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Bone Morphogenetic Protein-7 Inhibits Proximal Tubular Epithelial Cell Smad3 Signaling via Increased SnoN Expression

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Bone morphogenetic protein-7 (BMP-7) improves outcome in animal models of fibrotic renal disease by opposing transforming growth factor β 1 (TGF- β)-dependent fibrosis. However, the underlying mechanisms remain obscure. Here, we studied the effect of BMP-7 on response to TGF- β in the proximal tubular cell line HK-2 (PTC). BMP-7 specifically limited Smad3 but not Smad2 signaling. BMP-7 did not inhibit Smad3 phosphorylation or nuclear accumulation, nor did BMP-7 alter phosphorylated Smad3 dephosphorylation or degradation. However, BMP-7 treatment reduced Smad3 DNA binding to a consensus Smad binding element probe, and chromatin immunoprecipitation showed reduced Smad3 binding to the plasminogen activator inhibitor-1 promoter in PTCs treated with BMP-7 and TGF- β compared with TGF- β alone. Degradation of the transcriptional repressor SnoN has recently been shown to be necessary for Smad3 (but not Smad2) signaling. SnoN expression was transiently lost in PTCs after TGF- β stimulation, but BMP-7 prevented this. Furthermore, BMP-7 had no effect on Smad3 signaling after siRNA-mediated SnoN knock-down, whereas prevention of SnoN degradation with the proteasome inhibitor MG132 reproduced the inhibitory action of BMP-7 on Smad3 signaling. We conclude that BMP-7 prevents TGF- β -mediated loss of the transcriptional repressor SnoN and hence specifically limits Smad3 DNA binding, altering the balance of transcriptional responses to TGF- β in PTCs. These results provide an important mechanistic insight into a key regulator of TGF- β signaling. (*Am J Pathol* 2010, 176:1139–1147; DOI: 10.2353/ajpath.2010.090459)

Transforming growth factor β 1 (TGF- β) is a key profibrotic cytokine in the kidney and other solid organs.¹ However, TGF- β induces numerous cellular responses and, in ad-

dition to its profibrotic role, acts as a central orchestrator of development, wound healing, and cancer and a suppressor of inflammation and immune responses. The factors governing how cells read TGF- β signals are thus central to understanding pathology in many contexts.²

Bone morphogenetic protein-7 (BMP-7) has emerged as a key antifibrotic cytokine in the kidney. BMP-7 prevents fibrosis and antagonizes the effects of TGF- β in animal models including unilateral ureteric obstruction,³ nephrotoxic serum nephritis,⁴ collagen IV α 3-deficient mice (Alport's syndrome)⁵ MRL^{lpr/lpr} mice (lupus nephritis-like glomerulonephritis),⁵ and nephropathy associated with streptozotocin-induced diabetes.⁶ Accordingly, BMP-7 has attracted substantial interest as a potential therapy for chronic kidney disease.

Surprisingly, there are few data on mechanisms by which BMP7 opposes the profibrotic effects of TGF- β . In mesangial cells, BMP-7 inhibits nuclear accumulation of the key signaling molecule regulated by TGF- β , Smad3, after TGF- β stimulation.⁷ We have shown previously that BMP7 ameliorates proinflammatory cellular interactions in chronic kidney disease⁸ and inhibits monocyte-stimulated proximal tubular cell TGF- β generation.⁹ However, these effects do not fully explain the inhibition of the profibrotic effects of TGF- β seen in response to BMP-7 *in vivo*. In particular, reversal of TGF- β -dependent tubular cell epithelial to mesenchymal transition may be central to the action of BMP-7 in renal fibrosis,^{4,10} but the underlying mechanisms remain undefined. Cytokines from the TGF- β family elicit their biological functions by binding to a series of structurally related type I and type II receptors. The activated receptor complex recruits and serine-phosphorylates receptor-regulated Smad proteins (R-Smads, the intracellular signaling molecules of the TGF- β family). Phosphorylated R-Smads form complexes with the co-Smad (Smad4), accumulate in the nucleus, and

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alter gene transcription. Combinatorial interactions of type II and type I receptors occur, but the receptors and R-Smads phosphorylated by them can be functionally grouped into "TGF- β -like" and "BMP-like" (reviewed by Ross and Hill.¹¹). The R-Smads for TGF- β and related ligands are Smads 2 and 3, and for BMP-type ligands the R-Smads are Smads 1, 5, and 8.

The aim of the current study was to systematically examine the effect of BMP-7 on the signaling response to TGF- β . The data show that BMP7 specifically inhibits Smad3 but not Smad2 signaling in proximal tubular cells, leading to inhibition of profibrotic transcriptional responses elicited by TGF- β . The inhibition of the Smad3 transcriptional responses is due to reduction of Smad3 DNA binding, related to a failure to degrade the transcriptional repressor SnoN in BMP-7-treated cells. These results provide important mechanistic insight into the antifibrotic actions of BMP-7 in kidney disease.

Materials and Methods

Cell Culture

HK-2 cells (human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus 16 E6/E7 genes [PTCs]) were cultured as described previously.¹² With the exception of the cells used for transfection, cells were growth-arrested in serum-free medium for 48 hours before use in experiments. All experiments were performed in serum-free conditions.

Quantitative RT-PCR

Quantitative (q) RT-PCR was performed as described previously¹³ using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Fold changes in expression were calculated using the formula $2^{-(C_{t_{\text{sample 1}}} - C_{t_{\text{sample 2}}})}$, where C_t is the difference between the amplification threshold for the gene of interest and normalizer. P values were calculated by t -test using Microsoft Excel.

Transient Transfection and Reporter Gene Analysis

CAGA(4) Smad3 responsive reporter construct was a gift from Arisidis Moustakas.¹⁴ ARE and MF1 Smad2 responsive reporter constructs were a gift from Lalage Wakefield.¹⁵ pRL-CMV *Renilla* control plasmid was purchased from Promega (Madison, WI). Transient transfection and reporter gene analysis using HK-2 cells were performed as described previously.¹² For Smad3 experiments, 0.9 μg of the Smad3/4-specific reporter SBE-Luc was transfected with 0.1 μg of pRL-CMV *Renilla* to control for transfection efficiency. For Smad2-responsive experiments, 0.45 μg of the Smad2/4-specific promoter ARE-Luc was transfected together with 0.45 μg of its coplasmid MF1 and 0.1 μg of pRL-CMV *Renilla*. After lysis of the cells in Reporter Lysis Buffer (Promega), firefly and

Renilla luciferase content was quantified using the Dual-Glo Assay (Promega).

Immunoblotting

Cell extracts were prepared in SDS sample buffer and boiled for 5 minutes at 95°C before 10% SDS-polyacrylamide gel electrophoresis performed under reducing conditions, transfer to a nitrocellulose membrane (Amersham, Little Chalfont, Buckinghamshire, UK), incubation with primary antibody in PBS-0.1% (v/v) Tween-20, and then incubation with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Poole, UK). Protein was visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Immunofluorescence Microscopy

Subconfluent monolayers of cells grown in eight-well glass chamber slides were fixed in acetone/methanol (1:1, v/v) (Fisher Scientific, Pittsburgh, PA) for 10 minutes and then were washed in calcium/magnesium-free PBS, pH 7.4 (Invitrogen, Carlsbad, CA), blocked with 1% (w/v) bovine serum albumin (BSA)/HBSS, and washed in 0.1% (w/v) BSA/HBSS. The slides were incubated with primary antibody and then secondary antibody diluted in 0.1% BSA/HBSS for 2 hours at room temperature. The source and dilution of the antibodies were as follows: polyclonal mouse anti-Smad3 1:50 (sc-8332, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG 1:40 (Dako North America, Inc., Carpinteria, CA). The cells were washed extensively with 0.1% BSA in HBSS, mounted in Vectashield fluorescent mountant (Vecta Laboratories, Peterborough, UK), and examined on a Leica Dialux 20 fluorescent microscope [Leica Microsystems (UK) Ltd., Milton Keynes, UK].

Electrophoretic Mobility Shift Assay

Nuclear protein extraction and electrophoretic mobility shift assays for nuclear factor- κB were performed as described previously.¹⁶ In brief, cells were harvested in ice-cold PBS (pH 7.4) and pelleted by centrifugation. Cells were resuspended in ice-cold buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM/L MgCl_2 , 10 mM/L KCl, 0.5 mM/L dithiothreitol, and 0.2 mM/L phenylmethylsulfonyl fluoride) and incubated on ice for 10 minutes. The cell pellet was collected by centrifugation, resuspended in buffer B (20 mM/L HEPES-KOH [pH 7.9], 25% glycerol, 420 mM/L NaCl, 1.5 mM/L MgCl_2 , 0.2 mM/L EDTA, 0.3 mM/L dithiothreitol, and 0.2 mM/L phenylmethylsulfonyl fluoride), and incubated on ice for 20 minutes followed by a brief high-speed centrifugation ($12,000 \times g$ for 10 seconds at 4°C), and the resulting supernatants (nuclear extract) were collected. Smad Binding Element Oligonucleotide Probe (sc-2603) was bought from Santa Cruz Biotechnology, Inc. and annealed for use in the electrophoretic mobility shift assay. These double-stranded fragments were labeled with

[α^{32} P]dTTP (Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) using the Klenow fragment of DNA polymerase I. The composition of protein/DNA complexes was determined by supershift assays using rabbit polyclonal antibodies specific for Smad1 (9512, New England Biolabs, Ipswich, MA), Smad2 (3122, New England Biolabs), Smad3 (51-1500, Invitrogen), Smad4 (sc-7955, Santa Cruz Biotechnology, Inc.), and Smad 5 (9517, New England Biolabs).

Chromatin Immunoprecipitation

Cells were cross-linked with 1% formaldehyde at room temperature for 20 minutes and then were resuspended in lysis buffer (50 mmol/L HEPES-KOH, pH 7.5, 140 mmol/L NaCl, 1 mmol/L EDTA, pH 8, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS) before sonication to yield DNA fragments ranging in size from 200 to 500 bp (Bioruptor, Diagenode SA, Liège, Belgium). Approximately 25 μ g of protein of the cross-linked, sheared chromatin solution was used for immunoprecipitation. A small portion of each immunoprecipitation mixture was saved as input DNA (5%). Supernatants were diluted 10-fold in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 2 mmol/L EDTA, pH 8, 1% Nonidet P-40, and 0.5% sodium deoxycholate) and immunoprecipitated for 4 hours at 4°C with 5 μ g of rabbit anti-Smad3 with constant mixing. Control immunoprecipitations were performed at the same time with preimmune IgG. After addition of 60 μ l of protein A-Sepharose containing 1.5 μ g of sheared salmon sperm DNA, samples were incubated with constant mixing overnight at 4°C, centrifuged for 1 minute at 13,000 rpm, and washed three times with wash buffer I (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, pH 8, 150 mmol/L NaCl, and 20 mmol/L Tris-HCl, pH 8) and once with wash buffer II (0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, pH 8, 500 mmol/L NaCl, and 20 mmol/L Tris-HCl, pH 8). DNA was eluted in 1% SDS, 100 mmol/L NaHCO₃ for 1 hour. After proteinase K digestion, DNA was extracted with phenol: chloroform and ethanol-precipitated in the presence of 10 μ l of glycogen (5 mg/ml). DNA was amplified by real-time PCR using SYBR Green Master Mix. Primers were designed using Primer 3 software for the second and third Smad binding elements in the plasminogen activator inhibitor-1 (PAI-1) promoter, previously shown to cooperatively regulate the transcriptional response of PAI-1 to TGF- β .¹⁷ Primer sequences were as follows: second Smad binding element, -580CAGA: forward 5'-GGGAGTCAGCCGTGTATCAT-3' and reverse 5'-ACCTCCATCAAAACGTGGAA-3'; third Smad binding element -280CAGA, forward 5'-AGTCAACCTGGCAGGACATC-3' and reverse 5'-ACACCTCCCTCTCTGGGACT-3'.

Small Interfering RNA

Transfection of HK-2 cells with small interfering RNA (siRNA) was performed as described previously.¹³ siRNAs were purchased from Ambion (Austin, TX).

Statistical Analysis

Unless otherwise specified, data are presented for a representative experiment of at least three separate experiments giving consistent results. Statistical analysis between groups was performed using an unpaired Student's *t*-test and for multiple groups using analysis of variance, with a value of *P* < 0.05 considered to represent a significant difference. The data are presented as means of *n* biological replicates as indicated in the figure legends. Error bars indicate the SEM.

Results

Inhibition of TGF- β -Dependent Responses by BMP-7

TGF- β -induced fibrosis is Smad3-dependent, with key downstream targets including connective tissue growth factor, PAI-1, and TGF- β itself (reviewed by Roberts et al¹⁸). Initial experiments were performed to investigate inhibition of TGF- β -dependent transcriptional responses by BMP-7. HK-2 cells were incubated with 1 ng/ml TGF- β or control medium plus 50 ng/ml BMP-7 for 24 hours. Subsequent quantitative RT-PCR demonstrated increased expression of connective tissue growth factor, PAI-1, and TGF- β in response to TGF- β (Figure 1, A–C). BMP-7 alone did not significantly alter expression of connective tissue growth factor, PAI-1, or TGF- β , but BMP-7 significantly attenuated responses to TGF- β (Figure 1, A–C). PAI-1 is a powerful fibrogenic molecule that is not expressed in the normal kidney (reviewed by Eddy and Fogo¹⁹) and has a well characterized sustained transcriptional response to TGF- β .²⁰ Additional experiments demonstrated inhibition of PAI-1 expression when BMP-7 was added to TGF- β -treated cells at time points up to 8 days (Figure 1D).

Figure 1. Regulation of profibrotic TGF- β transcriptional targets by BMP-7. **A–C:** qRT-PCR analysis shows expression of profibrotic TGF- β -inducible genes in PTCs after 24 hours with or without 1 ng/ml TGF- β incubation with or without 50 ng/ml BMP-7. **A:** TGF- β . **B:** Connective tissue growth factor (CTGF). **C:** PAI-1. **D:** PAI-1 expression after 8 days of culture with or without 1 ng/ml TGF- β with or without 50 ng/ml BMP-7. *n* = 3, mean of duplicate determinations. Error bars indicate +SEM. Representative data from one of three experiments giving similar results. **P* < 0.01 versus control. ***P* < 0.05 versus TGF- β .

Figure 2. Regulation of Smad signaling response to TGF- β by BMP-7. HK-2 cells were transfected with Smad3-responsive (**A** and **B**) or Smad2-responsive (**C**) plasmids and then were incubated with BMP-7 for various times or doses, before incubation with 1 ng/ml TGF- β (black bar, control medium followed by TGF- β ; gray bars, BMP-7 followed by TGF- β) or control medium (white bars) for 6 hours. **A:** Effect on Smad3 signaling of time course of 50 ng/ml BMP-7 incubation. **B:** Effect on Smad3 signaling of 24 hours preincubation with a BMP-7 dose range of 0 to 2000 ng/ml. **C:** Effect on Smad2 signaling of a 24-hour preincubation with BMP-7 at a dose range of 0 to 2000 ng/ml. * $P < 0.05$ versus TGF- β . RLU, relative light units.

To further investigate alterations in TGF- β response caused by BMP7, transient transfection of reporter genes sensitive to Smad3 (CAGA) and Smad2 (ARE/MF1) was undertaken. BMP-7 inhibited subsequent Smad3 signaling response to TGF- β after 12 or more hours (Figure 2A) and at concentrations of 50 ng/ml or greater (Figure 2B). There was little difference in response to BMP-7 at doses above 50 ng/ml. In contrast, BMP-7 did not significantly alter Smad2 signaling at any of the doses or times examined (Figure 2C and data not shown).

BMP-7 Does Not Alter Smad3 Phosphorylation, Nuclear Accumulation, or Dephosphorylation

Inhibition of Smad3 but not Smad2 signaling suggested that BMP-7 acts beyond the point of TGF- β ligand-receptor interaction. Accordingly, the time course of Smad1, 2, and 3 phosphorylation in PTCs exposed to TGF- β and BMP-7 was examined, alone and in combination. Preliminary experiments showed that Smad2 and 3 phosphorylation was detectable for 24 hours after TGF- β treatment, with a maximal response by 1 hour (not shown). Although Smad1 phosphorylation was readily detected after BMP-7 treatment, confirming the signaling response to BMP-7, BMP-7 had no effect on Smad2 or Smad3 phosphorylation when cells were coincubated with TGF- β and

Figure 3. Time course of Smad phosphorylation and dephosphorylation in response to TGF- β and BMP-7. **A:** HK-2 cells were incubated with 50 ng/ml BMP-7 for 0 to 24 hours before incubation with 1 ng/ml TGF- β for 1 hour. Whole-cell lysates were immunoblotted with antibodies against Phospho-Smads (PSmad) 1, 2, 3, and total Smads 1 and 3. Stripping and reprobing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to confirm approximately equal loading. **B–D:** Time course of Smad1/3 dephosphorylation/degradation. HK-2 cells were incubated with control medium (**B** and **C**) or 50 ng/ml BMP-7 (**D**) for 24 hours before incubation with 1 ng/ml TGF- β for 30 minutes. Cells were washed extensively and incubated in cytokine-free control medium (**B**) or medium containing the Alk5 kinase inhibitor SB431542 (**C** and **D**) for time points up to 5 hours. Residual Phospho-Smad3 activity was detected by immunoblot before stripping and reprobing for total Smad3.

BMP-7 (Figure 3A). No change in Smad3 phosphorylation was seen in cells preincubated with BMP-7 and then exposed to TGF- β (data not shown). Transient Smad1 phosphorylation was also evident after TGF- β treatment (Figure 3A), in keeping with recent reports of transient Smad1/5 phosphorylation in epithelial cells in response to TGF- β .²¹

Nuclear import and export of Smad3, together with its dephosphorylation, ubiquitinylation, and degradation, are highly regulated (reviewed by Shi and Massague²²). Increased Smad3 degradation has been implicated in altered renal responsiveness to TGF- β in the kidney in a cell phenotype-specific fashion,²³ and in mesangial cells, nuclear accumulation of Smad3 is inhibited by BMP-7.⁷ To study possible Smad3 dephosphorylation or degradation in response to BMP-7, we examined the half-life of phosphorylated (Phospho)-Smad3. HK-2 cells were incubated with BMP-7 or control medium, and then Smad3 phosphorylation was stimulated with TGF- β . After 30 minutes, further Smad3 phosphorylation was inhibited using the Alk5 kinase inhibitor SB431542, and the rate of decline of Phospho-Smad3 was followed with immunoblotting. A similar rate of decline in Phospho-Smad3 was observed in control cells and cells treated with BMP-7

Figure 4. Nuclear accumulation of Smad3. HK-2 cells were incubated with 50 ng/ml BMP-7 or control medium for 24 hours before incubation with 1 ng/ml TGF- β or control medium for 1 hour. **A:** Immunoblotting of nuclear extracts for Phospho-Smad (PSmad) 1/3 and subsequent reprobing for c-Jun to confirm approximately equal loading. **B:** Immunofluorescent localization of Smad3. HK-2 cells were incubated with 50 ng/ml BMP-7 or control medium for 24 hours in eight-well chamber slides before incubation with 1 ng/ml TGF- β or control medium for 1 hour and detection of Smad3 by immunofluorescence microscopy.

(Figure 3, B–D), suggesting that Phospho-Smad3 dephosphorylation and degradation were not altered in response to BMP-7. Subsequently, nuclear accumulation of Phospho-Smad3 was assessed by immunoblotting and by fluorescence microscopy. Immunoblots of nuclear protein fractions suggested that BMP-7 did not alter nuclear accumulation of Smad3 (Figure 4A). Similarly, immunocytochemical analysis did not detect differences in TGF- β -dependent nuclear accumulation of Smad3 after BMP-7 treatment (Figure 4B).

BMP-7 Inhibits Smad3 DNA Binding

The above data suggest that BMP-7 predominantly limits Smad3 transcriptional responses to TGF- β , without altering TGF- β -dependent Smad phosphorylation and nuclear accumulation or the subsequent Phospho-Smad3 decay rate. Therefore, potential alterations in Smad3 DNA binding were investigated by an electrophoretic mobility shift assay. Probe retardation was readily detectable in protein extracts from TGF- β -treated cells, but this was attenuated in cells coincubated with BMP-7 (Figure 5A). To confirm the presence of Smad3 and Smad4 in the complexes formed, supershift experiments were performed with relevant antibodies (Figure

Figure 5. BMP-7 inhibits Smad3 DNA binding. **A** and **B:** Electrophoretic mobility shift assay with a consensus Smad binding element probe. HK-2 cells were incubated with BMP-7 or control (Ctrl) medium for 24 hours before incubation with 1 ng/ml TGF- β or control medium for 1 hour. **Bold arrow**, retarded probe; **arrow**, supershifted probe. **A:** Electrophoretic mobility shift assay performed with nuclear protein extract and consensus Smad binding element (SBE) probe. **B:** Supershift assay performed with antibodies to Smad3, 4, and 5, Sm, Smad. **C** and **D:** ChIP: Smad3 binding to the PAI-1 promoter. After chromatin immunoprecipitation with Smad3 antibody or pre-immune globulin, PAI-1 promoter Smad binding elements (SBE) were detected by qRT-PCR. Data are presented as Smad3-precipitated signal/pre-immune globulin-precipitated signal, normalized to control. **C:** HK-2 cells were incubated with TGF- β for time points to 24 hours before ChIP. **D:** HK-2 cells were incubated with BMP-7 and TGF- β as indicated for 6 hours before ChIP. RE, Relative Expression.

5B). Smad5 is reported to bind to consensus Smad3 elements, such that the potential for competitive inhibition of Smad3 binding by Smad5 exists.²⁴ However, no binding of Smad 5 was seen after addition of anti-Smad5 antibody (Figure 5B).

Subsequently, endogenous Smad3 DNA binding was evaluated by chromatin immunoprecipitation (ChIP). Induction of PAI-1 expression in the kidney is a major contributor to progressive renal fibrosis in chronic kidney disease (reviewed by Eddy and Fogo¹⁹). PAI-1 is transcriptionally activated by TGF- β with fast kinetics, and the PAI-1 promoter element contains several Smad binding elements, of which two cooperatively regulate the transcriptional response of PAI-1 to TGF- β .^{17,20} ChIP showed increased Smad3 binding to these two PAI-1 promoter Smad-responsive elements, with maximal Smad3 binding detected 6 hours after TGF- β stimulation (Figure 5C). BMP-7 alone did not significantly alter Smad3 binding to PAI-1 promoter sites, but inhibition of binding was seen when cells were coincubated with TGF- β and BMP-7 (Figure 5D).

Figure 6. BMP-7 prevents TGF- β -mediated down-regulation of the repressor of Smad3 transcription, SnoN. **A–C:** HK-2 cells were incubated with 1 ng/ml TGF- β , 50 ng/ml BMP-7, or both, for time points to 24 hours before immunoblotting of whole-cell extracts. Subsequently, blots were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm approximately equal loading. **A:** SnoN and Ski expression after TGF- β . **B:** SnoN expression after BMP-7. **C:** SnoN expression after TGF- β and BMP-7. **D** and **E:** HK-2 cells were incubated with 50 ng/ml BMP-7 or control medium for 24 hours before incubation with 1 ng/ml TGF- β or control medium for 30 minutes and immunoblotting of whole-cell extracts for SnoN. Subsequently, blots were reprobed for GAPDH to confirm approximately equal loading. **D:** Representative immunoblot of four experiments giving similar results. **E:** Densitometry of four independent experiments. **F:** HK-2 cells were transfected with SnoN siRNA together with Smad3 responsive (Scr) (CAGA luciferase) and control (*Renilla*) vectors for 48 hours. Subsequently, cells were incubated with BMP-7 or control medium for 24 hours and then incubated with TGF- β for 6 hours. Data are presented as firefly luciferase/*Renilla* luciferase, normalized to the TGF- β control. **G:** HK-2 cells were transfected with Ski siRNA together with Smad3-responsive (Scr) (CAGA luciferase) and control (*Renilla*) vectors for 48 hours. Subsequently, cells were incubated with BMP-7 or control medium for 24 hours and then incubated with TGF- β for 6 hours. Data are presented as firefly luciferase/*Renilla* luciferase, normalized to TGF- β control. RLU, relative light units.

BMP-7 Inhibits Smad3 DNA Binding by Preventing SnoN Degradation

Ski and SnoN are related proteins that bind to Smad proteins and to Smad binding elements in DNA and repress Smad-dependent transcriptional activation. Whereas Ski has broad TGF- β family repressor activity, with inhibitory action on BMP-dependent as well as TGF- β -dependent Smad signals, SnoN seems to be restricted to TGF- β -dependent Smad inhibition.²⁵ Recent work demonstrated that SnoN degradation is required for Smad3- but not Smad2-dependent transcription.²⁶ Accordingly, Ski and SnoN expression were examined in PTCs after TGF- β stimulation. Immunoblot studies showed reduced SnoN expression from 15 minutes to 6 hours after TGF- β stimulation, but from 12 or more hours after TGF- β stimulation, re-accumulation of SnoN was evident (Figure 6A). No changes in Ski expression were seen in response

Figure 7. Effect of proteasome inhibitor MG132 on SnoN expression, Smad3 signaling, and TGF- β -dependent PAI-1 expression. **A** and **B:** HK-2 cells were incubated with control medium or 1 ng/ml TGF- β for 30 minutes with or without 10 μ M MG132, and immunoblots of whole-cell extracts for SnoN are shown. Subsequently, blots were reprobed for GAPDH to confirm approximately equal loading. **A:** Representative immunoblot of four experiments giving similar results. **B:** Densitometry of four independent experiments. **C:** HK-2 cells were transfected with Smad3 and *Renilla* control reporter constructs for 48 hours, before incubation with control medium or 1 ng/ml TGF- β for 6 hours with or without 10 μ M MG132. Data are presented as firefly/*Renilla* luciferase activity, normalized to control. **D:** HK-2 cells were incubated with control medium or 1 ng/ml TGF- β for 24 hours with or without 10 μ M MG132 before detection of PAI-1 mRNA by qRT-PCR. * P < 0.001 versus control; ** P < 0.01 versus TGF- β . RLU, relative light units.

to TGF- β over the time course examined (Figure 6A). Subsequently, SnoN expression was evaluated in cells treated with TGF- β and BMP7, alone or in combination. BMP-7 alone did not alter SnoN expression (Figure 6B), but addition of BMP-7 prevented TGF- β -mediated SnoN degradation (Figure 6C). In additional experiments, cells were incubated for 24 hours with 50 ng/ml BMP-7 or control medium, followed by addition of 1 ng/ml TGF- β for 30 minutes. Preincubation with BMP-7 prevented loss of SnoN expression seen with TGF- β alone (Figure 6, D and E).

The above data are consistent with a reduction in Smad3 DNA binding due to a failure to degrade SnoN in BMP-7-treated cells. Accordingly, the effect of SnoN and Ski knockdown on modification of TGF- β signaling by BMP-7 was examined. Preliminary immunoblot and qRT-PCR studies confirmed >80% reduction in the expression of SnoN or Ski in PTCs transfected with the respective siRNA after 48 hours (not shown). In subsequent experiments, PTCs were co-transfected with siRNA and with the Smad3 reporter vector CAGA.⁴ Basal Smad3 reporter activity was not altered by SnoN knockdown. BMP-7 inhibited reporter response in control transfected cells but not in cells transfected with SnoN siRNA (Figure 6F). In contrast, Ski knockdown did not prevent inhibition of reporter activation by BMP-7 (Figure 6G).

Degradation of SnoN may be prevented by the proteasome and calpain inhibitor MG132.²⁷ The effect of MG132 on SnoN expression and Smad3 signaling in PTCs was studied. Immunoblotting showed that MG132 did not alter basal SnoN expression but that TGF- β -induced SnoN degradation was prevented by prior treat-

ment with MG132 (Figure 7A). MG132 treatment of cells transfected with CAGA⁴ reporter did not alter reporter activity. However, reporter activity in response to TGF- β was diminished in cells pretreated with MG132 (Figure 7B). Subsequently, induction of PAI-1 by TGF- β was studied in MG132-treated PTCs by qRT-PCR. PAI-1 induction by TGF- β was diminished by MG132 (Figure 7, C and D). Taken together, these data show that prevention of SnoN degradation reproduces the effect of BMP-7 on Smad3 signaling, whereas SnoN knockdown prevents it, and links preservation of SnoN expression in response to BMP-7 mechanistically to inhibition of Smad3 signaling by BMP-7.

Discussion

BMP-7 is widely expressed during embryonic life and plays a crucial role in renal development,²⁸ during which podocyte-derived BMP-7 is required for normal proximal tubular development.²⁹ Renal tubular BMP-7 expression is diminished in animal models of diabetic nephropathy.³⁰ PTCs express abundant BMP receptors,³¹ and recombinant BMP-7 improves renal fibrosis and accelerates return of renal function after relief of ureteric obstruction.³ BMP-7 counteracts the TGF- β -induced renal epithelial to mesenchymal transition and reverses chronic renal injury.⁴ Intriguingly, BMP-7 also opposes TGF- β -induced endocardial to mesenchymal transition in cardiac fibrosis,³² suggesting that BMP-7 may be of general importance in maintenance of the cellular phenotype in the face of excessive TGF- β stimulation. However, the mechanisms underlying these *in vivo* observations have remained largely obscure. Thus, the aim of this study was to discover the mechanisms by which BMP-7 inhibits TGF- β -dependent profibrotic changes in phenotype in PTCs.

TGF- β is a ubiquitous cytokine that functions in an autocrine or paracrine manner to elicit a multiplicity of effects, principally related to cell growth and extracellular matrix accumulation, which has been implicated widely in the pathogenesis of tissue fibrosis.³³ In renal disease, it has been implicated in directing fibrosis in both glomerular and interstitial compartments^{34–37} and is postulated to be a major biological signal that regulates the switch in tubular cell activities toward a profibrogenic phenotype.³⁸ The activated TGF- β -receptor complex phosphorylates Smad2 and 3, which transduce the TGF- β signal to the nucleus. Deletion of Smad3 prevents the TGF- β -induced epithelial to mesenchymal transition and attenuates fibrotic sequelae,¹⁸ suggesting that Smad3 signaling is the dominant pathway in terms of the profibrotic effects of TGF- β .

In the current study, we have found that BMP-7 specifically limits Smad3-dependent transcriptional activation in PTCs, via prevention of SnoN degradation, which is required for Smad3- but not Smad2-dependent transcriptional events (Figure 8, A–C).²⁶ Thus, we have uncovered a major pathway by which BMP-7 prevents profibrotic events in the kidney. It is interesting to compare our data with the action of BMP-7 in mesangial cells, in

Figure 8. Proposed mechanism of regulation of Smad3 signaling by BMP7. **A:** In the absence of TGF- β , Smads shuttle into and out of the nucleus. SnoN binds to Smad binding elements and prevents R-Smad binding. **B:** After ligand binding, active TGF- β receptor complex leads to R-Smad phosphorylation. R-Smad-Arkadia-SnoN complexes lead to SnoN degradation. R-Smad-Smad4 complexes accumulate in the nucleus and bind to DNA. **C:** BMP7 prevents loss of SnoN expression. R-Smad-Smad4 complexes accumulate in the nucleus, but DNA binding to consensus Smad binding elements is prevented by SnoN. Arkadia-dependent SnoN degradation appears necessary for Smad3 (or Smad2-exon3)-dependent responses, but not Smad1/Smad4-dependent responses or responses driven by Smad2-Smad4-FoxH1 complexes (see Levy et al²⁶).

which induction of the inhibitory Smad6 by BMP-7 leads to loss of TGF- β signaling and failure of nuclear Smad3 accumulation.⁷ This finding suggests that BMP-7 inhibits TGF- β signaling via multiple mechanisms. However, our data demonstrating a specific effect on Smad3, which is reversed by SnoN knockdown, suggest that in PTCs the dominant mechanism occurs via SnoN.

SnoN is a member of the *ski* family of oncoproteins that bind to the consensus Smad3 binding element (GTCTAGAC) and repress transcription.³⁹ SnoN has a specific inhibitory action on Smad3 DNA binding, and SnoN degradation is required for transduction of the Smad3 but not Smad2 signal.²⁶ SnoN plays a role in maintaining the repressed state of TGF- β -responsive genes in the absence of ligand and also in negative feedback regulation of TGF- β signaling.⁴⁰ SnoN expression is progressively reduced in the kidney after unilateral ureteric obstruction in mice, and loss of SnoN expression sensitizes tubular epithelial cells to the epithelial to mes-

enchymal transition.⁴¹ SnoN may be degraded by recruitment to the proteasome by Smad3,²⁷ and loss of SnoN expression in animal models of progressive renal fibrosis occurs via enhanced ubiquitin-mediated degradation.^{42,43} Interestingly, hepatocyte growth factor exerts an antifibrotic effect in the kidney by enhancing SnoN expression in PTCs.^{44,45} Taken together with our current data demonstrating that preservation of SnoN expression underlies the antifibrotic actions of BMP-7, it is possible to conclude that loss of SnoN expression is a key element of progressive renal fibrosis.

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