nature portfolio

Peer Review File

Efficient Recovery of Zebrafish Maternal-Zygotic Mutants from in vivo Expanded and Cryopreserved Germline Stem Cells

Corresponding Author: Professor Yonghua Sun

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)
In the manuscript by Wang et al., they describe the development of an efficient method for archiving of zebrafish mutant lines by spermatogonia stem cell (SSC) cryopreservation and then recovery of the mutants lines using a method that allows the development of but male and female recipients. This technique will be useful for generally line archiving, but they also demonstrate it utility for the production of fish lines that remove both the maternal and zygotic functions of a mutant (MZ mutants). Previous methods for production of MZ mutant lines are laborious and inefficient, so this new method will be of great interest to the community.
The main features of the method are to first enrich for SSC by transplanting SCC isolated for a particular mutant line, and transplanting them into a cyp11a2 mutant line whose resident germ cells have been removed. In the absence of cyp11a2 function, SSC proliferate but do not progress into meiosis, so gonads fill with SSC. This allows for a ≥100 fold increase in SSC numbers. They report an effect method for cryopreserving whole testis from the animals that allows for recovery of large numbers of live SSC following thawing. The second component of this method was to develop a recipient fish line for the recovery of the cryopreserved SSC, and particularly, a line that will result in the development of both males and females whose germline was derived solely for the transplanted SSC. They chose nanos2 mutant fish since nanos2 is required in both males and females for the maintenance of germline stem cells (spermatogonia and oogonial). Thus, only transplanted SSC would contribute to a lifelong germline stem cells in the recipient fish.
This is a novel and exciting method, that has been rigorously tested. It is also a method that should be easily adaptable for use by any lab that uses zebrafish as a research organism.

The only major critique I have is that the section title “Ovarian somatic niche is maintained in nanos2 mutants” (Lines 181-218) is not relevant to the conclusion that nanos2 mutants are the preferred recipient line for SSC transplantation. This section characterizes the gonads of nanos2 mutants, concluding that unlike previous reports, some nanos2 mutants can retain female characteristics through early adulthood and can even spawn a few oocytes. They also show that following loss of oocytes, the somatic cells retain female-like characteristics for a period of time before sex reversing to males. While this is very interesting, it is not directly relevant to the result that “nanos2-/- recipients enable efficient generation of donor-derived functional oocytes.” In the later experiment, the SSC transplantation happens at 4 dpf, well before the animals have determined their sex (and thus ovary vs. testis). The most likely explanation for why transplanting into nanos2 mutants vs. dnd germ cell KO can result in developmebt of females (nanos2 recipiets) instead of all males (dnd MO recipients) is that dnd morphants loose all germ cells before 24 hpf whereas nanos2 mutants have normal numbers of germ cells through early to mid-larval stages. Because germ cell numbers in mid-larval stages is a predictor or male vs. female development (i.e. low numbers leads to testis determination, and high numbers leads to ovary determination; Dai et al., 2015), nanos2+SSC animals will have more germ cells than dndMO+SSC animals so are more likely to develop as females. This section could likely be shortened or removed to better focus the manuscript on the mechanistic basis of recipient efficiency.

Minor critics:
1. “It is stated in abstract that this new method is the most effective method for preserving and recovering zebrafish mutants.” There is a good argue that sperm cryopreservation from otherwise normal animals is the most effective method for

cryopreserving and recovering mutant lines since it required minimal manipulations, and can be done with minimal training, reagents, or equipment. To be clear, the method described here is an amazing method for production of MZ mutants and for preserving mutants, but it takes more skill than sperm cryopreservation and recovery by in vitro fertilization. I think the statement is a distraction and unnecessary.

2. For rigor and reproducibility, please specify the source of anti-Nanos2 antibody in M&M and its primary reference.

3. Liner 417: For rigor and reproducibility, please indicate the allele numbers for each of the gene mutations used and references to the primary publications where they were characterized.

Reviewer #2

(Remarks to the Author)
This manuscript presents substantial improvements to germ cell transplantation techniques in zebrafish and can be highly evaluated for the following three major achievements.

First, by using the cyp11a2 mutant as a host, the authors established a system that enables extensive in vivo amplification of a small number of donor cells. This approach is particularly valuable for the expansion of cryopreserved germline stem cells, as demonstrated in this study.

Second, by employing the nos2 mutant as a host, the authors successfully generated not only male but also female recipients. This is highly significant, especially considering the rapid reconstruction of mutant lines from cryopreserved germ cells.

Third, by combining these systems, the authors succeeded in reconstructing maternal-zygotic mutants from cryopreserved germline stem cells within a short period. These achievements are supported by highly reliable and comprehensive datasets.

I list several minor comments below.

Line 70: There is also a published study in bitterling (Yamakawa et al., 2025 Sci Rep) demonstrating the generation of individuals from cryopreserved germ cells of an endangered species.

Line 121: The transplantation success rate in the control group appears to be lower than that reported in previous studies. This point requires further discussion.

Line 138: Please delete “predominantly.”

Line 178 and elsewhere: The terms “host” and “recipient” are used interchangeably throughout the manuscript. Please clarify whether these terms are intended to have different meanings. If they are synonymous, the terminology should be unified.

Line 197: Please also state whether oogonia were detected in the Ddx4 immunostaining analysis.

Line 335: Triploid hosts and germ cell-deficient hosts represent fundamentally different host types and should therefore be discussed separately.

Line 340: The manuscript suggests that germ cell-deficient hosts become male due to the absence of sex chromosomes in zebrafish. However, germ cell-deficient individuals also undergo masculinization in medaka, which possess a clearly defined sex-determining gene. This point requires reconsideration and further discussion.

Line 393: There is also a report demonstrating the generation of individuals from cryopreserved germ cells in an aquaculture species, such as ayu (Ichida et al., 2025 Aquaculture).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have address all of my comments and suggestions.

Reviewer #2

(Remarks to the Author)
This revised manuscript addresses my comments accurately, and the major issues have been substantially improved. I do

not find any particular problems with this version. I think it is a very well done piece of work.

Reviewer #3

(Remarks to the Author)
The authors have submitted an improved version of the manuscript addressing previous concerns

Point-by-point response to the reviewers

Reviewer #1 (Remarks to the Author):

In the manuscript by Wang et al., they describe the development of an efficient method for archiving of zebrafish mutant lines by spermatogonia stem cell (SSC) cryopreservation and then recovery of the mutants lines using a method that allows the development of but male and female recipients. This technique will be useful for generally line archiving, but they also demonstrate it utility for the production of fish lines that remove both the maternal and zygotic functions of a mutant (MZ mutants). Previous methods for production of MZ mutant lines are laborious and inefficient, so this new method will be of great interest to the community.

The main features of the method are to first enrich for SSC by transplanting SCC isolated for a particular mutant line, and transplanting them into a cyp11a2 mutant line whose resident germ cells have been removed. In the absence of cyp11a2 function, SSC proliferate but do not progress into meiosis, so gonads fill with SSC. This allows for a ≥100 fold increase in SSC numbers. They report an effect method for cryopreserving whole testis from the animals that allows for recovery of large numbers of live SSC following thawing. The second component of this method was to develop a recipient fish line for the recovery of the cryopreserved SSC, and particularly, a line that will result in the development of both males and females whose germline was derived solely for the transplanted SSC. They chose nanos2 mutant fish since nanos2 is required in both males and females for the maintenance of germline stem cells (spermatogonia and oogonial). Thus, only transplanted SSC would contribute to a lifelong germline stem cells in the recipient fish.

This is a novel and exciting method, that has been rigorously tested. It is also a method that should be easily adaptable for use by any lab that uses zebrafish as a research organism.

- We appreciate you for recognizing that our study presents a novel and exciting method, that has been rigorously tested, and is also a method that should be easily adaptable for use by any zebrafish lab.

The only major critique I have is that the section title “Ovarian somatic niche is maintained in nanos2 mutants” (Lines 181-218) is not relevant to the conclusion that nanos2 mutants are the preferred recipient line for SSC transplantation. This section characterizes the gonads of nanos2 mutants, concluding that unlike previous reports, some nanos2 mutants can retain female characteristics through early adulthood and can even spawn a few oocytes. They also show that following loss of oocytes, the somatic cells retain female-like characteristics for a period of time before sex reversing to males. While this is very interesting, it is not directly relevant to the result that “nanos2-/- recipients enable efficient

generation of donor-derived functional oocytes." In the later experiment, the SSC transplantation happens at 4 dpf, well before the animals have determined their sex (and thus ovary vs. testis). The most likely explanation for why transplanting into nanos2 mutants vs. dnd germ cell KO can result in developmebt of females (nanos2 recipiets) instead of all males (dnd MO recipients) is that dnd morphants loose all germ cells before 24 hpf whereas nanos2 mutants have normal numbers of germ cells through early to mid-larval stages. Because germ cell numbers in mid-larval stages is a predictor or male vs. female development (i.e. low numbers leads to testis determination, and high numbers leads to ovary determination; Dai et al., 2015), nanos2+SSC animals will have more germ cells than dndMO+SSC animals so are more likely to develop as females. This section could likely be shortened or removed to better focus the manuscript on the mechanistic basis of recipient efficiency.
Response: Thank you! We totally agree with you on this point, and we have moved this figure from the main figures to the supplementary data. In addition, we have revised and substantially shortened this section to better focus on the mechanistic basis for why *nanos2*$^{-/-}$ mutants serve as superior hosts for GSC transplantation (L175-L191).

Minor critics:
1. "It is stated in abstract that this new method is the most effective method for preserving and recovering zebrafish mutants." There is a good argue that sperm cryopreservation from otherwise normal animals is the most effective method for cryopreserving and recovering mutant lines since it required minimal manipulations, and can be done with minimal training, reagents, or equipment. To be clear, the method described here is an amazing method for production of MZ mutants and for preserving mutants, but it takes more skill than sperm cryopreservation and recovery by in vitro fertilization. I think the statement is a distraction and unnecessary.
Response: We thank the reviewer for this helpful comment and fully agree that sperm cryopreservation represents a technically simple and widely used approach for preserving mutant lines, as it requires minimal manipulation, training, reagents, and equipment also provide advantages for maintaining conventional mutant lines. The revised statement is as follows (L26-L28): Overall, our strategy provides a robust platform for preserving and restoring zebrafish homozygous mutants, opening new opportunities for zebrafish research.
2. For rigor and reproducibility, please specify the source of anti-Nanos2 antibody in M&M and its primary reference.
Response: Thanks for your suggestion. We have supplemented the source of the anti-Nanos2 antibody and its primary reference in the Materials and Methods section (L429-L432).

3. Liner 417: For rigor and reproducibility, please indicate the allele numbers for

each of the gene mutations used and references to the primary publications where they were characterized.

Response: Thanks for your suggestion. We have added the allele numbers for each gene mutation used in the study and the references to the primary publications where these mutants were characterized in (L377-L379), improving the rigor of the manuscript.

Reviewer #2 (Remarks to the Author):

This manuscript presents substantial improvements to germ cell transplantation techniques in zebrafish and can be highly evaluated for the following three major achievements.

First, by using the cyp11a2 mutant as a host, the authors established a system that enables extensive in vivo amplification of a small number of donor cells. This approach is particularly valuable for the expansion of cryopreserved germline stem cells, as demonstrated in this study.

Second, by employing the nos2 mutant as a host, the authors successfully generated not only male but also female recipients. This is highly significant, especially considering the rapid reconstruction of mutant lines from cryopreserved germ cells.

Third, by combining these systems, the authors succeeded in reconstructing maternal-zygotic mutants from cryopreserved germline stem cells within a short period. These achievements are supported by highly reliable and comprehensive datasets.

- We appreciate you for the highly positive evaluation and recognition of our work, that presents substantial improvements to germ cell transplantation techniques in zebrafish.

I list several minor comments below.

Line 70: There is also a published study in bitterling (Yamakawa et al., 2025 Sci Rep) demonstrating the generation of individuals from cryopreserved germ cells of an endangered species.

Response: Thanks for your suggestion. We have supplemented the reference (Yamakawa et al., 2025 Sci Rep) about the generation of individuals from cryopreserved germ cells of an endangered species (bitterling) in Line 64, enriching the background information of the study.

Line 121: The transplantation success rate in the control group appears to be lower than that reported in previous studies. This point requires further discussion.

Response: We sincerely appreciate this helpful comment. We agree that the transplantation success rate in the control group appears relatively lower than that reported in Marinović et al. (2019). We have added a detailed discussion addressing this point in Lines 307–310 of the revised manuscript.

Line 138: Please delete “predominantly.”

Response: Thanks for your suggestion. We have deleted the word

“predominantly” in Line 131 as suggested.

Line 178 and elsewhere: The terms “host” and “recipient” are used interchangeably throughout the manuscript. Please clarify whether these terms are intended to have different meanings. If they are synonymous, the terminology should be unified.
Response: Thanks for your suggestion. We have unified the terminology to “host” throughout the manuscript to avoid confusion.

Line 197: Please also state whether oogonia were detected in the Ddx4 immunostaining analysis.
Response: Thanks for your suggestion. We have supplemented the description in Line 197, stating whether oogonia were detected in the Ddx4 immunostaining analysis (L183-L184): with no oogonia detected according to Ddx4 immunofluorescence.

Line 335: Triploid hosts and germ cell-deficient hosts represent fundamentally different host types and should therefore be discussed separately.
Response: We thank the reviewer for this helpful suggestion. We agree that triploid hosts and germ cell-deficient hosts represent fundamentally different types of hosts and should be discussed separately. Accordingly, we have revised this section to clearly distinguish these two strategies. The revised text can be found on Lines 310–313.

Line 340: The manuscript suggests that germ cell-deficient hosts become male due to the absence of sex chromosomes in zebrafish. However, germ cell-deficient individuals also undergo masculinization in medaka, which possess a clearly defined sex-determining gene. This point requires reconsideration and further discussion.
Response: We thank the reviewer for pointing out this important issue. We agree that our previous wording was inaccurate. Germ cell-deficient zebrafish develop as males not because of the absence of sex chromosomes, but because depletion of germ cells during early development biases gonadal differentiation toward the male pathway. We have therefore revised the relevant text to clarify this point and removed the incorrect explanation related to sex chromosomes. The revised text can be found on Lines 317–320.

Line 393: There is also a report demonstrating the generation of individuals from cryopreserved germ cells in an aquaculture species, such as ayu (Ichida et al., 2025 Aquaculture).
Response: Thanks for your suggestion. We have supplemented the reference (Ichida et al., 2025 Aquaculture) about the generation of individuals from cryopreserved germ cells in an aquaculture species (ayu) in Line 65, further highlighting the application value of our method.