Transforming Growth Factor-β induced cross talk between p53 and a microRNA in the pathogenesis of Diabetic Nephropathy

Supriya D. Deshpande¹,², Sumanth Putta², Mei Wang², Jennifer Y. Lai³, Markus Bitzer³, Robert G. Nelson⁴, Linda L. Lanting² Mitsuo Kato² and Rama Natarajan¹,²

¹Irell & Manella Graduate School of Biological Sciences, ²Department of Diabetes and Division of Molecular Diabetes Research, Beckman Research Institute of the City of Hope, Duarte, CA. ³Internal medicine, University of Michigan, Ann Arbor, MI, ⁴National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona, USA.

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* Corresponding Authors
Email: RNatarajan@coh.org (Rama Natarajan)
mkato@coh.org (Mitsuo Kato)
Phone 626-256-4673; Fax 626-301-8136;

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ABSTRACT

Elevated p53 expression is associated with several kidney diseases including diabetic nephropathy (DN). However the mechanisms are unclear. We report that expression levels of transforming growth factor-β1 (TGF-β), p53 and microRNA-192 (miR-192), are increased in the renal cortex of diabetic mice, and this is associated with enhanced glomerular expansion and fibrosis relative to non-diabetic mice. Targeting miR-192 with locked-nucleic-acid modified inhibitors (LNA-192) in vivo decreases expression of p53 in the renal cortex of control and streptozotocin injected diabetic mice. Furthermore, mice with genetic deletion of miR-192 in vivo display attenuated renal cortical TGF-β and p53 expression when made diabetic, and reduced renal fibrosis, hypertrophy, proteinuria and albuminuria relative to diabetic wild-type mice. In vitro promoter regulation studies show that TGF-β induces reciprocal activation of miR-192 and p53, via the miR-192 target Zeb2, leading to augmentation of downstream events related to DN. Inverse correlation between miR-192 and Zeb2 was observed in glomeruli of human subjects with early DN consistent with the mechanism seen in mice. Our results demonstrate for the first time a TGF-β-induced feedback amplification circuit between p53 and miR-192 related to the pathogenesis of DN, and that miR-192-knockout mice are protected from key features of DN.
Diabetic Nephropathy (DN) is a microvascular complication that leads to kidney dysfunction and end stage renal disease. DN is characterized by renal glomerular hypertrophy, basement membrane thickening, and fibrosis due to accumulation of extracellular matrix (ECM) proteins like collagen (1-4). Transforming growth factor beta-1 (TGF-β) expression is increased in renal mesangial cells (MCs) in DN and acts as a pro-fibrotic agent inducing ECM accumulation, hypertrophy and cell survival. Clinical features of DN include proteinuria, albuminuria and progressive glomerular dysfunction (5-8).

Evidence suggests that the tumor-suppressor p53 plays a role in several kidney diseases and its inhibition confers a protective phenotype (9-14). It is also implicated in the pathogenesis of DN (15; 16), although the specific mechanisms are not clear. p53 has been shown to mediate podocyte apoptosis related to DN (14). p53 also acts in concert with TGF-β responsive Smads to promote the expression of pro-fibrotic genes such as plasminogen activator inhibitor-1 (12). In cancer cells, p53 binds to a p53 response-element (p53-RE) in the promoter of microRNA-192 (miR-192) to induce its expression, and miR-192 in turn mediates tumor-suppressive functions of p53 (17-19). miR-192 also regulates the MDM2-p53 autoregulatory axis in multiple myeloma cells (20). However, it is not known whether miR-192 regulates p53 or vice versa in the kidney and in MCs.

miRNAs are short non-coding RNAs that bind to the 3’UTR of target genes to repress their expression via post-transcriptional mechanisms (21; 22). Increasing evidence suggests that miRNAs can play a key role in the development of diabetic complications, especially DN (23). miR-192 is a key player downstream of TGF-β that increases collagen gene expression in MCs by targeting E-box repressors Zeb1/2 (5). TGF-β also triggers miRNA circuits involving miR-
miR-192 and miR-200b/c to increase TGF-β expression itself and accelerate DN (24). miR-192 expression is augmented by TGF-β or high-glucose (HG) treatment of MCs, podocytes and tubular cells (5; 25-27). Glomeruli from mouse models of diabetes and obstructive kidney disease, and renal biopsies from patients with certain kidney diseases have increased miR-192 expression that is related to fibrosis and renal complications (25; 28-31). TGF-β also induces a miRNA circuit involving miR-192 and miR-216a/217 that targets PTEN and activates Akt kinase (32). Inhibition of miR-192 in diabetic mice with locked-nucleic-acid-modified anti-miR-192 (LNA-anti-miR-192) oligonucleotides (oligos) reduces proteinuria and renal fibrosis associated with DN (33). Inhibition of TGF-β signaling by paclitaxel reduces miR-192 expression and associated renal fibrosis (34). Therefore several lines of evidence suggest that controlling miR-192 expression and its downstream pathways may be beneficial for treating DN (23).

We used in vivo and in vitro models to evaluate whether there is cross-talk regulation between miR-192 and p53 in the pathogenesis of DN. The proximal p53 promoter has an E-box (35) whose function has not been systematically examined. Since miR-192 targets Zeb2, we hypothesized that the p53 promoter E-box could be regulated by Zeb1/2, and consequently by miR-192. We report the operation of a feedback amplification circuit between p53 and miR-192 downstream of TGF-β signaling in MCs related to the pathogenesis of DN. Furthermore, we generated miR-192-knockout (KO) mice and found that they have decreased p53 levels and increased Zeb2 levels in renal glomeruli, and also display protection from key features of DN relative to wild-type (WT) mice.
RESEARCH DESIGN AND METHODS –

Animal studies. All animal studies were performed according to a protocol approved by the Institutional Animal Care and Use Committee at the Beckman Research Institute, City of Hope. C57BL/6 mice, miR-192-KO mice and litter-mate WT controls were made diabetic with multiple low dose streptozotocin (STZ) injections. Renal cortical sections from 2-week STZ-injected diabetic mice injected with LNA oligos were obtained as described (33). Kidneys from p53-KO mice were provided by Dr. Keigo Machida (University of Southern California). db/db and genetic control db/+ mice (10-12 weeks old) were from Jackson Laboratories (Bar Harbor, ME).

Primers used for generating miR-192-KO mice (Fig. 3A) were as follows –

a –5’-GGTATCGATCAAGGGTTCCGGGACCTGCTGTGGCTACC-3’,
b –5’-CGAAATCGATCTTGGCAGCCTGCTGACAACCCACCATTTCG-3’,
c –5’-TTGACTCGAGAGTCAGGGTGAGGGAGGTATCAAAGTCA-3’,
d –5’-TGTGAAGCTCTCTCAGCATACTGACTGCATGCACACCCACA-3’,
z –5’-GATTGGGAAGACAATAGCAGCAGGCA-3’,
f –5’-GCCAAAGCTTACCTAGGGATATCTAACCCTACCTCTTGCC-3’,
x –5’-CCCAGCGGCAACAGGACTGAGAGCGAGGAGGCTGGG-3’,
y –5’-TGTGAGATGCTCTGACAGGCAGGAGCAGTCAGGCAA-3’,
LoxP5’f –5’-AATTCCTGCAGCCCAATTCCCGATCATAT-3’.

Southern blot – Probe F –5’-ACATACAGGTGCTCTTCTGTGAAGAGCAGCATGT-3’,

LoxP3’r –5’-GCTTCACCGTGAGAGAGCGAGGAGCAGCATGT-3’.
Probe R –5’-TAGTGTAGGCTCTGGGCCCAAGAGTGTCAGACTA-3’.

**Cell culture and materials.** Primary mouse-MC (MMCs) were cultured from renal glomeruli (5). Recombinant human TGF-β1 was from R&D Systems, Inc. (Minneapolis, MN, USA). miR-192 mimics (192-M), negative control mimics (NC), Zeb2 siRNA (si-Zeb2) ON-TARGETplus SMART pool and ON-TARGETplus Non-targeting pool (Ctrl pool) were obtained from Dharmacon (Lafayette, CO). Cells were serum depleted for 48hr and then treated with TGF-β for indicated time periods.

**Quantitative RT-PCR (q-PCR).** Real time q-PCR analysis was performed as described (5). 18sII primers (Ambion) or Cyclophilin-A were used as internal controls and quantitative analysis performed using the ΔCt method. Sequence specific PCR primers used were as follows –

miR-192 5’-CTGACCTATGAAATTGACAGCC-3’;

p53 forward 5’-CGAAAGAAGACAGGCAGACTTTTCG-3’;

p53 reverse 5’-GAAGGTAAGGATAGGTCGGCGGTTC-3’;

Col1a2 forward 5’-CAGAACATCACCTACCACTGCA-3’;

Col1a2 reverse 5’-TTCAACATCGTTGGAACCCTG-3’;

Col4a1 forward 5’-GCCTTCCGGGCTCCTCAG-3’;

Col4a1 reverse 5’-TTATCACCAGTGGGTCCG-3’;

Cyclophilin A (CypA) forward 5’-ATGGTCAACCCCAACGTG-3’;

CypA reverse 5’-TTCTTGCTGTCTTTGGACTTTGTC-3’.
**Western Blot Analysis.** Protein extraction and subsequent analysis was performed as described (32). Antibodies used were: p53 and Cyclophilin A (Cell Signaling Technology, Beverly, MA), and α-Tubulin (Santa Cruz).

**Urine protein and albumin assays.** 24hr urine collection from mice, and protein measurements were performed as described (33). Albumin-creatinine ratio (ACR) in mouse urine samples were analyzed using Exocell Albuwell M and Creatinine companion kits (Exocell Inc, Philadelphia, PA).

**Human Study Subjects.** Kidney tissue was collected by biopsy from 46 Southwestern American Indians with type-2 diabetes who were enrolled in a randomized, placebo-controlled, clinical trial (ClinicalTrials.gov No. NCT00340678) (36; 37). This study was approved by the Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases. Each participant gave informed consent. Subjects were at least 18 years old at enrollment in the trial and underwent percutaneous kidney biopsy at the end of the randomized treatment period (~6 years) (37). Specimens were placed into RNA*later®* and stored at -20ºC until glomeruli were micro-dissected from biopsy cores (38).

**Small RNA preparation and quantification of miRNAs from human renal biopsies.** Total RNA was isolated from micro-dissected glomeruli using RNeasy kits (QIAGEN) to obtain the large RNA and flow-through small RNA fractions as per manufacturer’s protocols. To recover the small RNA fraction, the flow-through was further applied to RNeasy MiniElute Cleanup kit (QIAGEN) as per manufacturer’s protocol.

miRNA expression was obtained from TaqMan miRNA assays (Applied Biosystems). Small RNA from human glomeruli were reverse transcribed using TaqMan Megaplex RT primers and
further amplified by Megaplex PreAmp primers (Applied Biosystems) prior to q-PCR. miRNA expression, (Ct values) (threshold cycle), were normalized by U6 small nuclear RNA (snRNA), and RNU44 and RNU48 small nucleolar RNA (snoRNA). The normalized delta \( \Delta Ct \) was calculated by subtracting miRNAs’ Ct from geometric mean of snRNA’s and snoRNA’s Ct.

**mRNA microarray profiling and analysis (human glomeruli).** Total RNA isolated from micro-dissected glomeruli as described (39; 40) was reverse-transcribed and linearly amplified to be hybridized to Affymetrix HG-U133A microarrays for profiling mRNA expression using Affymetrix protocols. The microarray analysis was described before and Robust Multichip Average (RMA) was used to normalize the data (41)

**Correlation analysis (human glomeruli miRNA and mRNA)**

Pearson correlation was performed in the correlation analysis. miRNA \( \Delta Ct \) was correlated with mRNA hybridization log2 transformed intensity. Arbitrary fold change of miRNAs was calculated from \( 2^{\Delta Ct} \).

**Immunohistochemistry.** Immunostaining of paraffin-embedded renal cortical sections was performed as described (33). Antibodies used were: p53 (Novus Biological, Cell Signaling) and TGF-β (Santa Cruz). Hematoxylin and eosin, Periodic acid Schiff (PAS) and Masson’s trichrome staining were performed to analyze kidney structural function and ECM deposition (33).

**Plasmids and promoters.** miR-192 promoter constructs were provided by Dr. Carlo Croce (Ohio State University). The 0.7kb p53 promoter construct was provided by Dr. David Reisman (University of South Carolina). The WT mouse p53 promoter was made by amplifying the p53 genome region using primers, p53 forward 5’-
CTGAGATCTTACTTGTATGGCGACTATCCAG-3’ and p53 reverse 5’-GGCAAGCTTTCTGTAGTCGCTACCTACAGCC-3’ cloned into BglII and HindIII sites. The E-box mutant p53 promoter was made by site-directed mutagenesis using the following primers, p53 forward 5’-TTTCCCCCTCTACGAGCTCACCCTGG-3’ and p53 reverse 5’-CCAGGGTGAGCTCGTAGGAGGGAAA-3’. The p53 expression vector was from Dr. Emily Wang and the pCEP4 empty vector from Dr. David Ann (Beckman Research Institute of the City of Hope).

**Luciferase assays.** MMCs were transfected with reporter plasmids, NC, 192-M or si-Zeb2 using a Nucleofector and Basic Nucleofector Kit (Lonza) as described (5). Ratios of Firefly/Renilla were calculated and values were normalized to control conditions.

**Cell number and cellular protein levels.** MMCs were trypsinized and counted using a Coulter Counter with 100-µm aperture (Beckman). Cells were lysed and total protein content measured using the Bio-Rad Protein Assay (Bio-Rad) (32).

**Statistical Analyses.** PRISM Software (Graphpad, San Diego, CA) was used for data analysis. Results are expressed as mean ± s.e.m from multiple experiments. Student’s t-tests were used to compare two groups. Statistical significance was detected at the 0.05 level.
RESULTS

Increased p53 expression in the renal cortex of diabetic db/db mice relative to control db/+ mice.

We examined expression of p53 in Lepr<sup>db/db</sup> mice (model of type-2 diabetes) (db/db). Renal cortical lysates from 10-12 week old db/db mice showed a significant increase in p53 compared to lysates from control db/+ mice, as examined by western blot (Fig. 1A) and IHC (Fig. 1B). Glomerular area was also significantly increased in db/db mice compared to control db/+ mice (Fig. 1C). PAS staining showed a significant increase in mesangial matrix expansion in db/db compared to control db/+ mice (Fig. 1C). These results demonstrate that p53 expression is increased in cortical lysates and glomeruli of type-2 diabetic mice relative to control mice and this is associated with glomerular hypertrophy and fibrosis.

miR-192 regulates p53 expression in the renal cortex of control and STZ-diabetic mice

We next examined p53 expression in STZ-injected mice (model of type-1 diabetes). Since we hypothesized that p53 may be regulated by miR-192 by downregulation of Zeb2 to confer derepression at the E-box in the p53 promoter, we examined the effect of inhibiting miR-192 in vivo on p53 expression. We recently showed that inhibition of renal miR-192 with LNA-anti-miR-192 in non-diabetic and STZ-diabetic mice targets downstream signaling molecules and decreases pro-fibrotic gene expression, renal fibrosis, hypertrophy and proteinuria (32; 33). We now found that cortical lysates of non-diabetic mice injected with LNA-anti-miR-192 (C-LNA) showed significantly lower p53 expression compared to controls injected with normal saline (C-NS) (Fig. 2A). Cortical lysates of 2-week STZ-diabetic mice (STZ-NS) had significantly higher p53 expression compared to non-diabetic controls (C-NS) and this was significantly attenuated in
lysates from STZ-diabetic mice injected with LNA-anti-miR-192 (STZ-LNA) compared to control LNA (STZ-NC) (Fig. 2B). Immunostaining also revealed that p53 expression was significantly higher in glomeruli of STZ-NS and STZ-NC mice than C-NS mice, and this was significantly attenuated in glomeruli of STZ-LNA mice compared to STZ-NC (Fig. 2C), with similar trend in renal tubules of these mice. These results demonstrate that p53 expression is increased in cortical lysates and glomeruli of STZ-diabetic mice relative to respective control mice, and that inhibition of miR-192 decreases p53 expression in these mice.

**Generation of miR-192-knockout (miR-192-KO) mice**

In order to further determine the in vivo functional role of miR-192 expression under normal and diabetic conditions and evaluate cross-talk with p53, we generated mice deficient in miR-192 (miR-192-KO) and also examined whether miR-192 regulation of p53 is lost in these mice. The miR-192 gene was deleted in Sv 129 ES cells with a Neomycin resistant (Neo) gene cassette and Diphtheria toxin A (DTA). A 5kb upstream long arm of miR-192 was cloned between DTA and Neo. A short 2kb arm of miR-192 was cloned downstream of Neo (Fig. 3A). 129/SvJ ES cells were electroporated with the targeting vector. Out of 168 Neo-resistant colonies, 18 clones were homologous recombinants (Fig. 3B) and confirmed by Southern blot analysis. Digestion of genomic DNA at the StuI restriction site resulted in a 5.5kb KO allele and a 4.5kb WT allele band, while only the WT band was observed in negative controls (Fig. 3C). Three ES clones out of six recombinant clones yielded chimeric mice and germ-line transmission was obtained from one of these clones (Fig. 3D,E). Chimeric miR-192-KO mice were crossed with WT 129/SvJ Cre mice to generate neo-deleted heterozygotes (+/-), which were crossed again to generate homozygous (-/-) mice. Genotyping was performed using tail DNA and primers specific for the
WT allele (x), a common allele (y) and a deleted allele specific primer (z) (Fig. 3F,G). The miR-192-KO mice were healthy and viable, showed loss of miR-192 and also a reciprocal increase in the expression of its target Zeb2 in the kidney glomeruli (Fig. 3H) and MMCs (Fig. 3I) and no renal abnormalities relative to the genetic control WT mice.

**Relevance to Human DN**

Southwestern American Indians have a high incidence and prevalence of type-2 diabetes and those with diabetes often develop DN (42). To test the relevance of the miR-192-Zeb1/2 relationship in human DN, we profiled miR-192 levels in RNA isolated from glomeruli of kidney biopsies from 46 Southwestern American Indians with type-2 diabetes (37). The mean (SD) age of the patients was 44.7 (9.89) years, gender 80 % female, median [interquartile range (IQR)] GFR = 145.8 ml/min (112.74-183.1), and median (IQR) ACR = 42.0 mg/g (13.6-158.3). Interestingly, miR-192 expression was negatively correlated with the expression of its known targets, Zeb2 (R= -0.35, P-value=0.02) (Fig. 3J) and Zeb1 (R= -0.30, P-value=0.04), in these patients, consistent with our findings in mice and MMCs that miR-192 represses Zeb1/2 (5; 33) and in the miR-192-KO mice above.

**Reduced p53 expression in kidneys of miR-192-KO diabetic mice**

To determine the effect of miR-192-KO on p53 expression in normal and diabetic mice we analyzed cortical lysates from 2-week control and STZ-diabetic mice (WT and miR-192-KO). Cortical lysates from WT STZ-diabetic mice showed significantly higher p53 expression compared to WT controls, while cortical lysates from miR-192-KO STZ-diabetic mice did not compared to non-diabetic miR-192-KO controls (Fig. 4A). Since TGF-β signaling is activated under diabetic conditions and miR-192 increases TGF-β expression (24), we performed
immunostaining for TGF-β and p53 using cortical sections of these mice. Glomeruli of both WT STZ-diabetic and miR-192-KO STZ-diabetic mice showed increased TGF-β and p53 expression compared to non-diabetic controls (Fig. 4B). However, miR-192-KO non-diabetic and miR-192-KO STZ-diabetic mice showed significantly lower TGF-β and p53 expression compared to WT mice (Fig. 4B). In addition, significant increase in glomerular area was observed in WT-STZ diabetic mice but not in the miR-192-KO STZ-diabetic mice (Fig. 4C). Quantification of PAS positive staining in glomeruli revealed increased mesangial matrix levels in WT-STZ diabetic mice but not in the miR-192-KO STZ-diabetic mice (Fig. 4C). Increases in glomerular area and mesangial matrix expansion were also significantly attenuated in miR-192-KO STZ-diabetic mice compared to WT STZ-diabetic mice (Fig. 4C). These results indicate that kidneys of miR-192-KO STZ-diabetic mice have lower expression levels of TGF-β and p53 along with reduced glomerular area and mesangial matrix expansion relative to WT STZ-diabetic mice.

**Attenuation of key features of DN at 22-weeks in diabetic miR-192-KO mice**

Accumulation of ECM proteins, progressive proteinuria and albuminuria are more clearly evident at later stages of DN compared to early stages. Therefore, we examined diabetic mice at 22-weeks post diabetes onset. Similar to the 2-week STZ mice, cortical lysates from 22-week WT STZ-diabetic mice showed significantly higher p53 expression compared to non-diabetic controls (Fig. 5A). However, lysates from miR-192-KO STZ-diabetic mice did not show an increase in p53 expression compared to non-diabetic KO controls (Fig. 5A). Immunostaining showed that glomeruli from both WT STZ-diabetic and miR-192-KO STZ-diabetic mice had significantly higher TGF-β and p53 expression compared to respective controls from each group (Fig. 5B). However, the magnitude of increase in TGF-β and p53 expression was significantly
lower in *miR-192*KO STZ-diabetic than in WT STZ-diabetic mice. While WT diabetic mice showed significant increases in glomerular area, mesangial matrix expansion (PAS positive mesangial area) and interstitial fibrosis (trichrome positive area) compared to WT non-diabetic mice, at 22-weeks all these parameters were ameliorated in *miR-192*KO STZ-diabetic mice compared to WT STZ-diabetic mice (Fig. 5C,D). WT STZ-diabetic mice showed a significant increase in urine protein and albumin levels compared to WT controls at 19-weeks; however, both these parameters were attenuated in *miR-192*KO STZ-diabetic mice compared to WT STZ-diabetic mice (Fig. 5E,F). Overall these results indicate that TGF-β and p53 expression are attenuated at 22-weeks in *miR-192*KO STZ-diabetic mice and these mice show reduced severity in key patho-physiological parameters of DN.

**TGF-β induces transcriptional activation of p53 through miR-192 in MMCs**

TGF-β treatment increases *miR-192* expression in MMCs (5; 24; 32) and *miR-192* regulates p53 expression in certain cancer cells (20). However, it is not known whether *miR-192* regulates p53 expression in the kidney and in MMCs. To test this we used MMCs isolated from WT and *miR-192*KO mice. *miR-192*KO-MMCs showed significantly lower p53 expression compared to WT-MMCs (Fig. 6A). Reconstitution with 192-M increased p53 expression compared to NC in *miR-192*KO-MMCs (Fig. 6B). Furthermore, TGF-β induced p53 expression was lower in *miR-192*KO-MMCs compared to WT-MMCs (Fig. 6C).

The p53 promoter has an E-box and can be potentially repressed by E-box binding transcriptional repressors like Zeb2. Since *miR-192* targets and downregulates Zeb2 expression, we tested whether TGF-β can induce p53 promoter activity via *miR-192*. We performed luciferase assays in WT and *miR-192*KO-MMCs using a 700bp p53 promoter reporter construct.
(Fig. 6D). TGF-β significantly increased basal \( p53 \) promoter activity in WT-MMCs but not in \( miR-192 \)-KO-MMCs (Fig. 6E). Reconstitution with 192M (Fig. 6F) or inhibition of Zeb2 (si-Zeb2) (Fig. 6G) in \( miR-192 \)-KO-MMCs significantly increased \( p53 \) promoter activity compared to respective control oligos. The effect of TGF-β on the \( p53 \) promoter was also restored by transfecting 192-M or si-Zeb2 in \( miR-192 \)-KO-MMCs (Fig. 6F,G), supporting the notion that \( miR-192 \) or its target Zeb2 plays an important role in TGF-β induced \( p53 \) promoter activation.

Next, we performed luciferase assays in WT-MMCs transfected with a WT or an E-box mutant \( p53 \) promoter construct (Fig. 6H). TGF-β increased activity of the WT \( p53 \) promoter but not the E-box mutant \( p53 \) promoter (Fig. 6I), indicating that the E-box region plays an important role in regulating \( p53 \) promoter activity. These results demonstrate that TGF-β induces transcriptional activation of \( p53 \) which can be mediated by \( miR-192 \) targeting Zeb2 in MMCS.

**TGF-β induces transcriptional activation of \( miR-192 \) through \( p53 \) in MMCS**

The molecular pathway(s) by which TGF-β regulates \( miR-192 \) expression in MMCS remain unclear. \( p53 \) regulates \( miR-192 \) expression by binding to its promoter (17-19; 43). We examined whether \( p53 \) regulates \( miR-192 \) expression in MMCS by using WT and \( p53 \)-KO-MMCS. \( p53 \)-KO-MMCS showed significantly lower basal as well as TGF-β induced \( miR-192 \) expression compared to WT-MMCS (Fig. 7A). Reconstitution with a \( p53 \) expression vector (p53) significantly increased \( miR-192 \) expression compared to a control vector in both WT (Fig. 7B) and \( p53 \)-KO-MMCS (Fig. 7C). To further verify the involvement of \( p53 \), we tested the effect of TGF-β on three \( miR-192 \) promoter constructs, namely P1, 1871bp; P2, 245bp (both with an intact \( p53 \)-RE); and P3, 125bp with a \( p53 \)-RE deletion (Fig. 7D). TGF-β increased luciferase activity of all three promoter constructs in WT-MMCS (Fig. 7E). However, basal and TGF-β
induced activity of P3 was significantly lower compared to P1 and P2 (Fig. 7E). Reconstitution of p53-KO-MMCs with p53 significantly increased P1 and P2 promoter activity (Fig. 7F). Exogenous p53 also restored the effect of TGF-β on the miR-192 promoters (Fig. 7F). However there was no further enhancement with TGF-β relative to the untreated control, possibly due to a saturation effect of exogenous p53. These results demonstrate that TGF-β induced transcriptional activation of miR-192 is significantly regulated by p53 in MMCs.

**Attenuation in key features associated with DN in MMCs from miR-192-KO and p53-KO-mice**

We next tested the *in vitro* functional significance of our identified mechanism of reciprocal up-regulation of miR-192 and p53 by examining the expression of key ECM genes in WT, miR-192-KO-MMCs and p53-KO-MMCs. Compared to WT-MMCs, both miR-192-KO-MMCs and p53-KO-MMCs showed significantly lower or attenuated Col1a2 and Col4a1 gene induction following TGF-β treatment compared to WT-MMCs (Fig. 8A,B,C,D). TGF-β induced MC hypertrophy is another characteristic feature of DN. In WT-MMCs, TGF-β treatment significantly increased cellular hypertrophy (total cellular protein levels per total cell number) (Fig. 8E). However, this increase in hypertrophy was not observed in miR-192-KO-MMCs (Fig. 8E). Together, these results indicate that fibrosis and hypertrophy are attenuated in p53-KO-MMCs and miR-192-KO MMCs, suggesting that reciprocal upregulation of p53 and miR-192 following TGF-β treatment can amplify downstream functional events associated with DN (Fig. 8F).

**DISCUSSION**
Reports indicate that p53 induces miR-192 expression in cancer cells and miR-192 in turn regulates some of the tumor-suppressive roles of p53 (17-19; 43). Evidence also shows that miR-192 is upregulated in glomeruli of diabetic mice and in MCs treated with TGF-β or HG, and plays a role in the pathogenesis of kidney diseases including DN (3; 5; 24-27; 32-34). We demonstrate for the first time that miR-192 and p53 are co-regulated by a common pathway downstream of TGF-β signaling under diabetic conditions. We observed significantly higher p53 expression in the renal cortex of WT STZ-diabetic and db/db mice compared to respective controls. p53 expression was lower in kidneys of LNA-anti-miR-192 injected or miR-192-KO diabetic mice. On the other hand, miR-192 expression was lower in p53-KO MMCs. These data suggest that miR-192 and p53 up-regulate each other’s expression under diabetic conditions. Notably, proteinuria, albuminuria and increases in glomerular area, mesangial matrix expansion and fibrosis, were significantly attenuated in miR-192-KO STZ-diabetic mice compared to WT STZ-diabetic mice. To our knowledge, this represents the first study of DN in a mouse model deficient in a key miRNA known to have a functional role in renal cells. It is also known that p53 induces epithelial cell cycle arrest, podocyte apoptosis, kidney fibrosis, insulin resistance, and promotes kidney disease. Short-term inhibition of p53 has protective effects in many of these models (9-14). Our current studies suggest that reciprocal regulation of miR-192 and p53 might play a key role in the pathogenesis of DN. Translation elongation factor 1 alpha (EF-1α) is up-regulated by p53 in some cancer cell lines (44) and enhanced protein synthesis by EF-1α may be a key mechanism for the hypertrophy in kidney glomeruli from diabetic mice and MMC treated with TGF-β. Since mesangial matrix expansion correlates with renal dysfunction, proteinuria and albuminuria (2; 45), miR-192-p53-induced hypertrophy and fibrosis of glomerular MMCs may contribute to renal dysfunction during early stages of DN.
Our *in vitro* mechanistic studies using MMCs cultured from *miR-192-KO* and *p53-KO* mice elucidate a more direct signaling mechanism between TGF-β, *miR-192* and p53 under diabetic conditions. Basal and TGF-β induced p53 expression were lower in *miR-192-KO-MMCS*. Our results also showed that *p53* is up-regulated by *miR-192* via targeting of *Zeb2*. This mechanism (*Zeb1/2 targeted by *miR-192*) leads to the induction of collagen genes (*Col1a2* and *Col4a1*) and other miRNAs (*miR-216a/217* and *miR-200* family) (5; 24; 32). Importantly, our results from micro-dissected glomeruli of biopsies from human patients with early DN show a significant inverse correlation between glomerular *miR-192* expression and its direct target, *Zeb2*, confirming that *Zeb2* is a target of miR-192 even in human tissues. To our knowledge this is the first report demonstrating the inverse correlation between *miR-192* and *Zeb2* in human glomeruli samples. We found that basal and TGF-β induced *miR-192* expressions were lower in *p53-KO-MMCS* and that *miR-192* was up-regulated by p53 through the p53-RE in the *miR-192* promoter region. TGF-β mediated ECM gene induction was significantly attenuated in both *miR-192-KO-MMCS* and *p53-KO-MMCS*, highlighting the mechanistic and functional significance of this reciprocal regulation of *miR-192* and p53 on downstream effects in MMCs related to DN. Fig. 8F summarizes a novel mechanism by which TGF-β signaling induces reciprocal induction of *miR-192* and p53 expression to create an amplification loop (via *Zeb2* repression) that accelerates progression of DN.

We observed attenuation in proteinuria and albuminuria in *miR-192-KO* diabetic mice compared to WT diabetic mice. These results are consistent with previous results showing reduced proteinuria and albuminuria in diabetic mice injected with LNA-anti-miR-192 (33), suggesting that reduction of *miR-192* protects the kidney from injury caused by diabetes. Notably, the *miR-192-KO* mice did not depict any renal abnormalities or renal fibrosis, suggesting that loss of
miR-192 has no adverse effects in vivo. These results strongly support a key role for miR-192 in the progression of DN. Two studies showed that miR-192 expression was up-regulated in renal biopsies obtained from patients with other kidney diseases (28; 29). On the other hand, one study reported that miR-192 expression was decreased in RNA isolated from paraffin embedded kidney biopsies of a small number of patients with severe DN and declining glomerular filtration rates, and this inversely correlated with fibrosis (46). Differences in the specific nature of biopsy tissues tested or the time of biopsy collection could be responsible for these variations (47). Further studies that include larger diabetic cohorts and comparisons with biopsies from normal non-diabetic volunteers are needed to determine whether miR-192 levels are increased in early stages of human DN and then decline at later stages due to tubular hypertrophy or apoptosis.

Hepatocyte nuclear factor (HNF) and p53 are implicated for constitutive miR-192 promoter activity in proximal tubular cells, and TGF-β can decrease miR-192 expression in tubular cells lines (48; 49). Kim et al also reported that p53 inhibits epithelial-mesenchymal transition of cancer cell lines through miR-192, suggesting p53 as a critical regulator (18). Mutations in tumor-suppressors like p53 or other oncogenes might possibly alter the response to TGF-β in immortalized cell lines, or there are cell-type specific responses. We show here that p53 expression plays an important role in TGF-β induced transcriptional activation of miR-192 in primary MCs with functional relevance to DN. Therefore, diabetic conditions (including TGF-β and HG) can induce a reciprocal up-regulation of miR-192 and p53 in cells with WT miR-192 and p53 and lead to enhanced expression of ECM genes, fibrosis and hypertrophy. Our findings also suggest that inhibition of miR-192 expression may be an approach worth evaluating to slow down the progression of DN.
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SD and MK designed experiments, researched data and wrote the manuscript; SP, LL, MW researched data. JYL and MB researched the human glomerular biopsies data; RGN collected the human kidney tissue specimens, provided additional phenotypic data, and reviewed and edited the manuscript; RN designed experiments, wrote the manuscript and supervised the project. RN is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

FIG. 1. Increased p53 expression in the renal cortex of diabetic db/db mice relative to control db/+ mice.

A: Western blot and quantification of p53 expression from renal cortical lysates of 10-12 week old db/+ (n=5) and db/db mice (n=4). Representative immunostaining and quantification for B: p53 and C: PAS staining from cortical sections of db/+ and db/db mice, n=4 per group. *P<0.05, ***P<0.001; error bars, s.e.m; Scale bar, 20µm.

FIG. 2. miR-192 regulates p53 expression in the renal cortex of control and STZ-diabetic mice.

A: Representative western blot and quantification of p53 expression in cortical lysates of non-diabetic mice injected with normal saline (C-NS), negative control oligos (C-NC) or LNA-anti-miR-192 oligos (C-LNA) (2mg/kg) for 6hrs, n=3 per group. B: Representative western blot and quantification of p53 expression in cortical lysates of control mice injected with normal saline (C-NS, n=2) and STZ-diabetic for 2-weeks, injected with normal saline (STZ-NS, n=3), negative control oligos LNA-anti-miR-239b (STZ-NC, n=3) or LNA-anti-miR-192 oligos (STZ-LNA, n=4). C: Representative immunostaining and quantification of glomerular p53 from cortical sections of C-NS and STZ-NS mice, n=5 per group and STZ-NC, n=6 and STZ-LNA mice, n=7. *P<0.05, ***P<0.001; error bars, s.e.m; Scale bar, 20µm.

FIG. 3. Generation of miR-192-knockout (miR-192-KO) mice.

A: Schematic diagram showing design of the targeting vector used to generate miR-192-KO mice. B: Gel picture showing PCR screening for targeted miR-192-KO alleles from Neo-resistant
ES clones. Arrows, positive clones identified by PCR screening. C: Southern blot confirming recombinant clones. D: Schematic diagram showing genomic structures of WT and KO alleles and PCR approach. E: Gel picture confirming germ-line transmission of the miR-192 deleted allele. F: Schematic genomic structures of Neo deleted allele and PCR approach for identification of the deletion. G: Gel picture showing PCR analysis for genotyping performed using tail DNA. (+/+), WT; (+/-), heterozygous; or (-/-), homozygous mice. H-I: qRT PCR analysis of miR-192 and Zeb2 in (+/+ or (-/-) glomeruli (H) and MMCs (I), n=3. **P<0.01, ***P<0.001. (J): Scatter plot showing miR-192 and Zeb2 expression in glomeruli of type-2 diabetic human patients, n=46. Patient information is provided in the Results Section. R = -0.35, P value = 0.02.

FIG. 4. Reduced p53 expression in kidneys of miR-192-KO diabetic mice.

A: Western blot and quantification of p53 expression in cortical lysates of WT and miR-192-KO - control or STZ-diabetic (2-weeks) mice, control mice, n=3 per group, diabetic mice, n=5 per group. Representative immunostaining and quantification of (B) TGF-β, p53 and (C) Periodic Acid Schiff (PAS) staining from cortical sections of WT and miR-192-KO - control or STZ-diabetic mice, control mice n=3-5 per group, diabetic mice n=3-6 per group. Bar graph adjacent to (C) shows glomerular area and mesangial matrix expansion. *P<0.05, **P<0.01, ***P<0.001; error bars, s.e.m; Scale bar, 20µm.

FIG. 5. Attenuation of key features of DN at 22-weeks in diabetic miR-192-KO mice.

A: Western blot and quantification of p53 expression in cortical lysates of WT and miR-192-KO - control or STZ-diabetic (22-weeks) mice, control mice, n=3 per group, diabetic mice, n=4 per group. B: Representative immunostaining and quantification of TGF-β, p53, (C) PAS staining
and (D) Masson’s Trichrome staining from cortical sections of WT and miR-192-KO - control or STZ-diabetic mice, n=3-6 mice per group. E: Urine protein levels at 19-weeks post diabetes induction from WT and miR-192-KO - control or STZ-injected mice, (WT-C, n=6, WT STZ-diabetic, n=6, KO-C, n=3, KO-STZ, n=6) F: Bar graph showing relative ACR levels at 19-weeks post diabetes induction from urine samples of WT and miR-192-KO - control or STZ-diabetic mice, (WT-C, n=5, WT STZ-diabetic, n=5, KO-C, n=3, KO-STZ, n=5). *P<0.05, **P<0.01, ***P<0.001; error bars, s.e.m; Scale bar, 20µm.

FIG. 6. TGF-β induces transcriptional activation of p53 through miR-192 in MMCs.

qRT-PCR analysis of p53 in A: WT and miR-192-KO-MMCs under basal conditions, n=4, B: following transfection of miR-192-KO-MMCs with control oligos (NC, 10nM) or miR-192 mimic oligos (192-M, 10nM), n=3, and C: in WT and miR-192-KO-MMCs following TGF-β treatment (10ng/ml), n=3. D: Schematic genome structure of the p53 promoter region and the p53 promoter luciferase reporter construct. Luciferase assay results showing p53 promoter activity -/+ TGF-β (5ng/ml) in E: WT and miR-192-KO-MMCs, n=4, F: in miR-192-KO-MMCs following transfection with negative control (NC 10nM) or miR-192 mimic oligos (192-M 10nM), n=3 and G: in miR-192-KO-MMCs following transfection with control siRNA pool (Ctrl pool 10nM) or Zeb2 siRNA pool (si-Zeb2 10nM), n=3. H: Schematic genome structure of the WT and E-box mutant p53 promoter constructs. I: Luciferase assay results showing WT and E-box mutant p53 promoter activity in WT-MMCs, n=3. SD, serum depletion. *P<0.05, **P<0.01, ***P<0.001; error bars, s.e.m.
FIG. 7. TGF-β induces transcriptional activation of miR-192 through p53 in MMCs.

qRT-PCR analysis of miR-192 in WT and p53-KO-MMCs, A: under basal and TGF-β (10ng/ml) treated conditions, n=3. miR-192 expression following exogenous p53 expression in B: WT-MMCs, n=3 and C: p53-KO-MMCs, n=3, control - control vector, p53 - p53 expression vector, (100 - 200ng/ml). D: Schematic genome structure of the miR-192 promoter region and the three miR-192 promoter reporter constructs used - P1 (1871bp) and P2 (245bp) miR-192 promoter constructs with a p53 Response-Element, (p53-RE) and P3 (125bp), a p53-RE deletion mutant miR-192 promoter construct. Luciferase assay results showing miR-192 promoter activity -/+ TGF-β (5ng/ml) in, E: WT-MMCs, n=4 and F: following transfection of p53-KO MMCs with a control vector or p53 expression vector (100 - 200ng/ml), n=4. SD, serum depletion. *P<0.05, **P<0.01, ***P<0.001; error bars, s.e.m.

Figure 8 Attenuation in key features associated with DN in MMCs from miR-192-KO and p53-KO-mice.

qRT PCR analysis of Col1a2 and Col4a1 A, B: in WT or miR-192-KO-MMCs treated -/+ TGF-β (10ng/ml), n=3, and C,D : in WT or p53-KO-MMCs treated -/+ TGF-β (10ng/ml), n=3. E: Cellular hypertrophy levels -/+ TGF-β treatment (10ng/ml) of WT and miR-192-KO-MMCs, n=4. F: A schematic model of the mechanism of reciprocal regulation of miR-192 and p53 under diabetic conditions which can lead to hypertrophy and ECM accumulation associated with the pathogenesis of DN. *P<0.05, **P<0.01, ***P<0.001; error bars, s.e.m.
Figure 1 (Deshpande et al)

A

B

C

143x140mm (300 x 300 DPI)
Figure 2 (Deshpande et al)

A

B

C

172x198mm (300 x 300 DPI)
Figure 4 (Deshpande et al)

A

B

C

169x157mm (300 x 300 DPI)
Figure 6 (Deshpande et al)

A

B

C

D

E

F

G

H

I

165x135mm (300 x 300 DPI)
Figure 7 (Deshpande et al)

A

B

C

D

E

F

131x94mm (300 x 300 DPI)

Diabetes