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October 17th, 2021

Ref.: Letter of response with detailed answers (manuscript #wjsc 70949, Basic Study) - "***In vitro* induced pluripotency from urine-derived cells in porcine.**"

Dear Lian-Sheng Ma
Editor-in-Chief of World Journal of Stem Cells

We appreciate the corrections, comments and appropriate suggestions for the manuscript "I*n vitro* induced pluripotency from urine-derived cells in porcine". In this letter, we present the answers point by point to all the comments and questions raised by the reviewers. We have reviewed the full text and re-written when necessary, following the reviewer's suggestions, we now believe that the manuscript is greatly improved in content and will contribute to World Journal of Stem Cells readers. In addition, we are available for any further actions required.

Please find below our responses to reviewers' comments and suggestions, and the main modifications are highlighted in yellow in the revised version of the manuscript.

Reviewers' reports:

Reviewer #1 (02937551)

Urine-derived iPSCs or urine-derived stem cells have been recently reported in humans, nonhuman primates, rabbit and canine models. This paper reports the isolation and reprogramming of cells derived through the noninvasive collection of urine in a porcine model. It is important for agricultural traits,

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genetic improvement and *in vitro* and *in vivo* modeling of several diseases. The comments:

1. How to distinguish the expression of endogenous factors Oct4, Sox2, Nanog, and exogenic OSKM? How to calculate relative expression (calculation formula)?

AU: We thank Reviewer 1 for the comment and opportunity to clarify this important question. The primers used were designed to detect exclusively the porcine (endogenous) transcripts OCT4, SOX2 and NANOG, thus avoiding the detection of murine transcripts, whereas the exogenous (murine, STEMCCA vector was analyzed using the primers called "mOSKM", which did not detect porcine transcripts. The relative gene expression was performed by $2^{\Delta CT}$ (10.1006/meth.2001.1262) using GAPDH and B-ACTIN as housekeeping genes, as now better clarified in the text.

2. Figure 3 is too small to see clearly.

AU: Figure 3 has been rearranged for a better viewing, and we will be willing to provide a high quality and increased size image if requested.

3. Is D6 embryos in Figure 4 marked with a dot just a sample?

AU: We used the DOT sign to better visualize that a pool of 20 porcine blastocysts were used as a positive technical control for pluripotency-related gene expression.

4. The A-C superscript letters represent differences in Figure 5 is not clear. Figure 5 is wrongly marked as Figure 6.

AU: We thank the reviewer for the comment and correction. We have complemented Figure's 5 legend and the number has been corrected.

5. Supplementary Table 1. What does "s" stand for in primers sOct4? The abbreviation “pb” is incorrect. It should be “bp” (base pairs).

AU: The "s" was included at the beginning of the OCT4, SOX2, NANOG, GAPDH and β-ACTIN3 to describe that the primers are specific to the species in question, the **s**wine. We agree with the reviewer that this was confusing, therefore we have replaced the “s” with the "p" of porcine, and we corrected the abbreviation to “bp” and also included the information in the Supplem. Table 1 title.

6. Supplementary Table 2. hOSKM data is incorrect, please verify.

AU: We have corrected the Table 2.

Reviewer #2 (06079635)

The enclosed manuscript by the Bressan et al., reports the conversion of porcine urine-derived cells in to pluripotent cells. The present study is the first reported attempt to generate pluripotent cells from the cells isolated from urine. Such human equivalents to urine derived cells have previously been reported. This is an interesting study having potential applications for veterinary disease modeling and genetic improvements particularly in porcine model. Though similar reports of pluripotent cells generation from non-invasive origins does exist in humans, the importance of such studies in animals can not be discounted. The present study has applied previously established protocols of pluripotent cells generation but using the urine-derived cells of porcine. Few points that will aid in clarity of the manuscript are:

1. The importance of such work in porcine models needs to be adequately described in the introduction section.

AU: We thank the reviewer for the suggestion, and we have included the information and 6 new references in the introduction.

2. In-text citations should be uniform throughout the manuscript, e.g. 1st sentence of paragraph 2 of Introduction section contains author-date in-text citation and rest of the introduction has numbered references

AU: The correction was performed and the references were included following WJSC's instructions and Mendeley file.

3. Few abbreviations are used first time without explanation, e.g. NH in second last paragraph of Introduction section.

AU: We have reviewed and corrected the entire text.

4. Methodology section did not provide clarification as to why only female animals of reproductive age were used for urine collection.

AU: Female animals were used because they were available at our animal housing facility, and in special, because most of swine housing facilities have a greater number of females when compared to males, if any in some pig farms, due to the specific animal production characteristics for swine meat production. It has been reported the isolation of UDCs from males and females in other species, for example, in mice: 10.1038/srep23808. This is one advantage of using urine instead of milk, such as non- or less invasive cell isolation, in special regarding domestic animals (milk would be only physiologically, from females). Our study is the first using the swine model and UDCs; however, since it was not our main aim to compare females x males

UDCs isolation. We have re-written the sentence, and If the reviewer believes the manuscript still need further clarification, we may include the information about the greater number of females in pig farms in the discussion.

5. How endogenous and exogenous expression of reprogramming factors were distingusihed in PCR analysis? This needs to be elaborated.

AU: The primers used were designed to detect exclusively (specifically) the porcine transcripts OCT4, SOX2 and NANOG (endogenous), thus avoiding the detection of exogenous transcripts (mOSKM), which did not amplify porcine transcripts. We have included the information now in material and methods section.

6. FP, IP and LP are the abbreviations not explained in Results and Discussion section.

AU: The abbreviations were described firstly in material and methods section, and we have summarized again in the Result section now.

7. There should be elaboration of the types of cells present in porcine urine and if their heterogeneity could influence the reprogramming efficiency.

AU: We again agree with the reviewer, and we now included such discussion. New references were included citing possible cell types and the need to better characterize them.

8. Supplementary Table 5 is hard to understand as is presented, that needs to be elaborated further.

AU: Supplementary tables 4 and 5 contained formatting typos that were corrected. We have better described their titles and significance, and we hope them to be more adequate now.

Reviewer #3 (05446072):

1. The authors have used porcine urine for iPSC generation and need to mention its significance in regenerative medicine in detail. Also was there any difference in comparison to murine and human models already established.

AU: We thank reviewer 3 for the comments and suggestion. We have included new information in both introduction and discussion, we hope to be adequate now.

2. The choice of vectors need to be discussed in detail and how the reprogramming efficiency can be improved.

AU: The choice of lentiviral vector carrying the transcription factors OSKM was based on previous studies carried out by our research group in different species. In the swine model was possible to reprogram neonatal fibroblasts (10.1007/s12015-021-10198-8) or embryonic fibroblasts (10.1002/term.3143); however we also used for reprogramming cattle, human and mice cells (10.1186/s13287-020-01716-5). The integrative methodology (lentiviral OSKM) is widely used to induce somatic cells into a pluripotent state in domestic animals (10.4252/wjsc.v11.i8.491); however we truly believe that non-integrating methods would be a better choice if efficient. We have employed nonintegrative methodology with episomal vectors, however, we did not obtain success in reprogramming swine or human cell lineages. The discussion on the vector and possibilities to improve the reprogramming are now included in the discussion.

3. ICC image need to be presented better.

AU: Figure 3 has been rearranged for a better viewing, and we will be willing to provide a high quality and increased size image if requested.

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4. Was RNA integrity analysis carried out before downstream qPCR experiments were performed?

AU: All RNA samples were checked prior to cDNA synthesis regarding quality and quantity using a Nanodrop spectrophotometer. The information is now included in the text.

5. Please highlight any other novel aspect of the study apart from source of urine and how it presents a better model

AU: The highlights of the present study include: 1. the use of a suitable biomedical model; 2. The generation of iPSCs from a large domestic animal, which is not commonly reported; 3. The study of different cell lines in different passages, which is certainly one main strength of our experimental design, and the possibility to further use these cells to differentiate into germinative cells and gametes in vitro, once this model can be greatly explored for in vivo gene editing and reproductive studies. These have now been addressed in the manuscript.

Reviewer #4 (05203277)

The manuscript entitled 'In vitro induced pluripotency from urine-derived cells in porcine' is a good attempt to derived cells from urine and then transduced those using reprogramming factors to generate iPS cells. The authors showed the derivation and characterization of generated iPS cells using immunocytochemistry and gene expression approaches followed by in vitro differentiation. There are many reports are available on successful generation of porcine iPS cells generation from different type of cells but definitely Urine derived cells may be first report of current manuscript.

1. Recently many workers demonstrated to show the successful derivation of porcine iPS cells for example- using N2B27 base medium supplemented with FBS, LIF, activin A, vitamin C, knockout serum replacement and small molecule inhibitors such as GSK3 and Wnt. Similarly, Xu et al., 2019 demonstrated extended growth of porcine iPS cells using 2i condition media and these generated iPS cells were injected in early porcine embryo which subsequently developed into blastocyst where contributed competently to both ICM and trophectoderm cells. Authors have not tried these approaches to improve their efficiency.

AU: We appreciate the reviewer's 2 comments. We have indeed previously performed a multi-factorial experiment using LIF, bFGF or LIF+bFGF in another experiment (10.1007/s12015-021-10198-8). In our conditions, the FGF treatment presented more similar characteristics to pluripotency (including the maintenance of endogenous pluripotency gene expression during across passaging). Also, we have tried using 2i and non-integrative methodologies in parallel to lentiviruses; however, no success was obtained in more than 3 years of experiments (data not shown). Our next steps will include an inter-species comparison using RNASeq to identify further transcription factors involved in the reprogramming process; or else to explore +10 transcriptions factors at once; however, these experiments will suit better for future experimental designs.

2. The gold standard of iPS cells in differentiation ability *in vitro* and *in vivo*. In current study, in vitro differentiated cells not showed the expression of ecdoderm. Further, in vivo study has not conducted. Based upon these reports generated cells has not qualifying the iPS cells and suggested to use iPS cell-like cells.

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AU: We agree with reviewer 2, and therefore we have included the word "putative" in the abstract, first time in introduction and conclusion. Moreover, in most of the text we have replaced iPSCs with iPS cell-like cells (iPSCLCs).

We thank again the editor and reviewers for the time available to perform correction, comments and elegant suggestions. We carried out an extensive revision of the manuscript and accepted the reviewers' suggestions and comments, and we believe the manuscript is improved in the content and discussion.

Sincerely yours,

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