Survivin-Induced Abnormal Ploidy Contributes to Cystic Kidney and Aneurysm Formation

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Background—Cystic kidneys and vascular aneurysms are clinical manifestations seen in patients with polycystic kidney disease, a cilia-associated pathology (ciliopathy). Survivin overexpression is associated with cancer, but the clinical pathology associated with survivin downregulation or knockout has never been studied before. The present studies aim to examine whether and how cilia function (*Pkd1* or *Pkd2*) and structure (*Tg737*) play a role in cystic kidney and aneurysm through survivin downregulation.

Methods and Results—Cysts and aneurysms from polycystic kidney disease patients, *Pkd* mouse, and zebrafish models are characterized by chromosome instability and low survivin expression. This triggers cytokinesis defects and formation of nuclear polyploidy or aneuploidy. In vivo conditional mouse and zebrafish models confirm that *survivin* gene deletion in the kidneys results in a cystic phenotype. As in hypertensive *Pkd1*, *Pkd2*, and *Tg737* models, aneurysm formation can also be induced in vascular-specific normotensive *survivin* mice. *Survivin* knockout also contributes to abnormal oriented cell division in both kidney and vasculature. Furthermore, survivin expression and ciliary localization are regulated by flow-induced cilia activation through protein kinase C, Akt and nuclear factor-κB. Circumventing ciliary function by re-expressing survivin can rescue polycystic kidney disease phenotypes.

Conclusions—For the first time, our studies offer a unifying mechanism that explains both renal and vascular phenotypes in polycystic kidney disease. Although primary cilia dysfunction accounts for aneurysm formation and hypertension, hypertension itself does not cause aneurysm. Furthermore, aneurysm formation and cyst formation share a common cellular and molecular pathway involving cilia function or structure, survivin expression, cytokinesis, cell ploidy, symmetrical cell division, and tissue architecture orientation. **(*Circulation*. 2014;129:660-672.)**

Key Words: aurora kinase ■ blood flow ■ blood pressure ■ cardiovascular system ■ epithelium ■ endothelium, vascular

Polycystic kidney disease (PKD) is the most common hereditary kidney disorder, and formation of bilateral cystic kidneys is the hallmark of the disease. Among other extrarenal phenotypes, aneurysm formation is one of the deadliest vascular abnormalities observed in PKD patients. Unfortunately, there is no study that explains the formation of these "bulb-like structures" in both vasculatures and renal tubules. Abnormalities in primary cilia,[1–4] polyploidy,[5,6] and centrosomal number[5,7] have been independently studied in the vascular or renal systems. However, there is currently no unifying mechanism that explains these cellular phenotypes.

Clinical Perspective on p 672

Survivin is a chromosomal passenger involved in coordinating proper chromosomal events during mitosis.[8] Because of its clinical manifestation in cancer, overexpression of survivin has always been the main focus of medical research. Whereas overexpression of survivin is associated with cancer formation and progression, the *Survivin* knockout mouse model is not viable beyond 4.5 days post coitum.[9] Interestingly, *survivin* homozygote cells isolated at 4.5 days post coitum show a cellular polyploidy phenotype similar to that of *Pkd* cells.

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Our previous in vitro studies showed that vascular endothelial *Pkd* cell lines are characterized by survivin downregulation, resulting in abnormal spindle assembly checkpoint and polyploidy.[5] Here, we expanded our study through the use of in vivo mouse and zebrafish models to demonstrate that survivin knockout or knockdown is sufficient to induce the formation of bulb-like structures in the kidney tubule (cysts) and artery (aneurysms). Our studies further suggest that mechanosensory cilia regulate survivin expression and dictate the formation of cell ploidy. The asymmetrical cell division resulting from abnormal ploidy further undermines the establishment of tissue polarity or planar cell polarity, which is believed to be the underlying mechanism for tubule or artery dilatation. We thus propose a common cellular mechanism through survivin to explain both vascular and renal phenotypes in PKD.

Methods

Signed and informed consent to collect disposed human kidneys with PKD was obtained from the patients, and kidney collection protocols were approved by the Department for Human Research Protections of the Biomedical Institutional Review Board of the University of Toledo. The use of animal tissues was approved by the University of Toledo animal care and use committee.

Mouse Models

The following mouse models were used in our studies; *Mx1Cre, PdgfβCre, Tie2Cre, Pkd1^{flox}*, *Pkd2^{-}*, *Tg737^{Orpk}*, and *survivin^{flox}*. To accelerate the experimental cystic model, unilateral ureteral obstruction (UUO) was generated by tying a 6-0 silk suture against a 28G-gauge needle in the mice. Standard histology analyses were used to examine the kidneys. To accelerate experimental aneurysm formation, 0.25 mol/L calcium chloride was placed directly on the abdominal aorta of the mice for 10 minutes.

Cell Culture

Human and mouse primary tubular cells from distal collecting tubules were used in the present studies. For cell lines, we used previously generated mouse endothelial cells[3] and human renal epithelial cells.[4] In some experiments, human full-length survivin–green fluorescent protein was used, in addition to siRNA knockdown on protein kinase C (PKC), Akt, aurora-A, and survivin. These cells were then subjected to cell cycle, live imaging, karyotyping, immunostaining, or Western analysis.

Chromosomal Analysis

Chromosomes from a single cell were spread and hybridized with a cocktail of mouse or human fluorescence-labeled probes specific for individual chromosomes.[10] Data were analyzed with automated SKY View software (version 1.62). Because zebrafish chromosome-specific probes were not available, individual chromosomes were analyzed on the basis of the ideogram derived from the replication banding of *Danio rerio*.

Live Imaging study

Primary renal epithelial cells or vascular endothelial cells were transfected with or without *Survivin* siRNA. Hoechst dye was use to indicate the nucleus.

Zebrafish Study

Wild-type zebrafish AB strains were used for knockdown experiments with either control morpholino (*controlMO*: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) or *Pkd2* morpholino (*pkd2MO:* 5′-AGG ACG AAC GCG ACT GGG CTC ATC-3′). For rescue experiments, 100 pg purified full-length human *survivin* mRNA was either coinjected with *pkd2* morpholinos or injected alone into the 1- to 2-cell-stage embryos. In another case, 2.5 ng vascular endothelial growth factor (VEGF) was coinjected with *pkd2* morpholinos.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Effectiveness of the knockdown or overexpression in zebrafish was verified by reverse transcription–polymerase chain reaction. Total RNA was isolated from zebrafish embryos with TRIzol (Invitrogen, Inc) followed by DNase treatment (Roche, Inc). Superscript-II (Invitrogen, Inc) was used for cDNA synthesis. Reverse transcription–polymerase chain reaction is performed under the following cycling conditions: 95°C for 15 minutes and then 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute. The cDNAs were amplified using specific primers indicated in the Table.[11–14]

Pharmacological Agents

The pharmacological agents used in our studies include PKC inhibitor (5 µmol/L Bisindolylmaleimide XI hydrochloride, Sigma Inc), PKC activator (10 µmol/L forskolin, Sigma Inc), taxol (33.3 nmol/L, Sigma, Inc), nocodazole (0.1 µg/mL, Sigma, Inc), VEGF (2.5 ng, Prospec, Inc), and colcemid (50 µg/mL, Invitrogen Inc).

Data Analysis

Both surgical and nonsurgical kidneys were studied and compared among the mouse groups. All quantifiable data were reported as mean±SEM. Distribution analyses were performed on all data sets before any statistical comparisons to confirm normal data distribution (a bell-shaped curve distribution). Homogeneity of variance (homoscedasticity) was also verified within each data set. When the data set was not normally distributed or heterogeneous variance was detected, the distributions were normalized by log transformation. This approach produced normally distributed data sets. After distribution and variance analyses, data comparisons for >2 groups were performed with ANOVA followed by the Dunn multiple-comparison posttest analysis. Comparison between 2 groups was carried out with the Student *t* test. Whenever possible, paired experimental design was used in our studies to allow more powerful statistical analysis and the use of fewer mice in each study group. For all comparisons, power analyses were performed routinely to enable reliable conclusions, and comparisons with negative results had statistical powers of ≥0.8. Unless otherwise indicated, the difference between groups was statistically significant at $P<0.05$, and

Table. Primer Sequences

Description	Primer Sequence	Reference
Zebrafish *pkd2*	Forward: 5′-GGG ATA CGT GCT GTG GTT CTC-3′ Reverse: 5′-CAC GAT GAG CTC CAG TCG CGT-3′	11
Human *survivin*	Forward: 5′-AAG AAC TGG CCC TTC TTG GA-3′ Reverse: 5′-CAA CCG GAC GAA TGC TTT TT-3′	12
Zebrafish *survivin*	Forward: 5′-GGA GCG ACT TCG CAT CTA CAT-3′ Reverse: 5′-ACC TCA TCA CGA AAG TAG GCA ATC-3′	13
Zebrafish α-*tubulin*	Forward: 5′-GGA GCT CAT TGA CCT TGT TTT AGA TA-3′ Reverse: 5′-GCT GTG GAA GAC CAG GAA ACC-3′	14

significance is indicated in the graphs by asterisks to denote comparison with the wild-type control, nontreated, noninduced, or static group. The number of experimental replicates is indicated in the figures or figure legends. All statistical analyses were done with GraphPad Prism, version 5.0.

Results

Human and Mouse Polycystic Kidneys Are Characterized by Abnormal Ploidy and Survivin Downregulation

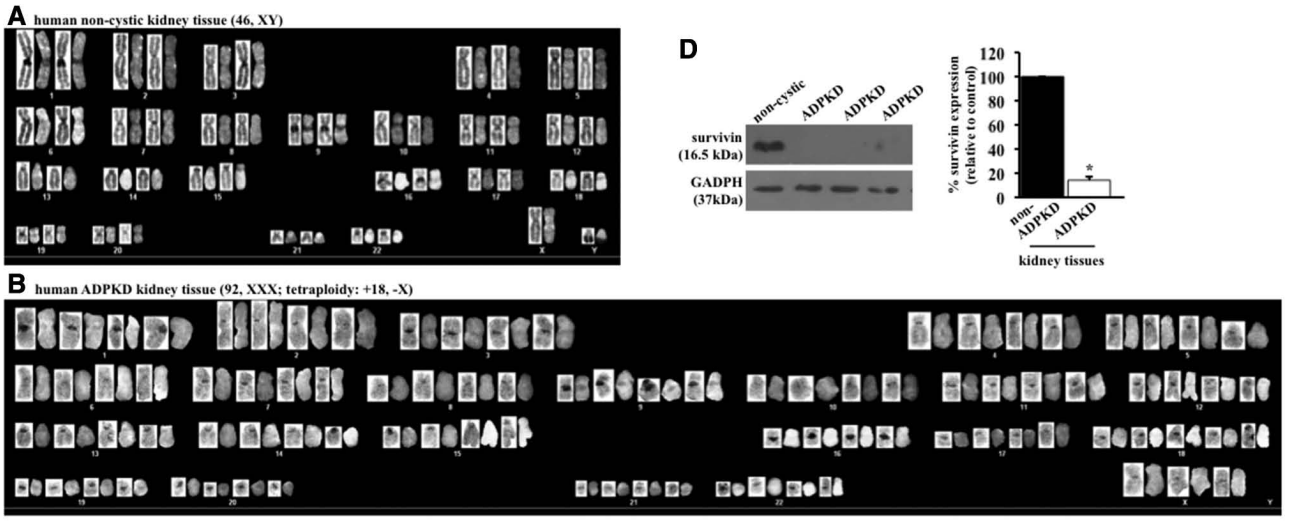
Compared with noncystic tissue (Figure 1A), karyotyping data of a single renal epithelium from PKD patients showed an abnormal ploidy (Figure 1B). We consistently observed an astonishingly high abnormality in the genetic composition in the samples acquired from PKD patients (Figure 1C). We recently showed that survivin is downregulated in *Pkd*-derived mouse vascular endothelia.[5] We therefore examined survivin expression levels in our patients' samples. All freshly isolated kidney samples from PKD patients consistently show a downregulation in survivin expression (Figure 1D).

Survivin Downregulation Is Sufficient to Promote Cystic Kidney Ex Vivo and In Vivo

Because *Survivin* knockout mouse dies at 4.5 days post coitum,[9] we crossed *survivin-flox* mice with kidney-specific *Cre* mice (*Mx1Cre*). We also performed UUO surgery as a renal injury model to examine the relationship between renal injury and cyst formation. We inactivated survivin (*Mx1Cre:Survivin*$^{flox/flox}$) in 1-week-old mice and analyzed the cystic kidney phenotypes in 4-week-old (Figure 2A-i) and 3-month-old (Figure 2A-ii) mice. At 5-week-old mice, the effects of *Survivin* knockout were most apparent in the injury model, in which the UUO kidneys were bulged and filled with fluid. Kidneys from 3-month-old *Mx1Cre:Survivin*$^{flox/flox}$ mice showed severe gross anatomic kidney defects. Cross-sectional analysis further showed that inactivation of *survivin* at 1 week of age was sufficient to induce kidney cyst formation at 5 weeks of age, although it was not as severe as those with UUO surgery (Figure 2B). Histology analysis using standard hematoxylin and eosin and fluorescent lectin staining confirmed a gross structure abnormality in *Survivin* knockout kidney, especially in the injury model, compared with wild-type age-matched kidneys undergoing the same surgery. Survivin inactivation resulted in a progressively more severe cystic kidney phenotype in older mice.

Survivin Downregulation Exacerbates Aneurysm Formation

The occurrence of aneurysm represents a major risk factor for morbidity and mortality associated with PKD.[15] To examine whether *Survivin* knockout would result in aneurysm, we induced aneurysm formation in endothelium-specific *Survivin* knockout (*PdgfβCre:Survivin*$^{flox/flox}$) mice. These mice were later euthanized to measure the aorta diameter at the site of the aneurysm surgery. Unlike wild-type mice, in which aorta diameter was only slightly enlarged after aneurysm surgery, *Survivin* knockout mice displayed a gross aortic aneurysm similar to that of *PdgfβCre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, or *Tg737*$^{Orpk/Orpk}$ mice after aneurysm surgery (Figure 3A). Histological analysis of the cross sections further confirmed a marked arterial enlargement and aneurysm



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kidney samples	normal	tetraploidy	aneuploidy	count (N)	% abnormal
non-ADPKD	34	0	2	36	6
ADPKD	35	12	11	58	40

Figure 1. Autosomal-dominant polycystic kidney disease (ADPKD) renal epithelia are characterized by abnormal ploidy level and survivin downregulation. **A**, Karyotyping was carried out in freshly isolated epithelial cells from non-ADPKD patients to visualize individual chromosomes (noncystic kidney). **B**, Characterization of individual chromosomes from a single renal epithelium isolated from an ADPKD patient indicated tetraploid and abnormal chromosomal composition. **C**, Overall karyotype analysis of individual cells confirmed the presence of abnormal genomic compositions (aneuploidy or polyploidy) in cells from ADPKD patients. **D**, Kidney tissues from ADPKD patients were also used to confirm survivin expression, and GADPH was used as loading control. Bar graph shows relative survivin expression levels. n=3 each for freshly isolated non-ADPKD and ADPKD kidneys.

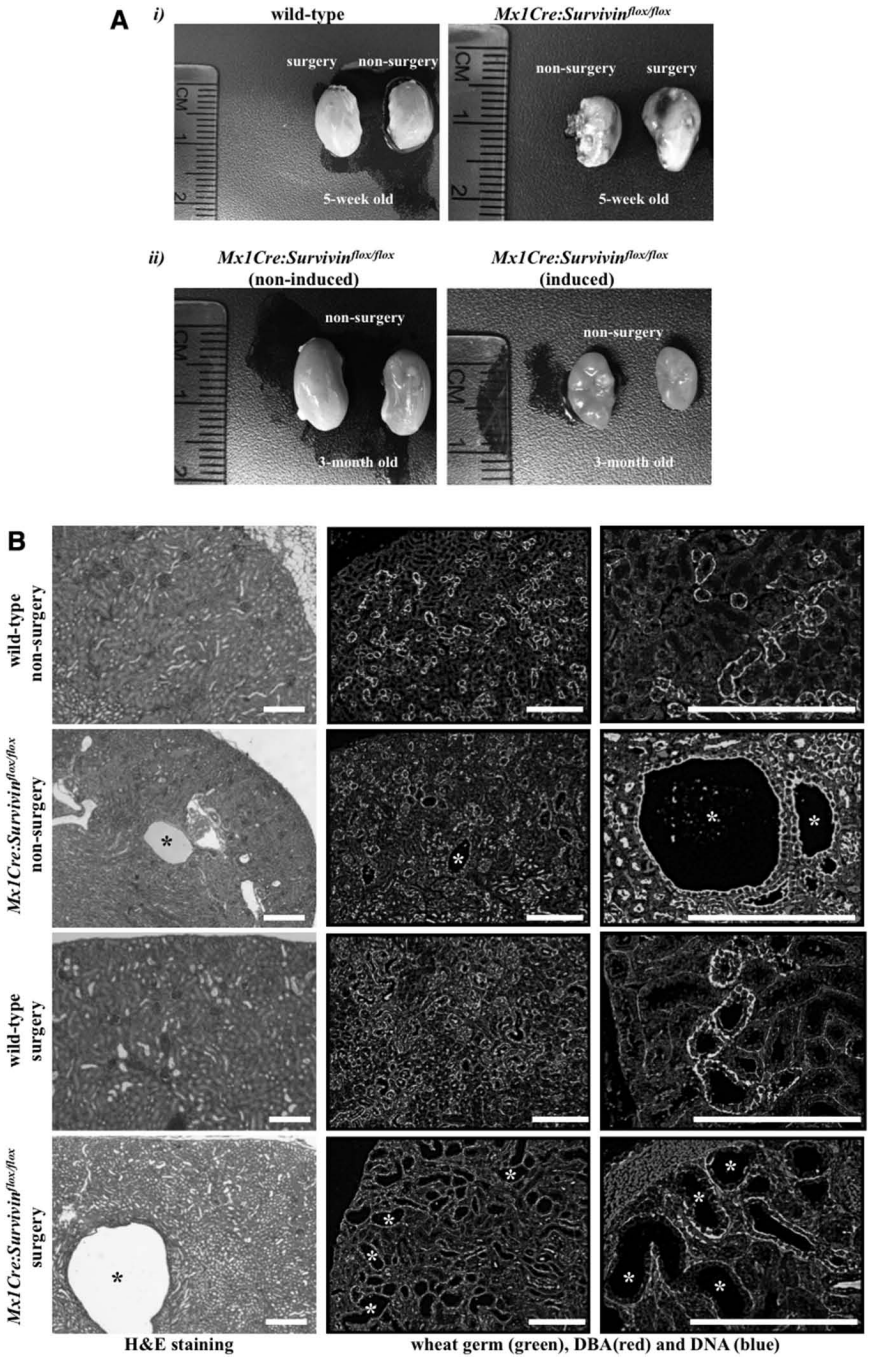
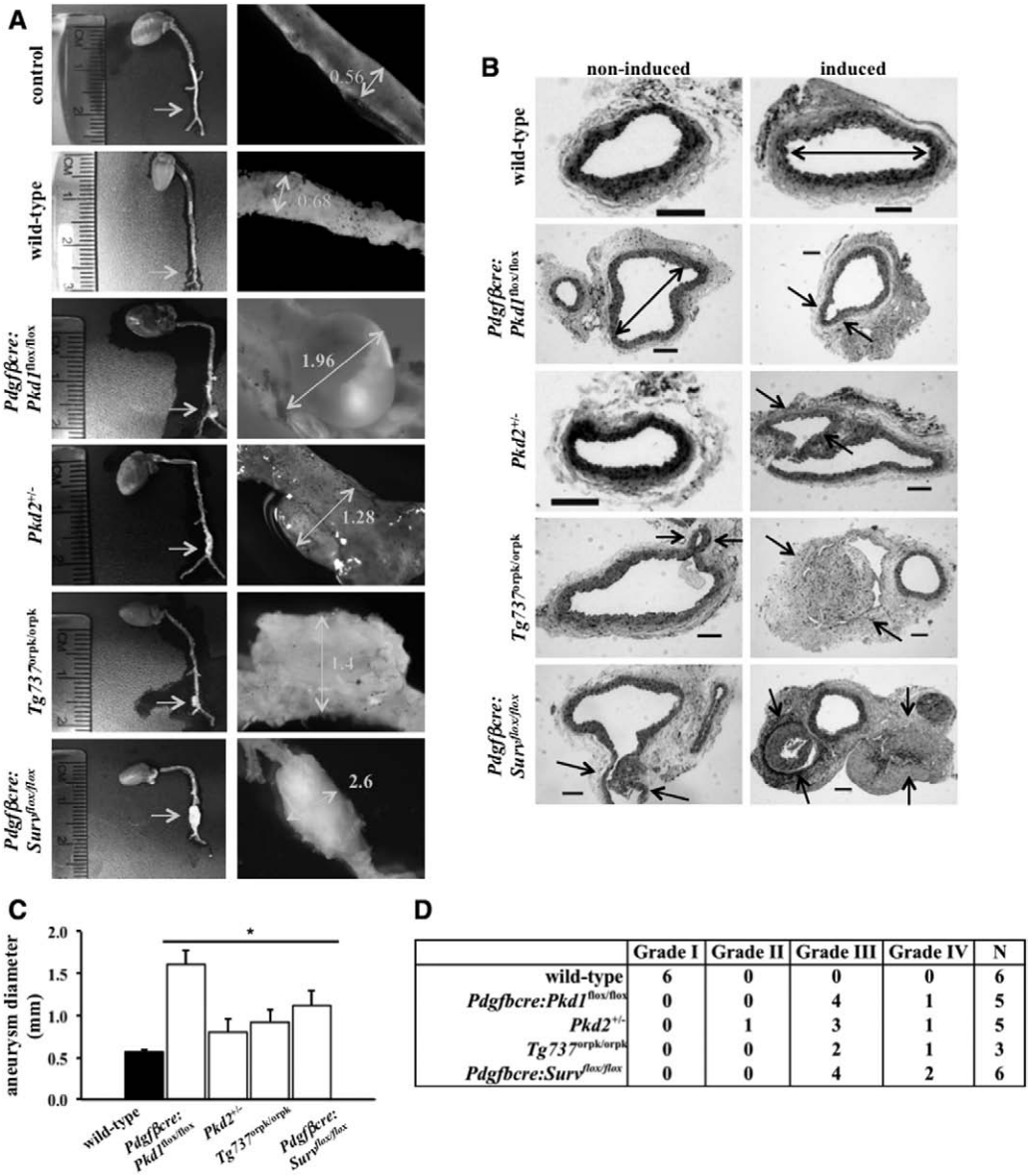


Figure 2. Survivin downregulation is sufficient to induce cystic kidney formation. **A-i**, Unilateral ureteral obstruction (UUO) was performed on either wild-type or *Survivin* knockout (*Mx1Cre:Survivin*$^{flox/flox}$) mice at 1 month of age, and mice were euthanized a week later. **A-ii**, *Mx1Cre:Survivin*$^{flox/flox}$-induced and noninduced littermates were euthanized, and their kidneys were compared 3 months later. Comparison of gross features revealed enlargement in *Survivin* knockout kidneys with an apparent bulged and fluid-filled kidney. **B**, Hematoxylin and eosin (H&E)–stained kidney sections from 5-week-old wild-type and *Mx1Cre:survivin*$^{flox/flox}$ mice with or without UUO surgery are shown. Florescence studies were performed with lectin Dolichos Biflorus Agglutinin (DBA) staining (red) and counterstained with wheat germ agglutinin (green) and DAPI (blue).

formation at the site of surgery from *PdgfβCre:Survivin*$^{flox/flox}$, *PdgfβCre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, and *Tg737*$^{Orpk/Orpk}$ mice (Figure 3B). Surprisingly, the *Pkd2*$^{+/-}$ mice also demonstrated a high propensity for aneurysm formation. Our data clearly indicated that similar to *Pkd1*, *Pkd2*, or *Tg737* inactivation, *Survivin* knockout resulted in aneurysm formation (Figure 3C). We next categorized the aneurysm types according to the classification by Daugherty et al.[16] Regardless of the genotypes, the mutant mice consistently showed a more severe grade than the wild-type mice (Figure 3D). Taking these results together, we proposed that vascular and kidney phenotypes of PKD may share a similar cellular mechanism through survivin.

Cellular Mechanism of Cystic and Aneurysm Formation Involves Polyploidy Formation Resulting From Abnormal Cell Division

To examine the mechanism by which survivin downregulation contributes to cystic kidney and vascular aneurysm, we performed live-cell imaging on renal epithelia (Figure 4A). As expected, we observed a symmetrical division in normal epithelial cell (Movie I in the online-only Data Supplement). Although survivin knockdown epithelium committed to enter cell division, severe cytokinesis defect was observed, resulting in failure to exit mitosis properly (Movie II in the online-only Data Supplement). This, in turn, led to polyploidy formation with cytomegaly and multinucleated phenotypes.



	Grade I	Grade II	Grade III	Grade IV	N
wild-type	6	0	0	0	6
Pdgfbcre:Pkd1$^{flox/flox}$	0	0	4	1	5
Pkd2$^{+/-}$	0	1	3	1	5
Tg737$^{orpk/orpk}$	0	0	2	1	3
Pdgfbcre:Surv$^{flox/flox}$	0	0	4	2	6

Figure 3. Aneurysm formation is an extrarenal phenotype of polycystic kidney disease. **A**, Aneurysm surgery was performed in mice at 1 month of age, and mice were euthanized at 3 months of age. The aortas were isolated and their diameters were measured at the surgical site. Unlike wild-type mice, *Pdgfβcre:Survivin*$^{flox/flox}$ mice showed a severe aneurysm induction to an extent similar to that shown in *Pdgfβcre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, and *Tg737*$^{orpk/orpk}$ mice. **B**, Representative cross sections of aortas at the aneurysm surgery site are shown in control wild-type, *Pdgfβcre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, *Tg737*$^{orpk/orpk}$, or *Pdgfβcre:Survivin*$^{flox/flox}$ mice with and without aneurysm surgery. Similar to *Pdgfβcre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, and *Tg737*$^{orpk/orpk}$ mice, *Pdgfβcre:Surv*$^{flox/flox}$ mice exhibited aneurysm formation and aortic dilation compared with wild-type mice. Arrows point to aneurysm formation, and double-headed arrows point to aorta diameter. n≥3 for each group and genotype. Bar, 200 μm. **C**, Bar graph shows averaged values for aorta diameter in wild-type, *Pdgfβcre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, *Tg737*$^{orpk/orpk}$, or *Pdgfβcre:Survivin*$^{flox/flox}$ mice. **D**, The grade of aneurysm is also tabulated.

Similar studies were performed on vascular endothelial cells (Figure 4B). Likewise, similar observations were obtained in control endothelia (Movie III in the online-only Data Supplement) and survivin knockdown endothelia (Movie IV in the online-only Data Supplement).

Polyploidy Formation Contributes to Abnormal Oriented Cell Division in Cystic Expansion and Aneurysm Formation

Oriented cell division dictates the maintenance of renal tubule diameter during tubular lengthening. Defects in this process will trigger renal tubular enlargement and cyst formation in *Pkd* rodent models.[17,18] We thus examined this possibility in *Survivin* mice. Unlike kidney sections from wild-type mice in which normal cell division orientation was parallel to the axis of kidney tubules, kidney sections from *Survivin* knockout mice (*Mx1Cre:Survivin*$^{flox/flox}$) revealed abnormal cell division and orientation pattern (Figure 5A). Both mitotic misorientation and abnormal cell division were very apparent in the *Survivin* knockout mice, particularly after UUO surgery. Abnormal cell divisions include enlarged nucleus, multinucleated cells, or asymmetrical mitosis. Our data further strengthened the argument that survivin shared a similar

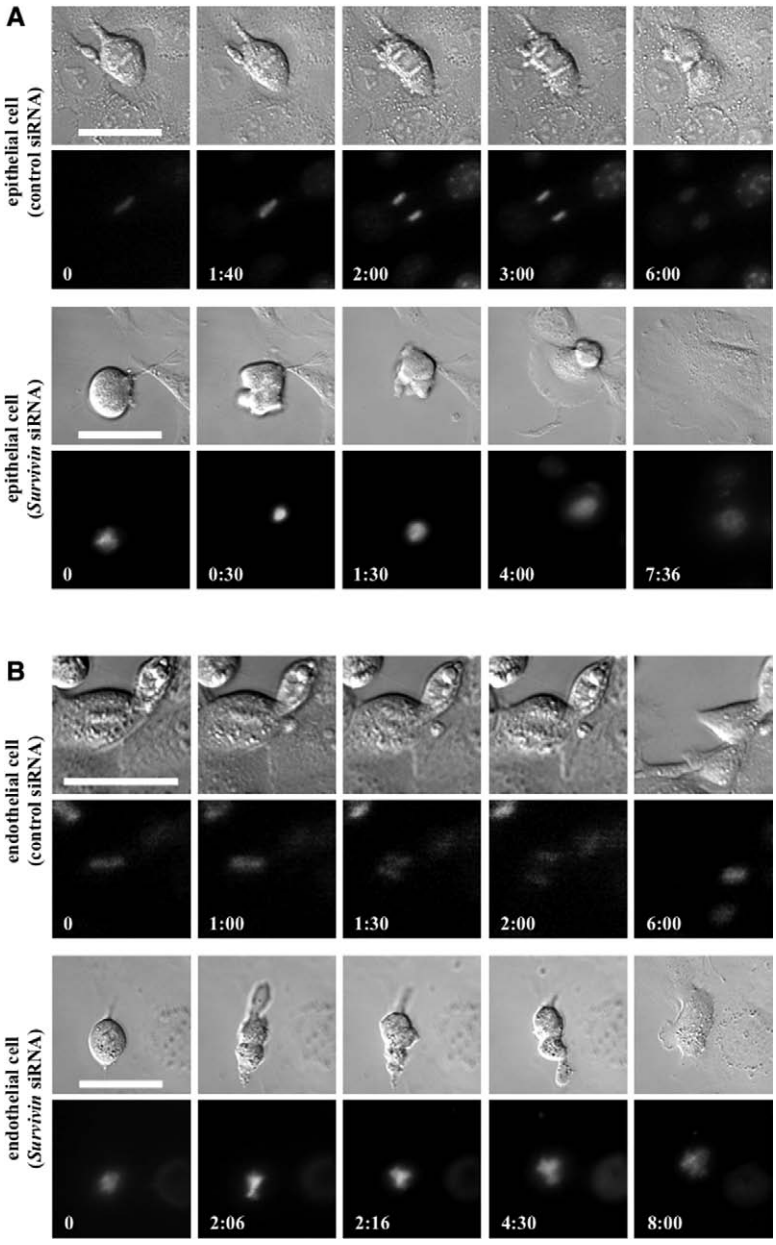


Figure 4. Survivin downregulation is associated with abnormal cytokinesis in primary cells of renal epithelia and vascular endothelial cells. **A**, To obtain the mechanistic insights of cytokinesis defect in renal epithelial cells, live-cell imaging analysis was performed. **B**, A similar study was also done in vascular endothelial cells. Control and survivin knockdown cells were loaded with Hoechst dye to examine nuclear division (**bottom**), whereas differential interference contrast (DIC) images were used to study cytokinesis (**top**). Time stamps indicate hours and minutes as illustrated in the Materials section and Movies I through IV in the online-only Data Supplement. Bar, 50 μm. n≥3 for each group and treatment.

cellular mechanism, as previously reported in polycystic kidney models.[17–19]

To further test our hypothesis that the pathogenesis of aneurysm and cystic kidney shared a common cellular mechanism, we examined for the first time how oriented cell division contributed to aneurysm formation in both *Survivin* and *Pkd* mouse models (Figure 5B). We studied cell division, cell-cell orientation, and division orientation in aortas from wild-type, *PdgfβCre:Pkd1*$^{flox/flox}$, *Pkd2*$^{+/-}$, *Tg737*$^{Orpk/Orpk}$, and *PdgfβCre:Survivin*$^{flox/flox}$ mice. Aorta sections from control wild-type mice displayed normal cell division orientation patterns; however, cell division orientation was slightly perturbed after aneurysm surgery. Similarly, aorta sections from *Pkd2*$^{+/-}$ mice displayed normal cell or cell division orientation, but they showed abnormal cell division after aneurysm surgery. On the other hand, aorta sections from *PdgfβCre:Pkd1*$^{flox/flox}$, *Tg737*$^{Orpk/Orpk}$, and *PdgfβCre:Survivin*$^{flox/flox}$ mice displayed abnormal cell division, cell-cell orientation, and cell division orientation with or without aneurysm surgery.

Primary Cilia Regulate Cell Division Through Survivin Expression

We previously showed that low survivin expression is associated with abnormal mitotic events in endothelial cells with cilia dysfunction.[5] To test our hypothesis that cilia function regulated survivin expression, we examined whether and how flow-induced cilia activation could regulate survivin expression. Wild-type, *Pkd1*$^{-/-}$, and *Tg737*$^{Orpk/Orpk}$ endothelial cells were subjected to fluid shear stress, and survivin expression was analyzed (Figure 6A). The differential expression of survivin between wild-type and cilia mutant cells was most obvious in the presence of fluid shear stress. Survivin expression increased after fluid flow in wild-type but not in cilia mutant cells. However, expression of aurora-A kinase was

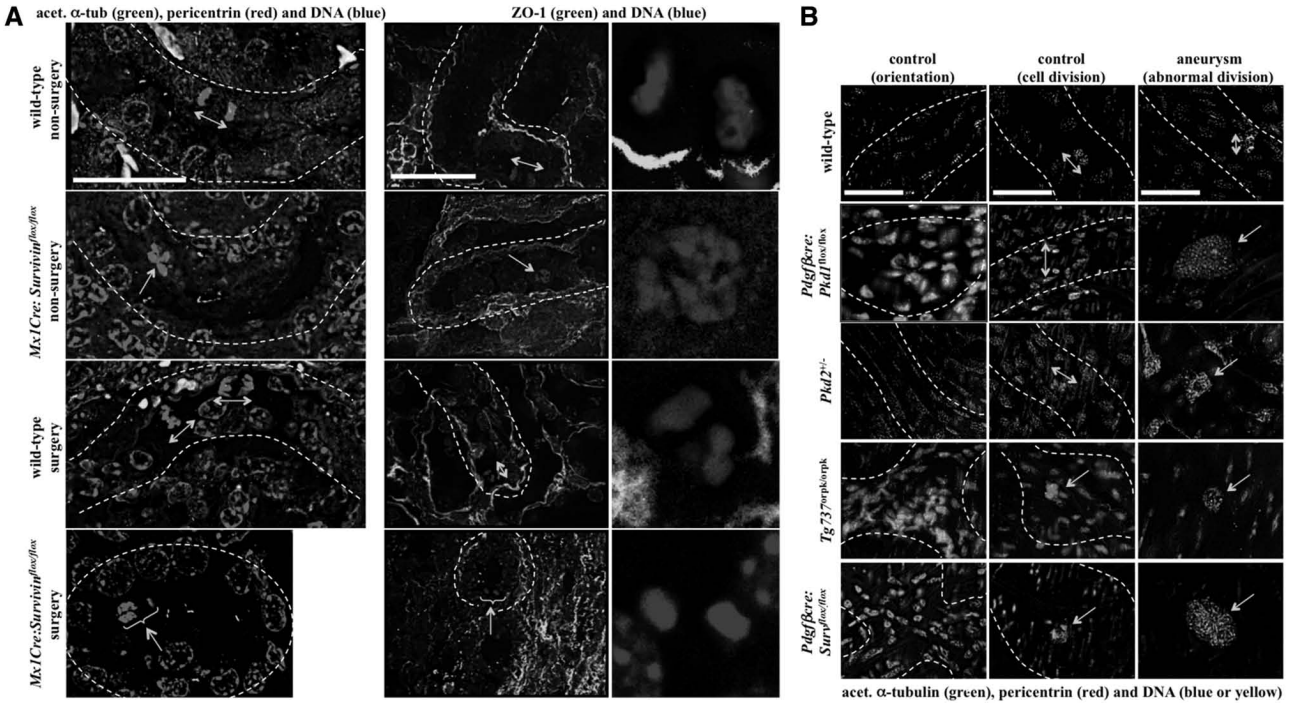


Figure 5. Abnormal cellular division orientation is associated with renal cystic and vascular aneurysm phenotypes. **A**, Kidney tubular sections from both *Mx1Cre:survivin*$^{flox/flox}$ mice, with or without unilateral ureteral obstruction surgery, showed abnormal cell division and division orientation with respect to the axis of the kidney tubule. ZO-1 staining was used to indicate renal tubule orientation, and a cell undergoing division within the region is further enlarged. **B**, Longitudinal abdominal aortic sections in nonsurgery (control) and aneurysm-induced (surgery) models were studied to analyze endothelial orientation and cell division. Nucleus from smooth muscle cells is shown in blue; the nucleus of a single intimal layer of endothelial tissue is pseudocolored in yellow. Abnormal randomized cell orientation is clearly visible. In all figures, division orientation relative to tubule/artery axis is shown in green double-headed arrows, and abnormal cell division is indicated by green arrows. n≥3 for each group and genotype. n≥100 for distribution of spindle orientation angle for each genotype and each treatment. Bar, 40 μm. Acet. α-tub indicates acetylated α-tubulin.

maintained at the same levels in all cells after fluid flow, indicating the specificity of flow-induced survivin expression. More surprising is that fluid flow induced survivin localization to primary cilia only in wild-type cells (Figure 6B). An increase in survivin expression and localization to cilia were not observed in cilia mutant cells in response to fluid shear stress, indicating that fluid flow was acting directly on cilia to induce survivin expression.

We next treated wild-type, *Pkd1*$^{-/-}$, and *Tg737*$^{Orpk/Orpk}$ cells with survivin or aurora-A inhibitors. At resting state (Figure 6C-i), such an inhibition resulted in centrosome overamplification with multiple stubby cilia formation. In dividing cells (Figure 6c-ii), inhibiting survivin or aurora-A induced mitotic arrest with profound defects in the bipolar spindle formation. Defects in resting and dividing cells were observed in wild-type cells, and they became more widespread in cilia mutant cells. Taken together, our data indicated that survivin expression was regulated by flow-induced cilia activation and that both survivin and aurora-A played critical roles in centrosomal number and cell division regulation.

Survivin Expression Is Regulated by PKC, Akt, and Nuclear Factor-κB

We previously demonstrated that PKC and Akt are downstream messengers of primary cilia.[5] Here, we asked whether cilia-induced survivin expression was also mediated by PKC or Akt. To assess whether Akt was downstream of PKC, we treated wild-type and cilia mutant cells with PKC inhibitor or PKC activator (Figure 7A). Expression of phosphorylated Akt (p-Akt) was significantly downregulated in all groups treated with PKC inhibitor compared with control nontreated groups. Moreover, p-Akt was significantly increased in cells treated with PKC activator. Consistent with a previous report,[20] our data support that Akt was downstream of PKC. We next analyzed whether Akt expression was dependent on cilia function (Figure 7B). Cilia activation by fluid flow caused a significant increase in p-Akt level in wild-type but not in cilia mutant cells. Moreover, this increase in p-Akt expression by fluid shear stress was repressed in wild-type cells treated with PKC inhibitor, indicating that Akt activation was dependent on cilia function and required PKC activity. In mutant cells, p-Akt expression was consistently and significantly depressed by PKC inhibitor. On the other hand, changes in aurora-A expression were not consistently observed in all groups after fluid shear stress or PKC inhibitor. We next examined whether aurora-A would regulate p-Akt, Akt, or survivin expression levels (Figure 7C). Our data demonstrated that although inhibiting aurora-A caused no apparent changes in p-Akt or Akt expression, the survivin level was slightly but not significantly altered. This suggested that aurora-A was neither regulated by fluid flow nor upstream of Akt.

It has been reported that Akt can regulate nuclear factor-κB (NF-κB), which is known to regulate survivin expression.[21,22] To investigate this possibility in our system, we studied both

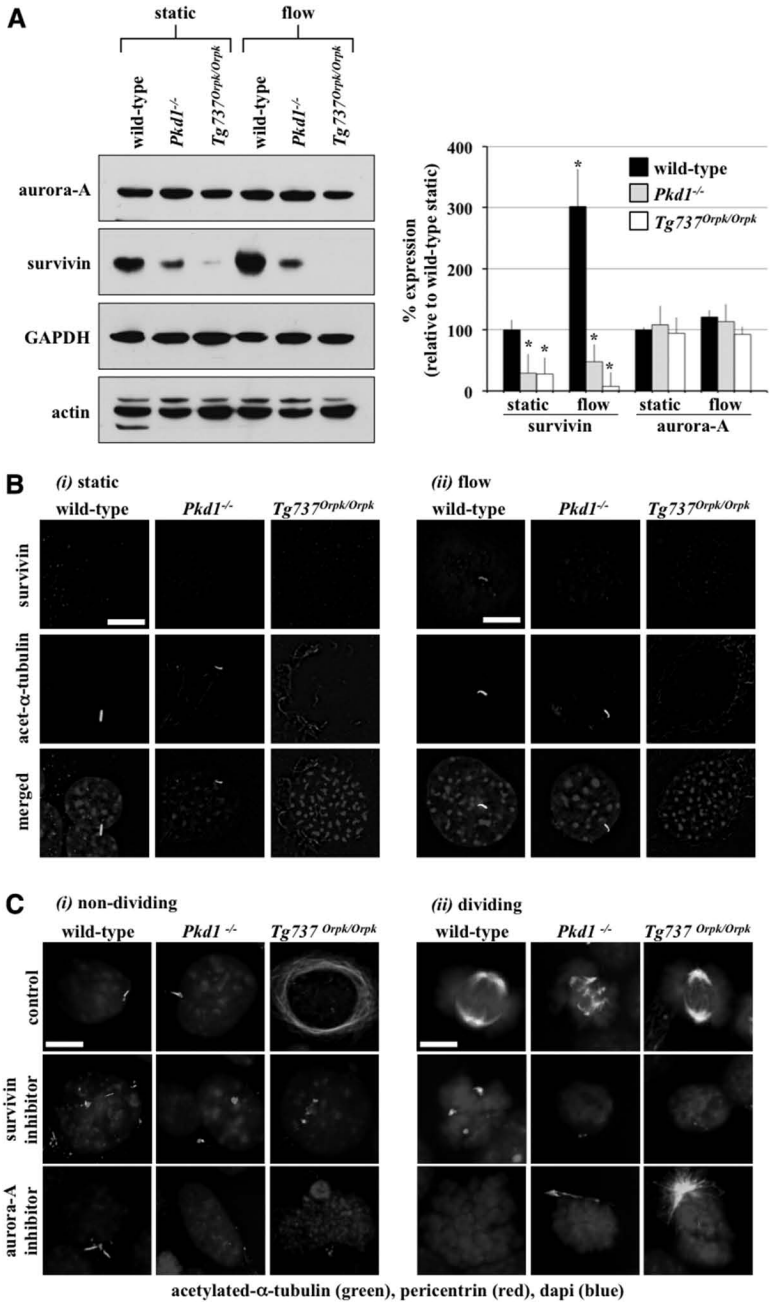


Figure 6. Cilia regulate cell division through survivin expression. **A**, Western blot analysis was used to study survivin and aurora-A expressions in wild-type and cilia mutant cells (*Pkd1*$^{-/-}$ and *Tg737*$^{Orpk/Orpk}$) in the presence or absence of fluid shear stress. GAPDH and actin were used as loading controls. Bar graph represents averaged survivin and aurora-A expressions. **B**, Acetylated α-tubulin (acet. α-tub) was used as a ciliary marker to indicate ciliary expression and localization of survivin in response to fluid shear in wild-type but not mutant cells. **C**, Cells treated with survivin or aurora-A inhibitors are characterized by multiple centrosomes, abnormal mitotic spindle, and mitotic arrest during cell division. Cells were stained with acetylated α-tubulin (green) and pericentrin (red) and captured at resting **(i)** and dividing **(ii)** stages of the cell cycle. Bar, 10 μm. n=3 for each cell type and treatment. Statistics was performed by comparing individual group with their corresponding wild-type static control groups.

NF-κB and phosphorylated NF-κB. We further inhibited aurora-A in the presence of fluid flow to confirm our earlier results and to study the relationship between aurora-A and NF-κB (Figure 7D). Our study corroborated our previous results that flow induces Akt phosphorylation[5] and that this induction was not affected by aurora-A inhibition. Consistent with our earlier studies, survivin expression was increased by flow, although this increase could be repressed by aurora-A inhibitor in the wild-type cells. More important, both NF-κB and phosphorylated NF-κB expressions were significantly increased in the presence of fluid flow in wild-type cells. No obvious changes of NF-κB and phosphorylated NF-κB expressions in response to fluid flow were observed in cilia mutant cells, although the basal level of NF-κB in the mutant cells was higher than in wild-type cells.

We thus far used various pharmacological agents to examine potential signaling pathways, amid some might have nonspecific or off-target effects. Therefore, we next used siRNA knockdown approaches to verify our proposed pathway (Figure 7E). Knockdown of PKC or Akt resulted in downregulation of p-Akt, aurora-A, and survivin expression. Aurora-A knockdown resulted in a decreased expression of aurora-A and survivin, whereas survivin knockdown showed a decrease in survivin expression only. The expression level of a common cell cycle marker, cyclin-B1, did not change after the siRNA studies, confirming that these knockdowns did not affect cell cycle and proliferation status in our cells. Taking these results together, we propose that the cilia-PKC–Akt–NF-κB pathway was involved in survivin expression and cell division regulation.

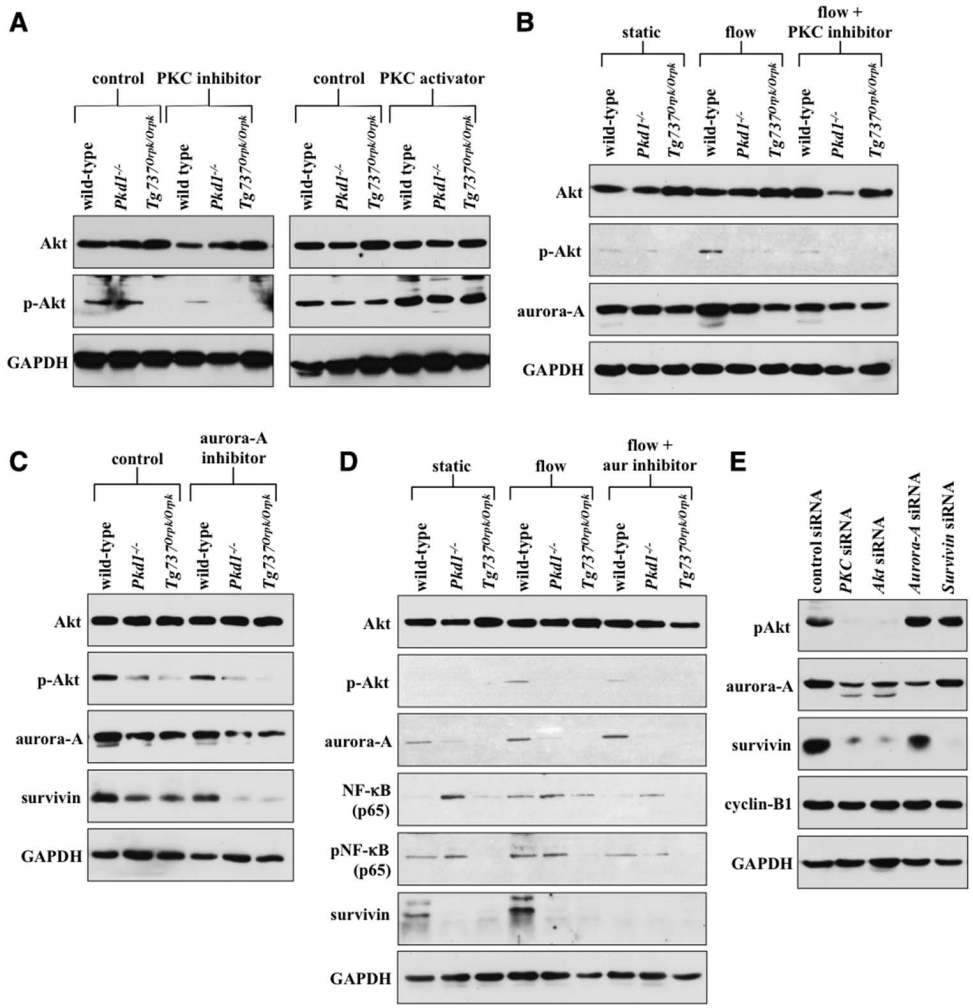


Figure 7. Protein kinase C (PKC)/Akt/nuclear factor-κB (NF-κB) signaling pathway regulates flow-induced survivin expression and cell division. **A**, After wild-type and cilia mutant (*Pkd1*$^{-/-}$ and *Tg737*$^{Orpk/Orpk}$) cells were treated with PKC inhibitor or activator, both Akt and phosphorylated (p-) Akt were analyzed. When treated with PKC inhibitor, all cell lines showed downregulation of p-Akt, whereas PKC activator treatment showed an increase in p-Akt compared with nontreated control cells. **B**, The effect of fluid flow on Akt and aurora-A expression was analyzed in the presence or absence of PKC inhibitor. When subjected to fluid shear, p-Akt expression was upregulated only in wild-type cells. Although p-Akt expression returned to basal levels after treatment with PKC inhibitor and fluid shear stress in wild-type cells, it stayed repressed in mutant cells. **C**, Treatment with aurora-A inhibitor resulted in a decrease in p-Akt, aurora-A, and survivin expression; however, these decreases were not significant compared with the control, nontreated group. **D**, Although the total Akt level was not changed, fluid shear stress significantly induced the expression of p-Akt in wild-type but not in mutant cells. Aurora-A expression was increased after fluid shear stress in wild-type cells; however, this increase was not significant compared with control. Both NF-κB and phosphorylated NF-κB (pNF-κB) expressions were increased after fluid shear stress only in wild-type cells, whereas mutant cells maintained a high basal level of NF-κB compared with static wild-type cells. Survivin expression was increased after shear stress in wild-type cells. **E**, Western blot analyses were conducted to confirm the signaling mechanism involving survivin expression by siRNA-mediated knockdown of PKC, Akt, aurora-A, or survivin. To further confirm the involvement of these signaling molecules in centrosome number and cell division abnormality, immunofluorescence and flow cytometry analyses are presented in the Materials section in the online-only Data Supplement, together with the statistics.

Re-Expression of Survivin Rescues PKD Phenotypes

Because *Survivin* knockout results in PKD phenotypes, it is expected that re-expression of survivin to normal levels should alleviate those phenotypes. To test this hypothesis, we used a zebrafish model for our in vivo studies. It has previously been reported that morpholino (*MO*)-induced depletion of *pkd2* causes profound developmental abnormalities, including cystic kidneys, curly tails, and pericardiac edema in zebrafish embryos.[11] We determined whether we could rescue the *Pkd2* morphants from these phenotypes by coinjecting mRNAs encoding the open reading frame of *survivin*. Because VEGF is known to induce survivin expression through the Akt–NF-κB pathway,[5,21] we also tested whether modulating this pathway by coinjecting VEGF would rescue PKD phenotypes. Our studies showed that coinjection of *survivin* mRNA or VEGF in morpholino knockdown of *pkd2* rescued the curly tail and cystic kidney phenotypes (Figure 8A). Overall data analysis showed that the rescue by *survivin* mRNA or VEGF was more apparent in younger (28 or 48 hours after fertilization) than older (72 hours after fertilization) fish. This was likely attributable to a decrease in the effectiveness or stability of injected survivin mRNA or VEGF as the fish developed to older stages. To examine whether *pkd2MO* zebrafish was associated with survivin downregulation as seen in the human

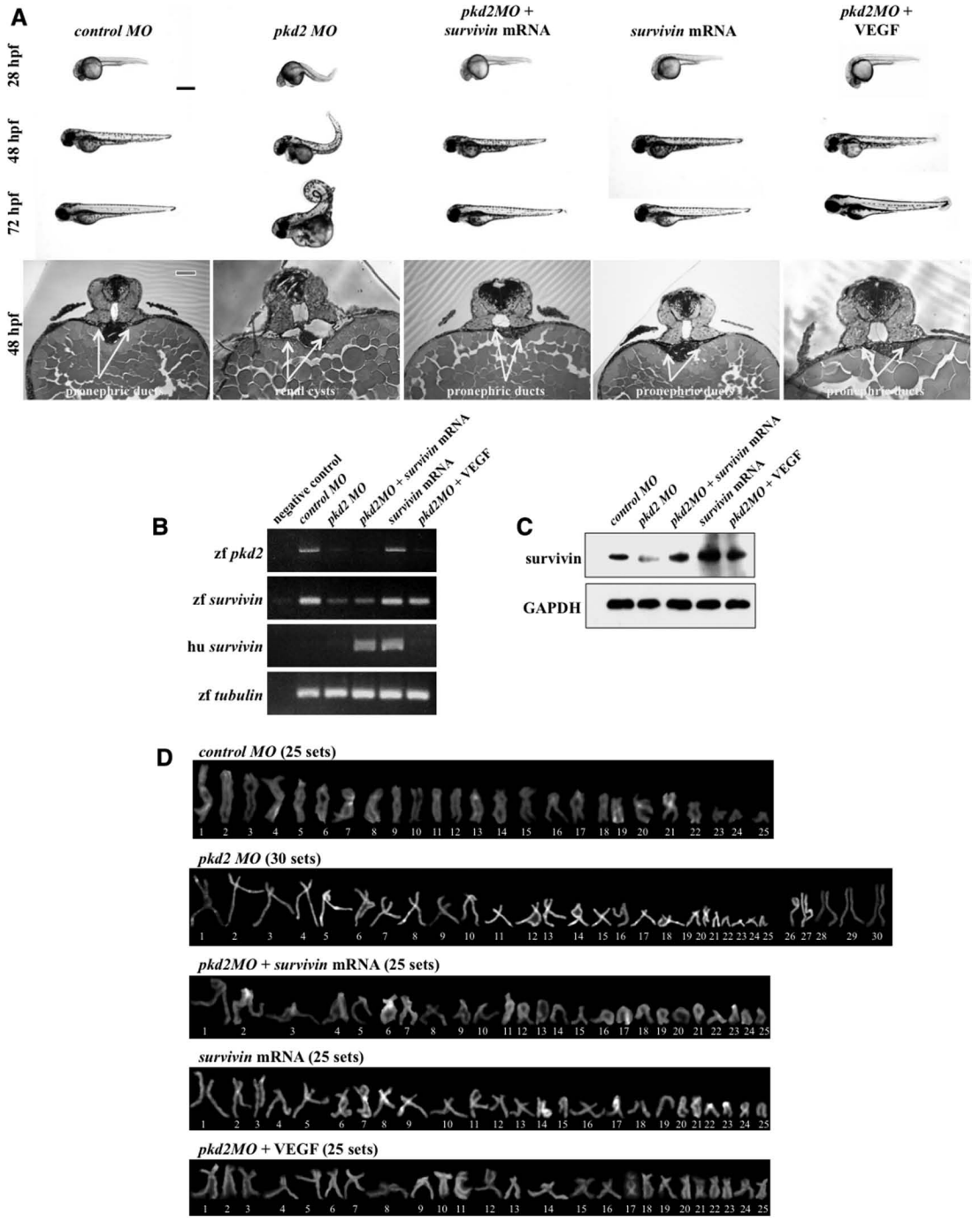


Figure 8. Survivin overexpression rescued polycystic kidney disease phenotypes in zebrafish. **A**, Zebrafish embryos were scored for phenotypic observations at different developmental stages. Shown here are representative images of zebrafish at 24, 48, and 72 hours postfertilization (hpf) injected with control morpholino (*MO)*, *pkd2 MO*, *pkd2 MO* plus *survivin* mRNA, *survivin* mRNA alone, or *pkd2 MO* plus vascular endothelial growth factor (VEGF). Abnormal phenotypes associated with *pkd2 MO* injections such as curly tail and renal cyst were rescued by *survivin* mRNA or VEGF injection into zebrafish embryos. Representative images of 48-hpf zebrafish sections are shown. The sections were stained with hematoxylin and eosin (**bottom**); and arrows point to pronephric structures. **B**, Reverse transcription–polymerase chain reaction (RT-PCR) was performed to examine zebrafish (zf) and human (hu) transcript levels for *survivin* and to confirm *pkd2* knockdown. Human survivin was introduced through mRNA injection. α-Tubulin was used as a loading control. **C**, Expression levels of survivin were analyzed in all the groups in which zebrafish and human survivin can be recognized by the same antibody. **D**, Analyses of individual chromosomes were performed in all groups of fish embryos to study chromosomal number. Black bar, 500 μm; red bar, 50 μm. All quantification and statistical analyses on Western blot, RT-PCR, and polyploidy level are presented in the Materials section in the online-only Data Supplement.

PKD and mouse models, we studied the levels of survivin transcript (Figure 8B) and protein (Figure 8C). The endogenous zebrafish *Pkd2* transcript levels were decreased in *pkd2MO*-, *pkd2MO* plus *survivin* mRNA–, or *pkd2MO* plus VEGF–injected embryos compared with *controlMO* fish. Injection of *survivin* mRNA alone did not alter the zebrafish

pkd2 transcript. Furthermore, the endogenous zebrafish *survivin* transcript levels were significantly decreased in the presence of *pkd2MO*, but this could be rescued by VEGF. Our data suggested that the PKD phenotypic rescue in *pkd2MO* plus VEGF–injected embryos was achieved via induction of endogenous zebrafish *survivin*, unlike in *pkd2MO* plus *survivin* mRNA–injected embryos, in which rescue depended on exogenous human *survivin*. Survivin protein was then quantified using an antibody that recognizes both human and zebrafish forms. Our analysis confirmed the decrease of survivin expression in *pkd2MO* fish and indicated that survivin expression could be rescued by human *survivin* mRNA or VEGF injection, leading to PKD phenotypic rescue.

To study how survivin rescued the PKD phenotypes, we investigated whether a molecular mechanism similar to human PKD and mouse models might involve abnormal polyploidy formation in zebrafish. Analysis of individual chromosomes confirmed a significant increase in polyploidy formation from cells derived from *pkd2MO* fish compared with those derived from *controlMO* fish (Figure 8D). This polyploidy increase could be partially rescued after coinjection with *survivin* mRNA. Overall, our data suggested that survivin played an important role in regulating ploidy, a common cellular contributor to PKD phenotypes.

Discussion

Our studies show that abnormal function of mechanosensory cilia leads to survivin downregulation, which is associated with abnormal ploidy formation and contributes to cystic kidney and vascular aneurysm phenotypes. We show for the first time that *Survivin* conditional knockout in the kidney or vascular tissues is associated with cyst or aneurysm formation, respectively. At least in the zebrafish model, re-expression of survivin can partially rescue PKD phenotypes. Overall, our studies demonstrate that primary cilia control renal and vascular architectures through survivin expression and symmetrical cell division along the longitudinal axis of the tissues.

Data from our PKD patients are supported strongly by the mouse and zebrafish studies, indicating that survivin downregulation triggers polyploidy formation, the predominant phenotype observed throughout our studies. In our controls, especially in human samples, some polyploidy was detected. This is most likely attributable to a physiological aging process characterized by cellular senescence.[23] A subpopulation of our precystic cells exhibited 8 N DNA content, suggesting that consecutive rounds of DNA replication without proper cell division are still possible in cells with a low survivin expression. This is consistent with the report that *survivin* deletion causes an overall decrease in cell number at the expense of DNA accumulation.[24] We further propose that polyploidy could potentially be used as an early marker in PKD. It is noteworthy that the sensitivity of the karyotyping technique is far superior to that of flow cytometry in single-cell analyses.[10] Thus, changes in chromosome number can easily be identified with absolute certainty in PKD patients before end-stage renal failure.

To ensure the clinical relevance of our findings in age- and sex-matched patients, we used noncystic and PKD human epithelial cell lines (Figure I in the online-only Data Supplement). We also used *Pkd2* and *Tg737* mouse models to verify our clinical data. Our mouse data supported the idea that polyploidy formation precedes cystic expansion and contributes to vascular aneurysm. As in human cell lines, all kidneys from *Pkd* mouse model samples were characterized by polyploidy and had a significant downregulation in survivin expression.

Given the evidence that kidney injury will trigger tubular epithelial cell proliferation,[25] in which survivin is required, it is not surprising that *Mx1Cre:Survivin*$^{flox/flox}$ mice exhibited more severe cystic kidneys in UUO-induced injury compared with their nonsurgical counterparts. In *Mx1Cre:Survivin*$^{flox/flox}$ mice with no surgery, the cystic kidney phenotype progressively became more severe with aging. Similar to *Pkd* mouse models, inactivation of vascular *Survivin* exhibited no apparent or consistent phenotype in 1-month-old adult mice. Especially during injury, however, survivin downregulation genetically or pharmacologically was directly linked to cystic kidney formation (Figure II in the online-only Data Supplement). To facilitate and accelerate vascular phenotype, we used a $CaCl_2$ aneurysm induction model. Not only in *survivin* but also in other *Pkd* mouse models, abnormal cell division or cilia function is sufficient to exacerbate the aneurysm phenotype and atherosclerotic plaques after the surgery. Homozygous *Survivin* knockout mice are also characterized by multinucleation, polyploidy, and apoptosis,[9] which are also seen in our renal epithelia and vascular endothelia of *Survivin*, *Pkd1,* and *Tg737*.

Downregulation of survivin was associated with apoptosis (Figure III in the online-only Data Supplement). More important, when blood pressure was monitored in our mutant mice, *Survivin* knockout mice surprisingly did not show an elevated blood pressure. This was consistent with the general understanding that hypertension itself does not account for aneurysm development.[26] Supporting this view, patients with PKD have a significantly greater chance of developing aneurysm than the general population with hypertension.[27]

Oriented cell division is involved in a variety of processes that contribute to organ shape and morphogenesis and are involved in coordinated cell division, differentiation, and spatial distribution. Elongation of the kidney tubule is also associated with oriented cell division, which when perturbed would result in cystic formation.[17–19] We show here not only that oriented cell division was perturbed in our *survivin*-inactivated renal tubules but also that the asymmetrical cell division phenotype was evident in dividing tubular cells. The mechanism of disturbed cell division and mitotic orientation was also confirmed for the first time in the arteries of *Survivin*, *Pkd1*, *Pkd2*, and *Tg737* mice. These mice show distorted cell division and mitotic spindle orientation, even before the aneurysm is formed, as also evidence from our studies on mitotic stress test and ploidy level in survivin knockdown epithelial and endothelial cells (Figures IV and V in the online-only Data Supplement). Overall, our comprehensive studies suggest that survivin downregulation is involved in the control of cell division, polypoidy, and asymmetrical division orientation. Furthermore, the control of cell division orientation defined a common mechanism for both cystic expansion and aneurysm formation in the *Pkd* and *survivin* mouse models.

Survivin, together with aurora-A kinase, regulates several distinct mitotic events such as the formation of mitotic spindle and cytokinetic ring.[8] We examined aurora-A kinase in our study because aurora-A has been shown to be localized to the basal body of primary cilia[28] and expressed abnormally in the cyst-lining renal epithelia.[29] Generally, neither survivin nor aurora-A was mislocalized in the mutant cells at different stages of cell division (Figure VI in the online-only Data Supplement). Our present data also suggest that aurora-A expression is not regulated by cilia activation through fluid flow. Nonetheless, our study reinforces the localization of aurora-A to the centriole in the resting stage and to the centrosome and midbody during cell division. More important, we demonstrated for the first time that inhibiting aurora-A function or expression would result in defects in cell division, ploidy, centrosomal amplification, cytokinesis, and mitotic spindle formation, all of which were phenotypes associated with survivin downregulation. This suggests that although aurora-A may not be part of the cilia-survivin pathway, aurora-A and survivin may function as molecular partners, reflecting their common roles in the contraction of the cytokinetic ring in regulating cell division (Figure VII in the online-only Data Supplement).

Not only is survivin expression increased after cilia activation, but our study also shows for the first time that its subcellular localization is differentially regulated from centriole to primary cilia after fluid shear stress. These localizations may reflect a novel survivin function in nondividing cells and may contribute to a larger pathological spectrum other than cancer or PKD. Moreover, our immunofluorescence analysis reveals the localization pattern of survivin during mitotic division, specifically during cytokinesis, reflecting its role in cytokinesis. This is also supported by live imaging studies in which survivin knockdown causes severe cytokinesis defects, resulting in cytomegaly and polyploidy phenotypes in both renal epithelia and vascular endothelia (see the movies in the online-only Data Supplement). Despite the finding that survivin expression was dysregulated in the cilia mutant cells, it is worth mentioning that survivin localization was not perturbed during cell division. To further decipher the signaling mechanisms between cilia and survivin expression, we examined the cilia–PKC–Akt–NF-κB–survivin/aurora-A pathway.

In an attempt to elucidate the physiological relevance of survivin downregulation in cystic kidney and vascular phenotypes in PKD, we used a zebrafish model to study the roles of survivin expression. Four novel insights are provided by these studies. First, *pkd2* knockdown is associated with survivin downregulation in zebrafish, which confirms our hypothesis that survivin expression is regulated by cilia function. Second, the rescue of PKD phenotypes associated with *pkd2* knockdown by re-expression survivin provides further evidence for the importance of survivin roles in PKD. Third, VEGF is an attractive modulator to induce survivin expression in *pkd2* knockdown fish. Fourth, *pkd2* knockdown contributes to polyploidy, a common mechanism representing PKD phenotypes as also seen in PKD patients and mouse models (Figure VIII in the online-only Data Supplement).

Conclusions

Our studies provide a novel aspect of the mechanism of pathogeneses of cystic kidney and aneurysm formation in PKD. Our present study shows for the first time that these phenotypes are contributed mainly by abnormal cilia function, resulting in dysregulation of survivin expression. Abnormal survivin expression further causes abnormal cytokinesis, which results in cell polyploidy, multimitotic spindle formation, and aberrant cell division orientation. The asymmetrical cell division, together with abnormal planar cell polarity, contributes to the expansion of tissue architecture, resulting in the formation of cystic kidney and vascular aneurysm. All in all, data from this study suggest that improving survivin expression could be a promising therapeutic target for kidney and vascular complications associated with PKD. Overall, our current working model would be as follows: primary cilia→PKC→Akt→NF-κB→survivin/aurora-A→cytokinesis→polyploidy→asymmetrical cell division/planar cell polarity→cystic kidney and vascular aneurysm (architecture expansion).

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Disclosures

None.

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CLINICAL PERSPECTIVE

Autosomal-dominant polycystic kidney disease (ADPKD) is a ciliopathy characterized by the formation of kidney cysts and vascular aneurysms. Surprisingly, these balloon-like structures in the kidney and blood vessel are greatly associated with one another. Although abnormal cilia function in detecting urine flow will result in kidney cyst formation, inability of cilia to sense blood flow can induce vascular aneurysm. The formation of these balloon-like structures is independent from blood pressure but is tightly regulated by survivin expression. During repair resulting from any physiological perturbation or insult, a proper expression level of survivin is required to maintain the overall architectural structure of an organ. ADPKD with abnormal cilia function fails to maintain this architectural structure because of a low survivin expression, which induces asymmetrical cell division. As a result of this random expansion during cell division, an elongated architecture of a nephron or vasculature will no longer be achieved. It is therefore not surprising that it takes a long period of time to form such structural abnormalities as renal cysts and vascular aneurysms. Our studies also raise some questions: Can a similar mechanism also occur in other organs besides renal and vascular systems in ADPKD? In addition, can we use survivin or cell ploidity as a biomarker to indicate disease progression or severity in ADPKD? More specifically, we have used spectral karyotyping, which only requires 1 single cell from our ADPKD patients to confirm their cellular polyploidity throughout our studies.