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Evolution of Developmental Control Mechanisms

Germ cells of the centipede *Strigamia maritima* are specified early in embryonic development

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ABSTRACT

We provide the first systematic description of germ cell development with molecular markers in a myriapod, the centipede *Strigamia maritima*. By examining the expression of *Strigamia vasa* and *nanos* orthologues, we find that the primordial germ cells are specified from at least the blastoderm stage. This is a much earlier embryonic stage than previously described for centipedes, or any other member of the Myriapoda. Using these genes as markers, and taking advantage of the developmental synchrony of *Strigamia* embryos within single clutches, we are able to track the development of the germ cells throughout embryogenesis. We find that the germ cells accumulate at the blastopore; that the cells do not internalize through the hindgut, but rather through the closing blastopore; and that the cells undergo a long-range migration to the embryonic gonad. This is the first evidence for primordial germ cells displaying these behaviours in any myriapod. The myriapods are a phylogenetically important group in the arthropod radiation for which relatively little developmental data is currently available. Our study provides valuable comparative data that complements the growing number of studies in insects, crustaceans and chelicerates, and is important for the correct reconstruction of ancestral states and a fuller understanding of how germ cell development has evolved in different arthropod lineages.

Introduction

The germ cells of an organism are the only cell type that contributes genetically to the next generation. Across the Metazoa, organisms segregate the germ line from the soma, either during embryonic development or in early larval life (reviewed in Extavour and Akam, 2003). The first cells that will give rise exclusively to the germ line are called the primordial germ cells (PGCs). The mechanism of germ line specification varies across taxa and can be viewed as a continuum characterized by two extremes. At one end is a class of mechanisms we call 'cytoplasmic'. These are characterized by the production of a special region of cytoplasm, either in the unfertilized egg or after fertilization in the early embryo, which is only inherited by a subset of embryonic cells that become the germ cells. This specialized cytoplasm is called germ plasm, and contains localized factors necessary and sufficient for germ line specification. At the opposite extreme is a class of mechanisms that we call 'induction'. In this class, the germ line is specified by signals sent from neighbouring cells. Evidence now available for mice and for the insect, *Gryllus bimaculatus*, shows that BMP signalling plays a role in induction (Donoughe et al., 2014; Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001).

In three of the four myriapod groups, classical literature describes a late origin of the PGCs from the coelomic pouches of the mesoderm (millipedes, symphylans and pauropods; Dohle, 1964; Tiegs, 1940, 1947). In the fourth myriapod group, the centipedes, classical studies on two species of *Scolopendra* identify a population of cells in the late embryonic/early post-embryonic gonad with distinctive cytological characteristics (larger nuclei and more abundant cytoplasm) that gives rise to the gametes, consistent with its identity as the centipede PGCs (Heymons, 1901). However, Heymons also reports an accumulation of cells earlier in development, at the posterior end of the embryonic rudiment, which later migrate anteriorly and become enclosed within the gonadal epithelium; and he identifies these earlier cells as the PGCs (Heymons, 1901). However, he notes that these cells are not distinguishable morphologically as PGCs until they reach the gonad, and provides little or no evidence for this description in the accompanying figures. Given the ambiguity, previous surveys of germ cell development have concluded that centipede PGCs arise late in embryogenesis from gonadal mesoderm (Extavour and Akam, 2003; Nieuwkoop and Sutasurya, 1981; but not in Johannsen and Butt, 1941). This highlights a general limitation of studies based on recognizing the germ cells cytologically. This limitation means it is not always possible to distinguish

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a late segregation of germ cells from merely a late acquisition of the distinctive cytological properties. The use of conserved molecular markers may circumvent this problem, and can give a more accurate estimate of the timing of PGC segregation (Tsunekawa et al., 2000; Wu et al., 2011; Yoon et al., 1997).

There are a growing number of studies on insects and crustaceans that are applying molecular techniques to elucidate the timing and mechanism of specification of the PGCs in these taxa (Calvo et al., 2005; Chang et al., 2002, 2006; Dearden, 2006; Ewen-Campen et al., 2013a, 2013b; Extavour, 2005; Gerberding et al., 2002; Gupta and Extavour, 2013; Khila and Abouheif, 2010; Lynch and Desplan, 2010; Nakao, 1999; Nakao et al., 2006; Oezhan-Kizil et al., 2009; Schroder, 2006). The myriapods are an ancient lineage of arthropods, and the living outgroup to the clade containing insects and all crustaceans (Rota-Stabelli et al., 2011). They are therefore phylogenetically well placed to determine ancestral states and the polarity of evolutionary change within the whole group of mandibulate arthropods. However, to our knowledge, at present there are no studies of germ cell development using molecular markers in any myriapod.

In this study we set out to establish, during embryogenesis of the centipede *Strigamia maritima*, when and where the germ cells first appear, and how they subsequently develop. We aimed to shed light on the mechanism of germ line specification in *Strigamia*, and to find evidence that would distinguish between a cytoplasmic and an inductive mode of germ line specification.

We use *Strigamia* orthologues of two conserved molecular markers of PGCs, *vasa* and *nanos*, to show conclusively that the PGCs are specified at least as early as the blastoderm stage, a much earlier embryonic stage than currently believed. This resolves an ambiguity in the older literature. Furthermore, we show that the PGCs accumulate at the blastopore; that they internalize through the closing blastopore, and not through the hindgut; and that they undergo a long-range migration to the embryonic gonad. Finally, we find a surprising localization of maternal Vasa protein within the germinal vesicle of developing oocytes, and suggest that this might act as a mechanism for localizing Vasa protein asymmetrically in early embryos.

Methods

Identification and cloning of germ line markers

A genome and adult and embryonic transcriptomes for *S. maritima* (genome release Smar_1.0) are available at http://www.ncbi.nlm.nih.gov/assembly/322118/. An annotated gene set is provided at EnsemblMetazoa (http://metazoa.ensembl.org/Strigamia_maritima/Info/Index) (Chipman et al., submitted). Cassandra Extavour and colleagues identified a set of 31 genes orthologous (one-to-one or one-to-many) to genes known to have roles in germ line specification or differentiation in other species during annotation of the *Strigamia* genome (Chipman et al., submitted). The names and Ensembl IDs of this set are provided in Supplementary Table 1. We designed gene-specific primers for 8 of these putative germ line markers and amplified PCR products from embryonic cDNA. The amplified fragments were cloned into pGEM-T Easy vector (Promega). The names, Ensembl IDs and primer sequences used for this set of 8 genes are provided in Supplementary Table 1. We screened these 8 possible germ line markers by examining their expression in stage 5 *Strigamia* embryos using in situ hybridization. Stage 5 was selected because the germ cells are cytologically differentiated (large round nuclei with diffuse chromatin) and easy to recognize at this stage. Of these 8 candidates, only the *Strigamia* orthologue of *vasa,* and one of the two *nanos* orthologues, *nanos2*, showed unambiguous staining of the putative PGCs.

Embryo collection, fixation and staging

Embryos were collected in the field from a population near Brora, Scotland and fixed as described previously (Brena and Akam, 2012). Accurate developmental series were obtained by examining the dynamics of a gene expression pattern, or a particular developmental process, within single clutches of *Strigamia* eggs. We took advantage of the degree of developmental synchrony within a clutch to obtain a close time series of embryos, which were placed in developmental order according to morphological criteria in Brena and Akam (2012), or using independent molecular markers, as indicated below.

Collection of adult females, ovary preparation and fixation

Adult female centipedes were collected at two different sites in the UK and maintained in the laboratory at 10–12 °C for several months. Females were collected from Brora, Scotland in June 2013 and from Brownsea Island, Poole, Dorset in October 2013. Ovaries were dissected from adult females in a solution of phosphate-buffered saline (1 × PBS) and 10 mM $MgCl_2$ (anaesthetic). Isolated ovaries were fixed immediately in 4% formaldehyde in 1 × PBS for 25 min at room temperature. After fixation, ovaries were washed 3 times for 5 min each in 1 × PBS and then transferred step-wise into methanol (25:50:75:100%), and stored at −20 °C.

RNA extraction and RT-PCR

For the RT-PCR experiment, RNA was extracted from pools of embryos from single clutches. Live eggs were returned to the lab on moist Petri dishes, as described in Brena and Akam (2012). To determine the stage of the clutches, two test embryos were removed from each clutch, fixed, DAPI stained to reveal nuclei and staged as in Brena and Akam (2012). The remaining eggs were snap frozen in liquid nitrogen and stored at −80 °C. RNA was extracted from the frozen samples using the RNeasy Mini Kit (Qiagen). Embryos were lysed and homogenized in buffer RLT according to manufacturer's instructions, except for RNA extractions from clutches at embryonic stages 1 and 2, to which 10 μg of poly-A carrier RNA (Qiagen) was added to the lysate before proceeding with the rest of the protocol. Residual genomic DNA contamination was removed by including the on-column DNase digestion step with the RNase-Free DNase Set in the manufacturer's protocol (Qiagen). RNA was eluted in 50 μl of RNase-free water. cDNA was synthesized using Expand Reverse Transcriptase (Roche) primed with random hexanucleotides (Roche), according to manufacturer's instructions (4 μl RNA in 20 μl reaction volume for all samples). Fragments were amplified by PCR (2 μl of cDNA prep in 25 μl PCR) using ThermoPrime Plus DNA polymerase (Thermo Scientific). The product of cDNA synthesis reaction in which no RNA had been added to the reaction was used as a no template control. Primer sequences used to detect *nanos2*, *vasa*, *bra1*, *en* and *tubulin* are provided in Supplementary Table 2. All primers were designed such that the amplified products crossed exon-intron boundaries. PCR products were visualized on 1% agarose gel with 0.1 ng/μl ethidium bromide. PCRs were run under the following conditions: an initial denaturation step of 95 °C for 8 min; followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min; and finally an extension step of 72 °C for 4 min.

in situ hybridization on embryos and adult ovaries

in situ hybridization reactions were carried out on whole mount embryos as described previously (Chipman et al., 2004),

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