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RESEARCH ARTICLE

Gα12 is required for renal cystogenesis induced by *Pkd1* inactivation

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ABSTRACT

Mutation of *PKD1*, encoding the protein polycystin-1 (PC1), is the main cause of autosomal dominant polycystic kidney disease (ADPKD). The signaling pathways downstream of PC1 in ADPKD are still not fully understood. Here, we provide genetic evidence for the necessity of Gα12 (encoded by *Gna12*, hereafter *Gα12*) for renal cystogenesis induced by *Pkd1* knockout. There was no phenotype in mice with deletion of *Gα12* (*Gα12*$^{-/-}$). Polyinosine-polycytosine (pI:pC)-induced deletion of *Pkd1* (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$) in 1-week-old mice resulted in multiple kidney cysts by 9 weeks, but the mice with double knockout of *Pkd1* and *Gα12* (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$) had no structural and functional abnormalities in the kidneys. These mice could survive more than one year without kidney abnormalities except multiple hepatic cysts in some mice, which indicates that the effect of Gα12 on cystogenesis is kidney specific. Furthermore, *Pkd1* knockout promoted Gα12 activation, which subsequently decreased cell–matrix and cell–cell adhesion by affecting the function of focal adhesion and E-cadherin, respectively. Our results demonstrate that Gα12 is required for the development of kidney cysts induced by *Pkd1* mutation in mouse ADPKD.

KEY WORDS: Polycystin-1, G proteins, Cadherin, and Polycystic kidney diseases

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the leading life-threatening genetic diseases. Kidney cysts start from the epithelia of kidney tubules, and slowly develop into multiple cysts. The integrity of nephrons is compromised by the enlargement cysts, which finally causes end-stage renal disease (ESRD). Mutation in *PKD1* (encoding polycystin-1, PC1) is found in 85% of ADPKD cases, where mutation in *PKD2* (encoding polycystin-2, PC2) accounts for 15% of cases (Sutters and Germino, 2003; Wilson, 2004; Zhou, 2009; Gallagher et al., 2010; Cornec-Le Gall et al., 2014). PC1 is a G-protein-coupled receptor (GPCR) and binds to all G proteins (Qian et al., 1997; Parnell et al., 1998; Yuasa et al., 2004). PC1 mediates cAMP-activated pathways, mammalian target of rapamycin (mTOR) (Shillingford et al., 2006; Novalic et al., 2012), planar cell polarity and E-cadherin/Wnt signaling (Huan and van Adelsberg, 1999; Luyten et al., 2010), and focal adhesions (Wilson et al., 1999; Israeli et al., 2010). In ADPKD, kidney cysts start *in utero* (Wilson, 2008). They gradually increase in size, filling with fluid that comes from glomerular filtrate in renal tubules or surrounding cells. By the age of 50 to 70, about half of people with ADPKD have chronic kidney disease (CKD) and renal failure. Dialysis or kidney transplant is the only treatment for them (Roitbak et al., 2004; Wilson, 2008). Some ADPKD patients also have other manifestations such as cardiovascular abnormalities, liver cysts, intracranial aneurisms, intestinal diverticuli and abdominal hernia (Morris-Stiff et al., 1997; Sutters and Germino, 2003; Wilson, 2004). There are several pathologic changes in the cystic epithelia of ADPKD, such as partial differentiation, dysregulation of proliferation and apoptosis, loss of cell polarity and dysfunction of cell–matrix and cell–cell interactions, focal inflammation and fibrosis (Cowley, 2004; Menon et al., 2011).

The G12 subfamily includes Gα12 and Gα13. Gα12 and Gα13 are widely expressed. They are 67% identical at the amino acid level. Their common signaling pathways include signaling through Rho kinase, MAPKs and Src kinase, and they couple to numerous receptors including angiotensin II, endothelin and others (Strathmann and Simon, 1991; Buhl et al., 1995; Katoh et al., 1998). Gα13 is crucial for blood vessel development as Gα13-null mice die at day E10 due to angiogenic defects (Gu et al., 2002). Gα12-deficient mice are viable and fertile without apparent abnormalities. However, Gα12 and Gα13 have overlapping functions. In mouse embryonic fibroblast cells, Gα12 and Gα13 have distinct roles in regulating cell migration (Gu et al., 2002; Goulimari et al., 2005). Gα12 is involved in regulation of Ca^{2+} (Huang et al., 2009), cell–cell junctions (tight junctions and adherens junctions) (Sabath et al., 2008), and integrin-related adhesions (Kong et al., 2009, 2010). *In vitro,* the cytoplasmic tail of PC1 selectively binds with Gα12 but not Gα13 (Yuasa et al., 2004). The direct PC1–Gα12 interaction is involved in controlling the apoptosis of renal cystic epithelial cells (Yu et al., 2011). Gα12 activation leads to cystic growth of kidney epithelial cells (Kong et al., 2009). Gα12 might have a fundamental role in renal cystogenesis caused by *Pkd1* mutation. To examine the hypothesis of whether Gα12 modulates renal cystic formation in ADPKD mice, we generated double-knockout mice for *Pkd1* and the gene encoding Gα12 (*Gna12*, hereafter *Gα12*) by crossing *Pkd1* inducible knockout mice with *Gα12*-knockout mice. Our results

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indicate that Gα12 is indeed a key signaling molecule downstream of PC1 and its absence protects the kidneys from cystogenesis in PC1-deficient mice.

RESULTS

Interaction of PC1 and Gα12 affects renal cystogenesis

We first determined how PC1 knockdown (by short hairpin RNA, shRNA) or Gα12 activation affected cystogenesis of kidney epithelial cells *in vitro*. Madin–Darby canine kidney (MDCK) cells expressing vector control and overexpressing PC1 grew spontaneously as tubular structures in a three-dimensional (3D) culture system. However, activation of Gα12, stimulated by thrombin, inhibited tubular formation and lead to cystogenesis (Fig. 1A). Cells expressing control shRNA cells formed cysts upon thrombin stimulation, similar to what we previously reported (Kong et al., 2009). PC1-silenced MDCK cells, however, formed cysts independently of thrombin stimulation (Fig. 1A).

We have previously reported that PC1 inhibited Gα12 activation through direct association (Yu et al., 2011). Gα12 could be crucial for kidney cystogenesis induced by *Pkd1* mutation. We performed an *in vivo* test of the hypothesis in mice with double-knockout of *Gα12* and *Pkd1* (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$). *Gα12*-knockout mice (*Gα12*$^{-/-}$) were alive and had no apparent abnormality, similar to a previous report (Gu et al., 2002). After *Pkd1* inactivation at 1 week of age, by polyinosine-polycytosine (pI:pC) injection to induce the Mx1Cre recombinase activity, multiple kidney cysts were observed in *Pkd1*-knockout mice (induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$) at 9 weeks of age, whereas wild type (WT, no injection of the

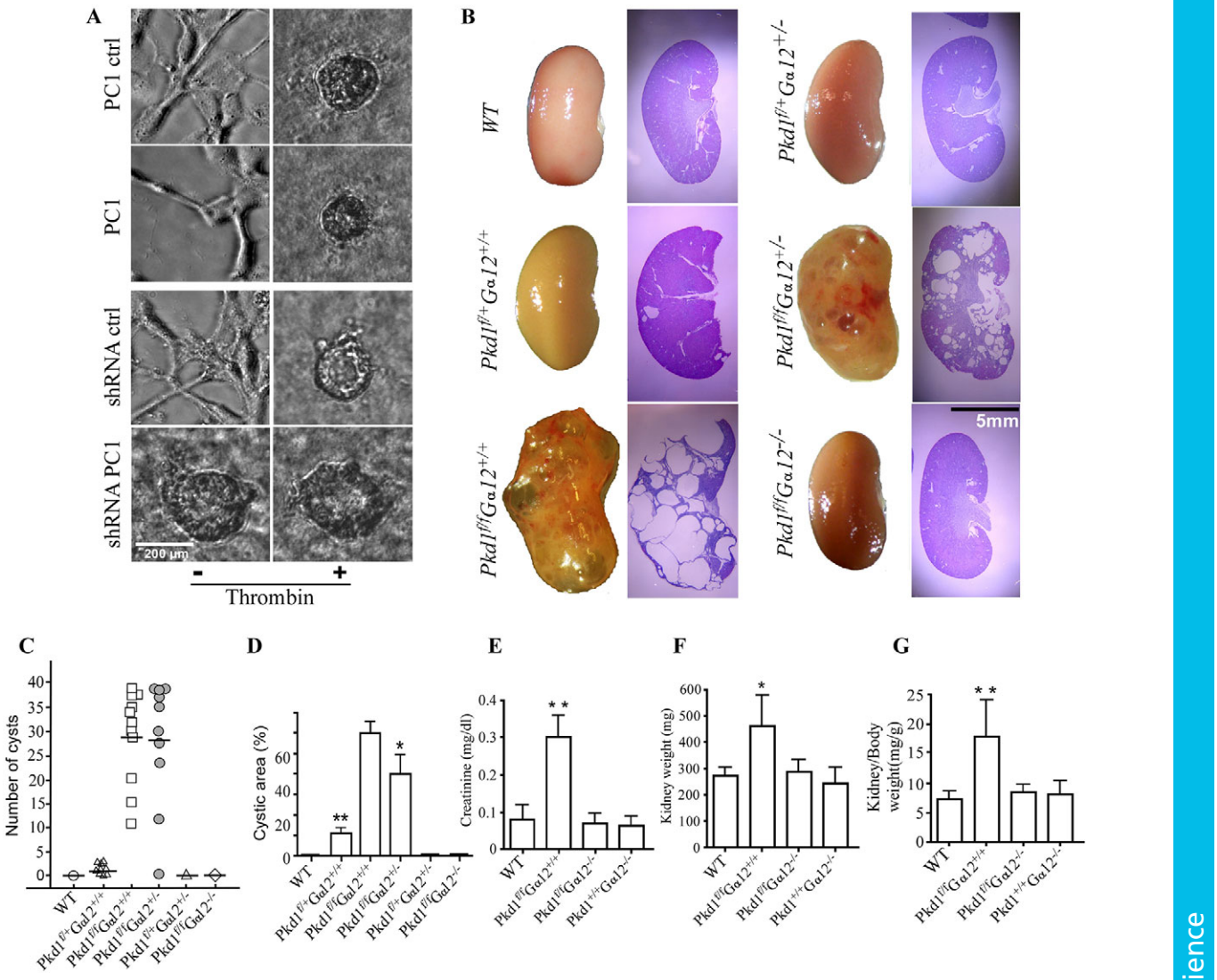

Fig. 1. Interaction of Gα12 and Pkd1 on kidney cystogenesis. (A) Overexpression of PC1 in MDCK cells induced tubule formation in 3D culture collagen I matrix. Knockdown of PC1 expression resulted in cyst formation. Activation of Gα12 (by thrombin stimulation) promoted cystic growth in PC1 overexpressing cells. (B) Inducible knockout of *Pkd1* in 1-week-old mice (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ *Gα12*$^{+/+}$) caused multiple cysts in kidneys after 9 weeks. Heterozygous deletion of *Pkd1* (*Mx1Cre*$^{+}$*Pkd1*$^{f/+}$ *Gα12*$^{+/+}$) produced mild kidney cysts. There were no abnormalities of kidneys in the double-knockout mice of *Pkd1* and *Gα12* (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ *Gα12*$^{-/-}$). The mice with heterozygous deletion of both *Gα12* and *Pkd1* (*Mx1Cre*$^{+}$*Pkd1*$^{f/+}$ *Gα12*$^{+/-}$) had normal kidneys. (C) The kidney cystic number and area (D) in *Pkd1*- and *Gα12*-knockout mice that are 9 weeks old. The mean of cyst number and area were calculated from three H&E stained slides of whole kidneys from *Mx1Cre*$^{+}$*Pkd1*$^{+/+}$ *Gα12*$^{+/+}$ (non-induced) (*n*=9), *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$ *Gα12*$^{+/+}$ (*n*=9), *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ *Gα12*$^{+/+}$ (*n*=11), *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ *Gα12*$^{-/-}$ (*n*=9), *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ *Gα12*$^{+/-}$ (*n*=10), and *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$ *Gα12*$^{+/-}$ (*n*=8) mice. The serum creatinine (E), kidney weight (F), and its ratio to the body weight (G) were measured to assess the renal function, the progress of the renal cysts. Values represent mean±s.d. of three whole kidney H&E slides. **P*<0.05; ***P*<0.01 (two-tailed *t*-test). Statistical analysis was performed as compared to *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ (D, E, F and G).

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inducer pI:pC) mice showed normal kidneys (Fig. 1B) as previously described (Takakura et al., 2008). The mice with induced *Pkd1* knockout in a *Gα12*-null background (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$) had normal appearing kidneys (Fig. 1B). Heterozygous deletion of Gα12 in these mice (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/-}$) led to fewer and smaller renal cysts than in *Pkd1*-knockout mice (induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$), which suggests that haploinsufficiency of Gα12 (*Gα12*$^{+/-}$) might not be adequate to completely prevent cyst formation but reduces the severity of cystic kidney disease. In addition, we observed less and smaller sized renal cysts in induced *Pkd1* heterozygous mice (*Mx1Cre*$^{+}$*Pkd1*$^{f/+}$*Gα12*$^{+/+}$), which were absent in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$*Gα12*$^{+/-}$ double heterozygotes (Fig. 1B,C). Morphometric measurements showed that the total cyst area followed a similar pattern. Extensive large renal cysts were observed in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice, but less renal cysts with smaller cyst area were observed in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$*Gα12*$^{+/+}$ mice (Fig. 1C,D). The kidney weight and the kidney to body weight ratio were also altered in these mice. Induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice had the highest serum creatinine and kidney to body weight ratio, whereas there was no statistical difference in creatinine levels and kidney weight between induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ and control mice (wild-type and non-induced *Mx1Cre*$^{+}$*Pkd1*$^{+/+}$*Gα12*$^{-/-}$ mice) (Fig. 1F,G). Under a microscope, large cysts were observed in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice but not in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ (Fig. 2).

In addition to renal cysts, ADPKD patients might have numerous other abnormalities such as brain aneurysms, heart defects and hepatic cysts (Sutters and Germino, 2003; Wilson, 2004; Gallagher et al., 2010). In induced *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$*Gα12*$^{+/+}$ 9-week-old mice, numerous small cysts were present on the hepatic surface (Fig. 3A). *Gα12*$^{-/-}$ mice appeared to have normal livers. In induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mice, hepatic cysts were present, similar to the liver cysts in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ (Fig. 3A). Most of *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice showed severe distress due to renal failure at the age of 3 to 4 months (Takakura et al., 2008), so these mice were killed when the distress appeared. Given that we noticed the absence of renal cysts in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mice, we chose these mice and related controls for a survival experiment. We closely monitored these mice until 14 to 15 months. These mice did not show any distress signs over the observation period except one mouse that died of a foot infection at the age of 9 months with unknown status of kidneys and liver

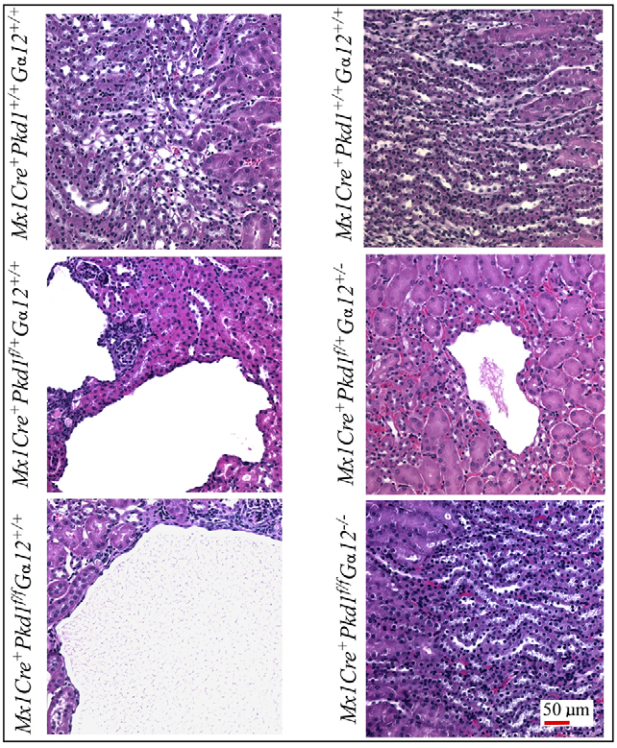

Fig. 2. High-resolution view of kidney tissue from mice. (A) Mice of the indicated genotype were intraperitoneally injected with of pI:pC at one week of age. The kidney samples were collected from 9-week-old mice, and stained with H&E.

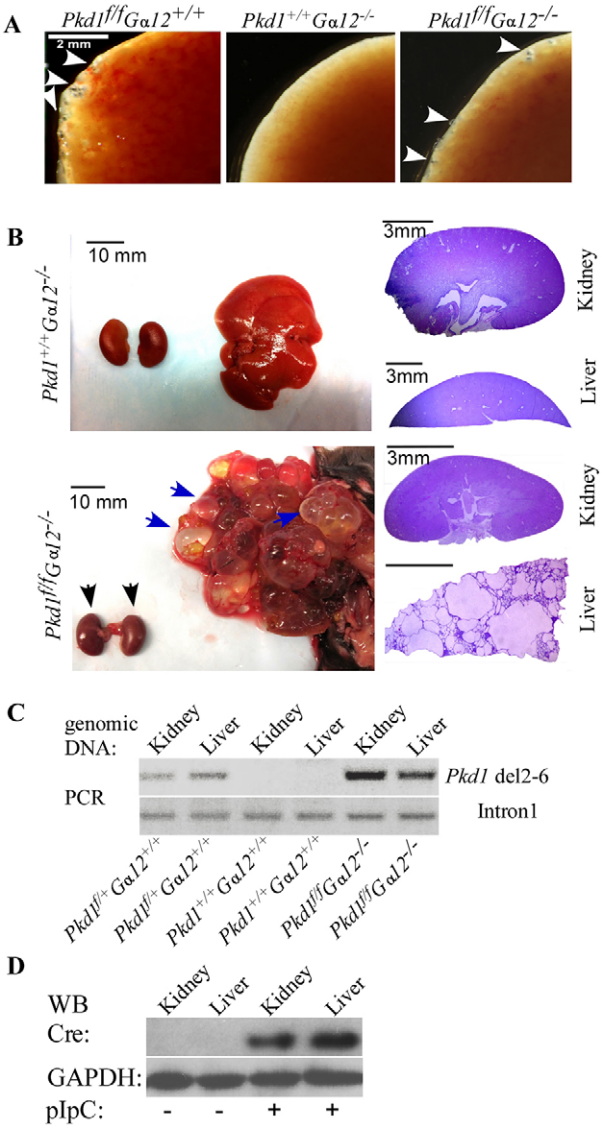

Fig. 3. Gα12 is not required for hepatic cystogenosis induced by *Pkd1* knockout. (A) Multiple small cysts (white arrowheads) developed in livers of 9-week-old mice with knockouts of *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ or *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ but not in *Pkd1*$^{+/+}$*Gα12*$^{-/-}$ mice. (B) *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mice survived for 14 to 15 months without kidney abnormalities (black arrows). Liver and kidney tissues were stained with H&E. The blue arrows indicate large liver cysts. (C) PCR confirmed the deletion of *Pkd1* exons 2–6 in genomic DNA from the liver and kidney tissues from the same mice. (D) In *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mice, the Cre recombinase protein was detected after injection of pI:pC (inducer). GAPDH served as loading control.

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change. In all other induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$mice, we did not see any kidney cysts. There was no difference in kidneys between noninduced *Mx1Cre*$^{+}$*Pkd1*$^{+/+}$*Gα12*$^{-/-}$ mice and induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mice. Both two kinds of mice did not show any kidney cysts (Fig. 3B). The deletion of *Pkd1* in the livers of these mice was further confirmed (Fig. 3C). The Cre expression was also confirmed in these tissues (Fig. 3D). Most of the induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$, *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ and *Mx1Cre*$^{+}$*Pkd1*$^{f/+}$*Gα12*$^{+/+}$ mice showed massive hepatic cysts (Table 1).

Pkd1 deletion increases Gα12 activation, which subsequently decreases the kidney epithelial cell-matrix adhesion

To examine the effect of PC1 on Gα12 activation in primary kidney epithelial cells, activated Gα12 was pulled down by GST–TPR as previously described (Yamaguchi et al., 2002). Deletion of *Pkd1* leads to the activation of Gα12 (Yu et al., 2011). Overexpression of PC1 reduced the levels of *Gα12* mRNA in human kidney epithelial cells (Fig. 4A). The levels of Gα12 were also elevated in cystic epithelia isolated from the kidneys of ADPKD patients (Fig. 4B). Active Gα12 is known to decrease the adhesion of MDCK cells to collagen I gel (Kong et al., 2009). In addition, overexpression of PC1 increases the attachment (adhesion) of MDCK cells on a collagen I gel, which was significantly impaired by Gα12 activation (Fig. 4C). Knockdown of PC1 also significantly reduced the adhesion of these cells to collagen I. The decrease was enhanced when Gα12 was activated by thrombin (Fig. 4D). In HEK293 cells, inducible expression of PC1 significantly increased the adhesion to collagen I, which was inhibited to a similar degree by thrombin or expression of constitutively active QLGα12 (Q229L mutation; Meyer et al., 2002). (Fig. 4E). In purified primary mouse tubular epithelial cells, deletion of *Pkd1* (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$) significantly reduced the adhesion, which was rescued to the WT level when *Gα12* was also knocked out (*Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$) (Fig. 4F).

To further investigate the relationship between PC1 and Gα12 on cell adhesion, we knocked down Gα12 with small interfering RNA (siRNA) in HEK293 cells (Fig. 4G). These Gα12-knockdown cells were no longer responsive to thrombin in regard to cell adhesion (Fig. 4H). Overexpression of PC1 increased cell adhesion, which was significantly reduced by thrombin-activated Gα12. However, the reduction caused by thrombin-stimulated Gα12 was abolished after Gα12 was knocked down (Fig. 4I). We transfected kidney epithelial cells with mutants of Gα12 that were all constitutively active. Mutants denoted O, Z and VV do not bind to PC1 whereas the mutants denoted OO, TT, KKK and GL are associated with wild-type PC1 [all the mutants were reported in detail previously (Yu et al., 2011)]. We transfected these Gα12 mutants into kidney epithelial cells with inducible expression of PC1 (Fig. 4J). In the absence of PC1 expression, all of these mutants caused a significant and similar reduction in cell adhesion (Fig. 4K). Overexpression of PC1 reduced the adhesion response of these cells elicited by the QLGα12 mutants OO, TT and KKK only and not in cells transfected with the mutants O, Z, VV (lacking the PC1-binding site) (Fig. 4L).

We previously showed that activation of Gα12 decreased focal adhesion formation of MDCK cells on a collagen I gel (Kong et al., 2009). Cell adhesion on collagen I is mostly mediated through integrin α2β1. We found that expression of PC1 increased the adhesion of MDCK and HEK293 cells to collagen I (Fig. 4E). There was no significant difference in surface expression of integrin α2β1 between PC1 overexpressing cells and control cells (Fig. 5A). Focal adhesion complexes play important roles in cell adhesion and migration. Besides integrins, focal adhesion kinase (FAK, also known as PTK2) and paxillin are also two key regulators of the formation of the focal adhesion complex. Similar to our previous findings (Kong et al., 2009), thrombin-stimulated Gα12 significantly reduced the phosphorylation of FAK at Y397 (Fig. 5B). Overexpression of PC1 hindered this effect by active Gα12, and increased phosphorylation of FAK at Y397 and at Y925 (Fig. 5C). However, thrombin activated Gα12 reduced the phosphorylation of FAK at Y407 and Y577, whereas expression of PC1 blocked this effect (Fig. 5C). Reduction of phosphorylated paxillin at Y118 was also observed with Gα12 activation; however, overexpression of PC1 did not seem to rescue this reduction (Fig. 5D). To assess the change of focal adhesions and stress fibers, we stained focal adhesions with vinculin and stress fibers with phalloidin in MDCK cells. In vector control cells, thrombin-activated Gα12 decreased focal adhesions (Fig. 5E, white arrowheads), but increased stress fibers (Fig. 5E, red arrowhead). PC1 expression increased focal adhesions and reduced stress fibers. Activated Gα12 reduced focal adhesions and enhanced stress fibers (Fig. 5E). Compared with control cells, knockdown of PC1 decreased focal adhesions but promoted stress fibers (Fig. 5F). Taken together, this result provides additional evidence that PC1 negatively regulates the effects of Gα12 on cell–matrix adhesion and stress fiber formation. PC1 affects the function of integrin α2β1 through Gα12 signaling.

Table 1. Mice with cysts in liver and/or kidneys

Gene Type	Months old	Mouse no.	Renal cysts	Hepatic cysts
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{-/-}$	14	4	0	3
Mx1Cre^{+}*Pkd1*$^{f/f-}$*Gα12*$^{-/-}$	15	5	0	4
Mx1Cre^{+}*Pkd1*$^{f/f-}$*Gα12*$^{-/-}$	9	1$^{\#}$	–	–
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{+/+}$	3	5	5	5
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{+/+}$	4	6	5	5
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{+/-}$	4	4	4	4
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{+/-}$	5	6	6	6
Mx1Cre^{+}*Pkd1*$^{f/f}$*Gα12*$^{+/-}$	7	2	2	2
Mx1Cre^{+}*Pkd1*$^{fl/+}$*Gα12*$^{+/+}$	5	3	3	3
Mx1Cre^{+}*Pkd1*$^{fl/+}$*Gα12*$^{+/+}$	6	5	5	3
Mx1Cre^{+}*Pkd1*$^{fl/+}$*Gα12*$^{+/+}$	8	2	2	2
Mx1Cre^{+}*Pkd1*$^{fl/+}$*Gα12*$^{-/-}$	15	8	0	6
Mx1Cre^{+}*Pkd1*$^{fl/+}$*Gα12*$^{+/-}$	15	7	0	7
Mx1Cre^{+}*Pkd1*$^{+/+}$*Gα12*$^{-/-}$	15	9	0	0
Mx1Cre^{+}*Pkd1*$^{+/+}$*Gα12*$^{+/-}$	15	8	0	0
Mx1Cre^{+}*Pkd1*$^{+/+}$*Gα12*$^{+/+}$	15	12	0	0

The experiment was ended after 15 months. If a mouse showed any distress mostly from renal cysts and renal failure, the mouse was killed before the end of experiment. #, died of foot infection; –, unknown status.

Pkd1 deletion or Gα12 activation results in the cleavage of E-cadherin and promotes the early form of N-cadherin in kidney epithelial cells

We have recently shown that deletion of *Pkd1* or Gα12 activation leads to the cleavage of E-cadherin, and disrupts adherens junctions in kidney epithelial cells (Xu et al., 2015). Here, we also found a change in the form of N-cadherin present after Gα12 activation or *Pkd1* deletion. Two forms of N-cadherin exist in certain epithelial cell types. The early form dominates in the cells that are widely dispersed and lack direct cell–cell contacts. When the cell density becomes high, tight cell–cell contacts are formed, and N-cadherin changes to its late form, with a lower molecular mass (Youn et al., 2006). In MDCK cells, we

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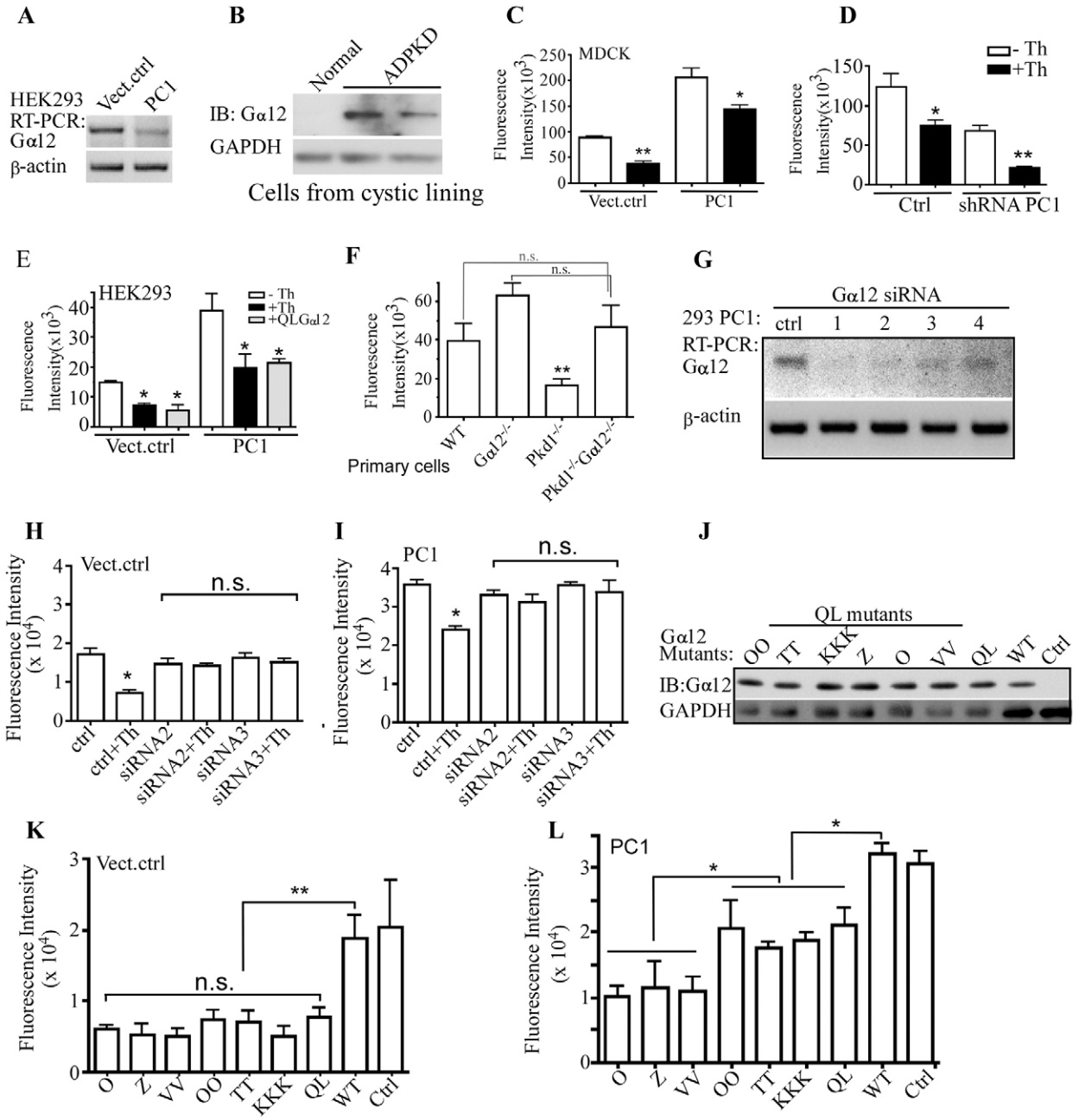

Fig. 4. *Pkd1* knockout increases activation of Gα12. (A) *Gα12* level in HEK 293 (mRNA). β-actin was the control for semi-quantitative RT-PCR. (B) Protein level of Gα12 in ADPKD human patient kidney tissue as determined by western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for the loading control. (C) Overexpression of PC1 increased the adhesion on collagen I gel, which was reduced by activation of Gα12. Knockdown of PC1 decreased the adhesion (D). +Th, with thrombin stimulation; -Th, without thrombin. (E) Expression of PC1 in HEK293 cells was induced by doxycycline. The adhesion of these cells on collagen I was significantly increased, which was reduced by Gα12 activation or expression of QLGα12 (+QLGα12, constitutively active mutant). (F) The primary kidney epithelial cells from ADPKD mice showed reduced adhesion. However, deletion of Gα12 did not lead to a significant change compared to wild type. (G–I) Gα12 expression was knocked down with siRNA (G), which abolished the reduction of adhesion by Gα12 (upon stimulation with thrombin) in HEK 293 cells (H) and upon ectopic expression of PC1 (I). (J–L) The indicated constitutively active Gα12 mutants were transfected into HEK 293 cells (J). OO, TT, KKK and QL mutants can associate with the cytoplasmic tail of PC1, and the Z, O and VV cannot associate with PC1. (K) All of these mutants decreased the adhesion of the cells. (L) After expression of PC1, the reduction of adhesion was significantly less in the mutants of OO, TT, KKK and QL, compared to the effect caused by the mutants Z, O and VV. Fluorescence intensity (Calcein AM) is proportional to the amount of attached cells on collagen I. The data are shown as mean±s.e.m. (*n*=3). **P*<0.05; ***P*<0.01; n.s., no significant difference (*P*>0.05) (*t*-test).

found that Gα12 activation led to an increase in the amount of the early form of N-cadherin, and downregulated the late form (Fig. 6A). Deletion of *Pkd1* also enhanced the amount of the early form (Fig. 6B). In ADPKD patients, all of the N-cadherin was of the early form except a very low level of the late form in one of the three kidney samples. However, in the normal control kidney tissue, the majority of N-cadherin was the late form (Fig. 6C).

DISCUSSION

Our data provide strong evidence that Gα12 activation promotes cystogenesis in the kidneys of ADPKD mice. In the absence of

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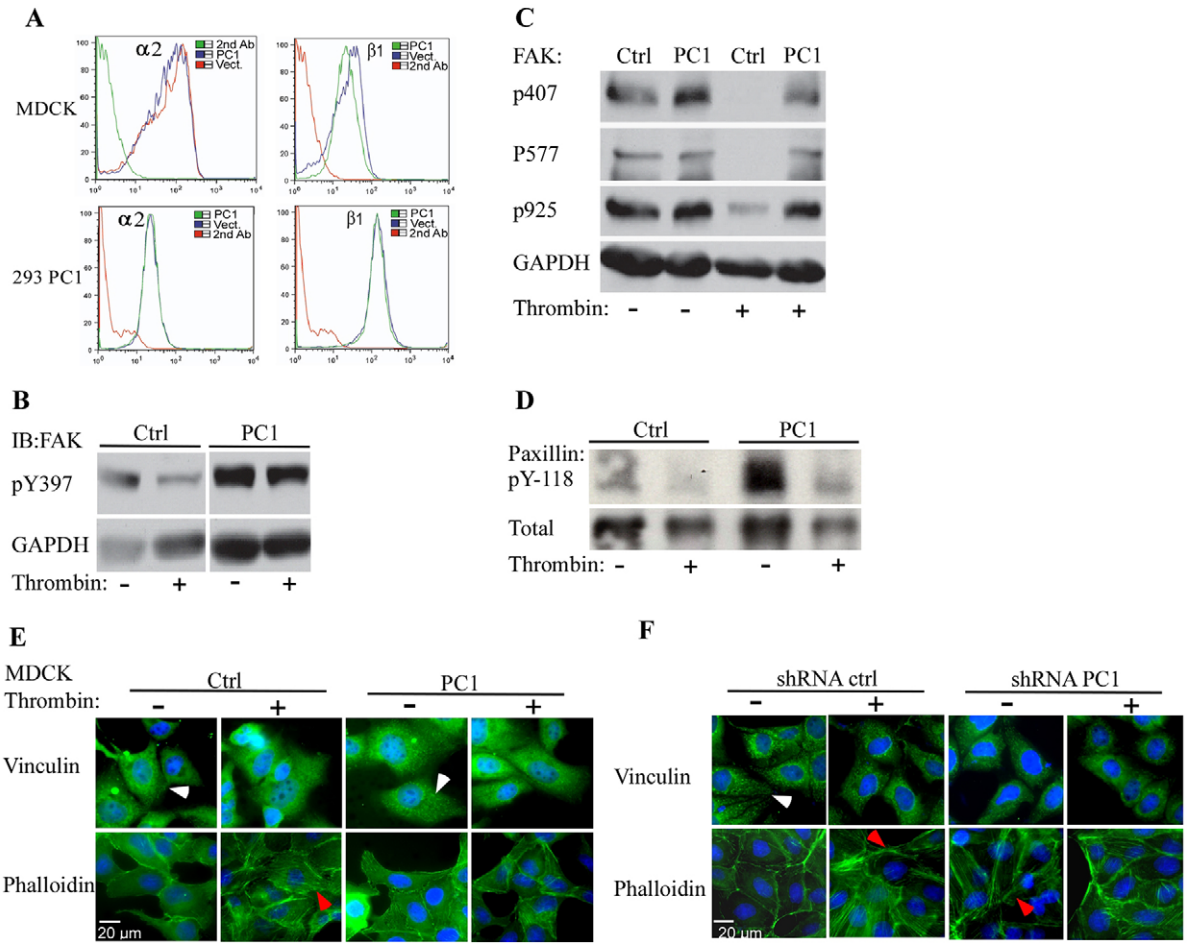

Fig. 5. PC1 and Gα12 affect focal adhesions and stress fibers. (A) Cell surface levels of integrin α2β1 upon ectopic expression of PC1 in MDCK and HEK293 cells as determined by flow cytometry. (B,C) FAK phosphorylation in MDCK cells as determined by western blotting. GAPDH, loading control. (D) Phosphorylation of paxillin at Y118 upon ectopic expression of PC1 or Gα12 activation (with thrombin). Loading control, total paxillin. (E,F) Focal adhesions (white arrowheads) and stress fibers (red arrowheads) were stained with vinculin and phalloidin, respectively, in MDCK cells expressing ectopic PC1 (E), and knocked down for PC1 (F). Blue color, nucleus staining with DAPI. The images shown here are representative of at least three different experiments.

Gα12, knockout of *Pkd1* could not induce renal cystogenesis in mice. After inactivation of *Pkd1*, Gα12 activity is increased, which subsequently leads to the changes in several aspects of kidney epithelial cells. These include disruption of cell–matrix adhesion (Kong et al., 2009) and cell–cell adhesion. Our results reveal that inhibition of Ga12 activation could block PKD renal cystogenesis, which could be used to develop a therapeutic target for ADPKD.

Development and expansion of multiple kidney cysts are the key pathological manifestation in ADPKD, which eventually destroys the nephrons and leads to deterioration of renal function. Mutation of *PKD1* is found in most ADPKD patients. PC1 protein is present throughout kidney epithelial cells, including at apical and basolateral sites (Sutters and Germino, 2003; Wilson, 2004; Gallagher et al., 2010; Cornec-Le Gall et al., 2014). PC1 is involved in different cellular functions in kidney epithelial cells, such as those mediated by Ca^{2+}, cAMP, JNK and AP-1, mTOR (Torres and Harris, 2009; Chang and Ong, 2012), JAK/STAT (Chauvet et al., 2004), integrins (Wilson and Burrow, 1999; Lee et al., 2015) and E-cadherin/Wnt (Huan and van Adelsberg, 1999). However, the key signaling pathway leading to disease remained unclear. Our findings indicate that Gα12 is a key signaling molecule for PC1, especially in pathological cystic development in ADPKD.

In 1-week-old mice, inducible deletion of *Pkd1* causes multiple kidney cysts developed at 3-4 weeks of age. These mice die of severe renal failure after about 3–4 months (Takakura et al., 2008). Although we did not see a significant difference in kidney cyst number between $Mx1Cre^{+}Pkd1^{f/f}G\alpha12^{+/+}$ and $Mx1Cre^{+}Pkd1^{f/f}G\alpha12^{+/-}$ mice (Fig. 1C), there was a significant difference in cyst area (Fig. 1D). This suggests that lacking one copy of *Gα12* allele is sufficient for inhibiting the expansion and enlargement of kidney cysts but cannot block the initiation of renal cysts. *Gα12*-knockout mice ($G\alpha12^{-/-}$) are phenotypically normal, whereas Gα13 knockout is lethal. However, the presence of at least one copy of the *Gα12* allele ($G\alpha12^{+/-}$) is required for survival of $G\alpha13^{-/-}$ mice (Yamaguchi et al., 2002). This suggests that there is a gene-dosage effect between *Pkd1* and *Gα12* that affects renal cystogenesis and the severity of ADPKD induced by inactivation of *Pkd1*. In our study, the induced $Mx1Cre^{+}Pkd1^{f/f}G\alpha12^{-/-}$ mice could survive more than 1 year without any kidney cysts. In ADPKD mice, knockout of *Pkd1* resulted in numerous cysts in the liver. We also found that *Gα12* knockout failed to rescue cystogenesis in the livers of induced $Mx1Cre^{+}Pkd1^{f/f}G\alpha12^{-/-}$ mice where normal kidneys, but large liver cysts, were seen. This finding indicates that cyst development in the liver is dependent on an alternative pathway that is different from that in the kidney. This finding is consistent with the finding that Soranefib coudl inhibit cyst development in human ADPKD cyst epithelial cells 3D culture but exacerbated liver cysts in PC2-defective mice (Yamaguchi et al., 2010; Spirli et al., 2012).

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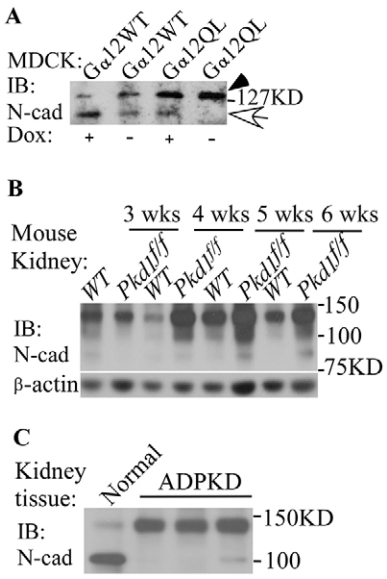

Fig. 6. The expression of two forms of N-cadherin. (A) Two different forms of N-cadherin were present in MDCK cells. In the cells transfected with WT Gα12, the major N-cadherin was the early mature form (white arrow), but in constitutively active Gα12QL cells, the major form was the late one (black arrowhead). (B) In *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{-/-}$ mouse kidney tissue, with increased age, the late form was also increased. (C) In the APPKD patient kidney tissue, the majority of N-cadherin was also the late form.

Cyst development in the kidney is dependent on Ras activation of B-raf homodimerization, whereas in liver cysts Ras activates B-raf–Raf1 hetrodimerization (Yamaguchi et al., 2010). Most ADPKD patients also have polycystic liver disease (PLD) (Chebib et al., 2016). However, some polycystic livers develop in the absence of polycystic kidneys. These isolated PLD instances are less frequent. The mutation of genes other than *PKD1* or *PKD2* is also contributory to PLD (Van Keimpema et al., 2011). In ADPKD patients, there is no relationship between the renal phenotype and the severity or growth of PLD, which indicates that modifiers are more important than the PKD gene to affecting the liver phenotype. Hence, it is possible that Gα12 is be involved in a signaling pathway that significantly influences only the renal phenotype, whereas a different G-protein regulates liver cystogenesis in ADPKD.

Total kidney volume (TKV) is used as a biomarker to assess the therapeutic effect of any regimen for ADPKD (Alam et al., 2015). Our results showed that the weight of kidneys and the ratio of kidney to body weight in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice increased. After deletion of *Gα12* in these mice, there was no difference in renal function and kidney weight from WT mice. Our findings demonstrate that the absence of *Gα12* blocked the development of multiple cysts induced by *Pkd1* inactivation. Tolvaptan (a vasopressin receptor antagonist) decreases the growth of total kidney volume (TKV) and maintains the glomerular filtration rate (GFR) in ADPKD for a short time period for the early stage of ADPDK (Boertien et al., 2015). It has passed a Phase III randomized, double-blind trial in Europe. The elucidation of PC1 signaling pathways in kidney epithelia will help develop a more effective regimen to block or slow down the development and expansion of kidney cysts in ADPKD. Given that *Gα12*-knockout mice are normal, we assume that inhibition of Gα12 in ADPKD could be a safe and effective target. As the kidney cystic area is much less in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/-}$ than in induced *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$*Gα12*$^{+/+}$ mice, partial inhibition of Gα12 might be sufficient to acquire a therapeutic response.

Disruption of cell–matrix adhesion is an important change in the renal epithelial cells in ADPKD (Wilson and Burrow, 1999). Focal adhesions regulate several biological processes in epithelial cells, such as cell–matrix interaction, migration and proliferation. The focal adhesion complex contains multiple proteins, such as integrins, talin, vinculin, and α-actinin, FAK, Src and paxillin etc (Sorenson and Sheibani, 1999; Zimerman et al., 2004; Zaidel-Bar et al., 2007). PC1 forms a complex with focal adhesion component proteins in renal epithelial cells (Wilson et al., 1999; Wilson and Burrow, 1999). Disorganized or disrupted focal adhesions in ADPKD renal epithelial cells are present in kidney cystogenesis (Geng et al., 2000). In ARPKD, the phosphorylation of both of Y319 and Y407 in FAK are inhibited (Israeli et al., 2010). We observed inhibited phosphorylation of FAK Y407 in MDCK cells. Gα12 activation decreases cell adhesions and migration by affecting integrins and other focal adhesion molecules (Kong et al., 2009). Deletion of integrin β1 in mice has recently been reported to block the development of kidney cysts and increase the survival rate of ADPKD mice (Lee et al., 2015). Decreased phosphorylation of FAK and paxillin by active Gα12 are likely to be an important component leading to reduced focal adhesions. Mammalian target of rapamycin complex 2 (mTORC2) is involved in controlling apoptosis and the cell cytoskeleton (Lieberthal and Levine, 2012a,b). Gα12 also participates in mTORC2 signaling (Shillingford et al., 2006). In addition, an increase in the amount of stress fibers by active Gα12 might also contribute to a change in focal adhesions and migration. β1 integrins are mostly located at the baso-lateral region. They regulate cell–matrix adhesion by binding to their ligands in the matrix, and cell–cell adhesion via their associated proteins (Kanasaki et al., 2008; Weitzman et al., 1995; Yeh et al., 2012). PC1 is present at baso-lateral and apical regions (Bukanov et al., 2002).

We have reported that Gα12 activation disrupted the adherens junction in kidney epithelial cell through shedding of E-cadherin (Xu et al., 2015). In a human kidney epithelial cell line, ectopic expression of PC1 enhanced the shedding of N-cadherin (data not shown). The early form of N-cadherin is consistent with rapid growth, whereas the late form of N-cadherin dominates when cell–cell contacts have been formed and there is less proliferation of epithelial cells (Youn et al., 2006). Our results showed that activation of Gα12 promoted the early form of N-cadherin even when cell–cell contacts had formed (Fig. 6A), which might cause the disruption of the integrity of cell–cell adhesion. The two forms of N-cadherin are slightly different in kidneys between ADPKD mice and patients (Fig. 6B,C). This could be from the species or the age difference. Mouse tissue was from 9-week-old mice, but the human kidney samples were collected from ADPKD patients suffering from renal cysts for dozens of years. In addition, we have shown previously that Gα12 activation increased the shedding of E-cadherin (Xu et al., 2015). Both E-cadherin and N-cadherin form homophilic interactions and regulate cell–cell contacts (Menke and Giehl, 2012; Bunse et al., 2013). In kidney epithelial cells, PC1 forms homophilic or heterophilic complexes for cell–cell adhesions (Streets et al., 2003). E-cadherin is one of the most important components in the PC1 complex. The association of E-cadherin molecules is very important for maintaining the integrity of E-cadherin-mediated adherens junctions in MDCK cells (Streets et al., 2009). N-cadherin and E-cadherin can also form heterodimers in adherens junctions through heterophilic binding (Prakasam et al., 2006), which suggests that their association might be very important for cystic epithelial cell–cell contacts, and also for contacts between cystic and surrounding normal epithelial cells. Reduced expression or loss of E-cadherin, but increase of N-cadherin ('cadherin switch')

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promotes epithelial cancer cell progression and metastasis (Straub et al., 2011). In ADPKD epithelial cells, the E-cadherin–N-cadherin switch is present during renal cyst development and progression (Roitbak et al., 2004). This switch could be mediated through a disintegrin and metalloproteinase 10 (Adam10) given that an Adam10-specific inhibitor could block the cystogenesis of MDCK cells (Xu et al., 2015).

In summary, Gα12 interacts with the cytoplasmic tail of PC1 in the apical region of renal epithelial cells. The knockout of *Pkd1* leads to the activation of Gα12. It then triggers the signaling pathways that promotes Adam10 activity, cleaves E-cadherin, changes the forms of N-cadherin, and affects cellular stress fibers and integrin-mediated focal adhesion. Nuclear translocation of catenin into the nucleus induces expression of its regulated proteins. Subsequently, all of these cause the change of cell morphology and growth behavior, which favor the cystic growth of renal epithelial cells (Fig. 7). Our findings reveal that Gα12 is the key signaling molecule for PC1 to initiate the pathological changes of cystic epithelial cells in ADPKD.

MATERIALS AND METHODS

Animals and kidney specimens

All of the mice were C57BL/6 strain. *Pkd1*-knockout mice ($Mx1Cre^{+}Pkd1^{f/f}$) were generated as described elsewhere (Takakura et al., 2008). Briefly, two LoxP sites were inserted between exon 2 and exon 6. Then these mice were crossed with *Mx1Cre* mice in which *Cre* recombinase was induced by an INF-inducible Mx1 promoter. *Gα12*-deficient mice ($Gα12^{-/-}$) were generated by replacement of exon 4 with a reverse Neo gene (Voyno-Yasenetskaya et al., 1996). Mice with double-knockout of *Pkd1* and *Gα12* were obtained by crossing $Mx1Cre^{+}Pkd1^{f/f}$ mice with $Gα12^{-/-}$ mice. The genotyping was performed by PCR on genomic DNA from mouse tails. The primers were as follows: *Gα12WT*, 5′-GTGCTCATCCTTCTTGGTTTCC-3′ and 5′-CGGG-TCGCCCTTGAAATCTGG-3′; *Gα12* mutant, 5′-GTGCTCATCCTTCTT-GGTTTCC and 5′-GGCTGCTAAAGCGCATGCTCC-3′; $Pkd1^{f/f}$, 5′-TTGCTGCCAGCTCTGTGTAT-3′ and 5′-CACAGCGGTAGGAAGAGG-AG-3′’ and *Mx1Cre*, 5′-TCCCAACCTCAGTACCAAGCCAAG-3′ and 5′-ACGACCGGCAAACGGACAGAAGCA-3′. Control littermates were the mice that did not have *Cre* or $Mx1Cre^{+}Pkd1^{f/f}$ mice that were not induced to express Cre recombinase. All these mice were labeled as $Mx1Cre^{+}Pkd1^{+/+}$. pI:pC (Sigma, St Louis, MO) was administrated to these mice by intraperitoneal injection in order to induce the expression of *Cre* recombinase, which then deleted *Pkd1*. We used 62.5 µg or 250 µg per mouse for 5 days, starting at 1 or 5 weeks old, respectively. The approval of the animal protocols was from the Standing Committee on Animals of Harvard Medical School. The gene type, $Mx1Cre^{+}Pkd1^{+/+}$ was for mice in which *Pkd1* was not deleted. $Mx1Cre^{+}Pkd1^{f/f}$, $Mx1Cre^{+}Pkd1^{f/+}$ represent mice with homegenous or heterogenenous, respectively, deletion of *Pkd1* after induced expression of Cre by injection of pI:pC. The mice (mixed male and female) were grouped randomly.

Dr Jing Zhou (Renal Division, Brigham and Women’s Hospital, Boston, MA) provided the kidney samples from control patients and ADPKD patients. All of the patient data were anonymous. The specimens were collected from certain diagnostic or therapeutic tissues. A written consent was given to each patient. The ethical committee and the institutional review board of Harvard Medical School approved the tissue collection. For all human tissue sample experiments, we confirm that all clinical investigation have been conducted according to the principles expressed in the Declaration of Helsinki.

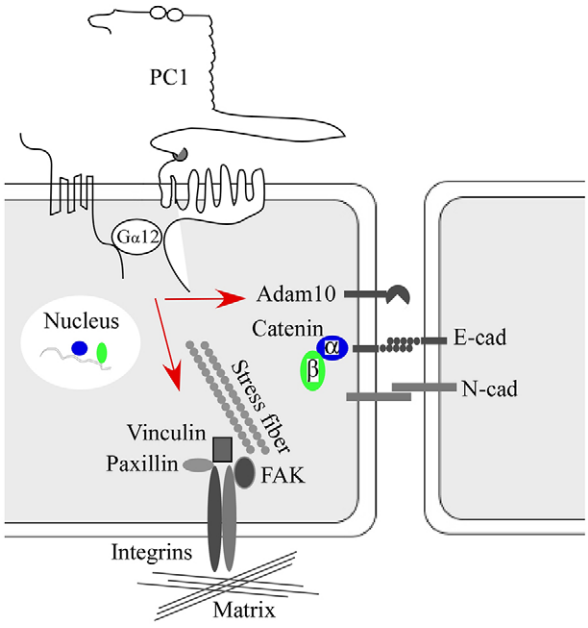

Fig. 7. Schematic diagram of the interaction between Gα12 and PC1 in kidney epithelial cells. At the apical area, Gα12 and PC1 are associated. Upon inactivation of PC1, Gα12 is activated, which causes the changes in E-cadherin-mediated adherens junction, N-cadherin, stress fibers and focal adhesion.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were stained with H&E. Then we used them for assessing pathological changes. Immunofluorescence analysis of cells was carried out as described previously (Kong et al., 2010). The secondary antibodies conjugated to Alexa Fluor 430 (green), and Alexa Fluor 532 (red) were from Invitrogen. Cre antibody (mAb 7.23) was from Abcam and was used at 1:500 dilution (Cambridge, MA). DAPI was from Vector Laboratories (Burlingame, CA). The images were obtained with a confocal microscopy (Leica TCS SP5). All other antibodies were described as in the previous report (Kong et al., 2009).

Three-dimensional cell culture

Primary mouse cells were collected as previously reported (Takakura et al., 2008). Cells from human cystic linings were from kidney cysts smaller than 3 cm size. After aspiration of cystic fluid, surgical dissected cystic linings were washed and treated with typsin-EDTA. Living, attached cells were collected for experiments. We used Gα12 and Gα12QL Madin-Darby canine kidney (MDCK) cell lines (Sabath et al., 2008). The 3D assay was detailed as reported previously (Kong, et al., 2009).

Cell adhesion and invasion

Cell attachment assay was performed as follows: sub-confluent MDCK cells were used for experiments. 50 µl of Calcein Am (Invitrogen) was added into 10 ml of DMEM (serum free), which was used to incubate the cells at 37°C for 30 min. Detachment buffer (Invitrogen) was used to collect cell from cultured dishes. Collagen-I solution (8.6 µg/ml) was used to coat the 96-well plate. A total of 2.5×10^{4} cells (100 µl serum-free DMEM) was used for each well. After 20–30 min, the suspended cells were gently washed away with PBS three times. The intensity of fluorescence was measured with a fluorimeter (Millipore, Bedford, MA). Cell spreading assay was performed as described previously (Kong, et al., 2009).

Immnuoblotting

Immunoprecipitation or pulldown was performed as previously (Sabath et al., 2008). The cell lysis and western blotting were carried out as reported (Kong, et al., 2009). Flow cytometry analysis was preformed as reported (Kong et al., 2009). Briefly, suspended cells were filtered with 40-µm sieves. Primary antibody was added into the cell at 4°C for 1 h. The second antibody, goat anti-mouse-IgG conjugated to FITC was from Biosource. NIH ImageJ software was used to quantify the density of bands in western blots. All of reagents and related antibodies were described in detail as reported previously (Kong, et al., 2009).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as follows. Total RNA was purified with TRIzol (Invitrogen).

5 µg of total RNA was reverse transcribed using the Transcriptor Reverse Transcriptase Kit (Roche).

Equal aliquots of cDNA were subsequently amplified for β-actin and Gα12. The primers for β-actin were: sense, 5′-CGCTAGTTGTAGATAA-CGGCTC-3′; antisense, 5-GCTTGCTGATCCACATCTGCTG-3; primers for Gα12 were: sense, 5′-GTTCTTGTCGATGCCCGAGACA-3′; antisense, 5′-TCACTGCAGCATGATGTCCTTC-3′.

Statistical analyses

STPLAN (University of Texas, MD Anderson Cancer Center) was used to determine the sample size and power calculations. The number of mice needed was estimated based on the following model for approximation: each IFN-inducer pI:pC *Mx1Cre*$^{+}$*Pkd1*$^{f/f}$ mouse kidney at P63 (9 weeks) weighs on average 0.48 g (s.d. of 0.1 g) based on what was published about this mouse model. We expect that crossing with *Gα12*$^{-/-}$ will decrease the kidney weight by at least 40% to 0.19 g (s.d. of 0.04 g) with significance of 0.05 (two sided). This will require 5.7 (~6) mice in each group to obtain 95% power to see the difference of 0.19 g in weight between the null and alternative hypothesis. The Mann–Whitney test, one-way ANOVA and Tukey's multiple-comparison test were used to analyze the data using GraphPad Prism 5 software. The equality of group variances was be tested using a Brown–Forsythe test or Bartlett's test. $P<0.05$ was considered significant.

GraphPad Prism (San Diego, CA) was used to perform statistical analysis. Significance was determined by using a *t*-test. $P<0.05$ was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

T.K. conceived and designed the study, and wrote the manuscript. W.Y and J.X.X. performed the majority of the experiments, analyzing data, preparing figures and editing of the manuscript. B.M.D, and J.Z. contributed to analyzing data and editing the manuscript. J.B. provided advice and assistance, and helped in critically reading and editing the manuscript. T.K managed the project, analyzed the data and carried out experiments. D.S. performed flow cytometry, immunostaining and took pictures for cell invasion and immunostaining. S.L., T.L., Q.W., M.T., W.Y., M.W. and I.E.B. aided in mouse genotyping, cell culture, PCR and western blotting, and assisted in animal dissections and husbandry. W.E.-J. collected human tissue and purified the cyst lining cells from ADPKD patients, and was responsible for editing and reviewing the manuscript and designing the statistics plan.

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