Inhibition of Activin Signaling Slows Progression of Polycystic Kidney Disease

Wouter N. Leonhard,* Steven J. Kunnen,* Anna J. Plugge,* Arja Pasternack,† Sebastian B.T. Jianu,* Kimberley Verraar,‡ Fatiha el Bouazzaoui,* Willem M.H. Hoogaars,§ Peter ten Dijke,‖ Martijn H. Breuning,* Emile De Heer,‡ Olli Ritvos,† and Dorien J.M. Peters*

Departments of *Human Genetics, ‡Pathology, and ‖Molecular Cell Biology and Cancer Genomics Centre Netherlands at the Leiden University Medical Center, Leiden, The Netherlands; †Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland; and §Department of Human Movement Sciences, Faculty of Behavior and Movement Sciences, Vrije Universiteit Amsterdam, MOVE Research Institute, Amsterdam, The Netherlands

ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD), characterized by the formation of numerous kidney cysts, is caused by PKD1 or PKD2 mutations and affects 0.1% of the population. Although recent clinical studies indicate that reduction of cAMP levels slows progression of PKD, this finding has not led to an established safe and effective therapy for patients, indicating the need to find new therapeutic targets. The role of TGF-β in PKD is not clearly understood, but nuclear accumulation of phosphorylated SMAD2/3 in cyst-lining cells suggests the involvement of TGF-β signaling in this disease. In this study, we ablated the TGF-β type 1 receptor (also termed activin receptor–like kinase 5) in renal epithelial cells of PKD mice, which had little to no effect on the expression of SMAD2/3 target genes or the progression of PKD. Therefore, we investigated whether alternative TGF-β superfamily ligands account for SMAD2/3 activation in cystic epithelial cells. Activins are members of the TGF-β superfamily and drive SMAD2/3 phosphorylation via activin receptors, but activins have not been studied in the context of PKD. Mice with PKD had increased expression of activin ligands, even at early stages of disease. In addition, treatment with a soluble activin receptor IIB fusion (sActRIIB-Fc) protein, which acts as a soluble trap to sequester activin ligands, effectively inhibited cyst formation in three distinct mouse models of PKD. These data point to activin signaling as a key pathway in PKD and a promising target for therapy.


Autosomal dominant polycystic kidney disease (ADPKD) is a frequent disorder affecting one in 1000 individuals, and it involves the polycystic kidney disease 1 (PKD1) gene, which is mutated in 85% of patients, and the PKD2 gene, which is mutated in 15% of patients.1–3 Other than extrarenal manifestations, such as liver cysts, pancreas cysts, hypertension, cardiovalvular abnormalities, and cerebral aneurisms, the kidney is the most severely affected organ.4 Patients develop thousands of renal cysts, causing anatomically distorted, enlarged, and fibrotic kidneys, which ultimately lead to renal failure around the age of 60 years old.5,6 When the levels of functional Polycystin 1 (PC1) or PC2, the gene products of PKD1 and PKD2, respectively, deviate too much from the normal levels, the likelihood of cyst formation increases.7–12 In addition, the context of the renal tissue is crucial in determining whether cyst formation occurs and at what growth rate this process takes place. Under normal...
conditions, the adult kidney is relatively resistant to cyst formation, but during renal development, renal injury, or continuous stress on renal tissue imposed by existing cysts, cells are prone to take part in cyst formation after they have lost PC function.13–18

Multiple signaling pathways, such as mammalian target of rapamycin, cAMP, Ca++, Wnt, STAT3, and Src/Ras/Raf/MEK/extracellular signal–regulated kinase among others, seem to be involved in driving cyst formation.19 In addition, we showed in a previous study that TGF-β signaling may also play a role in PKD.20 After binding of the TGF-β ligands (TGF-β1, -β2, or -β3) to the TGF-β type 2 receptor (TGF-βR2) TGF-β type 1 receptor (also termed activin receptor–like kinase 5 [ALK5]) is recruited and phosphorylated, which then phosphorylates SMAD2 and SMAD3. These phosphorylated SMAD2 and SMAD3 (pSMAD2/3) proteins form a complex with SMAD4, and this complex can enter the nucleus to initiate the transcription of various genes.21 In pathologic conditions, TGF-β signaling is known to drive fibrosis in various ESRDs.22–25 In cancer, TGF-β can either inhibit tumor formation or promote metastasis depending on the specific conditions in the tumor microenvironment. We previously found increased levels of nuclear pSMAD2 also in cystic epithelial cells, suggesting a possible role for TGF-β in these cells.20 Although TGF-β inhibited cyst formation in three-dimensional cyst cultures of ADPKD cells,26 the pleiotropic actions of TGF-β render it difficult to predict the exact role of TGF-β in the context of the polycystic kidney.

To better understand the role of TGF-β signaling in cyst formation and assess whether TGF-β signaling can be used as a therapeutic target to inhibit disease progression, we crossed kidney–specific, tamoxifen–inducible Cre–Pkd1lox/lox mice (iKspCre–Pkd1lox/lox) with mice in which exon 3 of the Alk5 gene is flanked by LoxP sites.13,27 As such, we generated iKspCre–Pkd1lox/lox, iKspCre–Pkd1lox/lox,Alk5fl/fl, Alk5lox/wt, and iKspCre–Pkd1lox/lox,Alk5lox/lox mice. Eleven weeks after tamoxifen administration at postnatal day 40 (P40) –P42 (i.e., these mice have mild tubular dilations), Alk5 expression was reduced in iKspCre–Pkd1lox/lox,Alk5lox/wt mice and reduced more in iKspCre–Pkd1lox/lox,Alk5lox/lox mice, indicating homozygous inactivation of Alk5 (Figure 1A). Next, we followed mice until the onset of end stage PKD (defined as blood urea [BU] concentration > 20 mmol/L). Both iKspCre–Pkd1lox/lox (referred to as Pkd1–cKO) and iKspCre–Pkd1lox/lox, Alk5lox/lox (referred to as Pkd1;Alk5–cKO) mice had a similar progression rate of PKD, and although there was a trend of the Pkd1;Alk5–cKO mice having slightly higher 2 kidney weight (2KW) -to-body weight ratios, this was not significant. Also, renal histology was not different, indicating that renal epithelial Alk5 expression does not play a significant role in the progression of PKD (Figure 1, B–D).

We next measured the expressions of SMAD2/3–dependent target genes by reverse transcriptase ligation–dependent probe amplification (RT-MLPA), which were massively upregulated in cystic kidneys of Pkd1–cKO mice compared with control mice (Figure 1E). Surprisingly, however, Pkd1;Alk5–cKO mice showed a similar upregulation, being only slightly less compared with that in Pkd1–cKO mice (Figure 1E). These results indicate that the role of renal epithelial Alk5 expression on cystogenesis or SMAD2/3–dependent signaling is limited and suggest the involvement of other pathways to account for these changes during cyst formation.

Activin Expression Is Increased in PKD, and Kidney Cells Respond to Activin

We previously found increased Inhibin-βA expression, which is a member of the TGF-β superfamily, in PKD samples.28 On dimerization, Inhibin-β chains can form different activin subtypes, which can drive SMAD2/3 phosphorylation through the ALK4 and ACTRII receptors.29 Here, we analyzed both Inhibin-βA and Inhibin-βB expressions and found that the expressions of both ligands were elevated at end stage PKD (defined as BU>20 mmol/L, which occurs approximately 16
weeks after tamoxifen administration) but also at early-stage PKD (11 weeks after tamoxifen administration, at which time the mice have mild tubular dilations), suggesting a possible role for activin signaling already at the early stages of cyst formation (Figure 2, A and B).

Cultured primary cells isolated from total kidneys from tamoxifen–treated Pkd1-cKO or Pkd1;Alk5-cKO mice and cultured Pkd1+/+ or Pkd1−/− immortalized proximal tubular epithelial cells were all capable to respond to activin A and TGF-β by inducing SMAD2 phosphorylation (Figure 2C). The response pattern of the primary kidney cells from tamoxifen–treated Pkd1;Alk5-cKO mice indicated reduced sensitivity to TGF-β, which is likely caused by the conditional ablation of Alk5 in the majority of cells (Figures 1A and 2C). Although these in vitro experiments do not recapitulate the complex signaling in the context of a cystic kidney, these results indicate that renal epithelial cells can potentially be stimulated by activins and TGF-β.

To further assess the role of activins in cystic kidneys, we aimed to inhibit activins in mouse models for PKD using a soluble activin ligand trap.

sActRIIB-Fc Inhibits Disease Progression in Two Mouse Models with Rapid PKD Progression

To explore the role of activin signaling in PKD and determine whether activins can be used as a therapeutic target for PKD, we used a soluble activin ligand trap (sActRIIB-Fc). We first used the Pkd1-cKO model, in which we inactivated Pkd1 by tamoxifen administration at P10–P12. Pkd1 inactivation matrix metalloproteinase-2 (Mmp2), and collagen, type 1, α1 (Col1α1) from kidneys of P40 tamoxifen–treated Pkd1-cKO mice (n=5) and Pkd1;Alk5-cKO mice (n=5) that were euthanized at the onset of renal failure caused by severe PKD (defined as having BU levels >20 mmol/L). Expression of the genes of Pkd1-cKO mice (n=5) without tamoxifen (No Tam Control) are also shown. Expression was measured by RT-MLPA. Hprt and ywhaz served as housekeeping genes to correct for cDNA input. Error bars indicate SDs. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 1. Conditional ablation of Alk5 in the renal epithelium does not affect PKD. (A) Renal expression of Alk5 in mice of different genotypes (iKspCre;Pkd1lox,lox;Alk5wt,wt, iKspCre;Pkd1lox,lox;Alk5lox,wt, and iKspCre;Pkd1lox,lox;Alk5lox,lox) treated with tamoxifen at P40–P42 and euthanized 11 weeks after tamoxifen. Adult iKspCre;Pkd1lox,lox;Alk5wt,wt mice without tamoxifen treatments served as controls (No Tam Control). Expression was measured by RT-MLPA. Hprt and ywhaz served as housekeeping genes to correct for cDNA input (n=5 mice per group). Error bars indicate interquartile ranges. (B) Renal survival analysis (a BU level of 20 mmol/L served as a cutoff point) of Pkd1-cKO (n=6) and Pkd1;Alk5-cKO (n=8) mice that were treated with tamoxifen at P40–P42. The ratio of KW to body weight expressed as a percentage (2KW/BW%) in the mice depicted in B. Error bars indicate interquartile ranges. (C) Representative hematoxylin and eosin–stained kidney sections from end stage polycystic kidneys of Pkd1-cKO and Pkd1;Alk5-cKO mice. Scale bar, 500 μm. (D) Expression of the SMAD2/3 target genes EDA splice form of fibronectin 1 [Fn1(EDA)], plasminogen activator inhibitor-1 (PAI1),
before P14 results in rapid cyst formation as opposed to Pkd1 inactivation after P14.13,14 As expected, at age P33, mice that received PBS had increased 2KW-to-body weight ratios, cystic indices (CIs), and BU levels, indicating deteriorating renal function (Figure 3, A–C). By contrast, mice that also received biweekly intraperitoneal (ip) injections of 3 or 10 mg/kg sActRIIB-Fc starting at P14 until the end of the experiment at age P33 showed improvement in all of these parameters and improvement of renal histology (Figure 3, Supplemental Figure 1).

To test if the cyst-reducing properties of sActRIIB-Fc are specific for just the conditional Pkd1–deletion model, we also used a different model for PKD. To this end, we also tested the efficacy of sActRIIB-Fc on hypomorphic Pkd1 nl,nl mice. These mice have in all of their cells only about 15%–20% of normal Pkd1 expression, and they already have small cysts at age P7; by the age of P20, they have developed massive PKD.10,32 In addition, we bred these mice on a different genetic background (i.e., the Pkd1 nl,nl mice in this study were F1 hybrids, having a fixed genetic background of exactly 50% C57BL6/J and 50% 129Ola/Hsd). We treated these mice with, in total, four ip injections of 0, 1, or 10 mg/kg sActRIIB-Fc between P10 and P21. Even this short treatment regimen showed significant improvements in renal function and size (Figure 4, Supplemental Figure 2).

The Improved Renal Health by sActRIIB-Fc Treatment Is Associated with Reduced Fibrosis and SMAD2/3-Dependent Signaling

The PKD phenotype is generally associated with increased proliferation and expression of SMAD2/3 target genes, which could be a reflection of increased fibrosis.20 We, therefore, measured the proliferation, apoptotic, and fibrotic indices of the P10 Pkd1-cKO mice. The proliferation indices were determined by taking the ratio of Ki67-positive to Ki67-negative nuclei of sections that were stained for this proliferation marker. The apoptotic index was measured in a similar fashion using sections stained for cleaved Caspase-3, which is a marker for apoptosis. The fibrotic index was measured by determining the amount of collagen fibers in the kidney tissue. The results showed significant improvements in all these indices compared to the PBS-treated group (Figure 4).

Figure 2. Increased Activin expression in PKD kidneys, and increased SMAD2 phosphorylation by Activin stimulation. (A) InhβA and (B) InhβB mRNA expression in kidneys from P40 tamoxifen–treated Pkd1-cKO mice euthanized 11 weeks after tamoxifen (mild PKD) or at the onset of renal failure (defined as having BU levels >20 mmol/L; severe PKD; n=5 mice per group). Expression of the genes of Pkd1-cKO mice without tamoxifen (No Tam Control) served as a reference. The expression was measured by qPCR. Hprt served as a housekeeping gene to correct for cDNA input. Error bars indicate interquartile ranges. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (C) Western blot and quantification of pSMAD2 and total SMAD2 on crude extracts from primary cells from a kidney of one P40 tamoxifen–treated Pkd1-cKO mouse (primary Pkd1-cKO) and one P40 tamoxifen–treated Pkd1;Alk5-cKO mouse (primary Pkd1;Alk5-cKO). Also, results of crude extracts of Pkd1+/+ (Pkd1+/+ proximal tubular epithelial kidney cells [PTECs]) and Pkd1−/− (Pkd1−/− PTECs) mouse PTECs are shown. All cell lines were either left unstimulated (US) or stimulated with 5 ng/ml TGF-β1 or 100 ng/ml activin A. Each stimulation experiment on the above-mentioned cell lines was performed at least three times. Representative experiments with two replicates of the same cell line of each condition are shown, and tubulin served as a loading control. pSMAD2-to-SMAD2 ratios are shown and were normalized to the US cells that were set to one. *P<0.05 compared with the US control; **P<0.01 compared with the US control; ***P<0.001 compared with the US control.
BASIC RESEARCH

females) were treated with tamoxifen at P10 and were additionally treated with 3 mg/kg sActRIIB-Fc (Figure 5, A and B). Taken together, these results indicate that the reduced cystic load achieved by sActRIIB–Fc treatment is associated with reduced SMAD2/3–dependent signaling and fibrosis.

**sActRIIB–Fc Treatment Slows PKD in Adult–Onset Pkd1-cKO Mice**

To investigate if activin inhibition could also slow disease progression in an adult-onset model for PKD, we conducted a survival experiment. We first treated male Pkd1-cKO mice with tamoxifen at P18–P20 to inactivate Pkd1. These mice have a young adult onset of PKD, with cyst formation from all tubular segments (Supplemental Figure 4) (W.N. Leonhard and D.J.M. Peters, unpublished data). After randomization of the mice in the different groups, biweekly treatments with PBS (PBS control) or 3 mg/kg sActRIIB–Fc were started at P46 (long treatment). In addition, one group of mice that received PBS from P46 was switched to 3 mg/kg sActRIIB–Fc treatment at P74 (late treatment). From approximately P78, the renal function of the mice was monitored weekly by measuring BU levels. BU of 20 mmol/L was used as a cut-off point to pinpoint the onset of renal failure. The median survival in days after the start of the long treatment regimen was 55 days in the PBS group, and it was significantly postponed to 97 days in the sActRIIB–Fc–treated group (Figure 6). sActRIIB–Fc treatments starting at 74 days did not significantly slow PKD, suggesting that the effect of sActRIIB–Fc is more prominent during the milder stages of PKD (Figure 6). Collectively, the improvement in renal health in three distinct models for PKD by sActRIIB–Fc treatment suggests that activin inhibition is an attractive approach to inhibit ADPKD.

**Additional Effects of sActRIIB–Fc**

Several studies showed that sActRIIB–Fc treatment increases body weight, which is primarily caused by increased muscle size because of the ability of sActRIIB–Fc to antagonize myostatin, which is a negative regulator for muscle growth. Because the 2KW-to-body weight ratio is influenced by the body weight, we summarized the body weights, KWs, 2KW-to-body weight ratios, and CIs of all mice in Supplemental Figure 5. Whereas the body weights at the start of each experiment generally were comparable between the groups, at the end of the experiments, the body weights of the sActRIIB–Fc–treated mice tended to be slightly higher. This was more prominent in the long–term sActRIIB–Fc–treated P18 Pkd1-cKO mice. Other than the effect on body weight, the data summary deposition in kidney sections using Sirius Red staining. The number of apoptotic cells increased in mice with Pkd1 deletion mice. (A–C) Pkd1-cKO mice (n=15 mice; eight males and seven females) were treated with tamoxifen at P10–P12 and euthanized at P33. Mice that were additionally treated with 3 mg/kg sActRIIB–Fc (n=9 mice; four males and five females) or 10 mg/kg sActRIIB-Fc (n=9 mice; four males and five females) from P14 to P33 had (A) improved renal function, (B) significantly lower KWs, and (C) lower CIs. (D) Shown are representative images of the renal histology of the different treatment groups. The mice treated with 3 or 10 mg/kg sActRIIB-Fc had improved renal histology compared with PBS-treated mice. Histology of all mice is shown in Supplemental Figure 1. Error bars indicate interquartile ranges. 2KW/BW%, ratio of KW to body weight expressed as a percentage; No Tam, no tamoxifen. Scale bar, 1 mm. **p<0.01; ***p<0.001.

Figure 3. Antagonizing activin signaling effectively inhibits cyst progression in conditional Pkd1 deletion mice. (A–C) Pkd1-cKO mice (n=15 mice; eight males and seven females) were treated with tamoxifen at P10–P12 and euthanized at P33. Mice that were additionally treated with 3 mg/kg sActRIIB–Fc (n=9 mice; four males and five females) or 10 mg/kg sActRIIB-Fc (n=9 mice; four males and five females) from P14 to P33 had (A) improved renal function, (B) significantly lower KWs, and (C) lower CIs. (D) Shown are representative images of the renal histology of the different treatment groups. The mice treated with 3 or 10 mg/kg sActRIIB-Fc had improved renal histology compared with PBS-treated mice. Histology of all mice is shown in Supplemental Figure 1. Error bars indicate interquartile ranges. 2KW/BW%, ratio of KW to body weight expressed as a percentage; No Tam, no tamoxifen. Scale bar, 1 mm. **p<0.01; ***p<0.001.

There were no statistically significant differences in proliferation between the tested groups (Figure 5, A and B). However, the fibrotic index was clearly reduced in 3- and 10-mg/kg sActRIIB–Fc–treated mice, which correlated with reduced expression of collagen, type 1, α1, and plasminogen activator inhibitor-1. However, compared with the PBS-treated mice, the expression of the EDA splice form of fibroactin 1 was only significantly reduced in mice treated with 10 mg/kg sActRIIB–Fc (Figure 5, C and D, Supplemental Figure 3). Because pSMAD2 protein levels in kidney extracts of the P10 Pkd1-cKO mice, as assessed by Western blotting, were below the detection limit, we were not able to connect the expression of these genes to the levels of pSMAD2/3 (data not shown). We, therefore, also immunoblotted kidney extracts of the Pkd1nl,nl mice that were treated with PBS or 1 or 10 mg/kg sActRIIB–Fc. pSMAD2 was only detected in PBS–treated Pkd1nl,nl mice, and it dropped below the detection limit in the sActRIIB–Fc–treated mice (Figure 5, E and F). Interestingly, total SMAD2 levels were also increased in Pkd1nl,nl mice compared with wild–type control mice, and they were reduced in the start of the long treatment regimen was 55 days in the PBS group, and it was significantly postponed to 97 days in the sActRIIB–Fc–treated group (Figure 6). sActRIIB–Fc treatments starting at 74 days did not significantly slow PKD, suggesting that the effect of sActRIIB–Fc is more prominent during the milder stages of PKD (Figure 6). Collectively, the improvement in renal health in three distinct models for PKD by sActRIIB–Fc treatment suggests that activin inhibition is an attractive approach to inhibit ADPKD.
At present, there is no approved, safe, and effective therapy for the treatment of ADPKD, although a few completed and ongoing clinical trials with the primary focus of inhibiting cAMP have shown clinical benefit in patients with ADPKD. Many other PKD-related signaling pathways are being studied as potential targets for the treatment of ADPKD. In this study, we focused on TGF-β and activins, which are members of the TGF-β superfamily, that signal through their receptors and can drive SMAD2/3 phosphorylation. Increased expression of SMAD2/3 target genes and extracellular matrix remodeling enzymes, expansion of the interstitial space followed by fibrosis, and the accumulation of psSMAD2 in cyst epithelial cells suggest their involvement in ADPKD. Because TGF-β is involved in driving fibrosis in different CKDs, its role likely has overlap with fibrosis in ADPKD. The role of TGF-β in driving cyst formation is less well studied, although in three-dimensional cyst cultures of ADPKD cells, TGF-β inhibited cyst formation. To study the specific role of TGF-β in renal epithelial cells in the context of a PKD phenotype, we ablated Tgf-βRI (Alk5) together with Pkd1 using a previously developed renal epithelial-specific and tamoxifen-inducible Cre system. As expected, primary cells from Pkd1-cKO mice were able to respond to TGF-β, whereas this response was abrogated in primary cells from Pkd1:Alk5-cKO mice. By contrast, in the PKD context in vivo, additional ablation of Alk5 only minimally affected the massive upregulation of SMAD2/3 target genes. We cannot exclude that other cell types may be involved in the increased expression of SMAD2/3 target genes in end stage PKD. Another explanation could be that Alk5-independent pathways contribute to these changes. Either way, our results indicate that renal epithelial expression of Alk5 does not contribute significantly to cyst progression in adult Pkd1-cKO mice. These data and the observed increased InhβA and InhβB expression in PKD pointed us to activins, which like the TGF-β ligands, are members of the TGF-β superfamily.

Activins are composed of two Inhibin-β chains and can stimulate SMAD2/3 phosphorylation via the activin type 2 receptors and ALK4. These ligands play key roles in cancer and wound repair programs. Of interest, mutations in the von Hippel–Lindau tumor suppressor gene stimulated hypoxia-inducible factor–dependent expression of activin B, which promoted tumor growth. However, whether activins stimulate or inhibit epithelial repair on damage and how they are confermed that sActRIIB-Fc treatment reduced KWSs and CIs or delayed the onset of renal failure.

We measured the weights of the quadriceps in the P10 Pkd1-cKO mice that were treated with 10 mg/kg sActRIIB-Fc and confirmed that muscle size was increased, which could explain the relative higher body weights of the sActRIIB-Fc–treated mice in this study (Supplemental Figure 6). Overall behavior and morphology was normal in sActRIIB-Fc–treated mice, and there were no obvious changes in liver, spleen, or pancreas tissues of the mice (data not shown). In addition, there was no sign of liver toxicity, because the alanine aminotransferase levels of the sActRIIB-Fc–treated mice in the survival experiment were similar or slightly lower compared with those in PBS–treated mice (Supplemental Figure 6).

DISCUSSION

The data presented in this study point to activins as novel players in driving the progression of PKD. Treatment with sActRIIB-Fc, which is capable of sequestering activins, inhibited cystogenesis in three distinct mouse models of PKD.
involved in fibrosis and cancer seem to differ among different tissues and also, depend on the specific context of the tissue, the interplay between stromal and parenchymal cells, and the expression of other members of the TGF-β superfamily. It is, therefore, difficult to predict the exact role of activins in the context of PKD. Because cyst formation is suggested to be a state of chaotic repair, it is tempting to speculate that the increased activin expression in PKD is, in fact, an underlying cause of this process. To test if blocking activin signaling could be a therapeutic strategy to treat ADPKD, we treated three different PKD mouse models with sActRIIB-Fc, which can be used as a soluble activin ligand trap. In all of these models, sActRIIB-Fc treatment was associated with slower cyst progression compared with that in PBS-treated littermates. Although in most analyses, there was a trend toward a dose-dependent response of the sActRIIB-Fc treatments, this did not reach statistical significance. The proliferation and apoptotic indices varied between the animals and were not clearly different between PBS– or sActRIIB-Fc–treated P10 Pkd1-cKO littermates. Although these data suggest that sActRIIB-Fc treatment did not significantly affect these processes, we cannot exclude that sActRIIB-Fc affects proliferation and/or apoptosis during any other time point throughout the course of the development of PKD. Whether this is the case remains to be investigated. However, the slower onset of PKD by sActRIIB-Fc treatment was associated with reduced SMAD2 expression, reduced SMAD2 phosphorylation, reduced SMAD2/3 target gene expression, and reduced collagen deposition, which are in line with the concept of the ability of sActRIIB-Fc to sequester activin ligands that are increased in PKD. However, sActRIIB-Fc is also known to sequester other ligands of the TGF-β superfamily, such as myostatin, growth and differentiation factor 11 (GDF11), and with lower efficiencies, a number of BMPs as well. In-house RNAseq data of mice with PKD showed that myostatin is not expressed in kidneys with or without PKD and that GDF11 expression is low and unchanged throughout the course of PKD progression (Malas, T et al., unpublished data). BMPs primarily

---

**Figure 5.** The improved renal phenotype by sActRIIB-Fc is associated with reduced SMAD2/3 signaling. (A) The proliferation index and (B) the apoptotic index were measured in renal samples of the P10 Pkd1-cKO mice in the indicated groups. (C) Collagen deposition was measured by Sirius Red staining (example images are shown in Supplemental Figure 3) from renal sections of the P10 Pkd1-cKO mice in the indicated groups. (D) The expression of EDA splice form of fibronectin 1 (Fn1[EDA]), plasminogen activator inhibitor-1 (PAI1), and collagen, type 1, α1 (Col1α1) from kidneys of the P10 Pkd1-cKO mice that were treated with 0, 3, or 10 mg/kg sActRIIB-Fc was measured by qPCR. The dotted lines indicate the expression levels of genotype– and age–matched Pkd1 floxed mice without tamoxifen. Hprt served as a housekeeping gene to correct for cDNA input. (E–G) Renal extracts from Pkd1<sup>nl,nl</sup> mice were immunoblotted and stained for pSMAD2, total SMAD2, or tubulin as a loading control. (E) A representative immunoblot of the indicated samples and protein expressions. (F and G) Quantification of (F) pSMAD2-to-tubulin ratios or (G) SMAD2-to-tubulin ratios. pSMAD2 was only detected in Pkd1<sup>nl,nl</sup> mice that were treated with PBS. For all quantifications, n=5 mice for all groups. These mice were representative of the actual averages on the basis of the ratio of KW to body weight expressed as a percentage. Error bars indicate SDs. n.d., Not detected; No Tam, no tamoxifen; WT, wild type. *P<0.05; **P<0.01; ***P<0.001; #the no tamoxifen control group had significantly lower numbers of apoptotic cells compared with each of the other groups (P<0.05); #PBS treatment compared with either 1- or 10-mg/kg sActRIIB-Fc-treated Pkd1<sup>nl,nl</sup> mice (P<0.05).
signal through SMAD1/5/8, which has been observed to not be significantly changed in two mouse models of PKD, suggesting that the cyst-reducing properties of sActRIIB-Fc are not likely mediated by its limited ability to sequester BMPs.20 sActRIIB-Fc treatment, indeed, increased muscle mass and body weight, which is likely because of the ability of sActRIIB-Fc to sequester the negative regulator of muscle growth myostatin. In addition, the ligand traps, including ActRlIA and ActRlIB variants, have been observed before to also prolong survival in cancer cachexia models, improve bone mineralization in models with established bone loss, suppress LPS–induced lung inflammation, improve obesity and obesity–linked metabolic disease, and correct ineffective erythropoiesis in mice with β-thalassemia by GDF11 inactivation.42–46 We, therefore, cannot fully exclude that the renal cyst reducing property of sActRIIB-Fc is actually secondary to effects of sActRIIB-Fc on other organs, such as the observed skeletal muscle hypertrophy. However, given the increase in the expression of activins within the cystic kidney and the reduction of SMAD2/3-dependent signaling and collagen deposition on sActRIIB-Fc treatment, our data point to a scenario in which the renal cyst–reducing property of sActRIIB-Fc is caused by its ability to trap activin ligands within the (pre)cystic kidney. How activins and their downstream signaling cascades are interconnected with other cyst–inducing pathways and how the combined processes induce the development of cysts are not yet clearly understood and remain to be elucidated.

Currently, several soluble receptor ligand traps are being tested in clinical trials for various purposes.47,48 One phase 2 trial to improve muscle function in patients with Duchenne Muscular Dystrophy using an sActRIIB-Fc variant had to be terminated because of side effects, which included bleeding of the nose and gum (NCT01099761). However, another 4-month phase 1 trial assessing the safety of an sActRIIA variant at a dosage of ≤1 mg/kg in healthy postmenopausal women reported that the treatments were safe and well tolerated.48 A number of phase 2 trials are currently being undertaken to further assess the therapeutic potential of the sActRIIA variant, primarily in diseases involving anemia. These studies will shed more light on the tolerability of treatments with such ligand traps.

Because patients with ADPKD likely require years of treatment, the treatment should have an excellent safety profile. The continuous improvements in the strategies to slow ADPKD are highly encouraging and will likely lead to a safe and effective therapy. Although it remains to be clarified if targeting activins can be applied safely in humans for longer periods, the effective inhibition of PKD in three mouse models by sActRIIB-Fc points to activins as potentially important targets for ADPKD treatment.

CONCISE METHODS

Mice and Treatments

Pkd1-cKO mice have a homozygously bred kidney–specific, tamoxifen–inducible Cre16 and two LoxP sites that flank exons 2–11 of the Pkd1 gene.13 For some experiments, these mice were crossed with mice having exon 3 of the Alk5 gene flanked by LoxP sites27 to obtain the indicated genotypes. These mice were all on full C57BL6/J genetic background. Oral tamoxifen administration to facilitate gene disruption was done as described previously.36 The mice received 200 mg/kg tamoxifen at P40–P42, 150 mg/kg tamoxifen at P18–P20, or 6 mg/kg tamoxifen at P10–P12. Tamoxifen administration at these dosages and ages is well tolerated on the basis of careful assessment of behavior, but the P10–P12 mice have reduced body weights caused by the tamoxifen treatments. Hypomorphic Pkd1nullnull mice have a PKG promoter–driven neomycin resistance gene that is flanked by LoxP sites in intron 1 of the Pkd1 gene. This neomycin resistance gene causes alternative splicing of the Pkd1 gene, resulting in reduced expression of Pkd1 wild–type transcripts, which leads to rapid cyst formation starting around P7.10,32 Male Pkd1nullnull mice on 129Ola/Hsd genetic background were crossed with female Pkd1nullnull mice on C57BL6/J genetic background to generate Pkd1nullnull F1 hybrids with a fixed genetic background of exactly 50% C57BL6/J and 50% 129Ola/Hsd. These F1 hybrids were used in the experiments. Because PKD in male P18 Pkd1-cKO mice progresses faster compared with PKD in female P18 Pkd1-cKO mice, we only used male mice for the survival experiment to reduce the number of mice needed for the experiment. For the rapid PKD models, male and female mice have a comparable progression rate, and both sexes were used.

The recombinant human sActRIIB-Fc protein is a fusion of a human Fc and the ActRIIB receptor, it was produced in house in CHO-S cell suspension cultures using chemically defined serum–free medium, and the protein was purified by affinity chromatography as described in detail in the work by Hulmi et al.49 Mice were treated twice a week.
with ip injections of 1, 3, or 10 mg/kg sActRIIB-Fc in PBS. As a control group, mice received ip injections of PBS twice a week.

Blood sampling and BU measurements were performed using Refflotron technology (Kerkhof Medical Service) as described previously.16

The local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the experiments performed.

**Gene Expression Analyses**

Kidneys from euthanized mice were removed, snap frozen in liquid nitrogen, and stored at −80°C until further processing. Kidneys were homogenized using MagnaLyser technology (Roche, Basel, Switzerland), and total RNA was isolated from kidney samples using Tri-Reagent (Sigma-Aldrich, St. Louis, MO). Gene expression was measured by either RT-MLPA or quantitative PCR (qPCR) as described previously.18

**RT-MLPA**

Briefly, 60–120 ng RNA was used to synthesize cDNA with strand-specific oligonucleotides in a single reaction with reverse transcription (Promega, Madison, WI). Forward probes (oligonucleotides with a universal sequence at the 5′ end) and reverse probes (oligonucleotides with a universal sequence at the 3′ end) were hybridized directly adjacent to each other onto the cDNA followed by a ligation step and PCR using a single universal sequence at the 5′ end. Probe sequences of the specific genes are available on request. Data were analyzed with LightCycler 2.0 (Roche, Basel, Switzerland), and total RNA was isolated from kidney samples using Magnalyser technology (Roche, Basel, Switzerland), and total RNA was isolated from kidney samples using Tri-Reagent (Sigma-Aldrich, St. Louis, MO). Gene expression was measured by either RT-MLPA or quantitative PCR (qPCR) as described previously.18

**qPCR**

cDNA synthesis was done with the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s protocol. qPCR was done on the LightCycler 480 II (Roche) using 2× FastStart SYBR-Green Master (Roche) according to the manufacturer’s protocol. Primer sequences are available on request. Data were analyzed with LightCycler 480 Software, version 1.5 (Roche). Gene expression was calculated using the LinRegPCR method as described previously50 and normalized to Hprt expression, giving the relative gene expression. Mean gene expression was measured by staining sections with Sirius Red: After deparaffinization, sections were stained with 0.2% phosphomolybdic acid (1 minute), 0.1% Sirius Red in picric acid (90 minutes), and then, saturated picric acid followed by standard ethanol/ xylool washes and mounting. All sections were stained simultaneously to avoid interexperimental variation, and all scanned images were processed exactly the same using Photoshop software (Adobe Systems, Inc., San Jose, CA). Specifically designed color palettes were used to first remove signal within cysts (leaving only the pixels from all tissue) and then, remove that of the tissue, except for the red Sirius Red signal (leaving only the Sirius Red signal). Large arteries were removed from the analysis. The ratio (Sirius Red pixels to complete tissue pixels) of the number of pixels was calculated as a percentage and defined as the fibrotic index. The proliferation index was determined on the basis of immunohistochemical staining with Ki67 (NCL Ki67p; Nova Castra) as described previously.31 The apoptotic index was determined in a similar fashion as the proliferation index but used rabbit anti-cleaved Caspase-3 (9661; Cell Signaling Technology, Danvers, MA). To determine the segmental origin of cysts in the kidneys of the P18 Pkd1−/− mice, we performed marker staining with rabbit anti-megalin (1:500; Pathology, Leiden University Medical Center, Leiden, The Netherlands) to detect proximal tubular cysts, goat anti-Tamm Horsfall protein (uro-modulin; 1:500; Organon Teknika-Cappel) to detect distal tubular cysts, or rabbit anti- aquaporin-2 (1:4000; Calbiochem, San Diego, CA) to detect collecting ducts as described previously.31

**Cell Culture and Western Blotting**

For in vitro analysis, primary cells were generated from the cortices of kidneys of tamoxifen-treated iKspCre;Pkd1lox,lox and iKspCre;Pkd1lox,lox;Alk5lox,lox mice. In addition, mouse Pkd1+/+ and Pkd1+/− proximal tubular epithelial cells were cultured and processed as described previously.31 Western blotting was performed on crude protein extracts using standard procedures as described previously with an antiphospho-SMAD2 antibody,51 an anti-α Tubulin antibody (CP06; Calbiochem), and a total SMAD2 antibody (3103; Cell Signaling Technology).20 For optimal performance using Western blot analysis of tissue extracts, TGX gradient gels were used from Bio-Rad (Hercules, CA). Densitometric analysis was carried out using Odyssey technology (LI-COR Biosciences, Lincoln, NE).

**Statistical Analyses**

Differences between survival curves were tested with the generalized Wilcoxon test. All other group comparisons were tested using two-tailed t tests.

**ACKNOWLEGMENTS**

We thank Dr. Stefan Karsson for providing us with Alk5 floxed mice.

This research was funded by Dutch Kidney Foundation grants NSN 140112 (to W.N.L. and A.J.P.) and IP11.34 (to W.N.L. and A.J.P.) and partially supported by Dutch Technology Foundation (Stichting Technische Wetenschappen) project 11823 (to W.N.L. and F.e.B.), which is part of The Netherlands Organization for Scientific Research,
The Netherlands Organisation for Scientific Research Earth and Life Sciences grant 820.02.016 (to S.J.K.), and Dutch Kidney Foundation consortium project CP10.12 (Developing Interventions to Hold Progression of Polycystic Kidney Disease; to K.V.).

DISCLOSURES

None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015030287/-/DCSupplemental.