC-I, 130b-I, let-7b-I, or double inhibitor (D-I; miR-130b inhibitor and let-7b inhibitor combined) (Fig. 3*E*). Inhibiting miR-130b in MMC increased *TGF-βR1* mRNA levels, whereas let-7b-I alone had no significant effect. No further increase in *TGF-βR1* expression was observed even with D-I compared with miR-130b-I alone, suggesting that miR-130b may be a more potent regulator of basal *TGF-βR1* levels than let-7b. However, this could also be due to lesser efficiency of the let-7b inhibitor. Taken together, these results suggest that TGF-β1 increases profibrotic genes, such as *Col4a1*, via up-regulation of TGF-βR1 mediated at least in part via down-regulation of miR-130b and let-7b, both of which target TGF-βR1 (Fig. 3*F*).

NF-YC Regulates the Promoter of the miR-130b Host Gene—Because the mechanisms involved in the regulation of miR-130b expression are unknown, we were interested in identifying some of the key players. We examined whether miR-130b expression depends upon the transcriptional regulation of its host gene. miR-130b forms a cluster with miR-301b within the first intron of the *2610318N02RIK* (*RIK*) gene (Fig. 4*A*). Relative to cells in the SD condition, MMC treated with TGF-β1 for 6 and 24 h showed a significant decrease in *RIK* mRNA expression levels. These results suggest that miR-130b may be co-regulated with its host gene, *RIK* (Fig. 4*A*). Interestingly, further investigation of the RIK promoter identified the presence of three potential nuclear transcription factor-Y (NF-Y) sites located at −200, −240, and −285 bp upstream from the transcription start site (Fig. 4*B*). To examine whether these NF-Y sites are important for RIK promoter activity, two luciferase

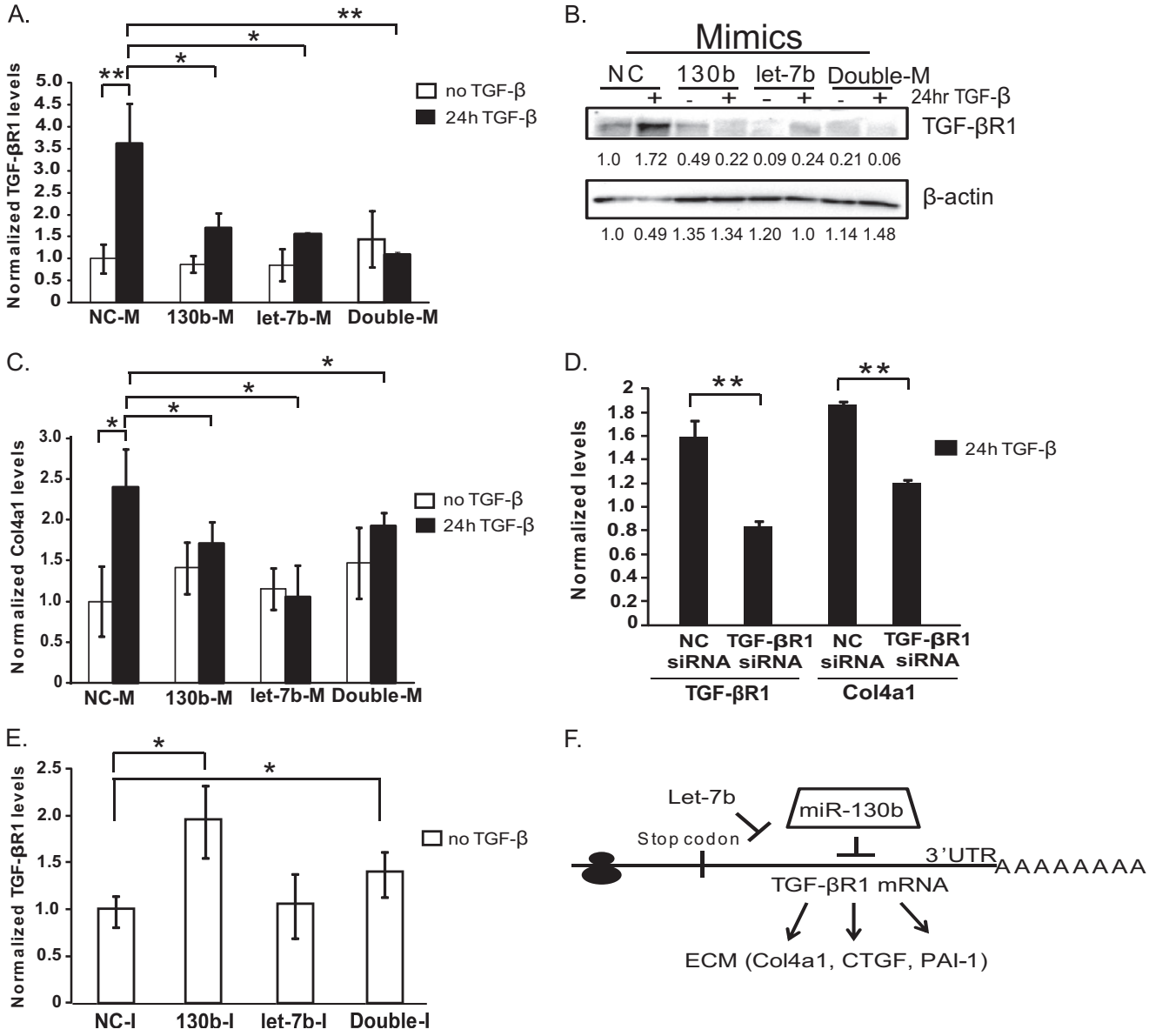


FIGURE 3. **miR-130b and let-7b down-regulate endogenous TGF-βR1 and Col4a1.** *A* and *C*, MMC transfected with 130b-M, let-7b-M, or double mimic (*Double-M*) display a TGF-β1-induced decrease of *TGF-βR1* (*A*) and *Col4a1* mRNA (*C*) expression compared with NC-M. *B*, similar to mRNA results, TGF-βR1 protein expression levels were significantly reduced with exogenous miR-130b-M, let-7b-M, and double-mimic conditions. *D*, TGF-β1-induced *TGF-βR1* and *Col4a1* mRNA levels were reduced in MMC transfected with TGF-βR1 siRNA oligonucleotides compared with NC siRNA oligonucleotides. *E*, MMC transfected with NC-I, 130b-I, let-7b-I, or double inhibitor (*Double-I*; where 130-I and let-7b-I were both transfected) resulted in increased *TGF-βR1* mRNA expression when miR-130b alone or in combination with let-7b (double inhibitor condition) was blocked. *F*, schematic model depicting miR-130b and let-7b directly binding to TGF-βR1 3′-UTR to regulate *TGF-βR1* and other downstream profibrotic genes, such as *Col4a1*, *CTGF*, and *PAI-1*. Results shown are mean ± S.E. (*error bars*) (n = 3 except Western blot (n = 2)). * and **, $p < 0.05$ and $p < 0.005$, respectively, *versus* NC conditions.

reporter constructs were generated (Fig. 4*B*), a WT-RIK luciferase reporter construct containing all three intact NF-Y sites and a RIK-Del construct in which all three NF-Y sites have been removed. MMC cells transfected with WT-RIK in the presence of TGF-β1 showed a significant decrease in RIK promoter activity, which was further abrogated in the RIK-Del reporter, suggesting that the absence of NF-Y sites leads to very low promoter activity (Fig. 4*B*). These results implicate the importance of the NF-Y sites in regulating the promoter activity of the *RIK* gene.

NF-Y consists of three subunits, NF-YA, NF-YB, and NF-YC, which are highly conserved from *Drosophila* to humans. NF-Y complexes have been reported to regulate the transcription of numerous genes related to the cell cycle, cancer, and various human diseases (39–45). All three subunits are important for the formation of NF-Y complexes and bind specifically to CCAAT motifs within the promoter region of target genes (45, 46). This CCAAT motif is one of the most common cis-acting elements in the promoter and enhancer region of a large number of eukaryotic genes (47). Furthermore, this specific motif has been shown to be enriched in several genes overexpressed in a variety of cancers, including breast, colon, thyroid, prostate, and leukemia (48). However, its role in DN is not known. To further examine the significance of these NF-Y sites on the RIK promoter, we tested the effect of loss of NF-YC using specific siRNAs. MMC cells were transfected with WT-RIK or RIK-Del

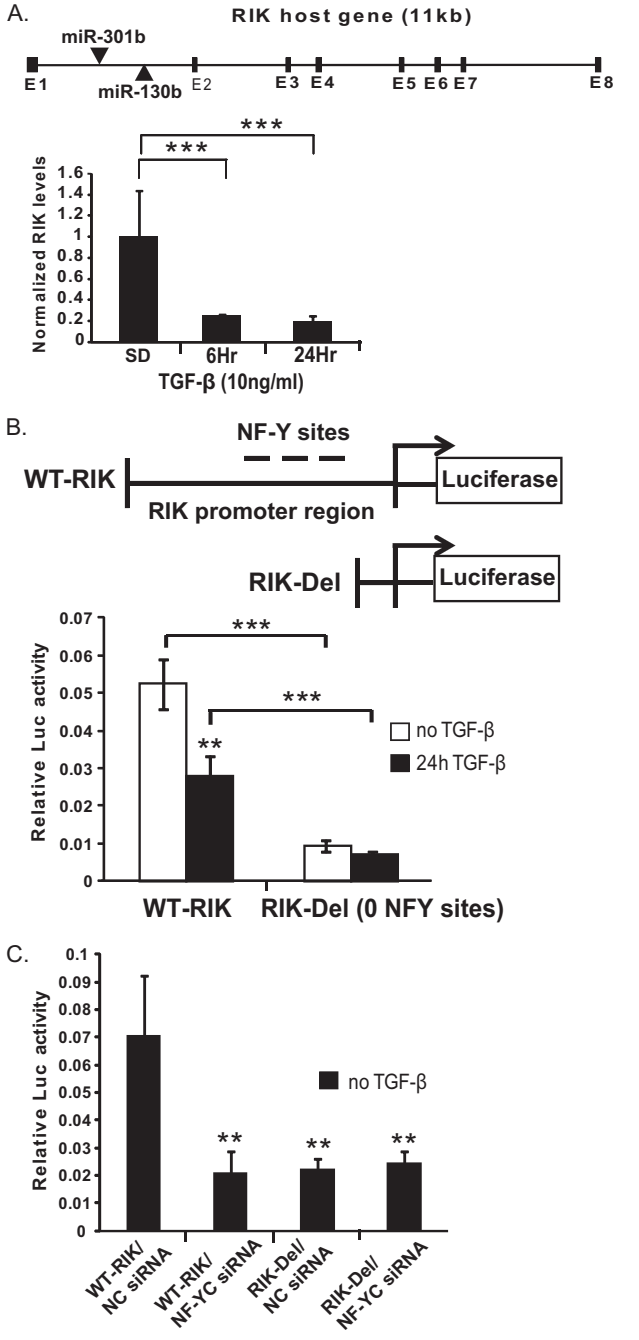


FIGURE 4. **NF-YC regulates the promoter of the miR-130b host gene.** *A*, quantitative PCR analysis of *RIK* expression levels in SD MMC treated without or with 10 ng/ml TGF-β1 for 6 and 24 h. *RIK* mRNA levels were significantly decreased throughout the time course. miR-130b gene location reveals that it forms a cluster with miR-301b between exon 1 and exon 2 of the *RIK* host gene. *B*, WT-RIK luciferase promoter construct depicting three NF-Y sites located −200, −240, and −285 bp upstream of the transcription start site of the *RIK* gene. The RIK-Del construct lacks these three NF-Y sites. MMC were transfected with WT-RIK or RIK-Del constructs and treated with or without TGF-β1 for 24 h. *C*, MMC were co-transfected with WT-RIK or RIK-Del and either NC siRNA (20 nM) or NF-YC siRNA (20 nM) oligonucleotides. WT-RIK promoter activity was significantly reduced with NF-YC siRNA compared with the NC siRNA condition. The RIK-Del construct showed very little activity with or without NF-YC siRNA. Mean ± S.E. (*error bars*) ($n = 3$). ** and ***, $p < 0.005$ and $p < 0.0005$, respectively, *versus* WT, NC, or SD conditions.

reporter constructs and either negative control (NC siRNA) or NF-YC siRNA (Fig. 4*C*). The promoter activity of WT-RIK was significantly reduced when NF-YC expression was knocked down with NF-YC siRNA compared with NC siRNA. Similarly, the absence of promoter NF-Y sites (RIK-Del construct) significantly abrogated any promoter activity with or without NF-YC siRNA. These results again implicate the importance of NF-Y sites in the regulation of RIK promoter activity.

NF-YC Regulates RIK and Other Downstream Components—Next, we wanted to determine whether any downstream targets of RIK and component miRNA, miR-130b, were affected by a decrease in *NF-YC* expression. MMC were transfected with either NC siRNA or NF-YC siRNA and treated with or without TGF-β for 24 h (Fig. 5). NF-YC siRNA decreased *NF-YC* mRNA expression significantly relative to control NC siRNA (Fig. 5*A*) and also reduced the expression of not only *RIK* but also miR-130b (Fig. 5, *B* and *C*). Conversely, the basal levels of *TGF-βR1* (the target of miR-130b) was increased by NF-YC siRNA compared with NC siRNA (Fig. 5*D*). These data reveal a novel feed-forward regulatory mechanism whereby decreased expression of *NF-YC* leads to a reduction in RIK promoter activity and thus RIK transcriptional activity, resulting in decreased miR-130b levels and ultimately increasing TGF-βR1 (Fig. 5*E*).

Expression of miR-130b and Key Parameters of DN in Glomeruli of Diabetic Mice—To determine the *in vivo* relevance of this signaling cascade, ↑ TGF-β1- ↓ NF-YC- ↓ RIK- ↓ miR-130b- ↑ TGF-βR1, on the progression of DN, we next examined the mRNA expression of these key mediators in the glomeruli of STZ-injected diabetic mice, 4 weeks after the onset. Similar to the observations in the *in vitro* MMC experiments, miR-130b, let-7b, and *RIK* showed decreased expression levels in the glomeruli of diabetic mice compared with their respective controls (Fig. 6, *A* and *B*). Conversely, *TGF-βR1*, *Col4a1*, *Col12a1*, *PAI-1*, and *CTGF* RNA expression levels were enhanced in the diabetic mice compared with control mice (Fig. 6, *C–E*). A significant increase in TGF-βR1 protein expression levels was also noted in the diabetic mice (Fig. 6*F*). Finally, immunohistochemistry staining showed a decrease in glomerular NF-YC expression in cortical sections of diabetic mice compared with control mice (Fig. 6*G*), reaffirming the notion of NF-YC as a key modulator of DN. Quantification of PAS- positive staining in glomeruli revealed a significant increase in mesangial matrix accumulation in diabetic mice compared with control mice (Fig. 6*H*). These results demonstrate that the signaling cascade found in *in vitro* experiments is also relevant in a mouse model of DN *in vivo*.

DISCUSSION

TGF-β1 is an important regulatory and profibrotic cytokine that affects the expression of numerous downstream miRNAs that play a role in the progression of DN (13–21, 33, 49, 50). A large body of work has been dedicated to identifying key miRNAs involved in the renal fibrotic processes because of their potential to serve as novel biomarkers of therapeutic targets for DN. In this study, we identified miR-130b as a new player and also studied the functional role of TGF-β1-induced down-regulation of miR-130b and expression of upstream effectors related to the pathogenesis of DN. miR-130b was evaluated in this study because it was found to be significantly down-regulated by TGF-β1 in MMC. Several reports have implicated a role for miR-130b in various cancers (34, 35, 51–54). However,

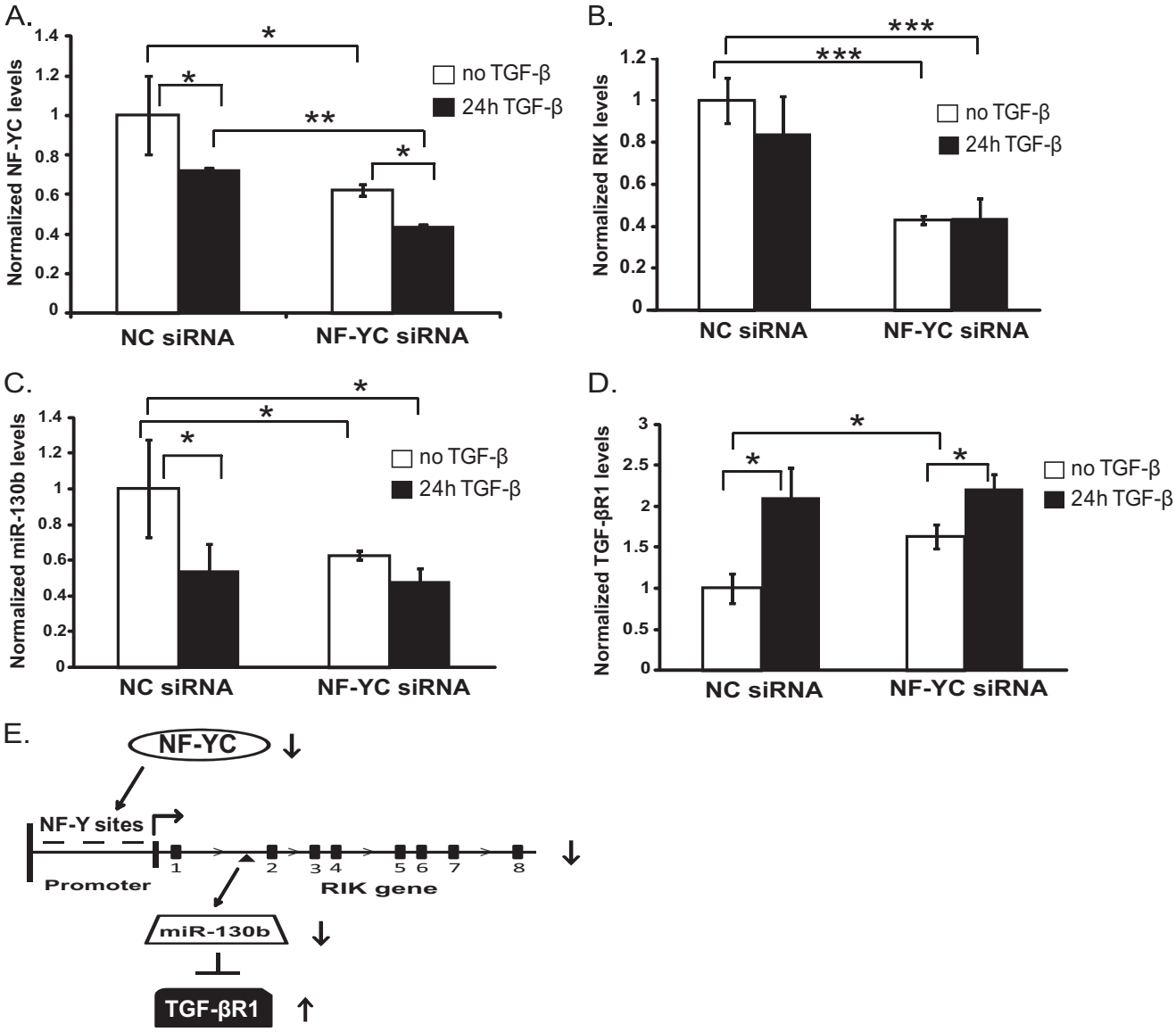
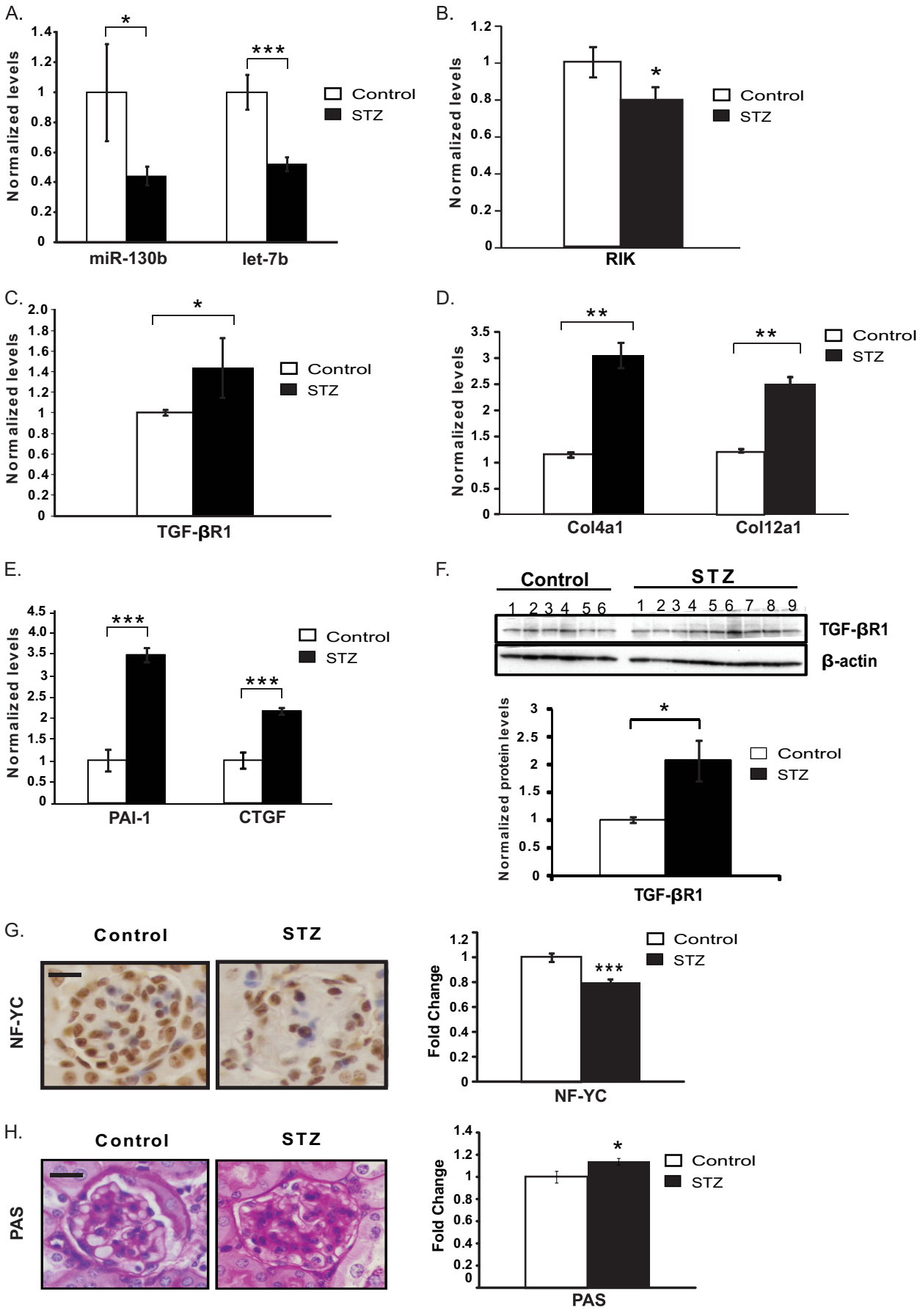


FIGURE 5. **NF-YC regulates RIK and other downstream components.** *A–C*, MMC transfected with NF-YC siRNA showed a significant decrease in basal and TGF-β1-induced mRNA expression of *NF-YC* and other downstream components (namely *RIK* and miR-130b) compared with the NC siRNA condition. *D*, conversely, basal levels of *TGF-βR1* mRNA expression were up-regulated in the presence of NF-YC siRNA. *E*, schematic diagram depicting how decreased expression of NF-YC in response to TGF-β1 leads to reduced binding of NF-YC to the CCAAT domains (NF-Y sites) within the RIK promoter, thereby decreasing RIK transcriptional activity and consequently decreasing the expression of miR-130b located within the *RIK* gene, ultimately resulting in increased TGF-βR1 expression. Mean ± S.E. (*error bars*) ($n = 3$). *, **, and ***, $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, *versus* NC conditions.

the role of miR-130b in DN has not been studied previously. In the present study, we demonstrate the participation of miR-130b in a novel signaling cluster and also the functional ability of TGF-β1 to initiate an amplifying cascade involving several key component players (NF-YC, RIK, miR-130b, and TGF-βR1) that results in the up-regulation of profibrotic factors associated with the accelerated progression of DN.

Preliminary data from high throughput RNA sequencing[3] revealed that miR-130b, let-7b, and miR-30e* were decreased in a TGF-β1-dependent manner in MMC. Further validation of these data in the current study showed that these miRNAs were down-regulated not only in TGF-β1-treated MMC but also in the glomeruli of diabetic mice (*in vivo* miR-30e* data not shown). miRNA mimic and inhibitor experiments confirmed TGF-βR1 to be a *bona fide* target of miR-130b and let-7b in MMC. The actions of miR-130b and let-7b on TGF-βR1 3′-UTR appear to be specific because mutant 3′-UTR constructs lacking the miR-130b and let-7b binding sites were unresponsive to mimics. Our data suggest that the down-regulation of miR-130b and let-7b by TGF-β1 can cooperatively enhance, at least in part, the TGF-βR1 signaling pathway and regulate subsequent downstream profibrotic factors, such as Col4a1. These results also highlight the redundancy among certain miRNAs, namely miR-130b and let-7b, both with the ability to target TGF-βR1 3′-UTR and influence the fibrotic phenotype. The data presented in this study suggest that miR-130b may be a more potent inhibitor of TGF-βR1, although more experiments are needed to confirm this.

[3] M. Kato and R. Natarajan, unpublished data.


A.
Normalized levels
miR-130b
let-7b
Control
STZ
B.
Normalized levels
RIK
C.
Normalized levels
TGF-βR1
D.
Normalized levels
Col4a1
Col12a1
E.
Normalized levels
PAI-1
CTGF
F.
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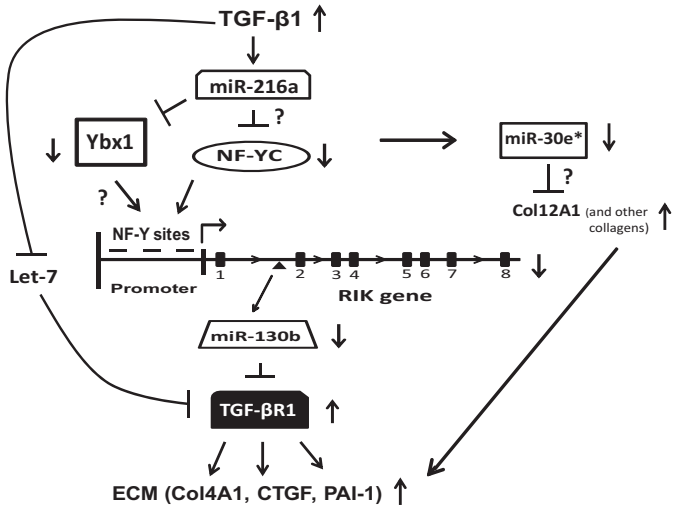

FIGURE 7. **Schematic model.** Under diabetic conditions where TGF-β1 levels are increased, NF-YC expression is down-regulated (possibly via up-regulated miR-216a), resulting in reduced binding of NF-YC to the NF-Y sites (CCAAT) within the RIK promoter. Alternatively, miR-216a can target and down-regulate Ybx1, which can result in reduced binding of Ybx1 to Y-box (CCAAT) within the RIK promoter. Decreased RIK promoter activity leads to reduced transcriptional activity of the *RIK* gene, thereby attenuating miR-130b expression, because miR-130b expression is dependent upon transcriptional activity of the host gene, *RIK*. This feed-forward amplifying cascade results in elevated expression of TGF-βR1 and subsequently other downstream profibrotic and ECM genes involved in mediating the progression of diabetic nephropathy, such as *Col4a1*, *CTGF*, and *PAI-1*. Down-regulation of miR-30e* might be a consequence of reduced NF-YC, a putative host gene for miR-30e* that can potentially target Col12a1.

miR-130b is located within the intron of the non-coding RNA, 2610318N02RIK (RIK) (UCSC Genome Browser), and we found that the expressions of both miR-130b and *RIK* were similarly down-regulated by TGF-β1. Further, miR-130b expression was dependent upon the transcriptional activity of the *RIK* host gene. To date, no known function has been reported for this *RIK* gene. Further analysis of the RIK promoter region revealed three functional NF-YC sites capable of regulating the promoter activity of the *RIK* gene. NF-Y complexes are composed of three individual subunits (namely NF-YA, NF-YB, and NF-YC), which bind to promoter CCAAT motifs. Understanding the mechanism by which TGF-β1 down-regulates NF-YC would be beneficial in further elucidating the importance of NF-YC as a protective factor in DN. Previously, our group demonstrated that miR-216a is induced by TGF-β1 and mediates TGF-β1-induced Akt kinase activation and collagen expression in MMC by targeting Ybx1 and Pten (49, 50). Because Ybx1 also recognizes Y-box (CCAAT) (48), it is possible that the decrease of *RIK* and miR-130b is caused by miR-216a-mediated down-regulation of Ybx1 (49) (Fig. 7). On the other hand, computational analysis revealed a potential target site for miR-216a in the NF-YC 3′-UTR. However, further experiments with miR-216a mimics and inhibitors are needed to fully substantiate NF-YC as a true target of miR-216a.

Interestingly, analysis of the *NF-YC* genome region also revealed an miRNA cluster consisting of miR-30e* and miR-30c-1 within intron 5, suggesting that *NF-YC* may be the host gene of miR-30e* and that the expression of miR-30e* may be dependent on NF-YC expression (Fig. 7). Further experiments to confirm this and also whether miR-30e* directly targets Col12a1 3′-UTR remain for a future study.

To further examine the *in vivo* relevance of our *in vitro* findings, we examined the mRNA expression levels of the key players within this novel pathway in the glomeruli of diabetic and control mice. Similar to our *in vitro* studies, miR-130b, let-7b, *RIK*, and NF-YC (immunohistochemistry staining) were down-regulated in the glomeruli of 4-week STZ-induced diabetic mice compared with control mice. Conversely, *TGF-βR1*, *Col4a1*, *PAI-1*, and *CTGF* profibrotic genes were up-regulated in the diabetic mice.

In summary, the current results demonstrate a novel amplifying cascade where TGF-β (diabetic conditions) is able to regulate the expression of various downstream key components, including NF-YC and RIK, to down-regulate miR-130b and thereby increase the expression of pathologic profibrotic genes, such as *TGF-βR1* and *Col4a1* (Fig. 7). These data also show a cooperation between miR-130b and let-7b and further add to our earlier reports demonstrating that several miRNAs induced by TGF-β1 can cooperate with each other and create amplifying circuits to fine-tune and augment the progression of DN (12, 14–16, 49, 50). There are clearly several alternative and parallel signaling pathways that further enhance the progression of DN, including Smads and other miRNAs. This collaboration among several key players could explain why our effects are relatively modest, indicating synergism among these multiple contributing pathways with the ability to cooperatively accelerate DN.

Notably, here, for the first time, miR-130b has been implicated as a potential protective miRNA in the pathology of renal fibrosis related to DN. Interestingly, it was previously reported that miR-130 can modulate adipocyte differentiation by repressing peroxisome proliferator-activated receptor γ, a key mediator of adipogenesis, thereby reducing adipogenesis and limiting the probability of obesity (55). This protective role of miR-130 in obesity may also translate to its protective role in DN, whereby increased levels of miR-130 impair the progression of adipogenesis and insulin resistance and decrease the potential for developing diabetes and DN. Conversely, the reduction of miR-130b has the potential to induce not only

FIGURE 6. **Expression of miR-130b and key parameters of DN in glomeruli of diabetic mice.** *A* and *B*, glomeruli were isolated from kidney cortical tissues from six mice in the control group and nine mice in the STZ-diabetic group. miR-130b, let-7b, and *RIK* mRNA expression levels were significantly decreased in glomeruli of STZ-injected diabetic mice (*STZ*) (4 weeks of diabetes) compared with vehicle-injected (*Control*) mice. *C–E*, conversely, *TGF-βR1*, *Col4a1*, *Col12a1*, *PAI-1*, and *CTGF* mRNA expression levels were significantly increased in glomeruli of these diabetic mice compared with control non-diabetic mice. Results shown are mean ± S.E. (*error bars*) ($n = 3$). *, **, and ***, $p < 0.05$, $p < 0.005$, and $p < 0.0005$, respectively, *versus* control conditions. Results were normalized to *CypA* internal control for regular genes and 18 S internal control for miRNAs. *F*, protein lysates were made from renal cortical tissues of STZ mice and control mice. TGF-βR1 protein expression levels were increased under diabetic conditions compared with control. β-Actin was used as a loading control for protein lysates. *G*, representative NF-YC immunostaining images and quantification using NF-YC antibody in cortical tissue from STZ-injected diabetic mice and control mice. NF-YC protein expression was significantly reduced in the STZ mice glomeruli compared with control mice. *H*, representative PAS staining images and quantification using cortical tissue from STZ-injected diabetic mice and control mice. Significantly increased PAS staining was observed in diabetic mice compared with control mice. Results shown are mean ± S.E. * and ***, $p < 0.05$ and $p < 0.0005$ ($n = 50$ glomeruli counted/condition). *Scale bar*, 20 μm.

obesity (or insulin resistance) but also DN. Therefore, reinstating the levels of miR-130b with oligonucleotide mimics during high TGF-β1 levels (diabetic conditions) would be a potential translational intervention for reducing renal fibrosis and slowing down the progression of DN. The reno-protective effects of miR-130b alone or in conjunction with other miRNAs *in vivo* could thus be further exploited for the development of better treatment strategies for DN and other fibrotic disorders.

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