Activin-like kinase 3 is important for kidney regeneration and reversal of fibrosis

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Molecules associated with the transforming growth factor β (TGF-β) superfamily, such as bone morphogenetic proteins (BMPs) and TGF-β, are key regulators of inflammation, apoptosis and cellular transitions. Here we show that the BMP receptor activin-like kinase 3 (Alk3) is elevated early in diseased kidneys after injury. We also found that its deletion in the tubular epithelium leads to enhanced TGF-β1-Smad family member 3 (Smad3) signaling, epithelial damage and fibrosis, suggesting a protective role for Alk3-mediated signaling in the kidney. A structure-function analysis of the BMP-Alk3–BMP receptor, type 2 (BMPR2) ligand-receptor complex, along with synthetic organic chemistry, led us to construct a library of small peptide agonists of BMP signaling that function through the Alk3 receptor. One such peptide agonist, THR-123, suppressed inflammation, apoptosis and the epithelial-to-mesenchymal transition program and reversed established fibrosis in five mouse models of acute and chronic renal injury. THR-123 acts specifically through Alk3 signaling, as mice with a targeted deletion for Alk3 in their tubular epithelidum did not respond to therapy with THR-123. Combining THR-123 and the angiotsensin-converting enzyme inhibitor captopril had an additive therapeutic benefit in controlling renal fibrosis. Our studies show that BMP signaling agonists constitute a new line of therapeutic agents with potential utility in the clinic to induce regeneration, repair and reverse established fibrosis.

BMP7, a member of the TGF-β superfamily, acts as an antagonist of TGF-β–mediated activity1–3. BMP7 binds to three type I receptors, Alk2, Alk3 and Alk6, and a type II receptor, BMPR2, and has distinct activities in different cell types4, showing anti-inflammatory and anti-apoptotic functions, as well as promoting bone formation5–6. It is essential that BMPs interact with both type I and type II receptors for signaling, and ligand-induced activation of intrinsic serine/threonine kinase activity triggers the phosphorylation of receptor-regulated Smads (r-Smads). Smad2 and Smad3 are phosphorylated by type I receptors of TGF-β and activin, whereas Smad1, Smad5 and Smad8 act downstream of BMP type I receptors5. High-affinity receptors for BMP7 are present in kidney epithelial cells and are speculated to mediate BMP7 actions in the kidney7–8.

End-stage renal diseases of various etiologies have a positive correlation with the degree of tubulointerstitial fibrosis9–13. Here we show that Alk3 functions to inhibit fibrosis by controlling inflammation, apoptosis and induction of an epithelial-to-mesenchymal (EMT) program. We generated peptide agonists of BMP signaling that bind to Alk3 and found that one such peptide, THR-123, controls fibrosis and induces kidney regeneration.

RESULTS

Alk3 is a negative regulator of fibrosis

Both renoprotective properties and anti-fibrotic effects for BMP7 have been shown in various kidney disease models14–16, and BMP7 expression is suppressed in acute and chronic kidney injury17–20. We evaluated the expression of several BMP7 targets in mice with chronic renal injury over time. Alk3 expression, among all co-receptors tested, uniquely peaked at 1 week after kidney injury, and this expression remained higher between 3 and 6 weeks after injury compared to the expression in control kidneys (Fig. 1a and Supplementary Fig. 1a,b). At week 9 after injury, Alk3 expression was lower in injured kidneys compared to control kidney (Fig. 1a), probably as a result of depletion of Alk3-expressing cells. In contrast, the expression of BMP7 decreased the most out of all the molecules tested after kidney injury, reaching its minimum expression at 3 weeks after injury, and this expression remained low at 9 weeks after injury (Fig. 1a and Supplementary Fig. 1a,b).

In mice with nephrotoxic serum-induced chronic kidney fibrosis (NTN) (Fig. 1b), phosphorylated Smad1 (p-Smad1) accumulated in the nuclei of the tubules at week 1 after injury. Notably, p-Smad1 labeling decreased at week 6 compared to week 1 after kidney injury (Fig. 1c).

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which was a similar trend as the one we observed with Alk3 expression (Fig. 1a). These results further suggest that the BMP7-Alk3 axis correlates negatively with renal epithelial injury and interstitial fibrosis. To functionally address this observation, we deleted the Alk3 receptor in the tubular epithelial cells using the γ-glutamyl transpeptidase (γGT)-Cre transgenic mice bred with mice with floxed alleles of Alk3 (Alk3floxflox), which resulted in the excision of exon 3 of the Alk3 gene, leading to the absence of Alk3 protein expression in the kidney tubules of these mice.

We confirmed the specificity of γGT-Cre expression using R26R-Rosa-LSL-lacZ mice.21 A reporter analysis revealed that γGT-Cre; R26R-Rosa-LSL-lacZ mice, in which lacZ expression, driven by the constitutively active R26R promoter, is enabled by Cre-mediated excision of a stop cassette upstream of lacZ, show stronger expression of lacZ in the proximal kidney tubules compared to littermate control mice (Cre−; R26R-Rosa-LSL-LacZ mice) (Fig. 1d). LacZ was not expressed in organs other than the kidney, including the liver and hematopoietic compartment, of the reporter mice (γGT-Cre; R26R-Rosa-LSL-lacZ mice) (Supplementary Fig. 1d and data not shown).

In γGT-Cre mice crossed with Alk3floxflox mice, Alk3 protein expression was absent in the kidney tubules of γGT-Cre−; Alk3floxflox mice compared to control Alk3floxflox mice without Cre recombinase activity (γGT-Cre−; Alk3floxflox mice).22,23 (Fig. 1e). NTN induced severe crescentic glomerulonephritis with interstitial fibrosis initially in all the groups of mice we analyzed (Fig. 1f). Six weeks after the induction of NTN, fibrosis was more severe in γGT-Cre; Alk3floxflox mice (mice in which Alk3 has been deleted) compared to control mice (Fig. 1f). F4/80+ macrophages express Alk3, and this expression remained intact despite the deletion of Alk3 in γGT-Cre mice, confirming that the accelerated fibrosis observed in these mice is reflective of the deletion of Alk3 in their proximal tubules (Supplementary Fig. 1d). Such accelerated fibrosis in the Alk3-deleted mice was associated with enhanced activation of the TGF-β pathway, as shown by the increased labeling of p-Smad2 in the nucleus of the tubular epithelial cells of these mice (Supplementary Fig. 1e). Renal function, as determined by measurements of blood urea nitrogen (BUN) concentration, was significantly higher in the Alk3 deleted mice with fibrosis when compared to the control mice (Fig. 1g). We did not observe this phenotype in γGT-Cre mice or in γGT-Cre; Alk3floxflox mice that we did not subject to NTN (Supplementary Fig. 1f.g).

Inflammation associated with macrophage influx and renal epithelial apoptosis are considered to be key instigators of renal fibrosis.24 Mice with a deletion of Alk3 in the kidney tubular epithelium showed a greater influx of macrophages in association with fibrosis (Supplementary Fig. 1h) and a greater number of tubular epithelial cells with co-localization of the epithelial marker E-cadherin and the mesenchymal marker S100 calcium binding...

**Figure 1** Alk3 and BMP7 expression inversely correlate in kidneys developing progressive injury and fibrosis. (a) Expression of the indicated genes in kidneys of mice before (day 0) and after NTN induction (1 week, 3, 6 and 9 weeks after induction). (b) Representative Masson’s trichrome staining (MTS) of kidneys from control mice and from mice at 1 and 6 weeks after NTN labeled with antibodies against p-Smad1. Scale bar, 50 μm. The black arrowheads point to nuclei positively stained for p-Smad1 stained nuclei. (c) Representative images of kidneys from control mice and from mice at 1 and 6 weeks after NTN labeled with antibodies against γdase, which was a similar trend as the one we observed with Alk3 expression (Fig. 1a). These results further suggest that the BMP7-Alk3 axis correlates negatively with renal epithelial injury and interstitial fibrosis. To functionally address this observation, we deleted the Alk3 receptor in the tubular epithelial cells using the γ-glutamyl transpeptidase (γGT)-Cre transgenic mice bred with mice with floxed alleles of Alk3 (Alk3floxflox), which resulted in the excision of exon 3 of the Alk3 gene, leading to the absence of Alk3 protein expression in the kidney tubules of these mice.

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protein A4 (FSP1, also known as S100A4), indicative of an EMT program, in comparison to wild-type mice (Fig. 1h).

**Design of the BMP signaling pathway agonists**

We designed cyclic peptide agonists of the BMP signaling pathway by identifying regions of the three-dimensional structures of TGF-β (refs. 25,26) and BMP7 (ref. 27) that were probably involved in the receptor interactions and by comparing side chain solvent accessibility with the regions of the TGF-β superfamily aligned sequences that have the highest variability27. To further refine the regions of interest, we used a structure-variance analysis program,28 which weighs physical and chemical residue properties at each position based on their correlation with an activity. The goal was to identify receptor-binding regions and then optimize the sequence for specific BMP activities. We then mapped the highest scoring residue positions onto the three-dimensional structure of BMP7 (ref. 27). Of the three structural regions we identified27, the peptides designed around the finger 2 loop were the most promising for drug design. These peptides are 16 residues long with a molecular weight of ~2 kDa and are cyclized using a disulfide bond between the first and eleventh residue positions to stabilize the loop and preserve a threedimensional conformation that is similar to the conformation in the finger 2 loop of BMP7 (Fig. 2a).

The preliminary screening for optimization was based on anti-inflammatory efficacy in an *in vitro* cell-based assay that used a human renal tubular epithelial cell line (HK-2). The assay tested the ability of compounds to reverse the increase in the production of the cytokine interleukin-6 (IL-6) that resulted from stimulation of the cells with TNF-α (Supplementary Fig. 1I). We performed a sequence-activity analysis using the structure-variance analysis program. After six optimization cycles, several compounds emerged as the lead candidates. We further evaluated one such peptide A4 (FSP1, also known as S100A4), indicative of an EMT program, in comparison to wild-type mice (Fig. 1h).

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First, we analyzed the specific binding of the extracellular domains (ECD) of the various type I BMP receptors to THR-123. We determined the binding of cold BMP7 to the immobilized receptor ECDs by competition with iodine-125 (*¹²⁵*I)-labeled BMP7 and analyzed this binding using a scatchard analysis to determine the effective dissociation constants of BMP7 for each of the receptor ECDs. To obtain an estimate of the effective dissociation constant of THR-123 for a particular receptor ECD, we multiplied the dissociation constant for cold BMP7 by the ratio of the half-maximal effective dose (50%) of THR-123 to the ED₅₀ for cold BMP7. The data showed that THR-123 competes with BMP7 for binding to Alk3 and, to some extent, Alk2 (data not shown), whereas we observed no competition between THR-123 and BMP7 in binding to Alk6 (Fig. 2b), suggesting that of the three known BMP type I receptors, Alk3 is the predominant target for THR-123. The observed ratio of ~10⁴ of competitive EC₅₀ binding of THR-123 with Alk3 to that of BMP7 with Alk3 is likely a result of three effects: first, BMP7 binds as a dimer to two type I receptor ECDs (which are cloned as two copies for each crystallizable fragment (Fc)), whereas monomeric THR-123 binds to one type I receptor only, and, therefore, its affinity is the square root of the observed dimer affinity. Second, because of the difference in the sizes of the molecules (the molecular weight of the BMP7 dimer is ~19 times greater than that of THR-123), the available surface area of the BMP7 monomer is approximately three times that of the THR-123 monomer. Third, when a weakly binding compound competes with a compound with stronger binding, the ratio of the EC₅₀ values overestimates the ratio of the Kᵣ values by a factor of 2. Although these results are not informative about the *in situ* binding affinity of the peptides to native BMP receptors, they do support the selective binding capacity of THR-123 to Alk3 and Alk2 but not to Alk6.

We tested the stability of THR-123 in whole blood and plasma of rats in vitro. In PBS-mannitol buffer, THR-123 remained stable at 400 min, which was the endpoint of the experiment (Fig. 2c). In rat plasma, THR-123 degrades slowly, with a half-life of 358 min, whereas in rat plasma, THR-123 degrades rapidly. In rat plasma, the half-life of THR-123 is ~50 min, whereas in rat plasma, THR-123 degrades rapidly. In rat plasma, the half-life of THR-123 is ~50 min.
whole blood, THR-123 degraded rapidly, with a half-life of 70 min (Fig. 2c). We evaluated the persistence of THR-123 in the systemic circulation of rats using 125I–labeled compound (125I–THR-123) administered intravenously (i.v.). In both the rat plasma and whole blood, THR-123 concentrations immediately decreased within 5 min (by almost 90%) of administration, suggesting a very short half-life of THR-123 in the 6 phase (initial phase of rapid decrease in drug plasma concentration, reflecting drug distribution from the circulation to tissues) (Fig. 2d). A β-phase (reflecting drug metabolism and excretion) assessment of the concentration of 125I–THR-123 indicated a half-life of 55–58 min (Fig. 2e). Six hours after i.v. administration of 125I–THR-123, the majority of the radioactivity remained localized to the kidney and bladder of the rats (Fig. 2f), suggesting that THR-123 accumulates in the kidney and is excreted, through the bladder, into the urine. Orally administered 125I–THR-123 localized primarily to the kidney cortex of the rats within 1 h after ingestion and peaked at about 3 h after ingestion (Fig. 2g). Twenty-four hours after ingestion, most of the 125I–THR-123 associated radioactivity was cleared from the rats’ kidneys (Fig. 2g).

**THR-123 inhibits inflammation, apoptosis and the EMT program**

Inflammation is a key feature in renal fibrosis. BMP7 has anti-inflammatory activity, which prompted us to investigate the effect of THR-123 on the expression of several pro-inflammatory cytokines in cells from a human renal tubular epithelial cell line (HK-2). BMP7 and THR-123 inhibited TNF-α–induced IL-6 production in a dose-dependent manner (Supplementary Fig. 2a). THR-123 also inhibited TNF-α–induced IL-8 and intercellular adhesion molecule 1 (ICAM1) production in HK-2 cells (Supplementary Fig. 1a), suggesting that, similar to BMP7, THR-123 has anti-inflammatory properties.

BMP7 has also been reported to protect tubular epithelial cells (TECs) from apoptosis. We analyzed TGF-β–induced apoptosis in TECs by annexin V labeling (Supplementary Fig. 2b). BMP7 and THR-123 had similar anti-apoptotic activity, whereas we did not detect any such anti-apoptotic activity when we used a control scrambled cyclic peptide (Supplementary Fig. 2b,c). Hypoxia-induced apoptosis of the TECs was also inhibited by both BMP7 and THR-123 (Supplementary Fig. 2c). Additionally, cisplatin-induced apoptosis of TECs was inhibited by THR-123 (Supplementary Fig. 2d).

BMP7 has been shown to inhibit the TGF-β–induced EMT program. Similarly, THR-123 also inhibited the TGF-β–induced EMT program in TECs (Supplementary Fig. 3). TGF-β inhibited E-cadherin expression, whereas both BMP7 and THR-123 restored this TGF-β–suppressed E-cadherin expression to normal (Supplementary Fig. 3b,c). A control cyclic scrambled peptide had no substantial effect on the EMT program (Supplementary Fig. 3a,b). The effect of THR-123 on the EMT program is associated with the phosphorylation of Smad1 and Smad5 (Supplementary Fig. 3d). The TGF-β–induced expression of mesenchymal features associated with EMT program, such as Snail (encoding snail) and Ctgf, was inhibited by THR-123 (Supplementary Fig. 3e). After 48 h of incubation with TGF-β and epidermal growth factor, the TECs showed mesenchymal features indicative of the EMT program (Supplementary Fig. 3f–h). The TGF-β–induced EMT program in these cells was reversed by treatment with either BMP7 or THR-123 (Supplementary Fig. 3f–h). Treatment with a control peptide did not have a notable effect on the induction of the EMT program (Supplementary Fig. 3f–h). The THR-123–induced reversal of the EMT program was associated with a restoration of E-cadherin expression (Supplementary Fig. 3g).
THR-123 induces regeneration and reverses fibrosis

We analyzed the effect of THR-123 on acute renal injury using the ischemic re-perfusion injury (IRI) model in mice. Seven days after IRI, untreated control mice showed renal morphology that was consistent with acute renal tubular necrosis and was characterized by tubular dilatation and flattened epithelial cells with eosinophilic homogenous cytoplasm (Supplementary Fig. 4a). Mice treated with THR-123 had significantly less tubular damage in their kidneys after IRI compared to control mice (P < 0.05), however BUN concentrations were similar in both groups of mice (Supplementary Fig. 4a).

Unilateral ureteral obstruction (UUO) is a well established model of severe renal interstitial injury and fibrosis (Supplementary Fig. 4b). Five days after UUO, these kidneys have a significantly greater interstitial volume compared to normal kidneys that have not been subjected to UUO (Supplementary Fig. 4b). Oral administration of THR-123 (5 mg or 15 mg per kg of body weight) inhibited an interstitial volume expansion in the kidneys of treated mice after UUO compared to the kidneys of untreated mice after UUO (Supplementary Fig. 4b). Seven days after UUO, the kidneys of all experimental mice had severe fibrosis with increased interstitial volume (Supplementary Fig. 4b). Intraperitoneal administration of BMP7 ameliorated the interstitial volume expansion of the kidneys of treated mice compared to control untreated mice (Supplementary Fig. 4c). Both intraperitoneal and oral administration of THR-123 inhibited fibrosis (Supplementary Fig. 4c). The lower tubular damage seen with THR-123 treatment compared to no treatment was associated with a decreased expression of matrix components such as fibronectin (encoded by Fn1) and type I collagen (encoded by Col1a1) (Supplementary Fig. 4d).

We next analyzed the effect of THR-123 on the NTN model in mice. Kidneys with NTN show severe crescentic glomerulonephritis with interstitial damage and fibrosis. Such lesions develop in a progressive manner in CD1 mice (Fig. 3a and Supplementary Fig. 5a). Six weeks after NTN induction, these mice had severe crescentic glomerulonephritis with severe interstitial damage and fibrosis (Fig. 3b). Treatment with THR-123 (initiated at 6 weeks after NTN induction) improved the glomerular lesion (sclerosis) and tubular atrophy and fibrosis (Fig. 3b) and was associated with decreased expression of matrix components such as fibronectin (Fn1) and type I collagen (Col1a1) (Supplementary Fig. 5b). BUN concentrations were decreased after THR-123 treatment (Fig. 3c). We identified tubular cells with the EMT program as being positive for both FSP1 and E-cadherin. Similar to previous reports, we observed the EMT program only in kidneys with NTN (Fig. 3d). THR-123 treatment significantly decreased the number of cells with an EMT program (Fig. 3d). NTN kidneys had greater numbers of macrophage-1 antigen (Mac-1) and F4/80” macrophages compared to control normal kidney, and THR-123 treatment inhibited the accumulation of these macrophages (Supplementary Fig. 5c,d). THR-123–treated kidneys presented with a greater accumulation of p-Smad1 and p-Smad5 than untreated kidneys, revealing a possible stimulation of the ALK5–mediating pathway (Supplementary Fig. 5e).

Alport syndrome is an inherited kidney disease caused by mutations in genes encoding type IV collagen proteins. Mice deficient in
Figure 5  Treatment with a combination of CPR and THR-123 inhibits the progression of fibrosis associated with advanced diabetic nephropathy. (a) Representative histological PAS (top) and MTS (bottom) staining of kidney sections of mice 7 months after STZ-induced diabetic nephropathy (n = 2), of mice at 8 months after diabetic nephropathy induction (n = 3), of mice at 8 months after diabetic nephropathy induction who were treated with CPR (n = 3) and of mice at 8 months after diabetic nephropathy induction who were treated with a combination of CPR and THR-123 (n = 4). Scale bar, top, 10 µm; bottom, 50 µm. (b) Morphometric analysis of the glomerular surface area (top left), the mesangial matrix (top right), tubular atrophy (bottom left) and relative interstitial volume (bottom right) of the indicated experimental groups. Data are mean ± s.e.m. **P < 0.01; NS, not significant (ANOVA). (c) BUN measurements in the indicated experimental groups. Data are mean ± s.e.m. *P < 0.05, **P = 0.08 (ANOVA). (d) Representative images of E-cadherin and FSP1 immunolabeling of kidneys from the indicated experimental groups and the percent of E-cadherin and FSP1 double-positive tubules. Arrows point to double-positive cells. Scale bar, 25 µm. Data are mean ± s.e.m. *P < 0.05, **P < 0.01 (ANOVA).

The α3 chain of type IV collagen (COL4A3KO) mice mimic the renal disease associated with Alport syndrome. At 16 weeks of age, kidneys from COL4A3KO mice showed increased glomerular abnormality, tubular atrophy and fibrosis when compared to kidneys from wild-type mice (Supplementary Fig. 6a). Although THR-123 treatment did not alter the glomerular abnormalities, it did significantly inhibit the tubular atrophy and interstitial fibrosis seen in COL4A3KO mice (P < 0.01) (Supplementary Fig. 6a,b). The BUN concentrations were higher in COL4A3KO mice compared to wild-type mice, and THR-123 treatment significantly lowered the BUN concentration in COL4A3KO mice (Supplementary Fig. 6b). In kidneys from COL4A3KO mice, the number of cells showing an EMT program was significantly higher than in kidneys from wild-type mice (P < 0.01) (Supplementary Fig. 6d). THR-123 treatment inhibited the acquisition of an EMT program in cells from the kidneys of COL4A3KO mice (Supplementary Fig. 6e). The macrophage infiltration in kidneys from COL4A3KO mice was higher compared to kidneys from wild-type mice, and THR-123 treatment inhibited this macrophage infiltration in COL4A3KO mice (Supplementary Fig. 6e). THR-123–treated kidneys from COL4A3KO mice were associated with an increase in the amount of p-Smad1 and p-Smad5 compared to untreated COL4A3KO mice (Supplementary Fig. 6f).

Next, we evaluated the efficacy of THR-123 in controlling diabetic nephropathy in mice. CD-1 mice injected with streptozotocin (STZ) showed glomerular mesangial matrix expansion with a larger glomerular surface area associated with interstitial damages at 6 months after injection when compared to non-injected control mice, suggesting advanced diabetic nephropathy in the injected mice (Fig. 4a and Supplementary Fig. 7a). Although neither BMP7 nor THR-123 inhibited a glomerular surface area increase, both BMP7 and THR-123 inhibited mesangium expansion in diabetic mice (Fig. 4b). Furthermore, THR-123 treatment (treatment from month 5 to month 6 after induction of diabetic nephropathy) substantially reversed the mesangial matrix expansion as compared to that seen in mice five months after induction of diabetic nephropathy (before THR-123 administration began) (Fig. 4b). Mice with diabetic nephropathy had greater tubular atrophy and interstitial volume compared to control mice (Fig. 4b). Treatment with BMP7 (starting at month 1 until month 6 after induction of diabetic nephropathy) or THR-123 (starting at month 5 until month 6 after induction of diabetic nephropathy) inhibited the tubular atrophy and interstitial volume expansion (Fig. 4b). THR-123 substantially reversed the tubular atrophy and interstitial volume expansion compared to untreated diabetic mice (Fig. 4b). The BUN concentrations were higher in mice with diabetic nephropathy compared to untreated control mice when measured at 5 and 6 months after the administration of STZ (Fig. 4c). Both BMP7 and THR-123 significantly reversed the renal dysfunction seen in mice with diabetic nephropathy (Fig. 4c), inhibited the induction of an EMT program (Fig. 4d) and reduced the macrophage infiltration (Supplementary Fig. 7b,c). Kidneys of THR-123–treated with diabetic nephropathy also showed a greater number of p-Smad1– and p-Smad5–positive nuclei compared to kidneys from untreated diabetic mice (Supplementary Fig. 7d).
Angiotensin-converting enzyme inhibitors (ACEis) are a group of well established drugs that are used to control the progression of several chronic progressive kidney diseases, including diabetic nephropathy. Therefore, we tested THR-123 and an ACEi (captopril (CPR)) in combination in mice with advanced renal fibrosis associated with diabetic kidney disease. Seven months after the induction of diabetes, the kidneys of mice with diabetic nephropathy showed greater glomerular surface area and mesangial matrix deposition than kidneys from untreated control mice (Figs. 4 and 5). CPR treatment or the CPR with THR-123 combination treatment was initiated in mice with severe diabetic nephropathy at 7 months after the induction of diabetic nephropathy. The glomerular surface areas were identical in all groups analyzed at this time point and at 8 months after the induction of diabetic nephropathy (Fig. 5a,b). CPR treatment did not inhibit the progression of mesangial matrix expansion in these experiments, but treatment with a combination of CPR and THR-123 significantly reduced the mesangial expansion and substantially reversed it compared to untreated control mice (Fig. 5b). At between 7 and 8 months (late stage) after the induction of diabetes, the kidneys of mice with diabetic nephropathy showed tubular atrophy and interstitial volume expansion (Fig. 5b). Treatment with CPR alone partially inhibited the tubulointerstitial alterations in the kidneys of these mice, whereas a combination of CPR and THR-123 completely inhibited the tubular atrophy and interstitial volume expansion (Fig. 5b). A BUN concentration analysis revealed that mice with diabetic nephropathy have marked renal function deterioration at between 7 and 8 months after the induction of diabetes (Fig. 5c). Treatment with CPR did not significantly inhibit the progressive loss of renal function (P = 0.08), but the combination therapy did (Fig. 5c). The EMT program was inhibited by treatment with CPR alone and also by the CPR and THR-123 combination treatment (Fig. 5d). Both CPR and the CPR and THR-123 combination therapy inhibited macrophage infiltration (Supplementary Fig. 7e). Treatment with CPR significantly inhibited apoptosis in the kidneys from diabetic mice (P < 0.05), and the CPR and THR-123 combination therapy had additive anti-apoptotic effects (P < 0.01) (Supplementary Fig. 7f). Kidneys from diabetic mice treated with CPR and THR-123 were associated with an increased accumulation of p-Smad1 and p-Smad5 (Supplementary Fig. 7g). Blood sugar concentrations and body weight were not altered in any of the groups analyzed compared to untreated diabetic mice of similar ages (Supplementary Fig. 7h).

**Loss of Alk3 prevents THR-123 activity**

The binding of THR-123 to the Alk3 receptor induced actions that mimic those induced by BMP7. Our experiments suggested that THR-123 functions to suppress renal injury and fibrosis by inhibiting inflammation, apoptosis and the EMT program. To functionally validate the target of THR-123 in exerting such renoprotective activities in mice, we tested the efficacy of THR-123 in the Alk3-deleted mice that we subjected to renal injury. We subjected the Alk3-deleted mice and their littermates (controls) to IRI. THR-123 inhibited renal injury in the control mice but did not have any therapeutic effect in the Alk3-deleted mice (Supplementary Fig. 8a). An Alk3-dependent action of THR-123 in the control mice was associated with a reduction in macrophage accumulation (Supplementary Fig. 8b) and decreased tubular apoptosis (Supplementary Fig. 8c).

We observed accelerated renal failure and fibrosis in Alk3-deleted mice with NTN compared to the control mice (Fig. 6a). We found that although THR-123 was successful in controlling renal injury and fibrosis in the control mice, it had no efficacy in the Alk3-deleted mice (Fig. 6a). Such therapeutic effects of THR-123 in control mice with NTN were associated with the inhibition of macrophage accumulation in the kidney and of the EMT program in the tubules, whereas THR-123 inhibited neither macrophage accumulation (Fig. 6b) nor the induction of an EMT program (Fig. 6c) in Alk3-deleted mice with NTN. THR-123 inhibited apoptosis in kidneys from control mice but had no effect on apoptosis in the kidneys from Alk3-deleted mice (Supplementary Fig. 8d). Finally, THR-123 restored renal function
in control mice with NTN, but we did not see any such renoprotective effect of THR-123 in Alk3-deleted mice (Fig. 6d).

**DISCUSSION**

The TGF-β superfamily proteins have considerable influence on the pathogenesis of renal fibrosis. Many of the molecules in this family, most notably TGF-β1 and TGF-β2, have been identified as positive regulators of fibrosis because of their ability to recruit myofibroblasts, facilitate the EMT program, influence inflammation and induce epithelial cell apoptosis. Although much focus has been placed on the positive role of TGF-β1 in fibrosis, BMP7 (another molecule in the TGF-β superfamily) was found to inhibit and reverse fibrosis. BMP7 action is realized through its anti-inflammatory, anti-apoptosis and EMT-suppressive actions. BMP7 counterbalances the actions of TGF-β1 through Smad-dependent pathways. In the kidney, TECs predominantly express the Alk3 receptor. Therefore, an ideal therapeutic candidate for renal fibrosis is one that binds to the Alk3 but not the Alk6 receptor and induces BMP signaling but does not induce osteogenic activity. Whereas the concentration of BMP7 decreases in the context of renal injury, the role of Alk3 in the progression of renal disease is unknown. Our results suggest that the expression of BMP7 and Alk3 inversely correlate with the progression of fibrosis, supporting the notion that BMP7 has a probable renoprotective role in opposition to the action of TGF-β1. Similarly, in this study we identify that Alk3 is also a positive regulator of renal health during injury. Alk3 responds to renal injury in a protective fashion, and its loss augments the progression of renal fibrosis. These results, coupled with the anti-fibrotic activity of BMP7, provide the necessary rationale to design a new class of molecules that serve as BMP signaling agonists through their binding to Alk3. We tested one such candidate molecule, THR-123, to determine its mechanism of action and therapeutic efficacy.

THR-123 suppressed the progression of kidney disease and substantially reversed established kidney fibrosis without inducing any osteogenic activity. Notably, whereas Alk3 receptor expression decreases during end-stage kidney disease, THR-123 continues to have substantial activity. This suggests that the diminished concentration of Alk3 in not a rate-limiting factor in the efficacy of THR-123. We show that a combination of THR-123 and CPR, an ACEi has an additive therapeutic effect in controlling the renal fibrosis associated with diabetic nephropathy. Collectively, our results indicate that THR-123 inhibits inflammation, apoptosis and the EMT program and reverses renal fibrosis. It is possible that the Alk3 receptor may also contribute to the action of THR-123, but experiments using the Alk3-deleted mice suggest that such Alk2-mediated activity, if present, is minimal.

In summary, our study suggests that the Alk3 receptor is a negative regulator of fibrosis when the kidney is injured. THR-123, a small-peptide BMP-signaling agonist that binds to Alk3 and activates the Smad pathway, has therapeutic efficacy when administered orally to mice with fibrosis. These renoprotective properties mirrored the action of BMP7, the natural ligand of Alk3, in the kidney. These preclinical studies offer insights into the use of BMP signaling pathway agonists in the treatment of acute and chronic kidney injury. Additionally, recent studies have suggested that Alk3 activity is key in mediating hepcidin action to keep serum iron concentrations low, and deficiency of Alk3 can result in an increase in serum iron concentrations leading to iron overload, mimicking human iron overload disorders known as hereditary hemochromatosis.

We speculate that THR-123 in this setting may serve the purpose of increasing Alk3 activity and reducing iron concentrations. Conversely, an antagonist of Alk3 could increase iron concentrations in the setting of anemia of chronic diseases. Future studies should explore such important therapeutic considerations.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

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**AUTHOR CONTRIBUTIONS**

R.K. conceptually designed the strategy for this study, participated in discussions, provided intellectual input, supervised the studies and wrote the manuscript. H.S. performed experiments and analyzed the data. M.Z. participated in the discussions and performed experiments. W.B., P.K., D.B., W.C., M.R., P.R., G.T., H.O., D.T., B.T. and V.S.L. performed experiments, analyzed the data and edited the manuscript and generated the figures. K.K. and V.S.L. supervised in vitro experiments, helped in the writing of the manuscript and generated the figures.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Mice. The Alk3flox/flox mice were bred with γGT-Cre mice to generate mice with a deletion of the Alk3 receptors in their proximal tubular cells. The γGT promoter used to generate the Cre-expressing mice was designed to be expressed predominantly in the kidney proximal and distal tubules, with undetectable expression in the liver. The mice were on a BALB/c background. COL4A3KO mice on a C57BL/6 background were previously described31. NTN, IRI, UUO and diabetic nephropathy models are described in Supplementary Methods section. The doses and routes for treatment of THR-123, BMP7 and CPR (and combination therapy) are detailed in the Supplementary Methods section. All animal experiments were reviewed and approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Synthesis and pharmacokinetics of THR-123. Radio-ligand receptor competitive binding assays of THR-123 for specific the individual type I receptors of Alk3 and Alk6 were performed using a highly purified ECD of Alk3 or Alk6 (expressed as a fusion protein with an Fc domain) immobilized on each well. A peptide analog or unlabeled BMP7 was added, which was followed by the addition of 125I-labeled BMP7. The radiolabeled BMP7 complex was counted in an automatic gamma counter. Unlabeled BMP7 served as a positive control in both assays. Quantification of the concentration of THR-123 in the systemic circulations of Wistar rats was performed by measuring the total radioactivity using an automatic gamma well counter. Elimination of THR-123 from the body after oral administration was evaluated by measuring the radioactivity in the kidneys of rats orally administrated 125I–THR-123 at a dose of 5 mg per kg of body weight.

Reagents. Monoclonal antibody to E-cadherin was purchased from BD Biosciences. Antibody to Mac-1 was purchased from AbD Serotec. Antibodies to F4/80 and BMP7 were purchased from Abcam. Antibodies to p-Smad1 and p-Smad5 were purchased from Cell Signaling Technology. Antibodies to Alk3 and E-cadherin were purchased from Santa Cruz Biotechnology. For the western blot analyses, antibodies to Alk3 was purchased from EMD Millipore. Measurements of BUN concentration were performed using the QuantiChrom Urea Assay Kit (BioAssay Systems) or the DIUR-500 QuantiChrom Urea Assay Kit (Gentaur). Measurements of urine albumin and urine creatinine were performed using the QuantiChrom Assay for albumin (DIAG-500) and creatinine (DICT-250) Kit (BioAssay System). Details of the morphometric analyses of the histological findings are listed in the Supplementary Methods section.

Quantitative PCR analysis. Total RNA was isolated from the kidney using a Trizol/Invitrogen PureLink RNA Mini Kit for RNA extraction. Ten nanograms of total RNA were used for generating complementary DNA using the TaqMan One-Step RT-PCR Master Mix (Applied Biosystems). Quantitative PCR was performed to analyze the gene expression profiles of the listed genes using ABI PRISM 7,000 (Applied Biosystems). The genes and primer sequences are listed in the Supplementary Methods section.

Detection of lacZ. Kidney samples (1 mm²) from 6-week-old R26R-Rosa-LSL-LacZ mice23 with or without Cre-recombinase expression were fixed at 4 °C for 4 h in 4% paraformaldehyde. Samples were washed three times with PBS at pH 7.3 and then stained overnight at 37 °C with lacZ staining buffer (1 mg ml⁻¹ X-gal, 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM MgCl₂, 0.02% NP-40 and 0.01% sodium deoxycholate in PBS). After washing with PBS at pH 7.3, the samples were embedded into paraffin. Sections (10μm) were then deparaffinized and counterstained with eosin. Statistical analyses. ANOVA followed by a Bonferroni-Dunn test for multiple comparisons of mouse samples was used to determine significance. A Student’s t test analysis was used for the single-parameter comparisons. Statistical significance was defined as P < 0.05. GraphPad Prism software was used for the statistical analyses.