Decision letter

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In the interests of transparency, eLife includes the editorial decision letter and accompanying author responses. A lightly edited version of the letter sent to the authors after peer review is shown, indicating the most substantive concerns; minor comments are not usually included.

Thank you for submitting your article "Generation of a novel growth-enhanced and reduced environmental impact transgenic pig strain" for consideration by *eLife*. Your article has been reviewed by three peer reviewers, and the evaluation has been overseen by a Reviewing Editor and Ian Baldwin as the Senior Editor. The following individuals involved in review of your submission have agreed to reveal their identity: Simon Lillico (Reviewer #1); Björn Petersen (Reviewer #3).

The reviewers have discussed the reviews with one another and the Reviewing Editor has drafted this decision to help you prepare a revised submission.

Summary:

The manuscript reports the production and characterization of pigs that were transgenic for several microbial enzymes expressed from the salivary glands. The expression of these enzymes is demonstrated, and the desired biological effects can be observed. Fecal nitrogen and phosphorous outputs were reduced by 20 to 46% , growth rate was improved by about 24% . The feed conversion rate was also improved by 12 to 14% . The authors claim that this is a promising approach to improve feed efficiency and to reduce emissions into the environment. Zhang et al., present a very interesting and important pig model for the expression of multiple microbial transgenes to improve the digestibility of pig feed and to reduce the excretion of N and P into the environment.

Essential revisions:

1) Although single cell clones positive for the transgene and EGFP were used for nuclear transfer, only 24 of the 35 piglets born were transgenic. The authors should discuss possible reasons why this happened.

2) 8 of these pigs survived to sexual maturity. 4 piglets contained the plasmid backbone (ampicillin gene), which the authors claimed was due to breaks in the transgene. This could simply be a consequence of random transgene integration and not transposase mediated integration and the authors should adjust their explanation to reflect this likely possibility. The other pigs contained either a single copy integration in the Legumain or CEP112 gene or a triple integration in an

intergenic region. It is unclear whether only four clones were used for nuclear transfer, and if so why these four clones were chosen. The authors should clarify these points.

3) The authors also need to give a brief description of the two genes (Legumain or CEP112) and if their expression was affected by the insertion of the transposon. Breeding was carried out with the line in which the transposon had integrated in CEP112, but no explanation is provided why this line was chosen.

4) In the feeding experiments the authors could show the functionality of the transgenes. Reduction of phosphorous output was however lower than pigs produced in 2001. The authors claim that this is the first time that multiple single copy genes were introduced into the pig germline. This is factually incorrect as several such pig lines have been produced for xenotransplantation e.g. using polycistronic vector constructs, PAC/Bac based constructs and multiple single transgenes placed in the porcine ROSA26 locus.

5) An extended breeding program was carried out resulting in 288 transgenic pigs, only a few of these were used for the experiments described. Why such a large number of animals? These cannot yet be used for food production.

6) The authors detected altered blood glucose levels; the authors should address potential causes, state whether this affects animal health?

7) The Introduction could be shortened and reduced to the most important facts.

8) In the Results section the authors should provide information about the cause for the death of the piglets. Out of 35 born Duroc piglets only 8 reached sexual maturity, why? The authors should also give the animal numbers of the piglets that survived, so that the reader could better track what happened to which pig and in which experiments was it used.

9) The pigs that harbored the Amp-resistance gene, did they serve as negative controls in Figure 2C,D, E? Did the authors find any differences between TG line 1 and 2? They should also give some information regarding the integration sites.

10) What role do Legumain and CEP112 play? Did they observe any adverse effects correlated to the integration site of the transgenes (only 6 piglets of line 2 survived)? Any implications the authors draw from their findings? They should discuss a targeted integration of the multi-transgene cassette into a safe harbor as described by Garrels et al., (2016) or Rieblinger et al., (2017).

11) Did the authors observe any differences in the meat composition of the transgenic pigs compared to wild type pigs? Do the authors expect any impact of the transgene expression on the microbiome of the pigs? The discussion part could be streamlined and reduced to the main findings and discussion points.

12) In the third paragraph of the Discussion section, were these data shown in the results? The authors should emphasize the importance of this pig model and should give some impressive calculations how much N and P could be saved worldwide.

13) The quality of some figures could be improved.

14) The authors should indicate from which pig line the figures are.

[Editors' note: further revisions were requested prior to acceptance, as described below.]

Thank you for resubmitting your work entitled "Novel transgenic pigs with enhanced growth and reduced environmental impact" for further consideration at *eLife*. Your revised article has been favorably evaluated by Ian Baldwin (Senior editor), a Reviewing editor, and three reviewers.

The manuscript has been improved but there are some smaller remaining issues that need to be addressed before acceptance, as outlined below:

Summary:

While it is acknowledged that the authors made a substantial effort to deal with the criticisms from the first round of reviews, a few issues remain to be solved prior to acceptance for publication. The authors have provided useful information in their rebuttal letter, but only parts of that have been integrated into the paper. The authors should carefully change that and add this information to the revised version of the paper.

Essential revisions:

Introduction: should read: “on soybean meals was decreased by.”

Introduction: should read: “have been established to date. Here, we established”

Subsection “Optimization and construction of a 2A-mediated salivary gland-specific multi-transgene”: Insertion of the transgene in a specific genomic locus can likely account for the lower expression efficiency (so called position effect).

Subsection “Generation of TG pigs”: this part is not easy to understand and needs clarification.

Subsection “Measurement of enzyme production in TG pigs”: should read “The results show that”

Subsection “Improved feed utilization and reduced nutrient emission in TG founders” should read “were significantly decreased in the TG pigs compared to that of the WT pigs. […] Fecal N and P excretion were decreased by”

Subsection “Enhanced growth performance in TG pigs”: the sentence is unclear and needs to be rephrased e.g. “To assess the growth performance, eight F1 TG pigs […] 50kg weights.”

Discussion section: should read: positive for the transgene.

Discussion section: “internal malformation in the digestive, circulatory or cardiopulmonary system. A previous study”

Discussion section: should read “in our study, the […] was in agreement with”

Discussion section: should read “high mortality rate, suggesting that the [...] may relate to the cloning technique.”

Discussion section: blank between “Kumar et al., 2013)” “The expression”

Subsection “Transgenic pigs”: add batch no. of the FBS, this is important when replicating the experiments.

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