MAD2B-mediated SnoN downregulation is implicated in fibroblast activation and tubulointerstitial fibrosis

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Tang H, Su H, Fan D, Ye C, Lei CT, Jiang HJ, Gao P, He FF, Zhang C. MAD2B-mediated SnoN downregulation is implicated in fibroblast activation and tubulointerstitial fibrosis. Am J Physiol Renal Physiol 311: F207–F216, 2016. First published April 27, 2016; doi:10.1152/ajprenal.00600.2015.—MAD2B, an anaphase-promoting complex/cyclosome (APC/C) inhibitor and a small subunit of DNA polymeraseζ, is indispensable for mitotic checkpoint control and DNA repair. Previously, we established that MAD2B is expressed in glomerular and tubulointerstitial compartments and participates in high glucose-induced podocyte injury. However, its role in other renal diseases remains elusive. In the present study, we aim to illustrate the potential role of MAD2B in the pathogenesis of renal fibrosis. By immunofluorescence and Western blotting, we found MAD2B expression is obviously increased in tubulointerstitial fibrosis (TIF) patients and unilateral ureteral obstruction (UUO) mice. It is widely accepted that resident fibroblasts are the major source of collagen-producing myofibroblasts during TIF. Therefore, we evaluated the level of MAD2B in fibroblasts (NRK-49F) exposed to transforming growth factor-β1 (TGFF-β1) by immunoblotting and revealed that MAD2B is upregulated in a time-dependent manner. Intriguingly, SnoN, a transcriptional repressor of the TGF-β1/Smad signaling pathway, is decreased in TGFF-β1-treated fibroblasts as well as the kidney cortex from TIF patients and UUO mice. Either in vitro or in vivo, local genetic depletion of MAD2B by lentiviral transfection could preserve SnoN abundance and suppress Smad3 phosphorylation, which finally dampens fibroblast activation, ECM accumulation, and alleviates the severity of TIF. However, the ubiquitin ligase APC/C is not involved in the MAD2B-mediated SnoN decline, although this process is ubiquitination dependent. In conclusion, our observation proposes that besides cell cycle management, MAD2B has a profibrotic role during fibroblast activation and TIF by suppressing SnoN expression. Targeting the MAD2B-SnoN pathway is a promising intervention for TIF.

MAD2B; SnoN; renal fibrosis; fibroblast activation; TGF-β1

CHRONIC KIDNEY DISEASE (CKD) has become a global issue, with increased mortality and substantial health costs. Getting a better understanding of its mechanisms and translating that into promising therapies are critical for the management of CKD. Renal tubulointerstitial fibrosis (TIF) is the final common pathway of CKD, no matter its underlying etiologies. TIF is a dynamic and complicated process involving various cell types and mediators. Myofibroblast activation and ensuing accumulation of extracellular matrix (ECM) with an expanded interstitial region are the core features of TIF (13). Until now, no consensus has been reached about the exact origin of myofibroblasts during TIF, but activated resident fibroblasts are generally regarded as an important source of interstitial myofibroblasts (15). However, the mechanism of resident fibroblast activation is poorly understood.

MAD2B (also referred to as MAD2L2 and hRev7), a protein of 211 amino acids, has emerged as an important regulator of the cell cycle (21) by acting as an APC/C inhibitor (1). In the pro-metaphase, MAD2B sequesters free CDH1 away from the anaphase-promoting complex/cyclosome (APC/C) and keeps APC/C inactive, while, once entering into anaphase, MAD2B is rapidly degraded by APC/C–CDC20, accompanied by CDH1 release, which ultimately results in APC/C activation and cell cycle arrest (12). Moreover, as a subunit of DNA polymeraseζ, MAD2B is also involved in replicating damaged DNA and regulating DNA repair activity (21). Therefore, MAD2B plays a crucial role in proper mitotic progression and faithful DNA replication. Accumulating evidence indicated that MAD2B is overexpressed in tumor genesis and tightly correlated with the progression and prognosis of these malignancies, such as colon cancer, epithelial ovarian cancer, and glioma (2, 19, 20, 31). Previously, we reported that hyperglycemia mediates neuronal apoptosis through inducing the production of MAD2B, which represents a novel mechanism of diabetic encephalopathy (17).

In the kidney, we proved MAD2B is presented in glomeruli and the tubulointerstitium. Also, MAD2B is upregulated in the renal cortex of diabetic nephropathy (DN) patients and mice. Additionally, MAD2B aggravates podocyte impairment via modulating the APC/C-cyclin pathway. Meanwhile, knocking down MAD2B ameliorates high glucose-induced podocyte injury, which indicates that MAD2B plays a pivotal role in podocyte injury during DN (25). However, the role of MAD2B in the development and progression of TIF, especially in fibroblast activation, has not been clarified.

Transforming growth factor-β1 (TGF-β1) is a well-recognized profibrotic molecule, and SnoN is one of the negative regulators of TGF-β1 signaling. It is known that SnoN can be ubiquitinated and degraded by the APC/C E3 ubiquitin ligase (4). As an APC/C repressor, whether MAD2B participates in fibrotic processes by governing SnoN expression is unclear. Here, we investigate the potential roles of MAD2B and its interplay with SnoN during TIF and fibroblast activation.

In the current study, we present new findings that MAD2B is involved in the activation of fibroblasts and the progression of TIF. Moreover, MAD2B downregulates SnoN, a repressor of TGF-β1 signaling, in an APC/C-independent and ubiquitination-dependent manner and is one of the underlying mechanisms accounting for MAD2B’s profibrotic property.
Fig. 1. Expression of MAD2B in the tubulointerstitial area is enhanced in tubulointerstitial fibrosis (TIF) patients and unilateral ureteral obstruction (UUO) mice. 

(A and B): Immunofluorescence staining (A) and summarized data (B) of MAD2B levels in tubulointerstitial region from TIF patients. Negative control was performed by replacing the primary antibodies with PBS (original magnification: ×200). Mild: mild TIF group; Moderate: moderate TIF group; Severe: severe TIF group; n = 4. *P < 0.05 VS. Mild.

(C and D): Immunohistochemical staining (C) and summarized data (D) of MAD2B levels in tubulointerstitial region from UUO and sham-operated (Sham) mice. Negative control was performed by replacing the primary antibodies with PBS (original magnification: ×400 and ×1,200); n = 6. *P < 0.05 vs. Sham.

(E and F): Representative Western blots (E) and summarized data (F) present relative MAD2B expression in the renal cortex of sham-operated and UUO mice at indicated day after operation; n = 8. *P < 0.05 vs. Sham.
MATERIALS AND METHODS

Ethics statement. All animal experimental procedures and human renal biopsy samplings carried out in this study were approved by the Ethics Committee of Huazhong University of Science and Technology. Animals were treated humanely, and all the procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) approved by the Animal Care and Use Committee of Tongji Medical College.

Human renal biopsy samples. Renal biopsies from patients with primary glomerular disease were performed as part of a routine clinical diagnostic investigation. The tissue was sampled by the Department of Nephrology, Union Hospital, Huazhong University of Science and Technology (Wuhan, China). The severity of TIF is classified as mild (<25%), moderate (25–50%), or severe (>50%) according to the percentage of fibrotic area (green/blue area) in the randomly selected interstitial fields under Masson staining. Secondary glomerular diseases and tubulointerstitial disorders were excluded.

Animal samples. Age-matched (12 wk) adult C57BL/6 mice were purchased from the animal center at Wuhan University. The unilateral ureteral obstruction (UUO) procedure was performed as previously described (6). Mice in the UUO group were euthanized at days 1, 3, and 7, respectively, after the operation. Sham-operated mice were euthanized at day 7 after surgery. The animals were perfused in the heart with heparinized saline solution before death.

Intrarenal lentivirus delivery. Recombinant lentivirus vectors harboring a short-hairpin (sh) RNA sequence targeting MAD2B (shRNA-MAD2B) were produced by JikaiGene (Shanghai, China). Two days before UUO model establishment, shRNA-MAD2B or their negative controls (scrambled) were delivered into the mice kidney by means of intraparenchymal injections. The procedure for intraparenchymal lentivirus delivery was performed as described before (9, 11). Briefly, after anesthesia the renal pedicle of the mice was temporarily occluded, and then a 31-G needle was inserted from the lower pole of the kidney parallel to the long axis and was carefully pushed toward the upper pole. As the needle was slowly moved backward, 100 μl of scrambled or MAD2B lentivirus shRNA (~1 × 10^5 IU/μl) was injected. Studies by us and other groups indicated that green fluorescent protein (GFP), the indicator of lentiviral transfection, was significantly increased in kidney parenchyma at 48 h after injection, and no toxicity was observed in mice treated with the lentiviral vector (9, 11).

![Fig. 2. Upregulated MAD2B is implicated in transforming growth factor (TGF)-β1-induced fibroblast activation. A and B: representative Western blots (A) and summarized data (B) present the relative protein levels of MAD2B, α-smooth muscle actin (SMA), and collagen III in NRK-49F cells exposed to 5 ng/ml TGF-β1 for the indicated times; n = 8. *P < 0.05 vs. Control (Ctrl). C and D: representative Western blots (C) and summarized data (D) displaying that MAD2B short-hairpin (sh) RNA transfection effectively inhibits TGF-β1-induced fibroblast activation as proved by decreased production of α-SMA and collagen III. scra: Scrambled shRNA; shMAD2B: MAD2B shRNA; n = 6. *P < 0.05 vs. Ctrl + scra. #P < 0.05 vs. TGF-β1 + scra.](http://ajprenal.physiology.org/Download/2017/10/20/32.246)
Cell culture, treatment, and transfection. Rat renal fibroblasts (NRK-49F) were obtained from the American Type Culture Collection (ATCC). NRK-49F cells were cultured in DMEM containing 4,500 mg/l glucose, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37°C in the presence of 5% CO2.

Once reaching 70–80% confluence, NRK-49F cells were serum starved for 12 h and then subjected to human TGF-β exposure (5 ng/ml, R&D Systems, Minneapolis, MN) for the indicated times before collection.

MAD2B shRNA and CDH1 small interfering (si) RNA were obtained from JikaiGene (Shanghai, China) and Ribobio (Guangzhou, China) to silence MAD2B and CDH1 gene expression in NRK-49F cells, respectively. The scrambled shRNA (JikaiGene) or scrambled siRNA (Ribobio) was used as a control. After synchronized for 12 h, NRK-49F cells were transfected with shRNA or siRNA according to the manufacturer’s instruction. Forty-eight hours after transfection, TGF-β1 (5 ng/ml) was mixed into the medium for the corresponding time to conduct the following experiments.

Histopathological and immunohistochemical analyses. Kidney tissues were fixed by 4% paraformaldehyde in PBS and embedded in paraffin. Tissue sections (3 μm thick) were stained with Masson’s trichrome for histopathological analysis. Immunoreactivity for MAD2B and SnoN proteins was determined by using a standard biotin-streptavidin-peroxidase method as described previously (25). The primary antibodies were rabbit anti-MAD2B antibody (1:100; Abcam, Cambridge, MA) and rabbit anti-SnoN (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was affinity-purified biotinylated goat anti-rabbit immunoglobulin G (Cortex Biochem, San Leandro, CA). The negative control was performed by replacing the primary antibodies with PBS. Nuclei were counterstained lightly with hematoxylin. The randomly chosen renal interstitial areas were analyzed by using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD), and integrated optical density was used to evaluate relative amount of positive staining.

Immunofluorescence staining. Indirect immunofluorescence staining was performed using an established procedure (7). Briefly, kidney cryosections were prepared and mounted onto poly L-lysine-coated slides and fixed in 4% paraformaldehyde in PBS for 30 min, then permeabilized with 0.3% Triton X-100 in PBS for 15 min, and blocked with 5% donkey serum for 30 min at 37°C. Thereafter, the slides were incubated with rabbit anti-MAD2B antibody (1:100; Abcam) overnight at 4°C. After PBS washing, the slides were incubated with Alexa 488-labeled secondary antibodies at room temperature for 1 h. The negative control was performed by replacing the primary antibodies with PBS. The images were captured by confocal microscopy at identical microscopic settings and analyzed by using Image-Pro Plus 6.0 software (Media Cybernetics). The integrated optical density was used to evaluate relative amount of positive staining.

Western blot analysis. Immunoblotting was performed as previously described (30). In short, proteins from mouse cortex or cultured cells were extracted with RIPA lysis buffer (Beyotime, Jiangsu, China). Protein concentration of cell lysates was measured with a protein assay dye reagent concentrate II (Bio-Rad, Hercules, CA). For Western blotting, 10 μg total protein was separated by electrophoresis in 10% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane and blocked in 5% milk in PBS-T for 1 h at room temperature. After blocking, the membranes were incubated with primary antibodies against MAD2B (1:1000; Abcam) and α-SMA (1:1000; Abcam) overnight at 4°C. After PBS washing, the membranes were incubated with HRP-conjugated secondary antibody (1:10,000; Abcam) for 1 h at room temperature. Finally, the bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The control for protein expression was β-actin (1:10,000; Santa Cruz Biotechnology). Blots were scanned and subjected to densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD). The integrated optical density was used to evaluate relative amount of positive staining.

Fig. 3. Local genetic depletion of MAD2B in kidney cortex alleviates myofibroblast activation in UUO mice. A and B: immunohistochemical staining (A) and summarized data (B) of α-SMA (marker of fibroblast transdifferentiation) level in tubulointerstitial region of Sham and UUO mice (original magnification: ×400); n = 4. C and D: representative Western blots (C) and summarized data (D) showing that abundance of α-SMA is reduced in the renal cortex of UUO mice with local MAD2B gene depletion compared with UUO mice injected with scra shRNA; n = 4. *P < 0.05 vs. Sham+scra. #P < 0.05 vs. UUO+scra.
BCA Protein Assay kit (Bio-Rad, Hercules, CA). After boiling for 5 min at 95°C in 5× loading buffer, an equal amount of protein (40 μg) was loaded onto an SDS-polyacrylamide gel for electrophoresis and then transferred to polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA). After blocking with 5% BSA for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-MAD2B antibody (1:1,000; Abcam), rabbit anti-p-Smad3 (1:1,000; Santa Cruz Biotechnology), rabbit anti-SnoN antibody (1:500; Protein Tech Group, Chicago, IL), rabbit anti-CDH1 antibody (1:1,000; Abcam), rabbit anti-collagen III antibody (1:1,000; Protein Tech Group), rabbit anti-smooth muscle actin (SMA) antibody (1:1,000; Protein Tech Group), and mouse anti-actin antibody (1:10,000; Santa Cruz Biotechnology). β-ACTIN was used as an internal control. After incubation with appropriate secondary antibodies, the immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat X-ray films. Densitometric analysis of the images obtained from X-ray films was performed by using Image J software (NIH, Bethesda, MD).

RNA extraction and quantitative RT-PCR. Total RNA was extracted from the cells by TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. The mRNA levels of target genes were analyzed by real-time quantitative RT-PCR as described previously (17). In short, aliquots of total RNA (1 μg) from each sample were reverse-transcribed into cDNA according to the manufacturer’s instructions (Bio-Rad). Then, equal amounts of the reverse transcriptional products were subjected to quantitative PCR by using SYBR Green dye (Applied Biosystems, Shanghai, China) and Applied Biosystems hardware and software (7500 RT-PCR System). The mRNA levels of target genes were normalized to the corresponding GAPDH mRNA levels. The 2−ΔΔCT method was used to calculate relative expression of the targeted genes. The primers used in this study were synthesized by Sangon Biotech (Shanghai, China), and the sequences were as follows: SnoN, forward 5′-GTGTCTGGAGTGGCTGTGGAA-3′ and reverse 5′-GCTGGGGTGTAAAAATGAATG-3′; MAD2B, forward 5′-TGCTTCGAGCCTTCATTCTT-3′ and reverse 5′-TGCTTCGAGCCTTCATTCTT-3′; and GAPDH, forward 5′-ATGGTGGTGAAGACGCCAGTA-3′ and reverse 5′-GGCACAGTCAAGGCTGAGAATG-3′.

Statistical analyses. All of the values are expressed as means ± SE. Significant differences between two groups were examined by using a paired t-test. P < 0.05 was considered as statistically significant.

Fig. 4. Local knockdown of MAD2B in kidney cortex minimizes ECM aggregation in UUO mice. A and B: immunohistochemical staining (A) and summarized data (B) of fibronectin (FN) levels in tubulointerstitial region of Sham and UUO mice (original magnification: ×200); n = 4. C and D: Masson’s trichrome staining (C) and summarized data (D) of %collagen in tubulointerstitial region of Sham and UUO mice (original magnification: ×400); n = 4. *P < 0.05 vs. Sham+scra. #P < 0.05 vs. UUO+scra.
RESULTS

Expression of MAD2B is enhanced in tubulointerstitial area of TIF patients and UUO mice. To explore the relevance of MAD2B and renal fibrosis, we investigated MAD2B protein expression in kidney biopsies from patients with primary glomerular diseases. The degree of TIF was evaluated by Masson staining and divided into mild, moderate, and severe groups. By immunofluorescence staining, we found that in humans, besides glomeruli, MAD2B is also expressed in the renal tubulointerstitial compartment. The intensity of tubulointerstitial MAD2B is positively related to the severity of TIF, and in severe TIF patients the levels of MAD2B is much higher than that in mild TIF groups \( (P < 0.05) \) (Fig. 1, A and B).

In UUO and sham-operated (control) mice, the kidney on the operated side was removed at 1, 3, or 7 days after surgery. By immunohistochemistry, significant nuclear and cytoplasmic staining in tubulointerstitial regions in mice was shown, the same staining pattern as in humans. Compared with the control group, MAD2B abundance in UUO mice was increased markedly in a time-dependent manner, which started to rise at 3

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**Fig. 5.** Expression of SnoN is reduced in the tubulointerstitial area of patients and UUO mice. A and B: immunohistochemical staining (A) and summarized data (B) of SnoN level in tubulointerstitial region from TIF patients. Negative control was performed by replacing the primary antibodies with PBS (original magnification: \( \times 400 \)); \( n = 4 \). TIF groups are defined as in Fig. 1. *\( P < 0.05 \) vs. Mild. C and D: immunohistochemical staining (C) and summarized data (D) of SnoN level in tubulointerstitial region of Sham and UUO mice. Negative control was performed by replacing the primary antibodies with PBS (original magnification: \( \times 400 \)); \( n = 7 \). *\( P < 0.05 \) vs. Sham. E and F: representative Western blots (E) and summarized data (F) presenting SnoN expression in the renal cortex from Sham-operated and UUO mice; \( n = 4 \). *\( P < 0.05 \) vs. Sham.
days after surgery and reached the peak on day 7. This finding was further verified by Western blotting (Fig. 1, C–F).

Uregulated MAD2B is implicated in TGF-β1-induced fibroblast activation. Currently, accumulating data support that resident fibroblasts are the dominant source of collagen-producing myofibroblasts (5, 8). Thus we next assessed the role of MAD2B in TGF-β1-stimulated fibroblasts. Fibroblasts (NRK-49F cells) were incubated with 5 ng/ml TGF-β1 for the indicated times and collected for subsequent analysis. As we can see in Fig. 2, A and B, in response to TGF-β1 MAD2B levels are elevated at 12 h of stimulus, accompanied by activation of myofibroblasts, which was proved by upregulated α-SMA (indicator of fibroblast transdifferentiation) and collagen III (indicator of ECM accumulation). Furthermore, depression of MAD2B by lentivirus shRNA can largely abrogate the TGF-β1-induced fibroblast activation, which infers that MAD2B is actively engaged in fibroblast activation triggered by TGF-β1 (Fig. 2, C and D).

Local genetic deletion of MAD2B in the kidney cortex alleviates TIF in UUO mice. To take advantage of the local lentivirus transfection technique, MAD2B in the renal cortex was regionally suppressed before the surgery. Our data showed after lentivirus delivery that the MAD2B level is reduced by ~40–50%. The most exciting results are that local genetic deletion of MAD2B can dramatically attenuate fibroblast activation in UUO mice, manifesting as decreased α-SMA (Fig. 3, A–D). Furthermore, local suppression of MAD2B also minimizes fibronectin (FN) abundance and ECM deposition, which was proved by immunostaining and Masson trichrome staining (Fig. 4, A–D).

SnoN, a negative regulator of TGF-β1, is reduced in TIF patients and UUO mice, which can be prevented by local MAD2B knockdown. Next, we explored the potential mechanisms accounting for MAD2B-associated TIF. As we know, SnoN, a well-defined TGF-β1/Smad transcriptional repressor, can be ubiquitinated by APC/C; thus we were intensely curious about whether MAD2B, an APC/C inhibitor, could interfere with TGF-β1-SnoN signaling. By immunohistochemistry, it was shown that SnoN is expressed in the renal tubulointerstitial compartment and its level is reduced in TIF patients and UUO mice (Fig. 5, A–D). Consistently, Western blotting indicates immunoreactivity of SnoN is markedly attenuated in UUO mice (Fig. 5, E and F).

Thereupon, we observed the link between MAD2B and SnoN via local lentivirus transfection to suppress MAD2B in the cortex before the unilateral ureteral ligation. After lentivirus injection, the MAD2B level is reduced by ~40–50%. Furthermore, genetic deletion of MAD2B can obviously prevent SnoN reduction as well as Smad3 phosphorylation (Fig. 6, A and B).

MAD2B genetic deletion attenuates the decline of SnoN protein in fibroblasts exposed to TGF-β1. In in vitro cultured fibroblasts, we first evaluated the mRNA transcription level of SnoN and found it is not affected by TGF-β1 stimulation (Fig. 7A). However, Fig. 7, B and C, shows that the SnoN protein level is reduced in fibroblasts after exposure to 5 ng/ml TGF-β1 from 1 h and sustained to 6 h, along with enhanced Smad3 phosphorylation. Knocking down MAD2B by lentivirus shRNA significantly attenuates SnoN protein reduction and Smad3 activation (Fig. 7, D and E). The results above are in line with our in vivo study.

The reduction of SnoN protein is not associated with APC/C/CDH1 ubiquitin ligase but related to other ubiquitin enzyme systems in fibroblasts exposed to TGF-β1. As we know, MAD2B is a negative regulator of the activity of APC/C/CDH1 E3 ubiquitin ligase; thus the downregulation of SnoN cannot be explained by the MAD2B-APC/C/CDH1 pathway during TGF-β1-initiated fibroblast activation. Also, CDH1 siRNA transfection could not reverse TGF-β1-induced SnoN degradation in NRK-49F cells exposed to 5 ng/ml TGF-β1 for 24 h (Fig. 8, A and B). Intriguingly, using an ubiquitin-activating enzyme (E1) inhibitor, PYR-41 (10 μM, pretreated for 30 min), to block posttranscriptional ubiquitination preserves SnoN abundance during TGF-β1 exposure (Fig. 8, C and D).

DISCUSSION

Since first identified in 1999, MAD2B is primarily considered to participate in APC/C-related mitosis control and DNA repair (3, 12, 16, 18, 28). Besides cell cycle regulation and error-free DNA maintenance, other functions of MAD2B are rarely known. In our earlier study, we demonstrated that MAD2B is extensively expressed in human and mouse kidneys as well as several renal cell lines. Additionally, we proved that MAD2B is implicated in podocyte injury under diabetic status (25). Currently, our data reveal that MAD2B expression is
upregulated in the renal tubulointerstitial area of patients with primary glomerular diseases, and the level of MAD2B protein is positively correlated to TIF severity. Consistently, in UUO mice, one of the generally accepted renal fibrosis animal models, the abundance of MAD2B in the kidney cortex is elevated from 3 days after surgery and continues to rise at day 7, and this enhancement is mainly taking place in tubulointerstitial areas. This finding suggests that MAD2B may have potential roles in TIF.

Although the origin of collagen-producing myofibroblasts is still in dispute, recent lineage tracing and fate determination studies propose that myofibroblasts derived from resident fibroblasts contribute importantly to ECM accumulation and TIF progression (14, 15). Thus we examined MAD2B expression in cultured fibroblasts and found that with TGF-β1 stimulation fibroblast MAD2B is dramatically upregulated in a time-dependent manner, which is accompanied by fibroblast activation, marked by α-SMA and collagen III accumulation. In addition, α-SMA and collagen III induction upon TGF-β1 exposure can be largely abrogated by MAD2B genetic deletion, which reveals a novel profibrotic role for MAD2B. Consistent with these in vitro results, we found that local knockdown of MAD2B in the kidney significantly alleviated TIF in UUO mice. Collectively, these data support the idea that MAD2B plays an essentially pathogenic role in fibroblast activation and subsequent TIF progression.

Fig. 7. Decline of SnoN protein in fibroblasts exposed to TGF-β1 can be reversed by MAD2B knockdown. A: summarized data presenting the relative mRNA level of SnoN in NRK-49F cells exposed to 5 ng/ml TGF-β1 for indicated times; n = 7, &P > 0.05 vs. Ctrl. B and C: representative Western blots (B) and summarized data (C) presenting the relative protein level of SnoN and p-Smad3 in NRK-49F cells exposed to 5 ng/ml TGF-β1 for indicated times; n = 6, *P < 0.05 vs. Ctrl. D and E: representative Western blots (D) and summarized data (E) displaying that MAD2B shRNA transfection alleviates TGF-β1-induced SnoN degradation and Smad3 phosphorylation in NRK-49F cells exposed to 5 ng/ml TGF-β1 for 24 h; n = 6, *P < 0.05 vs. Ctrl + scra. #P < 0.05 vs. TGF-β1 + scra.
MAD2B POSSESSES PROFIBROTIC CHARACTER BY SUPPRESSING SnoN

Fig. 8. The reduction of SnoN protein is not associated with APC/CDH1 ubiquitin ligase, whereas it is related to other ubiquitin enzyme systems in fibroblasts exposed to TGF-β1. A and B: representative Western blots (A) and summarized data (B) displaying that CDH1 small interfering (si) RNA transfection cannot reverse TGF-β1-induced SnoN degradation in NRK-49F cells exposed to 5 ng/ml TGF-β1 for 24 h. siCDH1, CDH1 siRNA; n = 5. *P < 0.05 vs. Ctrl+scra. #P < 0.05 vs. TGF-β1+scra. C and D: representative Western blots (C) and summarized data (D) displaying that SnoN reduction induced by TGF-β1 exposure in NRK-49F cells pretreated with 10 μM PYR-41 for 30 min is reversed; n = 5. *P < 0.05 vs. Ctrl. &P > 0.05 vs. Ctrl+PYR-41.

Next, we explored the underlying mechanisms involved in MAD2B-related fibrosis. SnoN, one of the negative regulators of TGF-β1 signaling, binds with the p-Smad2/3 complex and disrupts its interaction with Co-Smad4 and nuclear translocation, thereby preventing target gene transcription. Beyond that, SnoN recruits transcriptional corepressors, such as histone deacetylases (HDACs), to the promoter of TGF-β1-regulated genes or prevents the binding of coactivators to further dampen TGF-β1 signaling (10, 29). Accordingly, SnoN acts as a safeguard in health kidneys and prevents an excessive TGF-β1 response after TGF-β1 stimulation in cultured fibroblasts. In addition, the expression of SnoN protein in TIF patients and the UUO model is also suppressed, which is associated with the degree of TIF. In line with our findings, there are several other studies illustrating a rapid and significant decline of SnoN levels in obstructed kidneys, which is regarded as an amplification mechanism for TGF-β1 signaling (22, 27). Further studies by our and other laboratories show this downregulation is mainly due to enhanced ubiquitin-dependent degradation but not altered gene transcription or mRNA stability (26).

Several E3 ubiquitin ligases are reported to be responsible for SnoN degradation in response to TGF-β1, among which Smad-induced Smurf2 E3 ligase activation is most essential for SnoN ubiquitination and degradation (22, 23). However, Smads also stimulate the gene transcription of SnoN (24).

APC/C is another key E3 ligase accounting for SnoN ubiquitination. Since MAD2B governs the switch between APC/C<sup>CDH1</sup> and APC/C<sup>CDH1</sup> (<sup>12</sup>) and regulates the ubiquitination activity of APC/C, it is reasonable to speculate that MAD2B may play its profibrotic role via regulating the ubiquitin ligase activity of APC/C and consequently mediating SnoN degradation. Intriguingly, our results reveal that the decline of SnoN protein in TGF-β1-treated fibroblasts and UUO mice can be obviously alleviated by MAD2B knockdown, which is accompanied by diminished myofibroblast activation and ECM accumulation. The aforementioned data propose SnoN is negatively modulated by APC/C and then degraded in axon and hematological lineage cells (22, 27). Nevertheless, it seems not to be true during renal fibroblast activation since the increment of APC/C<sup>CDH1</sup> inhibitor MAD2B promotes SnoN degradation conversely. More importantly, knocking down the subunit of the APC/C ubiquitin ligase CDH1 could not reverse the decline of SnoN under TGF-β1 stimulation. This finding infers the interplay between MAD2B-APC/C-SnoN is cell type dependent and MAD2B...
regulation of SnoN is not via APC/C. However, blocking posttranscriptional ubiquitination can significantly preserve SnoN content, which indicates other ubiquitin enzyme systems may be involved in TGF-β1-induced SnoN degradation. Further investigation is needed to address this issue.

In summary, this is the first report concerning the pathogenic role of MAD2B in fibroblast activation and TIF progression. Here, we provide the evidence that tubulointerstitial MAD2B is upregulated, accompanied by reduced SnoN levels in TIF patients and obstructed mouse kidneys as well as the cultured fibroblasts exposed to TGF-β1. Genetic suppression of MAD2B can preserve the abundance of SnoN and eventually dampen fibroblast activation and ECM accumulation both in vivo and in vitro. Taken together, besides acting as a governor of cell cycle and DNA repair, MAD2B possesses a profibrotic character by depressing SnoN, a negative modulator of TGF-β1 signaling. In addition, MAD2B might be a promising therapeutic target for TIF.

REFERENCES

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.T., D.F., C.Y., and C.-T.L. performed experiments; H.T., H.S., H.-J.J., P.G., and F.-F.H. analyzed data; H.T., H.S., and C.Z. interpreted results of experiments; H.T., H.S., and C.Z. prepared manuscript; H.S. and C.Z. provided conception and design of research; H.S. and C.Z. edited and revised manuscript; C.Z. approved final version of manuscript.

GRANTS

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