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Impaired Hedgehog-GLI1 pathway activity underlies the vascular phenotype of Polycystic Kidney Disease

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Abstract

Polycystic Kidney Disease (PKD) has been linked to abnormal structure/function of ciliary proteins, leading to renal dysfunction. Recently, attention has been focused in the significant vascular abnormalities associated with PKD, but the mechanisms underlying this phenomenon remain elusive. Here, we seek to define the molecular events regulating the angiogenic imbalance observed in PKD.

Using Micro Computed Tomography (n=7) and protein expression analysis (n=5), we assessed the vascular density and the angiogenic profile of non-cystic organs in a well-established PKD rat model (PCK rat). Heart and lungs of PCK rats have reduced vascular density and decreased expression of angiogenic factors compared to WT. Similarly, PCK-VSMCs (n=4) exhibited lower levels of vascular markers. Then, using small interfering RNA (n=4), we determined the role of the ciliary protein fibrocystin in wild type vascular smooth muscle cells (WT-VSMCs), a critical component/regulator of vascular structure and function. Reduction of fibrocystin in WT-VSMCs (n=4) led to an abnormal angiogenic potential similar to that observed in PCK-VSMCs. Furthermore, we investigated the involvement of the Hedgehog signaling, a pathway closely linked to the primary cilium and associated with vascular development, in PKD. Mechanistically, we demonstrated that impairment of the Hedgehog signaling mediates, in part, this abnormal angiogenic phenotype. Lastly, overexpression of Gli1 in PCK-VSMCs (n=4) restored the expression levels of pro-angiogenic molecules.

Our data support a critical role of fibrocystin in the abnormal vascular phenotype of PKD and indicate that a dysregulation of Hedgehog may be responsible, at least in part, for these vascular deficiencies.

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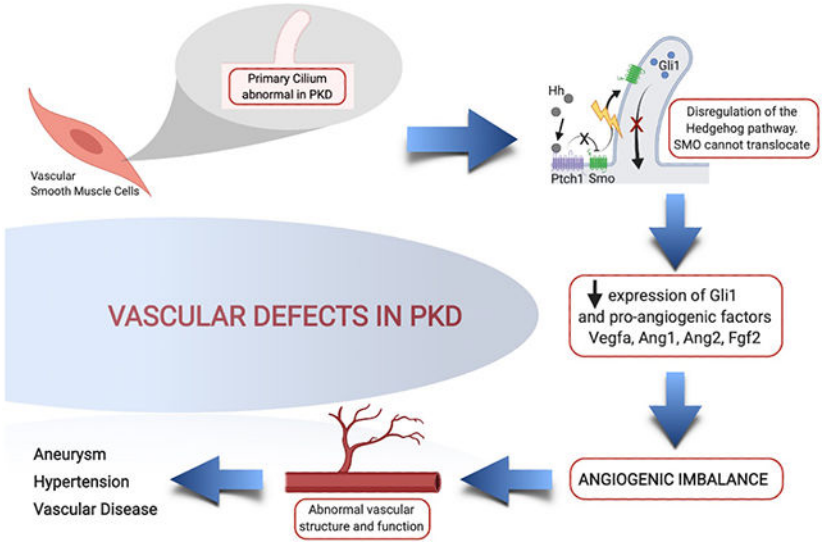
Disclosures. None.

Graphical Abstract



Keywords

Vascular biology; vascular smooth muscle; primary cilium; Hedgehog signaling pathway

Introduction

Polycystic kidney disease (PKD) is one of the most frequent, life-threatening inherited renal disorders. It accounts for 4%-10% of end stage renal diseases (ESRD) worldwide, after which patients require dialysis or transplantation.[1,2] PKD, whether in its autosomal dominant or autosomal recessive form (ADPKD or ARPKD, respectively), has been associated with abnormal function of proteins from the primary cilium.[2] PKD is characterized by defects that impair the normal function of the primary cilia, which are cellular organelles that determine the orientation, shape, and function of somatic cells.[2] Traditionally, PKD has been considered a reno-tubular disease, causing the development of cysts in the renal parenchyma, eventually leading to tissue compression and kidney failure.[2,3] Presently, therapies are mainly targeted to treat the consequences of renal failure and cystic expansion, such as pain, systemic hypertension, and ESRD. However, it is the cardiovascular alterations that account for the majority of morbidity and mortality in this population.[4,5] In fact, systemic arterial hypertension, the most common and important clinical problem in ADPKD, is a major risk factor for cardiovascular disease[4-7] and is known to lead to multi-end-organ damage like stroke and myocardial infarction.[4,8] Importantly, early treatment of hypertension slows the progression to end-organ damage and can reduce morbidity and mortality, underscoring the importance in understanding the pathophysiology behind vascular dysfunction that can direct early vascular treatment in PKD.

Using an ARPKD rat model, PCK rat,[9] our group has shown that PKD has impaired renal microvascular structure associated with decreased vascular density, abnormal expression of

vascular endothelial growth factor (VEGF) and its receptors as well as abnormal aortic endothelial function.[10,11] Importantly, we have also reported that the vascular defects observed in PCK rats appear even prior to the development of hypertension or measurable renal dysfunction, suggesting that the vascular impairments observed in early ARPKD are not secondary to renal dysfunction.[12]

Vascular Smooth Muscle Cells (VSMCs) are essential components of large blood vessels, such as arteries and veins, and are necessary for normal development, homeostasis and organ function.[13] Moreover, the primary cilium of somatic cells, including VSMCS,[14-16] regulates many cell processes, acting as a sensor of both biochemical and mechanical stimuli. Thus, it can be hypothesized that abnormalities in proteins related to the primary cilium, as seen in PKD, can be related to the vascular defects described. In fact, Lu et al. have recently reported that primary cilia of VSMCs mediate extra cellular matrix (ECM)-protein sensing and fluid-flow-induced mechanosensing.[15] The ECM is critical for the formation of new blood vessels, as it can regulate vascular shape and morphogenesis through many important signaling pathways.[17] However, the intracellular mechanisms involved remain unclear.

Mechanistically, the Hedgehog (Hh) pathway is intimately related to the function of the primary cilium and a critical regulator of vascular development.[18,19] Under normal conditions, the stimulation of the Hh pathway leads to the activation of several downstream genes, one of which is VEGF. While previous studies have evaluated the potential involvement of Hh in renal cystic disease,[20,21] the involvement of Hh in vascular PKD remains to be elucidated.

In this study, we expanded on these findings demonstrating a direct relationship between an abnormal production of the ARPKD protein, fibrocystin (FPC), and the angiogenic imbalance seen in ARPKD, and defining a novel role of the Hh-Gli1 pathway in the pathophysiology of this disease.

Methods

The authors declare that all supporting data are available within the article and its online Data Supplement.

[Extended Methods can be found in the Data Supplement]. The following references [22-25] are found in the Data Supplement only.

Experimental Design.

All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Mayo Clinic College of Medicine Institutional Animal Care and Use Committee (IACUC). Rats were divided into wild type (WT, Sprague-Dawley) and PKD (PCK rat model).[9] At 4 weeks of age, prior to changes in blood pressure or renal function,[10-12] animals were euthanized using CO_2 and tissues (lungs and heart) harvested to study their vasculature profile (immunohistochemistry and Western blotting) or to isolate VSMCs. Those rats used for micro Computed Tomography imaging – microCT – were euthanized with an overdose of isoflurane (5%).

Subsequently, to investigate the biology of VSMCs in PKD, we isolated VSMCs and studied their phenotype and function. Furthermore, to test the role of FPC in VSMCs, we performed siRNA studies to knockdown the expression of FPC in WT-VSMCs, followed by studies of protein expression of angiogenic factors. Finally, we investigated the role of the Hh pathway in PCK-VSMCs.

Statistical Analysis.

In vitro studies were performed using 4 different clones of VSMCs (isolated from 4 rats) and repeated independently 3 times. Data displayed as scatter dot plots show the median, whereas data displayed as bar graphs show the mean ± SEM. Statistical comparisons between groups were performed using non-parametric Kruskal-Wallis test or non-parametric Mann-Whitney test, as appropriate. Statistical tests were calculated using GraphPad Prism software 6.0. Statistical significance was established at two-tailed $p < 0.05$.

Results

Extra-renal non-cystic organs of PCK rats have impaired vasculature.

MicroCT scanning of the whole heart showed reduced vascular density in PCK rats compared to WT (Figure 1) as demonstrated by the myocardial vascular network highlighted in red. In addition, the number of stained vessels (vWf – von Willebrand factor – positive) in lungs as well as myocardium was significantly lower in PCK rats compared to WT (Figure 2A). The correlation analysis of heart and lungs staining (Spearman r = 0.7091, $p = 0.0268$) indicated that the number of vessels in the heart correlates well with the number of vessels in the lungs, showing that the vascular defects observed are systemic and not confined to just a single organ (Figure 2B). These observations were partially confirmed by the protein expression analysis of the endothelial marker CD31, the vascular endothelial growth factor receptors (VEGFRs), FLT1 (VEGFR1) and FLK1 (VEGFR2) as well as the inducible nitric oxide synthase (iNOS) in heart and lung tissues of young animals (Figures 3A **– heart,** 3B **– lungs**).

PCK-VSMCs show reduced function.—Rat VSMCs (Figure 4A) isolated from aortic tissues stained positive for α-SMA, as shown by flow cytometry (Figure 4A). Furthermore, the acetylated α–tubulin staining revealed the presence of the primary cilium in rat VSMCs cultured under starvation conditions (1% FBS, Figure 4B). Finally, in response to injury, VSMCs from PCK animals displayed significantly decreased migration (functionality) compared to WT, as shown by PCK-VSMCs covering a smaller area after injury (Figure 4C).

Protein expression analysis of PCK-VSMCs revealed a significant vascular dysfunction with reduced levels of VEGFA and FGF2 (Figure 4D).

Fibrocystin is important for the normal phenotype of rat VSMCs.—Pkhd1-targeted siRNA in WT-VSMCs resulted in a 62% decrease in FPC mRNA expression, while no changes in FPC levels were observed in the scrambled siRNA controls. Importantly, rat VSMCs that received Pkhd1-targeted siRNA, had a significant change in their phenotype

with decreased expression of pro-angiogenic markers both at the gene (Vegfa and Angiopoieting-1-Ang1-, Figure 4E) and protein level (VEGFA, Figure 4F), when compared to controls, showing a behavior comparable to rat VSMCs isolated from PCK animals (Figure 4D). Moreover, increased expression of FGF4 (siRNA: 1.66 ± 0.11 vs. control: 0.26 ± 0.08, $p < 0.05$) and caspase3 (siRNA: 0.90 ± 0.08 vs. control: 0.67 ± 0.06, $p < 0.05$) indicated a significant upregulation of proliferative and apoptotic pathways, demonstrating a shift in the protein expression profile that was similar to that observed in PCK-VSMCs. Importantly, no significant changes were seen in the biology of WT-VSMCs + scramble siRNA.

PCK-VSMCs show reduced Hh pathway activity.—In canonical Hh signaling, the Hh ligand binds to the cell receptor Patched1, that regulates the Smoothened (SMO) protein, prior to downstream activation of Gli-1 and downstream genes. Double immunofluorescent staining of aortic tissues revealed a significant reduction in GLI1 expression (in red) in the tunica media (Smooth Muscle Cells, in green) of PCK rats compared to WT (Figure 5A). Therefore, we examined Gli1 expression in cultured vascular smooth muscle cells. Smoothened Agonist (SAG) is a small molecule that binds directly to SMO, increasing its stability and promoting its maturation and translocation into the cilium.[26,27] In response to SAG, PCK-VSMCs showed a sustained decreased expression of Gli1, marker of activity of the Hh pathway when compared to WT cells (Figure 5B). In addition, immunofluorescent staining displayed a significant difference of GLI1 protein levels in PCK cells compared to WT (Figure 5C). Importantly, Gli1 and other angiogenic factors, such as Vegfa, Ang1 and Ang2, upregulated in WT-VSMCs in response to stimulation with SAG, were downregulated in PCK-VSMCs (Figure 5D). A similar behavior was observed in WT-VSMCs treated with SAG after Pkhd1 knockdown, showing a significant downregulation of the expression of Gli1 as well as other pro-angiogenic genes (Figure 5D).

Moreover, while in most WT cells induction of the Hh pathway with SAG led to clear colocalization of SMO (in green) with ARL13b (primary cilium, red, Figure 6A), quantification of staining results confirmed a severe impairment of Smoothened translocation into the cilium of PCK VSMCs at 6 and 24 hours after treatment with SAG, with a very small number of PCK cells that showed colocalization (Figure 6A).

Finally, Gli1 knockdown in WT-VSMCs resulted in a 50% decrease in Gli1 mRNA expression, which was associated with a significant decrease in VEGFA protein expression to levels comparable to PCK-VSMCs (Figure 6B). On the other hand, PCK-VSMCs that overexpressed the Gli1 plasmid showed a 740-fold increase in Gli1 mRNA expression. The upregulation of Gli1 in PCK-VSMCs could partially restore the expression levels of angiogenic markers, such as VEGFA and FGF2 (Figure 6C).

Discussion

The main conclusion of this study is that PKD has a primary defect in vascular structure and function, independent of clinically detectable renal dysfunction. This conclusion is supported by the following findings: 1) non-cystic organs in very young PCK rats have reduced vascular density associated with an abnormal angiogenic profile and impaired

vascular function, in the absence of changes in blood pressure or renal function, 2) VSMCs from young ARPKD animals have a defect in angiogenic factors, 3) a knockdown of FPC alters the angiogenic potential in VSMCs as seen in PKD, and 4) vascular therapeutic targeting with Gli-1 is able to, at least partially, correct the vascular abnormality.

Polycystic kidney disease affects a large proportion of patients in renal dialysis and is associated with the dysfunction of primary ciliary proteins. Both ADPKD as well as ARPKD have been mainly associated with abnormalities in the tubular portions of the nephron, leading to cyst formation. Furthermore, it has been suggested that cyst enlargement results to compression of the renal tissue, leading to renal dysfunction. Traditionally, the proliferation and expansion of cysts have been considered as the main responsible for the development of hypertension and other vascular complications, nourishing the idea that most of the abnormalities observed in PKD are just a secondary effect of the abnormal renal structure and function.

However, even though the renal affections have been traditionally considered the prime site of clinical manifestations, PKD has been mostly recently categorized as a multisystem disorder, with patients mainly suffering from cardiovascular complications,[4,28-30] increasing the interest in the vascular pathophysiology of PKD.[31-34] In fact, our group has previously shown that young (4-5 weeks old) PCK rats, even prior to changes in blood pressure or renal function,[12] have significant vascular abnormalities. Furthermore, we also reported an impaired relaxation response of the aorta (non-cystic organ) to acetylcholine, which functions as an activator of nitric oxide synthase (NOS), a defect corrected with NO donation.[12] In this study, we expand these observations and show that the derangements in vascular density is seen in multiple non-cystic organs (i.e. lungs and heart), together with an abnormal expression of endothelial (CD31, FLK1, FLT1) as well as pro-angiogenic (VEGFA, FGF2, ANG1 and ANG2) factors. These data suggest a significant decompensation in vascular stability, strongly supporting the presence of a primary vascular defect in PKD.

In the vasculature, the primary cilia of VSMCs can act as mechano-sensors, potentially regulating many cellular functions.[14,15,35] It can be hypothesized that a defect in the structure or function of the ciliary proteins (PC1 and PC2 in ADPKD, and FPC in ARPKD) can account for abnormalities in the structure and function of VSMCs.[15] Here, we show that a transient knockdown of fibrocystin, using siRNA, led to phenotypic alterations in the biology of WT-VSMCs in as little as 3 days. Importantly, these cells (WT Fc-siRNA) had a behavior comparable to cells isolated from PCK rats, providing evidence that an abnormal fibrocystin may be responsible for an abnormal, PKD-like, function of VSMCs. The pro-angiogenic potential was assessed by the analysis of the expression of pro-angiogenic markers, such as VEGFA, FGF2 and ANG1.

The precise pathways, from the abnormal cilia to target genes, like VEGFA or FGF2, that lead to the abnormalities in vascular structure and function in PKD have not yet been fully elucidated. In previous studies, we have described that PKD is associated with a lower expression of the VEGFA ligands, when compared to control.[11] Here, we extend these observations and propose that a dysregulation of the Hh pathway might contribute to the

smooth muscle cell dysfunction and vascular impairment observed in ARPKD. In fact, it has been previously described that the canonical Hh pathway is mainly ligand-activated at the primary cilium to regulate vascular development.[19] Thus, we believe that an abnormal cilium might be responsible for the impaired pro-angiogenic potential of PKD aortic smooth muscle cells due to a dysregulation of the Hh pathway. Previous studies have reported that enhanced Hh activity may have an important negative role in renal cystogenesis, promoting proliferation of cyst-lining epithelial cells.[20,21] In this study, our focus is on vascular smooth muscle cells and the importance of the primary cilium and the Hh pathway in vascular phenotype and function.

Here, we provide preliminary evidence for a critical role of GLI1, the main transcription factor activated by the Hh ligand, in the function of aortic SMCs. Our data suggest that the GLI1 protein may constitute a direct connection between fibrocystin and the downstream activation of angiogenic factors. Furthermore, our data suggest that the Hh signaling defects may be, at least in part, due to an impaired translocation of SMO[26,27,36] into the primary cilium, and consequently reduced levels of the transcription factor GLI1 and other pro-angiogenic factors, such as VEGFA, ANG1, ANG2 and FGF2. A plausible mechanism mediating the SMO mis-localization is the severe defects in intra-cilia transport present in PKD cells. This molecular event as well as the interaction of SMO with cilia-associated proteins is essential for translocation of this Hedgehog co-receptor and the activity of the pathway.[37-39]

Finally, the critical role of GLI1 in PKD is further supported by the recovery of function experiments, although preliminary, which may open a new potential therapeutic avenue in this disease.

Scope/Limitations

This study is mainly focused on the role of VSMCs in vascular PKD. We cannot exclude the potential contribution of endothelial cells and other cell types in this disease. Furthermore, it is very likely, and to some extent already proven by us[12] and others[40], the important role of ECs in PKD.

Experiments in this study were done in an “initially” considered ARPKD model, mostly based on the inheritance pattern, but with progression of disease resembling the human ADPKD. It should be mentioned, however, that this model has a specific defect in Fibrocystin (ARPKD), and not Polycystins (ADPKD). However, because both are ciliary proteins, whose functions are unknown, it can be speculated that some, if not all, of the findings from this study may apply to ADPKD. In summary, care should be exercised when extrapolating these results to the entire PKD spectrum of disease.

The involvement of other intracellular mechanisms, like calcium handling, phosphokinase A and others, can play a role in vascular PKD, and some of that involvement has already been shown in other aspects of PKD.[41,42]

Perspective

This study represents a paradigm shift in the pathophysiology of vascular PKD, providing evidence of primary (independent of renal dysfunction) and systemic (involving non-cystic organs) defects in vascular development and homeostasis, during the early stages of the disease. Our data suggests that a normal fibrocystin is critical for the adequate function of VSMCs. A better understanding of the mechanisms underlying the vascular defects in PKD may provide novel vascular therapeutic targets that can complement renal based therapeutic interventions being developed in PKD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and Significance

What Is New?

- Fibrocystin plays a critical role in the angiogenic potential of VSMCs.
- A dysregulation of the Hedgehog signaling might contribute to VSMCs dysfunction and vascular impairment in PKD.

What Is Relevant?

- In PKD, Hedgehog signaling defects may lead to reduced levels of pro-angiogenic factors and ultimately to hypertension and cardiovascular complications.

Summary

PKD has primary vascular defects that affect several key organs, with an underlying abnormality in Hedgehog signaling. GLI1 gain of function studies open new avenues for potential therapeutics opportunities. These therapies can be used to complement current interventions, such as those targeted to improve reno-tubular function.

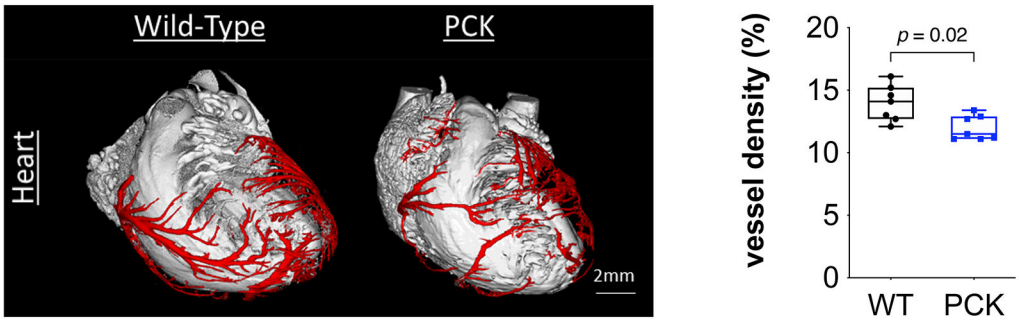

Figure 1. Cardiac vascular density in young PCK rats.

μCT images of heart vascular network in WT and PCK rats (n=7) and vascular density quantification. The analysis takes into account vessels with a diameter >20 μm. Data are displayed as box plot with median (*p*-value vs. WT, Mann-Whitney test).

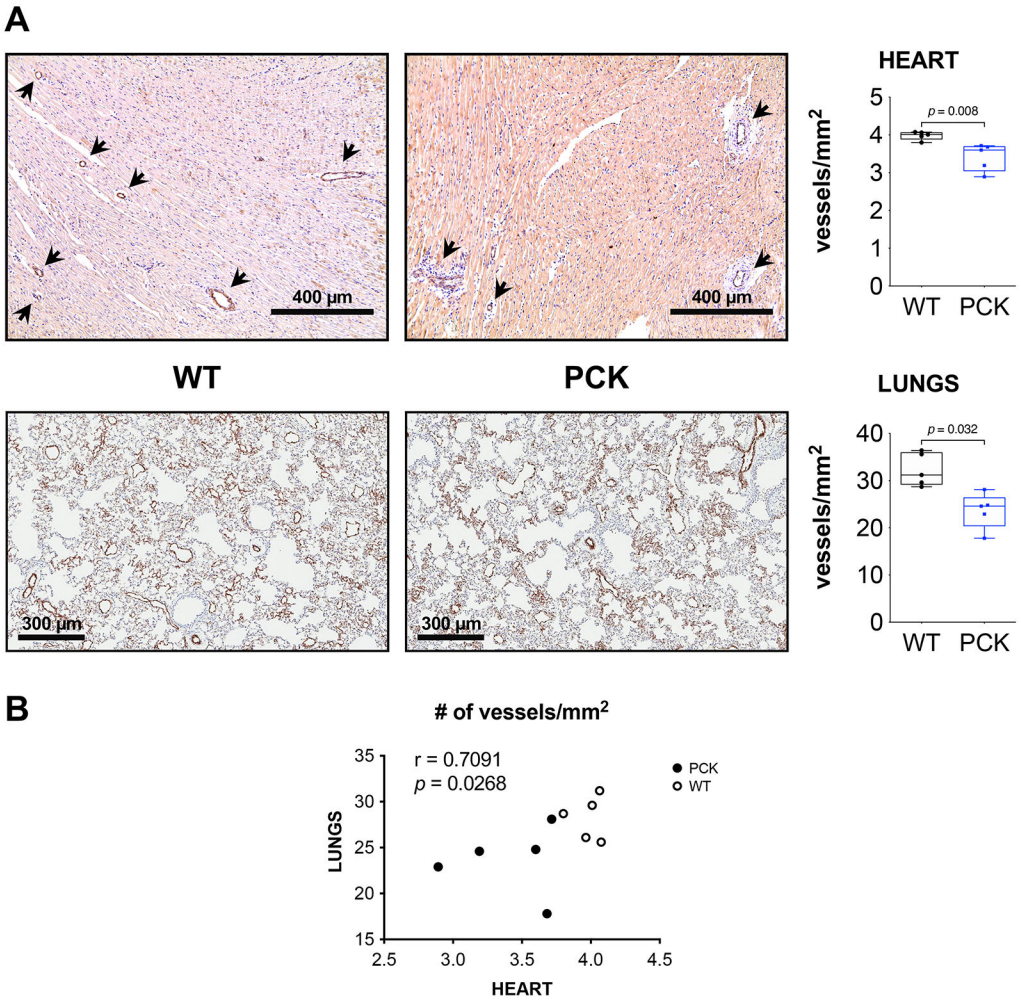

Figure 2. Immunohistochemical analysis of vascular density in non-cystic organs.

(**A**) Representative images of vWf staining of heart and lungs tissues from WT (**left**) and PCK (**right**) rats. The right panel shows the quantification, expressed as total number of vessels per mm^2 (n=5). Data are displayed as box plot with median (*p*-values vs. WT, Mann-Whitney test). (**B**) Correlation of number of vessels per mm^2 in heart and lung tissues among all samples analyzed. Data are displayed as scatter dot plots.

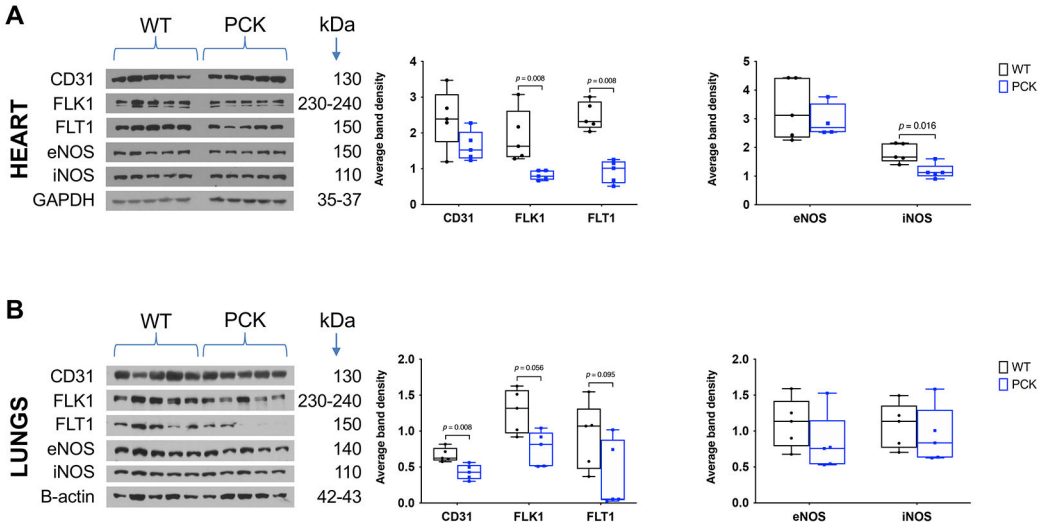

Figure 3. Vascular impairment of non-cystic organs in young PCK rats.

The protein expression of vascular markers, including CD31, FLK1, FLT1, eNOS and iNOS in heart (**A**) and lung (**B**) of 4-5 weeks old rats (n=5), shows an abnormal angiogenic profile and impaired vascular function in PCK rats compared to WT. Data are displayed as box plot with median (*p*-values vs. WT, Mann-Whitney test).

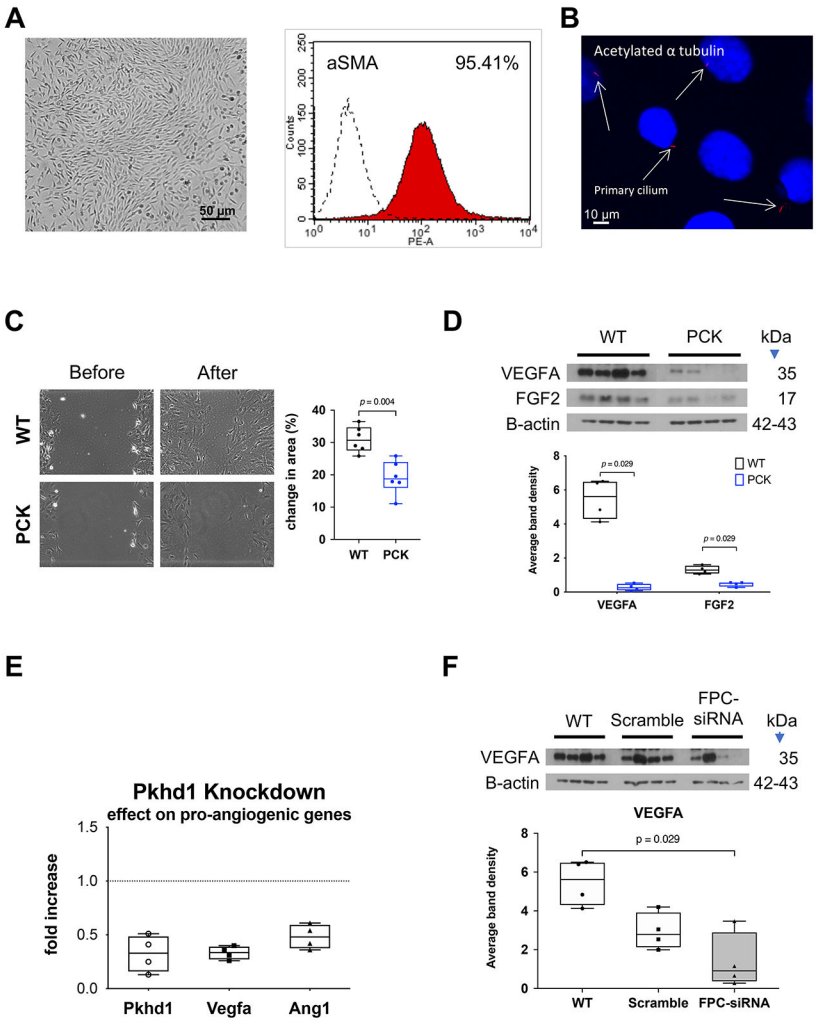

Figure 4. Characterization of rat vascular smooth muscle cells (VSMCs).

(**A**) Representative image showing the morphology of VSMCs isolated from the abdominal aorta of young rats (n=5). Rat VSMCs were positive for α-SMA, as shown by flow cytometry. (**B**) The acetylated *α*-tubulin staining revealed the presence of the primary cilium (arrows) in VSMCs cultured under starving conditions. (**C**) The analysis of the migration capacity displayed a significant dysfunction in PCK-VSMCs compared to WT (n=5). (**D**) Protein expression analysis of PCK-VSMCs showed reduced levels of VEGFA and FGF2 compared to WT (n=4).

Knockdown of fibrocystin in WT-VSMCs. (**E**) Pkhd1 knockdown induced a significant downregulation of pro-angiogenic genes, such as Vegfa and Ang1 (n=4). (**F**) WT-VSMCs transfected with Pkhd1-targeted siRNA displayed reduced VEGFA protein levels, comparable to those observed in PCK-VSMCs (n=4). Data are displayed as box plot with median (*p*-values vs. WT, Mann-Whitney test).

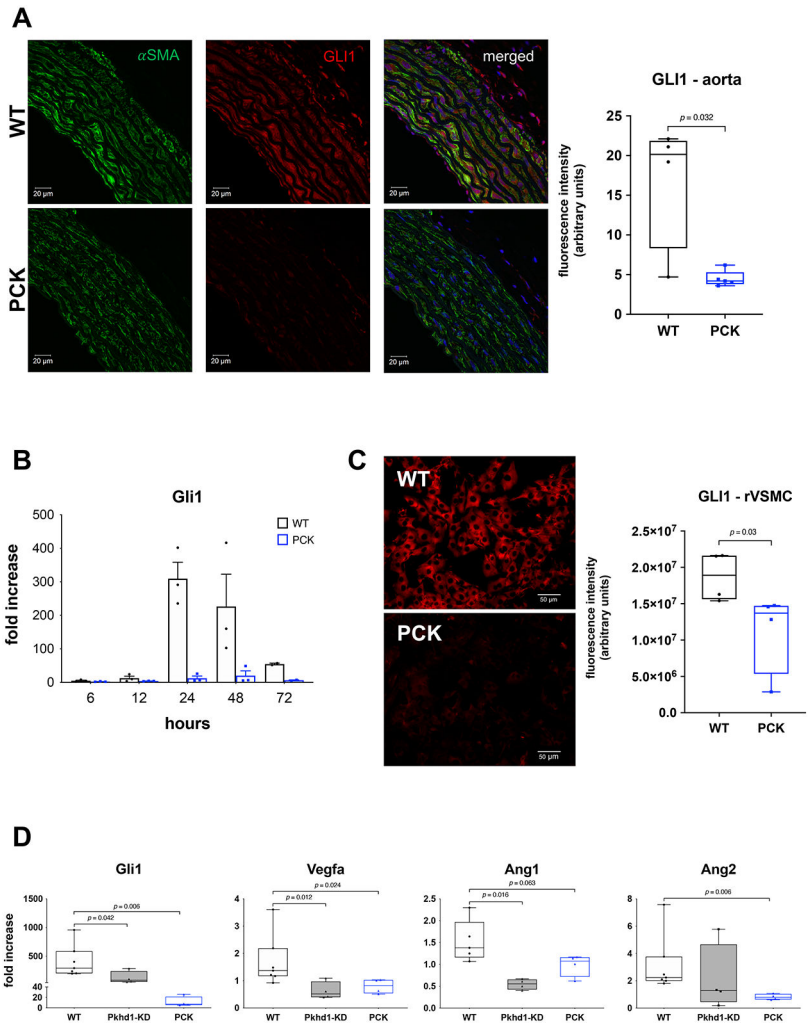

Figure 5. Impaired Hedgehog-Gli1 pathway in PCK-VSMCs.

(**A**) Representative images of immunofluorescent staining of aortic tissues from WT and PCK rats (n=5). Quantification of fluorescence intensities revealed a significant reduction of GLI1 expression (in red) in the tunica media (Smooth Muscle Cells, in green) of PCK rats compared to WT. Isolated VSMCs were, then, cultured in 1% FBS and stimulated with Smoothened Agonist (SAG, 100 nM) for 24 hours. (**B**) Analysis of the expression of GLI1 over time (6-72 hours) by real time RT-PCR (n=3). Real Time RT-PCR experiments have been run in triplicates. Data are displayed as mean ± SEM and expressed as fold increase vs. untreated VSMCs (without SAG). This experiment was performed to choose the best time point (highest Gli1 expression, as a measure of activation of the pathway). (**C**) Representative images and quantification of GLI1 staining of WT and PCK VSMCs (n=4), cultured in 1% FBS and stimulated with SAG for 24 hours. Pictures of the cultures were taken in three fields of view and total fluorescence intensities analyzed using ImageJ software. Mann-Whitney test was used to compare groups. (**D**) Analysis of the expression of Gli1 and other pro-angiogenic genes, Vegfa, Ang1 and Ang2, after stimulation with SAG (24 hours, n=4). Pkhd1-targeted siRNA in WT-VSMCs treated with SAG induced a significant downregulation of the genes studied to levels comparable to PCK-VSMCs. Real

Time RT-PCR experiments have been run in triplicates. Data are displayed as box plot with median and expressed as fold increase vs. untreated WT-VSMCs (without SAG). Kruskal Wallis test was used to compare groups. *p*-values vs. WT+SAG.

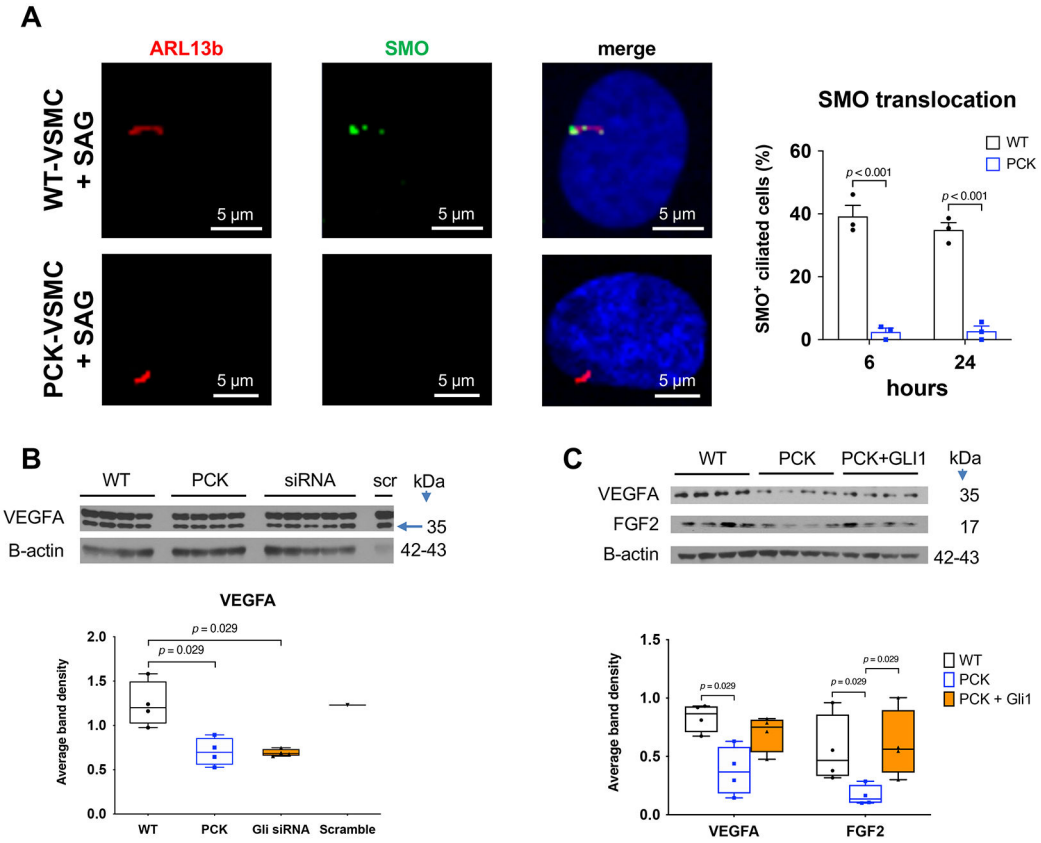

Figure 6. Smoothened translocation into the cilium and modulation of Gli1 expression in VSMCs.

(**A**) Representative immunofluorescent pictures of ARL13b (primary cilium, red) and SMO (Smoothened, green) in WT-VSMCs (top panel) and PCK-VSMCs (bottom panel), showing that in WT-VSMCs there is translocation of SMO (green) to the primary cilium (red). However, SMO does not translocate into the primary cilium after activation of the Hedgehog pathway in most PKD-VSMCs, as shown by the quantification (n=3). Data are displayed as mean ± SEM. TTEST was used to compare groups. *p*-values vs. WT. (**B**) VEGFA protein expression in WT-VSMCs transfected with Gli1-targeted siRNA (n=4). Scr: Scramble siRNA. (**C**) The upregulation of Gli1 in PCK-VSMCs partially restored the expression levels of pro-angiogenic markers, such as VEGFA and FGF2 (n=4). Data are displayed as box plot with median. Kruskal Wallis test with Dunn's post-test was used to compare groups. *p*-values vs. WT.