Dr Jaydev Upponi,

Science Editor

JoVE.

Dear Dr Upponi,

**Re: Revision of manuscript entitled “Evaluation of zebrafish kidney function using a fluorescent clearance assay”- JoVE52540R1.**

We have carefully considered the reviewers comments and have prepared a point-by-point document that addresses each comment in turn. In summary, we have successfully completed all the textual corrections and minor comments suggested by the reviewers. In addition, we have addressed many of their major concerns that we outline in the attached document.

Thank you for the valuable reviewers comments, I look forward to your reply.

With best wishes

Daniel Osborn

9th September 2014

**Reviewer 1:**

**Major concerns:**

***Justification of why the authors have chosen to target zebrafish bbs9 specifically and how their MO compares to previous published bbs9 MOs needs to be improved. The MO presented in this report gives a severe kidney phenotype, as observed by the reduced clearance of RD and the development of renal cysts, the latter being a phenotype that has not been described with two previously published bbs9 MOs (Veleri et al., 2012, PLoS One, 7, e34389).***

We have now included additional information explaining the rationale behind using bbs9 knockdown to demonstrate the rhodamine dextran assay. Firstly, Bbs9 encodes parathyroid hormone responsive B1 (PTHB1), where PTH has been implicated in renal cyst formation through the promotion of kidney epithelial cell proliferation. Secondly, Bbs9 has been shown to interact with other bbs proteins to form the core BBSome responsible for the formation of the primary cilium. Mutations in all 20 BBS genes, including BBS9 (representing 6% of BBS cases), have been shown to cause renal defects with no genotype-phenotype correlation. These points suggest that Bbs9 is likely to be a good candidate that when knocked down might display a kidney phenotype in zebrafish. We suggest that previous publication failed to document or comment on a renal phenotype because they mainly focus on the brain and eye phenotypes at stages where the renal cyst have yet to form. Pronephric cysts in zebrafish become apparent from 72 hpf onwards. At 72 hpf the cysts can only be convincingly shown by sections through the pronephric tubules to identify dilated kidney lumens. At 4 dpf (the stages we analyse), cysts can be observed down the microscope without the need to section embryos. Furthermore, we report cyst formation in 40% of embryos indicating that the phenotype does not have 100% penetrance so may have been missed in previous reports.

***To show that this assay can also be informative for less severe phenotypes, a dose-response curve of the bbs9 MO should be performed. What are the limitations of the assay in subtler phenotypes?***

This is a great suggestion from reviewer 1, however we feel this experiment is not necessary for a technical paper such as JoVE. Our remit was simply to demonstrate the use of the rhodamine dextran clearance assay to identify defects in kidney function in zebrafish. Here, I feel we go above and beyond to satisfy this.

***It would also benefit the reader to see that rescue of the phenotype with human BBS9 mRNA restores renal function using their assay, validating this assay for functional testing of allelic variants of unknown significance found in patients.***

Again, a great experimental suggestion. However, it is in our opinion that these experiments should be confined to more research based articles.

***It is unclear why the authors have chosen for a 100 pixel2 around the heart to analyze fluorescence intensity. Could the same results be achieved when measuring fluorescence in the entire embryo?***

Measurements of the heart avoids the autofluorescence associated with yolk cells, which might obscure the result if the whole embryo was used. Furthermore, we wanted to measure fluorescent content at a point in the system before the kidney, before filtration. We have added a note in our image processing section that clarifies this point: “We selected a fixed roi size of 100 px2 as this encompasses individual hearts between embryos. The heart was selected to perform measurements due to its large size that enables a convenient location to measure fluorescent content of the blood prior to entering the kidney”

***The authors measure mean gray values of the 100 pixel2 region, however should also normalize for background autofluorescence that could influence results, specifically for less penetrant phenotypes.***

Thank you for this suggestion. However, we feel this is an unnecessary step due to image acquisition parameters remaining constant between embryos and timepoints. This means that any autofluorescence in the heart will be accounted for and normalized between experimental embryos and controls. Furthermore, since each embryo is tracked independently any reduction is specific to that particular embryo where autofluorescence would remain consistent.

***It is unclear what the authors mean by 'control' to which they compare their bbs9 MO results. Are these embryos injected with scrambled MOs (preferred control) or a sham injection, or uninjected at single cell stage? Comparing the renal clearance in either sham-injected or uninjected animals is not an adequate control.***

Apologies for not including this in the original manuscript. Our control is a standard negative control morpholino, against an intronic mutation in human beta globin, injected in parallel at the same concentration as the *bbs9* morpholino. This is now included in the text.

**Reviewer 2:**

**Major concerns:**

***In the author's abstract and introduction, the description of pronephros anatomy is misleading. The pronephric kidney in the zebrafish is composed of two segmented nephron tubules, but the authors refer to these repeatedly as pronephric ducts (PND). For example, in the abstract (lines 49-50) the authors refer to the zebrafish kidney nephrons as "pronephric ducts". In the introduction (lines 109-110) the authors use the terms pronephric tubules stating, "Blood filters through the glomeruli into the pronephric tubules and into the pronephric ducts (PND). As Wingert et al., 2007 have described, the PND is just one distal segment of each pronephric tubule. Furthermore, the authors state in lines 111-112 that the PND are heavily ciliated, it is known that the proximal segment regions of the pronephric tubule also contains multiciliated cells (Ma and Jiang, 2006; Liu et al., 2006; Li et al., 2013). I think a bit more scrutiny on this subject is needed and furthermore that the aforementioned references should be included.***

Thank you to Reviewer 2 for pointing out this discrepancy. We have clarified these points in the main body of text and have avoided using the term pronephric duct by describing the pronephric tubules as a whole, we believe this avoids any confusion.