miR-192 Mediates TGF-β/Smad3-Driven Renal Fibrosis

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ABSTRACT

TGF-β/Smad3 promotes renal fibrosis, but the mechanisms that regulate profibrotic genes remain unclear. We hypothesized that miR-192, a microRNA expressed in the kidney may mediate renal fibrosis in a Smad3-dependent manner. Microarray and real-time PCR demonstrated a tight association between upregulation of miR-192 in the fibrotic kidney and activation of TGF-β/Smad signaling. Deletion of Smad7 promoted miR-192 expression and enhanced Smad signaling and fibrosis in obstructive kidney disease. In contrast, overexpression of Smad7 to block TGF-β/Smad signaling inhibited miR-192 expression and renal fibrosis in the rat 5/6 nephrectomy model; in vitro, overexpression of Smad7 in tubular epithelial cells abolished TGF-β1-induced miR-192 expression. Furthermore, Smad3 but not Smad2 mediated TGF-β1-induced miR-192 expression by binding to the miR-192 promoter. Last, overexpression of a miR-192 mimic promoted and addition of a miR-192 inhibitor blocked TGF-β1-induced collagen matrix expression. Taken together, miR-192 may be a critical downstream mediator of TGF-β/Smad3 signaling in the development of renal fibrosis.


TGF-β is a profibrogenic cytokine that mediates renal fibrosis positively by activating its downstream mediators called Smad2 and Smad3 but negatively by its inhibitory factor Smad7; however, the mechanisms as to how the Smads regulate the fibrogenic genes during renal fibrosis remain unclear.

MicroRNAs (miRNAs) are small, noncoding RNAs with approximately 22 nucleotides. The mature miRNAs can complementarily bind to the mRNA 3' untranslated region to regulate the gene expression by translational repression or induction of mRNA degradation. Increasing evidence shows that TGF-β may act by regulating miRNAs to exhibit its biological effects such as epithelial-to-mesenchymal transition (EMT), suggesting that TGF-β regulates the expression of these miRNAs to promote EMT.

Several miRNAs, including miR-192, -194, -204, -215, and -216, are highly expressed in the kidney, as compared with other organs. In the context of renal fibrosis, expression levels of miR-192 increased significantly in glomeruli isolated from diabetic mice. In vitro, miR-192 is induced by TGF-β1 and mediates TGF-β–induced collagen expression in mesangial cells (MCs) by downregulating ZEB2 expression. In contrast, a recent study also found that TGF-β1 suppresses miR-192 expression in human tubular epithelial cells (TEC) and loss of miR-192 promotes fibrogenesis in diabetic nephropathy. The discrepancy in these two studies with opposite findings and understanding of miR-192 in diabetic nephropathy necessitates further investigation of the potential role of miR-192 and the mechanisms that regulate miR-192 expression during renal fibrosis under various disease conditions. Thus, this study tested the hypothesis that TGF-β1 may act by stimulating Smad3 to regulate miR-192 expression during renal fibrosis.
tested in rodent models of obstructive and remnant kidney diseases induced in mice that lacked Smad3 or Smad7, had conditional knockout (KO) for Smad2, or overexpressed renal Smad7. In addition, TGF-β-induced miR-192 expression via the Smad3-dependent mechanism was determined in TECs overexpressing Smad7 or knocking down for Smad2 or Smad3 and in Smad2 or Smad3 KO mouse embryonic fibroblasts (MEFs).

RESULTS

Activation of TGF-β/Smad Signaling Is Essential for Upregulation of miR-192 During Renal Fibrosis In Vivo and In Vitro

We first examined miRNA expression in obstructive kidney disease by microarray and real-time PCR. As shown in Figure 1, compared with normal kidney, 120 miRNAs including miR-192 were significantly upregulated from mouse kidneys after unilateral ureteral obstruction (UUO) for 5 days (Figure 1A and B). This was associated with activation of TGF-β/Smad signaling and renal fibrosis, as reported previously.10 Because miR-192 has been shown to be important in diabetic glomerulosclerosis,8 we examined whether miR-192 was also important for tubulointerstitial fibrosis in the UUO kidney. Interestingly, real-time PCR revealed that deletion of Smad7, an inhibitor of TGF-β signaling, enhanced further renal miR-192 expression in obstructive kidneys when compared with Smad7 wild-type (WT) mice (Figure 1C), which, again, was associated with a further increase in TGF-β/Smad signaling and progressive renal fibrosis, as described previously.10 Similarly, in a rat model of remnant kidney disease in which progressive renal fibrosis has been shown to be TGF-β/Smad dependent,11 miR-192 expression was significantly increased (Figure 1D). In contrast, inhibition of TGF-β/Smad signaling by overexpressing renal Smad7 prevented upregulation of renal miR-192 (Figure 1D) and renal fibrosis, as described previously,10 suggesting a close link between activation of TGF-β/Smad signaling and miR-192 expression during renal fibrosis in various disease conditions.

We further delineated the relationship between TGF-β/Smad signaling and miR-192 expression in a rat TEC line (NRK52E) in vitro. As shown in Figure 2, A and B, addition of TGF-β1 was able to induce miR-192 expression in a time- and dosage-dependent manner, which was blocked by a neutralizing anti-TGF-β antibody. Pretreated NRK52E cells with cycloheximide, a protein synthesis inhibitor, did not prevent miR-192
expression induced by TGF-β1 (Figure 2C), indicating that upregulation of miR-192 by TGF-β1 did not require de novo protein synthesis; however, overexpression of Smad7 in NRK52E cells virtually abolished TGF-β1–induced miR-192 expression (Figure 2D), demonstrating an essential role for TGF-β/Smad signaling in miR-192 expression.

**TGF-β1–Induced miR-192 Expression Is Mediated by Smad3, not Smad2, In Vivo and In Vitro**

To dissect the specific role for TGF-β/Smad signaling, Smad2 or Smad3, in miR-192 expression, we examined mouse kidneys of UUO induced in Smad3 WT/KO mice or conditional Smad2 KO mice. Consistent with a previous report,12 mice null for Smad3 were protected against renal tubulointerstitial fibrosis such as collagen I matrix deposition and mRNA expression (Figure 3, A and B). By microarray and real-time PCR, compared with normal mice, miR-192 expression was significantly upregulated in the UUO kidney of Smad3 WT mouse but downregulated in Smad3 KO mice (Figure 3, C through E). To determine the role of Smad2 in miR-192 expression, we conditionally deleted Smad2 from the Smad2 floxed/floxed mice using a ultrasound-microbubble–mediated Cre recombinase technique.13 As shown in Figure 4A, PCR detected that >90% of floxed Smad2 allele was deleted from the kidney by the ultrasound-microbubble–mediated Cre recombinase, which was confirmed at the mRNA and protein expression levels by real-time PCR, Western blot, and immunohistochemistry with anti-Smad2 antibodies (Figure 4, B through D). In contrast to the results found in Smad3 KO mice, real-time PCR and immunohistochemistry detected that conditional deletion of Smad2 produced a nonprotective effect on tubulointerstitial fibrosis such as collagen I expression after UUO (Figure 4, E and F), which was associated with high levels of miR-192 expression in the fibrotic kidney with or without conditional Smad2 KO (Figure 4G).

The essential role for Smad3, not Smad2, in regulating miR-192 expression was further determined in vitro in NRK52E TEC cell line in which Smad2 or Smad3 expression were specifically knocked down by the small interfering RNA technique, as described in our previous studies.13–15 As shown in Figure 5A, TGF-β1–induced miR-192 expression was significantly blocked in Smad3 but not in Smad2 knockdown TECs. This observation was further confirmed in Smad2 or Smad3 KO MEF cells.16 MEFs lacking Smad3, not Smad2, were protected against TGF-β1–induced miR-192 mRNA expression (Figure 5B).

To delineate whether Smad3 specifically binds miR-192 promoter in response to TGF-β1, we performed in silico analysis (rVista 2.0, http://rvista.dcode.org/)17 to search for potential Smad-binding sites (SBSs) in miR-192 promoter. Studies by Sun et al.17 revealed a noncoding region with high cross-species conservation located at 2158 bp upstream of the miR-192 on human chromosome 11. Within this region, there is a potential transcription factor–binding site for Ets-1.17 In this study, we found not only an Ets-1 site but also three conserved SBSs at −800 to −3500 bp in the 5′ end of miR-192 in mouse and rat genomes (Figure 6A); however, only SBS2 is conserved in human miR-192 promoter (Figure 6, A and B). The presence of these potential SBSs suggests that Smad3 may interact with these binding sites to induce miR-192 expression. To confirm this finding, we used a chromatin immunoprecipitation

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**Figure 2.** TGF-β induces miR-192 expression in a time- and dosage-dependent manner in NRK52E cells. (A) Real-time PCR shows that TGF-β1 (2 ng/ml), not PBS, induces miR-192 expression in a time-dependant manner. (B) Real-time PCR demonstrates that TGF-β1 induces miR-192 expression at 1 hour in a dosage-dependent manner, being significant at 2 ng/ml. The induction of miR-192 expression by TGF-β1 is inhibited by a neutralizing TGF-β1 antibody (α-TGF-β1 Ab) but not by an isotype control antibody (CTL Ab). (C) Real-time PCR demonstrates that cycloheximide (Chx) does not suppress TGF-β1 induction of miR-192 expression. (D) Real-time PCR analysis demonstrates that Dox-induced overexpression of Smad7 in rat TEC suppresses TGF-β1–induced miR-192 expression. Each bar represents the mean ± SEM for at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus either the BSA control or time 0 (or dosage 0); #P < 0.05, ##P < 0.01 versus the control antibody or normal NRK52E at same time point.
miR-192 Is an Important Mediator of TGF-β Signaling in Renal Fibrosis In Vitro

The functional importance of miR-192 in TGF-β-induced fibrosis was determined in vitro by transiently transfecting the miR-192 mimic (premiR-192) or miR-192 inhibitor (anti–miR-192), respectively, into normal rat TECs (NRK52E). As shown in Figure 7, TGF-β1–induced expression of collagen I at both mRNA and protein levels was significantly enhanced by overexpression of the premiR-192 but inhibited by the anti–miR-192 treatment.

DISCUSSION

In this study, we found that miR-192 was highly upregulated in the diseased kidney with progressive renal fibrosis in a mouse model of UUO and in a rat model of remnant kidney disease. TGF-β1 was able to upregulate the miR-192 in rat TECs (NRK52E) in a time- and dosage-dependent manner. miR-192 was a mediator of TGF-β–induced renal fibrosis as evidenced by the findings that overexpression of miR-192 mimics enhanced TGF-β1–induced tubular collagen I expression, whereas inhibition of miR-192 by overexpressing anti–miR-192 blocked tubular collagen I expression in response to TGF-β1. Taken together, our results supported the findings of Kato et al.8 that miR-192 is upregulated by TGF-β1 and contributes to diabetic glomerulosclerosis. More important, this study, for the first time, identified the signaling mechanisms by which TGF-β signals through Smad3, not Smad2, to regulate positively miR-192 expression during renal fibrosis in vivo and in vitro.

In addition to the suggestion by Kato et al.8 that miR-192 is induced indirectly by TGF-β via ETS protein, we demonstrate in this study that Smad signaling is required for TGF-β–induced miR-192 expression during renal fibrosis, which is negatively regulated by Smad7. We found that TGF-β1 was able to induce miR-192 expression in TECs as
early as 1 hour, which was tightly associated with a rapid activation of TGF-\(\beta\)/Smad2/3 as early as 5 minutes with the peak at 30 minutes to 1 hour. Furthermore, we found that blockade of the de novo protein synthesis by addition of cycloheximide did not alter TGF-\(\beta\)-induced miR-192 expression in TECs, suggesting that TGF-\(\beta\) induces tubular miR-192 expression directly and does not require the new protein synthesis. Thus, activation of TGF-\(\beta\)/Smad signaling may directly regulate miR-192 expression in TECs, because miR-192 expression was tightly associated with activation of TGF-\(\beta\)/Smad2/3 in the fibrotic kidneys of UUO and remnant kidney disease. Inactivation of Smad2/3 by overexpressing Smad7 inhibits miR-192 expression and renal fibrosis in vivo in a rat remnant model of kidney disease and in vitro in TGF-\(\beta\)-stimulated TECs. Furthermore, deletion of Smad7 greatly enhanced TGF-\(\beta\)/Smad signaling in vivo and in vitro in TGF-\(\beta\)-stimulated TECs. The finding of Smad3, not Smad2, to regulate miR-192 expression in response to TGF-\(\beta\) may be associated with the ability of Smad3 to bind DNA sequences directly through its MH1 domain, whereas Smad2 has no DNA-binding sequences and activates transcription indirectly by binding to transcriptional co-activators or repressors. Additional evidence supporting the Smad3-dependent miR-192 expression

Figure 4. Conditional deletion of Smad2 produces no inhibitory effect on miR-192 expression and renal fibrosis in a mouse model of UUO. (A through D) Characterization of conditional Smad2 KO mice by ultrasound (US)-microbubble–mediated Cre recombination. (A) PCR detects that >90% of the floxed Smad2 (451 bp) is deleted from the kidney of Smad2 f/f mice when compared with the deleted allele (592 bp). Western blot (B), immunohistochemistry (C), and real-time PCR (D) show that ultrasound-mediated Cre/lox recombination results in a substantial deletion of Smad2 from the kidneys. (E and F) Immunohistochemistry (E) and real-time PCR (F) demonstrate that deletion of Smad2 from the kidneys (Smad2f/kO) does not protect against renal tubulointerstitial fibrosis such as collagen I (Col I) expression in day 7 UUO kidneys. (G) Real-time PCR results of miR-192 expression in Smad2 floxed/floxed (Smad2f/f) kidneys, Smad2f/kO kidneys (Smad2f/f mice treated with US-mediated Cre recombinase), and Smad2f/f kidneys treated with control vector (Smad2f/f/CV). Real-time PCR analysis demonstrates that specific deletion of Smad2 in kidney did not block miR-192 expression. Each bar represents the mean ± SEM for at least five mice. ***P < 0.001 versus normal mice. MW, molecular weight. Magnification, ×250.
comes from the findings that Smad3-binding elements were found in the 5' end of miR-192. Furthermore, by using ChIP assays, we successfully demonstrated that deletion of Smad3 but not Smad2 in MEF blocks TGF-β-induced miR-192 expression. Each bar represents the mean ± SEM for at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus either the BSA control or time 0 (or dose 0); ###P < 0.01, ####P < 0.001 versus the TGF-β1-treated samples of normal TECs or WT MEF.

Predicted and validated target genes for miR-192 have been published in several studies.8,24–28 During diabetic nephropathy, miR-192 has been shown to target both ZEB1 and ZEB2 in human TECs and only ZEB2 in mouse MCs.8,9 Both ZEB1 and ZEB2 are known to repress the E-cadherin transcription but to activate the vimentin expression during EMT.29 In addition, miR-192 has been demonstrated to contribute to the upregulation of collagen type I in mouse MCs and isolated glomeruli from diabetic mouse.8 In human TECs, miR-192 seems to associate with maintenance of the E-cadherin expression8; therefore, miR-192 may be functionally important in kidney diseases. Normally, miR-192 is preferentially expressed in kidney as compared with miRNA expression profiles in different...
significantly at 1 hour in normal rat TECs (NRK52E). In contrast, we found that miR-192 expression was reduced by TGF-β1 in human HK-2 TECs (Hong Kong University of Science and Technology, Hong Kong) and downregulated in human diabetic nephropathy. Thus, results from this study demonstrate a potential importance of miR-192 as a downstream mediator of TGF-β/Smad3 in renal fibrosis.

**CONCISE METHODS**

**Cell Culture**

The NRK52E normal rat TEC line was obtained from American Type Culture Collection and grown in DMEM/LG containing 0.5% FBS with human TGF-β1 (R&D Systems, Minneapolis, MN) at concentrations of 0.00, 0.25, 0.50, 1.00, 2.00, and 4.00 ng/ml in the presence or absence of a neutralizing TGF-β antibody (10 μg/ml; R&D Systems) for periods of 0, 1, 3, 6, 12, and 24 hours for miR-192 detection.

To investigate the negative regulating role of Smad7 in TGF-β-induced miR-192 expression, we used a stable doxycycline (Dox)-regulated Smad7-expressing NRK52E cell line and induced Smad7 transgene expression by addition of Dox at an optimal concentration (2 μg/ml) for 24 hours. To further dissect the specific role of Smad2 and Smad3 in TGF-β-induced miR-192 expression, we generated and characterized stable cell lines with Smad2 and Smad3 gene knockdown in NRK52E as described previously. To examine whether de novo protein synthesis was required for miR-192 expression, we pretreated cells with 20 μM cycloheximide (Sigma, St. Louis, MO) for 30 minutes before TGF-β1 stimulation and cultured them for periods as described previously.

**Transient Transfection of Cells with microRNAs**

NRK52E cells were reverse-transfected with premiR-192 or anti-miR-192 (Ambion, Austin, TX) at a final concentration of 30 nM by using siPORT NeoFX transfection reagent (Ambion) in six-well plates. The control experiments were transfected with scramble RNA ODN (30 nM/m; Ambion). Then the cells were stimulated with human TGF-β1 (R&D Systems) at 2 ng/ml for 3, 6, 12, and 24 hours in serum-free medium following our protocols.

**RNA Extraction and Quantitative Reverse Transcription–PCR Analysis**

Total RNA was isolated from the cultured cells and kidney tissues by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufac-
turer’s instructions. Template cDNA was prepared using reverse transcriptase, and miR-192 expression was quantified by real-time PCR by TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) with small nuclear RNA U6 as an endogenous control for normalization according to the manufacturer’s instructions. Gene expression profiles were normalized to U6 snRNA and calculated using the ΔΔCt (2−ΔΔCt) method.

Real-time reverse transcription–PCR of the fibrotic markers and Smad2 mRNA was performed as described previously.13 Ratios for mRNA/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were calculated using the ΔΔCt method for each sample and expressed as means ± SEM.

miRNA Expression Analyses
Total RNA (350 ng) was reverse-transcribed using TaqMan MicroRNA RT kit and Multiplex RT rodent primer pool (Applied Biosystems) according to the manufacturer’s instructions. cDNAs were added to master mix (Applied Biosystems) and applied to miRNA TaqMan low-density array rodent panels 2.0 (Applied Biosystems) for the simultaneous quantification of 375 miRNAs according to the manufacturer’s instructions. miRNA TaqMan assays were performed using 7900 HT Fast Real-Time PCR System (Applied Biosystems). Relative quantification was performed using ΔΔCt method, and the data were normalized with U6 and RNU48 (Applied Biosystems) as endogenous controls. Data were analyzed with Real-Time StatMiner (Integromics, Madrid, Spain). Significance was considered at P < 0.05.

ChIP Analysis
ChIP was performed by transcription factor ChIP kit according to the manufacturer’s instructions. In brief, cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C, quenched with glycine, and then sonicated using a Bioruptor (Diagenode, Liège, Belgium) to generate 300- to 600-bp DNA fragments. Immunoprecipitation was performed with the antibody against Smad3 (Upstate), and IgG was used as a control. Precipitated DNAs were detected by PCR using specific primers: SBS1 5′-AGGCAAGTGTCTTCTCGGT-3′ and 5′-GTTGGCAGAAGGTGTGTACT-3′, SBS2 5′-CAGTGTOCCAACATGCTC-3′ and 5′-GTTAGCAGGGAGAAGCAATG-3′, and SBS3 5′-CTTGGAGGATGAGATGC-3′ and 5′-GGCCAGCAGCTTAGACTCAC-3′. The reaction mix consisted of 1.5 μl of template, 5.0 μl of 2× Bio-Rad iQ SYBR Green supermix, 0.3 μl of forward primer, 0.3 μl of reverse primer, and 2.9 μl of ddH2O. qPCR was performed as described already for quantitative real-time PCR.

Obstructive Kidney Disease Model
A UUO kidney disease model was induced in eight Smad3 WT/KO mice,34 Smad7 WT/KO mice,35 Smad2 floxed mice,36 and conditional Smad2 KO mice by left ureteral ligation as described previously.37 Conditional Smad2 KO mice were generated in Smad2 floxed/floxed mice by an ultrasonound-microbubble-mediated Cre recombination technique as described previously13 and were characterized at the DNA level by PCR, miRNA level by real-time PCR, and protein expression levels by Western blot and immunohistochemistry with the anti-Smad2 antibody (Chemicon, Temecula, CA). Mice were killed on day 7 after the ligation. In addition, eight normal WT and eight normal transgenic mice were used as age-matched controls. The experimental procedures were approved by Animal Experimental Ethnic Committee of the Chinese University of Hong Kong.

Animal Model of Remnant Kidney Disease Treated with Smad7 Gene Transfer
A rat remnant kidney disease model was induced in male Sprague-Dawley rats by 5/6 subtotal nephrectomy as described previously.11 Immediately after the nephrectomy, groups of six animals received a ultrasound-microbubble–mediated, Dox-inducible Smad7 or empty vectors as described previously.11 All procedures were performed in accordance with institutional guidelines for animal care.

Western Blot Analysis
Western blot analysis was performed as described previously,14 with primary antibodies against collagen I (Southern Tech, Birmingham, AL) and GAPDH (Chemicon). After being incubated with an IRDyeTM800-conjugated secondary antibody (Rockland Immunochemicals, Gilbertsville, PA), the membranes were analyzed using Li-Cor/Odyssey infrared image system (LI-COR Biosciences, Lincoln, NE). Signal intensities from each Western blot were quantified by ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to GAPDH levels.

Statistical Analysis
Each experiment was repeated at least three times throughout the study. Data were expressed as means ± SEM and analyzed using one-way ANOVA with Newman-Keuls comparison program from GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

ACKNOWLEDGMENTS
This work was supported by grants from the Research Council of the Hong Kong SAR, China (GRF 768207 and 767508 and CUHK5/ CRF/09 to H.Y.L., and 763908 and 764109 to A.C.C.).

We thank Dr. Erwin P. Bottinger for the Smad3 WT and KO MEF cell lines, Dr. Wansheng Wang for assistance in characterizing conditional Smad2 KO mice, and Dr. Chen-Chun Hou for preparing rat remnant kidney tissues.

DISCLOSURES
None.

REFERENCES


