Mesenchymal FGFR1 and FGFR2 control patterning of the ureteric mesenchyme by balancing SHH and BMP4 signaling

Lena Deuper, Max Meuser, Hauke Thiesler, Ulrich Walter Heinrich Jany, Carsten Rudat, Herbert Hildebrandt, Mark-Oliver Trowe and Andreas Kispert
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MS ID#: DEVELOP/2022/200767

MS TITLE: Mesenchymal FGFR1 and FGFR2 control patterning of the ureteric mesenchyme by balancing SHH and BMP4 signaling

AUTHORS: Lena Deuper, Max Meuser, Hauke Thiesler, Ulrich Walter Heinrich Jany, Carsten Rudat, Herbert Hildebrandt, Mark-Oliver Trowe, and Andreas Kispert

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.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript is very well written, experiments are logical and the results highly impactful in the field of urinary system development. Phenotypic analyses of the Fgfr1/2cDKO-UM ureters are of high quality and the in vitro mechanistic studies using signaling pathway modulators carefully performed. All studies are well powered. In summary, the authors provide compelling evidence for their novel finding that the main role of FGF receptors in the periureteric mesenchyme is to modulate the activity of FGF signaling in the ureteric epithelium during development, as well as provide novel interpretations to the role of SHH signaling in ureter patterning.

Comments for the author

In this manuscript Deuper et al., investigate the role of two FGF receptors - Fgfr1 and Fgfr2 in the development and differentiation of mouse periureteric mesenchyme, which gives rise to the muscular wall of the ureter. For this purpose, they have generated a triple transgenic mouse line (Fgfr1/2cDKO-UM) in which the expression of Fgfr1 and Fgfr2 is conditionally deleted from the periureteric mesenchyme in Tbx18-Cre dependent manner, starting at the onset of ureter development (E10.5). Fgfr1/2cDKO-UM mice are perinatally lethal, so the authors focused their investigations to the early morphogenic stages of the ureter from E12.5 to E18.5. Using histological stains and molecular markers of the ureteric mesenchyme and epithelium the authors first meticulously characterized the changes in the patterning and differentiation of the muscular wall in control and cDKO-UM ureters. In their analyses they observed a significant expansion of the lamina propria and a delay in the differentiation of smooth muscle cells in Fgfr1/2cDKO-UM mice compared to littermate controls. No abnormalities were observed in the epithelial compartment of Fgfr1/2cDKO-UM ureters.
Functionally, the changes in the periureteric mesenchyme in Fgfr1/2cDKO-UM mice translated to impaired peristalsis and hydroureter formation at E18.5. To investigate the molecular changes underlying the impaired differentiation of the periureteric mesenchyme in Fgfr1/2cDKO-UM mice the authors performed a comparative microarray analysis, which revealed significant alterations in signaling pathways known to have key functions in ureter morphogenesis (SHH, BMP, WNT). Finally, employing ureter explant assays combined with treatment with small molecules that modulate SHH and/or BMP signaling pathway activities the authors demonstrated that abnormal activation of SHH and reduced BMP signaling was sufficient to explain the ureter developmental defect observed in Fgfr1/2cDKO-UM mice. Together, providing a mechanistic explanation to the formation of mesenchymal anomalies and hydroureter in Fgfr1/2cDKO-UM mice. The manuscript is very well written, experiments are logical and the results highly impactful in the field of urinary system development. Phenotypic analyses of the Fgfr1/2cDKO-UM ureters are of high quality and the in vitro mechanistic studies using signaling pathway modulators carefully performed. All studies are well powered. In summary, the authors provide compelling evidence for their novel finding that the main role of FGF receptors in the periureteric mesenchyme is to modulate the activity of FGF signaling in the ureteric epithelium during development, as well as provide novel interpretations to the role of SHH signaling in ureter patterning.

The reviewer has no major comments.

Minor comments.

1. ln. 104. Please move the citation to Figure S1A from the Introduction to the Results section.

2. Figure S5B. Please use a white line to indicate ureteric epithelium (ue) in the right side panel (E14.5 sections).

3. Figure 5F. Please correct the labeling to indicate which panel corresponds to the expression of Bmpr1b.

Reviewer 2

Advance summary and potential significance to field

In a recent publication the authors described an important role of FGF signaling in the ureter epithelium (Pax2-Cre as a driver; Meuser et al., 2022). The present paper now investigates a potential role of FGFR1 and FGFR2 in the surrounding mesenchyme by performing a tissue specific deletion using the Tbx18-Cre line. A detailed analysis showed a delay in mesenchymal cell differentiation, reduced peristaltic contractions and hydroureter development in mutant mice. Microarray experiments revealed upregulation of Shh, Wnt and RA signaling in E14.5 mutant tissue. Comparison with previous studies suggest that the phenotype may be caused by overactive FGF signaling in the UE compartment. Using explant cultures treated with FGFs, noggin and an Shh pathway activators the authors can recapitulate their in vivo findings. Together their data lead them to propose an interesting model in which mesenchymal FGFR2 acts as a molecular sink for FGFs to limit FGF signaling in the ureteric epithelium.

Comments for the author

This is a well written manuscript providing a thorough phenotypic characterization (although quantification is missing at times) and an interesting model concerning the role of FGFRs in the mesenchyme. However, some additional experiments are needed to validate the proposed model.

1. Figure S1 shows the expression of FGFR1/2 with weak staining in the UM and strong expression in the UE. The Tbx18-Cre line should in principle be specific for mesenchymal deletion, but ISH analysis should be used to confirm that UE specific FGFR1/2 expression remains unaltered (both qualitatively and quantitatively)
2. In line 277, the authors state that Spry1 is not expressed in the UM, but when checking their previous manuscript (Meuser et al., Fig1A) a signal is visible in UM (arguably much weaker than in the UE). Doesn't that mean that FGF signaling also occurs within the mesenchyme? Please clarify.
3. The model of mesenchymal FGFR1/2 acting as a molecular sink to limit epithelial FGF signaling is an interesting concept, but perhaps not strongly enough documented. The in situ hybridization analysis for Spry1 did not reveal dramatic changes upon mesenchymal FGFR deletion (Fig5C) and, in any case classical ISH is not considered to be quantitative. Moreover, qPCR analysis in Fig. 5D does not show a significant increase of Spry1. RNA-Scope analysis is supposed to be more quantitative and could be used to confirm their hypothesis.
What happens to other FGF downstream targets such as MKP3?
4. Another major conclusion of the manuscript is that mesenchymal FGFR deletion leads to a decrease of BMP signaling in the UM, but once again I feel this is not well documented. In line 247 the authors state 'clearly reduced expression of Id2 and Id4', but I have difficulties to see this in Figure 4G for Id4. The qPCR quantification of Bmp4 and Id2 in 4H also shows no significant reduction. Is there a reason why Id4 was not included for qPCR? RNA-Scope analysis may provide a more quantitative measure. Another way to show active BMP/pSMAD signaling would be to use pSMAD1/5 antibodies. Have the authors tried this?

Minor comments:

1. Fig S5D: please label the graphs with the ages analyzed.
2. Figure 5F: The figure shows twice the same label (Bmpr1a)
3. Figure 1F: The increased expression of Aldh1a2 at E18.5 is not obvious and the statement in line 152 " Aldh1a2…was more strongly expressed" is not valid. The data at earlier time points are more convincing (figure 2). Does this mean Aldh1a2 expression returns to normal levels at late stages of development?

First revision

Author response to reviewers' comments

Response to Reviewers:

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In summary, the authors provide compelling evidence for their novel finding that the main role of FGF receptors in the periureteric mesenchyme is to modulate the activity of FGF signaling in the ureteric epithelium during development, as well as provide novel interpretations to the role of SHH signaling in ureter patterning.

The reviewer has no major comments.
>> We would like to thank the reviewer for taking the time to carefully read and evaluate our manuscript. We are very happy about the strong words of appreciation on the quality and relevance of our work.<<

Minor comments.
1. ln. 104. Please move the citation to Figure S1A from the Introduction to the Results section.
>> We have moved the citation to Figure S1A to the first paragraph in the results section. <<

2. Figure S5B. Please use a white line to indicate ureteric epithelium (ue) in the right side panel (E14.5 sections).
>> We have used white lines to highlight the ureteric epithelium in Fig. 5SB. <<

3. Figure 5F. Please correct the labeling to indicate which panel corresponds to the expression of Bmpr1b.
>> Thanks for pointing out the mislabeling. We have corrected it and moved this sub-panel to Figure S9. <<

Reviewer 2 Advance summary and potential significance to field
In a recent publication the authors described an important role of FGF signaling in the ureter epithelium (Pax2-Cre as a driver; Meuser et al., 2022). The present paper now investigates a potential role of FGFR1 and FGFR2 in the surrounding mesenchyme by performing a tissue specific deletion using the Tbx18-Cre line. A detailed analysis showed a delay in mesenchymal cell differentiation, reduced peristaltic contractions and hydroureter development in mutant mice. Microarray experiments revealed upregulation of Shh, Wnt and RA signaling in E14.5 mutant tissue. Comparison with previous studies suggest that the phenotype may be caused by overactive FGF signaling in the UE compartment. Using explant cultures treated with FGFs, noggin and an Shh pathway activators the authors can recapitulate their in vivo findings. Together their data lead them to propose an interesting model in which mesenchymal FGFR2 acts as a molecular sink for FGFs to limit FGF signaling in the ureteric epithelium.

Reviewer 2 Comments for the author
This is a well written manuscript providing a thorough phenotypic characterization (although quantification is missing at times) and an interesting model concerning the role of FGFRs in the mesenchyme. However, some additional experiments are needed to validate the proposed model.
>> We would like to thank the reviewer for taking the time to carefully evaluate our manuscript. We are extremely happy about the words of appreciation on the quality and relevance of our study. We thank the reviewer for the suggestions to further improve our manuscript.

1. Figure S1 shows the expression of FGFR1/2 with weak staining in the UM and strong expression in the UE. The Tbx18-Cre line should in principle be specific for mesenchymal deletion, but ISH analysis should be used to confirm that UE specific FGFR1/2 expression remains unaltered (both qualitatively and quantitatively)
>> Yes, the reviewer is right, the Tbx18cre line is absolutely specific for the UM. We have shown this many times before and have provided a reference for that (Bohnenpoll et al., 2013).
To address the concerns of the reviewer, we have performed RNA in situ hybridization for Fgfr1 and Fgfr2 on the mutant ureters. We show in new Fig. S9D (in the context of analysis of FGFR signaling) that expression of Fgfr1 and Fgfr2 in the UE is unaltered. To show that Fgfr1 and Fgfr2 expression is quantitatively unchanged, we would need to cross in a reporter line, FACS sort the cells and perform mRNA quantification by RT-qPCR analysis. This effort cannot be done in the 3-month revision time. Moreover, it would need a large number of mice, which we could not justify. We kindly ask the reviewer to accept this limitation.

2.In line 277, the authors state that Spry1 is not expressed in the UM, but when checking their previous manuscript (Meuser et al., Fig1A) a signal is visible in UM (arguably much weaker than in the UE). Doesn't that mean that FGF signaling also occurs within the mesenchyme? Please clarify.
>> We respectfully disagree with the reviewer that Fig. 1B in the Meuser manuscript shows Spry1 expression in the UM. The low level signal for Spry1 in the UM at E14.5 and for Spry2 at E12.5 and E14.5 is background due to extended staining time. The few spots with stronger staining likely indicate vessels. Please compare the Spry1,2 stainings with the Fgfr2 staining in the same Figure which shows specific expression in the UM.
Of course, we cannot formally exclude that there is a very low level of Spry1 expression, hence of FGFR signaling in the UM but we deem it unlikely. However to accommodate the concern of the reviewer, we slightly modified a statement in the discussion section that relates to this problem: "Fourth and last, our recent study showed that expression of Spry1, a transcriptional target of FGF signaling activity, occurs in the UE dependent on epithelial FGFR2 signaling but cannot be reliably detected by in situ hybridization analysis in the UM (Meuser et al., 2022), indicating that mesenchymal FGFR1 and FGFR2 signaling elicits no or only a minor transcriptional response in this tissue".

3.The model of mesenchymal FGFR1/2 acting as a molecular sink to limit epithelial FGF signaling is an interesting concept, but perhaps not strongly enough documented. The in situ hybridization

analysis for Spry1 did not reveal dramatic changes upon mesenchymal FGFR deletion (Fig5C) and, in any case, classical ISH is not considered to be quantitative. Moreover, qPCR analysis in Fig. 5D does not show a significant increase of Spry1. RNA-Scope analysis is supposed to be more quantitative and could be used to confirm their hypothesis.
What happens to other FGF downstream targets such as MKP3?
>> We agree that the expression changes of the signaling systems in the Fgfr1/2cDKO-UM mutant ureters are not dramatic - in fact they are modest - but the combination of subtle changes leads to patterning defects that translate into hydroureter formation at birth.
With respect to ISH analysis of Spry1 expression in the UE at E14.5 (now Fig. S9A), we are convinced that Spry1 is upregulated. In the microarrays, Spry1 expression was +1.3 fold increased. We see a similar increase in our RT-qPCR assay (Fig. 5C). In our opinion, all three assays confirm (a slight) increase of Spry1 expression (in the UE) in Fgfr2cDKO-UM mutant ureters.
However, to address the concerns of the reviewer we additionally analyzed expression of Etv4 and Etv5, two well-known direct targets of FGFR signaling. Both genes were slightly increased (+1.2) in the microarray. Upregulation of both genes was detected by RT-qPCR, with Etv5 being significant. We included this new data in Fig. 5C. Notably, Etv4 and Etv5 cannot be detected by section in situ hybridization analysis in the ureter, while whole mount in situs are more sensitive and detect expression in the ureteric epithelium (new Fig. S9B,C). Please note that we moved the RNA in situ hybridization results formerly presented in Fig. 5 into new Fig. S9 to homogenously present the quantitative RT-qPCR results in the main figure.
All of these data together provide evidence that FGF signaling is weakly increased in the ureteric epithelium of Fgf1/2cDKO-UM ureters at E14.5.
We newly describe this section of the results part on p.9/10 as follows:
“To further substantiate this hypothesis, we analyzed expression of known direct targets of FGFR signaling, namely Spry1 (Hanafusa et al., 2002) (microarray: +1.3) as well as Etv4 and Etv5 {Firnberg, 2002 #305}{Liu, 2003 #306} (microarray: +1.2) in E14.5 Fgfr1/2cDKO-UM ureters by RNA in situ hybridization on sections. In fact, we found increased expression of Spry1 in the UE. Mesenchymal expression of Spry1 was undetectable by this method (Fig. S9A). Section in situ hybridization was not sensitive enough to detect expression of Etv4 and Etv5 in the ureter at this stage whereas in whole mounts an epithelial expression was apparent (Fig. S9, B and C). RT-qPCR analysis confirmed increased expression of Spry1, Etv4 and Etv5, with the latter reaching significance (Fig. 5C, Table S14B).“
We agree that RNAScope is a detection method that offers increased sensitivity. However, due to the monopoly situation of the discoverer/vendor (ACD) the kits and probes are very expensive amounting easily to 5000 Euro for a couple of probes. We are not in the financial situation to be able to afford these reagents at these costs at this point. We kindly request the reviewer to accept this limitation.

4. Another major conclusion of the manuscript is that mesenchymal FGFR deletion leads to a decrease of BMP signaling in the UM, but once again I feel this is not well documented. In line 247 the authors state ‘clearly reduced expression of Id2 and Id4’, but I have difficulties to see this in Figure 4G for Id4. The qPCR quantification of Bmp4 and Id2 in 4H also shows no significant reduction. Is there a reason why Id4 was not included for qPCR? RNA-Scope analysis may provide a more quantitative measure. Another way to show active BMP/pSMAD signaling would be to use pSMAD1/5 antibodies. Have the authors tried this?
>> As said above, the expression changes of the signaling systems (including BMP4) in the Fgfr1/2cDKO-UM ureters are not dramatic - in fact they are modest - but the combination of these subtle changes leads to patterning defects that translate into hydroureter formation at birth.
We performed the ISH analysis for Id2 and Id4 many times (n>=7) and are convinced about the reduced expression of both genes in the UM at E14.5, and a slight upregulation in the UE. In fact, we performed additional in situ hybridizations for Id4 and present a section which better supports our statement (Fig. 4G). Moreover, we are more cautious in describing these results by writing on p.9:
“Expression of Bmp4 appeared unchanged while Id2 and Id4 expression was reduced in the UM (Fig. 4G).“
We newly performed RT-PCR analysis for Id4 and found no change in expression as we did for Id2 in this assay. We suggest that this lack of expression change in the entire ureter is likely to reflect opposing changes of BMP4 signaling in the UM (BMP4 signaling down) and the UE (BMP4 signaling up). We carefully and cautiously stated this in the result section on p.9:

“Bmp4 expression was unchanged as was expression of Id2 and Id4. The latter may reflect opposing changes of BMP4 signaling in the UE and UM“ and in the discussion on p. 16:
“Our in situ hybridization results revealed reduced expression of the BMP target genes Id2 and Id4 in the UM of Fgfr1/2cDKO-UM embryos. Although the sensitivity of this method was not sufficient to detect changes of Id2/Id4 expression in the UE, it is conceivable that epithelial BMP4 signaling, hence, target gene expression, is enhanced considering the overall unchanged Id2/Id4 levels both in our microarrays and in RT-qPCR analysis of whole Fgfr1/2cDKO-UM ureters
Yes, we did immunofluorescence analysis for P-SMAD1,5,9. We did not see changes in the signal intensities between control and mutant ureters on sections at E14.5. However, we had to amplify the antibody reaction which abolishes the detection of weak expression changes.
And again, we agree that RNAScope is a detection method that offers increased sensitivity. However, due to the monopoly situation of the discoverer/vendor (ACD) the kits and probes are very expensive amounting easily to 5000 Euro for a couple of probes. We are not in the financial situation to be able to afford these reagents at these costs at this point.
We would like to stress again that the expression changes of the signaling systems in the Fgfr1/2cDKO-UM ureters are modest in particular with respect to FGFR and BMP4 signaling. This is the very reason, why we engaged in additional experiments, which clearly show that the mesenchymal FGFRs are likely to act as a sink for FGF ligands to limit activation of epithelial FGFR2 signaling (Figure 5) and that misbalanced SHH-BMP4 signaling accounts for the mesenchymal patterning defects in the Fgfr1/2cDKO-UM ureters (Figure 6). We kindly ask the reviewer to appreciate that the combination of these different experimental evidences provides a valuable justification for our interpretations and the model presented in Figure 7.

Minor comments:
1.Fig S5D: please label the graphs with the ages analyzed.
>> We put the labeling E12.5 and E14.5 at the top of the whole figure to make it clear that all assays shown in A-D relate to these stages. To better indicate these stages in panel D, we place E12.5 and E14.5 into the graphs. Due to space constraints, we could not label the y-axis in both panels. >>

2.Figure 5F: The figure shows twice the same label (Bmpr1a).
>> Thanks for pointing out the mislabeling. We have corrected it and moved this sub-panel to Figure S9D. <<

3.Figure 1F: The increased expression of Aldh1a2 at E18.5 is not obvious and the statement in line 152 “ Aldh1a2…was more strongly expressed” is not valid. The data at earlier time points are more convincing (figure 2). Does this mean Aldh1a2 expression returns to normal levels at late stages of development?
>> No, Aldh1a2 levels do not return to normal levels at late stages of development. This can be clearly seen in our analysis of E15.5 + 6 days explant cultures where the layer of Aldh1a2/ALDH1A2-positive cells is clearly thickened (Fig. S7).
The statement that “Aldh1a2, a marker for fibroblasts of the inner lamina propria, was more strongly expressed in the mutant..“ was linked to the first part of the sentence before which read „Considering the dilatation of the ureter...“ Unfortunately, we put a point behind the first sentence which made this relation unclear. To avoid this ambiguity, we now write:
„Considering the dilatation of the ureter, markers of this differentiated cell type appeared either unchanged (ACTA2, TAGLN, Myocd, Myh11), weakly (Tnnt2, Tagln, Actg2) or strongly (Ckm) reduced in their expression (Fig. 1E,F). Expression of Aldh1a2, a marker for fibrocytes of the inner lamina propria, appeared increased in the mutant, whereas Col1a2, a marker for outer adventitial fibrocytes, was unchanged (Fig. 1F), again taken the ureter dilatation into account. <<

Second decision letter

MS ID#: DEVELOP/2022/200767

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AUTHORS: Lena Deuper, Max Meuser, Hauke Thiesler, Ulrich Walter Heinrich Jany, Carsten Rudat, Herbert Hildebrandt, Mark-Oliver Trowe, and Andreas Kispert
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

The advances of the revised paper to the field of developmental biology of the urogenital system remain the same as in the original submission. In addition, they are further strengthened by the few additional experiments that have been added to the revised manuscript and support the original statements and interpretations.

Comments for the author

The authors have addressed all of the concerns with their revision. There are no additional concerns.

Reviewer 2

Advance summary and potential significance to field

The present paper demonstrates that the two FGF receptors FGFR1 and FGFR2 are required within the ureteric mesenchyme to act as a molecular sink for FGFs.
Deletion of FGFR1/2 leads to increased signaling within the UE compartment which in turn leads to a delay in mesenchymal cell differentiation, reduced peristaltic contractions and hydroureter development in mutant mice. The study thus further underlines the importance of precise regulation of the FGF pathway to ensure normal ureter development.

Comments for the author

The authors have addressed all my concerns and the additional data in the revised version support their claims. The more careful phrasing of the conclusions is highly appreciated.