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Complexities in Bombyx germ cell formation process revealed by Bm-nosO (a Bombyx homolog of nanos) knockout

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ABSTRACT

Inheritance (sequestration of a localized determinant: germplasm) and zygotic induction are two modes of metazoan primordial germ cell (PGC) specification. vasa and nanos homologs are evolutionarily conserved germline marker genes that have been used to examine the ontogeny of germ cells in various animals. In the lepidopteran insect Bombyx mori, although the lack of vasa homolog (BmVLG) protein localization as well as microscopic observation suggested the lack of germplasm, classical embryo manipulation studies and the localization pattern of Bm-nosO (one of the four nanos genes in Bombyx) maternal mRNA in the egg raised the possibility that an inheritance mode is operating in Bombyx. Here, we generated Bm-nosO knockouts to examine whether the localized mRNA acts as a localized germ cell determinant. Contrary to our expectations, Bm-nosO knockout lines could be established. However, these lines frequently produced abnormal eggs, which failed to hatch, to various extent depending on the individuals. We also found that Bm-nosO positively regulated BmVLG expression at least during embryonic stage, directly or indirectly, indicating that these genes were on the same developmental pathway for germ cell formation in Bombyx. These results suggest that these conserved genes are concerned with stable germ cell production. On the other hand, from the aspect of BmVLG as a PGC marker, we showed that maternal Bm-nosO product(s) as well as early zygotic Bm-nosO activity were redundantly involved in PGC specification; elimination of both maternal and zygotic gene activities (as in knockout lines) resulted in the apparent lack of PGCs, indicating that an inheritance mechanism indeed operates in Bombyx. This, however, together with the fact that germ cells are produced at all in Bm-nosO knockout lines, also suggests the possibility that, in Bombyx, not only this inheritance mechanism but also an inductive mechanism acts in concert to form germ cells or that loss of early PGCs are compensated for by germline regeneration: mechanisms that could enable the evolution of preformation. Thus, Bombyx could serve as an important organism in understanding the evolution of germ cell formation mechanisms; transition between preformation and inductive modes.

1. Introduction

Animal germ cells are specified by either cytoplasmic inheritance (preformation) or by zygotic induction (epigenesis). The former examples are seen in model organisms such as Drosophila, C. elegans, and Xenopus. In the eggs of these animals, specialized cytoplasms called germ plasms are present, which dictate the formation of germ cell precursors (primordial germ cells-PGCs). Zygotic induction refers to the mode in which cell-to-cell interaction is involved and is well-known in mice (Extavour and Akam, 2003).

The existence of germ plasm has been suggested by embryo manipulations in some insect species. Remarkably in dipteran Drosophila, by ectopically assembling germ plasm by molecular genetic means, it was possible to demonstrate that the germ plasm (called pole plasm since it exists near the posterior pole of the oocyte) harbors a germ cell determinant (Ephrussi and Lehmann, 1992). Pole plasm is also reported to be observed in some higher insect orders such as Lepidoptera and Hymenoptera in addition to Diptera. In contrast, unequivocal germ plasm has so far not been detected in basally branching hemimetabolous insect orders, in which epigenesis appears

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to occur (Extavour and Akam, 2003; Nakamura and Extavour, 2016; Quan and Lynch, 2016).

Studies in Drosophila have identified several germ plasm components, some of which are highly evolutionarily conserved, leading to their utilization in various germ cell studies in various animal species including insects (Calvo et al., 2005; Dearden, 2006; Donnell et al., 2004; Khila and Abouheif, 2008; Lall et al., 2003; Lin and Chang, 2009; Lynch and Desplan, 2010; Mito et al., 2008; Nakao, 1999; Nakao et al., 2008; Rezende-Teixeira et al., 2009; Schroder, 2006; Tanaka and Hartfelder, 2009; Zhurov et al., 2004).

vasa and nanos encode such molecules. Antibodies against the Vasa protein, an RNA helicase with various functions, are used to detect germ plasm in many organisms. nanos, which encodes an RNA binding protein with C2H2 zinc finger motifs, is involved in the regulation of translation and plays essential roles in germ cell development including germ cell specification, germ-line stem cell maintenance, and oocyte development. In Drosophila, it is known to promote germ line cell fate by suppressing pathways for somatic development (Extavour and Akam, 2003; Gavis and Lehmann, 1994; Hay et al., 1988; Hayashi et al., 2004; Kobayashi et al., 1996; Lasko and Ashburner, 1990).

The germ cell development of Bombyx mori, a lepidopteran insect, has been relatively well-studied. In this insect, the existence of germ plasm was, although not microscopically detectable, suggested by classical embryo manipulation studies (Miya, 1958). Upon availability of germ cell markers such as vasa and nanos, the ontogeny of PGCs was examined molecularly. In situ hybridization using a vasa homolog (BmVLG) probe revealed that the PGCs were first detected as a group of cells located at the posterior side of ventral midline of the germ band after the germ band differentiates from the extraembryonic tissue anlage, consistent with previous microscopic study (Nakao, 1999); the maternal BmVLG transcript, initially distributed evenly within the germ anlage, is degraded during early embryogenesis, except for in PGCs. Then, an antibody against the BmVLG protein was raised to examine its tissue distribution pattern during embryogenesis, which did not suggest the existence of germ plasm despite the fact that the antibody clearly recognized germ plasms of not only Drosophila, but also the sawfly, Athalia rosae, which supported the idea that the germ plasm is not present in Bombyx (Nakao et al., 2006). Subsequently, developmental profiles of nanos homologs were examined, which complicated the situation. In Bombyx, four nanos genes were identified from the search in the genomic sequence. These are designated as Bm-nosM, N, O, and P. Each of these genes were expressed in different patterns, suggesting their functional differentiation. Of these, Bm-nosO appeared to be the most relevant in germ cell development and is the subject of this study. In situ hybridization using a Bm-nosO probe revealed that Bm-nosO transcripts are dispersed along the ventral midline with a focus on the posterior side, some of which appeared to be taken up by future PGCs, suggesting that these might represent germ plasm and that Bm-nosO might be a germ cell determinant (Nakao et al., 2008). Here, we investigated the function of Bm-nosO by RNAi knockdown and gene knockout, specially focusing on whether Bm-nosO functions as a germ cell determinant, i.e. whether a preformation mode of PGC specification operates in Bombyx. The results are presented and their implications discussed.

2. Results

2.1. Bm-nosO embryonic RNAi affects PGC formation and oogenesis

Our embryonic RNAi method injects dsRNAs during two-to-three hours AEL (After Egg Laying). Previous studies suggested that periplasmic maternal RNA was efficiently targeted by this procedure: we observed dsRNA-mediated degradation of maternal RNAs before cleavage energids (nuclei and associated cytoplasm) begin to enter the periplasm, which occurs at approximately eight hours AEL. Thus, we assume that the RNAi targeted maternal RNA as well as the zygotic

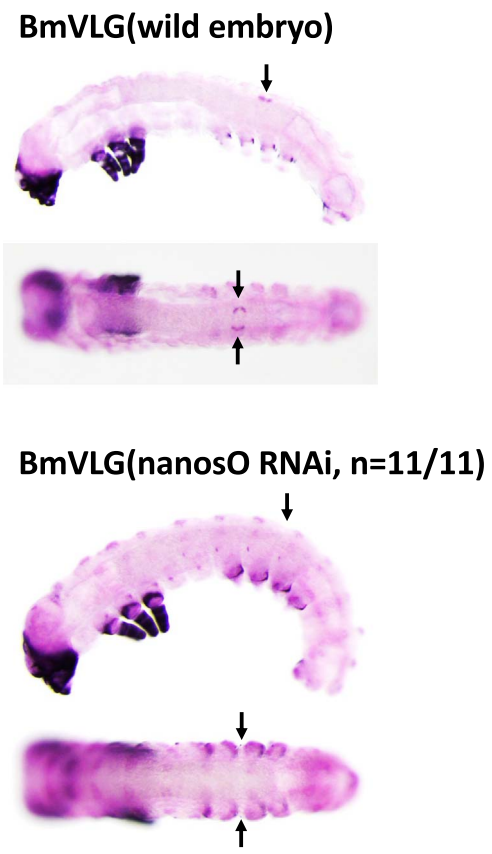

Fig. 1. Embryonic BmVLG expression was not detected in Bm-nosO RNAi embryos. Wild type or Bm-nosO dsRNA-injected embryos were fixed at 4–5 days AEL and subject to in situ hybridization using a BmVLG probe. BmVLG expression was not detected in Bm-nosO RNAi embryos (arrows).

transcript (Nakao, 2012). Wild type and RNAi-treated eggs were dissected at four days AEL (just after blastokinesis), embryos were isolated, fixed, and subjected to in situ hybridization using DIG-labelled BmVLG RNA as a probe. In all the RNAi-treated eggs (n = 20), a BmVLG signal was not observed, suggesting the lack of germ cells or at least the lack of BmVLG-positive germ cells (Fig. 1) (Also see below). Some of the treated eggs successfully hatched and grew to adulthood. These insects were dissected and their reproductive organ formation was examined. While we could not detect alternation in the testis morphology in most cases (n = 19/23: 19 cases out of 23 examined; remaining four cases appeared to show a slight reduction in size and slight deformation but the significance is unclear), in the female, among individuals that apparently showed normal ovary formation, we observed individuals that possessed ovarioles containing very few oocytes (n = 6/21), which, in our experience, has never been observed in wild types (Fig. 2). While these results suggested that Bm-nosO is involved in germ cell formation, the high frequency of RNAi

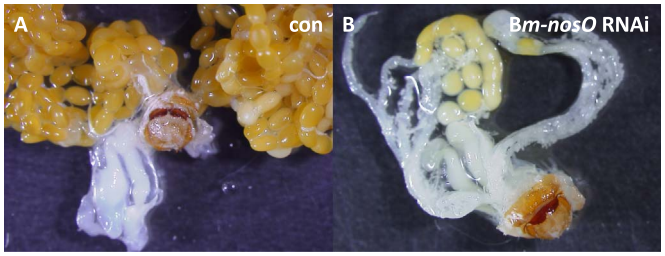

Fig. 2. Effects of Bm-nosO RNAi on oogenesis. Bm-nosO dsRNA-injected embryos were allowed to develop to adulthood and oogenesis was examined. (A) An ovary from an uninjected embryo. (B) An ovary from an injected embryo. A very small number of oocytes are seen, together with empty ovarioles in (B).

treated individuals exhibiting apparently normal gonad formation appeared to be contradicted with the observation that BmVLG expression is lacking in all the Bm-nosO RNAi embryos, which in turn suggested the lack of embryonic germ cells. It should also be noted that in this RNAi experiment, we did not closely inspect the morphology of oocytes, thus, whether there was any alteration or not was not determined (see below).

2.2. Generation and phenotype of Bm-nosO knockouts

The RNAi studies described in the previous section have suggested the involvement of Bm-nosO in the development of germ cells. However, RNAi procedures suffer from a drawback in the completeness of the suppression of gene activities and also cannot distinguish an effect of the maternal transcript from the zygotic one. To resolve these issues, we next decided to use TALEN-mediated gene knockout to examine Bm-nosO gene functions. Previous studies indicated that TALEN-mediated gene editing works effectively in Bombyx (Daimon et al., 2014; Takasu et al., 2013a, 2013b). Two pairs of TALEN vectors were prepared so as to remove most of the coding sequence in order to maximize the removal of target gene activities (Fig. 3-1). *In vitro* synthesized RNAs from these vectors were injected into two-to-five hours AEL eggs (before cellularization). Injected eggs were allowed to hatch and reared to adulthood (G0). Imagoes obtained were crossed to wild types and G1 eggs were obtained, a fraction of which were expected to harbor gene-edited alleles. Genomic DNAs from approximately 20 eggs from each cross were subjected to PCR analysis to examine the existence of gene-edited alleles, and we detected positive crosses (Supplementary Fig. 1). At this stage, we noticed a fraction of the eggs from these positive injected female/wild type male crosses had morphological anomalies. Eggs from positive crosses were allowed to hatch and the resulting imagoes were subject to screening of their genotype. Male and female heterozygotic Bm-nosO knockouts were thus obtained. These heterozygotes were then crossed to obtain homozygotes. Remarkably, nosO- knockout lines were established from such homozygotic crosses and the subsequent analyses could be performed (Fig. 3-2,3-3).

2.3. Phenotypes of nosO knockouts

We closely examined phenotypes of Bm-nosO knockouts for alleles #14 and #45 (Fig. 3-3). Female insects heterozygous for Bm-nosO knockout (Bm-nosO-) alleles laid eggs in a manner indistinguishable from wild type females. For female nosO- homozygotes, a considerable proportion of the eggs (20–80% depending on individuals) had anomalies in not only outside, but also inside (cytoplasm), morphologies and the number of eggs laid was lower (Figs. 4, 5). To examine the number of eggs produced more directly, homozygous females were dissected just after emergence and the number of mature oocytes those females contained was counted. The results are shown in Fig. 6-2. The mean number of oocytes from females homozygous for Bm-nosO- derived from Bm-nosO- homozygous mothers (mat-zyg- in Fig. 6-1) was significantly reduced compared to wild type females, with the mean number of oocytes from female Bm-nosO- homozygotes derived from Bm-nosO- heterozygous mothers (mat+zyg-) in between. These results suggested that Bm-nosO activity is not absolutely required for the formation of germ cells and, may also suggest that the maternal Bm-nosO transcript could contribute to the formation (specification) of germ cells. It should be noted that in addition to these quantitative differences, a qualitative difference was also found; among female homozygotes, we observed individuals that possessed ovarioles containing very few oocytes (14: n = 0/37, 45: n = 3/30; the lack of the observation of this phenotype in #14 line may be due its genetic background), which phenocopied Bm-nosO RNAi-treated insects (Supplementary Fig. 2).

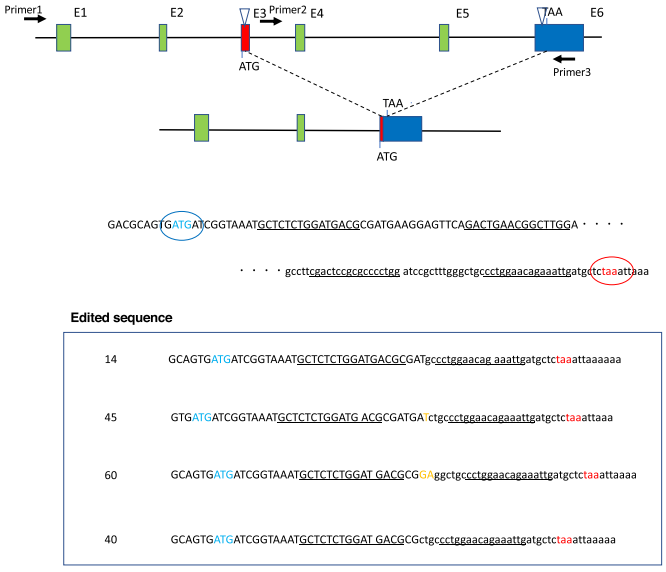

Fig. 3. TALEN-mediated editing of the Bm-nosO locus. (3-1) Schematic representation of the editing. Open triangles indicate the positions of target sequences. Successful editing should lead to the removal of most of the coding sequences. E1~E6 denotes exon1~exon6. Positions of PCR primers used for screening are also indicated (arrows). (3-2) Upstream and downstream TALEN target sequences (underlined) located in the vicinity of the start and stop codons (circled in blue and red, respectively). (3-3) Edited sequences. Four independently edited sequences are shown as examples. 5′ sequences of the upstream target half site and 3′ sequences of the downstream site were joint by a spacer of as few as about 10 nucleotides or fewer. Nucleotides that do not appear in the genome are highlighted in yellow.

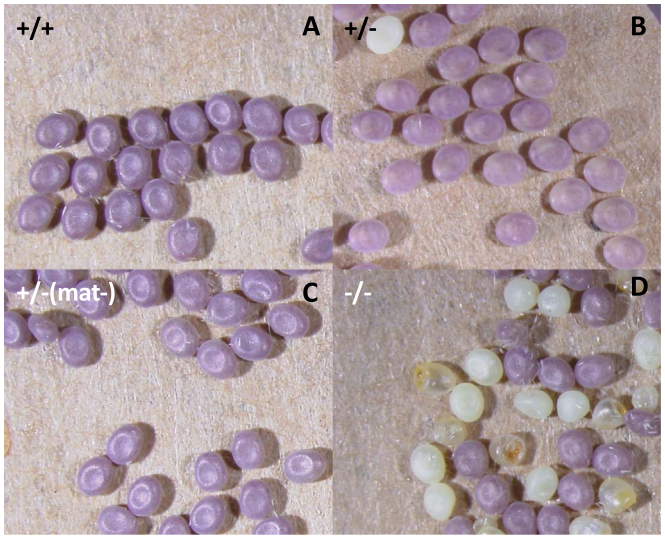

Fig. 4. Eggs laid by wild type, Bm-nosO knockout, or wild/Bm-nosO knockout heterozygotes developed at approximately 5 days. Grey-colored eggs represent the development of serosa. (A)Wild type eggs. (B) Eggs laid by wild/Bm-nosO knockout heterozygotic females. (C) Eggs laid by wild/Bm-nosO knockout heterozygotic females derived from a Bm-nosO knockout homozygotic female, devoid of the maternally supplied Bm-nosO product. (C) Eggs laid by Bm-nosO knockout homozygotes. Eggs of heterozygotes are indistinguishable from those of wild types in appearance. Many aberrant eggs are seen among the eggs produced by Bm-nosO homozygotic females. Most of the white (unfertilized) eggs seen in (D) have anomalies of the cytoplasmic appearance (see also Fig. 5).

To further investigate the possibility of whether the maternal genotype affects the morphology and number of eggs produced, the germ cell formation of Bm-nosO- heterozygous females derived from Bm-nosO- homozygotic female and wild type male crosses (mat-zyg+) were compared with that of wild types. No differences in oocyte

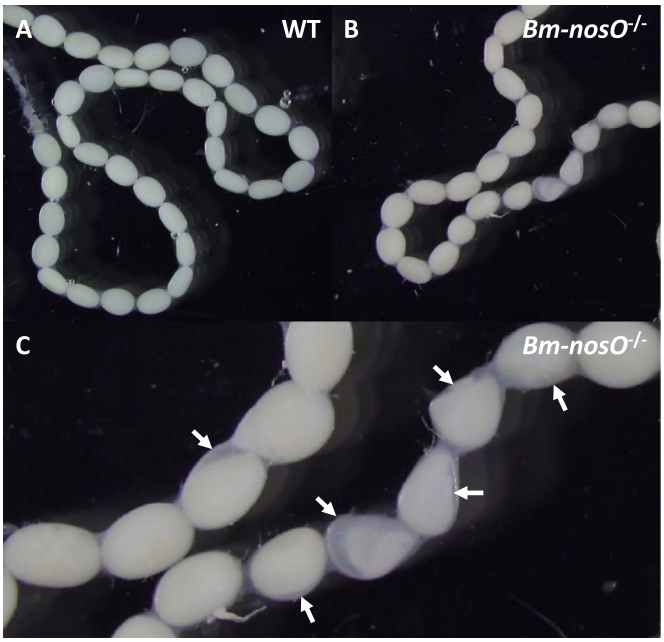

Fig. 5. Morphology of near-mature oocytes in wild type (A) and Bm-nosO homozygotic females (B, C). (C) Enlarged view of a part of (B). Many oocytes in homozygotic females have anomalies of outside and inside morphologies (arrows).

morphologies from wild types were found and produced eggs appeared normal (Fig. 4C) and hatched normally. Surprisingly, however, the number of oocytes within female insects derived from the former crosses (mat-zyg+) was significantly higher compared to that of wild types. This result not only suggested that zygotic expression alone is sufficient for normal oogenesis but also the existence of mechanisms that compensate for the loss of maternal activities concerning oogenesis. However, the role of localized maternal mRNA remained elusive.

As for male germ cell formation, although we could not examine the morphologies of spermatocytes or sperm, we could not detect any difference in the fertilization abilities of Bm-nosO- homozygotic and wild type males. Thus, further analyses concerning spermatogenesis was not conducted.

2.4. Zygotic Bm-nosO localization does not occur prior to cellularization of the energids

Our prime interest in this study was whether the inheritance mode of PGC formation occurs in Bombyx. As described above, this was suggested previously by the localization pattern of Bm-nosO transcript in wild type Bombyx eggs. The results described in the previous section did not provide enough evidences to resolve issue. The observation that germ cell formed in knockout lines at all suggested against the operation of the inheritance mode that is dependent on Bm-nosO and inferred the existence of other mechanisms. Thus, the issue is now the relative contribution of Bm-nosO dependent inheritance mode, if it exists at all. The observation that the number of oocytes increased in Bm-nosO homozygous females because of the presence of the maternal Bm-nosO gene (compare oocyte number in mat-zyg- lines and mat+zyg- lines) suggested for the operation of Bm-nosO-dependent inheritance mode. On the other hand, normal oogenesis in Bm-nosO- heterozygous females derived from Bm-nosO knockout mothers (mat-zyg+) dwarfed the possibility of maternal contribution of Bm-nosO. Here, however, when interpreting the result from the viewpoint of whether inheritance mode operates in Bombyx, we must be careful of the role of zygotic Bm-nosO, i.e. the possibility that the zygotic transcript may also undergo localization and also contribute to the operation of the inheritance mode. In fact, we observed previously that after fertilization, the signal of the localized Bm-nosO transcript appeared to intensify gradually. If this is indeed the case, the above result concerning female heterozygotes could be interpreted more positively in relation to the occurrence of an inheritance mode. To clarify this issue, we next examined whether the Bm-nosO zygotic transcript localizes in eggs. Bm-nosO homozygous females were crossed to wild type males, the resulting eggs were allowed to develop, and the embryos were examined for Bm-nosO expression. We detected no localized Bm-nosO transcript before or just after cellularization (Fig. 7-1B). It was after gastrulation that a Bm-nosO signal was first detected. This result suggested that the zygotic Bm-nosO transcript does not localize before cellularization, indicating that maternal Bm-nosO product alone was responsible for the localized mRNA.

2.5. Maternal Bm-nosO activity is involved in PGC formation

To obtain more direct information on Bombyx germ cell specification mechanisms, we next utilized another early PGC marker, BmVLG. As described in the previous section, Bm-nosO RNAi resulted in the elimination of BmVLG signal. To ascertain if this was true, we examined BmVLG expression in Bm-nosO homozygotes and found that embryonic germ line-specific BmVLG expression was eliminated (Supplementary Fig. 3). Thus, the result of RNAi study was confirmed, and the result in the previous section that germ cells can form in Bm-nosO null lines, agreed well with the observation in the RNAi study that, despite the lack of BmVLG expression in all the Bm-nosO RNAi embryos, RNAi-treated embryos frequently formed germ cells normally.

We next investigated whether or how the maternal Bm-nosO transcript affects BmVLG expression in early stage PGCs or the formation of early stage PGCs by examining the expression of BmVLG in embryos derived from Bm-nosO homozygotic females and wild type males (mat-zyg+). The maternal BmVLG transcript, initially distributed evenly within the germ anlage, is degraded during early embryogenesis, except for in PGCs. Thus, after cellularization, but before gastrulation, PGCs are clearly marked by the remaining BmVLG transcript. We expected, if maternal Bm-nosO is responsible for this type of BmVLG regulation, that PGCs should not be marked by BmVLG expression or PGCs should not be visible in early stage embryos derived from these crosses (mat-zyg+), if indeed Bm-nosO zygotic expression does not occur at this stage. In such early embryos, we observed a small number of PGCs marked by BmVLG expression (Fig. 7-1K). This phenomenon could be explained by two ways; BmVLG expression in PGCs at this stage was independent of the Bm-nosO activities at this stage or Bm-nosO indeed is concerned with Bm-VLG expression at this stage and this effect (BmVLG expression) was brought about by zygotically expressed Bm-nosO function. To distinguish these possibilities, we next examined early BmVLG expression in the Bm-nosO null condition. In Bm-nosO- homozygotic embryos derived from Bm-nosO- homozygotic parents (mat-zyg-), PGCs were not detected by BmVLG expression (Fig. 7-1L). This result indicated that zygotic Bm-nosO expression was responsible for the formation of BmVLG-positive PGCs in the heterozygote (mat-zyg+), i.e. zygotic Bm-nosO expression is involved in PGC specification. Then, we speculated whether maternal Bm-nosO is also involved in PGC specification. To answer this, we examined BmVLG expression in early stage embryos derived from crosses of Bm-nosO- homozygotic males and heterozygotic females. Since half of these embryos, with the maternal Bm-nosO transcript, have the zygotic Bm-nosO transcript and half do not, if maternal Bm-nosO is not involved in PGC specification, early PGCs should not be detected by BmVLG stain in half the embryos. In all the embryos examined, we detected PGCs (n = 52/52, Fig. 7-1M, N), suggesting that maternal Bm-nosO is also involved in PGC specification. This was in good agreement with the apparently small number of PGCs observed in the heterozygote (mat-zyg+).

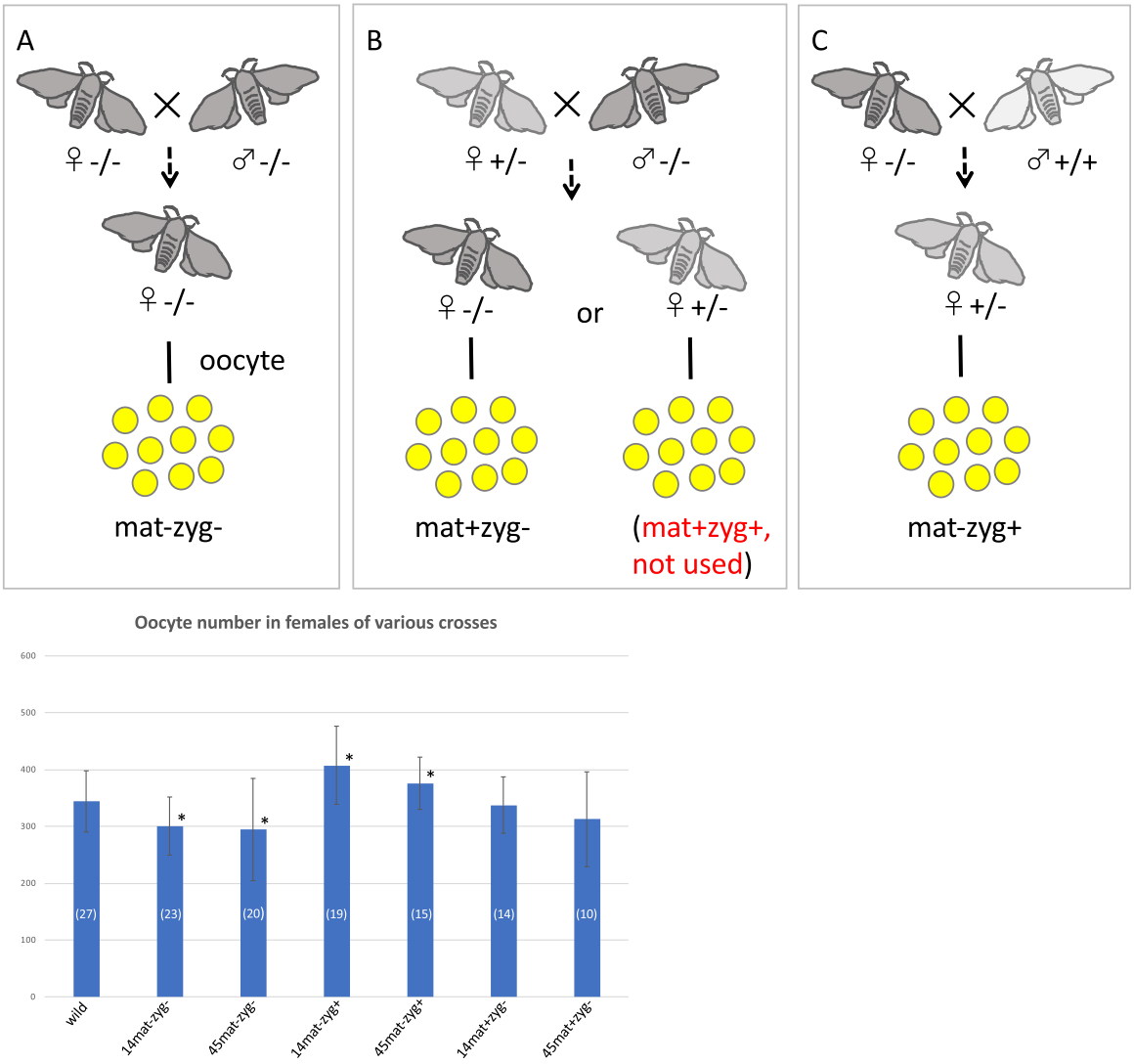

Fig. 6. (6-1) Schematic representation of crosses used in oocyte number counting experiments as described below. (6-2) Oocyte number in females of various crosses. Females of various crosses (indicated) were dissected and the number of mature oocytes were counted. mat+/- concerns whether the females were derived from eggs positive or negative for the Bm-nosO maternal product, i.e. Bm-nosO knockout allele homozygotic or not. zyg+ /- concerns whether the females have (+) or do not have (-) the Bm-nosO knockout allele. mat-zyg- females derived from crosses of Bm-nosO knockout allele homozygotic males and females. mar-zyg+ females derived from crosses of Bm-nosO knockout allele homozygotic females and wild type males. mat+zyg- females were selected from females derived from crosses of Bm-nosO knockout allele heterozygotic males and females. See text for results. The number in each bar represents the number of samples analyzed. Asterisks indicate the sample groups in which the difference in the mean value, as compared to that of wild types, was significant (the p-values are equal to or less than 0.05).

3. Discussion

We previously identified four nanos genes from Bombyx; Bm-nanosM, N, O, P (Nakao et al., 2008). These genes have distinct expression patterns, which suggests their functional difference. Of these, Bm-nosO seemed to be the most relevant to germ cell formation since its maternal transcript appears to be localized at the site of the egg periplasm where future germ cells occur. In the present study, we examined the function of Bm-nosO by RNAi and TALEN-mediated knockout. The results showed that Bm-nosO is involved in oogenesis as well as the regulation of another germ cell marker, BmVLG (Bombyx vasa homolog). Bm-nosO- female homozygotes frequently laid eggs with aberrant morphology, which failed to hatch. Knockout females just after emergence also possessed fewer mature oocytes. The frequency of the occurrence of aberrant eggs differed between individuals. The number of oocytes harbored by these females is also different between individuals; this is also the case with wild type females. However, in one of the two knockout lines examined in detail, we observed ovarioles containing a very small number of oocytes, which has never been observed in wild type females but observed in Bm-nosO RNAi treated samples. These individual differences may be attributable to differences in the genetic or epigenetic background.

The severe oocyte number reduction phenotype is reminiscent of the Drosophila nos mutant phenotype and may reflect the germ-line stem cell maintenance function (Bhat, 1999). However, aberrant oocyte morphology has, to our knowledge, not yet been reported in Drosophila. At present, it is unclear which function of Bm-nosO caused this mutant phenotype. However, since the Bombyx oocyte structure is distinct from Drosophila (Nakao, 2012), it may have some relationship with this phenotype. On the other hand, downregulation of BmVLG expression in the BmnosO knockout, which suggests that these genes are on the same pathway for the formation of germ cells, appears to be related to the known function of Drosophila nanos in suppressing somatic cell fate (Hayashi et al., 2004). In any case, some of the knockout eggs hatch normally and produced fertile offspring, enabling generations to succeed in the Bm-nosO null condition. This may reflect the functional redundancy of Bombyx nanos genes. In addition, together with the unstable nature of oocyte production in Bm-nosO

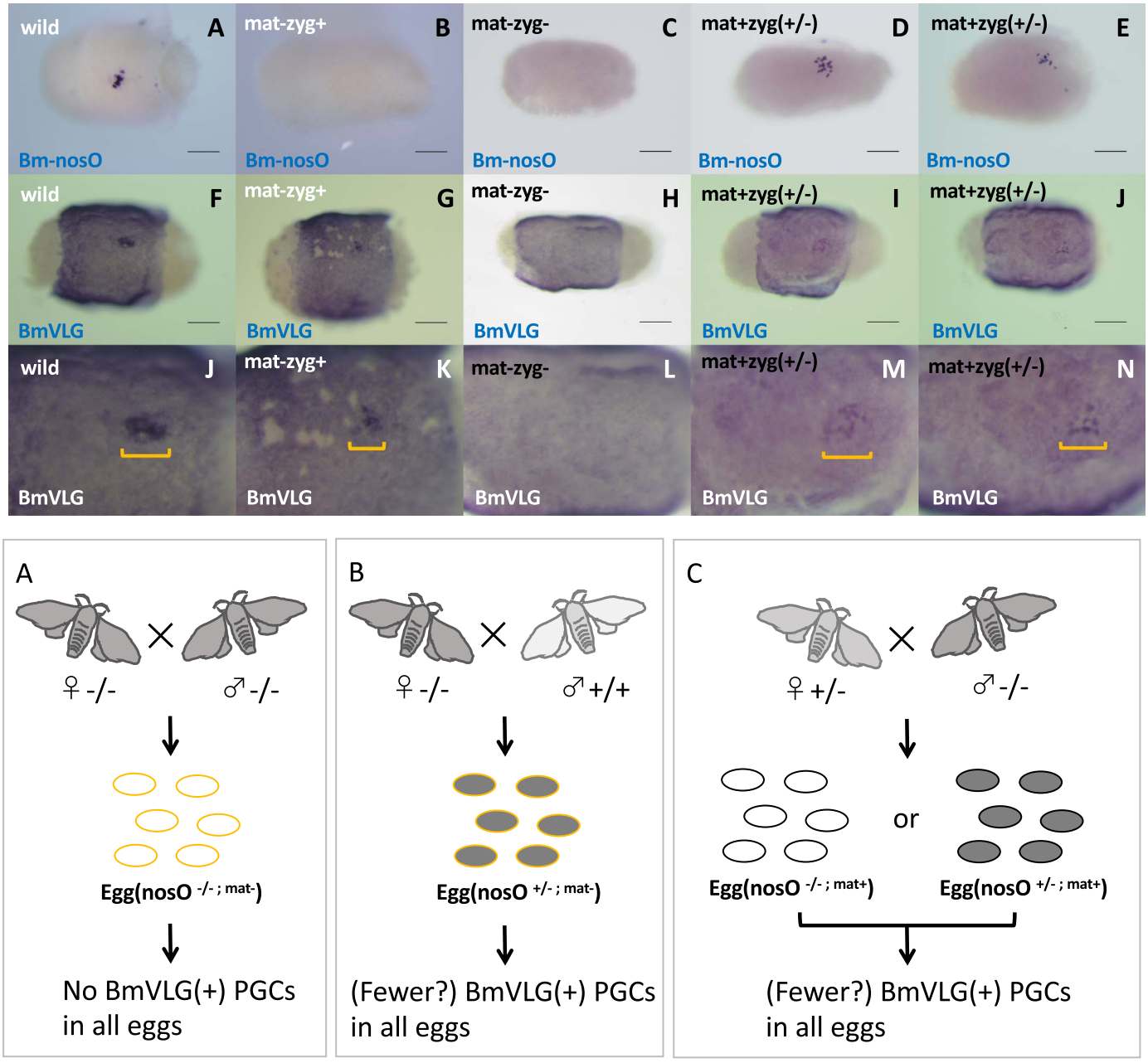

Fig. 7. (7-1) Bm-nosO(A-E) and BmVLG(F-N) expression in embryos. Embryos derived from crosses of wild types (A, F, J), Bm-nosO- homozygotic females and wild type males (B, G, K), Bm-nosO-/Bm-nosO- females and males (C, H, L), and Bm-nosO-/wild type females and males (two examples: D, I, M and E, J, N) were fixed at approximately 20 h AEL and subjected to in situ hybridization using DIG-labelled probes. J-N are enlarged views of the central regions of F-J, respectively, to highlight BmVLG-positive cells. Brackets in J, K, M, and N indicate the locations of BmVLG-positive cells. The number of BmVLG-positive cells appears to be smaller in K, M, and N than in J, whereas no BmVLG-positive cells were detected in L (also see text). (7-2) Summary of results described above.

knockouts as described above, the evolutionarily conserved expression of nanos and vasa homologous genes may function in stabilizing germ cell production.

To date, definite proof of preformation is lacking in Bombyx. The prime interest in our study of Bm-nosO was whether its maternally localized transcript within eggs is involved in the specification of PGCs. We thought it could be answered by generating Bm-nosO knockouts. Female Bm-nosO- heterozygotes (mat-zyg+) derived from Bm-nosO homozygotic mothers (derived from eggs devoid of maternal transcript) produced eggs in a manner indistinguishable from wild type females in terms of morphology, although, curiously, the number of mature oocytes was higher. This suggested that the Bm-nosO maternal transcript is not involved in the specification of germ cells. We further proceeded to investigate the function of Bm-nosO by examining early PGC formation using BmVLG as a marker gene. We found maternal Bm-nosO RNA is involved the formation of early stage PGCs expressing BmVLG formed soon after cellularization. We also found zygotic Bm-nosO activity is also involved in the formation of PGCs at this stage and simultaneous elimination of both Bm-nosO activities (as in Bm-nosO knockout lines) results in the absence of such (BmVLG positive) PGCs. This suggests that there is a temporal overlap between the expression of maternal and zygotic Bm-nosO activity, which contributed to the formation of early PGCs by a redundant mechanism. The most likely explanation for the formation of PGCs by unlocalized zygotic Bm-nosO expression is that there are some additional localized cues similar to that provided by Bm-nosO maternal transcript, which, together with zygotic Bm-nosO activities, dictate the PGC formation at this stage. Without Bm-nosO activity, BmVLG positive PGCs may undergo

apoptosis. In any case, these indicated that inheritance mode indeed operates in Bombyx. Of note, however, as described above, germ cells form without apparent lack of PGCs normally observed at this stage, strongly suggesting that, in addition to this Bm-nosO-dependent inheritance mode, some other germ cell formation mechanisms operate in Bombyx. This could be an inductive mode: in Bombyx inheritance and inductive modes may act in concert to form germ cells in normal circumstances and the inheritance mechanism revealed in this study might function to stabilize germ cell formation (see above), which might be a driving force in the evolution of this inheritance mechanism.

The occurrence of dual modes in one organism has been discussed in the recent reports from an evolutionary point of view (Seervai and Wessel, 2013). Such occurrence enables the transition between the two modes: the transition from ancestral inductive mode to inheritance mode, which appeared to occur multiple times during evolution, and their reversal (Extavour and Akam, 2003; Lynch et al., 2011). Such a continuum of modes between the extreme inheritance and inductive mode was suspected in the sea urchin (Seervai and Wessel, 2013). In the wasp Pimpla also, it has been demonstrated that gonads were populated with germ cells even though the no pole cells formed after destruction of the germline determinant (oosome), suggesting the possibility of the employment of dual modes (Achtelig and Krause, 1971). Scrutiny of germ cell formation mechanisms using modern molecular biological techniques are awaited in these cases. In Bombyx, to validate the possibility of the operation of dual mode suggested in this study, it will be necessary to show that residual germ cell formation activities in Bm-nosO knockout lines are not due to some other inheritance mechanisms, which act in parallel with Bm-nosO dependent inheritance mechanism; an issue which, due to the lack of appropriate PGC marker, we could not clarify in this study.

From another point of view, the phenotype observed in this study could be relevant to 'germline regeneration': germline compensation mechanisms could be invoked by the loss of early PGCs (Dannenberg and Seaver, 2018; Takamura et al., 2002). In this case, such back-up mechanisms could manifest only after removal of PGCs and might not operate during normal development. However, this mechanism may also enable the evolution of a preformation mode. The possibility of such regeneration mechanisms in Bombyx could be supported by the observation in this study that the oocyte number increase observed in mat- /zyg+ individuals.

In conclusion, Bombyx PGC formation appears to be a complex process, even the existence of multiple modes of germ cell specification mechanisms are possible. The study of germ cell formation mechanisms in Bombyx could contribute to the understanding of the evolutionary mechanisms of transition between germ cell specification modes. In addition, as described above, Bombyx possesses four nanos genes with distinct expression patterns. This and other interesting properties concerning embryogenesis appear to be broadly shared in lepidoopterans (Carter et al., 2015; Lynch, 2014; Nakao, 2012, 2016; Quan and Lynch, 2016; Schmidt-Ott and Lynch, 2016). Thus, we believe this study contributes to the understanding of germ cell formation mechanisms and embryogenesis not only in Bombyx, but more generally in lepidopterans, since it has been hypothesized, although not without controversy, that in the evolution of embryogenesis, axis formation is closely related to the evolution of germ cell formation mechanisms (Dixon, 1994; Evans et al., 2014; Johnson and Alberio, 2015; Whittle and Extavour, 2016, 2017).

4. Materials and methods

4.1. Silkworm strains, rearing, and development

The *B. mori* strain *pnd-2* was used in this study. Silkworms were reared on an artificial diet (Nippon Nosanko). Refer to Nagy et al. (1994) for a general description of early *Bombyx* development.

4.2. Generation of Bm-nosO knockouts

The Bm-nosO locus contains six exons designated as E1 to E6. The start codon resides in E3 and the stop codon in E6 (Fig. 3–1). The target sequences for TALENs were searched for in the neighborhood of start and stop codons, respectively, using a TALEN targeter (https://boglab.plp.iasate.edu/). Generation of TALEN-mediated knockouts was performed as previously described (Takasu et al., 2013; Daimon et al., 2014). For construction of TALEN expression plasmids, pBlue-TAL scaffold plasmid was used. G0 individuals were screened for the presence of the edited allele by PCR using three primers (Primers 1, 2, and 3). Primer 1 is a forward primer located inside the intron immediate 5′ of E1, primer 2 is also a forward primer between E3 and E4, and primer 3 is a reverse primer downstream of the stop codon (Fig. 3-1). Successful gene targeting removes the primer 2 binding site. As a result of gene targeting, the combination of primers 1and 3 produces about 780 bp fragment from edited alleles instead of about 450 bp fragment from the wild type allele, whereas the combination of primers 2 and 3 produces about 450 bp fragment only from wild type alleles by PCR. Therefore, the presence of edited alleles should be known by the presence of a 780 bp fragment. After screening, more than 10 positive crosses were obtained, of which four homozygotic lines were established. Genomic DNAs were isolated from those and PCR fragments spanning edited regions amplified using primers 4 and 5 were cloned and sequenced. As expected, successful removal of most of the coding regions was confirmed. Sequences of primers are described below.

4.3. Embryo fixation, in situ hybridization, and RNAi

Embryo fixation, in situ hybridization, and RNAi were performed as previously described (Nakao, 1999, 2012; Nakao et al., 2006).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.10.012.

References

Achtelig, M., Krause, G., 1971. Experiments on uncleared egg of Pimpla turionellae L. (Hymenoptera) for functional analysis of oosome region. Wilhelm Roux Arch. Entwickl. Mech. Org. 167, 164–182.

Bhat, K.M., 1999. The posterior determinant gene *nanos* is required for the maintenance of the adult germline stem cells during Drosophila oogenesis. Genetics 151, 1479–1492.

Calvo, E., Walter, M., Adelman, Z.N., Jimenez, A., Onal, S., Mari-notti, O., James, A.A., 2005. Nanos (nos) genes of the vector mosquitoes, *Anopheles gambiae*, *Anopheles stephensi* and *Aedes aegypti*. Insect Biochem. Mol. Biol. 35 (7), 789–798.

Carter, J.-M., Gibbs, M., Breuker, C.J., 2015. Divergent RNA localisation patterns of maternal genes regulating embryonic patterning in the butterfly Pararge aegeria. PLoS One 10 (12), e0144471. http://dx.doi.org/10.1371/journal.pone.0144471.

Daimon, T., Kiuchi, T., Takasu, Y., 2014. Recent progress in genome engineering techniques in the silkworm, Bombyx mori. Dev. Growth Differ. 56, 14–25.

Dannenberg, L.C., Seaver, E.C., 2018. Regeneration of the germline in the annelid Capitella teleta. Dev. Biol. 440, 74–87.

Dearden, P.K., 2006. Germ cell development in the Honeybee (Apis mellifera); vasa and nanos expression. BMC Dev. Biol. 6, 6.

Dixon, K.E., 1994. Evolutionary aspects of primordial germ cell formation. CIBA Found. Symp. 182, 92–120.

Donnell, D.M., Corley, L.S., Chen, G., Strand, M.R., 2004. Caste determination in a polyembryonic wasp involves inheritance of germ cells. Proc. Natl. Acad. Sci. USA 101, 10095–10100.

Ephrussi, A., Lehmann, R., 1992. Induction of germ cell formation by *oskar*. Nature 358, 387–392.

Evans, T., Wade, C.M., Chapman, F.A., Johnson, A.D., Loose, M., 2014. Acquisition of germ plasm accelerates vertebrate evolution. Science 344 (6180), 200–203.
Extavour, C.G., Akam, M.E., 2003. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 130, 5869–5884.
Gavis, E.R., Lehmann, R., 1994. Translational regulation of nanos by RNA localization. Nature 369 (6478), 315–318.
Hay, B., Jan, L.Y., Jan, Y.N., 1988. A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell 55, 577–587.
Hayashi, Y., Hayashi, M., Kobayashi, S., 2004. Nanos suppresses somatic cell fate in Drosophila germ line. Proc. Natl. Acad. Sci. USA 101, 10338–10342.
Johnson, A.D., Alberio, R., 2015. Primordial germ cells: the first cell lineage or the last cells standing? Development 142 (16), 2730–2739.
Khila, A., Abouheif, E., 2008. Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. Proc. Natl. Acad. Sci. USA 105, 17884–17889.
Kobayashi, S., Yamada, M., Asaoka, M., Kitamura, T., 1996. Essential role of the posterior morphogen nanos for germline development in Drosophila. Nature 380, 708–711.
Lall, S., Ludwig, M.Z., Patel, N.H., 2003. Nanos plays a con- served role in axial patterning outside of the Diptera. Curr. Biol. 13 (3), 224–229.
Lasko, P.F., Ashburner, M., 1990. Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. Genes Dev. 4, 905–921.
Lin, G.-W., Chang, C.-C., 2009. Cloning and developmental characterization of four vasa genes, Apvasa1-4, in the parthenogenetic and viviparous pea aphid Acyrthosiphon pisum. Mech. Dev. 126, S252.
Lynch, J.A., 2014. Diversity of molecules and mechanisms in establishing insect anterior–posterior polarity. Curr. Opin. Insect Sci. 1, 39–44.
Lynch, J.A., Desplan, C., 2010. Novel modes of localization and function of nanos in the wasp Nasonia. Development 137, 3813–3821.
Lynch, J.A., Ozuak, O., Khila, A., Abouhelf, E., Desplan, C., Roth, S., 2011. The phylogenetic origin of oskar coincided with the origin of maternally provisioned germ plasm and pole cells at the base of Holometabora. PLoS Genet. 7, e1002029.
Mito, T., Nakamura, T., Sarashina, I., Chang, C.C., Ogawa, S., Ohuchi, H., Noji, S., 2008. Dynamic expression patterns of vasa during embryogenesis in the cricket Gryllus bimaculatus. Dev. Genes Evol. 218, 381–387.
Miya, K., 1958. Studies on the embryonic development of the gonad in the silkworm, *Bombyx mori*L. Part I. differentiation of germ cells. J. Fac. Agric. Iwate Univ. 3, 436–467.
Nagy, L., Riddiford, L., Kiguchi, K., 1994. Morphogenesis in the early embryo of the Lepidopteran *Bombyx mori*. Dev. Biol. 165, 137–151.
Nakamura, T., Extavour, C.G., 2016. The transcriptional repressor Blimp-1 acts downstream of BMP signaling to generate primordial germ cells in the cricket Gryllus bimaculatus. Development 143, 255–263.
Nakao, H., 1999. Isolation and characterization of a *Bombyx vasa*-like gene. Dev. Genes Evol. 209, 312–316.
Nakao, H., 2012. Anterior and posterior centers jointly regulate *Bombyx* embryo body segmentation. Dev. Biol. 371, 293–301.
Nakao, H., 2016. Hunchback knockdown induces supernumerary segment formation in Bombyx. Dev. Biol. 413, 207–216.
Nakao, H., Hatakeyama, M., Lee, J.M., Shimoda, M., Kanda, T., 2006. Expression pattern of Bombyx vasa-like (BmVLG) protein and its implications in germ cell development. Dev. Genes Evol. 216, 94–99.
Nakao, H., Matsumoto, T., Oba, Y., Niimi, T., Yaginuma, T., 2008. Germ cell specification and early embryonic patterning in Bombyx mori as revealed by nanos orthologues. Evol. Dev. 10 (5), 546–554.
Quan, H., Lynch, J.A., 2016. The evolution of insect germline specification strategies. Curr. Opin. Insect Sci. 13, 99–105.
Rezende-Teixeira, P., Rosa, M.C., Palomino, N.B., Machado-Santelli, G., 2009. Gene expression profile in germ line and its involving in development of Rhynch- osciara americana. Mech. Dev. 126 (S99), 03–P109.
Schmidt-Ott, U., Lynch, J.A., 2016. Emerging developmental genetic model systems in holometabolous insects. Curr. Opin. Genet. Dev. 39, 116–128.
Schroder, R., 2006. vasa mRNA accumulates at the posterior pole during blastoderm formation in the flour beetle Tribolium castaneum. Dev. Genes Evol. 216, 277–283.
Seervai, R.N.H., Wessel, G.M., 2013. Lessons for inductive germline determination. Mol. Reprod. Dev. 80, 590–609.
Takamura, K., Fujimura, M., Yamaguchi, Y., 2002. Primordial germ cells originate from the endodermal strand cells in the ascidian Ciona intestinalis. Dev. Genes Evol. 212, 11–18. http://dx.doi.org/10.1007/s00427-001-0204-1.
Takasu, Y., Sajwan, S., Daimon, T., Osanai-Futahashi, M., Uchino, K., Sezutsu, H., Tamura, T., Zurovec, M., 2013a. Efficient TALEN construction for Bombyx gene targeting. PLoS One 18, e73458.
Tanaka, E.D., Hartfelder, K., 2009. Sequence and expression pattern of the germ line marker vasa in honey bees and stingless bees. Genet. Mol. Biol. 32, 582–593.
Whittle, C.A., Extavour, C.G., 2016. Refuting the hypothesis that the acquisition of germ plasm accelerates animal evolution. Nat. Commun. 7, 12637.
Whittle, C.A., Extavour, C.G., 2017. Causes and evolutionary consequences of primordial germ-cell specification mode in metazoans. Proc. Natl. Acad. Sci. USA 114, 5784–5791.
Zhurov, V., Terzin, T., Grbic, M., 2004. Early blastomere determines embryo proliferation and caste fate in a polyembryonic wasp. Nature 432, 764–769.