Remodeling of organelles and microtubules during spermiogenesis in the liverwort *Marchantia polymorpha*

Naoki Minamino, Takuya Norizuki, Shoji Mano, Kazuo Ebine and Takashi Ueda
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Original submission

First decision letter

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MS TITLE: Remodeling of organelles and microtubules during spermiogenesis in the liverwort *Marchantia polymorpha*

AUTHORS: Naoki Minamino, Takuya Norizuki, Shoji Mano, Kazuo Ebine, and Takashi Ueda
ARTICLE TYPE: Research Article

Dear Dr. Ueda,

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Yours sincerely,

Yka Helariutta
Handling Editor
Development

Reviewer 1

Advance summary and potential significance to field

In this paper, the authors aimed to define developmental stages of spermiogeneis in the liverwort M. polymorpha, based on morphological observation using dissected spermatid cells treated with cell wall-degrading enzymes. They defined five stages (stages 1-5) of spermiogenesis, in addition to the one (stage 0) representing pre-spermiogenic state. Then, the authors performed immunostaining to visualize the state of poly-glutamate modification of tubulin proteins, which is known to occur in the axonemes of animals and some green algae, and found that tubulins constituting both flagella and the spline undergo dynamic changes in poly-glutamate modification. Subsequently, the authors analyzed membrane dynamics during the spermiogenesis using transgenic lines expressing fluorescently-tagged marker proteins. This latter part is an extension of a previous study by the same authors (Minamino, 2017), which revealed cell-autonomous removal of plasma membrane proteins, as well as reorganization of organelles into vacuole-like compartments, presumably mediated by autophagy. In the present study, the authors visualized this process in higher resolution and for more compartments, and revealed development of a single large vacuole and a single Golgi apparatus in each cell and their loss in the later stages. They also revealed distinct temporal dynamics between nuclear envelope and endoplasmic reticulum during the late stages of spermiogenesis. Overall, this study presents a nice cell biological description of spermiogenesis in M. polymorpha, and potentially useful for discovery and characterization of key molecules in this genetically amenable model bryophyte species.

Comments for the author

A major concern on this paper is the lack of mechanistic insights and clear conceptual advance, which would be expected for a paper in Development. Functional analyses of at least one key gene/protein for the described processes, by genetic and/or pharmacological manipulation are to be performed.

Minor points:
Line 51, "reproductive cells, which are generally classified into male and female gametes". In a wider viewpoint, oogamy and distinction of gametes as male or female are not truly general in the concepts of sexual reproduction. Need more detailed description about gamete forms.
Line 58, "several species including Arabidopsis". If it refers to species, please say "Arabidopsis thaliana" (in italic).
Line 103, The meaning of "unified standard" is not clear.
Lines 119 and 123, Do "round" and "spherical" refer to different nuclear morphologies?
Line 129, "Altered modification of microtubules". Is the modification altered? The authors seem to have analyzed presence or absence of one form of modification.
Line 220, "a set of PM proteins". Not clear what does the "set" refer to.
Line 237, "depending on proteins" is not understandable.
Line 292, "in the basal land plant". If this specifically refers to M. polymorpha, pleas say so.
Line 315. "We investigated the reorganization of endomembrane organelles with the progression of spermiogenesis; the results are summarized in Figure 7". Fig.7 shows a timetable of endomembrane and organelle dynamics, with illustrations only showing cell and nuclear morphologies. Illustrations including endomembrane and organelle dynamics as well as tubulin modification would be more helpful.

Reviewer 2

Advance summary and potential significance to field

Minamino et al
Remodeling of organelles and microtubules during spermiogenesis in the liverwort Marchantia polymorpha

This manuscript describes organelle modifications during spermiogenesis in the liverwort Marchantia polymorpha and provides a description of stages based on nuclear and cell body morphology combined with flagellar formation (gene expression patterns cannot be used as

transcription largely ceases during sperm formation). The manuscript is well organized and will provide a useful benchmark for the community.

Comments for the author

Major comment
It would be useful to include how the number and morphology of the other organelles, the mitochondria and chloroplasts, change with those organelles studied in this paper. Changes in both the mitochondria and chloroplasts have been described previously; e.g. Carothers 1975 (CAROTHERS, Z. B., 1975 Comparative studies on spermatogenesis in bryophytes. Biological Journal of the Linnean Society 7, Supplement 1: 71-84) provides a nice overview. Given the interrelationships between the microtubules and these organelles this would be useful data and could be incorporated into the final diagram as well (Figure 7), such that most of the (known) organelles of the cell are presented.

Minor comments:

Page 2, line 32: should clarify what is meant by plants here. Sperm have evolved mutliple time independently among the eukaryotes; in the case here, sperm evolved within the Streptophtya, in an ancestral alga that gave rise to a few lineages of charophycean algae and land plants; motility of the sperm was subsequently lost in the seed plants.
Page 4, line 111: would be nice to cite Ikeno here, who described this in detail over a century ago (IKENO, S., 1903 Beiträge zur Kenntnis der pflanzlichen Spermatogenese: Die Spermatogenese von Marchantia polymorpha. Beihefte zum Botanischen Centralblatt 15: 5-88.)

Response to reviewers' comments

We thank the reviewers for the constructive comments and suggestions, which were very helpful in guiding us to improve the manuscript.

Response to Reviewer #1:
A major concern on this paper is the lack of mechanistic insights and clear conceptual advance, which would be expected for a paper in Development. Functional analyses of at least one key gene/protein for the described processes, by genetic and/or pharmacological manipulation are to be performed.

According to this comment, we newly analyzed the functions of a key machinery of membrane trafficking, the ESCRTs complex, in spermiogenesis in *M. polymorpha*. The main roles of the ESCRTs complex are to mediate the formation of intralumenal vesicles (ILVs) at the multivesicular endosome and in the sorting of cargos to be degraded into the ILVs during the endocytic degradation of membrane proteins. Recent studies have shown that the ESCRTs complex also mediates membrane sealing in multiple cellular events, including the repair and reorganization of the nuclear envelope. Because we found that plasma membrane proteins are rapidly endocytosed and degraded during the early stages of spermiogenesis, we speculated that the ESCRTs complex could also play a role in spermiogenesis. We perturbed ESCRT-III function by expressing dominant-negative forms of ESCRT-III components, and found that inhibition of ESCRTs function results in hampered motility and shaping of spermatozoids. We also found that an ESCRT-III subunit delocalizes from the nuclear envelope to the diverticulum, a unique structure that extends from the nuclear envelope with unknown function. These results suggest that the ESCRTs complex plays essential roles during spermiogenesis in *M. polymorpha* through the regulation of endocytic degradation and/or nuclear envelope reorganization. We included these newly obtained data and related discussion in the revised manuscript (p. 8, l. 263–p. 10, l. 322; p. 14, l. 446–p. 15, l. 471). We also added brief background related to the ESCRTs complex in the Introduction section (p. 4, l. 97–l. 114).

Line 51, "reproductive cells, which are generally classified into male and female gametes". In a wider viewpoint, oogamy and distinction of gametes as male or female are not truly general in

the concepts of sexual reproduction. Need more detailed description about gamete forms.

Thank you for pointing this out. We have modified this sentence accordingly (p. 2, l. 50– l. 51).

Line 58, "several species including Arabidopsis". If it refers to species, please say "Arabidopsis thaliana" (in italic).

We modified the text accordingly (p. 2, l. 58).

Line 103, The meaning of "unified standard" is not clear.

We modified this part to avoid ambiguity (p. 4, l. 113).

Lines 119 and 123, Do "round" and "spherical" refer to different nuclear morphologies?

We had used “round” and “spherical” to represent the same general morphology. To avoid confusion, we have now used “spherical” consistently throughout the paper.

Line 129, "Altered modification of microtubules". Is the modification altered? The authors seem to have analyzed presence or absence of one form of modification.

We examined only the glutamylation of tubulin, and found that the extent of glutamylation changes during spermiogenesis. We have revised the wording in this subheading to avoid ambiguity (p. 5, l. 140).

Line 220, "a set of PM proteins". Not clear what does the "set" refer to.

We removed “a set of” in the revised manuscript (p. 7, l. 231).

Line 237, "depending on proteins" is not understandable.

We modified this sentence to better clarify our meaning (p. 8, l. 246–l. 247).

Line 292, "in the basal land plant". If this specifically refers to M. polymorpha, pleas say so.

This sentence was modified as suggested. Thank you (p. 11, l. 366).

Line 315. "We investigated the reorganization of endomembrane organelles with the progression of spermiogenesis; the results are summarized in Figure 7". Fig.7 shows a timetable of endomembrane and organelle dynamics, with illustrations only showing cell and nuclear morphologies. Illustrations including endomembrane and organelle dynamics as well as tubulin modification would be more helpful.

Thank you for this suggestion. We have added illustrations showing the changes in the organization of organelles and microtubules. The modified figure is presented as Figure 8 in the revised manuscript.

Response to the comments of Reviewer 2:
It would be useful to include how the number and morphology of the other organelles, the mitochondria and chloroplasts, change with those organelles studied in this paper. Changes in both the mitochondria and chloroplasts have been described previously; e.g. Carothers 1975 (CAROTHERS, Z. B., 1975 Comparative studies on spermatogenesis in bryophytes. Biological Journal of the Linnean Society 7, Supplement 1: 71-84) provides a nice overview. Given the interrelationships between the microtubules and these organelles this would be useful data and could be incorporated into the final diagram as well (Figure 7), such that most of the (known) organelles of the cell are presented.

We thank the reviewer for this suggestion. According to the suggestion, we included a diagram demonstrating the reorganization of the mitochondria and plastid, referring to the Carothers

(1975) article suggested by the reviewer and our own manuscript that is currently under revision for another journal (p. 12, l. 391). The new figure is presented as Figure 8 in the revised manuscript.

Page 2, line 32: should clarify what is meant by plants here. Sperm have evolved mutliple time independently among the eukaryotes; in the case here, sperm evolved within the Streptophtya, in an ancestral alga that gave rise to a few lineages of charophycean algae and land plants; motility of the sperm was subsequently lost in the seed plants.

We appreciate this comment. We removed the corresponding part from the abstract due to the word number limit, and have instead described the taxa that generate motile sperm in further detail in the Introduction section (p. 2, l. 54–l. 56).

Page 4, line 111: would be nice to cite Ikeno here, who described this in detail over a century ago (IKENO, S., 1903 Beiträge zur Kenntnis der pflanzlichen Spermatogenese: Die Spermatogenese von Marchantia polymorpha. Beihefte zum Botanischen Centralblatt 15: 5-88.)

We appreciate this suggestion very much. The suggested article is now cited in the revised manuscript (p. 4, l. 120–l. 121).

Resubmission

First decision letter

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AUTHORS: Naoki Minamino, Takuya Norizuki, Shoji Mano, Kazuo Ebine, and Takashi Ueda
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The developmental stages defined in this paper are significant and provide an appropriate reference for explaining temporal changes in the spermatogenesis in bryophytes. The new attempts of authors take the study on bryophyte spermatogenesis, which was centered on morphological description using TEM, to a new stage. I understand that this paper has already been improved and made many corrections through several peer reviews.

Comments for the author

I have some suggestions and questions for additional improvement.
(1)
Fig. 1B shows the process of formation of eight spermatozoids from one spermatogenous cell, but the difference in cell shape and size can be misleading. Since it is not unequal division, division of spermatogenous cells should give rise to four cells of the same size. The relative size of each organelle does not reflect the actual one. The nucleus and plastids occupy a larger area of the cytoplasm. Please revise Fig. 1B.

(2)
I have a question about experiments on microtubule dynamics.

If the post-translational glutamylation of tubulins is a progressive change, there may be a difference in staining intensity between the base and the tip of the growing flagella. In Fig 2, only the tip of the growing flagella appears to be stained with tubulin antibodies. Is this the evidence that the post-translational modification is a progressive change, or is there a large amount of unmodified free tubulin molecules at the tip of growing flagella? In any case, please explain why only the tip is stained. It's not absolutely necessary, because there is evidence of western blotting, but it will be an appropriate control to show that there is no change in stainability by using an antibody that recognizes the N-terminal of tubulins or a general polyclonal antibody.

Reviewer 2

Advance summary and potential significance to field

With the revised manuscript, the authors seem to have addressed most of the concerns raised during the first reviewing process. The work still remains rather descriptive, however, the detailed and careful cell biology analysis of spermiogenesis in Marchantia presented in this study valuable in that it could serve as the basis for future molecular studies.

Comments for the author

I have one comment to the newly added data:
Lines 308-312
"however, transgenic plants expressing MpVPS2a-mGFP at a moderate level did not exhibit marked defects in the number and morphology of spermatozoids released into water, distinct from spermatids of MpSNF7a-mGFP and MpVPS241-152-mGFP plants. We could therefore observe the subcellular localization of ESCRT-III during spermiogenesis in these plants. "

ESCRT c-terminal fusions seem to cause toxic effects in other species. Without a genetic complementation, it is not clear whether the observed localisation really reflects the native localisation of MpVPS2a during spermiogenesis. The authors could maybe tone down the conclusion and/or state this point. A quantification similar to Figure 7F will be feasible and help to strengthen the claim that VPS2a-mGFP is at least not causing a dominant-negative effect.

Reviewer 3

Advance summary and potential significance to field

Process of gametes differentiation before the sexual process is largely unknown in molecular terms in plants (starting with the algal level) - as it was addressed mostly by the microscopy approaches until now. As a starting point a well-defined framework clearly defining stages of this process for male gametes in model liverwort was missing - a condition necessary for the future comparative and integrative studies from different labs. This report addresses exactly this point using quite challenging detailed observation of this process starting from the spermatogenous cells as a 0 stage via four transition stages resulting in mature stage 5 spermatozoids. Stages are described in an unambiguous manner allowing scholars working in this field to adopt this system. Great contribution of this report is then that, using different cytoskeletal and endomembrane markers, authors describe basic dynamics of compartments during the spermiogenesis. As this comprise mostly cellular simplification and elimination processes, they in response to the first round of review reason -that ESCRT machinery should be involved. Using ectopic DN versions of proteins expression approaches they demonstrate that this is the case. This report will be used as a starting point for the future molecular mechanisms inquiry in this field.

Comments for the author

For the readership it might be interesting, even in this type of report, to shortly address why simplification and elimination of cellular components is happening specifically during spermatogenesis and what are the chances that part of that might be evolutionarily related or a result of the convergent evolution (homoplasy).