nature portfolio

Peer Review File

Genetically engineered pig heart transplantation in non-human primates using 10-gene edit to demonstrate clinical translation of xenotransplantation

Corresponding Author: Professor Muhammad Mohiuddin

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors aimed to summarize their preclinical experience in orthotopic pig to non-human primate transplantation using 10-genes edited pig hearts.
Based on four transplantations with an animal survival ranging from 55 to 225 days the authors conclude that this study demonstrates long-term cardiac xenotransplant survival from 10 GE donor pigs into baboons but also highlights the detrimental effect of CMAHKO on non- human primate models. However, an additional human gene expression (CD46, TBM, EPCR, DAF, CD47, and HO-1) may have protected and helped prolong xenograft survival. Therefore,10GE xenografts might be more suitable for clinical xenotransplantation than in NHPs.
Therefore further human experiments are needed to answer these questions, besides the two human compassionate use cases and decedent model experiments, for demonstrating proof-of-principle.

It is certainly a pleasure to review the manuscript the manuscript on preclinical xenoheart transplantation of the group who is leading the field in cinical xenoheart transplantation.
The manuscript is well written and the content of high interest to bring xenoheart transplantation further to the clinics.
However, there are major concerns that need attention:
The major concern of the manuscript is that the graft survival of the 10 gene edited pig xenograft is inferior to that of the 7-gene edited pig heart.
This certainly raises the question of the quality of stable gene expression of all 10 genes.
The authors should show this for all pig hearts that they have used in the study.

- Reference 1 refers to the first clinical transplantation of a porcine heart into a human by the group and not to the second transplantation, which to the best of my knowledge has not yet been published.
-line 81: CD46, CD55, and CD59 should be explained to the general reader at the time of the first use in the manuscript. The authors are inconsistent. Later they use hDAF instead of CD55…

-In describing the surgical procedure, the authors refer to “Goerlich CE, et 494 al. A Standardized Approach to Orthotopic (Life-supporting) Porcine CardiacXenotransplantation in a Nonhuman Primate Model. Transplantation, (2023)”, which does not describe how the right arterial anastomosis was performed . Did they use the Lower-Shumway technique or the Cooley-Barnard modification?
- The mononuclear cell infiltration in the case (B9314) with acute cellular rejection should be further analyzed by CD4 and CD8 staining.
-Please provide representative images of the presence and absence of antibody and complement deposition in the hearts
- The fat deposition should be confirmed by a more specific staining such as Sudan III.
This result should also be added to Table 2.
-The donor number for recipient B33500 is inconsistent: What is correct A315-1 or A315-3 ?
-What was the target level of MMF. Where the authors able to achieve this consistently in all animals
- How do the authors explain the occurrence of thrombotic microangiopathy in transplanted hearts, is it due to CMAHKO or the presence of porcine CMV?.
-Why did the authors stop monitoring PTT and d-dimers in animal B33500 ?

-Table 1 suggests that the ejection fraction in animal B33500 was 75% on POD 225. However, Table 3 A shows the last echo recording on day 200 in this animal. What were the echo findings between day 200 and day 225?
-How was the blood pressure monitored and treated to counteract myocardial hypergrowth?
-Interestingly the hypertrophy in these animals did not progress over time , but developed early within the first two weeks. How do the authors explain this?
-The authors should add PTT monitoring in the Method section.
- The manuscript would benefit from a discussion of conducting preclinical studies without CMAHKO and clinical cases with CMAHKO. What are the current FDA requirements in this regard?
- To elaborate on this further, wouldn't it be more convincing if the non-CMAHKO were to achieve a success of 1 year in the pre-clinical pig to NHP studies before further clinical trials are continued?

Reviewer #2

(Remarks to the Author)
Summary: This manuscript describes the results of 4 pig-to-baboon orthotopic heart transplants that are claimed in the title to have led to the clinical translation of xenotransplantation. The four baboons showed quite variable outcomes, with none of the xenografts surviving more than about 8 months and one rejected as early as 55 days. Survivals of the 4 xenografts were 55,105,127 and 225 days and all seemed to undergo some form of graft damage/rejection. These results compare unfavorably to results reported previously by this group and the Munich group with fewer genetic modifications, in which orthotopic life-supporting cardiac xenograft survival >6 months was consistently achieved. Thus, it is unclear what conclusions the authors hope to draw from the present study. Most importantly, there is no clear indication of which unique gene modifications have been added to the pigs used for these 4 transplants that distinguishes them from previous studies by this group. Overall, the manuscript is poorly written with insufficient information and inappropriate interpretation of the results, with many unclear sentences and errors.

Specific Comments:

Abstract:
“Culminating in two required proof of concept for FDA-approved compassionate use transplants..”. The meaning of this sentence is undecipherable.

“Consistent survival was achieved”. What is meant by consistent? Survival was quite variable and short in some cases.

“We argue ..survival of 10-GE pig hearts is comparable to prior 7-GE donors..”. The data presented in the abstract do not support this argument. Even if they did, why would this be important? The idea behind adding more GEs is to improve survival, not achieve equivalent results to grafts with fewer GEs.

Introduction:
The introduction should highlight what modifications have been added to the “10GE” pig compared to the “9GE” and “7GE” pigs previously published by this group. This is not discernable in the Introduction. Instead, a highly opinionated summary of the various GEs is provided without evidence supporting the advantage of the various GEs. Statements such as (lines 67-68, in reference to B4GLANT2KO and CMAHKO) “required knockout based on the evidence of extensive research over the past 30 years” are not justified by any data.
The title claims that the 10-GE orthotopic heart transplants in this study led to the clinical translation of xenotransplantation. However, it is not explicitly stated whether or not these transplants were performed prior to the clinical xenotransplants. If they were not, then the title is misleading and should be modified.
Lines 84-85: The authors presumably mean C1q esterase inhibitor, not “C1 esterase” inhibitor.

Results:
Genetic modifications were assessed by Western blot on individual donors on tail biopsies. Why are these relevant? The relevant information would be obtained from analyses of endothelial cells of each donor. If these were cloned animals, differences in EC expression of GMs may be highly relevant to outcomes.

Lines 110-111: “Stable” gene expression is claimed on the donor hearts. This implies longitudinal assessment, but no data are shown. How was gene expression assessed in hearts? On what structures? How was stability shown?

Lines 122-124 in reference to Figure 1: Survival of 10-GE hearts was “less than previously reported using 7-GE xenografts” but “longer than reported using 9-GE xenografts”. No references are provided here for these previous reports. How can highly variable results in 4 10-GE recipients be said to be longer or shorter than those in these previous studies?

Line 129: The statement that troponin levels “decreased to a consistent low-level background” is not borne up by the data in the figure. Levels are shown for only 3 of the 4 animals, two of which maintained high troponin levels for most of the duration of follow-up.

Line drawings should include markers indicating the data points. Without markers, it is impossible to discern when samples were obtained during long periods when no inflection is shown for a line. For example, a rising straight line between 50 and 100 days could indicate that no data were obtained in the intervening period and the value increased suddenly or,

alternatively, that there was a steady increase in values from samples obtained at frequent intervals. The addition of symbols marking data points would overcome this uncertainty.

Lines 131-134 contains statements about T and B cell depletion and recovery. However, these statements are not backed up by data showing absolute concentrations of each cell type in the blood. Instead, percentages of each cell type are shown in Figures 2B and 2C and no indication is given of what the denominator is (whole WBCs, lymphocytes?). In any case, the absolute concentrations of each cell type are required in order to allow assessment of cell recovery.

Figure 2D is difficult to interpret. Were the initial samples taken before any induction treatment was given? In contrast to the statement that there did not appear to be any significant trends, all animals appear to show persistent elevations in IL-6 and TNF above this “baseline”.

Lines 143-144 describe cardiac dimensions as “relatively stable”, discounting the fact that 3 of 4 animals showed elevations in left ventricular dimensions after transplant.

Figure 5A is uninterpretable. It contains numerous data points, which are much greater in number than numbers of animals transplanted. Are these all from transplanted animals? If so, which ones? All orthotopic or some heterotopic? At what time points in relation to transplant and graft rejection? Was the same pig target assessed with each sample? Was this a Gal KO pig with no other GEs? The IgG levels should be compared between 7GE and 10GE recipients. If the conclusion is that recipients of 9GE and 10GE had higher non-Gal antibody levels induced by the transplant than 7GE transplants, the data should be presented in a manner that allows this important conclusion to be drawn.

Lines 187-188: The global statement that xenotransplants “have demonstrated that iterative and progressive gene edits have yielded prolonged xenograft survival” is not backed up by data. Indeed, the data in the current manuscript compared to historical data suggest that progressive gene edits may worsen outcomes.

Lines 202-203: “we demonstrate prolonged survival of 10-GE cardiac xenografts in baboons, up to 225 days, which is more than 9-GE but less than 7GE xenografts”. This statement is misleading, since only one animal survived to 225 days and the others were much shorter. The statement implies that survival was prolonged in more than one animal. What are the 9-GE and 7-GE animals to which these are compared? Only 2 of each type are showin in Figure 1. How can such comparisons be made with such small numbers? Did they all receive orthotopic transplants? Were they carried out concurrently with the same regimen as the 10-GE transplants? Have the two 7-GE and 9-GE animals in Figure 1 been published previously? If so, this should be stated and references provided. If not, the details of these transplants should be provided in this manuscript in the same manner as those for the 10-GE transplants.

Statements in lines 236-240, dismissing the significance of CMV infection of the donor, are not warranted on the basis of the data in the manuscript. As noted above, there were persistent elevations in IL-6 and TNF in several animals, but the authors have discounted these data, and no attempt is made to correlate these levels with CMV status.

Given the small numbers of animals in the 7,9 and 10-GE groups, the Discussion overall is far too speculative about the potential benefits and harms produced by each genetic modification. Overall, I have great difficulty concluding anything from this paper.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors need to be congratulated on a very thorough reivew which has the manuscript significantly improved.
I still think that the additional histological staining should be easily possible for the requested standard marker.

Reviewer #2

(Remarks to the Author)
The manuscript has been significantly improved by the edits and new information added. I still have a few concerns:

The paper states in the Abstract and elsewhere (e.g.line 246) that pCMV did not influence graft survival. This is an overinterpretation of minimal data, since only one CMV-negative pig was included in the cohort of 4 donors.

The authors now provide important expression data on the genetic modifications of the pigs. These data suggest that transgene expression is highly variable from donor to donor, especially in the explants. Some comment should be made on these results in terms of relation to time of loss or pathology on the explanted grafts.

Baseline T cell counts seem very low in these animals. Could these be underestimated for some reason? Had treatment already been initiated when these counts were obtained?

Line 200: CD47 is described as an “anti-apoptotic regulatory protein”. This is an unusual description. CD47 is generally

considered to be an inhibitor of macrophage-mediated phagocytosis due to its ability to trigger the inhibitory protein SIRPalpha on macrophages and it is in this context that the human gene has been added to pigs.

Line 220: “Anti-pig PAECs” should be “pig PAECs”.

Line 241: “introduction of pCMV infection” should be changed to “reactivation of pCMV”.

Line 243 “pCMV transmission” should be changed to “pCMV reactivation”.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The question of reviewer 2 is absolutely justified. In my first review, I asked about the stable expression of the transgenes, and the authors explained that it was stable for all transgenes (“ We did see stable gene expression of all human transgenes in the 10-GE donor pigs..”) They now (on p.6, lines 115-117)admit that there is variation. The key question that reviewer 2 asks is whether these differences are relevant to the outcome of transplanted organs. The information given in on p.6, lines 115-117 is indeed insufficient in this respect.

Reviewer #2

(Remarks to the Author)
Some of the remaining concerns have been addressed. However, the important issue of gene expression in relation to outcome of individual grafts has not been addressed by the non-grammatical sentence added on p.6, lines 115-117. The data interpretation and sentence are uninformative. The question I raised in my review was whether the expression of any particular transgene(s), which varied from pig to pig, correlated with outcome, as the outcomes were quite variable. The data warrant much more careful analysis.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for the clarification.

Dear Editor,

Please see our response to the queries from the reviewers. We have made all our efforts to answer the reviewer's questions. We hope that the reviewer will find them satisfactory.

Thanks

Muhammad M Mohiuddin, MD

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors aimed to summarize their preclinical experience in orthotopic pig to non-human primate transplantation using 10-genes edited pig hearts.
Based on four transplantations with an animal survival ranging from 55 to 225 days the authors conclude that this study demonstrates long-term cardiac xenotransplant survival from 10 GE donor pigs into baboons but also highlights the detrimental effect of CMAHKO on non- human primate models. However, an additional human gene expression (CD46, TBM, EPCR, DAF, CD47, and HO-1) may have protected and helped prolong xenograft survival. Therefore,10GE xenografts might be more suitable for clinical xenotransplantation than in NHPs.
Therefore further human experiments are needed to answer these questions, besides the two human compassionate use cases and decedent model experiments, for demonstrating proof-of-principle.

It is certainly a pleasure to review the manuscript the manuscript on preclinical xenoheart transplantation of the group who is leading the field in cinical xenoheart transplantation.
The manuscript is well written and the content of high interest to bring xenoheart transplantation further to the clinics.

Thank you for your kind words and for recognizing our research work in the cardiac xenotransplantation field. This study reports long-term cardiac xenotransplant survival of 10 GE donor pig hearts along with using CD40 antibody-based immunosuppression in non-human primates (NHPs). Due to the role of a possible '4th xenoantigen' related to CMAHKO, further studies are needed in human experiments in order to demonstrate the suitability of 10-GE pig hearts for clinical xenotransplantation.

However, there are major concerns that need attention:
The major concern of the manuscript is that the graft survival of the 10 gene edited pig xenograft is inferior to that of the 7-gene edited pig heart.
This certainly raises the question of the quality of stable gene expression of all 10 genes. The authors should show this for all pig hearts that they have used in the study.

We agree that cardiac xenograft survival of 10 GE pig hearts was not as long as previously reported for 7 GE pig hearts. This could be due to the CMAHKO in the 10GE pig hearts, which has been discussed in the manuscript (page 11, lines 231-236).

We did see stable gene expression of all human transgenes in the 10-GE donor pigs used in this study by Immunohistochemistry (IHC), flow cytometry and western blot, and we have included it in the supplementary figure 1.

- Reference 1 refers to the first clinical transplantation of a porcine heart into a human by the group and not to the second transplantation, which to the best of my knowledge has not yet been published.

Thank you for making a note of it. We want to cite only one paper for the first patient. A correction has been made. A citation for a news article that reported a news piece and an abstract presented and published in the meeting is cited. (page 3, lines 50-51)

-line 81: CD46, CD55, and CD59 should be explained to the general reader at the time of the first use in the manuscript. The authors are inconsistent. Later they use hDAF instead of CD55…
Thank you for the suggestion. We agree that most of the readers are not familiar with these genes, so we have described these genes and used the acronyms consistently.

-In describing the surgical procedure, the authors refer to "Goerlich CE, et 494 al. A Standardized Approach to Orthotopic (Life-supporting) Porcine CardiacXenotransplantation in a Non-human Primate Model. Transplantation, (2023)", which does not describe how the right arterial anastomosis was performed . Did they use the Lower-Shumway technique or the Cooley-Barnard modification?

Thanks for identifying this important omission, we used the modified Shumway procedure of atrial anastomosis. This is now added to the methods. (page 15 , lines 316-318)

- The mononuclear cell infiltration in the case (B9314) with acute cellular rejection should be further analyzed by CD4 and CD8 staining.

Thank you for the suggestion. We will take this suggestion seriously and will analyze these respective populations of CD4 and CD8s in our future experiments. Unfortunately, at this time, we do not have CD4 and CD8 staining for these animals available.

-Please provide representative images of the presence and absence of antibody and complement deposition in the hearts

We have only included the IHC staining for antibody and complement from one animal because we did not see any difference between the 4 animals.

- The fat deposition should be confirmed by a more specific staining such as Sudan III. This result should also be added to Table 2.

Thank you for the suggestion. At this time, we do not have Sudan III staining.

-The donor number for recipient B33500 is inconsistent: What is correct A315-1 or A315-3 ?

Thank you for making a note of it. The correct number is A315-3, and changes have been made wherever required.

-What was the target level of MMF. Where the authors able to achieve this consistently in all animals

MMF levels have historically not been measured in our experiments as MMF levels have not been shown to correlate with immunosuppression efficacy, and their routine measurement is controversial. This has additionally been proven in the clinical setting; therefore, the University of Maryland Medical Center transplant program does not routinely measure levels. However, in prior experiments, we measured MMF levels and found them consistent when administering MMF via the IV route. We have administered MMF through the IV route in these baboons.

Jean-Baptiste Woillard, Franck Saint-Marcoux, Caroline Monchaud, Rym Youdarène, Lucie Pouche, Pierre Marquet,

Mycophenolic mofetil optimized pharmacokinetic modelling, and exposure-effect associations in adult heart transplant recipients,

Pharmacological Research, Volume 99, 2015, Pages 308-315, https://doi.org/10.1016/j.phrs.2015.07.012.

- How do the authors explain the occurrence of thrombotic microangiopathy in transplanted hearts, is it due to CMAHKO or the presence of porcine CMV?.

Thanks for pointing this out and it is a valid question. We think its most likely due to the CMAHKO but have no way of dissecting it out unless we do a direct comparison between a group with and one without CMAHKO. Currently, we only have CMAHKO available through our collaborators.

-Why did the authors stop monitoring PTT and d-dimers in animal B33500 ?

We routinely monitor ACT to maintain the heparin infusion rate. We somehow missed the evaluation of these parameters, perhaps due to the quality issue of the samples.

-Table 1 suggests that the ejection fraction in animal B33500 was 75% on POD 225. However, Table 3 A shows the last echo recording on day 200 in this animal. What were the echo findings between day 200 and day 225?

Just before euthanasia, heart function showed good biventricular function, estimated EF 75% grossly with some mild RV dilation without wall thickening. Table 1 is a summary only. In contrast, Figure 3a plots echocardiographic data obtained by formal assessment by an independent cardiologist using methods described in the methods section. This has been clarified in the manuscript.

-How was the blood pressure monitored and treated to counteract myocardial hypergrowth?

This is a great point, and we have traditionally not treated higher blood pressure with pharmaceutical agents as we have shown that transplants done without blood pressure control did not exhibit myocardial hypertrophy in a limited study (references below). We know Dr Reichart / Brenner group has successfully done that and we are considering blood pressure control for our next series of transplants. We have added this to the discussion.

Goerlich CE, Griffith B, Hanna P, Hong SN, Ayares D, Singh AK, Mohiuddin MM. The growth of xenotransplanted hearts can be reduced with growth hormone receptor knockout pig donors. J Thorac Cardiovasc Surg. 2023 Feb;165(2):e69-e81. doi: 10.1016/j.jtcvs.2021.07.051. Epub 2021 Sep 4. PMID: 34579956; PMCID: PMC8894505.

Mohiuddin MM, Goerlich CE, Singh AK, Zhang T, Tatarov I, Lewis B, Sentz F, Hershfeld A, Braileanu G, Odonkor P, Strauss E, Williams B, Burke A, Hittman J, Bhutta A, Tabatabai A, Gupta A, Vaught T, Sorrells L, Kuravi K, Dandro A, Eyestone W, Kaczorowski DJ, Ayares D, Griffith BP. Progressive genetic modifications of porcine cardiac xenografts extend survival

to 9 months. Xenotransplantation. 2022 May;29(3):e12744. doi: 10.1111/xen.12744. Epub 2022 Mar 31. PMID: 35357044; PMCID: PMC10325874.

-Interestingly the hypertrophy in these animals did not progress over time , but developed early within the first two weeks. How do the authors explain this?

Again a very important observation. We do not have an explanation of this, but it could possibly be a result of the difference of blood pressure between the baboon and the donor pig, causing a physiologic mismatch. Another important area to investigate in our future experiments. We have added this to the discussion.

-The authors should add PTT monitoring in the Method section.

This has been added. (Page 17, line 362)

- The manuscript would benefit from a discussion of conducting preclinical studies without CMAHKO and clinical cases with CMAHKO. What are the current FDA requirements in this regard?

Thank you for the suggestion. We have included a discussion in the manuscript. (Page 11, lines 273-276). There is no specific FDA guidance for the use of CMAHKO or any other gene modification. The investigators have to demonstrate the benefit of gene modifications for the humans.

- To elaborate on this further, wouldn't it be more convincing if the non-CMAHKO were to achieve a success of 1 year in the pre-clinical pig to NHP studies before further clinical trials are continued?

We agree that non-CMAHKO xenograft could survive up to one year or more. Unfortunately, non-CMAHKO donor pigs are not available for us to use therefore, we are unable to do such studies. CMAHKO is required for clinical transplantation due to preformed antibodies in humans, so most genetically modified pigs have CMAHKO.

Reviewer #2 (Remarks to the Author):

Summary: This manuscript describes the results of 4 pig-to-baboon orthotopic heart transplants that are claimed in the title to have led to the clinical translation of

xenotransplantation. The four baboons showed quite variable outcomes, with none of the xenografts surviving more than about 8 months and one rejected as early as 55 days. Survivals of the 4 xenografts were 55,105,127 and 225 days and all seemed to undergo some form of graft damage/rejection. These results compare unfavorably to results reported previously by this group and the Munich group with fewer genetic modifications, in which orthotopic life-supporting cardiac xenograft survival >6 months was consistently achieved. Thus, it is unclear what conclusions the authors hope to draw from the present study. Most importantly, there is no clear indication of which unique gene modifications have been added to the pigs used for these 4 transplants that distinguishes them from previous studies by this group. Overall, the manuscript is poorly written with insufficient information and inappropriate interpretation of the results, with many unclear sentences and errors.

Thank you very much for recognizing our previously published work in cardiac xenotransplantation. As we have previously demonstrated, the iterative and progressive genetic modification from 3 gene edits to 7 gene modifications in donor pigs has improved or prolonged xenograft survival in non-human primates (NHPs). However, further gene modification or deletion of CMAH (which may be important for clinical xenotransplantation as humans have preformed antibodies) in donor pigs exposes another 4th antigen. NHPs might have antibodies against 4th antigens, which ultimately may affect xenograft survival in NHPs. While this is a limited series, this is the first of its kind and will inform clinical trials in cardiac xenotransplantation. This pig may not be perfect for transplants in NHP but is presumed beneficial for future human transplantation.

Specific Comments:

Abstract:
"Culminating in two required proof of concept for FDA-approved compassionate use transplants..". The meaning of this sentence is undecipherable.

The sentence has been modified for clarity. (Page 2, lines 30-31).

"Consistent survival was achieved". What is meant by consistent? Survival was quite variable and short in some cases.

The sentence has been modified for clarity. (Page 2, lines 36-38)

"We argue ..survival of 10-GE pig hearts is comparable to prior 7-GE donors..". The data presented in the abstract do not support this argument. Even if they did, why would

this be important? The idea behind adding more GEs is to improve survival, not achieve equivalent results to grafts with fewer GEs.

The sentence has been modified for a clear and better understanding. (Page 2, lines 41-44)

Introduction:
The introduction should highlight what modifications have been added to the "10GE" pig compared to the "9GE" and "7GE" pigs previously published by this group. This is not discernable in the Introduction. Instead, a highly opinionated summary of the various GEs is provided without evidence supporting the advantage of the various GEs. Statements such as (lines 67-68, in reference to B4GLANT2KO and CMAHKO) "required knockout based on the evidence of extensive research over the past 30 years" are not justified by any data.
The title claims that the 10-GE orthotopic heart transplants in this study led to the clinical translation of xenotransplantation. However, it is not explicitly stated whether or not these transplants were performed prior to the clinical xenotransplants. If they were not, then the title is misleading and should be modified.

Thank you for highlighting the context; we have described the gene modification of 10 GE pig hearts and their justification for each modification is also discussed.

All the orthotopic heart transplants in baboons from 10 GE donor pigs were performed before the first clinical cardiac xenotransplantation.

Lines 84-85: The authors presumably mean C1q esterase inhibitor, not "C1 esterase" inhibitor.

Thank you for noting this, and changes have been made. (Pages 5 and 14; lines 91 and 292)
Results:
Genetic modifications were assessed by Western blot on individual donors on tail biopsies. Why are these relevant? The relevant information would be obtained from analyses of the endothelial cells of each donor. If these were cloned animals, differences in EC expression of GMs may be highly relevant to outcomes.

Donor pigs were cloned animals. We have cultured the endothelial cells from the porcine aorta. We have performed flow cytometry from pAECs, and stable gene expression was observed. Supplementary figure S1(B) has been included. However, we think Isolation and cell culture can alter expression profiles and phenotype of primary cells. So, the expression pattern in cultured cells may not be a true indicator of expression or expression level at the time of transplant. Expression in non-transplanted tissue samples could be a better option. Therefore, we have included a figure for human gene expression in non-transplanted kidneys (Supplementary Figure 1A).

Lines 110-111: "Stable" gene expression is claimed on the donor hearts. This implies longitudinal assessment, but no data are shown. How was gene expression assessed in hearts? On what structures? How was stability shown?

Thank you for the suggestion. Unfortunately heart biopsy sample at the time of xenotransplantation was not collected. However, gene expression at the time of necropsy was collected, and consistent human gene expression was observed in all 4 animals. Supplementary Figure S1(C &D) has been included.

Lines 122-124 in reference to Figure 1: Survival of 10-GE hearts was "less than previously reported using 7-GE xenografts" but "longer than reported using 9-GE xenografts". No references are provided here for these previous reports. How can highly variable results in 4 10-GE recipients be said to be longer or shorter than those in these previous studies?

Reference has been cited for the previous study. The mean survival for 10GE donor hearts in baboons was 128±36 days, less (222 ± 42 days) than 7GE but higher (89.5±5.5 days) than 9 GE. (Page 6, lines 130)

Line 129: The statement that troponin levels "decreased to a consistent low-level background" is not borne up by the data in the figure. Levels are shown for only 3 of the 4 animals, two of which maintained high troponin levels for most of the duration of follow-up.

Regarding the statement about the troponin levels, the authors are referring that they rise immediately after cardiac xenotransplantation and go down to a low level until there is xenograft dysfunction. Troponin levels for all 4 animals are shown.

Line drawings should include markers indicating the data points. Without markers, it is impossible to discern when samples were obtained during long periods when no inflection is shown for a line. For example, a rising straight line between 50 and 100 days could indicate that no data were obtained in the intervening period and the value increased suddenly or, alternatively, that there was a steady increase in values from samples obtained at frequent intervals. The addition of symbols marking data points would overcome this uncertainty.

Thank you for the suggestion. Line graphs have been replaced with newer line graphs with markers.

Lines 131-134 contains statements about T and B cell depletion and recovery. However, these statements are not backed up by data showing absolute concentrations of each cell type in the blood. Instead, percentages of each cell type are shown in Figures 2B and 2C and no indication is given of what the denominator is (whole WBCs,

lymphocytes?). In any case, the absolute concentrations of each cell type are required in order to allow assessment of cell recovery.

Thank you for the suggestion. We have replaced the figure and absolute numbers of CD3+ T and CD20+ cells have been added.

Figure 2D is difficult to interpret. Were the initial samples taken before any induction treatment was given? In contrast to the statement that there did not appear to be any significant trends, all animals appear to show persistent elevations in IL-6 and TNF above this "baseline".

Line graphs have been replaced with newer line graphs with markers for better clarity. Yes, initial samples were taken at -7 days before starting immunosuppression.

Lines 143-144 describe cardiac dimensions as "relatively stable", discounting the fact that 3 of 4 animals showed elevations in left ventricular dimensions after transplant.

Thanks for noticing this. In our long-term survivor, the thickness was not noted until the graft started to be rejected. In the other three grafts, we noticed a gradual increase in thickness.

Figure 5A is uninterpretable. It contains numerous data points, which are much greater in number than numbers of animals transplanted. Are these all from transplanted animals? If so, which ones? All orthotopic or some heterotopic? At what time points in relation to transplant and graft rejection? Was the same pig target assessed with each sample? Was this a Gal KO pig with no other GEs? The IgG levels should be compared between 7GE and 10GE recipients. If the conclusion is that recipients of 9GE and 10GE had higher non-Gal antibody levels induced by the transplant than 7GE transplants, the data should be presented in a manner that allows this important conclusion to be drawn.

Figure 5 A demonstrates the donor-specific anti-pig non-gal antibodies (APA) at all times after orthotopic cardiac xenotransplantation in the baboon recipients receiving 7, 9, and 10 GE pig hearts. The cumulative mean of the APA in the baboon recipient receiving 7 GE pig hearts was lower than 9 and 10 GE. A figure for longitudinal anti-pig antibodies (IgG and IgM) is shown in Supplementary Figure S2) after cardiac xenotransplantation.

Lines 187-188: The global statement that xenotransplants "have demonstrated that iterative and progressive gene edits have yielded prolonged xenograft survival" is not backed up by data. Indeed, the data in the current manuscript compared to historical data suggest that progressive gene edits may worsen outcomes.

We agree that iterative and progressive gene edits should improve xenograft survival, and, in fact, cardiac xenograft survival of 3 GE to 7GE donor hearts in NHPs has improved. However, deletion of CMAH (which may be important for clinical xenotransplantation as humans have preformed antibodies) in donor pigs exposes another 4th antigen. NHPs might have antibodies against 4th antigens, which ultimately may affect xenograft survival in NHPs.

Lines 202-203: "we demonstrate prolonged survival of 10-GE cardiac xenografts in baboons, up to 225 days, which is more than 9-GE but less than 7GE xenografts". This statement is misleading, since only one animal survived to 225 days and the others were much shorter. The statement implies that survival was prolonged in more than one animal. What are the 9-GE and 7-GE animals to which these are compared? Only 2 of each type are show in in Figure 1. How can such comparisons be made with such small numbers? Did they all receive orthotopic transplants? Were they carried out concurrently with the same regimen as the 10-GE transplants? Have the two 7-GE and 9-GE animals in Figure 1 been published previously? If so, this should be stated and references provided. If not, the details of these transplants should be provided in this manuscript in the same manner as those for the 10-GE transplants.

We agree that the survival in all four animals ranged from 55 to 225 days. Mean survival for 10GE donor hearts in baboons was 128±36 days. 9 GE donor pigs are CMAHKO and have additional human CD47 and HO1 expression, whereas 7 GE donor pigs have GHRKO in addition to GGTA1, β4GalNT2 hTBM, and hEPCR. All baboons had an orthotopic cardiac xenotransplantation. Survival of 7GE and 9GE orthotopic donor pig heart transplants in baboons was published before and citation has been included.
I hope reviewers will appreciate the cost of performing these experiments, the availability of these novel pigs, and, therefore, our inability to perform these experiments in a larger cohort. We are also unable to to decipher the function of each gene in these multi-gene modified pigs. We are confident, though, that these modifications, that are made considering their use in humans, will prove to be beneficial in clinical xenotransplantation.

Statements in lines 236-240, dismissing the significance of CMV infection of the donor, are not warranted on the basis of the data in the manuscript. As noted above, there were persistent elevations in IL-6 and TNF in several animals, but the authors have discounted these data, and no attempt is made to correlate these levels with CMV status.

Thank you very much for noting these observations. Authors have noted this pattern, baboon recipients receiving CMV-negative (n=1) xenograft had slightly less IL-6 and TNFa levels as compared to CMV-positive (n=3). However, no statistically significant difference was found.

Given the small numbers of animals in the 7,9, and 10-GE groups, the discussion overall is far too speculative about the potential benefits and harms produced by each genetic modification. Overall, I have great difficulty concluding anything from this paper.

As indicated above, we do agree with the reviewer that it is difficult to evaluate the role of these gene modifications conclusively. However, our vendor of these pigs, who is one of the two companies providing pigs for human studies has decided on using these 10 GE pigs for the first clinical trials. So far there is no report available describing the use of these pigs in NHP. Though we agree that the study has small numbers of animals, however, this is the first report on the cardiac xenograft survival in NHPs from 10 GE donor pigs which have already been used by us in expanded access cardiac xenotransplantation in two human patients. We report for the first time, the longest 225 days (mean and median 128±36 and 116 days) of cardiac xenograft survival from 10 GE donor pigs in NHPs.

Dear Editor,

Thank you very much for giving up an opportunity to respond to the reviewer's comments.

Please see the reply to each comment below.

Thanks again.

Thanks

Kind Regards

Sincerely

Muhammad M Mohiuddin, MD

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors need to be congratulated on a very thorough review which has the manuscript significantly improved.

I still think that the additional histological staining should be easily possible for the requested standard marker.

Thank you very much for your kind words and appreciation. We have done our best to improve the manuscript and are glad that our efforts were noticed.

We have done our best to incorporate reviewers' suggestions to the extent we can. We wish additional histological staining could have been included for standard markers, but believe that such staining would not have added anything more than what we have presented in the current manuscript. H&E and Immunohistochemistry are included. We take this suggestion seriously and will include these stainings in our future experiments.

Reviewer #2 (Remarks to the Author):

The manuscript has been significantly improved by the edits and new information added. I still have a few concerns:

Thank you very much for your kind words and appreciation. We have done our best to improve the manuscript and are glad that our efforts were noticed.

The paper states in the Abstract and elsewhere (e.g.line 246) that pCMV did not influence graft survival. This is an overinterpretation of minimal data, since only one CMV-negative pig was included in the cohort of 4 donors.

Thank you for the suggestion. We have made changes to the manuscript. See line 38 on page 2 and line 248-49, on page 12.

The authors now provide important expression data on the genetic modifications of the pigs. These data suggest that transgene expression is highly variable from donor to donor, especially in the explants. Some comment should be made on these results in terms of relation to time of loss or pathology on the explanted grafts.

Thank you for the suggestion. We have made changes to the manuscript. (See line 115-117, Page 6)

Baseline T cell counts seem very low in these animals. Could these be underestimated for some reason? Had treatment already been initiated when these counts were obtained?

That is true that these baboons have lower T cell numbers. But T cell number varies from baboon to baboon. None of these animals received any other treatment before starting the experiments.

Line 200: CD47 is described as an “anti-apoptotic regulatory protein”. This is an unusual description. CD47 is generally considered to be an inhibitor of macrophage-mediated phagocytosis due to its ability to trigger the inhibitory protein SIRPalpha on macrophages and it is in this context that the human gene has been added to pigs.

We have corrected the description. (See line 200 Page 10)

Line 220: “Anti-pig PAECs” should be “pig PAECs”.

Thank you for noting this, and changes have been made. (See line 221 Page 11)

Line 241: “introduction of pCMV infection” should be changed to “reactivation of pCMV”.

Thank you for noting this, and changes have been made. (See line 242 Page 11)

Line 243 “pCMV transmission” should be changed to “pCMV reactivation”.

Thank you for noting this, and changes have been made. (See line 244 Page 12)

Dear Editor,

Thank you very much for giving up an opportunity to respond to the reviewer's comments.

Please see the reply to each comment below.

Thanks again.

Thanks

Kind Regards

Sincerely

Muhammad M Mohiuddin, MDReviewers' comments:

Reviewer #1 (Remarks to the Author):

The question of reviewer 2 is absolutely justified. In my first review, I asked about the stable expression of the transgenes, and the authors explained that it was stable for all transgenes (" We did see stable gene expression of all human transgenes in the 10-GE donor pigs..") They now (on p.6, lines 115-117)admit that there is variation. The key question that reviewer 2 asks is whether these differences are relevant to the outcome of transplanted organs. The information given in on p.6, lines 115-117 is indeed insufficient in this respect.

We are sorry that we were not clear in our previous reply. Human transgenes were expressed as anticipated but the level of expression varied though not significantly. We think that a larger cohort is needed to elucidate the potential effect of transgenes on xenograft survival.

As you have suggested we have added a sentence on page 6 on lines 131-132. Which reads as "Human transgene expression levels of each gene in 10 GE varied in donor animals and no correlation was observed with xenograft survival."

Reviewer #2 (Remarks to the Author):

Some of the remaining concerns have been addressed. However, the important issue of gene expression in relation to outcome of individual grafts has not been addressed by the non-grammatical sentence added on p.6, lines 115-117. The data interpretation and sentence are uninformative. The question I raised in my review was whether the

expression of any particular transgene(s), which varied from pig to pig, correlated with outcome, as the outcomes were quite variable. The data warrant much more careful analysis.

We are sorry that we were not clear in our previous reply.

Transgenic donor pigs were produced by Revivicor, where they use somatic cell nuclear transferases (SCNT) techniques to generate genetically engineered cloned pigs. Expression of human transgenes was confirmed by immunohistochemistry and western blot which are shown in the Supplementry figure (Figure S1).

Variable human gene expression of individual transgenes (CD46, DAF, EPCR TBM CD47, and HO1) was observed but we did not see any correlation between transgene expression and xenograft survival. However, a larger cohort is needed to determine any potential effects.

For better clarity, we have also changed the sentence on line 116-17 on page 6. Which reads as "Expression of hCD47, hCD46, and hHO1 transgene was weak in explanted xenograft while hTBM and hEPCR genes expression was increased in IHCs." At this time we cannot comment on the specific role of expression levels of each gene.

As you have suggested we have added a sentence on page 6 on lines 131-132. Which reads as "Human transgene expression levels of each gene in 10 GE varied in donor animals and no correlation was observed with xenograft survival."

We have also discussed these results in discussion line 211-220, page 10. Which reads as: 10-GE also expresses six human transgenes which include complement inhibitors(CD46 & DAF), anticoagulation (EPCR &TBM), anti phagocytosis/ apoptotic (CD47), and anti-inflammatory genes (HO-1). As reported by us earlier, human multi-transgene in genetically engineered pigs appears to confer superior xenograft outcomes and reduce prevailing immediate postoperative xenotransplantation challenges[33]. In this study, human transgene expression of each gene varied among 10GE donor animals and they did not correlate with xenograft survival. A larger cohort is needed to elucidate the potential effect of the expression level of each gene on graft survival. This investigation is not possible with a limited number of transplants reported here".

33. Chan JL*, et al.* Encouraging experience using multi-transgenic xenografts in a pig-to-baboon cardiac xenotransplantation model. *Xenotransplantation* 24, (2017)

Dear Editor,

Thank you very much for giving up an opportunity to respond to the reviewer's comments.

Please see the reply to each comment below.

Your manuscript entitled "10-genes edit genetically engineered pig's heart transplantation in non-human primates: Studies that led to clinical translation of xenotransplantation" has now been seen again by our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Medicine.

Thank you very much for considering publishing our manuscript. We appreciate it. We believe this will benefit the readers and advance the xenotransplantation field.

We therefore invite you to revise your paper one last time to address the remaining concerns of our reviewers. At the same time, we ask that you edit your manuscript to comply with our format requirements and to maximise the accessibility and therefore the impact of your work.

Thank you very much for allowing us to answer the queries of the reviewers.

* Please see the attached document for editorial requests for the final version (.docx file). Please ensure a completed version of this file is uploaded as a Related Manuscript with your final submission.

Please see the attached file for the reply to each editorial request. We have answered all the questions and complied with all the requirements.

* Please review our final submission file checklist to ensure all necessary files are present with your final submission and to avoid delays in accepting your manuscript.

Please see the attached checklist.

Thanks

Kind Regards

Muhammad M Mohiuddin, MD