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The Zebrafish Kupffer's Vesicle: A Special Organ in a Model Organism to Study Human Diseases

Mónica Roxo-Rosa and Susana Santos Lopes

Abstract

The Kupffer's vesicle (KV) is a small, ciliated organ transiently present during embryogenesis of the zebrafish and other teleosts. The KV is required to the establishment of visceral laterality, such as the heart on the left side, being also known by the name left-right organizer (LRO). The LRO is found in other vertebrates, including mice, rabbits, frogs and human embryos. Among these, the KV became an excellent model organ to investigate the early left-right events during development and in disease. Many ciliary molecular players associated to the human disease primary ciliary dyskinesia have been tested in the zebrafish looking at KV cilia and its downstream effects on flow and left-right markers. Additionally, given its morphology and molecular features, we proposed the KV as a model organ to study the molecular mechanisms of the renal cyst inflation that occurs in the autosomal dominant polycystic kidney disease. Although having no connection to the kidney, the KV mimics a renal cyst because it is a fluid-filled vesicle, lined by monociliated epithelial cells that express polycystin-2, which knockdown leads to the organ luminal enlargement through changes in ion/water epithelial transport. Here, we explore the usefulness of the zebrafish KV to model these diseases.

Keywords: Kupffer's vesicle (KV), left-right (LR), left-right organizer (LRO), primary ciliary dyskinesia (PCD), autosomal dominant polycystic kidney disease (ADPKD)

1. The Kupffer's vesicle

The KV is an organ transiently present in the early embryonic life of the fish to establish internal body laterality [1, 2]. It derives from the dorsal forerunner cells (DFCs), a cluster of cells that migrate together from shield developmental stage until the end of the epiboly stage during zebrafish development. The DFC cluster later forms a lumen, and cilia start to protrude from the cells apically towards the new space. Thus, the KV organ will be formed by only one cell layer surrounding a lumen (**Figure 1**). The lumen is also progressively filled with fluid as it opens up through a mechanism involving cystic fibrosis transmembrane conductance regulator (CFTR, OMIM-602421), as described before [3–5]. CFTR is a chloride channel

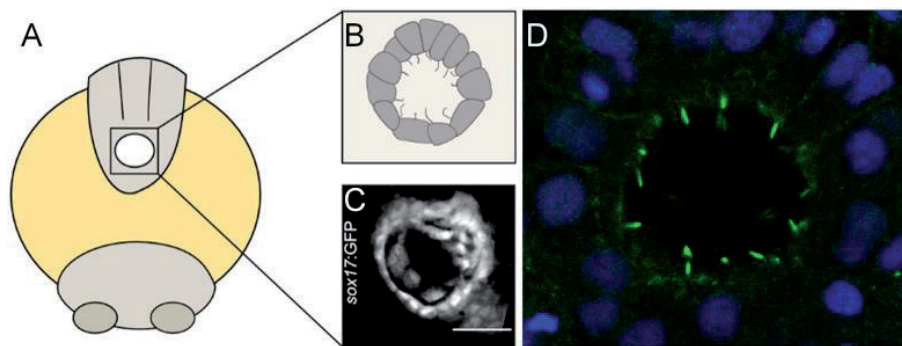


Figure 1.

The zebrafish Kupffer's vesicle. (A) It is imaged from the dorsal side of the embryo in the tail region from 6 to 14 hours post fertilization (hpf). (B) KV is formed by one layer of monociliated cells. (C) In sox17:GFP transgenic line, KV-lining cells are labeled with GFP. (D) Cross section through the KV midplane acquired by confocal fluorescent microscopy shows the cilia stained with acetylated alpha-tubulin in green and nuclei stained with DAPI in blue.

which absence or dysfunction causes cystic fibrosis [6–8]. As the KV enlarges, the solitary cilium from each KV cell starts to beat, and by ten somite stage (ss), there are on average 80% motile cilia and 20% immotile cilia in a total of 60 cells [9]. Despite it presents different shapes and sizes, the left-right organizer (LRO) has a conserved function among vertebrates, which is to break initial symmetry of embryonic body plans. As detailed below there are still many gaps in the developmental process of left-right (LR) establishment that need to be tested. Due to its genetic amenability and optical transparency, the zebrafish has gained impact in the LR field as one of the best models for testing early LR establishment questions [2, 9–15]. So far, it is the only animal system where we can manipulate the LRO without damaging or sacrificing the animal, thus letting it develop until organ localization can be visualized [2]. This allows for causal conclusions to be drawn in the same individual fish, which is a powerful advantage in developmental biology.

2. Primary cilia dyskinesia

2.1 The disease

Primary ciliary dyskinesia (PCD) is a rare congenital and heterogeneous disorder (OMIM: 244400) with an estimated prevalence of around 1:10,000 according to Rubbo and Lucas [16], which is thought to be higher in consanguineous populations [17]. However, other references in the literature consider a much lower prevalence, affecting approximately 1 in 20,000 individuals [18]. It is thought that the correct prevalence of the disease is unknown because many patients remain undiagnosed. PCD is characterized by a deficient mucociliary clearance, which is caused by the lack of motile cilia in the respiratory epithelia, uncoordinated ciliary pattern, or a total lack of ciliary motion giving rise to static cilia. PCD leads to chronic respiratory infections, and the earlier it is diagnosed, the better prognostic the patients will have. Its clinical features usually begin at birth with respiratory distress followed by a wet cough in early childhood and evolve to include bronchiectasis and chronic sinusitis. In the worst scenario, PCD may lead to lung lobectomy [19].

However, PCD is not just a respiratory disease because half of the PCD cases are associated with situs inversus and heterotaxy, and in these latter cases, there is a high correlation with congenital heart disease [16]. Male sterility is common in adults because normal sperm flagella are special motile cilia and in PCD patients

may present defective motility. Female ectopic pregnancies have also been reported due to the presence of defective motile cilia lining the fallopian tubes [20]. PCD usually follows an autosomal recessive inheritance pattern [17].

To date, mutations in more than 35 genes were identified to cause PCD. These genes are mainly coding for axonemal proteins that are required for cilia motility or for proteins that are needed for the assembly or transport of axonemal components [18, 21–23].

Our body is LR asymmetrical. In humans, the heart, spleen, and pancreas are localized on the left, while most of the liver and gallbladder are placed on the right side of our body axis. The fact that patients with immotile cilia presented defects in the location of their internal organs was not obvious to discern and raised many questions in the 1970s.

Bjorn Afzelius, who devoted great interest to this topic, postulated that the existence of an embryonic organ covered by motile ciliated epithelia should explain the laterality defects seen in patients with the immotile cilia syndrome [24]. However, until today the complete molecular mechanism is still to be fully demonstrated.

2.2 The KV as a model organ to study PCD

Previous early experiments on animal models, namely, in mice mutants named *inversus viscerum*, have shown that mutants that have no ciliary motility in the primitive node showed defective situs [25]. Imaging of the mouse nodal cilia helped to establish this causal link. These were first imaged in fixed samples by performing in situ hybridization with a riboprobe for LR-dynein (*lrd*) [25]. Soon after, mice nodal cilia and nodal flow were filmed and observed in live embryos for the first time by Nonaka et al. [26]. These authors compared wild-type (WT) embryos with homozygous mutants for KIF3b that formed no cilia and had no nodal flow. However, a definitive evidence that the nodal flow was relevant came from the elegant experiment, where Nonaka et al. [27] reversed the fluid flow that was generated by the nodal motile cilia using an artificial flow in immotile mutants. This study revealed that the correct direction of the fluid flow was crucial for the correct establishment of LR. Since then many mouse mutants were generated and studied [28, 29]. Research on other model organisms, such as frogs and zebrafish, followed in order to pursue the molecular mechanisms of laterality (e.g., [2, 30]). Among all the models in place, the zebrafish and its KV offer unique advantages to the LR field that we will explain and focus here.

The KV is ventrally positioned deep in the embryo, close to the yolk (**Figure 1**), but nevertheless it is accessible for live imaging from the dorsal side. This is only possible due to its optical clarity. The embryo is extremely transparent allowing filming the cilia inside the KV in a live intact zebrafish embryo that is simply mounted in soft agarose and embryo medium. This is strikingly different from any other vertebrate model. Mice require extracting the embryos at 7.5 days after coitus from the mother's uterus, and then dissected embryos are mounted in a medium prior to removal of the Reichert's membrane that covers the node, to then allow to film the cilia that lay inside the node [26]. In *Xenopus*, it is needed to dissect a frog embryo at stage 14 and perform dorsal explant cultures [30]. Therefore, in both these model organisms, the manipulations needed to expose the node for live imaging will later lead to the death of the animal, before the organs are placed in their asymmetric destinations. This problem impedes causal conclusions in the same individuals.

Zebrafish KV, due to its optical properties for live imaging, has been extremely useful for the study of nodal flow dynamics (**Figure 2**). How flow forces could trigger the first signals in the KV cells has been intensively explored in this model by our lab, both using experimental and theoretical approaches [2, 31–33].

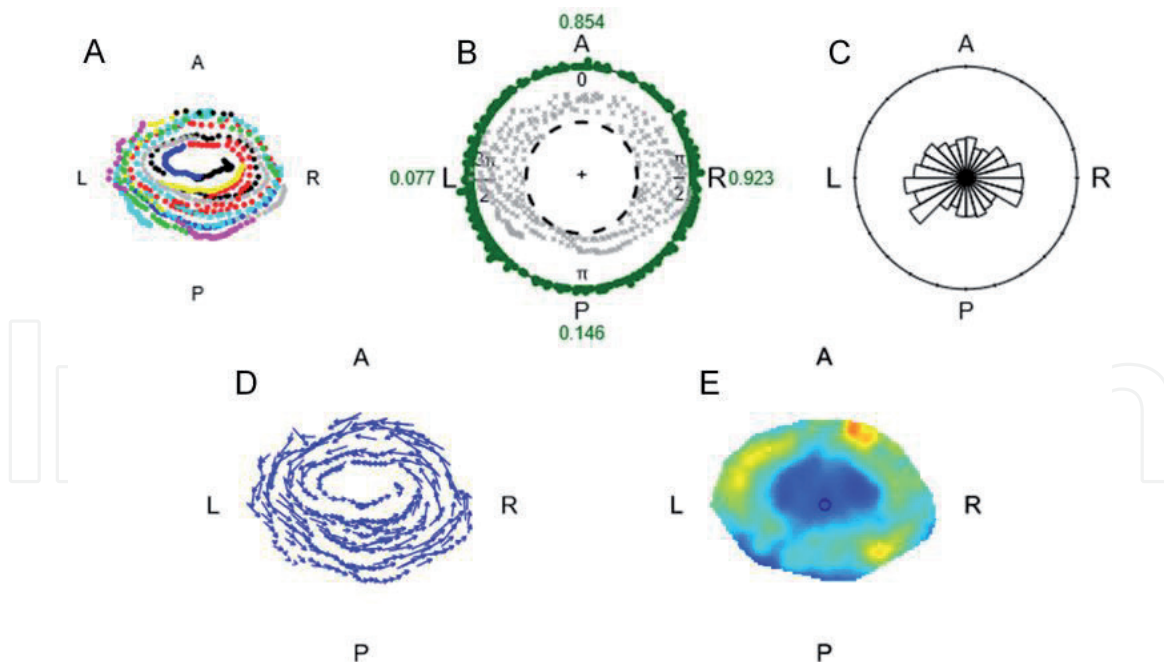


Figure 2.

Experimental fluid flow measurements and their different readouts. (A) Native particle tracking where each second has a different color to rapidly show where are the slowest regions of flow in the KV. (B) Rayleigh tests were performed considering a null hypothesis of a bias distribution toward a given KV quadrant, and p values are indicated as green numbers. Note that only particles moving at a distance of $r > 0.5$ from the center of the KVs were considered (r is the normalized distance from the center ($r = 0$) to the wall ($r = 1$) of the KV). (C) Rose maps show where flow is stronger in a radial manner. (D) Vector maps denote the actual flow forces in a vectorial manner. (E) Heat maps of flow speed showing detailed regions within each KV. The pseudocolor scale represents flow speed in micrometers per second, where red represents high speed versus low speed in blue. A, anterior; P, posterior; R, right; L, left.

We focused this review on the LR studies that deal with early events that occur in the KV organ. The nature of the signal that is perceived by the KV/node cells is not yet resolved. It can still be mechanical or chemical (or both) as debated for many years, since the first mouse studies [25, 26, 34]. It is now consensual that fluid flow is important, either by its force or by transporting molecules or molecules inside extracellular vesicles [34–36].

Zebrafish studies on the speed of flow *per* KV regions have shown that flow has a stereotyped pattern that is biologically relevant for LR establishment [2]. When we stopped motility of cilia everywhere except in the right half of the KV, we could demonstrate that this led to larva with situs inversus [9]. Although this seems to advocate for a mechanosensation process, it is still possible that some chemical component exists. For example, secretion of molecules caused by shear stress triggered by the local ciliary beating. This hypothesis is currently under investigation in our lab, and it was put forward in a theoretical recent study led by our collaborator mathematicians. In this study we predicted the shear stress to be greater on the anterior-dorsal side of the KV, mainly caused by the presence of a dorsal cluster of cilia [32].

The KV system has also been used to demonstrate that cilia length impacts greatly on fluid flow speed [31, 37, 38]. Other labs have also contributed to these biophysical characterizations, such as the Amack lab by analyzing the cell shape changes that lead to the dorsal cluster of cilia [13], reported earlier by Kreiling et al. [39]. The Vermot lab by pioneering the studies on KV flow [40] and recently by studying cilia tilt along the 3D KV [12] and its resultant theoretical flow forces. Contradictory interpretations emerged from our lab [9] and Vermot lab [12] concerning the number of immotile cilia in the KV and the consequent ability for the animal to sense flow in a mechanosensory way. This subject needs further

experiments to be solved as our labs used different imaging protocols to determine the number of immotile cilia. Another important contribution trying to understand the immediate output of the flow came from the Sun and Bruckner labs, where Yuan et al. have reported asymmetric intracellular calcium transients that seem to be stronger on the left side and become absent upon *pkd2* knockdown (the gene encoding polycystin 2) [41].

In summary, studies in the KV allowed for the biophysical characterization of fluid flow and have shown that disruption of these properties always lead to defective gene expression of *dand5*, the first asymmetric gene to appear in zebrafish [35]. The expression of *dand5* is thought to be dependent on flow forces and will directly impact on *nodal/spaw* later expressed in the lateral plate mesoderm. However, the nature of the crucial signal that initiates this conserved genetic cascade is still to be demonstrated.

3. Autosomal dominant polycystic kidney disease

3.1 The disease

Autosomal dominant polycystic kidney disease (ADPKD) has been reported to affect 1 in 400–1000 newborns worldwide. However, a recent population-based study in European Union puts ADPKD in the group of rare diseases, having an estimated prevalence rate of less than 1 in 2000 individuals [42]. In any case, ADPKD is for sure the most common genetic cause of renal failure [42], representing a major health problem, with an important socioeconomic impact worldwide.

It is caused by mutations in the human genes *PKD1* (OMIM-601313) and *PKD2* (OMIM-613095). These encode two ciliary proteins, polycystin-1 and polycystin-2, respectively. *PKD1* mutations account for about 78% of ADPKD patients and are associated with more severe phenotypes. In contrast, a milder disease is observed in the patients presenting *PKD2* mutations (about 15% of ADPKD patients) [43]. In 2016, *GANAB* (OMIM-104160) was identified as a third gene that, when mutated, causes ADPKD [44]. This encodes the catalytic α subunit of glucosidase II, a resident enzyme of the endoplasmic reticulum involved in the *N*-glycosylation of membrane proteins [44]. Together, PKD1 and PKD2 form a ciliary mechanosensor-calcium channel complex in the renal epithelium that is essential for the intracellular calcium homeostasis [45, 46]. On the other hand, the glucosidase II subunit α is critical for the proper maturation and ciliary *versus* cell membrane localization of both PKD1 and PKD2 proteins [44]. Through not fully understood mechanisms, the absence of functional ciliary PKD1/PKD2 complex triggers the formation and inexorable expansion of multiple cysts in all segments of the nephrons [45, 46]. This ciliopathy also affects other organs and systems. The extrarenal manifestations of ADPKD include liver and pancreatic cysts, hypertension, vascular problems, and abdominal hernias [43].

The kidney cyst inflation with water and ions and continuous expansion throughout the patient life is assured by an abnormal transepithelial fluid secretion toward their lumen [47]. CFTR plays a central role in the ADPKD cyst inflation [48–53]. Early in the cystogenesis process, CFTR becomes abnormally activated in ADPKD cyst-lining cells [48–53]. Supporting the involvement of CFTR in ADPKD cyst inflation, it was shown that the fluid accumulation within cysts involves CFTR-like chloride currents [53] and it is slowed down either through inhibition or knockdown of CFTR [49–53]. Additionally, a milder renal phenotype was observed in patients affected by both ADPKD and cystic fibrosis [47].

The in vivo mechanisms involved in the abnormal activation of CFTR during kidney cyst inflation are still emerging. It is known that the lack of calcium homeostasis raises the intracellular levels of cAMP in ADPKD cells [47]. As CFTR activation requires its prior cAMP-dependent phosphorylation by PKA [5, 7, 54, 55], it has been suggested that CFTR is a downstream effector of the raised levels of cAMP, in cyst growth [47]. This led to several studies testing drugs targeting renal cAMP production. However, so far only one drug showed an effect in slowing down the enlargement of cysts in adult ADPKD patients, the Tolvaptan [56]. Acting as a vasopressin V2 receptor antagonist, Tolvaptan lowers the intracellular cAMP levels of the cyst-lining cells and, therefore, the CFTR activity [47].

An evidence is growing that renal cyst formation starts in utero and that hypertension in childhood correlated well with disease severity. This is changing the old paradigm that considers ADPKD has a late-onset disease [57]. However, renal cysts still become clinically detectable only in adulthood, when the disease is fully established. The renal volume determination by imaging techniques (ultrasound, CT, and T2-weighted NMR scans) is the available tool to assess the disease progression. Although the majority of patients remain a- or pauci-symptomatic until adulthood, the natural progression of the disease leads to the total destruction of the renal parenchyma by the countless large cysts. Ultimately, about 50% of patients end up requiring renal replacement therapy in their sixth decade of life. The recent approval of Tolvaptan in Japan, Canada, and European Union brought some hope to patients [48]. However, it shows a moderate effect in slowing down the cyst enlargement [56]. Moreover, its side effects include polydipsia, polyuria, nocturia, pollakiuria [56], and significant liver enzyme elevation [58] limiting the eligible patients to those having <50 years, CKD stages 1–3, and rapidly progressing disease [43]. This means that the identification of biomarkers for the early events of ADPKD is an unmet need of the field. These are needed for the early diagnosis of the disease and accurate prediction of renal function decline and may bring novel therapeutic targets for ADPKD.

There is, therefore, an urgent need to investigate the cellular and biochemical pathways involved in kidney cytogenesis with innovative conceptual and methodological approaches. In this review, we will put forward the zebrafish model and its KV as an unconventional but promising organ model to find such biomarkers.

3.2 The zebrafish *pkd* genes

The zebrafish genome presents seven *pkd* and *pkd-like* genes: *pkd1* (also known as *pkd1a*), *pkd1b*, *pkd1l1*, *pkd1l2a*, *pkd1l2b*, *pkd2*, and *pkd2l1* [59]. In situ hybridizations showed that both *pkd1* and *pkd2* are expressed in the zebrafish pronephros at 24 hpf. In contrast, none of the other five *pkd* genes were found to be expressed in the developing pronephros [59].

The combined knockdown of the paralogues *pkd1a* and *pkd1b*, i.e., the orthologues of human *PKD1*, by injection of specific morpholinos against their mRNA which specifically block their translation, resulted in dorsal axis curved embryos [60]. An identical phenotype was observed by us and others in *pkd2* knocked-down embryos (*pkd2*-morphants) (**Figure 3**) [1, 11, 61, 62]. This phenotype was shown to be associated with changes in extracellular matrix upon the loss of these polycystins and may correlate to the vascular problems observed in ADPKD patients [60]. It ended up to being a good control of the efficiency of the knockdown of those genes in the embryo.

Additionally, the knockdown of *pkd2* leads to the formation of pronephric dilations which clearly impaired the fluid homeostasis of the animals. Indeed, *pkd2* morphants presented severe edema [11, 61, 62]. However, those pronephric dilations did not form individualized cystic structures that bud off from the tubules. Although

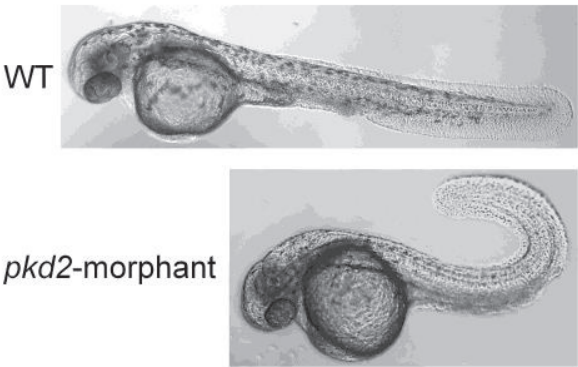


Figure 3. Curly-up tail curvature characteristic of *pkd2* morphants. Lateral view of WT and *pkd2*-morphant zebrafish embryos at 48 hpf.

many times named as kidney cysts, these pronephric dilations do not recapitulate the vesicular architecture of the ADPKD cysts [11, 61, 62]. On the other hand, the available zebrafish *pkd2* mutant, the curly-up (*cup*^{-/-}) mutant, did not develop kidney cysts or dilations [11, 62]. Our data suggested that this could be a result of the maternal contribution of *pkd2* mRNA present during early embryonic stages of *cup*^{-/-} mutants [1]. The combined knockdown of *pkd1a* and *pkd1b* also resulted in the formation of kidney cysts, but, in this case, the phenotype was observed in only 10–20% of the embryos [60]. Therefore, we consider the zebrafish pronephros limited to the study of the molecular mechanisms involved in the ADPKD cystogenesis.

Although KV does not express *pkd1a*, *pkd1b*, *pkd1l2a*, *pkd1l2b*, or *pkd2l1*, by in situ hybridizations, it was shown that KV cells do express both *pkd2* [1, 59] and *pkd1l1* [59]. Clearly showing the important role of Pkd2 in the LR axis establishment, *pkd2* morphant presented laterality defects [1, 11, 62, 63]. Indeed, we showed that 33 and 21% of these embryos have right-sided and central hearts, respectively [1]. Schottenfeld and co-authors demonstrated that the laterality defects of *pkd2* morphants were comparable to those observed in *cup*^{-/-} mutants [11]. Supporting its role in the laterality axis establishment, we detected Pkd2 protein expression through the KV-lining cells, intracellularly and along their cilia [1]. We now show in **Figure 4** that these cells do also express Pkd1l1 (polycystin 1 like 1) along their cilia. A similar expression pattern was described by Kamura et al. for this gene in the KV of medaka fish [64]. These data suggests that the Pkd1l1 is the partner of Pkd2 in the zebrafish KV cells [59], as it was also proposed for the medaka KV [64]. Supporting this hypothesis, the lack of *pkd1l1* expression caused laterality defects in medaka embryos [64].

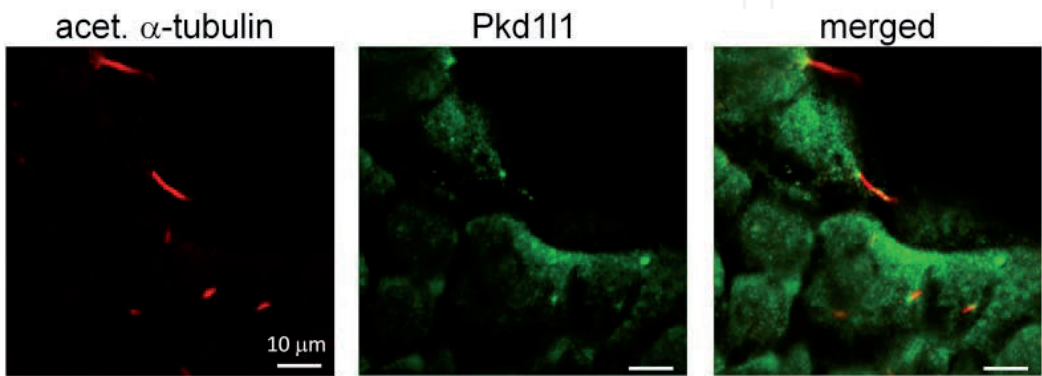


Figure 4. Confocal images for the immunolocalization of Pkd1l1 in KV cells at the 10–11 ss in WT embryos. Pkd1l1 is detected throughout the cells' cytoplasm and along their cilia. Pkd1l1 is in green and acetylated α -tubulin in red. Scale bars: 10 μ m.

3.3 The KV as a model organ to study the ADPKD cyst inflation

In our perspective, a good model to study the *in vivo* molecular mechanisms involved in the ADPKD cysts inflation should:

1. Be a fluid-filled vesicle.
2. Be lined by ciliated cells that must express Pkd2/Pkd1 and CFTR.
3. Exhibit a physiological fluid flow induced Ca^{2+} -signaling mediated by Pkd2.
4. Depend on CFTR activity for its lumen inflation.
5. Allow the easy knockdown of Pkd2/Pkd1 and CFTR.
6. Allow the easy access to the organ luminal volume.
7. Above all, the knockdown of PKD1/PKD2 must increase the CFTR-mediated fluid secretion into the lumen, mimicking the ADPKD cyst inflation process.

The KV is neither related to the kidney nor to the renal function. However, as it fulfills the mentioned requirements, a few years ago we proposed the zebrafish KV as a *bona fide* model organ of the cyst inflation process [1].

As already mentioned, this fluid-filled vesicle organ is lined by monociliated cells [1, 2, 9]. The KV cilia are motile and generate a fluid flow that is essential for the organ function [2, 9]. These are different from the primary cilia of the nephron epithelium or the cilia present in the luminal surface of *pkd2*^{WS25/-} mice ADPKD cysts [65]. Nevertheless, as primary cilia of the nephron segments, the KV cilia do express Pkd2 [1]. Interestingly, this channel is crucial for the asymmetric intracellular calcium transients that occur in the KV cells [41]. Opposing to the renal primary cilia, KV cilia are not expected to express Pkd1 [59]. However, as demonstrated in **Figure 4**, they express Pkd1l1 which is thought to be the Pkd2 partner in this organ [59, 64]. We expect Pkd1l1 knockdown to cause an abnormal LR patterning, similarly to phenotype observed in *pkd1l1*-medaka morphants [64]. By using a morpholino against CFTR, we were able to corroborate the findings of Navis et al. [4] showing that the knockdown of CFTR fully impairs the proper KV inflation [1].

The transparency of zebrafish embryos makes the KV of transgenic lines presenting GFP staining in KV-lining cells accessible by confocal live microscopy [1]. These include *ras*:GFP [1, 66], *foxj1*:GFP [1, 67], *sox17*:GFP (**Figure 5**) [68], or *TgBAC(cftr-GFP)pd1041* [4] transgenic embryos. In this way, the KV allows the measurement of its whole volume as a live readout of CFTR activity, with smaller volumes, meaning reduced CFTR activity [1].

The most important feature of the model is the fact that on average the knockdown of PKD2 leads to ~1.6 times larger KV volumes than the CFTR activation. Using a pharmacological approach, we showed that the *pkd2* morphants' KV enlargement is assured by a CFTR-mediated fluid secretion into the KV lumen, mimicking the ADPKD cyst inflation process [1].

This model was an important innovation in testing drugs that may modulate the CFTR-dependent KV inflation. We will publish soon our results with Tolvaptan among other drugs. These may give us clues on new drugs that may prevent ADPKD cyst enlargement.

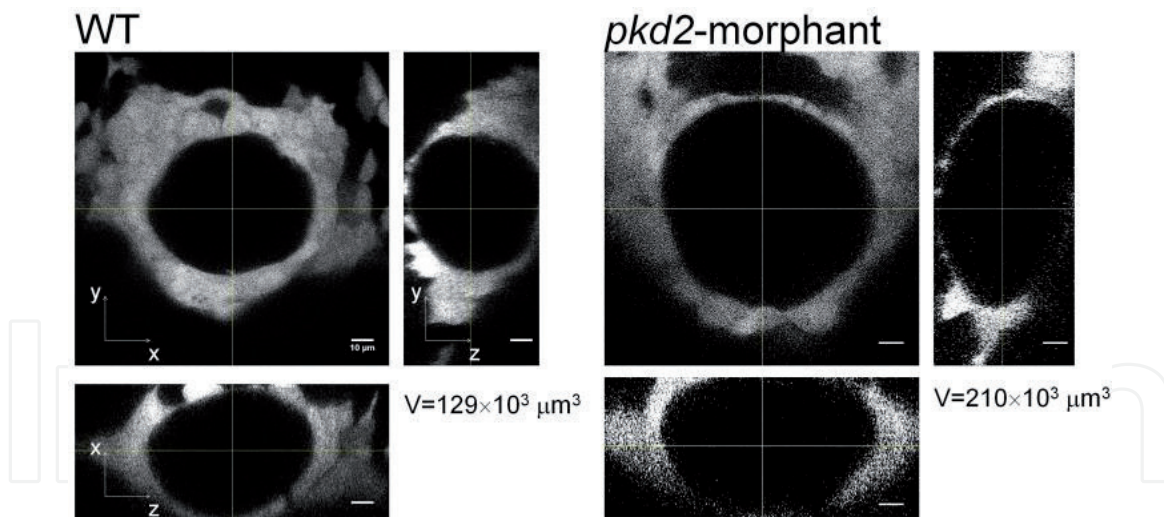


Figure 5.
KV volume. Confocal live-microscopy scans of the whole KV of 10–11 ss sox17:GFP transgenic embryos. The middle focal plane along the xy axis and the respective orthogonal views (along xz and yz axes) are shown for WT and pkd2-morphant representative embryos.

4. Conclusions

The zebrafish KV offers unique features that allow the proper study of the in vivo molecular mechanisms of both PCD and ADPKD. In the first disease, the KV has already emerged as an ideal system for its uniqueness in allowing to manipulate early genes and physical properties and then following up the LR pattern of the final organs. In the latter disease, it offers an excellent in vivo model for screening compounds and genes that may slow down cyst enlargement through CFTR inhibition.

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Conflict of interest

The authors declare no conflict of interest.

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