Curcumin inhibits cystogenesis in autosomal dominant polycystic kidney disease cells via altering JAK2/STAT3 activity.

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ABSTRACT

The Janus Kinase and Signal Transducers and Activators of Transcription (JAK-STAT) is a major pathway controlling proliferation, differentiation and immunity. Its mis-regulation contributes to the development of numerous diseases including the common genetic disorder Autosomal Dominant Polycystic Kidney Disease. Curcumin is a potent anti-inflammatory phytochemical that limits cystic disease, yet the underlying mechanisms are largely unknown. We have studied the effects of curcumin on the formation of cysts in organotypic three-dimensional assays. Curcumin, potently inhibits the growth of cysts in a dose- and time-dependent manner. Mechanistically, curcumin reduces the levels of tyrosine-phosphorylated STAT3 without affecting total STAT3 protein levels. JAK2, is a major tyrosine kinase known to cause various diseases, is inactivated in the presence of curcumin. Inactivation of JAK2 is mediated by its translocation into intracellular aggresomes. The role of JAK2 in ADPKD was investigating immunohistochemically using anti-JAK2 antibodies in a murine model of ADPKD ($Pkd1^{nl/nl}$). In the normal kidney, JAK2 staining is restricted to tubular epithelial cells and vascular cells with lesser staining in bowman's capsule. By contrast, mesenchymal cells and fibroblasts showed no staining. In the diseased kidney, JAK2 is strongly localised in cyst-lining epithelial and vascular cells while exhibiting some interstitial localisation. These results suggest that curcumin is a potent inhibitor of the major kinase JAK2, thus limiting cystic growth and protecting the renal epithelium from cystic development. Interestingly, JAK2 expression, monitored at the protein level by immunohistochemistry, appears higher in cystic epithelia, indicating that JAK2 may be a key contributor to disease.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a devastating multi-organ disease, affecting over 9 million people worldwide, lacking a cure. It accounts for approximately 10% of patients with renal failure. Genetically, it arises predominately due to mutations in the Pkd1 or Pkd2 genes, encoding for the polycystin-1 and polycystin-2 proteins. ADPKD is characterised by the progressive growth and enlargement of renal cysts, typically leading to renal failure by middle age. Mutations in polycystins also cause a strong vascular phenotype associated with hypertension and aneurysms (1,2) which together with cysts in other organs, shows that ADPKD is a systemic disease. The complexity of changes in ADPKD have made the identification of a cure difficult. Currently Tolvaptan, a V2-vasopressin receptor antagonist, is the only approved therapy; however, it carries significant side effects precluding its use in the majority of patients (3). Therefore, a major biomedical challenge is to identify central druggable pathways for the treatment of ADPKD.

We have previously shown that the evolutionarily-conserved JAK-STAT pathway is abnormally activated in, and contributes to, ADPKD (4). Others have also shown that JAK-STAT is misregulated in ADPKD, and likely to drive disease (5-8). In addition to its clear involvement in ADPKD, JAK-STAT is misregulated in a number of other diseases such as arthritis and cancer and anti-JAK-STAT therapies have therefore been developed. Combining our previous and current work with that of other groups we predict that JAK-STAT inhibition may be of therapeutic benefit in ADPKD. Curcumin (diferuloylmethane) is known to inhibit JAK-STAT activity. It is a natural phenol and major component of turmeric, derived from the rhizomes of the plant Curcuma longa (9). It exhibits strong antioxidant, anti-tumour and anti-inflammatory pharmacological properties (10), without appreciable side-effects. Curcumin has been shown to limit the growth of cysts in murine models of ADPKD (11). Moreover, it is currently being trialled in young patients and children with ADPKD (clinicaltrials.gov). The precise mechanism(s) utilised by curcumin to confer kidney protection are however unknown.

To characterise how curcumin inhibits cystic growth we perform biochemical assays which show that it acts directly on renal epithelial cells via inhibition of the major tyrosine kinase JAK2. To analyse JAK2 expression we have assayed its distribution in wild-type and ADPKD murine kidneys at two different time-points of disease. These data show that curcumin inhibits the JAK2 kinase, which is highly expressed in the cystic epithelium.

RESULTS

Curcumin inhibits cystic growth *in vitro*.

Inhibition of cystic growth, previously illustrated in murine ADPKD models (11), shows that curcumin is capable of protecting the kidneys *in vivo*. However, whether it acts directly on kidney cells or indirectly via involving other cell types is currently unknown. To address this, three-dimensional organotypic cyst assays of renal

tubular epithelial cells, derived from ADPKD patients, were employed (Fig 1). Three cell lines were investigated, OX161 (Fig 1A), SKI-001 (Fig 1B) and mouse derived ADPKD cells F1 Pkd1$^{-/-}$ (Fig 1C). Pictures of the cysts were taken at day 0, day 2, 6 and 9. We found that 100µM and 50µM of curcumin were sufficient to cause a statistically significant reduction in the size of cysts over time in all cell lines tested, when compared to DMSO-treated cells. Curcumin caused both a dose- and time-dependent reduction in cyst diameter with a pattern consistent in all three independent ADPKD lines investigated. These data show that curcumin is a potent inhibitor of cystic growth via targeting renal tubular epithelial growth in a cell-autonomous manner.

Curcumin potently inhibits STAT3 tyrosine phosphorylation.

Studies in non-renal systems have suggested that curcumin might be a JAK-STAT inhibitor (12-14), however whether curcumin inhibits the JAK-STAT pathway in renal cells is unknown. To address this, human ADPKD-derived epithelial cells were treated with curcumin at 50µM for 6 hours, this concentration was used because it is the lowest dose that consistently caused a reduction in cystic growth (Figure 1). We found that curcumin acted as a potent inhibitor of activity causing an almost complete inhibition of tyrosine phosphorylation of STAT3 following oncostatin M treatment (OSM at 10ng/ml – all OSM treatment subsequently will be at this fixed concentration) (Figure 2A); OSM was used to activate STAT3 tyrosine phosphorylation (15). These data therefore demonstrate that curcumin is a STAT3 inhibition in a variety of renal epithelial cells.

To gain a deeper understanding of the kinetics of the observed inhibition, we performed a dose-curve and found that as low as 25µM of curcumin was enough to cause at least 50% reduction in the phosphorylation of STAT3 when compared with DMSO vehicle-control (Figure 2B and quantification). We treated cells with 10µM of curcumin, but this did not significantly affect STAT3 tyrosine phosphorylation (data not shown). Next to understand how quickly curcumin can inhibit STAT3 we performed a time-curve experiment. 1 hour of curcumin treatment (50µM) is sufficient to inhibit STAT3 phosphorylation (Fig 2C). Taken together, these experiments suggested that 50µM of curcumin is sufficient to inhibit JAK-STAT signalling, the fact that the inhibition happens within 1 hour suggests that curcumin may block JAK-STAT directly.

Curcumin inhibits tyrosine phosphorylation of STAT3 via JAK2.

The underlying mechanism to explain how curcumin inhibits STAT3 in ADPKD is unknown. Our biochemical studies showed that total STAT3 protein levels are unaffected following curcumin treatment (Fig 2), therefore curcumin is likely to work upstream of STAT3 and specifically it might affect the kinases that phosphorylate it. To investigate this we studied JAK2, a tyrosine kinase upstream of STAT3 responsible for phosphorylating STAT3. Curcumin within 1 hour caused a drastic decrease in detergent-soluble JAK2, which was almost complete at 100µM and 50µM and only partial at 25µM of curcumin (Fig 3 and quantification). The effect of curcumin on JAK2 (Figure 3) mirrors those of STAT3 phosphorylation inhibition (Fig 2B). Given that JAK2 is required for STAT3 phosphorylation, we reasoned that the mechanism by

which curcumin inhibits STAT3 activity, at least in part, is via inhibition of JAK2.

Curcumin does not interfere JAK2 levels but makes JAK2 insoluble.

Next, we wished to understand how curcumin affects JAK2. Since the levels of soluble JAK2 are reduced in cells treated with curcumin, we hypothesized that one possible mechanism of action may involve causing JAK2 to degrade. To investigate this, we treated renal tubular epithelial cells with MG132, which is a proteasome inhibitor and thus addition of MG132 is likely to stabilise proteins destined to be degraded via the proteosome. We expected MG132 will cause JAK2 to become stabilised if JAK2 was targeted to the proteasome following curcumin treatment. However, MG132 in the presence of curcumin did not rescue JAK2, suggesting that JAK2 is not being proteasomally processed in curcumin treated cells (Fig. 4A). Moreover, STAT3 phosphorylation was not changed following inhibition of the proteasome, further suggesting that proteosomal targeting is not part of the mechanism of action of curcumin (Fig 4A). To ensure that MG132, was indeed inhibiting the proteasome we blotted for ubiquitin, the level of protein ubiquitination should increase visibly when the proteasome is inhibited sufficiently. Indeed, we found that total levels of ubiquitinated proteins were increased in MG132 treated cells (Fig 4A), thus validating the results. It is of interest to mention that treatment of cells with curcumin also caused an accumulation of ubiquitinated proteins, suggesting that curcumin interferes with the processing of, at least some, proteins (Fig 4A). We also inhibited the lysosome (using Baffilomycin A), given that JAK2 has been shown to be lysosomally processed in other settings, however we did not detect any appreciable effects (data not shown). Collectively these data demonstrate that curcumin inhibits JAK2/STAT3 in a proteasome-independent manner.

Curcumin has previously been shown to cause precipitation of proteins into aggresomes (16,17). To test whether JAK2 was moved into an insoluble aggregated state, we next performed immunocytochemistry in cells treated either with DMSO control or 1, 3 or 6 hours treatment with 50µM of curcumin. JAK2 was localised at the plasma membrane (arrow, Fig 4B, top left panel) in vehicle-treated cells. However, 1 hour of curcumin treatment was sufficient to cause JAK2 to move into puncta and aggregatate, these aggregates known as aggresomes became fewer but larger at 3 and 6 hours post curcumin treatment (Fig 4B). Our data therefore suggest that curcumin causes JAK2 to become inactivated by moving into an insoluble aggregated state.

JAK2 is highly expressed by renal tubular cystic epithelial cells *in vivo*.

Next, we wondered if JAK2 may be involved in the development of cysts *in vivo*. To determine whether renal epithelial cells express JAK2, we stained kidney sections from the $Pkd1^{nl/nl}$ mouse model (18) and associated wild-type littermate control kidneys with anti-JAK2 antibodies. Kidneys from 5 weeks old mice, with intermediate disease, and 10 weeks old with advance disease were studied, as previously described (4). We found that JAK2 is widely expressed by cortical renal epithelial cells and its expression appears predominately cytoplasmic with no detectable expression by interstitial fibroblasts (Fig. 5, top). During early

stages of ADPKD kidney JAK2 is strongly expressed by cystic tubules, vessels and to a lesser extent by non-cystic, otherwise normal, tubular epithelial cells (Fig. 5, middle). As the disease advances at 10 weeks of age (as evidenced by increased number of larger cysts and interstitial expansion), JAK2 expression by cystic epithelial cells appears to be ever stronger, with some expression by interstitial cells and a lesser expression by bowman's capsule cells (Figure 5, bottom). Expression of JAK2 in vessels remains strong as disease progresses (Fig. 5, bottom). Hence, JAK2 expression is temporally and spatially coincident with cyst development in the ADPKD kidney.

Discussion

Over the years, phytochemicals have been used to inhibit, prevent or reverse disease processes. Curcumin specifically was found to have strong anti-cystic effects in murine models of ADPKD (11). Whether curcumin acts primarily on renal epithelial or vascular cells is however unclear, making the interpretation of the *in vivo* results (11) difficult. Moreover, extra complication arises due to the fact that curcumin is currently under trial (phase 4) for its potential ability to modify the vascular phenotype in children and young individuals with ADPKD. Our work identifies that curcumin directly affects renal tubular epithelial cells, without requiring interactions with the vasculature, thus providing some answers. However, expression studies show that JAK2 is also expressed by vascular cells, thus not precluding a role of curcumin in vessels. Further studies are therefore required to determine the effects of curcumin on JAK inhibition in the vasculature.

The use of curcumin as a therapy has both advantages and disadvantages. One advantage is the possibility of long term use, due to lack of appreciable side effects (19). However, curcumin's reduced bioavailability (20,21), makes it less attractive when compared with small molecule inhibitors. Indeed, our study identifies JAK2 as a therapeutic target in ADPKD. Because of the involvement of JAK2 in multiple diseases, several inhibitors have been developed, including the approved Ruxolitinib, Tofacitinib and Baracitinib (22-25). Additional JAK inhibitors are in clinical trials, thus making JAK-STAT a therapeutically tractable pathway.

Our protein immunohistochemistry studies show diffuse expression of JAK2 primarily in tubular epithelial cells and cystic epithelium. Given however that ADPKD is a disease of a systemic nature, with cysts in other organs, a full survey of JAK2 expression is required to determine precisely the cellular localisation of JAK2 in each tissue.

Overactivation of JAK2 can lead to malignancy (26). In our study we find that expression of JAK2 in wide spread in cortical tubules, however no dysplastic phenotype in seen ADPKD, with cysts arising from nephrons that otherwise look normal. This suggests that activation of JAK2 in ADPKD leads to a change in the maintenance of epithelial differentiation and turnover. This is supported by our previous work which showed that increased JAK-STAT activity led to enhanced proliferation in renal tubular epithelial cells (4).

Mechanistically curcumin causes JAK2 to move into an insoluble fraction consistent with generation of multi-molecular structures or aggresomes. Therefore, we propose a model whereby curcumin takes JAK2 temporarily out of action by causing it to aggregate, without causing JAK2 to degrade which is an irreversible consequence. Temporarily inhibiting JAK2 may explain why curcumin is not cytotoxic, allowing JAK2 to become reactivated when needed. Likewise, others have reported that curcumin-treated cancer cells respond by inhibiting STAT3, a curcumin-induced effect which is fully reversible (27).

How curcumin causes abnormally activated JAK2 to aggregate is currently unknown. Based on previous literature we hypothesize that

curcumin disturbs JAK2 intramolecular interactions. Curcumin has been shown to be neuroprotective, by preserving gross intramolecular conformation of beta amyloids, while disrupting the intermolecular arrangements, a phenomenon which is very similar to the effects of Zn^{2+} ions, as reported previously (17). Likewise, curcumin can alter the structure of lipoproteins by interacting with the soluble intermediates, thus altering their aggregation patterns (16). Similarly, A20, which is an anti-inflammatory zinc-finger protein is shown to cause aggregation of NFκB (an inflammatory transcription factor) and mediate vascular protection (28). We therefore propose that a similar mechanism may be in place also in renal tubular epithelial cells to cause aggregation of JAK2.

In conclusion curcumin is shown to be a potent inhibitor of cystic growth *in vitro* by inhibiting JAK2, which is highly expressed in the ADPKD murine kidneys. Therefore, our work suggests that specific JAK2 inhibitors need to be investigated in ADPKD.

METHODS

Cell lines. Conditionally-immortalized ADPKD-derived lines (OX161 and SKI-001) (29) are tubular epithelial cells isolated from human kidneys and immortalized by transduction at an early passage (P1-4) with a retroviral vector containing a temperature-sensitive large T antigen and the catalytic subunit of human telomerase (30). The F1-Pkd1 WT renal epithelial cells were previously isolated from kidney papillae of the Pkd1fl/fl mouse (B6.129S4-Pkd1tm2Ggg/J; the Jackson Laboratory) and were immortalized with the lentiviral vector VVPW/mTert expressing the mTert; to delete Pkd1 and produce F1/Pkd1-/- cells they were subsequently transfected with VIRHD/HY/SiIntβ1/2363 lentivectors followed by hydromycin selection (31).

Cyst assays. Cyst assays were performed as previously described (4). In brief, for ADPKD cystic cell lines, 2 human lines namely OX161, SKI-001, and one mouse line F1-Pkd1$^{-/-}$ were used. OX161 and SKI-001 were kept at 33°C F1-Pkd1-/- were at 37°C. Healthily growing cells were trypsinised and re-suspended in DMEM media containing 10% FBS (no antibiotics) and counted using a haemocytometer. Subsequently 2x10^{4} cells per well were mixed with basement Matrigel (354230, BD biosciences). Prior to use the Matrigel was left to thaw on ice and once cells were added it was immediately loaded onto the 96 well plate, allowing it to polymerise. 100µl of DMEM media supplemented with 10% FBS was added to each well and cells returned to cell incubator for 24 hours to allow them to recover. After 24 hours, the media was aspirated and replaced with fresh media (DMEM + 10% FBS) supplemented with either vehicle (DMSO) or curcumin (Sigma). The cells were photographed before every media change and every two days, the media was replaced with fresh media containing either curcumin or DMSO.

Immunohistochemistry/immunofluorescence. Cells grown on coverslips were fixed with ice-cold methanol prior to blocking in 2% milk/TBST for 30 minutes and incubated with primary antibodies overnight at 4°C. Antibodies used was anti-rabbit JAK2 (3230S, cell Signalling, D2E12). Cells were washed and incubated with secondary which was an anti-rabbit AF594 (A-11037, Invitrogen (1:250) antibody. Microscopy was carried out using a confocal microscope. Immunohistochemistry using a JAK2 antibody (3230S, Cell Signalling) was performed as previously described (4).

Western blotting and curcumin treatment. Cells were analysed by Western blotting with previously reported protocols (32-35). In brief, cells were lysed using ice-cold Lysis Buffer (50mM Tris (pH 7.4) 250mM NaCl, 0.3% Triton X-100, 1mM EDTA) supplemented with protease inhibitor cocktail (Roche), freeze-thawed and sonicated. Whole cell lysates were boiled in 2xLaemmeli sample buffer for 5 minutes. Samples were resolved by SDS-PAGE and transferred using the Mini-PROTEAN system (Bio-Rad). Primary antibodies were anti-β-actin (ab8226, Abcam), anti-phosphorylated STAT3 (p-STAT3) (9145, Cell Signalling), anti-STAT3 (12640, Cell Signalling). Curcumin (08511-10MG, Sigma) was resuspended in DMSO and used at stated doses, DMSO served as vehicle control.

Animals. *Pkd1*$^{nl/nl}$, harboring an intronic neomycin-selectable marker, or wild type littermate controls were used (4,36). *Pkd1*$^{nl/nl}$ or control mice

were sacrificed at 5 or 10 weeks of age and kidneys collected, formalin-fixed and paraffin embedded. 5-micron section were used for histological examination. All mouse experiments were done under the authority of a U.K. Home Office license.

Statistical analysis. Data were analysed using Prism GraphPad and Non-parametric, two-tailed, Mann-Whitney T-tests or one-way ANOVA were performed. Results with a P value of 0.05 or lower were considered statistically significant.

Conflict of interest. None.

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Figures and legends

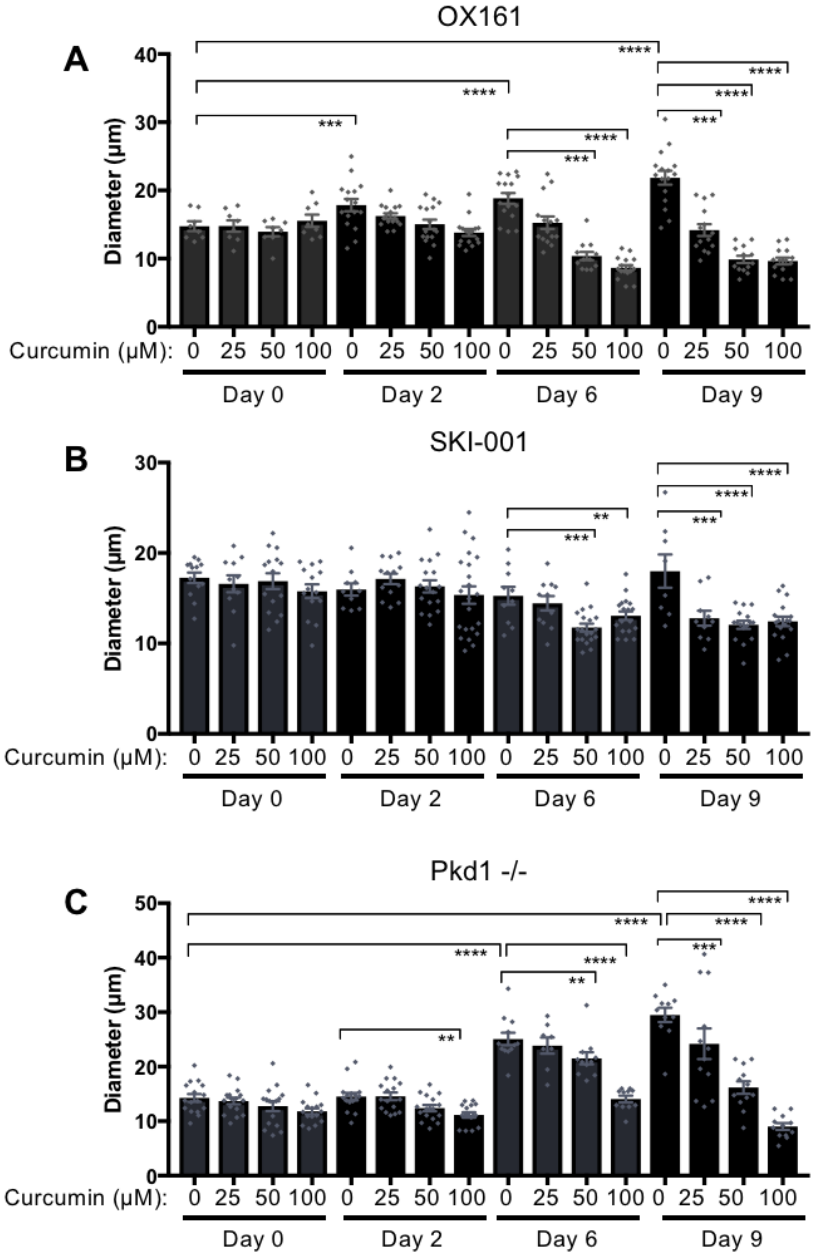

Figure 1: Curcumin slows down cyst growth *in vitro*. ADPKD-derived human epithelial cells were grown in BD-Matrigel to allow the formation of cysts to grow over time. All cysts formed were photographed at day 0, 2, 6 and 9; using an Olympus inverted microscope. Curcumin at 100, 50, 25 and 10µM or DMSO vehicle-control was added and cyst growth measured in **A** human OX161 **B** human SKI-001 and **C** mouse derived Pkd1$^{-/-}$ cell lines. One-way Anova with Bonferroni corrections was carried out and values lower that 0.05 were considered statistically significant. Symbol meaning: * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤0.0001.

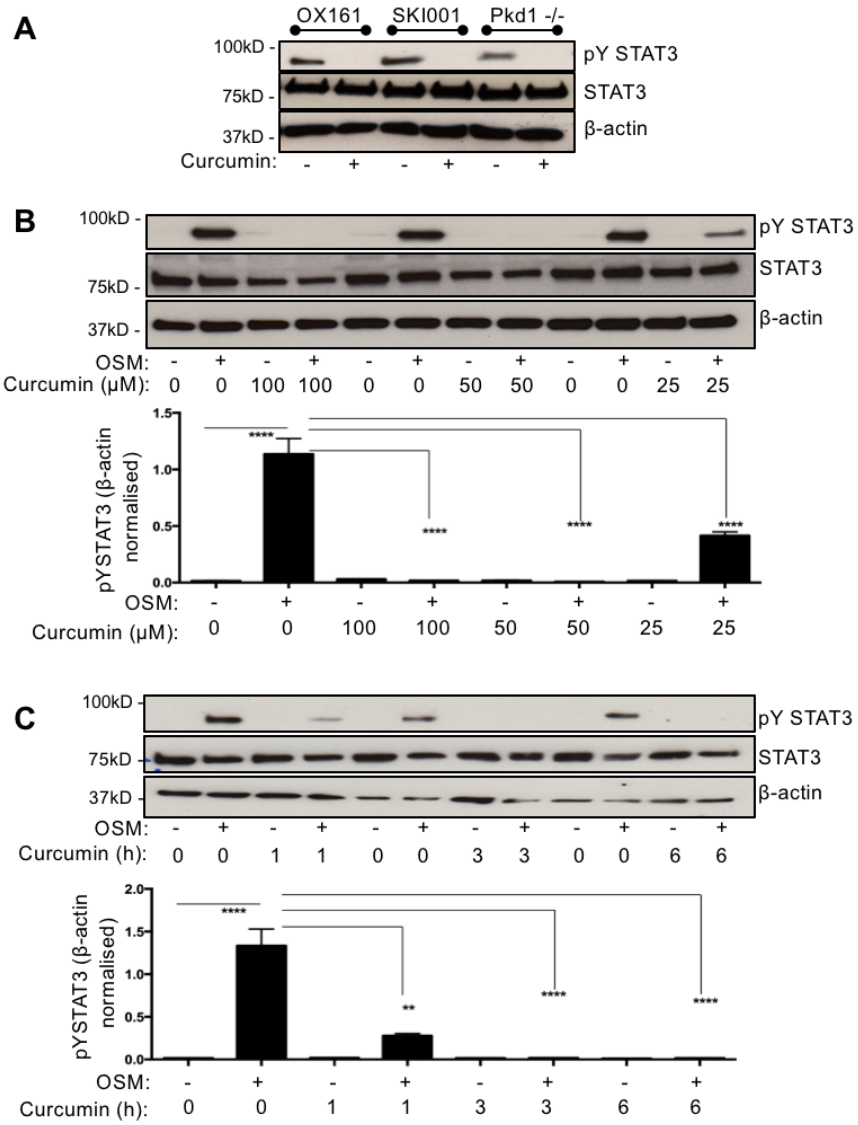

Figure 2: Curcumin is a potent STAT3 inhibitor. A. Oncostatin M (OSM, 10ng/ml) was used to stimulate phosphorylation of STAT3. Western blotting was performed (in human ADPKD-derived epithelial cells OX161, SKI-001 and mouse F1 Pkd1$^{-/-}$) anti-STAT3 phospho-tyrosine 705 antibody (pY STAT3), total STAT3 (STAT3) and β-actin which served as an internal loading control were used. **B.** SKI001 cells treated with either 100, 50 or 25μM of curcumin were subjected to blotting for STAT3, pYSTAT3 and β-actin control. Densitometric quantification of three independent blots was carried out. One-way Anova with Bonferroni corrections was performed, and values lower that 0.05 were considered statistically significant. C. SKI001 cells were treated with 100μM of curcumin for 1 or 3 or 6 hours and stimulated either with water vehicle control or 10ng/ml of OSM. pYSTAT3, STAT3 and β-actin were investigated by blotting. Quantification of three independent blots was carried out. One-way Anova with Bonferroni corrections was performed, and values lower that 0.05 were considered statistically significant. Symbol meaning: * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤0.0001.

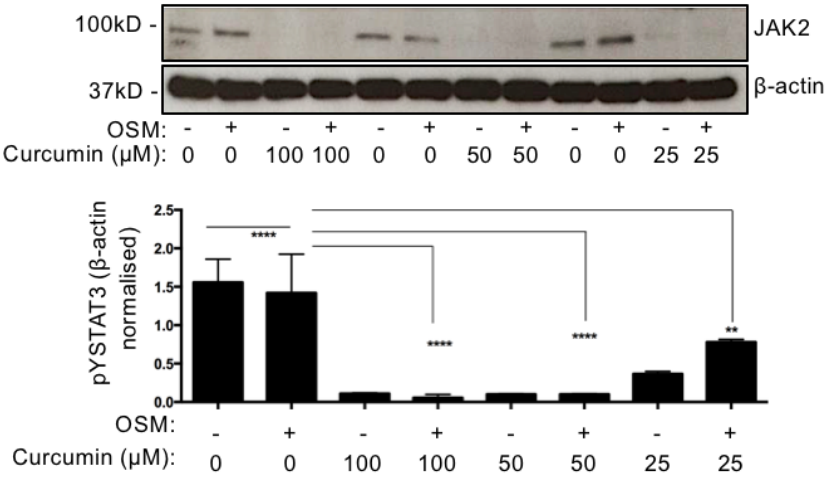

Figure 3: Curcumin inhibits JAK2. Western blotting of SKI001 lysates exposed to 100 or 50 or 25μM of curcumin was carried out, anti-JAK2 and β-actin were blotted. Quantification of three independent blots was carried out. One-way Anova with Bonferroni corrections was carried out and values lower that 0.05 were considered statistically significant. Symbol meaning: * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤0.0001.

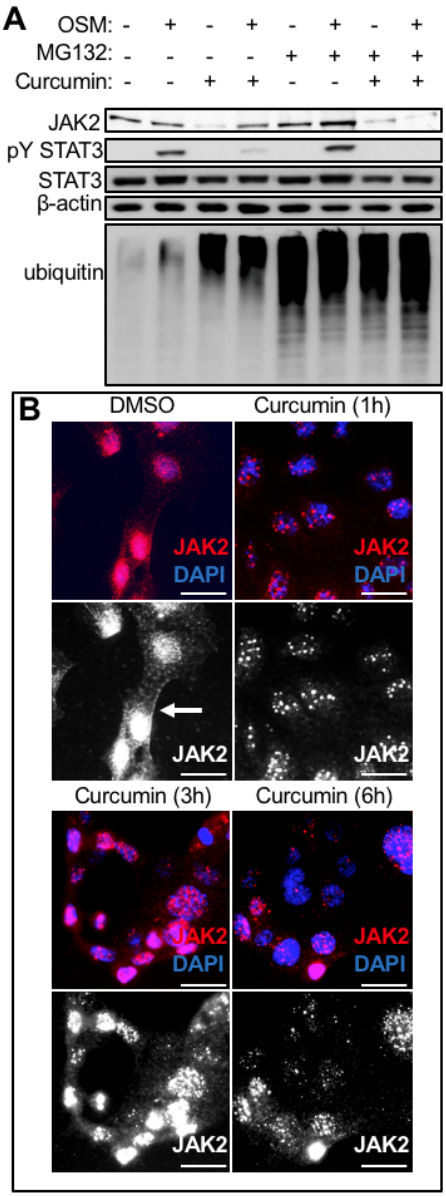

Figure 4: Curcumin controls JAK2 localisation. **A.** SKI001 cells were treated with OSM (10ng/ml), or MG132 (50µM) or curcumin (50µM) for 3 hours and lysates were subjected to immunoblotting. Antibodies against JAK2 pYSTAT3, STAT3, ubiquitin (indicator of proteasome inhibition control) and β-actin (internal loading control) were studied. **B.** SKI001 cells were either treated with DMSO vehicle control for 3 hours (3h) or curcumin for 1h or 3h or 6h, they were then snap frozen on ice-cold methanol, stained with anti-JAK2 rabbit antibody, followed by an anti-rabbit AF594 and imaged in an inverted fluorescent microscope. Red is anti-JAK2 (also in greyscale in lower panel), blue is nuclear counterstain (DAPI). Scale bars are 25µm.

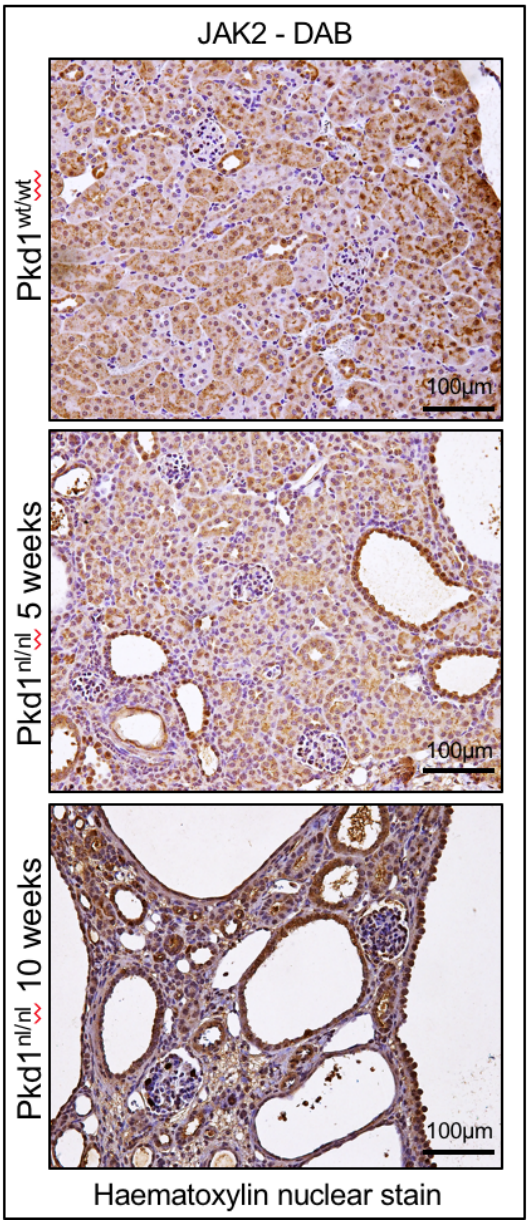

Figure 5: Cystic epithelial cells are JAK2 positive. Kidneys sections of an orthologous ADPKD murine model, Pkd1$^{nl/nl}$, at 5 and 10 weeks of age (5 mice in each group) were used to stain for JAK2. Immunohistochemistry of JAK2 was performed and representative images at 20x magnification are shown. The nuclei are counterstained with haematoxylin. Scale bars are 100µm.