

BMP7- induced-Pten inhibits Akt and prevents renal fibrosis

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Abstract:

Bone morphogenetic protein-7 (BMP-7) counteracts pro-fibrotic effects of $\text{TGF}\beta_1$ in cultured renal cells and protects from fibrosis in acute and chronic renal injury models. Using the unilateral ureteral obstruction (UUO) model of chronic renal fibrosis, we investigated the effect of exogenous-rhBMP-7 on pro-fibrotic signaling pathways mediated by $\text{TGF}\beta_1$ and hypoxia. Mice undergoing UUO were treated with vehicle or rhBMP-7 (300 $\mu\text{g}/\text{kg}$ i.p.) every other day for eight days and kidneys analysed for markers of fibrosis and SMAD, MAPK, and PI3K signaling. In the kidney, collecting duct and tubular epithelial cells respond to BMP-7 via activation of SMAD1/5/8. Phosphorylation of SMAD1/5/8 was reduced in UUO kidneys from vehicle-treated animals yet maintained in UUO kidneys from BMP-7-treated animals, confirming renal bioactivity of exogenous rhBMP-7. BMP-7 inhibited *Collagen I α 1* and *Collagen III α 1* gene expression and Collagen I protein accumulation, while increasing expression of *Collagen IV α 1* in UUO kidneys. Activation of SMAD2, SMAD3, ERK, p38 and PI3K / Akt signaling occurred during fibrogenesis and BMP-7 significantly attenuated SMAD3 and Akt signaling in vivo. Analysis of renal collecting duct (mIMCD) and tubular epithelial (HK-2) cells stimulated with $\text{TGF}\beta_1$ or hypoxia (1% oxygen) to activate Akt provided further evidence that BMP-7 specifically inhibited PI3K / Akt signaling. PTEN is a negative regulator of PI3K and BMP-7 increased PTEN expression in vivo and in vitro. These data demonstrate an important mechanism by which BMP-7 orchestrates renal protection through Akt inhibition and highlights Akt inhibitors as anti-fibrotic therapeutics.

Keywords:

Fibrosis, Anti-fibrotic, PTEN, Akt, $\text{TGF}\beta_1$, hypoxia

Introduction:

Bone morphogenetic protein -7 (BMP-7 , or osteogenic protein (OP)-1) is a 35kDa member of the transforming growth factor beta (TGF β) family of secreted extracellular proteins. In addition to its role in regulating bone and cartilage formation, BMP-7 is essential for renal morphogenesis, stimulating condensation of the metanephric mesenchyme around the ureteric bud, leading to the formation of collecting duct epithelia through promotion of mesenchymal to epithelial transition (MET).¹⁻⁴ BMP-7 null mice die soon after birth due to renal hypoplasia associated with reduced ureteric bud branching, loss of metanephric mesenchyme, cessation of nephrogenesis and a marked reduction of glomerular density.^{2, 5} BMP-7 expression is retained in adult kidneys and is thought to be critical for maintaining tubular epithelial integrity.⁶⁻¹³ During renal disease, interaction of BMP-7 with distinct serine / threonine kinase receptors is prevented by binding to endogenously generated antagonists such as gremlin, noggin and USAG-1 (Uterine sensitization-associated gene -1, also referred to as Sclerostin domain containing -1 , *Sostdc1*),¹³ facilitating damage to the tubular epithelium. Several studies have reported that introduction of exogenous recombinant BMP-7 or reactivation of endogenous BMP-7 signaling through peptide mediated receptor activation can prevent progression of renal disease.^{7, 8, 10, 14-17}

BMP-7 is reno-protective in acute and chronic renal injury models (reviewed in¹⁸) including ischemia reperfusion injury (IRI),^{7, 14} unilateral ureteral obstruction (UUO),^{8, 19-21} 5/6 nephrectomy,^{3, 22} lupus nephritis, nephrotoxic serum nephritis (NTN), Collagen type 4 α 3-null 'Alport' mice,²³ and STZ-induced diabetic nephropathy.^{24, 25} BMP-7 is efficacious in other fibrotic diseases including cardiac,²⁶ liver,^{27, 28} corneal,²⁹ and silica induced pulmonary fibrosis.³⁰ Preservation of renal function, normalisation of serum creatinine, reduction of extracellular matrix accumulation, and reduction in pro-inflammatory mediators have been demonstrated.^{7, 8, 14, 19, 23, 31} However, the molecular mechanisms involved in this protection have not been fully defined. In this report, we describe the effect of BMP-7 on key signaling pathways underlying fibrogenesis.

TGF β ₁ is the prototypic pro-sclerotic cytokine which is aberrantly up-regulated during fibrotic disease. It is responsible for the induction and accumulation of interstitial matrix, which is considered the pathological hallmark of all kidney injuries regardless of the aetiology.³² TGF β ₁ promotes pro-fibrotic responses of renal epithelia and pericytes and activates resident fibroblasts.³³ TGF β ₁ binds type I and type II serine / threonine receptors on the plasma membrane activating receptor-associated SMAD proteins (R-SMADs), predominantly SMAD2 and SMAD3. Phosphorylated R-SMADs associate with co-SMAD-4 and bind specific SMAD binding elements in gene promoters. SMAD3 mediated signaling plays a central role in renal fibrogenesis³⁴⁻³⁷ and SMAD3 knockout mice are protected against STZ-induced diabetic glomerulopathy,³⁸ and UUO-induced tubulointerstitial fibrosis.³⁹ In addition, TGF β ₁ signals through non-SMAD proteins to effect a variety of cellular changes, including the mitogen activated protein kinases (MAPK) p38 and ERK, and phosphoinositide -3 kinase (PI3K).^{10, 40, 41} These pathways may further enhance or oppose SMAD signaling in a context dependent manner.^{42, 43}

BMP-7 is predominantly expressed in cortical and corticomedullary proximal tubular cells, distal convoluted tubules, collecting duct epithelia and glomerular podocytes,^{6, 7, 10, 13, 14, 25, 31, 44, 45} and signals through three type I serine / threonine receptors (termed activin-like kinase (Alk) receptors; Alk 3 (BMPR-1A), Alk6 (BMPR-1B) and Alk2 (Type 1A activin receptor) and one type II receptor (BMPRII) to phosphorylate SMAD1, SMAD5, and SMAD8.^{10, 17, 46-48} SMADs 1, 5, and 8 also bind SMAD4 and translocate to the nucleus to activate gene transcription. Alk3 is predominantly expressed in renal tubular epithelial cells, with Alk6 expressed in osteoblasts¹⁶ and Alk2 in cartilage.^{10, 31} The importance of Alk3 in mediating the protective effects of renal BMP-7 signaling was borne out by the enhanced pro-fibrotic effects of TGF β ₁ observed upon deletion of Alk3 in the tubular epithelium.¹⁶

The principle drivers of injury in UUO include TGF β ₁ and hypoxia.⁴⁹ In this report, we describe the effect of BMP-7 on hypoxia- and TGF β ₁-induced signaling pathways in the unilateral ureteral

obstruction (UUO) model of renal fibrosis. We report that BMP-7 inhibits activation of PI3K signaling via Akt in addition to inhibition of SMAD3 *in vivo*. We demonstrate that BMP-7 specifically inhibits TGF β ₁- and hypoxia-induced PI3K activity in renal collecting duct and tubular epithelial cells via up-regulation of the PI3K inhibitor phosphatase and tensin homolog (PTEN).

Results:

BMP-7 preserves pSMAD1/5/8 activity in fibrotic kidneys

Phosphorylation of SMAD1/5/8 was analysed in contralateral (CTL) and obstructed (UUO) kidneys to determine whether exogenous recombinant human BMP-7 (rhBMP-7) administered via i.p. injection could activate BMP-7 signaling in renal tissue. In vehicle treated animals, pSMAD1/5/8 was detected in the contralateral kidney (veh-CTL) but lost during development of fibrosis in the corresponding obstructed kidneys (veh-UUO) ($P<0.001$, Figure 1A and Supplemental Table 1). In contrast, in animals treated with rhBMP-7, pSMAD1/5/8 was detected in the obstructed kidneys (BMP-7-UUO) suggesting maintenance of BMP-7 responsive pathways, confirming functional activity of exogenously applied rhBMP-7 in the kidney (Figure 1A).

As a further readout for activation of BMP-7 pathways, expression of the BMP-7-target gene *Id-1* was analysed. In vehicle-UUO kidneys, *Id-1* mRNA was significantly down-regulated ($P<0.01$, Figure 1A and Supplemental Table 1), mirroring loss of SMAD1/5/8 activity in these kidneys, however there was no significant decrease of *Id-1* in BMP-7-UUO kidneys confirming preservation of BMP-7 regulated pathways (Figure 1B).

Expression of *Bmp-7* mRNA was significantly down-regulated in vehicle-UUO kidneys compared with vehicle-CTL kidneys (78.4% reduction, $P<0.001$) while BMP-7-UUO kidneys maintained *Bmp-7* mRNA expression (56.8% reduction, $P<0.001$) (Figure 1B). BMP-7 antagonist *Usag1* mRNA was significantly increased in vehicle-UUO ($P<0.05$), consistent with loss of BMP-7 signaling in veh-UUO, whereas

Usag1 mRNA was not increased in BMP-7-UUO kidneys consistent with maintenance of BMP-7 signaling. BMP-7 antagonist *Grem1* mRNA was not changed in response to UUO but was significantly increased in BMP-7-CTL kidneys ($P<0.05$ compared to either veh-CTL or BMP7-UUO, Figure 1B).

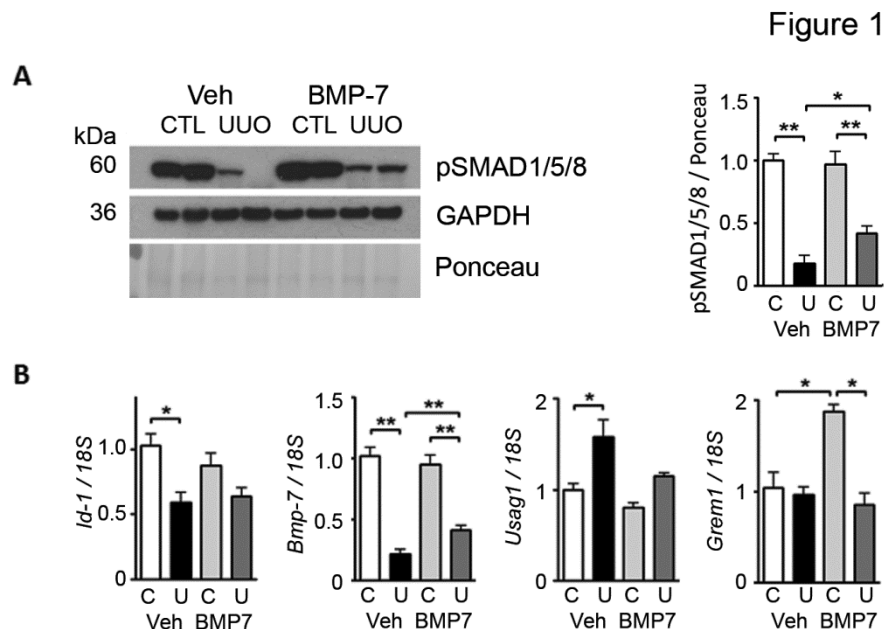


Figure 1: Exogenous BMP-7 activates SMAD1/5/8 signaling in fibrotic kidneys in vivo. **A** Western blot of 20µg whole cell lysate from vehicle (veh) or BMP-7 treated contralateral (CTL, C) and 8d-post obstructed (UUO, U) kidneys probed for phosphorylated SMAD1/5/8 and GAPDH, $n=5$ vehicle and $n=4$ BMP-7 groups, representative blots shown. Graph shows densitometric analysis of pSMAD1/5/8 normalised to ponceau. **B** Quantitative PCR analysis of *Id-1*, *Bmp-7*, *Usag1*, and *Gremlin 1* (*Grem1*) gene expression normalised to 18S mRNA levels. Graphs show fold-change in mRNA expression with Veh-CTL arbitrarily set to 1, $n=5$ in vehicle and BMP-7 groups, statistical analysis performed using One-way ANOVA and Bonferroni's multiple comparison test, $*P<0.05$, $**P<0.001$.

BMP-7 reduces extracellular collagen, but not α -SMA, accumulation in vivo

The protective effect of BMP-7 on the development of fibrosis in vivo was investigated by quantifying extracellular matrix (ECM) accumulation in contralateral and obstructed kidneys from

vehicle- or BMP-7-treated mice. A significant increase in sirius red staining of newly deposited collagen fibrils was observed in vehicle-UUO kidneys compared with vehicle-CTL kidneys ($P<0.01$), in contrast there was no detectable increase in sirius red-stained collagen in BMP-7-UUO kidneys ($P>0.05$, Figure 2A & B and Supplemental Table 1) confirming reduced ECM accumulation.

Despite reducing collagen accumulation, BMP-7 had no effect on UUO-induced α -SMA protein ($P<0.05$ in veh-UUO and BMP-7 UUO compared to respective contralateral kidneys, Figure 2A&B) or mRNA expression ($P<0.001$, in veh-UUO and BMP-7-UUO compared to respective contralateral kidneys, Figure 2C and Supplemental Table 1). Additionally, BMP-7 had no effect on *Fsp-1* mRNA expression as a marker of interstitial injury in fibrotic kidneys ($P<0.001$ in veh-UUO and BMP-7-UUO compared to respective contralateral kidneys, Figure 2C and Supplemental Table 1).

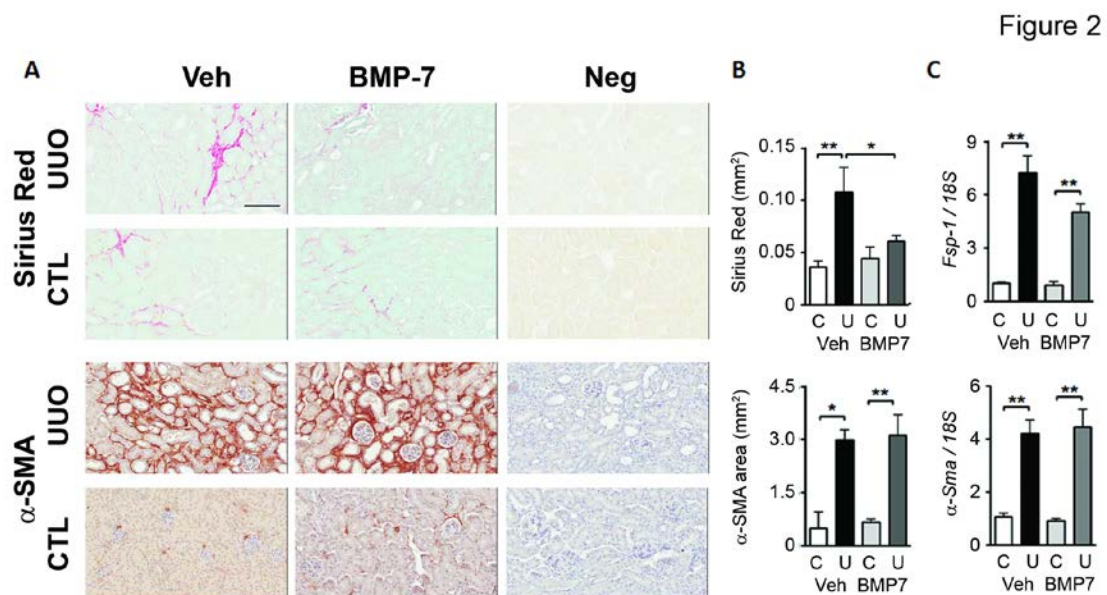


Figure 2: Exogenous BMP-7 reduces renal fibrosis and collagen deposition, but not fibroblast activation, in vivo. **A** Extracellular matrix deposition in contralateral (CTL, C) and 8d-post obstructed (UUO, U) kidneys, from mice treated with vehicle (Veh) or BMP-7 as described in Methods, detected by Sirius Red or immunohistochemical staining for α -SMA, magnification x20, scale bar 100 μ m. Neg: negative control with sections processed in absence of primary antibody. **B** Morphometric analysis

of positive IHC stained area (mm²). **C** Quantitative PCR analysis of *Fsp-1* and *α-Sma* mRNA normalised to *18S* mRNA. n=5 in vehicle and BMP-7 groups. Statistical analysis performed using One-way ANOVA with Bonferroni's multiple comparison post-test, *P<0.05, **P<0.001.

BMP-7 reduces renal fibrosis in vivo via reduced Collagen I and III and enhanced Collagen IV

To determine the specific collagen types that were altered by BMP-7 treatment, Collagen Iα1, IIIα1, and IVα1 proteins were assessed by immunohistochemistry (Figure 3A and B). Collagen Iα1 was significantly increased in veh-UUO kidneys (*P*<0.01) whereas there was no significant increase observed in BMP-7-UUO kidneys (*P*>0.05, Figure 3A and B and Supplemental Table 1). Similarly, *Col Iα1* mRNA was significantly increased in veh-UUO compared to veh-CTL kidneys (*P*<0.001, Figure 3C). While there was a significant increase in *Col Iα1* mRNA between BMP-7-CTL and BMP-7-UUO kidneys (*P*<0.001, Figure 2C), *Col Iα1* gene expression was significantly reduced in both contralateral and obstructed kidneys of BMP-7-treated animals compared with vehicle treated animals (*P*<0.001).

Collagen IIIα1 protein was significantly increased in BMP-7-UUO kidneys compared to BMP-7-CTL kidneys (*P*<0.01), and unlike Collagen Iα1, there was no significant difference in the quantity of Collagen IIIα1 protein between veh-UUO kidneys and BMP-7-UUO (*P*>0.05, Figure 3A & B and Supplemental Table 1). However, induction of *Collagen IIIα1* mRNA in UUO kidneys was significantly impaired (*P*=0.002) in animals treated with BMP-7 (*P*<0.001 in veh-UUO and BMP-7 UUO versus respective CTL kidneys, Figure 3C).

Collagen IVα1 protein was not significantly increased in UUO kidneys compared with CTL kidneys of either group (*P*>0.05, Figure 2A & B and Supplemental Table 1). However, *Col IVα1* mRNA was significantly increased in BMP-7-UUO kidneys compared with vehicle-UUO kidneys (*P*<0.001, Figure 3C).

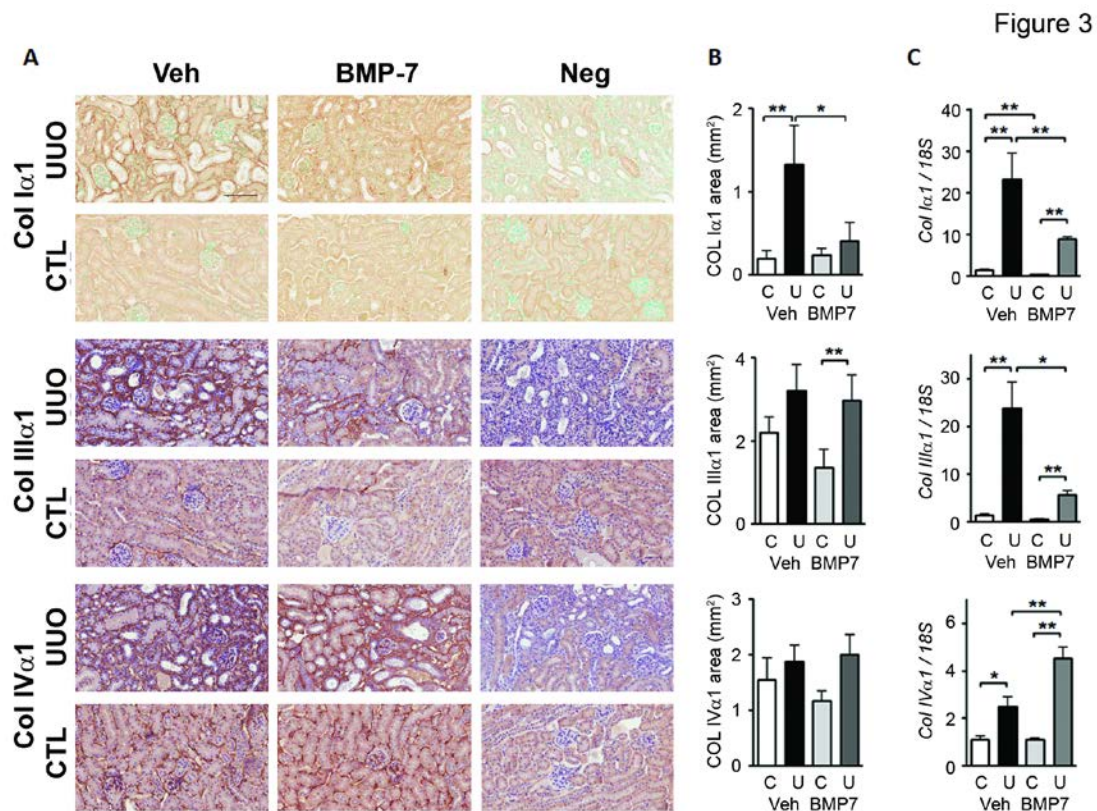


Figure 3: Exogenous BMP-7 reduces Collagen I and III and enhances Collagen IV expression in vivo.

A Extracellular matrix deposition in contralateral (CTL, C) and 8d-post obstructed (UUO, U) kidneys, from mice treated with vehicle (Veh) or BMP-7 as described in Methods, detected by immunohistochemical staining for Col Iα1, IIIα1, and IVα1, magnification x20, scale bar 100μm. Neg: negative control with sections processed in absence of primary antibody. **B** Morphometric analysis of positive IHC stained area (mm²). **C** Quantitative PCR analysis of *Col Iα1*, *Col IIIα1*, and *Col IVα1* mRNA normalised to 18S mRNA. n=5 in vehicle and BMP-7 groups. Statistical analysis performed using One-way ANOVA with Bonferroni's multiple comparison post-test, *P<0.05, **P<0.001.

BMP-7 inhibits phosphorylation of SMAD3 but not SMAD2 in vivo

To determine the effects of BMP-7 on TGFβ₁-mediated signaling pathways in vivo, activation of canonical signaling, via SMAD2 and SMAD3 (Figure 4 and Supplemental Table 1), and non-canonical

signaling, via ERK, p38 and Akt, was analysed (Figure 5 and Supplemental Table 1). While BMP-7 had no effect on the activation and phosphorylation of SMAD2 in UUO kidneys, phosphorylation of SMAD3 was completely ablated in both contralateral and UUO kidneys of BMP-7 treated animals ($P<0.001$, Figure 4). This is the first report of inhibition of SMAD3 by BMP-7 in whole kidneys in vivo and corroborates the reported BMP-7-inhibition of SMAD3 in mesangial cells in vitro.⁴³

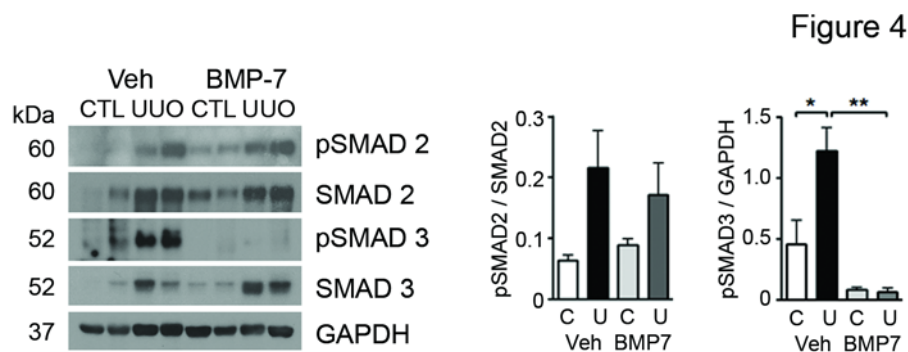


Figure 4: Exogenous BMP-7 inhibits SMAD3 activation in diseased kidneys but does not alter SMAD2 signaling in vivo. Western blot of 20µg whole cell lysate from vehicle or BMP-7 treated contralateral (CTL, C) or corresponding 8d-post UUO (U) kidneys probed for total and phosphorylated SMAD2 and SMAD3. Graphs show densitometric analysis of each phosphorylated protein normalised to its respective total isoform or GAPDH. n=5 in vehicle and BMP-7 groups, representative blots shown, statistical analysis performed using One-way ANOVA and Bonferroni's multiple comparison test, * $P<0.05$, ** $P<0.001$.

BMP-7 inhibits phosphorylation of Akt but not ERK or p38 MAPK in vivo

Renal fibrosis in vivo was associated with activation of PI3K signaling as determined by phosphorylation of Akt on Serine⁴⁷³ and Threonine³⁰⁸. Activation of PI3K signaling was significantly impaired in BMP-7-UUO kidneys compared with vehicle-UUO kidneys ($P<0.001$, Figure 5A and Supplemental Table 1). To determine whether this inhibition had effects further downstream of Akt,

the phosphorylation status of the Akt target GSK3 β was investigated. UVO significantly increased the phosphorylation, and thus inactivation, of GSK3 β ($P<0.05$ in vehicle kidneys) and this was significantly reduced in BMP-7 treated UVO kidneys ($P<0.01$, Figure 5A).

In contrast, BMP-7 did not alter the activation status of ERK or p38, both of which were significantly up-regulated in response to UVO (pERK: $P<0.001$ in vehicle kidneys and $P<0.01$ in BMP-7 kidneys compared to respective contralateral kidneys, p38: $P<0.01$ in BMP7-UVO kidneys versus BMP7-CTL, Figure 5B and Supplemental Table 1). Our findings suggest that the protective role of BMP-7 in vivo is mediated via inhibition of both SMAD3 and Akt signaling.

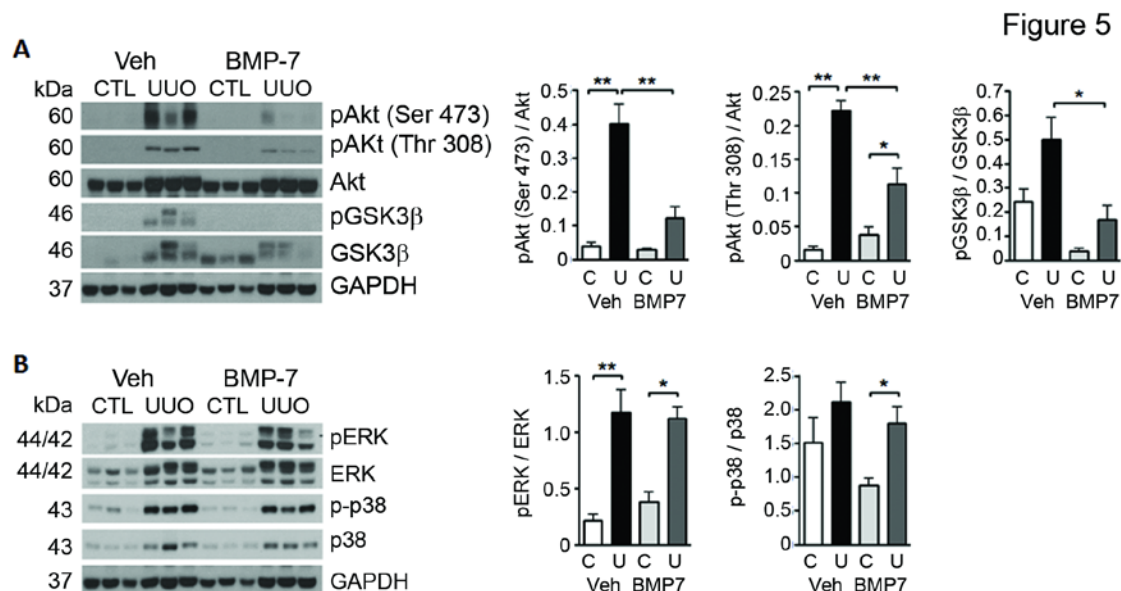
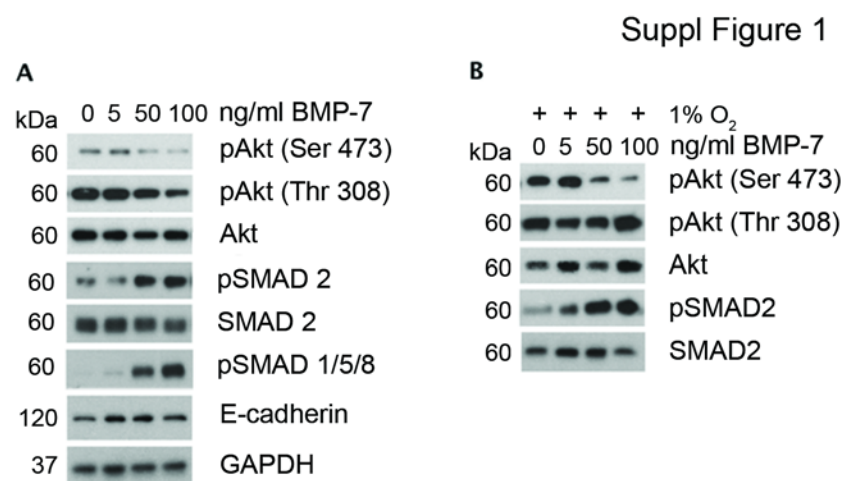


Figure 5: Exogenous BMP-7 inhibits phosphorylation of Akt and GSK3 β during fibrosis but does not alter ERK or p38 MAPK signaling in vivo. Western blot of 20 μ g whole cell lysate from vehicle or BMP-7 treated contralateral (CTL, C) or corresponding 8d-post UVO kidneys (U) probed for total and phosphorylated **A** Akt and GSK3 β , and **B** ERK and p38 MAPK. Graphs show densitometric analysis of each phosphorylated protein normalised to its respective total isoform. $n=5$ in vehicle and BMP-7 groups, representative blots shown, statistical analysis performed using One-way ANOVA and Bonferroni's multiple comparison test, * $P<0.05$, ** $P<0.001$.

BMP-7 inhibits TGF β ₁- and hypoxia-induced-Akt signaling in renal epithelia

To investigate the effects of BMP-7 on Akt signaling in renal epithelial cells in culture, collecting duct (mIMCD) and proximal tubular (HK-2) cells were treated with 5-100ng/ml BMP-7. BMP-7 inhibited phosphorylation of Akt-Ser⁴⁷³ and Akt-Thr³⁰⁸ in a dose dependent manner in mIMCD and induced expression of E-cadherin (Supplemental Figure 1A). HK-2 tubular epithelial cells were stimulated with hypoxia (1% O₂) to activate Akt signaling, BMP-7 inhibited phosphorylation of Akt-Ser⁴⁷³ in a dose dependent manner but not Akt-Thr³⁰⁸ (Supplemental Figure 1B). Interestingly, in both cell types BMP-7 dose-dependently increased phosphorylation of SMAD2 (Supplemental Figure 1A&B).



Supplemental Figure 1: BMP-7 inhibits Akt activation in renal collecting duct (mIMCD) and tubular epithelia (HK-2) in a dose dependent manner. **A** Western blot of 20 μ g whole cell lysate from

mIMCD cells treated with BMP-7 (0-100ng/ml for 150 minutes) and probed for phosphorylated and total Akt, SMAD2, SMAD 1/5/8 and E-Cadherin, GAPDH was analysed as a loading control. **B**

Western blot of 20 μ g whole cell lysate from HK-2 cells pre-treated with rhBMP-7 (0-100ng/ml for 30 minutes) followed by 1% O₂ for 60 minutes. Blots were probed for phosphorylated and total forms of Akt and SMAD2. The experiments were repeated twice, representative blots shown.

To determine the effects of BMP-7 on TGF β_1 -induced signaling in vitro, collecting duct epithelia (mIMCD) were co-stimulated with 100ng/ml BMP-7 and 5ng/ml TGF β_1 . BMP-7 inhibited TGF β_1 -induced phosphorylation of Akt at Ser⁴⁷³ but not at Thr³⁰⁸ ($P<0.05$, Figure 6A and Supplemental Table 1). TGF β_1 induced phosphorylation of GSK3 β ($P<0.05$, Figure 6A) and reduced E-cadherin protein ($P<0.01$, Figure 6A), although BMP-7 did not alter the phosphorylation status of GSK3 β it restored E-cadherin protein expression ($P<0.05$ versus TGF β , Figure 6A). BMP-7 had no effect on TGF β_1 -induced phosphorylation of ERK and p38 (Figure 6B).

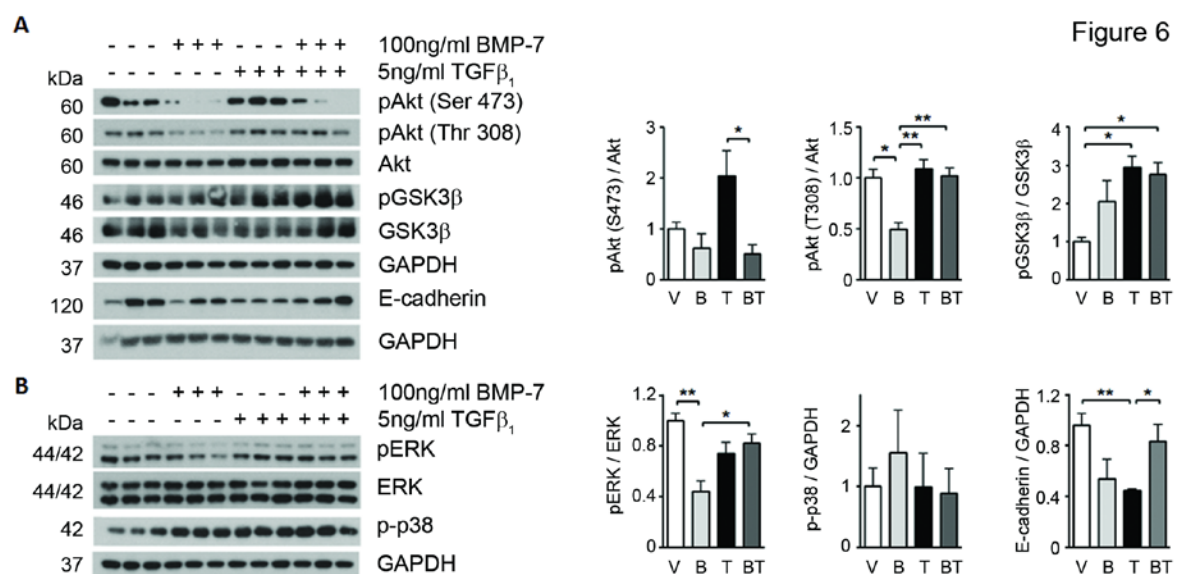


Figure 6: BMP-7 inhibits TGF β_1 -induced Akt activation and restores E-cadherin expression in collecting duct epithelial cells. Western blot of 20 μ g whole cell lysate from mIMCDs pre-treated with vehicle or rhBMP-7 (100ng/ml for 30 minutes) followed by vehicle or TGF β_1 (5ng/ml for 60 minutes) probed for phosphorylated and total isoforms of **A** Akt and GSK3 β , and E-cadherin, and **B** ERK and p-38 MAPK. GAPDH was analysed as a loading control. Graphs show densitometric analysis, V: Vehicle, B: BMP-7, T: TGF β_1 , BT: BMP-7 & TGF β_1 . Treatments were performed in triplicate and experiments repeated three times, statistical significance was determined by one-way ANOVA and Bonferroni's multiple comparison test, * $P<0.05$, ** $P<0.001$.

BMP-7 inhibits Akt signaling via up-regulation of PTEN

Activation of PI3K signaling involves conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃) enabling subsequent phosphorylation of Akt.

Phosphatase and tensin homologue (PTEN) negatively regulates PI3K signaling by converting PIP₃ back to PIP₂.⁵⁰ In UUO kidneys, gene expression of *Pten* was significantly reduced in response to UUO in vehicle treated animals ($P<0.05$, Figure 7A) consistent with the observed phosphorylation of Akt (Figure 5A). However, the opposite was observed in UUO-kidneys from BMP-7-treated animals where *Pten* expression was significantly up-regulated compared to contralateral kidneys ($P<0.05$, Figure 7A), consistent with the observed dephosphorylation of Akt in BMP-7-UUO kidneys (Figure 5A).

Similarly in collecting duct cells, BMP-7 induced expression of *Pten* ($P<0.05$, Figure 7B). Interestingly, when collecting duct epithelia were co-stimulated with BMP-7 and TGFβ₁ expression of *Pten* was further amplified ($P<0.05$ versus individual treatments, Figure 7B). This synergistic effect of BMP-7 and TGFβ₁ on *Pten* expression correlates with the reduction of Akt phosphorylation observed in the BMP-7-UUO kidneys (Figure 5A).

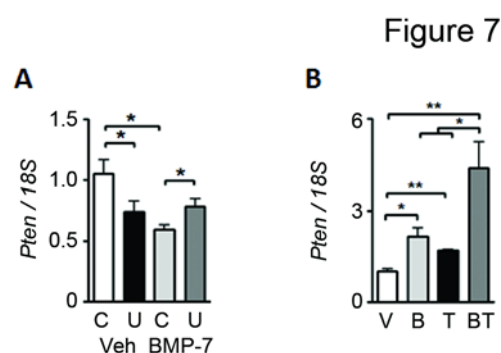


Figure 7: BMP-7 increases expression of Akt negative regulator *Pten* in vivo and in vitro.

Quantitative PCR analysis of *Pten* mRNA normalised to 18S expression in **A** contralateral (C) and obstructed (U) kidneys from vehicle-treated (Veh) and BMP-7-treated animals, n=5 for vehicle and

BMP-7 groups, **B** collecting duct epithelial cells treated with vehicle (V), BMP-7 (B, 100ng/ml), TGF β_1 (T, 5ng/ml), or combined BMP-7 and TGF β_1 (BT). Treatments were performed in triplicate and the experiments repeated three times. Statistical analysis was determined by one-way ANOVA and Bonferroni's multiple comparison test, *P<0.05, **P<0.001.

Discussion:

A protective role for BMP-7 has been reported in a number of renal injury models however, the molecular mechanisms by which BMP-7 exerts this protection have focussed primarily in vitro on the effect of BMP-7 on TGF β_1 -induced extracellular matrix (ECM) accumulation,^{10, 51} and E-cadherin expression in cultured renal cells¹¹, and genomic methylation.⁵²⁻⁵⁷ Here we describe specific effects of BMP-7 on TGF β_1 -signaling and show in vivo and in vitro that BMP-7 not only inhibits activation of SMAD3 but also inhibits Akt resulting in loss of pro-fibrotic signals and protection from fibrogenesis.

Exogenous rhBMP-7 activated SMAD1/5/8 signaling within the obstructed kidney, maintaining the expression of the BMP-7 target gene *Id1* (Figure 1). Gene expression of *Bmp-7* was significantly down-regulated in response to injury in vehicle treated animals, similar to reports in human renal disease⁵⁸⁻⁶⁰, however rhBMP-7 induced a significant induction of *Bmp-7* mRNA expression suggesting that BMP-7 can regulate its own expression in a positive feed forward loop (Figure 1B).

BMP-7 prevented the up-regulation of *Usag1* expression observed in veh-UUO kidneys (Figure 1B) suggesting that BMP-7 can regulate its bioactivity by inhibiting expression of its antagonists.

Additionally, in BMP-7-CTL kidneys *Grem1* expression was significantly up-regulated, perhaps in an attempt to regulate the bioactivity of exogenous BMP-7 under physiologic conditions. This induction of *Grem1* was not observed in BMP-7-UUO kidneys suggesting the involvement of active BMP-7 under pathophysiologic conditions as a protective mechanism against fibrosis.

Remarkably, BMP-7 abrogated fibrosis and ECM deposition despite an inability to alter myofibroblast activation. Similar induction of α -SMA, the hallmark marker of the myofibroblast, was observed in fibrotic kidneys from both vehicle- and BMP7-treated animals suggesting that BMP-7 works downstream of myofibroblast activation to block ECM production (Figure 2). Myofibroblast numbers are increased during renal fibrosis and they are considered to contribute to collagen and ECM production in fibrotic diseases.⁶¹ Interestingly, Takeji et al reported that α -SMA null mice had significantly worse renal disease, in two separate in vivo models, than mice wild-type for α -SMA expression, concluding that myofibroblasts play a role in suppressing renal fibrosis. Given their findings, it is perhaps not surprising that the beneficial effects of BMP-7 require that myofibroblast activation and α -SMA expression remains intact.⁶²

Induction of *Collagen I α 1* and *III α 1* gene expression during fibrosis was abrogated by BMP-7-treatment and a significant reduction of Collagen I α 1 protein accumulation was observed in BMP-7-UUO kidneys (Figure 3). Collagen accumulation during fibrosis is dependent not only on increased synthesis of proteins but also on decreased collagen turn-over. BMP-7 increases collagen degradation through up-regulation of matrix metalloproteinases (MMP)-2 and MMP-9²³ and down-regulation of plasminogen activator inhibitor-1 (PAI-1).⁵¹ We propose that the reduced type I collagen accumulation observed in BMP-7-UUO kidneys is likely due not only to reduced production but enhanced degradation of ECM molecules. TGF β ₁-induced Collagen I and III expression is mediated via activation of SMAD3,^{50, 63} therefore inhibition of SMAD3 activity by BMP-7 in vivo (Figure 4) may explain the reduced gene expression of Col I α 1 and III α 1 observed in BMP-7-UUO kidneys (Figure 3). Our findings support those of Wang and Hirschberg in cultured renal mesangial cells whereby a BMP-7-SMAD5-dependent up-regulation of SMAD6 inhibited nuclear accumulation of phospho-SMAD3 leading to loss of TGF β ₁-induced *Col I α 1* gene expression.^{43, 64} Our results highlight that BMP-7 inhibits SMAD3 mediated TGF β ₁ signaling in vivo and expands the repertoire of

cell responses from the previously reported mesangial cells to collecting duct (Figure 6) and tubular epithelial cells (Supplemental Figure 1).

In contrast to the inhibitory effects of BMP-7 on SMAD3, BMP-7 had no effect on ERK and p38 MAPK signaling pathways in vivo or in cultured epithelia, consistent with the findings of Wang and Hirschberg in mesangial cells.⁴³ However, BMP-7 significantly reduced phosphorylation of PI3K, Akt and GSK3 β . In veh-UUO kidneys, both phosphorylated Akt-Ser⁴⁷³ and -Thr³⁰⁸ were increased, however both phosphorylation events were inhibited in the presence of BMP-7 (Figure 5). In vitro, BMP-7 inhibited TGF β ₁-induced phosphorylation of Akt-Ser⁴⁷³ in collecting duct epithelial cells (Figure 6) and hypoxia-induced phosphorylation of Akt-Ser⁴⁷³ in proximal tubular epithelial cells (Supplemental Figure 1). However, only the initial phosphorylation event at Ser⁴⁷³ was successfully inhibited by BMP-7 whereas phospho-Akt -Thr³⁰⁸ was still detected in cultured cells in vitro. This may be due to increased secretion of endogenous TGF β ₁ or other cytokines that may overcome BMP-7's ability to inhibit PI3K signaling in vitro.

Under physiologic conditions, active GSK3 β phosphorylates β -catenin causing its ubiquitination and degradation. In response to renal injury in veh-UUO kidneys (Figure 5) or to TGF β ₁ (Figure 6), phosphorylation and inactivation of GSK3 β results in stabilisation and nuclear translocation of β -catenin inducing the expression of its target gene *Snai1*.^{65, 66} Snail is an E-cadherin E-box transcriptional repressor which decreases E-cadherin protein as part of the epithelial injury process.⁶⁷ Inactivation of GSK3 β , observed during renal injury, inhibits interaction between GSK3 β and Snail, resulting in nuclear translocation of Snail and repression of E-cadherin transcription^{65, 66}. In the presence of BMP-7, GSK3 β remains active and phosphorylates Snail promoting ubiquitin-mediated degradation of Snail⁶⁵ and stabilisation of E-cadherin expression. Our results in collecting duct cells confirmed this regulation, where TGF β ₁ increased phosphorylation of GSK-3 β and reduced expression of E-cadherin protein (Figure 6). Furthermore addition of BMP-7 reversed the TGF β ₁ down-regulation and restored E-cadherin protein expression. Our findings further delineate the

mechanism by which BMP-7 impacts cellular signaling pathways to orchestrate protection from fibrogenesis.

PTEN dephosphorylates PIP₃ to PIP₂ antagonizing PI3K signaling. Loss of PTEN leads to accumulation of PIP₃ and activation of Akt. Conversely, over-expression of PTEN leads to accumulation of PIP₂ and subsequent inhibition of PI3K signaling and Akt phosphorylation. A recent publication by Samarakoon et al reported that expression of PTEN was lost in several models of renal injury including UUO, aristocholic acid induced nephropathy (AAN), and streptozotocin-mediated injury.⁶⁸ We now report that BMP-7 can prevent the loss of PTEN during UUO-induced fibrosis ensuring that activation of Akt does not occur (Figure 7).

Akt has been implicated in renal fibrogenesis previously, however this is the first report of BMP-7 as an inhibitor of Akt signaling. Crosstalk between Akt and SMAD signaling pathways has been reported by a number of independent studies, Kattla et al reported that while TGFβ₁-activation of PI3K and SMAD3 occurred independently, inhibition of Akt, by LY294002 or Akt inhibitor II or PTEN over-expression, abrogated TGFβ₁-induced pro-fibrotic responses in renal epithelial cells.⁶⁹ Similarly Bakin et al reported that Akt was required for TGFβ₁-induced EMT of mammary epithelial cells.⁷⁰ Finer et al reported that Akt mediated pro-fibrotic changes in a mouse adriamycin nephropathy model. By treating mice with a specific inhibitor of PI3K they observed reduced phospho-Akt-Ser⁴⁷³ in glomeruli and tubular epithelial cells which abrogated fibrosis and proteinuria in response to adriamycin.⁷¹ We previously reported that the protective effect of Lipoxin A₄ in a model of rapidly progressing renal fibrosis was associated with reduced activation of Akt and reduced Collagen I accumulation in the obstructed kidney.⁷² Runyan et al reported that inhibition of PI3K, by LY294002 or over-expression of a dominant negative kinase deficient Akt construct, abrogated TGFβ₁-induced collagen I expression via reduced SMAD3 activity.⁵⁰ Connective tissue growth factor (CTGF) mediates the pro-fibrotic effects of TGFβ₁ through interaction with β3-integrins, resulting in activation of Akt and subsequent induction of fibronectin in primary human mesangial cells.⁷³ BMP-7 binds and inhibits

CTGF⁷⁴ highlighting a further potential mechanism through which BMP-7 may inhibit Akt activation to prevent fibrosis.

Our data shows that exogenous BMP-7 activates SMAD1/5/8 signaling in the kidney as determined via maintenance of pSMAD1/5/8 and *Id-1* gene expression in BMP-7-UUO kidneys (Figure 1), BMP-7 inhibits collagen type I and III gene and protein expression in renal injury (Figure 2&3) via inhibition of SMAD3 and Akt signaling (Figures 4-6), and that these effects are mediated by up-regulation of PTEN (Figure 7). In addition to inhibition of SMAD3, BMP-7 promoted activation of SMAD2-mediated signaling (Supplemental Figure 1), consistent with previous reports describing a protective role for SMAD2 against fibrogenesis.⁷⁵ This report delineates the key role that Akt plays in mediating the pro-fibrotic changes that occur during kidney injury and describes how BMP-7 can up-regulate PTEN to prevent activation of Akt signaling. We highlight the potential of Akt inhibition as a therapeutic approach to preventing progression of renal disease. It will be interesting to investigate whether Akt inhibitors, developed as anti-cancer and anti-inflammatory therapeutics⁷⁶ may also be useful as anti-fibrotic agents.

Methods:

Unilateral ureteral obstruction (UUO):

UUO was performed on 8-10 week old C57BL/6, FVB mice and kidneys harvested at 8 days.⁴⁹

Animals were randomized into two groups (n=5 each) receiving vehicle (20mM histidine, 140mM NaCl, Roche) or 300µg/kg recombinant human BMP-7 (R&D systems) i.p. on days 0, 2, 4, and 6. One third of each contralateral and obstructed kidney was formalin fixed, one third methyl Carnoy's fixed, and one third divided for RNA and protein extraction. The animal study was performed in accordance with ethical guidelines of UCD AREC and under license from the Department of Health.

Immunohistochemical analysis of kidney tissue:

4µm thick sections were prepared from formalin and Methyl Carnoys fixed paraffin embedded kidneys. Sirius red stain of collagen was detected on formalin fixed tissue.⁴⁹ IHC for type I, type III, type IV Collagen (Southern Biotech) and α-SMA (Sigma) was performed on Methyl Carnoy's fixed tissue.⁷⁷ Sections were scanned using Aperio Scanscope and analysed morphometrically using Aperio Imagescope software.⁴⁹

Cell culture:

Murine inner medullary collecting duct (mIMCD) and human tubular epithelial (HK-2) cells were cultured as described.^{78, 79} Cells were serum starved for 24 hours prior to stimulation with 0-100ng/ml rhBMP-7, 5ng/ml TGFβ₁ (both R&D systems), or 1% oxygen (Coy hypoxia chamber) for 60 minutes before lysis with either TRIzol or Cellytic.⁴⁹

Quantitative real time PCR:

2µg RNA was reverse-transcribed using 200U superscript reverse transcriptase II and the cDNA analysed for *Collagen type Iα1*, *IIIα1*, *IVα1*, *α-Sma*, *Fsp-1*, *Id-1*, *Bmp-7*, and *Pten* using Taqman primer probes and SYBR green analysis of *Usag1* (forward: GCATTTTCAGTAGCACTGGAC and reverse: ATGTATTTGGTGGACCGCAG), and *Grem1* (forward: TCATTGTGTTCCATGTGCCT and reverse: CAGCCCTCAGAGTTACCTCC).⁸⁰ qPCR results were analysed using ΔΔCt normalised against 18S rRNA and calibrated against vehicle treated contralateral kidneys or vehicle-treated cells.

Western blot protein analysis:

20µg denatured whole cell lysates were probed with primary antibodies against α-SMA, pSMAD1 (Ser^{465/465}), 5 (Ser^{463/365}), 8 (Ser^{426/458}), SMAD5, pSMAD2 (Ser^{465/467}), SMAD2, pERK1/2 (Thr²⁰²/Tyr²⁰⁴), ERK, p-p38 (Thr¹⁸⁰/Tyr¹⁸²), p38, pAkt (Ser⁴⁷³ and Thr³⁰⁸), Akt, pGSK3β (Ser⁹) and GAPDH (Cell Signaling), pSMAD3 (Ser^{423/425}), SMAD3 (Abcam), followed by incubation with appropriate secondary HRP-IgG antibody. Bands were detected by chemiluminescence.

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Disclosure:

There are no competing financial interests.

| Figure | Target | Veh | | | | | BMP-7 | | | | |
|--------|---|------|------|-------|------|---------------------|-------|------|------|------|----------------------|
| | | CTL | | UUO | | <i>P</i> vs Veh-CTL | CTL | | UUO | | <i>P</i> vs BMP7-CTL |
| | | mean | SEM | mean | SEM | | mean | SEM | mean | SEM | |
| 1A | pSMAD1/5/8 / SMAD5 | 2.89 | 0.37 | 0.49 | 0.17 | <0.001 | 1.96 | 0.22 | 1.74 | 0.43 | n.s. |
| 1B | <i>Id-1</i> mRNA | 1.03 | 0.09 | 0.59 | 0.08 | <0.01 | 0.88 | 0.09 | 0.64 | 0.07 | n.s. |
| 1B | <i>Bmp-7</i> mRNA | 1.02 | 0.10 | 0.22 | 0.05 | <0.001 | 0.95 | 0.10 | 0.41 | 0.05 | <0.001 |
| 1B | <i>Usag1</i> mRNA | 1.00 | 0.06 | 1.58 | 0.18 | <0.05 | 0.80 | 0.05 | 1.15 | 0.03 | n.s. |
| 1B | <i>Grem1</i> mRNA | 1.04 | 0.17 | 0.96 | 0.08 | n.s. | 1.87 | 0.08 | 0.85 | 0.13 | <0.01 |
| | | | | | | | | | | | |
| 2B | Sirius red (mm ²) | 0.04 | 0.01 | 0.11 | 0.02 | <0.01 | 0.04 | 0.01 | 0.06 | 0.01 | n.s. |
| 2B | α -SMA (mm ²) | 0.48 | 0.46 | 2.98 | 0.30 | <0.05 | 0.67 | 0.07 | 3.11 | 0.59 | <0.05 |
| 2C | α -SMA mRNA | 1.07 | 0.12 | 4.21 | 0.51 | <0.001 | 0.91 | 0.08 | 4.47 | 0.65 | <0.001 |
| 2C | <i>Fsp-1</i> mRNA | 1.00 | 0.05 | 7.21 | 0.96 | <0.001 | 0.90 | 0.20 | 5.03 | 0.43 | <0.001 |
| | | | | | | | | | | | |
| 3B | Collagen I α 1 (mm ²) | 0.35 | 0.13 | 1.22 | 0.49 | <0.01 | 0.32 | 0.08 | 0.59 | 0.24 | n.s. |
| 3B | Collagen III α 1 (mm ²) | 2.19 | 0.39 | 3.21 | 0.63 | n.s. | 1.35 | 0.44 | 2.98 | 0.62 | <0.01 |
| 3B | Collagen IV α 1 (mm ²) | 4.38 | 0.77 | 5.26 | 1.05 | n.s. | 3.54 | 0.35 | 4.69 | 0.82 | n.s. |
| 3C | <i>Collagen I α1</i> mRNA | 1.30 | 0.35 | 23.16 | 8.86 | <0.001 | 0.31 | 0.05 | 8.78 | 0.56 | <0.001 |
| 3C | <i>Collagen III α1</i> mRNA | 1.26 | 0.30 | 23.83 | 5.42 | <0.001 | 0.47 | 0.09 | 5.62 | 0.81 | <0.001 |
| 3C | <i>Collagen IV α1</i> mRNA | 1.08 | 0.14 | 2.47 | 0.41 | <0.05 | 1.09 | 0.05 | 4.51 | 0.50 | <0.001 |
| | | | | | | | | | | | |
| 4 | pSMAD2 / SMAD2 | 0.06 | 0.01 | 0.22 | 0.06 | n.s. | 0.09 | 0.01 | 0.17 | 0.05 | n.s. |
| 4 | pSMAD3 / GAPDH | 0.45 | 0.20 | 1.22 | 0.19 | <0.01 | 0.08 | 0.02 | 0.06 | 0.03 | n.s. |
| | | | | | | | | | | | |
| 5A | pAkt Ser ⁴⁷³ / Akt | 0.04 | 0.11 | 0.40 | 0.06 | <0.001 | 0.03 | 0.01 | 0.12 | 0.03 | <0.05 |
| 5A | pAkt Thr ³⁰⁸ / Akt | 0.01 | 0.00 | 0.20 | 0.02 | <0.001 | 0.03 | 0.01 | 0.10 | 0.02 | <0.05 |
| 5A | pGSK3 β / GSK3 β | 0.24 | 0.05 | 0.05 | 0.09 | n.s. | 0.04 | 0.01 | 0.17 | 0.06 | n.s. |
| 5B | pERK / ERK | 0.21 | 0.05 | 1.17 | 0.20 | <0.001 | 0.38 | 0.09 | 1.12 | 0.10 | <0.01 |
| 5B | p-p38 / p38 | 1.51 | 0.37 | 2.11 | 0.29 | n.s. | 0.87 | 0.11 | 1.80 | 0.24 | <0.01 |
| | | | | | | | | | | | |
| 7A | <i>Pten</i> mRNA | 1.05 | 0.12 | 0.74 | 0.09 | <0.05 | 0.60 | 0.04 | 0.79 | 0.06 | <0.05 |

| Figure | Target | Veh | | BMP-7 | | <i>P</i> vs Veh | TGFβ1 | | <i>P</i> vs Veh | BMP-7 & TGFβ1 | | <i>P</i> vs Veh | <i>P</i> vs TGFβ ₁ |
|--------|-------------------------------|------|------|-------|------|-----------------|-------|------|-----------------|---------------|------|-----------------|-------------------------------|
| | | mean | SEM | mean | SEM | | mean | SEM | | mean | SEM | | |
| 6A | pAkt Ser ⁴⁷³ / Akt | 1.00 | 0.12 | 0.62 | 0.28 | n.s. | 2.04 | 0.50 | n.s. | 0.50 | 0.18 | <0.05 | <0.05 |
| 6A | pAkt Thr ³⁰⁸ / Akt | 1.00 | 0.08 | 0.50 | 0.06 | <0.01 | 1.09 | 0.09 | n.s. | 1.02 | 0.08 | n.s. | n.s. |
| 6A | pGSK3β / GSK3β | 1.00 | 0.09 | 2.04 | 0.06 | n.s. | 2.94 | 0.29 | <0.05 | 2.76 | 0.30 | <0.05 | n.s. |
| 6A | E-cadherin / GAPDH | 0.96 | 0.09 | 0.54 | 0.15 | n.s. | 0.44 | 0.01 | <0.01 | 0.83 | 0.13 | n.s. | <0.05 |
| 6B | pERK / ERK | 1.00 | 0.06 | 0.44 | 0.08 | <0.001 | 0.74 | 0.09 | <0.05 | 0.82 | 0.07 | n.s. | n.s. |
| 6B | p-p38 / p-38 | 1.00 | 0.29 | 1.56 | 0.69 | n.s. | 0.99 | 0.55 | n.s. | 0.89 | 0.40 | n.s. | n.s. |
| | | | | | | | | | | | | | |
| 7B | <i>Pten</i> mRNA | 1.01 | 0.07 | 2.15 | 0.29 | <0.05 | 1.69 | 0.04 | <0.001 | 4.39 | 0.85 | <0.001 | <0.05 |

Supplemental Table 1: Statistical analysis for quantitative PCR, morphometric analysis of immunohistochemical staining, and densitometric analysis of western blot protein analysis for Figures 1-7.

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