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Author: Formica, C.
Title: Molecular mechanisms involved in renal injury-repair and ADPKD progression
Issue Date: 2020-09-10
Reducing YAP expression in Pkd1 mutant mice does not improve the cystic phenotype

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Abstract

The Hippo pathway is a highly conserved signalling route involved in organ size regulation. The final effectors of this pathway are two transcriptional co-activators, Yes-associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (WWTR1 or TAZ). Previously, we showed aberrant activation of the Hippo pathway in Autosomal Dominant Polycystic Kidney Disease (ADPKD), suggesting that YAP/TAZ might play a role in disease progression. Using Antisense Oligonucleotides (ASOs) in a mouse model for ADPKD, we efficiently downregulated Yap levels in the kidneys. However, we did not see any effect on cyst formation or growth. Moreover, the expression of YAP/TAZ downstream targets was not changed, while WNT and TGF-β pathways downstream targets Myc, Acta2 and Vim, were more expressed after Yap knock-down. Differences in YAP/TAZ expression pattern in vivo, as well as in vitro experiments using Yap or Taz mutant renal cells, suggest that YAP/TAZ might play cell-type-specific roles in kidneys. In vitro experiments revealed that knock-out of Yap is associated with cytoskeleton changes and aberrant expression of Itga1 and Itgav. Although highly impaired, cyst formation and swelling were not prevented by Yap nor Taz knock-out in vitro indicating that YAP levels reduction is not a viable strategy to modulate PKD progression.
Introduction

The Hippo pathway is a highly conserved signalling route involved in the regulation of key cellular processes like proliferation, apoptosis and differentiation, which ultimately results in the regulation of organ size. The pathway is named after the core pathway component, the kinase Hippo, which has two homologues in mammals: mammalian sterile 20-like protein kinases 1 and 2 (MST1/2). MST1/2 together with large tumour suppressors 1 and 2 (LATS1/2) can phosphorylate the pathway effectors Yes-associated protein (YAP) and its parologue Transcriptional co-activator with PDZ-binding motif (WWTR1 or TAZ), resulting in their retention into the cytoplasm. When the Hippo pathway is inactive, YAP and TAZ are unphosphorylated and can shuttle to the nucleus where they can work as transcriptional co-activators driving the transcription of genes involved in proliferation and apoptosis\(^1,2\).

Indeed, elevated YAP/TAZ protein levels and nuclear localisation have been observed in multiple human cancers\(^1,3-6\).

In a previous study, we showed altered Hippo signalling in Autosomal Dominant Polycystic Kidney Disease (ADPKD)\(^7\). ADPKD is characterised by progressive deterioration of kidney function as a consequence of the formation of thousands of epithelium-derived cysts, leading to renal failure beyond mid-life. In the majority of the cases, ADPKD is caused by a mutation in either the \(PKD1\) or \(PKD2\) gene, which encode for Polycystin 1 (PC1) and Polycystin 2 (PC2) respectively\(^8,9\). We observed strong nuclear accumulation of YAP in dilated tubules and cysts of several orthologous mouse models, which was accompanied by up-regulation of the YAP transcriptional targets \(Birc-3\), \(Ctgf\), \(Inhba\) and \(Fjx1\). This was further observed also in human ADPKD cystic kidneys and cystic liver tissues\(^7\). Interestingly, YAP and TAZ have been described to interact with and regulate the activity of many other transcription factors implicated in APDKD, such as TEAD, GLIS3, SMAD, ERBB4, TP73 and RUNX2\(^10-17\). In addition, canonical WNT signalling, as well as, the stability of the PC1 and PC2 proteins can be modulated by Hippo signalling, placing YAP and TAZ in a central position in the regulation of PKD associated molecular pathways\(^18-21\). Therefore, we hypothesise that reducing nuclear localisation of YAP may slow down the renal cystic disease.

In this study, we used Antisense Oligonucleotides (ASOs) to specifically downregulate YAP level in \(iKspPkd1^{del}\) mice to modulate renal cyst growth. However, we did not see any effect of YAP downregulation on cyst formation or growth. This suggests that knockdown of \(Yap\) is not sufficient to slow disease progression in a mouse model for ADPKD.
Materials and Methods

Cell culture
Wild-type (Wt) mouse inner medulla collecting duct cells from ATCC (mIMCD3, CRL-2123™ ATCC®, City of Manassas, VA, USA) and Madin-Darby canine kidney (MDCK) cells (CCL-34™; ATCC) were commercially available. Briefly, cells were maintained at 37°C, and 5% CO₂ in DMEM/F-12 with GlutaMAX (#31331-093; Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 100 U/mL Penicillin-Streptomycin (#15140-122; Gibco, Life Technologies), 10% Fetal bovine serum (#S1860; Biowest, Nuaille, France). Cell cultures were monthly tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit. For 3D cyst assay, cells were grown in Matrigel as described previously. Briefly, cells were mixed with Matrigel (#354230; Corning, NY, USA) supplemented with 10% rat tail collagen I (kindly provided by OcellO B.V., Leiden, ZH, The Netherlands) and seeded in 96-wells. Cells were cultured in normal condition for 72 h and subsequently stimulated with forskolin (#344270, Calbiochem, Millipore B.V., Amsterdam, NH, The Netherlands) or DMSO for 72 h. Cells were collected for immunohistochemistry (IHC) or RNA extraction.

Generation of knock-out cell lines
Generation of the Pkd1 knock-out cell line mIMRFNPDKD5E4 was described before by making use of the FokI nucleases (RFN) method, described by Tsai et al. in mIMCD3. A comparable method was used to generate the Yap1 knock-out cell lines, using RFN guide RNAs for Yap1 exon 2. Sequencing of the selected clones mIMRFNYap9 and mIMRFNYap14, revealed an 8bp out of frame deletion in one allele and a 22bp out of frame deletion in the other allele for clone mIMRFNYap9 and revealed 13bp and 26bp out of frame deletions for clone mIMRFNYap14.

Using a similar method we set out to make knock-out cell lines for Wwtr1 (202: ENSMUST00000120977.1), by targeting exon 2 or exon 4. However, after screening 200 single clones, we noticed that mIMCD3 has three Wwtr1 alleles, and we were unable to identify any clone with an out of frame deletion in all three alleles. Therefore, we switched strategy: using two guide RNAs to delete exon 3 to be sure that every Cas9 induced deletion leads to a Wwtr1 mRNA with an out of frame deletion. CRISPR/Cas9 RNA-guides were designed targeting introns 2 and 3 with the online tool of the Zhang lab (https://zlab.bio/guide-design-resources) to obtain a genomic exon 3 deletion. In the end, single clones were isolated using G418 selection. In none of the 250 clones analysed exon 3 deletion was observed in three alleles. We applied the same exon deletion strategy to MDCK cells. We designed guide RNAs to delete exon 4 of the canine Wwtr1 gene (201: ENSCAFT00000013268.4). In 4 out of 40 clones, deletion of exon 4 was found in both alleles, leading to a frameshift in the Wwtr1 mRNA.

To generate Yap1/Pkd1 double knock-outs, mIMCD3 cells guide RNAs were designed and
cloned into a vector containing a hygromycin selection gene, to facilitate the deletion of exon 3 Yap1 gene in the Pkd1 knock-out cell line mMRFPKD5E4. After co-transfection with eSpCasCsy and hygromycin selection (0.1 mg/ml), approximately 75 single colonies were analysed: 2 clones had deletions on both Yap1 alleles and were verified using RT-PCR and sequencing. For detailed protocols, see Supplementary Methods.

**Experimental animals and study design**

All the animal experiments performed have been approved by the local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture. Inducible kidney-specific Pkd1 deletion mice (iKspPkd1<sup>1<sup>1<sup>th</sup></sup>) and tamoxifen administration have been described before<sup>25</sup>. Pkd1 gene has been knocked-out at post-natal day 18 (PN18). 32 male mice have been divided into two experimental groups of 16 animals each: one received scrambled antisense oligonucleotide (ASO), the other received Yap-specific ASO. Both groups received an injection of 100 mg/kg of ASO via i.p. injection, starting two weeks after Pkd1 inactivation (PN18 + 2 weeks), once a week, until sacrifice (PN18 + 8 weeks). ASOs were provided by IONIS Pharmaceuticals (Carlsbad, CA, USA). Both ASOs were 16mer S-constrained ethyl gapmers with a 3-10-3 chimeric design and a phosphorothioate backbone. Yap ASO sequence was: 5’-AACCAACTATTACTTC-3’; scrambled ASO sequence was: 5’- GGCCAATACGCCGTCA-3’. The Yap ASO was selected from leads identified following in vitro screens which were then evaluated in vivo for renal activity and tolerability. Scrambled ASO did not bind to any known target and was included as a control for non-specific effects. At sacrifice, both kidneys were collected and used for IHC or snap-frozen for RNA and protein extraction. Blood urea nitrogen level (BUN) was measured using the Reflotron Plus (Roche Basel, Switzerland). Three age-matched Wt mice were also included for IHC purposes.

**Immunofluorescence, Immunohistochemistry and Western blotting**

Cells were grown on coverslips and fixed in 4% paraformaldehyde for 15 min, at room temperature. Then, cells were permeabilised in 0.2% Triton-X100 in PBS for 10 min, at room temperature. Cells were blocked in 5% non-fat dried milk in PBS for 1 hour and then incubated with Phalloidin Atto 594 (1:1500) in 2% BSA in PBS. Immunofluorescence slides were mounted with Vectashield containing DAPI and pictures were taken on the Leica DMS500 B microscope.

For IHC, formalin-fixed paraffin-embedded kidneys or cysts in Matrigel were sectioned at 4µm thickness. Sections were stained with Haematoxylin-Eosin (H&E), Periodic acid-Schiff (PAS) or with these antibodies: rabbit anti-YAP (1:800 for kidneys and 1:1000 for 3D cysts; #14074; Cell Signaling Technology, Danvers, MA, USA); rabbit anti-TAZ (1:500 for kidneys and 1:1000 for 3D cysts; #4883; Cell Signaling Technology); rabbit anti-Ki-67 (1:3000, Novocastra, Leica Biosoysts, Wetzlar, Germany).
For Western blot, snap-frozen kidneys were homogenised using the Magnalyser technology (Roche) in Ripa buffer supplemented with protease inhibitors cocktails (#05892970001; Roche). Antibodies used: rabbit anti-YAP (1:1000; #14074; Cell Signaling Technology), rabbit anti-TAZ (1:1000; #4883; Cell Signaling Technology), mouse anti-GAPDH (1:5000; #97166; Cell Signaling Technology). Secondary antibodies: goat-anti-rabbit IRDye 800CW (1:10000; #926-32211; LI-COR Biosciences; Lincoln, NE, USA) and goat-anti-mouse IRDye 680RD (1:10000; #926-32220; LI-COR Biosciences).

Quantification of Ki-67 positive cells
Formalin-fixed paraffin-embedded kidneys were sectioned at 4 µm thickness and stained overnight at room temperature with rabbit anti-Ki-67 antibody and counterstained with haematoxylin. Sections were acquired using Philips Ultra Fast Scanner at 20x magnification factor and pictures of 15 random areas of the kidney were taken. ImageJ software (public domain software, NIH, USA) was used to measure the Ki-67 positive area and the haematoxylin positive area. The relative Ki-67 area was calculated as a percentage of the ratio of Ki-67 positive area over haematoxylin positive area.

Gene expression analysis
Total RNA was isolated from cultured cells or snap-frozen kidneys using TRI Reagent (#T9424; Sigma-Aldrich; St. Louis, MO, USA) according to manufacturer’s protocol, and gene expression analysis was performed by quantitative PCR (qPCR) as described previously26. Briefly, cDNA synthesis was done using Transcriptor First Strand cDNA Synthesis Kit (#04897030001; Roche) according to the manufacturer’s protocol. qPCR was done in triplicate on the LightCycler 480 II (Roche) using 2x FastStart SYBR-Green Master (#04913914001; Roche) according to the manufacturer’s protocol. Data were analysed with LightCycler 480 Software, Version 1.5 (Roche). Gene expression was normalised to the housekeeping gene *Hprt* or *GAPDH*. For primer sequences see Supplementary Table 1.

Quantification of cysts swelling
Cells were seeded in Matrigel in triplicate and cultured for four days. Subsequently, cells were stimulated with forskolin or DMSO as a control, for three days. At the end of the stimulation, the plates were imaged using a transmitted light microscope (Leica AXIO Observer.A1) at a magnification factor of 5x. One picture per well was taken, and the experiment was repeated four times. Using ImageJ, the circumference of the cysts was manually assessed. Around 90 cysts per well in triplicate have been evaluated, and the average cyst size was calculated for the four independent experiments. The average cyst size of the forskolin-treated cells was divided by the average cyst size of the DMSO treated cells (i.e. Wt cells + FSK / Wt cells - FSK) and compared between genotypes.
Statistical Analysis
Data were analysed using GraphPad Prism 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com)
Results

**Effect of YAP knock-out on cyst formation in vitro**
To study the effect of YAP on cyst growth, we generated mIMCD3 Yap knock-out (KO) cells and cultured them in Matrigel. Epithelial cells grown in this condition, spontaneously develop cystic structures with a visible lumen. After stimulation with forskolin, the cysts start to swell (Figure 1a). Interestingly, Yap KO cells (two different clones) showed impaired cyst formation, with only sporadic lumen formation. Most structures were very disorganised, resembling a tumour-like mass. Stimulation with forskolin did not result in swelling of the tumour-like agglomerates. Only the sporadic cysts that already had developed a lumen before forskolin treatment, increased in size. These data indicate that cyst growth per se is not impaired (Figure 1a), suggesting that Yap KO impairs cyst formation, but not growth, *in vitro*.

**mIMCD3 cells double knock-out for Yap and Pkd1 do not form cysts in vitro**
We are interested in the role of YAP in the context of ADPKD. Therefore, we generated mIMCD3 cells knock-out for Pkd1 as well as cells double KO for Yap and Pkd1. Pkd1 KO cells, when grown in Matrigel, form cysts that respond to forskolin stimulation (Figure 1a)\(^23\). However, Yap/Pkd1 double KO cells showed impaired cyst formation, as observed before for single Yap KO cells. Again, we saw sporadic forskolin-responsive cysts while the majority of cells grew in tumour-like agglomerates, suggesting that Yap KO, alone or together with Pkd1 KO, causes impaired cyst formation in cell culture (Figure 1a).

**Effect of TAZ knock-out on cyst formation in vitro**
YAP has a paralogue protein, TAZ (or WWTR1). To study the role of TAZ in cyst formation *in vitro*, we tried to generate mIMCD3 cells knock-out for Taz. To our surprise, we could not obtain any viable knock-out clone for Taz. We screened hundreds of clones and, although we found evidence for CRISPR/Cas guide RNA activity, we were not able to produce Taz KO clones suggesting that full deletion of Taz, but not Yap, in mIMCD3 cells is lethal. Thus, YAP and TAZ might play specific roles in the different cells of the kidney. Indeed, differences in the expression of YAP and TAZ in the different renal segments are also observed *in vivo*. Staining of sequential sections of Wt kidneys showed a complementary pattern of expression for YAP and TAZ, with tubule segments strongly positive for one protein but not the other (Supplementary Figure 1).

Next, we tried to generate TAZ KO MDCK cells. MDCK cells, when grown in Matrigel, can form cystic structures that respond to forskolin stimulation, as observed in mIMCD3 cells (Figure 1b). We could successfully achieve homozygous deletion of TAZ, and when cultured in Matrigel, MDCK TAZ KO cells were able to form cysts. Interestingly, although cysts were formed, the size of cysts was reduced in TAZ KO compared to MDCK Wt cells (Figure 1b). Nevertheless, the relative increase in the size of TAZ KO cysts upon forskolin stimulation
was comparable to that observed in Wt (Figure 1c). Thus, TAZ KO does not prevent cyst formation and growth in vitro, and we found a cell-type difference in TAZ dependency.

Figure 1. 3D cyst assay using Wt and mutant cells

(a) Light microscopy of mIMCD3 Wt and mutant cells before and after forskolin stimulation (respectively -FSK and +FSK). H&E staining of formalin-fixed, paraffin-embedded cysts grown in Matrigel after forskolin stimulation (bottom row). (b) Light microscopy of MDCK Wt and mutant cells before and after forskolin stimulation. H&E staining of formalin-fixed, paraffin-embedded cysts grown in Matrigel after forskolin stimulation (bottom row). White scale bar 200 µm; black scalebar 50 µm. (c) Bar graph representing the cyst swelling ratio calculated in MDCK Wt cells versus two different TAZ KO clones. Every symbol represents one independent experiment. Mean with ±SD. Mann-Whitney test. n.s. not significant.
The localisation of YAP and TAZ in 3D cysts assay

Since nuclear YAP accumulation was previously seen in cyst-lining epithelia, we investigated whether knocking out Yap or Taz has an influence on each other’s subcellular localisation during cyst growth. Staining of Wt and mutant cells that were grown in Matrigel revealed that both YAP and TAZ are expressed in the cytoplasm and the nuclei of Wt cysts (mIMCD3 and MDCK). Particularly, mIMCD3 cells formed small cysts with a relatively thick wall but also larger stretched cysts. These cysts had a thin epithelial layer and showed more often nuclear YAP and TAZ staining (Figure 2a). In Yap KO cells, and Yap/Pkd1 double KO cells, nuclear TAZ staining was observed, and it was not limited to stretched cysts but also visible in the tumour-like agglomerates. This was especially clear in the tumour-like agglomerates before forskolin treatment (Supplementary Figure 2), suggesting that lack of YAP increases TAZ shuttling (Figure 2a). In MDCK cells, 3D cysts assay lead to the formation of cysts with an overall flatter wall, and both nuclear and cytoplasmic YAP and TAZ localisation were visible within the same cyst. In TAZ KO cells, YAP expression does not clearly differ from the localisation observed in Wt cells (Figure 2b). Thus, Yap KO seems to affect TAZ localisation in vitro, but not vice versa.

Figure 2. Immunostaining of Wt and mutant cells in forskolin-treated 3D cysts
(a) Representative IHC of formalin-fixed, paraffin-embedded cysts of forskolin-treated Wt and mutant mIMCD3 cells and (b) forskolin-treated Wt and mutant MDCK cells, stained for YAP and TAZ. For mIMCD3 Yap/Pkd1 KO and MDCK TAZ KO cells, cysts from multiple fields of view are shown separated by a white line. White arrowheads indicate cytoplasmic localisation of the proteins; black arrowheads indicate nuclear localisation of the proteins. Scale bar 50 µm.
YAP knock-down using ASOs does not improve cystic phenotype in vivo
We showed in the past that cyst-lining epithelia have intense nuclear YAP localisation, both in Pkd1-mutant mouse models and in ADPKD patients\(^7\). Therefore, we hypothesised that YAP could actively contribute to cyst formation or cyst growth, through upregulation of target genes involved in cell proliferation and apoptosis.
To check the effect of YAP on the cystic phenotype in vivo, we knocked-down Yap using ASOs in young adult iKspPkd1\(^{del}\) mice. The Pkd1 gene was inactivated in 18 day old mice (PN18), and 2 weeks after gene inactivation they were injected i.p., with Yap specific ASO (n=16) or scrambled ASO (n=16), every week until sacrifice. Mice were sacrificed 8 weeks after gene inactivation (PN18 + 8 weeks) (Figure 3a).
The Yap ASO treatment resulted in about 70% reduction of Yap gene expression levels without affecting Taz expression, confirming its efficacy and specificity in vivo (Figure 3b). YAP reduction was confirmed at the protein level, while TAZ protein expression was unchanged by the ASO treatment (Figure 3c-e).
Analysis of kidney size, by measuring two kidney weight/body weight ratios, and of renal function, using BUN levels, revealed comparable disease progression in the two experimental groups (Figure 4b, c). PAS staining revealed tubule dilation and cyst formation in different segments of the kidneys, both in Yap ASO and scrambled ASO treated mice, suggesting that Yap knock-down did not affect cyst formation in vivo (Figure 4a).

YAP and TAZ downstream targets expression are not changed by Yap knock-down in vivo
YAP and TAZ are transcriptional co-activators and can translocate into the nucleus where they can drive gene expression. To study the effect of Yap knock-down on the expression of its target genes, we quantified the expression of known YAP/TAZ targets, Wtip, Ajuba, Cyr61 and Amotl2\(^27\). Despite the consistent Yap reduction at the mRNA level, the expression of target genes is not changed in Yap ASO treated compared to scrambled ASO treated mice (Figure 4d). Additionally, we evaluated the expression of Ki-67, a marker for cell proliferation, as YAP and TAZ can regulate transcriptional programs that control cell proliferation\(^27\). The Ki-67 positive areas in Yap ASO treated mice were not significantly different from those observed in scrambled ASO treated mice (Figure 4e, f). In conclusion, the knockdown of Yap does not affect the expression of the downstream targets we tested.

WNT and TGF-β pathways seem to be more active in Yap ASO mice
It is well known that both YAP and TAZ can interact with the final effectors of the WNT and TGF-β pathways\(^18\). For this reason, we checked the expression levels of several target genes regulated by β-catenin (Axin2 and Myc) and Smads (Acta2, Col1a1, Vim, Fn1, Pai1 and Mmp2). We observed increased Myc expression in Yap ASO treated mice compared to scrambled ASO, but only a trend for Axin2 expression (Figure 5a). Moreover, we saw significantly increased expression of alpha-smooth muscle actin (Acta2) and vimentin (Vim)
and a consistent trend for collagen 1 alpha-1 (Col1a1), fibronectin (Fn1), plasminogen activator inhibitor-1 (Pai1) and matrix metallopeptidase 2 (Mmp2) (Figure 5b). Thus, although not conclusive, these results suggest that WNT and TGF-β pathways are more active upon Yap knock-down.
REDUCING YAP EXPRESSION IN PKD1 MUTANT MICE DOES NOT IMPROVE THE CYSTIC PHENOTYPE

**Figure 4. Effect on PKD progression of in vivo downregulation of Yap with ASOs**

(a) Representative Periodic acid-Schiff (PAS) staining of renal tissue from mice treated with scrambled ASO and Yap ASO. Scale bar 200 µm. (b) Quantification of kidney size using two kidney weight/body weight ratio. n.s. not significant (c) Blood urea nitrogen (BUN) level at the sacrifice. n.s. not significant (d) Gene expression (fold-change) of YAP/TAZ targets at the sacrifice in mice treated with Yap ASO and scrambled ASO. Each symbol represents a mouse. Mean with ± SD. n.s. (not significant) refers to all the genes in the graph, t-test. (e) Quantification of Ki-67 positive area. Each symbol represents a mouse. Mean with ± SD. n.s. not significant, t-test. (f) Representative pictures of renal tissue stained for Ki-67. Scale bar 50 µm.

**Yap KO affects cytoskeleton integrity and integrins expression in vitro**

Following up on the aberrant phenotype observed in Yap KO cells grown in Matrigel, we decided to characterise these cells further. We stained the actin cytoskeleton using fluorescent dye-conjugated phalloidin, a well-known marker for filamentous actin. Wt mIMCD3 cells showed a normal cytoskeleton with clear cortical cytoskeletal and stress fibres present throughout the cell body. Also, TAZ KO cells showed a normal cytoskeleton, similar to Wt cells. Conversely, Yap KO cells showed aberrant cytoskeleton and rarely stress fibres, suggesting that the effect on the actin structure was a consequence of Yap gene disruption (Figure 5c).

Additionally, gene expression of integrin-alpha 1 (Itga1) and -alpha v (Itgav) was significantly reduced in Yap KO cells, before and after forskolin stimulation, compared to Wt cells. This was not observed in TAZ KO cells, in which the level of Itga1 was significantly increased compared to Wt cells (Figure 5d). These results might explain the loss of polarity observed in Yap KO cells leading to impaired cyst formation and development of tumour-like structures in 3D cultures.
Figure 5. WNT and TGF-β pathway targets \textit{in vivo}, and characterisation of mutant cell lines

\textbf{(a)} Gene expression (fold-change) of WNT pathway targets \textit{Axin2} and \textit{Myc} at the sacrifice in mice treated with \textit{Yap} ASO and scrambled ASO. Each symbol represents a mouse. Mean with ± SD. ** \( P < 0.01 \), n.s. not significant, t-test. \textbf{(b)} Gene expression (fold-change) of TGF-β pathway targets, \textit{Acta2}, \textit{Col1a1}, \textit{Vim}, \textit{Fn1}, \textit{Pai1} and \textit{Mmp2}, at the sacrifice in mice treated with \textit{Yap} ASO and scrambled ASO. Each symbol represents a mouse. Mean with ±SD. * \( P < 0.05 \), ** \( P < 0.01 \), t-test. If no significance is indicated, the comparison is not significant. \textbf{(c)} Representative immunofluorescence of Wt and mutant mlMCD3 cells and Wt and mutant MDCK cells. In red, the actin cytoskeleton is stained using phalloidin Atto 594. In blue, nuclei are stained using Vectashield containing DAPI. Scale bar 20µm. \textbf{(d)} Gene expression normalised on housekeeping genes (\textit{Hprt} for mlMCD3 cell lines and \textit{GAPDH} for MDCK cell lines) of Integrins alpha-1 and alpha-v. Cells are grown in Matrigel and allowed to form cysts. Subsequently, forskolin is added to the medium (+FSK) or DMSO as control (-FSK). Cells are then collected for RNA extraction, and gene expression of the integrins mentioned above is evaluated. For mutant cells, two different clones are included. Each symbol represents an independent experiment. Mean with ± SD. Asterisks indicates significance of the comparison of mutant cell lines versus the Wt in the same stimulation group (Wt-FSK vs. mutant-FSK and Wt+FSK vs. mutant+FSK) * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \). § indicates the significance of the comparison of the same cell line in the two stimulation group (Wt-FSK vs. Wt+FSK or mutant-FSK vs. mutant+FSK). ANOVA significance test with Tukey’s multiple comparisons test.
Discussion

ADPKD is the fourth most common renal disease that requires renal replacement therapy\(^{29}\). Indeed, the majority of patients with ADPKD will develop the end-stage renal disease (ESRD), severely impacting patients’ lives and representing a substantial economic burden to the healthcare system\(^ {30}\). Thus, finding viable intervention strategies aimed to slow down the disease progression is of paramount importance.

In a previous study, we showed that the Hippo pathway’s effector YAP was more active in the cyst-lining epithelia both in murine and human renal tissues with PKD\(^7\). When in the nucleus, YAP can modulate the transcription of genes involved in the regulation of proliferation and apoptosis\(^{3,4,6}\). Therefore, we hypothesised that reducing nuclear localisation of YAP might slow down the renal cystic disease. In this study, we used ASOs to selectively knock-down the expression of \(Yap\) in young adult \(iKspPkdl^del\) mice. We reached about 70% reduction in gene expression, indicating that ASOs can be a viable strategy to effectively and selectively downregulate a target in kidneys in models for PKD, as also shown for a few other targets\(^{31,32}\). Our data clearly indicate that \(Yap\) knock-down using ASOs in a mouse model for ADPKD does not improve the cystic phenotype. Indeed, also \textit{in vitro}, \(Yap\) KO did not impair the growth of the sporadic cysts that were able to form, suggesting that proliferation is not affected.

Considering that TAZ (or WWTR1) levels are not changed by \(Yap\) ASO, we hypothesised that TAZ could be compensating for \(Yap\) knock-down. Indeed, expression levels of the target genes are not changed by \(Yap\) knock-down, and TAZ shows a clear nuclear localisation in most of the renal tubules, both cystic and dilated ones. This suggests that TAZ is compensating for the loss of YAP and might be contributing to cyst growth, hence targeting TAZ together with \(Yap\) might be a viable strategy to inhibit cyst progression. However, we also showed that TAZ KO did not result in impaired cyst formation nor cyst growth in a 3D cyst assay, arguing against it. This is further supported by \textit{in vivo} results, since mice with a constitutive or conditional \(Taz\) KO, develop mild cysts even in the absence of a \(Pkd1\) mutation\(^ {18,33}\). Moreover, \textit{Merrick et al.} showed that TAZ physically interacts with the C-terminal tail of PC1 (PC1-CTT) in HEK293 cells. They also proved in zebrafish, that the bone phenotype and curly tail observed after injection of \(Pkd1\) morpholino were rescued by co-injection of PC1-CTT mRNA but not if also \(Taz\) was knocked out\(^ {21}\). Altogether, these results suggest that PC1 and TAZ participate in common signalling routes, and reducing or depleting \(Taz\) levels might worsen PKD progression.

In contrast to our work, a recent paper showed that \(Yap\) KO, especially together with \(Taz\) KO, was able to reduce PKD progression mildly in \(Pkd1\) deficient mice. However, the mouse model used was based on leaky Cre recombinase activity, suggesting that gene inactivation already
occurred during development, rather than in adult mice\textsuperscript{34}. Indeed, constitutive \textit{Yap} KO can lead to impaired kidney development, which might affect subsequent cyst development\textsuperscript{35}. Another difference with our study is that we did not use a genetic deletion of \textit{Yap}, but we decided to use a strategy based on antisense oligonucleotides that could potentially be translated to the clinic. The use of ASOs allows to reduce YAP levels consistently, but not to altogether abolish the expression. Additionally, by treating young adult mice that develop cysts relatively slowly and in every renal segment, we mimic the disease observed in patients even more.

Although YAP and TAZ have some overlapping functions, they also partly interact with and are regulated by different partners, indicating that YAP and TAZ also have unique functions\textsuperscript{36}. Interestingly, we could generate \textit{Yap} KO but not \textit{Taz} KO mIMCD3 cells, suggesting that TAZ plays a crucial role in mIMCD3 cells and potentially in specific segments of the kidney. Indeed, we observed differential expression patterns for YAP and TAZ in the various renal segments (Supplementary Figure 1), corroborating the idea that the two transcriptional co-activators also have distinct functions in the kidneys. However, if and how modulation of YAP levels affects the activity of TAZ in kidneys, and vice versa, is not clear and should be further investigated.

YAP and TAZ are transcriptional co-activators that can modulate a variety of biological processes. They have been associated with fibrogenesis and epithelial-to-mesenchymal transition \textit{in vivo} and \textit{in vitro} via interaction with both TGF-\(\beta\) and WNT signalling pathways. Particularly, when the Hippo pathway is active, YAP and TAZ are phosphorylated and restrained in the cytoplasm where they can interact with SMADs and \(\beta\)-catenin, preventing their nuclear translocation and transcriptional activity\textsuperscript{28}. In our study, we observed increased expression of some of the downstream targets of WNT and TGF-\(\beta\) pathways in \textit{Yap} ASO treated mice. This might suggest that reduced YAP levels cause an imbalance in the regulation of these interacting signalling pathways, either because YAP cannot physically interact with SMADs and \(\beta\)-catenin anymore, or due to increased activation of TAZ overcompensating for YAP loss. Indeed, YAP has been shown to inversely regulate TAZ protein levels in a process conserved from mouse to human, and we also observed increased nuclear TAZ in \textit{Yap} KO cells grown in 3D\textsuperscript{37}. Based on this, \textit{Yap} knock-down might result in overactivation of TAZ, in turn causing increased activation of WNT or TGF-\(\beta\) pathways, which are well known for promoting cyst formation in ADPKD\textsuperscript{38,39}. Such a process might explain why we did not see any amelioration of the phenotype with \textit{Yap} knock-down \textit{in vivo}, although further studies are necessary to unveil the exact molecular mechanisms.

YAP/TAZ were initially identified as downstream effectors of the Hippo pathway. Nevertheless, in the last years a variety of upstream regulators, dependent or independent of the Hippo
pathway, have been identified affecting YAP/TAZ sub-localization. These include mechanical signals via cell-cell contacts, polarity proteins and cadherin-β-catenin complexes, focal adhesions, extracellular matrix (ECM) elasticity and cytoskeletal tension\textsuperscript{40}, as well as several mitogens like epidermal growth factor (EGF), lysophosphatidic acid (LPA), sphingosine-1 phosphate, insulin and the protease thrombin, which can control YAP/TAZ localisation\textsuperscript{41-45}. Indeed, we also showed that in Yap KO mIMCD3 cells, expression of Itga1 and Itgav is impaired and that the architecture of the cytoskeleton is aberrant. In 3D cysts, this results in non-polarized growth of the cells that fail to form a lumen and grow as a tumour-like mass. In vivo, mice mutant for proteins involved in the formation of cell-cell junctions, focal adhesions and related to cytoskeletal assembly, develop renal cysts\textsuperscript{46}. Thus, the aberration of any of the processes mentioned above can lead to cyst formation. For these reasons, the modulation of Hippo pathway effectors YAP and TAZ to intervene on cyst progression might not be a viable option.

In conclusion, although we cannot exclude that the Hippo pathway is involved in cyst growth, we believe that the strong nuclear YAP localisation observed in cyst-lining epithelia is more a consequence of cell stretching or stiffer ECM or mitogen-induced signalling rather than a driving force for cell proliferation. Indeed, downregulation of YAP using ASOs did not affect cell proliferation nor the cystic phenotype in our Pkd1 KO mouse model. Moreover, due to its profound interconnection with other signalling pathways, such as WNT and TGF-β, a therapeutic intervention for PKD based on the modulation of YAP levels might not be feasible, at least with the current knowledge.
Funding

This work was supported by funding from the Dutch Kidney Foundation [NSN P12.18 to S.K.]; Dutch government [LSHM15018 to C.F.]; People Program (Marie Curie Actions) of the European Union’s Seventh Framework Program FP7/2007-2013 under Research Executive Agency Grant Agreement [317246 to C.F.]; the DIPAK Consortium, which is an inter-university collaboration in the Netherlands established to study Autosomal Dominant Polycystic Kidney Disease and to develop treatment strategies for this disease, sponsored by the Dutch Kidney Foundation [CP10.12, CP15.01] and Dutch government [LSHM15018]; and IPSEN Farmaceutica BV, the Netherlands, which provided an unrestricted grant.

Acknowledgements

The authors would like to thank Janne Plugge and Hester Bange for technical assistance.

Authors contributions


Conflict of interests

The authors declare no competing or financial interests.

Data Availability Statement

No additional data were generated beyond the data presented in the manuscripts and its supplements.
References


Mcb.01620-08 (2009).


Reducing YAP expression in PKD1 mutant mice does not improve the cystic phenotype


Supplementary Figures

Supplementary Figure 1. YAP and TAZ expression in Wt kidneys
Representative IHC for YAP and TAZ on sequential slides of Wt mice kidneys at post-natal day 100. The staining shows a complementary expression pattern of YAP and TAZ in the various segments of the kidneys, with tubule segments strongly positive for one protein but not the other. Arrowheads show the same tubules stained for the two different proteins. Scale bar 200 µm.

Supplementary Figure 2. YAP and TAZ staining of Wt and mutant mlMCD3 cells
Cells are grown in Matrigel and allowed to form cysts. In the pictures, Wt and mutant mlMCD3 cells are shown, which were not stimulated with forskolin. Representative IHC of formalin-fixed, paraffin-embedded cysts stained for YAP and TAZ shows intense nuclear TAZ staining in Yap single and double KO cells. White arrowheads indicate cytoplasmic localisation of the proteins; black arrowheads indicate nuclear localisation of the proteins. Scale bar 50 µm.
Supplementary methods

Generation of knock-out cell lines

Generation of the Pkd1 knock-out cell line mIMRFNPKD 5E4 was described before\(^1\) by making use of the FokI nuclease (RFN) method, described by Tsai et al.\(^2\) in mouse kidney, medulla/collecting duct cell line mIMCD3 (mIMCD3, ATCC® CRL-2123™). A comparable method was used to generate the Yap1 knock-out cell lines. In short, the RFN guide RNAs for Yap1 exon 2 were selected using ZifIT (http://zifit.partners.org/ZifIT/Disclaimer.aspx) and cloned into vector pSQT1313neo. This is a modified version of pSQT1313 (Addgene #53370), in which we replaced the ampicillin gene of pSQT1313 by the kanamycin/neomycin resistance cassette of pEGFP-N1 (Clontech). This was done to facilitate G418 selection of clones that have taken up pSQT1313neoRFN and enrich for clones that carry a Yap1 exon 2 deletion. The RFN-guide RNA clone was co-transfected with pSQT1601 (Addgene #53369) a plasmid expressing the Csy4 and dCas9-FokI fusion proteins. mIMCD3 cells were grown to 80% confluency in a 9 cm petri dish and transfected with 2µg Yap1ex2RFN and 8µg pSQT1601 DNA using Lipofectamin 2000 (Invitrogen). G418 (0.5mg/ml) selection was applied after 48 hours. After 7 days, cells were re-plated at a density of ~50 cells per 9 cm plate. In total 60 single colonies were picked and analysed using PCR with primers flanking the RFN target sites. PCR products were digested with restriction-endonuclease BpmI, which cuts between the Yap1ex2 RFN target sites. From 7 clones that showed undigested PCR products, demonstrating a deletion of the BpmI, restriction site on both alleles, the PCR products were subcloned using the TOPO® cloning kit (Invitrogen). Fifteen subclones were analysed by Sanger sequencing. The sequences for clone mIMRFNYap9 revealed an 8bp out of frame deletion in one allele and a 22bp out of frame deletion in the other allele and clone mIMRFNYap14, revealed 13bp and 26bp out of frame deletions.

Using a similar method we set out to make knock-out cell lines for Wwtr1 (202: ENSMUST00000120977.1), by targeting exon 2 or exon 4. However, after screening 200 single clones, we noticed that mIMCD3 has three Wwtr1 alleles and we were unable to identify any clone with an out of frame deletion in all three alleles. We switched strategy: using two guide RNAs to delete exon 3 to be sure that every Cas9 induced deletion leads to a Wwtr1 mRNA with an out of frame deletion. CRISPR/Cas9 RNA-guides were designed targeting introns 2 and 3 with the online tool of the Zhang lab (https://zlab.bio/guide-design-resources) to obtain a genomic exon 3 deletion. RNA guides were cloned into pSQT1313neo, both guide RNAs flanked and separated from each other by Csy4 recognition sites, and co-transfected with eSpCasCsy, a modified eSpCas9(1.1) (Addgene #71814) which expresses both the high specificity SpCas9 protein together with the Csy4 RNASe. Single clones were isolated using G418 selection. In none of the 250 clones analysed exon 3 deletion was observed in three alleles. Sequencing of many clones revealed, deletion of exon 3 in two alleles and indels were observed at the Cas9 cutting sides of the guide RNA recognition
sides in the third allele, but these events did not lead to a deletion of exon 3. From these results we conclude that knocking-out three alleles in mIMCD3 leads to cell death. We applied the same exon deletion strategy to MDCK cells (ATCC® CCL-34™). We designed guide RNAs to delete exon 4 of the canine Wwtr1 gene (201: ENSCAFT00000013268.4), and cloned these into pSQT1313neo. After co-transfection with eSpCasCsy and G418 selection 40 single colonies were analysed using PCR. In 4 clones a deletion of exon 4 in both alleles was observed. RT-PCR and sequencing on RNA isolated of these clones, revealed deletion of exon 4 leading to a frameshift in the Wwtr1 mRNA and knocking-out both alleles. Finally, we generated a Pkd1/Yap1 double knock-out in mIMCD3. Guide RNAs were designed and cloned into a vector containing a hygromycin selection gene, to facilitate the deletion of exon 3 Yap1 gene in the Pkd1 knock-out cell line mIMRFNPKD 5E4. After co-transfection with eSpCasCsy and hygromycin selection (0.1mg/ml), approximately 75 single colonies were analysed: 2 clones had deletions on both Yap1 alleles, and were verified using RT-PCR and sequencing.

References


Supplementary Tables

Supplementary Table 1. List of primer sequences used for qPCR

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<th>Mouse</th>
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