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14. ABSTRACT Hypertension and aneurysm are a prevalent problem in our society. Because the risk of these vascular diseases is too important to ignore, our studies are designed to provide exciting and original concepts to examine the etiologies of hypertension and aneurysm with regard to mechanosensory organelles (primary cilia). Especially within polycystic kidney disease, untreated hypertension can worsen kidney function. Aneurysm rupture in PKD patients also remains a devastating complication that often could result in stroke and death. Our studies therefore aim at investigating new ideas in understanding hypertension and aneurysm formation in PKD.					
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Polycystic kidney disease (PKD) is characterized by formation of fluid-filled cysts in both kidneys. PKD patients will eventually have renal failure, with subsequent dialysis or renal transplant. The genes mutated in PKD include Pkd1 and Pkd2, encoded for polycystin-1 and polycystin-2, respectively. Many studies have shown that the baseline circulating NO is much lower in PKD patients. This suggests possible vascular dysfunction. The purpose of our research is to investigate cilia function in the vascular endothelial cells. More important, hypertension has been a critically important risk factor for cardiovascular diseases in PKD patients, which occur early in these individuals, compared to their age-matched cohorts, and still remains the most frequent cause of mortality even when their renal function is still normal. With our expertise and tools available in our laboratory, we thus hypothesize that cilia in the cilia play crucial and important roles in regulating PKD pathology. Specifically, we will provide the first insights into physiological functions and cellular pathways of primary cilia in vasculatures and in PKD.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Cardiovascular, cilia, ciliopathy, ciliotherapy, endothelia, epithelia, polycystic kidney disease, polycystin-1, polycystin-2, primary cilia

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The main goal of the project is to determine the roles of primary cilia in PKD. This goal is divided into two major sub-aims, and our Statement of Work (SOW) is as follow.

Aim 1 (months 1-30). We will study mechanosensory function of endothelial cilia in hypertension.

Aim 1.1 (months 1-12): We will measure blood pressure in cilium mutant mice in vivo.

Aim 1.2 (months 13-30): We will examine signaling mechanisms of cilia & their effects on blood pressure.

Aim 2 (months 7-36). We will study mechanosensory function of endothelial cilia in vascular aneurysm.

Aim 2.1 (months 7-20): We will quantify aneurysm formation in cilium mutant mice in vivo.

Aim 2.2 (months 20-36): We will identify signaling mechanisms of cilia & their consequence on aneurysm.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Activities

Our major activities are as follow:

- To achieve four main goals as outlined in Aims 1 and 2.
- To train postdoctoral fellows and graduate students on the project.
- To remedy Helicobacter contamination in our mouse colonies.
- To further seek potential translational aspect of the project.

2. Specific Objectives

Our *scientific objectives* are:

- To study mechanosensory function of endothelial cilia in hypertension.
We accomplished this objective by the dividing the objective into 2 sub-aims:
Aim 1.1: We measured blood pressure in cilium mutant mice in vivo.
Aim 1.2: We examined signaling mechanisms of cilia & their effects on blood pressure.
- To study mechanosensory function of endothelial cilia in vascular aneurysm.
We accomplished this objective by the dividing the objective into 2 sub-aims:
Aim 2.1: We quantified aneurysm formation in cilium mutant mice in vivo.
Aim 2.2: We identified signaling mechanisms of cilia & their consequence on aneurysm.

Our *training objectives* are to launch an independent career for our postdoctoral fellows in academic research and to provide educational-rich environment for our graduate student to be successful in the biomedical research. Our strategies include excellent scientific training and professional development plans, discussed elsewhere below.

3. Significant Results and Key Outcomes

Aim 1.1: Measurement of blood pressure

In our first year, we have measured the systolic and diastolic blood pressure by non-invasive blood pressure system - tail cuff method with the aid of a computerized system (CODA system, Kent Scientific, Connecticut, USA). Measurements were performed at the baseline 3-times per week for 2 weeks after previous 3 days of training for each mouse. On each day of blood pressure measurement, 2 sets of 18 measurements were obtained including three measurements of training or acclimation. The measurements were averaged for each mouse with at least three mice for each genotype. All animals were tested by an investigator blinded to the genotypes of the animals. The data from the tail cuff method was also verified with limited studies with a more invasive, surgically implanted telemetry probe (data not shown).

When blood pressure was monitored in our mutant mice, *Survivin* knockout mice surprisingly did not show an elevated blood pressure (**Figure 1**). However, *Pkd1* and *Tg737* mice were hypertensive. Supporting this view, patients with PKD have a significantly greater chance to develop hypertension than general population.

Part of Aim 1 was to set up mouse models for Aim 2. To generate vascular-specific knockout, we have used and confirmed *PdgfbCre* and *Tie2Cre* mice (**Figure 2**). Briefly, one-week old pups were injected intra-peritoneally with 62.5 μ g of 50 μ L polyinosinic:polycytidylic ribonucleic acid (pI:pC) every day for five consecutive days. *Tie2Cre* or *PdgfbCre* mouse was bred with *Gt(ROSA)26Sor* (*Rosa26*) mouse, resulting in either *PdgfbCre*•*Rosa26* or *Tie2Cre*•*Rosa26* genotype. The *Rosa26* mouse was used as a control. These 3 mouse groups were induced to activate Cre which acts on *Rosa26* allele. The *Rosa26* genetic background is used as a reporter system to verify and validate the efficiency of the *Cre* mice. The *Rosa26* includes its inducible fluorescence reporter system; i.e. all endothelia lining the vasculatures have red fluorescence (non-induced). The red fluorescence will be replaced with green fluorescence upon *Cre* recombinant (induction). Abdominal aortas were isolated, stained with nuclear marker (blue), and imaged for their green/red fluorescence (**Figure 2a**). Quantitation analysis of vascular-lining endothelia indicates high efficacy of both *PdgfbCre* and *Tie2Cre* backgrounds to delete a specific gene in vascular lining endothelia (**Figure 2b**). N=4 for each genotype and treatment.

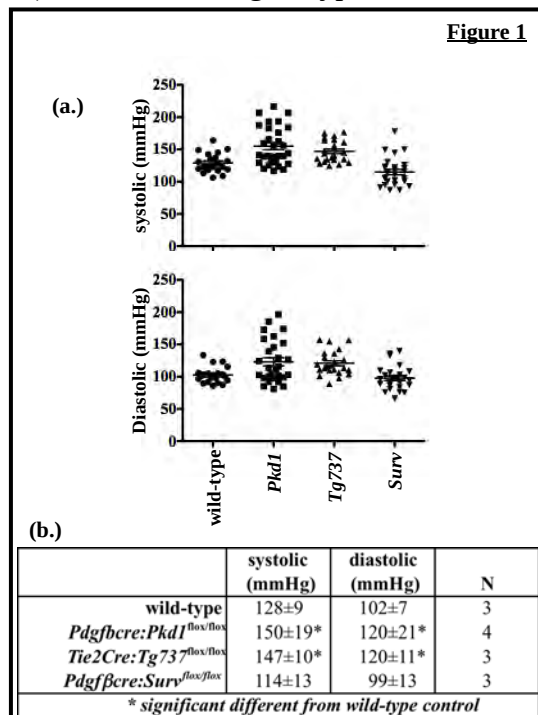


Figure 1. Blood pressure was measured in wild-type and conditional *Pkd1*, *Tg737*, and *Survivin* (*Sur*) mice for a 2-week period (a). The averaged measurements of blood pressure during this 2-week period were tabulated (b).

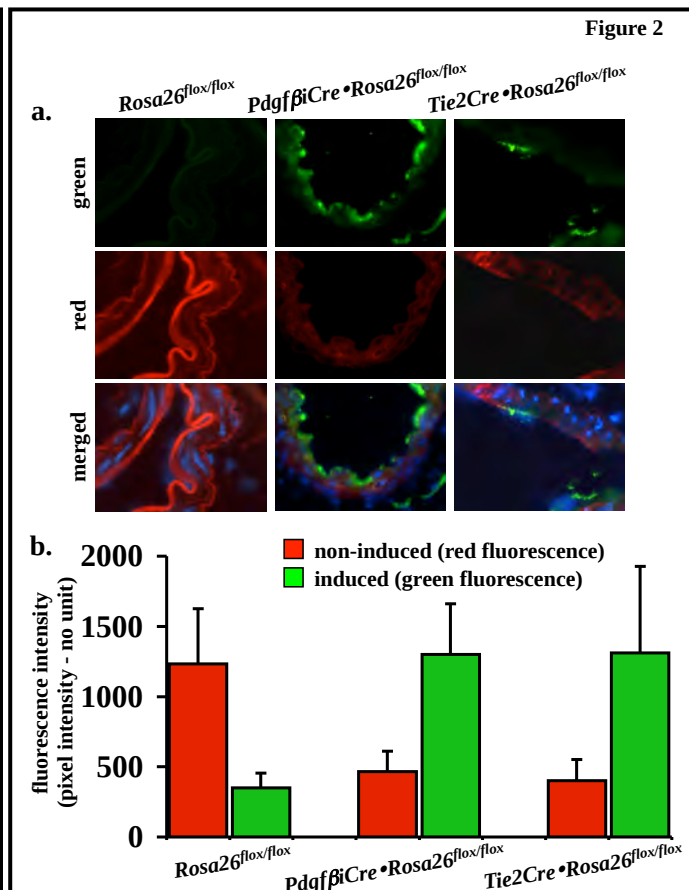


Figure 2. Both *Tie2Cre* and *PdgfbCre* models had high efficiency in gene deletion to turn vascular endothelia from red to green fluorescence.

Aim 1.2: Examine signaling mechanisms of cilia

To identify if formation of ciliary signaling was regulated by GM3S, Bicc-1 or PC2, we generated knockdown cell lines for the respective genes (*St3gal5*, *Bicc-1* or *Pkd2*). We tested knockdown efficiencies of these genes using four different knockdown sequences (**Figure 3**). In the case of *Bicc-1*, we used both chemical siRNA transfection and shRNA viral infection approaches. All stably knockdown cell lines were verified for their corresponding protein expressions, and *Bicc-1* #B, *St3gal5* #D, and *Pkd2* #D shRNA knockdown cell lines were selected for further studies. Because the shRNA expression was tagged with fluorescence GFP, we confirmed the stability of the shRNA integration in these knockdown cell lines after several passages by immunofluorescence microscopy and flow-cytometry. We next examined if Bicc-1 and PC2 regulated overall expression of GM3S. To understand the global effect of the knockdown genes at the cell level, we performed immunoblot studies to understand the interrelationship between the ciliary and cytoplasmic proteins. In particular, Bicc-1, GM3S and PC2 have been detected at the cell body. miRNA-17 (Mir-17) was included in this experiment, because Mir-17 has been known to modulate PC2 and Bicc-1. Our studies indicated while knockdown of *Mir-17* induced Bicc-1 expression level, knockdown of *Bicc-1*, *St3gal5*, or *Pkd2* could have an effect on the global expression of each other (**Figure 4**).

Aim 2.1: Quantify aneurysm formation

Compared to normal tissue, karyotyping data of a single renal epithelium from PKD patients showed an abnormal ploidy. We consistently observed an astonishingly high abnormality in the genetic composition in the samples acquired from PKD patients. We recently showed that survivin is down-regulated in *Pkd*-derived mouse vascular endothelia. We therefore examined survivin expression levels in our patients' samples. All freshly isolated kidney samples from PKD patients consistently show a down-regulation in survivin expression. Because *Survivin* knockout mouse dies at 4.5 days *post coitum*, we crossed *survivin-flox* mouse with kidney-specific *Cre* mouse (*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 one-week old mice and analyzed the cystic kidney phenotypes in five-week and three-month old mice. At five-weeks old, the effects of *Survivin* knockout were most apparent in injury model, in which the UUO kidneys were bulged and filled with fluid. Kidneys from three-month-old *Mx1Cre:Survivin^{flox/flox}* mice showed severe gross anatomical kidney defects. Cross-section analysis further showed that inactivation of *survivin* at one-week old was sufficient to induce kidney cyst formation at five-weeks old, although it was not as severe as those with UUO surgery. Histology analysis using standard H&E and fluorescent lectin staining confirmed a gross structure abnormality in *Survivin* knockout kidney, especially in the injury model, compared to wild-type age-matched kidneys undergoing the same surgery. *Survivin* inactivation resulted in a progressively more severe cystic kidney phenotype in older mice.

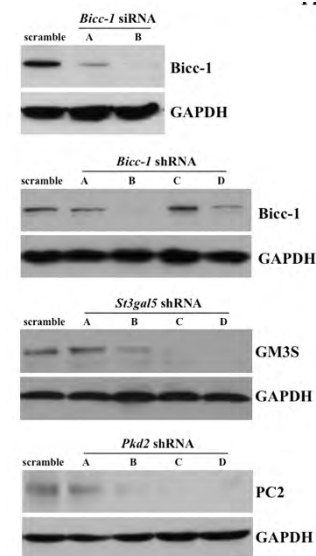


Figure 3. Knockdown efficiency was examined with silencing different regions of each gene, and siRNA was also tested as an earlier approach. Total cell lysate from scramble (control) and various siRNAs or shRNA-GFP lentivirus sequences (A, B, C, D) of each gene were analyzed.

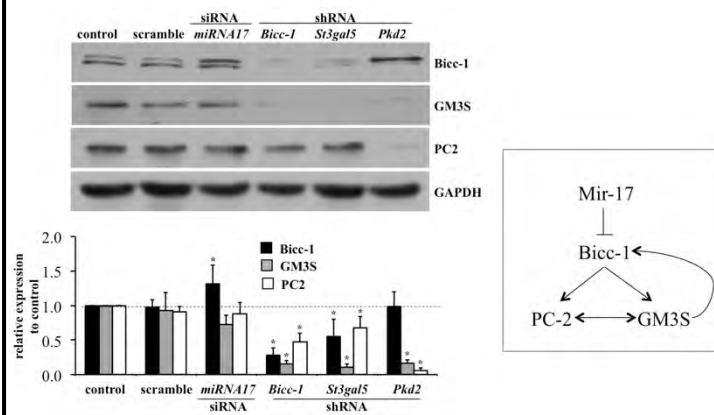


Figure 4. To examine if there is any interrelationship between the proteins located in the cilia, protein expressions were analyzed in wild-type (control), scramble, *miRNA17*, *st3gal5*, *Bicc-1* and *Pkd2* knockdown cells. A flow diagram shows the proposed mechanistic pathway for the identified ciliary proteins. Asterisks denote significant difference between groups.

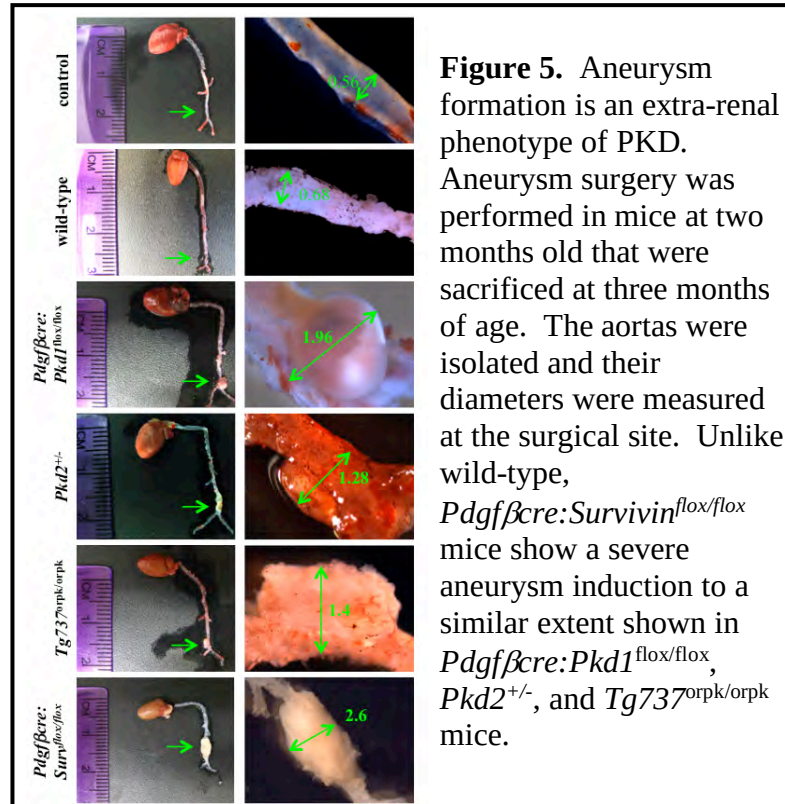


Figure 5. Aneurysm formation is an extra-renal phenotype of PKD. Aneurysm surgery was performed in mice at two months old that were sacrificed at three months of age. The aortas were isolated and their diameters were measured at the surgical site. Unlike wild-type, *Pdgfrcre:Survivin^{flox/flox}* mice show a severe aneurysm induction to a similar extent shown in *Pdgfrcre:Pkd1^{flox/flox}*, *Pkd2^{+/-}*, and *Tg737^{orpk/orpk}* mice.

The occurrence of aneurysm represents a major risk factor for morbidity and mortality associated with PKD. To examine whether *Survivin* knockout would result in aneurysm, we induced aneurysm formation in endothelial-specific *Survivin* knockout (*PdgfβCre:Survivin^{flox/flox}*) mice. These mice were later sacrificed to measure the aorta diameter at the site of the aneurysm surgery. Unlike wild-type mice, in which aorta diameter was only slightly enlarged following aneurysm surgery, *Survivin* knockout mice displayed a gross aortic aneurysm similar to that of *PdgfβCre:Pkd1^{flox/flox}*, *Pkd2^{+/-}* or *Tg737^{Orpk/Orpk}* mice following aneurysm surgery (**Figure 5**). Histological analysis of the cross sections further confirmed a marked arterial enlargement and aneurysm formation at the site of surgery from *PdgfβCre:Survivin^{flox/flox}*, *PdgfβCre:Pkd1^{flox/flox}*, *Pkd2^{+/-}*, and *Tg737^{Orpk/Orpk}* mice. 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. We next categorized the aneurysm types according to the classification by Daugherty¹⁶. Regardless of the genotypes, the mutant mice consistently showed a more severe grade than the wild-type mice. Taken together, we proposed that vascular and kidney phenotypes of PKD may share a similar cellular mechanism through survivin.

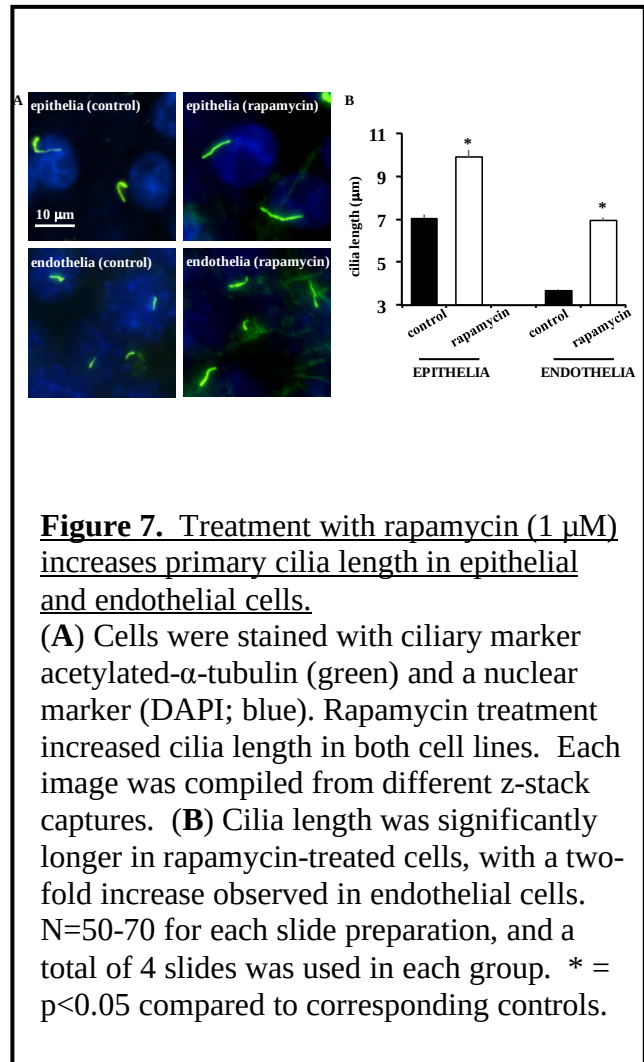
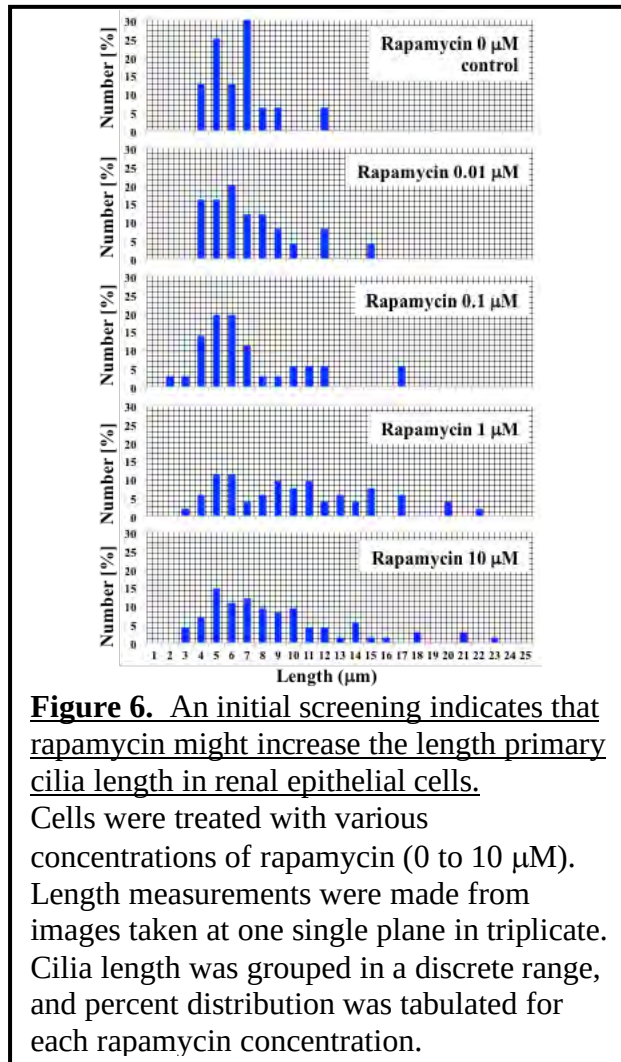
To examine the mechanism by which survivin down-regulation contributes to cystic kidney and vascular aneurysm, we performed live-cell imaging on renal epithelial. As expected, we observed a symmetric division in normal epithelial cell. Although survivin knockdown epithelium committed to enter cell division, severe cytokinesis defect was observed, resulting in failure to exit mitosis properly. This, in turn, led to polyploidy formation with cytomegaly and multi-nucleated phenotypes. Similar studies were performed on vascular endothelial cells. Likewise, similar observations were obtained in control and survivin knockdown endothelia.

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. We thus examined this possibility in *Survivin* mouse. 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. Both mitotic misorientation and abnormal cell division were very apparent in the *Survivin* knockout mice, particularly following UO surgery. Abnormal cell divisions include enlarged nucleus, multi-nucleated cells, or asymmetric mitosis. Our data further strengthened the argument that survivin shared a similar cellular mechanism as previously reported in polycystic kidney models.

Aim 2.2: Identify signaling mechanisms of cilia

To find concentration of rapamycin that might affect the length of primary cilia, we carried out initial screening of rapamycin at a range of 0 μ M to 10 μ M in renal epithelial cells. Cilia length analysis using immunofluorescent-staining of acetylated α -tubulin shows the changes in cilia length distribution with different rapamycin concentrations (**Figure 6**). Treatment with rapamycin at a concentration of 1.0 μ M gave an optimal and consistent percentage of cilia with longer lengths. Concentration of 1.0 μ M was therefore selected for the rest of our experiments. We next confirmed the effect of rapamycin by acquiring the images three-dimensionally for a more precise measurement to account for cilia that appear at different focal planes. In renal epithelial cells, average cilia length was $7.05 \pm 0.15 \mu$ m. When treated with rapamycin (1.0 μ M) for 20 hours, cilia

length increased to $9.90 \pm 0.33 \mu\text{m}$ showing an increase of almost $3.0 \mu\text{m}$. In vascular endothelial cells the effect of rapamycin on cilia length was more pronounced. Compared to an average cilia length of $3.67 \pm 0.04 \mu\text{m}$ in control endothelial cells rapamycin treatment increased cilia length to $6.94 \pm 0.16 \mu\text{m}$, almost twice the length of normal cilia. Statistical analysis showed significant differences in cilia length between the control vs. rapamycin-treatment in epithelial and endothelial cells (**Figure 7**).



As previous studies have shown, primary cilia responding to fluid flow can be observed through an influx of extracellular calcium. For live-cell acquisition during flow experiments, cytosolic calcium level was measured with fura-2-AM a cell permeant calcium-specific indicator. After baseline measurement, cells were subjected to their optimal shear-stress and fura-2 fluorescence was captured at 510 nm. We observed an increase in the cytosolic calcium levels, which can be seen in representative pseudocolored images which correlate to cytosolic calcium levels (**Figure 8**). Our data show that rapamycin treatment enhances calcium influx after induction of shear stress in epithelial and endothelial cells (**Figure 9A**). Comparisons of peak calcium levels between control and rapamycin treated cells show significant increase in the maximum levels of calcium that is achieved upon exposure to shear stress (**Figure 9B**).

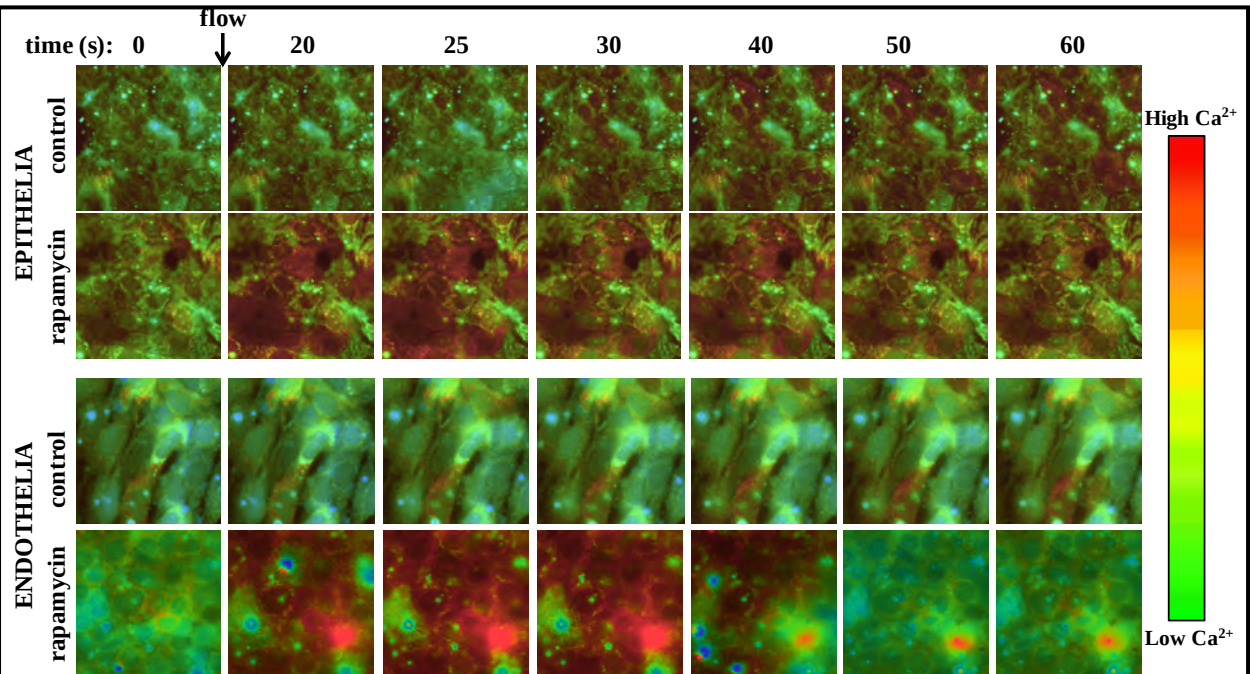


Figure 8. Fluid-shear stress induces calcium influx.

Representative Fura images at different time points from each group are shown, and the arrow indicates the start of fluid flow. Color bars indicate cytosolic calcium levels from low (green) to high (red).

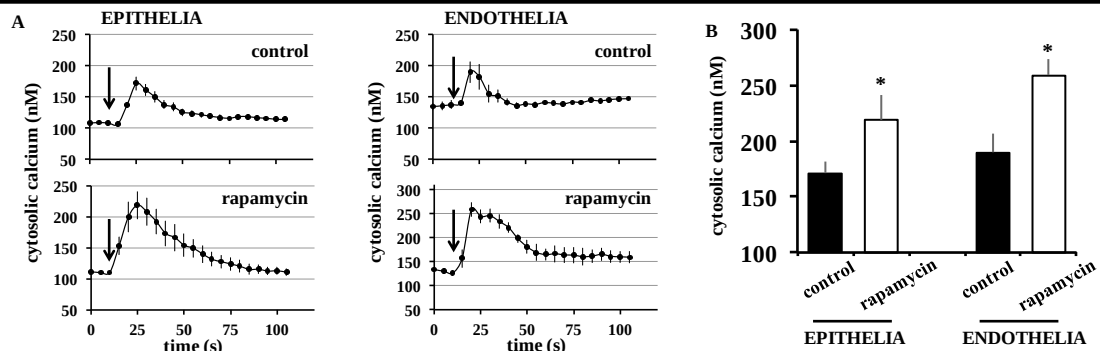


Figure 9. Flow-induced calcium influx into the cytoplasm is increased in cells treated with rapamycin.

(A) Intracellular calcium was measured in response to fluid-shear stress. The arrows indicate start of fluid flow. N=50 cells for each preparation, and a total of 4 preparations was used in each group. (B) Cilia function is assessed as peak of calcium influx in response to fluid-shear stress. Average peak calcium levels in control and rapamycin-treated cells are shown. Calcium peak is used to determine cilia function in response to fluid-shear stress. Cilia function is significantly greater in rapamycin-treated than in control cells. N=50 cells for each preparation, and a total of 4 preparations was used in each group. * = $p < 0.05$ compared to corresponding controls.

4. Other Achievements (unpublished)

The original proposal was to study blood vessels within the ciliopathy mouse models, which we have accomplished. During the course of the study, PI and his team also observe that the ciliopathy mouse models have cardiomyopathy. Upon further examinations, the ciliopathy heart is characterized by cardiomyopathy.

Cardiomyopathy is a heart disease that prevents heart muscle to sufficiently pump blood to the entire body. There are different types of cardiomyopathy. In our ciliopathy (PKD) mouse model, we observe left ventricular hypertrophic cardiomyopathy associated with low ejection fraction. What makes this model unique is that the heart is also characterized with arrhythmia. To our knowledge, ciliopathy has never been described as a cardiomyopathy [1], although cilia could function as a cardiomyocyte mechanosensor that is required for cardiac hypertrophy [2]. Worth noted is that ciliopathic patients have left ventricular hypertrophic heart [3, 4] and may have thus been predisposed to cardiomyopathy [5]. Below are the characteristics of cardiomyopathy observe in our mouse models. We strongly believe such characteristics are scientifically important to be further verified.

In vascular endothelia-specific knockout (*Tie2Cre•Pkd2*), we observed left ventricular hypertrophy (**Figure 2**). Because *Tie2Cre•Pkd2* mouse is hypertensive, we are currently generating a heart-specific knockout (*MHCiCre•Pkd2*) to differentiate if cardiomyopathy is a result from a secondary vascular effect (hypertension) and/or a primary heart effect (cilia dysfunction). Our preliminary assessment shows that both *Tie2Cre•Pkd2* and *MHCiCre•Pkd2* mice have cardiomyopathy (**Figure 10**).

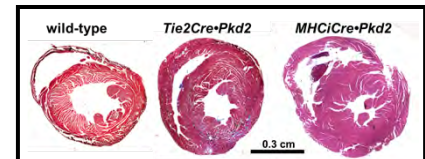


Figure 10.

Masson's Trichrome staining were performed for structural morphology analyses in *Pkd2^{flx}* (wild-type), *Tie2Cre•Pkd2* (endothelia-specific) and *MHCiCre•Pkd2* (myocardia-specific). Aside from hypertrophy, Trichrome staining also show severe fibrosis in cell-specific *Pkd2* knockout.

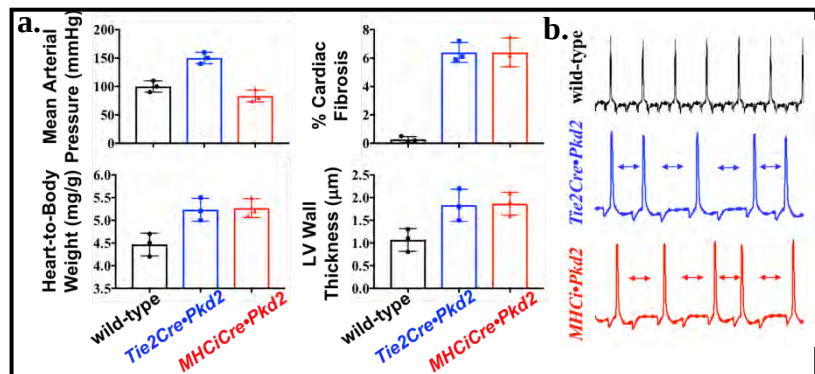


Figure 11. a) Preliminary data to assess myocardiopathy indicate that cell-specific *Pkd2* knockout may alter blood pressure, heart-to-body weight ratio, myocardial fibrosis and left ventricular (LV) wall thickness. **b)** Interestingly, arrhythmia was detected in these mice as indicated by the arrows.

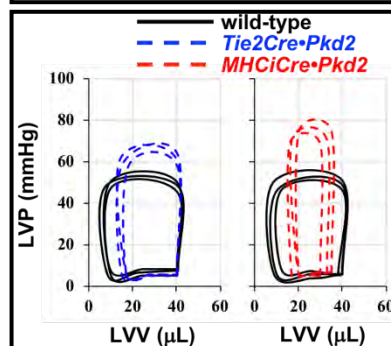


Figure 12. The relationship between the left ventricular pressure (LVP) and left ventricular volume (LVV) was analyzed as an index of heart function. A complete set of parameters for left ventricle function is not shown.

Our initial assessment indicates that hypertension is not required for left ventricular (LV) hypertrophy as *MHCiCre•Pkd2* mice are not hypertensive but share similar myocardial characteristics as hypertensive *Tie2Cre•Pkd2* mice (**Figure 11a**). These characteristics include larger heart-to-body weight ratio, cardiac fibrosis and LV wall thickness. This implies that primary cilia play an important role in cardiomyopathy, at least in *MHCiCre•Pkd2* mice. Interestingly, when electrocardiogram (ECG) was measured, we noted that both *Tie2Cre•Pkd2* and *MHCiCre•Pkd2* mice are characterized with arrhythmogenic cardiomyopathy as indicated by uneven ECG pacing (**Figure 11b**).

When left ventricle functions were measured and plotted in the pressure-volume (PV) loop plot (**Figure 12**), abnormal left ventricle function was detected in both *Tie2Cre•Pkd2* and *MHCiCre•Pkd2* mice. What becomes more interesting is that the characteristics of ventricle functions seem to be very different between *Tie2Cre•Pkd2* and *MHCiCre•Pkd2* cardiomyopathy hearts. Of note is that the ejection fraction and cardiac output seem to be much smaller in *MHCiCre•Pkd2* than *Tie2Cre•Pkd2* hearts.

References

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Expansion Award (Funding Opportunity Number: W81XWH18PRMRPEA)

The above unpublished observation was submitted for a potential continuing funding via the Expansion Award program. Unfortunately, our proposal was not selected for continuing funding. As a result, we will have to halt this exciting project to further our understanding on the roles of cilia on cardiomyopathy. Of note is that the impact of our continuing studies includes a potential for a new therapeutic approach to treat cardiomyopathy. But, this is only possible after we know more on the etiology of the cardiomyopathy. For example, if primary cilia dysfunction is involved in cardiomyopathy, we might want to look for a more direct intervention to rescue cilia function in cardiomyopathic patients.

The PI (nauli@chapman.edu) requests for a potential avenue to continue our studies as our manuscripts for potential drug delivery to cilia are currently under-review in *Science Translational Medicine* (Manuscript ID: aaw0637), *Nano Letters* (Manuscript ID: nl-2018-04138s) and *Current Biology* (Manuscript ID: CURRENT-BIOLOGY-D-18-01696). Please see appendices for the submissions to these journals.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training and professional development were provided to

- Dr. Kimberly Atkinson (postdoctoral fellow): ~9 months
- Dr. Ashraf Mohieldin (postdoctoral fellow): ~24 months
- Dr. Rajasekharreddy Pala (postdoctoral fellow): ~30 months
- Mr. Rinzhin Sherpa (PhD student): ~29 months

Scientific training plans include the following.

Weekly one-on-one meeting: In addition to ensuring execution of proposed experiments, the purpose of this meeting is to ensure that all required materials, reagents and equipment are available for the trainees to use. For example, if access to facility equipment ever becomes limited to the trainees, such limitation will be immediately resolved. The PI will contact our facility manager for a resolution. This resolution may include to prioritize our trainees’ projects first. In case of broken equipment (mostly to happen), a timeline will be made as to when the equipment to be fixed. This weekly meeting also allows discussion for troubleshooting and to ensure all proper controls are included in the experiments between Ashraf and myself. If needed, the PI and committee members (for student) will discuss alternative experiments that can be done in the meantime.

Monthly laboratory presentation: The purpose of the presentation is to gain feedback from students, postdoctoral fellows and/or collaborators. The presentation will reinforce that trainees have properly analyzed their data in a timely manner. During this meeting, previous benchmark for scientific accomplishment will be assessed; a new benchmark will be formulated. Such a presentation forum is also conducive to discuss data interpretation and potential research direction. This monthly presentation can provide experience for the trainees to present their work and to articulate questions from the audience. The trainees will also attend monthly presentation to learn about others’ research projects. Interaction among laboratory members will potentially provide new scientific ideas.

Annual research day participation: Aside from the monthly laboratory presentation, the trainees are encouraged to involve in the annual research day hosted by the school and/or institution. The annual research day involves one to two keynote speakers, professional pharmacy students, graduate students and postdoctoral fellows in addition to the faculty around the campus.

Trainees are encouraged to submit an abstract. The abstract will be reviewed and assigned for either oral or poster presentation. The main purpose of such presentation is to nurture trainees' presentation skills toward a much larger audience and to also prepare the trainees for a presentation in a national and international meeting.

Attending national and international meeting: The PI believes that attending national or international meeting regularly will enhance scientific development of the trainees. It is part of our research program to have our entire laboratory members continuously exposed to new knowledge within and outside our research fields. Such meeting can also serve the purpose to interact with other scientists within or outside our cilia/PKD field. Since joining the laboratory as a postdoctoral fellow in 2016, for example, Ashraf has attended 3 meetings: The Experimental Biology 2016 in San Diego, April 2-6, 2016; Cilia 2016 meeting in Amsterdam, The Netherlands on October 4-7, 2016; FASEB PKD meeting in Big Sky, Montana June 25-30, 2017. The Experimental Biology meeting allowed Ashraf to meet Dr. John Yates and his laboratory members to learn about proteomic studies that Ashraf proposed in this application. The Cilia 2016 and FASEB PKD meetings allowed Ashraf to better understand the roles of cilia, exosomes and ciliopathies.

Scientific rigor and data analysis: Our laboratory takes experimental rigor very seriously. Whenever it is feasible, our trainees including the PI will perform experiments in a pair - serving as double-blind operators. This is especially required by the mentor for all *ex vivo* and *in vivo* experiments. Another aspect of scientific rigor includes statistical analysis. The mentor has introduced all laboratory members including the PI to Dr. Amir Ahsan (Dept of Mathematics and Physics), who has agreed to serve as an independent statistician and, more importantly, trainees' advisory committee (especially for our students). Thus, the data analysis performed by the trainees will be supported by a strong and valid statistical analysis. Important toward the trainees' professional growth, Dr. Ahsan has been working with our trainees throughout their training.

Animal training (as needed): Because double-blind operators are required by our laboratory, many trainees have gained some experience serving as a blind operator to randomize mice when they assisted their teammates in their studies. Trainees will need additional training before s/he participates in their own *in vivo* studies. The Animal Care and Use training will compose of two levels: training at a personal level and at the experiential level from the IACUC or vivarium staff/manager. At the personal level, trainees will learn from online courses within our campus about animal welfare and IACUC compliance, techniques in animal care and use, occupational health and management. trainees will also use the online training of Collaborative Institutional Training Initiative (CITI). When necessary, Jackson laboratory (JAX) also sponsors a wide variety of courses, conferences, workshops and webinars that can be beneficial for trainees to join. At the experiential level, our vivarium manager or staff will provide laboratory animal tours and use of equipment. The manager/staff will reiterate on the "replacement, refinement and reduction" strategy for the purpose of research rigor and reproducibility, recording animal information in the databases, disaster preparedness, occupational health programs and the approved IACUC protocols.

Courses: All trainees are recommended and student is required to the following courses.

- PHS 601- Research Ethics and Regulations
- PHS 614- Biologics
- PHS 636- Proteomics

Professional development plans include the following.

Attending national and international meeting: The purpose of such meetings is to allow trainees an opportunity for networking. For example, such a strategy has allowed Ashraf to meet Dr. Yates during the Experimental Biology meeting. Since then, we have fostered the collaborative effort between the two laboratories. The professional networking is also important toward trainees' professional growth, and it serves as an opportunity for any potential academic research employment for Ashraf.

Advising graduate student research: All post-doctoral fellows at our institution are research focused without classroom teaching assignments or other administrative duties. Post-doctoral trainees are welcome to shadow PI when PI is advising graduate students. When post-doctoral trainees have observed enough different mentoring and interaction between PI and students, they will be given an opportunity to take on a graduate student. Their interactions with the graduate student will be closely supervised by the PI. It is expected that post-doctoral trainees will not only be an excellent experimentalist but also be an outstanding mentor and teacher. This strategy has been proven to work. Many of my postdoctoral fellows who are now faculty in academic institutions, hospitals or private companies have a great mentoring skill, a skill that was learned during their postdoctoral training.

Meeting with seminar speakers: Our school has departmental scientific seminars throughout the year. When outside seminar speakers are invited, we will continue to provide the opportunity for our trainees to meet with the speakers one-on-one. The purpose of such meeting is to allow trainees to gain a more intimate interaction with different scientists. This also serves as a platform to enhance trainees' personal growth and interpersonal communication skills.

Facilitating professional independent: Aside from fulfilling scientific benchmark such as high-quality research publications, the PI has been committed to facilitate trainees' professional independent through several other ways. For most of the technical skills and methods proposed in his application, trainees will be trained within our laboratory and surrounding laboratories, including the committee members' laboratories. For more specialized skills, I will encourage trainees to attend various workshops. I will continue providing funding for trainees to attend various workshops and training. For example, Ashraf has attended individualized workshop to study ciliary exosomes using a combined high-pressure freezing (HPF) and freeze-fracture transmission electron microscopy (FFTEM) technique. He obtained a personalized training and access to the system at Liquid Crystal Institute, Kent State University, OH. This resulted in a publication where Ashraf is the first-authored [Sci Rep. 2015 Nov 2;5:15982]. He also recently received training from Dr. John Yates' laboratory at The Scripps Research Institute in La Jolla, CA.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

All results have been published and/or deposited into the National Library of Medicine. Based on the Science Citation Index (SCI) indicator, our studies have been cited for a total of **3,621** times. This results in the *h*-index of the PI of 26. Please see appendix.

In addition to depositing our work for free public access, we also regularly update our website [<https://sites.chapman.edu/cilia/>]. Scientific movies, interviews, figures and laboratory accomplishments are included in our website. All our trainees have a full access to the website to update their personal interests and accomplishments. We have about **1,932** downloads of the scientific papers, movies and/or figures from the website. Please see appendix.

To reach members of communities with interests in our studies, we have participated in various lectures, national and international meetings. Some samples are shown here.

1. Cilium-dependent calcium signaling; 7th Annual Symposium on Polycystic Kidney Disease; Harvard Medical School, Boston, MA; May 2014
2. Sensory Primary Cilia, from understanding ciliopathy to searching ciliotherapy; University of Kansas Medical Center, Kansas City, KS; September 2014
3. Roles of Sensory Primary Cilia in Disease and Therapy; University of Cincinnati School of Medicine, Cincinnati, OH; March 2015
4. Primary cilium as potential therapeutic target in cardiovascular diseases; Loma Linda University Medical School, Loma Linda, CA; December 2015
5. Cilia-targeted therapy in polycystic kidney disease; University of Irvine Medical School, Orange, CA; December 2015 (Nephrology Grand Rounds)
6. Ciliotherapy as a potential treatment for polycystic kidney disease; Mayo Clinics, Rochester, MN; April 2017
7. Role of calcium signaling in PKD; PKD FASEB, Big Sky, MT; June 2017
8. Functional mechanisms of primary cilia; IUPS 38th World Congress. Rio de Janeiro, Brazil; August 2017

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

All our goals and objectives have been successfully accomplished. This report would serve as our last one. However, the PI (nauli@chapman.edu) would like to request a potential continuing support to further our scientific research and biomedical training for our student and postdocs. Please note that as the results of the previous support, we have been able to provide high impact science, some of which are still under review in Science Translational Medicine (IF=16.8), Nano Letters (IF=12.1) and Current Biology (IF=8.8). Please see appendices for our submissions to these journals.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Based on our studies, we now know the underlying mechanism of aneurysm and hypertension. For the first time, our studies offer a unifying mechanism that explains both vascular phenotypes due to primary cilia dysfunction. Although primary cilia dysfunction accounts for aneurysm formation and hypertension, hypertension itself does not cause aneurysm. Furthermore, aneurysm formation involves cellular and molecular pathways involving cilia function or structure which triggers survivin expression, cytokinesis, cell ploidy, symmetrical cell division, and tissue architecture orientation. The completion of this project may present primary cilia as a novel therapeutic target for vascular hypertension.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Our study provides a huge impact in the clinical discipline with regards to potential treatments, not only toward patients with cardiovascular disorders but those with renal diseases. Polycystic kidney disease (PKD) 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. During repair resulting from any injury, a proper expression level of survivin is required to maintain the overall architectural structure of an organ (such as blood vessels and kidneys). 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 (kidney) or vasculature (blood vessel) 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 PKD? In addition, can we use survivin or cell ploidy as a biomarker to indicate disease progression or severity in PKD? More specifically, we have used spectral karyotyping, which only requires 1 single cell from our PKD patients to confirm their cellular polyploidy throughout our studies.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report. All our results from the studies are free for public access.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to Report. There were no changes in the project or its direction.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Actual problem: On August 18, 2017, our Office of Research contacted Ms. Susan Dellinger to formally request for a 12-month extension without funds to continue our studies. We submitted a Statement on Intervention and Impact on Research Activity from our Vivarium Manager. The main reason for our request for extension without funds was that our mouse colony was infected with *Helicobacter* in early 2017. During the treatment process, our colony had difficulties with breeding and pup production, which had hampered the progress of our research. Our request for an extension was subsequently approved.

Actions to remedy the problem: During the *Helicobacter* eradication process (4 months), our colony had difficulties with breeding and pup production. In May/June 2017, we were able to initiate breeding of the colony to obtain appropriate genotypes in July/August. Per our vivarium policy, we therefore implemented *Helicobacter* Eradication Plan on our mouse colony in early 2017. And, this plan is as follow:

Helicobacter Treatment Diet

The *Helicobacter* medicated diet is a complete rodent chow and was fed as the sole diet. Feed was switched over gradually to minimize adverse effect in breeders. It was geared towards the average mouse eating one 5-gram medicated wafer or feed/day.

The formulation of the feed is as follows:

- Omeprazole: 0.02 mg/tab
- Metronidazole: 1.00 mg/tab
- Amoxicillin: 3.00 mg/tab
- Clarithromycin: 0.50 mg/tab

Housing

- Mice were housed in ventilated cages in a paired or trio mating system.
- Environmental parameters included a 12h: 12h light: dark cycle; 50-60% relative humidity and temperature maintained at 68-76°F.
- Noise was minimized as much as possible
- Males was removed from cage 14 days post observation of vaginal plus.
 - Males might carry a high bacterial burden per literature.
 - This would also prevent the dam from becoming pregnant at postpartum estrus. Her resources are to be dedicated to the newborn pups.
- As soon as pups were weaned, they were placed on regular food and placed in a different room.
- It was noted that animal husbandry played a significant role in the success of eradication of this pathogen that was transmitted via the fecal-oral route or via fomites.
- Cage changes occurred three times a week (MWF) and glove changes occurred between every cage.

Introducing the Diet

- The diet of the pregnant female (male/female pair) was gradually changed so that by day 7, the standard breeder diet would have been replaced by a bacon-flavored, nutritionally balanced, grain based medicated diet (Bioserv). Pregnant mice were kept on the feed until the pups were weaned at ~19 days (or until pups were capable of being weaned, because some of our transgenic mice could not be weaned up until day 28). The pups were then placed into a Helicobacter-free room or environment.

Diagnostics

- Sample analysis for Helicobacter spp. was done by collecting fresh fecal material on a cage by cage basis and submitting to a commercial laboratory.
- PCR evaluation was done pretreatment (to confirm positive dam), prior to parturition (to confirm negative dam, at weaning (to confirm negative dam & litter) and at one-month post weaning at a minimum.
- Whole animal sentinel evaluations were completed at 9 weeks or at completion of treatment period.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

There is no major deviation in our expenditures. At the end of the project period, however, we projected that there would be a couple of thousand dollars at less cost than anticipated. This is primarily due to publication costs that we are still unable to pay waiting for the final acceptance statement from the editorial office.

The PI (nauli@chapman.edu) therefore would like to request that the remaining fund will still be allowed to pay for the publication costs.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report. There is no use or care of human subjects in our proposal.

Significant changes in use or care of vertebrate animals

Nothing to Report. Helicobacter was eradicated from the colony as described above.

Significant changes in use of biohazards and/or select agents

Nothing to Report. There is no significant change in the use of biohazards. No select agent was put in our proposal.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

All journal publications below have acknowledged the federal support.

1. Aboualaiwi WA, Muntean BS, Ratnam S, Joe B, Liu L, Booth RL, Rodriguez I, Herbert BS, Bacallao RL, Fruttiger M, Mak TW, Zhou J, **Nauli SM**. Survivin-induced abnormal ploidy contributes to cystic kidney and aneurysm formation. Circulation. 2014 Feb 11;129(6):660-72.
2. Liu T, Jin X, Prasad RM, Sari Y, **Nauli SM**. Three types of ependymal cells with intracellular calcium oscillation are characterized by distinct cilia beating properties. J Neurosci Res. 2014 Sep;92(9):1199-204.

3. Nauli AM, Sun Y, Whittimore JD, Atyia S, Krishnaswamy G, **Nauli SM**. Chylomicrons produced by Caco-2 cells contained ApoB-48 with diameter of 80-200 nm. Physiol Rep. 2014 Jun 6;2(6).
4. Jin X, Muntean BS, Aal-Aaboda MS, Duan Q, Zhou J, **Nauli SM**. L-type calcium channel modulates cystic kidney phenotype. Biochim Biophys Acta. 2014 Sep;1842(9):1518-26.
5. Abdul-Majeed S, Mell B, **Nauli SM**, Joe B. Cryptorchidism and infertility in rats with targeted disruption of the Adamts16 locus. PLoS One. 2014 Jul 1;9(7):e100967.
6. Aal-Aaboda M, Alhaddad H, Osowik F, **Nauli SM**, Sari Y. Effects of (R)-(-)-5-methyl-1-nicotinoyl-2-pyrazoline on glutamate transporter 1 and cysteine/glutamate exchanger as well as ethanol drinking behavior in male, alcohol-preferring rats. J Neurosci Res. 2015 Jun;93(6):930-7.
7. Mohieldin AM, Haymour HS, Lo ST, AbouAlaiwi WA, Atkinson KF, Ward CJ, Gao M, Wessely O, **Nauli SM**. Protein composition and movements of membrane swellings associated with primary cilia. Cell Mol Life Sci. 2015 Jun;72(12):2415-29.
8. Patel S, Garapati C, Chowdhury P, Gupta H, Nesamony J, **Nauli S**, Boddu SH. Development and evaluation of dexamethasone nanomicelles with potential for treating posterior uveitis after topical application. J Ocul Pharmacol Ther. 2015 May;31(4):215-27.
9. Atkinson KF, Kathem SH, Jin X, Muntean BS, Abou-Alaiwi WA, Nauli AM, **Nauli SM**. Dopaminergic signaling within the primary cilia in the renovascular system. Front Physiol. 2015 Apr 16;6:103.
10. Al Omran AJ, Saternos HC, Liu T, **Nauli SM**, AbouAlaiwi WA. Live Imaging of the Ependymal Cilia in the Lateral Ventricles of the Mouse Brain. J Vis Exp. 2015 Jun 1;(100):e52853.
11. Mohieldin AM, AbouAlaiwi WA, Gao M, **Nauli SM**. Chemical-Free Technique to Study the Ultrastructure of Primary Cilium. Sci Rep. 2015 Nov 2;5:15982.
12. Mohieldin AM, Zubayer HS, Al Omran AJ, Saternos HC, Zarban A, **Nauli SM**, AbouAlaiwi WA. Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension. Curr Hypertens Rev. 2016;12(1):57-67.
13. Atkinson KF, **Nauli SM**. pH sensors and ion Transporters: Potential therapeutic targets for acid-base disorders. International Journal of Pharma Research & Review. 2016 March 01; 5(3):51-58.
14. Kathem SH, AbouAlaiwi WA, Zi X, **Nauli SM**. Capillary Endothelia from Two ADPKD Patients are Polyploidy. Annals of clinical cytology and Pathology. 2016 April 25; 2(2):1022.
15. Grimes DT, Keynton JL, Buenavista MT, Jin X, Patel SH, Kyosuke S, Vibert J, Williams DJ, Hamada H, Hussain R, **Nauli SM**, Norris DP. Genetic Analysis Reveals a Hierarchy of Interactions between Polycystin-Encoding Genes and Genes Controlling Cilia Function during Left-Right Determination. PLoS Genet. 2016 Jun 6;12(6):e1006070.
16. **Nauli SM**, Pala R, Kleene SJ. Calcium channels in primary cilia. Curr Opin Nephrol Hypertens. 2016 Sep;25(5):452-8.
17. Doerr N, Wang Y, Kipp KR, Liu G, Benza JJ, Pletnev V, Pavlov TS, Staruschenko A, Mohieldin AM, Takahashi M, **Nauli SM**, Weimbs T. Regulation of Polycystin-1 Function by Calmodulin Binding. PLoS One. 2016 Aug 25;11(8):e0161525.
18. Zarban A, Saternos HC, Kalinoski AL, Liu L, **Nauli SM**, and AbouAlaiwi WA. Localization and Distribution of Primary Cilia in the Adult Mouse Heart. Heart and Cardiology: Open Access. 2016 Nov; 2(2): 1

19. Sherpa RT, Atkinson, KF, Ferreira VP, **Nauli SM**. Rapamycin increases length and mechanosensory function of primary cilia in renal epithelial and vascular endothelial cells. Int Educ Res J. 2016 Dec; 2(12): 91.
20. Shamloo K, Chen J, Sardar J, Sherpa RT, Pala R, Atkinson KF, Pearce WJ, Zhang L, **Nauli SM**. Chronic Hypobaric Hypoxia Modulates Primary Cilia Differently in Adult and Fetal Ovine Kidneys. Front Physiol. 2017 Sep 20;8:677.
21. Omran AJA, Saternos HC, Althobaiti YS, Wisner A, Sari Y, **Nauli SM**, AbouAlaiwi WA. Alcohol consumption impairs the ependymal cilia motility in the brain ventricles. Sci Rep. 2017 Oct 20;7(1):13652.
22. Pala R, Alomari N, **Nauli SM**. Primary Cilium-Dependent Signaling Mechanisms. Int J Mol Sci. 2017 Oct 28;18(11).
23. Shamloo K, Chen J, Sardar J, Sherpa RT, Pala R, Atkinson KF, Pearce WJ, Zhang L, **Nauli SM**. Chronic Hypobaric Hypoxia Modulates Primary Cilia Differently in Adult and Fetal Ovine Kidneys. Front Physiol. 2017 Sep 20;8:677.
24. Pala R, Zeng Y, Pattnaik S, Busi S, Alomari N, **Nauli SM**, and Liu G. Functionalized Silver Nanoparticles for Sensing, Molecular Imaging and Therapeutic Applications. Current Nanomedicine. 2018; 8 (1): 1-17 [In Press]
25. Pala R, Mohieldin AM, Sherpa RT, Kathem SH, Shamloo K, Luan Z, Zhou J, Zheng HG, Ahsan A, **Nauli SM**. Treatment of vascular hypertension with remote control of primary cilia movement and function by magnetic Nanoparticles. Science Translational Medicine [Submitted on November 2018; **Impact Factor of 16.8**]
26. Pala R, Mohieldin AM, Sherpa RT, Kathem SH, Shamloo K, Luan Z, Zhou J, Zheng HG, Ahsan A, **Nauli SM**. Personalized nanotherapy by specifically targeting cell organelles to improve vascular hypertension. Nano Letters [Currently under revision; **Impact Factor of 12.1**]
27. Mohieldin AM, Pala R, Shamloo K, Sherpa RT, Alanazi M, Alanazi A, Ahsan A, AbouAlaiwi WA, Moresco JJ, Yates JR, **Nauli SM**. Proteomic analysis reveals that mechanically responsive ciliary exosomes contribute to syndromic ciliopathy. Current Biology [Submitted on December 2018; **Impact Factor of 8.8**]

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

1. **Nauli SM**, Sherpa RT, Reese CJ, Nauli AM. Mechanosensory and Chemosensory Primary Cilia in Ciliopathy and Ciliotherapy. John Wiley and Sons, Inc. c2017; Chapter 5, p.75-99 (ISBN: 978-1-118-96614-3)
2. **Nauli SM**, Mohieldin, AM, Alanazi M, Nauli AM. Mechanobiology in Health and Disease: Mechanobiology of primary cilia in the vascular and renal systems. Academic Press. c2018; Chapter 10 (ISBN: 9780128129524)

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Ashraf MM, Haymour HS, Lo S, Oliver W, **Nauli SM**. Dynamics structures of ciliary length and bulbs are mechanically regulated. Keystone Symposia. Cilia, Development and Human Disease. March 2-7, 2014
2. Muntean BS, Jin X, AbouAlaiwi W, Williams FE, Gunning WT, Liu L, Nauli AM, **Nauli SM**. CaV1.2 interaction with polycystin-2 in primary cilia is required for cardiac function. O11. 2014 Graduate Research Forum. The University of Toledo. March 17-18, 2014
3. **Nauli SM**. Biomechanics properties and structures of primary cilia. Gordon Research Conference: cilia, mucus, and mucociliary interactions. Galveston, Tx; February, 2015.
4. Alomran AJ, Saternos HC, Liu T, **Nauli SM**. and AbouAlaiwi WA. Live imaging of the ependymal cilia in the lateral ventricles of the mouse brain. Gordon Research Conference: cilia, mucus, and mucociliary interactions. Galveston, Tx; February, 2015.
5. Mohieldin AM, Haymour HS, Lo ST, AbouAlaiwi WA, Gao M, Wessely O, **Nauli SM**. Dynamics structure and protein composition of ciliary bulb in a primary cilium. Gordon Research Conference: cilia, mucus, and mucociliary interactions. Galveston, Tx; February, 2015. [**Carl Storm Underrepresented Minority Fellowship, Ashraf Mohieldin**]
6. Sherpa RT, **Nauli SM**. Effects of Tolvaptan on Mechanosensory Primary Cilia. J FASEB. 30 (1): B164 (1219.2) April 2016. [**BPS Department & Chapman University Travel Award; Rinzhin Sherpa**]
7. Kathem SH, **Nauli SM**. Mechanosensory Function of Cav1.2 CalciumChannel in Renal Primary Cilia. J FASEB. 30 (1): A46 (1188.3) April 2016. [**ASPET Travel Award; Sarmed Kathem**]
8. Pala R, Mohieldin AM, **Nauli SM**. Confirmation of ciliary calcium in response to fluid-shear stress. June 2017; PKD FASEB, Big Sky, MT.
9. Mohieldin AM, Pala R, Moresco J, Yates JR, **Nauli SM**. Proteomic Analysis of Mechanically Induced Ciliary Protein Compositions. June 2017; PKD FASEB, Big Sky, MT.
10. Sherpa RT, Pala R, **Nauli SM**. Modulation of ciliary length is dependent on high levels of cAMP in the cilioplasm generated by adenylyl cyclase. June 2017; PKD FASEB, Big Sky, MT. [**Chapman Univ. Travel Award**]
11. Shamloo K, Pala R, Zhou J, **Nauli SM**. Analyzing cardiac function in PKD mouse model. June 2017; PKD FASEB, Big Sky, MT. [**Chapman Univ. Travel Award**]
12. Alanazi M, Mohieldin AM, **Nauli SM**. BMPR2 and Tfr1 Interact to Each Other. J FASEB. LB626 (C299) April 2018. [**Chapman Univ. Travel Award**]
13. Alanazi A, Mohieldin A, **Nauli S**. Determining the Expression Level of Mir-17 in Renal Epithelia. Proceedings of American Association for the Advancement of Science. 37(1): 88 June 2018 [**Travel Award from the Cultural Mission of Royal Embassy of Saudi Arabia**]

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

All our peer-reviewed articles have been deposited into the National Library of Medicine [<https://www.ncbi.nlm.nih.gov/pubmed/>].

In addition to depositing our work for free public access, we also regularly update our website [<https://sites.chapman.edu/cilia/>]. This website allows blogging and contacting our trainees, downloading scientific figures, movies, papers, requesting for scientific artwork or other matters directly to the PI and/or trainees.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

As evidence from our publications, we have collaborated with many laboratories. In some instances, trainees from other laboratories were spent time to be trained for various activities, such as calcium imaging. We will continue to have different laboratories to access our expertise and use all the equipment in our laboratory.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report. The results from our research have been disseminated for free of use by the public. Access to our expertise and laboratory equipment are also provided with no charge.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention,

diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

The only product that we have generated is the animal models. We have generated *Tie2Cre•Pkd2* and *Tie2Cre•Ift88* mice. These mice show vascular hypertension and aneurysm, and they are used in our proposed studies. Interestingly, we also observe left ventricular hypertrophy in these mice. We therefore have requested potential continuing funding via the EXPANSION program to study if cardiomyopathy is a result from a secondary vascular effect (hypertension) and/or a primary heart effect (cilia dysfunction). Our pending manuscripts also offer potential clinical intervention in these mice. The PI (nauli@chapman.edu) requests if a small budget can be allocated to look at heart defects in the animal models that we have established via DoD funding.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name: Dr. Kimberly Atkinson
Project Role: postdoctoral fellow
Nearest person month worked: ~9
Contribution to Project: Dr. Atkinson has validated the effects of pH in our experimental conditions.
Funding Support: DoD (PR130153)

Name: Dr. Ashraf Mohieldin
Project Role: postdoctoral fellow
Nearest person month worked: ~24
Contribution to Project: Dr. Mohieldin has performed cilia bending studies to look at various signaling pathway. He also served as double-blind operator in many of our *in vivo* studies.
Funding Support: DoD (PR130153)

Name: Dr. Rajasekharreddy Pala
Project Role: postdoctoral fellow
Nearest person month worked: ~30
Contribution to Project: Dr. Pala has performed most of our *in vivo* studies including blood pressure measurement. He also observes heart abnormality and arrhythmia.
Funding Support: DoD (PR130153)

Name: Mr. Rinzhin Sherpa
Project Role: postdoctoral fellow
Nearest person month worked: ~24
Contribution to Project: Mr. Sherpa has performed most of our calcium imaging studies. He also served as double-blind operator in many of our *in vivo* studies.
Funding Support: DoD (PR130153)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The PI currently has one active grant (R56 HL131577) that ends on August 2019. The R56 Award will provide limited, interim research support for our animal housing and salary of our postdocs. The R56 serves as our bridge funding since our funding (DoD PR130153) expired in October 2018. Note that investigators do not apply for an R56 grant. R56 is awarded for a high-priority research usually is very limited in scope. Given the evidence for the productivity of our previous studies, the PI (nauli@chapman.edu) wishes that the U.S. Army Medical Research has such a program to support the continuation of our research.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendices:

Three manuscripts that have recently been submitted for publications. Proves of submission are included here.

**Science Translational Medicine (Manuscript ID: aaw0637)
Impact Factor of 16.8**

**Nano Letters (Manuscript ID: nl-2018-04138s)
Impact Factor of 12.1**

**Current Biology (ID: CURRENT-BIOLOGY-D-18-01696)
Impact Factor of 8.8**

Submission Confirmation for Treatment of vascular hypertension with remote control of primary cilia movement and function by magnetic nanoparticles

no-reply@aaas.org

Wed 11/14/2018 10:32 AM

To: Nauli, Surya <nauli@chapman.edu>;

Manuscript Title: Treatment of vascular hypertension with remote control of primary cilia movement and function by magnetic nanoparticles

Author: Nauli

Manuscript Number: aaw0637

Dear Dr. Nauli:

We have received your submission entitled "Treatment of vascular hypertension with remote control of primary cilia movement and function by magnetic nanoparticles".

You can see the status of your manuscript at any time by logging into your account at the Science Journals Submission and Information Portal at <https://cts.sciencemag.org>. Your manuscript number is noted above. Your manuscript is now undergoing an initial screening to determine whether it will be sent for in-depth review. If the manuscript is sent to review, its status will change to "To Review".

We encourage you to login and link your account to your ORCID ID, an identifier that facilitates correct attribution of your publications. To learn more about ORCID or to obtain an ORCID ID, visit their site at <http://orcid.org>.

Thank you for submitting your work to Science Advances.

Best regards,
The Science Advances Editorial Team

Manuscript nl-2018-04138s assigned to Editor

Nano Letters <onbehalfof@manuscriptcentral.com>

Fri 10/19/2018 5:44 AM

To: Nauli, Surya <nauli@chapman.edu>;

19-Oct-2018

Manuscript ID: nl-2018-04138s

Dear Dr. Nauli:

Thank you for submitting your manuscript entitled, Personalized nanotherapy by specifically targeting cell organelles to improve vascular hypertension to Nano Letters.

Your manuscript has been assigned to the following Editor:

Dr. Guangjun Nie
CAS Key Laboratory
nie-office@nanolett.acs.org

Please address all future correspondence regarding this manuscript to the above editor.

ACS Publications uses CrossCheck's iThenticate software to detect instances of similarity in submitted manuscripts. In publishing only original research, ACS is committed to deterring plagiarism, including self-plagiarism. Your manuscript may be screened for similarity to published material.

You will be notified shortly if the manuscript does not pass the pre-screen phase.

Best wishes,

Nicole Alivisatos, Ph.D.
Coordinating Editor, Nano Letters
Email: alivisatos-office@nanolett.acs.org
Fax: 510-705-1980,

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recipient, you have received this communication in error. Please notify the sender immediately by e-mail, and delete the original message. Thank you.

Re: A manuscript number has been assigned to Proteomic analysis reveals that mechanically responsive ciliary exosomes contribute to syndromic ciliopathy.

Mohieldin, Ashraf

Tue 12/4/2018 1:16 PM

To: Nauli, Surya <nauli@chapman.edu>;

FYI...

Sincerely,

Ashraf Mohieldin, PhD
Post-Doctoral Fellow
Harry and Diane Rinker Health Science Campus
9401 Jeronimo Road, Irvine, CA 92618
Office: (714) 516-5447 | Fax: (714) 516-5481
Email: Mohieldi@chapman.edu

From: em.current-biology.0.5fac64.e5691e42@editorialmanager.com <em.current-biology.0.5fac64.e5691e42@editorialmanager.com> on behalf of Current Biology Editorial Office <em@editorialmanager.com>

Sent: Monday, December 3, 2018 11:22:34 AM

To: Mohieldin, Ashraf

Subject: A manuscript number has been assigned to Proteomic analysis reveals that mechanically responsive ciliary exosomes contribute to syndromic ciliopathy.

Dear Dr Mohieldin,

Your manuscript entitled "Proteomic analysis reveals that mechanically responsive ciliary exosomes contribute to syndromic ciliopathy." has been assigned the following manuscript reference number: CURRENT-BIOLOGY-D-18-01696. Please reference this number in all future correspondence.

Thank you for submitting your work to Current Biology.

Best wishes

Mary Devane
Senior Editorial and Features Administrator
Tel: +44 (0)20 7424 4506
Email: m.devane@elsevier.com

Maxine Herman-Oakley

Associate Editorial and Features Administrator

Tel: +44 (0)20 7424 4510

Email: maxine.oakley@elsevier.com

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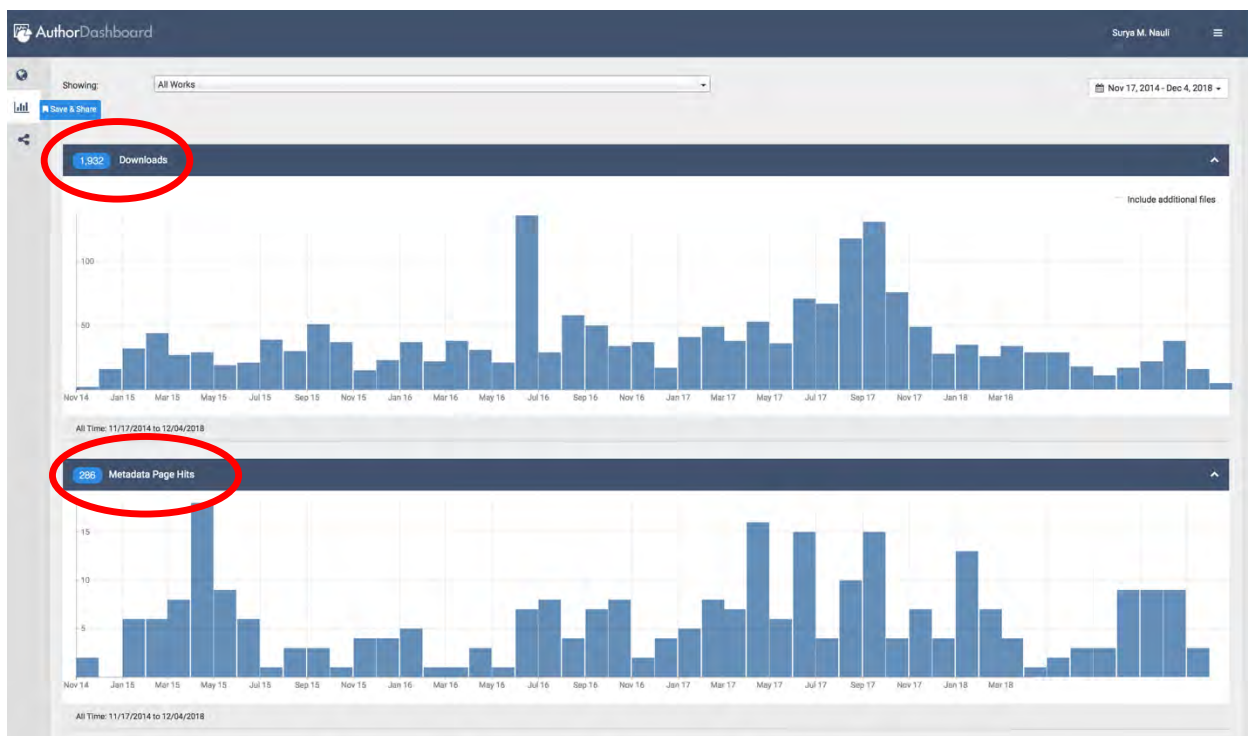
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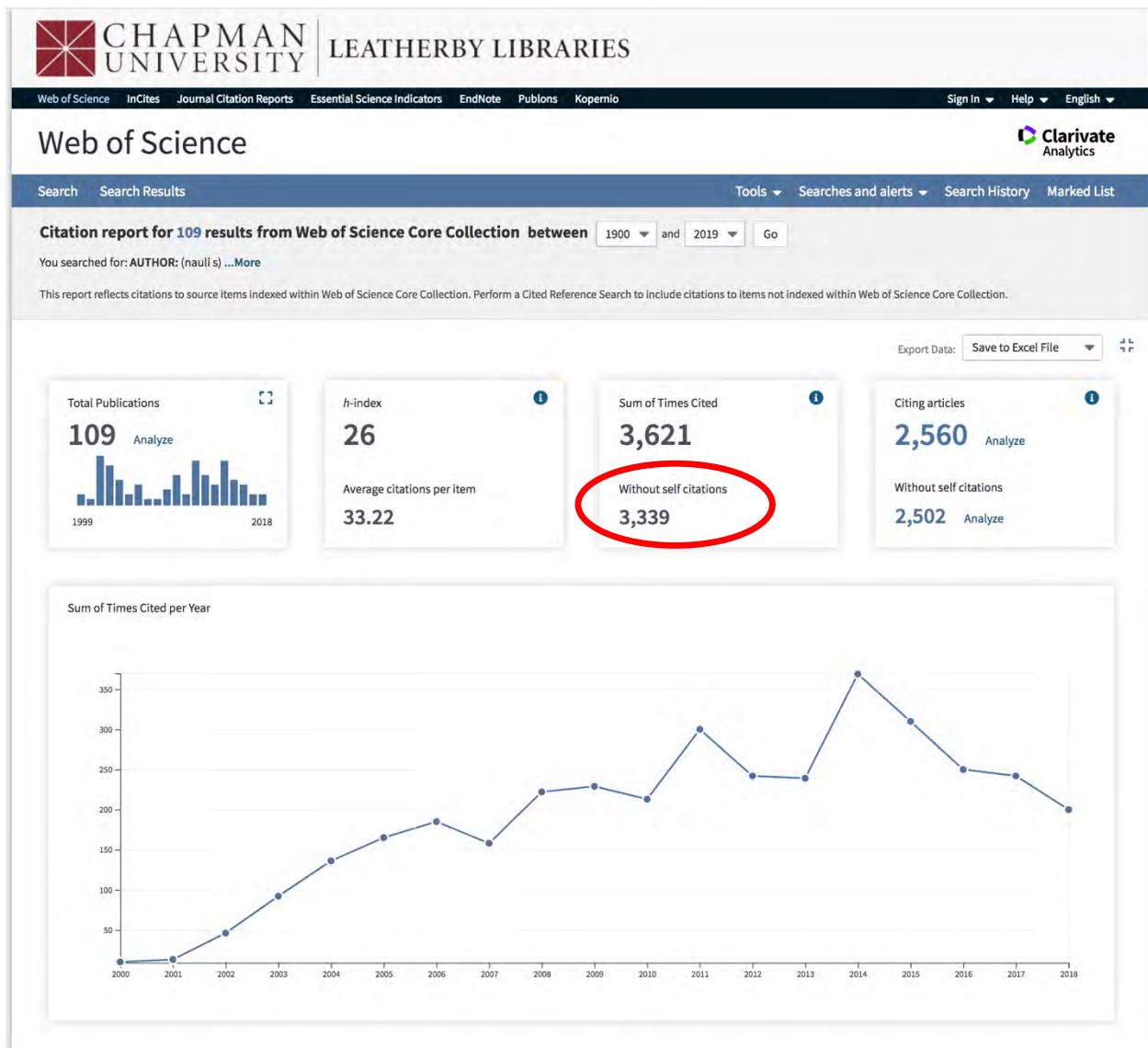
Appendix:

Website access and download activities from our personal website [<https://sites.chapman.edu/cilia/>] as a means for public, outside the scientific communities' access. Shown below is access for each month (Nov 2014 to Dec 2018) - when the funding starts to ends.



Appendix:

Screenshot below is to show that our manuscripts have been cited for a total of 3,339 time (without self-citations).



Appendices:

Below are samples of data or reports that we have published in the peer-reviewed journals.

Three Types of Ependymal Cells With Intracellular Calcium Oscillation Are Characterized by Distinct Cilia Beating Properties

Tongyu Liu, Xingjian Jin, Rahul M. Prasad, Youssef Sari, and Surya M. Nauli*

Department of Pharmacology, University of Toledo, Toledo, Ohio

Ependymal cells are multiciliated epithelial cells that line the ventricles in the adult brain. Abnormal function or structure of ependymal cilia has been associated with various neurological deficits. For the first time, we report three distinct ependymal cell types, I, II, and III, based on their unique ciliary beating frequency and beating angle. These ependymal cells have specific localizations within the third ventricle of the mouse brain. Furthermore, neither ependymal cell types nor their localizations are altered by aging. Our high-speed fluorescence imaging analysis reveals that these ependymal cells have an intracellular pacing calcium oscillation property. Our study further shows that alcohol can significantly repress the amplitude of calcium oscillation and the frequency of ciliary beating, resulting in an overall decrease in volume replacement by the cilia. Furthermore, the pharmacological agent cilostazol could differentially increase cilia beating frequency in type II, but not in type I or type III, ependymal cells. In summary, we provide the first evidence of three distinct types of ependymal cells with calcium oscillation properties. © 2014 Wiley Periodicals, Inc.

Key words: cilia; cerebrospinal fluid; calcium

Cilia are generally classified as solitary nonmotile and bundled motile organelles (Abou Alaiwi et al., 2009a; Nauli et al., 2011). Motile and nonmotile cilia have been implicated in fundamental processes of development and disease. Motile cilia can be found in the ependymal cells, forming a lining in the brain ventricles and central canal of the spinal cord. Ependymal cells are ciliated, simple cuboidal, epithelium-like glial cells that move cerebrospinal fluid (CSF) along the ventricles (Del Bigio, 1995). Abnormal ependymal cilia result in hydrocephalus induced by anomalous flow of CSF (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010).

Ependymal cells also play an important role in regulating pluripotent neural stem cells (Rietze et al., 2001). Beating of ependymal cilia is required for normal CSF flow, which functions as a guide for specific directional migration of new neurons (Sawamoto et al., 2006). The coupling between ependymal cilia beating and hydrodynamic forces has been proposed to regulate planar cell

polarity during development or stroke (Guirao et al., 2010; Mirzadeh et al., 2010; Devaraju et al., 2013). In addition, ependymal cilia play major roles in CSF dynamics, cerebral fluid balance, secretion, toxin metabolism, and many other functions (Genzen et al., 2009; Appelbe et al., 2013). Although ependymal cells regulate CSF flow, which regulates many neuronal processes, different types of ependymal cells have not been distinguished. We show here, based on the cilia beating frequency and beating angle, that ependymal cells can be distinctly categorized into three types. Furthermore, each type of ependymal cell is uniquely localized within the ventricle.

MATERIALS AND METHODS

All animal experiments were approved by the University of Toledo's Institutional Animal Care and Use Committee (IACUC). The wild-type mice were euthanized with carbon dioxide for 5 min. After craniotomy, the whole brain was removed. The sagittal slice was dissected with a thickness of about 100 μ m and was immediately embedded in Dulbecco's modified Eagle's medium (DMEM; Cellgro Corning Life Sciences-Mediatech, Manassas, VA) at 39°C in the presence of 95%/5% O₂/CO₂ mixture.

Immunofluorescence Microscopy

The brain slice was fixed in phosphate buffer containing 3% paraformaldehyde and 2% sucrose for 10 min. Mouse primary antibody antiacetylated α -tubulin was used at a dilution of 1:5,000 (Sigma, St. Louis, MO). The brain slice was

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: NIH, contract grant number: DK080640; Contract grant sponsor: DOD, contract grant number: PR130153; Contract grant sponsor: University of Toledo.

*Correspondence to: Surya M. Nauli, PhD, Department of Pharmacology, University of Toledo, MS 1015, Health Education Building, Room 274, 3000 Arlington Ave., Toledo, OH 43614.

E-mail: surya.nauli@utoledo.edu

Received 15 January 2014; Revised 7 April 2014; Accepted 7 April 2014

Published online 9 May 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23405

incubated in primary antibody solution overnight. Secondary antibody fluorescein anti-mouse IgG was used at a dilution of 1:500 (Vector Laboratories, Burlingame, CA), and the brain slice was incubated with secondary antibody solution for 1 hr. Before observation under a TiU fluorescent microscopy (Nikon, Tokyo, Japan), the section was dyed with DAPI for 5 min (Abou Alaiwi et al., 2014).

Measurement of Cilia Beating Frequency

The prepared brain slice was kept in a customized glass-bottom plate covered with 500 μ l DMEM containing 2% B27 at 39°C. In some cases, 0.25% ethanol was added to the media. The video of cilia beating was captured with a TiU high-resolution differentiation interference contrast microscope. The capture rate of the video was 5 msec for a minimum of 1 sec (200 frames per sec).

Measurement of Fluid Movement and Volume Replacement

Because of the transparency of the buffer solution, we used 200-nm latex beads (Molecular Probes, Eugene, OR) to help analyze speed in the solution movement. The velocity of fluid movement was calculated by tracing one single nanobead flowing across the third ventricle wall. The overall fluid volume moved by ependymal cilia was calculated with the following formula: volume replacement ($\mu\text{m}^3/\text{stroke}$) = fluid movement velocity ($\mu\text{m}^3/\text{sec}$)/cilia beating frequency (stroke/sec).

Calcium Signal Recording

To record cytosolic calcium oscillation, the brain slice was incubated with 20 $\mu\text{g}/\text{ml}$ fluo-2 (TEFLabs, Austin, TX) for 30 min at 39°C. The tissue was then transferred to a glass-bottom plate covered with 500 μ l DMEM containing 2% B27 (Gibco, Rockville, MD) at 39°C. In some cases, 0.25% ethanol was added to the media. The video of calcium oscillation was recorded at a capture rate of 5 msec for a minimum of 1 sec (200 frames per sec), with excitation and emission wavelengths of 488 nm and 515 nm, respectively.

RESULTS

Ependymal Cells Can Be Classified Into Three Types Based on Their Cilia Beating Frequency

We cut the mouse brain in a sagittal plane to enhance our observation of the entire third ventricle. To verify our high-resolution differential interference contrast and fluorescence microscope systems, we examined the presence of ependymal cilia in the third ventricle (Fig. 1). Ependymal cilia were confirmed with a ciliary marker, acetylated- α -tubulin. Although in the control permeabilized brain no fluid movement was observed in nonbeating ependymal cilia (Supp. Info. Movie 1), we could observe the direction of fluid movement via oil ink in a freshly prepared brain ex vivo (Supp. Info. Movie 2).

We next attempted to quantify the cilia beating frequency. Based on our observation of individual ependymal cells from 87 independent experiments, we were surprised to notice that there were wide variations in the beating fre-

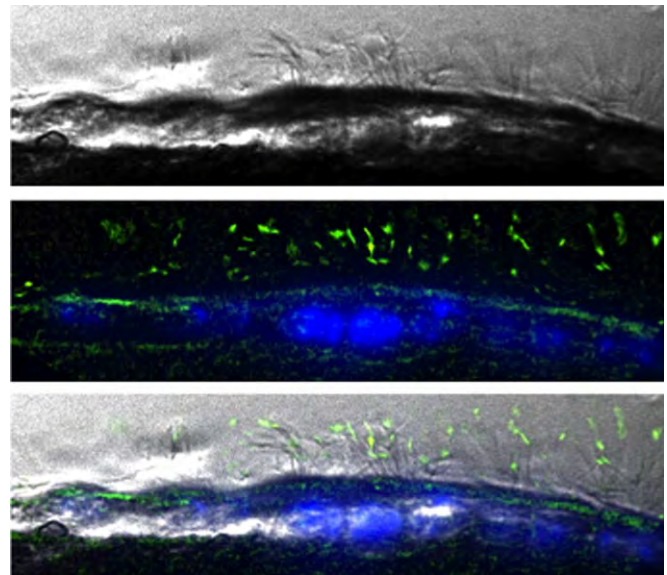


Fig. 1. The presence of ependymal cilia in mouse brain. Shown here are ependymal cells from the third ventricle of a mouse brain. The brain section was stained with anti-acetylated- α -tubulin, a ciliary marker (green), and counterstained with DAPI, a nucleus marker (blue). Individual differential interference contrast (**top**), fluorescence (**middle**), and merged (**bottom**) images are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

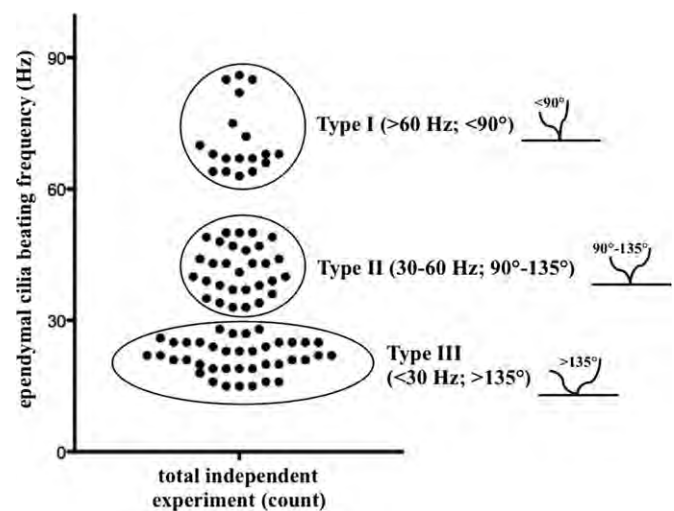


Fig. 2. Categories of ependymal cells in mouse brain. Based on a total of 87 independent experiments or preparations (represented as dots), ependymal cells could be classified into three types (grouped within circle or oval lines). Type I ependymal cilia had the highest beating frequency (>60 Hz), with a ciliary beating angle of less than 90° . Type II ependymal cilia had a medium beating frequency (30–60 Hz), with a ciliary beating angle between 90° and 135° . Type III ependymal cilia had the slowest beating frequency (<30 Hz), with a ciliary beating angle of greater than 135° . The cartoon on the right depicts the beating angles of ependymal cilia.

quencies of ependymal cilia (Fig. 2). We could accurately assign each ependymal cell to one of three classifications depending on its ciliary beating. Type I ependymal cells

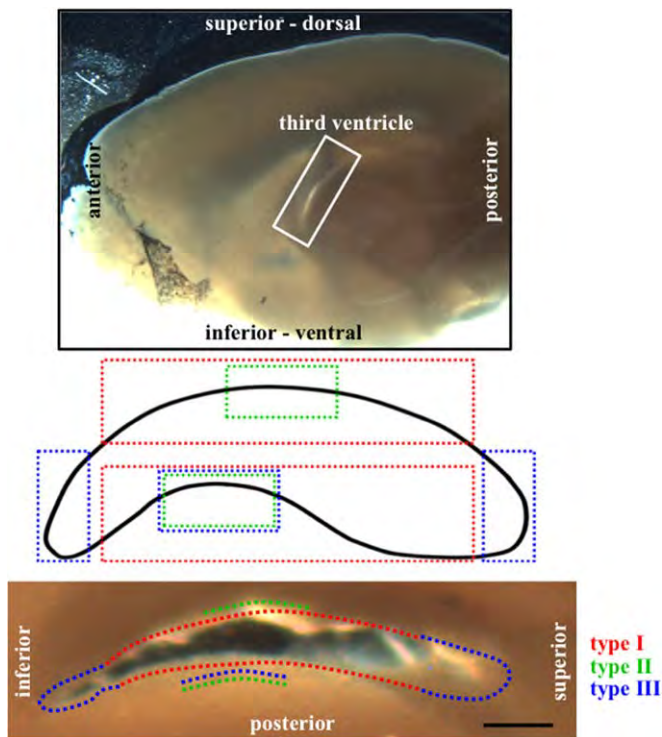


Fig. 3. Differential ependymal cell types and localization. Based on the ependymal cilia beating frequency, different ependymal cell types were enriched at certain locations within the third ventricle. A freshly cut brain section was imaged to show the third ventricle region (top). The cartoon (middle) and brain section (bottom) of the third ventricle depict localizations of different ependymal cells from a sagittal view. Scale bar = 30 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

had the highest beating frequency (>60 Hz) and had a beating angle of less than 90° (Supp. Info. Movie 3). Type II ependymal cells had a medium beating frequency (30–60 Hz) and had a beating angle between 90° and 135° (Supp. Info. Movie 4). Type III ependymal cells had the slowest beating frequency (<30 Hz) and had a beating angle of more than 135° (Supp. Info. Movie 5).

To understand the difference in localizations among these ependymal cells, we mapped the distributions of ependymal cell types within the third ventricle (Fig. 3). Our mapping analysis indicated that type I cells were widely distributed along the ventricle walls, but they were absent at both inferior and superior corners of the third ventricle. Type II cells were observed mainly on the anterior wall of the ventricle, but they could also be found at the posterior wall of the ventricle. Type III cells were distributed almost exclusively in the inferior and superior corners of the third ventricle. Of note is that we could find all three cell types at the lower wall of the third ventricle.

Cilia Beating Frequency Is Age Independent in Third Ventricles

Because ependymal cilia have been proposed to move CSF to support migration of various substances

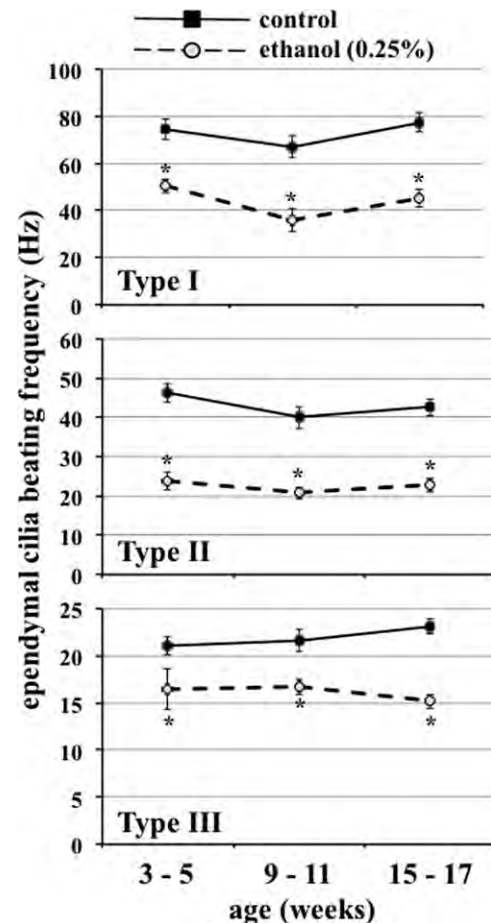


Fig. 4. Ependymal cilia beating frequency in different mouse age groups. All types of ependymal cilia in control and alcohol-treated mouse third ventricles were studied at different ages. Although beat frequency of ependymal cilia was not affected by age, acute alcohol treatment significantly decreased cilia beating frequency in all types of ependymal cells. At least three independent preparations were used for each ependymal cell type and age group.

(Banizs et al., 2005; Sawamoto et al., 2006), we next sought to understand the role of aging in ependymal cilia beating. Mice were grouped according to age: 3–5 weeks, 9–11 weeks, and 15–17 weeks. The data indicate that we could distinctively classify the ependymal cilia beating into three types regardless of the age groups (Fig. 4). More importantly, there was no evidence that age was a factor in regulating ependymal cilia beating.

Ependymal Cilia Beating Can Be Repressed by Ethanol

To confirm further that our classification of ependymal cells was valid and consistent, we performed chemical screening on the cilia beating frequency. Ethanol at a concentration of 0.25% provided us with the most consistent changes in ependymal cilia beating. The data indicated that ethanol repressed ependymal cilia beating regardless of the age group (Fig. 4). Most importantly, ethanol repressed

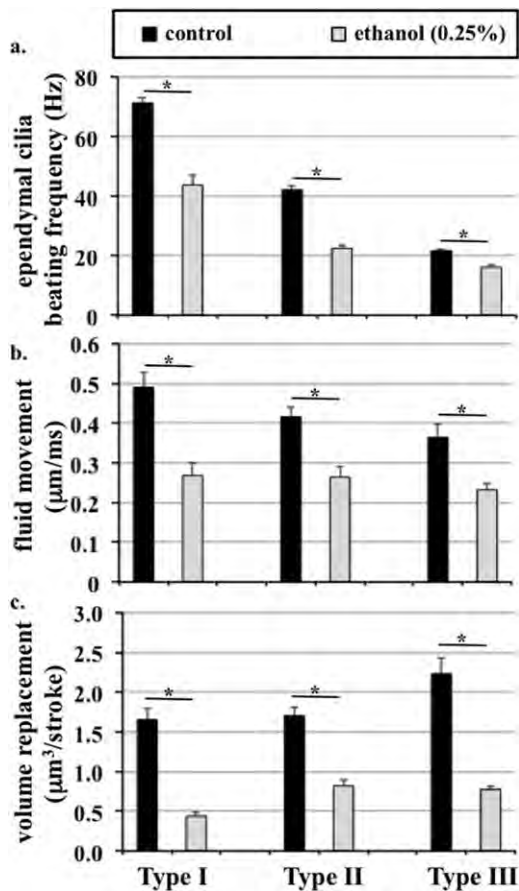


Fig. 5. Effects of alcohol on the dynamics of mouse third ventricle. The ex vivo brain slice was incubated without (control) or with (ethanol) alcohol for 5 min. **a:** Compared with control, alcohol treatment significantly decreased cilia beating frequency. **b:** This resulted in a decrease in fluid movement, as indicated by speed of fluid movement surrounding the ependymal cilia. **c:** Further calculation of the volume replacement and cilia beating indicated that, compared with control, alcohol significantly decreased volume replacement for each stroke of cilia beating. This indicates that alcohol not only decreases the frequency of ependymal cilia beating but also reduces the efficiency of each cilia stroke. At least 10 independent preparations were used for each ependymal cell type and treatment group.

ependymal cilia beating (Fig. 5a), resulting in a significant decrease in fluid movement velocity around ependymal cilia (Fig. 5b). Because of the transparency of the fluid media, we used nanobeads to guide us in measuring the speed of the fluid movement (Supp. Info. Movie 6). Given the fluid movement velocity, we estimated the volume replacement for each stroke of cilia beating efficiency. Our calculation indicated that ethanol not only repressed cilia beating frequency but also decreased the efficiency of ependymal cilia to move fluid per each stroke (Fig. 5c).

Calcium Signaling by Ependymal Cilia Can Be Altered by Alcohol

Fluid-shear stress resulting from fluid movement above a layer of cells can generate intracellular calcium

signaling (Abou Alaiwi et al., 2009b; Jin et al., 2013). We therefore examined the calcium signaling within the third ventricle (Fig. 6a). As expected, we did not see any apparent calcium oscillation in fixed brain sections (Supp. Info. Movie 7). In the absence (Supp. Info. Movie 8) or presence (Supp. Info. Movie 9) of ethanol, however, calcium oscillation was observed on ependymal cells. The frequency of calcium oscillation was not changed either by mock (PBS or control) or by ethanol treatment (Fig. 6b). Although the frequency of calcium signal in each ependymal cell type was unchanged, the amplitude of calcium signal was significantly repressed in the ethanol group compared with the control groups of each of the corresponding ependymal cell types (Fig. 6c).

DISCUSSION

We report here for the very first time that there are three distinct types of ependymal cells uniquely and specifically positioned within the third ventricle. We classified them based on their cilia beating frequency as type I (>60Hz), type II (30–60 Hz), and type III (<30 Hz). The beat frequency for each type of ependymal cilia is age independent. We also report here that ependymal cells are characterized by calcium oscillations, the frequency and amplitude of which are the same in all ependymal cell types. Our chemical testing indicates that alcohol has a profound effect on the beating frequency of the ependymal cilia, resulting in a significant decrease in fluid movement and volume replacement. Although alcohol did not change the frequency of calcium oscillation in the ependymal cells, the amplitude of calcium oscillation was significantly repressed.

Even with the advancement in the technology of high-speed digital imaging (Lehtreck et al., 2009), there has been no report on different types of ependymal cells. We thus believe that our study is the first to identify distinct ependymal cilia, which is fundamentally important to gain basic understanding of ependymal physiology. For example, many substances that are known to alter cilia beating (Sisson et al., 1991; Sisson, 1995) may fail to show an effect in ependymal cells, especially when ependymal cells are randomly analyzed (Smith et al., 2013). To validate our point further, 1% ethanol was reported to have no effect on the beating frequency of ependymal cilia (Smith et al., 2013). After we classified the ependymal cilia into three types, however, our data clearly showed that ethanol as low as 0.25% had a definitive effect on ependymal cilia beating frequency.

Our study also revealed a unique aspect of calcium signaling in ependymal cells. It was previously thought that cardiac myocytes were the only cells that naturally have a pacing calcium oscillation. We used a fluorescence high-speed digital imaging system to demonstrate that, like myocytes, ependymal cells also have an oscillating intracellular calcium pattern. The frequency and amplitude of this calcium oscillation are similar among all three types of ependymal cells, indicating that the calcium may reflex functional states rather than types of the ependymal

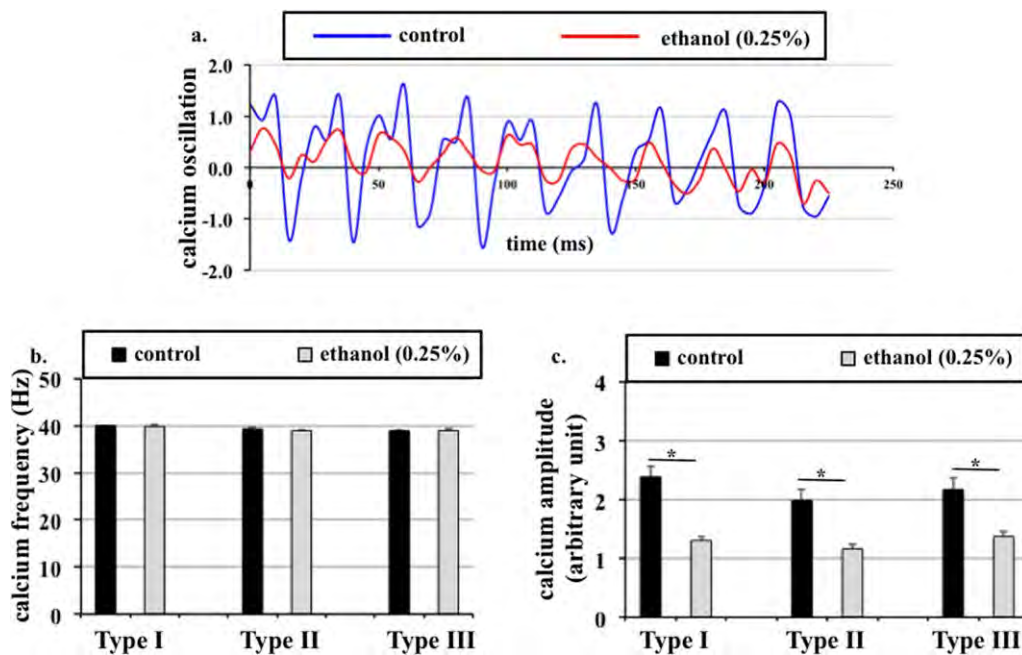


Fig. 6. Effects of alcohol on calcium oscillation in mouse brain ependymal cells. After being loaded with calcium indicator fluo-2, the ex vivo brain slice was incubated without (control) or with (ethanol) alcohol for 5 min. **a:** Intracellular calcium of ependymal cells was measured every 5 msec, as indicated by the representative blue and red lines. **b:** There was no difference in calcium oscillation frequency

between control and alcohol-treated groups. **c:** However, the amplitude of calcium oscillation was significantly repressed in alcohol-treated groups compared with control groups in all types of ependymal cells. At least five independent preparations were used for each ependymal cell type and treatment group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells. Furthermore, the frequencies of ciliary beating and calcium oscillation on ependymal cells might not be associated with one another. Consistent with this idea, an ethanol-induced decrease in ciliary beating frequency did not alter the frequency of calcium oscillation.

Despite the fact that the characteristics of cilia beating are very different among these three types of ependymal cilia, it is important to note that our understanding of these cells is just starting to unfold. For example, cilostazol could differentially increase cilia beating frequency in type II but not in type I or type III cells (Supp. Info. Fig. 1). Cilostazol is a specific inhibitor for phosphodiesterase-3, an enzyme that metabolizes cAMP to AMP, and it also regulates intracellular calcium (Kawanabe et al., 2012). It has been known that cAMP and calcium could regulate cilia beating frequency (Nguyen et al., 2001; Monkkonen et al., 2008; Genzen et al., 2009), but the differential effect of cilostazol and many other pharmacological agents requires further in-depth study to advance our understanding of the molecular and cellular biology of different ependymal cell types.

It is also worth mentioning that, among the three types of ependymal cells, type III cells were the most efficient at moving fluid volume with each ciliary stroke. Although their frequency of ciliary beating was the slowest, type III cells had the largest angle of stroke. The angle of stroke might therefore contribute significantly to mov-

ing fluid volume. However, it is important to mention that ciliary beat frequency was also a critical contributing factor in moving fluid volume, as seen with the ethanol treatment. Treatment with ethanol decreased fluid replacement significantly, primarily because of a slowing of ciliary beating.

Alcohol can produce a variety of detrimental effects in the central nervous system, leading to a wide range of impairments. Within minutes of alcohol consumption, the alcohol in the CSF reaches the same level as that in the blood (Kiianmaa and Virtanen, 1978; Agapejev et al., 1992; Huang and Huang, 2007). However, the effect of alcohol on each type of ependymal cell had never before been examined, although abnormal ependymal cilia are associated with ventricle enlargement associated with hydrocephalus (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010). Consistent with this notion, the brains of alcoholics are known to have an increase in the size of the ventricles, causing hydrocephalus ex vacuo (de la Monte, 1988). The use of 0.25% of ethanol in our study was within the range of alcohol levels in the CSF as observed in humans and various animal models (Kiianmaa and Virtanen, 1978; Agapejev et al., 1992; Huang and Huang, 2007). Thus, our study also reflects a serious clinical implication in alcohol abusive behavior with regard to ventricle-lining ependymal cells, in addition to providing fundamental basic

scientific understanding of ependymal cilia and calcium signaling.

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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- κ B. 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*, *Pdgfr β Cre*, *Tie2Cre*, *Pkd1^{fllox}*, *Pkd2⁻*, *Tg737^{Orpk}*, and *survivin^{fllox}*. 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 cells³ 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 used 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 μ mol/L Bisindolylmaleimide XI hydrochloride, Sigma Inc), PKC activator (10 μ mol/L forskolin, Sigma Inc), taxol (33.3 nmol/L, Sigma, Inc), nocodazole (0.1 μ g/mL, Sigma, Inc), VEGF (2.5 ng, Prospeg, Inc), and colcemid (50 μ g/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 $\pm$ 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 <i>pkd2</i>	Forward: 5'-GGG ATA CGT GCT GTG GTT CTC-3'	11
	Reverse: 5'-CAC GAT GAG CTC CAG TCG CGT-3'	
Human <i>survivin</i>	Forward: 5'-AAG AAC TGG CCC TTC TTG GA-3'	12
	Reverse: 5'-CAA CCG GAC GAA TGC TTT TT-3'	
Zebrafish <i>survivin</i>	Forward: 5'-GGA GCG ACT TCG CAT CTA CAT-3'	13
	Reverse: 5'-ACC TCA TCA CGA AAG TAG GCA ATC-3'	
Zebrafish α - <i>tubulin</i>	Forward: 5'-GGA GCT CAT TGA CCT TGT TTT AGA TA-3'	14
	Reverse: 5'-GCT GTG GAA GAC CAG GAA ACC-3'	

significance is indicated in the graphs by asterisks to denote comparison with the wild-type control, nontreated, nontreated, 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*-*lox* 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^{lox/lox}*) 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^{lox/lox}* 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.

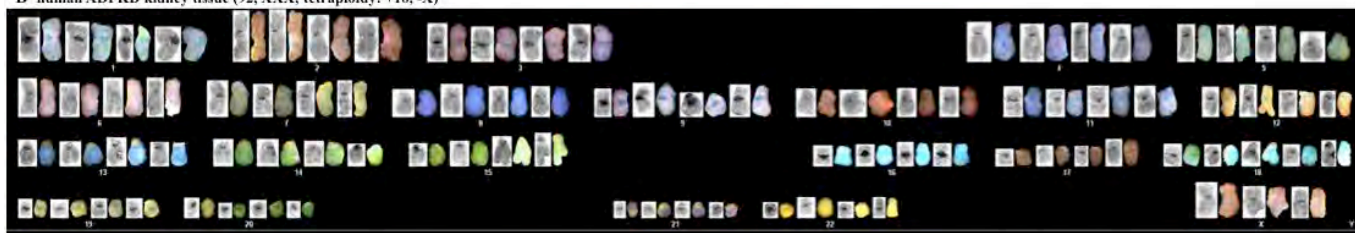
Survivin Downregulation Exacerbates Aneurysm Formation

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 (*Pdgfr β Cre:Survivin^{lox/lox}*) 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 *Pdgfr β Cre:Pkd1^{lox/lox}*, *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

A human non-cystic kidney tissue (46, XY)



B human ADPKD kidney tissue (92, XXX; tetraploidy: +18, -X)



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

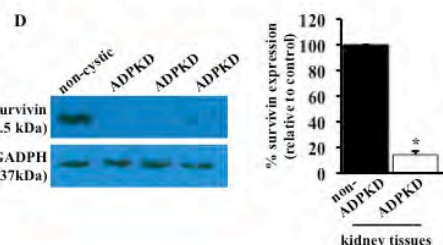


Figure 1. Autosomal-dominant polycystic kidney disease (ADPKD) renal epithelia are characterized by abnormal ploidy level and survivin downregulation. **A**, Karyotyping was carried out in freshly isolated epithelial cells from non-ADPKD patients to visualize individual chromosomes (noncystic kidney). **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.

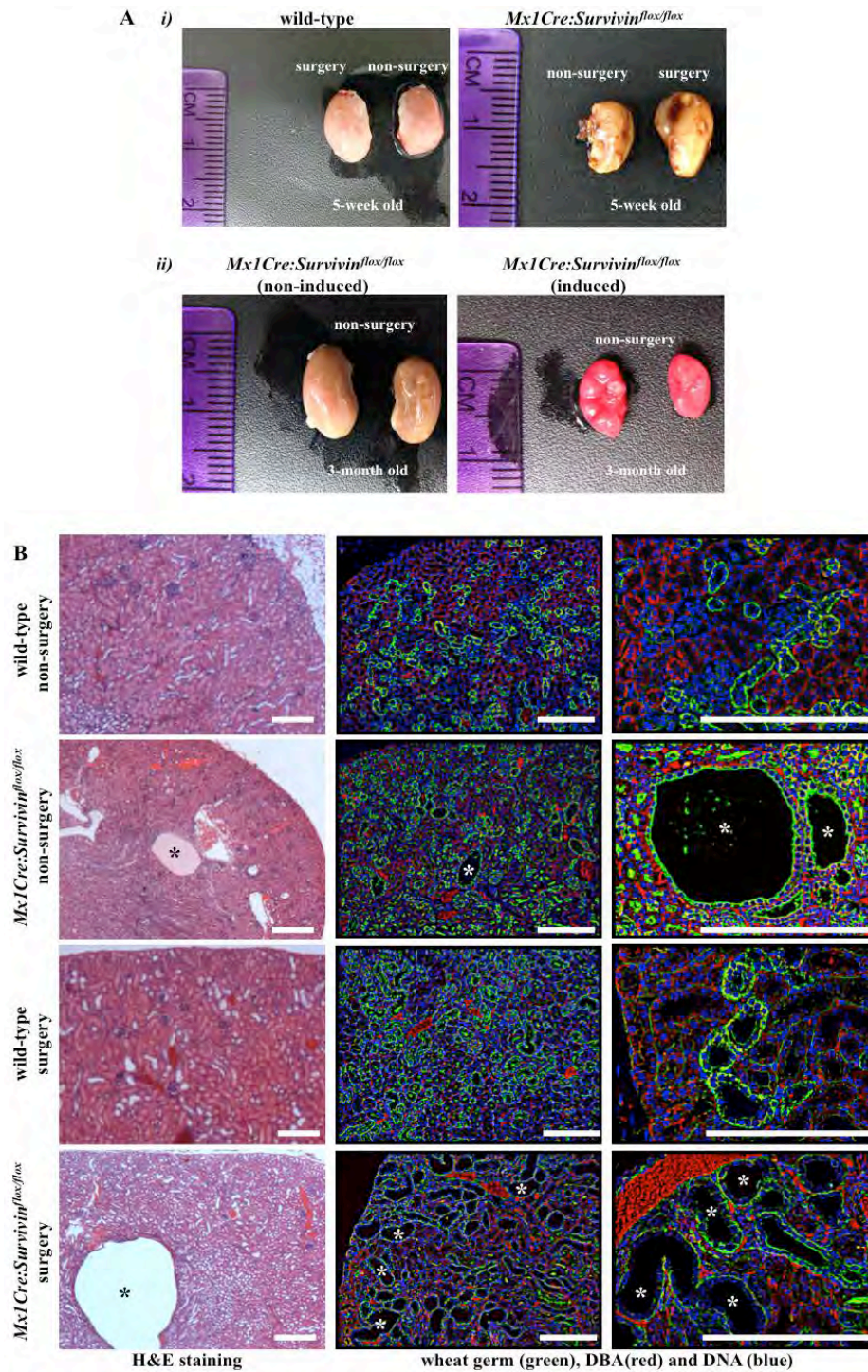


Figure 2. Survivin downregulation is sufficient to induce cystic kidney formation. **A-i**, Unilateral ureteral obstruction (UO) was performed on either wild-type or *Survivin* knockout (*Mx1Cre:Survivin^{flox/flox}*) mice at 1 month of age, and mice were euthanized a week later. **A-ii**, *Mx1Cre:Survivin^{flox/flox}*-induced 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 UO surgery are shown. Fluorescence 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 *Pdgfr β Cre:Survivin^{flox/flox}*, *Pdgfr β Cre: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.

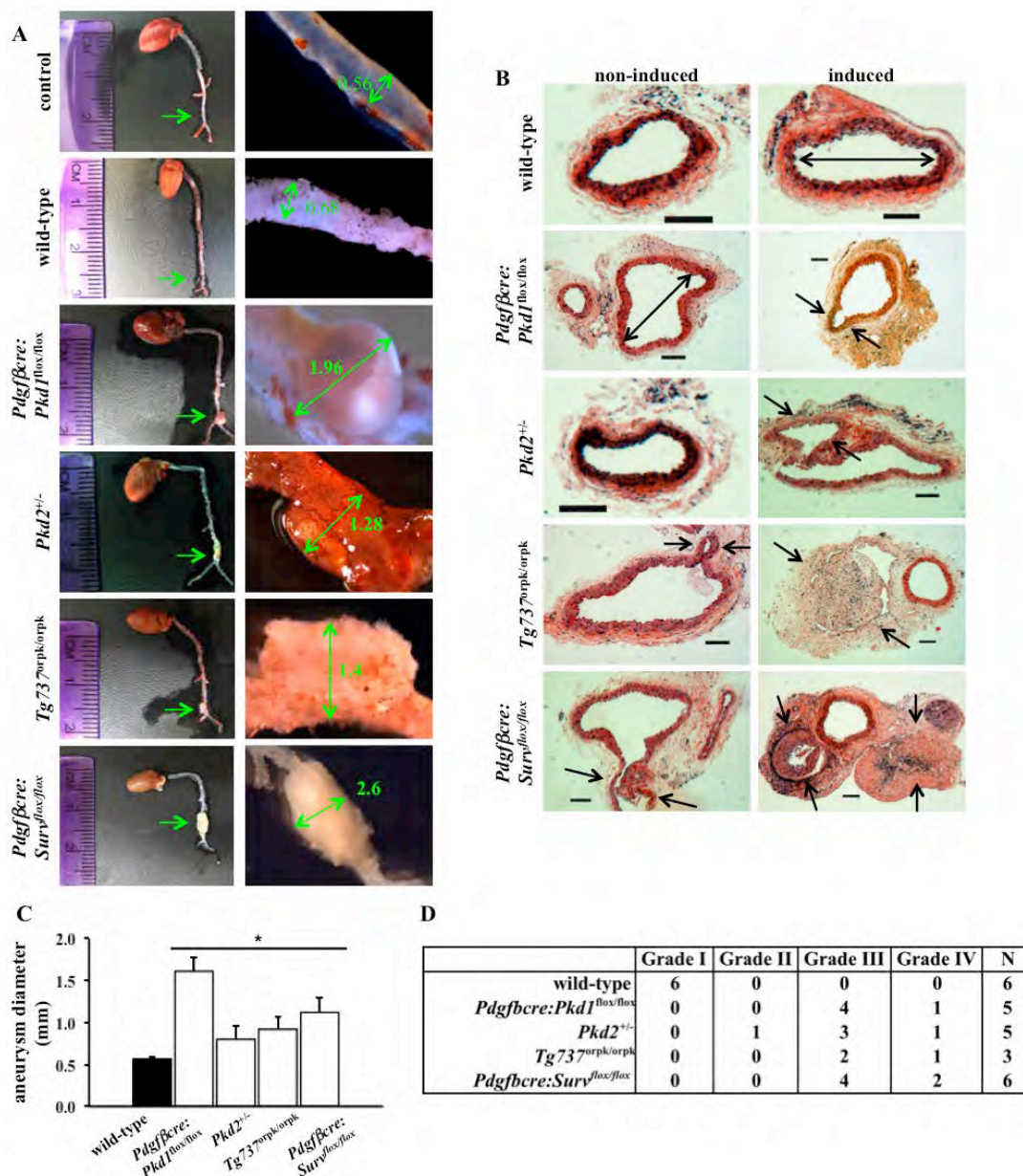


Figure 3. Aneurysm formation is an extrarenal phenotype of polycystic kidney disease. **A**, Aneurysm surgery was performed in mice at 1 month of age, and mice were euthanized at 3 months of age. The aortas were isolated and their diameters were measured at the surgical site. Unlike wild-type mice, *Pdgfr β cre;Surv^{flx/flx}* mice showed a severe aneurysm induction to an extent similar to that shown in *Pdgfr β cre;Pkd1^{flx/flx}*, *Pkd2^{+/-}*, and *Tg73^{orp/orp}* mice. **B**, Representative cross sections of aortas at the aneurysm surgery site are shown in control wild-type, *Pdgfr β cre;Pkd1^{flx/flx}*, *Pkd2^{+/-}*, *Tg73^{orp/orp}*, or *Pdgfr β cre;Surv^{flx/flx}* mice with and without aneurysm surgery. Similar to *Pdgfr β cre;Pkd1^{flx/flx}*, *Pkd2^{+/-}*, and *Tg73^{orp/orp}* mice, *Pdgfr β cre;Surv^{flx/flx}* 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. $n \geq 3$ for each group and genotype. Bar, 200 μ m. **C**, Bar graph shows averaged values for aorta diameter in wild-type, *Pdgfr β cre;Pkd1^{flx/flx}*, *Pkd2^{+/-}*, *Tg73^{orp/orp}*, or *Pdgfr β cre;Surv^{flx/flx}* 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* knock-out mice (*Mx1Cre:Survivin^{flx/flx}*) 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

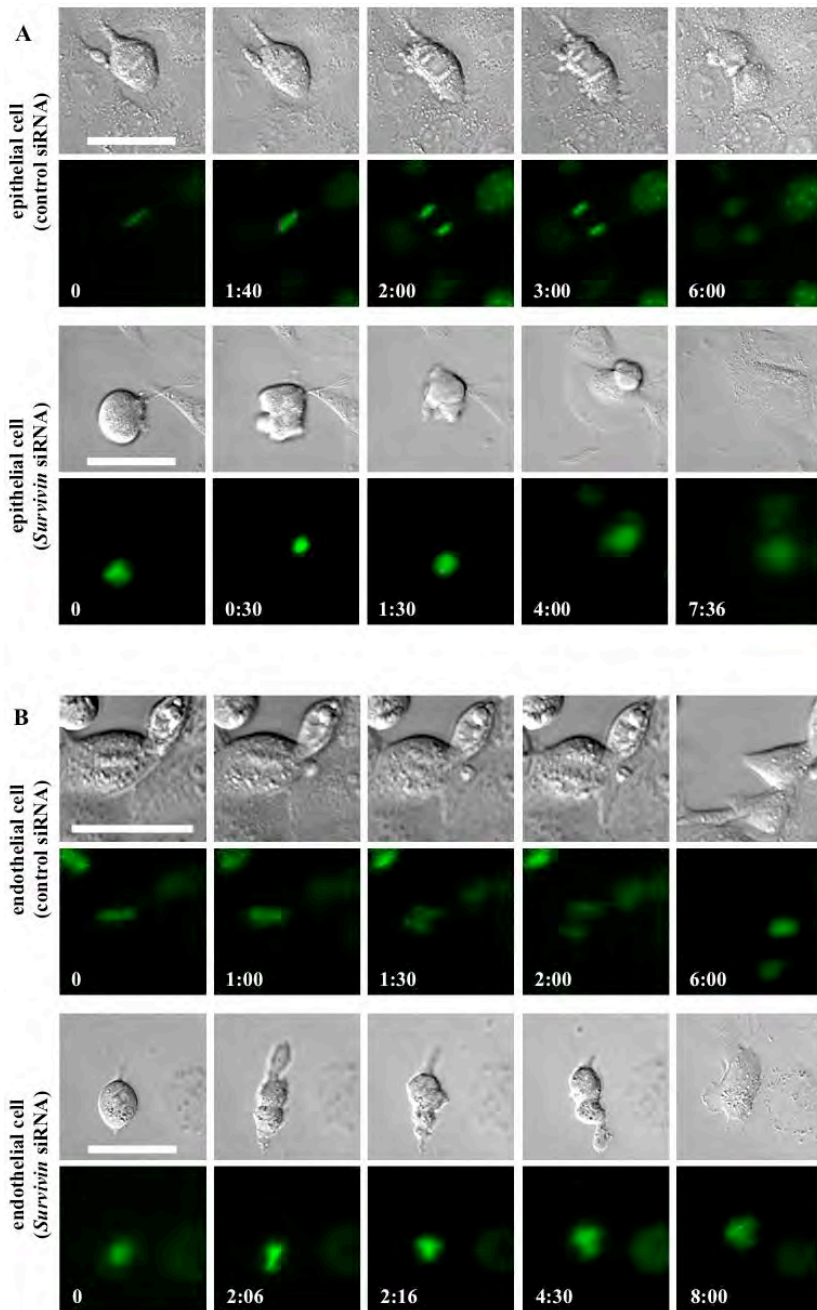


Figure 4. Survivin downregulation is associated with abnormal cytokinesis in primary cells of renal epithelia and vascular endothelial cells. **A**, To obtain the mechanistic insights of cytokinesis defect in renal epithelial cells, live-cell imaging analysis was performed. **B**, A similar study was 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 μ m. $n \geq 3$ 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, *Pdgfr β Cre:Pkd1^{fllox/fllox}*, *Pkd2^{+/-}*, *Tg737^{Orpk/Orpk}*, and *Pdgfr β Cre:Survivin^{fllox/fllox}* 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 *Pdgfr β Cre:Pkd1^{fllox/fllox}*, *Tg737^{Orpk/Orpk}*, and *Pdgfr β Cre:Survivin^{fllox/fllox}* 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

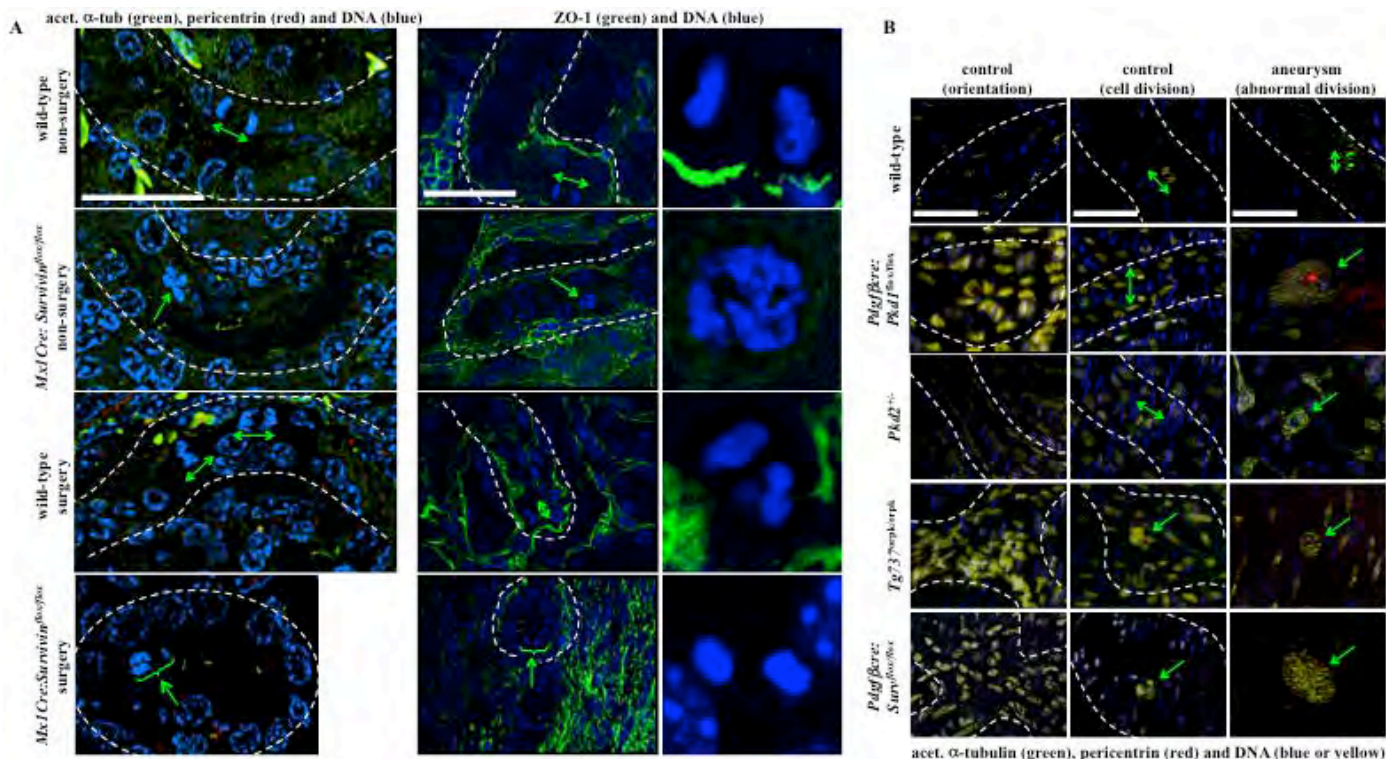


Figure 5. Abnormal cellular division orientation is associated with renal cystic and vascular aneurysm phenotypes. **A**, Kidney tubular sections from both *Mx1Cre:Survivin^{lox/lox}* mice, with or without unilateral ureteral obstruction surgery, showed abnormal cell division and division orientation 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. $n \geq 3$ for each group and genotype. $n \geq 100$ for distribution of spindle orientation angle for each genotype and each treatment. Bar, 40 μ m. 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 *Tg73^{Orpk/Orpk}* cells with survivin or aurora-A inhibitors. At resting state (Figure 6C-i), such an inhibition resulted in centrosome over-amplification 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- κ B

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- κ B (NF- κ B), which is known to regulate survivin expression.^{21,22} To investigate this possibility in our system, we studied both

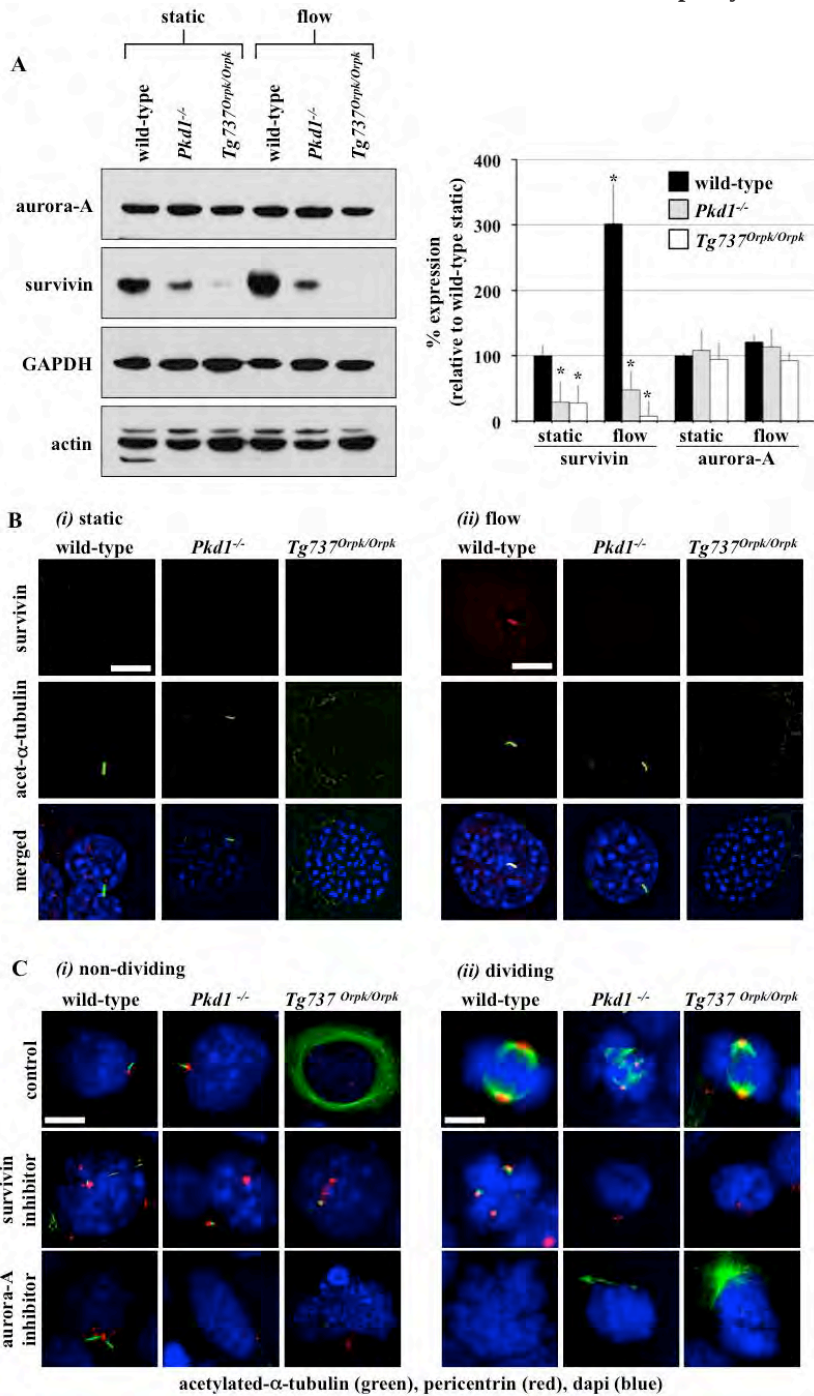


Figure 6. Cilia regulate cell division through survivin expression. **A**, Western blot analysis was used to study survivin and aurora-A expressions in wild-type and cilia mutant cells (*Pkd1*^{-/-} and *Tg737*^{Orpk/Orpk}) in the presence or absence of fluid shear stress. GAPDH and actin were used as 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 phosphorylation⁵ 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.

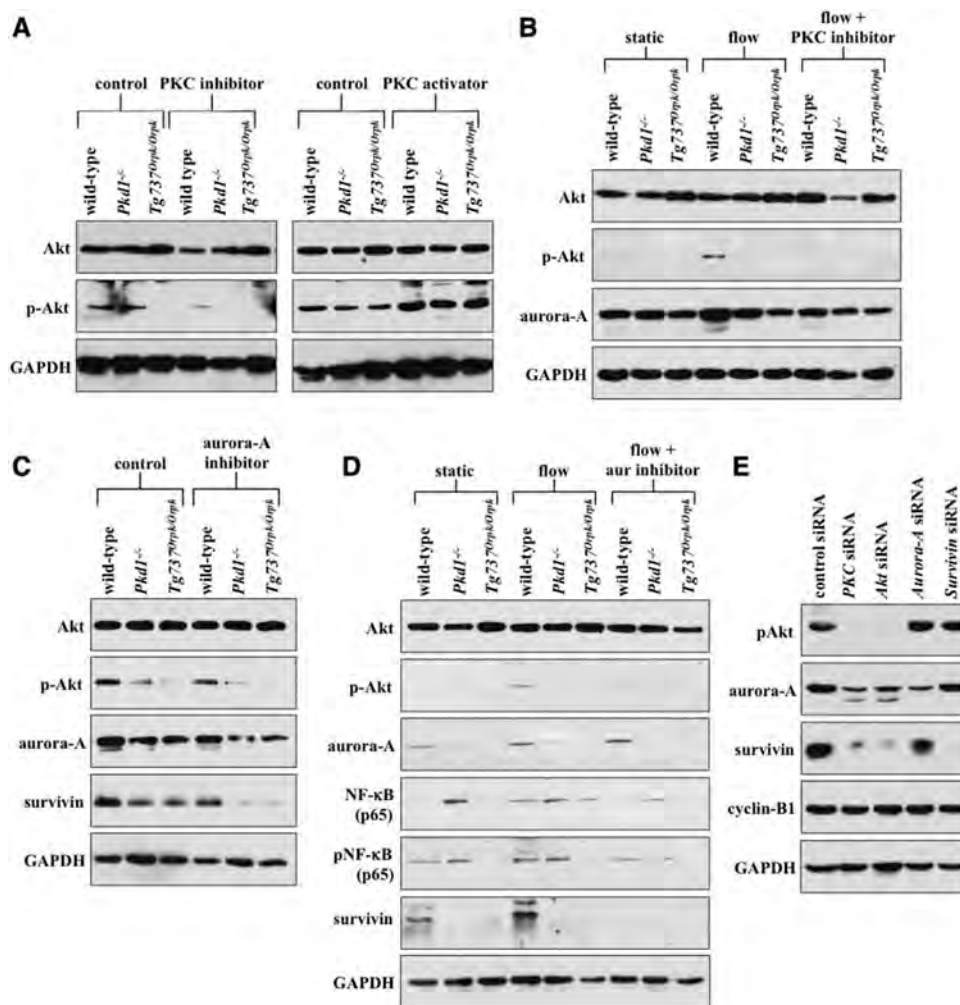


Figure 7. Protein kinase C (PKC)/Akt/nuclear factor- κ B (NF- κ B) signaling pathway regulates flow-induced survivin expression and cell division. **A**, After wild-type and cilia mutant (*Pkd1*^{-/-} and *Tg737^{Orpk/Orpk}*) cells were treated with PKC inhibitor or activator, both Akt and phosphorylated (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- κ B and phosphorylated NF- κ B (pNF- κ B) expressions were increased after fluid shear stress only in wild-type cells, whereas mutant cells maintained a high basal level of NF- κ B 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- κ B 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 *pkd2**MO* zebrafish was associated with survivin downregulation as seen in the human

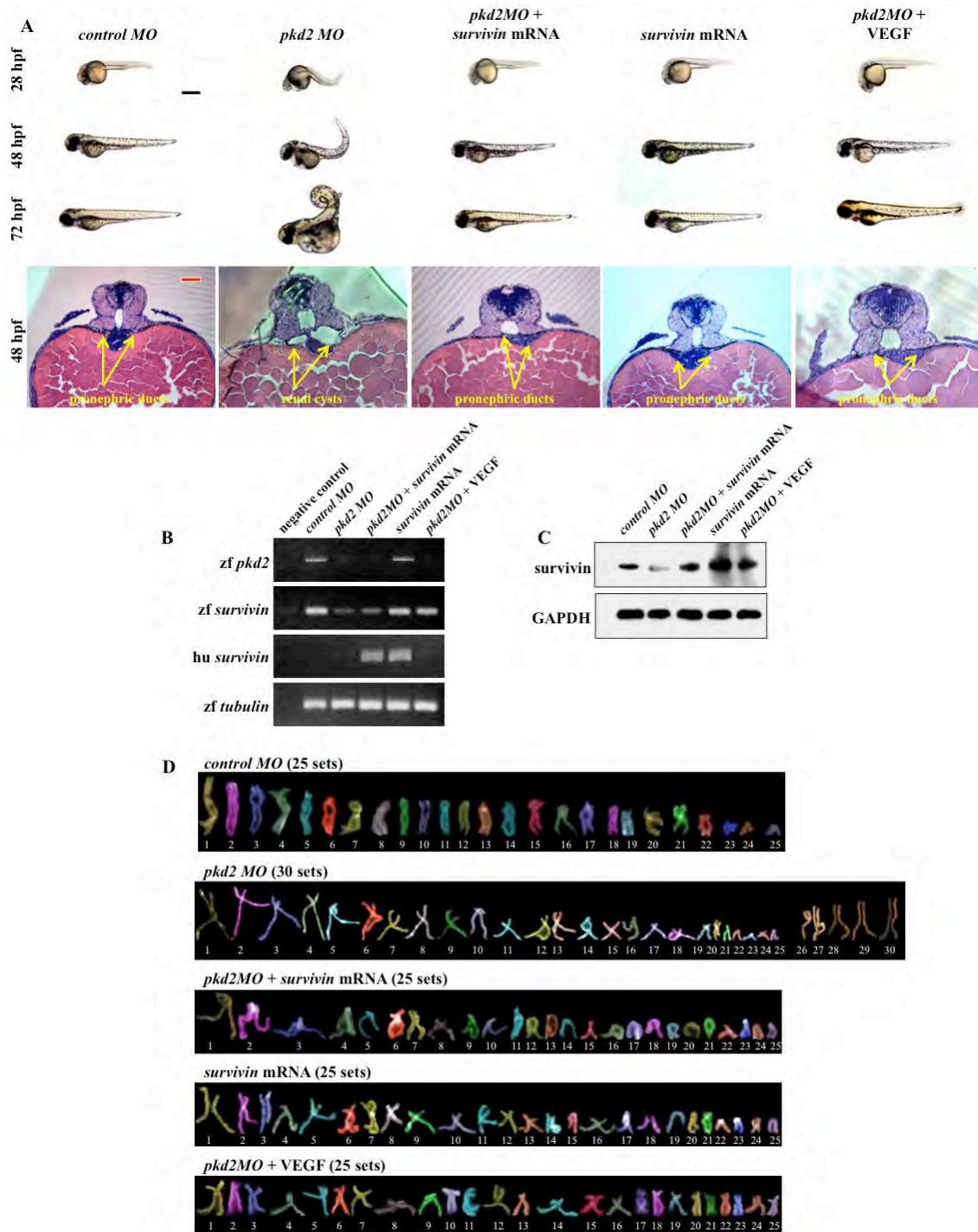


Figure 8. Survivin overexpression rescued polycystic kidney disease phenotypes in zebrafish. **A**, Zebrafish embryos were scored for phenotypic observations at different developmental stages. Shown here are representative images of zebrafish at 24, 48, and 72 hours postfertilization (hpf) injected with 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 cysts 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 μ m; red bar, 50 μ m. 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

*pkd2*MO-, *pkd2*MO plus *survivin* mRNA-, or *pkd2*MO plus VEGF-injected embryos compared with *control*MO 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^{fllox/fllox}* mice exhibited more severe cystic kidneys in UUO-induced injury compared with their nonsurgical counterparts. In *Mx1Cre:Survivin^{fllox/fllox}* 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, polyploidy, 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 cilia²⁸ 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- κ B-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 pathogenesis 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- κ B→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 ploidy 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 polyploidy throughout our studies.

SUPPLEMENTAL MATERIAL

Survivin-induced abnormal ploidy contributes to cystic kidney and aneurysm formation

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SUPPLEMENTAL METHODS

Human tissues and cell lines

Signed and informed consent to collect disposed human tissues was obtained from the ADPKD (autosomal dominant polycystic kidney disease) and non-ADPKD patients. The protocols for tissue collection were approved by the Department for Human Research Protections of the Biomedical Institutional Review Board of The University of Toledo. Kidney tissues were collected from both ADPKD and non-ADPKD patients undergoing kidney transplant surgery. The non-cystic (male) and cystic (two males and one female) kidneys were obtained randomly from our hospital. Thus, these samples were not age-matched, but they are freshly collected within one hour after kidney removal from each patient. To ensure proper comparisons of human samples in our studies, we also used well-characterized human cell lines isolated from age- and sex-matched normal and PKD renal epithelia. Importantly, the PKD epithelial cells have been characterized to have abnormal cilia function. These cells also contain a marker of proximal nephron origin, which has previously been confirmed to have Q4004X mutation in *Pkd1* gene¹.

Genetic mouse models

All animal studies were approved by The University of Toledo animal care and use committee. We used both traditional and conditional transgenic mouse models in our present studies. We have previously obtained traditional transgenic *Pkd*-mouse models *Pkd2*^{+/-} and *Tg737*^{Orpk/Orpk} from Drs. Somlo² and Yoder³, respectively. We used *survivin*^{fllox/fllox} and *Pkd1*^{fllox/fllox} conditional mouse models that were previously generated in our laboratories^{4, 5}. To generate kidney-specific knockout, we used *Mx1Cre* mice previously confirmed with a *ROSA* model⁵. Briefly, one-week

old pups were injected intra-peritoneally with 62.5 µg of 50 µL polyinosinic:polycytidylic ribonucleic acid (pI:pC) every day for five consecutive days. In some cases, mice were subjected to renal injury at one-month old. Mice were sacrificed at either five-weeks or three-months of age, and their kidney morphologies were analyzed. To generate vascular-specific knockout, we used *PdgfrβCre* mice previously confirmed with a *ROSA* model⁶. One-week old pups were injected intra-peritoneally with 250 µg of 50 µL tamoxifen every day for five consecutive days. In some cases, mice were subjected to vascular surgery at two-months old. Mice were sacrificed at three-months old, and their vascular morphologies were analyzed.

UUO and aneurysm inductions

Mouse health was confirmed before and after each surgical procedure. During the surgery, a mask connected to isoflurane regulator was placed over the nose and mouth of the mouse. 3-5% isoflurane was used to induce anesthetics followed by 1-3% isoflurane depending on the response to toe pinching and eye reflex. After shaving the hair around the incision site, skin was scrubbed with a betadine surgical scrub in circular motion from center out followed by 70% isopropyl alcohol. After the procedure was completed, the incision site was closed in a single layer using 4-0 silk suture. The skin was then closed using surgical staples (Clay Adams Autoclips). Mouse was monitored every 15 minutes until awake, followed by daily for 4 days post surgery. Only if the mouse was in pain, buprenorphine at 2.5/kg SQ twice a day was administered. Pain was determined by weight loss, lack of activity and failure to eat or drink. Penicillin G Procaine at 40,000 units/kg IM once a day was given if an infection was suspected. One sign of an infection would be discharge around the incision site.

To induce an experimental cystic model, unilateral ureteral obstruction (UUO) was generated by tying a 6-0 silk suture against a 28G needle in one-month old mice. After removal of the needle, a narrowing of total ureter diameter was achieved. The incision was sealed using a 4-0 silk suture followed by application of surgical staples to close the layer of skin. The mice were sacrificed one week later. To induce an experimental aneurysm model, sterile cotton gauze containing saline (control) or 0.25 M calcium chloride (aneurysm induction) was placed directly on the abdominal aorta for 10 minutes in two-month-old mice. Mice were euthanized at three-months old, at which time the heart and aorta were harvested, photographed, sectioned, and H&E stained to measure the diameter of the aneurysm lesions.

In both renal and vascular studies, we used respective UUO and CaCl_2 as models to facilitate and accelerate renal cyst and vascular aneurysm formation. These types of surgery models have been widely used to study kidney⁷ and vascular⁸ phenotypes. The advantages of such models include a rapid assessment of transgenic rodent models and provide a more precise location of injury, especially for mild aneurysm, which is very hard to spot within the vascular beds. In addition, these models are also characterized by an increased rate of cellular proliferation in kidney repair⁷ and neovascularization⁸. This, in turn, will facilitate studying planar cell polarity, especially in non-PKD tissue. Consistent with this idea, it has been shown that cyst formation requires increased rates of cell proliferation⁹, in which survivin is required. Although such operations may be less ideal in regard to mechanism and pathology comparison, we have thus collected data from zebrafish and PKD patients to further support our hypothesis. Of note is that increased cell proliferation is a major requirement of phenotypic development in human ADPKD kidney¹⁰ and vascular endothelia¹¹.

Blood pressure measurement

Systolic and Diastolic blood pressure were monitored by non-invasive blood pressure system - tail cuff method with the aid of a computerized system (CODA system, Kent Scientific, Connecticut, USA). Measurements were performed at the baseline 3-times per week for 2 weeks after previous 3 days of training for each mouse. On each day of blood pressure measurement, 2 sets of 18 measurements were obtained including three measurements of training or acclimation. The measurements were averaged for each mouse with at least three mice for each genotype. All animals were tested by an investigator blinded to the genotypes of the animals. The data from the tail cuff method was also verified with limited studies with a more invasive, surgically implanted telemetry probe (data not shown).

Cell culture

Both primary cultures and cell lines were used in the present studies. The use and isolation of mouse primary tubular cells from distal collecting tubules have been described previously in detail¹². For cell lines, we used previously generated endothelial cells from embryonic aortas. Various endothelial markers have been confirmed in these cells^{13, 14}. More importantly, the mutant cell lines did not respond to fluid-shear stress, a characteristic of *PKD* cells with abnormal cilia function^{1, 14}. All of these cells were subjected to cell sorting to obtain only those with normal chromosomal numbers. However, the rate of polyploidy or aneuploidy remains relatively constant in our mutant cells or human PKD cells. If we were to sort for polyploidy, they would not propagate further in culture. If we were to sort for diploidy, the polyploidy would appear within six passages, or even earlier. Thus, we believe that the “normal” PKD cells

will eventually become polyploidy or aneuploidy in a matter of time. In some cases, fluid-shear stress was applied to the surface of fully differentiated and confluent cells as previously described^{13, 14}.

Transfection study

For survivin-GFP experiments, human full-length survivin construct was inserted into pEGFPc1 (*Clontech, Inc.*) as previously described¹¹. Cells were transiently transfected with the construct with Fugene 6, according to the manufacturer's instructions (*Roche Diagnostics, Inc.*). About 24 hours after transfection, cells expressing GFP were randomly selected for immunolocalization studies. For siRNA knockdown experiments, we purchased pre-designed commercially available siRNAs that have been previously characterized for PKC (*Santa Cruz Biotechnology, Inc.*; cat.#sc-29449), Akt (*Cell Signaling Inc.*; cat.#6211), aurora-A (*Ambion, Inc.*; cat.#s202070) and survivin (*Ambion, Inc.*; cat.#s62463). All siRNAs were used at 10 μ M and transfected into cells using Lipofectamine RNAiMAX following the manufacturer's protocol (*Invitrogen Inc.*).

Organ cultures

For *in vitro* analysis, we isolated kidneys from wild-type E15.5 mice. Individual kidney was then cultured on the transwell filter. DMEM culture medium containing 4 mM glutamine, 1X penicillin/streptomycin, 5 mg/mL insulin, 5 mg/mL transferrin and 5 ng/mL selenium was used in the absence or presence of 50 μ M EM-1451 (*Erimos Pharmaceuticals, Inc.*) or 100 μ M VE-465 (*Merck, Inc.*). These concentrations were determined to be optimal conditions through dose-response studies (data not shown). The medium was supplied from underneath the transwell or the base of the filter to feed the kidney tissue. The medium was changed every day after

micrography of the kidneys with Nikon TE2000 microscope equipped with a color camera. Unless otherwise indicated, all culture media were obtained from *Fisher, Inc.*

Western analysis

Cultured cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, and mouse kidneys and human kidney tissues were homogenized with RIPA buffer. Intracellular contents were collected by centrifugation at 100 *g* for 10 minutes. Total cell lysates were analyzed with a standard 8% SDS-PAGE. For protein isolation and Western analysis from zebrafish embryos, pooled embryos injected with control *MO*, *pkd2MO*, *pkd2MO* plus *survivin* mRNA, *survivin* mRNA alone, or *pkd2MO* plus VEGF were dechorionated and deyolked manually at 28 hpf stage and lysed with RIPA buffer containing protease inhibitor cocktail (*Roche, Inc.*). After determination of protein concentration (BCA assay kit, *Fisher Scientific, Inc.*), 50 µg of protein extract was loaded onto a 12% SDS-PAGE gels and transferred to nitrocellulose membranes.

The following primary antibodies were used for Western. pNF-κB (1:250), Akt (1:1,000), p-Akt (1:250), GAPDH (1:1,500), and cyclin-B1 (1:1,000) were obtained from *Cell Signaling, Inc.*; NF-κB (1:500) from *Santa Cruz Biotechnology Inc.*; aurora-A (1:1,000) from *BD Transduction, Inc.*; survivin (1:500) from *Novus Biologicals, LLC*; and actin (1:1,000) from *Sigma, Inc.*

Immunostaining study

Both actively dividing and fully differentiated cells were stained with various antibodies to study protein translocations and subcellular localizations. Longitudinal sections from mouse kidneys and aortas were also studied for cell division and mitotic spindle orientation. Briefly, cells and

tissues were fixed with 4% paraformaldehyde in 2% sucrose solution for 10 minutes at room temperature. After primary and secondary antibodies had been applied, samples were sandwiched on the microscope slide. They were observed with an inverted Nikon Ti-U microscope and analyzed three dimensionally with Metamorph 7.0. We have recently published a more detailed protocol and analysis of the longitudinal sections¹⁵. In some cases, a standard H&E and lectin staining was carried out. For zebrafish, whole-mount immunocytochemistry was performed using JB4 resin according to the manufacturer's instructions (Polyscience, Inc., Warrington, Philadelphia). Four-micron sections were cut by a Leica RM2255 microtome (Leica, Buffalo Grove, Illinois). Phenotypes were scored with a Nikon SMZ800 stereomicroscope equipped with a QImaging MicroPublisher 5.0 RTV color CCD camera operated by QCapture software.

Some kidney sections were immunostained with ZO-1, DBA (*dolichos biflorus* agglutinin) and WGA (wheat-germ agglutinin). ZO-1 recognizes tight junctions of renal epithelia; DBA is a lectin used as a marker for renal collecting ducts; WGA is a lectin found in all convoluted tubules as well as glomeruli in the kidney. We used these markers to generate contrast and indicate individual tubules in the renal phenotypic analyses. While ZO-1 was used in our planar cell polarity to highlight directions of tubular axes, DBA and WGA were used to indicate cyst or lumen areas. For the planar cell polarity analysis, we only counted dividing cells and measured the angle of nuclear division orientation relative to the orientation of the tubular axis as revealed by ZO-1 staining. For lumen/cyst areas, we measured lumen areas in DBA and/or WGA-positive cysts in both wild-type and *Mx1Cre:Survivin^{flox/flox}* control and surgical kidney sections. Image acquisition and analysis were done with Metamorph 7.0.

Anti-survivin (1:500), aurora-A (1:1,000), NF- κ B (1:500) antibodies were used for immunostaining. Antibodies purchased from *Vector Laboratories, Inc.* include rhodamine-labeled DBA (1:100), fluorescein-labeled WGA (1:100), rhodamine-conjugated phalloidin (1:500), and DAPI (1X). Other antibodies include pericentrin (1:1,000; *Covance, Inc.*), acetylated- α -tubulin (1:1,000; *Sigma, Inc.*), and ZO-1 (1:100; *ABCam, Inc.*).

Flow cytometry analysis

Cultured cells or freshly isolated cells from human, mouse and zebrafish were used in flow cytometry experiments. In some cases, cells were incubated with 30 μ M 5-bromo-deoxyuridine (BrdU; *Sigma, Inc.*) for 15 minutes and rinsed with 1X PBS. The DNA was then denatured at 97 °C for 15 minutes and quickly chilled in an ice-water bath for 15 minutes. Anti-BrdU Alexa Fluor488 antibody was used at dilution of 1:100 (*Invitrogen, Corp.*). In apoptotic experiments, FITC conjugated annexin V antibody was used at dilution 1:100 (*Invitrogen, Inc.*). Propidium iodide at concentration of 20 μ g/ml (PI; *Sigma, Inc.*) with or without counter-staining of phospho-Histone3B antibody (*Sigma, Inc.*) at dilution 1:100 was used in other experiments. Cells were analyzed with C6 Flow Cytometer (*Accuri Cytometers, Inc.*) using FL1, FL2 or FL3 detector to measure different fluorophore intensities.

Chromosomal analysis

Individual cells were isolated and incubated with 0.05 mg/ml colcemid solution for 30 minutes at 37 °C. The cells were incubated with 0.56% KCl solution for 45 minutes at 37 °C, followed by fixing with 3:1 (v/v) methanol:acetic acid. Individual cells were dropped onto a pre-cleaned

glass slide and counter stained with DAPI. After sequential digestion with RNase and pepsin, the chromosomal DNA on the slide was denatured in 70% formamide and then hybridized with a cocktail of mouse or human SKY paint probes tagged with various nucleotide analogues according to the procedure recommended by *Applied Spectral Imaging*, Inc. (ASI; Vista, CA). The images from combinations of five different fluorophores were developed and analyzed. Rhodamine, Texas-Red, Cy5, FITC and Cy5.5 were captured with a spectral cube and interferometer module installed on an Olympus microscope. Spectral-Karyotypes were carried out using SKY View software (Version 1.62).

For zebrafish chromosome spreading, eggs were collected at the two-cell stage and injected with controlMO, *pkd2MO*, *pkd2MO* plus *survivin* mRNA, *survivin* mRNA alone, or *pkd2MO* plus VEGF. Because these cells were actively dividing and developing into embryos at this period, three hours incubation was sufficient for the morpholinos, mRNA or VEGF to take effect. These three-hours postfertilization (hpf) eggs contained about 1,000 cells. Next, 0.02% colchicine was added for an additional four hours before the choriones were removed with protease solution. De-chorionated embryos were then incubated in 0.56% KCl solution for 40 minutes and fixed with 3:1 (v/v) methanol:acetic acid solution for 20 minutes. Chromosomes were spread on the slide for analysis after DAPI staining. Because zebrafish chromosome-specific probes for chromosomal spectral karyotype analysis are not available, we characterized individual chromosomes based on the ideogram derived from the replication banding of *Danio rerio*¹⁶.

Live-imaging study

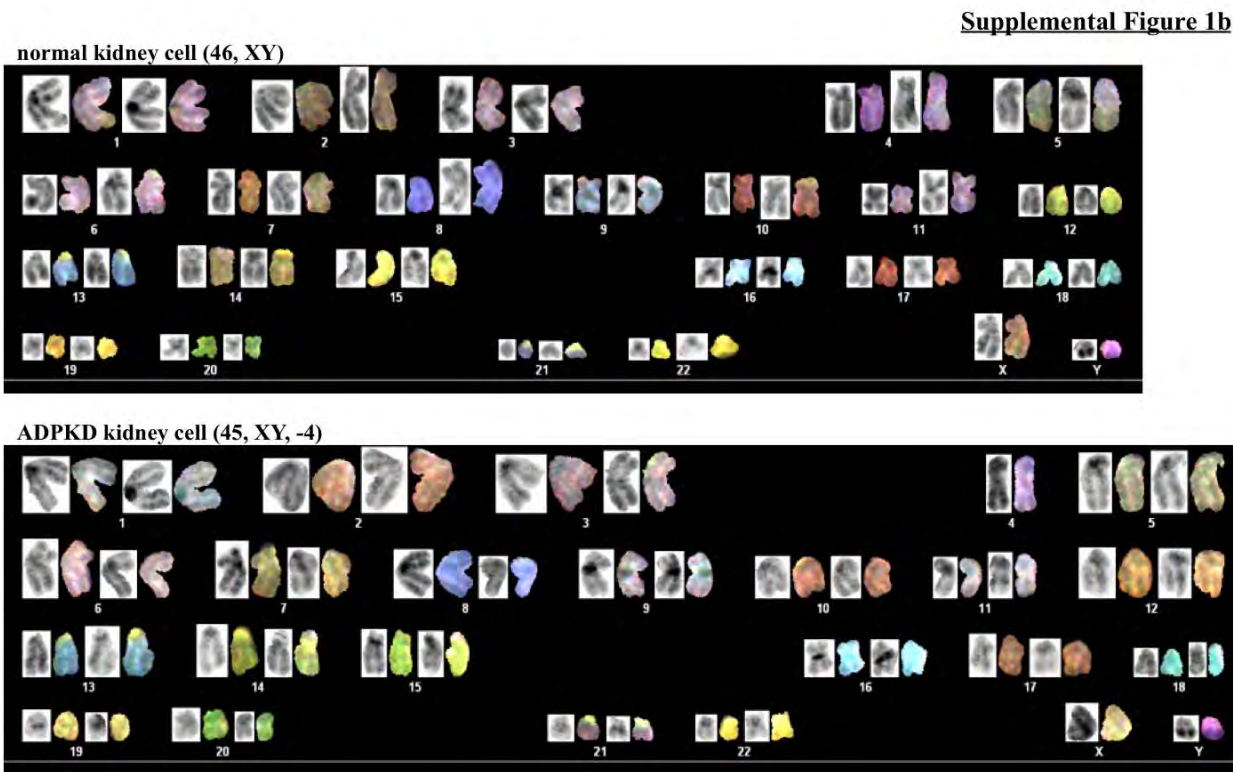
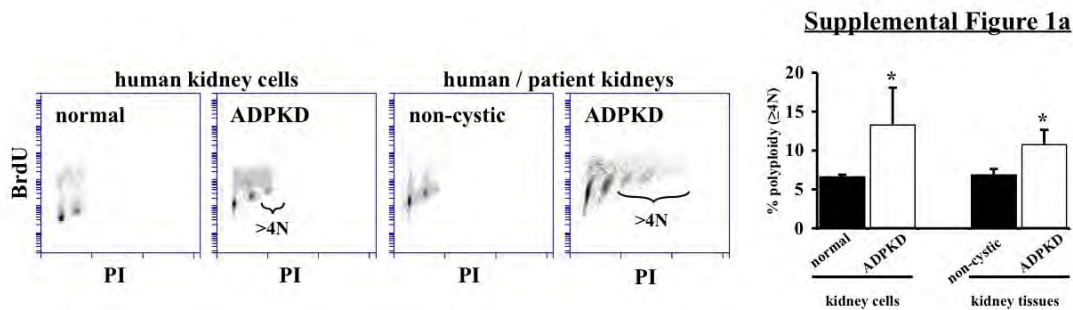
Primary renal epithelial cells or vascular endothelial cells were transfected with or without *Survivin* siRNA. Cells were then incubated with membrane-permeable and DNA-specific dye, Hoechst for 20 minutes. One Randomly selected cells from each group were studied and recorded with a Nikon TE2000 microscope equipped with an environmental chamber. Hoechst fluorescence and high-resolution differential interference contrast images were captured every 2-5 minutes at exposure time of 500 and 100 milliseconds, respectively. For better focusing, the microscope was equipped with XY-axis motorized flat top inverted stage, automatic focusing RFA Z-axis drive, and custom-designed vibration isolation platform. For a better-controlled environment, the body of the microscope was enclosed inside a custom-built chamber to control CO₂, humidity, heat and light.

Zebrafish study

Wild-type (wt) zebrafish AB strains were maintained according to standard protocols used to maintain and raise wt strains and embryos. Embryos were cultured at 28.5°C in 0.0045% phenylthiourea in a Danieau buffer to inhibit pigmentation. Zebrafish embryos were microinjected at the 1-2 cell stage with 1 mM antisense morpholino (*MO*) oligonucleotides obtained from GeneTools (Philomath, OR). A volume of 1 nL of *pkd2MO* (translational blocking *MO*) that targets against the 5'UTR of *pkd2* (*pkd2MO*: 5'-AGG ACG AAC GCG ACT GGA GCT CAT C-3') was injected using a Narishige IM-9B microinjector (East Meadow, New York) controlled by a Narishige NAI-2N micromanipulator (East Meadow, New York) to deliver 9.0 ng into the embryo. As a control, zebrafish embryos were injected with 1-2 nL of *controlMO* (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') to deliver 6-12 ng into the embryo. Note

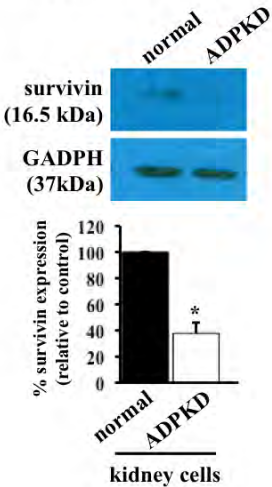
that both of these *MOs* have been previously utilized¹⁷⁻¹⁹. Un-injected control embryos were also used to verify the studies (data not shown). For rescue experiments, full-length human survivin gene (without GFP) was cloned in the survivin vector as described previously¹¹. After linearization, the plasmids were transcribed *in vitro* with T7 RNA polymerase using the mMMESSAGE mMACHINE kit from *Ambion* (Austin, TX, USA). 100 pg of purified *survivin* mRNA were either co-injected with *pkd2* morpholinos or alone into the 1-2 cell-stage embryos. In another case, 2.5 ng vascular endothelial growth factor (VEGF) was co-injected with *pkd2* morpholinos.

SUPPLEMENTAL FIGURE & FIGURE LEGENDS

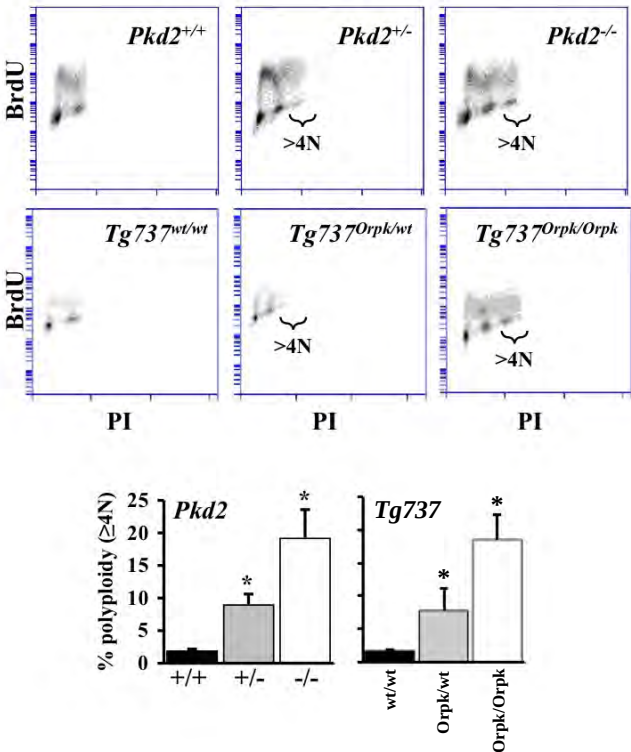


kidney cell line	normal	tetraploidy	aneuploidy	count (N)	% abnormal
non-ADPKD	9	0	2	11	18
ADPKD	20	1	6	27	26

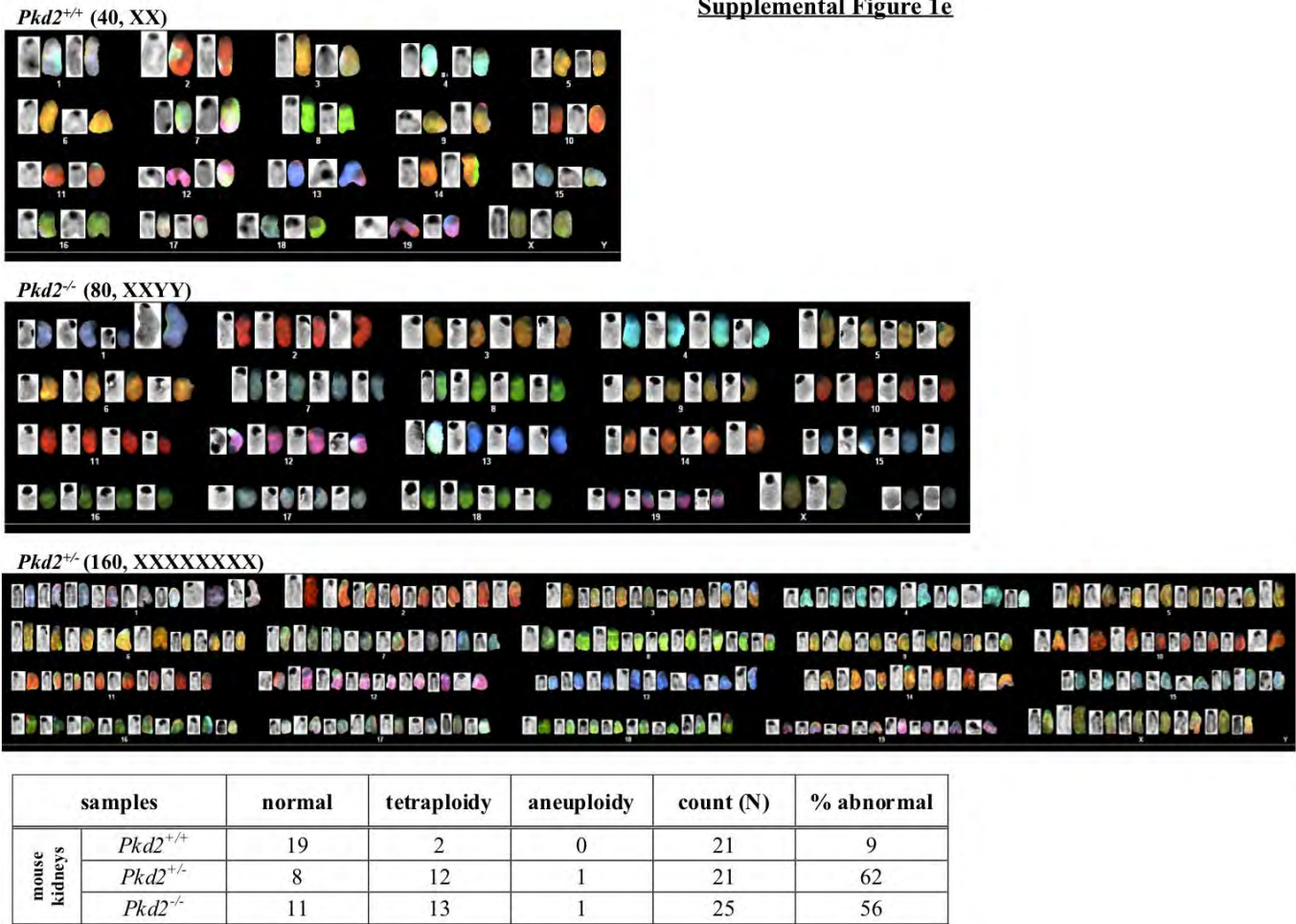
Supplemental Figure 1c



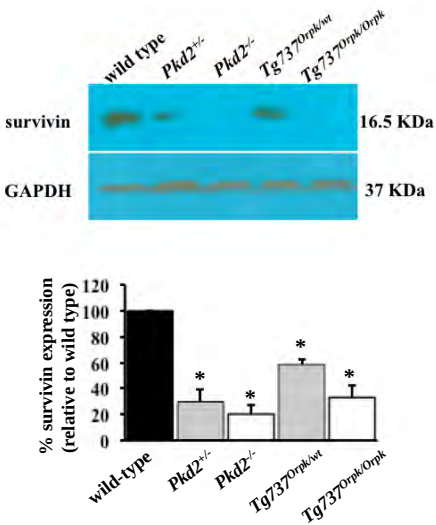
Supplemental Figure 1d



Supplemental Figure 1e



Supplemental Figure 1f

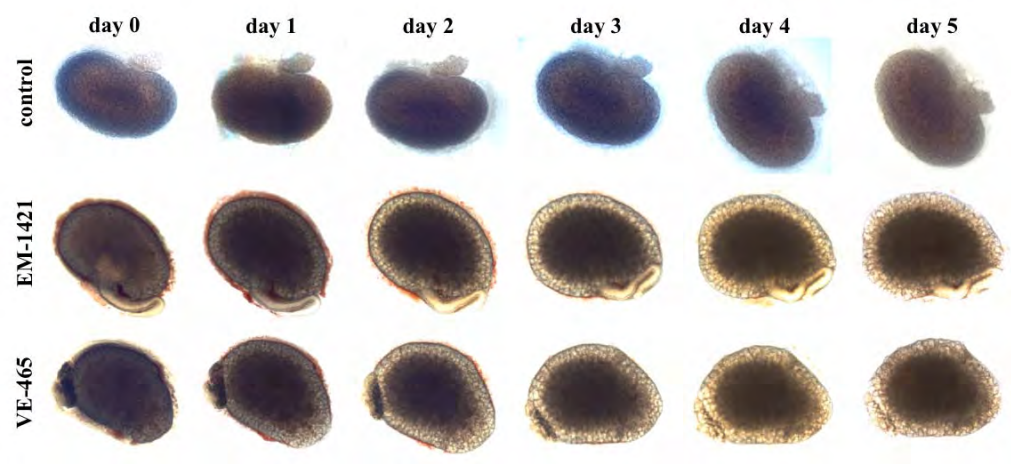


Supplemental Figure 1. Human ADPKD and mouse *Pkd* kidney epithelia are characterized by abnormal ploidy level and survivin down-regulation.

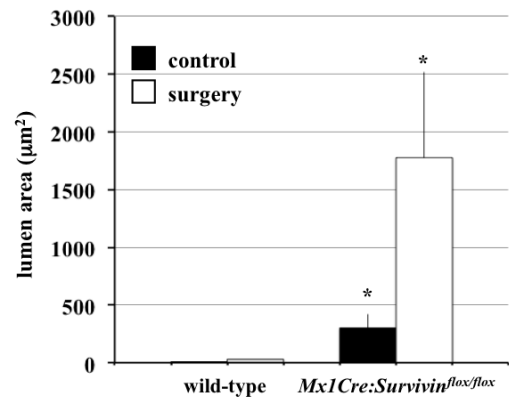
(a) Previously characterized human cell lines from normal and ADPKD kidneys were analyzed for their cellular divisions with flow cytometry by BrdU and PI staining. We first pre-sorted and collected only diploid cells (2N). Over the next 5-10 passages, we observed that compared to non-cystic cells, ADPKD-derived cells have a greater chance of producing genetic instability in their chromosomal composition. Bar graph shows that epithelia from both ADPKD kidney cells contain a greater polyploidy with DNA content of >4N compared to non-ADPKD or normal ones. (b) Further karyotype analysis of individual cells confirms the presence of abnormal genomic compositions (aneuploidy or polyploidy) in ADPKD cells. (c) Human cell lines were also used to confirm survivin expression, and GAPDH was used as loading control. Bar graph shows relative survivin expression levels. (d) Representative BrdU and PI labeling profiles reveal an increase in cell polyploidy in kidneys from both heterozygous (*Pkd2*^{+/-} or *Tg737*^{Orpk/wt}) and homozygous (*Pkd2*^{-/-} or *Tg737*^{Orpk/Orpk}) mice compared to the corresponding wild-type (*Pkd2*^{+/+} or *Tg737*^{wt/wt}). Bar graph shows that epithelia from both *Pkd2* and *Tg737* kidneys, compared to their wild-type kidneys, significantly contain greater polyploidy with DNA content of >4N. (e) Karyotyping was carried out in freshly isolated cells from *Pkd2*^{+/+}, *Pkd2*^{+/-} and *Pkd2*^{-/-} kidneys to visualize individual chromosomes. A simple chromosome count indicates the presence of polyploidy cells in both *Pkd2*^{+/-} and *Pkd2*^{-/-} kidneys. Further characterization of individual chromosomes with fluorescence probes indicates the polyploid and aneuploid nature of *Pkd2*^{+/-} and *Pkd2*^{-/-} kidneys. (f) Embryonic kidneys were pooled and analyzed for survivin expression, and GAPDH was used as loading control. Bar graph shows relative survivin

expression levels. For flow cytometry and Western experiments, $N \geq 4$ for independent kidney isolations for each genotype (analyzed with ANOVA test followed by Dunn's Multiple Comparison posttest analysis) and each human cell line (analyzed with paired Student *t*-test).

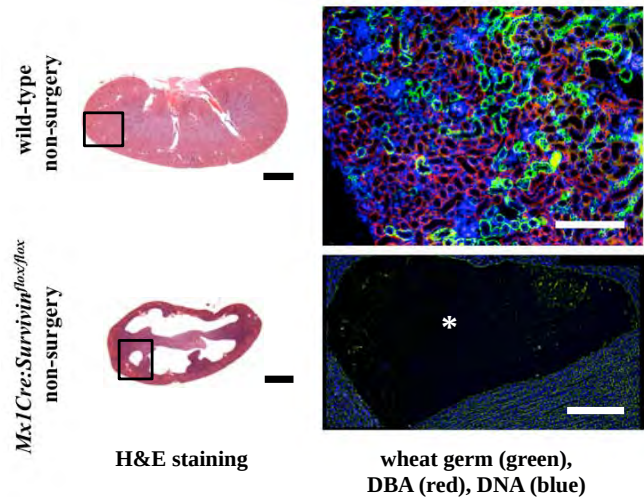
Supplemental Figure 2a



Supplemental Figure 2b



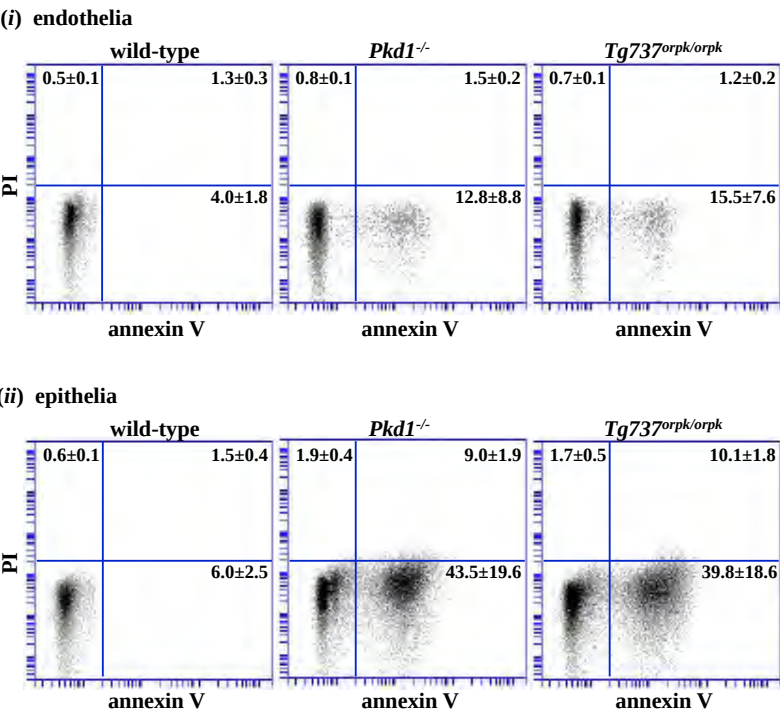
Supplemental Figure 2c

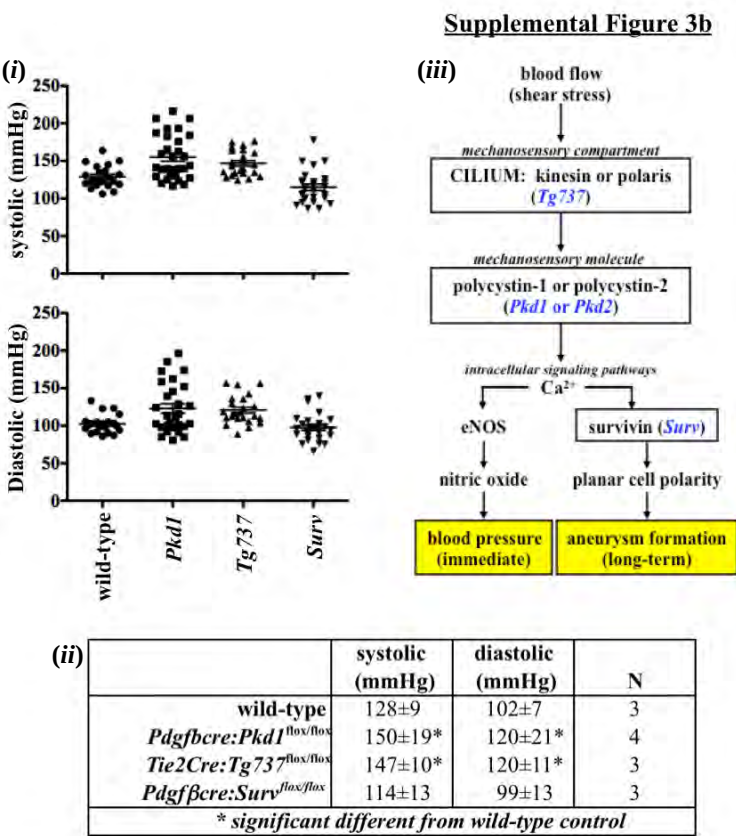


Supplemental Figure 2. Survivin downregulation is sufficient to induce cystic kidney formation.

(a) Embryonic kidneys from E15.5 of the same litter were isolated and cultured on transwell filters. After the first micrographs were taken at day 0, cultures were then treated with vehicle as control, 50 μ M EM-1451 or 100 μ M VE-465 to induce abnormal cell division by inhibiting survivin or aurora-A kinase, respectively. Kidney cultures were micrographed daily for a total of six days. Cyst-like phenotype was apparent as early as day 2. (b) Bar graph shows quantitation of lumen sizes (cyst areas) in wild-type and *Survivin* knockout kidneys. A total of 30-53 lumen areas were measured from three randomly selected kidneys in each group. (c) Paralleled kidney sections were obtained from three-months old mice for H&E and fluorescence analyses. Box in the H&E kidney corresponds to an approximate area for further fluorescence study. White asterisk indicates renal cyst. $N \geq 3$ for kidney analysis in each group and genotype (analyzed with ANOVA test followed by Dunn's Multiple Comparison posttest analysis). White bar=200 μ m; black bar=1 mm.

Supplemental Figure 3a

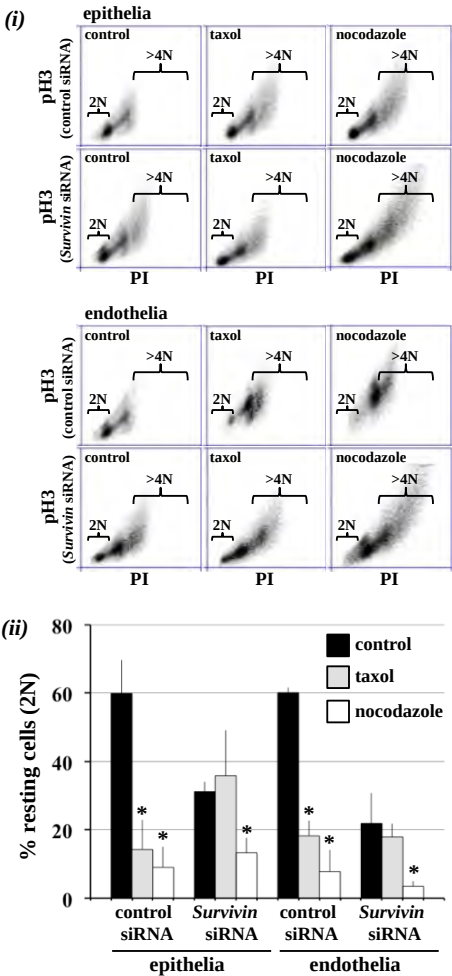




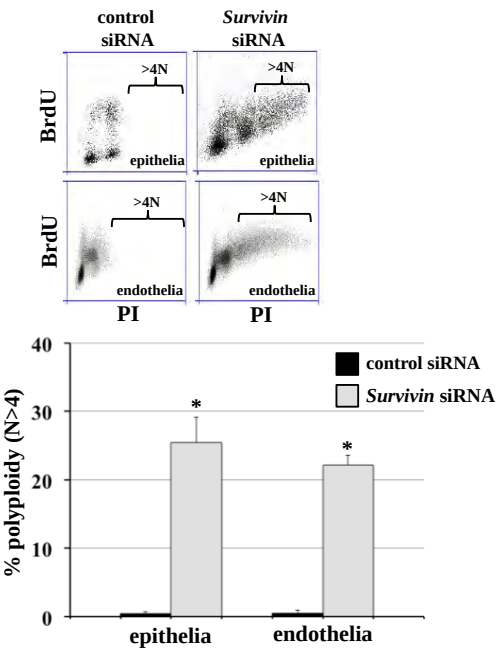
Supplemental Figure 3. Apoptosis and blood pressure measurements.

(a) Compared to the corresponding control wild-type cells, apoptosis is significantly greater in vascular endothelial **(i)** and renal epithelial **(ii)** of *Pkd1* and *Tg737* mutant cells. Annexin V is used as an early apoptotic marker, and propidium iodide (PI) is used as a necrotic marker. **(b)** *Pdgfbcre:Survivin^{flx/flx}* mice do not have elevated systolic or diastolic blood pressure. Blood pressure was measured in wild-type and conditional *Pkd1*, *Tg737*, and *Survivin* (*Sur*) mice for a 2-week period **(i)**. The averaged measurements of blood pressure during this 2-week period were tabulated **(ii)**. A working model is presented showing the roles of primary cilia in blood pressure and aneurysm formation **(iii)**.

Supplemental Figure 4a



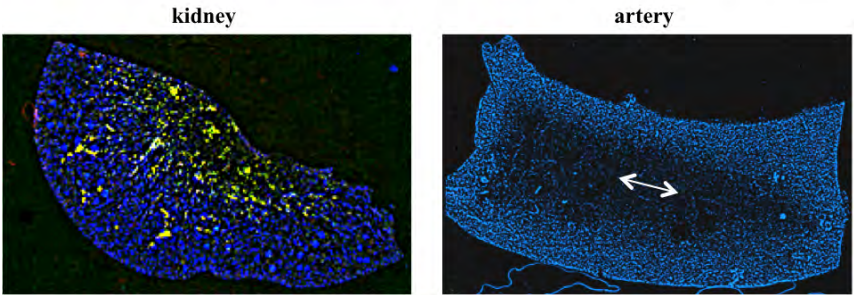
Supplemental Figure 4b



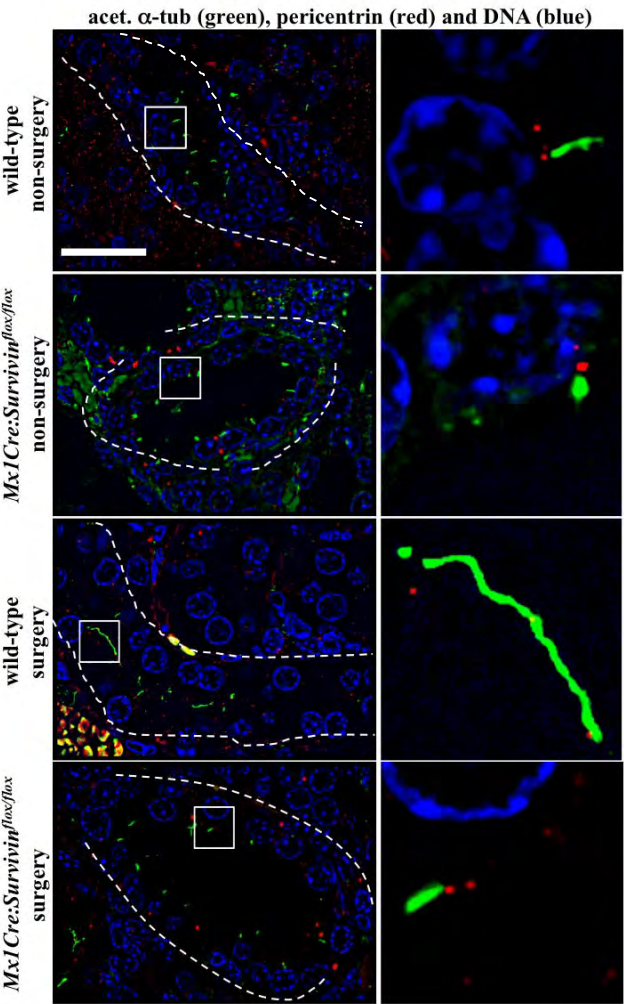
Supplemental Figure 4. Survivin down-regulation is associated with abnormal cytokinesis in primary cells of renal epithelia and vascular endothelial cells.

(a-i) Mitotic-stress test indicates abnormal cytokinesis associated with knockdown of a chromosomal passenger protein (survivin). Cell-cycle arrest was induced with taxol or nocodazole to stabilize or depolymerize mitotic spindles, respectively. Those cells with abnormal chromosomal passenger protein expression were not inhibited in cell arrest-induced by taxol. Phospho-histone3b (pH3) and PI were used to differentiate those cells that were not arrested by taxol. **(a-ii)** Mitotic stress was measured by analyzing changes in resting cells (2N), which are PI- and pH3-negative, from the total cell population. The test would thus examine if survivin down-regulation is responsible for a cell escaping cell-cycle arrest in the presence of taxol. The results from the mitotic-stress test indeed indicate that knockdown of survivin expression was characterized by abnormal cytokinesis. Although most normal cells could be arrested by nocodazole (microtubule depolymerizer) and taxol (microtubule stabilizer), survivin knockdown cells could only be arrested by nocodazole. This is consistent with previous reports showing that cells with abnormal chromosomal passenger complex, such as survivin, would not be arrested by taxol. **(b)** To validate that *Survivin* knockdown would induce abnormal cytokinesis leading to polyploidy, cell-cycle profile was analyzed with PI and BrdU to study cellular proliferation in epithelial and endothelial cells. Bar graph shows that compared to corresponding control groups, survivin knockdown-epithelia and endothelia contain significantly more polyploidy with DNA content of $>4N$. Bar=50 μm . $N \geq 3$ for each group and treatment (analyzed with ANOVA test followed by Dunn's Multiple Comparison posttest analysis).

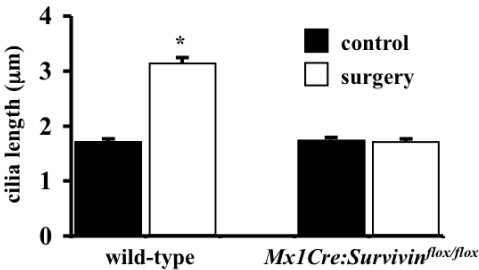
Supplemental Figure 5a



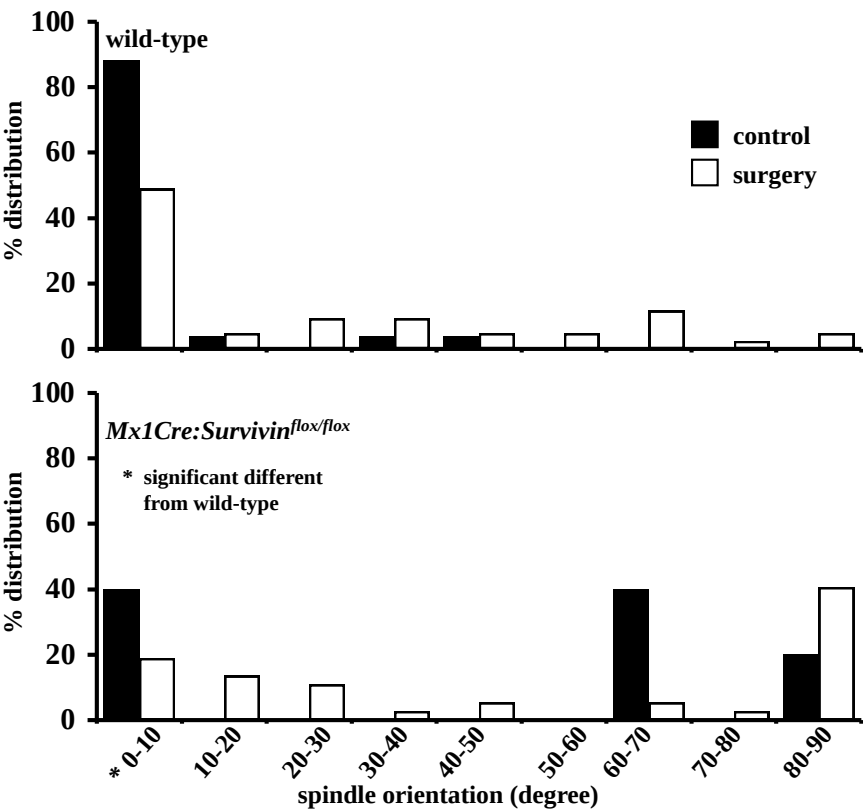
Supplemental Figure 5b



Supplemental Figure 5c

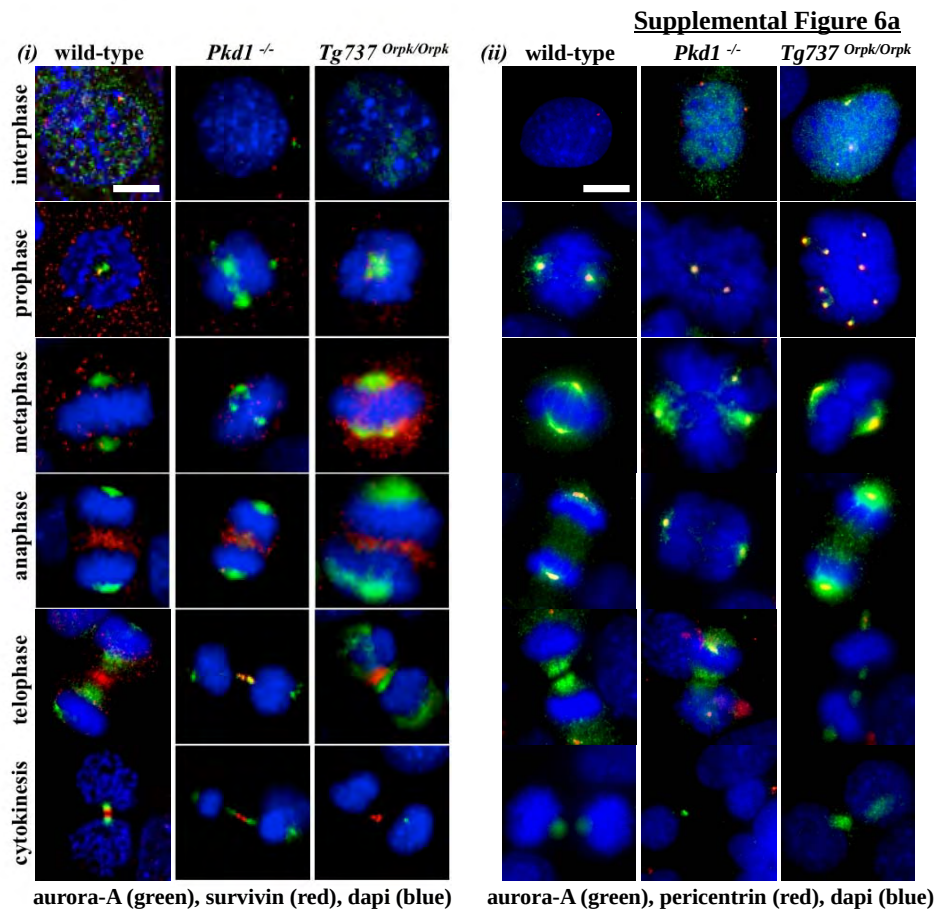


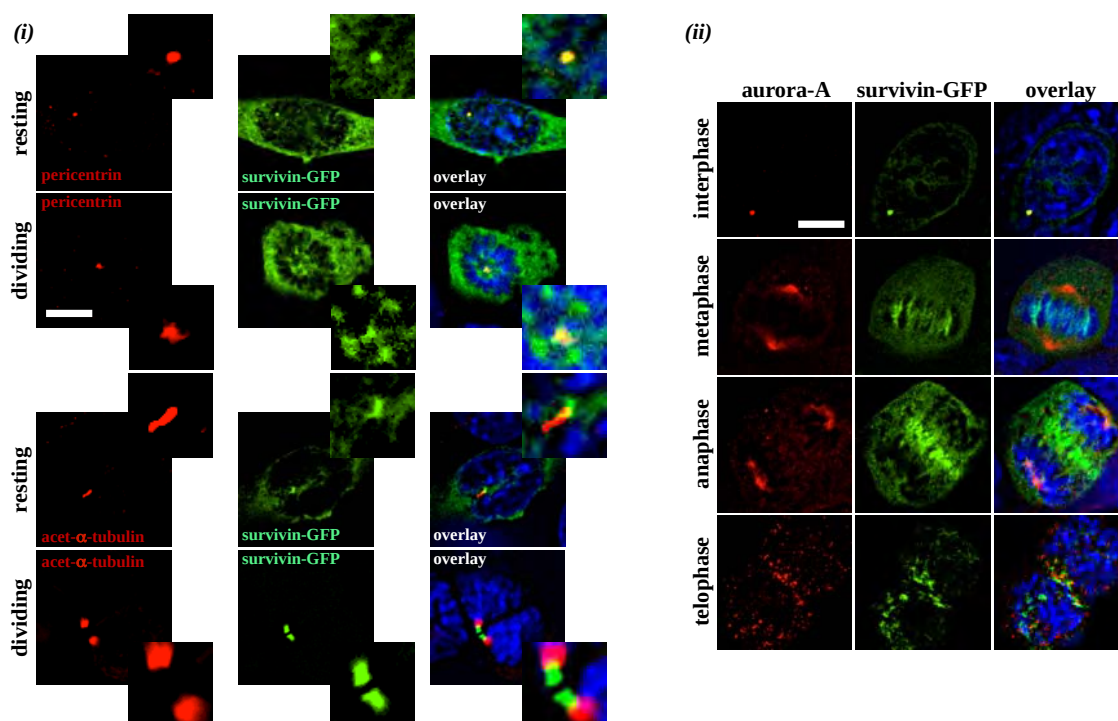
Supplemental Figure 5d



Supplemental Figure 5. Abnormal cellular division orientation is associated with renal cystic and vascular aneurysm phenotypes.

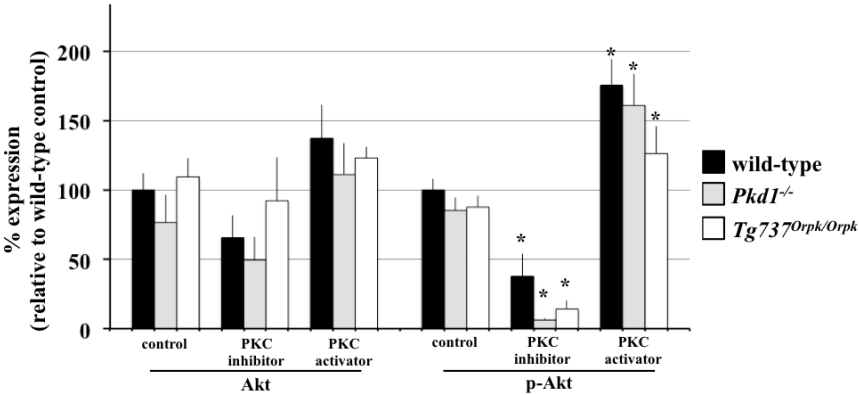
(a) Mouse kidney and artery longitudinal sections stained with acetylated- α -tubulin (green), pericentrin (red), and DAPI (blue) were used to examine cell division orientation. (b) Longitudinal kidney tubular sections from wild-type and *Mx1Cre:survivin*^{flox/flox} mice with or without UUO surgery were used to study structural length of primary cilia. Longer cilia were observed in tubular sections of wild-type UUO kidneys. White box indicates the enlargement of the region within the kidney lumen. (c) The length of cilia in wild-type kidney tubules was significantly increased following UUO surgery but not in *Mx1Cre:survivin*^{flox/flox} mice. (d) The frequency distribution of cell-division orientation angle (degrees) was tabulated in control and surgical kidneys in both wild-type and *Mx1Cre:survivin*^{flox/flox} mice. Angles of cell-division orientation in *Mx1Cre:survivin*^{flox/flox} mice show a shift towards the higher angle groups in both control and surgical kidneys. N $\geq$ 3 for each group and genotype. N $\geq$ 100 for distribution of spindle orientation angle for each genotype and each treatment. N $\geq$ 1,600 for cilia length measurement. Bar=40 μ m.



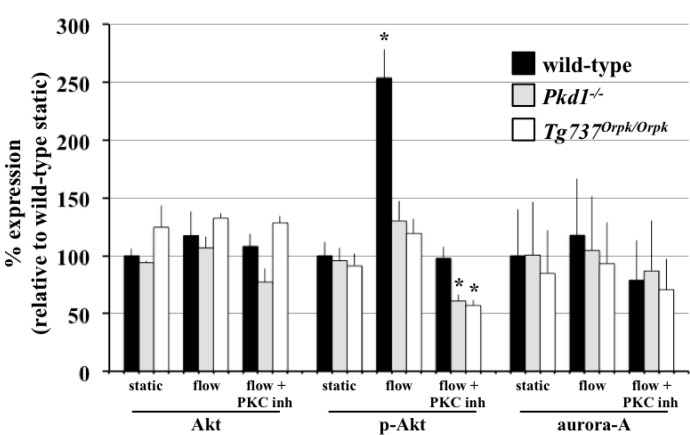
Supplemental Figure 6b**Supplemental Figure 6.** Confirmations of survivin antibody and survivin/aurora-A localization.

(a) Cells were stained with aurora-A (green) and survivin (*i*; red) or pericentrin (*ii*; red). To study subcellular localization of aurora-A and survivin, images were captured at different cell-cycle stages of interphase, prophase, metaphase, anaphase, telophase and cytokinesis. Aurora-A and survivin are localized to the centriole in interphase, the centrosome in prophase and the mid-body during telophase and cytokinesis. (b) Cells were transfected with survivin-GFP (green) and stained with pericentrin/acetylated- α -tubulin (*i*; red) or aurora-A (*ii*; red). Subcellular localizations of survivin/aurora-A were studied in resting and dividing cells. Insert shows area of enlargement for survivin/pericentrin/acetylated- α -tubulin localization. Bar=10 μ m.

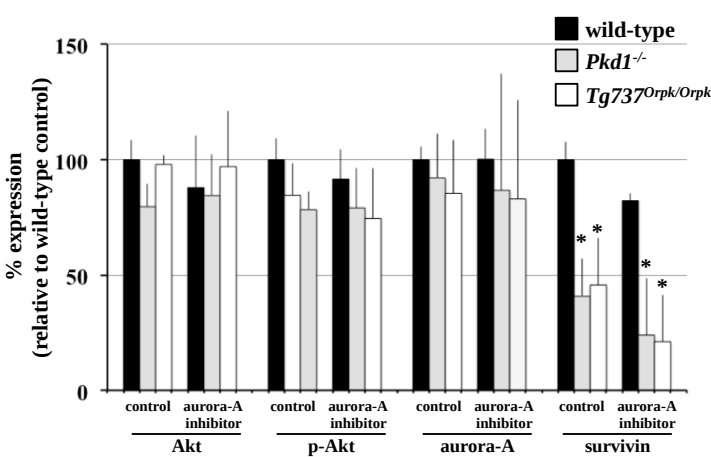
Supplemental Figure 7a



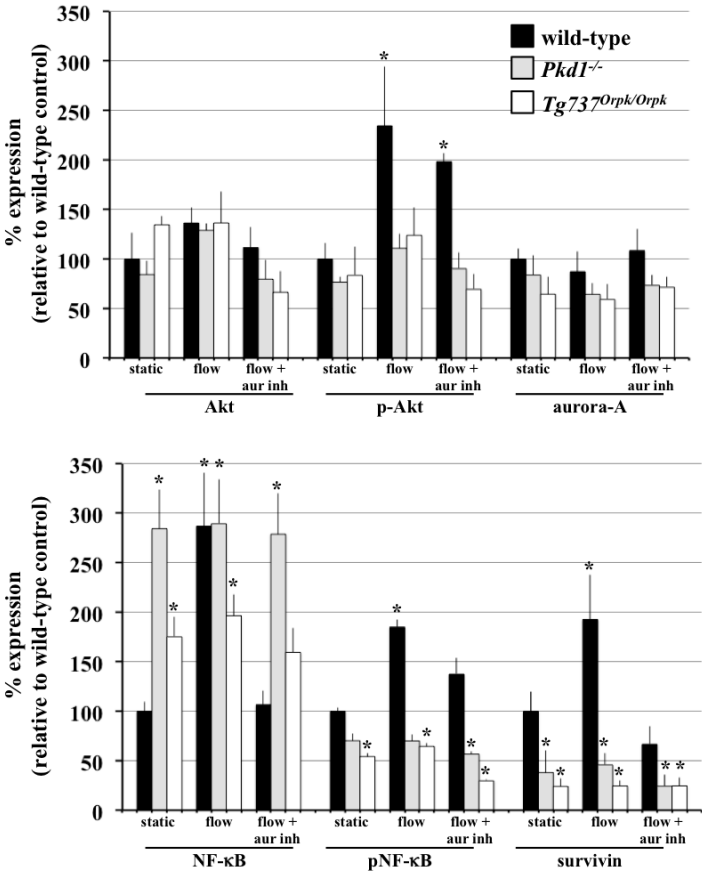
Supplemental Figure 7b



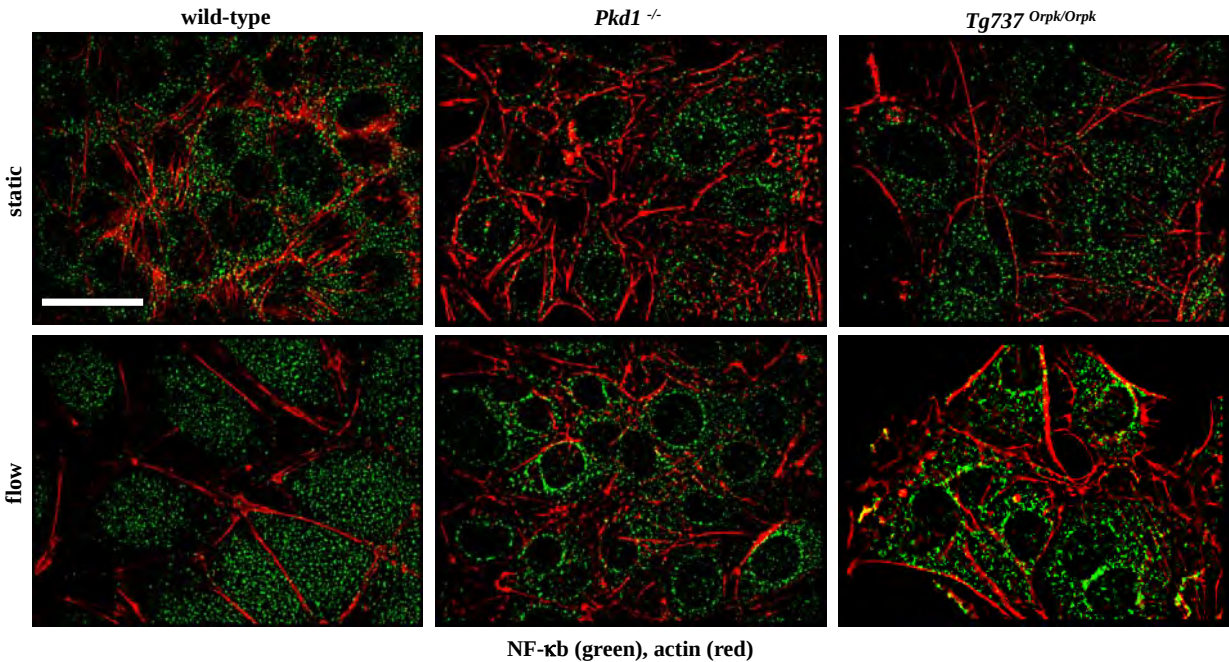
Supplemental Figure 7c



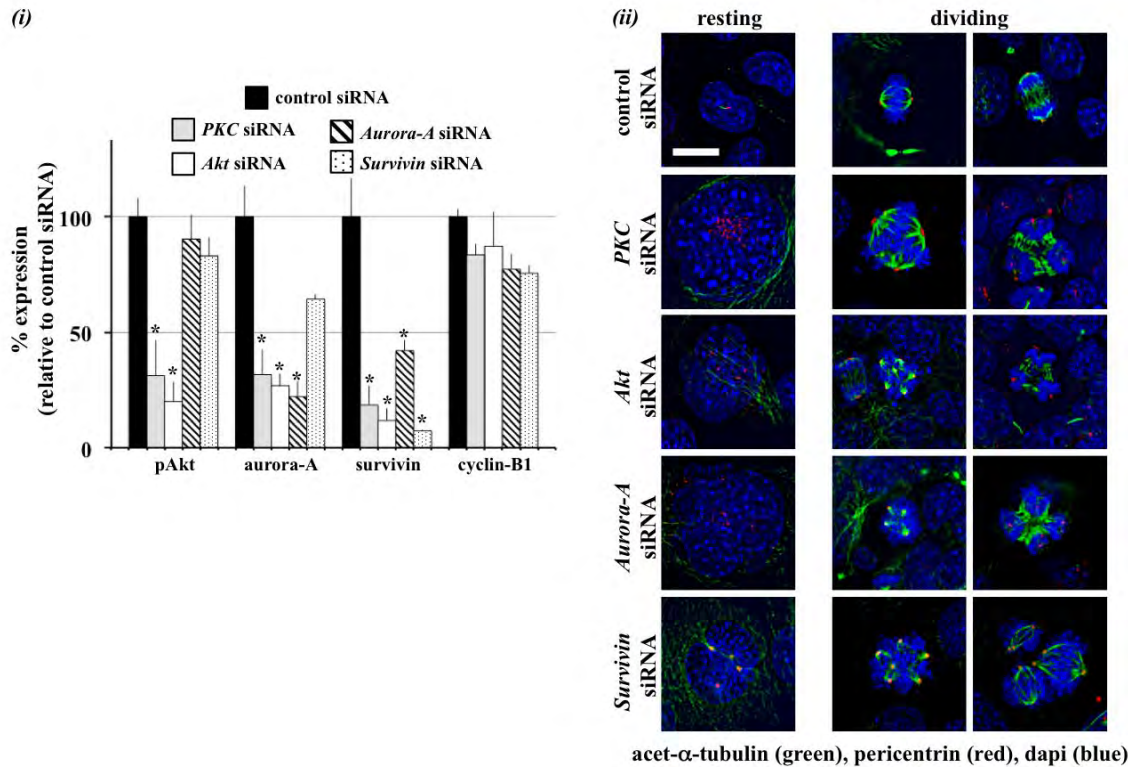
Supplemental Figure 7d



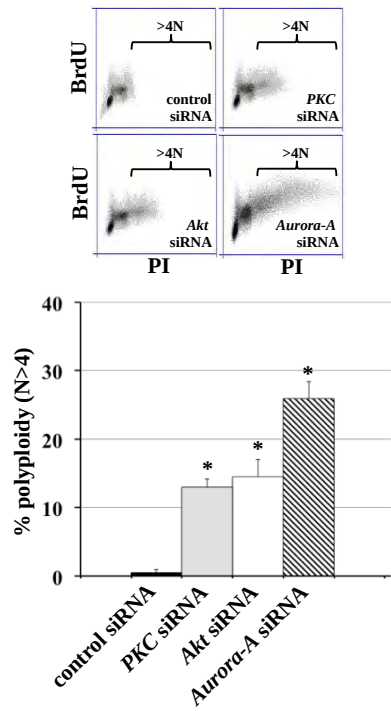
Supplemental Figure 7e



Supplemental Figure 7f



Supplemental Figure 7g



Supplemental Figure 7. PKC/Akt/NF- κ B 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 p-Akt were analyzed. When treated with PKC inhibitor, all cell lines showed down-regulation of p-Akt, while PKC activator treatment showed an increase in p-Akt compared to non-treated 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 up-regulated only in wild-type cells. While p-Akt expression returned to basal levels following 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 from the control, non-treated group. (d) While total Akt level was not changed, fluid-shear stress significantly induced expression of p-Akt in wild-type but not in mutant cells. Aurora-A expression was increased following fluid-shear stress in wild-type cells; however, this increase was not significant from control. Both NF- κ B and pNF- κ B expressions were increased following fluid-shear stress only in wild-type cells, while mutant cells maintained a high basal level of NF- κ B compared to static wild-type cells. Survivin expression was increased following shear-stress in wild-type cells. (e) NF- κ B nuclear translocation was confirmed with immunostaining study. Cells were stained with NF- κ B p65 (green), actin (red) to examine subcellular localization of NF- κ B before and after fluid flow. In contrast to mutant cells, wild-type cells show NF- κ B translocation from cytoplasm to nucleus when subjected to fluid-shear stress. (f-i) Western blot analyses were conducted to confirm the signaling mechanism involving survivin expression by siRNA-mediated knockdown of PKC, Akt, aurora A, or survivin. (f-ii)

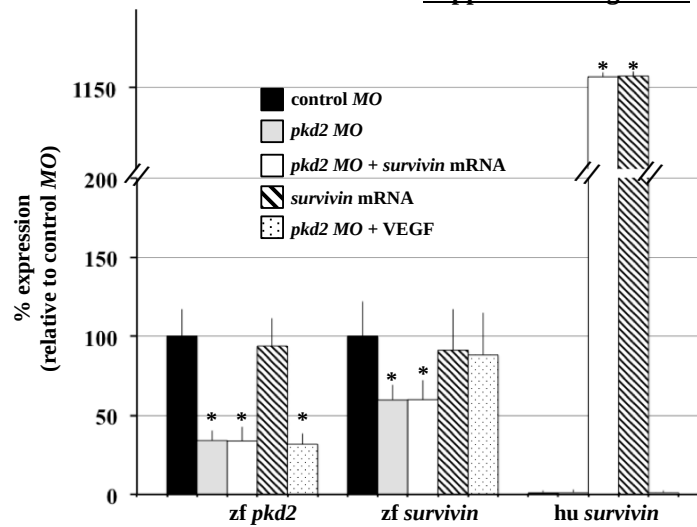
To further confirm the involvement of these signaling molecules in centrosome number and cell division abnormality, immunofluorescence analysis was performed using acetylated- α -tubulin and pericentrin. (g) Flow cytometry was done to analyze ploidy level in PKC, Akt or aurora-A knockdown cells. Bar=40 μ m. $N \geq 3$ for each group and treatment. All Western blot analyses were done by comparing treatment groups to their corresponding wild-type control, non-treated groups. Data analysis was performed with ANOVA test followed by Dunn's Multiple Comparison posttest analysis

Supplemental Figure 8a

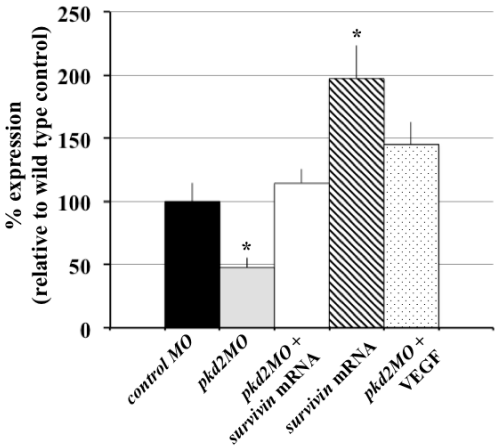
overall phenotypic observations

28 hpf	n/total (%)	curly tail	renal cyst
control <i>MO</i>		0/61 (0)	N/A
<i>pkd2 MO</i>		55/70 (79)	N/A
<i>pkd2</i> + <i>survivin</i> mRNA		36/95 (38)	N/A
<i>survivin</i> mRNA		0/53 (0)	N/A
<i>pkd2</i> + VEGF		22/67 (33)	N/A
48 hpf	n/total (%)	curly tail	renal cyst
control <i>MO</i>		0/45 (0)	0/25 (0)
<i>pkd2 MO</i>		36/46 (78)	24/29 (83)
<i>pkd2</i> + <i>survivin</i> mRNA		24/63 (38)	10/26 (38)
<i>survivin</i> mRNA		0/42 (0)	0/25 (0)
<i>pkd2</i> + VEGF		30/67 (45)	14/28 (50)
72 hpf	n/total (%)	curly tail	renal cyst
control <i>MO</i>		0/45 (0)	0/27 (0)
<i>pkd2 MO</i>		36/46 (78)	23/26 (88)
<i>pkd2</i> + <i>survivin</i> mRNA		28/61 (46)	19/28 (68)
<i>survivin</i> mRNA		0/42 (0)	0/21 (0)
<i>pkd2</i> + VEGF		45/67 (67)	19/26 (73)

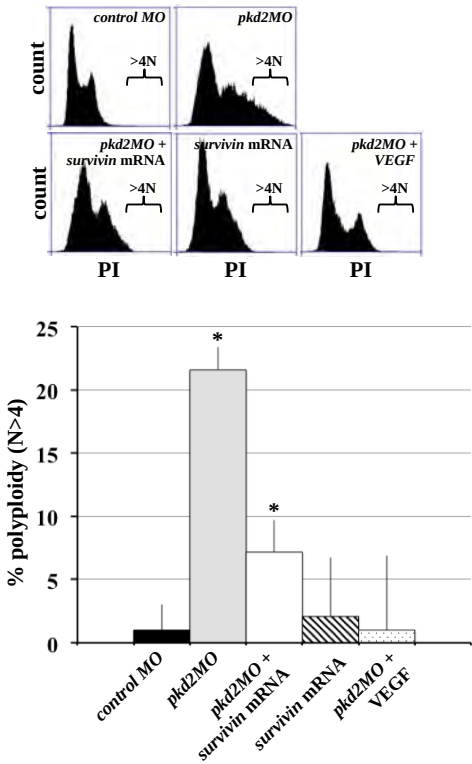
Supplemental Figure 8b



Supplemental Figure 8c



Supplemental Figure 8d



Supplemental Figure 8. Survivin overexpression rescued PKD phenotypes in zebrafish.

(a) An overall phenotypic was tabulated for wild-type, curly tail and renal cyst phenotypes in zebrafish injected with either control *MO*, *pkd2* *MO*, *pkd2* *MO* plus *survivin* mRNA, *survivin* mRNA alone, or *pkd2* *MO* plus VEGF. (b) 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) Polyploidy level was also validated and quantified with flow cytometry by isolating cells from each group of zebrafish. $N \geq 4$ for each group and treatment. All Western blot and RT-PCR analyses were done by comparing injected groups to the control *MO* group. Data analysis was performed with ANOVA test followed by Dunn's Multiple Comparison posttest analysis

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SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Normal cell division in renal epithelial cell.

The movie shows a cell undergoing a normal mitotic event. The movie was analyzed by superimposing DIC and Hoechst fluorescence images, which were taken every five minutes with 40X magnification.

Movie S2. Failure of cytokinesis induced polyploidy formation in survivin knockdown renal epithelial cell.

The movie shows that abnormal cytokinesis leads to polyploidy formation. The survivin knockdown cell is able to initiate cell division but is unable to execute cytokinesis properly. The cell then shows various membrane blebbing followed by the formation of a cytomegalic cell with multiple nuclei. Images were captured every two minutes with 40X magnification.

Movie S3. Normal cell division in vascular endothelial cell.

The movie shows a cell undergoing a normal mitotic event. The movie was analyzed by superimposing DIC and Hoechst fluorescence images, which were taken every two minutes with 40X magnification.

Movie S4. Failure of cytokinesis induced polyploidy formation in survivin knockdown vascular endothelial cell.

The movie shows that abnormal cytokinesis leads to polyploidy formation. The survivin knockdown cell is able to initiate cell division but is unable to execute cytokinesis properly. The cell then shows various membrane blebbing followed by the formation of a cytomegalic cell with

multiple nuclei. Of note is the presence of another multinucleated cell within the field of view. Images were captured every two minutes with 40X magnification.

Primary Cilium Regulates CaV1.2 Expression Through Wnt Signaling

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Primary cilia are sensory organelles that provide a feedback mechanism to restrict Wnt signaling in the absence of endogenous Wnt activators. Abnormal Wnt signaling has been shown to result in polycystic kidney disease (PKD) although the exact mechanism has been debated. Previously, we reported that the calcium channel CaV1.2 functions in primary cilia. In this study, we show that CaV1.2 expression level is regulated by Wnt signaling. This occurs through modulation of mitochondrial mass and activity resulting in increased reactive oxygen species which generate oxidative DNA lesions. We found that the subsequent cellular DNA damage response triggers increased CaV1.2 expression. In the absence of primary cilia where Wnt signaling is upregulated, we found that CaV1.2 is overexpressed as a compensatory mechanism. We show for the first time that CaV1.2 knockdown in zebrafish results in classic primary cilia defects including renal cyst formation, hydrocephalus, and left-right asymmetry defects. Our study shows that suppressed Wnt signaling prevents CaV1.2 expression ultimately resulting in PKD phenotypes. Thus, CaV1.2 expression is tightly regulated through Wnt signaling and plays an essential sensory role in primary cilia necessary for cellular homeostasis.

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Wnt signaling is an important regulator of cellular development and proliferation. In the absence of Wnt ligands, a complex consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 β (GSK3 β) induces β -catenin for ubiquitylation by SCF E3 ligases and eventual proteasomal degradation (Aberle et al., 1997). Wnt signal transduction occurs when secreted Wnt ligands bind Frizzled receptors resulting in phosphorylation of LRP5/6. The Axin-APC-GSK3 β complex is then recruited to LRP5/6 at the cell membrane which prevents β -catenin from being degraded. The accumulated β -catenin translocates to the nucleus and activates transcription of Wnt target genes (Muntean et al., 2012).

Primary cilia are non-motile sensory organelles present as a single copy on most differentiated cells in the body. Calcium signaling through primary cilium is essential for renal epithelial homeostasis (Nauli et al., 2003; Jin et al., 2013). Cilia extend from the cell surface through the basal body via intraflagellar transport (Moyer et al., 1994). The most common pathologies resulting from cilia dysfunction include polycystic kidney (Wilson, 2004), hypertension (Nauli et al., 2008; AbouAlaiwi et al., 2009), aneurysm (AbouAlaiwi et al., 2013), hydrocephalus (Carter et al., 2012), and left-right asymmetry defects (Norris, 2012).

Abnormal Wnt signaling has also been linked to polycystic kidney disease (PKD) (Lancaster et al., 2009). For example, increased cytosolic and nuclear β -catenin accumulation has been shown in various cilia mutant cells (Gerdes et al., 2007; Lancaster et al., 2011). Thus, primary cilia are thought to provide a feedback mechanism that restricts Wnt signaling in the absence of appropriate ligands (Gerdes et al., 2007; Lancaster et al., 2009, 2011).

We recently showed that voltage-gated L-type calcium channel CaV1.2 localized to primary cilia in renal epithelia (Jin et al., 2013). Because Wnt signaling has also been reported to modulate mitochondrial physiology (Yoon et al., 2010), we hypothesized that primary cilia play a role in Wnt regulation of mitochondria through CaV1.2. We show that although CaV1.2 is not required for cilia formation, Wnt increases mitochondria mass and activity in CaV1.2 deficient renal epithelial cells. This increases mitochondria reactive oxidative species (ROS) and DNA damage, resulting in PKD phenotypes. Thus, our study suggests that primary cilia may play a role in CaV1.2 expression level through Wnt regulation of mitochondria.

Materials and Methods

The experimental use of zebrafish was approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). The use of lentiviral components was approved by the Institutional Biosafety Committee of The University of Toledo.

Cell culture

Immortalized mouse renal epithelial wild-type and *Tg737^{orp/k}* cells were cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin/streptomycin (Corning Cellgro) at 39°C in 5% CO₂, as previously described (AbouAlaiwi et al., 2013). Prior to experiments, cells were treated with 100 ng/ml recombinant Wnt3a (R&D Systems, Minneapolis, MN) for 3 days and serum starved for 24 h.

RNAi knockdown cells

shRNA lentiviral vectors specific to *Cacna1c* (Origene; pGFP-C-shLenti clone ID: TL500242) were transfected into HEK293T cells. Viral supernatants were collected after 48 h, centrifuged, and passed through a 0.45 μ m filter. Cells were then spin-inoculated with pseudoviral particles containing 8 μ g/ml polybrene at 2,500 rpm for 30 min at 30°C and then cultured for up to 1 week. CaV1.2 knockdown was verified through Western blot analysis.

Brian S. Muntean and Xingjian Jin contributed equally to this work.

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TABLE 1. shRNA sequences

Descriptions	Sequences
Scrambled control	5'-TGACCACCCTGACCTACGGCGTGCAGTGC-3'
<i>Cacna1c</i>	5'-TCAGAGTGCCTCACTGTTCTCGTGACCT-3'

Stable knockdown cell lines were obtained through puromycin selection. The following shRNA sequences were used (Table 1).

Immunostaining studies

Cells were grown to confluence on collagen-coated glass coverslips and differentiated in serum-free media for 24 h. Cells were then fixed in 4% paraformaldehyde in PBS containing 2% sucrose, permeabilized in 10% triton X-100, incubated sequentially with primary followed by secondary antibodies for 1 h each, and finally mounted on a slide with DAPI hard set mounting media (Vector Laboratories, Burlingame, CA). The following primary antibody dilutions were used: acetylated- α -tubulin 1:10,000 (Sigma-Aldrich, St. Louis, MO) and CaV1.2 1:50 (Alomone Labs, Jerusalem, Israel). Anti-mouse Texas Red and anti-rabbit FITC fluorescent conjugated secondary antibodies were used at 1:500 (VectorLabs).

Mitochondrial studies

MitoTracker Green FM and MitoTracker Red CMXRos (Cell Signaling Technology) were incubated with cells at 100 nM for 30 min at 37°C. MitoSOX (Life Technologies) was incubated with cells at 5 μ M for 10 min at 37°C. After staining, cells were washed three times with PBS and analyzed immediately through microscopy or flow cytometry. For microscopic analysis, cells were grown on custom glass-bottom cell culture plates and imaged under a Nikon Eclipse TE2000-U microscope controlled by MetaMorph software with a 100 $\times$ objective lens. For flow cytometry studies, cells were detached with trypsin, washed, and analyzed.

DNA damage assessment

Oxidative DNA lesions were detected with an 8-oxoguanine antibody (Santa Cruz). Detached cells were fixed in 4% formaldehyde for 10 min at 37°C and permeabilized in ice-cold 90% methanol for 30 min on ice. After washing with PBS, cells were incubated in PBS containing anti-8-oxoguanine antibody (1:50), 0.5% Tween-20, and 5% FBS for 1 h. Cells were washed and incubated in PBS containing anti-mouse Texas Red antibody (1:500), 0.5% Tween-20, and 5% FBS for 1 h. Cells were then washed and analyzed with flow cytometer.

Mitochondrial DNA and mRNA measurement

Total cellular DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen) and used for detection with PCR primers listed below to quantify the nuclear (*18S rRNA*) to mitochondrial DNA (*CoI*) ratio as described (Brown and Clayton, 2002; Bai et al., 2004). Total cellular RNA was obtained using TRIzol (Life Technologies) and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR detection of expression genes was performed using the primers listed below comparing mitochondrial encoded oxidative phosphorylation genes (*ATP5 γ* and *CytC*) to nuclear encoded β -Actin as described (Yoon et al., 2010) (Table 2).

Western blot analysis

Cells were scraped from culture plates in the presence of RIPA buffer supplemented with Complete Protease Inhibitor (Roche,

TABLE 2. Primer sequences

Descriptions	Sequences
<i>CoI</i> F	5'-GCCCCAGATATAGCATTCCC-3'
<i>CoI</i> R	5'-GTTTCATCTGTTCTCTGCTCC-3'
<i>18S rRNA</i> F	5'-TAGAGGGACAAGTGGCGTTC-3'
<i>18S rRNA</i> R	5'-CGCTGAGCCAGTCAAGTGT-3'
<i>ATP5γ</i> F	5'-AGTTGGTGTGGCTGGATCA-3'
<i>ATP5γ</i> R	5'-GCTGCTTGAGAGATGGGTTC-3'
<i>CytC</i> F	5'-GGAGGCAAGCATAAGACTGG-3'
<i>CytC</i> R	5'-TCCATCAGGGTATCCTCTCC-3'
β -actin F	5'-TGTACCAACTGGGACGACA-3'
β -actin R	5'-GGGGTGTGAAGGTCTCAA-3'

New York, NY), incubated on ice with frequent vortexing, and centrifuged. Supernatants were subjected to protein quantification and PAGE on 6–10% SDS gels followed by wet transfer to PVDF membranes and detection using β -catenin 1:1,000, CaV1.2 1:200, NF- κ B p65 1:200, and GAPDH 1:1,000 (Cell Signaling Technology, Danvers, MA).

Zebrafish

Adult wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) and used for breeding. Embryos were injected with 1 mM antisense translation blocking morpholino oligos (MO; GeneTools) at the 1–2 cell stage. Zebrafish embryos were then cultured at 28.5°C in sterile egg water (Muntean et al., 2010). The following MO sequences were used: *control* MO: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3', *cav1.2* MO: 5'-ACA TGT TTT TGC TTT CAT TTA CCA T-3', *pkd2* MO: 5'-AGG ACG AAC GCG ACT GGA GCT CAT C-3'. Knockdown of CaV1.2 was verified through Western blot analysis. Briefly, zebrafish embryos were dechorionated at 28 h postfertilization and homogenized in RIPA buffer to obtain protein extracts. Western was performed on 50 μ g total protein using CaV1.2 (1:200) and GAPDH (1:1,000) antibodies.

Histological examination was used to measure renal cyst formation and hydrocephalus at 3 days postfertilization. Embryos were fixed in a PBS solution containing 4% paraformaldehyde and 2% sucrose overnight at 4°C, dehydrated through an ethanol gradient, and embedded in JB4 resin (Polysciences, Inc., Warrington, PA) as specified in manufacturer's protocol. A Reichert Jung microtome was used to cut 5 μ m sections which were subsequently hematoxylin and eosin stained. Heart looping was assessed at 48 h postfertilization by positioning zebrafish on their dorsal axis and recording heart beat to reveal the respective relative locations of the atrium and the ventricle.

Data analysis

Data are reported as the mean $\pm$ standard error of the mean. All image analysis was performed using ImageJ. All flow cytometry data were analyzed with BD Accuri C6 software and were presented without any compensation gating. All data were analyzed using IBM SPSS Statistics Version 21 software by performing the student t-test for two group comparison or ANOVA test followed by Tukey's post-test for three or more group comparison. Statistical significance is reported with a statistical power greater than 0.8 at $P < 0.05$.

Results

CaV1.2 is not required for primary cilia assembly

We recently reported that the voltage gated L-type calcium channel CaV1.2 localized to primary cilia in bovine LLC PK cells (Jin et al., 2013). We performed immunostaining to verify this finding in mouse renal epithelial cells (Fig. 1). The mouse *Tg737^{orp/orpk}* cell line contains a hypomorphic mutation in an intraflagellar transport gene (*Ift88*) that is required for cells

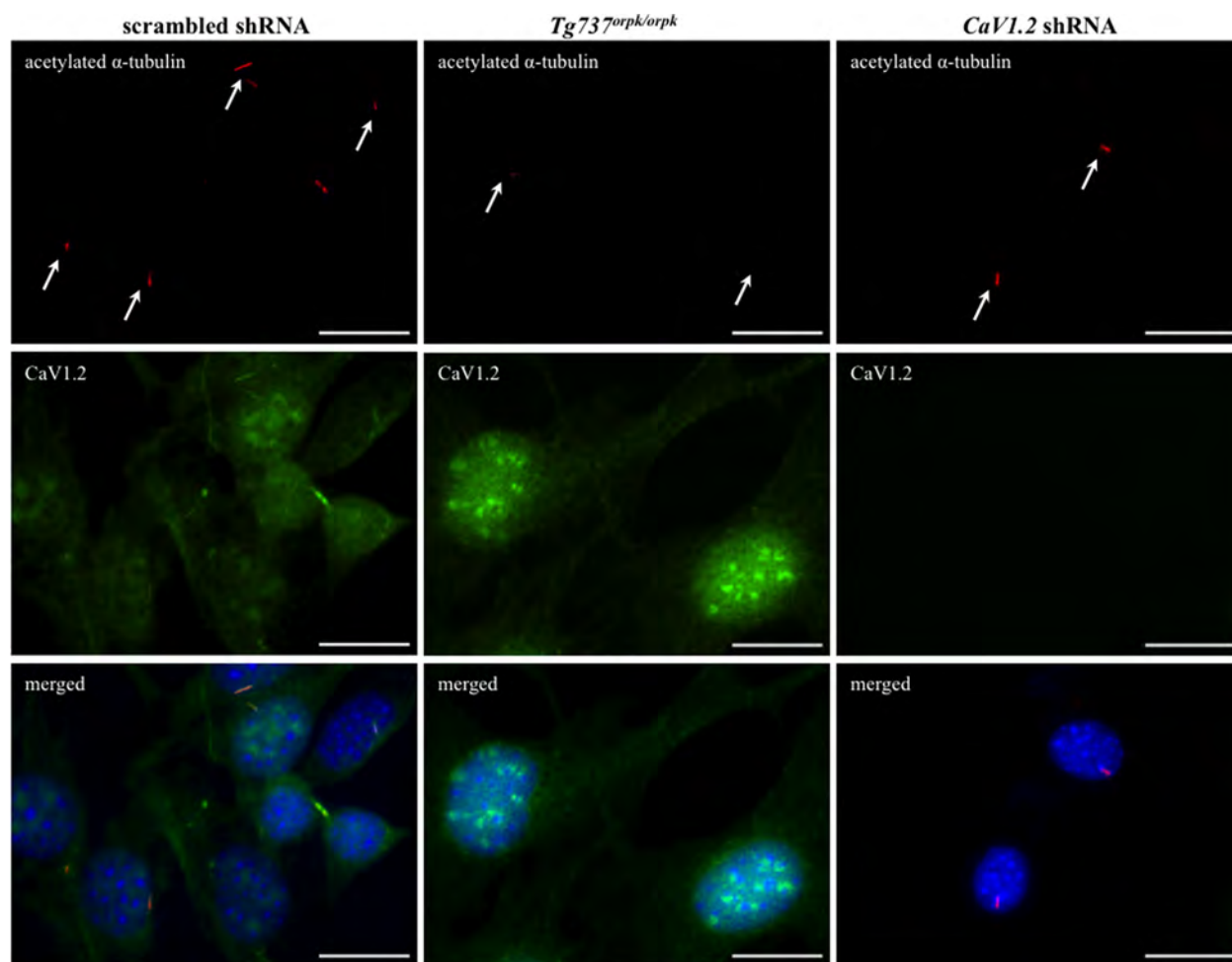


Fig. 1. Localization of CaV1.2 to renal epithelial cilia is not required for primary cilia assembly. Immunofluorescence revealed that CaV1.2 localized to primary cilia in renal epithelial cells (scrambled shRNA) when compared with cilia-deficient cells (*Tg737^{orp/ork}*). The presence of primary cilia was confirmed in CaV1.2 shRNA cells. Acetylated- α -tubulin was used as a ciliary marker. Arrow indicates the presence of primary cilium, except in cilia-deficient cells. Bar = 20 μ m.

to assemble primary cilia (Moyer et al., 1994). Thus, the *Tg737^{orp/ork}* system is a well-established model for studying cells without longer primary cilia, as verified through our immunostaining. We next asked if CaV1.2 played a role in primary cilia assembly. We generated a stable CaV1.2 shRNA knockdown mouse renal epithelial cell line and immunostaining studies revealed that primary cilia were similar to that of scrambled shRNA.

Wnt3a induces mitochondrial biogenesis in CaV1.2-deficient but not cilia-deficient cells

Wnt signaling has recently been reported to regulate mitochondrial physiology (Yoon et al., 2010). To assess mitochondrial mass, cells were stained with Mito Tracker Green (MTG) and observed live using fluorescence microscopy. When treated with recombinant Wnt3a, mitochondrial mass increased (Fig. 2a). However, the mitochondrial mass in *Tg737^{orp/ork}* cells was unchanged after Wnt3a treatment. We next performed this experiment in CaV1.2 shRNA cells and the results were similar to that of the scrambled control. To

quantify these findings, MTG fluorescence was recorded using flow cytometry which confirmed our fluorescent observation (Fig. 2b). Our MTG studies were further validated using a common technique by comparing mitochondrial DNA (*Coi*) to nuclear DNA (*18S rRNA*) (Brown and Clayton, 2002; Bai et al., 2004). As expected, Wnt3a did indeed statistically increase mitochondrial biogenesis in scrambled and CaV1.2 shRNA but not in *Tg737^{orp/ork}* cells (Fig. 2c). Our immunofluorescence study showed that Wnt3a did not alter CaV1.2 localization to cilia (Table 3).

Wnt3a increases mitochondrial activity in CaV1.2-deficient cells while decreasing activity in cilia-deficient cells

We next asked if Wnt3a would have an effect on mitochondrial oxidative phosphorylation (activity) in *Tg737^{orp/ork}* cells. Similar to before, we stained cells with Mito Tracker Red (MTR). Unlike MTG, MTR staining is dependent on the mitochondrial membrane potential (Poot and Pierce, 2001; Pendergrass et al., 2004). Therefore, increased staining

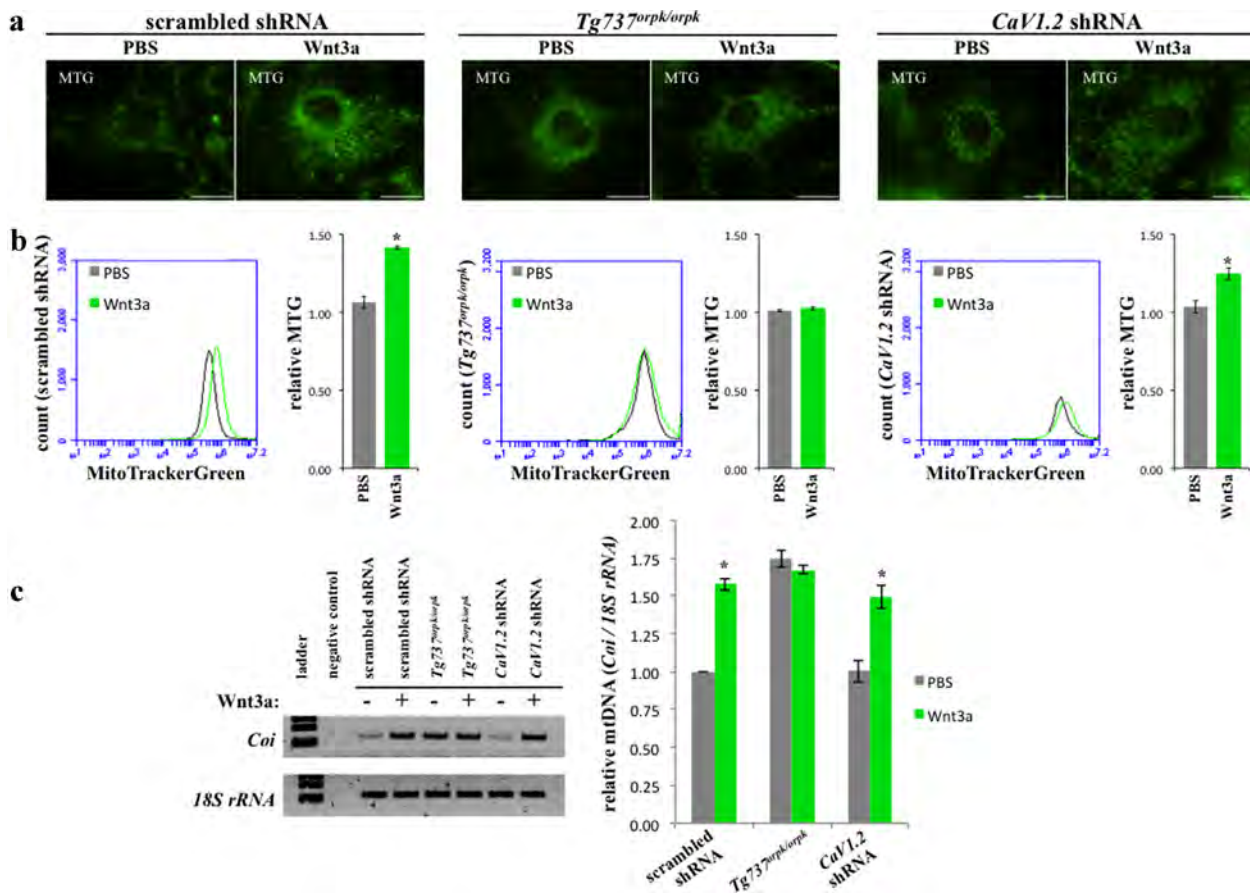


Fig. 2. Wnt3a induces mitochondrial biogenesis in *CaVI.2* shRNA but not *Tg737^{orp/orp}* cells. **a:** Mitochondrial mass was assessed by staining cells with Mito Tracker Green. Wnt3a was found to induce mitochondrial mass in scrambled and *CaVI.2* shRNA cells but had no effect on *Tg737^{orp/orp}* when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Mitochondrial DNA was measured using PCR by taking the ratio of a mitochondrial gene (*Coi*) to a nuclear gene (*18S* rRNA) ($N = 3$).

correlates to increased oxidative phosphorylation. As expected, Wnt3a increased MTR staining in scrambled and *CaVI.2* shRNA cells when observed using fluorescence microscopy (Fig. 3a). However, mitochondrial activity decreased in *Tg737^{orp/orp}* cells. We again quantified our findings using flow cytometry (Fig. 3b). Wnt3a significantly increased mitochondrial activity in scrambled and *CaVI.2* shRNA while significantly decreasing activity in *Tg737^{orp/orp}* cells. To verify these results, we compared expression of two key mitochondrial encoded oxidative phosphorylation genes (ATP Synthase 5 γ I and Cytochrome c) relative to that of nuclear encoded β -actin (Fig. 3c).

TABLE 3. *CaVI.2* ciliary localization

	% <i>CaVI.2</i> localization to cilia	N
PBS (control)		
Scramble shRNA	91.1	45
<i>Tg737^{orp/orp}</i>	91.7	36
<i>CaVI.2</i> shRNA	0.0	41
Wnt3a (100 ng/ml)		
Scramble shRNA	90.4	52
<i>Tg737^{orp/orp}</i>	92.3	39
<i>CaVI.2</i> shRNA	0.0	46

Wnt3a increases ROS and DNA damage in *CaVI.2*-deficient but not in cilia-deficient cells

An inevitable consequence of oxidative phosphorylation is the generation of reactive oxygen species (ROS) (Boveris et al., 1972; Boveris and Chance, 1973). MitoSOX is a cell permeable red fluorescent indicator specific for mitochondrial ROS. We therefore stained cells with MitoSOX and observed a significant increase in mitochondrial ROS in scrambled and *CaVI.2* shRNA after treatment with Wnt3a (Fig. 4a). A significant decrease in staining was observed in *Tg737^{orp/orp}* cells (Fig. 4b). Genomic DNA can be damaged by ROS to form DNA lesions resulting from mismatched repairs (Kasai et al., 1984). Thus, we quantified the levels of 8-Oxoguanine, a common DNA lesion formed by mismatched Adenine (Kasai, 1997). Treatment with Wnt3a was found to increase 8-Oxoguanine in scrambled and *CaVI.2* shRNA while no change was observed in *Tg737^{orp/orp}* cells (Fig. 4c).

Cilia modulates Wnt signaling to regulate *CaVI.2* expression

As previously reported, Wnt3a treatment induced β -catenin expression in all cells (Aberle et al., 1997). We confirmed this in our system, including in *Tg737^{orp/orp}* and *CaVI.2* shRNA cells (Fig. 5). Consistent with previous study (Corbit et al., 2008),

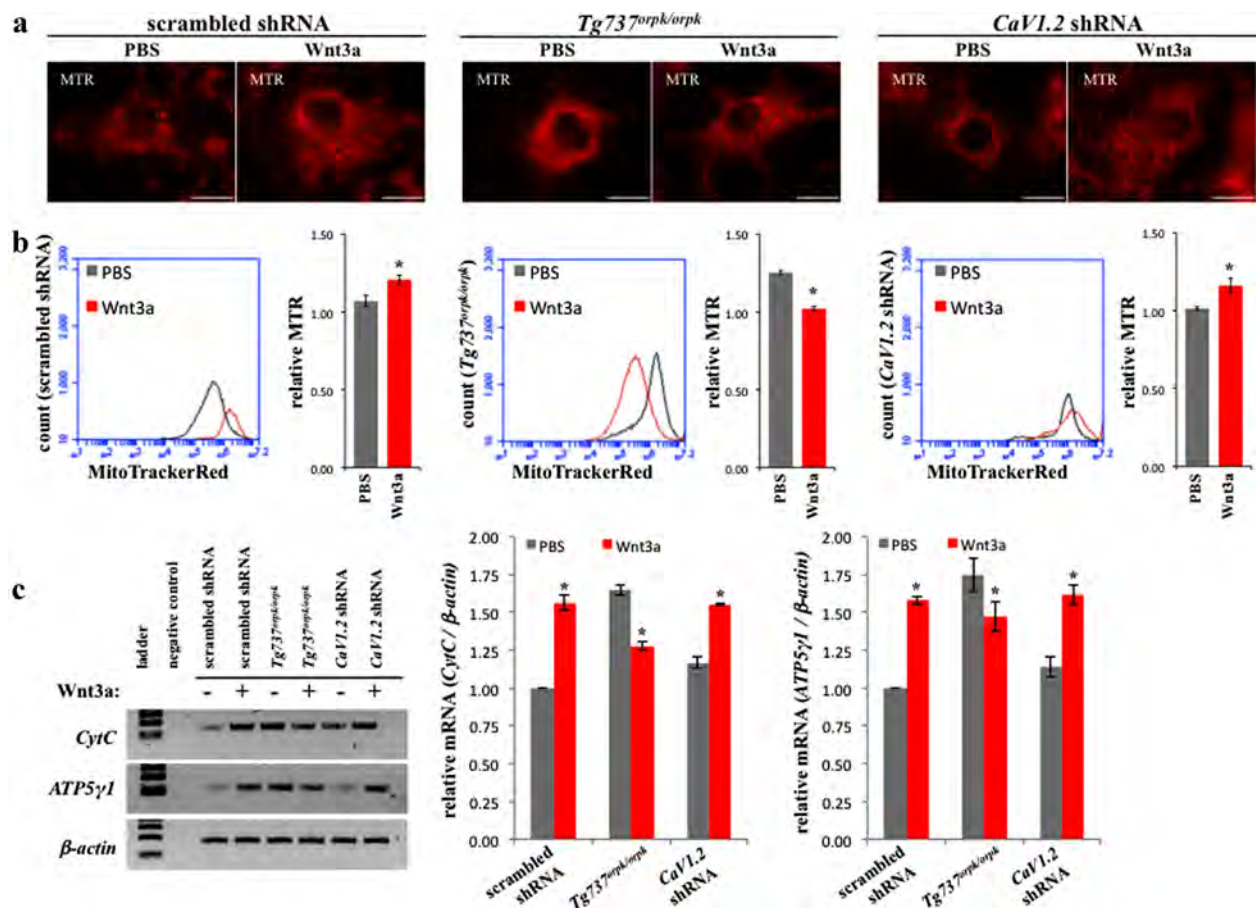


Fig. 3. Wnt3a increases mitochondrial activity in *CaV1.2* shRNA but decreasing activity in *Tg737orp/orpk* cells. **a:** Mitochondrial oxidative phosphorylation was used to indicate activity through Mito Tracker Red staining. Wnt3a was found to increase oxidative phosphorylation in scrambled and *CaV1.2* shRNA cells while decreasing oxidative phosphorylation in *Tg737orp/orpk* when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Two mitochondrial mRNAs encoded oxidative phosphorylation genes (CytC and ATP5 γ 1) were measured using PCR normalized to nuclear encoded β -actin (N = 3).

Tg737orp/orpk cells showed a higher basal level of β -catenin than control. Further, Wnt3a treatment increased *CaV1.2* expression in scrambled shRNA while decreasing *CaV1.2* in *Tg737orp/orpk* cells. Of note is that *CaV1.2* expression was not detectable in *CaV1.2* shRNA cells, confirming knockdown of *CaV1.2* in our stable cell line.

The DNA damage response (DDR) is a cellular mechanism to recover from DNA lesions, such as 8-Oxoguanine (Kasai et al., 1984; Jackson and Bartek, 2009). One arm of this cell survival pathway is the activation of nuclear factor κ B p65 (NF κ B p65) (Janssens and Tschopp, 2006). Through Western blot analysis, we also found that Wnt3a induced NF κ B p65 expression in scrambled and *CaV1.2* shRNA (Fig. 5). On the other hand, *Tg737orp/orpk* cells expressed a high basal level of NF κ B p65 which decreased in response to Wnt3a. In addition, *CaV1.2* expression was found to correlate with NF κ B p65.

CaV1.2 knockdown zebrafish develop PKD phenotypes

We have shown that *CaV1.2* localizes to primary cilia and have now elucidated the mechanism by which *CaV1.2* expression is regulated in renal epithelial cells. To assess the biological significance of *CaV1.2* expression, we used antisense

morpholinos to knockdown *CaV1.2* in zebrafish. Knockdown of the ciliary calcium channel polycystin-2 in zebrafish has been reported to result in PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry (Obara et al., 2006). Our study showed that knockdown of *pkd2* increased *CaV1.2* expression (Fig. 6). This slight increase in *CaV1.2* was significant compared to the control morpholino. Interestingly, similar phenotypes were observed in *CaV1.2* morpholino (*cav1.2* MO) zebrafish. Compared with a non-specific control morpholino (control MO) injection, *cav1.2* MO zebrafish developed renal cysts (Fig. 7a), hydrocephalus (Fig. 7b) and various heart-looping defects (Fig. 7c). As generally accepted (Bakkers, 2011), left-right asymmetry was assessed by measuring the relative position of the cardiac atrium and ventricle with respect to the dorsal axis (Supplemental Movie 1).

Discussion

Non-motile primary cilia have been found to play a critical role in Wnt signaling by restricting β -catenin accumulation. Overexpression of polycystin-1 (a ciliary signaling receptor) inhibits GSK3 β and stabilizes β -catenin (Kim et al., 1999).

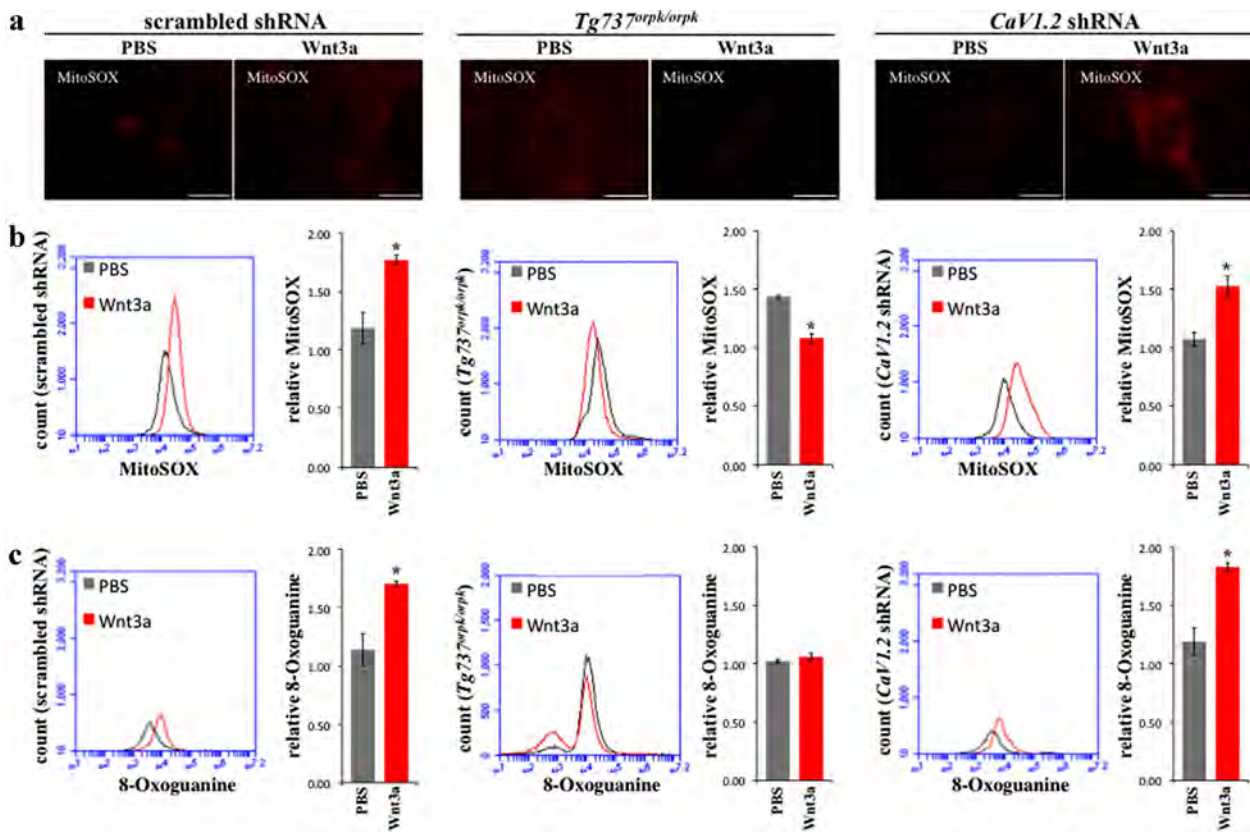


Fig. 4. Wnt3a increases ROS and DNA damage in *CaV1.2* shRNA but not in *Tg737orp/orpk* cells. **a:** Mitochondrial ROS was assessed by staining cells with MitoSOX. Wnt3a was found to increase ROS in scrambled and *CaV1.2* shRNA cells while decreasing ROS in *Tg737orp/orpk* cells when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Wnt3a increased formation of the oxidative DNA lesion 8-Oxoguanine in scrambled and *CaV1.2* shRNA cells but had no effect on *Tg737orp/orpk* cells (N = 3).

Polycystin-2 (encoded by *Pkd2*) is calcium channel forming protein found in primary cilia. In *Pkd2*^{-/-} embryos, cilia length was found to be decreased while β -catenin was upregulated (Kim et al., 2009). Interestingly, transgenic mice overexpressing β -catenin also developed cystic kidneys (Saadi-Kheddouci et al., 2001). Further, *LRP6*^{-/-} (a component of the Wnt receptor complex) mouse embryos die in utero with cystic kidneys (Pinson et al., 2000). Thus, primary cilia and Wnt signaling play a crucial role in PKD (Corbit et al., 2008).

Given that Wnt signaling also modulates mitochondrial physiology (Yoon et al., 2010), we examined the role of cilia in regards to mitochondria. *Tg737orp/orpk* contains an intron insertion at the 3' end of the intraflagellar transport 88 (*Ift88*) gene which results in a hypomorphic mutation that prevents ciliogenesis (Moyer et al., 1994). We used *Tg737orp/orpk* cells as a model for a cilia-deficient system. Through immunostaining, we confirmed the absence of cilia in *Tg737orp/orpk* cells compared with control (Fig. 1). The voltage-gated L-type calcium channel *CaV1.2* also localized to primary cilia. We generated a stable *CaV1.2* shRNA cell line and observed no changes in primary cilia compared with control. Further, treatment with Wnt3a had no effect on cilia number or length in scrambled or *CaV1.2* shRNA cells (data not shown). Thus, *CaV1.2* does not seem to play a role in ciliogenesis.

Mitochondrial biogenesis, oxidative phosphorylation, and generation of reactive oxidative species (ROS) were increased in response to Wnt3a in control renal epithelial cells

(Figures 2–4). The elevated levels of oxidative stress increased the formation of DNA lesions and the cellular DNA damage response (DDR). An interesting aspect of this response was an increase in expression of *CaV1.2* (Fig. 5). In *CaV1.2* knockdown cells, Wnt3a induced a similar effect on mitochondria and DDR. This data suggests that *CaV1.2* is a downstream effector in regard to Wnt signaling. In cilia-deficient cells, Wnt3a was unable to induce mitochondrial biogenesis and decreased mitochondrial activity, ROS production, and DDR. *CaV1.2* was found to be overexpressed in cilia-deficient cells as a compensatory mechanism; however, its expression decreased following Wnt3a treatment. Therefore, cilia length plays a role in regulating *CaV1.2* expression through modulation of Wnt signaling.

Defective primary cilia, indicated by either depletion of key ciliary proteins or fundamental changes in structure/length, results in PKD phenotypes (Wilson, 2004). Here we show that *CaV1.2* is a biologically significant ciliary protein. In the absence of *CaV1.2* in zebrafish (Fig. 6), PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry defects were observed (Fig. 7). Moreover, *CaV1.2* was found to be overexpressed in *pkd2* knockdown zebrafish. This is intriguing given that both *CaV1.2* and PC2 are calcium channel forming proteins in the primary cilium, which further suggests a role for *CaV1.2* in PKD pathogenesis. Therefore, *CaV1.2* not only localizes to renal epithelial primary cilia, but it is also required for cilia function.

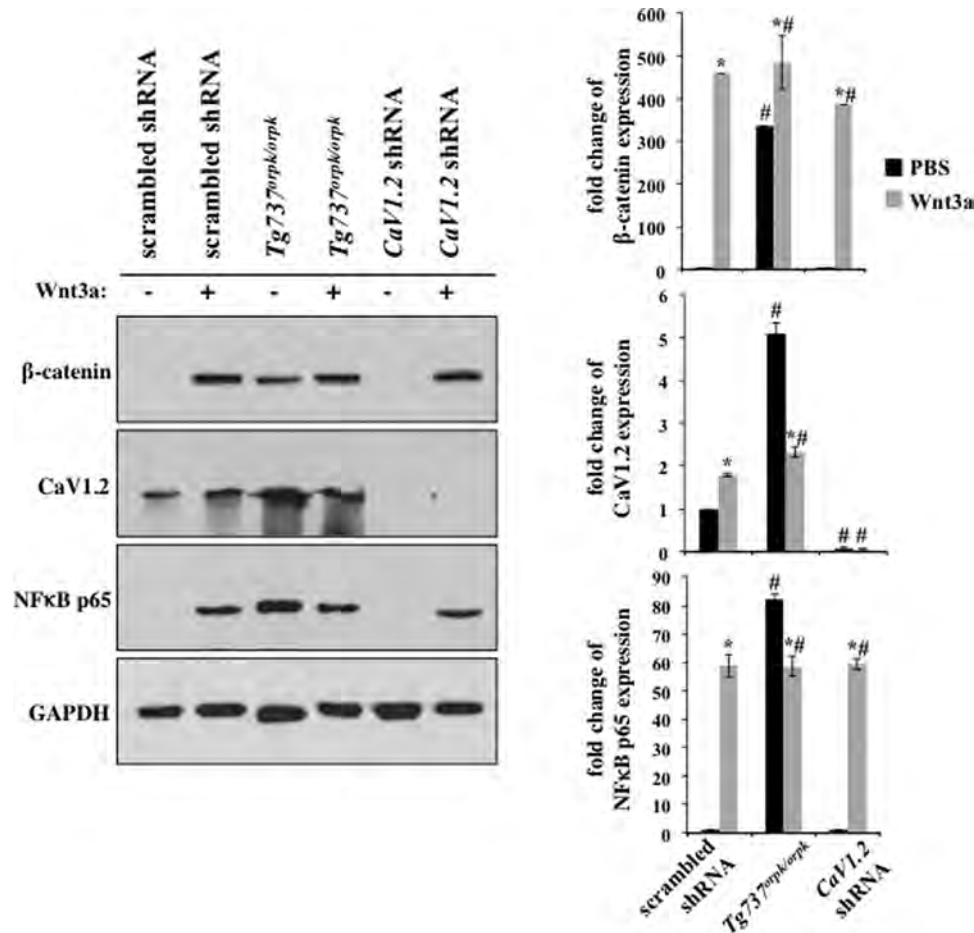


Fig. 5. Cilia modulates Wnt signaling to regulate CaV1.2 expression. Western blotting was performed on cellular protein extracts. Wnt3a treatment increased β -catenin accumulation in all cells, however, Tg737^{orp/orp} cells displayed high basal levels of β -catenin. NF- κ B p65 was blotted as a readout for DNA damage response (DDR) to ROS induced DNA lesions. Wnt3a induced NF- κ B p65 expression in scrambled and CaV1.2 shRNA cells but decreased NF- κ B p65 in Tg737^{orp/orp} cells. CaV1.2 was overexpressed in Tg737^{orp/orp} cells. Wnt3a treatment induced CaV1.2 expression in scrambled shRNA while decreasing expression in Tg737^{orp/orp} cells. Data normalized to GAPDH for analysis (N = 3). Asterisks indicate significant difference from the corresponding control group ($P < 0.05$). # signs denote significant difference from the corresponding scrambled shRNA group ($P < 0.05$).

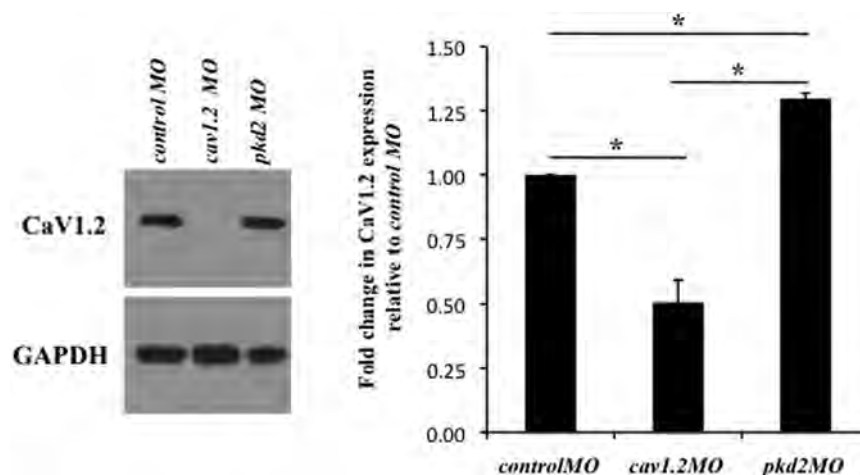


Fig. 6. pkd2 knockdown increases CaV1.2 expression in zebrafish. Embryonic zebrafish protein extracts were obtained at 28 h postfertilization. Western blotting showed that cav1.2 MO effectively reduced CaV1.2 expression compared with control MO. pkd2 MO zebrafish were used to further verify morpholino specificity. Results were quantified through one-way ANOVA with Tukey post-test. Statistical significance is reported with a mean difference at the 0.05 level and denoted with an asterisk (N = 3).

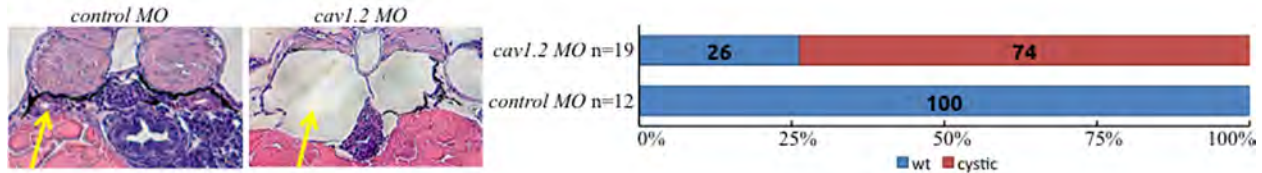
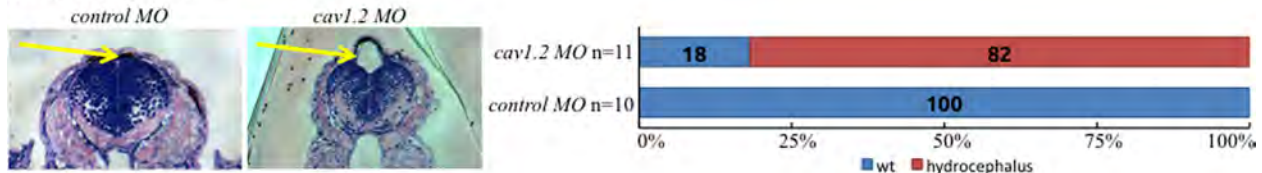
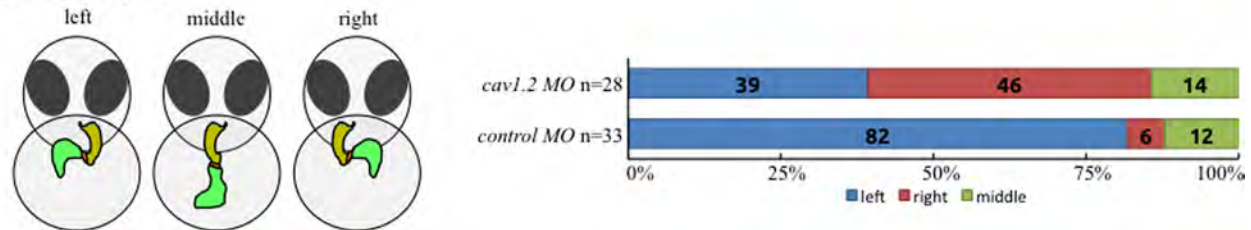
a renal cyst**b hydrocephalus****c heart looping**

Fig. 7. CaV1.2 knockdown zebrafish develop renal cysts, hydrocephalus, and left-right asymmetry defects. CaV1.2 was knocked down in zebrafish (cav1.2 MO) using morpholino microinjection and compared to a scrambled control morpholino (control MO) to assess phenotypes. Renal cyst formation (a) and hydrocephalus (b) were measured at 3 days postfertilization through standard H&E staining as indicated by arrows. Left-right asymmetry was determined by measuring heart looping (c) at 2 days postfertilization under live microscopy.

Although it has been known that abnormal Wnt signaling leads to renal cyst formation (Lancaster et al., 2009), the underlying mechanism has been unclear. One proposed explanation is that Wnt signaling regulates renal cell proliferation and planar cell polarity to maintain renal tubule homeostasis (Happe et al., 2009). Our present study further suggests that cilia regulate Wnt signaling which ultimately controls CaV1.2 expression. Therefore, in the absence of CaV1.2, ciliary function is compromised leading to formation of renal cysts. In addition, our study also shows a previously unrecognized relationship between primary cilia and mitochondrial function. Overall, we propose that Wnt3a induces β -catenin accumulation in a cilia-dependent manner. This in turn increases mitochondrial biogenesis, oxidative phosphorylation, and generation of ROS that cause genomic DNA damage. The DDR triggers NF κ B p65 expression ultimately resulting in increased CaV1.2 expression.

Acknowledgments

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Primary Cilium Regulates CaV1.2 Expression Through Wnt Signaling

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Primary cilia are sensory organelles that provide a feedback mechanism to restrict Wnt signaling in the absence of endogenous Wnt activators. Abnormal Wnt signaling has been shown to result in polycystic kidney disease (PKD) although the exact mechanism has been debated. Previously, we reported that the calcium channel CaV1.2 functions in primary cilia. In this study, we show that CaV1.2 expression level is regulated by Wnt signaling. This occurs through modulation of mitochondrial mass and activity resulting in increased reactive oxygen species which generate oxidative DNA lesions. We found that the subsequent cellular DNA damage response triggers increased CaV1.2 expression. In the absence of primary cilia where Wnt signaling is upregulated, we found that CaV1.2 is overexpressed as a compensatory mechanism. We show for the first time that CaV1.2 knockdown in zebrafish results in classic primary cilia defects including renal cyst formation, hydrocephalus, and left-right asymmetry defects. Our study shows that suppressed Wnt signaling prevents CaV1.2 expression ultimately resulting in PKD phenotypes. Thus, CaV1.2 expression is tightly regulated through Wnt signaling and plays an essential sensory role in primary cilia necessary for cellular homeostasis.

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Wnt signaling is an important regulator of cellular development and proliferation. In the absence of Wnt ligands, a complex consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 β (GSK3 β) induces β -catenin for ubiquitylation by SCF E3 ligases and eventual proteasomal degradation (Aberle et al., 1997). Wnt signal transduction occurs when secreted Wnt ligands bind Frizzled receptors resulting in phosphorylation of LRP5/6. The Axin-APC-GSK3 β complex is then recruited to LRP5/6 at the cell membrane which prevents β -catenin from being degraded. The accumulated β -catenin translocates to the nucleus and activates transcription of Wnt target genes (Muntean et al., 2012).

Primary cilia are non-motile sensory organelles present as a single copy on most differentiated cells in the body. Calcium signaling through primary cilium is essential for renal epithelial homeostasis (Nauli et al., 2003; Jin et al., 2013). Cilia extend from the cell surface through the basal body via intraflagellar transport (Moyer et al., 1994). The most common pathologies resulting from cilia dysfunction include polycystic kidney (Wilson, 2004), hypertension (Nauli et al., 2008; AbouAlaiwi et al., 2009), aneurysm (AbouAlaiwi et al., 2013), hydrocephalus (Carter et al., 2012), and left-right asymmetry defects (Norris, 2012).

Abnormal Wnt signaling has also been linked to polycystic kidney disease (PKD) (Lancaster et al., 2009). For example, increased cytosolic and nuclear β -catenin accumulation has been shown in various cilia mutant cells (Gerdes et al., 2007; Lancaster et al., 2011). Thus, primary cilia are thought to provide a feedback mechanism that restricts Wnt signaling in the absence of appropriate ligands (Gerdes et al., 2007; Lancaster et al., 2009, 2011).

We recently showed that voltage-gated L-type calcium channel CaV1.2 localized to primary cilia in renal epithelia (Jin et al., 2013). Because Wnt signaling has also been reported to modulate mitochondrial physiology (Yoon et al., 2010), we hypothesized that primary cilia play a role in Wnt regulation of mitochondria through CaV1.2. We show that although CaV1.2 is not required for cilia formation, Wnt increases mitochondria mass and activity in CaV1.2 deficient renal epithelial cells. This increases mitochondria reactive oxidative species (ROS) and DNA damage, resulting in PKD phenotypes. Thus, our study suggests that primary cilia may play a role in CaV1.2 expression level through Wnt regulation of mitochondria.

Materials and Methods

The experimental use of zebrafish was approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). The use of lentiviral components was approved by the Institutional Biosafety Committee of The University of Toledo.

Cell culture

Immortalized mouse renal epithelial wild-type and *Tg737^{orp/k}* cells were cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin/streptomycin (Corning Cellgro) at 39°C in 5% CO₂, as previously described (AbouAlaiwi et al., 2013). Prior to experiments, cells were treated with 100 ng/ml recombinant Wnt3a (R&D Systems, Minneapolis, MN) for 3 days and serum starved for 24 h.

RNAi knockdown cells

shRNA lentiviral vectors specific to *Cacna1c* (Origene; pGFP-C-shLenti clone ID: TL500242) were transfected into HEK293T cells. Viral supernatants were collected after 48 h, centrifuged, and passed through a 0.45 μ m filter. Cells were then spin-inoculated with pseudoviral particles containing 8 μ g/ml polybrene at 2,500 rpm for 30 min at 30°C and then cultured for up to 1 week. CaV1.2 knockdown was verified through Western blot analysis.

Brian S. Muntean and Xingjian Jin contributed equally to this work.

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TABLE 1. shRNA sequences

Descriptions	Sequences
Scrambled control	5'-TGACCACCCTGACCTACGGCGTGCAGTGC-3'
<i>Cacna1c</i>	5'-TCAGAGTGCCTCACTGTTCTCGTGACCT-3'

Stable knockdown cell lines were obtained through puromycin selection. The following shRNA sequences were used (Table 1).

Immunostaining studies

Cells were grown to confluence on collagen-coated glass coverslips and differentiated in serum-free media for 24 h. Cells were then fixed in 4% paraformaldehyde in PBS containing 2% sucrose, permeabilized in 10% triton X-100, incubated sequentially with primary followed by secondary antibodies for 1 h each, and finally mounted on a slide with DAPI hard set mounting media (Vector Laboratories, Burlingame, CA). The following primary antibody dilutions were used: acetylated- α -tubulin 1:10,000 (Sigma-Aldrich, St. Louis, MO) and CaV1.2 1:50 (Alomone Labs, Jerusalem, Israel). Anti-mouse Texas Red and anti-rabbit FITC fluorescent conjugated secondary antibodies were used at 1:500 (VectorLabs).

Mitochondrial studies

MitoTracker Green FM and MitoTracker Red CMXRos (Cell Signaling Technology) were incubated with cells at 100 nM for 30 min at 37°C. MitoSOX (Life Technologies) was incubated with cells at 5 μ M for 10 min at 37°C. After staining, cells were washed three times with PBS and analyzed immediately through microscopy or flow cytometry. For microscopic analysis, cells were grown on custom glass-bottom cell culture plates and imaged under a Nikon Eclipse TE2000-U microscope controlled by MetaMorph software with a 100 $\times$ objective lens. For flow cytometry studies, cells were detached with trypsin, washed, and analyzed.

DNA damage assessment

Oxidative DNA lesions were detected with an 8-oxoguanine antibody (Santa Cruz). Detached cells were fixed in 4% formaldehyde for 10 min at 37°C and permeabilized in ice-cold 90% methanol for 30 min on ice. After washing with PBS, cells were incubated in PBS containing anti-8-oxoguanine antibody (1:50), 0.5% Tween-20, and 5% FBS for 1 h. Cells were washed and incubated in PBS containing anti-mouse Texas Red antibody (1:500), 0.5% Tween-20, and 5% FBS for 1 h. Cells were then washed and analyzed with flow cytometer.

Mitochondrial DNA and mRNA measurement

Total cellular DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen) and used for detection with PCR primers listed below to quantify the nuclear (*18S rRNA*) to mitochondrial DNA (*CoI*) ratio as described (Brown and Clayton, 2002; Bai et al., 2004). Total cellular RNA was obtained using TRIzol (Life Technologies) and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR detection of expression genes was performed using the primers listed below comparing mitochondrial encoded oxidative phosphorylation genes (*ATP5 γ* and *CytC*) to nuclear encoded β -Actin as described (Yoon et al., 2010) (Table 2).

Western blot analysis

Cells were scraped from culture plates in the presence of RIPA buffer supplemented with Complete Protease Inhibitor (Roche,

TABLE 2. Primer sequences

Descriptions	Sequences
<i>CoI</i> F	5'-GCCCCAGATATAGCATTCCC-3'
<i>CoI</i> R	5'-GTTTCATCTGTTCTCTGCTCC-3'
<i>18S rRNA</i> F	5'-TAGAGGGACAAGTGGCGTTC-3'
<i>18S rRNA</i> R	5'-CGCTGAGCCAGTCAAGTGT-3'
<i>ATP5γ</i> F	5'-AGTTGGTGTGGCTGGATCA-3'
<i>ATP5γ</i> R	5'-GCTGCTTGAGAGATGGGTTC-3'
<i>CytC</i> F	5'-GGAGGCAAGCATAAGACTGG-3'
<i>CytC</i> R	5'-TCCATCAGGGTATCCTCTCC-3'
β -actin F	5'-TGTACCAACTGGGACGACA-3'
β -actin R	5'-GGGGTGTGAAGGTCTCAA-3'

New York, NY), incubated on ice with frequent vortexing, and centrifuged. Supernatants were subjected to protein quantification and PAGE on 6–10% SDS gels followed by wet transfer to PVDF membranes and detection using β -catenin 1:1,000, CaV1.2 1:200, NF- κ B p65 1:200, and GAPDH 1:1,000 (Cell Signaling Technology, Danvers, MA).

Zebrafish

Adult wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) and used for breeding. Embryos were injected with 1 mM antisense translation blocking morpholino oligos (MO; GeneTools) at the 1–2 cell stage. Zebrafish embryos were then cultured at 28.5°C in sterile egg water (Muntean et al., 2010). The following MO sequences were used: *control* MO: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3', *cav1.2* MO: 5'-ACA TGT TTT TGC TTT CAT TTA CCA T-3', *pkd2* MO: 5'-AGG ACG AAC GCG ACT GGA GCT CAT C-3'. Knockdown of CaV1.2 was verified through Western blot analysis. Briefly, zebrafish embryos were dechorionated at 28 h postfertilization and homogenized in RIPA buffer to obtain protein extracts. Western was performed on 50 μ g total protein using CaV1.2 (1:200) and GAPDH (1:1,000) antibodies.

Histological examination was used to measure renal cyst formation and hydrocephalus at 3 days postfertilization. Embryos were fixed in a PBS solution containing 4% paraformaldehyde and 2% sucrose overnight at 4°C, dehydrated through an ethanol gradient, and embedded in JB4 resin (Polysciences, Inc., Warrington, PA) as specified in manufacturer's protocol. A Reichert Jung microtome was used to cut 5 μ m sections which were subsequently hematoxylin and eosin stained. Heart looping was assessed at 48 h postfertilization by positioning zebrafish on their dorsal axis and recording heart beat to reveal the respective relative locations of the atrium and the ventricle.

Data analysis

Data are reported as the mean $\pm$ standard error of the mean. All image analysis was performed using ImageJ. All flow cytometry data were analyzed with BD Accuri C6 software and were presented without any compensation gating. All data were analyzed using IBM SPSS Statistics Version 21 software by performing the student t-test for two group comparison or ANOVA test followed by Tukey's post-test for three or more group comparison. Statistical significance is reported with a statistical power greater than 0.8 at $P < 0.05$.

Results

CaV1.2 is not required for primary cilia assembly

We recently reported that the voltage gated L-type calcium channel CaV1.2 localized to primary cilia in bovine LLC PK cells (Jin et al., 2013). We performed immunostaining to verify this finding in mouse renal epithelial cells (Fig. 1). The mouse *Tg737^{orp/orpk}* cell line contains a hypomorphic mutation in an intraflagellar transport gene (*Ift88*) that is required for cells

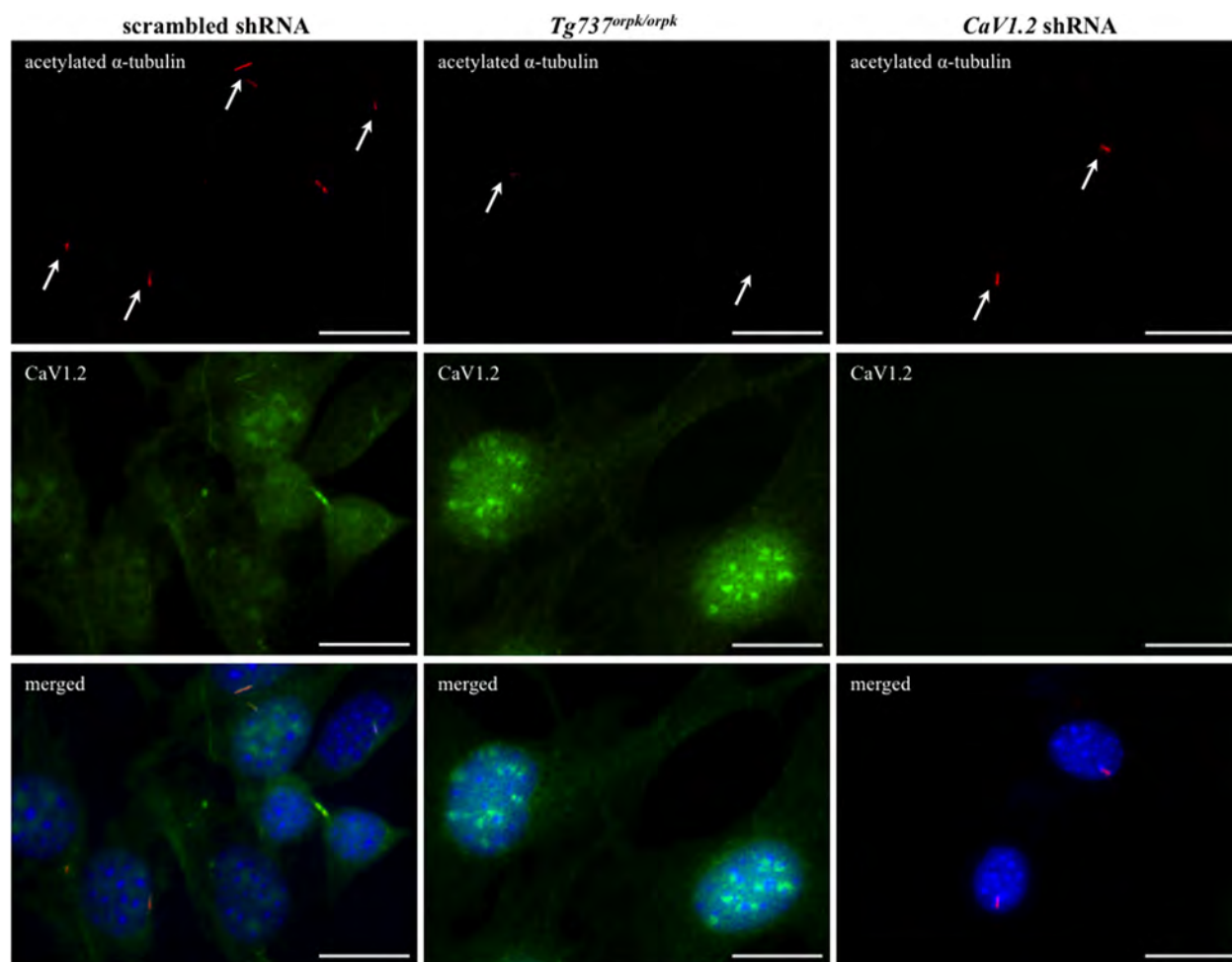


Fig. 1. Localization of CaV1.2 to renal epithelial cilia is not required for primary cilia assembly. Immunofluorescence revealed that CaV1.2 localized to primary cilia in renal epithelial cells (scrambled shRNA) when compared with cilia-deficient cells (*Tg737^{orp/ork}*). The presence of primary cilia was confirmed in CaV1.2 shRNA cells. Acetylated- α -tubulin was used as a ciliary marker. Arrow indicates the presence of primary cilium, except in cilia-deficient cells. Bar = 20 μ m.

to assemble primary cilia (Moyer et al., 1994). Thus, the *Tg737^{orp/ork}* system is a well-established model for studying cells without longer primary cilia, as verified through our immunostaining. We next asked if CaV1.2 played a role in primary cilia assembly. We generated a stable CaV1.2 shRNA knockdown mouse renal epithelial cell line and immunostaining studies revealed that primary cilia were similar to that of scrambled shRNA.

Wnt3a induces mitochondrial biogenesis in CaV1.2-deficient but not cilia-deficient cells

Wnt signaling has recently been reported to regulate mitochondrial physiology (Yoon et al., 2010). To assess mitochondrial mass, cells were stained with Mito Tracker Green (MTG) and observed live using fluorescence microscopy. When treated with recombinant Wnt3a, mitochondrial mass increased (Fig. 2a). However, the mitochondrial mass in *Tg737^{orp/ork}* cells was unchanged after Wnt3a treatment. We next performed this experiment in CaV1.2 shRNA cells and the results were similar to that of the scrambled control. To

quantify these findings, MTG fluorescence was recorded using flow cytometry which confirmed our fluorescent observation (Fig. 2b). Our MTG studies were further validated using a common technique by comparing mitochondrial DNA (*Coi*) to nuclear DNA (*18S rRNA*) (Brown and Clayton, 2002; Bai et al., 2004). As expected, Wnt3a did indeed statistically increase mitochondrial biogenesis in scrambled and CaV1.2 shRNA but not in *Tg737^{orp/ork}* cells (Fig. 2c). Our immunofluorescence study showed that Wnt3a did not alter CaV1.2 localization to cilia (Table 3).

Wnt3a increases mitochondrial activity in CaV1.2-deficient cells while decreasing activity in cilia-deficient cells

We next asked if Wnt3a would have an effect on mitochondrial oxidative phosphorylation (activity) in *Tg737^{orp/ork}* cells. Similar to before, we stained cells with Mito Tracker Red (MTR). Unlike MTG, MTR staining is dependent on the mitochondrial membrane potential (Poot and Pierce, 2001; Pendergrass et al., 2004). Therefore, increased staining

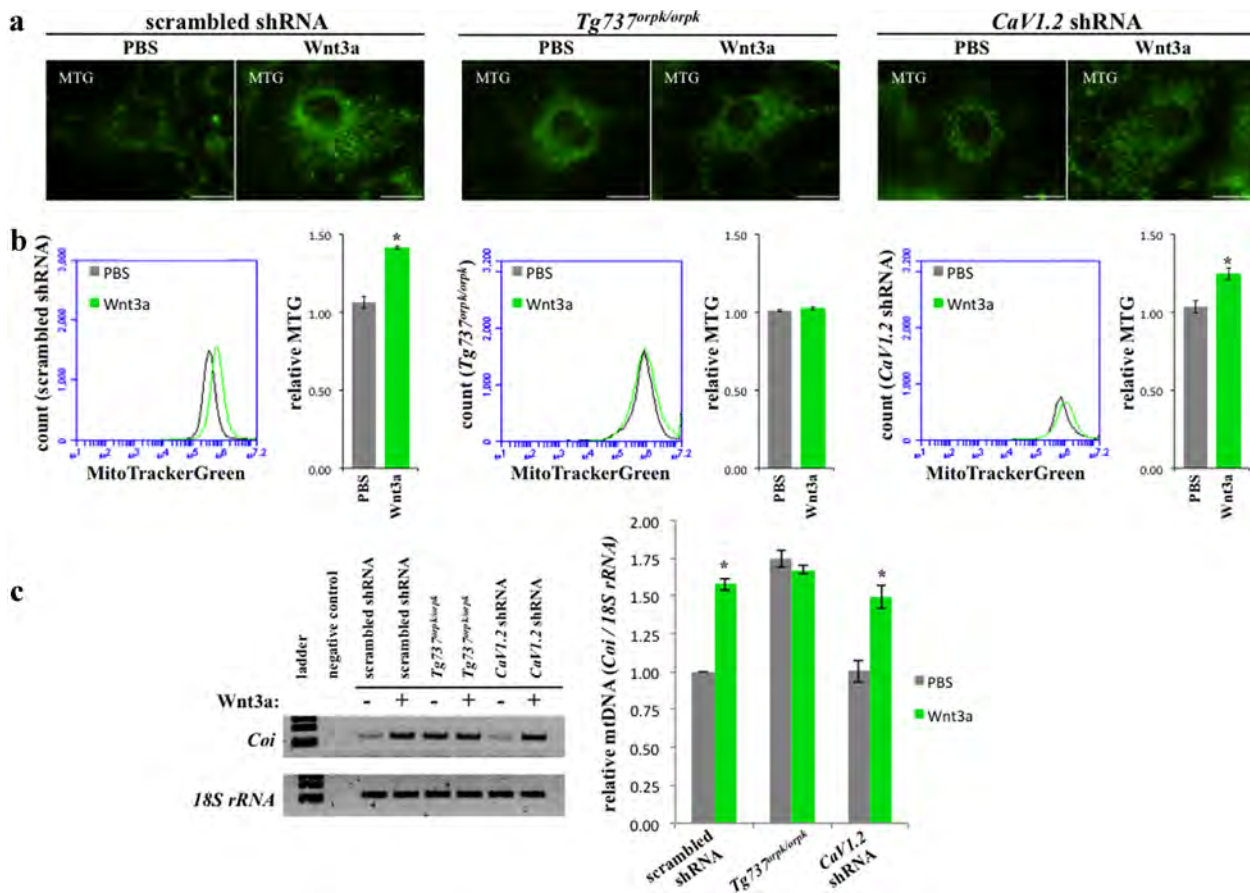


Fig. 2. Wnt3a induces mitochondrial biogenesis in *CaVI.2* shRNA but not *Tg737^{orp/orp}* cells. **a:** Mitochondrial mass was assessed by staining cells with Mito Tracker Green. Wnt3a was found to induce mitochondrial mass in scrambled and *CaVI.2* shRNA cells but had no effect on *Tg737^{orp/orp}* when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Mitochondrial DNA was measured using PCR by taking the ratio of a mitochondrial gene (*Coi*) to a nuclear gene (18S rRNA) (N = 3).

correlates to increased oxidative phosphorylation. As expected, Wnt3a increased MTR staining in scrambled and *CaVI.2* shRNA cells when observed using fluorescence microscopy (Fig. 3a). However, mitochondrial activity decreased in *Tg737^{orp/orp}* cells. We again quantified our findings using flow cytometry (Fig. 3b). Wnt3a significantly increased mitochondrial activity in scrambled and *CaVI.2* shRNA while significantly decreasing activity in *Tg737^{orp/orp}* cells. To verify these results, we compared expression of two key mitochondrial encoded oxidative phosphorylation genes (ATP Synthase 5 γ I and Cytochrome c) relative to that of nuclear encoded β -actin (Fig. 3c).

TABLE 3. *CaVI.2* ciliary localization

	% <i>CaVI.2</i> localization to cilia	N
PBS (control)		
Scramble shRNA	91.1	45
<i>Tg737^{orp/orp}</i>	91.7	36
<i>CaVI.2</i> shRNA	0.0	41
Wnt3a (100 ng/ml)		
Scramble shRNA	90.4	52
<i>Tg737^{orp/orp}</i>	92.3	39
<i>CaVI.2</i> shRNA	0.0	46

Wnt3a increases ROS and DNA damage in *CaVI.2*-deficient but not in cilia-deficient cells

An inevitable consequence of oxidative phosphorylation is the generation of reactive oxygen species (ROS) (Boveris et al., 1972; Boveris and Chance, 1973). MitoSOX is a cell permeable red fluorescent indicator specific for mitochondrial ROS. We therefore stained cells with MitoSOX and observed a significant increase in mitochondrial ROS in scrambled and *CaVI.2* shRNA after treatment with Wnt3a (Fig. 4a). A significant decrease in staining was observed in *Tg737^{orp/orp}* cells (Fig. 4b). Genomic DNA can be damaged by ROS to form DNA lesions resulting from mismatched repairs (Kasai et al., 1984). Thus, we quantified the levels of 8-Oxoguanine, a common DNA lesion formed by mismatched Adenine (Kasai, 1997). Treatment with Wnt3a was found to increase 8-Oxoguanine in scrambled and *CaVI.2* shRNA while no change was observed in *Tg737^{orp/orp}* cells (Fig. 4c).

Cilia modulates Wnt signaling to regulate *CaVI.2* expression

As previously reported, Wnt3a treatment induced β -catenin expression in all cells (Aberle et al., 1997). We confirmed this in our system, including in *Tg737^{orp/orp}* and *CaVI.2* shRNA cells (Fig. 5). Consistent with previous study (Corbit et al., 2008),

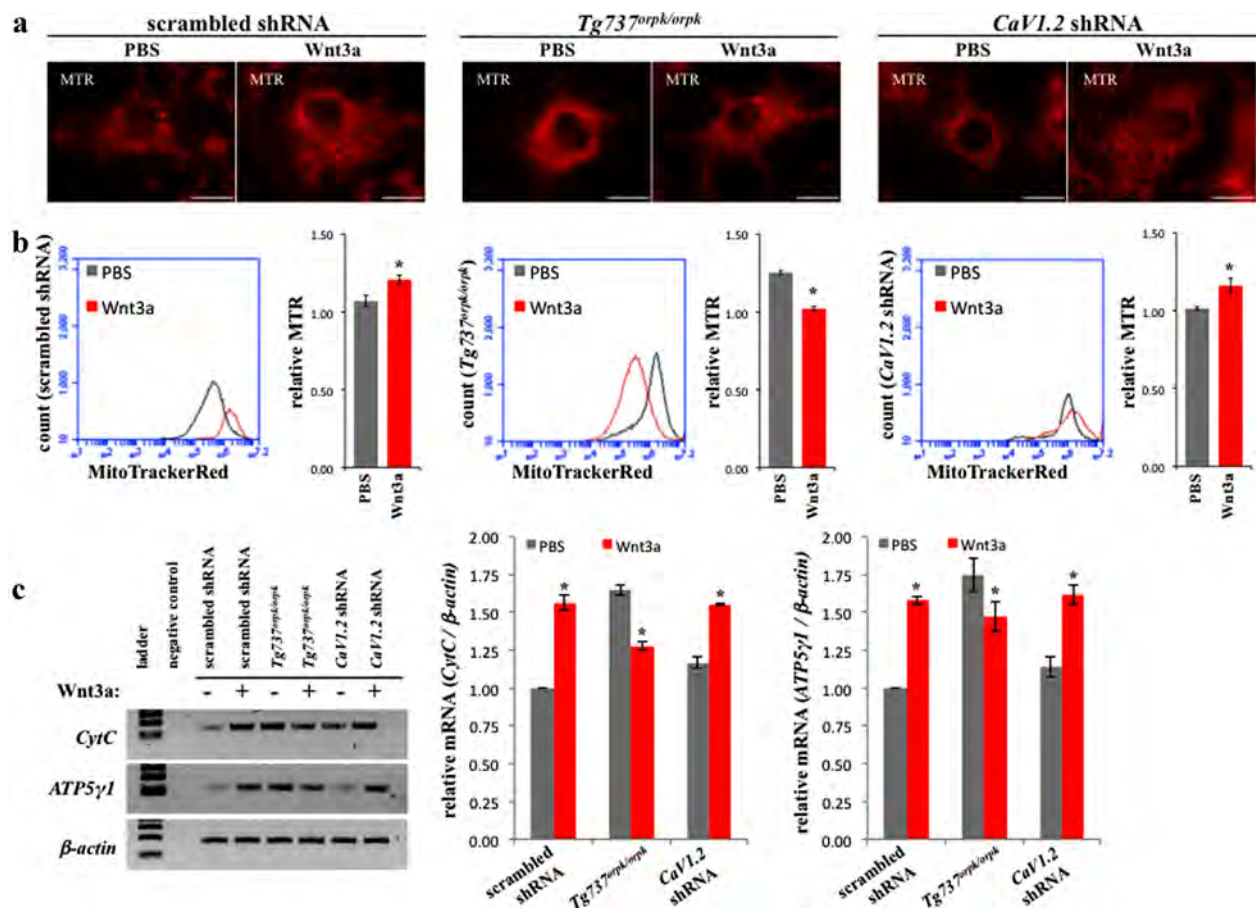


Fig. 3. Wnt3a increases mitochondrial activity in *CaV1.2* shRNA but decreasing activity in *Tg737orp/orpk* cells. **a:** Mitochondrial oxidative phosphorylation was used to indicate activity through Mito Tracker Red staining. Wnt3a was found to increase oxidative phosphorylation in scrambled and *CaV1.2* shRNA cells while decreasing oxidative phosphorylation in *Tg737orp/orpk* when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Two mitochondrial mRNAs encoded oxidative phosphorylation genes (CytC and ATP5 γ 1) were measured using PCR normalized to nuclear encoded β -actin (N = 3).

Tg737orp/orpk cells showed a higher basal level of β -catenin than control. Further, Wnt3a treatment increased *CaV1.2* expression in scrambled shRNA while decreasing *CaV1.2* in *Tg737orp/orpk* cells. Of note is that *CaV1.2* expression was not detectable in *CaV1.2* shRNA cells, confirming knockdown of *CaV1.2* in our stable cell line.

The DNA damage response (DDR) is a cellular mechanism to recover from DNA lesions, such as 8-Oxoguanine (Kasai et al., 1984; Jackson and Bartek, 2009). One arm of this cell survival pathway is the activation of nuclear factor κ B p65 (NF κ B p65) (Janssens and Tschoop, 2006). Through Western blot analysis, we also found that Wnt3a induced NF κ B p65 expression in scrambled and *CaV1.2* shRNA (Fig. 5). On the other hand, *Tg737orp/orpk* cells expressed a high basal level of NF κ B p65 which decreased in response to Wnt3a. In addition, *CaV1.2* expression was found to correlate with NF κ B p65.

CaV1.2 knockdown zebrafish develop PKD phenotypes

We have shown that *CaV1.2* localizes to primary cilia and have now elucidated the mechanism by which *CaV1.2* expression is regulated in renal epithelial cells. To assess the biological significance of *CaV1.2* expression, we used antisense

morpholinos to knockdown *CaV1.2* in zebrafish. Knockdown of the ciliary calcium channel polycystin-2 in zebrafish has been reported to result in PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry (Obara et al., 2006). Our study showed that knockdown of *pkd2* increased *CaV1.2* expression (Fig. 6). This slight increase in *CaV1.2* was significant compared to the control morpholino. Interestingly, similar phenotypes were observed in *CaV1.2* morpholino (*cav1.2* MO) zebrafish. Compared with a non-specific control morpholino (control MO) injection, *cav1.2* MO zebrafish developed renal cysts (Fig. 7a), hydrocephalus (Fig. 7b) and various heart-looping defects (Fig. 7c). As generally accepted (Bakkers, 2011), left-right asymmetry was assessed by measuring the relative position of the cardiac atrium and ventricle with respect to the dorsal axis (Supplemental Movie 1).

Discussion

Non-motile primary cilia have been found to play a critical role in Wnt signaling by restricting β -catenin accumulation. Overexpression of polycystin-1 (a ciliary signaling receptor) inhibits GSK3 β and stabilizes β -catenin (Kim et al., 1999).

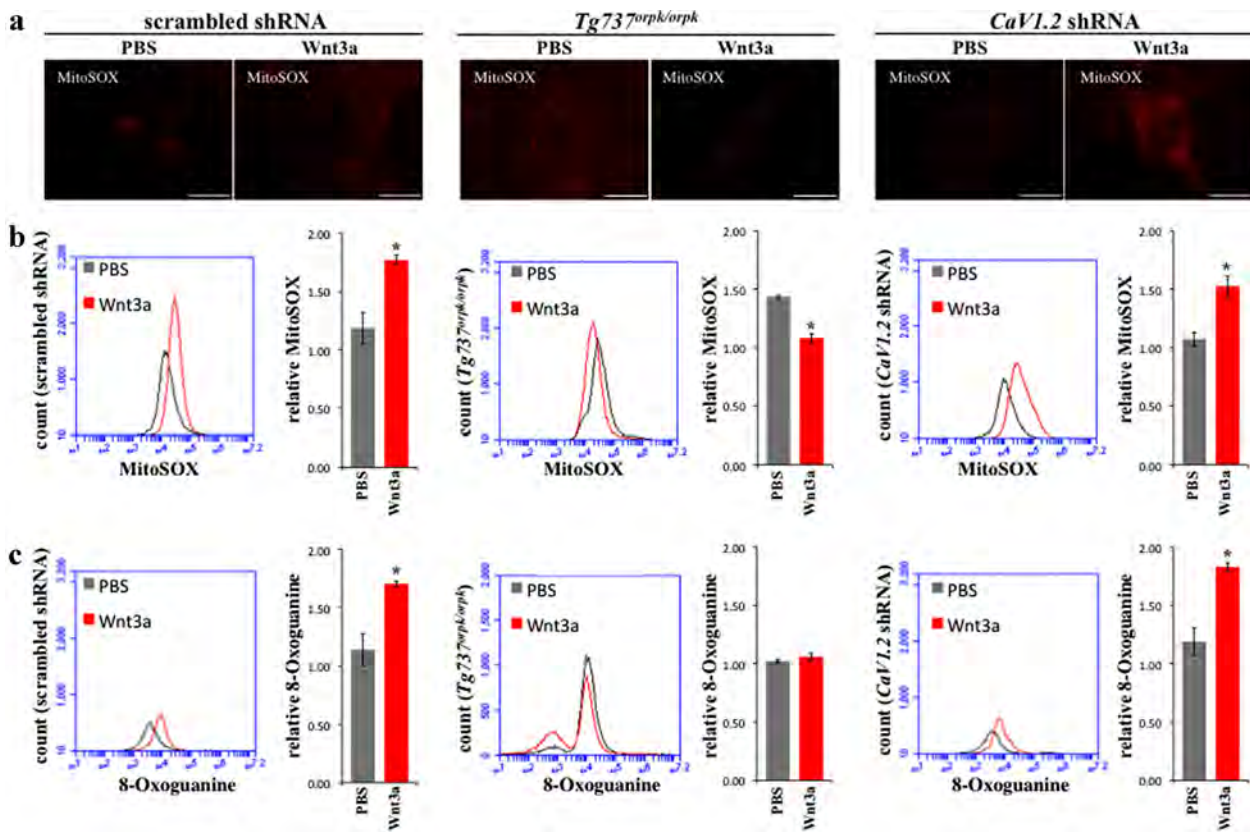


Fig. 4. Wnt3a increases ROS and DNA damage in *CaV1.2* shRNA but not in *Tg737orp/orpk* cells. **a:** Mitochondrial ROS was assessed by staining cells with MitoSOX. Wnt3a was found to increase ROS in scrambled and *CaV1.2* shRNA cells while decreasing ROS in *Tg737orp/orpk* cells when examined using fluorescence microscopy (bar = 20 μ m). **b:** Results were quantified using flow cytometry. **c:** Wnt3a increased formation of the oxidative DNA lesion 8-Oxoguanine in scrambled and *CaV1.2* shRNA cells but had no effect on *Tg737orp/orpk* cells (N = 3).

Polycystin-2 (encoded by *Pkd2*) is calcium channel forming protein found in primary cilia. In *Pkd2*^{-/-} embryos, cilia length was found to be decreased while β -catenin was upregulated (Kim et al., 2009). Interestingly, transgenic mice overexpressing β -catenin also developed cystic kidneys (Saadi-Kheddouci et al., 2001). Further, *LRP6*^{-/-} (a component of the Wnt receptor complex) mouse embryos die in utero with cystic kidneys (Pinson et al., 2000). Thus, primary cilia and Wnt signaling play a crucial role in PKD (Corbit et al., 2008).

Given that Wnt signaling also modulates mitochondrial physiology (Yoon et al., 2010), we examined the role of cilia in regards to mitochondria. *Tg737orp/orpk* contains an intron insertion at the 3' end of the intraflagellar transport 88 (*Ift88*) gene which results in a hypomorphic mutation that prevents ciliogenesis (Moyer et al., 1994). We used *Tg737orp/orpk* cells as a model for a cilia-deficient system. Through immunostaining, we confirmed the absence of cilia in *Tg737orp/orpk* cells compared with control (Fig. 1). The voltage-gated L-type calcium channel *CaV1.2* also localized to primary cilia. We generated a stable *CaV1.2* shRNA cell line and observed no changes in primary cilia compared with control. Further, treatment with Wnt3a had no effect on cilia number or length in scrambled or *CaV1.2* shRNA cells (data not shown). Thus, *CaV1.2* does not seem to play a role in ciliogenesis.

Mitochondrial biogenesis, oxidative phosphorylation, and generation of reactive oxidative species (ROS) were increased in response to Wnt3a in control renal epithelial cells

(Figures 2–4). The elevated levels of oxidative stress increased the formation of DNA lesions and the cellular DNA damage response (DDR). An interesting aspect of this response was an increase in expression of *CaV1.2* (Fig. 5). In *CaV1.2* knockdown cells, Wnt3a induced a similar effect on mitochondria and DDR. This data suggests that *CaV1.2* is a downstream effector in regard to Wnt signaling. In cilia-deficient cells, Wnt3a was unable to induce mitochondrial biogenesis and decreased mitochondrial activity, ROS production, and DDR. *CaV1.2* was found to be overexpressed in cilia-deficient cells as a compensatory mechanism; however, its expression decreased following Wnt3a treatment. Therefore, cilia length plays a role in regulating *CaV1.2* expression through modulation of Wnt signaling.

Defective primary cilia, indicated by either depletion of key ciliary proteins or fundamental changes in structure/length, results in PKD phenotypes (Wilson, 2004). Here we show that *CaV1.2* is a biologically significant ciliary protein. In the absence of *CaV1.2* in zebrafish (Fig. 6), PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry defects were observed (Fig. 7). Moreover, *CaV1.2* was found to be overexpressed in *pkd2* knockdown zebrafish. This is intriguing given that both *CaV1.2* and PC2 are calcium channel forming proteins in the primary cilium, which further suggests a role for *CaV1.2* in PKD pathogenesis. Therefore, *CaV1.2* not only localizes to renal epithelial primary cilia, but it is also required for cilia function.

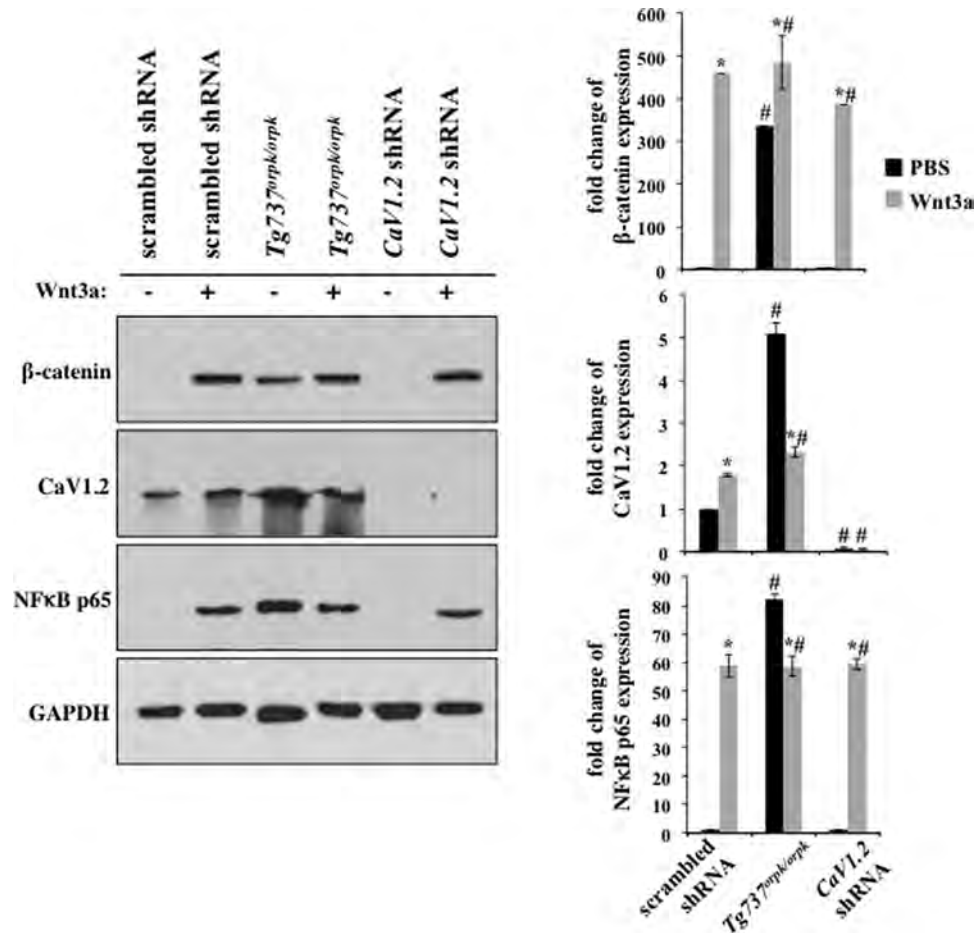


Fig. 5. Cilia modulates Wnt signaling to regulate CaV1.2 expression. Western blotting was performed on cellular protein extracts. Wnt3a treatment increased β-catenin accumulation in all cells, however, Tg737^{orp/orp} cells displayed high basal levels of β-catenin. NF-κB p65 was blotted as a readout for DNA damage response (DDR) to ROS induced DNA lesions. Wnt3a induced NF-κB p65 expression in scrambled and CaV1.2 shRNA cells but decreased NF-κB p65 in Tg737^{orp/orp} cells. CaV1.2 was overexpressed in Tg737^{orp/orp} cells. Wnt3a treatment induced CaV1.2 expression in scrambled shRNA while decreasing expression in Tg737^{orp/orp} cells. Data normalized to GAPDH for analysis (N = 3). Asterisks indicate significant difference from the corresponding control group ($P < 0.05$). # signs denote significant difference from the corresponding scrambled shRNA group ($P < 0.05$).

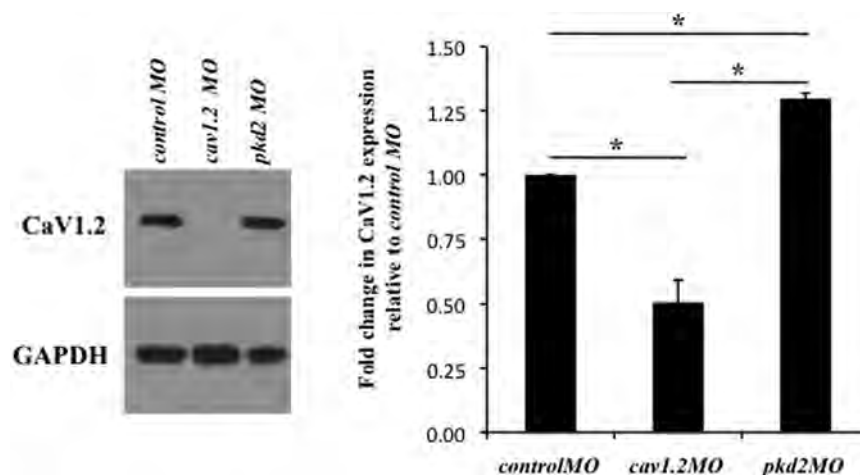


Fig. 6. pkd2 knockdown increases CaV1.2 expression in zebrafish. Embryonic zebrafish protein extracts were obtained at 28 h postfertilization. Western blotting showed that cav1.2 MO effectively reduced CaV1.2 expression compared with control MO. pkd2 MO zebrafish were used to further verify morpholino specificity. Results were quantified through one-way ANOVA with Tukey post-test. Statistical significance is reported with a mean difference at the 0.05 level and denoted with an asterisk (N = 3).

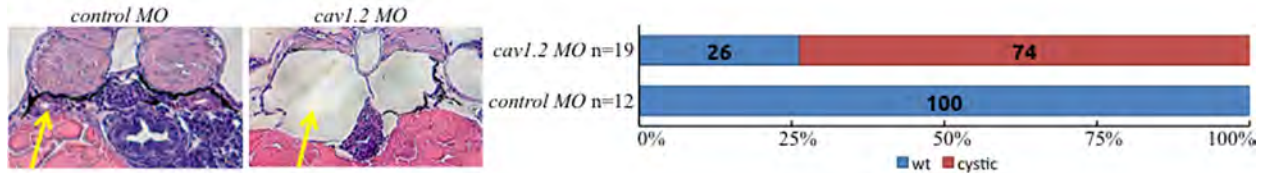
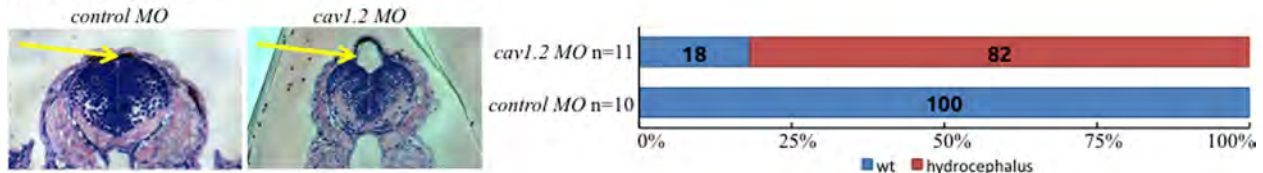
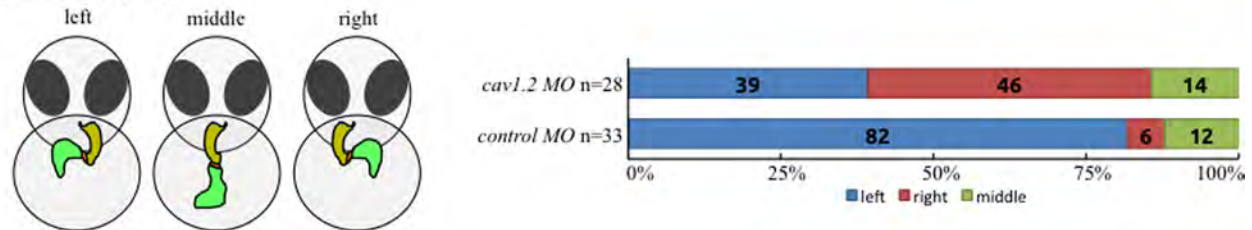
a renal cyst**b hydrocephalus****c heart looping**

Fig. 7. CaV1.2 knockdown zebrafish develop renal cysts, hydrocephalus, and left-right asymmetry defects. CaV1.2 was knocked down in zebrafish (cav1.2 MO) using morpholino microinjection and compared to a scrambled control morpholino (control MO) to assess phenotypes. Renal cyst formation (a) and hydrocephalus (b) were measured at 3 days postfertilization through standard H&E staining as indicated by arrows. Left-right asymmetry was determined by measuring heart looping (c) at 2 days postfertilization under live microscopy.

Although it has been known that abnormal Wnt signaling leads to renal cyst formation (Lancaster et al., 2009), the underlying mechanism has been unclear. One proposed explanation is that Wnt signaling regulates renal cell proliferation and planar cell polarity to maintain renal tubule homeostasis (Happe et al., 2009). Our present study further suggests that cilia regulate Wnt signaling which ultimately controls CaV1.2 expression. Therefore, in the absence of CaV1.2, ciliary function is compromised leading to formation of renal cysts. In addition, our study also shows a previously unrecognized relationship between primary cilia and mitochondrial function. Overall, we propose that Wnt3a induces β -catenin accumulation in a cilia-dependent manner. This in turn increases mitochondrial biogenesis, oxidative phosphorylation, and generation of ROS that cause genomic DNA damage. The DDR triggers NF κ B p65 expression ultimately resulting in increased CaV1.2 expression.

Acknowledgments

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension

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Abstract: Primary cilia are sensory organelles that extend from the cell surface and sense extracellular signals. Endothelial primary cilia protruding from the inner surface of blood vessel walls sense changes in blood flow and convert this mechanosensation into an intracellular biochemical/molecular signal, which triggers a cellular response. Primary endothelial cilia dysfunction may contribute to the impairment of this response and thus be directly implicated in the development of vascular abnormalities such as hypertension and aneurysms. Using both *in vitro* techniques as well as *in vivo* animal models, we and others have investigated fluid flow mechanosensory functions of endothelial cilia in cultured cells, animal models and autosomal dominant polycystic kidney disease (ADPKD) patients. More in-depth studies directed at identification of the mechanisms of fluid flow sensing will further enhance our knowledge of cilia-dependent vascular pathology. Although the current treatments aimed at treating the cardiovascular symptoms in ADPKD patients successfully slowed the progression of cyst growth, there is growing evidence which suggests that drugs which interfere with primary cilia function or structure could reduce cardiovascular complications in ADPKD. This review is to summarize the most recent studies on primary endothelial cilia function in the vascular system and to present primary cilia as a novel therapeutic target for vascular hypertension.

Keywords: Cardiovascular, cell division, fluid-shear, hypertension, primary cilia.

INTRODUCTION

It has been over a century since primary cilia have been visualized, but the study of their sensory role is, comparatively, a new field [1]. Like other cellular organelles i.e. nucleus, Golgi bodies, mitochondria etc. cilia can be considered as a separate entity and have the following specialized functions:

- Mechanosensation [2-4].
- Shear-stress sensation [5-7].
- Nodal flow generation [8-10].
- Nodal flow sensation [11, 12].
- Osmosensation [13-15].
- Chemosensation [16-19].
- Light sensation [20-22].
- Smell sensation [23, 24].
- Gravitational sensation [25].
- Developmental regulation [26-28].
- Fluid clearance [29, 30].
- Sperm motility [31-33].
- Fluid transportation [34-36].
- Oocyte transportation [37, 38].
- Sound-wave sensation [39-41].

In order to study a cilium, it must be divided into five distinct structural blocks – the ciliary membrane, the soluble compartment, the axoneme, the ciliary tip and the basal body complex (Fig. 1a). The primary ciliary membrane has a distinct lipid bilayer composition and is continuous with the cell membrane at the transition fiber. It houses various membrane mechanosensory receptors, ligand-activated chemoreceptors and ion channels to support mechanosensation, chemosensation and ion influx in response to extracellular stimuli. The soluble or matrix compartment also called the cilioplasm is composed of fluid materials to support signaling activities. The axoneme, which emanates from the basal body, is composed of nine pairs of microtubules forming heterodimer structure. Apart from delivering cellular components in and out of the ciliary shaft, it plays a significant role in maintaining a long ciliary structure. The ciliary tip contains specialized proteins, but

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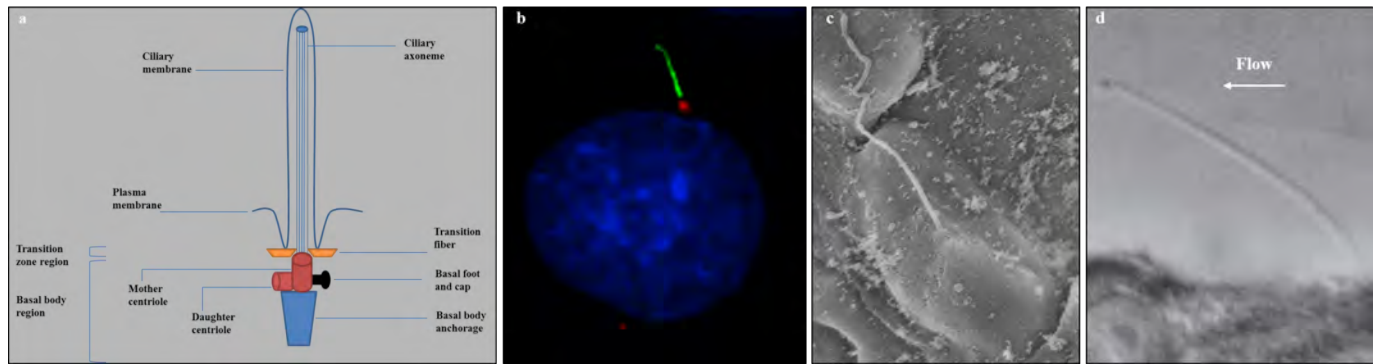


Fig. (1). Mechanosensory cilium. A primary cilium is a cellular organelle extending from the apical surface of many cell types. The mechanosensory cilia act as antennas to receive and transmit extracellular fluid-shear stress into cellular biochemical signals. **(a)** A cilium is composed of a ciliary membrane, a soluble matrix, a ciliary axoneme, and a basal body complex. The *ciliary membrane* contains various sensory proteins. The *soluble matrix* contains many regulatory proteins that are involved in signal transduction networks. The *ciliary axoneme* is surrounded by the nine pairs of microtubules that are thought to control the length of a cilium. The *basal body complex* is the anchoring point for axonemal microtubules. **(b)** Primary cilia found on the surface of endothelial cells can be localized by a simple immunostaining experiment using antibody against acetylated α -tubulin (green) to label primary cilia and pericentrin (red) to label centriole or basal body. The nucleus is counterstained with DAPI to label DNA. **(c)** Mouse primary epithelial cilia can be studied *in vivo* by scanning electron microscopy (SEM) to reveal kidney epithelial cilia protruding from the surface of kidney epithelial cells towards the lumen of kidney tubules. **(d)** Primary cilia are sensory organelles that sense fluid-shear stress on the apical surface of cells. Fluid flow that produces enough drag-force on the cells will bend sensory cilia. Part of this figure is reproduced from [152].

their roles are still unknown. The basal body complex is composed of the mother and daughter centrioles connected by rootlets and emanate from the basal body anchorage. The mother centriole is the basal body from which the cilium protrudes [42].

Primary Cilia is a term commonly used to denote “9+0” non-motile cilia. These have nine parallel doublet microtubules (denoted by ‘9’) and a central pair of microtubule is absent within the central sheath (denoted by ‘0’). Length of a primary cilium can vary from 2-50 μm and it has a diameter of approximately 0.25 μm [42]. Primary cilia are found on several mammalian cell types i.e. endothelia (Fig. 1b) [43-46], epithelia (Fig. 1c) [47-49], neurons [50-53], osteoblasts [54-56], fibroblasts and others [57-59]. Thus, abnormal ciliary proteins and/or abnormal ciliary function can be associated with various pathological conditions collectively known as ciliopathies; which include polycystic kidney disease (PKD), nephronophthisis, bardet biedl syndrome, left-right asymmetry defect, oral facial syndrome, obesity, hypertension and others [60]. Primary cilia can be activated by bending through perfusing cells with fluid-shear stress (Fig. 1d) or by mechanical stress [61]. Bending cilia by apical fluid perfusion with physiologically relevant flow rate [2, 62] or by suction through a micropipette [63] can activate primary cilia. Consequently, mechanosensory ciliary proteins such as polycystin-1 [5], polycystin-2 [7], transient receptor potential-4 [64], fibrocystin [65], and probably many others convert this mechanical force into an intracellular biochemical signal [66, 67].

PRIMARY ENDOTHELIAL CILIA AS BLOOD PRESSURE SENSORS

The control of the circulatory system depends largely on the mechanical properties of blood vessels in both normal

and pathological conditions. The continuous change in blood vessel diameter triggered by contraction and relaxation of vascular smooth muscle cells is important for normal blood flow [68-71].

Different blood vessels are subjected to changes in blood pressure as well as changes in the hemodynamic forces which induce distinct responses within the vascular tree and allows for tissue perfusion. Resistant arteries have the most influence on blood flow and they are subjected to the continuous effect of blood flow and pressure. An increase in blood pressure induces a vasoconstriction known as the myogenic tone, which facilitates the action of the sympathetic nervous system [72]. When transmural blood pressure increases, vessel diameter is reduced [73-75]; while faster flow velocity (shear stress) increases blood vessel diameter [74, 76, 77]. Vascular endothelial cells lining the inner wall of blood vessels can sense changes in blood flow and pressure and convert these mechanical changes into changes of smooth muscle tone [74, 78].

The pulsatile nature of blood flow through the vasculature exerts different types of mechanical forces such as shear stress, cyclic strains and hydrostatic pressure that can impact the physiology of the blood vessel wall. Being the inner most layer and in direct contact with blood flow, the endothelial cell layer bears the most frictional forces induced by the flow of blood. Though these forces are practically impossible to differentiate *in vivo*, they can be distinguished from one another in *in vitro* and *ex vivo* studies [79-81]. In this review, we will focus on shear stress which is the most studied mechanical force in cilia research.

We have previously demonstrated the presence as well as the function of endothelial primary cilia as fluid mechanosensor *in vitro* in mouse aortic endothelial cells, *ex*

vivo in isolated mouse arteries and *in vivo* in mouse models as well as in blood vessels from human patients [5, 7]. The presence as well as the size of primary endothelial cilia varies with respect to the level of fluid shear stress or fluid turbulence. For example, blood vessels with relatively low fluid shear stress have longer cilia while blood vessels with high fluid shear stress are devoid of cilia or have shorter cilia simply due to the inability of cilia to tolerate high levels of shear stress, which could induce its disassembly. Consequently, the ciliary mechanosensory function in those areas of high fluid shear stress could be minimized or substituted by other vessel components such as the glycocalyx [82]. Sensory proteins localized to primary endothelial cilia such as polycystins can detect any sudden increase in blood pressure or shear stress inside the blood vessel [5, 7]. This in turn, leads to the influx of calcium ions to the cilium followed by an increase in intracellular calcium concentration. As a result, primary cilia enable cells to translate this extracellular fluid mechanics into a complex intracellular signaling cascade. This signaling cascade eventually leads to the activation of endothelial nitric oxide synthase (eNOS), an endothelial enzyme that synthesizes nitric oxide gas. Nitric oxide diffuses from endothelial cells to the surrounding smooth muscle cells and promotes vasodilation [83-85]. Both polycystin-1 and polycystin-2, an 11 transmembrane protein with a long extracellular domain and a cation channel with 6 transmembrane domains, respectively are expressed in the endothelial and vascular smooth muscle cells of all major blood vessels [86]. We previously reported the loss of response to fluid-shear stress in mouse endothelial cells with *knockdown* or *knockout* of *PKD2* [7]. In addition to the mouse data, polycystin-2 null endothelial cells generated from *PKD2* patients that do not show polycystin-2 in the cilia are unable to sense fluid flow. Therefore, mutations in both *PKD1* and *PKD2* have been shown to contribute to hypertension [87], in part by the failure to convert an increase in mechanical blood flow into cellular NO biosynthesis [5, 7]. In summary, by sensing any increase in fluid shear stress, primary cilia can activate different cellular mechanisms in order to lower total peripheral resistance and consequently, blood pressure.

It is crucial to discern the physiological mechanism behind mechanosensory role of primary cilia in order to clearly understand the correlation between primary cilia abnormalities and cardiovascular pathology and in particular hypertension. A new mechanism has been proposed that involves polycystin-1 and polycystin-2 [5, 7]. Endothelial cells without primary cilia were isolated from *Tg737^{Orpk/Orpk}* mice to examine the mechanosensory role of primary cilia. In addition and to confirm the role of polycystin-1 and polycystin-2 as mechanosensory proteins, endothelial cells were collected from PKD mice and ADPKD human patients. Polycystins are absent in the cilia of these cells and present in *Tg737* mice cells, but localized at the base of the “stubby” primary cilia. Different magnitudes of fluid shear stress were applied to these cells. Though shear induced cytosolic calcium was increased in normal endothelial cells, diseased artery and mutant endothelial cells did not show any calcium response to fluid shear stress [5, 7]. To verify cilia function specifically against fluid shear stress, a new technique involving artery perfusion in glass capillary was utilized [7,

88]. It was observed that though cilia react normally when other mechanical stimuli are applied, response to fluid shear stress is highly altered.

To dissect the molecular mechanisms downstream of cilia function, different biochemical and pharmacological inhibitors were used in these studies to block downstream molecular targets in endothelial cells. When EGTA is used to block extracellular calcium, both cytosolic calcium and nitric oxide production was inhibited. In addition, L-NAME (*N*^G-nitro-L-arginine methylester), an eNOS inhibitor, was found to block shear induced nitric oxide synthesis but not cytosolic calcium increase. This indicates the necessity of extracellular calcium influx for the downstream generation of nitric oxide. To examine calcium dependent mechanism of nitric oxide biosynthesis, calphostin C, a PKC inhibitor and W7, a calmodulin antagonist were employed. Results showed that PKC and calmodulin function downstream of calcium pathway and blocking either of these will inhibit nitric oxide synthesis. When pharmacological blockers Akt inhibitor II, LY-294,002, and wortmannin were applied, role of Akt was confirmed in shear induced nitric oxide production. These studies in addition to others demonstrate an interaction between mechanosensory polycystin-1 and polycystin-2 and that absence of this interaction results in disturbance of localization of polycystin-2 to primary cilia and hence, disturb ciliary functions. In summary, these studies lead us to propose that, after sensing fluid shear stress, polycystin-1 interacts with and activates polycystin-2, a calcium channel which induces an intracellular calcium influx. This is followed by activation of PKC and formation of calcium-calmodulin complex. Our data also indicate that Akt/PKB is also involved in regulation of eNOS activation and the generation of nitric oxide in response to fluid shear (Fig. 2a and b).

ROLE OF PRIMARY CILIA IN THE CARDIOVASCULAR SYSTEM

Despite its well-known and demonstrated function as a mechanosensory organelle in the cardiovascular system, the role of primary cilia in this system is still a widely debated topic. Recent studies have successfully demonstrated the presence of primary cilia throughout the cardiovascular system. Primary cilia have been observed in endocardia [6, 89], arterial endothelia [2, 7], venous endothelia [90], corneal endothelia [44, 46], arterial smooth muscle cells [91] and airway smooth muscle cells [92].

Primary Cilia and Heart Development

Recent studies have demonstrated an important role of cardiac primary cilia in harmonizing heart development, and defects in these cilia are associated with inherited heart disease. Different types of cilia are present in different chambers throughout heart development to control cardiogenesis. For example, sensory nodal cilia play an important role in regulating signaling mechanisms essential for the establishment of left-right asymmetry, a process for regulating heart morphogenesis. In addition, primary cilia are present on the surface of cardiomyocytes during heart development and houses different receptor complexes which coordinate various signaling mechanisms that are important

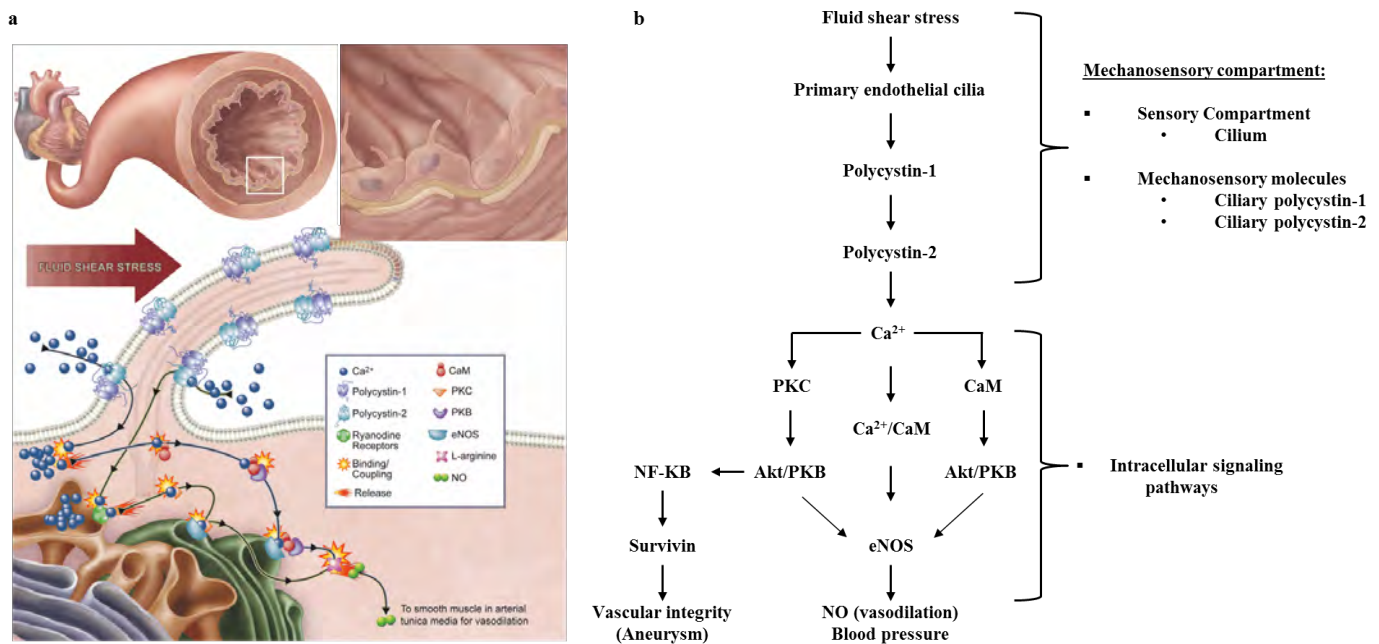


Fig. (2). Endothelial primary cilia as regulators of blood pressure through nitric oxide production and vascular architecture through survivin. (a and b) Nitric oxide synthesis and survivin expression depend on the presence of functional sensory cilia housing sensory complex, polycystin-1 and polycystin-2. Bending of primary endothelial cilia by fluid flow activates polycystin complex and initiates a series of intracellular signaling cascades resulting in the synthesis and release of NO gas, which vasodilates smooth muscles in the vasculature. These intracellular signaling cascades include calcium influx, calcium-calmodulin, protein kinase C, Akt/PKB and eNOS. Survivin downregulation is involved in aneurysm formation. Fluid shear stress induced the activation of polycystin-1/2 complex followed by increase in intracellular calcium influx. Akt and NF-κB are downstream effectors of PKC pathway in regulating flow-induced survivin expression. The increase in p-NF-κB expression is accompanied by an increase in survivin expression, which is critical for normal endothelial cell division and consequently vascular integrity. Modified from [152, 153] with permission.

for proper heart morphogenesis and development. Cilia mutants have been characterized by heart developmental defects such as ventricular and atrial septum defects, myocardial wall disorganization and thinning and double-outlet right ventricles due to mutations in *pkd1* or *pkd2*, the causative genes of ADPKD [93]. Moreover, knockout mice of cilia structural genes such as *ift88* and *kif3a* are also characterized by severe cardiac phenotypes such as hypoplasia of the endocardial cushions, reduction in ventricular trabeculation and enlarged pericardium [94]. This suggests that these cardiac phenotypes could be contributed mainly by ciliary dysfunction and that primary cardiac cilia are critical for normal heart development. More importantly, dysfunctional cilia are associated with congenital heart disease. Several ciliopathies such as nephronophthisis (NPHP), Meckel syndrome (MKS), Bardet-Biedl syndrome (BBS), and Joubert syndrome (JBTS) are usually associated with cardiac defects such as septum defects and aortic stenosis [95].

Cardiac development is controlled by complex regulatory networks of both inductive and inhibitory signals from both the heart as well as the surrounding tissue which coordinate different stages of heart development. An example is the Hedgehog (Hh) signaling pathway which regulates the L-R asymmetry in vertebrates and promotes the activation of several transcription factors involved in various cellular mechanisms during tissue homeostasis and development. It

has been shown that primary cilia can regulate the Hh signaling pathway in several cell types by functioning as a unique compartment for the continuous turnover of Hh signaling components. As a result, defects in primary cilia assembly and consequently Hh signaling components turnover can lead to a variety of developmental pathologies including congenital heart defects [96]. Another example of a signaling network which plays a critical role during heart development is the TGFβ/Bone Morphogenic Protein (BMP) network. It has been recently shown that primary cilia can regulate the canonical TGFβ signaling network through the activation of transcription factors Smad2/3 [97]. Furthermore, it has been shown that the TGFβ ligand, TGFβ-1 can stimulate the differentiation of stem cells into cardiomyocytes and that *Tg737^{Orpk/Orpk}* mouse embryonic fibroblasts are characterized by diminished TGFβ activity, suggesting a direct role of cardiac primary cilia in regulating TGFβ signaling during cardiomyogenesis. Taken together, these studies prompted us to speculate that primary cilia may function as signaling hubs facilitating the cross-talk between the different signaling networks that regulate heart development.

Primary Endothelial Cilia and Vascular Architecture

The morphology and function of blood vessels are continuously altered in response to blood flow which is mainly detected by the vascular endothelium. An aneurysm

is a balloon-like bulge in the wall of blood vessels. Studies on both ADPKD patients and animal models indicate that polycystins are essential for normal blood vessel's structure and development [98]. Aneurysm formation is one of the deadliest vascular abnormalities in PKD patients. The occurrence of aneurysm represents a major risk factor for morbidity and mortality associated with PKD [99]. Mice with *Pkd* mutations develop hemorrhages and aneurysms [100]. Vascular aneurysms are associated with tissue remodeling due to abnormal proliferation of the endothelial cell layers in response to complex hemodynamic changes in fluid shear stress [101]. We recently demonstrated that aneurysm formation is more severe in *survivin*, *pkd1* and *Tg737* vascular-specific knockout (*Pdgfr β Cre:Survivin^{flox/flox}*, *Pdgfr β Cre:Pkd1^{flox/flox}* and *Pdgfr β Cre:Tg737^{flox/flox}*) mice than in wild type mice [102]. An abnormality of primary cilia structure (polaris) or function (polycystin-1) is associated with downregulation of survivin expression, which is associated with abnormal mitotic events in endothelial cells [103]. This in turn can lead to abnormal cytokinesis triggered by polyploidy formation and ultimately contributes to vascular aneurysm [102]. Thus, the inability of primary endothelial cilia to respond to fluid shear stress can contribute to the exacerbation of aneurysm formation in these mice models.

Protein Kinase C (PKC) and Akt are downstream signaling messengers of primary cilia. Studies on endothelial cells have verified that survivin expression following cilia activation is regulated by PKC, Akt and Nuclear Factor- κ B (NF- κ B). Akt is downstream of PKC and can regulate NF- κ B, which is known to regulate survivin expression. Taken all the above together, we propose a Cilia-PKC-Akt-NF- κ B pathway involved in survivin expression and cell division regulation [102, 104-107]. All in all, the inability of mechanosensory cilia to detect fluid shear stress is associated with down-regulation of survivin leading to abnormal cytokinesis triggered by polyploidy formation and finally contributing to vascular aneurysm (Fig. 2b) [102].

Primary Cilia in Corneal Endothelium

The corneal endothelium located beneath the anterior chamber of the eye is orchestrated into a two-dimensional layer of hexagonal cells and plays critical role in maintaining corneal transparency. The presence of primary cilia in the corneal endothelium has been demonstrated in various mammalian species [44]. In humans, corneal endothelial cilia protruding into the anterior chamber of the eye have similar structure to the "9+0" primary cilia, but their function remains somehow unclear, although evidence for an osmoregulatory or a chemosensory function exists. Moreover, it has been postulated that primary cilia of the mouse corneal endothelium play key role towards proper morphogenesis of the characteristic hexagonal shape or pattern of the corneal endothelium during development and disassemble following tissue homeostasis during adult stage pointing towards a role of corneal endothelial primary cilium in corneal endothelial development [108].

More importantly, a link between primary cilia and intraocular pressure regulation has been recently demonstrated through studying a rare X-linked congenital

syndrome known as pediatric glaucoma or Lowe syndrome [109]. It has been previously shown that the *Oculocerebrorenal syndrome of Lowe (OCRL)* gene encoding an inositol polyphosphate 5-phosphatase, which is mutated in Lowe syndrome is involved in vesicle trafficking to the primary cilium [110]. More recently, a potential role of primary cilia in the trabecular meshwork which regulates intraocular pressure in the eye has been demonstrated. In this study, it was shown that primary cilia are important sensors of intraocular pressure changes and that in glaucoma, defective trabecular meshwork cells have shortened primary cilia due to increased intraocular pressure and fluid flow [109]. Consequently, this shortening is associated with increased expression of TNF α , TGF β and GLI1 genes. Primary cilia response to increased intraocular pressure is mainly mediated through interaction between OCRL and transient receptor potential vanilloid 4 (TRPV4), a ciliary mechanosensory channel. Although these findings significantly advance the current understanding of how intraocular pressure is regulated, they provide further evidence and support for our proposed role of primary endothelial cilia as both blood pressure sensor and regulator.

Primary Cilia and Vascular Smooth Muscle Cell Function

Vascular smooth muscle cells (VSMCs) localized in the medial layer of the arterial wall play critical roles in maintaining and regulating blood vessel tone, blood pressure and blood flow [111]. VSMCs express a unique group of proteins with specialized contractile functions and have very low proliferative profile during adult stages. The environment in which VSMCs reside is mainly composed of collagen and elastic fibers as extracellular matrix (ECM) components. Normally, VSMCs are not exposed directly to fluid shear stress simply because the inner lining endothelial cell layer provide the contacting surface with blood flow and consequently shields the VSMC layer. However, in cases of endothelial cell layer injury or denudation, VSMCs become directly exposed to fluid shear stress levels similar to the endothelial cell layer in intact vascular regions. It is critical to understand the mechanisms by which VSMCs sense and transduce the stimuli of shear stress into intracellular biochemical signals which allows better understanding of vascular disease. Studies reviewing the effects of fluid shear stress on VSMCs revealed the presence of shear specific mechanosensors such as cell membrane-related receptors, cell surface glycocalyx, ion channels and integrins as well as the presence of shear-specific secondary signaling messengers such as NO, Ca⁺² and MAPKs. These messengers in turn regulate the expression of shear-responsive genes which control various VSMCs responses such as proliferation, differentiation, apoptosis and migration.

A sensory role of primary cilia in VSMCs similar to its role in several other cellular systems has not been demonstrated until recently [112]. It has been shown that primary cilia are localized to VSMCs in a preferentially oriented pattern with respect to the artery axis as well as to the ECM and they house mechanosensory proteins that are responsive to ECM proteins and to cell-ECM interaction. These initial observations suggest a mechanochemical

sensory role of VSMCs primary cilia in the vasculature. In addition, primary cilia on the surface of VSMCs may act as fluid mechanosensors regulating intracellular Ca^{+2} influx and VSMCs migration in response to fluid shear stress. Primary cilia and cell surface glycocalyx are both displaced in response to fluid flow and affect cell surface receptors as well as integrins. Both cell contraction and migration requires disassembly of integrin-mediated focal adhesion [113]. Therefore, it remains to be discovered if VSMCs primary cilia can sense interstitial flow and play a role in controlling VSMCs contraction and migration. In addition, VSMCs *in vivo* are exposed to other hemodynamic forces such as stretch, and pressure at the same time, which may act in concert to regulate mechanosensitive signaling pathways controlling vascular function and disease. In summary, it is safe to assume that if VSMCs' primary cilia function or structure is perturbed, it will contribute to vascular disease and hypertension.

HYPERTENSION AND HUMAN ADPKD

ADPKD is an example of a ciliopathy or a genetic disorder resulting from the abnormal function and/or structure of primary cilia. Even with successful renal transplant or replacement therapy, patients with ADPKD eventually die due to cardiovascular complications, including hypertension, aneurysms, aortic root dilation, dissection, vascular ectasia, mitral valve prolapse and abnormal function of the microvascular bed [99, 114-116], heart failure and congestive heart disease [115-121]. Aside from the kidney phenotype, the frequencies of phenotypic manifestations in patients with ciliopathy include congestive heart failure/hypertension (78%), hepatic cysts (75%), diverticulosis (70%), ovarian cysts (40%), cardiac valve disorders (25%), inguinal hernias (15%) and intracranial aneurysms (10%) [107]. Our group is one of the first to report cilia function using mouse models and patient samples to explain cystic kidney phenotypes [2, 122] and to report cilia function in nitric oxide (NO) biosynthesis [5, 123]. Plasma concentration of the vasodilator nitric oxide has been found to be lowered in ADPKD patients compared to healthy volunteers [124]. This demonstrates a strong link between endothelial dysfunction and ADPKD. Another study reported similar results where the level of ADMA (asymmetric dimethylarginine), a marker of an inhibitor of nitric oxide synthase, was found to be elevated in PKD patients [125].

The correlation between hypertension and kidney volume occurs in the early childhood stages of ADPKD. The development of hypertension in ADPKD patients is associated with increased renal volume as well as increased left ventricular mass index. Similarly, it has also been suggested that high blood pressure can advance cyst growth [126, 127]. Interestingly, hypertension appears in children before they confront any significant reduction in glomerular filtration rate or development of ADPKD [128, 129]. Now that ADPKD is strongly correlated with dysfunction of ciliary proteins and/or abnormal cilia structure [2, 130, 131], this prompted us to propose that, endothelial dysfunction in ADPKD patients may be attributed to the inability of

primary endothelial cilia to sense fluid-shear stress, or to an anomaly in any other downstream signaling mechanism. Taken together, this suggests that the pathogenesis of hypertension is a risk factor of endothelial primary cilia dysfunction at least partially independent from kidney function in ADPKD.

On the other hand, renal cyst enlargement in ADPKD adults has been ascribed to contribute in part to hypertension. Enlargement of renal cysts leads to the stimulation of both circulating as well as the intrarenal renin-angiotensin-aldosterone system (RAAS) system due to the compression of the cyst-adjacent parenchyma on the renal vasculature resulting in areas of renal ischemia and vascular changes [132, 133]. Activation of RAAS in PKD has been substantiated in both clinical studies [134] and murine models [135, 136]. Other components of the RAAS, including angiotensinogen, angiotensin converting enzyme (ACE), angiotensin II receptor and angiotensin II peptide have also been detected in cysts and dilated tubules in ADPKD kidneys [137]. In addition to hypertension, angiotensin contributes significantly to cyst growth and expansion, increased endothelin levels, fibrosis and increased sympathetic activity. Sympathetic activity can stimulate RAAS and angiotensin can stimulate sympathetic nervous system as well [138, 139]. Moreover, activation of the RAAS has been found in normotensive and hypertensive PKD patients, regardless of their blood pressure and renal function [140]. Not surprisingly, the high levels of circulating angiotensin II in PKD patients have been shown to contribute to the development of vascular hypertrophy, which is further implicated in vascular remodeling [141]. The effect of blockage of the renin-angiotensin-aldosterone system with Angiotensin Converting Enzyme inhibitors (ACE-I) and angiotensin receptor antagonists on renal volume and kidney function is discussed in the next section. A study involving eleven kidney transplant cases of hypertensive PKD patients accounted an improved blood pressure in only six patients [142]. Another report demonstrated that PKD patients with hypertension continue to show cardiovascular complications after renal transplantation [143]. These studies clearly suggest that, though kidney transplantation is beneficial for improving some cardiovascular issues, it is not sufficient to control hypertension in PKD patients. Whether hypertension contributes to diminished renal function or deteriorated kidney function exacerbates hypertension is still a debated topic that warrants further discussion and there is a wide scope of more research to clearly understand the cause and effect phenomenon undergoing in this case.

AVAILABLE AND ONGOING TREATMENT OPTIONS FOR CARDIOVASCULAR DISEASES IN ADPKD

A recent review involving 1877 ADPKD patients' records showed that the use of antihypertensive therapy in ADPKD patients have been increased from 32% in 1991 to 62% in 2008. A similar increase in the use of RAAS inhibitors was also reported (from 7 % to 42 %) [144]. But given that cardiovascular complications are the most

common cause of death in ADPKD patients, it is justified that researches are going on to a greater extent for innovation of novel therapeutics.

Alike regular hypertensive patients, reduced salt and caffeine intake, smoking cessation, regular exercise, and optimal water consumption have been suggested and found beneficial in hypertensive PKD patients [145, 146]. Pathophysiology of hypertension in this disease suggests the usefulness of ACE inhibitors. ACE-I enalapril is found to control blood pressure but it has a consistent antiproteinuric effect [133]. In a different study, 3% of patients displayed a reversible increase in serum creatinine after initiation of ACE-I use [147, 148]. So, ACE-Is should be used cautiously in ADPKD patients. The RAAS based pathophysiology of hypertension foretells the effectiveness of Angiotensin Receptor Blockers (ARBs). A randomized trial compared candesartan (ARB) with amlodipine (calcium channel blocker) and both were found equally effective in controlling blood pressure in PKD patients [149]. However, there is no other study exhibiting similar outcome and should be explored further. Dual blockade of RAAS with ACE-Is and ARBs has also been suggested and being tested in two clinical trials [150]. The most recent release of the results of the HALT study among 558 hypertensive participants with ADPKD revealed that the combination of lisinopril, an ACE-I and telmisartan, an ARB did not significantly alter the rate of increase in total kidney volume in the early stages of ADPKD. However, they indicated that aggressive blood-pressure control might slow kidney cyst growth and reduce left-ventricular-mass index compared to normal blood pressure control [151]. Beta-blockers (metoprolol, atenolol) and calcium channel blockers (amlodipine) are found to be effective in controlling hypertension in PKD patients. Though metoprolol did not alter glomerular filtration rate (GFR) or urinary albumin excretion, effects of atenolol on kidney function is still unknown. Calcium channel blockers are not recommended for use as a first line therapy in PKD patients, as this may cause a significant reduction in GFR. Theoretically, diuretics are recommended in PKD patients only as an additional therapy with ACE-Is as diuretics activate RAAS. Moreover, patients treated with diuretics showed increased urinary excretion of proteins [150].

We and others have independently studied abnormality in primary cilia in the vascular and renal systems in PKD. We hypothesized that cilia in the vascular system play important roles in regulating blood pressure and vascular integrity through the expression of mechano- or chemosensory receptors. Supporting our hypothesis is that patients with abnormal cilia function (PKD) also show a greater incidence of hypertension and other cardiovascular problems. In theory, drugs that interfere or alter cilia sensory function could prevent or delay this high blood pressure increase. This idea is based on our most recent chemical-screening studies showing that dopamine receptor is localized in endothelial cilia and that this ciliary receptor has dual chemo and mechano-sensing roles in endothelial cells. We showed that “activated” dopamine receptor-type 5 in the cilia could switch its chemosensing function to mechanoreceptor, while “non-activated” receptor has a primary function as a chemoreceptor. Activation of ciliary dopamine receptor

could bypass mechano-ciliary polycystin complex through a mechanism that increases the cilia length. The extension of cilia probably serves to increase the sensitivity of these sensory organelles to fluid flow, in which dopamine receptor-type 5 acts as the mechanoreceptor.

CONCLUSION

As more evidence is presented to support the direct involvement of primary cilia in the cardiovascular system, this necessitates large-scale screening studies for drugs to investigate whether targeting primary cilia function or structure towards hypertension results in better outcomes.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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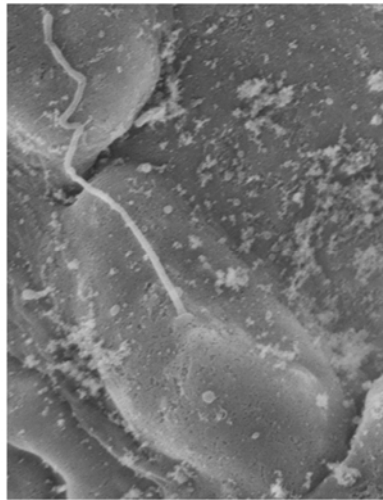
Graphical Abstract

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Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension

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Primary cilia are mechanosensory organelles extending from the cell surface to sense cues in the extracellular environment and transduce signals into the cell producing a response that regulate a physiological phenomenon like blood pressure.

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Localization and Distribution of Primary Cilia in the Adult Mouse Heart

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Localization and Distribution of Primary Cilia in the Adult Mouse Heart

Comments

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Localization and Distribution of Primary Cilia in the Adult Mouse Heart

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Abstract

Although primary cilia have been shown to play crucial roles in the development of embryonic mouse heart, their presence and function in adult mouse heart remains controversial. In this study, the presence of primary cilia in adult mouse heart was investigated. The presence of primary cilia was initially demonstrated in the surface of cardiac cells of mouse hearts from both young and adult mice by immunostaining with acetylated α -tubulin, a ciliary structural marker. The presence of cardiac primary cilia in 1-, 3-, 6- and 12-month old mice was further confirmed by staining heart tissues with an antibody against pericentrin, a marker for the basal body or the centriole. Ciliary polycystin-2 is a calcium channel and mechanosensory molecule, was demonstrated for the first time to localize to primary cilia of cardiac cells in both early and late stages of mouse adulthood, thus proposing a role of primary cilia in adult mouse heart calcium signaling. Primary cilia presence in different heart chambers was further confirmed by immunostaining. Furthermore, the abundance and length of cardiac primary cilia during mouse adulthood in three different age groups (< 3 month, 3-6 month, and >6 month old mice) was studied and found out that cardiac cells with primary cilia in the first age group (< 3 month) accounted for 37% of the total number of cells; however, the number of primary cilia in the 3-6 month and > 6 month age groups accounted for 29% and 25%, respectively, suggesting that primary cilia abundance is age dependent.

Keywords: Primary cilia; Cardiac myocytes; Mechanosensation.

Introduction

Primary cilium is a sensory organelle that senses mechanical signals on the apical membrane of cells [1-4]. Mechanical signals that produce enough force on the top of cells will bend and activate sensory cilia. These biomechanical properties play a very important role in visceral organs that support bodily fluid perfusion, including the heart. Of note is that traditional mouse models of homozygous cilia mutants do not survive at birth, pointing towards a possibility of heart dysfunction among others [5-10]. Aside from the kidney phenotype, the frequencies of phenotypic manifestations of patients with ciliopathy include congestive heart failure/hypertension (78%), hepatic cysts (75%), diverticulosis (70%), ovarian cysts (40%), cardiac valve disorders (25%), inguinal hernias (15%) and intracranial aneurysms (10%) [11].

Polycystic kidney disease (PKD), affecting 1 in 1,000 individuals, is an example of a ciliopathy resulting from the abnormal function and/or structure of primary cilia due to mutation in *Pkd2* gene encoding for polycystin-2 (PC2). Even with a successful renal transplant or replacement therapy, patients with PKD will eventually die due to cardiovascular complications, including heart failure and congestive heart disease [12-18]. Cardiovascular complications, including heart failure, contribute significantly to morbidity and mortality of PKD patients [19]. Although PKD has been categorized as a ciliopathy [20], the role of cilia in the heart has never been studied.

Polycystin-2 is involved in mechanosensation in the primary cilium of renal epithelia [21, 22], vascular endothelia [23, 24], cholangiocyte epithelia [25], and embryonic neural cells [26]. Most recently, polycystin-2 has been proposed to function as pressure sensor within the vascular system [27]. Cilia have direct roles in heart development. *Pkd2* is required for the development of left-right asymmetry of the heart [28]. Cilia are also important for the development and function of the heart. Different mutations of cilia have led to abnormal cardiac development. For example, ventricular dilation and abnormal outflow tract development are seen at E11.5 in *IFT-88*-null mouse where cilia are absent. Cobblestone mutant mice, a hypomorphic allele of the gene *IFT-88*, have also shown numerous heart defects including persistent truncus arteriosus and hyperplasia of the myocardium at E14.5 and E16.5. Myocyte contraction is initiated by calcium influx through voltage gated L-type calcium channels (CaV1.2) triggering calcium-induced calcium release from the sarcoplasmic reticulum. Zebrafish with mutated *CaV1.2* do not have a functional beating heart [29, 30]. Furthermore, a recent study demonstrated that CaV1.2 and PC2 are co-localized to primary cilia of cardiac myocytes and that the loss of function of CaV1.2 and PC2 complex is manifested by cardiac edema and hypertrophy in zebrafish [2].

Primary cilia with 9+0 axoneme in myocytes of different species such as chicken, lizard, rabbit and mice

were first described by Rash and colleagues decades ago [31]. Primary cilia in embryonic human heart were seen in epicardial, myocardial, and endocardial cells while adult human heart showed few cilia in non-muscular cells of the myocardial layer. Moreover, embryonic mouse heart at embryonic day E9.5 and E12.5 displays primary cilia in different areas. At E9.5, cilia are seen in left and right atria primordia, endocardial layer around ventricular trabeculations, endothelial cells of the atrial side of the endocardial cushion and mesenchymal cells within endocardial cushion (ECC). They are also found in early compact myocardial layer but with less abundance. At E12.5, cilia are found in locations similar to those in E9.5 in addition to the epicardial layer; however, they are less in number in the atrial endocardial layer [28]. Cilia length in embryonic mouse hearts ranges between 2-5µm in wild type animals according to Slough and colleagues [28]; however, others have also reported cilia at the E12.5 stage as 1-2µm length.

Although the presence of primary cilia in myocytes has been known for over 40 years [31], the presence and function of primary cilia in the adult cardiac system is still controversial. In fact, there is currently very little information about the roles of cilia in the adult heart function. Thus, cilia are mistakenly thought to be vestigial organelles with passive, nonfunctional remnants in the cardiac system. In this study, we demonstrate for the first time the presence of primary cilia in the adult mature mouse heart. We further propose primary cilia as a new organelle required for myocyte signaling and contraction. Furthermore, primary cilia could potentially be a novel and attractive therapeutic target for various cardiac diseases. Thus, our studies have a broad implication in basic science and in future therapeutic discoveries.

Materials and Methods

All experiments involving research animals are approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). Wild type (WT) C57BL/6 mouse strain is used in all experiments. Mice are euthanized by asphyxiation using carbon dioxide for 5 minutes. Cervical dislocation was used as a second method of euthanasia to confirm death.

Immunofluorescence Microscopy

After dissecting the animals, mice hearts are excised and fixed in about 15 ml of 10% formalin overnight. Fixed tissues are embedded in paraffin and sectioned longitudinally at 4 µm thickness. Paraffin-embedded heart sections are baked in an oven at 60°C for 2 hours to remove the paraffin wax. Slides are then restored and rehydrated using xylene substitute (Sigma, Inc.) and a series of ethanol (100% 3x3 min, 90% 1x3 min, and 70% 1x3 min).

Antigen retrieval is performed by incubating the tissue sections in Proteinase K (A.G. Scientific, Inc.) in 20mM Tris-HCL (1:50) for 20 minutes followed by incubation in 30% H₂O₂ for 15 minutes. Slides are then washed with 1XPBS (HyClone Laboratories, Inc.) for 5 minutes and tissues are permeabilized and blocked using 1% triton X-100 (Fisher Scientific) in 10% FBS in PBS for 1 hour. Primary mouse anti-acetylated- α -tubulin antibody 1:500 (Sigma, Inc.) and primary rabbit anti-polycystin-2 antibody (PC2) 1:100 (Santa Cruz Biotechnology, Inc.) are used as primary cilia markers. Primary rabbit anti-pericentrin antibody 1:500 (Covance, Inc.) is used to stain the basal body of cilia or centriole. Slides are then washed three times with 1XPBS for 5 minutes. Secondary fluorescein anti-mouse IgG 1:500 (Vector Labs, Inc.), secondary texas-red anti-rabbit IgG 1:500 (Vector Labs, Inc.), and fluorescein Wheat Germ Agglutinin (WGA) 1:500 (Vector Labs, Inc.) are added for 1 hour after washing with 1XPBS three times for 5 minutes. Before observation under a fluorescent microscope (Nikon TiU), the section was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 minutes to stain the nucleus/DNA. To minimize photo bleaching, the sections were imaged immediately under minimum exposure time.

Cilia Number

The presence of primary cilia is confirmed with anti-acetylated- α -tubulin antibody and cilia numbers are recorded as a percentage, by dividing the number of cells with primary cilia by the total number of cells (represented by DAPI or WGA staining) in each field of vision under a fluorescent microscope (Nikon TiU). At least three wild-type mice from each age group (<3months, 3-6months, and >6months old) are used in every experiment. Over 400 cells are counted in each group. Percentages are then averaged and compared between the three groups using SPSS software.

Cilia Length Measurement

Primary cilia structure is mainly composed of acetylated microtubules. Primary cilia length is measured by direct immunofluorescence staining using anti-acetylated α -tubulin antibody (1:1,000; *Sigma, Inc.*). Cilia length (μ m) is recorded using confocal microscope (Leica Microsystems) and measured using calibrated image acquisition and analysis MetaMorph software. For statistical purposes, at

least three wild-type mice are used in each experiment from three different age groups (<3months, 3-6months, and >6months old). More than 30 cilia are measured in each group. Lengths are averaged and compared between the three groups using SPSS software.

Dissection of Heart Chambers

In addition to staining the whole heart tissue, we also stained the individual heart chambers in some experiments. In order to dissect the heart chambers, the whole heart was excised first and positioned in a way similar to its position in the body. The right atrium is easily identified and cut at the top right of the heart and the left atrium is located to the top left of the heart but in a slightly lower plane than the right atrium. In order to dissect the ventricles, the heart is cut below the roots of aorta and pulmonary artery to expose the left ventricle and left ventricle chambers, respectively. Right ventricle is easily identified and cut from its attachment with left ventricle since it has the thinner layer wall. After dissecting right ventricle, left ventricle with atrioventricular septum attached to it is exposed. Left ventricle is isolated after cutting the borderline of septum along its attachment to the left ventricle.

Statistics

All images are analyzed using MetaMorph software. All quantifiable data are reported as mean $\pm$ SEM. Comparisons between means are performed using one way ANOVA test followed by Tukey post-test analysis and statistical significance implies $p < 0.05$. All data analysis is done using SPSS software.

Results

Primary Cilia are Present in Adult Mouse Heart

In order to verify the presence of cilia in adult mouse heart, whole-heart tissue-sections from adult mice from three different age groups (<3 months, 3-6 months, and >6 months old) are stained with anti-acetylated- α -tubulin antibody (red), a well-known ciliary marker, and Wheat Germ Agglutinin (WGA, green), a carbohydrate-binding protein used to mark the plasma membrane of cells in the cardiac tissues. DAPI (blue) is used to counterstain DNA/nucleus. The presence of primary cilia is confirmed in all age groups (Figure1).

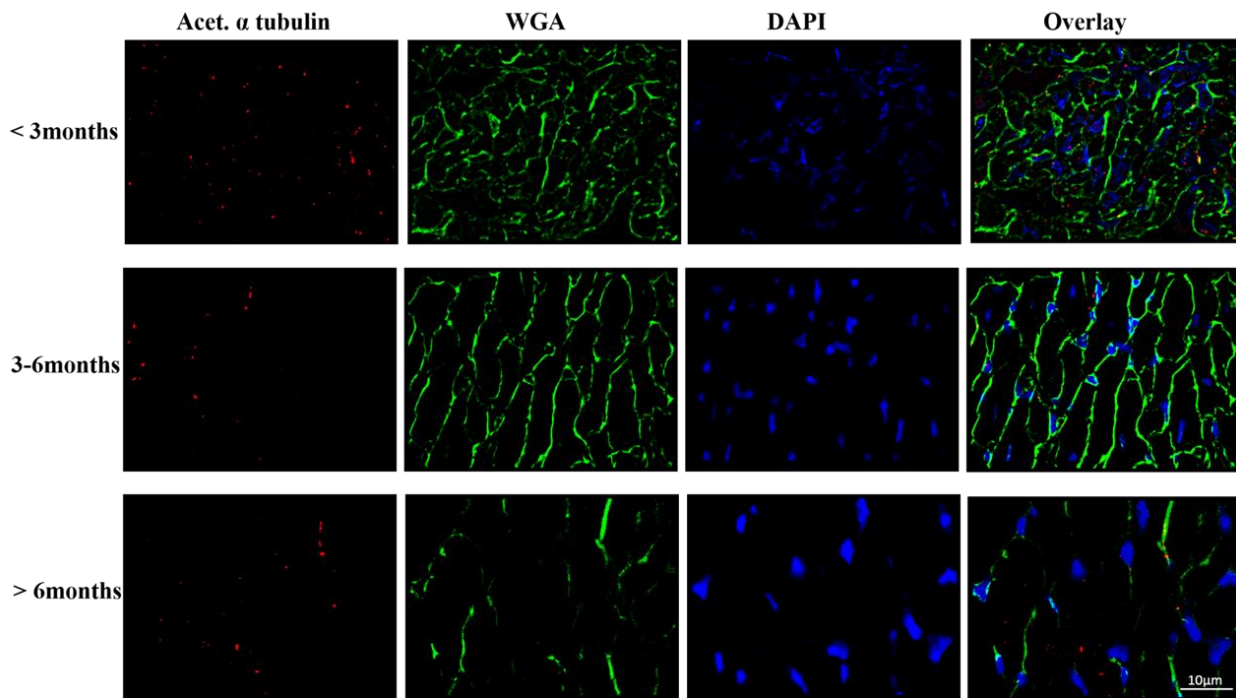


Figure 1: Primary cilia are present in adult mouse heart. Representative images from the whole heart showing primary cilia from different age groups of adult mice. Heart sections stained with anti-acetylated- α -tubulin antibody (red), a ciliary marker, and WGA (green), a plasma membrane marker and DAPI (blue) is used to counterstain DNA/nucleus. Images were captured at 60X 1.5- magnifications.

Primary cilia emanate from and are anchored to the cell body by the basal body or the centriole.

To further confirm the presence of cilia in adult mouse heart, anti-acetylated- α -tubulin antibody staining is accompanied with anti-pericentrin antibody (a marker for the centriole at the base of cilia). Representative images of the co-localization of anti-acetylated- α -tubulin (green) and

anti-pericentrin (red) antibodies at the cardiac primary cilia are obtained from 1-, 3-, 6-, and 12-month old mice (Figure 2).

For clarification purposes, the anti-pericentrin and WGA staining performed in the previous experiments were merged and the co-localization with anti-acetylated- α -tubulin (red) within cardiac cells is clearly seen (Figure 3).

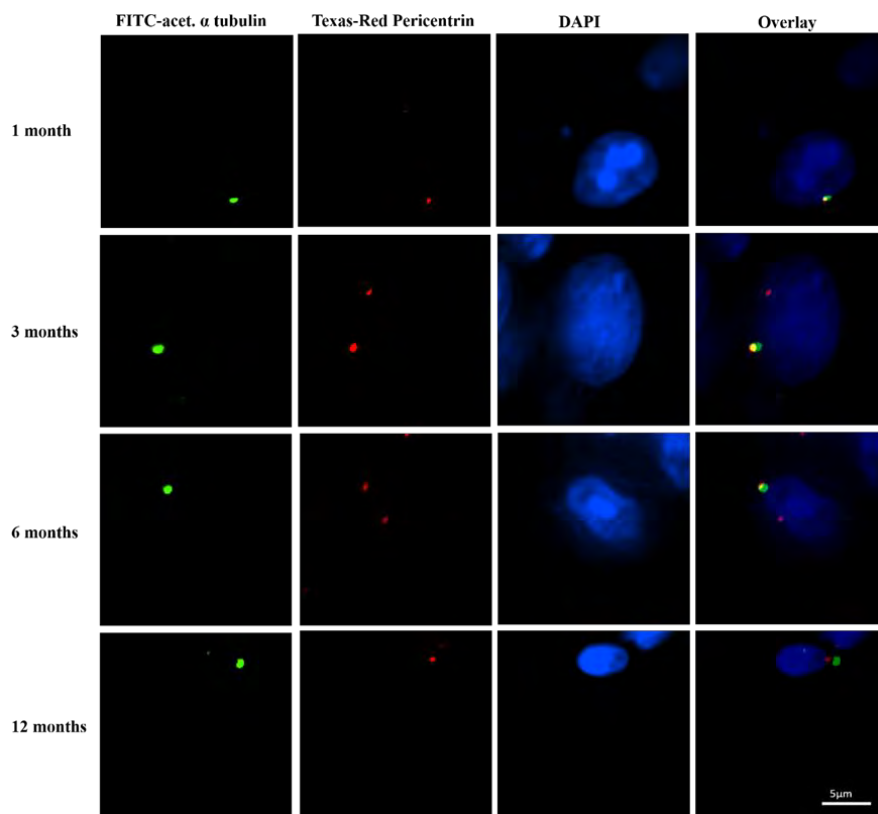


Figure 2: Confirmation of primary cilia presence in adult mouse heart with anti-pericentrin antibody. Co-localization of anti-acetylated- α -tubulin (green) and anti-pericentrin (red) antibodies at the cardiac primary cilia at different stages in mouse adulthood is shown through representative images from the whole heart. Images are captured at 100X magnification.

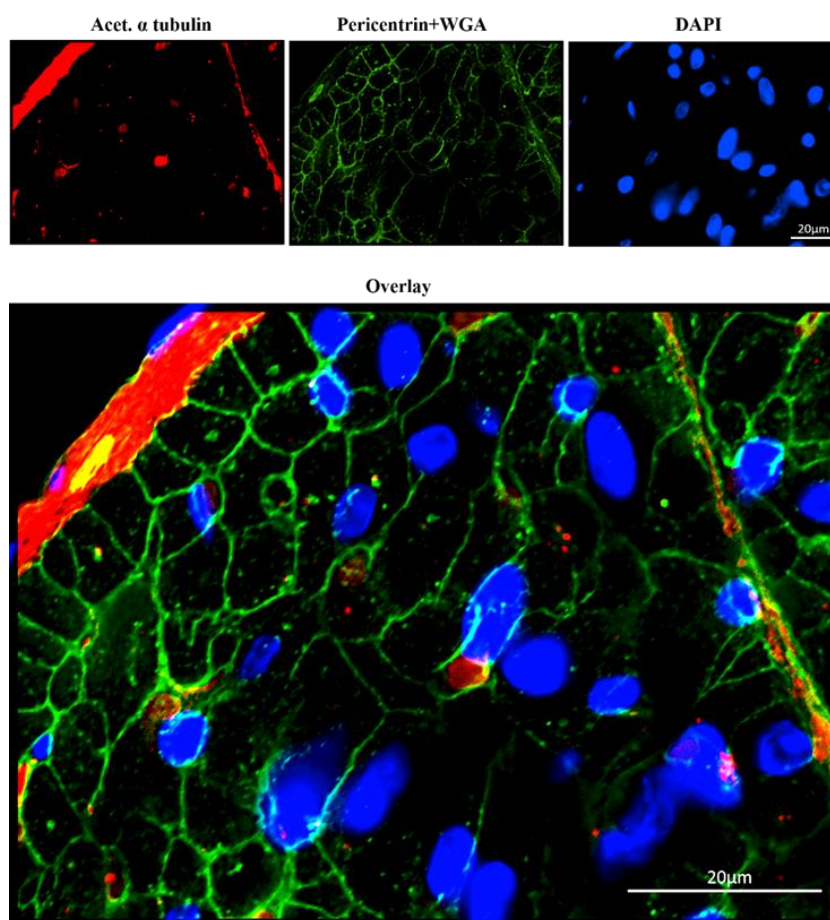


Figure 3: Confirmation of primary cilia presence in adult mouse heart with combined staining of WGA and anti-pericentrin antibody. Representative images from the whole heart showing the co-localization of anti-acetylated- α -tubulin and anti-pericentrin antibodies in the presence of WGA, a plasma membrane marker. DAPI (blue) is used to counterstain DNA/nucleus. Image is captured at 100X magnification.

Polycystin-1 and polycystin-2 are mechanosensory proteins that are known for their ciliary localization in different tissue- and cell-types throughout the body [21]. Polycystin-2 functions as a calcium channel and has been shown to play a role in calcium signaling in various cell types such as kidney and endothelia [23]. In order to test the hypothesis that polycystin-2 is a calcium channel involved in adult cardiac myocytes contraction, polycystin-2 localization to primary cilia of cardiac myocytes in the adult heart tissue sections was analyzed. This also provides an additional confirmation of cardiac primary cilia presence. Immunohistochemistry is performed by staining heart tissues with anti-polycystin-2 antibody. Representative images of the co-localization of

anti-acetylated- α -tubulin (green) and anti-polycystin-2 (red) antibodies are attained from 1-, 3-, 6-, and 12-month old mice (Figure 4).

After confirming the cilia presence with different markers in the adult mouse heart sections, the presence of cilia in the isolated heart chambers i.e. left auricle (LA), left ventricle (LV), right auricle (RA) and right ventricle (RV) was then examined. The different heart chambers were isolated and stained with anti-acetylated- α -tubulin antibody (red) as a cilia marker, WGA (green) to mark the plasma membrane of the cells and DAPI (blue) to counterstain DNA/nucleus. Figure 5 shows representative images of the primary cilia in all heart chambers.

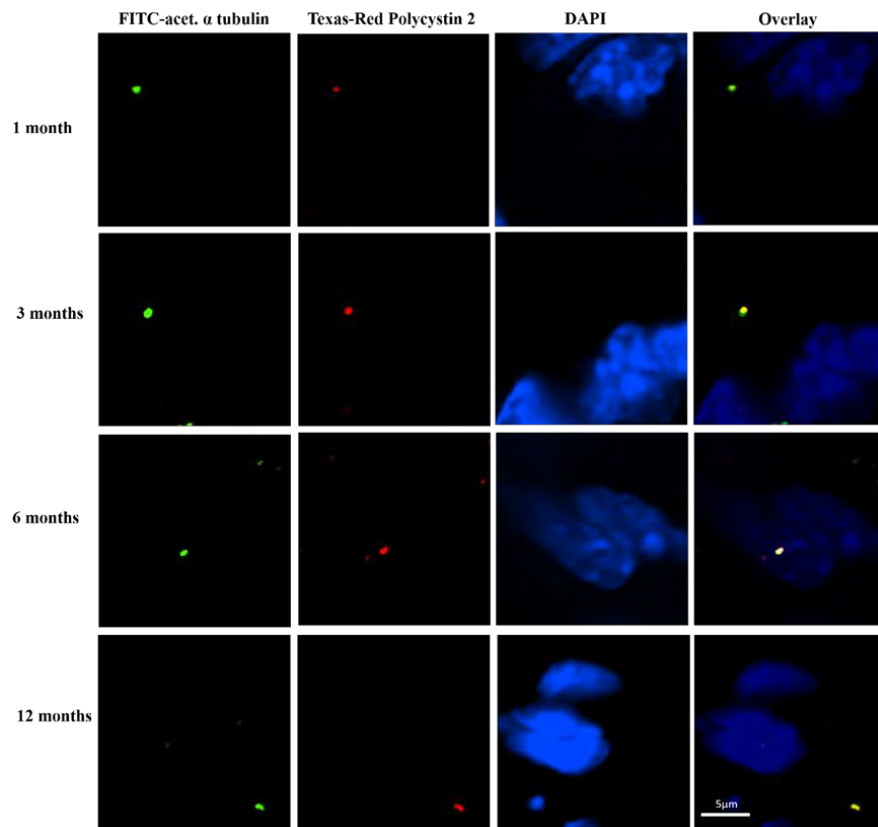


Figure 4: Polycystin-2 is localized to primary cilia of adult mouse heart. Representative images obtained from the whole heart of 1-, 3-, 6-, and 12-month old mice showing the co-localization of polycystin-2 (red) and acetylated- α -tubulin (green) at cardiac primary cilia. DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification.

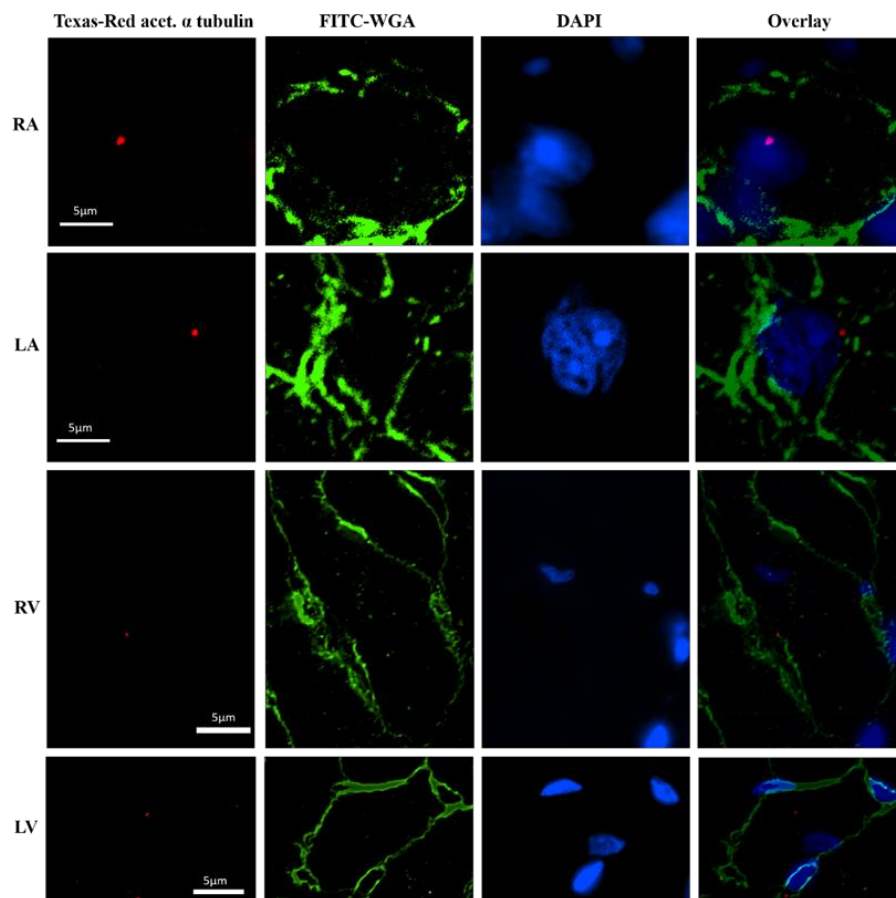


Figure 5: Primary cilia are present in adult mouse heart chambers. Representative images from individual heart chambers showing cardiac primary cilia in the different heart chambers of adult mouse tissues stained with anti-acetylated- α -tubulin antibody (red) and WGA (green). DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification. RA= Right atrium, LA=Left atrium, RV=Right ventricle, LV=Left ventricle.

Similar to previous experiments and after confirming the cilia presence in heart chambers with anti-acetylated- α -tubulin antibody, the presence of cilia with anti-pericentrin antibody was then further confirmed. So,

heart chambers are stained with anti-acetylated- α -tubulin (green) and anti-pericentrin (red) antibodies. Figure 6 shows representative images of the co-localization of the two markers to the cilia in all the heart chambers.

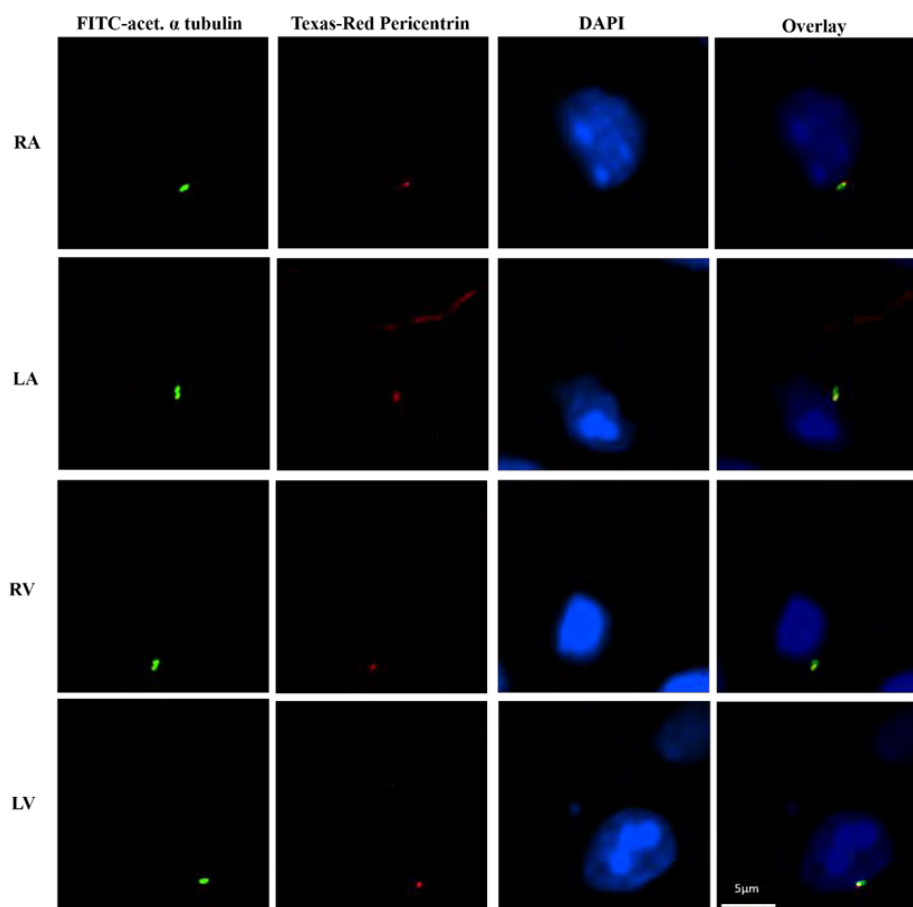


Figure 6: Confirmation of primary cilia presence in adult mouse heart chambers with anti-pericentrin antibody. Representative images from individual heart chambers showing the co-localization of anti-acetylated- α -tubulin (green) and anti-pericentrin (red) antibodies at cardiac primary cilia in the atria and ventricles. DAPI (blue) is used to counterstain DNA/nucleus. Images are captured at 100X magnification. RA= Right atrium, LA=Left atrium, RV=Right ventricle, LV=Left ventricle.

Primary cilia number in adult mouse heart

After confirming the existence of primary cilia in adult mouse heart with several markers, the abundance of primary cilia among different age groups (<3 months old, 3-6 months old, and >6 months old) was studied. The number of cilia as a percentage of the total number of cells within the same field of vision was recorded. In the first age group (<3 months old), cardiac myocytes with primary cilia

accounted for about 37% of the total number of cells. Interestingly, the abundance of primary cilia declined to 29% and 25% in the second (3-6 months old) and third (>6 months old) age groups, respectively. Although our study shows that there is a decline in primary cilia abundance with increased age in adult mice heart, this decline in cilia number is only statistically significant between the first group (< 3 months) and the third group (> 6 months) (Figure 7).

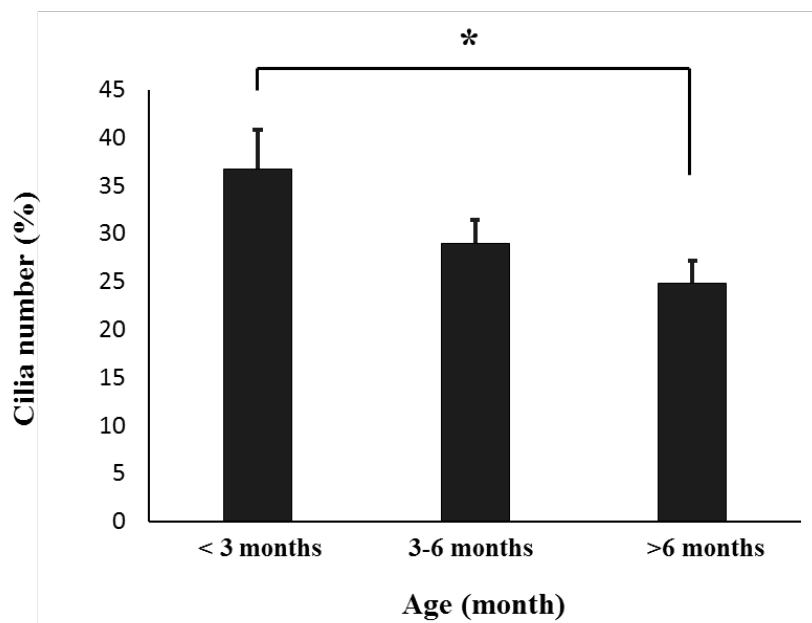


Figure 7: Primary cilia abundance in adult mouse heart is age dependent. Cilia number in the whole heart is represented in three age groups (<3 months, 3-6 months, and >6 months old mice) and our study shows a significant decrease in their abundance with age specifically between the first group (< 3 months) and the third group (> 6 months), $p<0.021$.

Primary cilia length in adult mouse heart

Studies on primary cilia in the renal epithelial system show that cilia length is highly regulated, with the cilia being longer in larger lumen and shorter in smaller lumen[32]. Many studies have also found that cilia length can be regulated under different physiological conditions[33]. Moreover, the length of primary cilia has

been shown to play a significance role in their sensory function. So, cilia length was the next parameter that was studied. To accomplish this goal, cilia length from three different age groups (<3 months, 3-6 months, >6 months old mice) is measured and compared between the groups. Our data show that the length of primary cilia does not change significantly during different stages of mice adulthood with an average length of approximately 1 μ m (Figure 8).

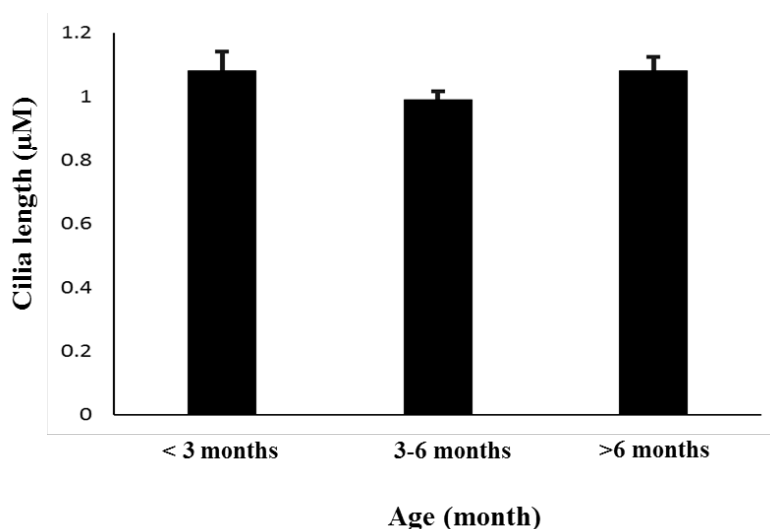


Figure 8: Primary cilia length in adult mouse heart is age independent. Primary cilia length does not change with age during mouse adulthood and the average length is around 1 μ m, $p<0.27$.

Discussion

Primary cilia in embryonic mouse heart have been reported several times to play crucial roles in cardiac development and function [28]. However, little is known and doubts are rising regarding the presence of primary cilia in adult mouse heart and their function. In order to confirm the presence of primary cilia in adult mouse heart, many

approaches can be used such as electron microscopy, immunohistochemistry, or functional studies through knockdown or knockout approaches of genes that encode ciliary proteins. In our study, the presence of primary cilia in adult mouse heart and their potential role in the cardiovascular system through immunohistochemistry was investigated.

Our studies initially aimed at examining mouse heart sections from different adult age groups by staining with an antibody against acetylated- α -tubulin, a well-known ciliary marker, and WGA, a carbohydrate binding protein used to mark cells' plasma membrane. Our data confirms the presence of primary cilia within heart cells in adult mice aged between <3 months, 3-6 months, and >6 months old. This confirms the presence of primary cilia in adult mouse heart in early and late stages of adulthood indicating a possible function of cilia in the heart during mouse adulthood.

To rule out the possibility that our staining with anti-acetylated- α -tubulin antibody was not due to non-specific binding or artifacts and to further confirm cardiac primary cilia presence, adult mouse heart tissues from different stages in mouse adulthood were stained with anti-pericentrin antibody, a marker for the basal body of cilia from which cilia project, in addition to the staining with anti-acetylated- α -tubulin antibody (a ciliary marker). Figure 2 shows representative images of the co-localization of the two markers to cardiac primary cilia from 1-, 3-, 6-, and 12-month old mice. This additionally confirms that primary cilia continue to be present during mouse adulthood and suggests that they may still play a role in adult mouse heart in addition to their role during embryonic stage.

To further confirm the presence of primary cilia and get a better grasp of their possible function in adult heart, heart sections were stained with antibody against polycystin-2, a calcium channel found to be localized to cilia in different cell types mediating many calcium dependent pathways. Figure 4 provides representative images obtained from 1-, 3-, 6-, and 12-month old mice that show the co-localization of anti-polycystin-2 and anti-acetylated- α -tubulin antibodies to cardiac primary cilia, proposing a possible role of cilia in the adult heart through calcium signaling. Based on previous and ongoing studies from our laboratory, we propose that primary cilia might regulate myocardial contractility and blood flow within the heart chambers through maintaining sufficient calcium influx into cardiac myocytes. Also, their role could be similar to the one reported in endothelial cells where the activation of polycystin-2 increases the influx of calcium and triggers a series of calcium dependent signaling pathways.

Moreover, our data indicated the presence of primary cilia in all heart chambers. Figure 6 provide representative images of the co-localization of anti-acetylated- α -tubulin and anti-pericentrin antibodies to cardiac primary cilia in all heart chambers. The presence of primary cilia in all heart chambers might suggest a common function of primary cilia in each chamber. More importantly, the

presence of primary cilia in an area with high shear stress such as the left ventricle contradicts some previous studies that report primary cilia reabsorption in response to shear stress. However, this might be explained partially by the very short and stubby nature of primary cilia in these chambers where the magnitude of shear stress is extremely high for the cilia to maintain their presence.

Furthermore, we studied the abundance of cardiac primary cilia during mouse adulthood. Cilia number was recorded as a percentage by dividing the number of cells with primary cilia by the total number of cells in each field of vision. Figure 7 in our results section compares the cilia abundance in adult mouse heart among three age groups (< 3 months, 3-6 months, and >6 months old). Our data shows that cells with primary cilia in early mouse adulthood comprise approximately one third of the total number of cells. Although this shows that not all cells in the heart are ciliated, this percentage of abundance of primary cilia must indicate a role of cilia in adult mouse heart. A decline in cilia number with increased age was seen in the older groups. This could be a sign that primary cilia abundance is age dependent.

Finally, Cilia length was the next parameter to be examined in our study due to its association with their sensory function. Cilia were confirmed with anti-acetylated- α -tubulin antibody and the length of primary cilia in adult mouse heart was measured and compared between three age groups (< 3 months, 3-6 months, and >6 months old). As shown in figure 8, we reported the average cilia length to be around 1 μ m and do not fluctuate much during adulthood. This shows slightly shorter cilia than the ones seen at the embryonic stage which could be simply a response of cilia towards the high shear stress inside the heart during adulthood and a mechanism by which cilia survive such a harsh condition.

Conclusions

In summary, our studies provide a novel insight into the localization and distribution of primary cilia in adult mouse heart. Without doubt, there are many more interesting future experiments that are revealed by our study. For example, studies of primary cilia abundance and cilia length in the different heart chambers are still needed in order to verify any difference in the distribution of cilia between heart chambers. Moreover, for better understanding of the role of primary cilia in adult mouse heart, other ciliary protein localizations and functional studies are warranted among which are CaV1.2, a voltage gated calcium channel, polycystin-1 and others. Finally, an *in vivo* model of cilia in the heart may reveal some translational importance of our studies.

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Declaration of Interest

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RAPAMYCIN INCREASES LENGTH AND MECHANOSENSORY FUNCTION OF PRIMARY CILIA IN RENAL EPITHELIAL AND VASCULAR ENDOTHELIAL CELLS

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ABSTRACT

Primary cilia are biophysically-sensitive organelles responsible for sensing fluid-flow and transducing this stimulus into intracellular responses. Previous studies have shown that the primary cilia mediate flow-induced calcium influx, and sensitivity of cilia function to flow is correlated to cilia length. Cells with abnormal cilia length or function can lead to a host of diseases that are collectively termed as ciliopathies. Rapamycin, a potent inhibitor of mTOR (mammalian target of rapamycin), has been demonstrated to be a potential pharmacological agent against the aberrant mTOR signaling seen in ciliopathies such as polycystic kidney disease (PKD) and tuberous sclerosis complex (TSC). Here we look at the effects of rapamycin on ciliary length and function for the first time. Compared to controls, primary cilia in rapamycin-treated porcine renal epithelial and mouse vascular endothelial cells showed a significant increase in length. Graded increases in fluid-shear stress further indicates that rapamycin enhances cilia sensitivity to fluid flow. Treatment with rapamycin led to G0 arrest in porcine epithelial cells while no significant change in cell cycle were observed in rapamycin-treated mouse epithelial or endothelial cells, indicating a species-specific effect of rapamycin. Given the previous *in vitro* and *in vivo* studies establishing rapamycin as a potential therapeutic agent for ciliopathies, such as PKD and TSC, our studies show that rapamycin enhances ciliary function and sensitivity to fluid flow. The results of our studies suggest a potential ciliotherapeutic effect of rapamycin.

KEYWORDS: cilium, kidney, mechanosensation, shear-stress, vascular.

Introduction

The primary cilium is a solitary cellular organelle that protrudes from the apical cell membrane. Studies on cilia-dependent mechanosensation have shown that the primary cilium acts as a transducer of fluid-shear stress into intracellular signaling (Masyuk et al., 2006, Nauli et al., 2003, Nauli et al., 2008). Along with its mechanosensory function, the primary cilium houses a variety of receptors, ion channels, transporter proteins, and other protein complexes involved in signal transductions, such as the Hedgehog (Huang and Schier, 2009), Wnt (Corbit et al., 2005), planar cell polarity (Ross et al., 2005) and platelet-derived growth factor (Schneider et al., 2005) pathways.

Abnormalities in cilia structure or function lead to a spectrum of diseases called ciliopathies. Ciliopathies can affect a variety of organs due to the ubiquitous presence of primary cilia in different organ systems and their role as a signaling hub (Lancaster and Gleeson, 2009). The mechanosensory function of primary cilia occurs in various organs, such as renal nephron (Nauli et al., 2006, Siroky et al., 2006, Liu et al., 2005), hepatic biliary system (Masyuk et al., 2006), pancreatic duct (Cano et al., 2006) and vasculature (Abou Alaiwi et al., 2011, Abou Alaiwi et al., 2009). Shortened primary cilia have been shown to result in polycystic kidneys (Lin et al., 2003, Yoder et al., 1995), and a reduction in mechanosensory function of the cilia has also been reported to promote polycystic kidney phenotypes (Abou Alaiwi et al., 2014, Nauli et al., 2008, Nauli et al., 2003). The ability to sense and respond to extracellular stimulation is thought to be the basis for homeostatic adaptation and critical for normal development of various organs. When the primary cilium is dysfunction, cells cannot respond properly to environmental cues leading to ciliopathy phenotypes.

The primary cilium responds to flow-induced bending with calcium entry through mechanically sensitive channels. Studies have shown that bending of the cilium in cultured cells results in an increase in intracellular calcium concentration (Jin et al., 2014, Praetorius and Spring, 2001). The large increase in cytosolic calcium may activate calcium dependent processes that range from cell proliferation to cell death (Berridge et al., 2000). For this reason, calcium fluorimetry has been used as a means of quantifying cilia function, i.e. response to fluid-shear stress (Liu et al., 2005, Siroky et al., 2006) and pharmacological agents (Kathem et al., 2014, Abdul-Majeed and Nauli, 2011).

Polycystic kidney disease (PKD) has been associated to the inability of renal epithelia (Xu et al., 2007) or vascular endothelia (Nauli et al., 2008) to carry out a calcium influx in response to fluid-flow. Aberrant activation of mTOR (mammalian target of rapamycin) has also been reported in PKD (Shillingford et al., 2010, Shillingford et al., 2006, Pema et al., 2016). The loss of function in the TSC complex has been associated with development of renal cysts (Armour et al., 2012, Bonnet et al., 2009). In normal cells, the cilia regulate the mTOR pathway through polycystin-1 (PC1) mediated suppression. PC1 is localized to the cilia and is critical in the formation of the polycystin flow-sensing complex in the cilia. When PC1 is inactivated, cyst-lining epithelial cells show activation of mTOR as measured by phosphorylation of mTOR and S6 kinase, a downstream effector. This has led to the use of mTOR inhibitors, such as rapamycin, as potential drug candidate to target the aberrant mTOR activation in PKD.

Although the role of rapamycin on cilia length and function has never been studied, mTOR pathway may modulate cilia length and function, pointing to a potential effect of rapamycin on cilia length and function (Yuan et al., 2012). The relationship between mTOR and cilia also suggests that primary cilia are dynamic organelles that can be modulated through the mTOR intracellular signaling pathways. Because *in vivo* evidence has shown that rapamycin can reduce cyst growth and preserve renal function (Stayner et al., 2012, Shillingford et al., 2010, Tao et al., 2005), our present study aims to examine the effects of rapamycin-induced changes in cilia length and function. Our studies further evaluate if rapamycin would have an effect in vascular system in addition to the kidney. The use rapamycin also offers positive outcomes in different species, including mouse, rat, and pig (Annes et al., 2012). This indicates that mTOR pathway has a cross-species conserved element. To test this possibility of rapamycin effect on primary cilia, we use both pig and rodent cultured cells. Therefore, our hypothesis is that rapamycin will alter cilia length and function in porcine renal epithelial and mouse vascular endothelial cells.

Materials and Methods

Cell culture

Porcine renal epithelial cells from proximal tubule (LLCPK) and mouse vascular endothelial (ET) cells were cultured to a confluent monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. For our cell proliferation studies, mouse inner medullary collecting duct (mIMCD) cells were cultured using the same conditions as mentioned above. The generations of LLCPK, ET and mIMCD cells have been previously described (Hull et al., 1976, Nauli et al., 2008, Rauchman et al., 1993). Once confluent, cultured cells were incubated with media containing 2% FBS. We used less serum at this time to further induce cell differentiation. Fully differentiated cells tend to have optimal cilia length, and changes in cilia length can be measured more consistently. In some cases, the media contained different concentrations of rapamycin (0.01, 0.1, 1 and 10 μM) for 20 hours. For control experiments, vehicle alone was added to cells in the same manner and volume.

Cilia length analysis

Primary cilia consist of acetylated microtubule structures and were measured by direct immunofluorescence with anti-acetylated α-tubulin staining in the absence or presence of 20-hour incubation with 1 μM rapamycin. The cells were fixed for 10 minutes (4% paraformaldehyde/2% sucrose in PBS) and permeabilized for 5 minutes (10% triton X-100). Acetylated α-tubulin (1:10,000 dilution, Sigma Aldrich, St. Louis, MO) and fluorescein isothiocyanate (FITC)-conjugated (1:1000 dilution, Vector Labs Burlingame, CA) antibodies were each incubated with the cells for 1 hour at 37 °C. Slides were then mounted with DAPI (Southern Biotech, Birmingham, AL) hard set mounting media. Nikon Eclipse Ti-E inverted microscope with NIS-Elements imaging software (version 4.30) was used to capture images of primary cilia. Automated image acquisition was conducted in 100X magnification fields and Z-stacks (0.1 μm slices) to create a large 3D image. This was done to select cilia from a confluent monolayer since it mimics the physiological structure of the epithelial and endothelial cells.

Cytosolic calcium analysis

Cells were grown on glass-bottom plates to enable live microscopy imaging. After incubation for 20-hour without or with 1 μ M rapamycin, cells were loaded with 5 μ M Fura2-AM (TEFLabs, Austin, TX) at 37°C for 30 min. Cells were then washed with DPBS (Dulbecco's Phosphate-Buffered Saline) and observed under a 40 $\times$ objective lens with a Nikon Eclipse Ti-E microscope controlled by Elements software. Cytosolic calcium was observed by recording calcium-bound Fura excitation fluorescence at 340/380 nm and emission at 510 nm. Baseline calcium was observed for 2 minutes prior to data acquisition. Fluid-shear stress was then applied to cells utilizing an Instech P720 peristaltic pump with an inlet and outlet setup. The fluid was perfused on the glass-bottom plates at shear stress of 0.2, 0.6 or 1.0 dyne/cm² for epithelial cells and 2.0, 5.0 or 8.0 dyne/cm² for endothelial cells. After each experiment, the maximum calcium signal was obtained by perfusion of ATP (10 μ M) to confirm cell viability. Conditions for all experiments were maintained at 37°C and 5% CO₂ in a stage top cage incubator (okoLab, Burlingame, CA). Calcium analysis was then followed a standard calculation as previously described (Upadhyay et al., 2014).

Cell cycle analysis

To investigate a possibility of rapamycin effect on cell growth, cells were harvested with and without 1 μ M rapamycin treatment. Cells were then fixed using 70% ethanol and incubated with propidium iodide (PI, 50 μ g/ml), a DNA-intercalating fluorescent molecule, for 30 min at 37°C. To investigate cell cycle more accurately, cells were synchronized by physical separations based on their size and density. Cells were then grown for 36 hours and incubated with 10 μ M BrdU (Invitrogen, Eugene, OR) for 1 hour at 37°C and 5% CO₂. Cells were trypsinized and fixed with 70% ethanol overnight at -20°C. After fixation the cells were permeabilized with 0.5% triton X-100 in PBS for 10 minutes. To denature DNA, a solution of 4N HCl with 1.0% triton X-100 in ddH₂O was used to incubate the cells for 20 minutes at room temperature followed by a quick neutralization step using 0.1M sodium borate, pH 8.5. The cells were then treated with Alexa 488 conjugated BrdU antibody (Invitrogen, Eugene, OR) at 1:25 dilution in PBS for 1 hour at 37°C. Samples were stored in the dark for the antibody incubation and all steps afterwards. The cells were then stained with 50 μ g/ml PI for 30 min at 37°C. Cell analysis was carried out with flow cytometry BD FACSverse with BD FACSuite software.

Statistical analysis

Cilia length measurement consisted of n = 50-70 for each slide preparation, and a total of 4 slides was used in each group. Cytosolic calcium measurements consisted of n = 50 for each plate, and a total of 4 plates were used in each group. Cell cycle analysis consisted of n = 4 and a sample size of 10,000 for each individual experiment. All data are reported as mean $\pm$ standard error of mean with statistical power greater than 0.8 at p < 0.05. Data were analyzed utilizing ANOVA test followed by Tukey post-test for multiple groups. Analysis of data was performed with Prism GraphPad 5 software.

Results

To find concentration of rapamycin that might affect the length of primary cilia, we carried out initial screening of rapamycin at a range of 0 mM to 10 mM in renal epithelial cells. Cilia length analysis using immunofluorescent-staining of acetylated α -tubulin shows the changes in cilia length distribution with different rapamycin concentrations (Figure 1). Treatment with rapamycin at a concentration of 1.0 μ M gave an optimal and consistent percentage of cilia with longer lengths. Concentration of 1.0 μ M was therefore selected for the rest of our experiments. We next confirmed the effect of rapamycin by acquiring the images three-dimensionally for a more precise measurement to account for cilia that appear at different focal planes (Supplemental Movie). In renal epithelial cells, average cilia length was 7.05 $\pm$ 0.15 μ m. When treated with rapamycin (1.0 μ M) for 20 hours, cilia length increased to 9.90 $\pm$ 0.33 μ m showing an increase of almost 3.0 μ m. In vascular endothelial cells the effect of rapamycin on cilia length was more pronounced. Compared to an average cilia length of 3.67 $\pm$ 0.04 μ m in control endothelial cells rapamycin treatment increased cilia length to 6.94 $\pm$ 0.16 μ m, almost twice the length of normal cilia. Statistical analysis showed significant differences in cilia length between the control vs. rapamycin-treatment in epithelial and endothelial cells (Figure 2).

As previous studies have shown, primary cilia responding to fluid flow can be observed through an influx of extracellular calcium (Siroky et al., 2006; Liu et al., 2005). For live-cell acquisition during flow experiments, cytosolic calcium level was measured with fura-2-AM a cell permeant calcium-specific indicator. After baseline measurement, cells were subjected to their optimal shear-stress and fura-2 fluorescence was captured at 510 nm. We observed an increase in the cytosolic calcium levels, which can be seen in representative pseudocolored images which correlate to cytosolic calcium levels (Figure 3). Our data show that rapamycin treatment enhances calcium influx after induction of shear stress in epithelial and endothelial cells (Figure 4A). Comparisons of peak calcium levels between control and rapamycin treated cells show significant increase in the maximum levels of calcium that is achieved upon exposure to shear stress (Figure 4B).

Previous studies in our laboratories have indicated a correlation between cilia length and its ability to sense shear stress (Abdul-Majeed et al., 2012; Upadhyay et al., 2014). To examine if increase in cilia length from the rapamycin-treated

cells would result in a greater sensitivity to fluid shear-stress, we used lower levels of shear stress and measured intracellular calcium influx in response to the simulation. In our epithelial cells we chose a physiologically relevant range of shear stress from 0.2 to a maximum of 1.0 dyne/cm² (Figure 5A). In our control cells we observe a calcium response starting at shear-stress levels $\geq$ 0.6 dyne/cm². At the same level of shear-stress, rapamycin treated epithelial cells show a much higher influx of calcium and the calcium response is even present in a low shear-stress of 0.2 dyne/cm² which is not seen in control cells. However, the trend of increased shear-stress inducing higher cytosolic calcium is seen in control cells, consistent with the idea that cells respond better to an optimal shear-stress level (Doerr et al., 2016; Nauli et al., 2008). This trend is also seen in endothelial cells, which line the vasculature and are exposed to higher levels of shear stress *in vivo* (Figure 5B). In our experiments with endothelial cells we chose to observe the effect of physiologically relevant range of shear stress starting from 2 dyne/cm² to a maximum of 8 dyne/cm². Rapamycin treated cells were able to initiate a calcium response at shear stress $\geq$ 2.0 dyne/cm² while control cells only responded at shear stress $\geq$ 6.0 dyne/cm². Even the cytosolic calcium increase was reduced in control cells at different shear stress compared to rapamycin treated cells. Significant differences were observed between the shear stress-induced calcium influx in both epithelial and endothelial cells when compared to their respective control at varying levels of shear stress (Figure 5C).

Different models have been proposed for mTOR as a central regulator of cell metabolism, growth, proliferation and survival. Studies have shown that inhibition of the mTOR pathway using rapamycin leads to a G0/G1-phase arrest in yeast cells and mammalian lymphocytes (Fingar et al., 2004). It is widely known that cilia formation and elongation occurs during the resting phase. The same effect can be achieved by serum starvation, which results in cilia growing to their full extent (Nauli et al., 2013). With rapamycin known to be an anti-proliferative agent, we next examined the effect of rapamycin treatment on the cell growth distribution and whether growth arrest played a role in ciliary length increase. Significant differences were observed only in epithelial cells, where a higher percentage of cells were non-dividing compared to control (Figure 6). This same effect was not observed in endothelial cells and might be attributed to the difference in species (pig vs. mouse) or cell types (renal epithelia vs. vascular endothelia).

To investigate the possibility of species or cell type contribution on the differential effect of rapamycin, we included mouse renal epithelial cells in our study. BrdU was used to more specifically label dividing cells, and it was coupled with propidium iodide to stain total DNA in each cell. Consistent with previous study, we observed significant effect of rapamycin in cell cycle. However, the effect of rapamycin was only seen in porcine renal epithelial cells (Figure 7A) and not in mouse renal epithelial cells (Figure 7B) or mouse vascular endothelial cells (Figure 7C). A previous study has shown that inhibition of discrete mTOR downstream effectors may also be dependent on rapamycin concentration. The report suggests that a higher dose in the micromolar range of rapamycin was needed to completely block G1 cell cycle progression and that too was dependent on the sensitivity of the cells to rapamycin (Chatterjee et al., 2015). Thus, a potential effect of rapamycin to reduce cell division in endothelial cells remains possible at a higher concentration.

Discussion

Primary cilia are critical sensory organelles that respond to flow in lumen-lining cells by initiating calcium influx into the cytoplasm. Using cytosolic calcium as a readout of ciliary function Upadhyay et al. were able to show that cilia sensitivity to fluid-shear stress correlates with the length of cilia (Upadhyay et al., 2014). Our current studies indicate that rapamycin increases cilia length and enhances cilia sensitivity. We therefore hypothesize that rapamycin might increase cell sensitivity in response to fluid-shear stress through cytosolic calcium increase (as a readout) by enhancing cilia length and function. This, in turn, might have clinical relevance in preserving organ functions (Figure 8). In particular, many rodent models with no or short cilia length result in ciliopathy phenotypes, including cystic kidney, developmental delay, intellectual disability, and many others (Wallmeier et al., 2016; Ha et al., 2016; Lu et al., 2016; Zhang et al., 2011). Because cilia formation and function have been associated with cell cycle (AbouAlaiwi et al., 2011; Plotnikova et al., 2009), we also investigated the roles of rapamycin in cell division. Our studies indicate that the effect of rapamycin on cell cycle may be species-dependent, although we cannot rule out that different species may have different sensitivity toward rapamycin.

Novel insights provided by *in vitro* studies and animal models may be utilized into designing treatments that improve ciliary structure/function. In the case of PKD, treatment strategies have included drugs that target disrupted mechanisms such as intracellular calcium, cAMP, CFTR chloride channels, or mTOR signaling (Torres, 2010; Yang et al., 2008; Magenheimer et al., 2006). Previous studies show that the mTOR pathway is aberrantly activated in PKD cells (Pei, 2010; Shillingford et al., 2006; Shillingford et al., 2010). mTOR is a serine/threonine kinase that provides the catalytic subunit for two distinct multi-protein complexes, mTORC1 and mTORC2. mTORC1 acts as a metabolic sensor and is regulated by availability of amino acids, growth factors and energy stores. Activation of mTORC1 promotes both cell growth and proliferation. mTORC2 has been shown to function as an important regulator of the cytoskeleton organization, cell survival and polarity (Lieberthal and Levine, 2012; Loewith et al., 2002). West-

ern blot analysis of orthologous animal models and humans with PKD show increased activation, i.e. phosphorylation of mTORC1 downstream targets and the 4E-BPs (Zafar et al., 2010). Rapamycin binds to FKBP12 and can inhibit both mTORC1 and, to a lesser extent, mTORC2 signaling (Zeng et al., 2007, Sarbassov et al., 2006). Rapamycin may induce glomerulonephritis and intratubular cast formation in protein overload nephropathy (Coombes et al., 2005, Marti and Frey, 2005). Nonetheless, treatment with rapamycin slows down cyst progression and improves renal function in animal models of PKD (Shillingford et al., 2006, Tao et al., 2005, Wahl et al., 2006, Zafar et al., 2010). Although the effect of reducing cyst burden by rapamycin has not been consistent in all animal models or in trials with PKD patients, substantial evidence linking aberrant mTOR activity to cystogenesis suggests that mTOR might be one of complex intertwined pathways in the pathogenesis of PKD and other ciliopathies.

We use both porcine and mouse cells in the present studies. The porcine cells were generated previously and have been used because humans are more closely related to pigs than to rodents, particularly with regard to renal physiology (Heussner and Dietrich, 2013, Hull et al., 1976). These porcine cells also display inherent characteristics of structural organization and transepithelial transport functions similar to those renal epithelia observed under *in vivo* conditions within the proximal tubules. Thus, it is thought that these porcine cells might represent an alternative to human primary cells. The mouse cells were generated previously from aorta and retain its vascular properties and endothelial markers (Abou Alaiwi et al., 2011, Brookes et al., 2013, Nauli et al., 2008). The functional studies of these cells have also shown that these cells retain cellular responses to various mechanical forces and pharmacological agents. In addition, these cells have normal directional migration, monolayer permeability and cytoskeletal organization (Jones et al., 2012).

Endothelial cells, which line the inner lumen of blood vessels, depend on cilia to sense the shear stress. Observations of vascular endothelial cells lacking cilia show that without functional cilia, there is no calcium influx in response to fluid flow (Nauli et al., 2008). This prevents the vasculature to adapt with variable blood flow levels since the bending of primary cilia also regulates the production of nitric oxide (NO), an important vasodilator (Nauli et al., 2008). Abnormalities in cilia function have been associated with defective NO production resulting in hypertension (Rahbari-Oskoui et al., 2014), vascular aneurysm (Aboualawi et al., 2014) in addition to cyst formation in the kidneys (Eder and Schrier, 2009).

In our study, we observed that rapamycin has an effect in the cilia of porcine epithelial and mouse endothelial cells. The results show that in addition to increasing cilia length, rapamycin-treated cells showed a higher cytosolic calcium response when challenged to fluid flow. This is in agreement with previous studies done with dopaminergic agents that enhanced cilia length and function (Upadhyay et al., 2014). Typically, ciliogenesis occurs during the G0/G1 phase when the centriole becomes the basal body that serves as a foundation for attachment of ciliary subunits. With the help of interflagellar transport (IFT) proteins the cilia keeps elongating till it reaches its determined length. This induction of optimum ciliary length can be observed in cilia of cells subjected to serum deprivation to induce a G0/G1 arrest. Because rapamycin is an antiproliferative agent, we did cell cycle analysis to determine if our concentration of 1 μ M rapamycin caused any changes to the cell cycle distribution, which could have affected our ciliary length observations. Our FACS results show that only porcine epithelial cells show a slight increase population of cells in the G0/G1 phase. To examine whether this was a cell line specific or species-specific response we used mouse renal epithelia and observed no significant changes in cell cycle distribution.

In healthy renal epithelia or vascular endothelia, primary cilia bend when exposed to physiological flow and initiate an increase in cytosolic calcium in addition to initiating modulation of other signaling pathways important for normal tissue homeostasis. Using influx of extracellular calcium as a marker of cilia function in response to fluid flow, we were able to observe an increase in ciliary function with rapamycin treatment.

Rapamycin has been effective in decreasing cyst progression but its effect on the cilia structure/function has not received much attention. Primary cilia have been established as important flow sensing organelles and shortened cilia fail to translate the mechanical stimulation to downstream intracellular responses. Recently studies showed that primary cilia can regulate mTOR activity (Aznar and Billaud, 2010, Boehlke et al., 2010). MDCK cells grown under permanent fluid flow for several days showed decreased mTOR activity compared to cilia-less Kif3a knockdown cells. This *in vitro* study demonstrated that flow-induced bending of the cilium activates Lkb1-mediated phosphorylation of AMPK at the basal body of the cilium, inhibiting mTORC1 activity and regulating cell size (Boehlke et al., 2010). The ability of cilia to silence mTOR and the reciprocal increase in cilia length by mTOR inhibition indicate that the cilia-mTOR pathway may have much more in common than previously thought. Although the rationale of rapamycin treatment for PKD or TSC hinges on the aberrant mTOR pathway, our study shows that rapamycin plays a role in cilia length/function providing tentative evidence that rapamycin could potentially be used as a ciliotherapy. In healthy cells there is a defined range of cilia length and it is dependent on the shear stress experienced by the system. In places with high levels of shear stress,

such as the vasculature, cilia are usually shorter. In the renal nephron where the filtrate flows comparatively slowly, the epithelial cells have longer cilia that can extend outward into the lumen to sense flow, which is unhindered by the drag forces near the wall.

Our studies offer a fundamental idea that rapamycin has an effect on primary cilia. Whether effect of rapamycin on cilia is direct or indirect, future studies are warranted for a better in-depth understanding to extrapolate the results from animal cells into clinical practice. Importantly, our results require further research to understand the pathways connecting the primary cilium, mTOR signaling and cystogenesis in ciliopathies.

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Figure legends

Figure 1

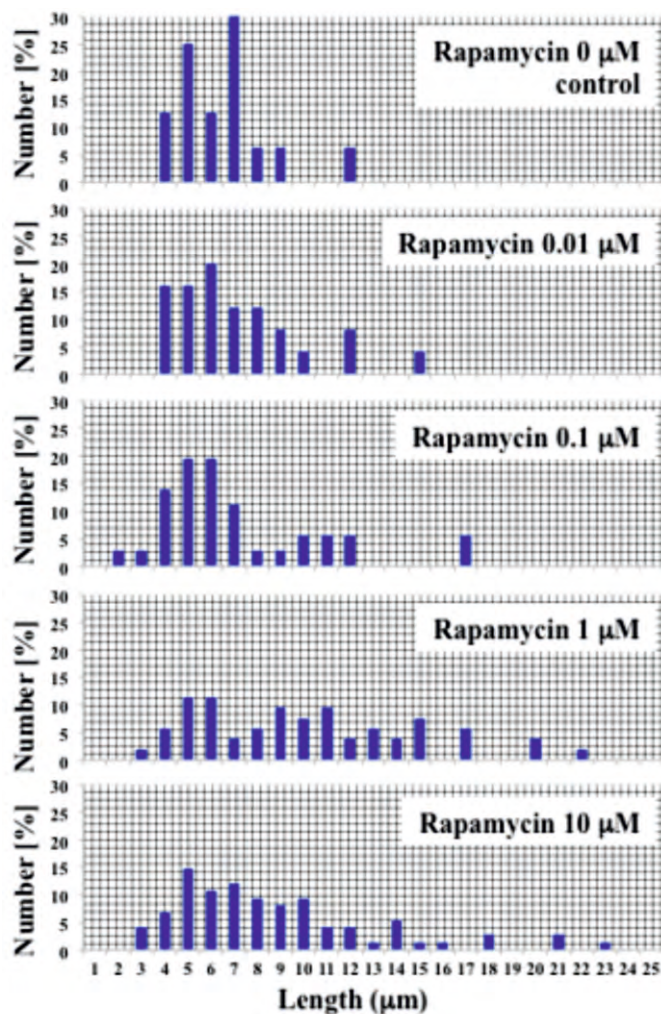


Figure 1. An initial screening indicates that rapamycin might increase the length primary cilia length in renal epithelial cells.

Cells were treated with various concentrations of rapamycin (0 to 10 mM). Length measurements were made from images taken at one single plane in triplicate. Cilia length was grouped in a discrete range, and percent distribution was tabulated for each rapamycin concentration.

Figure 2

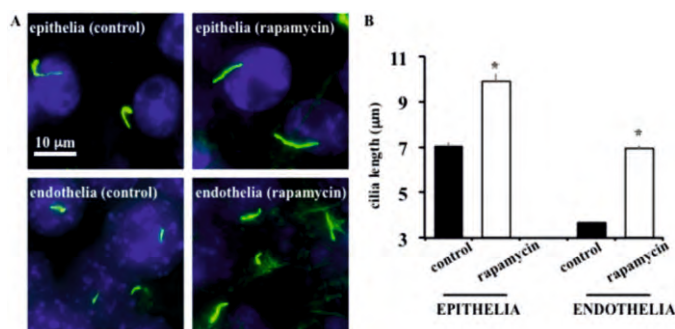


Figure 2. Treatment with rapamycin (1 µM) increases primary cilia length in epithelial and endothelial cells.

(A) Cells were stained with ciliary marker acetylated- α -tubulin (green) and a nuclear marker (DAPI; blue). Rapamycin treatment increased cilia length in both cell lines. Each image was compiled from different z-stack captures. (B) Cilia length was significantly longer in rapamycin-treated cells, with a two-fold increase observed in endothelial cells. N=50-70 for each slide preparation, and a total of 4 slides was used in each group. * = $p < 0.05$ compared to corresponding controls.

Figure 3

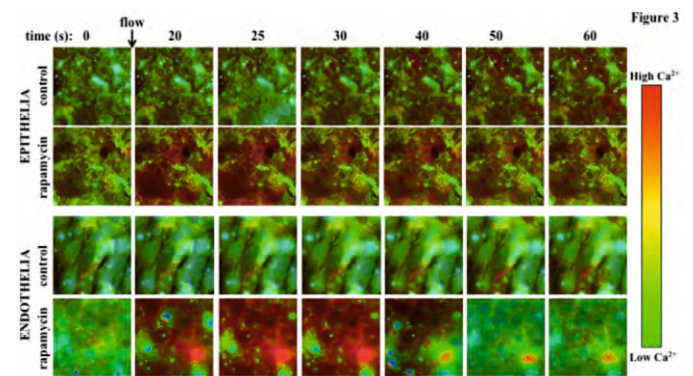


Figure 3. Fluid-shear stress induces calcium influx.

Representative Fura images at different time points from each group are shown, and the arrow indicates the start of fluid flow. Color bars indicate cytosolic calcium levels from low (green) to high (high).

Figure 4a

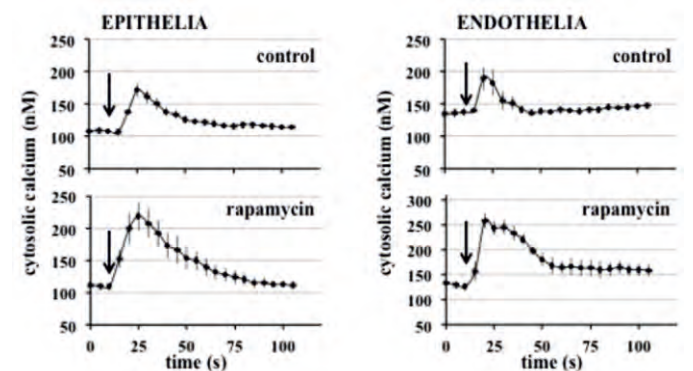


Figure 4b

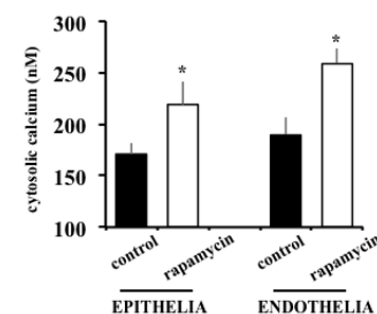


Figure 4. Flow-induced calcium influx into the cytoplasm is increased in cells treated with rapamycin.

(A) Intracellular calcium was measured in response to fluid-shear stress. The arrows indicate start of fluid flow. N=50 cells for each preparation, and a total of 4 preparations was used in each group. (B) Cilia function is assessed as peak of calcium influx in response to fluid-shear stress. Average peak calcium levels in control and rapamycin-treated cells are shown. Calcium peak is used to determine cilia function in response to fluid-shear stress. Cilia function is significantly greater in rapamycin-treated than in control cells. N=50 cells for each preparation, and a total of 4 preparations was used in each group. * = $p < 0.05$ compared to corresponding controls.

Figure 5a

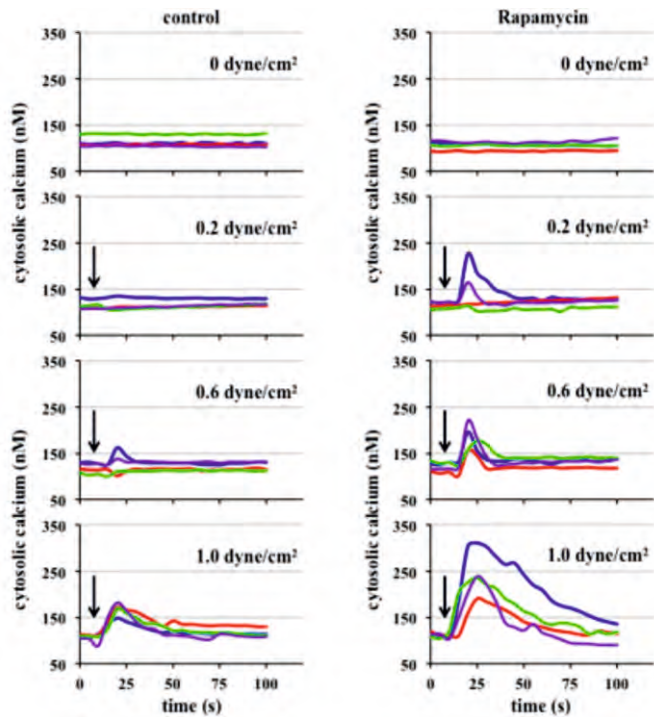


Figure 5b

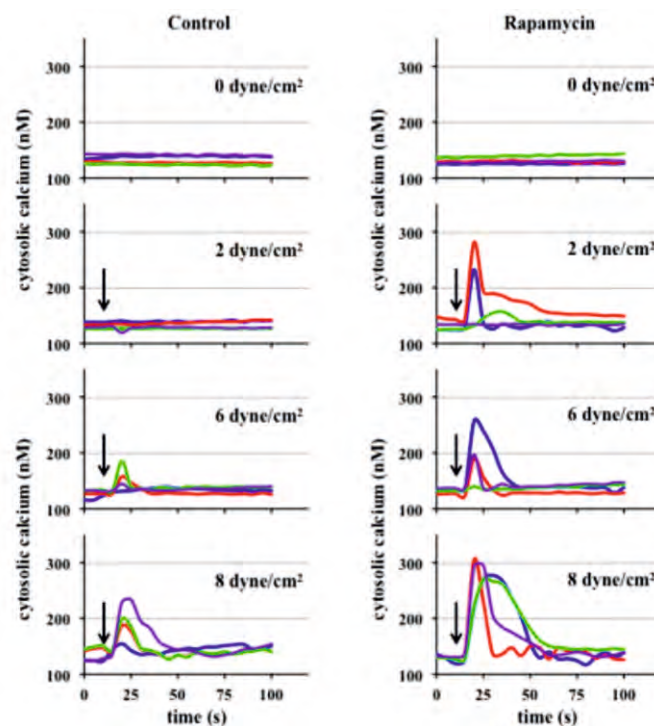


Figure 5c

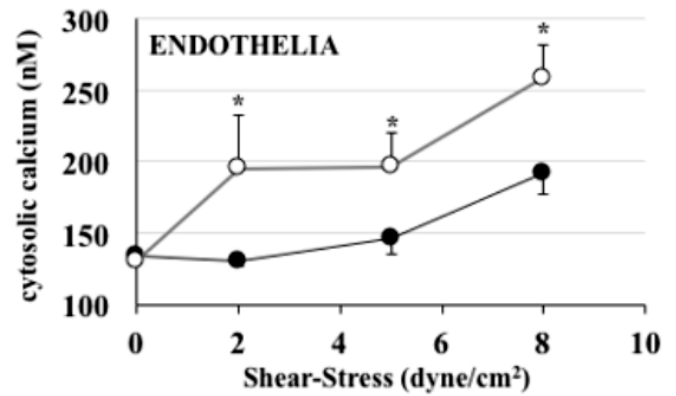
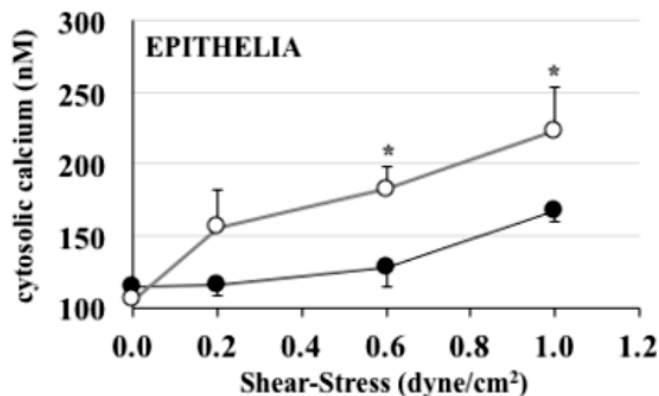


Figure 5. Rapamycin-treated cells show higher calcium influx in response to increasing various shear-stress forces.

In the time-lapse graph, baseline calcium levels were measured, and shear stress was applied at ~10 seconds (arrows). For epithelial cells a physiologically relevant range of 0-1.0 dyne/cm² was used and for endothelial cells which experience higher ranges maximum shear stress used was 8 dyne/cm². (A) In renal epithelial cells, rapamycin treated cells showed higher levels of peak calcium in response to shear stress of varying magnitude. A shear stress of 0.2 dyne/cm² was enough to cause calcium influx in rapamycin-treated cells. (B) Same effect was observed in vascular endothelial cells where rapamycin treatment increased the shear stress-induced calcium influx compared to control cells. (C) Peak calcium levels are shown on the graph at different levels of shear stress. In epithelial cells, significant increase in calcium influx was observed at shear stress levels ≥ 0.6 dyne/cm² in rapamycin-treated vs. control cells. Endothelial cells also show significant increase in calcium influx when exposed to different levels of shear stress. For each calcium measurement, and a total of 4 experiments with N=50 for each was used. * = $p < 0.05$ compared to corresponding controls.

Figure 6

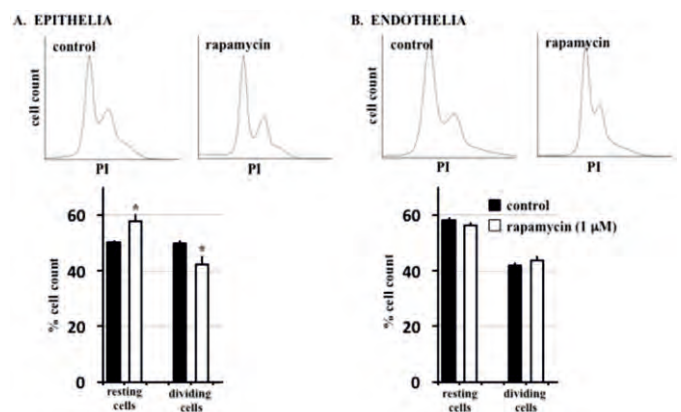


Figure 6. Treatment with rapamycin shows a minimal effect on the cell growth.

Cells were stained with propidium iodide (PI), a DNA intercalating agent, for flow cytometry analysis. (A) Epithelial cells showed a slight but significant increase in cells at the resting (G0) stage and lower percentage of cells undergoing mitosis when treated with rapamycin. (B) For endothelial cells, no significant variation in cell cycle was seen in rapamycin-treated cells compared to control. * = $p < 0.05$ compared to corresponding controls.

Figure 7

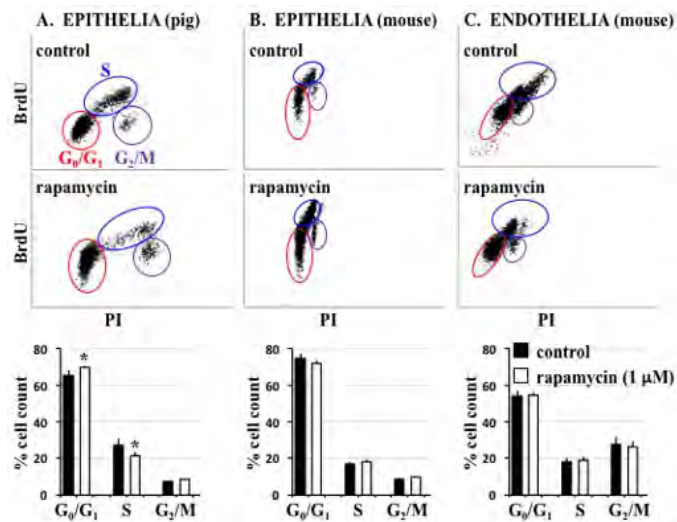


Figure 7. Treatment with rapamycin causes G₁ phase arrest in porcine cells but not in mouse-derived cells.

In our cell cycle assay, BrdU incorporation was used to resolve cell cycle phases. (A) In epithelial cells treated with rapamycin the cell population was shifted more towards a G₀/G₁ phase by a significant amount. The rapamycin treated cells also had lower levels of BrdU incorporation indicating a G₁ phase arrest. The population of cells in S-phase were found to be significantly reduced after rapamycin treatment. For mice epithelial (B) and endothelial (C) cells, no significant variation in cell cycle was seen in rapamycin-treated cells compared to control. * = p < 0.05 compared to corresponding controls.

Figure 8

Hypothetical Model:

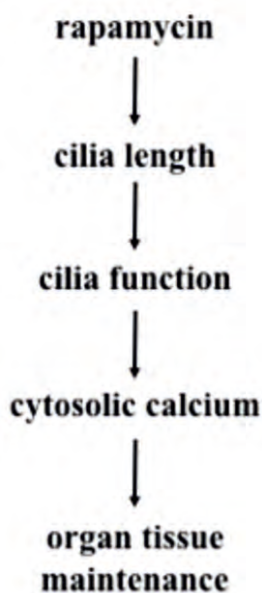


Figure 8. Hypothetical working model of rapamycin on cilia length and function.

Rapamycin increases cell sensitivity in response to fluid-shear stress. A greater sensitivity or response to shear stress is postulated to be a result of longer primary cilia. This, in turn, increases cilia function as denoted by an increase in shear-induced cytosolic calcium flux. Optimal length and function of primary cilia are thought to be required for proper architecture of tissue maintainane.

Supplemental Movie

Shown here is a representative approach of our data analysis on cilia length. To enable a more accurate measurement of cilia, the images of primary cilia (green) and nucleus (blue) were taken three-dimensionally. This allows measurement of cilia that are within a single focal plane and at a different focal plane.



Chronic Hypobaric Hypoxia Modulates Primary Cilia Differently in Adult and Fetal Ovine Kidneys

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Hypoxic environments at high altitude have significant effects on kidney injury. Following injury, renal primary cilia display length alterations. Primary cilia are mechanosensory organelles that regulate tubular architecture. The effect of hypoxia on cilia length is still controversial in cultured cells, and no corresponding *in vivo* study exists. Using fetal and adult sheep, we here study the effect of chronic hypobaric hypoxia on the renal injury, intracellular calcium signaling and the relationship between cilia length and cilia function. Our results show that although long-term hypoxia induces renal fibrosis in both fetal and adult kidneys, fetal kidneys are more susceptible to hypoxia-induced renal injury. Unlike hypoxic adult kidneys, hypoxic fetal kidneys are characterized by interstitial edema, tubular dispartition and atrophy. We also noted that there is an increase in the cilia length as well as an increase in the cilia function in the hypoxic fetal proximal and distal collecting epithelia. Hypoxia, however, has no significant effect on primary cilia in the adult kidneys. Increased cilia length is also associated with greater flow-induced intracellular calcium signaling in renal epithelial cells from hypoxic fetuses. Our studies suggest that while hypoxia causes renal fibrosis in both adult and fetal kidneys, hypoxia-induced alteration in cilia length and function are specific to more severe renal injuries in fetal hypoxic kidneys.

Keywords: cilium, kidney injury, mechanosensor, pO₂, sheep, shear-stress, renal fibrosis

INTRODUCTION

Hypoxic environments in humans can result in the high-altitude renal syndrome (HARS) (Arestegui et al., 2011). HARS is characterized by reduced renal plasma flow (Becker et al., 1957), microalbuminuria (Becker et al., 1957), proteinuria (Jefferson et al., 2002; Chen et al., 2011), hyperuricemia (Jefferson et al., 2002) and systemic hypertension (Jefferson et al., 2002; Gilbert-Kawai et al., 2016). The kidneys have a fundamental role in adaption to regulate body fluids, electrolyte and acid-base homeostasis during acclimatization to high altitude and in mountain sickness syndromes. Kidneys also respond to hypoxic diuresis and natriuresis through inhibition of renal tubular sodium reabsorption, in addition to erythropoietin production (Haditsch et al., 2015).

Kidneys are very susceptible to lowered oxygen tensions (Mayer, 2011; Fu et al., 2016). In acute hypoxia, kidneys exhibit defense mechanisms centered around the activation of hypoxia-inducible

factor (HIF) (Minet et al., 2000; Rosenberger et al., 2006). HIF up-regulates pro-angiogenic factors that protect against capillary rarefaction. HIF also increases expression of matrix metalloproteinases that effect repair and protect against fibrosis by degrading extracellular matrix. In chronic hypoxia, however, HIF mRNA is destabilized, shutting-down the kidney's initial protective mechanisms (Uchida et al., 2004). The expression of pro-angiogenic factors is repressed, and the expression of dys-angiogenic factors is increased. This may thus result in capillary rarefaction and potential fibrosis.

Primary cilia are sensory organelles that sense extracellular milieu along the tubule of a nephron. Abnormal cilia length and function result in polycystic kidneys (Nauli et al., 2003, 2006; Xu et al., 2007). Renal cilia also display length and function alterations following kidney injury. Primary cilia play a crucial role in cisplatin-induced tubular apoptosis by regulating tubular apoptotic pathway (Wang et al., 2013). Primary cilia also undergo abnormal changes in renal transplant biopsies with acute tubular injury (Hayek et al., 2013). During injury, renal cilia decrease in length in both humans and mice (Verghese et al., 2008, 2011). The shortened cilia length serves to attenuate cilia-mediated signaling pathways in response to extracellular stress, which promotes infiltration of neutrophils and macrophages in the kidney (Prodromou et al., 2012). However, the effects of chronic hypobaric hypoxia on primary cilia have yet to be investigated.

Changes in fluid-shear stress generated by urine movement can also contribute to kidney injury if the primary cilia are not functioning normally. Thus, the change in urinary flow associated with nephropathies has been proposed to be a potential insult for tubular cells leading to disorganization of the tubular epithelium (Maggiorani et al., 2015).

Based on the evidence above, it has been proposed that cilia play important roles in sensing environmental cues caused by injury and in the repair process for reestablishing a new epithelial layer of differentiated cells (Bell et al., 2011; Wang and Dong, 2013). By using a series of human renal transplant biopsies, it was found that acute tubular necrosis is associated with more than two-fold longer cilia 1-week after kidney injury, and normalization of cilium length occurred at a later stage. These results indicate that cilia function could be a clinically relevant indicator of kidney injury and repair in patients with kidney transplantation (Wang and Dong, 2013).

Despite the fact that hypoxic environment at high altitude may have significant effects on the kidneys, the histological analysis of renal architecture has never been examined especially in the fetus. Given the background information, our premises indicate that hypoxia causes renal injury/damage and that renal injury often includes altered cilia structure/function. Thus, our hypothesis is that hypoxia causes altered cilia structure/function. Here, we examined the effects of hypoxic high-altitude on the kidneys in adult and fetal sheep. We also took this opportunity to study cilia length-function relationship in response to chronic hypobaric hypoxia, because it has been reported that HIF could alter the structural length of primary cilia in hypoxic *in vitro* models (Verghese et al., 2011; Ding et al., 2015; Lavagnino et al., 2016; Resnick, 2016).

METHODS

The sheep were purchased from Nebeker Ranch, Inc. (Lancaster, CA). The normoxic control animals were maintained at sea level throughout the gestation period (300 m). To induce chronic high-altitude hypoxia, the animals were then transported at 30 days of gestation to the Barcroft Laboratory, White Mountain Research Station at Bishop, CA (3,801 m). The hypoxic animals stayed in Bishop for 110 days. Both normoxic control and hypoxic animals were fed Alfalfa hay in a "keyhole" feeder in addition to providing a mineral block *ad libitum*. The animals were transported from Bishop to the laboratory immediately before the studies (Dasgupta et al., 2012; Thorpe et al., 2013).

Two-years-old pregnant sheep and near-term 146 gestational day fetal lambs were used. Please note that sheep gestation typically lasts ~150 days. The sheep were anesthetized with thiamylal (10 mg/kg, i.v.) followed by inhalation of 1.5–2.0% halothane. An incision was made in the abdomen, and kidneys were isolated and immediately placed in either 10% buffer formalin or the phosphate-buffered sucrose solution (30% sucrose, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 KH₂PO₄, 1 mM CaCl₂•2H₂O and 0.5 mM MgCl₂•6H₂O at pH 7.4). All procedures and protocols were conducted in full compliance with the Animal Welfare Act, followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at Loma Linda University, CA.

Tissue Processing

Kidney cross-sections of the cortex and medulla were dehydrated with isopropyl alcohol and infiltrated with parañ n in a tissue processor (Excelsior AS, Thermo Scientific, Inc.). Tissues were allowed to solidify in a base mold and then snapped into plastic tissue cassettes using a parañ n molding processor (HistoStar, Thermo Scientific, Inc.). Samples were cut to 5 µm thickness sections with a microtome (HM 355 S, Thermo Scientific, Inc.), and continuous "ribbon" of the sections were formed. Sections were carefully transferred onto a 40°C water dish for about 3–5 min to flatten and avoid wrinkles in the sectioned tissues.

Tissue slices were placed on the charged microscope slides, and deparañ nization was performed by placing the slides in a 60°C oven for 30 min in order to melt any extra parañ n. Slides were rinsed twice with a xylene solution for 5 min each, followed by hydration through a series of washing for 1 min each in decreasing alcohol concentrations (100, 95, 80, 70%). Slides were then submerged in the distilled water for 3 min for the staining process.

Tissue Staining

For H&E staining, tissues were stained with hematoxylin for 5 min, rinsed with tap water for 1 min and dipped for 1 sec in acid alcohol (200 ml of 50% alcohol containing 500 µL HCl 5N). Tissues were then stained with Eosin for 2 min and rinsed with distilled water for 1 min followed by dehydration with 95% and 100% alcohol each for 1 min and cleaning with xylene for 2 min. Coverslip was mounted onto a slide with xylene compatible mounting media.

For periodic acid Schiffs (PAS) staining, staining kit (Polysciences, Inc.) was used and experiments were performed based on the Company's protocol. Briefly, tissues were oxidized in 0.5% periodic acid for 5 min. After washing with deionized (DI) water, tissue sections were placed in Schiff's reagent for 15 min then rinsed with 0.55% potassium metabisulfite. Slides were washed in tap water for 10 min to allow the color to develop in the tissues, which were then counterstained with acidified Harris Hematoxylin for 30 s. Slides were washed with tap water until the tissues turned to a blue color. After dehydration with 95 and 100% alcohol each for 1 min and clearing with xylene for 2 min, the coverslips were mounted onto the slides with a xylene compatible mounting media.

For Masson's Trichrome staining, a staining kit (Polysciences, Inc.) was used according to the manufacture's instructions. Briefly, tissues were incubated in Bouin's fixative solution at 60°C for 1 h and rinsed with warm tap water for 2–3 min until the yellow color disappeared. Fresh Weigert's iron hematoxylin working solution was prepared by mixing Weigert's hematoxylin A and Weigert's hematoxylin B in a 1:1 ratio. Tissues were incubated in the working solution for 20 min and rinsed for 5 min with distilled water. Tissues were then stained with Biebrich Scarlet-Acid Fuchsin solution for 15 min and rinsed with tap water to remove any extra solution-residue. After rinsing with distilled water, the tissues were soaked in Phosphotungstic / Phosphomolibdic acid for 10 min, transferred to Aniline Blue for 7 min and washed with distilled water until the slides were clear. Tissues were then soaked in 1% acetic acid for 1 min and washed with distilled water for 1 min. After dehydration with 95 and 100% alcohol each for 1 min followed by cleaning with xylene for 2 min, the coverslips were mounted onto the slides with a xylene compatible mounting media.

For immunofluorescence staining, heat-induced epitope retrieval was performed using a pressure cooker and Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0). Tris-EDTA buffer was first boiled in the pressure cooker to 100°C. Tissues were then maintained in the pressure cooker at about 121°C and 15 psi for 8 min. The pressure cooker and slides were then chilled quickly with cold tap water. The slides were rinsed three times with phosphate buffer saline (PBS) and blocked in PBS solution containing 5% bovine serum albumin for 15 min in a humidifying chamber. Using our standard protocol (Loghman-Adham et al., 2003; Nauli et al., 2003, 2006), tissues were stained with the ciliary marker acetylated- α -tubulin (1:1,000 dilution; Sigma-Aldrich, Inc.), distal tubular marker bolichos biflorus agglutinin (DBA; 4:1,000 dilution; Vector Laboratory, Inc.), proximal tubular marker lotus tetragonolobus lectin (LTL; 4:1,000 dilution; Vector Laboratory, Inc.), and nucleus marker DAPI (Vector Laboratory, Inc.).

Primary Cell Culture

Isolation of renal epithelia from fresh tissues has been previously described in detail (Loghman-Adham et al., 2003; Nauli et al., 2003, 2006). After extensive washing in phosphate-buffered sucrose solution, the cortex and medullary regions of kidney were dissected into pieces and incubated with collagenase followed

by 0.05% trypsin/0.53 mM EDTA at 37°C for 15–20 min. After vortexing vigorously for 5 min, ice-cold HBSS containing 10% FBS was added to inactivate the trypsin. The cells were further released from the fibrous basement membrane by trituration, washed twice with HBSS. Cells were then sorted based on their surface markers for DBA or LTL and resuspended in fresh culture medium. To allow attachment, the cells was grown in DMEM containing 10% FBS and supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenium, 36 ng/ml (10^{-7} M) hydrocortisone, 10^{-8} M triiodothyronine, 10 ng/ml EGF, and 50 ng/ml PGE1, as well as 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 37°C humidified incubator ventilated with 5% CO₂–95% O₂. The culture medium was changed every 2–3 days until confluency was reached.

Cilia Function and Length Measurements

Bending primary cilia with fluid-flow activates the cilium and increases cytoplasmic calcium in renal epithelial cells (Praetorius and Spring, 2001; Liu et al., 2005; Jin et al., 2014). Thus, intracellular calcium was measured with Fura2-AM [to study cilia function] as previously described (Nauli et al., 2013). Briefly, after pre-incubation with 5 μ M Fura2-AM for 30 min at 37°C, the tissues were equilibrated for at least a minute. The optimal shear-stress of 0.8 dyne/cm² was used to monitor changes in cytosolic calcium as previously described (Nauli et al., 2013). Ionomycin (1 μ M) was used at the end of each experiment to confirm Fura-2 loading and determine minimal and maximal ratiometric fluorescence signals. Cells were then analyzed for cilia length by staining with the ciliary marker acetylated- α -tubulin by staining with the ciliary marker acetylated- α -tubulin as previously described (Loghman-Adham et al., 2003; Nauli et al., 2003, 2006).

Data Analysis

All images represent the extracted information from the digital pictures. Images were captured through a Nikon Ti-E microscope. The Nikon NIS Elements for Advanced Research software was used for image capture and analysis, including automatic object recognition, image scanning and color binary segmentation. Scale bars were provided in all figures to indicate the actual image reduction size. All morphometric data were reported as mean $\pm$ SEM. Distribution analyses were performed and presented on all data sets to verify normal data distributions. After distribution and variance analyses, data comparisons for more than two groups were performed using ANOVA test followed by a Tukey *post-hoc* analysis. The correlation between cilia length and cilia function was analyzed with the ordinary least squares (OLS) regression of y on x, because the ordinary least products (OLP) would not allow analysis of covariance as a technique for testing the equality of slopes or intercepts in linear regressions (Ludbrook, 2012). Pearson correlation coefficients were therefore used to analyze the significance of differences in the coefficients correlating primary cilia length and function. Asterisks (*) denote with statistical significance differences at $p < 0.05$ relative to corresponding control groups as indicated in the figures.

A total of 12 sheep and 12 lambs was used; for each normoxic or hypoxic group, 6 sheep and 6 lambs were used ($N = 6$ sheep and $N = 6$ lambs for normoxia; $N = 6$ sheep and $N = 6$ lambs for hypoxia). From each animal, both kidneys were collected for different studies. Each pair of kidneys were randomly selected for an immediate fixation in formalin or trypsinization for cell culture. From each kidney, a minimum of 10 experimental replicates was sampled. All statistical analysis was done with *GraphPad Prism*, version 5.0b.

RESULTS

The normoxic control animals had maternal and fetal arterial PO_2 (PaO_2) of 102 ± 2 and 25 ± 1 mmHg, respectively. The hypoxic maternal and hypoxic fetal PaO_2 were 60 ± 2 and 19 ± 1 mmHg, respectively.

Fetal Hypoxic Kidneys Were Characterized by Interstitial Medullary Edema

Representative images of H&E stained renal tissue sections from normoxic and hypoxic kidneys are presented (Figure 1A). In all kidney sections, light microscopy analysis indicated that the cortex regions of the kidneys had normal glomeruli. There were no significant differences in glomerulus size between normoxic and hypoxic kidneys (Figure 1B). Capillary loops of the glomeruli were also normal, and the number and morphology of endothelial cells were comparable between normoxic and hypoxic tissues.

In normoxic fetal kidneys, all tubules were closely spaced in the interstitium. Tubules were lined with a single layer of epithelia and well-organized nuclei. In hypoxic fetuses, the renal cortex demonstrated some evidence of mesangial and intercapillary cell proliferation. The renal medulla showed apparent atrophic tubules, interstitial fibrosis and edema, and tubular dispartition characterized by greater inter-tubular spaces and separation of tubules filled with edema (Figure 1C).

While all tubules in adult sheep were closely spaced in the interstitium and lined by a single layer of cells with well-organized nuclei, some abnormalities were observed in the chronically hypoxic kidney. The hypoxic kidneys showed some proliferation of mesangial cells in the cortex and slight tubular edema in the medullary region. Otherwise, the surrounding tubules appeared normal in both normoxic and hypoxic adult kidneys.

Hypoxia Modulated Medullary Tubular Basement Membrane Thickness

PAS staining was done to highlight basement membranes of glomerular capillary loops and tubular epithelia. Renal tissue sections from normoxic and hypoxic kidneys are shown in the representative images (Figure 2A). There were no significant differences in basement membranes thicknesses of glomerular capillary loops between normoxic and hypoxic adult kidneys.

Basement membrane thicknesses of the tubules were significantly less in hypoxic fetal kidneys compared to normoxic fetal kidneys (Figure 2B). Although a reverse trend was

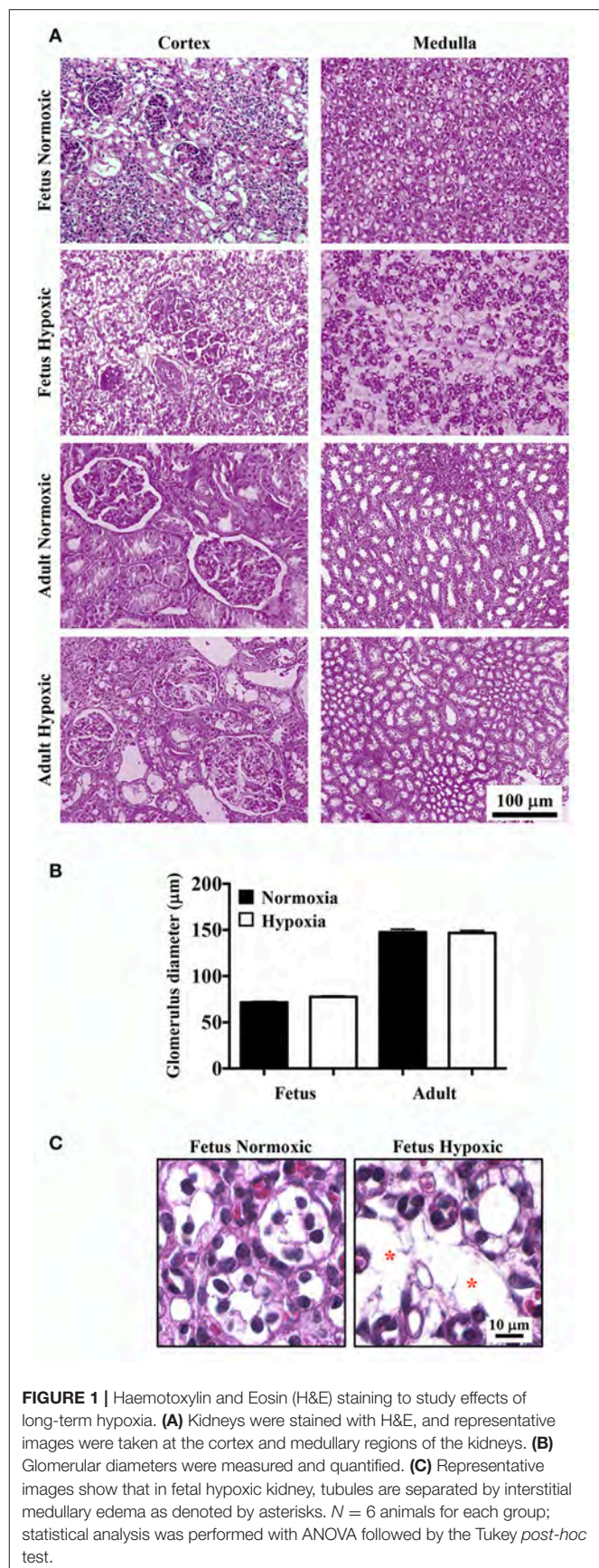


FIGURE 1 | Haematoxylin and Eosin (H&E) staining to study effects of long-term hypoxia. (A) Kidneys were stained with H&E, and representative images were taken at the cortex and medullary regions of the kidneys. (B) Glomerular diameters were measured and quantified. (C) Representative images show that in fetal hypoxic kidney, tubules are separated by interstitial medullary edema as denoted by asterisks. $N = 6$ animals for each group; statistical analysis was performed with ANOVA followed by the Tukey *post-hoc* test.

observed in adult kidneys, it was not statistically significant in adult normoxic or hypoxic kidneys. To refine the statistical analysis, tubular membrane thicknesses were re-measured by discriminating the tubular origins. In proximal tubules, no significant difference in tubular membrane thickness was observed (Figure 2C). In distal collecting tubules, tubular membranes were significantly thicker in hypoxic than normoxic adult kidneys. As expected, tubular membrane thicknesses were significantly less in hypoxic compared to normoxic fetal kidneys.

As observed in the H&E staining, hypoxic fetal kidneys showed interstitial medullary edema, fibrosis, tubular disparition and atrophy characterized by greater inter-tubular space (Figure 2D).

Long-Term Hypoxia Induced Renal Fibrosis

To examine potential fibrosis in the kidneys, Masson's Trichrome staining was performed to highlight deposition of collagen and fibrin fibers. Renal tissue sections from normoxic and hypoxic kidneys are shown in representative images (Figure 3A). The percentage of blue-stained area was calculated using a binary threshold program, which indicated significant fibrosis in hypoxic compared to normoxic kidney tissues (Figure 3B). In normoxic tissues, there was some collagen fibers that were normally distributed around renal tubules. In hypoxic tissues, normal distributions of collagen fibers were observed, but abnormal depositions of collagen/fibrin fibers were also observed throughout tubular epithelia and interstitial spaces.

As observed in the H&E and PAS staining, hypoxic fetal kidney showed interstitial medullary edema characterized by more inter-tubular space (Figure 3C). As also seen in previous staining, hypoxia modulated lumen size of distal tubules in both fetal and adult kidneys (Figure 3D). No significant changes in lumen size were observed in proximal tubules.

Fetal Hypoxic Kidney Was Characterized by Longer Renal Epithelial Primary Cilia

It remains uncertain if hypoxia can maintain and stabilize the primary cilia or it would inhibit primary cilia formation (Verghese et al., 2011; Ding et al., 2015; Lavagnino et al., 2016; Resnick, 2016). To investigate the effect of chronic hypoxia, primary cilia were labeled with the cilia marker acetylated- α -tubulin. Representative images of renal tissue sections from normoxic and hypoxic kidneys are shown for staining of cilia and tubular markers. Distal collecting (Figure 4A) and proximal (Figure 4B) tubular markers were used to identify cilia length in respective tubules. Cilia measurements were the represented in the bar graphs to compare the distributions of the cilia length (Figure 4C). While hypoxia did not significantly alter cilia length in adult kidneys, fetal kidneys were very susceptible to hypoxia (Figure 4D). Cilia length was significantly longer in hypoxic fetal kidneys than normoxic fetal kidneys.

Of note, that the distal collecting tubules had larger lumen sizes in adult than fetal kidneys. This may be associated with longer cilia in adult than fetal distal collecting tubules. For the first time, our studies show that the length of primary cilia in distal collecting tubules become longer during maturation, while

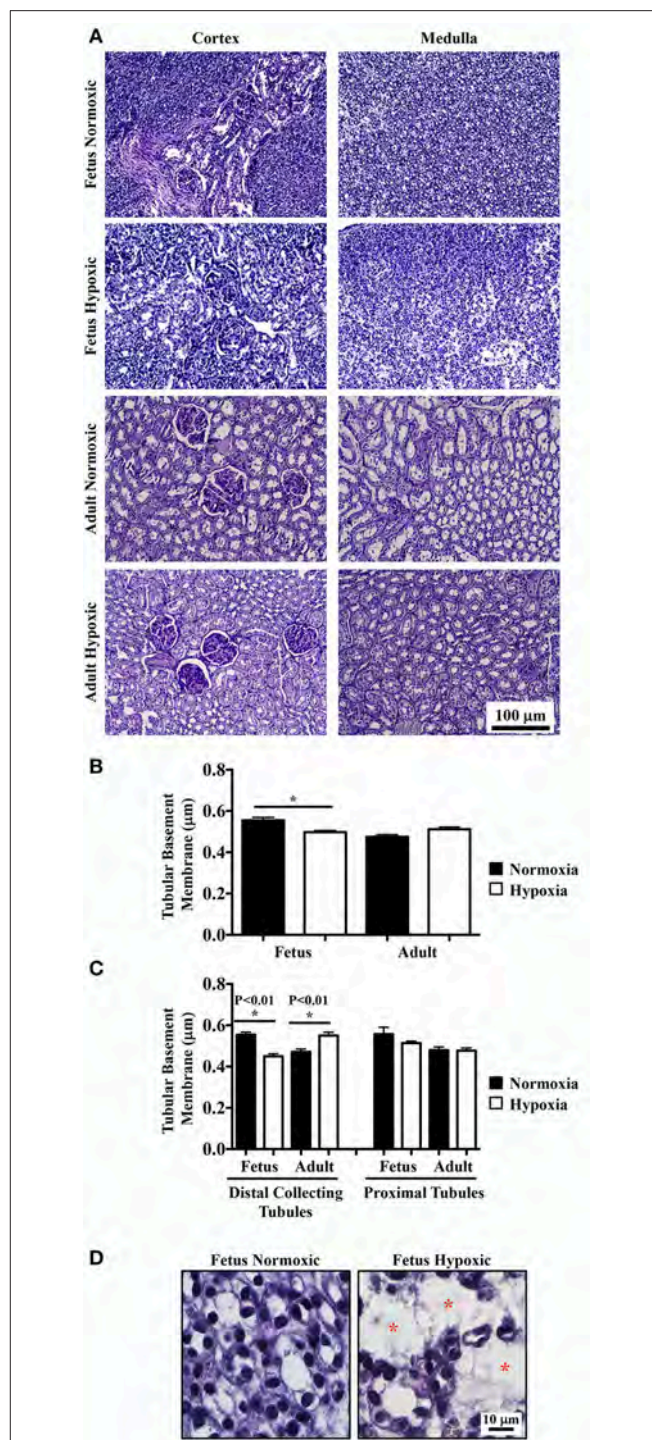


FIGURE 2 | Periodic acid-Schiff (PAS) staining to study effects of long-term hypoxia. (A) Kidneys were stained with PAS, and representative images were taken at cortex and medullary regions of the kidneys. (B) Tubular basement membrane thickness was measured and quantified. (C) More detail analysis of basement membrane was achieved by separation between distal collecting and proximal tubules. (D) Representative images show that in fetal hypoxic kidney, tubules were separated by interstitial medullary edema as denoted by red asterisks. *Indicates significant differences between normoxic and hypoxic groups. $N = 6$ animals for each group; statistical analysis was performed with ANOVA followed by the Tukey *post-hoc* test.

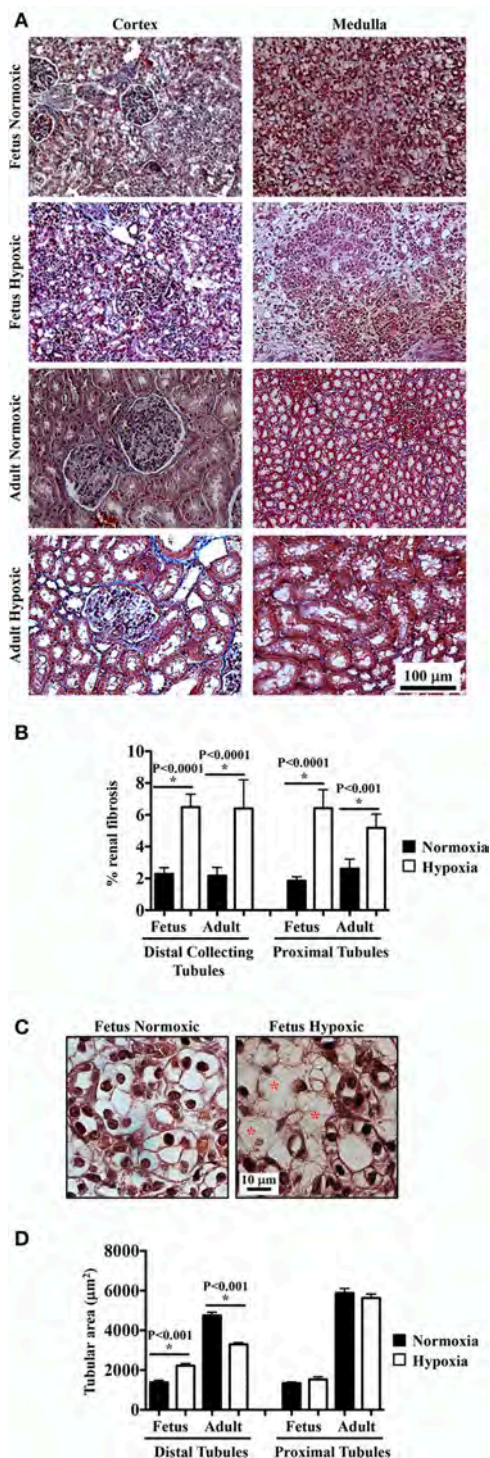


FIGURE 3 | Masson's Trichrome staining to study effects of long-term hypoxia. **(A)** Kidneys were stained with Masson's Trichrome, and representative images were taken at cortex and medullary regions of the kidneys. **(B)** Interstitial renal fibrosis was measured and quantified. **(C)** Representative images show that in fetal hypoxic kidney, tubules were separated by interstitial medullary edema as denoted by red asterisks. **(D)** Tubular areas were measured, quantified and compared. *Indicates significant differences between normoxic and hypoxic groups. $N = 6$ animals for each group; statistical analysis was performed with ANOVA followed by the Tukey *post-hoc* test.

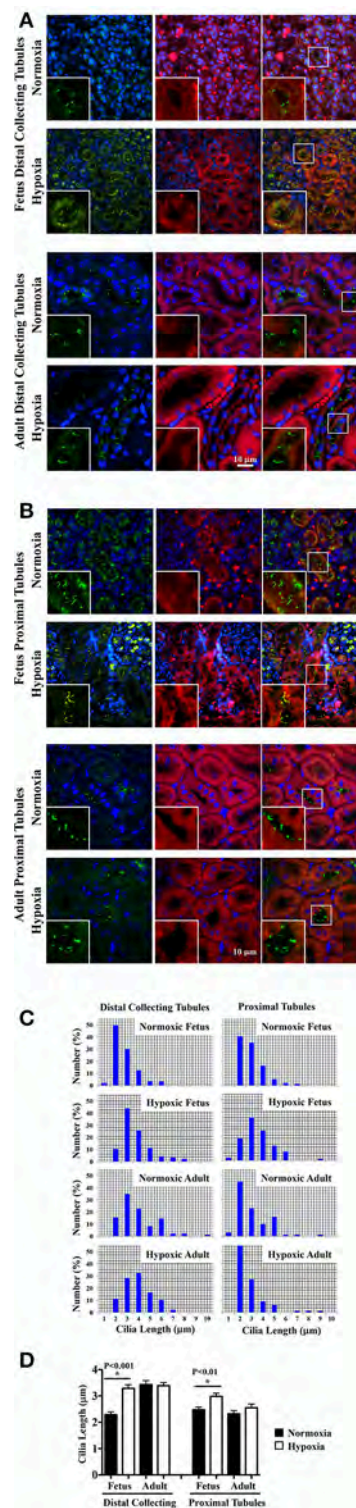


FIGURE 4 | Immunofluorescence staining to study effects of long-term hypoxia on cilia length *in vivo*. **(A)** Kidneys were stained with ciliary marker (acetylated- α -tubulin; green), distal collecting marker (DBA; red) and nucleus marker (DAPI; blue). **(B)** Instead of DBA, kidneys were stained with proximal tubular marker (LTL; red). White boxes show enlargement of the images to show cilia length. *(Continued)*

FIGURE 4 | Continued

depict the magnified view of primary cilia. **(C)** The length of primary cilia was measured and represented in the bar graph to depict length distribution within each group. **(D)** Cilia length was quantified. *Indicates significant differences between normoxic and hypoxic groups. $N = 6$ animals for each group; statistical analysis was performed with ANOVA followed by the Tukey *post-hoc* test.

there is no change in proximal tubule cilia length from fetuses to adults.

Fetal Hypoxic Kidney Had Greater Sensitivity in Response to Fluid-Shear Stress

Renal primary cilia are mechanosensory organelles that sense filtrate moving within the tubules. To examine the possibility that hypoxia could alter mechanosensory of cellular responses, renal epithelia were isolated, cultured, stained with a cilia marker and challenged with shear-stress. Representative images of these cells from normoxic and hypoxic kidneys reveal changes in cilia length (**Figure 5A**). On average about 80% of cells had cilia, and there were no apparent differences in cilia formation between normoxic and hypoxic tissues, or between fetal and adult kidneys. Cilia measurements were then tabulated to compare their distributions (**Figure 5B**). Cilia measurements were also tabulated to analyze the impact of hypoxia on cilia lengths (**Figure 5C**). While hypoxia did not significantly alter cilia length in adult cells, cilia length was significantly longer in hypoxic than normoxic fetal cells. Interestingly, trend in cilia length changes was similar for *in vitro* and *in vivo* preparations. When the overall cilia length *in vivo* kidney (**Figure 4**) or *in vitro* cell culture (**Figure 5**) were averaged, it was apparent that the *in vitro* cell culture produced much longer cilia than observed *in vivo* ($6.35 \pm 0.28 \mu\text{m}$ vs. $2.84 \pm 0.12 \mu\text{m}$; $p < 0.00005$).

To examine the effect of chronic hypoxia on mechanosensory cilia function, cells were challenged with 0.8 dyne/cm^2 shear-stress. Changes in cytosolic calcium were averaged and plotted in line graphs (**Figure 6A**). When peaks of cytosolic calcium were examined, hypoxia significantly enhanced mechanosensory function in fetal epithelial cells (**Figure 6B**). In contrast, hypoxia did not significantly alter mechanosensory sensitivity in adult cells.

Hypoxia-Induced Longer Cilia Were Associated with Greater Cilia Function

It has been hypothesized that longer cilia are more sensitive to fluid-shear stress (Abdul-Majeed et al., 2012; Upadhyay et al., 2014). To examine the effect of hypoxia on cilia length-function relationship, both *in vivo* (**Figure 4**) and *in vitro* (**Figure 5**) cilia lengths were used to assess changes in cilia function. When *in vivo* cilia length was plotted against cilia function, no apparent length-function association was observed (**Figure 7A**; $R^2 = 0.42$). Because hypoxia did not alter cilia length in adult kidneys, length-function relationship was next analyzed only in

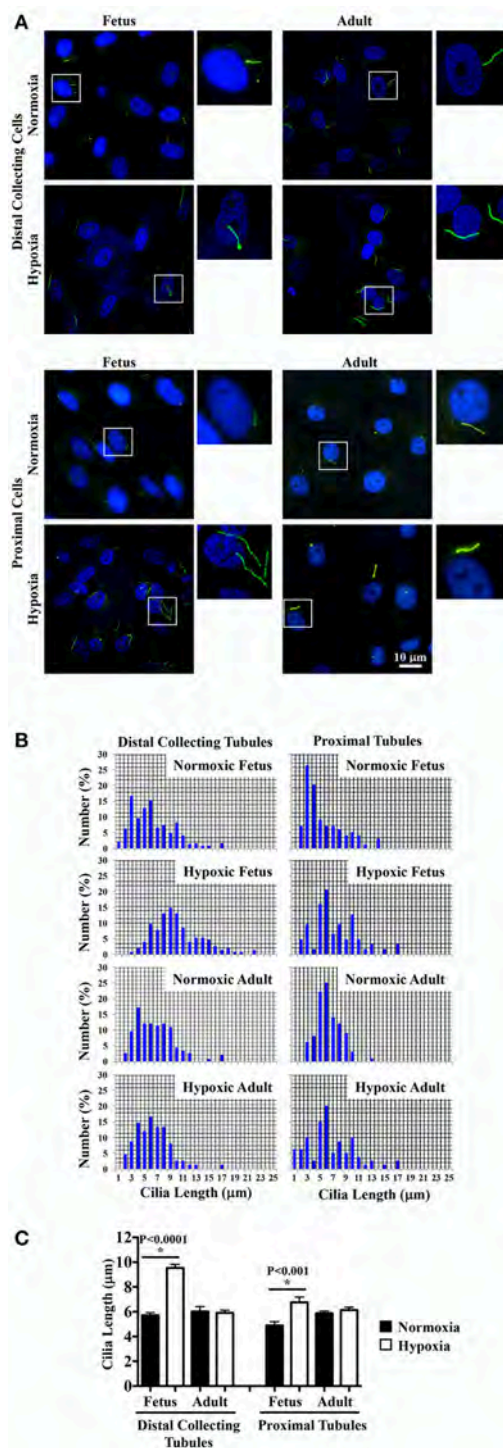
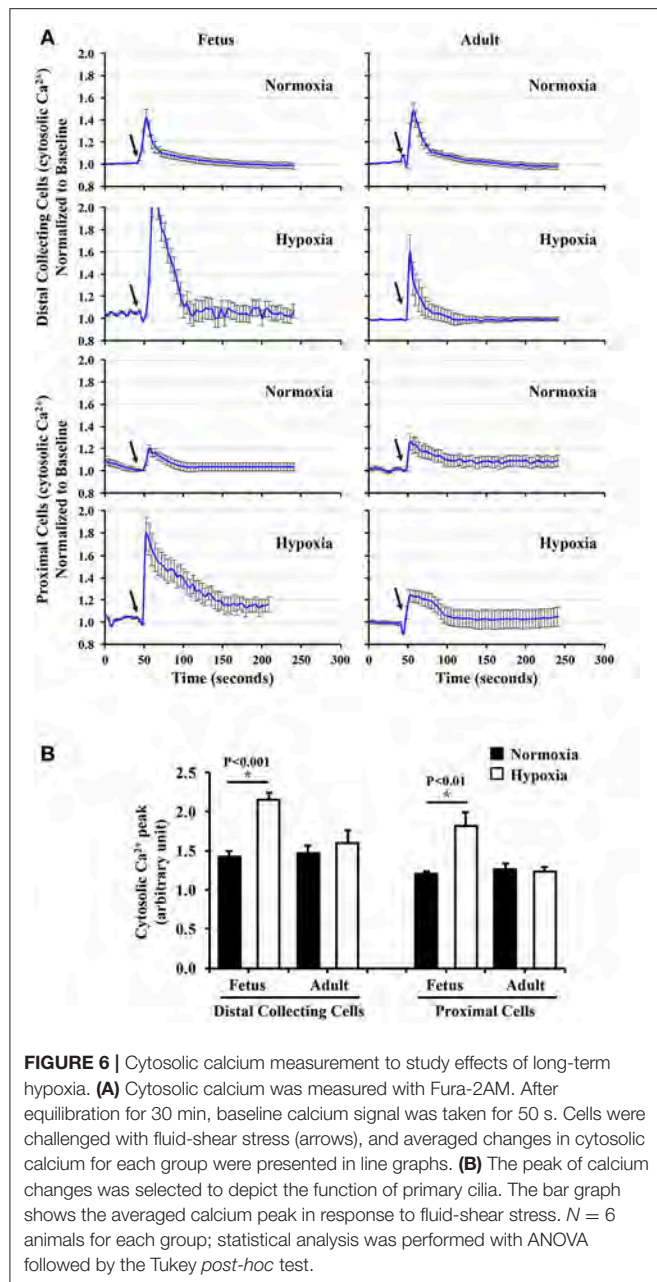


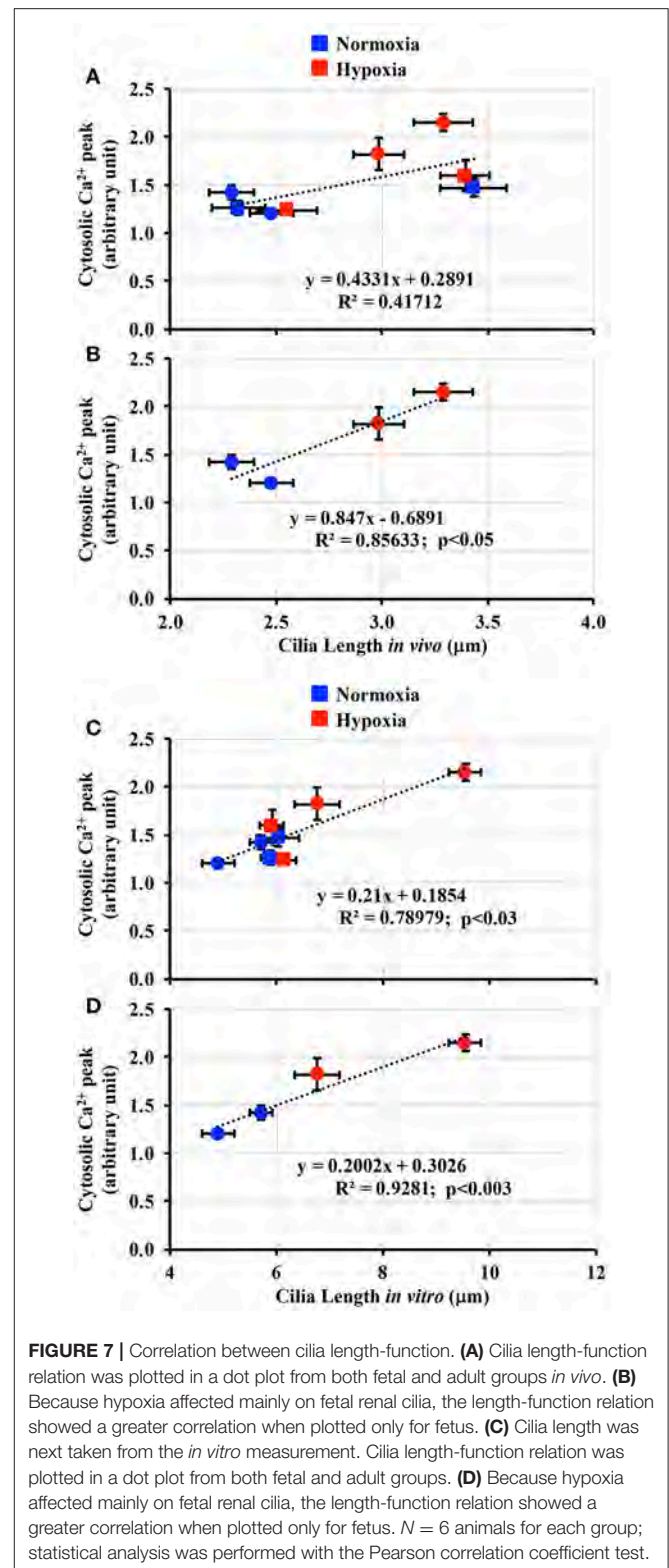
FIGURE 5 | Immunofluorescence staining to study effects of long-term hypoxia on cilia length in *in vitro* cultures. **(A)** Ovine renal epithelia were isolated and stained with ciliary marker (acetylated- α -tubulin; green) and nucleus marker (DAPI; blue). White boxes show enlargement of the images to depict the presence of primary cilia. **(B)** The length of primary cilia was measured and tabulated in the bar graph to depict length distribution within each group. **(C)** Cilia length was quantified. *Indicates significant differences between normoxic and hypoxic groups. $N = 6$ animals for each group; statistical analysis was performed with ANOVA followed by the Tukey *post-hoc* test.



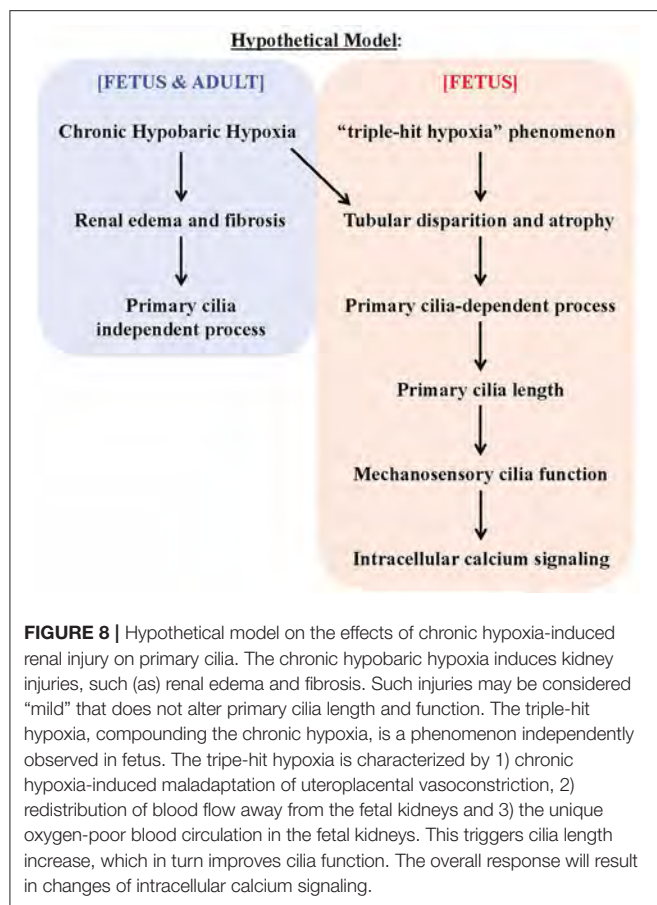
fetal kidneys. This revealed significant correlation within length-function relationships (**Figure 7B**; $R^2 = 0.86$). When *in vitro* cilia length was analyzed, the cilia function was significantly correlated with cilia length following either inclusion (**Figure 7C**; $R^2 = 0.79$) or exclusion (**Figure 7D**; $R^2 = 0.93$) of adult kidneys.

DISCUSSION

Although kidneys are important for regulation of body fluid, pH, electrolyte, hormone and overall metabolism, the effects of chronic hypobaric hypoxia on fetal kidneys have never been examined. We therefore study the effects of chronic hypoxia on fetal and adult sheep kidneys within the cortex and medullary



regions. Effects of hypoxia on mechanosensory primary cilia are also evaluated. Our studies suggest that: (1) there are no significant differences in glomerulus size between normoxic and hypoxic kidneys; (2) the hypoxic kidneys show proliferation



of mesangial cells, medullary edema and fibrosis; (3) hypoxia modulates changes in tubular basement membranes; and (4) compared to adult kidneys, fetal kidneys are more susceptible to hypoxia-induced tubular disparition and atrophy, characterized by alterations in primary cilia and function.

A previous study has demonstrated enlargement of renal glomeruli in hypoxic children (Naeye, 1965). However, our studies do not support glomerulus enlargement in the hypoxic fetal or adult sheep kidneys. This discrepancy could potentially be due to an age-specific effect. For example, glomerular enlargement only occurs after the first month of life, while the size of glomeruli is normal at birth in those hypoxic children (Naeye, 1965). Since all of these children die as a result of unknown illness, it is also possible that other genetic and environmental factors contributed to the glomerulus enlargement. For example, an oxonic acid diet in rats is known to cause hyperuricemia through elevated plasma renin activity (Eraranta et al., 2008). Glomerular hypertrophy is observed in oxonic acid-induced hyperuricemia and can be prevented by ACE inhibitor therapy in the rats (Nakagawa et al., 2003).

Abnormal mesangial cells, edema and fibrosis are observed in our hypoxic kidneys. This is possibly a result from interstitial renal injury due to chronic hypobaric hypoxia. Of note, the deposition of collagen and fibrin fiber that functions as a by-product of a reparative process, is commonly used as an index of

various renal injury (Ma et al., 1998; Kaissling and Le Hir, 2008; Forbes et al., 2012). Although hypoxia is one of the stimuli that drives chronic kidney disease (Fu et al., 2016), our studies further reveal that hypoxia could alter the thickness of tubular basement membranes. Interestingly, hypoxia only induces changes in basement membranes of distal collecting tubules. This could be due to medullary regions that operate within a relatively lower range of pO_2 , and it is therefore more susceptible to hypoxic injury than proximal regions (Heyman et al., 1997; El Sabbahy and Vaidya, 2011). Greater susceptibility of medullary regions to hypoxia might thus contribute to an increase in collecting distal tubular basement membrane of adult kidneys. Unlike adult medullary tissues, however, hypoxia actually causes a decrease in medullary basement membrane in fetal kidney. This could possibly be due to tubular disparition and atrophy, which are very apparent in the fetal medulla. The thinning in basement membrane in medullary tissues might therefore be associated with a degeneration of tubular structure, as observed in fetal hypoxic kidneys.

Based on the histological analyses, fetal kidneys are more susceptible to hypoxia, possibly due to a “triple-hit hypoxia” phenomenon. In addition to the hypobaric hypoxia in the atmosphere, fetal kidneys are impacted by three additional factors (triple-hit). First, chronic hypoxia induces vasoconstriction in sheep uterine arteries during gestation (Hu et al., 2012; Xiao et al., 2013). This maladaptation of the uteroplacental circulation can result in reduced tissue perfusion and further exacerbate the effects of hypoxia. Second, in hypoxic fetal sheep there is a rapid drop in fetal heart rate and a rise in mean arterial blood pressure that redistributes blood flow to the heart and brain at the expense of the renal circulation. Hypoxia therefore reduces renal blood flow, resulting in renal hypoperfusion (Robillard et al., 1986; Green et al., 1997). Third, the unique fetal circulation system allows oxygen-rich blood from the aorta to mix with oxygen-poor blood before reaching renal circulation (Nakamura et al., 1987). Thus, the fetal circulation system reduces oxygenation support to the kidneys.

Our results also indicate that greater susceptibility to hypoxia in fetal kidneys could be associated with changes in primary cilia length and function throughout the nephrons. Primary cilia are sensory organelles rising from the apical surface of most mammalian cells. Cilia are activated during bending by fluid-flow, which in turn initiates an intracellular calcium response (Praetorius and Spring, 2001; Liu et al., 2005; Nauli et al., 2013). Previous studies demonstrate a link between cilia length and hypoxia-inducible mechanisms. In tendon cells, hypoxia inhibits primary cilia formation and cellular mechano-responsiveness (Lavagnino et al., 2016). However, studies in different hypoxic models using renal epithelial cells indicate that primary cilia are longer and that expression of HIF maintains primary cilia length (Verghese et al., 2011; Ding et al., 2015). Interestingly, the renal epithelial cilia become more flexible during hypoxia (Resnick, 2016), and it thus might alter cilia function. Consistent with this view, a correlation between cilia length and function in response to chronic hypoxia is observed in our studies. Interestingly, greater

cilia length in hypoxic fetal kidney *in vivo* are maintained as measured in *in vitro* cell culture. This could be due to epigenetic changes occurring during hypoxia. Of note, long-term hypoxia during gestation has been reported to cause epigenetic adaptation in the sheep (Dasgupta et al., 2012; Chen et al., 2014).

Our studies indicate that chronic hypobaric hypoxia could induce renal injury (Figure 8). Renal hypoperfusion as a result from the “triple-hit hypoxia” phenomenon seen in fetus would further induce tubular disparition and atrophy, a process that modulated changes in cilia length and function. This, in turn, induces an increase in intracellular calcium fluxes in response to fluid-shear stress. Our results potentially point to a complex renal injury caused by chronic hypoxia with regards to primary cilia.

It has been reported that kidney compensation induced by a reduction of renal mass results in primary cilia elongation, and this elongation is associated with an increased production of reactive oxygen species (Han et al., 2016). In addition, renal primary cilia lengthens after acute tubular necrosis (Verghese et al., 2009). The contribution of primary cilia from acute to chronic injury is also confirmed when a ciliary protein is inactivated. In this case, renal damages are more pronounced following ischemia/reperfusion, and this induces microcyst formation (Bastos et al., 2009). In addition to the association of human renal carcinoma and primary cilia (Ding et al., 2015), acute injury can induce chronic kidney disease through cyst formation in the absence of normal renal cilia (Patel et al., 2008).

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- Furthermore, mechanosensory function of cilia is abnormal in chronic kidney disease patients (Nauli et al., 2006; Xu et al., 2007). Based on these findings, we speculate that the increases in hypoxia-induced cilia-related calcium signaling in hypoxic fetal kidneys serves a potential mechanism associated with renal injury. Without doubt, future studies on the hypoxia-induced changes in intracellular calcium are warranted.

AUTHOR CONTRIBUTIONS

KS analyzed data, contributed to drafting the manuscript and oversaw the entire progress. JC and RS performed the calcium studies. JS and RP performed cilia measurements and kidney staining. KA assisted in sample collections. WP and LZ provided the kidney samples. LZ and SMN finalized the manuscript and oversaw the research project.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SCIENTIFIC REPORTS

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Alcohol consumption impairs the ependymal cilia motility in the brain ventricles

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Ependymal cilia protrude into the central canal of the brain ventricles and spinal cord to circulate the cerebral spinal fluid (CSF). Ependymal cilia dysfunction can hinder the movement of CSF leading to an abnormal accumulation of CSF within the brain known as hydrocephalus. Although the etiology of hydrocephalus was studied before, the effects of ethanol ingestion on ependymal cilia function have not been investigated *in vivo*. Here, we report three distinct types of ependymal cilia, type-I, type-II and type-III classified based upon their beating frequency, their beating angle, and their distinct localization within the mouse brain-lateral ventricle. Our studies show for the first time that oral gavage of ethanol decreased the beating frequency of all three types of ependymal cilia in both the third and the lateral rat brain ventricles *in vivo*. Furthermore, we show for the first time that *hydin*, a hydrocephalus-inducing gene product whose mutation impairs ciliary motility, and polycystin-2, whose ablation is associated with hydrocephalus are colocalized to the ependymal cilia. Thus, our studies reinforce the presence of three types of ependymal cilia in the brain ventricles and demonstrate the involvement of ethanol as a risk factor for the impairment of ependymal cilia motility in the brain.

The motile ciliated cells are ubiquitous in the lung airways, the fallopian tubes in the uterus, the efferent ducts in the testes and sperm tail, as well as in the brain ventricles and spinal cord canal. Primary ciliary dyskinesia (PCD), male infertility and hydrocephalus are phenotypes that result from motile cilia dysfunction. Hydrocephalus is a combination of neurological and neuropathological manifestations characterized by an imbalance between the production and absorption of CSF. This disparity leads to an extreme accumulation of fluid causing the brain ventricular cavities and the subarachnoid spaces to expand in response to the increase in CSF^{1,2}. Although hydrocephalic symptoms are constant, the disease is classified into two types based upon the etiology- congenital and non-congenital. Congenital, also known as neonatal hydrocephalus occurs due to genetic abnormalities in combination with other conditions such as intraventricular hemorrhage and/or ethanol abuse during pregnancy^{3–7}. Non-congenital hydrocephalus occurs after birth resulting from an injury or infection of the brain ependyma^{2,8,9}.

The ependymal cilia help distribute and circulate the CSF from its origins in the choroid plexus located in the lateral and the third ventricles. The CSF flows in a unilateral direction starting in the lateral ventricle, then moving through the third ventricle, and then the fourth ventricle where it passes into the subarachnoid space insulating the brain and spinal cord. The direction of flow depends upon the ependymal cellular orientation, which in turn is dependent upon the motile cilia^{2,10,11}.

The motile cilia dynamics deteriorate in hydrocephalic conditions for several reasons. First, any mutation within the essential proteins responsible for ciliogenesis or cilia ultrastructure will lead to a disruption in cilia motility¹². There are several markers for the motile cilia, each localizing to specific areas. Either a mutation in one of these protein markers or an altered localization is associated with motile cilia dysfunction¹³. *Hydin* is a hydrocephalus-inducing gene that encodes for a central pair protein within the axoneme of motile cilia. A mutation in *hydin* leads to dislocation and eventual loss of the central pair, which disturbs the movement of cilia leading to hydrocephalus^{14,15}. *Hy3/hy3* mutant mice show early hydrocephalous onset and an enlargement of the lateral and third ventricles, as a result of abnormal CSF flow caused by the impairment of cilia movement.

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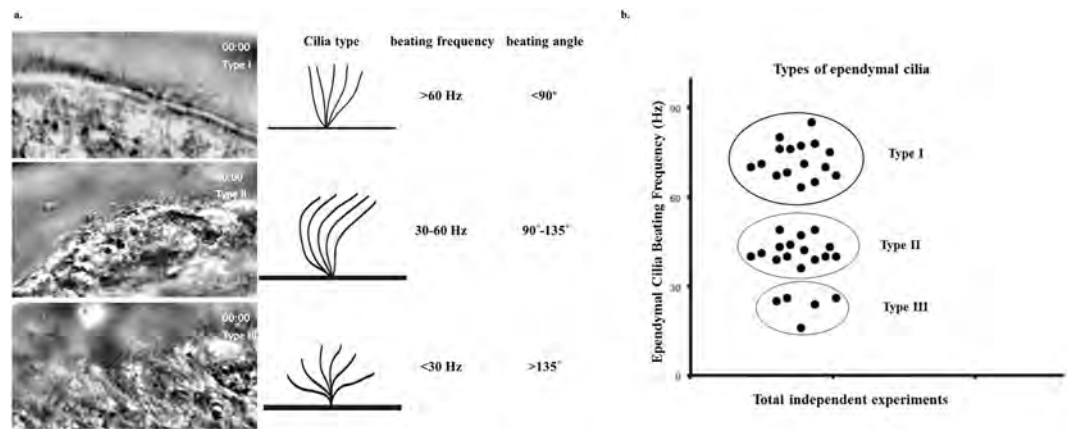


Figure 1. Ependymal cilia of the brain lateral ventricle are classified into three types based on their beating frequency and angle. (a) Shown here are representative DIC images of the ependymal cells of the mouse brain lateral ventricle with the three types of motile cilia. The images of the ependymal cilia are taken from a time-lapse movie during a live imaging experiment. (b) Classification of ependymal cilia is based upon their beating frequency and angle. Each dot represents an independent experiment. Type I cilia are the fastest and has a beating frequency >60 Hz with a beating angle less than 90°. Type II beating frequency is between 30–60 Hz with a beating angle between 90°–135°. Type III cilia have the slowest beating frequency <30 Hz and a beating angle >135°.

The beating frequency of ependymal cilia in the *hydin* mutant mice declines significantly due to changes in the location of the central pair microtubules leading to ineffective fluid movement¹³.

Polycystin-2 (PC-2) is a membrane-associated protein known to be expressed in primary cilia and plays an important role in the mechanosensory and ciliogenesis functions of motile cilia¹⁶. Polycystin-2 is also associated with sperm development and motility. *Pkd2* mutant *Drosophila* develops male sterility as a result of declining sperm movement¹⁷. Polycystin-2 serves as a sensory protein that detects changes in fluid flow within the respiratory ciliated cells and contributes to the development of brain planar cell polarity (PCP) and prevention of hydrocephalus¹⁸.

Ethanol consumption has been reported to reduce respiratory performance due to its significant effect on mucociliary clearance. Recent evidence suggest a dose-dependent relationship between alcohol use and thinner cortex and ventricular expansion even at lower alcohol concentrations and in healthy individuals as well¹⁹. Previous studies from our lab had investigated the dynamics of ependymal cilia in the third ventricle and found a decrease in beating among the cilia within the third ventricle after ethanol incubation *ex vivo*²⁰. In the current study, we show, through cilia beating frequency and beating angle, that ependymal cells in the brain lateral ventricle can be distinctly categorized into three types with each type uniquely localized in the ventricle. Furthermore, we show for the first time that oral gavage of ethanol alters the beating of ependymal cilia in the rat brain lateral and third ventricles. Thus, our studies suggest a direct link between ethanol dependence and hydrocephalus involving ependymal cilia function.

Clinical Implications and Significance

Dysfunctional cilia have been associated with a large number of diseases characterized by pleiotropic symptoms collectively referred to as “ciliopathies”. Hydrocephalus is a ciliopathy characterized by CSF accumulation in the brain caused by genetic mutations that lead to impairment of ciliary motility. Variations in ciliary motility can be influenced by changes in ciliary beating patterns as well as ciliary beating frequencies, both of which correlate with the patients’ genotype. Hence characterizing the different types of ependymal cilia based on variations in ciliary motility may provide a guide for identifying the mechanism responsible for ciliopathies in the brain. This is the first report to demonstrate the involvement of ethanol as a risk factor for cilia motility impairment in the brain.

Several factors are responsible for causing hydrocephalus such as brain injury, infection, or genetic factors coupled with a secondary stressor. A common secondary stressor is maternal alcohol consumption while pregnant; research on brain abnormalities have shown hydrocephalic symptoms in some offspring prenatally exposed to alcohol³. However, despite a wealth of literature, the mechanism linking alcohol consumption to hydrocephalus remains unknown. Treatment for hydrocephalus is not started until the child is born and typically requires surgery to alleviate the symptoms. Unfortunately, surgical intervention is not always effective long-term^{4,21}. Thus, elucidating the mechanism of hydrocephalus pathophysiology may provide the necessary information to develop more effective pharmacological compounds that could target cilia motility as a therapeutic option²².

Results

Live imaging of ependymal cilia. We developed a novel technique to allow close observation of the movement of ependymal cilia within the brain ventricles. By obtaining a sagittal plane section from the mouse brain, we can expose the ependymal cilia with minimum reduction in vitality and image quality by using a high-resolution differential interference contrast (DIC) microscopy system producing live images and movies (Fig. 1a).

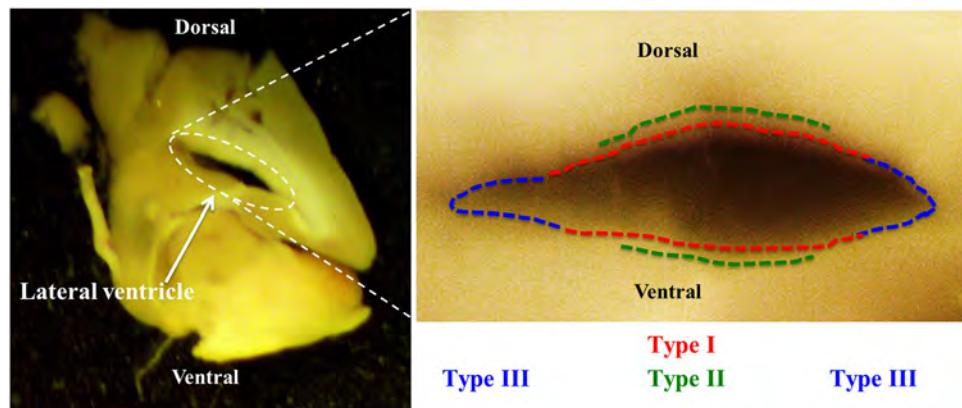


Figure 2. Each type of ependymal cilia has specific localization within the brain lateral ventricle. This figure shows a sagittal section view of the lateral ventricle (left). Each type of the ependymal cilia (shown on the enlarged area) is localized within specific area in the lateral ventricle based on the beating frequencies and angle of movement.

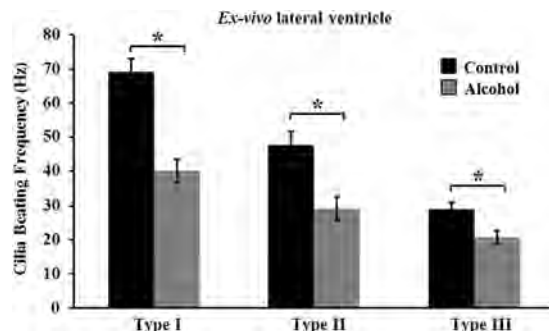


Figure 3. Ethanol decreased cilia beating *ex vivo*. Treatment of *ex vivo* brain slices with 0.25% ethanol for five minutes shows a significant reduction in the beating frequency of all three types of ependymal cilia. Up to 22 independent preparations were used and the presence of an asterisk (*) denotes significant difference at $p < 0.05$.

A step-by-step procedure detailing the sectioning, imaging, and staining of our brain sections was published recently²³.

Ependymal cilia are classified into three types based on their beating frequency and angle.

The live movie analysis of the cilia beating in the lateral ventricle revealed remarkable variations in the beating frequencies. This enabled us to classify the cilia into three distinct types with regards to their beating frequency, movement angle and pattern (Fig. 1b). Type I cilia beat the fastest at >60 Hz and have the least volume replacement stroke with a beating angle of $<90^\circ$ (Movie 1). The beating frequency of type II cilia was 30–60 Hz with a beating angle between 90° and 135° (Movie 2), while type III cilia have the slowest beating frequency of <30 Hz but the largest volume replacement stroke with a beating angle of $>135^\circ$ (Movie 3). The ciliary beating in the lateral ventricle mimics that of the cilia within the third ventricle²⁰. It is worth mentioning that the three types of ependymal cilia are located in specific locations within the lateral ventricle. We mapped the distributions of ependymal cell types within the lateral ventricle. Following our mapping analysis, we demonstrated that type I cells are mostly distributed along the ventricle dorsal and ventral walls, but they are absent from both corners of the lateral ventricle. Type II cells are mainly distributed in the middle of the dorsal/upper wall of the ventricle, but they could also be found in the middle of the ventral/lower wall of the ventricle. Type III cells are distributed almost exclusively at the corners of the lateral ventricle (Fig. 2).

Ex-vivo exposure to ethanol decreases ependymal cilia beating in the brain lateral ventricle.

The *ex-vivo* ethanol experiment was performed to examine the accuracy of the ependymal cell classification, as well as to investigate whether the effect of ethanol is consistent within all three types of ependymal cilia. In addition, it enabled close observation of the effect of oral gavage of ethanol on the ependymal cilia in controlled environmental settings. Data from the *ex-vivo* experiments indicate that there is a significant decline in cilia motility after incubating the brain sections in 0.25% ethanol for 5 minutes. By analyzing and comparing the movement of the three types of cilia before and after the ethanol treatment, we found a decrease in cilia movement occurring in all three types (Fig. 3 and Movies 3–6).

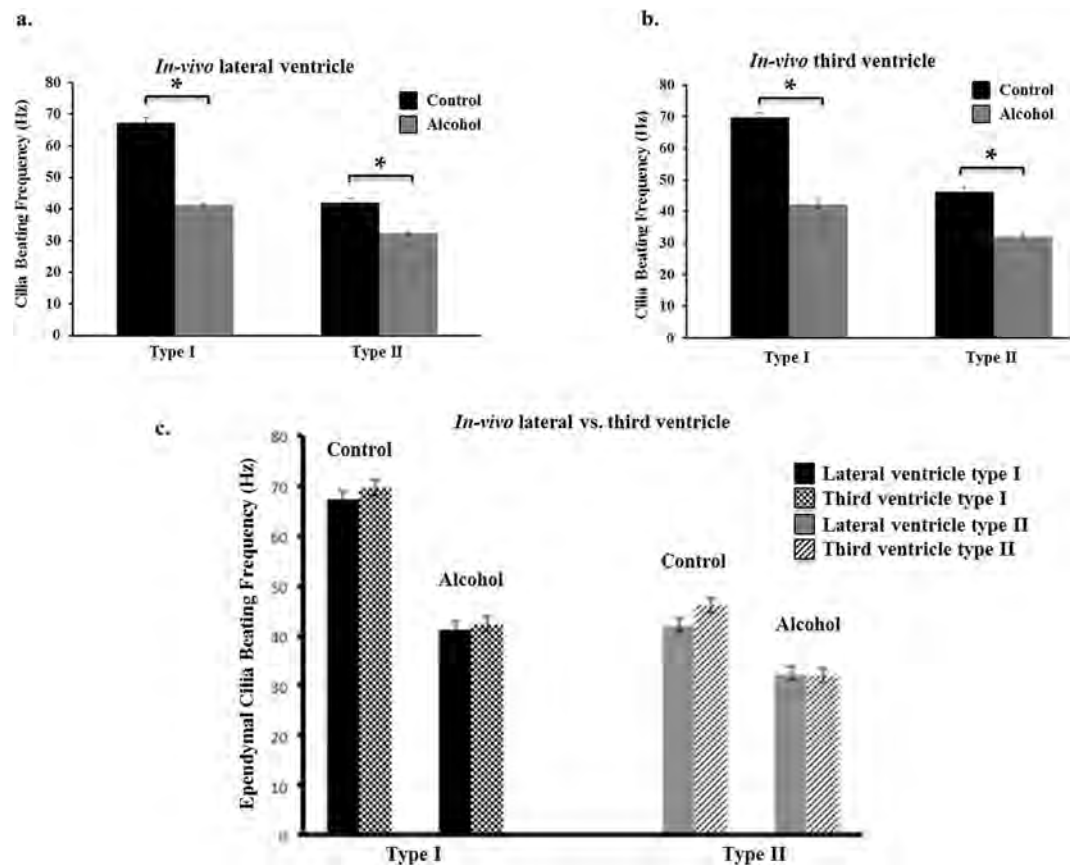


Figure 4. Alcohol drinking altered the dynamics of ependymal cilia in the brain. Two-month old Wistar rats were given either water or 95% ethanol (alcohol) at a 6 g per kg of body weight for seven days. After acute oral treatment with alcohol, the rat's brain was dissected to examine the dynamics of ependymal cilia in both the lateral and third ventricles. Alcohol drinking caused a significant decrease in the beating frequency of the ependymal cilia of both, (a) the lateral and (b) third ventricles compared to the control group. (c) Further comparison within the control groups and the alcohol-drinking groups between the dynamics of the ependymal cilia in the lateral ventricle vs. the third ventricle demonstrated no significant difference between the ciliary beating frequencies.

Ethanol drinking decreases ependymal cilia beating in the brain lateral and third ventricles *in vivo*. To confirm the physiological relevance of the *ex vivo* ethanol treatments, the effect of oral gavage of ethanol on ciliary beating frequency and ependymal cilia function were further investigated. Our data on ependymal cilia motility clearly demonstrated a significant decrease in the ependymal cilia dynamics after acute oral gavage of ethanol, which is supported by our previous findings (Fig. 4a,b). Studies into both the lateral and the third ventricles confirmed that cilia motility was affected by oral gavage of ethanol in the same manner (Fig. 4c and Movies 7–14).

Hydin and polycystin-2 are expressed in the ependymal cilia. To verify our high-resolution differential interference contrast and fluorescence microscope systems, we examined the presence of ependymal cilia in the lateral ventricle. Ependymal cilia were confirmed with a ciliary marker, acetylated- α -tubulin (Supplementary Figure S2). In an effort to explore the mechanism or the structural differences that could explain the variation in movement and beating patterns among the three types of cilia, the ciliary localization of several key structural proteins were investigated for the first time by immunofluorescence staining. Hydin, a central ciliary axonemal protein, presented as a good marker to distinguish between the three types of cilia since it is located in the central pair of the motile cilia. Eventually, we were able to localize hydin in the ependymal cilia for the first time by immunofluorescence microscopy in the lateral ventricle. Hydin's ciliary localization was confirmed in the lateral and third ventricles by co-staining with the ciliary marker acetylated- α -tubulin (Supplementary Figures S1 and S2). Polycystin-2's known expression in airway motile cilia lead to the hypothesis that it may also localize to the motile cilia of the ependymal cilia¹⁸. Data from our immunofluorescence experiments demonstrated the localization of polycystin-2 to the cilia in the lateral ventricle (Supplementary Figure 6c). Hydin and Polycystin-2 were localized in all of the three types of the ependymal cilia. Future studies are warranted to examine other motile cilia proteins, which may be specific to each type of cilia such as CCDC115, CCDC114, and ARMC4 that encode for outer dynein arm proteins²⁴.

Discussion

We report, for the first time, the classification of ependymal cilia in the mouse brain lateral ventricle and confirmed the previous classification of the ependymal cilia in the third ventricle²⁰. The ependymal cilia are divided among three distinct types in regard to their beating frequency and beating angle. Type I has a beating frequency greater than 60 Hz and a movement angle domain of less than 90°, type II has a beating frequency between 30–60 Hz and an angle between 90°–135°, and type III has a beating frequency less than 30 Hz and an angle greater than 135°. Although the three types vary in velocity and fluid volume movement due to differences in the beat patterns and speed, the three types of cilia are distributed equally throughout the lateral ventricle to maintain efficient circulation of the CSF. Our pharmacological screening confirms that *ex vivo* treatment of brain slices with 0.25% ethanol resulted in a reduced cilium beating frequency thus contributing to a decrease in the velocity and volume of fluid movement. More importantly, our studies show that ethanol ingestion in rats leads to an impairment of ependymal cilia beating and function in both the lateral and third brain ventricles *in vivo*. To our knowledge, this is the first report to study the *in vivo* effect of oral gavage of ethanol on the physiology of ependymal cells in the brain lateral and third ventricles.

Several studies introduce advanced methods for performing and analyzing high speed digital imaging of ependymal cilia^{25,26}; however, classification of ependymal cells has not been reported before. To date, studies have indicated chemicals such as ethanol and its metabolite, acetaldehyde, have no effect on motile cilia beating frequency in concentrations between 0.1% and 1%^{27,28}. However, based on our new ependymal cilia classification parameters, *ex-vivo* studies on treated ependymal cell tissue, using a minimum concentration of 0.25% ethanol, reveal that ethanol causes a significant decrease in the ciliary beating frequency.

As previously mentioned, disruption in ependymal cilia function is one of the main reasons for the ventricle enlargement observed in hydrocephalous. Chronic ethanol consumption leads to numerous destructive effects on the brain, including an increase in the size of the brain ventricles by 31–71%, the hallmark of hydrocephalus^{29,30}. Several studies have been undertaken to investigate the effects of ethanol on the brain in cases of chronic alcoholism; however, none have focused on the ependymal cilia in particular. In our study, we confirmed our findings of a reduction in ciliary beating frequency from the *ex-vivo* ethanol treatment. We also found, for the first time, that ciliary beating frequency *in-vivo* was significantly reduced, triggering a decrease in ciliary performance. This could potentially provide an explanation for the occurrence of hydrocephalus in patients suffering from acute alcoholism. Previous studies examining the effects of ethanol drinking and other toxic agents on motile cilia function have been mainly performed either on excised tissue or on primary ciliated epithelial cells grown directly from fresh tissue^{31,32}. However, none of these studies examined the effect of ethanol consumption directly on the motility and function of ependymal cilia in the brain ventricles. It has been postulated that exposure of the airway epithelium to volatilized ethanol from the bronchial circulation initially leads to a rapid increase in cilia beat frequency followed by a desensitization of ciliary stimulatory response³². Our studies reinforce the deleterious effects of ethanol consumption on the sensory function of ependymal cilia and provide a potential explanation for the brain-related symptoms that are associated with ethanol drinking such as headaches, difficulty walking, blurred vision, slurred speech, slowed reaction times, and others.

It is well established that cilia are specialized sensory compartments on the apical surface of most cells to sense and transmit information from the extracellular matrix to the cell interior. To perform its unique sensory roles, a high density of specialized proteins, such as receptors, ion channels, and other signaling modules localize in the ciliary compartment³³. As reported in this study, our classification of ependymal cilia is based on differences within the movement patterns and velocity of beating. In an attempt to explain the variation in beating frequencies and angles with regards to the ultrastructure of the three types of motile cilia, we used antibodies against different proteins that are linked to the ciliary membrane or that recognize posttranslational modifications of ciliary axonemal molecules that can be used to differentiate between different types of motile cilia. In our study, we used ciliary markers that could potentially help us identify the unique pattern of different motile cilia types or shed light on the function of these ciliary markers.

Hydin is a central axonemal microtubule protein required for ciliary motility. Although the morphology of ependymal cilia in the brains of mutant animals is normal, one of the two central microtubules lacks a specific projection and the cilia are unable to bend normally, ciliary beat frequency is reduced, and the cilia tend to stall. As a result, these cilia are impaired and incapable of generating fluid flow. Thus, causing the phenotypes associated with hydrocephalus such as fluid accumulation in the brain¹³. Although *hydin* mutation has been shown to be directly associated with the pathology of hydrocephalus, to our knowledge, there has been no report showing the localization of hydin to the ependymal cilia in the brain. We were able to localize hydin for the first time throughout the entire axoneme of the cilia. Localization of hydin in the brain ventricle could assist in the diagnosis and the treatment of hydrocephalus³⁴.

Polycystic kidney disease 1 (*Pkd1*) and *Pkd2* genes encoding for polycystin-1 (PC-1) and polycystin-2 (PC-2) form a mechanosensory complex in the primary cilia of kidney and vascular endothelial cells^{35–37}. The mechanosensory proteins are recently shown to be present in primary cilia of radial glia cells (RGCs). Deletion of *Pkd1* or *Pkd2* in central and peripheral nervous system neuronal and glial cell precursors affected planar cell polarity (PCP) and development in RGCs and ependymal cells³⁸. This study suggested that PC-1 and PC-2 mechanosensory proteins contribute to the brain development, PCP, and hydrocephalus prevention. Here, we further show that polycystin-2 is localized and detected in all three types of ependymal cilia in the brain ventricles. An understanding of the molecular mechanism leading to the pathogenesis of hydrocephalus, which could potentially vary by location of the ependymal cilia type, could help develop genetic tools for the diagnosis and treatment of hydrocephalus. This study identifies cellular and molecular components that could mediate hydrocephalus formation in ependymal cells.

Methods

The Institutional Animal Care and Use Committee (IACUC) of The University of Toledo approved all of the procedures for animal use in accordance with the guidelines of the Institutional Animal Care and Use Committee at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Live imaging. For ependymal cilia live imaging, wild type mice strain C57BL/6 were euthanized by asphyxiation using a CO₂ gas chamber for five minutes. Cervical dislocation was used as a second method of euthanasia to confirm death. A craniotomy was performed to collect the whole brain. A thin sagittal plane section was obtained and immediately placed in a glass-bottom petri dish containing 37°C Dulbecco's Modified Eagle Medium (DMEM)/High-Glucose (HyClone Inc.), 10% fetal bovine serum (FBS) (Fisher Scientific Inc.) and 1% penicillin/streptomycin solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin. To provide the fresh live tissue section with the proper environmental conditions to survive, the microscope's enclosed chamber temperature has been adjusted to 37°C with a gas mixture of 95%/5% O₂/CO₂. The cilia movement was recorded using an eclipse TE2000 microscope equipped with a 60x objective oil immersion lens and a differential interference contrast filter. Time-lapse images were captured and analyzed using Metamorph imaging software at a 1 × 1 binning with a 5–10 msec exposure time with at least 200 frames per second.

Beating frequency measurement. After recording the live cilia movement, each movie was analyzed by counting the number of cilia beats per one second. The motile cilia fluid dynamics have two stages, the power stroke where the cilia arc becoming horizontal to the cell surface, and the recovery stroke where the cilia reneges its initial upright position. The power and recovery stroke of ependymal cilia are counted as one complete beat cycle. In order to accurately estimate the number of beats, the time-lapse movie may be slowed to a manageable speed while using a cell counter to count the number of beatings. Thus number of cilia beatings is counted in one minute using a cell counter and the frequency of beatings is calculated by multiplying the exposure time at which the video is recorded by the number of frames or time-lapse images acquired to get the number of sec. (Example: exposure time 5 msec. 200 frames = 1,000 msec or 1 sec). The number of cilia beatings over a one-second time interval (Example: cilia beats 50 times in a 200 frames video recorded at exposure time of 5 msec *i.e.* 5 msec × 200 frames = 1 sec; now divide 50 beats by 1 sec = 50 Hz). The ciliary beating angle is then calculated by evaluating the path taken by the ependymal cilia during both the power and recovery strokes. This is performed according to a previously described method, with minor modifications²³. Briefly, a horizontal line along the ependymal edge and a vertical line through the midline position of the cilia at the start of the power stroke were drawn on an acetate sheet placed over the monitor. The position of the cilium is then plotted frame by frame as it moves forward during both the power and the recovery strokes. Finally, the ciliary beating angle is calculated from the maximum deviation of the cilium from the midline during the power stroke as well as the recovery stroke. At least 5–15 mice were used in each experiment and up to 20 preparations were counted from each mouse brain.

Determination of different types of cilia within the lateral ventricle. To determine the specific location of each type of cilia and their relation to each other, a video for each type was recorded, marking the region of each video within the ventricle. To determine the type of cilia, after recording and analyzing the videos, the number of beats per second was counted and the angle of movement was measured. Subsequently a map showing the distribution of the cilia types in the ventricle walls is drawn.

Ex-Vivo ethanol treatment. After dissection, a thin sagittal plane section of the mouse brain was obtained and incubated in 1 ml 37°C DMEM/High-Glucose media. Then, the videos of the beating cilia were captured at an exposure rate of 5 milliseconds for at least 200 frames per second. To investigate the effect of ethanol in the ependymal cilia, the same section was incubated in 0.25% ethanol (190-proof, Decon Lab Inc.) in 37°C DMEM/High-Glucose media for 5 minutes. The cilia beats were recorded from the same areas examined prior to the ethanol addition. The movies were analyzed by counting the ciliary beating frequency for both the control and the ethanol-treated tissue.

In-vivo ethanol treatment. Two-month-old Wistar rats were received from Harlan Laboratories Company and housed in the Department of Laboratory Animal Resources at the University of Toledo. All animals were kept in standard plastic tube cage with free access to food and water. The animal room's temperature and relative humidity was maintained at 25°C and 50%, respectively with a 12-hour light/dark cycle. The body weight was monitored daily for the rats under treatment.

The animals were divided into two different groups. The control group, totaling six rats, was treated with water by oral gavage for seven days. The rats were deprived of food for two hours prior to oral gavage. The treatment group of six rats were given 95% ethanol, the equivalent of 190-proof by oral gavage. The dose of ethanol is 6 g per kg of body weight divided by the density of ethanol and diluted in deionized water to the total volume of 3 ml.

Immunofluorescence microscopy. The brain sections were fixed in a phosphate buffered saline (PBS) solution containing 4% paraformaldehyde (PFA) (Electron Microscopy Science Lab, Inc.) and 2% sucrose (Sigma, Inc.) for 10 minutes. The brain slices were incubated in a solution of 0.1% Triton-X (Fisher Scientific, Inc.) in 1X PBS for 5 minutes. Mouse primary antibodies, anti-acetylated α -tubulin (Sigma, Inc.), anti-hydin (Novus Biologicals, Inc.) or anti-polycystin 2 (Santa Cruz Biotechnology, Inc.) were used at a dilution of 1:5,000, 1:30, and 1:250, respectively in a 10% FBS in 1X PBS solution for one hour at room temperature (RT) or overnight at 4°C. The brain slices were then incubated in the secondary antibodies, fluorescein anti-mouse IgG or texas-red anti-rabbit IgG (Vector Labs, Inc.), at a dilution of 1:500 in a 10% FBS in 1X PBS solution for 1 hour at RT. Before observation under a fluorescent microscope, the sections were counterstained with

4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Inc.) for 5 minutes to stain the nucleus/DNA. To minimize photobleaching, the sections were imaged immediately with the minimum exposure time possible.

Statistics

All the images and movies were captured and analyzed using Metamorph software. All quantitative data were displayed as mean $\pm$ SEM. Statistical analysis using student t-test was performed to compare the effect of ethanol on the dynamics of ependymal cilia within the ventricles between ethanol-treated and control groups. All statistical results were considered significant at a significance level of $p < 0.05$ and denoted by an asterisk (*).

Data availability statement. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

A.A.J. performed the study and drafted the manuscript; H.C.S. and Y.A.S. assisted in the experiments and edited the manuscript, A.W. facilitated in editing the figures, W.A.A., S.M.N. and Y.S. wrote the manuscript and provided technical advice to overcome challenges in cilia imaging, improving the protocol and technical troubleshooting.

Additional Information

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Review

Primary Cilium-Dependent Signaling Mechanisms

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Abstract: Primary cilia are hair-like organelles and play crucial roles in vertebrate development, organogenesis, health, and many genetic disorders. A primary cilium is a mechano-sensory organelle that responds to mechanical stimuli in the micro-environment. A cilium is also a chemosensor that senses chemical signals surrounding a cell. The overall function of a cilium is therefore to act as a communication hub to transfer extracellular signals into intracellular responses. Although intracellular calcium has been one of the most studied signaling messengers that transmit extracellular signals into the cells, calcium signaling by various ion channels remains a topic of interest in the field. This may be due to a broad spectrum of cilia functions that are dependent on or independent of utilizing calcium as a second messenger. We therefore revisit and discuss the calcium-dependent and calcium-independent ciliary signaling pathways of Hedgehog, Wnt, PDGFR, Notch, TGF- β , mTOR, OFD1 autophagy, and other GPCR-associated signaling. All of these signaling pathways play crucial roles in various cellular processes, such as in organ and embryonic development, cardiac functioning, planar cell polarity, transactivation, differentiation, the cell cycle, apoptosis, tissue homeostasis, and the immune response.

Keywords: calcium; cilioplasm; cytoplasm; sensory function; signaling

1. Introduction

Cilia are slender microtubule-based sensory organelles that protrude from the apical membrane in many cell types; approximately 800 ciliary proteins have been identified [1,2]. These multifunctional proteins are synthesized in the cytosol and transported to the cilia by intraflagellar transport (IFT) [3]. Cilia sense environmental cues and play vital roles in organ development. Based on their motility, cilia can be classified as motile and non-motile (also referred to as primary cilia). Motile cilia are usually bundled organelles known to be required for mucus clearance, cerebrospinal fluid flow, sperm motility, and leftward flow at the embryonic node, among other functions [4]. Defects in this motility are known to be associated with diseases such as primary ciliary dyskinesia, including Kartagener syndrome. On the other hand, non-motile cilia are solitary sensory organelles. Defects in the sensory function or structure of non-motile cilia are associated with diseases termed ciliopathies, including polycystic kidney. Based on their structure and motility, a special hybrid type of cilia, known as nodal cilia, has been identified. Nodal cilia are solitary motile organelles. Among all types of cilia, nodal cilia are currently known to play the earliest physiological role during embryonic development. Dysfunction in nodal cilia could potentially result in internal thoracic and abdominal organ configuration from normal (situs solitus) to mirror-image configuration (situs inversus), randomized configuration (situs ambiguous), or configuration with duplication (situs isomerism).

2. Cilia Structure

The cilium structure contains the microtubule-based cytoskeleton core unit called the axoneme (Figure 1). The axonemal structure is comprised of nine pairs of peripheral microtubules that are post-translationally acetylated to support the long cilia structure. In motile cilia, these nine pairs of microtubules are arranged in circumferentially with a central pair of microtubules (9+2). In the non-motile primary cilia, the axoneme structure usually lacks the central pair of microtubules structure (9+0 arrangement) and many other machineries [5]. In the nodal cilia, the axoneme also lacks the central pair of microtubules (9+0 arrangement), but it requires both dynein arms and radial spokes for motility [6]. The axonemal microtubule structures support the movement of IFT along cilia shaft. IFT includes kinesin-based anterograde and dynein-powered retrograde transport [7–9]. A cilium extends from a basal body, which is composed of two centrioles. One of the centrioles is known as the mother centriole, to which the ciliary axoneme is rooted beneath the cell membrane. In addition to its vital structural role, the basal body connected to the transition-fiber proteins is thought to regulate protein entry and exit from the ciliary compartment [10,11]. Protein entering and exiting the cilium is believed to be regulated by a barrier at the ciliary base that encompasses the transition zone (Y-shaped linkers) [12,13]. The Y-shaped linkers, also called Y-ankle, Y-linkers, or simply Y-links, extend from the outer microtubule doublets of the transition zone to the ciliary membrane, generating a very stable membrane domain resistant to detergent [14]. The triplet microtubules at the transition zone are transitioning to doublet microtubules at the ciliary axoneme. The ciliary membrane of the transition zone houses many proteins. The majority of these protein functions are largely unknown and have yet to be established. Some proteins are specifically confined within the transition zone only at a specified time and are later translocated out to the ciliary membrane. For example, many G-protein-coupled receptors are first targeted within the transition zone prior to translocation to the ciliary membrane [15–18]. The ciliary membrane is therefore known to have many receptor proteins, ion channels, protein transporters and sensory proteins to support the sensory function of the cilia. On the other hand, cilioplasm is constituted within the soluble compartment where many of the signaling proteins are localized.

A general classification of cilia structure is very complex because of the discoveries of a “9+4” axoneme in Hensen’s node of a rabbit and a “3+0” axonemal structure in a protozoan [19,20]. Nonetheless, each part of this cilia structure is crucial to support various signaling molecules. Some of the more established cilia-dependent signaling pathways are described below. Because the roles of primary cilia are diverse, the signaling pathways by cilia are more complex than previously thought. There is currently no literature that summarizes all signaling pathways by primary cilia; in this report, we therefore take the opportunity to provide a brief and concise summary of all known and potential signaling pathways that depend on primary cilia structure and function.

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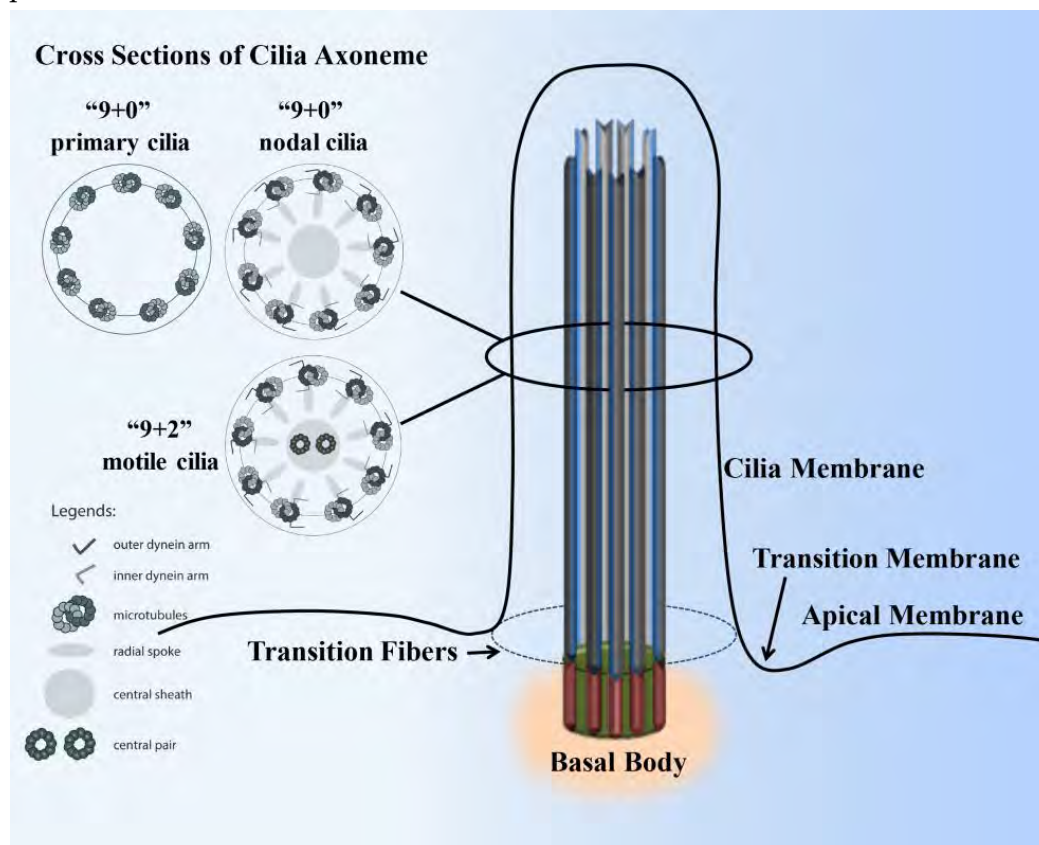


Figure 1. Structure of a cilium. The cilium is a hair-like structure composed of the ciliary membrane, cilioplasm, axoneme, and basal body. This sensory organelle is membrane bound and contains multiple microtubules running along its length. The ciliary membrane and axoneme make up the upper part of the cilium. The axoneme has nine peripheral microtubule doublets. The axoneme may have two central microtubules (9+2 vs. 9+0 axoneme). The 9+2 cilia usually have dynein arms and radial spokes that link the microtubule doublets and are motile, whereas most 9+0 cilia lack dynein arms, radial spoke, and central sheath and are nonmotile (primary cilia). Some 9+0 cilia only lack the central microtubule and are motile (nodal cilia). The axoneme is enclosed in the ciliary membrane, which is distinct from the cell membrane. Between the cilia and the cell membrane, there is a hypothetical transition membrane surrounding the transition fibers. The transition fibers at the junction of the basal body act as a filter for molecules that can pass into or out of the primary cilium.

3. Calcium Signaling

The association between cilia and calcium (Ca^{2+}) signaling has been recognized for decades but had only focused on the motile cilia [21,22]. Recently, many studies have demonstrated that, in different cell types, primary cilia are involved in the regulation of intracellular Ca^{2+} signaling by two major ciliary-associated proteins (polycystin-1 (PC1) and polycystin-2 (PC2)) [23–26]. In renal epithelial and vascular endothelial primary cilia, PC2 is co-localized with PC1 and functions as a component of a mechanosensory complex [23,24,26]. PC2 activation results in increased intracellular Ca^{2+} . The bending of the primary cilium causes cytoskeletal deformation and bending induced membrane stretching at the base of the primary cilium [27]. This, in turn, helps extracellular Ca^{2+} influx through PC2, an event that further stimulates the release of Ca^{2+} from the intracellular stores through the stimulation of ryanodine receptors (Figure 2A) [28]. A more recent advance to study intracellular Ca^{2+} is through the use of genetic markers that specifically encode Ca^{2+} -reporter proteins targeted to the cilium. This technique allows for the differentiation of intracellular Ca^{2+} signaling between the cilioplasm and the cytoplasm. Ca^{2+} signals within the cilium mechanically induced

by shear stress have also been studied by combining biosensor technology with mechanical flow systems [28–31]. Using a sensor targeted to primary cilia cells, it is shown that deflecting the cilium with fluid shear force causes an increase in ciliary Ca^{2+} [28–31].

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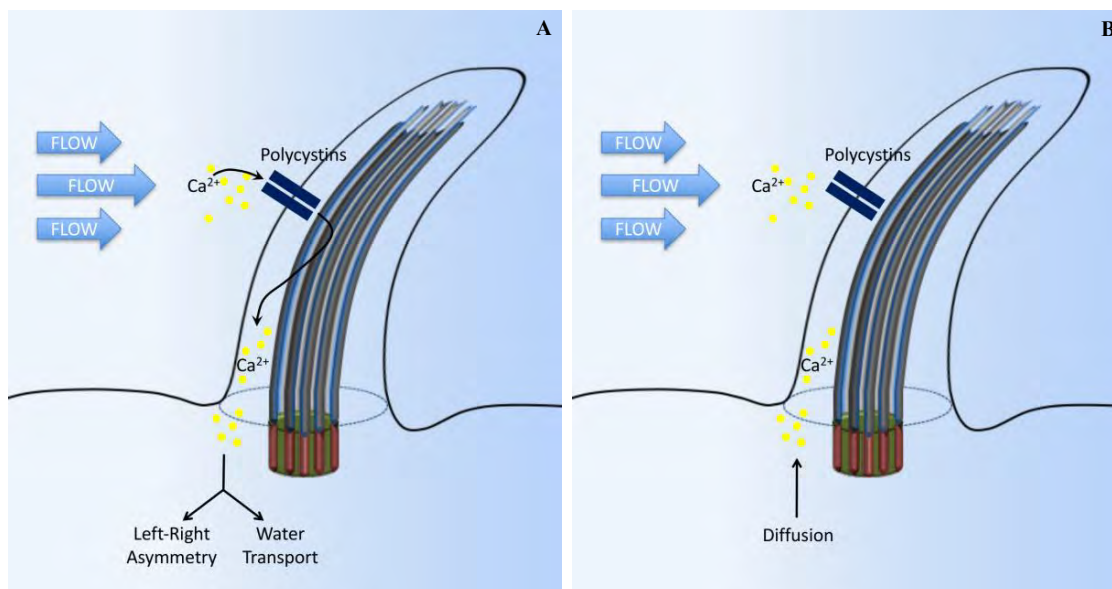


Figure 2. Cilia-dependent calcium signaling. (A) The standard established model suggests that primary cilia respond to force through mechanosensation. In this original model, fluid flow bends the cilium, which triggers the opening of calcium-sensitive channel proteins and allows Ca^{2+} to enter the cilium. Intracellular signaling cascades are activated by the Ca^{2+} influx, leading to altered gene expression on the left side of the embryo, or promoting water transport in the kidneys. (B) In another model, ciliary bending in response to fluid force does not open Ca^{2+} channels. Instead, Ca^{2+} influx, observed in previous experiments, might have been due to diffusion from the cell body or caused by a damaged cilium membrane in response to extreme levels of shear force.

Aside from PC2, a primary cilium also contains other transient receptor potential (TRP) channels [35–39]. These TRP channels may also conduct Ca^{2+} signaling. Each transient receptor potential cation channel occurs with one or a significant alteration in primary Ca^{2+} , arguing against the hypothesis that channels in the primary cilium would have a significant effect on global cytoplasmic Ca^{2+} [32]. Mechanically pulling of the cilium [35–39] led to a rapid increase in TRP2, a cilia-dependent propagating cilia cilium, while the cilium is the regulatory Ca^{2+} level. It is proposed that the cilium can become rapidly raised and ciliary membrane [40,41]. In addition, several bodies (Figure 2B) [35–39] that TRP3 contribute to the response to the mechanical fluid flow in cilia [42]. In zebrafish, left-right asymmetry of zebrafish cilia and muscle cells and vascular endothelial [23,25,30,42–44] be due to genetically encoded cilia and muscle cells and vascular endothelial [23,25,30,42–44]. A recent study shows that TRPP3, also known as the PKD2-L1 polycystin ion channel, has atypical Ca^{2+} regulation [39]. This study shows that TRPP3 is partially selective to Ca^{2+} influx. This influx is inhibited by higher internal Ca^{2+} concentrations. Moreover, the C-terminal EF-hands and coiled-coil domains of TRPP3 do not contribute to Ca^{2+} -induced potentiation and inactivation. It is proposed that the unique characteristics of the TRPP3 enable Ca^{2+} to enter but not leave the small ciliary compartments [39].

Apart from TRP channels, the L-type Ca^{2+} channel also modulates the cystic kidney phenotype, hydrocephalus, and left-right organization defects [45,46]. L-type Ca^{2+} channel knockdown in a zebrafish model facilitates the formation of these ciliopathic phenotypes and the L-type Ca^{2+} channel presence in renal epithelial cilium is confirmed by immunocytochemistry. This confirms the importance of Ca^{2+} signaling in the mechanosensory roles of the primary cilium. This L-type Ca^{2+} channel expression also regulates through Wnt signaling [40]. Although the exact mechanism is still unknown, it is thought that the modulation of intracellular mass and activity results in increased reactive oxygen species that generates oxidative DNA lesions. The subsequent cellular DNA damage response triggers increased $\text{CaV}1.2$ expression [46].

determined several trafficking mechanisms that regulate transport between the endoplasmic reticulum, plasma membrane, and ciliary membrane [40,41]. In addition, several laboratories independently show that TRPP2 contributes to responses to the mechanical fluid flow in cholangiocytes, embryonic node, left–right asymmetry of zebrafish, smooth muscle cells, and vascular endothelia [23,25,30,42–44].

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4. Hedgehog Signaling

The evolutionarily conserved Hedgehog (Hh) signaling plays an essential role in many aspects of vertebrate embryonic development and stem cell maintenance. Hh signaling is disrupted in a spectrum of human tumors, such as in basal cell carcinoma and medulloblastoma. Although the precise intracellular process of Hh signaling has slightly diverged between species, involving different mechanisms and protein components, primary cilia have an essential role in Hh signaling in both vertebrate and invertebrate development [47,48]. The association between Hh and cilia was first established from genetic screening in the mouse based on the neural tube patterning of mouse embryos. This screening identifies a set of genes that are required for normal Hh signaling and the formation of primary cilia.

The cilium has been suggested to function as the Hh transduction hub [49]. Hh signaling involves multiple inhibitory interactions. In the absence of Hh ligand (Figure 3A), Hh transmembrane receptor Patched1 (PTCH1, Hh 12-transmembrane receptor) is located at the base of a primary cilium and inhibits the activity of seven transmembrane smoothened proteins (SMO) through an unidentified signaling cascade. Meanwhile, suppressor of fused (SUFU) and three zinc-finger-containing transcriptional factors (Gli1, Gli2, and Gli3) interact with each other within the cilium. This leads to the inhibition of Gli transcriptional repressor (Gli3) formation and stimulation of gli transcriptional activators (Gli1 and Gli2) [47,50]. Thus, the transcriptional activation by Hh is largely driven by Gli1 and Gli2.

In the presence of an Hh ligand (Figure 3B), PTCH1 is activated. This triggers PTCH1 to move out of the cilium and releases the inhibition of SMO, allowing SMO translocation into the cilium [51,52]. Activated SMO co-receptor translocates into the cilium and activates Gli (Gli activator) by releasing the inhibition of SUFU. Furthermore, the Gli activator is transported to the cell nucleus to turn on the expression of other genes [53–55]. The repressor complex composed of SUFU and kinesin-like KIF7 sequesters Gli2 and Gli3 in the cilium tip. Sequential phosphorylations of Gli2 and Gli3 by protein kinase A (PKA), glycogen 3b (Gsk3b), and casein kinase 1 (Ck1) create a phosphopeptide motif on Gli2 and Gli3 that is recognized by Skip/Cullin1/F-box E3 ubiquitin ligase β -TrCP (β -transducin repeat-containing protein) [56]. The addition of ubiquitin then directs the Gli proteins to the proteasome for limited (Gli3) or complete (Gli2) degradation by the proteasome. This in turn regulates the abundance of the Gli2 and Gli3 repressor forms that ultimately inhibit Hh target genes in the

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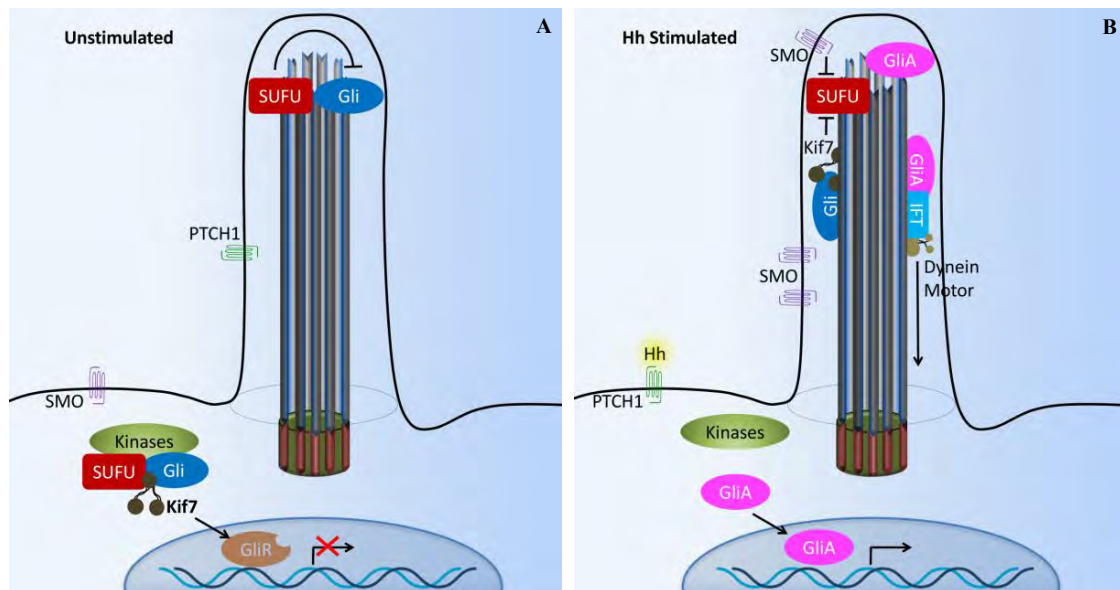


Figure 3: Cilia-dependent Hedgehog signaling. (A) The Hedgehog (Hh) pathway in vertebrates utilizes cilia as a signal transduction compartment. In the absence of the Hh ligand, the PTCH1 receptor localizes to the cilium and is thought to block the entry of SMO into cilia. The kinesin Kif7 (the C23S2 homologue) localizes to the base of the cilium, where it may form a complex with Gli transcription factors and other pathway components. Kif7 at the cilium base prevents Gli proteins' enrichment within the cilium and promotes processing of GliRs. (B) Upon Hh binding, SMO moves to the ciliary membrane and Kif7 translocates into the primary cilium, thereby promoting Gli accumulation at the ciliary tip. Kif7 and SMP in the cilia may also block the function of SUFU. Activated Gli (GliA) is transported out of the cilium by the dynein motor and intraflagellar transport particles (IFT). X and T symbols denote inhibition of gene transcription and protein function, respectively.

In the presence of an Hh ligand (Figure 3B), PTCH1 is activated. This triggers PTCH1 to move out of the cilium and releases the inhibition of SMO, allowing SMO translocation into the cilium [51,52]. Activated SMO coreceptor translocates into the cilium and activates Gli (Gli activator) by releasing the inhibition of SUFU. Furthermore, the Gli activator is transported to the cell nucleus to initiate Wnt pathways (canonical and non-canonical, Figure 4) [63]. The Wnt signaling pathway is evolutionarily conserved in many species, ranging from flies to human, and the signaling is mediated within primary cilia. Wnt signaling is involved in cell migration, planar cell polarity, neural patterning, skeletal system development, and organogenesis. In cultured cells and zebrafish embryos, ciliary proteins have been shown to regulate both non-canonical and canonical Wnt signaling pathways [64–67]. Wnt binding to membrane-bound receptors (frizzled) can activate both canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) Wnt signaling.

In the presence of a canonical Wnt signal (Figure 4A), cytosolic levels of β -catenin are high due to the inhibition of the β -catenin destruction complex [55,63,68,69]. This complex is composed of Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 β (GSK-3 β). The β -catenin destruction complex operates within the β -TrCP/SCF-dependent ubiquitin–proteasome pathway. In the absence of Wnt, the phosphorylation of β -catenin by CK1 and GSK-3 β is thought to act as a trigger for degradation. This process occurs at the basal body. Furthermore, disheveled (DSH) regulates canonical Wnt signaling through direct inhibition of GSK-3 β kinase activity [70].

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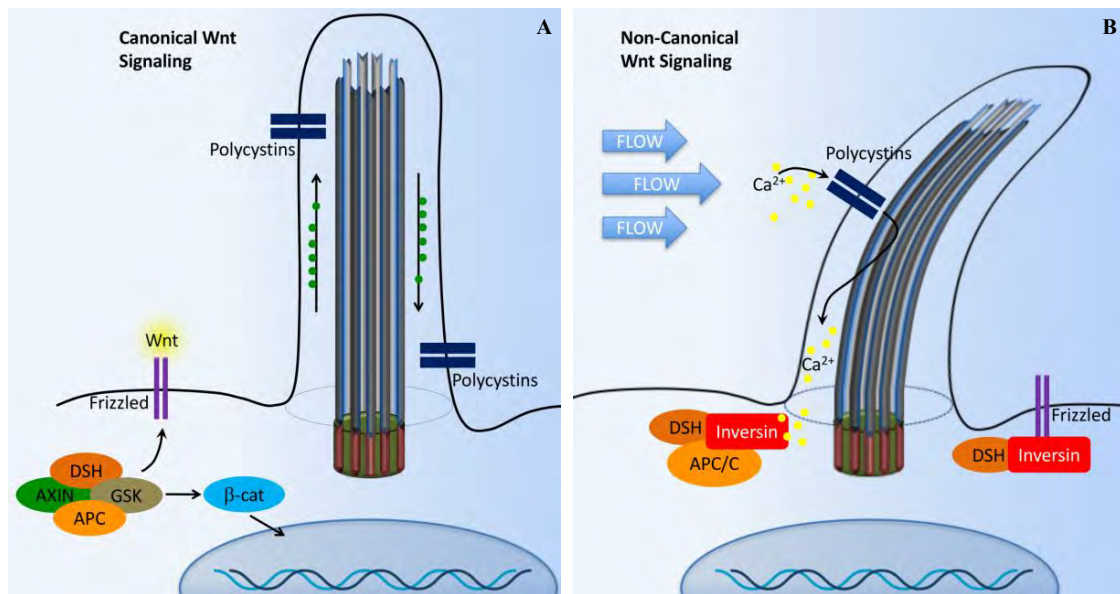


Figure 4. Cilia-dependent Wnt signaling. The cilium/basal body may function as a regulatory switch to control the canonical and non-canonical Wnt signaling pathways. (A) In the absence of fluid flow, canonical Wnt signaling predominates. Wnt ligand binds to the coreceptor frizzled, dishevelled (DSH) is recruited to frizzled, and glycogen synthase kinase-3 (GSK3) is inactivated. β -catenin (β -cat) translocates to the nucleus, where it acts as a transcriptional co-activator with members of the Lef and TCF family and induces transcription of Wnt target genes such as cMYC, AXIN2, or LIGAM. (B) In non-canonical Wnt signaling, the mechanosensation by fluid flow causes intracellular Ca^{2+} increase and an increase in inversin expression. Inversin resides in multiple locations in the cell as well as at the base of the cilium. Inversin targets cytoplasmic DSH for anaphase-promoting complex/cyclosome (APC/C)-dependent ubiquitylation and degradation, making it unavailable for canonical Wnt signaling.

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The non-canonical Wnt pathway is less well characterized. It is thought that the non-canonical pathway is independent of β -catenin, but dependent on DSH (Figure 4B). The non-canonical Wnt signaling is involved in controlling tissue functions and maintaining tissue architectures by modulating cell migration and orientation. The control of cells in the plane of a tissue, also known as planar cell polarity (PCP) pathway, was first established in *Drosophila* [71]. The mechanosensation-dependent calcium (Ca^{2+}) pathway is believed to be an important initiator for non-canonical Wnt signaling. DSH is recruited to the plasma membrane to prevent the proteasomal degradation of DSH in a β -catenin-independent manner. Several studies have implicated many other ciliary and basal body proteins in the regulation of Wnt signaling [67,72–74]. Inversin, in particular, interacts with DSH and targets the cytoplasmic fraction of DSH for degradation. Inversin is thought to function as a molecular switch required for non-canonical Wnt signaling and the suppression of β -catenin activity [67]. Inversin thus regulates the balance between the canonical and non-canonical Wnt signaling. Notably, inversin also functions to separate protein pools among the basal body or cilium, cytoplasm, adherens junction, and nuclear proteins [67,75]. However, it is currently unknown how inversin navigates its role among those protein pools.

A recent study shows that several Wnt ligands can bind to PKD1 and that PKD1/TRPP2 can mediate the effects of other Wnts, not only in the kidney but also in other organs where Wnt/ Ca^{2+} activity is present [76]. This study further suggests that the PKD1/TRPP2 channel complex can function as a ligand-activated channel. Specifically, Wnt3A, a typical canonical Wnt, activates TRPP2, suggesting that Wnts could induce Ca^{2+} influx regardless of their ability to signal through β -catenin as long as they can bind to PKD1 and PKD1/TRPP2 is present in the target cell.

6. PDGFR Signaling

Platelet-derived growth factor (PDGF) and their receptors (PDGFRs) play pivotal roles in cell survival, growth control, proliferation, cell migration, embryonic development, and the maintenance of tissue growth. The PDGFR is a receptor tyrosine kinase that mediates signaling events through primary cilia [77]. Abnormal PDGF signaling causes a range of diseases, including cancer development, cardiovascular inflammation, or fibrosis [78]. PDGFR α -signaling is coordinated by the primary cilium in mouse cultured embryonic fibroblasts [79,80]. PDGFR α is a tyrosine-kinase receptor located within the ciliary membrane. In normal fibroblasts and the fibrosarcoma cell line, the binding of ligand PDGF-AA activates the dimerized PDGFR- $\alpha\alpha$ receptor, which is present in the extracellular flow, and induces downstream cellular responses via MEK/ERK cascade signaling pathways (Figure 5). These pathways specifically lead to cellular growth, cytoskeletal development, and cellular migration and their differentiation [55,81,82]. Subsequently, PDGFR α -signaling through the fibroblast primary cilium may also be important in tissue homeostasis, while defects in this pathway could lead to tumorigenesis [83]. It has been reported that β -catenin may form a complex with PDGFR α to modulate cell migration [82].

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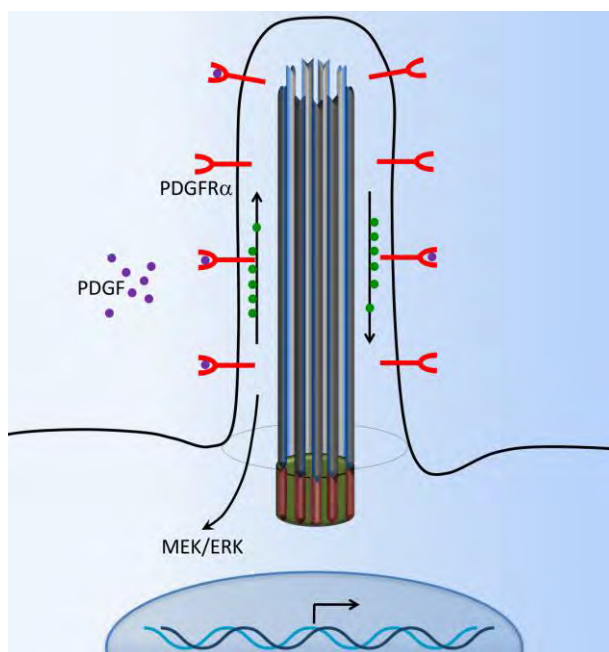


Figure 5. Cilia-dependent PDGFR signaling. The G-protein coupled receptors such as platelet-derived growth factor receptor (PDGFR α) are located at the cilia membrane. The PDGFR pathway is initiated with PDGF ligand binding to their receptors and inducing cellular responses through downstream signaling pathways such as the MEK/ERK cascade.

7. Notch Signaling

Notch signaling plays a key role in various aspects of patterning and cell fate choice in neurogenesis and maintenance of adult tissue growth and development. Notch signaling balances neurogenesis and maintenance of adult tissue growth and development. Notch signaling balances progenitor cells with differentiated neurons; it regulates the binary cell fate choice of progenitors as they segregate into different neuronal subtypes [84–89]. In mammals, Notch refers to four different kinds of receptors, Notch1–4. The Notch receptor has a single transmembrane domain with a large extracellular portion associated with Ca^{2+} and a short intracellular portion. There is evidence suggesting that some Notch signaling may depend on primary cilium for the signal transduction [90,91].

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The Notch3 receptor interacts with Presenilin-2, an enzyme responsible for Notch cleavage and activation (Figure 6). The Notch3 receptor has also been shown to localize in the ciliary membrane while Presenilin-2 is localized to the ciliary basal body [92]. Zebrafish with defects in basal body proteins (such as bbs1 and bbs4) have an increased expression of Notch targets. In these zebrafish models, the Notch receptor accumulates in the late endosomes, and consequent lysosomal degradation of Notch receptor is impaired [90]. The Notch signaling pathway plays a central role in left-right asymmetry by regulating cilium length and thus plays a crucial role in cilium length control [93,94]. Finally, Notch signaling also promotes trafficking of the sonic hedgehog (Shh) signaling mediators into primary cilium, thereby enhancing the responsiveness of neural progenitor cells to Shh [95].

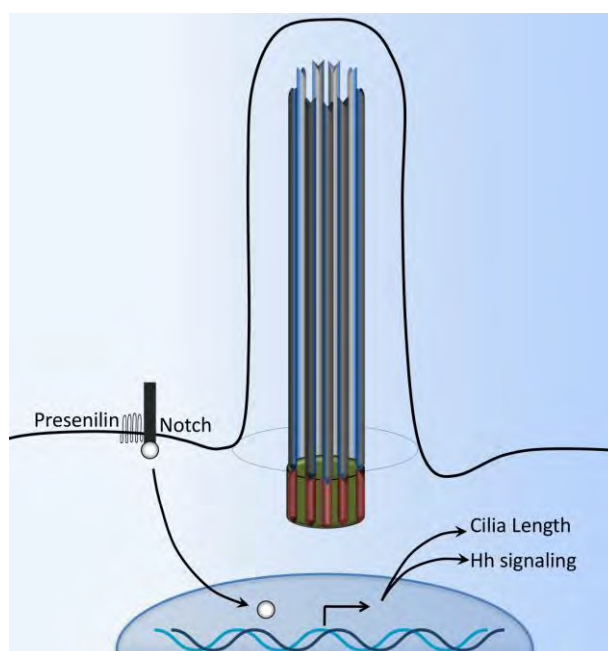


Figure 6. Cilia-dependent Notch signaling. A single-transmembrane Notch forms a receptor complex with the eight-transmembrane presenilin. Upon activation, presenilin mediates the intramembranal cleavage of notch containing the cdc10/Ankyrin repeats. The Notch intracellular domain is then released to the nucleus to activate various gene transcriptions.

8. TGF- β Signaling

Transforming growth factor beta (TGF- β) plays major roles in bone development and maintenance metabolism by regulating cellular proliferation, differentiation, matrix deposition, and cell migration [96,97]. TGF- β /Bone morphogenic protein (BMP), which is localized in the extracellular bone matrix, is activated during osteoclast-mediated resorption. The TGF- β /BMP bone matrix, is activated during osteoclast-mediated resorption. The TGF- β /BMP complex is released in response to loading or injury such as a bone fracture [98–101]. Recent studies have elegantly demonstrated that a discrete spatial group of TGF- β 1 and TGF- β 2 receptors is localized within and around the primary cilium in embryonic fibroblast and stem cells [102–104]. Both TGF- β 1 and TGF- β 2 receptors localize to the ciliary tip and endocytic vesicles at the ciliary base (Figure 7). TGF- β stimulation increases clathrin-mediated receptor localization and activation of SMAD2/3 and ERK1/2 at the ciliary base, which is increased in stem cells undergoing cardiomyogenesis [104]. TGF- β 1-induced recruitment of human mesenchymal stem cells (MSCs) is regulated by the primary cilium. Furthermore, the cilium mediates this response through distinct spatial localization of TGF- β receptors and signaling components at the primary cilium, whereby TGF- β 1-mediated activation of SMAD3 takes place at the ciliary base (Figure 7). Signaling through the primary cilium is necessary for the sensing of a TGF- β 1 chemotactic gradient in human bone MSCs, demonstrating a novel mechanism of TGF- β signal regulation and recruitment in stem cells.

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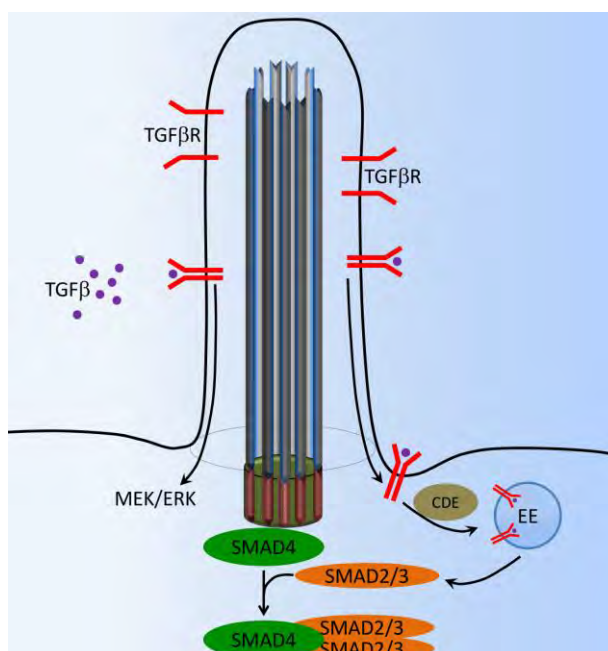


Figure 7. Cilia-dependent TGF- β signaling. In the presence of TGF- β , activated TGF- β receptors (TGF- β 1 and TGF- β 2) translocate from the ciliary membrane to the ciliary pocket (CiPo) for clathrin-dependent endocytosis (CDE) and activation of SMAD transcription factors 2/3 (SMAD2/3) in early endosomes (EE) at the ciliary base. SMAD2/3 forms a trimeric complex with SMAD4 for nuclear translocation and activation of TGF- β target genes. Ciliary TGF- β signaling also involves the activation of ERK1/2.

9. mTOR Signaling

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase functioning as a central regulator in cellular metabolism, cell growth, cell proliferation, cell cycle progression, and survival. mTOR associates with different proteins to form the rapamycin-sensitive (mTORC1) and -insensitive (mTORC2) complexes, respectively. mTOR signaling and primary cilia are associated with the hyper-proliferation of renal tubular cells, which induces the formation of renal cysts [106]. The most intensive studies about the primary cilium and mTOR in kidney research lie in the polycystic kidney-associated proteins [107]. The mTOR subcellular signaling involves many proteins, and the detailed subcellular localization of mTOR family members is largely unclear except that Hamartin (TSC1) is localized to the base of the primary cilium (Figure 8) [108]. In a recent study, the LKB-1-AMPK-mTOR signaling pathway is proposed in the kidney for the flow-dependent downregulation of the mTOR pathway. This pathway is necessary for the proper control of cell size by autophagy downregulation of the mTOR pathway. This pathway is necessary for the proper control of cell size by autophagy [109]. It is proposed that the primary cilia prevent cyst progression and kidney hypertrophy and proliferation [110], and PC1 inhibits mTOR activity through the regulation of tubular cell hypertrophy and proliferation [110], and PC1 inhibits mTOR activity through the regulation of tuberous sclerosis complex (TSC2) localization [111].

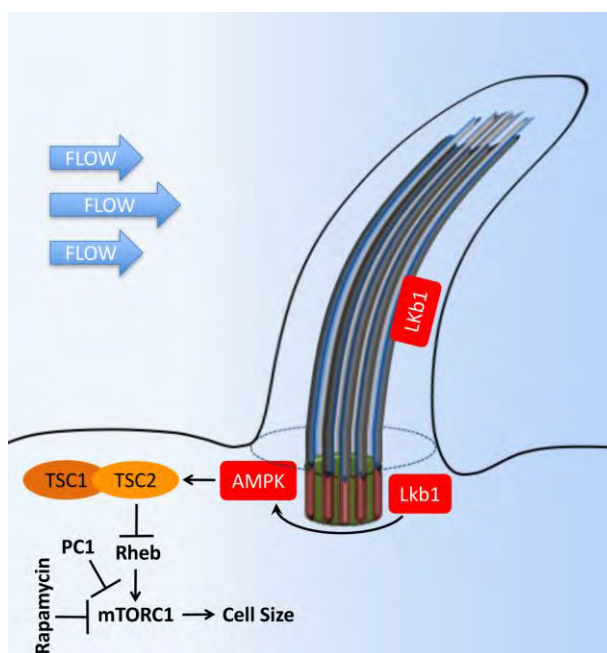


Figure 8. Cilia-dependent mTOR signaling. Flow shear stress mediates the bending of primary cilia and inhibition of mTORC1. Tumor suppressor kinase (LKB1) and AMP-dependent protein kinase (AMPK) are found in the cilia and basal body. Upon cilium bending by fluid flow, LKB1 is activated and transported into the basal body, where AMPK is phosphorylated. Furthermore, AMPK phosphorylates TSC2 to activate the TSC1–TSC2 complex. In turn, TSC1–TSC2 stimulates the GTPase activity of Rheb, preventing it from activating mTORC1. Otherwise, AMPK could also directly antagonize mTORC1. Polycystin-1 (PC1) may also exert its inhibitory effect on the mTORC1 pathway through its known interaction with TSC2. The T bars denote inhibition of protein function.

10. OFD1 and Autophagy Signaling

Autophagy is a process by which cells break down cytoplasmic contents. Oral facial digital syndrome 1 (OFD1) localizes to centrioles and centriolar satellites (Figure 9A). At the basal autophagy level, OFD1 and the autophagosome marker LC3 are co-localized at the basal body [112]. In starvation-induced autophagy, OFD1 is degraded (Figure 9B); the decreased OFD1 level upon serum starvation is contingent on both the autophagy-related (ATG) protein ATG5 and lysosome function. Fascinatingly, OFD1 degradation is specific to the centriolar satellite pool of this protein as OFD1 levels at the centrioles remain unchanged following serum starvation. OFD1 at the centriolar satellites suppresses ciliogenesis and that starvation-induced autophagy removes OFD1 from centriolar satellites.

Another study explores the effect of ciliogenesis on autophagy [113]. Starvation-induced autophagy and autophagosome biogenesis are dysfunctional in cell lines with abnormal IFT20 or IFT88, proteins required for cilia formation. Additional experiments show that signaling by the Hh pathway, components of which are recruited to the cilia by IFT1, is required for cilia-induced autophagy. Increasing Hh signaling tends to induce autophagic flux, while decreasing Hh will prevent autophagy. This mechanism is abnormal in IFT20 or IFT88 mutants. These data suggest that primary cilia promote autophagosome formation.

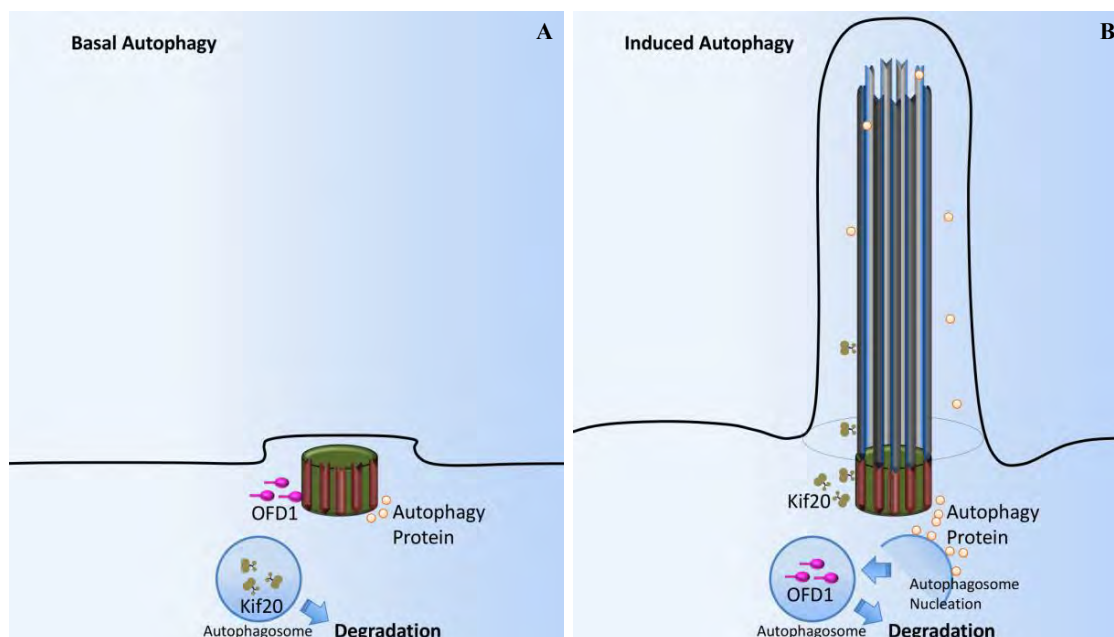


Figure 9. Cilia-dependent OFD1/Autophagy signaling. (A) In basal autophagy, ciliogenesis is prevented by the sequestering of IFT20 in autophagosomes and its autophagic degradation. OFD1, a protein that constrains cilium formation at centriolar satellites, is not affected by basal autophagy and ciliogenesis is therefore impaired. (B) Autophagy induced by serum deprivation promotes ciliogenesis through inactivation of OFD1, while sparing IFT20. Autophagy proteins are also confined through inactivation of OFD1, while sparing IFT20. Autophagy proteins are also confined to this sensory organelle, probably contributing to cilium organization. At the basal body, signals from the cilium stimulate autophagosome nucleation and OFD1 degradation.

11. Other Cilia-Dependent Signaling Pathways

11. Other Cilia-Dependent Signaling Pathways

G-protein coupled receptors (GPCRs) constitute a substantially diverse family of seven-transmembrane receptors that transduce extracellular signals to the cell through all proteins that regulate the activity of various cellular and physiological signaling networks. GPCRs also localize to rodent neuronal cilia, including the serotonin subtype-6 receptor (5-HT₆), somatostatin receptor 3 (SSTR3) that regulate the activity of various cellular and physiological signaling networks. GPCRs also localize to rodent neuronal cilia, including the serotonin subtype-6 receptor (5-HT₆), somatostatin receptor 3 (SSTR3), and melanin-concentrating hormone receptor 1 (MCH1R) [18,114,115]. Although receptors were found on neuronal cilia a long time ago, the functional roles of these GPCRs are still elusive. The elusive role of ciliary kisspeptin receptor (KISS1R) in the onset of puberty and adult reproductive function is observed in gonadotropin-releasing hormone (GnRH) neurons [116]. While the selective ablation of cilia from GnRH neurons affects neither cell migration nor sexual maturation, in the mice, the loss of the cilia in GnRH neurons seems to alter neuronal signaling. All these findings demonstrate that ciliary localization of a GPCR can have different effects on cellular metabolic function compared to being distributed throughout the membrane.

Overexpression of the serotonin subtype-6 receptor (5-HT₆) in cilium induces abnormally long cilia in rat brains that also have significantly reduced dendritic branches [117]. This effect may be associated with signaling through type 3 adenylyl cyclase (AC) and important ciliary AC and cAMP, which have been found to impact cilia length and further control neuronal cell development [118].

Another GPCR that has been associated with cilia signaling is dopamine receptor 2 (DOP2R), which has been found to impact cilia length and further control neuronal cell development [118]. Dopamine is a cilia length regulator in the hypothalamus and its specific regulation of the cilia length is involved in different tissues length in the body. The family of dopamine binding GPCRs that are involved in the regulation of cilia and flagella length [119]. One of the many regulators of cilia length is the orphan GPR32, a rhodopsin-like GPCR that couples to G_q/α to inhibit AC and cAMP. GPR22 regulates cilia

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the orphan GPR22, a rhodopsin-like GPCR that couples to $G_{\alpha i}/\alpha_o$ to inhibit AC. GPR22 regulates cilia length and structure as well as left–right asymmetry in zebrafish [120]. The expression of the dopamine receptor in renal epithelial and vascular endothelial primary cilia has further led to the hypothesis in the mechanism and signaling of dopamine involved cilia-specific signaling [121,122]. Activation of the ciliary dopamine receptor induces vasodilation via changes in the chemical and mechanical properties of primary cilia through changes in the cilia length. Another regulator of cilia length is oculocerebrorenal syndrome of Lowe 1 (OCRL1), a lipid phosphatase that has been shown to modulate cilia length in renal epithelial cells. OCRL1 knockdown leads to elongation of cilia and reduced intracellular Ca^{2+} release in response to ATP [123].

12. Conclusions and Perspectives

It is important to note that while the cellular and molecular pathways described above have been studied in the context of cilia structure or function, there is evidence that some pathways could also take place independent of cilia. For example, it has been suggested that cilia are not involved in Wnt signaling [124,125]. When a Wnt ligand binds to its receptor, the effector β -catenin translocates to the nucleus [126,127]. Although the roles of many ciliary proteins have been shown to alter the translocation of β -catenin to the nucleus [63,65], there is contradicting evidence about the roles of cilia on the Wnt signaling, ranging from inactivation to overactivation of the pathway [128]. Likewise, Hedgehog (Hh) signaling has also been shown to function independently of cilia [129]. It is shown that Hh regulation of Gli protein levels by Sufu is cilium-independent [129], despite the fact that many Hh pathway components are localized to cilia. It is therefore conceivable to assume that the cellular signaling described above could mechanistically involve both cilium-dependent and cilium-independent signal transduction pathways.

In summary, the primary cilium remains a vital organelle responsible for integrative signaling from extracellular signals in order to exert the physiological functions within a cell. It would certainly be a generalization to assume that a single type of cilium would serve most of the needs of every cell. The key to our understanding of cilia function in cellular signaling might be to differentiate the roles of cilia in different cells, tissues, and organs. Although abnormal ciliary proteins cause ciliopathies, which present a diversity of clinical features, we do not have a clear idea as to how the cilia signaling compartment plays an ultimate role in the pathogenesis. There needs to be further research on primary cilia as this neglected organelle continues to disclose its internal mysteries.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/11/2272/s1.

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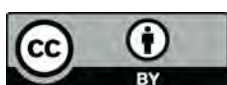
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Functional probes for cardiovascular molecular imaging

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Abstract: Cardiovascular diseases (CVDs) are a severely threatening disorder and frequently cause death in industrialized countries, posing critical challenges to modern research and medicine. Molecular imaging has been heralded as the solution to many problems encountered in individuals living with CVD. The use of probes in cardiovascular molecular imaging is causing a paradigmatic shift from regular imaging techniques, to future advanced imaging technologies, which will facilitate the acquisition of vital information at the cellular and molecular level. Advanced imaging for CVDs will help early detection of disease development, allow early therapeutic intervention, and facilitate better understanding of fundamental biological processes. To promote a better understanding of cardiovascular molecular imaging, this article summarizes the current developments in the use of molecular probes, highlighting some of the recent advances in probe design, preparation, and functional modification.

Keywords: Cardiovascular disease (CVD); molecular imaging; probe

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Introduction

Cardiovascular diseases (CVDs) are considered the most threatening disorder among the global population and are a source of challenge in modern research and medicine (1). There is an urgent need to formulate sensitive and reproducible non-invasive technologies for early disease detection, more specific biomarkers and personalized treatment of CVDs. Many traditional medical imaging methodologies, such as computed tomography (CT), ultrasound, and magnetic resonance imaging (MRI), have been regularly employed to facilitate early diagnoses. These

methods serve as a means to supervise early onset of CVDs. Since molecular differentiation is the basis of pathogenesis, traditional cardiovascular imaging technology has aimed at detecting a specific molecular target and fundamental biological development in CVDs (2). Accordingly, in recent years, molecular imaging technologies have been generally accepted in the role of rapid proliferating research interests that significantly promote the non-invasive diagnostic imaging for the conversion of anatomical description to detect specific tissue epitopes and monitor the fundamental biological developments at the cellular and subcellular level (3). Molecular imaging was created as a new branch

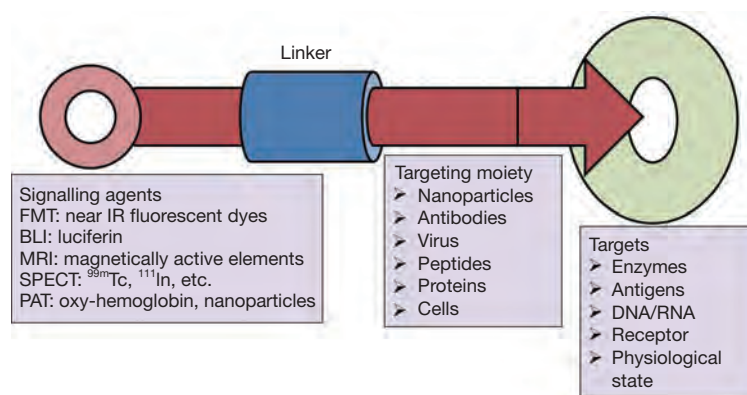


Figure 1 Schematic representation of molecular imaging probes. A molecular imaging probe is synthesized by using a linker to connect an imaging agent with a targeting moiety. FMT, fluorescence molecular tomography; BLI, bioluminescence imaging; MRI, magnetic resonance imaging; SPECT, single-photon emission computed tomography; PAT, photoacoustic tomography.

of biomedical science by merging the areas of imaging technology and probe utilization, thus enabling the direct/indirect spatio-temporal evaluation of molecular markers. In addition to this capability, molecular imaging can provide critical information for advanced diagnosis, appropriate treatment, better prognosis, improved staging, and better management—adding a significantly greater effectiveness to personalized medicine overall (4).

Cardiovascular molecular imaging technologies have been integrated with advanced scientific tools; this merger potentially allows for both morphological and functional imaging in cardiovascular pathophysiology. The emergence of cardiovascular molecular imaging provides detailed molecular and cellular level visualization that allows for early diagnostics and advanced therapeutics practices, which will additionally facilitate a greater understanding of the fundamental biological developments of CVDs. The mutual contribution among inter-disciplinary branches of science, such as molecular biology, chemistry, nanotechnology, and imaging technology, has effectively enhanced the rapid growth in the cardiovascular molecular imaging technologies and has allowed for the development of a large number of attractive new probes in the last decade. The appropriate probes should possess imaging, targeting, and therapeutic functions in order to facilitate the appropriate remedies in the battle against CVDs (2). In this section, we will first briefly describe the general working principles of molecular imaging-probe design, and then summarize the current state-of-the-art in probe-assisted cardiovascular molecular imaging.

Typical molecular imaging probes

Molecular imaging probes are agents that effectively facilitate visualization, quantification and characterization of biological processes in living systems. A molecular imaging probe typically comprises three key components: (I) an imaging agent for the corresponding imaging modality; (II) a targeting moiety that recognizes the intended molecular/cellular target; and (III) a linker connecting the imaging agent with the targeting moiety (Figure 1). The imaging agents used include radionuclides for positron emission tomography (PET) and single-photon emission computed tomography (SPECT), gadolinium (Gd) chelates or superparamagnetic iron oxide nanoparticles (SPIO) for MRI, fluorophores or quantum dots (QDs) for optical imaging, and microbubbles for ultrasound imaging. The targeting moiety, which specifically coordinates with the biomarker of a specific biological process, is also globally termed as a targeting ligand. The linker employed in the design of molecular imaging probes helps in merging the imaging agent with the targeting moiety, and has a significant impact on the pharmacokinetics of the molecular imaging probe. Concerning the latter point, many other distinct features are thus required to optimize the imaging quality; on the other hand, however, some probes do not even have the 3 essential components mentioned (5).

Favorable molecular imaging probes possess useful harness capabilities to gain concomitant anatomic, chemical, and physiological analytical report with high sensitivity, specificity and consistency. To diagnose the biochemical activity of CVDs, particularly at an initial stage, probes need to monitor

on the clinical change of a much lower amount of biomarker. Therefore, greater sensitivity is a critical requirement for a potential imaging probe. Amplification strategies such as augmented relaxivity of magnetic substrates or quenching of fluorochromes are the key strategies for developing sufficiently sensitive probes for clinical application. These probes can show a specific physical change that profoundly favors the signal amplification when the probes are spaced close together. In addition, due to the presence of numerous corroding enzymes and proteases in serum/tissue, maintenance of the intact structure of the imaging probes is a prerequisite to achieving high-quality images. The quantitative analysis of the image validity to assure the precession interpretation of physiological and pathological processes of the CVDs, along with the quality of molecular images, can be potentially affected by the *in vivo* stability of an imaging probe after administration (5).

Initially, the deposition of the imaging probes at the targeted site is required to allow the probes to actively interact with particular biomarkers like enzymes, receptors, and transporters, all which participate in different biological activities correlated with specific molecular/cell compartments. The specific interaction between biomarkers and imaging probes distinctly promotes the acquisition of information from the biological processes at the molecular level, which is effectively used to investigate and understand the distinct biological mechanisms underlying a specific disease. Additionally, the specificity between the imaging probe and the target site can potentially reduce the non-specific site interaction, significantly increasing the feasibility for quantification analysis of imaging output. Antibodies, peptides, aptamers and small organic molecules, have been widely and successfully employed as targeting agents for specific biomolecules in cardiovascular molecular imaging. The use of targeted imaging probes has the capacity to facilitate valuable insights into the pathophysiology of CVDs and to facilitate the development of novel therapeutic strategies (6).

Molecular imaging probes can be considered a special class of pharmaceuticals, especially some theranostics (the fusion of therapeutic and diagnostic approaches). Thus, potentiality and safety are the two most important concerns for molecular imaging probes. Generally, molecular imaging probes are administrated in low dose and their pharmacological manifestation can be negligible. However, the biological effects caused by an imaging probe still require close monitoring. The toxicity of molecular imaging probes is highly dependent on the probes' physicochemical

properties, which include the size, surface charge/coating materials, probe dosage, and duration to probe exposure. In order to enhance the quality of patients' lives, there is urgent need to synthesize more biocompatible and less potentially harmful imaging probes (7,8).

Strategy for molecular imaging probes design: interdisciplinary efforts

Progress in molecular imaging highly depends on the designing of advanced probes that can effectively detect biological activities on the cellular and molecular level. The design and development of molecular imaging probes require interdisciplinary efforts from the molecular, biological, chemical, imaging and nanotechnology fields (Figure 2). These interdisciplinary efforts can begin with the identification of biomarkers that are applicable to human CVD. Following this, physicochemical expertise is needed to synthesize molecular imaging probes capable of interacting to the target, and to properly optimize the devices for imaging. Multifunctional imaging probes based on nanotechnology can then extend the limits of currently available molecular imaging and allow precision diagnosis, contributing greatly to the advancement of personalized medicine. However, optimism in this new technology must be tempered by consideration of its potentially harmful elements. For instance, many of the fluorophores, contrasting agents and tracers, could be toxic in nature and the right material and specific concentration needs to be first identified before a molecular imaging probe can be deemed safe to administer to patients. More specifically, a gamut of information including cytotoxicity, tissue toxicity, *in vivo* toxicity and mutagenicity, require documentation in relation to molecular imaging probes before these probes are considered suitable for biomedical application. In this regard, some fluorophores such as indocyanine green and fluorescein have had their toxicity profile's fully elucidated, and subsequently have been approved by the Food and Drug Administration (9).

Biomarker-basis of molecular imaging

Biomarkers can act as indicators of health by correlating with disease in physiological measurement, or as determiners of disease complexity, diagnosis and prognosis. In addition, biomarkers also provide signals of fundamental metabolic or pathophysiological activities, or provide pharmacological responses to interventions. The major

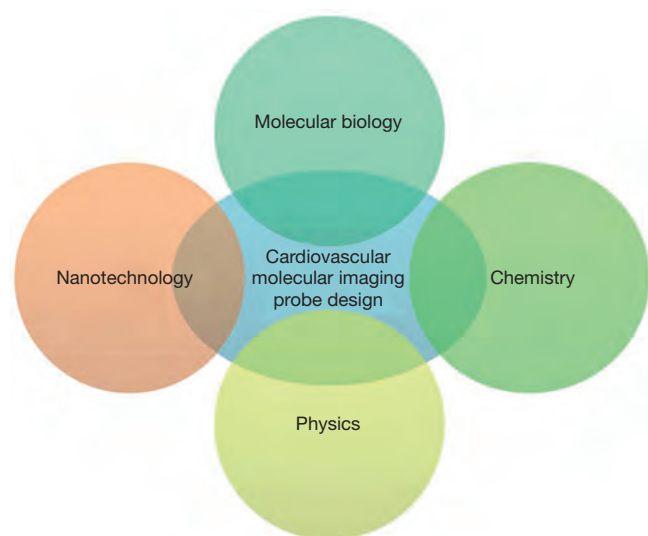


Figure 2 The designing and development of molecular imaging probes require interdisciplinary efforts from molecular, biological, chemical, physical imaging and nanotechnology sciences.

contributors to biomarker discoveries are molecular profiling studies in cardiovascular medicine, which are based on finding correlations between a molecular signature and a corresponding CVD behavior. Generally, molecular biomarkers include altered/mutant genes, proteins, lipids, and small metabolite molecules, which profiling studies then correlate with a biological behavior or a clinical consequence (10).

The selection of molecular imaging targets should meet certain criteria: They should specifically participate in the disease in question, particularly in CVD, and they should show the difference with respect to the progress and development of the disease. Biomarker determination and selection is therefore the initial, success-limiting step, in the designing of novel molecular imaging probes. As genomic and proteomic information from CVDs becomes increasingly available, databases to find new cellular/molecular biomarkers will provide a plethora of new targets for molecular imaging of CVDs (11). For example, vascular cell adhesion molecule-1, a well-validated marker of endothelial activation of atherosclerosis, could serve as a target for molecular imaging of atherosclerosis (12). Integrins such as $\alpha_v\beta_3$, expressed by neovessels, are similarly employable for molecular imaging of atherosclerosis, and several ^{18}F -labeled affinity ligands have already been developed. The evidence above suggests that the molecular imaging of CVDs based on biomarkers is reaching beyond

anatomy to encompass the assessment of aspects of molecular biology related to the pathogenesis of CVDs.

However, CVDs are highly complex and a set of biomolecules are unlikely to be able to detect most of the individual sensitivities related to developing CVD. The development of cardiovascular biomarkers then, naturally needs to overcome various obstacles. Research challenges include the development of probes for molecular imaging that will provide sensitive and robust measurement of thrombosis, infarction, angiogenesis, tissue oxygenation, activation and adhesion of immune cells. The molecular imaging probes also need a scalable synthesis using safe manufacturing processes and the necessary accompanying toxicology studies for human use. Much progress is being made in this field, and the rapidly expanding toolset of available molecular imaging probes will make it possible to non-invasively reveal the biological processes governing CVDs.

Chemistry-molecular probes synthesis

The design and development of a biologically active probe is of utmost importance to realizing the great potential of molecular imaging. By achieving the identification of select molecular biomarkers appropriate for the diagnosis of CVD, advanced chemistry has paved the way in molecular imaging probe synthesis. Specifically, click chemistry has made breakthroughs in a wide array of molecular imaging probe application developments. Within this field, it has become a routine strategy to fine-tune the synthesis of novel molecular imaging probes and to enhance their pharmacokinetic and pharmacodynamics profiles.

Generally, a 1:1 targeting moiety affinity towards the biomarker is considered suitable for molecular imaging. Biochemical amplification strategies are desirable to enhance the imaging signal in the cases where biomarker expression levels are low. The selected targeting moiety should have good affinity, particularly towards the selected biomarker. It also should possess a chemical anatomy that can transport different labels: isotopes for nuclear imaging, Gd for MRI or fluorescence, and dyes for optical imaging. For imaging technologies with intrinsic low sensitivity such as MRI, probes carrying large amounts of Gd allow for high molecular sensitivity (11).

The probe's *in vivo* pharmacokinetics, like adsorption, distribution, metabolism, and excretion, are gaining critical attention for their molecular imaging probe design potential. In general, the widely accepted factors that are believed to be significant in the probe's *in vivo* pharmacokinetics are its

particular physicochemical properties, including ionization constant, lipophilicity and stability. A molecular probe must remain stable enough in the blood circulation in order to effectively access the specifically targeted site with sufficient concentration and time for molecular imaging. Longer systemic circulation does provide greater target exposures that are highly suitable for therapeutic purposes. In cases where the molecular imaging probes are developed to image intracellular tissues, the probe needs to penetrate the cell membrane. Because ionization is a significant parameter that potentially increases/affects the solubility and membrane permeability of a molecule, most of the molecular imaging probes have been developed with ionizable groups and have different charges within the physiological pH range. Moreover, the recent developments in high-performance liquid chromatography/mass spectrometry instrumentation and procedures also provide an array of alternative techniques for determining the metabolites of the imaging probe—a capability which can amply facilitate necessary modification of the imaging probe and potentially enhance its *in vivo* stability (5,13).

Physics and advanced imaging devices

In CVDs, molecular imaging can diagnose, treat and monitor disease progress. Still, major modifications to these regular procedures need to be adopted in order to upgrade the imaging technologies in light of the concurrent development of more sophisticated molecular probes. At the time of writing, a large number of imaging modalities are under preclinical research, with some of them effectively being ready for investigation at the clinical stage. The predominant imaging modalities can be generally categorized as either anatomical imaging modalities like CT and ultrasound, or functional imaging modalities like PET, single-photon emission computed tomography (SPECT), optical imaging, molecular MRI, and magnetic resonance spectroscopy. From an engineering perspective, each imaging modality would benefit from an increased sensitivity and improved temporal and spatial resolution (2,4).

Anatomical imaging can provide anatomical information that is potentially altered by changes in the anatomy at the time of disease, while functional imaging modalities can determine biochemical activities of CVDs *in vivo* (2). Each imaging modality can be characterized by its own weaknesses and strengths. By exploiting the combined strengths of different imaging methods, a novel imaging technology might prove to be an attractive alternative to the

already mentioned imaging technologies, thereby advancing the capacity to image both small animals and humans. Multimodality imaging was designed by merging different imaging techniques including anatomical, functional and molecular imaging. The sets of information of specific target sites from each imaging technique have been combined to create hybrid images that show characteristics superior to all the previously mentioned imaging technologies. Generally, multimodality imaging modalities are selected to provide synergistic, complementary, or clinically relevant data beyond that furnished by any one of the single-modality methods. For example, co-registration with MRI images provides the anatomic landscape for localizing the functional or molecular information generated by PET (14).

The ideal multimodal imaging approach should provide high precision information like the exact localization, extent, molecular change and metabolic activities of the targeted site, in addition to specifically highlighting the pathogenomic changes which eventually lead to disease. While this is still a comparatively young field, it is reasonable to expect that sometime in the future, the multimodal approach will have significantly advanced cardiovascular molecular imaging technology by formulating novel multifunctional probes. Fresh insights in hybrid imaging technology have already appreciably contributed to translational research in cardiovascular medicine with small and large animals. Nonetheless, the human anatomy's complex interlinking of organ function, (e.g., when the heart beat is affected by lung motion) yet demands further advancement be made in areas of cardiovascular imaging, such as motion detection devices (15-17).

Nanotechnology is a promising platform for multifunctional molecular imaging probe design

Nanotechnology is a multidisciplinary field and has made great strides forward in recent years. The term “nanotechnology” can generally be applied to any fabrication of new materials with a scale smaller than one hundred nanometers. Unique characteristic features like optical, electronic, magnetic, and chemical reactivity are associated with nanomaterials solely because of their nanoscale sizes, big surface area, and shapes, which can allow for various biomedical applications including molecular imaging. A wide variety of nanoparticles have been used as molecular probes with each imaging modality demanding nanoparticles with specific properties for contrast production. For example, SPIO have long

been investigated and are considered to have remarkable potential in MRI contrast enhancement. QDs, which show tunable wavelength photoluminescence from the visible region to near infrared (in accordance with their size), are among the most widely studied nanoparticles for optical imaging (18,19).

Nanoparticle imaging probes offer many advantages compared to conventional small-molecule-based imaging probes. These advantages include producing excellent contrast, lengthy circulation time, and integrating multiple properties. For example, hundreds (or more) imaging labels (fluorescence tags, radionuclides, and other biomolecules) or a group of labels for different imaging modalities, can be merged to formulate a novel single nanoparticle, thus providing drastic signal amplification. In addition, multiple and potentially different, targeting ligands (antibodies, peptides, aptamers, and small molecules) on the nanoparticle show increased binding affinity and specificity (20).

Recently, theranostics have been rapidly developing technology which can be employed as platforms for placing different functionalities not only for molecular imaging functions, but for targeted drug delivery as well. The combination of different imaging labels, targeting ligands, and therapeutics could permit the effective and controlled drug delivery to the targeted site, while providing noninvasive, quantitative monitoring in real time. Application of such multifunctional nanomaterial will potentially elevate the diagnostic validation and design of therapeutic practice, which can then in turn facilitate the prediction of clinical outcomes, realizing the hope of personalized and advance medicine (21). In recent years, the development of theranostic-based nanomedicines has gradually progressed due to the availability of better knowledge about molecular imaging technologies and the processes that occur at the basic molecular and cellular level during cell-exposure to certain therapeutic aids. The intimate understanding of mesenchymal stem cells (MSCs) and related mediated cancer therapy, has facilitated the formulation of theranostic probes which contain umbilical cord-derived MSCs conjugated with triple fusion genes containing the herpes simplex virus truncated thymidine kinase, Renilla luciferase and red fluorescent protein. By using near infrared imaging technology, the real-time activities of the cells can be monitored by bioluminescence signals provided by Renilla luciferase and red fluorescent protein. Hence, the formulated theranostic probe can effectively inhibit breast cancer progression by inducing tumor cell apoptosis and suppressing angiogenesis, in

addition to facilitating the tracking of cell delivery and tumor response to MSCs and molecular and cellular activities in tumor cells (22). Similarly, other theranostic probes have been developed by conjugating MSCs with citrate-coated SPIO and investigated for their efficient labeling of human MSCs without transfection agents. The formulated theranostic probes showed no adverse effect in cell proliferation, presentation of typical cell surface marker antigen, and differentiation into the adipogenic and osteogenic lineages. Overall, the synthesized theranostic probes demonstrated very efficient capacity for intracellular magnetic labels for *in vivo* stem cell tracking by MRI (23).

The most promising applications of nanopatform-based imaging probes appears to be in cardiovascular medicine. Imaging probes might be able to overcome the barriers of the biological system to accumulate the drug at the targeted site because certain barriers of the biological system potentially affect the drug entry and delivery (24). Still, there remains a significant concern as to the potential adverse effects of human exposure to nanoparticles. The biological distribution and circulation of nanoparticles within biological tissues, and immune responses like phagocytosis, opsonization and endocytosis of nanoparticles, are the specific considerations which seriously need to be evaluated before the field of nanoparticles can be specialized into nanomedicine proper (19). In the design of probes for molecular imaging, several functionally active nanomaterials that have been employed have very low biocompatibility which can subsequently lead to quick excretion, instability, low circulating time, and potential toxicity. Hence, significant precaution and validation should be taken before adopting the nanomaterial in a biomedical application. PEGylation is widely known as a coating technology that is used effectively for nanoparticle formulation and can potentially enhance the blood circulation time by modulating the protein adsorption to the nanoparticle. The prolonged availability of the nanomaterial in the blood stream significantly augments the accumulation of higher concentrations of prescribed nanomaterial in the targeted site. Moreover, the surface-functionalized nanoparticles with polyethylene glycol (PEG), promote the bio-conjugation with various ligands including drug molecules. Several steps have been taken to reduce the risks posed by nanomaterials in order to enable the novel formulation of highly biocompatible and less toxic nanomedicines (e.g., silica nanoparticles) (25).

Other important questions concerning the application of nanomaterial in molecular imaging still need to be

answered before responsible clinical application is possible. These issues include a more thorough understanding of the following: the change that occurs in the association or conjugation between the nano-carrier and the drug molecules, particularly their pharmacokinetics, biodistribution and potential adverse effects of the nanotherapeutic nanomedicine; chance of change of the safety profile of the nanomaterial at the time of conjugation or surface functionalization; the possibility to reduce the toxicity of the polymeric nanoparticles which accumulate considerably at the targeted site and are made up of non-biodegradable polymer and greater than the size of the renal discharge threshold; and the adverse effects (and accompanying prevention measures), that could arise when nanomaterials cross the blood-brain barrier. The above topics are hitherto understudied; thus, clarifying them is critically significant to optimizing the selectivity, efficacy and safety of nanomaterials in clinical practice. Healthy interdisciplinary competition involving the scientific fields of physics, chemistry, biology, nanotechnology, engineering and medicine could contribute to each discipline's mutual development and the possible design of effective, cutting-edge nanomedicine for the diagnosis and treatment of clinical complications (26,27).

Potential developers of molecular imaging probes need to be aware of the specific properties of functional nanomaterials before they can be repurposed as effective nanoplatforms. The probes should be developed with optimality in features such as surface coating, targeting properties, and extent of biocompatibility, in order to create an assembly with the suitable contrast/therapeutics.

Optical imaging

Optical imaging of live tissues through photonic technology has emerged as a significant tool in advanced biomedical research. Optical imaging can be defined as a technique in which light in ultraviolet, visible or infrared ranges is used to visualize intact organisms. Certain optical imaging techniques like intracoronary optical coherence tomography, Raman spectroscopy and near infrared spectroscopy, are widely employed to inspect vascular cellular and lipid components in patients suffering from CVDs. Fluorescent probes are used to image the molecular phenotype more precisely. Near infrared fluorophores, which have an excitation and emission wavelength of 630 and 1,000 nm, have been employed in optical imaging technologies. Properties like high diffusibility, molecular targeting,

and marking for angiogenesis make these fluorophores exceptional candidates for optical imaging technology (28,29). The optical imaging itself has a few advantages over rapid visualization protocols and other simpler methodologies. Fluorescence molecular tomography (FMT) and bioluminescence imaging (BLI) are the two major optical molecular imaging methods that have been employed to diagnose complications of the cardiovascular region. FMT is a recent innovation that permits greater spatial localization of the fluorescence signal, while BLI is another powerful tool for visualizing temporal and spatial development of cardiovascular complications in real time (30).

FMT

In the biomedical research area, microscopic fluorescence techniques are playing a major role in the study of molecular and cellular development in cell and tissue cultures. Using sophisticated labeling methods, the enhanced spatial resolution provided by fluorescence techniques, facilitates easy comparison between cellular dimensions and site targeted imaging. Currently, two- and three-dimensional (3D) optical imaging has been employed to investigate the biological activities in intact organisms like mammals. However, this method has come up against certain limitations such as poor light propagation in tissue, and difficulty in reconstruction of exact spatial information due the high scattering and absorbing capacity of biological tissues. As a result, the detection of the signal on the surface of the tissues will be stronger than the signal detected inside the embedded tissues because of the optical properties of the surrounding tissues. In an attempt to overcome these challenges, FMT techniques have been used to map the 3D distribution of a fluorescent probe or concentration of protein (31). FMT can be defined as any advanced tomographic technique which models the imaging in near infrared regions and facilitates the 3D quantitative detection of a fluorescence signal distribution in the live tissues of the small animal at any depth. Following this, nearly 50,000 to 100,000 pairs of source detectors have been used to measure the diffusion models of photon propagation in turbid media (32). The particularly high sensitivity of these optical imaging techniques potentially permits the detection in tissue of even low concentrations of targeted molecules. Furthermore, these methods are comparatively cost effective, which supports the biomedical research community by reducing their expense in research and development procedures (31). However, the effectiveness

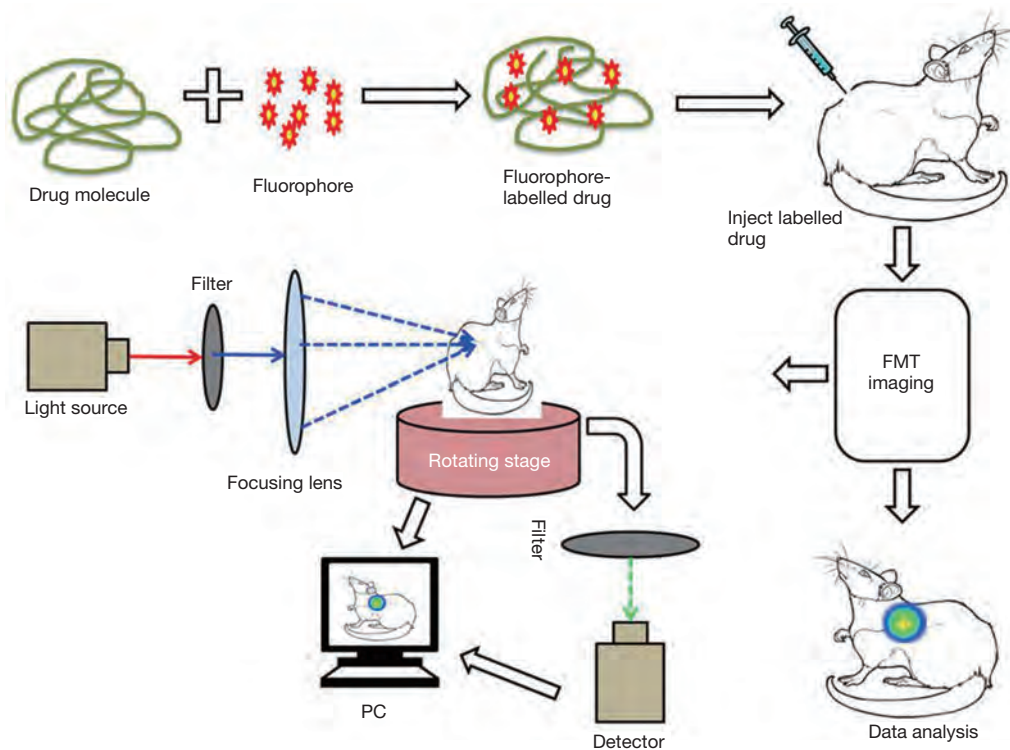


Figure 3 A schematic representation of the major components of the FMT system. FMT, fluorescence molecular tomography.

of the imaging agents in the *in vivo* condition could be highly dependent on certain factors such as probe targeting, activation, pharmacokinetics, biocompatibility and photophysics, which are all still not yet fully understood. Advancements in biomedical research have begun to investigate these variable factors as they concern the application of optical reporters for *in vivo* imaging (33).

A basic explanation of how the FMT method works is as follows. Light passes to the tissues of the animal body at different projections and signals are detected at multiple points in the tissue. The imaging prototype is generated using cylindrical geometry and charge-coupled device (CCD) detection methods. According to the recorded raw emission and excitation information, a synthetic assessment is developed and applied to the inversion code for the reconstruction of absolute fluorochrome density in the animal tissue (Figure 3). With the current available acquisition setup, the determination of threshold fluorochrome is approximately 200 femtomoles in the target tissues volume deep in a phantom enhances the optical properties of the tissues (34). In a separate experiment, a novel handheld device for the 3D fluorescence molecular tomography was described. The device is characterized

by its bendable structure and miniaturized size (nearly 10 mm in diameter) which was particularly effective as an intraoperative tool for imaging the internal tissues and facilitating the image guide surgery (35). FMT can overcome the limitations encountered by traditional fluorescent imaging technology, such as poor images at shallow tissues depths due to scattering of signal, by increasing the number of source detectors, resulting in comparatively less background noise and high submillimeter resolution at a depth of 7.5 mm (36).

BLI

Novel approaches and technologies that facilitate the early detection of infections, even before the observation of visible clinical manifestations, result in effective diagnosis and treatment, by easing the selection of therapeutic procedures that start at these earlier stages of infection. Fluorescence molecular imaging extends several opportunities to explore the biological processes in intact organisms. Similarly, BLI is a method which works on a sensitive detection of visible light produced during the oxidation reaction between luciferase enzymes and the molecular substrate

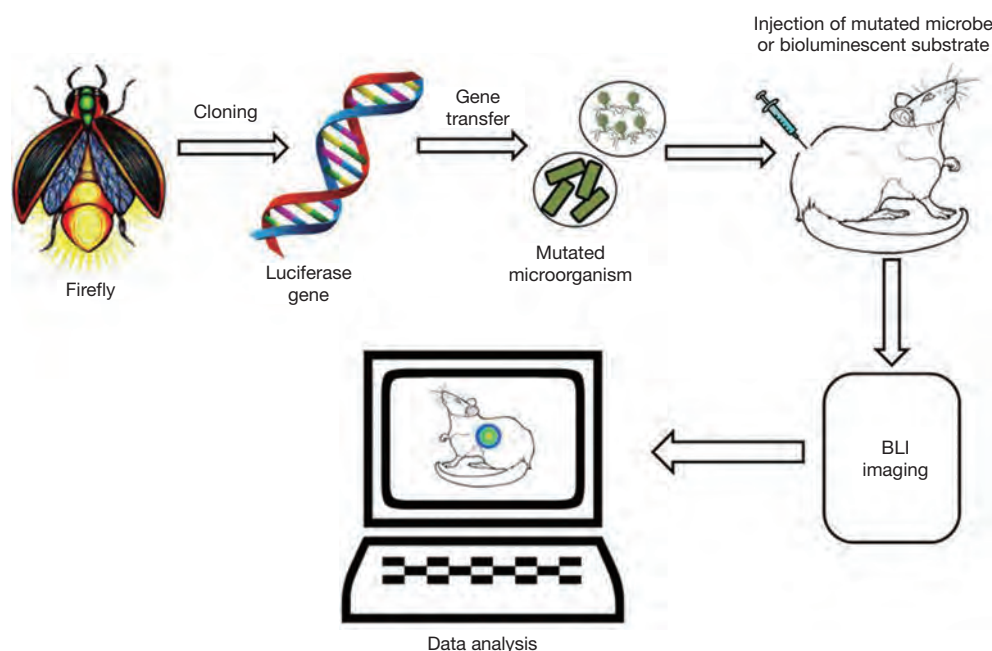


Figure 4 Schematic representation of bioluminescent imaging. BLI, bioluminescence imaging.

(30,37). Bioluminescence is the natural capacity some organisms have to convert chemical energy into light energy. Currently, biological researchers are employing this natural phenomenon to visualize intact biological tissues, using a technique called BLI. The basic principle of this technology is the detection of photons emitted from the tissues in the living organisms. In the fluorescence imaging methods, usually light absorption is required to emit light at longer wavelengths; in BLI, light absorption is not required for imaging. For the biological process of bioluminescence to occur, luciferase enzymes are required to be in the presence of luciferin (substrate) and oxygen. In some cases, the enzyme also needs other cofactors like ATP and Mg^{2+} for successful bioluminescence reactions. Most often bioluminescence emits light at a wavelength of nearly 560 nm, which allows for the imaging of deeper tissue layers with higher resolutions (*Figure 4*). This method is widely applied in the biomedical areas of monitoring transgene expression, development of infection, toxicology, progression of viral infections and gene therapies (38,39).

The major advantage of the BLI method is its relatively simple implementation which permits the monitoring of a disease with continual quantification of progression of infections, all without scarifying the experimental animal. Moreover, this method decreases the number of experimental animals required in testing because multiple

measurements can be recorded within the same animal over the course of infection and treatments, which also minimizes the complication of biological variations. Recent BLI methods have used a variety of luciferase enzymes including firefly luciferase, Renilla luciferase, Gaussia luciferase, Metridia luciferase, Vargula luciferase and bacterial luciferase. Of these luciferase enzymes, firefly, Renilla and bacterial luciferases have been widely employed for optical imaging. The BLI method has gained significantly more importance than its counterpart, fluorescence molecular imaging, mostly due to FMI's lack of endogenous bioluminescent reaction in mammalian tissues to offer background free images, in addition to the prevalence of fluorescently active compounds in the biological tissues which could affect the target resolution. BLI technology also has different modalities like steady-state bioluminescent imaging, multi-reporter bioluminescent imaging, multi-component bioluminescent imaging and bioluminescence, as a supplementary imaging technique (40). The greater signal-to-noise ration due to the absence of intrinsic bioluminescence background in animal tissues, has promoted BLI as an interesting tool for monitoring biological development of a living animal in real time (41). As such, BLI has been mostly used for small animal studies to monitor and correlate the survival, engraftment and migration of a range of cell populations.

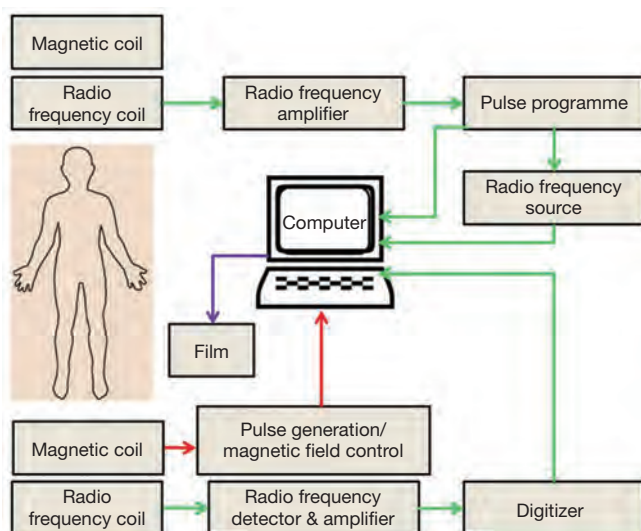


Figure 5 A schematic diagram of functional MRI scanning. MRI, magnetic resonance imaging.

MRI

The basic principle of the MRI works upon the inherent magnetic properties of tissue and the ability to employ these properties to produce tissue contrast. Single protons, which induce the magnetic movement in omnipresent hydrogen atoms, produce the magnetic resonance images. As is well-known, when an electric charge in the movement that creates magnetic field, spinning protons on small magnetic field and can be conceived of as little magnet. When a patient is placed in the bore of a large magnet (MRI scanner), hydrogen protons align with the externally applied static magnetic field to generate a net magnetization vector. Most of the protons will distribute randomly on a quantum level, either with or against the scanner static magnetic fields. On the other hand, a little increase in the spins aligns protons with the field causing net tissue magnetization (*Figure 5*). The time required for this alignment is determined by the longitudinal relaxation time. This longitudinal relaxation time varies between the tissues it is employed to provide contrast for (42). MRI can provide high-spatial resolution anatomic images with intense living tissues' contrast by using the difference in relaxation times of protons in different biochemical environments. The collaboration of high spatial resolution and contrast, facilitates the anatomic responses of many disease complications to be visualized in animal tissues. MRI can be used to monitor different physiologic parameters like diffusion, permeability and change in blood oxygenation

levels after neuronal activation. A recent advancement in nanotechnology is the addition of passive contrasting agents like iron-oxide nanoparticles which can further enhance the contrast (43). MRI molecular imaging data can be evaluated according to the amount of signal change, the area of the tissue displaying contrast enhancement, and the calculation of relaxation time change or direct detection of contrasting agents by multi-nuclear imaging and/or spectroscopy (44).

The MRI method of molecular imaging has certain limitations however, like lower sensitivity than that of nuclear imaging. Nevertheless, certain nanomaterials such as micelles, liposomes, iron oxide nanoparticles and emulsion can be used to deliver contrasting agents like Gd chelates in larger amounts to the targeted sites. In certain cases, iron oxide nanoparticles can be used as contrasting agent in MRI technology (45,46). The application of molecular MRI in vascular imaging is mainly focused on determining the significant components of atherosclerotic plaque, along with adhesion molecules, plaque hemorrhage, ventricular volume or blood velocity, and plaque macrophage content (47,48). MRI molecular imaging comprises a wide range of technologies like site-targeted contrasting agents, drug delivery vehicles, controllable MRI probes, and direct mapping of tissue metabolite (44). However, due to the magnetic field interference caused by devices like defibrillators and pacemakers, MRI cannot be employed on some patients with implantable devices.

SPECT

SPECT is a noninvasive technique that can facilitate 3D functional information with greater resolution, sensitivity and specificity. All nuclear-based imaging technology, including SPECT, relies on the injection of trace amounts of molecules labeled with radioactive isotopes. The decaying of radiolabeled molecules results in the emission of photons. These photons are detected by a position-sensitive detector which can give the arrival location of the photon and its energy. It cannot provide the information about the travelling path of the photons as it requires generating projections of the emitting body. A collimator, a device used to produce a parallel beam of rays or radiation, is inserted between the patient and the detector which in turn shapes the stream of emitting photons into a beam. This allows the detector to locate the photon associated to a line in space along which the decay occurred. Usually, a collimator allows to pass the photons to the detector in a certain angle rest of the photons are removed. This results in the projection

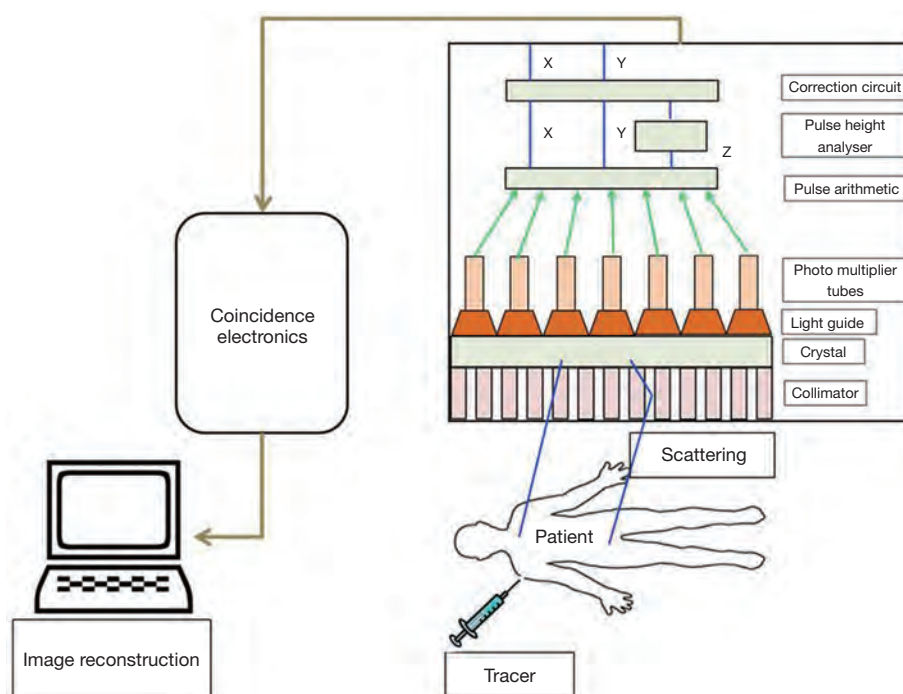


Figure 6 Image-capturing system using Single-photon emission computed tomography.

of the image at the expense of the discards of most of the photons (49). Customarily, SPECT records the 2D nuclear medicine images obtained at different positions around the patient and gives an estimated 3D radioactivity distribution by employing the tool to reconstruct from multiple projections. SPECT stands out from the other methods of medical imaging modalities due to its special features of instrumentation and image reconstruction (Figure 6) (50).

Generally speaking, energy resolution widely affects the quality of an image. In principle, images should be only constituted with primary photons which are emitted from a decaying radionuclide, and not the secondary scattered photons. A device possessing better energy resolution should be able to discriminate the primary photons from the scattered photons and also provide the better image contrast and more precise quantification of radionuclide amount and distribution inside an animal. By using this feature of SPECT in myocardial perfusion imaging, the area of hypoperfusion can be discriminated from its neighboring normal perfusion region by enhancing image contrast (51). SPECT can also be used to detect coronary artery diseases (CAD) even at the time of asymptomatic or moderate CAD. To support this application of SPECT, research has been conducted by the American College

of Cardiology Foundation and the American Society of Nuclear Cardiology to assess if SPECT could be used to rate uncertain detection and risk of CAD in asymptomatic patients at initial periods of disease complication. The experimental results demonstrated that, with a retrospectively determined group of asymptomatic patients at moderate CAD risk, SPECT had great potential in detection and risk stratification of CAD. Moreover, the test results showed that average annual mortality was 4% in patients with the high-risk scan but only showed 1.6% in patients with normal scanning. This proves that SPECT imaging can detect the complications in asymptomatic patients and can help in bypass surgery even in the nonoccurrence of symptoms (52). As can be seen, SPECT is a highly sensitive, easy and fast-labeling imaging technology with significant spatial resolution. It can facilitate convenient quantification *in vivo* and *in vitro*, provide real-time monitoring of cell viability, and enable imaging over long intervals.

Photoacoustic tomography (PAT)

PAT, also known as optoacoustic imaging, is an emerging imaging technology which holds great promise for

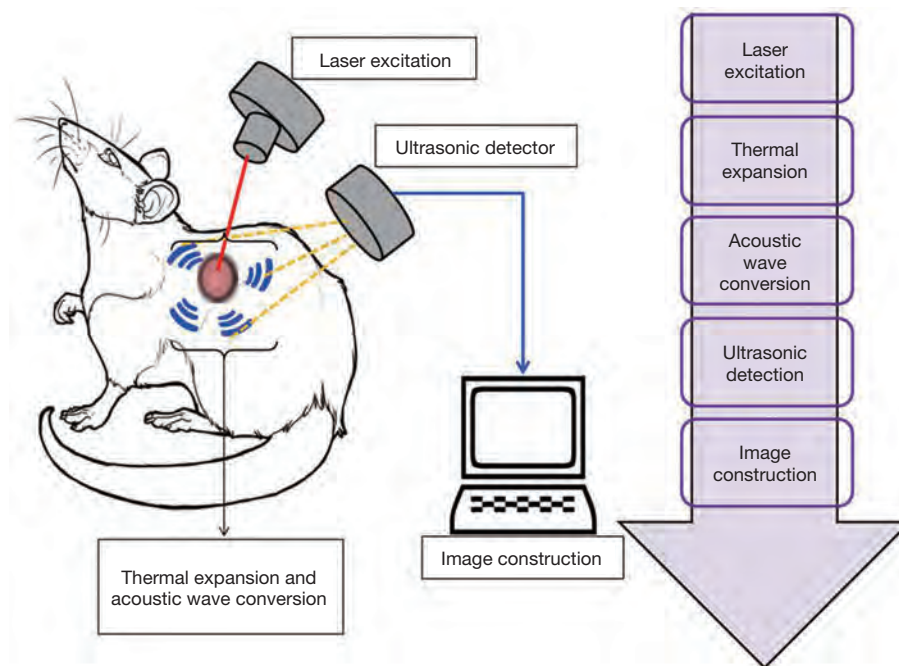


Figure 7 Schematic representation of working principle of photoacoustic imaging.

preclinical research and clinical treatment. In every imaging technology, the fundamental constraint is the limiting effect of light diffusion on spatial resolution in deep tissue. In contrast to these methods, photoacoustic imaging employs a laser light generated ultrasound, and so has emerged as a promising new technology which can potentially overcome the challenges found in traditional optical imaging. PAT works with the principle of photoacoustic effect where the absorbed optical energy is converted into acoustic energy. Biological tissues are lit by nanosecond laser pulses, raising the temperature at localized places and generating a wideband of ultrasound pulses due to thermal expansion. The light-excited ultrasound pulses spread in the biological tissues and clarify the detection boundary surrounding the biological tissues in ultrasonography (*Figure 7*) (53). The special properties of acoustic waves, which scatter much less than optical waves in tissues, are what makes PAT technology distinct from other optical imaging technologies. Interestingly, PAT is able to produce high-resolution images in both optically ballistic and diffusive regimes. Moreover, at the time of optical absorption even with single origination, PAT can readily utilize the advantages of rich endogenous and exogenous optical contrasts. For imaging vascular structures, endogenous oxy- and deoxy-hemoglobin are used as anatomical and functional contrasts.

Certain dyes, nanoparticles and reporter genes are also used as exogenous contrasts for molecular imaging (33,54-56). PAT imaging has a wide range of applications like glucose metabolism imaging, deep tissue imaging of fluorescent proteins, evaluating the prolong period of biodistribution of an optical contrast agent, and video rate cross sectional imaging (57-60). Due to the advancements in science and technology, PAT has been evolving quickly towards greater spatial resolution, higher frame rates and higher sensitivity detection. Moreover, the continuous progress in PAT has also accelerated and augmented contributions from biology, chemistry and nanotechnology. In summary, PAT has several applications including performing anatomical, functional, molecular and fluid-dynamic imaging at different system levels, and plays a significant role in essential bio-research and clinical practice.

Conclusions

As a new branch of the biomedical sciences, molecular imaging was born under the merging of two areas: imaging and probe technology. Based on the probes allowing direct/indirect spatio-temporal evaluation of molecular markers, molecular imaging can provide critical information for much earlier detection of disease and is expected

to have a significant impact in personalized medicine through providing more appropriate treatment, better prognosis, improved staging and better management. This review focused on the development of probes for future applications in novel cardiovascular targeted imaging strategies. The future for probes in cardiovascular molecular imaging rests on, among other innovations, the development of targeted biological molecules, reporter gene methods to enhance gene therapy, tagging cells to track stem cell transplantation, and labeling small-molecule probes with sensitive isotopes. Realizing the potential of these promising probes is likely to play a significant role in both diagnostic and prognostic functions, and the investigation of therapeutic practices.

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Footnote

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