nature portfolio

Peer Review File

Title

Generation of human-pig chimeric renal organoids using iPSC technology

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Fujimori et al outlines their progress in developing human-pig chimeric organoids. Their hypothesis is that it will be possible to develop a chimeric kidney, derived from both pig and human progenitors and that this organ will be both functional and provide less of a rejection barrier than porcine organs for clinical xenotransplantation. The first step in this ambitious objective is the development of porcine-human renal organoids derived from foetal pig kidneys and human iPSC that have been induced to form nephron progenitor cells.

The main focus of this manuscript is to describe, in a systematic way, the steps they have taken to produce a human-porcine renal organoid. They state that they are the first group to achieve this milestone. The major steps in this process were:

• They lent heavily on their published work developing a human-mouse organoid

• Different media appears to have different influences on the proportion of chimerism and the development of renal organoid formation

• Wnt and FGF9 signalling appears to be important as the optimal results were obtained when CHIR99021 and FGF9 were added to the media

• Not only chimerism was achieved but structural components such as glomeruli and tubules developed in a somewhat co-ordinated manner

• With further modification the investigators were successful in developing human-pig chimeric organoids with the presence of tubular structures and to a lesser extent glomeruli.

• They also confirm that human-pig chimeras can develop from human NPC that have been injected into the sub-capsular space of porcine foetal kidneys.

Whilst these finding are or interest, and essential, if chimeric kidneys are to be developed there are a few points if highlighted would provide more guidance to the reader:

• Whist the description of the steps taken, describing the different culture steps, is clear in the Results section. It is clear from reading the Methods that underneath this description there is a lot of complexity in developing iPSC and the organoids. It would help the reader considerably if there was a chart outlining the time lines and how these individual components intersect. This could be included as a supplementary figure.

• The authors state that WNT and FGF9 signalling are essential in the development chimeric renal organoids. This is based on the findings of adding CHIR99021 and FGF9 to the media. It would be interesting to use other techniques to show how this affects cell signalling.

• Whilst I agree that the authors have demonstrated that they can produce human-porcine renal organoids, there are several caveats to this statement. Certainly this is done with far less efficiency than for those obtained in a mouse system. Also as the authors point out glomeruli were present in very few numbers. Also it would appear that the proportion of human NPC

present in mouse foetal kidneys diminish over time (see Figures 5b and c). This diminution has occurred in only a 4 day window.

There were other issues easily address:

In supplementary Figure 1c the authors state that they show human-mouse chimeric organoids as well as foetal mouse and human NPC organoids. However, only the human-mouse chimeric organoid is shown.

The discussion section would benefit from the addition of a paragraph highlighting the limitations of the study. Also there should be a paragraph stating the next steps and what are the remaining challenges in the development of a fully function chimeric organ. These would include the differences in maturation between the pig and human foetus, the steps from organoid development to a differentiated organ and how to determine if the development of such an organ will provide meaningful survival benefits over a genetically modified pig organ.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors generate human-pig chimeric renal organoids using iPSC technology, The paper is well-structured, logically-presented and can be of interest to a wide range of journal readers. I simply have a few minor concerns.

Question 1 ：What is the magnification of the image below ab in Fig 2? The images that involve enlarging are blurry, including the one below Fig 3d.

Question 2 ：The KU80 stain appears as a strip in Figure 3d. Is this typical?

Question 3 ：The images of organoids in Fig 1b and Fig 3c show that the central part is black. Whether the whole organoid is active, how to ensure the activity of the cells in the middle of the organoid?

Question 4: Whether chimeric organoids produced in this way have a function, such as reabsorption or filtration? The additional experiments to identify the chimeric organoids' function will make the manuscript better.

Reviewer #3 (Remarks to the Author):

This study tested several medium compositions to differentiate mouse, human, and pig nephron progenitor cells into kidney organoids. The culture testing was conducted over a relatively short period of 7 days. Starting with two different medium conditions for the reaggregation phase, three different conditions were tested for the subsequent maturation stage. Thus, a total of six culture conditions were tested. Briefly, the optimal condition was low CHIR and FGF9 for reaggregation, and a short period of low CHIR and FGF9 for the maturation step. This condition has been previously published in a study on human kidney organoid differentiation from pluripotent stem cells (PMID: 26458176). Therefore, the present work is consistent with prior studies.

While the major finding was to validate prior work, the authors could have better clarified the significance and novelty of the present study. The chimeric approach with mouse and pig cells is interesting. However, the advancements based on the above testing are not entirely clear. While the authors emphasize the potential of human-pig chimeric kidney generation in the introduction, the final figure shows the implantation of human cells into mice, not pigs. Overall, the clarity of writing is somewhat low, making it difficult to fully appreciate the significance of this study. The following are my suggestions for improvement:

1. Figure 1b does not provide a quantitative assessment. Additional quantitative results would strengthen this conclusion.

2. Perhaps the major challenge the authors faced is the lack of significant improvement in the culture conditions to generate kidney organoids. Previous studies have already demonstrated the crucial roles of WNT and FGF9 for the reaggregation step and further differentiation. The authors may want to develop new ideas to show progress in the generation of human-pig chimeric kidneys. Maybe the creation of pig kidney organoids is novel? The introduction can be significantly improved by clarifying the significance of the research topics directly related to their results, in depth.

3. While both human and pig cells are present in the organoids, it is unclear if these cells formed chimeric glomeruli and tubules. The provided images show distinct clusters of human or pig cells in Figure 4. For example, in Figure 4c, human cells do not express ECAD, while adjacent pig cells do express ECAD. It is, thus, unclear if human and pig cells created nephron structures together. Separate channel images may help with clarity?

4. Figures 3c and 3d also need quantitative assessment with statistical analysis.

5. Figure 5 does not seem to add significant value to this work. The formed tubules are few, and glomeruli seem absent.

6. The long-term stability and maturity of kidney organoids are key topics in this research field. Additional experiments to address these aspects may enhance the novelty of this paper.

Point-by-Point Responses to reviewers' comments

We thank the reviewers for evaluating our manuscript (COMMSBIO-24-2361) entitled

"**Generation of human-pig chimeric renal organoids using iPSC technology**".

We found the insightful comments made by the reviewers to be very helpful, and we addressed all the issues raised. Below, the reviewers' comments are presented in bold, and the changes made to the revised manuscript are indicated in blue italics.

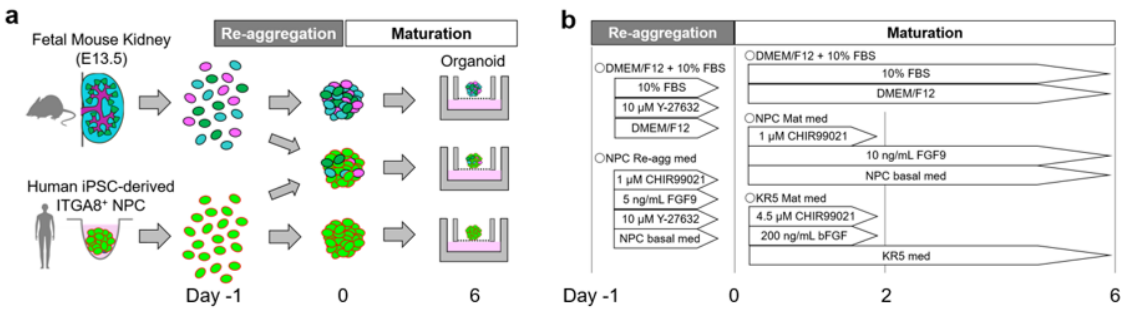
We hope that you will find the new data and revised manuscript suitable for publication in *Communications Biology*.

Response to the comments by Reviewer #1:

1) Whist the description of the steps taken, describing the different culture steps, is clear in the Results section. It is clear from reading the Methods that underneath this description there is a lot of complexity in developing iPSC and the organoids. It would help the reader considerably if there was a chart outlining the time lines and how these individual components intersect. This could be included as a supplementary figure.

Thank you for your comments. In response, we have made the article more reader-friendly by presenting the scheme of the organoid fabrication process and the incorporation of additives at each step in a divided manner. Furthermore, we have included a time course for better clarity and understanding.

Fig. 1



(Figure legends) (Page 37, Line 749-752)

Fig. 1 Screening of culture conditions using human-mouse chimeric renal organoids.

a. *Experimental scheme for the generation and examination of chimeric renal organoids.*

b. *Experimental scheme depicting the tested culture conditions and time course for chimeric renal organoid fabrication.*

2) The authors state that WNT and FGF9 signalling are essential in the development chimeric renal organoids. This is based on the findings of adding CHIR99021 and FGF9 to the media. It would be interesting to use other techniques to show how this affects cell signalling.

Thank you for your valuable comments. Indeed, investigating the role of Wnt and FGF9 signaling in kidney development using alternative methods is intriguing. However, it is important to note that multiple research groups have already clearly demonstrated the significance of both signals in kidney development. The relevant part in the manuscript is indicated below.

On the other hand, although the mechanisms of action and the developmental stages affected by these signals were not explicitly described in the manuscript, we have added the following to the Discussion section to address this concern.

(Discussion) (Page 15, Line 293-302)

In particular, a significant improvement in the human cell ratio within chimera organoids was observed when NPC_Re-agg, containing CHIR99021 and FGF9, was used as a re-aggregation culture medium (Fig. 2e). Taguchi et al. demonstrated that the addition of low-concentration CHIR99021 and FGF9 is crucial in inducing differentiation from posterior intermediate mesoderm to metanephric nephron progenitors using human iPSCs[28]. FGF9 has been reported to contribute to the maintenance and proliferation of human NPCs[30]. Previous studies on renal development using rodents have also reported that the formation of the metanephric mesenchyme critically relies on the FGF receptors, while the maintenance of the metanephric mesenchyme necessitates FGF9/FGF20[30,31]. Therefore, it is believed that the FGF9 signaling pathway significantly contributes to the survival and development of not only mouse renal cells but also human NPCs in chimera organoids.

3) Whilst I agree that the authors have demonstrated that they can produce human-porcine renal organoids, there are several caveats to this statement. Certainly this is done with far less efficiency than for those obtained in a mouse system. Also as the authors point out glomeruli were present in very few numbers. Also it would appear that the proportion of human NPC present in mouse foetal kidneys diminish over time (see Figures 5b and c). This diminution has occurred in only a 4 day window.

Thank you for your comments. We apologize for any confusion caused. To clarify, we first have added the culture conditions applied to each experimental scheme, and have made revisions to the main text.

As you correctly pointed out, our previous findings demonstrated a decrease in the number of human cells in the human-mouse chimeric renal organoids over the culture period with conventional culture conditions (Supplementary Fig. 1b-d). However, when the culture conditions were optimized, specifically, the combination of NPC_Re-agg and NPC_Mat, we observed a significant improvement in suppressing the decrease of human cells in the human-mouse chimeric renal organoids (Fig. 2a-e). Likewise, in Figure 5, applying a similar culture condition, we observed a suppression of the decrease and even an increase in the number of human cells (correctly, human “NPCs”) injected into the subcapsular region of the mouse kidney. To clearly demonstrate the increase of human NPC in the mouse renal subcapsular space, we conducted additional experiments, quantitative analysis, and statistical analysis (Fig. 5d).

Furthermore, the selected culture conditions showed excellent rates of chimera formation in both human-pig and human-mouse chimeric kidney organoid models (Fig. 4f), and clear chimera formation in the human NPC-injected mouse kidney model (Fig. 5e, f).

Based on these results, we have successfully addressed the limitations of previous studies in the field of heterogeneous chimeric kidney/organoid models, including the decrease in human cell numbers and inadequate chimera formation, with the culture conditions discovered in this study.

Fig. 1

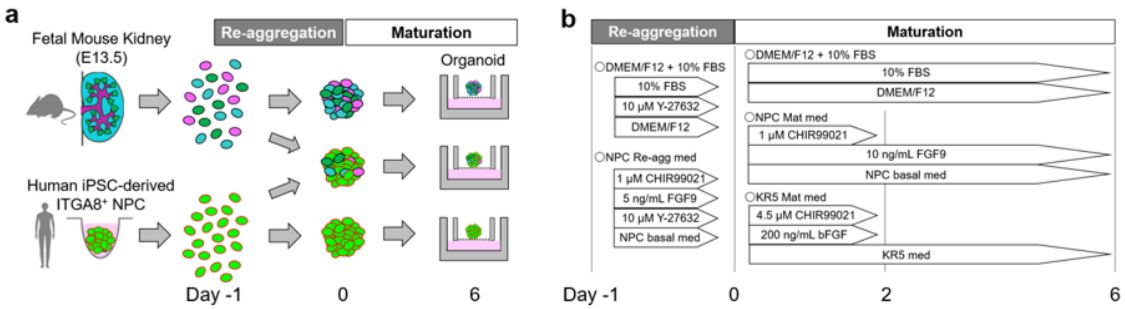
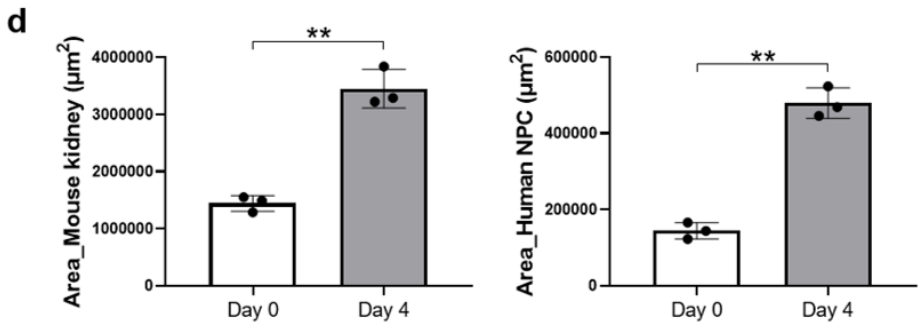


Fig. 5



(Figure legends) (Page 37, Line 749-752 and Page 40, Line 819-820)

Fig. 1 Screening of culture conditions using human-mouse chimeric renal organoids.

a. *Experimental scheme for the generation and examination of chimeric renal organoids.*

b. *Experimental scheme depicting the tested culture conditions and time course for chimeric renal organoid fabrication.*

Fig. 5 Evaluation of human NPC-injected fetal mouse kidney using specific culture conditions.

d*. Quantitative analysis of the area of human NPC-injected fetal mouse kidneys and the injected human NPC (n= 3 independent experiments; mean ± s.d.; **P< 0.01; two-tailed t-test).*

(Results) (Page 6, Line 106-115)

Therefore, we further explored culture conditions that would enable the survival, differentiation, and maturation of three organoid models: human-animal chimeric renal organoids and their constituent species-specific renal organoids (e.g., human renal organoids and mouse or pig renal organoids). We initiated an investigation into the culture conditions using human-mouse chimeric renal organoids as a starting point (Fig. 1a, b). During the formation of chimeric renal organoids, the reaggregation medium and maturation medium have conventionally been FBS-supplemented DMEM/F12[20]. In this study, we selected previously employed media for differentiation and maturation of NPCs, specifically "NPC_Re-agg" medium for the reaggregation medium, and "NPC_Mat" medium and "KR5_Mat" medium[15,16] for the maturation medium[26–29], and conducted a comparative investigation (Fig. 1a, b).

(Results) (Page 12, Line 253-Page 13, Line 258)

Microscopic examination of the injected fetal mouse kidneys immediately after injection revealed that the morphology of the recipient kidneys remained intact, and the amount of EGFP-labeled human NPCs injected beneath the renal capsule surface was observed (Fig. 5b). By the fourth day of culture, it was quantitatively confirmed that the recipient fetal mouse kidneys had increased in size, and the human NPCs injected beneath their capsule also exhibited proportional enlargement (Fig. 5c, d).

(Methods) (Page 26, Line 535-548)

Area-based cell composition analysis

Immunostained sections of frozen organoids were captured using a fluorescence microscope. Using the IN Cell Developer Toolbox (Cytiva), the fluorescence intensity of the organoid slice images was used to calculate the positive areas of ECAD, WT1, CK8, Ku80, and DAPI. The positive area of each marker was divided by the DAPI-positive area to calculate the proportion of each constituent cell type within the organoid. For cell composition analysis, three sections from the central slice of the organoid specimen and two additional sections located 50 μm apart from anterior and posterior directions were used.

The fetal mouse kidneys injected with human NPCs expressing EGFP were subjected to whole-tissue imaging using the EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.) to acquire brightfield and fluorescence images. The image analysis software, ImageJ, was utilized to quantitatively determine the total area of the fetal kidneys in the brightfield images, as well as to quantify the regions of EGFP-positive human NPC in the fluorescence images.

All analyses were conducted through three independent experiments.

4) In supplementary Figure 1c the authors state that they show human-mouse chimeric organoids as well as foetal mouse and human NPC organoids. However, only the human-mouse chimeric organoid is shown.

I appreciate your pointing out the mistake in our writing. Based on your comment, we have made the following corrections.

(Supplementary Figure legends)

Supplementary Figure 1. Evaluation of human-mouse chimeric renal organoids generated using existing protocols.

c. Immunostaining images of human-mouse chimeric renal organoids generated using existing protocols. Scale bar represents 200 μm.

5) The discussion section would benefit from the addition of a paragraph highlighting the limitations of the study. Also there should be a paragraph stating the next steps and what are the remaining challenges in the development of a fully function chimeric organ. These would include the differences in maturation between the pig and human foetus, the steps from organoid development to a differentiated organ and how to determine if the development of such an organ will provide meaningful survival benefits over a genetically modified pig organ.

Thank you for your feedback. As you correctly pointed out, we have added the challenges of this study in the Discussion section. The optimization of culture conditions during the early stages of kidney development has been demonstrated to overcome interspecies differences, as shown by the results of this study. However, long-term maturation and acquisition of renal function have not been achieved, and we have added the following clarification on this point. Additionally, we have included a mention of the future consideration of conducting chimera kidney culture experiments using a human-pig combination.

Furthermore, we have also added a statement highlighting the immunological advantages of humanized or human-replaced pig organs in clinical settings compared to current xenotransplantation.

(Discussion) (Page 17, Line 354-Page 18, Line 384)

While a similar investigation using the human-pig combination has not been conducted in this study, these findings could serve as a pivotal milestone on the pathway towards the future generation of xenogeneic chimera kidneys with potential clinical immunological advantages compared to genetically edited xeno-kidneys that still retain inherent risks of immune rejection.

There are still remaining challenges to fully exploiting the potential of our human-xenogeneic chimeric renal models to acquire renal function. Long-term maturation of the chimeric renal organoid and the human NPC-injected chimeric kidney necessitates sufficient nutrient and oxygen supply to the internal structure; furthermore, from a physical standpoint, blood inflow to the glomeruli is necessary for them to acquire filtration capacity. In other words, we believe that transplantation into the animal body and vascular invasion are essential for functional acquisition in both renal models[42]. In fact, in our previous study, we confirmed the feasibility of urine production

through the transplantation of mouse-rat chimeric kidney models created by injecting fetal rat renal cells into fetal mouse kidneys with endogenous NPCs that can be eliminated through drug induction, and transplanting the chimeric kidneys into immunodeficient adult mice[16].

However, despite our recent attempts to transplant human-mouse chimeric renal organoids into the subcapsular region of host immunodeficient mice, differentiation and maturation of the grafts pose challenges to the full realization of our chimeric renal organoids. Sharmin et al. have demonstrated that even human NPCs, when transplanted into the subcapsular region of mouse kidneys, can form functional glomeruli with Bowman's space. They co-cultured human NPCs with mouse embryonic spinal cord to promote differentiation and maturation, followed by transplantation of these two components together. Furthermore, they co-transplanted agarose rods soaked in VEGF solution to induce host vascularization[43]. The implementation of such techniques for post-transplantation renal development and host vascularization promotion may offer valuable solutions to the problem of generating chimeric renal organoid that are functional. Another suitable approach may involve transplanting chimeric renal organoids with vascular networks obtained through pre-cultivation in artificially scaffolded environments such as microphysiological systems utilizing fluidic dynamics.

Similar technical challenges are anticipated for the acquisition of renal function in xenogeneic kidney models with human NPC injection. In particular, the introduction of the system for removing endogenous NPCs in fetal kidneys, as mentioned earlier, would be a crucial element to expedite research towards the creation of functional humanized xenogeneic kidneys.

Response to the comments by Reviewer #2:

1) What is the magnification of the image below ab in Fig 2? The images that involve enlarging are blurry, including the one below Fig 3d.

Thank you for your comment. In response to your comment, we have replaced the enlarged images you mentioned with higher resolution versions. Additionally, we have included a scale bar. These modifications have been made to ensure clarity and accuracy.

Fig. 2

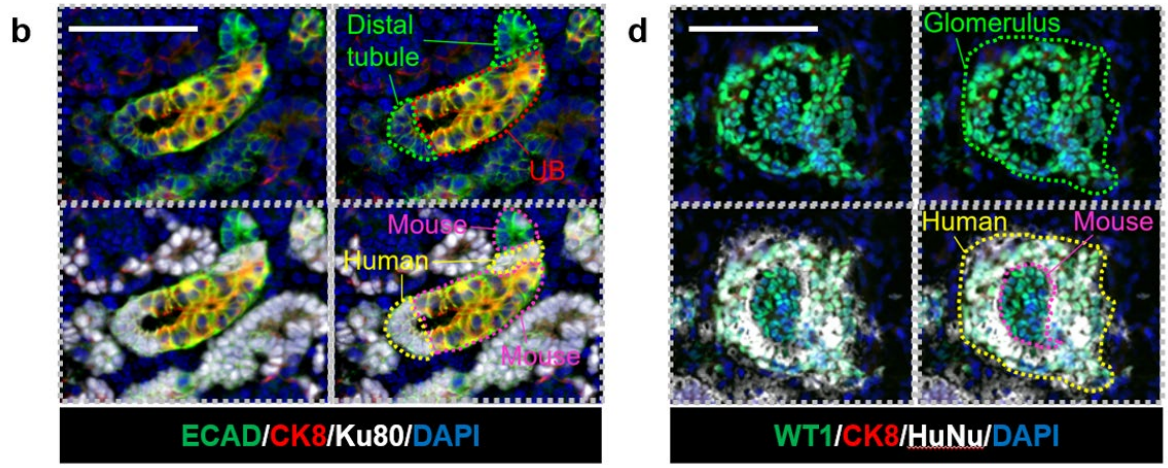
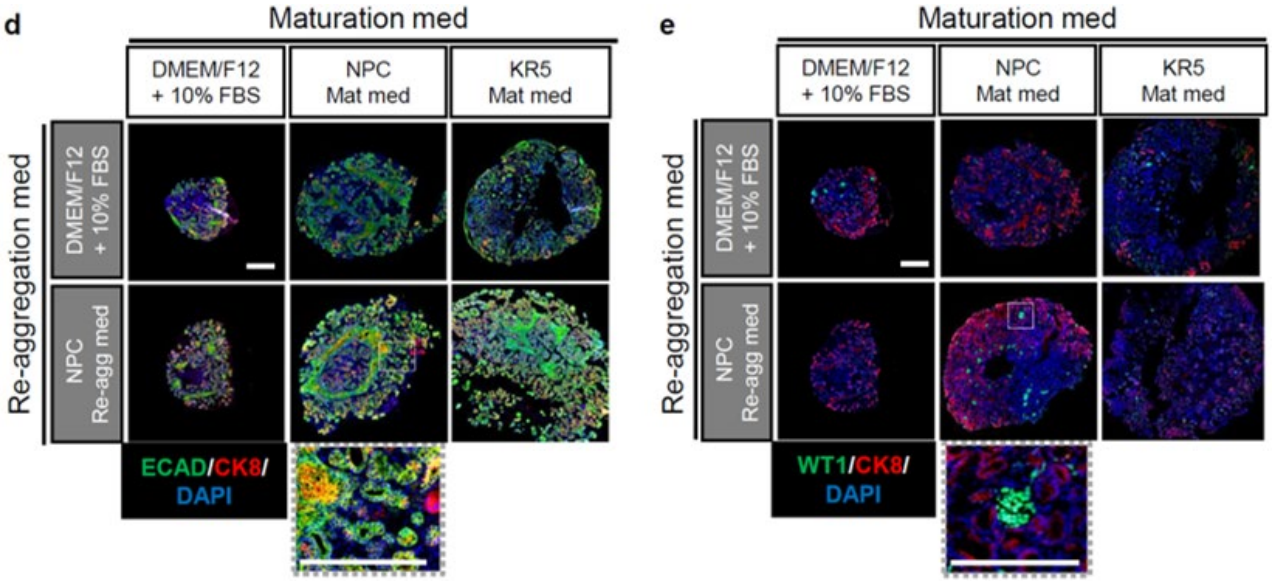


Fig. 3



(Figure legends) (Page 37, Line 758-765 and Page 38, Line 784-787)

Fig. 2 Evaluation of human-mouse chimeric renal organoids formed under different culture conditions.

a, b*. Immunostaining images of chimeric renal organoids at Day 6 that were stained using antibody against a distal tubule marker, a ureteric bud marker, and a human cell marker. Scale bars represent 200 μm (**a**: original images) and 100μm (**b**: magnified images).*

c, d*. Immunostaining images of chimeric renal organoids at Day 6 that were stained using antibody against a glomerulus marker, a ureteric bud marker, and a human cell marker. Scale bars represent 200 μm (**c**: original images) and 100 μm (**d**: magnified images).*

Fig. 3 Generation and evaluation of fetal pig kidney organoids.

d*. Immunostaining images of pig kidney organoids at Day 6 that were stained by antibody against a distal tubule marker and a ureteric bud marker. Scale bar represents 200 μm.*

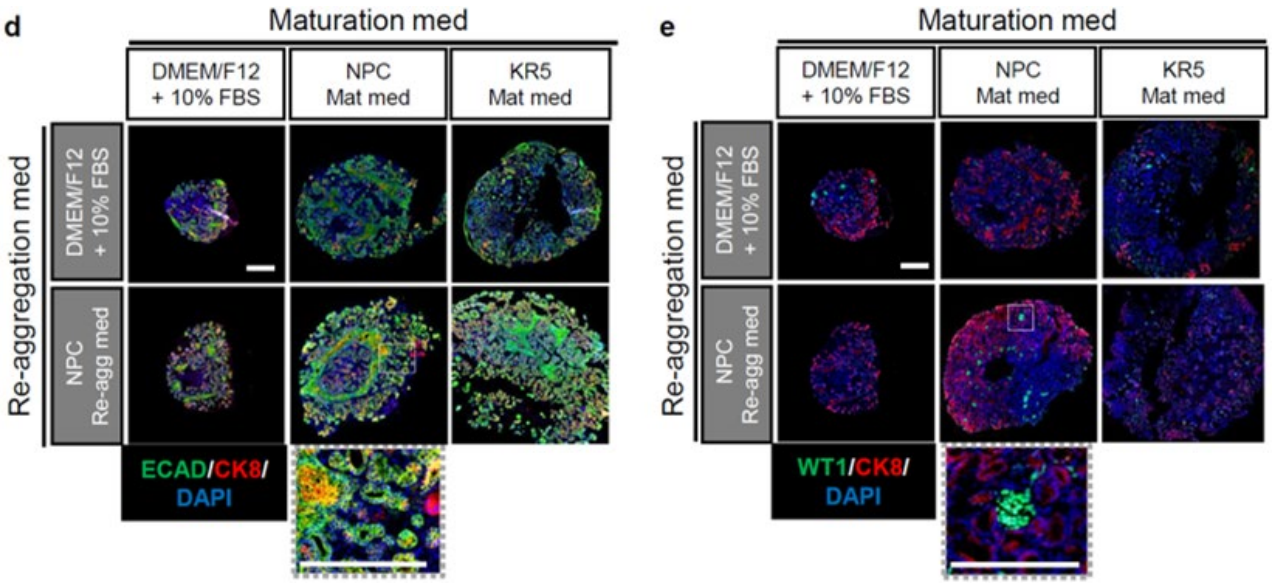
e*. Immunostaining images of pig kidney organoids at Day 6 that were stained by antibody against a glomerular marker and a ureteric bud marker. Scale bar represents 200 μm.*

2) The KU80 stain appears as a strip in Figure 3d. Is this typical?

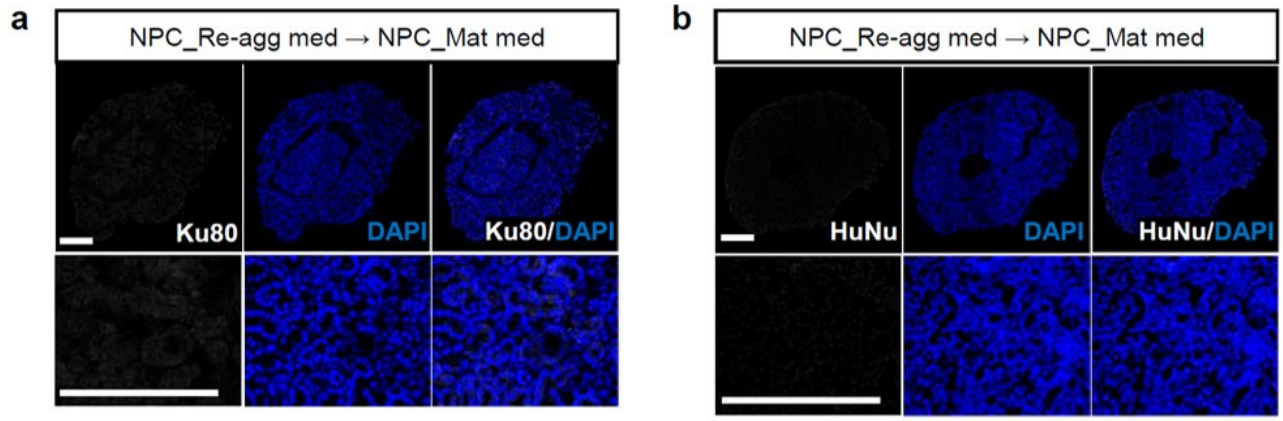
We appreciate your comment. The "strip" you mentioned is not a Ku80-positive signal but rather a false positive artifact that appears as a white strip due to folding during the sample sectioning process. We have replaced this image with a more suitable one.

We believe that demonstrating the Ku80 negativity of the porcine renal organoid is not the main focus of Figure 3d, and it could potentially lead to misunderstandings. Therefore, we have moved only the Ku80 staining image to Supplementary Figure 3 and revised the Results section as below.

Fig. 3



Supplementary Figure 3



(Supplementary Figure legends)

Supplementary Figure 3. Evaluation of fetal pig kidney organoids generated using identified culture conditions.

a, b*. Immunostaining images of pig kidney organoids at Day 6 that were stained by antibody against human cell markers, Ku80 (**a**) and HuNu (**b**). Scale bar represents 200 μm.*

(Results) (Page 10, Line 190-193)

However, all porcine fetal kidney organoids were confirmed to possess kidney structures positive for WT1, ECAD, and CK8 (Fig. 3d, e), while they were negative for human cell markers, Ku80 and HuNu (Supplementary Fig. 3a, b).

3) The images of organoids in Fig 1b and Fig 3c show that the central part is black. Whether the whole organoid is active, how to ensure the activity of the cells in the middle of the organoid?

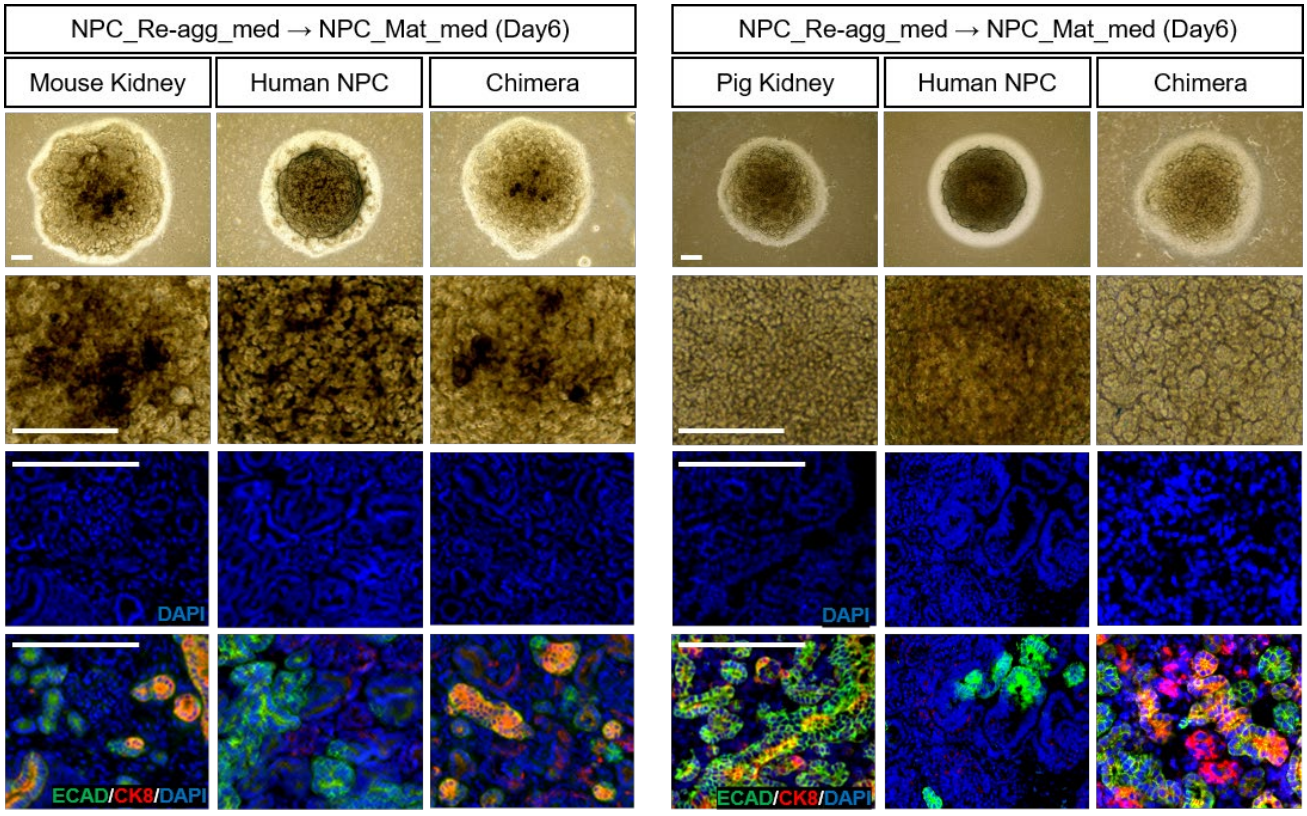
Thank you for your comment. It is indeed true that larger organoids tend to have sparser central regions if they lack blood vessels/flow, leading to insufficient supply of nutrients and oxygen.

However, our organoids are not as large in size and do not undergo long-term culturing, so we believe there are no issues regarding nutrient and oxygen supply even with liquid-air interface culture. Our samples assume a semi-spherical shape, causing reduced light penetration in the central region of the organoid, which appears dark. Therefore, the black area in the center of our organoids is unrelated to a decrease in cell viability. This can be confirmed from the DAPI staining image, which indicates the presence of viable cells in the central region (Fig. 2a-d and Fig. 3d, e; also, see the following images).

Attached below are the sliced images of the central and middle portions of the organoids. It can be observed that, in addition to DAPI staining, various renal structures are abundantly present, supporting the assessment that the central area of the organoids exhibits sufficient activity.

(It should be noted that partial loss in the central region of the organoid can occasionally be observed due to factors such as bubble entrapment or ice crystal formation during freeze embedding of the samples.)

Images of the central and middle portions of the organoids



4) Whether chimeric organoids produced in this way have a function, such as reabsorption or filtration? The additional experiments to identify the chimeric organoids' function will make the manuscript better.

We have provided evidence of a certain level of development and maturation of human-pig chimeric renal organoids through additional experiments, primarily by immunostaining for various developmental markers expressed in each nephron segment (Fig. 4d and Supplementary Fig. 4a, b).

The acquisition of renal function in organoids is a major challenge, even for our study. It has been observed that organoids cultured at the air-liquid interface reach a maturation plateau after approximately 10 days, beyond which cell survival becomes difficult. Although our study demonstrates the maturity of kidney organoids at Day 6, even if the culture period is extended up to 10 days, we are unable to produce functional organoids with reabsorption and filtration capabilities.

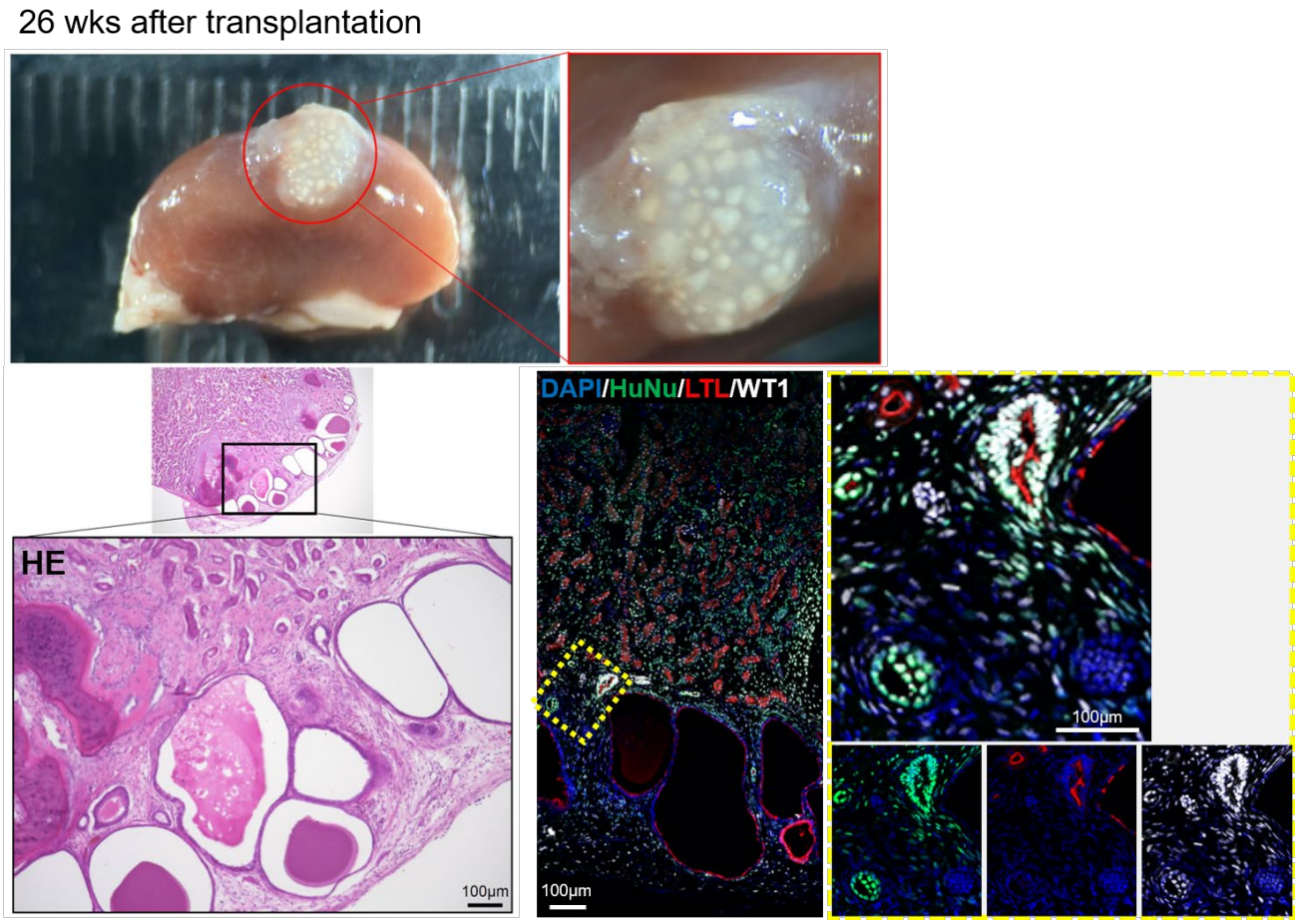
In organoid models without blood influx, nutrient supply and oxygen supply are insufficient, and in order for glomeruli to acquire filtration capabilities, blood influx to the glomeruli is necessary

also from a physical standpoint. In other words, we believe that transplantation into animal hosts and vascular infiltration are essential for functional acquisition of kidney organoids.

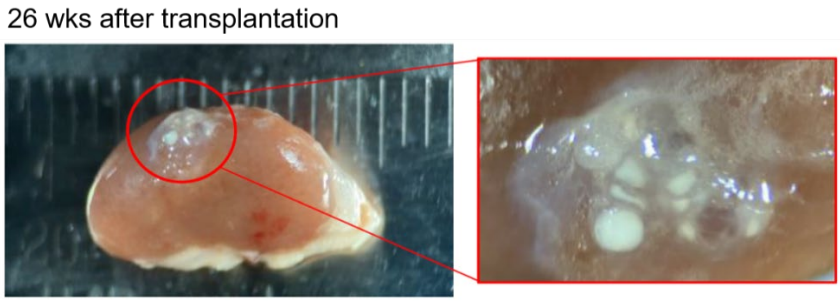
Therefore, we performed transplantation of human-mouse chimeric renal organoids into the subrenal capsule of immunodeficient mice. Even after 26 weeks of transplantation, the presence of HuNu-positive human cells was confirmed, but it was not observed that a significant number of chimera structures were formed. Furthermore, multiple white granular structures, likely representing unintended cells, were formed within the organoids, and the desired nephron structures such as glomeruli were only present in minimal quantities. It was also confirmed that the white granular structures are negative for HuNu.

A similar formation of multiple white granular structures was observed when the transplanted cells were changed to fetal mouse renal organoids using the same experimental design, suggesting that these unintended granules are derived from fetal mouse kidneys.

<u>Human-mouse chimeric renal organoids transplantation</u> into the subrenal capsule of immunodeficient mice

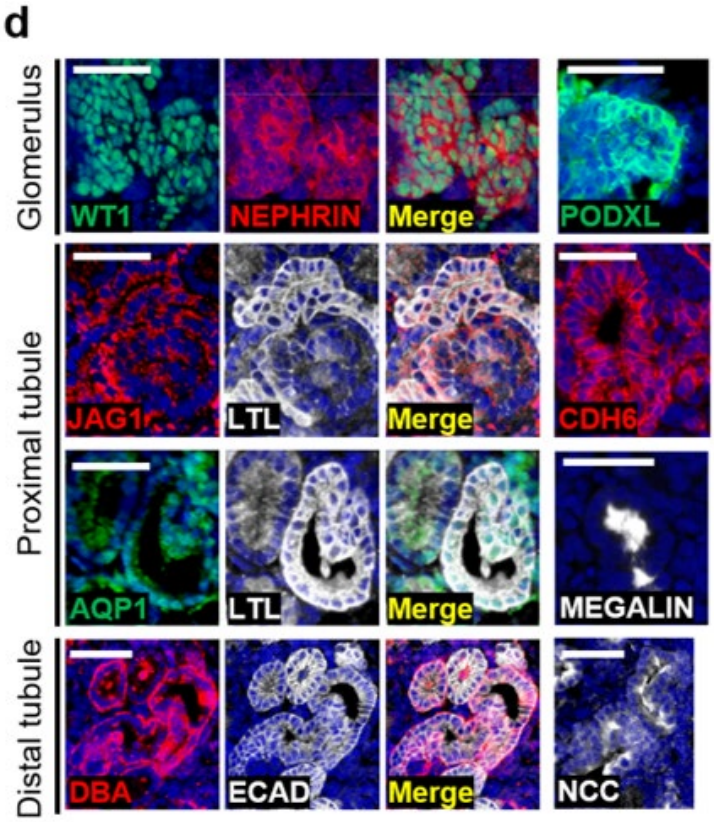


<u>Fetal mouse renal organoids transplantation</u> into the subrenal capsule of immunodeficient mice



Based on these results, we consider further maturation and functional acquisition of chimeric renal organoids as the next challenge. We have added the following statement to the Discussion section of our paper.

Fig. 4

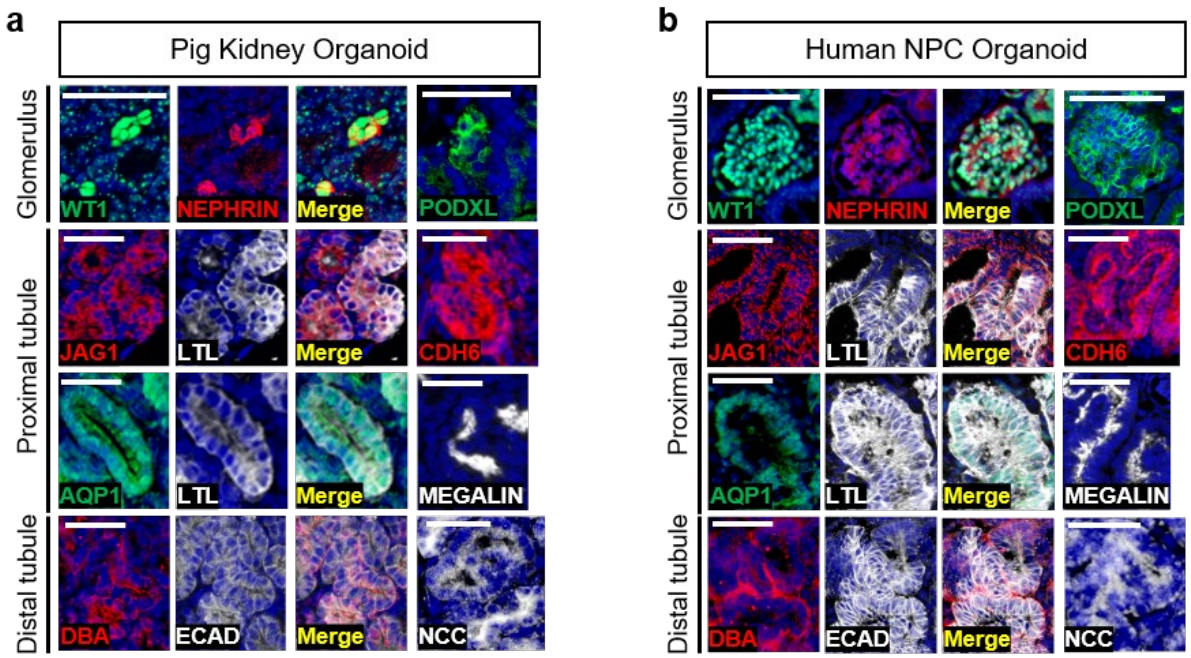


(Figure legends) (Page 39, Line 799-800)

Fig. 4 Generation and evaluation of human-pig chimeric renal organoids.

d*. Images of human(3)-pig(1) chimeric renal organoids immunostained by antibodies at various developmental stages of each nephron segment. Scale bars represent 100 μm.*

Supplementary Figure 4



(Supplementary Figure legends)

Supplementary Figure 4. Developmental evaluation of fetal pig kidney organoids and human NPC organoids cultured for 6 days under the identified culture conditions.

a, b. Immunostaining images of fetal pig kidney organoids (a) and human NPC organoids (b) stained by antibodies at various developmental stages of each nephron segment. Scale bar represents 100 μm.

(Results) (Page 11, Line 222-230)

In terms of both human cell inclusion rate and chimeric structure formation rate, immunostaining analysis of chimeric renal organoids at the optimal human-pig mixing ratio of 3:1 revealed the expression of various glomerular markers, proximal tubule markers, and distal tubule markers, indicating the presence of late developmental stage in the generated chimeric renal organoids (Fig. 4d). Similar staining patterns were observed in fetal pig kidney organoids and human NPC-derived organoids cultured under the same conditions (Supplementary Fig. 4a, b). These results suggest that the selected culture conditions facilitate the coexistence and coincident development of human and pig renal cells, leading to the induction of certain levels of differentiation and maturation in the organoids.

(Discussion) (Page 17, Line 359-Page 18, Line 384)

There are still remaining challenges to fully exploiting the potential of our human-xenogeneic chimeric renal models to acquire renal function. Long-term maturation of the chimeric renal organoid and the human NPC-injected chimeric kidney necessitates sufficient nutrient and oxygen supply to the internal structure; furthermore, from a physical standpoint, blood inflow to the glomeruli is necessary for them to acquire filtration capacity. In other words, we believe that transplantation into the animal body and vascular invasion are essential for functional acquisition in

both renal models[42]. In fact, in our previous study, we confirmed the feasibility of urine production through the transplantation of mouse-rat chimeric kidney models created by injecting fetal rat renal cells into fetal mouse kidneys with endogenous NPCs that can be eliminated through drug induction, and transplanting the chimeric kidneys into immunodeficient adult mice[16].

However, despite our recent attempts to transplant human-mouse chimeric renal organoids into the subcapsular region of host immunodeficient mice, differentiation and maturation of the grafts pose challenges to the full realization of our chimeric renal organoids. Sharmin et al. have demonstrated that even human NPCs, when transplanted into the subcapsular region of mouse kidneys, can form functional glomeruli with Bowman's space. They co-cultured human NPCs with mouse embryonic spinal cord to promote differentiation and maturation, followed by transplantation of these two components together. Furthermore, they co-transplanted agarose rods soaked in VEGF solution to induce host vascularization[43]. The implementation of such techniques for post-transplantation renal development and host vascularization promotion may offer valuable solutions to the problem of generating chimeric renal organoid that are functional. Another suitable approach may involve transplanting chimeric renal organoids with vascular networks obtained through pre-cultivation in artificially scaffolded environments such as microphysiological systems utilizing fluidic dynamics.

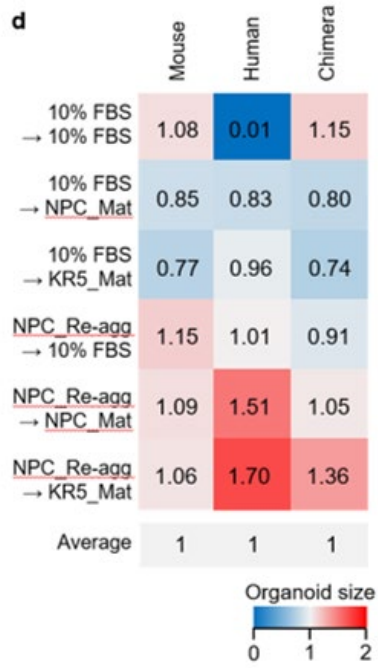
Similar technical challenges are anticipated for the acquisition of renal function in xenogeneic kidney models with human NPC injection. In particular, the introduction of the system for removing endogenous NPCs in fetal kidneys, as mentioned earlier, would be a crucial element to expedite research towards the creation of functional humanized xenogeneic kidneys.

Response to the comments by Reviewer #3:

1) Figure 1b does not provide a quantitative assessment. Additional quantitative results would strengthen this conclusion.

We appreciate your feedback. We have added quantitative analysis data of the organoid area under each condition, and accordingly, we have modified the text as shown below.

Fig. 1



(Figure legends) (Page 37, Line 754-756)

Fig. 1 Screening of culture conditions using human-mouse chimeric renal organoids.

d*. Quantitative analysis of the sizes of fetal mouse kidney organoids, human NPC organoids, and chimeric renal organoids composed of human NPC and fetal mouse kidney, under various culture conditions.*

(Results) (Page 7, Line 122-124)

From the morphological analysis, it was quantitatively confirmed that all three organoid models exhibited larger organoid sizes when NPC_Re-agg was used as a reaggregation medium, followed by NPC_Mat or KR5_Mat as a maturation medium (Fig. 1d).

(Methods) (Page 26, Line 550-Page 27, Line 553)

Morphological analysis of organoids

The brightfield images of the organoids were acquired using the EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.). The ImageJ analysis software was utilized to quantitatively determine the area of the organoids in the brightfield images.

2) Perhaps the major challenge the authors faced is the lack of significant improvement in the culture conditions to generate kidney organoids. Previous studies have already demonstrated the crucial roles of WNT and FGF9 for the reaggregation step and further differentiation. The authors may want to develop new ideas to show progress in the generation of human-pig chimeric kidneys. Maybe the creation of pig kidney organoids is novel? The introduction can be significantly improved by clarifying the significance of the research topics directly related to their results, in depth.

Thank you for your valuable feedback. As you mentioned, the importance of Wnt and FGF9 in renal development has already been reported. In response, we have further supplemented the Discussion section by citing additional relevant studies.

(Discussion) (Page 15, Line 293-300)

In particular, a significant improvement in the human cell ratio within chimera organoids was observed when NPC_Re-agg, containing CHIR99021 and FGF9, was used as a re-aggregation culture medium (Fig. 2e). Taguchi et al. demonstrated that the addition of low-concentration CHIR99021 and FGF9 is crucial in inducing differentiation from posterior intermediate mesoderm to metanephric nephron progenitors using human iPSCs[28]. FGF9 has been reported to contribute to the maintenance and proliferation of human NPCs[30]. Previous studies on renal development using rodents have also reported that the formation of the metanephric mesenchyme critically relies on the FGF receptors, while the maintenance of the metanephric mesenchyme necessitates FGF9/FGF20[30,31].

(References)

[28] A. Taguchi, Y. Kaku, T. Ohmori, S. Sharmin, M. Ogawa, H. Sasaki, R. Nishinakamura, Redefining the In Vivo Origin of Metanephric Nephron Progenitors Enables Generation of Complex Kidney Structures from Pluripotent Stem Cells, Cell Stem Cell 14 (2014) 53–67. https://doi.org/10.1016/j.stem.2013.11.010.

[30] H. Barak, S.-H. Huh, S. Chen, C. Jeanpierre, J. Martinovic, M. Parisot, C. Bole-Feysot, P. Nitschké, R. Salomon, C. Antignac, D.M. Ornitz, R. Kopan, FGF9 and FGF20 Maintain the Stemness of Nephron Progenitors in Mice and Man, Developmental Cell 22 (2012) 1191–1207. https://doi.org/10.1016/j.devcel.2012.04.018.

[31] D.P. Poladia, K. Kish, B. Kutay, D. Hains, H. Kegg, H. Zhao, C.M. Bates, Role of fibroblast growth factor receptors 1 and 2 in the metanephric mesenchyme, Developmental Biology 291 (2006) 325–339. https://doi.org/10.1016/j.ydbio.2005.12.034.

Regarding the novelty of porcine renal organoids, we reported the use of cryopreserved fetal porcine kidneys in a separate publication in April of this year (Int. J. Mol. Sci. 2024, 25(9), 4793; https://doi.org/10.3390/ijms25094793). However, at the time we posted this article on bioRxiv, our publication was already ahead, and furthermore, our group remains the only one successful in creating porcine renal organoids.

As mentioned earlier, although the importance of the culture conditions we discovered in human, mouse, and pig kidney development was already known, the fact that these culture conditions enable coexistence and co-development of human-mouse and human-pig chimeras beyond species barriers represents a significant scientific achievement. We believe that this breakthrough represents a major milestone in future research on interspecies chimera organogenesis.

To clearly emphasize the novelty and innovation in our research, we have made the following modifications to the manuscript.

(Abstract) (Page 2, Line 28-30)

This study provides evidence that optimizing culture conditions enables the early-stage kidney development beyond species barriers, thus laying the foundation for accelerating research on humanized xenogeneic kidney fabrication for clinical purposes.

(Introduction) (Page 3, Line 57-Page 4, Line 70)

As mentioned above, we have obtained evidence suggesting the future clinical utility and potential of the humanized xeno-kidney, combining human renal cells with embryonic kidneys from a different species. However, the chimerism rate and maturity of human cells are still insufficient, necessitating the identification of a specific environment that enables coexistence and co-development regardless of species. Therefore, as a new chimera model aimed at exploring culture conditions that promote the coexistence and co-development of cells from humans and other animal species, "chimeric renal organoids" were successfully generated by co-culturing dissociated fetal kidneys and human NPCs at the single-cell level[20].

However, with the current culture techniques, even the chimeric renal organoids combining human and mouse cells did not exhibit sufficient competency to coexist and co-develop. Furthermore, studies utilizing pluripotent stem cells from different species have suggested the presence of a barrier inhibiting interspecies chimera formation[21], along with reported variations in the rate of organogenesis among interspecies animals[22]. These suggest that there are remaining challenges in effectively achieving interspecies chimera formation.

(Introduction) (Page 4, Line 74-80)

Pigs are considered the most ideal organ xenograft donor, and have several advantages for chimeric kidney production due to their similar organ size, physiological functions resembling humans, rapid growth, and ease of organ procurement[23]. In xenotransplantation of pig organs, challenges in immune rejection continue to be addressed, and our ultimate goal is to overcome immunological barriers by creating humanized pig kidneys. Thus, the generation of chimeric renal organoids using human NPCs and porcine kidney cells represents a crucial step towards the future development of humanized pig kidney organogenesis for clinical application.

(Discussion) (Page 14, Line 270-275)

This study established a cultivation system enabling cross-species nephrogenesis in the early stages of kidney development by refining the existing culture conditions for human-mouse chimeric renal organoids. This optimized culture system allows for coexistence of both the animal and human cellular components at least during the early stage of nephrogenesis and facilitates more efficient interspecies chimera formation. Furthermore, the identified optimized culture conditions were found to be suitable for the formation of porcine fetal kidney organoids.

(Discussion) (Page 18, Line 385-Page 19, Line 389)

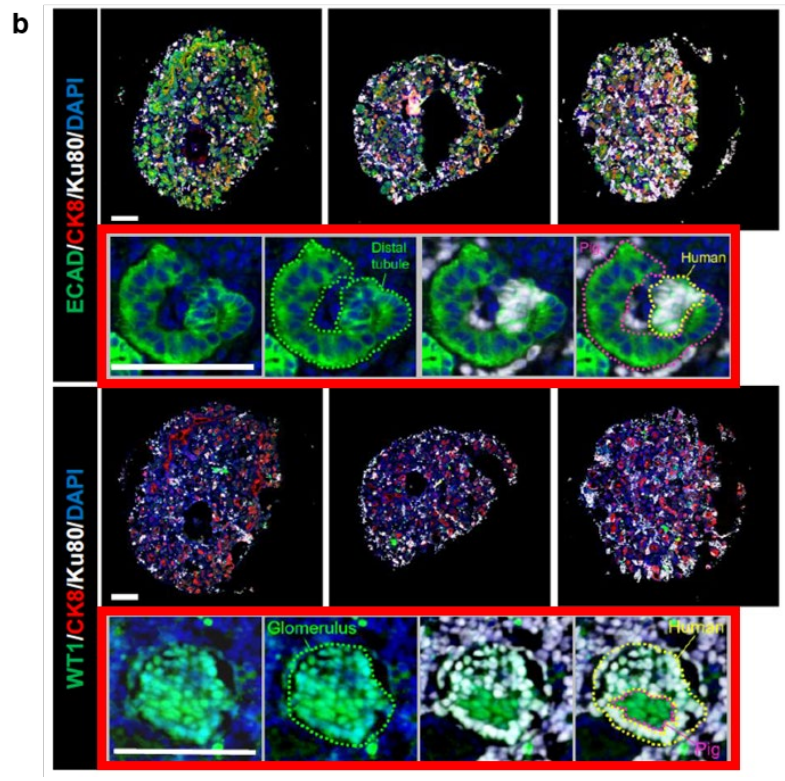
In summary, this study provides evidence showing that the cross-species renal development can be achieved by optimizing cultivation conditions, using human, mouse, and pig renal cells. While further advancement of renal development to acquire kidney function remains a future challenge, the established cultivation technique holds great potential for accelerating future research on humanized pig kidney generation, aimed at clinical applications.

3) While both human and pig cells are present in the organoids, it is unclear if these cells formed chimeric glomeruli and tubules. The provided images show distinct clusters of human or pig cells in Figure 4. For example, in Figure 4c, human cells do not express ECAD, while adjacent pig cells do express ECAD. It is, thus, unclear if human and pig cells created nephron structures together. Separate channel images may help with clarity?

We apologize for any misleading impression caused by the Figure 4 images, especially Figure 4c image, where the fluorescence intensity of ECAD staining in the human cell compartment was partially insufficient, giving the impression of non-expression of ECAD. However, in reality, the human cell compartment also exhibits ECAD positivity.

To ensure clarity for the readers and avoid any misinterpretation, we have replaced the Figure 4 images with a more appropriate one (highlighted by the red box in the lower panel).

Fig. 4



4) Figures 3c and 3d also need quantitative assessment with statistical analysis.

Thank you for your comments and suggestions. We have carefully reviewed our figures and understand that your comment likely refers to Fig 3d/e or Fig 4c/d.

In response to your concerns, regarding Figure 3, we have newly included quantitative analysis of the cellular composition ratios of each nephron segment in fetal pig kidney organoids formed under different culture conditions.

For Figure 4, we conducted additional experiments using human-pig chimeric renal organoids. We performed quantitative and statistical analyses on the obtained data.

Furthermore, we have made the necessary revisions to the manuscript accordingly.

Fig. 3

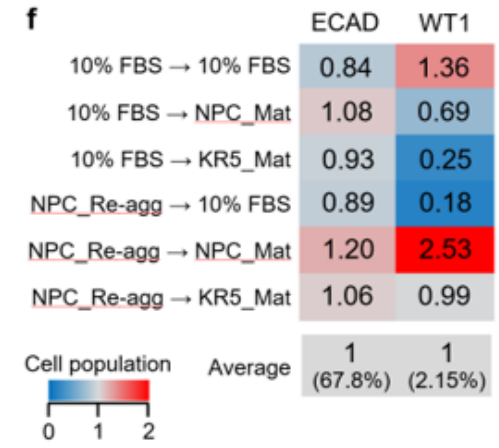
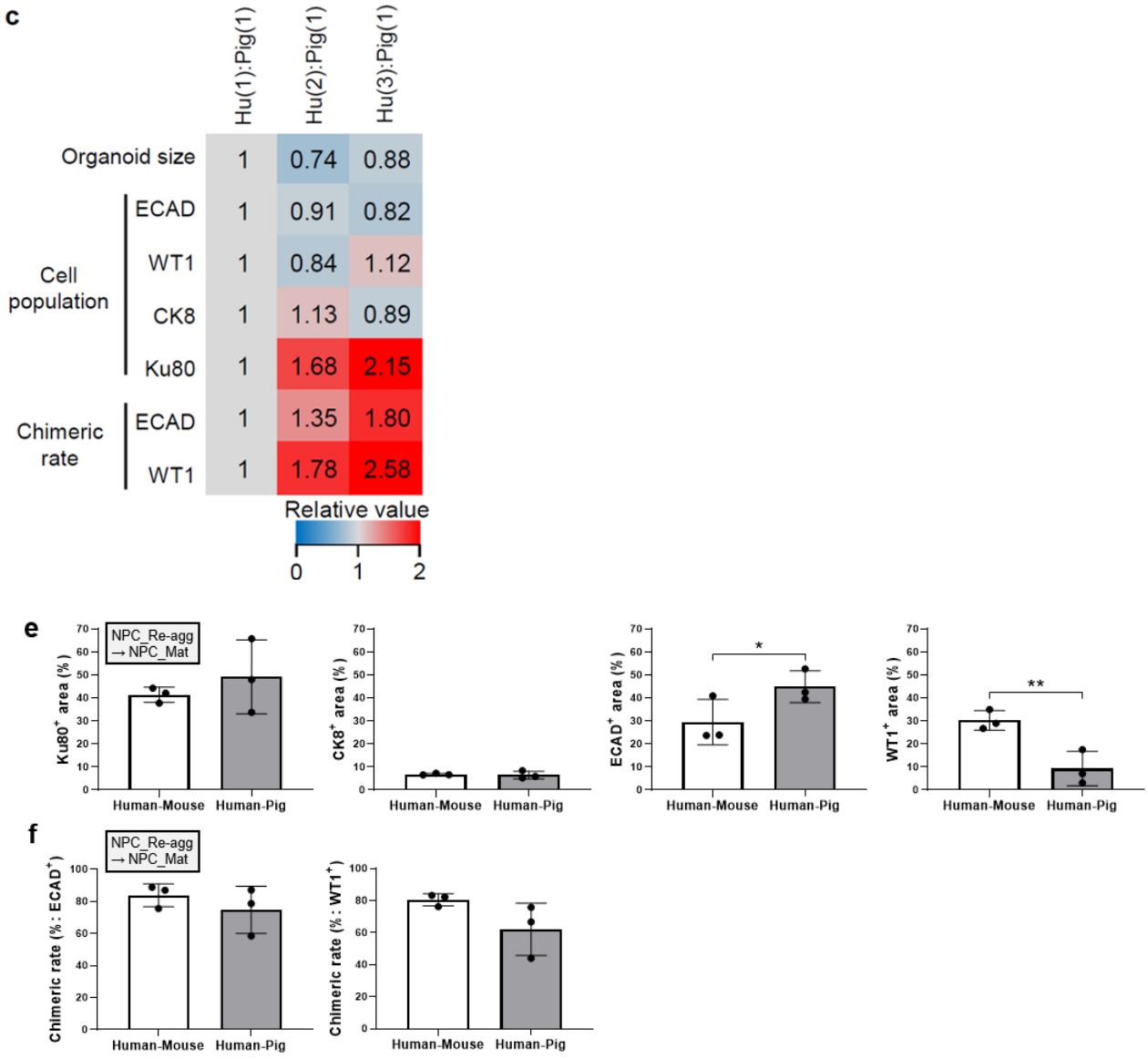

f
ECAD
WT1
10% FBS → 10% FBS
0.84
1.36
10% FBS → NPC_Mat
1.08
0.69
10% FBS → KR5_Mat
0.93
0.25
NPC_Re-agg → 10% FBS
0.89
0.18
NPC_Re-agg → NPC_Mat
1.20
2.53
NPC_Re-agg → KR5_Mat
1.06
0.99
Cell population
Average
1 (67.8%)
1 (2.15%)
0 1 2

Fig. 4


c
Hu(1):Pig(1)
Hu(2):Pig(1)
Hu(3):Pig(1)
Organoid size
1
0.74
0.88
Cell population
ECAD
1
0.91
0.82
WT1
1
0.84
1.12
CK8
1
1.13
0.89
Ku80
1
1.68
2.15
Chimeric rate
ECAD
1
1.35
1.80
WT1
1
1.78
2.58
Relative value
0 1 2
e
NPC_Re-agg → NPC_Mat
Ku80+ area (%)
CK8+ area (%)
ECAD+ area (%)
WT1+ area (%)
*
**
Human-Mouse
Human-Pig
f
NPC_Re-agg → NPC_Mat
Chimeric rate (%: ECAD+)
Chimeric rate (%: WT1+)
Human-Mouse
Human-Pig

(Figure legends) (Page 38, Line 788-789 and Page 39, Line 797-811)

Fig. 3 Generation and evaluation of fetal pig kidney organoids.

f*. Quantitative analysis of cell composition ratio for fetal pig kidney organoids under various culture conditions.*

Fig. 4 Generation and evaluation of human-pig chimeric renal organoids.

c*. Quantitative analysis of size, cellular composition ratio, and chimeric structure formation rate using human-pig chimeric renal organoids at different human-pig cell mixing ratios.*

d*. Images of human(3)-pig(1) chimeric renal organoids immunostained by antibodies at various developmental stages of each nephron segment. Scale bars represent 100 μm.*

e*. Cellular composition analysis based on images of organoids after immunostaining. The quantitative data of human-pig chimeric renal organoids were obtained from chimeric organoid images with a human-pig cell mixing ratio of 3:1. The quantitative data of human-mouse chimeric renal organoids were reutilized from Figure 2e (n= 3 independent experiments; mean ± s.d.; *P< 0.05, **P< 0.01; two-tailed t-test).*

f*. Quantitative analysis of chimera formation rate. The left graph indicates the chimera rate in images of stained distal tubules, while the right graph indicates the chimera rate in images of stained glomeruli. The quantitative data of human-pig chimeric renal organoids were obtained from chimeric organoid images with a human-pig cell mixing ratio of 3:1. The quantitative data of human-mouse chimeric renal organoids were reutilized from Figure 2f (n= 3 independent experiments; mean ± s.d.; two-tailed t-test).*

(Results) (Page 10, Line 193-196)

Moreover, irrespective of the culture condition, ECAD-positive structures predominated over WT1-positive ones, and a bias towards the distal tubules was observed in nephron segments (Fig. 3d-f). The combination of reaggregation medium NPC_Re-agg and maturation medium NPC_Mat yielded the highest number of WT1-positive cells (Fig. 3d-f).

(Results) (Page 11, Line 217-221)

The quantitative analysis demonstrated that, regardless of variations in the mixing ratio of human and pig cells, the size of organoids remained largely consistent (Fig. 4b, c). No significant differences were observed in the composition ratio of each nephron segment or UBs. However, the composition ratio of human cells increased and interestingly the rate of chimeric renal structure formation increased in a human cell mixing ratio-dependent manner (Fig. 4b, c).

(Results) (Page 11, Line 231-Page 12, Line 237)

Comparison between human-pig and human-mouse chimeric renal organoids at a 3:1 mixing ratio revealed characteristic cellular compositions, where the human-pig chimeric organoids exhibited a higher proportion of ECAD-positive distal tubules and a lower proportion of WT1-positive glomeruli compared to human-mouse chimeric organoids (Fig. 4e). However, no significant

differences were observed in the rate of chimeric structure formation (Fig. 4f). These findings suggest that, from the perspective of chimeric structure formation, human-pig chimeric renal organoids are comparable to human-mouse chimeric renal organoids.

(Methods) (Page 26, Line 550-Page 27, Line 553)

Morphological analysis of organoids

The brightfield images of the organoids were acquired using the EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.). The ImageJ analysis software was utilized to quantitatively determine the area of the organoids in the brightfield images.

5) Figure 5 does not seem to add significant value to this work. The formed tubules are few, and glomeruli seem absent.

Thank you for your valuable comments. We acknowledge that the low abundance of tubules and glomeruli in Figure 5 is expected due to the early developmental stage. However, we believe that the significance lies in demonstrating the coexistence and co-development of mouse ureteric bud and human NPC in a cap-like structure, as depicted in Figure 5.

During kidney development, it is crucial for the NPC to form a cap-like structure surrounding the ureteric bud during the initial stages. Previous reports have not been able to confirm clear chimeric cap-like structures like the one shown in Figure 5, which could explain the lack of subsequent progress in renal development. Although we acknowledge that the acquisition of renal function has not been confirmed in our study, we consider the ability to form characteristic structures of early kidney development across species barriers as a significant advancement in the field of xenogeneic chimera organogenesis research.

(Results) (Page 12, Line 240-250)

Targeting clinical applications, we next conducted investigations to validate the applicability of the selected culture conditions for the establishment of humanized xenogeneic kidneys. In our previous study, we confirmed that human NPCs can mature and connect with mouse UBs, reaching the RV stage when cultured in MEMα medium supplemented with FBS[16]. However, the engraftment efficiency of human NPCs was low, and the formation of chimeric structures was limited. Additionally, it is essential for kidney development that NPCs form a cap-like structure surrounding the UB during its early stages. Our previous reports lacked conclusive evidence of a distinct human-mouse chimeric cap-like structure, which may have hindered further progression of kidney development[16]. Therefore, in this study, we investigated whether the engraftment and chimera formation abilities of human NPCs could be enhanced, as well as foster the formation of chimeric cap-like structures, by culturing them in selected conditions using xenogeneic embryonic kidneys after injection (Fig. 5a).

(Results) (Page 13, Line 263-267)

These results demonstrate that the selectively chosen culture conditions contribute to the survival and chimera formation ability of human NPCs injected into mouse fetal kidneys beneath the subrenal capsule. Furthermore, the resulting chimeric structures exhibited characteristic features of early kidney development, signifying the achievement of a milestone on the pathway to future acquisition of renal functionality in humanized xenogeneic kidneys.

(Discussion) (Page 14, Line 270-275)

This study established a cultivation system enabling cross-species nephrogenesis in the early stages of kidney development by refining the existing culture conditions for human-mouse chimeric renal organoids. This optimized culture system allows for coexistence of both the animal and human cellular components at least during the early stage of nephrogenesis and facilitates more efficient interspecies chimera formation. Furthermore, the identified optimized culture conditions were found to be suitable for the formation of porcine fetal kidney organoids.

(Discussion) (Page 17, Line 348-358)

NPCs are known to exist beneath the renal capsule during the fetal period. In order to generate humanized xenogeneic kidneys for future clinical applications, we endeavored to inject human NPCs beneath the renal capsule of fetal mice. In this study, we successfully identified culture conditions that improve the survival and chimera formation of human NPCs injected beneath the renal capsule, thereby improving not only the formation of chimeric renal structures but also confirming the presence of cap-like structures characteristic of early kidney development. This indicates the incorporation of human NPCs into early mouse development and holds promise for further kidney development. While a similar investigation using the human-pig combination has not been conducted in this study, these findings could serve as a pivotal milestone on the pathway towards the future generation of xenogeneic chimera kidneys with potential clinical immunological advantages compared to genetically edited xeno-kidneys that still retain inherent risks of immune rejection.

6) The long-term stability and maturity of kidney organoids are key topics in this research field. Additional experiments to address these aspects may enhance the novelty of this paper.

Thank you for your valuable comments. Indeed, long-term stability and maturation of kidney organoids are important challenges in the field. To our knowledge, no previous studies have achieved long-term maturation and successful acquisition of reabsorption and filtration abilities in kidney organoids.

Similarly, in our study, we have observed that organoids cultured at the air-liquid interface reach a maturation plateau at approximately 10 days, and beyond that point, it becomes difficult for the cells to survive. While the renal organoids presented in our study are at Day 6, even if the

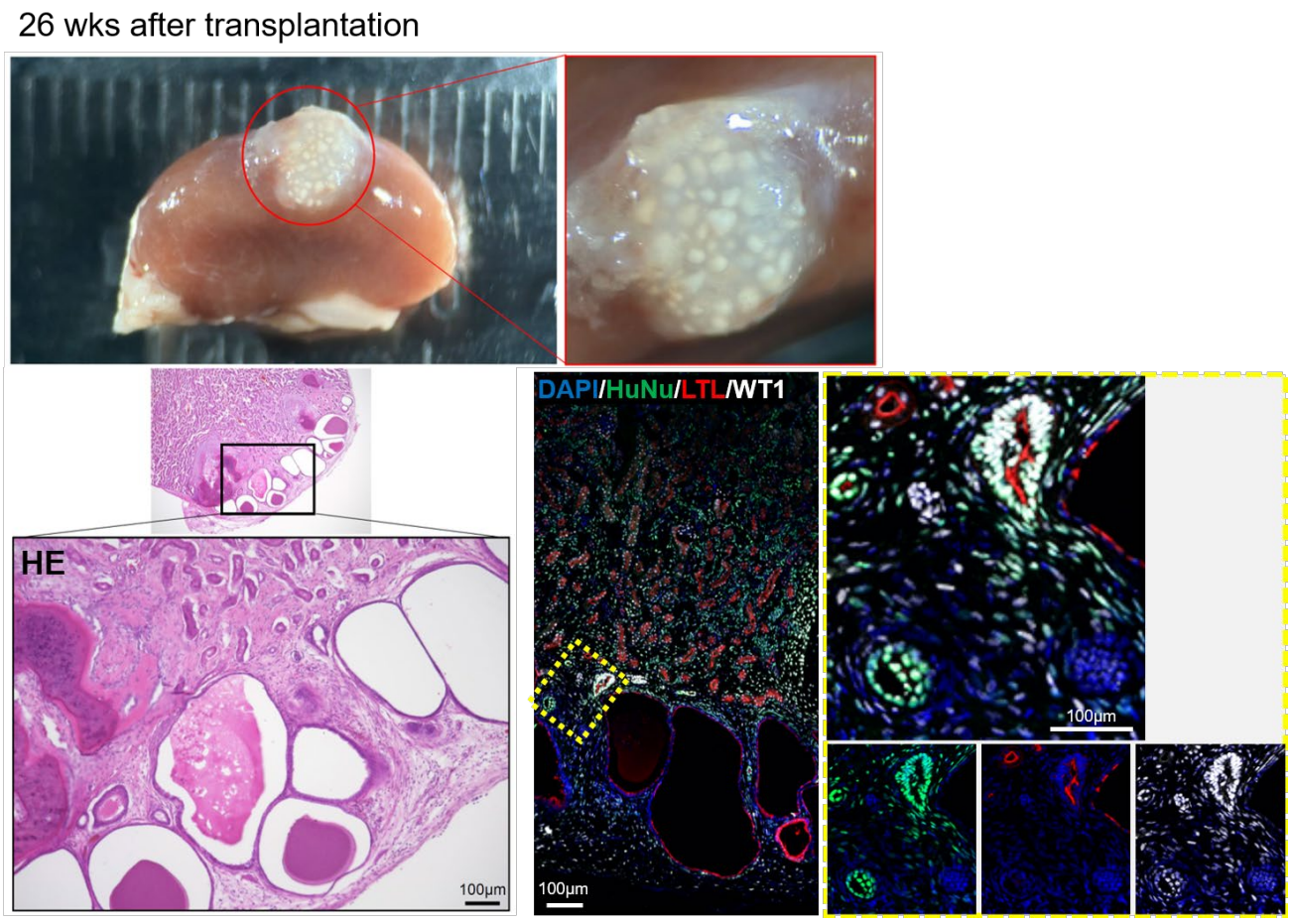
culture period is extended up to 10 days, functional organoids capable of reabsorption and filtration abilities cannot be achieved.

In organoid models without blood influx, nutrient supply and oxygen supply are insufficient, and in order for glomeruli to acquire filtration capabilities, blood influx to the glomeruli is necessary also from a physical standpoint. In other words, we believe that transplantation into animal hosts and vascular infiltration are essential for functional acquisition of kidney organoids.

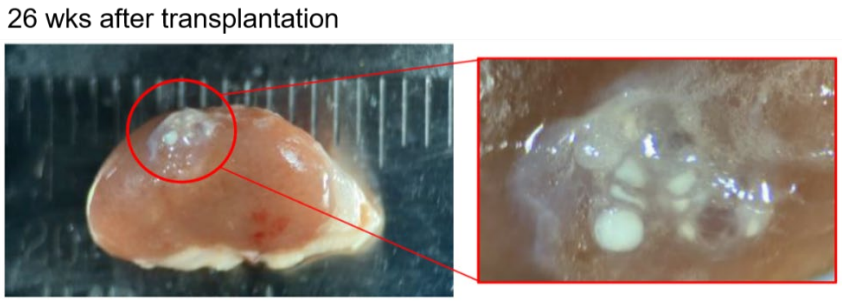
Therefore, we performed transplantation of human-mouse chimeric renal organoids into the subrenal capsule of immunodeficient mice. Even after 26 weeks of transplantation, the presence of HuNu-positive human cells was confirmed, but it was not observed that a significant number of chimera structures were formed. Furthermore, multiple white granular structures, likely representing unintended cells, were formed within the organoids, and the desired nephron structures such as glomeruli were only present in minimal quantities. It was also confirmed that the white granular structures are negative for HuNu.

A similar formation of multiple white granular structures was observed when the transplanted cells were changed to fetal mouse renal organoids using the same experimental design, suggesting that these unintended granules are derived from fetal mouse kidneys.

<u>Human-mouse chimeric renal organoids transplantation</u> into the subrenal capsule of immunodeficient mice



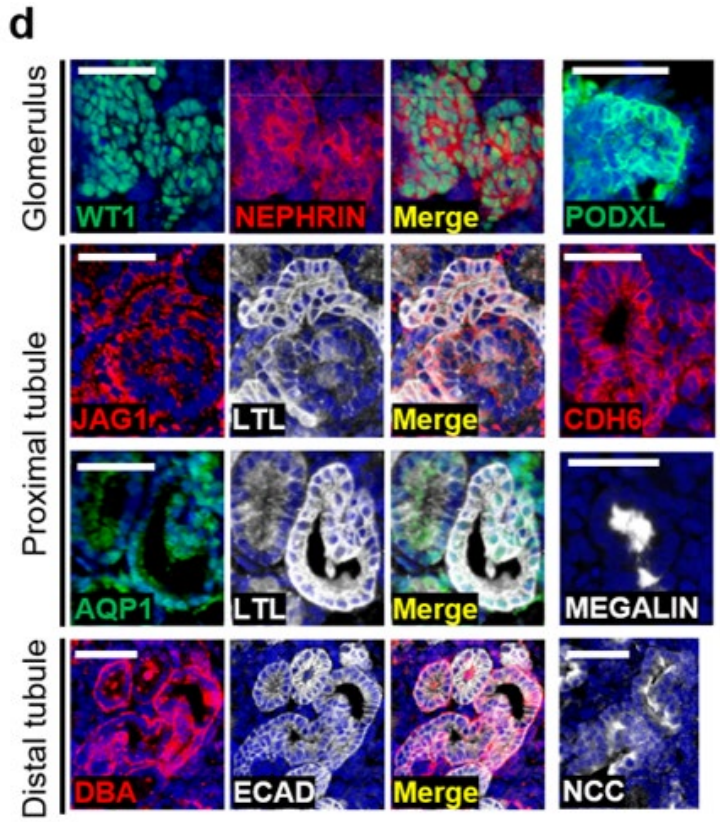
<u>Fetal mouse renal organoids transplantation</u> into the subrenal capsule of immunodeficient mice



Based on these results, we consider further maturation and functional acquisition of chimeric renal organoids as the next challenge. We have added the following statement to the Discussion section of our paper.

Additionally, through our additional experiments, we demonstrated the expression of various developmental markers in three nephron segments through immunostaining. This suggests that human-pig chimera kidney organoids show certain levels of development and maturation (Fig. 4d and Supplementary Fig. 4a, b).

Fig. 4

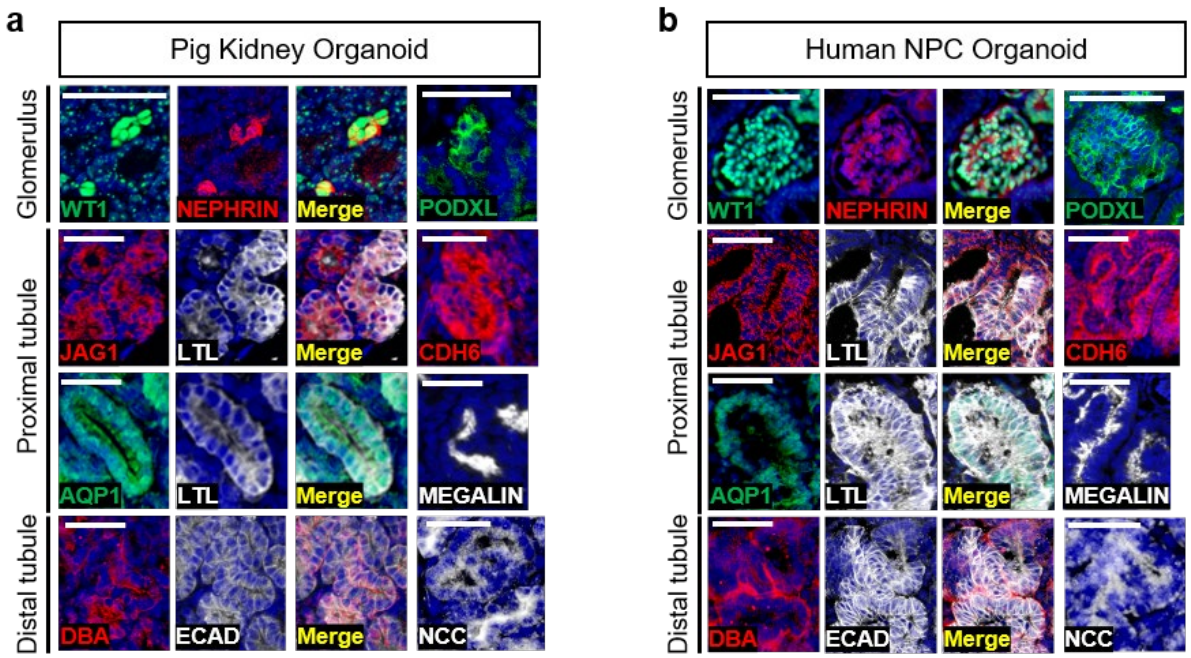


(Figure legends) (Page 39, Line 799-800)

Fig. 4 Generation and evaluation of human-pig chimeric renal organoids.

d*. Images of human(3)-pig(1) chimeric renal organoids immunostained by antibodies at various developmental stages of each nephron segment. Scale bars represent 100 μm.*

Supplementary Figure 4



(Supplementary Figure legends)

Supplementary Figure 4. Developmental evaluation of fetal pig kidney organoids and human NPC organoids cultured for 6 days under the identified culture conditions.

a, b. Immunostaining images of fetal pig kidney organoids (a) and human NPC organoids (b) stained by antibodies at various developmental stages of each nephron segment. Scale bar represents 100 μm.

(Results) (Page 11, Line 222-230)

In terms of both human cell inclusion rate and chimeric structure formation rate, immunostaining analysis of chimeric renal organoids at the optimal human-pig mixing ratio of 3:1 revealed the expression of various glomerular markers, proximal tubule markers, and distal tubule markers, indicating the presence of late developmental stage in the generated chimeric renal organoids (Fig. 4d). Similar staining patterns were observed in fetal pig kidney organoids and human NPC-derived organoids cultured under the same conditions (Supplementary Fig. 4a, b). These results suggest that the selected culture conditions facilitate the coexistence and coincident development of human and pig renal cells, leading to the induction of certain levels of differentiation and maturation in the organoids.

(Discussion) (Page 17, Line 359-Page 18, Line 384)

There are still remaining challenges to fully exploiting the potential of our human-xenogeneic chimeric renal models to acquire renal function. Long-term maturation of the chimeric renal organoid and the human NPC-injected chimeric kidney necessitates sufficient nutrient and oxygen supply to the internal structure; furthermore, from a physical standpoint, blood inflow to the glomeruli is necessary for them to acquire filtration capacity. In other words, we believe that transplantation into the animal body and vascular invasion are essential for functional acquisition in

both renal models[42]. In fact, in our previous study, we confirmed the feasibility of urine production through the transplantation of mouse-rat chimeric kidney models created by injecting fetal rat renal cells into fetal mouse kidneys with endogenous NPCs that can be eliminated through drug induction, and transplanting the chimeric kidneys into immunodeficient adult mice[16].

However, despite our recent attempts to transplant human-mouse chimeric renal organoids into the subcapsular region of host immunodeficient mice, differentiation and maturation of the grafts pose challenges to the full realization of our chimeric renal organoids. Sharmin et al. have demonstrated that even human NPCs, when transplanted into the subcapsular region of mouse kidneys, can form functional glomeruli with Bowman's space. They co-cultured human NPCs with mouse embryonic spinal cord to promote differentiation and maturation, followed by transplantation of these two components together. Furthermore, they co-transplanted agarose rods soaked in VEGF solution to induce host vascularization[43]. The implementation of such techniques for post-transplantation renal development and host vascularization promotion may offer valuable solutions to the problem of generating chimeric renal organoid that are functional. Another suitable approach may involve transplanting chimeric renal organoids with vascular networks obtained through pre-cultivation in artificially scaffolded environments such as microphysiological systems utilizing fluidic dynamics.

Similar technical challenges are anticipated for the acquisition of renal function in xenogeneic kidney models with human NPC injection. In particular, the introduction of the system for removing endogenous NPCs in fetal kidneys, as mentioned earlier, would be a crucial element to expedite research towards the creation of functional humanized xenogeneic kidneys.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have taken on board all the reviewers comments. They have done additional work, provided new information that improves the manuscript and provided more detail in the discussion and introduction that makes the articles more accessible to a wider readership.

They have responded satisfactorily to my comments.

Reviewer #2 (Remarks to the Author):

All my concerns have been addressed well. The revised version of the manuscript is alright for me.

Reviewer #3 (Remarks to the Author):

The authors addressed my concerns.