Repression of let-7 by transforming growth factor-β1-induced Lin28b up-regulates collagen expression in glomerular mesangial cells under diabetic conditions

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Directed by Professor Shin-Wook Kang

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ABSTRACT

Repression of let-7 by transforming growth factor-β1-induced Lin28b up-regulates collagen expression in glomerular mesangial cells under diabetic conditions

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Background: Diabetic nephropathy (DN), the leading cause of end-stage renal disease worldwide, is characterized pathologically by glomerular basement-membrane thickening, mesangial expansion and cellular hypertrophy, and accumulation of extracellular matrix (ECM) proteins such as collagen and fibronectin. Transforming growth factor-β1 (TGF-β1) plays an important role in ECM accumulation in DN. However, to date, the molecular mechanism of TGF-β1 induced ECM accumulation is not fully elucidated. Evidences show that miRNAs play mediatory roles in the progression of DN. Recently, the miRNA let-7 was suggested to be involved as a negative regulator of profibrotic processes in several disease states. In this study, I aimed to investigate whether let-7 directly targets ECM genes to regulate glomerular mesangial fibrosis, and

also whether the up-regulation of Lin28, the negative regulator of let-7, is involved in the down-regulation of let-7 in response to TGF-β1 in mesangial cells and under diabetic conditions.

Methods: *In vitro*, mouse glomerular mesangial cells (MMCs) serum depleted for 48 hours and then treated with TGF-β1 for the indicated time periods. TGF-β1 treated MMCs were harvested for RNA and protein isolation to investigate the effect of TGF-β1 on let-7 family miRNAs, target genes, and Lin28b. To evaluate the effect of let-7b regulation on target genes, MMCs were transfected with miRNA mimic or hairpin inhibitor oligonucleotides. Transfected cells were harvested at indicated times for RNA and protein isolation. To verify whether col1a2 and col4a1 are direct targets of let-7b, the col1a2 and col4a1 3′UTRs containing the let-7b binding site were cloned downstream of luciferase in a reporter vector. Luciferase activity was measured in MMCs co-transfected with 3'UTR reporter vectors and let-7b mimic or hairpin inhibitor oligonucleotides. In order to evaluate the role of the potential Smad-binding element (SBE) in TGF-β1 induced Lin28b up-regulation, the proximal promoter and promoter deletion constructs of the mouse *Lin28b* gene were cloned into a luciferase reporter. Chromatin immunoprecipitation assays were performed to examine whether TGF-β1 activated Smad2/3 binds to the SBE in the proximal promoter with TGF-β1 treatment.
In vivo, C57BL/6 mice were injected with 50 mg/kg of streptozotocin intraperitoneally on 5 consecutive days. Control mice were injected with diluent. Each group was comprised of eight mice. All mice were sacrificed at 6 weeks post induction of diabetes. Ten to twelve weeks old type 2 diabetic *db/db* mice and genetic control non-diabetic *db/+* mice were also sacrificed and glomeruli were sieved from renal cortical tissue for RNA extraction.

Results: miRNA let-7 family members (let-7b/c/d/g/i) were down-regulated in

TGF-β1-treated MMCs along with up-regulation of col1a2 and col4a1. Ectopic expression of let-7b in TGF-β1-treated MMCs attenuated the col1a2 and col4a1 up-regulation. Conversely, let-7b inhibitors increased col1a2 and col4a1 levels. Co-transfection of MMCs with mouse col1a2 or col4a1 3'-UTR luciferase constructs and let-7b inhibitors increased luciferase activity. However, constructs with let-7 target site mutations were un-responsive to TGF-β1. TGF-β1 induced 3'UTR activity was attenuated by let-7b mimics, suggesting col1a2 and col4a1 are direct targets of let-7b. In addition, Lin28b, a negative regulator of let-7 biogenesis, was up-regulated in TGF-β1-treated MMCs. Luciferase assays showed that the *Lin28b* promoter containing potential Smad-binding site responded to TGF-β1, which was abolished in constructs without the Smad site. Additionally, chromatin immunoprecipitation assays showed TGF-β1-induced enrichment of Smad2/3 at the *Lin28* promoter together suggesting that Lin28b is transcriptionally induced by TGF-β1 through the Smad site. Furthermore, let-7b levels were decreased, while Lin28b, col1a2 and col4a1 levels were increased in glomeruli of type 1 and 2 diabetic mice compared to nondiabetic controls, demonstrating *in vivo* relevance of this Lin28/let-7/collagen axis.

Conclusion: In conclusion, these results characterize Lin28b as a new target of TGF-β1 in the kidney. In addition, they suggest a novel role for the Lin28/let-7 pathway in mediating the effects of TGF-β1 on ECM genes in mesangial cells under diabetic conditions.

Key words: microRNA, diabetic nephropathy, TGF-β1, Lin28b, let-7, collagen, extracellular matrix

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I. INTRODUCTION

The prevalence of diabetes is increasing worldwide and is also associated with several long-term complications, including diabetic nephropathy (DN).[1, 2] More than 40% of patients with diabetes progress to DN which is the major cause of end-stage renal disease (ESRD).[3-5] In addition, DN is a significant risk factor for developing macrovascular diseases such as atherosclerosis, hypertension, and stroke.[4, 6]

The major characteristics of DN include glomerular basement membrane thickening, mesangial expansion and hypertrophy, and accumulation of extracellular matrix (ECM) proteins such as collagen and fibronectin.[7-10] Glomerular fibrosis, caused by ECM accumulation in the glomerulus, is strongly associated with progressive decline of renal function which can lead to

ESRD.[11-13] In addition, fibrotic changes in the glomerulus are not effectively reversed with most currently available treatment modalities.[14] Therefore, understanding the mechanisms associated with the pathogenesis of glomerular fibrosis is important for the development of much needed better therapies for DN.

Transforming growth factor-β1 (TGF-β1) is a major regulator of profibrotic events in various cell types and pathologic conditions including DN.[15-17] Increased expression of TGF-β1 in multiple renal cell types is associated with kidney fibrosis under diabetic conditions.[18, 19] TGF-β1 is transcriptionally up-regulated in mesangial cells as well as tubular cells treated with high glucose.[20-24] Moreover, the progression of DN was attenuated by neutralizing TGF-β1, further demonstrating a major role for TGF-β1 in DN progression.[13, 22] TGF-β1 acts via its cell surface receptors and signaling pathways to activate downstream Smad transcription factors to drive gene expression changes related to renal fibrosis.[25, 26]

Accumulating evidence has revealed that microRNAs (miRNAs) can play critical roles in the actions of TGF-β1 and the progression of DN.[27-30] miRNAs are a family of endogenously produced, short noncoding RNAs. They silence the expression of target genes via post-transcriptional mechanisms through base pairing to the 3'UTRs of the target mRNAs to inhibit mRNA translation or promote mRNA degradation.[31-33] Expression levels of several miRNAs have been shown to be altered by TGF-β1 treatment in mesangial cells, and, in addition, key miRNAs are misregulated in the renal cortex and glomeruli of animal models of diabetes, implicating a functional role for miRNA in DN pathophysiology.[32-39]

Recently, the miRNA let-7 was suggested to be involved as a negative

regulator of profibrotic processes in several disease states. Let-7 has been shown to modulate ECM deposition in breast, pancreas, and oral cancer cells.[40-42] In addition, a decrease in let-7 was found in lung tissue samples from idiopathic pulmonary fibrosis patients.[43] Recently, downregulation of let-7 has also been associated with renal fibrosis.[44, 45] However, the mechanisms by which let-7 is down-regulated and its direct effects on ECM genes in mesangial cells are not known. In this study, I aimed to investigate whether let-7 directly targets ECM genes to regulate glomerular mesangial fibrosis, and also whether the up-regulation of Lin28, the negative regulator of let-7, is involved in the down-regulation of let-7 in response to TGF-β1 in mesangial cells and under diabetic conditions.

II. MATERIALS AND METHODS

1. Animals

All animal studies were conducted according to a protocol approved by the Institutional Animal Care and Use committee of the Beckman Research Institute of City of Hope and Yonsei University College of Medicine. Type 2 diabetic *db/db* mice and genetic control non-diabetic *db/+* mice (10 – 12 weeks old, eight per group), were obtained from Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6 mice (Jackson Laboratories) were injected with 50 mg/kg of streptozotocin (STZ) intraperitoneally on 5 consecutive days. Control mice were injected with diluent. Each group was comprised of eight mice. Tail vein blood glucose levels were measured to confirm diabetes (fasting glucose >300 mg/dl). All mice were sacrificed at 6 weeks post induction of diabetes.

2. Cell culture and materials

Mouse glomerular mesangial cells (MMCs) were obtained from renal cortex of C57BL/6 mice. After removing the kidneys, glomeruli were isolated by differential sieving, and isolated glomeruli were incubated in collagenase and trypsin-ethylenediaminetetraacetic acid (EDTA) (GIBCO Laboratories, Bethesda, MA, USA). The identification of mesangial cells was performed by their characteristic stellate appearance in culture and was confirmed by immunofluorescent microscopy for the presence of actin, myosin, and Thy-1 antigen and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA, USA). Mesangial cells were maintained in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) supplemented with L-glutamine, HEPES 7 mmol/L, and 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA) and were incubated at 37℃ in humidified 5% CO_2 in air. Passages 5 through 7 were used for experiments. Recombinant human TGF-β1 was from R&D System

(Minneapolis, MN, USA). let-7b mimic oligonucleotides (let7b-M), negative control mimics (NC-M), let-7b hairpin inhibitor oligonucleotides (let7b-I), and negative control inhibitors (NC-I) were obtained from Dharmacon (Lafayette, CO, USA). Cells were serum depleted for 48 hours and then treated with TGF-β1 or high glucose (HG, 25 mM) for the indicated time periods.

3. Glomeruli isolation

Glomeruli were isolated from freshly harvested kidneys by a sieving technique. Renal capsules were removed and the cortical tissue of each kidney was separated by dissection. The cortical tissue was then carefully strained through a stainless sieve with a pore size of 150 μm by applying gentle pressure. Enriched glomerular tissue below the sieve was collected and transferred to another sieve with a pore size of 75 μm. After several washings with cold PBS, the glomerular tissue remaining on top of the sieve was collected. The pooled glomeruli were centrifuged and pellet collected for RNA extraction. Each glomeruli sample was composed of tissue pooled from 2 mice.

4. Transfection

Cells were transfected with siRNA or miRNA oligonucleotides using an Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer's protocols. MMCs were trypsinized and resuspended in Basic Nucleofection Solution at 1×10^7/ml. Subsequently, 100 μl of cell suspension (1×10^6 cells) was mixed with miRNA mimic or hairpin inhibitor oligonucleotides (Thermo Fischer Scientific Inc., Waltham, MA, USA) as indicated. Transfection was done using the Basic Nucleofector Kit for Primary Smooth Muscle Cells (Lonza). Nucleofection was performed with program U25. Transfected cells were harvested at indicated times for RNA and protein isolation. For overexpression vector transfections, MMCs were cultured in 10cm culture dishes at a concentration of 1×10^6 cells. Transfection was

conducted for recombinant Lin28b expression plasmid pCMV-Lin28b (Genecopia Inc., Rockville, MD, USA) according to the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection manual. Plasmid pCMV-GFP expressing EGFR was used as vector control. The plasmids were diluted with serum free DMEM medium. After 5 minute incubation under ambient temperature, Lipofectamine 2000 was added to the diluted plasmids, respectively. The mixture was successively incubated for 20 minutes under ambient temperature before being added to the culture plate with MMCs. The obtained mixture added MMCs were incubated in 5 % CO_2 at 37 ℃. The culture medium was exchanged to RPMI 1640 medium containing 10 % fetal bovine serum after 5 hours and the MMCs were incubated for another 48 hours before harvest.

5. Real-time quantitative PCR

RNA was extracted using miRNeasy columns (Qiagen, Inc. Valencia, CA, USA). miRNA expression analysis was performed by using the qScript miRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) and PerfeCTa SYBR Green Supermix (Quanta Biosciences). GeneAmp RNA PCR kit (Applied Biosystems, Carlsbad, CA, USA) and POWER SYBR Green mix (Applied Biosystems) was used for mRNA quantification. Extracted mature miRNAs were first polyadenylated with poly (A) polymerase followed by reverse transcription into cDNA using oligo-dT primer with universal tag. miRNAs were amplified using specific mature miRNA sequences as forward primers and the universal primer provided in the kit as reverse primer. Real-time qPCRs were performed on the 7500 real-time PCR system (Applied Biosystems). PCR primer sequences were as follows: col1a2 forward, 5’-CAGAACATCACCTACCACTGCAA-3’; col1a2 reverse, 5’-TTCAACATCGTTGGAACCCTG-3’; col4a1 forward, 5’-GCCTTCCGGGCTCCTCAG-3’; col4a1 reverse,

5’-TTATCACCAGTGGGTCCG-3’; Lin28b forward, 5’-GGCCTTTGATTCAGAAACGG-3’; Lin28b reverse, 5’-CTTGCATGAGGTAGACTTCCC-3’.

6. Western blot analysis

Cells were lysed in in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. Lysate was centrifuged at 10,000 × *g* for 10 minutes at 4℃, and the supernatant was stored at -70 ℃. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 50 µg protein were treated with Laemmli sample buffer, and then heated at 100 ℃ for 5 minutes, and electrophoresed 50 µg/lane in a 10% acrylamide denaturing SDS-polyacrylamide gel (Bio-Rad). Proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA). The membrane was incubated in blocking buffer A [1× phosphate-buffered saline (PBS), 0.1% Tween-20, and 8% nonfat milk] for 1 hour at room temperature and then incubated overnight at 4 ℃ with appropriate antibodies. Antibodies against collagen 1 and collagen 4 were from Santa Cruz Biotechnology (Dallas, Tx, USA) and Abcam (Cambridge, MA, USA) respectively. Lin28b, and ß-actin antibodies were from Cell Signaling (Beverly, MA, USA).

7. Plasmids and promoters

The mouse *Lin28b* promoter (P1) was made by amplifying the -2.5kbp region of *Lin28b* proximal promoter genome region using primers forward 5’-ctagcccgggctcgagTGATGAGATTGTGTCTGCAACT-3’ and reverse 5’-gatcgcagatctcgagGGTCTAATGTGCTTTCCTTCTT-3’. This was cloned into pGL4.10[luc2] vector (Promega Corp., Madison, WI, USA). Several

deletion constructs of the *Lin28b* proximal promoter (P2 to P4) were also constructed by the following procedures. P2 deletion construct of the *Lin28b* promoter was made by amplifying the -2.5kbp to -2.4kbp *Lin28b* proximal genome region using the primers: forward 5'-ctagcccgggctcgagTGATGAGATTGTGTCTGCAACT-3' and reverse 5'-gatcgcagatctcgagGCATAAAGAGAGGGAAATAGAGG-3'. For P4 deletion, amplification of the -640bp *Lin28b* proximal genome region was performed using primers: forward 5'-ctagcccgggctcgagATCAGGTTGAAATTAGTTGTGCTT-3' and reverse 5'-gatcgcagatctcgagGGTCTAATGTGCTTTCCTTCTT-3'. Lower cases denote sequences used for recombination. Cloning was done using the In-Fusion® HD cloning kit (Clontech Laboratories, Inc., Mountain View, CA, USA). P3 deletion was prepared by deleting the upstream region of the 2.5kbp length P1 promoter by digestion with BglII and subsequent ligation. Mouse col1a2 3'UTR and col4a1 3'UTR regions were amplified by PCR using primers shown below. Amplified col1a2 3'UTR and col4a1 3'UTR were digested with XhoI and NotI, and cloned into the NotI-XhoI site of psiCheck2 vector (Promega Corp.). For mutagenesis of the let-7 binding site in the col1a2 3'UTR and col4a1 3'UTR region (designated MT), two primers listed below were used. Primers for PCR of col1a2 3'UTR region are: forward 5'-ggttctcgagGTGAACTCAACCTAAATTAAAAACC-3' and reverse 5'-cctagcggccgcTTTAAACATCTCACATATACAAAATAGG-3'. Primers for col4a1 3'-UTR region are: forward 5'-aggcctcgagTATGATGCTCGCCTCTGCCA-3' and reverse 5'-gtacgcggccgcAGCTTACAGGTTTATTAGTGGTAC-3'. Lower cases denote sequences added for cloning. Primers for col1a2 3'UTR and col4a1 3'UTR let-7 binding site mutation are: sense 5'-AGGTTTGAACTCGAGGAAGACACTTTG-3' and antisense 5'-CAAAGTGTCTTCCTCGAGTTCAAACCT-3'. Primers for col4a1 3'UTR

let-7 binding site mutation are: sense 5’-ATATCCTGTGTCTCGAGCTGTCCACTA-3’ and antisense 5’-TAGTGGACAGCTCGAGACACAGGATAT-3’. The mutated sites are underlined.

8. Luciferase assays

Cells (1×10^6/transfection) were transfected with plasmids, miRNA mimic, miRNA hairpin inhibitor oligonucleotides, or negative controls (Thermo Fischer Scientific Inc.) using an Amaxa Nucleofector. After 48 hours, luciferase activities were measured according to the manufacturer’s protocol. For 3’UTR reporter experiments, psiCHECK2 luciferase reporter plasmids (col1a2 3’UTR, col1a2 3’UTR-M, col4a1 3’UTR, col4a1 3’UTR-M) were cotransfected and the ratio of *Renilla*/firefly luciferase activities were calculated. The pGL4-Lin28b promoter reporter plasmids (P1, P2, P3, and P4) were cotransfected with internal control vector, pRL-TK (*Renilla* luciferase; Promega), and the ratio of firefly/*Renilla* activities calculated. The values were normalized to control conditions (normalized luciferase activity). For TGF-β1 treatment, transfected cells were treated with TGF-β1 (10 ng/ml) for 24 hours.

9. Chromatin immunoprecipitation (ChIP) assays

MMCs were treated with TGF-β1 (10 ng/ml) and then cross-linked with 1% formaldehyde for 30 minutes, washed twice with cold phosphate-buffered saline, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1× protease inhibitor mixture; Applied Science). The cross-linked chromatin was sheared by sonication one to three times for 30 seconds each at 40% maximum setting of the sonicator (Branson Sonifier model 250; Branson Ultrasonics, Danbury, CT, USA) followed by centrifugation for 10 minutes. One-tenth of the total lysate was used for total genomic DNA as “Input DNA” control. Supernatants were collected and diluted in buffer (1% Triton X-100, 2

mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1, 1× protease inhibitor mixture) followed by immunoclearing with 1 μg of sheared salmon sperm DNA, 10 μl of rabbit IgG, and 20 μl of protein-A-Sepharose (Upstate Biotechnology, Lake Plaacid, NY, USA) for 1 hour at 4 ℃. Immunoprecipitation was performed for 15 hours at 4 ℃ with 2-5 μg with antibodies against Smad2/3 (Cell Signaling). Precipitates were washed sequentially for 10 minutes each in TSE I buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), TSE II (TSE I with 500 mM NaCl), and buffer III (0.25 MLiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed twice with TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) and extracted twice with 1% SDS containing 0.1 M $NaHCO_3$. Eluates were pooled and heated at 65 ℃ for at least 6 hours to reverse the formaldehyde cross-linking. DNA fragments were purified with Qiagen Qiaquick spin kit. The purified ChIP-enriched DNA was used as a template for real-time qPCR; the Smad binding element (SBE) in the promoter region of *Lin28b* was amplified with primers forward 5'-ctagcccgggctcgagTGATGAGATTGTGTCTGCAACT-3' and reverse 5'-gatcgcagatctcgagGCATAAAGAGAGGGAAATAGAGG-3'. ChIP-qPCR results (normalized enrichment) were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to input DNA.

10. Immunohistochemistry

Slices of the kidney were fixed in 10% neutral-buffered formalin, processed in the standard manner, and 5 μm-thick sections of paraffin-embedded tissues were utilized for immunohistochemical staining. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black & Decker vegetable steamer. Slides were blocked with Dako protein block (Dako, Carpinteria, CA, USA), and incubated overnight at 4 ℃ with Lin28b antibody

(1:50; Santa Cruz) which were diluted to the appropriate concentrations with 2% casein in bovine serum albumin. After washing with Dako wash solution followed by hydrogen peroxide treatment for 5 minutes, PBS wash, a secondary antibody was added for 20 minutes, and the slides were washed and incubated with a tertiary PAP complex for 20 minutes incubated with anti-rabbit secondary antibody conjugated with a peroxidase polymer (Dako). Incubation was done with 3,3'-diaminobenzidine for 5 minutes. After counterstaining with hematoxylin, the slides were mounted. Periodic acid–Schiff (PAS) staining was performed to analyze ECM deposition. The Lin28b and PAS positively stained areas were determined by examining 10 glomeruli in all mice at 400× magnification.

11. Statistical analysis

Statistical analysis was performed using PRISM software (Graph-Pad, San Diego, CA, USA) for data analysis. Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed by the Mann-Whitney U-test. $P < 0.05$ was considered statistically significant. All data were expressed as means and S.E.

III. RESULTS

1. TGF-ß1 expression is up-regulated in MMCs under diabetic conditions

First, in order to confirm that TGF-β1 is up-regulated in MMCs under diabetic conditions, MMCs were cultured with either normal glucose (NG, 5.5 mM) or high glucose (HG, 25 mM) for 72 hours. The expression of *TGF-ß1* mRNA was significantly up-regulated by HG relative to NG treatment (Figure 1A). To further validate the *in vivo* significance of TGF-β1 up-regulation under diabetic conditions in MMCs, TGF-ß1 expression levels were evaluated in glomeruli from diabetic mice in comparison to their non-diabetic controls. TGF-ß1 expression levels were significantly higher in glomeruli from STZ-induced type 1 diabetic mice (STZ) compared to non-diabetic control mice (CTR) (Figure 1B). A similar up-regulation in TGF-ß1 expression levels were found in glomeruli from type 2 diabetic *db/db* mice compared to non-diabetic control mice (*db/+*) (Figure 1C). Taken together these data verify that TGF-β1 expression is increased under diabetic conditions in MMCs both *in vitro* and *in vivo*, validating its pathophysiologic role in DN.

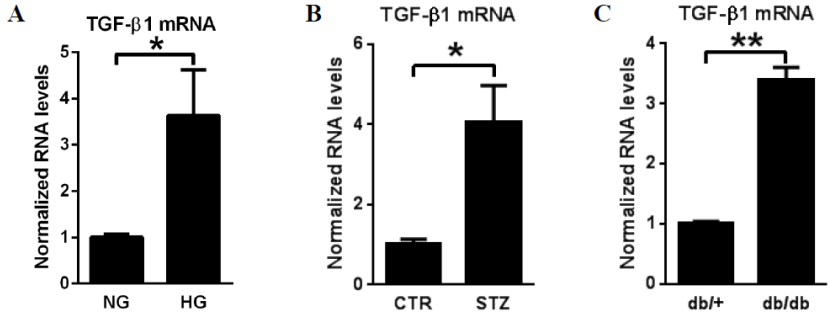

Figure 1. TGF-β1 is up-regulated in MMCs under diabetic conditions. *A, TGF-ß1* mRNA levels in MMCs treated with normal glucose (NG, 5.5 mM) or high glucose (HG, 25mM). *(A)* Significant increase of *TGF-ß1* mRNA levels were detected at 72 hours after HG treatment compared to NG. Results were

normalized with internal control 18S (mean and S.E. n=3). *B and C, TGF-ß1* mRNA expression in glomeruli. *(B)* Significant increase in *TGF-ß1* mRNA in glomeruli from STZ mice compared to those from CTR mice. *(C)* Significant increase in *TGF-ß1* mRNA in glomeruli from *db/db* mice compared to those from genetic control *db/+* mice. Glomeruli were isolated by a sieving technique from 8 mice in each group. Results were normalized with internal control 18S (mean and S.E.). * P <0.05, ** P <0.01.

2. Let-7 expression is down-regulated in MMCs treated with TGF-β1

To evaluate whether the let-7 family miRNAs can directly modulate ECM genes related to glomerular mesangial fibrosis, the expression of let-7 family members (let-7 b/c/d/g/i) in serum depleted (SD) MMCs treated with or without TGF-β1 were compared. The expression levels of all these let-7 family members were significantly down-regulated with TGF-β1 treatment, with the effect of TGF-β1 being most prominent on let-7b and let-7c compared to other let-7 family members (Figure.2A). Because let-7 family members share the same seed sequence, they are expected to target common genes. Therefore, let-7b, being one of the most highly expressed let-7 family members in MMCs, was evaluated in subsequent experiments to represent the let-7 family miRNAs. let-7b was significantly down-regulated in MMCs treated with TGF-β1 at a dose of 10 ng/ml or higher (10-20 ng/ml; Figure 2B). Time course studies showed a decrease of let-7b as early as 1 hour of TGF-β1 treatment which was significant by 6 hr (Figure 2C). These results imply that let-7 has a functional role in TGF-β1 signaling in MMCs.

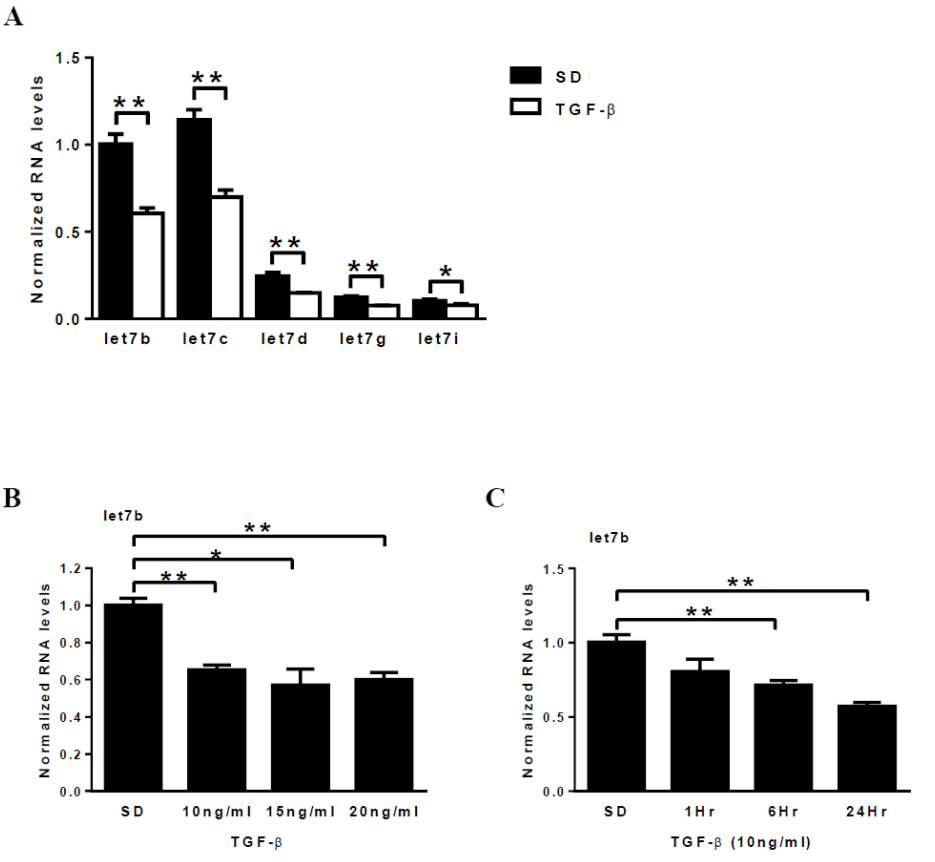

Figure 2. TGF-β1 decreases the expression of let-7 family members in MMCs. *A*, quantitative real-time PCR validation of some let-7 family members (b/c/d/g/i) in serum depleted (SD) MMCs treated without or with TGF-β1 (10 ng/ml) for 24 h. let-7 family members were significantly down-regulated with TGF-β1 treatment relative to SD (mean and S.E. n=3). Results were normalized with internal control U6. * P <0.05, ** P <0.01 compared with SD. *B and C,* let-7b levels (assessed by quantitative real-time PCR) in MMCs treated with TGF-β1. (*B*) Dose response effect of TGF-β1 on let-7b levels. let-7b levels were significantly decreased with indicated concentrations of TGF-β1 treatment (24 h, mean and S.E. n=3). * P <0.05, ** P <0.01 compared with SD. (*C*) Time course study of let-7b. Significant decrease was detected at 6 and 24 hours of TGF-β1

treatment compared with SD (10 ng/ml, mean and S.E. n=3). ** $P<0.01$ compared with SD.

3. Let-7b regulates col1a2 and col4a1 expression in MMCs

In order to evaluate possible functions of let-7 in TGF-β1 signaling, a miRNA target prediction algorithm (TargetScan) to screen potential target genes of the let-7b family miRNAs was used. Interestingly, *col1a2* and *col4a1*, both of which are known ECM genes, were identified as potential direct targets (Figure 3A and 3B). When MMCs were treated with TGF-β1, *col1a2* and *col4a1* mRNA expression levels were significantly increased as anticipated in line with their known role in ECM accumulation in MMCs (from 6 to 24 hr; Figure 3C and 3D).

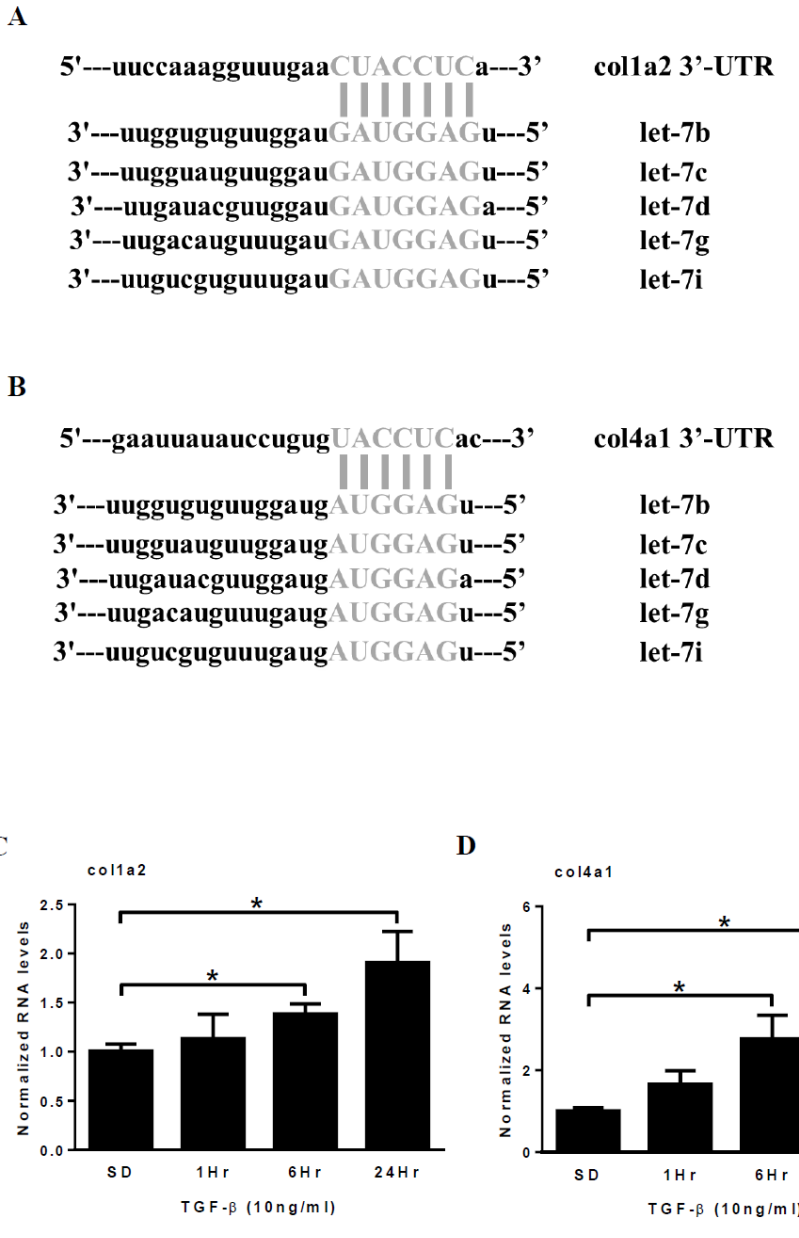

Figure 3. Collagen I α-2 (col1a2) and collagen IV α-1 (col4a1) 3'UTRs are potential targets of let-7 family. *A and B*, sequence alignment of let-7 family binding sites in mouse col1a2 3'-UTR and col4a1 3'-UTR. Target scan

predicted *col1a2* 3'-UTR (*A*) and *col4a1* 3'-UTR (*B*) as potential targets of let-7 family members. Nucleotide locations of the binding sites are shown. *C and D,* col1a2 and col4a1 levels in MMCs treated with TGF-β1. *col1a2* (*C*) and *col4a1* (*D*) mRNA levels were significantly increased at 6 and 24 hours of TGF-β1 treatment compared with serum depleted MMCs without TGF-β1 treatment (SD) (mean and S.E. n=3). * $P<0.05$ compared with SD.

Next, to investigate whether let-7 plays a role in TGF-β1 induced accumulation of col1a2 and col4a1, MMCs were transfected with let-7b mimic oligonucleotide (let7b-M), or with let-7b hairpin inhibitor oligonucleotides (let7b-I) that inhibit let-7b, or the corresponding negative control oligonucleotides. Treatment of negative control mimic (NC-M) transfected MMCs with TGF-β1 significantly increased the expression of *col1a2* and *col4a1* mRNAs (Figure 4A and 4B) and protein (Figure 4C to 4E) levels compared to NC-M transfected MMCs without TGF-β1 treatment, and these up-regulations were significantly attenuated in let7b-M transfected cells treated with TGF-β1 (Figure 4A to 4E).

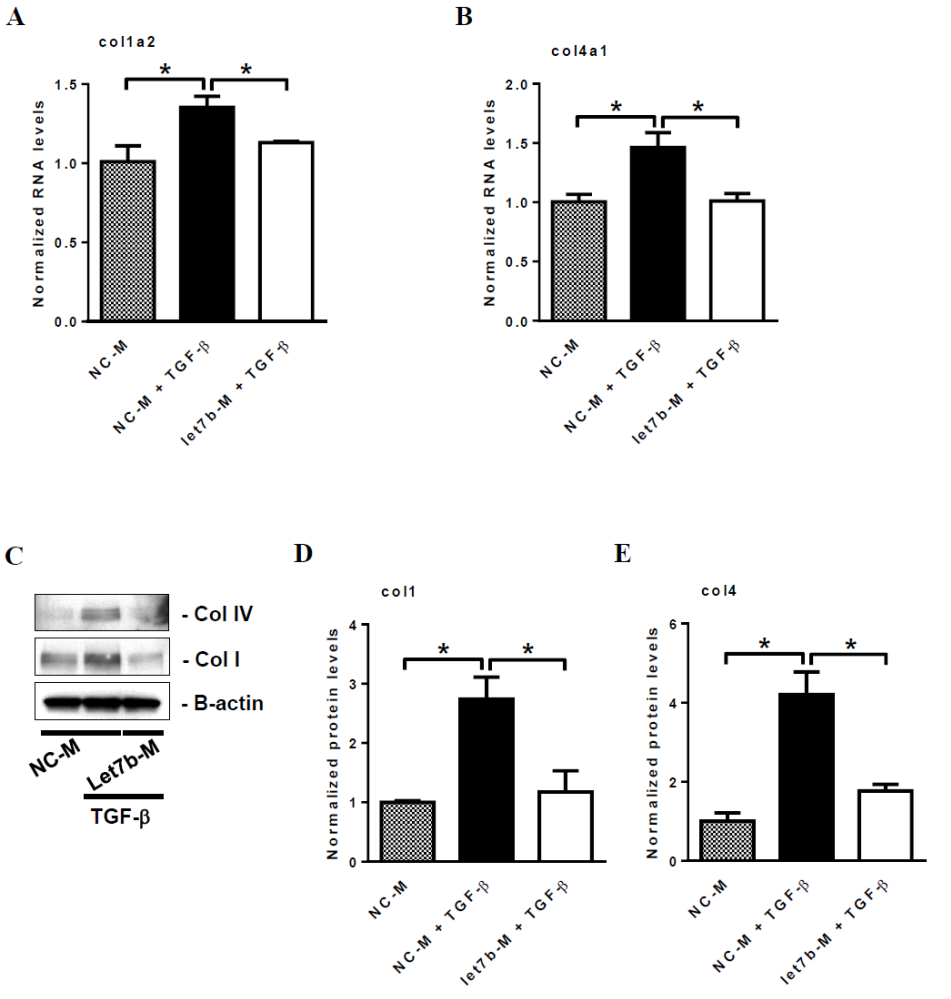

Figure 4. Attenuation of TGF-β1 induced col1a2 and col4a1 expression by ectopic let-7b expression in MMCs. *A and B,* expression of *col1a2* and *col4a1* mRNA levels in MMCs treated with TGF-β1 after transfection with let-7b mimic oligonucleotide (let7b-M). Treatment of MMCs with TGF-β1 up-regulated *col1a2* (*A*) and *col4a1* (*B*) mRNA levels (NC-M + TGF-β) compared to NC-M (negative control mimic) which were attenuated by let7b-M transfection (let7b-M + TGF-β). Results were normalized with internal control 18S (mean and S.E. n=3). * P <0.05. *C-E,* immunoblotting analysis of col1a2

and col4a1 protein levels in MMCs treated with TGF-β1 after transfection with let7b-M. *C*, representative blots. TGF-β1 treatment significantly up-regulated col1a2 (*D*) and col4a1 (*E*) protein levels (NC-M + TGF-β) compared to NC-M, which were each attenuated by let7b-M transfection (let7b-M + TGF-β). Results were normalized with internal control β-actin (mean and S.E. n=3). * P <0.05.

Moreover, transfection of MMCs with let7b-I significantly up-regulated the mRNA (Figure 5A and 5B) as well as protein (Figure 5C to 5E) levels of col1a2 and col4a1. These results clearly show that TGF-β1 induced down-regulation of let-7b can augment the expression of col1a2 and col4a1 in MMCs.

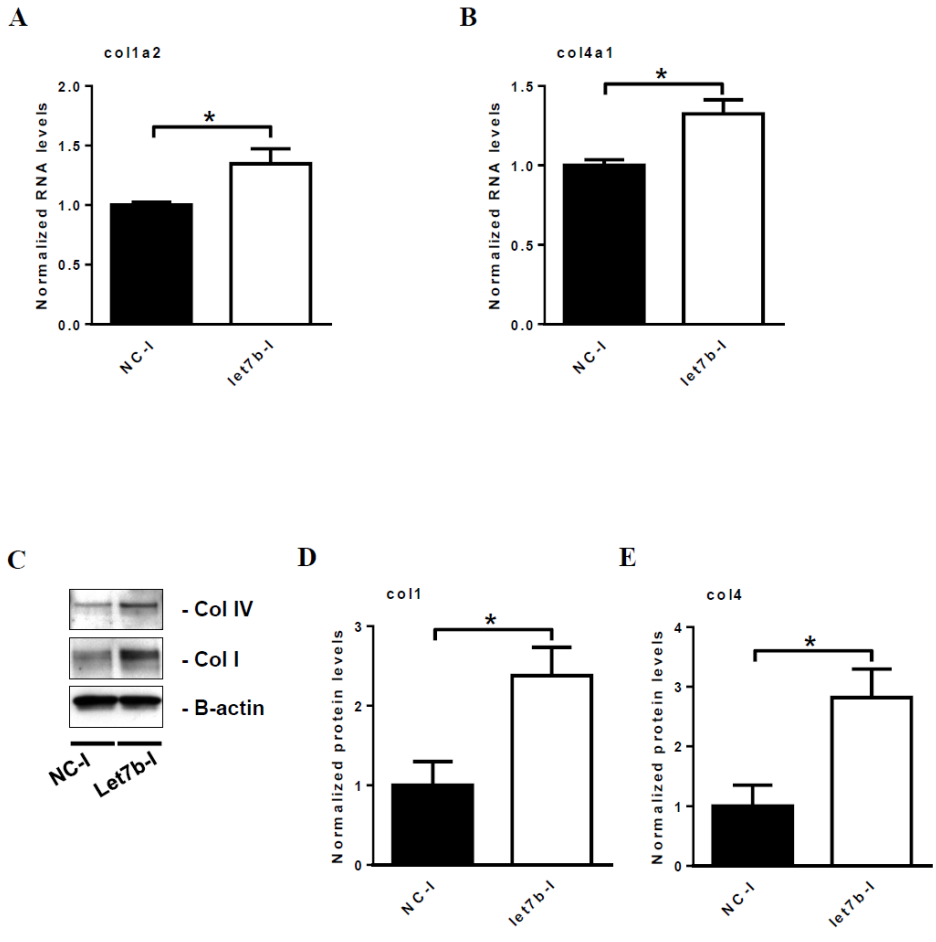

Figure 5. Up-regulation of col1a2 and col4a1 by let-7b inhibition in MMCs. *A and B*, expression of *col1a2* and *col4a1* mRNA in MMCs transfected with let-7b hairpin inhibitor oligonucleotides (let-7b-I). Transfection with let-7b-I significantly increased *col1a2* (*A*) and *col4a1* (*B*) mRNA levels compared to NC-I (negative control inhibitor). Results were normalized with internal control 18S (mean and S.E. n=3). * P <0.05. *C-E*, immunoblotting analysis of col1a2 and col4a1 protein levels in MMCs transfected with let7-b-I. Transfection with let7-b-I significantly increased col1a2 (*D*) and col4a1 (*E*) protein levels compared to NC-I. Results were normalized with internal control β-actin (mean

and S.E. n=3). * P <0.05.

4. col1a2 and col4a1 are bona fide targets of let-7b

To verify whether col1a2 and col4a1 are direct targets of let-7b, the col1a2 and col4a1 3′UTRs containing the let-7b binding site were cloned downstream of luciferase in a reporter vector (Figure 6A and 6D). let7b-I-transfected cells showed significantly increased luciferase activity of col1a2 3'UTR reporter vector (col1a2 3'UTR) compared to NC-I transfected cells (Figure 6B). However, this increase was not observed in cells co-transfected with col1a2 luciferase reporter vectors with base substitutions (mutations) in the let-7b binding site of the 3'UTR (col1a2 3'UTR-MT; Figure 6B). In addition, TGF-β1 treatment of MMCs transfected with col1a2 3'UTR increased the luciferase activity which was significantly attenuated by co-transfection with let7b-M relative to NC-M (Figure 6C). Similarly, let-7b-I transfected cells also showed increased luciferase activity of the col4a1 3'UTR reporter vector (col4a1 3'UTR), which was not evident with the col4a1-3'UTR-MT (Figure 6E). Let7b-M transfection also blocked TGF-β1 induced luciferase activity in col4a1 3'UTR transfected MMCs (Figure 6F). Taken together, these results clearly demonstrate that col1a2 and col4a1 are direct targets of let-7b in MMCs.

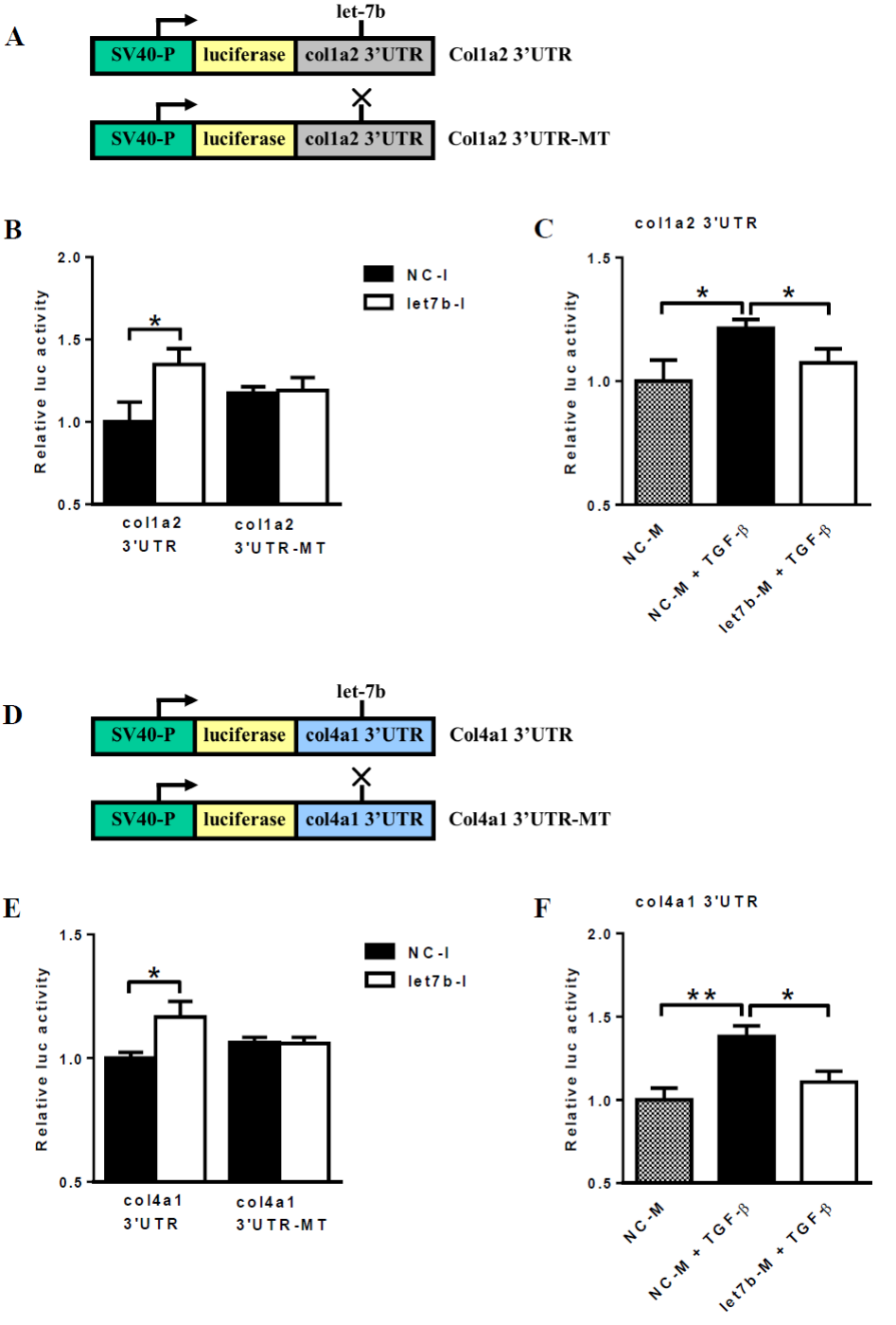

Figure 6. The 3' untranslated regions (UTRs) of col1a2 and col4a2 are bona

fide targets of let-7b. *A and D,* luciferase (Luc) reporters for mouse col1a2 3'-UTR or col4a1 3'-UTR. Plasmids containing Luc, col1a2 3'-UTR (col1a2 3'-UTR) or col4a1 3'-UTR (col4a1 3'-UTR) were used as reporters of let-7b. Mutations were introduced into the let-7 family binding sites in the col1a2 3'-UTR (col1a2 3'UTR-MT) or col4a1 3'-UTR (col4a1 3'UTR-MT) by site direct mutagenesis. *B and E,* relative Luc activity of MMCs transfected with reporter plasmids with or without mutations. Luc activity was significantly increased in col1a2 3'-UTR (*B*) and col4a1 3'-UTR (*E*) by co-transfection with let-7b hairpin inhibitor oligonucleotides (let7b-I). However, this increase in Luc activity was not found in MMCs transfected with col1a2 3'UTR-MT (*B*) or col4a1 3'UTR-MT (*E*) reporter (mean and S.E. n=3). * P <0.05. *C and F,* relative Luc activity of TGF-β1 treated col1a2 3'-UTR or col4a1 3'-UTR transfected MMCs with or without co-transfection of let-7b mimic oligonucleotides (let7b-M). Luc activity was significantly increased in col1a2 3'-UTR (*C*) or col4a1 3'-UTR (*F*) transfected MMCs treated with TGF-β1 (NC-M + TGF-β) compared to negative control mimic co-transfected MMCs without TGF-β1 treatment (NC-M). Co-transfection with let-7b mimic oligonucleotides (let7b-M) significantly attenuated this increase in Luc activity (mean and S.E. n=3). * P <0.05 and ** P<0.01.

5. Let-7b is down-regulated in the glomeruli of diabetic mice

To determine the *in vivo* relevance, the association between let-7b and collagen expression was further verified in diabetic animal models. let-7b expression levels were significantly decreased in renal glomeruli from streptozotocin-induced type 1 diabetic mice (STZ) compared to nondiabetic control mice (CTR) (Figure 7A). In parallel, the expression levels of *col1a2* and *col4a1* mRNA showed significant increases in glomeruli from STZ compared to CTR (Figure 7B and 7C). Similarly, in glomeruli from type 2 diabetic *db/db* mice (db/db), let-7b expression levels were significantly decreased (Figure 7D)

while *col1a2* and *col4a1* mRNA levels were increased compared with nondiabetic control mice (db/+) (Figure 7E and 7F). These results suggest that inverse correlations found between let-7b and collagen genes *in vitro*, are also evident *in vivo* in glomeruli from diabetic animal models.

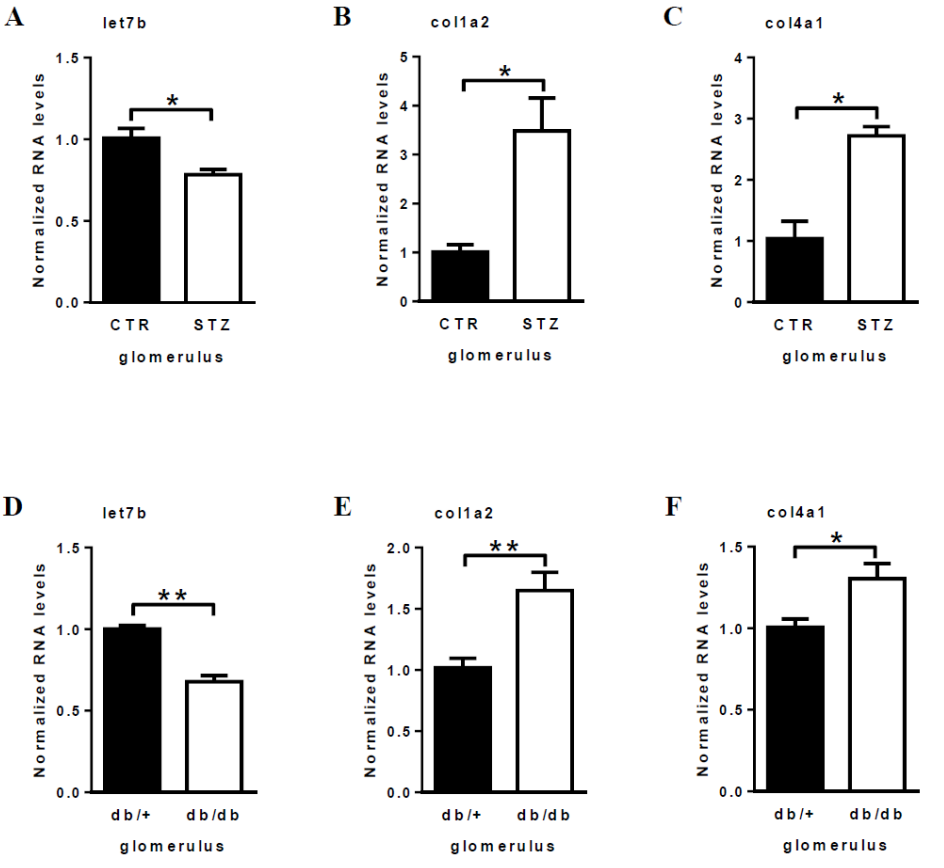

Figure 7. Let-7b is down-regulated and, in parallel, col1a2 and col4a2 are up-regulated in glomeruli from type 1 (STZ-induced) and type 2 (*db/db*) diabetic mice relative to the respective control mice. *A* and *D*, let-7b expression levels in type 1 and type 2 mice glomeruli. let-7b levels were significantly decreased in type 1 (*A*) and type 2 (*D*) diabetic (STZ and *db/db*) mice glomeruli relative to control non-diabetic (CTR and *db/+*) mice. Results were normalized

with internal control U6 (mean and S.E. n=6). * P<0.05, ** P<0.01. *B and E, col1a2* expression in glomeruli from STZ and *db/db* mice. *col1a2* mRNA levels were significantly increased in STZ (*B*) and *db/db* (*E*) mice glomeruli relative to CTR and *db/+* mice. *C and F, col4a1* expression in glomeruli from STZ and *db/db* mice. *col4a1* mRNA levels were increased significantly in STZ (C) and *db/db* (F) mice glomeruli relative to CTR and *db/+* mice. Results were normalized with internal control 18S (mean and S.E. n=6). * P <0.05, ** P<0.01.

6. Lin28b expression is up-regulated by TGF-β1

Next, the molecular mechanism underlying TGF-β1 induced let-7 down-regulation in MMCs was further investigated. Lin28a and Lin28b have been shown to regulate let-7 biogenesis in developmental processes and in cancer cells.[46, 47] They bind to let-7 selectively and block let-7 biogenesis.[48] Therefore, whether these proteins also play a role in let-7 down-regulation induced by TGF-β1 in MMCs under diabetic conditions were examined. *Lin28a* mRNA was not detectable in MMCs, and the expression levels of Lin28a were not altered by TGF-β1 treatment (data not shown). However, the expression levels of *Lin28*b mRNA were significantly up- regulated by TGF-β1 treatment (6 to 24 hr; Figure 8A) which paralleled the down-regulation of let-7b (Figure 2C). *Lin28b* mRNA levels were increased by TGF-β1 in a dose-dependent manner (from 10 to 20 ng/ml; Figure 8B). Protein levels of Lin28b also showed a significant increase with TGF-β1 treatment in MMCs (from 6 to 24 hr; Figure 8C and 8D). Together these results suggest that up-regulation of Lin28b may be a mechanism for TGF-β1 induced down-regulation of let-7b in MMCs.

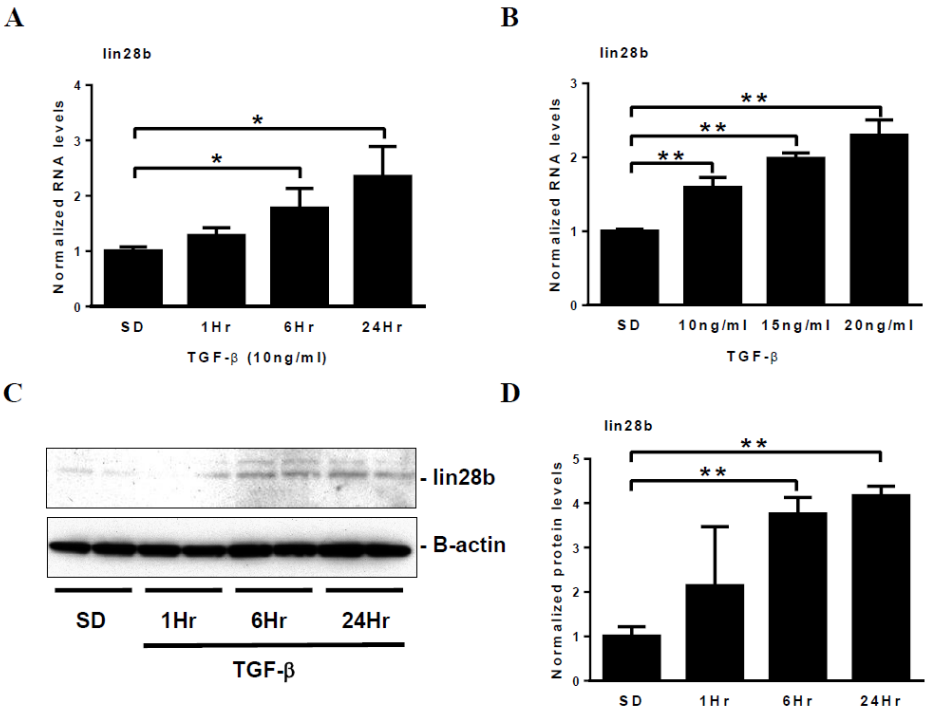

Figure 8. Lin28b expression is up-regulated in TGF-β1 treated MMCs. *A and B*, expression levels of *Lin28b* mRNA in TGF-β1 treated MMCs. (*A*) Time course study of Lin28b. Significant increase of *Lin28b* mRNA was detected at 6 hours and 24 hours of TGF-β1 treatment compared with serum depleted MMCs without TGF-β1 treatment (SD) (10 ng/ml, mean and S.E. n=3). (*B*) Dose response of Lin-28. *Lin28b* mRNA levels significantly increased with indicated concentrations of TGF-β1 treatment (24 hr, mean and S.E. n=3). * P <0.05, ** P<0.01 compared with SD. *C and D*, immunoblotting analysis of MMCs for Lin28b protein levels after treatment with TGF-β1 (10ng/ml for 1 to 24 h). (*D*) TGF-β1 significantly increased protein levels of Lin28b at 6 and 24 hours (normalized with internal control β-actin) (mean and S.E. n=3). ** P <0.01 compared with SD.

7. Sustained overexpression of Lin28b up-regulates col1a2 and col4a1 in MMCs

To further confirm Lin28b plays a role in col1a2 and col4a1 up-regulation, the functional relevance of Lin28b up-regulation was determined by using MMCs stably transduced with vectors that express full length mouse *Lin28b* mRNA under the control of a CMV promoter (pCMV-Lin28b). MMCs transfected with GFP were used as controls (pCMV-GFP). Lin28b overexpression resulted in a significant up-regulation of col1a2 as well as col4a1 further verifying that the up-regulation of Lin28b in MMCs could induce col1a2 and col4a1 accumulation in MMCs (Figure 9A and 9B).

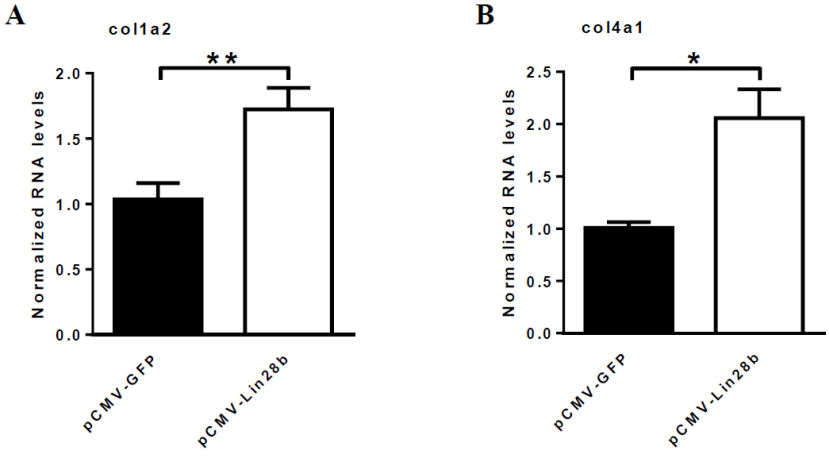

Figure 9. Regulation of col1a2 and col4a1 in MMCs stably overexpressing Lin28b. (A) Significant increase of *col1a2* mRNA was detected in pCMV-Lin28b transfected MMCs compared to pCMV-GFP transfected MMCs (mean and S.E. n=6). (B) Significant increase of *col4a1* mRNA was detected in pCMV-Lin28b transfected MMCs compared to pCMV-GFP transfected MMCs (mean and S.E. n=6). * P <0.05, ** P<0.01.

8. Lin28b expression is transcriptionally regulated by TGF-β1 activated Smad2/3

Interestingly, a potential SBE in the proximal promoter of the mouse *Lin28b* gene was noticed. This potential SBE sequence was conserved in several species including rat and human (Figure 10A). To evaluate the role of this SBE in TGF-β1 induced Lin28b up-regulation, the proximal promoter and promoter deletion constructs of the mouse *Lin28b* gene into a luciferase reporter were cloned (Figure 10B). This *Lin28b* proximal promoter responded to TGF-β1 treatment showing significant increase in luciferase activity (Figure 10B, P1). Deletion constructs (P2 and P3) that still included the SBE were also responded to TGF-β1, whereas while a deletion mutant (P4) lacking the SBE was not responsive to TGF-β1 (Figure 10B). Next, whether TGF-β1 activated Smad2/3 binds to the SBE in the proximal promoter with TGF-β1 treatment was examined. ChIP assays showed that the occupancy of Smad2/3 at the SBE in the proximal promoter of *Lin28b* was significantly increased at 1 hr after TGF-β1 treatment (Figure 10C). These results demonstrate that the SBE is essential for TGF-β1 response, suggesting for the first time that TGF-β1 leads to transcriptional up-regulation of *Lin28b* through activation of Smads.

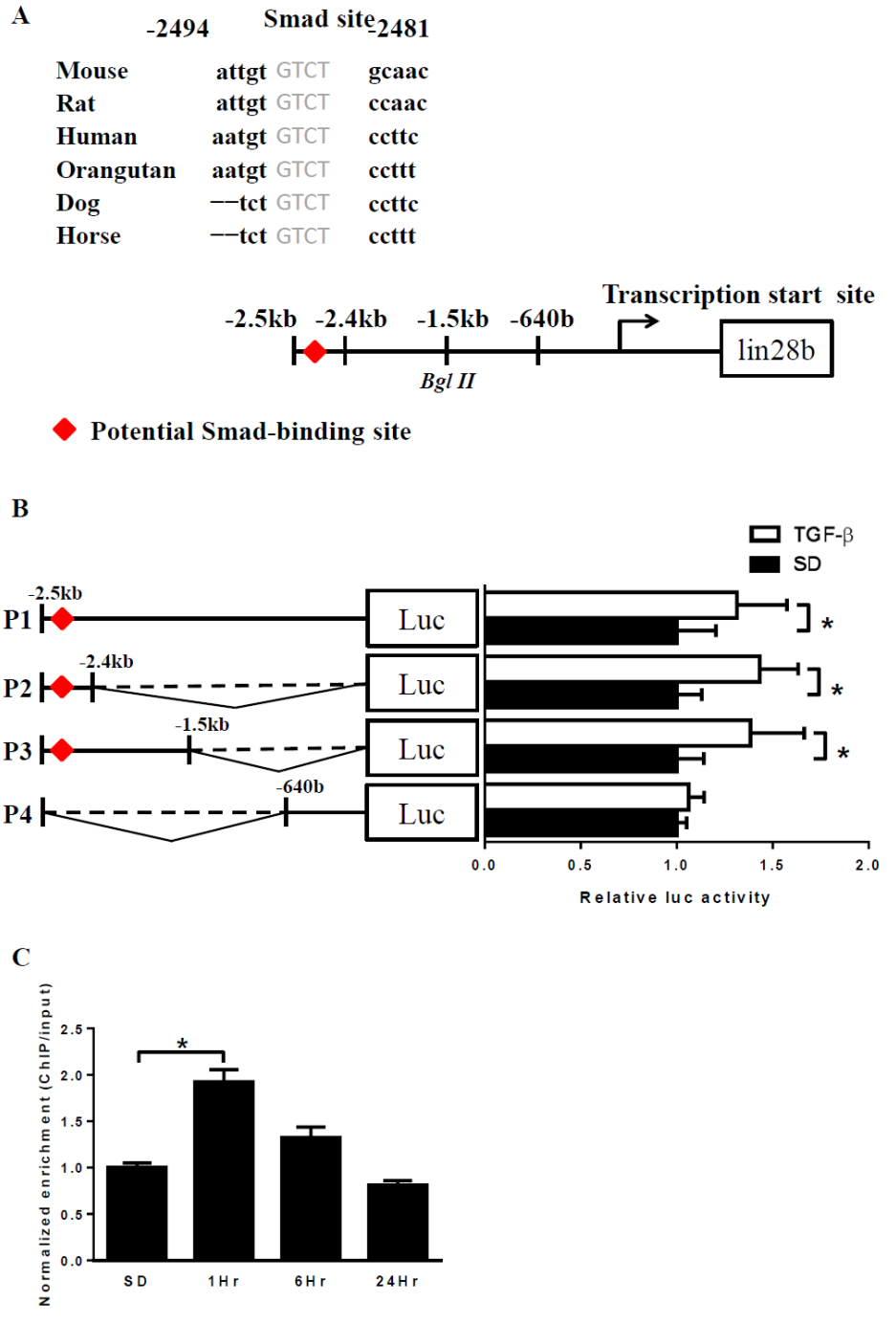

Figure 10. TGF-β1 induces transcriptional activation of *Lin28b* through the

promoter Smad binding element (SBE). *A*, comparison of the proximal promoter sequence of the *Lin28b* gene in several species (mouse, rat, human, orangutan, dog, horse). A conserved consensus sequence of a potential SBE was found in the *Lin28b* promoter. *B*, schematic genome structure of the four *Lin28b* promoter reporter constructs used: P1 (-2.5k), P2 (-2.5~-2.4k), P3 (-2.5~-1.5k) *Lin28b* promoter constructs with a SBE, and P4 (-640), a SBE deletion mutant *Lin28b* promoter construct. Reporter constructs having an intact SBE (P1-P3) show a significant increase in luciferase activity with TGF-β1 treatment compared to SD. However, luciferase activity was not significantly altered by TGF-β1 in the SBE deleted reporter construct (P4) compared to SD (mean and S.E. n=6). * P <0.05. *C*, ChIP analysis of Smad2/3 occupancy at the *Lin28b* promoter SBE. The same primers used for cloning P2 (the SBE) were also used for ChIP analysis. Significant increase in Smad2/3 occupancy was observed at 1 hour after TGF-β1 treatment in MMCs (mean and S.E. n=3). * P<0.05.

9. Lin28b expression is up-regulated in the diabetic glomerulus

To further confirm that the inverse correlation between let-7 and Lin28b is relevant *in vivo* in DN, Lin28b levels were evaluated in diabetic animal models. *Lin28b* mRNA expression levels were significantly increased in renal glomeruli from type 1 diabetic STZ mice compared to nondiabetic CTR (Figure 11A), which paralleled the observed decrease in let-7b levels in the glomeruli of STZ mice compared to CTR (Figure 7A). Similarly, in glomeruli from type 2 diabetic *db/db*, *Lin28b* mRNA expression levels were significantly increased compared with nondiabetic *db/+* (Figure 11B). Immunohistochemical staining showed that Lin28b expression was significantly increased in glomeruli from STZ mice compared to CTR (Figure 11C, 11D, and 11G), as well as in glomeruli from *db/db* mice compared to *db/+* (Figure 11E, 11F, and 11G). PAS staining verified a significant increase in mesangial matrix expansion in STZ mice compare to CTR (Figure 11H, 11I, and 11L), as well as in glomeruli from

db/db mice compared to *db/+* (Figure 11J, 11K, and 11L). Taken together, these results demonstrate that the correlations found between let-7, Lin28b, and collagen *in vitro* are also present *in vivo* in the glomeruli of diabetic animal models.

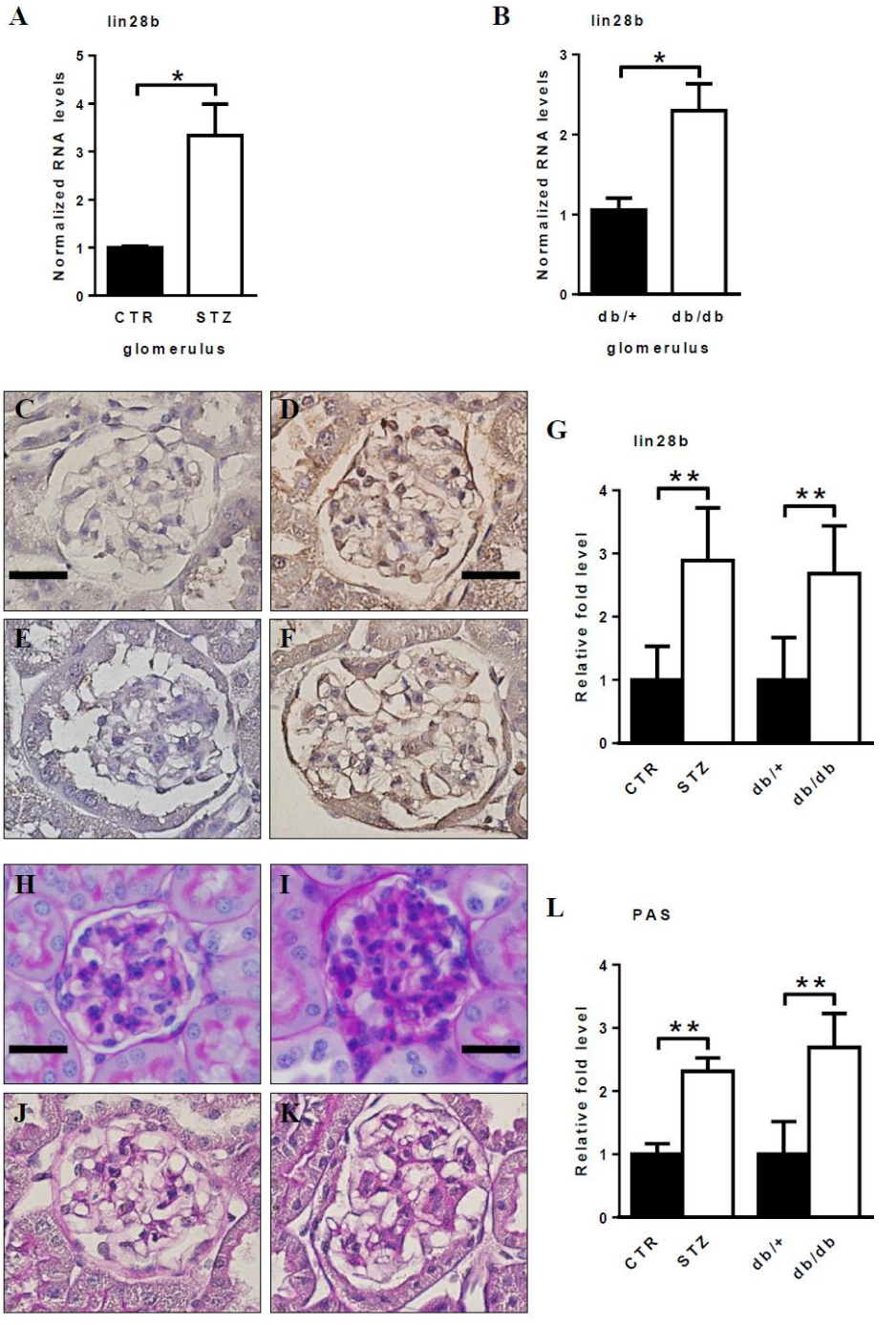

Figure 11. Lin28b expression is up-regulated in the glomeruli from type 1

(STZ-induced) and type 2 (*db/db*) diabetic mice. *A and B, Lin28b* mRNA levels were significantly increased in type 1 (*A*) and type 2 (*B*) diabetic (STZ and *db/db*) mice glomeruli relative to the respective non-diabetic controls (CTR and *db/+*) mice (mean and S.E. n=6). * $P<0.05$. *C to F*, representative IHC staining of Lin28b in glomeruli from type 1 and type 2 diabetic mice and respective controls (scale bar, 20 μm). IHC staining of Lin28b was significantly increased in type 1 STZ (*D*) and type 2 *db/db* (*F*) diabetic mice glomeruli relative to control non-diabetic CTR (*C*) and *db/+* (*E*) mice. *G*, relative fold level of Lin28b positive area. *H to K*, representative PAS staining of glomeruli from type 1 and type 2 diabetic mice (scale bar, 20 μm). Mesangial matrix expansion was significantly increased in type 1 STZ (*I*) and type 2 *db/db* (*K*) diabetic mice glomeruli relative to control non-diabetic CTR (*H*) and *db/+* (*J*) mice. *L*, relative fold level of PAS positive area. To quantify Lin28b and PAS positive stained area, the positive stained areas/glomerular areas (%) were measured using Image J (National Institute of Health). For each animal, 10 glomeruli were evaluated (Mean and S.E.). * $P<0.05$, ** $P<0.01$.

IV. DISCUSSION

In the current study, I have shown that the let-7 family miRNAs play a protective role against TGF-β1-induced collagen accumulation in mesangial cells, and that col1a2 and col4a1 are direct targets of the let-7 family. The down-regulation of let-7 family members by TGF-β1 resulted in up-regulation of the let-7 targets col1a2 and col4a1 in MMCs. Also, that this down-regulation of let-7 was associated with an increase in the expression of Lin28b, a key inhibitor of let-7 biogenesis was shown for the first time. Furthermore, the transcriptional induction of Lin28b by TGF-β1 through the activation of Smads was demonstrated, thus identifying a new target of TGF-β1 in the kidney with functional relevance to fibrosis. Together, these results reveal novel roles for the combined actions of let-7 and Lin28b in controlling TGF-β1-induced collagen accumulation in DN.

TGF-β1 is a known profibrotic agent that plays key pathological roles in the accumulation of ECM proteins in renal cells under diabetic conditions through the activation of its downstream targets.[20, 22, 24-26] The Smad transcription factors have been most extensively investigated as effectors of TGF-β1 receptor activation and signaling in DN progression.[20, 23, 26, 49-51] TGF-β1 activates Smad2 and Smad3 which are substrates for TGF-β type 1 receptor serine/threonine kinase activity. The activated Smads form a complex with the common mediator Co-Smad 4, which then translocates to the nucleus to increase the transcription of target genes, including collagens.[26, 52-56] However, recently, other post-transcriptional mechanisms involving miRNAs have been found to play a key role in TGF-β1 induced ECM accumulation.[32] For example, TGF-β1 induced miR-192 and miR-200 family members inhibit E-box repressors (ZEB1/2) which relieves repression at upstream E-boxes to induce col1a2 and col4a1 as well as TGF-β1.[57, 58] In addition, miR-216a and miR-217, up-regulated by TGF-β1, were shown to mediate the down-regulation of PTEN, a phosphatase which inactivates phosphatidylinostitol 1, 4, 5-triphosphate to

inhibit the phosphorylation of Akt, thereby resulting in the activation of the Akt pathway and mesangial cell hypertrophy.[38, 59] The up-regulation of miR-200b/c by TGF-β1 was also found to target FOG2, a factor which inhibits phosphatidylinositol 3-kinase activity, and thereby contribute to the activation of Akt and ERK kinases, key pathways in mesangial cell ECM accumulation under diabetic conditions.[60] Several other miRNAs have been implicated in DN pathogenesis.[32-39] Adding to this repertoire of miRNAs associated with DN, the current results show that the down-regulation of a whole family of miRNAs which are highly expressed in glomerular mesangial cells, likely due to up-regulation of their inhibitor (Lin28b), can further contribute to glomerular fibrosis in response to TGF-β1 and in DN. These findings suggest that, in addition to the direct activation of the Smad transcription factors, TGF-β1 leads to the concerted regulation of a group of miRNAs to further fine-tune fibrotic gene expression and augment its pathophysiologic effects in DN.

Recent reports have suggested that the let-7 family members participate in regulating TGF-β1 induced fibrosis in various cell types and disease conditions.[40-43] let-7a was found to be down-regulated in skin samples of patients with systemic scleroderma, a skin disorder characterized by fibrosis of the skin and internal organs.[61] Further, in renal tubular epithelial cells, TGF-β1 was recently shown to down-regulate let-7b which up-regulated the let-7 target TGF-β receptor 1 (TGFBR1) resulting in an augmentation of TGF-β1 induced actions via TGFBR1.[45] Further supporting these actions of let-7, another report showed that lipoxins attenuate renal tubular fibrosis by inducing let-7c which down-regulated its target TGFBR1.[44] In line with these reports, the current finding that the let-7 family miRNAs can also directly target the final ECM gene products (col1a2 and col4a1) of the TGF-β1 cell signaling pathway shows that the let-7 family negatively modulates ECM accumulation by regulating multiple players in the pathway starting from the receptor down to the final gene product. Based on these data showing that let-7 family members act on multiple steps of the TGF-β1 cell signaling pathway, the high cellular level of

let-7 miRNAs may play an important protective role as an efficient natural suppressor of the TGF-β1 pathway in mesangial cells and other renal cells during DN.

The known regulator of let-7 biogenesis, Lin28, has been widely investigated as an oncogene.[50, 62-64] Although mostly down-regulated in adult tissue cells, overexpression of Lin28 has been observed in numerous advanced stage tumors, including ovarian cancer, prostate cancer, hepatocellular carcinoma, and hematologic malignancies.[47, 65-67] In addition, Lin28 expression significantly correlates with clinical outcomes and prognosis of various cancer types.[68, 69] The results of this study show, for the first time, that in addition to its role in cancer progression, Lin28 may also participate in the progression of organ damage and fibrosis in metabolic diseases like diabetes. Epidemiologic studies have repeatedly shown an increased risk of cancer in patients with diabetes and metabolic syndrome. Several proposals have been addressed to explain this relationship. However, the biologic link between metabolic disease and malignancy is still not fully understood. Although mesangial cells do not undergo malignant transformation into cancer cells, the findings of this investigation could serve as a possible molecular explanation for this metabolic disease-cancer relationship. The fact that, prior to progressive glomerular ECM accumulation, mesangial cell proliferation occurs in the natural course of DN may further support this notion.[8]

Interestingly, an intact SBE in the promoter region of *Lin28b* was shown to be involved in the transcriptional regulation of the *Lin28b* gene by TGF-β1, suggesting a role for Smad activation in the up-regulation of Lin28 by TGF-β1. This was further supported by ChIP results which showed that Smad 2/3 occupancy at this SBE in proximal promoter of *Lin28b* was clearly increased after TGF-β1 treatment. In addition to the canonical signaling pathway in which TGF-β1 activated Smad 2/3 leads to their binding and transcriptional activation of ECM gene promoters, the current results demonstrate that the activated Smad

2/3 may also work to down-regulate the natural suppressors of ECM genes and thereby enhance the efficiency of TGF-β1 actions. This notion, that these Smads not only directly up-regulate ECM genes but also down-regulates suppressor of ECM genes, could help explain the clinical findings showing that the progression of renal fibrosis is persistent in DN and that current medical strategies are not fully efficacious in preventing fibrotic organ damage. Further investigations aimed at examining the consequences of experimentally modulating Lin28 levels are necessary to evaluate this.

In this study, Smad 2/3 occupancy at the *Lin28b* promoter SBE was increased shortly after TGF-β1 treatment which was followed by a rapid decrease. Based on these findings, it is likely that, in response to TGF-β1 treatment, *Lin28b* transcription is initiated first by Smad binding to the promoter region SBE, and that the later observed increases in Lin28b may involve other mechanisms of transcription activation. Notably, in another study it was found that Smad occupancy in the SBE of the miR-192 promoter was increased rapidly after TGF-β1 treatment, giving way to subsequent SBE binding of Akt-activated p300 which led to promoter chromatin histone lysine acetylation and sustained miR-192 expression for longer time periods.[37] It is possible that similar chromatin remodeling mechanisms may also operate in during TGF-β1 induced transcription of *Lin28b* at later time periods.

In summary, the current results demonstrate that TGF-β1 induced down-regulation of let-7 family miRNAs up-regulate the expression of their targets col1a2 and col4a1 in MMC. In addition, the increase in Lin28b expression, which is transcriptionally regulated by TGF-β1 activated Smad 2/3 and the *Lin28b* promoter SBE, plays a role in this down-regulation of let-7 family miRNAs under diabetic conditions (Figure 12). These results characterize Lin28b as a new target of TGF-β1 in the kidney, as well as a novel role for the Lin28/let-7 pathway in mediating the effects of TGF-β1 on ECM genes in mesangial cells under diabetic conditions.

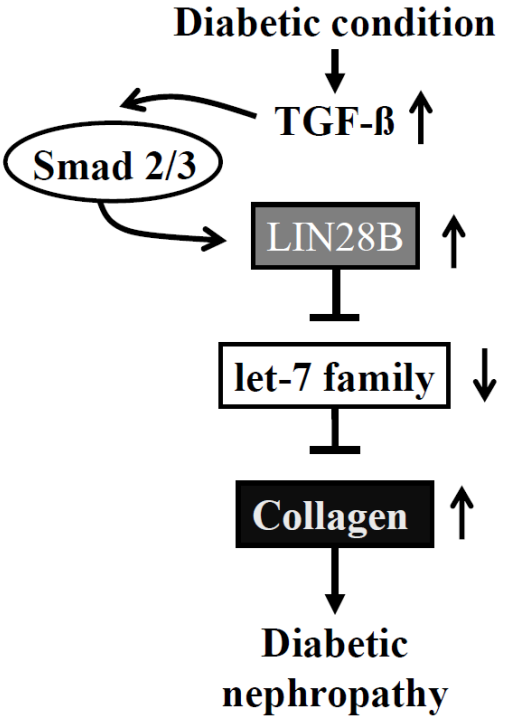

Figure 12. Schematic model depicting Lin28b and let-7 family dependent mechanism of collagen accumulation in the pathogenesis of diabetic nephropathy. TGF-β1 induced by diabetic conditions up-regulates Lin28b through the activation of Smad 2/3. Lin28b induced down-regulation of let-7 family miRNAs facilitates the up-regulation of collagens (let-7 targets), leading to glomerular ECM accumulation, a main feature of diabetic nephropathy.

V. CONCLUSION

To further clarify the mechanism of TGF-β1 induced glomerular mesangial fibrosis under diabetic conditions, I have proven that the let-7 family miRNAs have a protective role against TGF-β1-induced collagen expression. In addition, Smad induced transcriptional activation of Lin28b was also shown to play a role in this TGF-β1-induced let-7 family dependent mechanism of collagen accumulation in mesangial cells.

1. Significant increase of *TGF-ß1* mRNA levels was detected at 72 hours after HG treatment compared to NG. In addition, *TGF-ß1* mRNA was significantly up-regulated in glomeruli from *db/db* mice compared to those from genetic control *db/+* mice. Similarly, *TGF-ß1* mRNA was clearly up-regulated in glomeruli from STZ mice compared to those from CTR mice.
2. Let-7 family members were significantly down-regulated with TGF-β1 treatment relative to SD in cultured MMCs.
3. Compared with serum depleted MMCs without TGF-β1 treatment *col1a2* and *col4a1* mRNA levels were significantly increased after TGF-β1 treatment.
4. Treatment of MMCs with TGF-β1 up-regulated *col1a2* and *col4a1* mRNA levels compared to NC-M which were attenuated by let7b-M transfection. Correspondingly, TGF-β1 treatment significantly up-regulated col1a2 and col4a1 protein levels compared to NC-M, which were each attenuated by let7b-M transfection.
5. Transfection with let-7b-I significantly increased *col1a2* and *col4a1* mRNA levels compared to NC-I. Correspondingly, transfection with let7-b-I significantly increased col1a2 and col4a1 protein levels compared to NC-I.
6. Luciferase activity was significantly increased in col1a2 3'-UTR and col4a1 3'-UTR by co-transfection with let-7b hairpin inhibitor oligonucleotides. However, this increase in luciferase activity was not found in MMCs transfected with col1a2 3'UTR or col4a1 3'UTR reporters with let-7 binding site mutations. Luciferase activity was significantly increased in col1a2 3'-UTR

or col4a1 3'-UTR transfected MMCs treated with TGF-β1 compared to negative control mimic co-transfected MMCs without TGF-β1 treatment. Co-transfection with let-7b mimic oligonucleotides significantly attenuated this increase in luciferase activity.

7. Let-7b levels were significantly decreased in type 1 and type 2 diabetic (STZ and *db/db*) mice glomeruli relative to control non-diabetic (CTR and *db/+*) mice. Whereas, *col1a2* and *col4a1* mRNA levels were significantly increased in STZ and *db/db* mice glomeruli relative to CTR and *db/+* mice.

8. Significant increase of *Lin28b* mRNA was detected after TGF-β1 treatment compared with serum depleted MMCs without TGF-β1 treatment. Correspondingly, TGF-β1 significantly increased protein levels of Lin28b with TGF-β1 treatment.

9. Significant increase of *col1a2* and *col4a1* mRNA was detected in pCMV-Lin28b transfected MMCs compared to pCMV-GFP transfected MMCs.

10. Reporter constructs having an intact SBE showed a significant increase in luciferase activity with TGF-β1 treatment compared to SD. However, luciferase activity was not significantly altered by TGF-β1 in the SBE deleted reporter construct compared to SD. In addition, a significant increase in Smad2/3 occupancy was observed at the Lin28b promoter SBE after TGF-β1 treatment in MMCs.

11. *Lin28b* mRNA levels were significantly increased in type 1 and type 2 diabetic (STZ and *db/db*) mice glomeruli relative to the respective non-diabetic control (CTR and *db/+*) mice. In addition, IHC staining of Lin28b was significantly increased in type 1 STZ and type 2 *db/db* diabetic mice glomeruli relative to control non-diabetic CTR and *db/+* mice. In parallel, mesangial matrix expansion was significantly increased in type 1 STZ and type 2 *db/db* diabetic mice glomeruli relative to control non-diabetic CTR and *db/+* mice.

In conclusion, TGF-β1 induces let-7 family miRNA down-regulation which attenuates the up-regulation of let-7 family miRNA targets col1a2 and col4a1 in MMCs. In addition, Lin28b expression is transcriptionally regulated by TGF-β1

activated Smad 2/3 and the *Lin28b* promoter SBE, which plays a role in this down-regulation of let-7 family miRNAs under diabetic conditions. These results prove a novel pathway including Lin28 and let-7 in the pathophysiologic mechanisms of TGF-β1 induced ECM gene accumulation in mesangial cells under diabetic conditions.

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ABSTRACT (IN KOREAN)

당뇨 조건 하에서 TGF-β1에 의한 Lin28b 및 let-7 발현 변화가 사구체 메산지움세포의 콜라겐 생성에 미치는 영향

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배경: 당뇨병성 신병증은 전세계적으로 말기 신부전증의 가장 중요한 원인 질환이며, 병리학적으로 사구체 기저막 및 메산지움세포의 비후, 그리고 콜라겐, 파이브로넥틴과 같은 세포바깥바탕질 (extracellular matrix) 단백의 축적을 특징으로 갖는다. 세포바깥바탕질 단백 축적의 병리기전에 있어서 transforming growth factor-β1 (TGF-β1)가 중요한 역할을 하는 것으로 알려져 있으나, 현재까지 TGF-β1에 의한 섬유화 진행의 정확한 분자생리학적 기전은 밝혀지지 못한 실정이다. 최근의 연구들에 의하여 miRNA가 당뇨병성 신병증을 포함한 만성 질환의 진행에 중요한 역할을 하는 것으로 밝혀지고 있다. 그런데, miRNA let-7이 여러 질환에 있어서 섬유화 진행을 억제하는 역할이 있음이 최근 들어 밝혀진 바 있다. 이에 따라, 본 연구를 통하여 당뇨병성 신병증의 진행에 있어서 let-7이 세포바깥바탕질 단백의 발현을 효율적으로 억제하여 메산지움의 섬유화 과정에 관여하는지를 밝히고자 한다. 또한, let-7의

발현을 조절하는 것으로 알려진 Lin28이 당뇨 환경하의 메산지움세포에서 TGF-β1에 의한 let-7의 발현 조절에 관여하는 기전을 조사하고자 한다.

방법: 세포 실험을 위하여 생쥐 사구체 메산지움세포를 TGF-β1로 처리하여 RNA 및 단백질을 추출하였으며, 이를 바탕으로 메산지움세포에서 TGF-β1이 let-7 family miRNA, miRNA의 표적 유전자, 그리고 Lin28의 발현에 미치는 영향을 조사하였다. let-7b가 표적 유전자에 미치는 여향을 확인하기 위하여 let-7b mimic 및 hairpin inhibitor oligonucleotide로 메산지움세포를 유전자 이입하는 실험을 진행하였다. 콜라겐 1 α-2 (col1a2)와 콜라겐 4 α-1 (col4a1)이 let-7b의 실제 표적임을 증명하기 위하여 let-7b 결합 부위를 포함한 col1a2와 col4a1 3'UTR를 luciferase reporter 벡터를 이용하여 유전자복제하였으며, 3'UTR reporter vector와 let-7b mimic 또는 hairpin inhibitor oligonucleotides를 동시에 유전자 이입한 메산지움세포에서 luciferase 활성도를 측정하였다. TGF-β1에 의한 Lin28b의 발현 증가 과정에 있어서 *Lin28b*의 promoter에 위치한 Smad-binding element (SBE)의 역할을 규명하기 위하여, 생쥐 *Lin28b* 유전자의 proximal promoter 및 유전자결손을 유발시킨 promoter를 luciferase reporter 벡터를 이용하여 유전자복제하였다. 또한, TGF-β1에 의하여 활성화된 Smad2/3이 실제로 proximal promoter의 SBE에 결합하는지를 확인하기 위하여 chromatin immunoprecipitation assay를 시행하였다.

동물 실험을 위하여 streptozotocin으로 당뇨를 유발시킨

C57BL/6 생쥐 및 10주에서 12주령의 *db/db* 생쥐를 사용하였다. 각각의 동물에서 사구체를 채집하였으며, 이를 이용하여 RNA를 추출하여 실험에 사용하였다.

결과: TGF-β1로 처리한 메산지움세포에서 let-7 family miRNA (let-7b/c/d/g/i)가 유의하게 감소하였으며, col1a2와 col4a1은 의미있게 증가하였다. TGF-β1로 처리한 메산지움세포에서 let-7b를 과발현시켰을 경우, 증가하였던 col1a2와 col4a1이 유의하게 감소함을 확인할 수 있었다. col1a2 또는 col4a1 3'UTR luciferase construct와 let-7b inhibitor oligonucleotide를 동시에 유전자 이입한 메산지움세포에서는 luciferase의 활성도가 증가함을 관찰할 수 있었다. 반면, let-7 결합 부위에 돌연변이를 유발한 col1a2와 col4a1 3'UTR luciferase construct는 TGF-β1 처리에도 luciferase의 활성도가 증가하지 않았다. 또한, let-7b mimic 유전자 이입은 TGF-β1 처리에 의하여 증가한 luciferase의 활성도를 의미 있게 감소 시켰는데, 이를 통하여 col1a2와 col4a1이 let-7b의 실제 표적임을 증명하였다. let-7의 발현을 조절하는 것으로 알려진 Lin28b의 발현 양이 TGF-β1 처리에 의하여 증가함을 확인할 수 있었는데, luciferase assay를 이용하여 Lin28b promoter의 SBE를 포함한 construct는 TGF-β1 처리로 활성도가 증가한 반면, SBE를 포함하지 않은 construct는 반응을 나타내지 않음을 관찰하였다. 또한, 당뇨 생쥐의 사구체에서 let-7b의 발현이 감소하는 반면 cola1a2, col4a1, 그리고 Lin28b의 발현이 증가함을 확인할 수 있어서 생체 내에서의 Lin28과 let-7의 발현 양상 변화를 통한 콜라겐 축적 기전을 확인할 수 있었다.

결론: 당뇨병성 신병증의 진행에 있어서 TGF-β1에 의한 사구체 메산지움 섬유화 과정에 Lin28b 및 let-7이 중요 역할을 하는 것으로 생각되며, 이는 TGF-β1의 섬유화 기전에 새로운 세포전달체계로서 의미가 있을 것으로 생각된다.

핵심 되는 말: microRNA, 당뇨병성 신병증, TGF-β1, Lin28b, let-7, 콜라겐, 세포바깥바탕질