**Abstract**

**Background**: Autosomal dominant polycystic kidney disease (ADPKD) is a common cause of inherited renal failure that results from mutations in \( PKD1 \) and \( PKD2 \). The disorder is characterized by focal cyst formation that involves somatic mutation of the wild type allele in a large fraction of cysts. Consistent with a two-hit mechanism, mice that are homozygous for inactivating mutations of either \( Pkd1 \) or \( Pkd2 \) develop cystic kidneys, edema and hemorrhage and typically die in midgestation. Cystic kidney disease is unlikely to be the cause of fetal loss since renal function is not required to complete gestation. One hypothesis is that embryonic demise is due to leaky vessels or cardiac pathology.

**Methodology/Principal Findings**: In these studies we used a series of genetically modified \( Pkd1 \) and \( Pkd2 \) murine models to investigate the cause of embryonic lethality in mutant embryos. Since placental defects are a frequent cause of fetal loss, we conducted histopathologic analyses of placentas from \( Pkd1 \) null mice and detected abnormalities of the labyrinth layer beginning at E12.5. We performed placental rescue experiments using tetraploid aggregation and conditional inactivation of \( Pkd1 \) with the Meox2 Cre recombinase. We found that both strategies improved the viability of \( Pkd1 \) null embryos. Selective inactivation of \( Pkd1 \) and \( Pkd2 \) in endothelial cells resulted in polyhydramnios and abnormalities similar to those observed in \( Pkd1^{-/-} \) placentas. However, endothelial cell specific deletion of \( Pkd1 \) or \( Pkd2 \) did not yield the dramatic vascular phenotypes observed in null animals.

**Conclusions/Significance**: Placental abnormalities contribute to the fetal demise of \( Pkd^{-/-} \) embryos. Endothelial cell specific deletion of \( Pkd1 \) or \( Pkd2 \) recapitulates a subset of findings seen in \( Pkd \) null animals. Our studies reveal a complex role for polycystins in maintaining vascular integrity.

**Introduction**

Autosomal dominant polycystic kidney disease (ADPKD) is a common cause of inherited renal failure [1]. Affected individuals develop progressive renal cyst formation and enlargement that ultimately destroys normal kidney architecture. Mutations in \( PKD1 \) are responsible for 85% of all cases while mutations in \( PKD2 \) account for the remainder [2]. Both forms of the disease are associated with a similar array of extra renal manifestations, including liver cysts and cardiovascular complications such as intracranial aneurysms and mitral valve prolapse [3].

The protein products of \( PKD1 \) (Polycystin-1, PC1) and \( PKD2 \) (Polycystin-2, PC2) are thought to function in a common signaling pathway that has yet to be completely elucidated [4,5]. PC1 is predicted to be a large, non-kinase membrane receptor with a multi-component extracellular N-terminus, 11 transmembrane domains and an intracellular C-terminus that is critical for signal transduction. PC2 is a \( Ca^{2+} \)-permeable, TRP-like channel that interacts with the C-terminus of PC-1 forming a receptor channel complex that has been hypothesized to mediate mechanosensory signal transduction by primary cilia in several cell types including renal epithelial cells and endothelial cells [6,7,8].

Although both PC1 and PC2 are expressed in nearly every tissue that has been studied, the manifestations of gene loss in humans are relatively restricted and focal in nature. For example, in the kidney, microdissection studies of early stage human PKD
kidneys demonstrate that cyst formation is a localized process that affects only a subset of cells in the renal tubules [9]. A two hit model of disease is consistent with this observation and is supported by genetic analyses of cystic tissues where somatic mutations affecting the wild type allele can be detected in a large fraction of cysts [10,11].

Studies of mice with targeted mutations of Pkd1 and Pkd2 are in keeping with this model [10,12,13]. Pkd1 and Pkd2 heterozygous mice present with few if any renal cysts but develop dramatic cystic disease when the other allele undergoes somatic deletion at a high rate [14,15,16,17]. In addition, germline homozygous mutants develop severely cystic kidneys and die in utero at mid-gestation [14,15,16,18,19,20,21,22,23]. Polycystinios, severe edema and hemorrhage are universal findings in homozygous mutants of either locus by E12.5.

Taken together, the data from mouse models suggests that ADPKD is recessive at the molecular level and that embryonic loss of either Pkd1 or Pkd2 is incompatible with viability. Interestingly despite the high incidence of Pkd mutations, there have been no reports of live-born humans with inactivating, biallelic PKD1 or PKD2 mutations. In the only example reported in the literature, a marriage between affected first-degree cousins with PKD1-linked disease resulted in a higher than expected rate of fetal loss [24]. The authors concluded that biallelic mutation of PKD1 was the likely cause of embryonic lethality.

Why does polycystin loss result in fetal death? It is unlikely that cystic renal disease is the cause of intrauterine demise since renal function is not required to complete gestation. The uniform presence of vascular fragility and edema in Pkd null mice along with reports of cardiac defects in some models has led to the hypothesis that the demise of these embryos is primarily cardiovascular, due to a combination of leaky vasculature and possibly cardiac defects [18,20,21,22,23,25]. This is in keeping with the expression of polycystins throughout the embryonic and adult cardiovascular systems as well as in both endothelial cells and vascular smooth muscle cells [7,8,18,26].

In this study we address the cause of lethality in Pkd null embryos. We show that polycystins are expressed in the murine placenta and that placental abnormalities are a likely cause of fetal demise in polycystin null animals. We also used floxed alleles of Pkd1 and Pkd2 to investigate the roles of polycystins in various placental cells types. Tetraploid aggregation as well as conditional knock out experiments with both Meox2 and Tie2-Cre recombinases suggest that Pkd1 and Pkd2 play a functional role in both trophoblast and endothelial cell compartments of the placenta. Surprisingly, endothelial loss of Pkd1 or Pkd2 induced by a Tie2-Cre recombinase does not recapitulate the vascular collapse observed in null animals.

Results

Pkd1 and Pkd2 are expressed in Murine Placenta

We used RT-PCR to document the expression of Pkd1 and Pkd2 in murine placentas between E10.5 and E14.5 (Figure 1A). Placental expression of PC1 and PC2 could also be detected by Western analysis by E12.5 (Figure 1 B and C).

Characterization of Pkd1+/− Null Placentas

Defects in the structure and/or function of the chorioallantoic placenta are recognized to be common causes of mid-to late gestational loss of pregnancy. Since Pkd1 and Pkd2 were expressed in placental tissue we considered the possibility that placental failure might be the cause of fetal demise in Pkd null embryos [27,28]. We systematically examined the placentas of Pkd1+/−, Pkd1+/− and Pkd1−/− mice at gestational ages E10.5 through E15.5 in both mixed (129Sv/BL/6) and congenic C57BL/6 backgrounds. At E10.5–E11.5, the decidua basalis and spongiosus layer of all genotypes were similar though the caudal layer of Pkd1−/− placentas appeared slightly less developed with polyhydramnios apparent as early as E11.5 (Figure 1 D and E). At later time points, the polyhydramnios became more severe and abnormalities in Pkd1−/− placentas became progressively more noticeable. In Figure 2 null placentas harvested from C57BL/6 congenic E12.5 embryos were stained for isolectin B4 (IB4), a marker that highlights the extracellular matrix surrounding fetal blood vessels [29]. We observed disorganized fetal arterioles and capillary networks and Pkd1−/− placentas exhibited a statistically significant decrease in the number of vascular branches when compared with wild type controls (Figure 2 A, C, D and E). The trophoblast lined maternal vascular channels also appeared dilated (Figure 2A, arrows in Figure 2 C and D). Small areas of separation, hemorrhage and necrosis with fibrin deposition were occasionally observed at the maternal-fetal interface. In addition, antibodies to alpha smooth actin (α-SMA) detected fewer pericytes, possibly reflecting a decrease in the number of fetal vessels (Figure 2B). Despite a decrease in vascular complexity (Figure 2E), at E12.5, there was no significant difference in either the total placental area (Pkd1−/−; 5.57±0.42 mm² vs. Pkd1+/+; 6.22±0.27 mm², P = .24) or in the labyrinth area between Pkd1−/− and controls. (Pkd1−/−; 2.85±.27 vs. Pkd1+/+; 3.4±.24, P = .16).

There was variability in the onset of severe placental defects, with congenic C57BL/6 Pkd1−/− mice generally exhibiting more severe abnormalities at earlier time points compared with the mixed background. This is consistent with the observation that congenic C57BL/6/Pkd1−/− embryos also die at earlier time points. Pkd2 null placentas exhibit similar histologic abnormalities [30].

Placental Rescue Experiments

In order to explore whether a placental defect might be responsible for intrauterine demise, we performed placental rescue experiments using two strategies. First, we aggregated wild type tetraploid embryos with diploid embryos derived from Pkd1−/− intercrosses [31]. In this method, tetraploid, wild type cells contribute preferentially to extraembryonic placental tissues including trophoblasts while the embryo is derived exclusively from potentially mutant diploid cells. Our results were consistent with a partial rescue of lethality since 3/7 embryos harvested at E17.5 were viable Pkd1−/−, which is remarkable since Pkd1 null embryos rarely survive past E15.5 (Table S1). Of the remaining embryos, three were Pkd1−/+, and one was Pkd1+/+. Consistent with the enhanced viability of the “rescued” Pkd1−/− null embryos, we observed variable improvement in the placental histology (Figure 3 C and D). As expected Pkd1−/− fetuses had severe cystic kidney disease, edema and polyhydramnios despite the lack of fetal incorporation of tetraploid cells (Figure 3 A and B).

As a second complementary approach, we crossed a floxed allele of Pkd1 (Pkd1f/f (floxed)) to Pkd1+/−, Meox2-Cre mice to produce embryos in which the floxed Pkd1 allele was inactivated in the E6.5 embryo but not in the placental trophoblasts or extra-embryonic endoderm lineages [15,32]. Pkd1f/f, Meox2-Cre mice also exhibited cystic kidneys, edema, and polyhydramnios (Figure 4A) but a substantial fraction survived to birth (~16/102, expected ~25/102). Those that survived died shortly after birth of apparent respiratory failure. The hearts of edematous Pkd1f/f, Meox2-Cre pups appeared normal with intact valves and septa, making this organ an unlikely cause of either their edema or death.
Examination of Pkd1cond/2, Meox2-Cre+ placentas likewise showed a variable degree of improvement in placental architecture, suggesting a partial rescue. In Figure 4B and C (middle panels), the Pkd1cond/2, Meox2-Cre+ placenta was similar to wild type, with respect to the density and complexity of placental vessels. In contrast, another placenta of the same genotype exhibited fewer fetal vessels and more dilated vascular spaces (Figure 4B and C, right panels). This was consistent with the observation that only a subset of Pkd1cond/2, Meox2-Cre+ embryos survived to birth. On average, however, Pkd1cond/2, Meox2-Cre+ placentas had a lower density of fetal vessels compared with controls (Figure 4E) but there was no difference in the size of any placental layer. In order to confirm the pattern of Meox2-Cre activity in the fetal placenta, we carried out crosses using mice carrying the ROSA26R reporter transgene [33]. The Meox2-Cre recombinase activity was restricted to the fetal portion of the placenta, overlapping with the vasculature as marked by PECAM 1 expression (Figure 4D). There was no obvious difference in Cre recombinase activity between various placentas and Pkd1+/-; Rosa26R did not stain with β-galactosidase.

Endothelial cell Deletion of Pkd1 or Pkd2 Results in Reduced Viability and Placental Defects

These findings raised the possibility that the abnormalities detected in Pkd1 and Pkd2 null placentas might be due to a requirement for these genes not only in trophoblasts but also in the vascular compartment of the labyrinth layer. In order to examine this possibility we ablated Pkd1 in fetal endothelial cells using a Tie2-Cre transgene [34]. When we crossed Pkd1cond/cond and Pkd1cond/+; Tie2-Cre+ mice we found a reduction in live born Pkd1cond/+; Tie2-Cre+ animals (referred to as Pkd1endo+), indicating that endothelial cell loss of polycystin-1 results in the embryonic demise of a significant fraction of animals (Table 1). If pregnancies were harvested before E18.5, however, mendelian ratios were restored.

We generated a novel conditional allele of Pkd2 in order to carry out analogous experiments (Figure S1 and S2, Table S2). Endothelial cell specific deletion of Pkd2 (Pkd2cond/-) similarly resulted in embryonic demise (Table 2). We note that in matings of Pkd2cond/cond and Pkd2cond/+; Tie2-Cre+ mice, we consistently observed a lower than expected fraction of Pkd2cond/+ progeny.

Figure 1. Coordinate Expression of PC1 and PC2 in Murine Placentas. A. Expression of Pkd1 and Pkd2 in E10.5–E14.5 placentas assayed by quantitative RT-PCR, normalized to RPL13. N = 4 placentas were analyzed at E10.5 and E14.5 and N = 3 placentas at the other time points. B. Immunoprecipitation of PC1 from placentas harvested at E12.5 and E14.5 and E12.5 embryos using a chicken antibody directed at the PC1 C-terminus. The asterisk denotes a non-specific band previously described [46]. Pkd1−/+ placentas were not used as a negative control since they would be expected to contain maternal PC1. C. Expression of PC2 by Western analysis in E10.5–E14.5 placentas. Expression level was normalized to actin. N = 4 placentas analyzed at each time point. Inset shows a representative Western blot. D. Representative Pkd1+/+ and littermate control (Pkd1+/−) embryos harvested at E11.5. Asterisk indicates polyhydramnios. E. Quantification of amniotic fluid. Increased amniotic fluid can be detected in Pkd1−/+ embryos as early as E11.5. ** p < .01, *** p < .001. doi:10.1371/journal.pone.0012821.g001
Figure 2. Abnormal labyrinth layer in Pkd1−/− placentas. A. Low power (upper panels) and high power (lower panels) views of congenic C57BL/6, E12.5 placentas stained with haematoxylin-eosin (H&E). B. Pericytes in E12.5 placentas stained with antibodies to alpha smooth actin (α-SMA). The density of pericytes in Pkd1−/− placentas is reduced, consistent with a decrease in the density of fetal vessels. C. Low power (upper panels) and high power (lower panels) views of E12.5 placentas stained with isoelectin B4 (IB4), highlighting the matrix surrounding fetal vessels. Arrowheads denote fetal vessels. Arrows indicate maternal vascular spaces. The null placenta has fewer fetal vessels and dilated maternal spaces. D. Fetal vessels in Pkd1+/+ and Pkd1−/− 12.5 dpc placentas stained with fluorescent-labeled isoelectin B4 (green) and laminin (red). Nuclei stained with DAPI are blue. White arrowheads indicate fetal vessels that are outlined by IB4 and laminin. Arrows indicate maternal vascular spaces. There is a decrease in the number of fetal vessels. E. Vessel density is decreased in the labyrinth layer of Pkd1−/− placentas compared with Pkd1+/+ controls (37.6 ± 1.1 branches/mm² vs. 46.3 ± 2.3 respectively) * p<.05.

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Figure 3. Placental Rescue by Tetraploid Aggregation. A, B. Haematoxylin-eosin stained sections of E17.5 tetraploid rescue n4/Pkd1−/− embryos show classic Pkd1 null phenotype with renal cysts (Panel A) and pancreatic cysts (Panel B). This is because tetraploid wild type cells are not incorporated into the fetus. “Cy” denotes cysts. C. Haematoxylin-eosin stained placental sections harvested from viable E17.5 wild type (Pkd1+/+) or Pkd1 mutant (n4/Pkd1−/−) embryos. The defect in the labyrinth layer appears improved in this placenta. D. IB4 staining of representative sections from E17.5 Pkd1+/+ (left panels) and n4/Pkd1−/− placentas (right panels). There was variable improvement in the placental phenotype with the n4/Pkd1−/− placenta in the upper panel appearing to be more like the wild type placenta.

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Since Pkd2cond/+ and Pkd2cond/cond mice are phenotypically normal and born at the expected frequencies (Table S2), the most likely explanation is that the Pkd2 locus and the Tie2-Cre transgene are genetically linked but far enough apart for some recombination to occur. We detected a further reduction in live born Pkd2cond/cond; Tie2-Cre+ (Pkd1endo2) vs. Pkd2cond/+ progeny (P = .01, chi-square test) that was not present when pregnancies were harvested before E15.5. These findings suggest that there is fetal lethality of Pkd1endo2 embryos superimposed on segregation distortion.

We examined Pkd1endo2 and Pkd2endo2 placentas along with littermate controls and found a phenotype similar to that described for Pkd1 and Pkd2 null animals (Figure 5). Isolectin B4 and laminin staining served to highlight a significant decrease in the density of fetal vessels with a simplified vascular branching pattern and dilated maternal spaces. In addition, staining for alpha-smooth muscle actin appeared more sparse and disorganized when compared with control placentas. There was no significant difference in the size of placental layers (labyrinth or spongiotrophoblast) between Pkd1endo− or Pkd2endo− placenta versus controls.

Phenotype of mice lacking endothelial cell Polycystins

Unexpectedly, the phenotypic consequences of endothelial cell loss of either Pkd1 or Pkd2 appeared to be primarily restricted to extra-fetal tissues. Pkd1endo− and Pkd2endo− embryos universally developed polyhydramnios (Figure 6, Figure S2) and could be easily distinguished from wild type littermates. Only a small fraction of mutant animals, however, exhibited hemorrhages (Figure 6 E and F). In striking contrast to the Pkd1 and Pkd2 null mice, which universally had edema, none of the Pkd1endo− or Pkd2endo− embryos were edematous (Figure 5A, Figure S2F). We also failed to find any obvious cardiac or renal abnormalities in several Pkd1endo− and Pkd2endo− embryos that were sectioned (Figure 6D, Figure S2G). We maintained a cohort of the Pkd1endo− mice (n = 15) for up to one year and observed no differences in the rate of sudden death. Autopsies performed on a subset of the

Figure 4. Conditional Inactivation of Pkd1 using Meox2 Cre recombinase. A. Conditional inactivation of Pkd1 using a Meox-2 Cre recombinase expressing mouse line results in the typical polycystin null phenotype, reflecting high activity of the recombinase in the embryo. The top panel shows the gross appearance of Pkd1cond/cond; Meox2-Cre+ (Pkd1Meox2-Cre) and Pkd1cond/+; Meox2-Cre− (Pkd1+/*+) neonates (top panel). Pkd1Meox2-Cre+ mutants are severely edematous (*). The bottom panels show haematoxylin-eosin stained sections of cystic kidney (left) and cystic pancreas (right). “Cy” denotes cysts. B, C. Sections of E14.5 placentas from Pkd1cond/+; Meox2-Cre- (Pkd1+/+) or Pkd1cond/cond; Meox2-Cre- (Pkd1cond/cond; Meox2-Cre-) embryos stained with haematoxylin-eosin (B) or IB4 (C). Two representative examples of Pkd1Meox2-Cre placenta show different degrees of rescue when compared with a Pkd1+/+ littermate control. The Meox-2Cre recombinase activity would be expected to spare placental trophoblasts and cells derived from extra-embryonic endoderm lineages. D. In order to determine the spatial pattern of placental Meox-2 Cre activity, frozen sections of Pkd1cond/+; Meox2-Cre-, Rosa26R E14.5 placentas were stained with β-galactosidase (left panel) or antibodies to Pecam-1 (right panel). The dotted white line outlines the stained area. Nuclei are stained with DAPI (blue). Cre activity appears to overlap with anti-Pecam 1 staining, suggesting activity in fetal vessels that comprise the placental labyrinth layer. E. Vessel density is decreased in the labyrinth layer of Pkd1Meox2-Cre placenta compared with Pkd1+/+ controls (37±2.6 branches/mm² vs. 47±2.3 respectively) ** p <.01.

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Table 1. Deletion of Pkd1 in endothelial cells.

| Offspring genotype | Expected ratio | Obtained Ratio (Perinatal Period) | Obtained Ratio (13.5–18.5 d.p.c) |
|---------------------|----------------|----------------------------------|----------------------------------|
| Pkd1cond/cond; Tie2-Cre+ (Pkd1endo−) | 25% | 16.9% (n = 47) | 23.3% (n = 45) |
| Pkd1cond/−; Tie2-Cre− | 25% | 27.3% (n = 76) | 23.8% (n = 46) |
| Pkd1cond+/−; Tie2-Cre+ | 25% | 26.2% (n = 73) | 24.4% (n = 47) |
| Pkd1cond+; Tie2-Cre+ | 25% | 29.5% (n = 82) | 28.5% (n = 55) |

Table 2. Deletion of Pkd2 in endothelial cells.

| Offspring genotype | Expected ratio | Obtained Ratio (Perinatal Period) | Obtained Ratio (8.5–15.5 d.p.c) |
|---------------------|----------------|----------------------------------|----------------------------------|
| Pkd2cond/cond; Tie2-Cre+ (Pkd2endo−) | 25% | 4.3% (n = 4) | 19.2% (n = 14) |
| Pkd2cond/− | 25% | 35.5% (n = 33) | 41.1% (n = 30) |
| Pkd2cond+/−; Tie2-Cre+ | 25% | 44.1% (n = 41) | 28.7% (n = 21) |
| *Pkd2cond+/+ | 25% | 16.1% (n = 15) | 11% (n = 8) |

*The lower than expected percentage of Pkd2cond/+ progeny suggests that Pkd2 and the Tie2-Cre transgene are linked but far enough apart for some recombination to occur. There are fewer Pkd2cond/+ Tie2-Cre+ (Pkd2endo−) progeny compared with Pkd2cond/+ (P = .01, chi-square test).

mutants found no obvious vascular abnormalities including aortic or intracranial aneurysms.

**Efficiency of Tie2-Cre Recombinase**

Although 100% of Pkd1endo− and Pkd2endo− embryos exhibited polyhydramnios and placental abnormalities in utero, there was variability in the severity of the phenotype with almost 2/3 of Z/AP line carrying the in utero polyhydramnios and placental abnormalities. Endothelial cells we bred test for the efficiency of differences in the degree of Cre recombinase activity. In order to studies. Alternatively phenotypic variability could be due to modifiers operating in the mixed background used for these observations could be the presence of unidentified genetic alterations. We performed a reciprocal analysis using two independent strategies to rescue the complexity of fetal vascular networks along with dilated fetal and maternal spaces. We used two independent strategies to rescue PC1/PC2 receptor-channel complex in regulating placental morphogenesis.

In this report we demonstrate that polycystins play a role in the development of complex vascular networks with fewer pericytes and trophoblasts. As in the developing kidney, the formation of the placental labyrinth layer involves complex reciprocal interactions between trophoblasts and incipient vessels. The reduced complexity of the labyrinth layer in Pkd1 or Pkd2 mutants, suggests an additional function for the PC1/PC2 receptor-channel complex in regulating placental morphogenesis.

Interestingly, the phenotype that we observed in Pkd2 mutant placenta is similar to the defect described for PDGFB (platelet derived growth factor β) or PDGFRB (PDGF receptor β) null placenta [29]. Placentas from these animals exhibited dilated embryonic blood vessels with fewer pericytes and trophoblasts but no reduction in size of either the labyrinth or spongiosotrophoblast layers. These abnormalities are attributed to a failure of pericyte recruitment to fetal vessels, which in turn is presumed to affect the morphogenesis of complex vascular networks that comprise the placental labyrinth layer. Whether loss of either Pkd1 or Pkd2 in the fetal placenta affects PDGF signaling will require further investigation.

One ubiquitous feature of all Pkd1 and Pkd2 targeted alleles is the presence of dramatic polyhydramnios in null embryos.
Surprisingly deletion of either Pkd1 or Pkd2 in endothelial cells alone was sufficient to recapitulate this phenotype. We were unable to identify additional renal, pulmonary or cardiac pathology that might explain these findings. Since placental membranes are thought to play a role in amniotic fluid dynamics, one possibility is that the polyhydramnios in these embryos is related to the placental abnormalities described above [38].

We note that our findings differ from those recently published using another targeted Pkd1 null allele [39]. These authors were unable to detect a placental defect and concluded that polyhydramnios, which precedes cyst formation, is caused by abnormal proximal tubular function and excessive loss of renal solutes. Our data, however, are not consistent with this conclusion since we see polyhydramnios as early as E11.5, prior to filtration by the metanephric kidney [40]. In addition, Pkd1/endo- embryos lack a demonstrable renal phenotype yet they have significant polyhydramnios, making it unlikely that this is renal in origin. Finally although these authors reported that gross morphometry was no different in Pkd1 null placentas, more sensitive measures confirm a reproducible defect in the complexity and density of fetal vessels in the placental labyrinth.

One surprising aspect of our work is the relatively limited impact of selective endothelial inactivation of either polycystin in the intact animal. Both Pkd1 and Pkd2 are expressed throughout the cardiovascular system and null animals have a dramatic vascular phenotype manifest by edema and vascular hemorrhage [18,20,25]. Moreover these proteins have been detected in the primary cilia of cultured endothelial cells where they are reported to be involved in shear stress sensing thereby activating a variety of signaling pathways that result in nitric oxide production [7,8]. Yet, selective inactivation of either Pkd1 or Pkd2 in endothelial cells using a Tie-2 Cre recombinase is not sufficient to reproduce all

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**Figure 5.** Deletion of Pkd1 or Pkd2 in endothelial cells results in abnormal placental histology. A, B. Representative E14.5 Pkd2endo- and littermate control (Pkd2endo+) placental sections stained with PAS. Area within the square of Panel A is located in the labyrinth layer (lt), and is shown at larger magnification in Panel B. “st” = spongiotrophoblast. Arrow indicates a dilated vascular space that is likely maternal. C, D. Representative sections of E18.5 Pkd1endo- (Panel C, right), E14.5 Pkd2endo- (Panel D, right) and littermate control placentas (Pkd1endo+ and Pkd2endo+) left panels, C and D, respectively stained as indicated. Both Pkd1endo- and Pkd2endo- placentas have fewer fetal vessels and investing pericytes when compared with controls. Arrowheads and arrows denote fetal vessels and maternal vascular spaces, respectively. E. Quantification of vessel density in the placental labyrinth. For Pkd1endo- vs. Pkd1endo+, the number of vessel branches is 45.3±2 and 57.9±2.9, respectively. For Pkd2endo- vs. Pkd2endo+ the number of branches is 45.3±2 and 45.3±2.9. P **p<.01.

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features of vascular collapse. Most notably, Pkdendoanimals lack edema, which has been presumed to be due to leaky junctions between endothelial cells [20].

There are several conceivable explanations for these contradictory observations. It is possible, for example, that endothelial cells which escape Cre-mediated Pkd gene inactivation have sufficient activity to avert a more dramatic vascular phenotype. This seems unlikely given the high degree of Cre recombinase activity that we observed in all tissues examined as well as the greatly reduced level of polycystin expression that was present in endothelial cells isolated from Tie2-Cre specimens. In addition we note that others have used this Tie2-Cre recombinase line to demonstrate essential endothelial cell functions of other genes [41,42,43]. Alternatively, it is theoretically possible that the edematous phenotype is caused by the loss of polycystin in the lymphatic system. However, investigators have also used this Tie2-Cre recombinase to specifically delete genes required for lymphatic development [44]. We cannot exclude a primary cardiac cause for the fetal edema and hemorrhages or a circulating factor released by a cell other than one of endothelial origin. The apparent lack of significant cardiac abnormalities in severely edematous embryos, however, would make the former less likely.

One final possible explanation for our results arises from the observation that polycystins are likely involved in heterologous cell-cell interactions. The complete vascular phenotype observed in null embryos may result only when Pkd genes are simultaneously inactivated in multiple cell/tissue types. Alternatively, loss of polycystin in a functionally interacting cell type like smooth muscle cells may have a different effect on endothelial cell function than loss in endothelial cells themselves. Further studies using other Cre recombinases alone and in combination will be necessary to address these possibilities. While the present studies have not yet fully explained the observed vascular phenotype, they do reveal the unexpectedly complex role these proteins likely play in maintaining vascular integrity.

**Figure 6. Phenotypes Associated with Endothelial cell loss of Polycystin 1 or 2.** A. Gross appearance of E14.5 Pkd1endo (right) and littermate control (left). The Tie2-Cre (Pkd1endo) embryo has polyhydramnios indicated by the asterisk. In the lower panel the yolk sac was removed and neither embryo has edema. B, C. Quantification of Amniotic Fluid demonstrates that both Pkd1endo (B) and Pkd2endo (C) embryos have significant polyhydramnios, *p < 0.05; *** p < 0.001. D. Haematoxylin-eosin staining of kidney and heart from an E16.5 Pkd1endo embryo and control. There are no obvious abnormalities in the Pkd1endo embryos. E. Gross appearance of an E14.5 Pkd2endo embryo with multiple areas of hemorrhage (right). A littermate control is also shown (left). Arrowheads indicate areas of hemorrhage. F. Haematoxylin-eosin staining of hemorrhagic areas in the head (upper panel) and back (lower panel). High power view of areas in dotted square shown at right. Arrowheads indicate free red blood cells. doi:10.1371/journal.pone.0012821.g006
Materials and Methods

Ethics Statement

All studies were performed using protocols approved by the University Animal Care and Use Committee (Protocol MO05M318), and mice were housed and cared in pathogen-free facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and meet federal (NIH) guidelines for the humane and appropriate care of laboratory animal.

Endothelial Cell Ablation of Pkd1 or Pkd2

Pkd1 null alleles and conditional alleles have been previously described [15,45]. We inactivated Pkd1 or Pkd2 in endothelial cells using transgenic mice expressing Cre recombinase under the control of the Tie2 promoter (Tie2-Cre line; Masashi Yanagisawa, UT Southwestern) [34]. Pkd1+/+ heterozygotes were crossed to Tie2-Cre transgenic mice to generate Pkd1+/+, Tie2-Cre+ males. These males were crossed to Pkd1cond/cond females to generate Pkd1cond/+ males (Pkd1endo2). For Pkd2, we mated Pkd2cond/cond homozygous females to the Pkd2+/+, Tie2-Cre+ males to create Pkd2cond/+ females, Pkd2cond/cond male and Pkd2cond/+ male (Pkd2endo2). Genotyping was performed from tail DNA using the REDExtract-N-Amp tissue PCR Kit (SIGMA) according to the manufacturer’s protocol. Pkd1+/+ and null alleles were genotyped as described previously [15]. Pkd2 alleles were genotyped as described in supplementary methods S1.

Quantitative PCR Analysis

Total RNA was isolated from individual placentas using the Qiagen RNA extraction kit. Five micrograms of total RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen). Quantitative PCR (Stratagene Mx3005P) for Pkd1 and Pkd2 expression was performed using the SYBR green PCR master mix (Applied Biosystems). Samples were run in triplicate and an RT negative sample was used to control for DNA contamination. A water control was also included. Annealing temperatures and primer sequences are as follows: Pkd1, 60°C: 5'-TCTGGATGGGCTTCAGCAA and 5'-AGCGGGAAGGCAGTGGAT; Pkd2, 60°C: 5'-AGACTTCTC- GGTGTATAACGCAAA and 5'-CACCCGTTGCTGGGAACT. Values were normalized to those of RPL13 (ribosomal protein L13).

Immunoprecipitation/Western Blotting

Samples were homogenized in lysis buffer (20 mM Na phosphate [pH 7.2], 150 mM NaCl, 1 mM EDTA, 1% glycerol, 1% Triton X-100 and protease inhibitor cocktail (Roche). The homogenate was incubated for 1 hour on ice and cleared of debris by centrifugation at 15,000 x g for 10 min at 4°C. Six milligrams of protein in 1 ml was used for immunoprecipitation (IP) with 1 ul of a polyclonal chicken antibody (chicken anti-CC) directed at the mouse PC1 C-terminus. Western blots were probed with rabbit antisera directed against the PC2 C-terminus (PC2 anti-CT, 1:1000) or the PC1 C-terminus (PC1 anti-CC, 1:1000). Antisera to PC1 and PC2 have been described [46,47].
Placental sections were prepared and stained with peroxidase conjugated IB4 (Sigma L5391) using published methods [29]. Placental pericytes were stained with the α-smooth muscle actin immunohistochemistry kit (IMMH2, from Sigma) using the manufacturer’s protocol. Anti-Laminin antibodies (Sigma L9393) were used at a final concentration of 1:400. Frozen placental sections were stained with Anti-Pecam antibody (BD Pharmingen, MEC13.3) diluted to 1:500. Indirect fluorescence microscopy was performed using a Nikon Eclipse E600 and images were captured using a SPOT-RT monochrome camera (SPOT Diagnostic Instruments). At least 4 sections were examined for each genotype except in the case of tetraploid rescue.

Placental Morphometry

At least 4 independent pairs of placentas were used for comparison: 1) Pkd1+/− vs. Pkd1+/+ at E12.5 2) Pkd1+/− vs. Pkd1+/+ at E14.5 3) Pkd2cond−/− vs. Pkd2cond+/- at E14.5 and 4) Pkd1neo+/−, Mox2-Cre vs. Pkd1neo+/+, Mox2-Cre at E14.5. Placentas were fixed, paraffin embedded, hemisected and 5 μm sections were prepared. Two separate placental sections representing the middle section of the placenta were hybridized with a Tbpβ riboprobe (Dr. JC Cross, University of Calgary) to distinguish the spongiotrophoblast and the overlying the labyrinth region. Digital photographs at 1X were taken and NIH Image J software was used to measure total placental area, labyrinth and spongiotrophoblast areas. Three independent measurements were taken for each layer and section. In order to assay vessel density, hemisected placentas were stained with IB4 as above. Six to twelve photographs at 20X magnification were taken from two different sections (middle region) representing more than 90% of the labyrinth. The vessels were counted using NIH Image J software that equally distributes 208 crosses (comprising a .96 mm2 grid) over the photograph. A blinded observer counted the vessels touching each cross. Comparisons between groups were performed using the two-sided unequal Student’s t-test (Excel, Microsoft).

Endothelial Cell Isolation and Analysis of Cre Deletion

Minced E13.5 embryos were digested with collagenase II (2 mg/ml) and DNase I to generate a single cell suspension. After centrifugation, the cell pellet was washed, and passed through a 40 μm cell strainer. The cells suspension was incubated with Anti-Pecam (CD31, BD Biosciences) coated Dynabeads (Dynal) per manufacturer’s recommendations. The beads were separated using a magnetic particle concentrator. Western analysis for PC2 was performed and the relative expression was calculated by correcting for loading and then normalizing to Pkd2 (100% expression). For Pkd1, genomic DNA was prepared from endothelial cells and a 3 primer PCR amplification for 25 cycles was performed with SYBR green Master Mix (Applied Biosystems) using primers and conditions previously described [15]. Both Pkd1 wild type (WT) and conditional alleles give a 180 bp band while the null or deleted (KO) allele yields a 250 bp band. The relative intensity of the two bands was determined using the Molecular Imager System™ (Bio-Rad) and used to calculate the relative Pkd1 deletion (intensity KO band/−intensity WT band + KO band).

Supporting Information

Supplementary Methods S1

Found at: doi:10.1371/journal.pone.0012821.s001 (0.05 MB DOC)

Figure S1 Generation of Floxed and Mutant alleles of Phd2. A. Schematic representation of the Phd2 targeting construct (TC), Phd2 locus (Gene) and resulting allelic series. Phd2neo(11−13) (Phd2Neo3/+) refers to the floxed allele, Phd2neo(11−13) (Phd2tm1.1Tjw) results from deletion of the Neomycin cassette. We refer to this allele as Phd2Neo3/+. The restriction maps for AijIII and BglII are as indicated. The location of PCR primers a, b, and c in the various Phd2 alleles is shown along with the size of the corresponding PCR products. The red and yellow triangles represent loxP and FRT sites, respectively and grey ovals correspond to exons. B. Genomic Southern showing germ line transmission of Phd2neo(11−13) allele. The “−” signifies a mouse carrying the targeted allele and “−−” is a wild type littermate. DNA from the offspring of highly chimeric mice was digested with either AijIII (top) or BglII (bottom) and hybridized with 5′ or 3′ probes (position depicted in panel A). In each case the probe detects the wild type band and a larger band as expected for the appropriately targeted locus. Internal probes (position depicted in panel A) only hybridize to the bands recognized by the external probes indicating that there were no random integration events. C. Genotyping with a 5-primer PCR strategy. Primers a, b and c were used to identify wild type, floxed and deleted alleles. A representative ethidium bromide stained gel is shown. Primers a and b amplify a 232 bp band from the wild type allele and a 318 bp band in the floxed allele. Primers a and c are far apart and do not amplify a product from genomic DNA in either allele. In the deleted allele the primer b site is lost and a 143 bp band is amplified from primers a and c. D. The Phd2neo(11−13) mutant allele is transcribed. The intron/exon structure of wild type and mutant (Phd2(11−13)) alleles along with the location of primers f, h, i shown schematically. Cre recombinase is predicted to result in a frame shift and stop codon “UAG” in exon 14. RTPCR products amplified from embryos of the indicated genotypes are shown. “−” is the water control. Primers f and i yield a 900 bp and a 496 bp product from wild type and mutant alleles respectively. Both bands are seen in a heterozygote, Phd2neo(11−13). The faint band indicated by the asterisk is likely due to heteroduplex formation. Primers h and i, contained within exon 15, amplify a 105 bp band from both alleles. E. The Phd2(11−13) mutant allele does not yield protein. Total lysates derived from embryos of the indicated genotypes were used to prepare Western blots. Both the N-terminal and C-terminal PC-2 antibodies fail to detect polycystin-2 in the Phd2(11−13) mutant. Alpha tubulin was used as a loading control. F. Cell lysates were prepared from various embryos and human PKD2 transfected HEK cells (positive control). PC-2 was immunoprecipitated and then detected with either N-terminal or C-terminal antibodies as indicated. PC-2 cannot be immunoprecipitated from Phd2(11−13) homozygotes with either the N-terminal or C-terminal antibody. The star indicates a non-specific band that was not visualized when total lysates were probed with the N-terminal antibody (data not shown). Found at: doi:10.1371/journal.pone.0012821.s002 (3.61 MB TIF)
**References**

1. Gabow PA (1993) Autosomal dominant polycystic kidney disease. N Engl J Med 329: 332–342.
2. Parfrey PS, Bear JC, Morgan J, Cramer BG, McNamamon PJ, et al. (1990) The diagnosis and prognosis of autosomal dominant polycystic kidney disease. N Engl J Med 323: 1093–1090.
3. Pirson Y. Extrarenal manifestations of autosomal dominant polycystic kidney disease. Adv Chronic Kidney Dis 17: 173–180.
4. Harris PC, Torres VE (2009) Polycystic kidney disease. Annu Rev Med 60: 321–337.
5. Gallagher AR, Germino GG, Somlo S. Molecular advances in autosomal dominant polycystic kidney disease. Adv Chronic Kidney Dis 17: 118–130.
6. Nauli SM, Kawanabe Y, Kaminski JJ, Pearce WJ, Ingber DE, et al. (2008) Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. Circulation 117: 1161–1171.
7. Abou-Aliawi WA, Takahashi M, Mell BR, Jones TJ, Ratnam S, et al. (2009) Ciliary polycystin-2 is a mechanosensitive cation channel involved in nitric oxide signaling cascades. Circ Res 105: 860–869.
8. Baert L (1978) Hereditary polycystic kidney disease (adult form): a microdissection study of two cases at an early stage of the disease. Kidney Int 13: 519–525.
9. Wainwright TJ, He N, Wang K, Liang Y, Parfrey P, et al. (2000) Mutations of PKD1 in ADPKD2 cysts suggest a pathogenic effect of trans-heterozygous mutations. Nat Genet 25: 143–144.
10. Priy Y, Wainwright TJ, He N, Wang K, Liang Y, et al. (1999) Somatic PKD2 mutations in individual kidney and liver cysts support a “two-hit” model of cystogenesis in type 2 autosomal dominant polycystic kidney disease. J Am Soc Nephrol 10: 1524–1529.
11. Peters DJ, Sandkuijl LA (1992) Genetic heterogeneity of polycystic kidney disease in Europe. Contrib Nephrol 97: 129–139.
12. Qian F, Wainwright TJ, Onuchic LF, Germino GG (1996) The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type 1. Cell 87: 979–987.
13. Wu G, D’Agati V, Cai Y, Markowitz G, Park JH, et al. (1998) Somatic inactivation of ADPKD2 results in polycystic kidney disease. Cell 93: 177–188.
14. Piontek KB, Huo DL, Grinberg A, Liu L, Bedja D, et al. (2004) A functional floxed allele of Pkd1 that can be conditionally inactivated in vivo. J Am Soc Nephrol 15: 3035–3043.
15. Wu LW, Fan X, Rasouli N, Babakhaholu H, Law T, et al. (1999) Late onset of renal and hepatic cysts in Pkd1-targeted heterozygotes. Nat Genet 21: 160–161.
16. Piontek K, Menezes LF, Garcia-Gonzalez MA, Huo DL, Germino GG (2007) A critical developmental switch defines the kinetics of kidney cyst formation after loss of Pkd1. Nat Med 13: 1490–1495.
17. Boixeda L, Mulroy S, Webb S, Fleming S, Brindle K, et al. (2001) Cardiovascular, skeletal, and renal defects in mice with a targeted disruption of the Pkd1 gene. Proc Natl Acad Sci U S A 98: 12174–12179.
18. Lu W, Peisel B, Babakhaholu H, Pardova A, Geng L, et al. (1997) Perinatal lethality with kidney and pancreas defects in mice with a targeted Pkd1 mutation. Nat Genet 17: 179–181.
19. Kim K, Drummond I, Ibrahimov-Besktorova O, Klinger K, Arnaout MA (2000) Polycystin 1 is required for the structural integrity of blood vessels. Proc Natl Acad Sci U S A 97: 1731–1736.
20. Muto S, Aiba S, Saito Y, Nakao K, Nakamura K, et al. (2002) Pigletization improves the phenotype and molecular defects of a targeted Pkd1 mutant. Hum Mol Genet 11: 1741–1742.
21. Wu G, Markowitz GS, Li L, D’Agati VD, Factor SM, et al. (2000) Cardiac defects and renal failure in mice with targeted mutations in Pkd2. Nat Genet 24: 75–78.
22. Pennekamp P, Karicher C, Fischer A, Schweickert A, Skybabin B, et al. (2002) The ion channel polycystin-2 is required for left-right axis determination in mice. Curr Biol 12: 938–943.
23. Paterson AD, Wang KR, Lupea C, St George-Hyslop P, Priy V (2002) Recurrent fetal loss associated with bilineal inheritance of type 1 autosomal dominant polycystic kidney disease. Am J Kidney Dis 40: 16–20.
24. Bruck S, Woodford LM (2003) Mouse models of polycystic kidney disease: molecular and therapeutic insights. Am J Physiol Renal Physiol 285: F1034–F1049.
25. Qian Q, Li M, Cai Y, Ward CJ, Somlo S, et al. (2003) Analysis of the polycystins in aortic vascular smooth muscle cells. J Am Soc Nephrol 14: 2290–2297.
26. Gonzalez-Perrett S, Kim K, Ibarra C, Damiano AE, Zotta E, et al. (2001) Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease (ADPKD), is a Ca2+-permeable nonselective cation channel. Proc Natl Acad Sci U S A 98: 1182–1187.
27. Gonzalez-Perrett S, Kim K, Ibarra C, Damiano AE, Zotta E, et al. (2001) Polycystin-2, the protein mutated in autosomal dominant polycystic kidney disease, is a Ca2+-permeable nonselective cation channel. Proc Natl Acad Sci U S A 98: 1182–1187.
28. Ong AC, Ward CJ, Butler RJ, Biddolph S, Bowker C, et al. (1999) Coordinate distribution of tetraploid cells in mouse tetraploid embryos: a tool to distinguish embryonic vs. extra-embryonic nuclear transfer. Dev Biol 212: 124–136.
29. Allen E, Krellt AB, Keighren M, Fleethart JH, West JD (1995) Restricted expression in murine tissues via alterations in nuclear hormone receptors. Hum Mol Genet 4: 2675–2684.
30. Allen E, Paterson AD, Wang KR, Lupea D, Biddolph S, Bowker C, et al. (1999) Coordinate distribution of tetraploid cells in mouse tetraploid embryos: a tool to distinguish embryonic vs. extra-embryonic nuclear transfer. Dev Biol 212: 124–136.
31. James RM, Krellt AB, Keighren M, Fleethart JH, West JD (1995) Restricted expression in murine tissues via alterations in nuclear hormone receptors. Hum Mol Genet 4: 2675–2684.
34. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, et al. (2001) Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol 230: 230–242.
35. Lobe CG, Koop KE, Kreppner W, Lemeli H, Gertsenstein M, et al. (1999) Z/AP, a double reporter for cre-mediated recombination. Dev Biol 208: 281–292.
36. Watson ED, Cross JC (2005) Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) 20: 180–193.
37. Rossant J, Cross JC (2001) Placental development: lessons from mouse mutants. Nat Rev Genet 2: 338–348.
38. Beall MH, van den Wijngaard JP, van Gemert MJ, Ross MG (2007) Regulation of amniotic fluid volume. Placenta 28: 824–832.
39. Ahrabi AK, Jouret F, Marbaix E, Delporte C, Horie S, et al. Glomerular and proximal tubule cysts as early manifestations of Pkd1 deletion. Nephrol Dial Transplant 25: 1067–1078.
40. Zamboni L, Upadhyay S (1981) Ephemeral, rudimentary glomerular structures in the mesonephros of the mouse. Anat Rec 201: 641–644.
41. Cartelino A, Labbe S, Gallini R, Zanetti A, Balconi G, et al. (2003) The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. J Cell Biol 162: 1111–1122.
42. Allende ML, Yamashita T, Proia RL (2003) G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. Blood 102: 3665–3667.
43. Fu J, Gerhardt H, McDaniel JM, Xia B, Liu X, et al. (2008) Endothelial cell O-glycan deficiency causes blood/lymphatic misconnections and consequent fatty liver disease in mice. J Clin Invest 118: 3725–3737.
44. Srinivasan RS, Dillard ME, Lagutin OV, Liu FJ, Tsai S, et al. (2007) Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. Genes Dev 21: 2422–2432.
45. Bhunia AK, Pioutek K, Boletta A, Liu L, Qian F, et al. (2002) PKD1 induces p21(raf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. Cell 109: 157–168.
46. Yu S, Hackmann K, Gao J, He X, Pioutek K, et al. (2007) Essential role of cleavage of Polycystin-1 at G protein-coupled receptor proteolytic site for kidney tubular structure. Proc Natl Acad Sci U S A 104: 18688–18693.
47. Boletta A, Qian F, Osuchic LF, Bhunia AK, Phakleekitcharoen B, et al. (2000) Polycystin-1, the gene product of PKD1, induces resistance to apoptosis and spontaneous tubulogenesis in MDCK cells. Mol Cell 6: 1267–1273.