FGFR2 signaling enhances the SHH-BMP4 signaling axis in early ureter development

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MS TITLE: FGFR2 signaling enhances the SHH-BMP4 signaling axis in early ureter development

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I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

This manuscript examines the role of Fgfr1/2 signaling within the developing ureter epithelium and finds critical actions required for proper urothelial stratification. The predominant of the two receptors in this context appears to be Fgfr2. The authors go on to show likely downstream targets of Fgfr2 leading to a complex axis of signaling that includes reciprocal actions in epithelium and

mesenchyme. The data are strongly backed up by experiments knocking down the distal pathways and recapitulating the abnormal phenotypes and by stimulating the downstream targets and partially rescuing the defects. Overall, the manuscript is strong and has significant merit offering new mechanistic insights into the actions of Fgfr signaling in ureter development.

Comments for the author

While the manuscript has merit, there are some concerns as listed below:

- While the authors examine developmental expression of the appropriate ligands for Fgfr2 (namely Fgf7 and Fgf10), they do not examine expression of ligands that could bind to Fgfr1 (Fgf7 and 10 bind fairly exclusively to the epithelial isoform of Fgfr2 but not Fgfr1).
- Can the authors explain the reason for the partial lethality of the mutants, which increases as they go from a one allele loss of Fgfr2 to two allele loss (both with a two allele loss of Fgfr1)?
- I appreciate that it was a tour-de-force for the authors to create so many combinations of Fgfr1 and Fgfr2 deletions, but they do not report what happens to the ureter with either a homozygous loss of Fgfr1 or Fgfr2 (with the no loss of the other receptor). From the data shown, it does appear that Fgfr2 has a stronger effect than Fgfr1 as all ureters with a two allele loss of Fgfr2 have more ureter differentiation defects; thus, it would be interesting to see if this were re-capitulated with just a homozygous loss of Fgfr2.
- When the authors talk about Fgfr2cko, do all of these mice have a one allele loss of Fgfr1? It would be good to be explicit about the genotypes of those mice
- It would be helpful if the authors could show some high power H&E staining images from Fig 3 particularly when describing the apparent urothelial stratification defects.
- For Fig 6, where the authors discuss the decrease in epithelial stratification with increasing doses of Cyclopamine and Noggin, it would be helpful to see H&E stained images (including high power images)

Reviewer 2

Advance summary and potential significance to field

This is a really interesting study looking at the epithelial-mesenchymal pathways that control ureter differentiation. The authors use a Pax2Cre line to generate mutants in Fgfr1 and Fgfr2, which have been shown to be important in patterning of ureteric bud branching via Sprouty signaling, as well as in muscle and urothelial differentiation. Interestingly, inactivation of Fgfr2 signaling in the epithelium of the nephric duct, the precursor of the ureter results in abnormal differentiation of fibrocytes (first layer of mesenchyme surrounding the epithelium) and loss of the p63-expressing populations, which include Basal and Intermediate cells.

A careful examination of the phenotypes and inclusion of a table showing number of embryos examined, and whether phenotypes are are bilateral/vs unilateral, as described below would be a great addition, and could be a source of information for those working on genetic diseases that cause LUT defects.

Comments for the author

The paper focuses on Shh and Bmp4, which are relatively known pathways, it would be nice to know more about the deltaNp63 role in this process. The authors see loss of Fosb, Fos, which may be linked to AP-1 signaling, which could be important for regulating basal cells for example (as they are in skin basal cells).

It would be good to include alterations in the expression of the appropriate smads to confirm hypothesis that Bmp4 signaling is altered in mutants and to show which cell types respond to the BMP4 signal in this case.

These findings are important and could have implications for understanding genetic disorders in humans. To that end, the paper needs a table carefully summarizing the phenotypes even if they are not further investigated in the study. This should include bilateral descriptions of: Nephric duct insertion (E9 if available), ureter insertion into bladder (is it inserted, or is it in the appropriate

position roughly), obstruction, sex of embryos from visual inspection (E13.5 and later). Should document the presence of shortened ureters (Fig. 1) and renal hypoplasia (maybe present in Fig. 1 A, 4th sample from the left). The table should also clarify the same detail for the Fgfr1 mutant vs the Fgfr2 mutant vs compound mutants, and should include numbers of embryos examined.

Loss of expression could signify loss of cell types or expression loss of a particular cell type. Please indicate which are likely to be occurring. For instance, Aldh1a3; is it lost because basal cells are lost or because expression is down-regulated? Please address this for other targets discussed in the paper.
Does P63 expression ever come on? Does the phenotype compare to the P63 phenotypes observed in the bladder?

First revision

Author response to reviewers' comments

Response to reviewers

Reviewer 1 Advance Summary and Potential Significance to Field:

This manuscript examines the role of Fgfr1/2 signaling within the developing ureter epithelium and finds critical actions required for proper urothelial stratification. The predominant of the two receptors in this context appears to be Fgfr2. The authors go on to show likely downstream targets of Fgfr2 leading to a complex axis of signaling that includes reciprocal actions in epithelium and mesenchyme. The data are strongly backed up by experiments knocking down the distal pathways and recapitulating the abnormal phenotypes and by stimulating the downstream targets and partially rescuing the defects. Overall, the manuscript is strong and has significant merit offering new mechanistic insights into the actions of Fgfr signaling in ureter development.

>> We are very grateful that the reviewer appreciates the quality our work and sees its merits. We are thankful for his/her critical advise and have changed our manuscript accordingly. <<

Reviewer 1 Comments for the Author:
While the manuscript has merit, there are some concerns as listed below:

•While the authors examine developmental expression of the appropriate ligands for Fgfr2 (namely Fgf7 and Fgf10), they do not examine expression of ligands that could bind to Fgfr1 (Fgf7 and 10 bind fairly exclusively to the epithelial isoform of Fgfr2 but not Fgfr1).

>> In fact, we did not analyze the expression of other FGF ligands since our rationale was to analyze for the conservation of the *Fgf7/10-Fgfr1/2* module described to be present in the bladder primordium. Since we assumed that FGFR2 is the decisive receptor for urothelial development in the ureter (see arguments below), we deemed this restriction appropriate.
Nonetheless, we performed an additional in situ hybridization analysis of FGF ligand genes. We did not find specific expression of other FGF ligand genes in the UE and UM at E12.5 to E16.5. We include these data in the new Figure S1. (Please note that we overdeveloped our in situs to detect weak expression domains of FGF ligand genes. This led in some cases to strong background staining which, however, was homogenous and contiguous with that of surrounding tissues, such as the kidney).

We now write in our results:
"Expression of *Fgf7* and *Fgf10*, whose encoded proteins predominantly bind to the epithelial (IIIb) isoform of FGFR2 (Igarashi et al., 1998), occurred weakly in the UM, particularly at E14.5 (Fig. 1A). We did not detect specific expression of other genes encoding FGF ligands including those that have been reported to bind to the epithelial isoform of FGFR1 or the mesenchymal (IIIc) isoform of FGFR1 and/or FGFR2 {Iwata, 2009 #310}{Hui, 2018 #311}, in the UM or the UE from E12.5 to E16.5

(Fig. S1). Importantly, *Spry1* and *Spry2*, target genes of the FGF signaling pathway (Hanafusa et al., 2002), were strongly expressed in the UE at E12.5 and at E14.5 (Fig. 1B). These findings suggest that mesenchymal FGF7/10 predominantly activate epithelial FGFR2 signaling in early ureter development."<<

•Can the authors explain the reason for the partial lethality of the mutants, which increases as they go from a one allele loss of Fgfr2 to two allele loss (both with a two allele loss of Fgfr1)?

>> No, at this point we cannot provide a definitive explanation for the lethality of the conditional *Fgfr1* mutants and its enhancement by further loss of *Fgfr2*. However, we would like to mention that the *Pax2-cre* line used in our conditional gene targeting experiments also recombines outside the nephric duct epithelium (and its derivatives) particularly strongly in the midbrain-hindbrain region and the branchial arches at E9.5 (Kuschert et al., Dev. Biol. 229, 128-140). These expression domains are likely to give rise to vessels in the brain but also to the second heart field from which the atria, the right ventricle and the outflow region derive. Given the known role of FGF signaling in the second heart field region, deletion of *Fgfr1* and/or *Fgfr2* might contribute to lethality due to cardiac defects and circulatory insufficiencies, respectively.
In the revised version of our manuscript we take up this point in a new (first) paragraph in the discussion that deals with the specificity of the cre line used, the relative significance of *Fgfr1* and *Fgfr2 i*n the urothelial development and extraurothelial phenotypic manifestations.<<

•I appreciate that it was a tour-de-force for the authors to create so many combinations of Fgfr1 and Fgfr2 deletions, but they do not report what happens to the ureter with either a homozygous loss of Fgfr1 or Fgfr2 (with the no loss of the other receptor). From the data shown, it does appear that Fgfr2 has a stronger effect than Fgfr1 as all ureters with a two allele loss of Fgfr2 have more ureter differentiation defects; thus, it would be interesting to see if this were re-capitulated with just a homozygous loss of Fgfr2.

>> Thanks for appreciating the enormous amount of mouse work we had to do for this study!
Due to our breeding strategy (*Pax2-cre/+;Fgfr1fl/+;Fgfr2fl/+* x *Fgfr1fl/fl;Fgfr2fl/fl*), we did not recover conditional single mutants (*Pax2-cre/+;Fgfr1fl/fl* and *Pax2-cre/+;Fgfr2fl/fl)* for analysis. Generation of these single mutants would require additional breeding efforts which would exceed the time limit for revision of the manuscript.
Although we agree with the reviewer that analysis of these conditional single mutants would formally define the individual relevance of *Fgfr1* and *Fgfr2* in urothelial development in a conclusive manner, we think for a number of reasons that our current analysis already supports that *Fgfr2* is the key player in this context. We specifically would like to point out that we analyzed *Spry1/Spry2* expression in all mutant combinations and found that expression is only lost when both alleles of *Fgfr2* are gone (new Figure S2). Moreover, we would like to point to a previous study in which another cre line (*Hoxb7cre*) was used to individually delete *Fgfr1* and *Fgfr2* from the ureteric bud. This study had clearly shown that only loss of *Fgfr2* leads to ureteric and renal defects. We list these points as well as additional ones in the first paragraph of our revised discussion which reads:
"Due to our breeding strategy, we recovered only *Fgfr1-Fgfr2* compound mutants for phenotypic analysis. Although we cannot formally exclude a (minor) contribution of heterozygous loss *Fgfr1* to the observed phenotypic changes of the ureter in embryos with homozygous loss of *Fgfr2*, we are convinced that control of early ureter development is exerted almost exclusively by FGFR2. First, *Fgfr2* is much more strongly expressed in the UE from E12.5 to E14.5 than *Fgfr1*. Second, complete loss of *Fgfr1* with combined loss of one allele of *Fgfr2* did not result in changes of FGF signaling, i.e. *Spry1/2* expression, in the UE whereas complete loss of *Fgfr2* did. Third, complete loss of *Fgfr2* but not of *Fgfr1* resulted in severe ureteric cytodifferentiation defects. Fourth, FGF7 and FGF10, the two ligands with expression in the UM, signal predominantly through the epithelial isoform of FGFR2 (Igarashi et al., 1998; Jans, 1994; Ornitz and Itoh, 2015) while specific expression of FGF ligands that preferentially signal through FGFR1 was not detected in the early ureter. Fifth, previous studies using a *Hoxb7cr*e line for recombination in the ureteric bud lineage did not detect defects in the urogenital system of *Hoxb7cre/+;Fgfr1*$^{fl/fl}$ embryos whereas *Hoxb7cre/+;Fgfr2*$^{fl/fl}$ embryos exhibited renal hypo(dys)plasia due to reduced branching morphogenesis, thinning of the early ureter, and hydroureter, highly reminiscent of the phenotypic changes observed in our *Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/fl}$ embryos (Sims-Lucas et al., 2011; Zhao et al., 2004)."<<

•When the authors talk about Fgfr2cko, do all of these mice have a one allele loss of Fgfr1? It would be good to be explicit about the genotypes of those mice.

>> The reviewer is right, all the *Fgfr2cKO* embryos bear a loss of two alleles of *Fgfr2* and an additional loss of one allele of *Fgfr1*. We are now more precise in our wording and write in paragraph 3 of our results:
"We used for this and all subsequent assays *Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/fl}$ (from now on termed *Fgfr2cKO)* embryos since they exhibited the same ureteric cytodifferentiation defects as *Pax2-cre/+Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ embryos but presented in a normal Mendelian ratio."<<

•It would be helpful if the authors could show some high power H&E staining images from Fig 3, particularly when describing the apparent urothelial stratification defects.

>> We assume that the pdf file offered for review was not of the size necessary for zooming in for details. We now provide a higher magnification image of the HE and CDH1 stainings of the ureter sections from E12.5 to E16.5 in the new Fig. S4. <<

•For Fig 6, where the authors discuss the decrease in epithelial stratification with increasing doses of Cyclopamine and Noggin, it would be helpful to see H&E stained images (including high power images).

>> We have documented HE stainings and higher magnifications of these HE stainings as well as of CDH1 stainings and show them in the new Fig. S7.<<

Reviewer 2 Advance Summary and Potential Significance to Field:

This is a really interesting study looking at the epithelial-mesenchymal pathways that control ureter differentiation.
The authors use a Pax2Cre line to generate mutants in Fgfr1 and Fgfr2, which have been shown to be important in patterning of ureteric bud branching via Sprouty signaling, as well as in muscle and urothelial differentiation.
Interestingly, inactivation of Fgfr2 signaling in the epithelium of the nephric duct, the precursor of the ureter results in abnormal differentiation of fibrocytes (first layer of mesenchyme surrounding the epithelium) and loss of the p63-expressing populations, which include Basal and Intermediate cells.
A careful examination of the phenotypes and inclusion of a table showing number of embryos examined, and whether phenotypes are bilateral/vs unilateral, as described below would be a great addition, and could be a source of information for those working on genetic diseases that cause LUT defects.

>> Thank you very much for stressing the relevance of our study for the analysis of the pathways that control early ureter development; and thank you very much for pointing out experimental and textual ways to further improve the quality of our manuscript. <<

Reviewer 2 Comments for the Author:

•The paper focuses on Shh and Bmp4, which are relatively known pathways, it would be nice to know more about the deltaNp63 role in this process.

>> We respectfully disagree with the reviewer that the paper focuses on Shh and Bmp4. We focus on *Fgfr2* function in the epithelium. We describe the phenotypic changes and identify reduced expression and activity of the Shh/Bmp4 signaling module in *Fgfr2cKO* ureters. Our further analysis proves that the reduction of this module indeed accounts for the observed phenotypic defects in the *Fgr2cKO* ureters. Hence, we provide compelling evidence for a functional Fgf7/10-Fgfr2-Shh-BMP4 axis in early ureter development.
Work by several labs has shown that loss of ΔNP63 expression leads to the formation of a single-layered urothelium consisting largely of S-cells (Cheng et al., 2006; Pignon et al., 2013; Weiss et al., 2013)), thereby mimicking the phenotypic changes in *Fgfr2cKO* ureters. We have previously

shown that ΔNP63 expression is lost in ureters with conditional loss of Bmp4 (Mamo et al., 2017). Hence, ΔNP63 is a mediator of Bmp4 function in the urothelium but acts several layers downstream of *Fgfr2*. We have already discussed these published results in our initial submission.
We think that a further detailed analysis of the cellular and molecular function of ΔNP63 is beyond the scope and the focus of this study, and would not contribute to a better understand the FGFR2 signaling in the ureteric epithelium. <<

•The authors see loss of Fosb, Fos, which may be linked to AP-1 signaling, which could be important for regulating basal cells for example (as they are in skin basal cells).

>> Yes, we noted reduced expression of AP1 components in the microarray analysis of *Fgfr2cKO* ureters at E14.5. However, expression of *Fosb, Egr1 and Fos* was not detected in the control or was unchanged in the mutant by in situ hybridization (Fig. S5) preventing us from further analyzing the role of AP signaling downstream of *Fgfr2*. This was retrospectively justified by our finding that the activation of the Shh-Bmp4 module largely rescues the loss of *Fgfr2* in the epithelium, and that reduction of Shh/Bmp4 signaling phenocopies the changes.
If at all, AP1 signaling may present only a minor mediator of epithelial *Fgfr2* function. Moreover, a careful genetic analysis of AP1 signaling function in the early ureter is a project of its own, which is certainly out of range of our current resources and efforts.
However, we now explain the rationale to omit AP1 from further analysis in the revised manuscript by writing:
“Since we could not confirm expression (changes) of AP1 components and targets in the UM of mutants (Fig. S5), we excluded this pathway from further investigation.” <<

•It would be good to include alterations in the expression of the appropriate smads to confirm hypothesis that Bmp4 signaling is altered in mutants and to show which cell types respond to the BMP4 signal in this case. These findings are important and could have implications for understanding genetic disorders in humans.

>> Thank you very much for this suggestion. We have newly performed a detailed analysis of expression of mediators of BMP4 signaling by immunohistochemical detection of P-P38, P-SMAD1,5,9, P-AKT and P-ERK1,2 as well as ISH analysis of *Id* genes (direct transcriptional targets of BMP signaling). We show that P-SMAD1,5,9 and P-AKT are reduced as are *Id* genes in the UM and UE at E14.5 supporting our notion that BMP4 signaling is reduced in *Fgfr2cKO* ureters.
We present these compelling new data in Figure 4E,F and write in the results:
“Moreover, expression of *Id* genes (*Id2, Id3, Id4*), direct transcriptional targets of BMP signaling (Hollnagel et al., 1999; Liu and Harland, 2003), was reduced both in the UE and UM of *Fgfr2cKO* embryos at E14.5 (Fig. 4E). Since BMP4 signaling is mediated by different cytoplasmic effector proteins in the developing ureter (Mamo et al., 2017), we analyzed their activated, i.e. phosphorylated forms by immunohistochemistry. We found reduced expression of P-SMAD1/5/9 in the UM, and of P-AKT in the UE at E14.5 while P-ERK1/2 and P-P38 expression was unaffected (Fig. 4F, Fig. S6)..”<<

•To that end, the paper needs a table carefully summarizing the phenotypes even if they are not further investigated in the study. This should include bilateral descriptions of: Nephric duct insertion (E9 if available), ureter insertion into bladder (is it inserted, or is it in the appropriate position roughly), obstruction, sex of embryos from visual inspection (E13.5 and later). Should document the presence of shortened ureters (Fig. 1) and renal hypoplasia (maybe present in Fig. 1 A, 4th sample from the left). The table should also clarify the same detail for the Fgfr1 mutant vs the Fgfr2 mutant vs compound mutants, and should include numbers of embryos examined.

>> It might have slipped the attention of this reviewer but we already had a table showing the number of embryos analyzed in this study (before Table S2, now Table S1) and a table describing the ureter changes observed in the mutants at E18.5 (before Table S3, now Table S2).
Nonetheless, we are happy to follow the suggestion of the reviewer and have therefore invested quite some effort and time to expand the introductory evaluation of the stage-dependent genotype distribution and phenotypic changes of the urogenital system at E18.5 for the different genotypes with respect to kidney size, ureter length, uni-bilateral hydroureter occurrence and sex distribution in the revised version of our manuscript.

For the genotype distribution, we write in paragraph2 our result:
“We mated *Pax2-cre;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/+}$ males with *Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ females and analyzed the genotype distribution at different time points of embryogenesis. At all stages, *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/+}$ embryos were found at approximately half of the expected frequency, and *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ embryos at a quarter indicating that homozygous loss of *Fgfr1* accounts for lethality before E12.5 which is further enhanced by removal of *Fgfr2* function (Table S1).”
For our largely expanded analysis of the genotype-dependent phenotypic changes of E18.5 urogenital systems we now write:
“Morphological inspection of whole urogenital systems at the end of embryonic development, at E18.5, revealed that conditional loss of two and more alleles of *Fgfr1* and *Fgfr2* led with variable severity and penetrance to sex-independent hydroureter formation (Fig. 2A; Table S2A). Approximately 40% of *Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/+}$ (n=32) and 30% of *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/+}$ urogenital systems (n=11) presented with mild unilateral hydroureter whereas *Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/fl}$ (n=26) and *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ (n=8) urogenital systems had an increased occurrence (approx. 85%) of strong bilateral hydroureter. In the latter two genotypes, we detected one case each of ureter/kidney agenesis. Loss of both alleles of *Fgfr2* (*Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/fl}$; *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$) was additionally affected with uni- or bilateral dilatation of the epididymis, while kidney size and ureter length was strongly reduced in *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ urogenital systems only (Fig. 2A; Table S2A). Histological analysis confirmed hydroureter formation upon loss of two or more alleles of *Fgfr1* and/or *Fgfr2*, which however, did not translate into hydronephrosis in any of the genotypes (Fig. 2B, Fig. S3A). Moreover, we newly performed ink injection experiments and histological analyses to analyze the patency of the ureter and its junction with the bladder. This provides a mean to judge nephric duct insertion and distal ureter maturation since defects in these programs would affect the ureter-bladder connectivity and hence result in physical obstruction or deviation from the normal path. We describe this analysis in the results by writing:

“To test for patency of the ureter and its junctions, we injected ink into the renal pelvis of isolated urogenital systems and observed its flow to the bladder upon mild hydrostatic pressure. In most of the embryos with conditional loss of two or three alleles of *Fgfr1* and/or *Fgfr2 (Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl//+}$; *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/+}$; *Pax2-cre/+;Fgfr1*$^{fl/+}$*;Fgfr2*$^{fl/fl}$) the ureteric lumen was contiguous and the distal ureter inserted normally in the dorsal bladder neck. In 60% of *Pax2-cre/+;Fgfr1*$^{fl/fl}$*;Fgfr2*$^{fl/fl}$ urogenital systems (n=5), the ink did not reach the bladder, either due to insertion of the distal ureter into the urethra (1 out of 5) or due to ureteropelvic junction obstruction (n=2) (Table S2B). Histological analysis of the ureter-bladder connection of these specimens confirmed these findings (Fig. S3B). We conclude that loss of *Fgfr2* is associated with strong hydroureter formation. Additional loss of *Fgfr1* contributes to kidney hypoplasia, and increased physical obstruction along the ureter and its junctions.”

We also would like to mention that many years ago, Zhao et al (Dev Biol 276, 403-415) analyzed the role of *Fgfr1* and *Fgfr2* in the ureteric bud epithelium using a conditional approach with a Hoxb7cre line and floxed alleles of *Fgfr1* and *Fgfr2*. They stated in their abstract: “Absence of fgfr1 from the ureteric bud (fgfr1UB-/- results in no apparent renal abnormalities. In contrast, fgfr2UB-/- mice have very aberrant ureteric bud branching, thin ureteric bud stalks, and fewer ureteric bud tips”. To our satisfaction and relief, our conditional approach with the Pax2-cre driver which recombines in the nephric duct and its derivatives, confirmed the lack of phenotypic changes in *Fgfr1* conditional mutants and the occurrence of kidney hypodysplasia, hydroureter and a thinning of the ureteric epithelium in *Fgfr2*-deficient urogenital systems. In the first paragraph of our discussion, we now refer to this study in more detail since it nicely complements and supports our own results.<<

•Loss of expression could signify loss of cell types or expression loss of a particular cell type. Please indicate which are likely to be occurring. For instance, Aldh1a3; is it lost because basal cells are lost or because expression is down-regulated? Please address this for other targets discussed in the paper.

>>We agree that loss of marker expression does not necessarily mean loss of a specific cell type. In our analysis of E18.5 *Fgfr2cKO* ureters, we detected a very strong reduction of the basal marker KRT5 and the I-cell marker ΔNP63. We now more specifically write:
"In mutants with a complete loss of *Fgfr2,* the mono-layered epithelium expressed the S-cell marker UPK1B while KRT5- and ΔNP63-expressing B- and I-cells were largely absent."
For the lamina propria marker ALDH1A2, we do not further address whether lamina propria fibrocytes are absent, and therefore write:
"…expression of a marker of the *lamina propria* (ALDH1A2) was absent in ureters with complete loss of *Fgfr2* function (Figure 2C,D, row 1-5). And later :
"We conclude that loss of *Fgfr2* in the UE compromises differentiation of I- and B-cells but also affects the development of *lamina propria* fibrocytes in the UM."
Our time course analysis of markers (Figure 3) showed that differentiated cell types are established after E14.5 (B-cells at E16.5, S-cells at E15.5, lamina cells after E16.5).
We analyzed our microarray candidates at E12.5 and E14.5 when the ureteric epithelium is completely undifferentiated. Reduced expression of *Aldh1a3* and *Shh* at these stages, therefore, merely tells that they are reduced but not that a differentiated cell type is lost. <<

•Does P63 expression ever come on? Does the phenotype compare to the P63 phenotypes observed in the bladder?

>> Our immunofluorescence analysis did not detect ΔNP63 positive cells *in Fgfr2cKO* ureters at E14.5, and only one to two cells per cross section at E15.5 and E16.5 (see Figure 3). At E18.5, we found a couple of ΔNP63-positive cells in the undilated Pax2cre/+;Fgfr1fl/+; Fgfr2fl/fl specimen at E18.5 (Figure 2). Moreover, we found a strong reduction but not a loss of ΔNP63-positive cells in E13.5 ureter explants of *Fgfr2cKO* ureters grown for 4 days in culture (Figure 5). Together, this clearly shows that ΔNP63 expression, and hence, the formation of intermediate cells, is delayed and reduced but not completely abrogated in *Fgfr2cKO* ureters.
Due to the strong defects of the urogenital system of mice with complete loss of *Fgfr2*, and the associated phenotypic burden, we did not attempt to recover litters from our matings at postnatal stages for analysis.

Mice with conditional loss of ΔNP63 in the cloacal and ureteric epithelium display a mono-layered urothelium consisting of S-cells (Cheng et al., 2006; Pignon et al., 2013; Weiss et al., 2013). However, they do not show urothelial hypoplasia and mesenchymal defects as found in *Fgfr2cKO* ureters. While lack of stratification in *Fgfr2cKO* ureters is certainly caused by reduced activation of ΔNP63 due to reduced BMP4 signaling, lack of ΔNP63 does not explain these other phenotypes.
We have already discussed the role of ΔNP63 as a mediator of the *Fgfr2cKO* phenotype in the discussion of our manuscript:
"*Fgfr2cKO* ureters displayed a mono-layered urothelium consisting of S-cells. This phenotype is highly reminiscent of that seen in the bladder and ureters of mice with conditional loss of ΔNP63 in the respective epithelial primordium (Cheng et al., 2006; Pignon et al., 2013; Weiss et al., 2013). Failure to activate ΔNP63 in *Fgfr2cKO* ureters, therefore, likely accounts for the lack of stratification and B-cell differentiation in the mutant urothelium.
Expression and lineage tracing analysis uncovered that S- and B-cells are terminally differentiated cell types that arise from a common progenitor by an I-cell intermediate. The I-cells were recognized as $\Delta NP63^{+}$ cells lacking high expression of UPKs and KRT5 (Bohnenpoll et al., 2017a; Gandhi et al., 2013). Differentiation of S-cells in absence of ΔNP63 shows that stratification is not a prerequisite for S-cell differentiation, and suggests that S-cell differentiation is normally inhibited in I-cells by ΔNP63.

Mice with conditional loss of *Smo* or *Bmp4* in the UM do not activate ΔNP63 in the urothelium, and lack stratification and B- and S-cell differentiation (Bohnenpoll et al., 2017c; Mamo et al., 2017). In *Fgfr2cKO* ureters, *Shh*, and consequently, *Bmp4* expression is reduced but not lost, suggesting that ΔNP63 expression and stratification requires higher levels of SHH and BMP4 signaling than S-cell differentiation. This notion is supported by the restoration of ΔNP63 expression in *Fgfr2cKO* ureters by purmorphamine and BMP4 treatment on the one hand, and a relatively higher decrease of I-cells than S-cells by increasing doses of cyclopamine and NOGGIN in wildtype ureters on the other hand. Administration of BMP4 to early kidney explants leads to UPK expression in collecting duct cells (Mills et al., 2017; Wang et al., 2009) indicating that BMP4 is required and sufficient to activate S-

cell differentiation. It is conceivable that ectopic induction of I-cell differentiation and of ΔNP63 expression, respectively, requires higher levels of BMP4 and/or additional positive signals, similar to the situation in other epithelia (Terakawa et al., 2016). Alternatively, concurrent repression of an inhibitor may allow induction of ΔNP63".<<

Second decision letter

MS ID#: DEVELOP/2021/200021

MS TITLE: FGFR2 signaling enhances the SHH-BMP4 signaling axis in early ureter development

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript examines the role of Fgfr1/2 signaling within the developing ureter epithelium and finds critical actions required for proper urothelial stratification. The predominant of the two receptors in this context appears to be Fgfr2. The authors go on to show likely downstream targets of Fgfr2 leading to a complex axis of signaling that includes reciprocal actions in epithelium and mesenchyme. The data are strongly backed up by experiments knocking down the distal pathways and recapitulating the abnormal phenotypes and by stimulating the downstream targets and partially rescuing the defects.

Comments for the author

The authors have addressed all of my prior concerns.

Reviewer 2

Advance summary and potential significance to field

Understanding the basis of urothelial differentiation in the ureter will be important for developing regenerative strategies and for distinguishing differences between urothelial cells lining the. bladder and those lining the ureters.

Comments for the author

The authors have made a considerable effort to address the issues raised by reviewers and the manuscript is much improved.