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Evolution of Developmental Control Mechanisms

Asymmetric localization of germline markers *Vasa* and *Nanos* during early development in the amphioxus *Branchiostoma floridae*

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ABSTRACT

The origin of germline cells was a crucial step in animal evolution. Therefore, in both developmental biology and evolutionary biology, the mechanisms of germline specification have been extensively studied over the past two centuries. However, in many animals, the process of germline specification remains unclear. Here, we show that in the cephalochordate amphioxus *Branchiostoma floridae*, the germ cell-specific molecular markers *Vasa* and *Nanos* become localized to the vegetal pole cytoplasm during oogenesis and are inherited asymmetrically by a single blastomere during cleavage. After gastrulation, this founder cell gives rise to a cluster of progeny that display typical characters of primordial germ cells (PGCs). Blastomeres separated at the two-cell stage grow into twin embryos, but one of the twins fails to develop this *Vasa*-positive cell population, suggesting that the vegetal pole cytoplasm is required for the formation of putative PGCs in amphioxus embryos. Contrary to the hypothesis that cephalochordates may form their PGCs by epigenesis, our data strongly support a preformation mode of germ cell specification in amphioxus. In addition to the early localization of their maternal transcripts in the putative PGCs, amphioxus *Vasa* and *Nanos* are also expressed zygotically in the tail bud, which is the posterior growth zone of amphioxus. Thus, in addition to PGC specification, amphioxus *Vasa* and *Nanos* may also function in highly proliferating somatic stem cells.

Introduction

The segregation of germline cells from the somatic cell lineages during animal development is considered an effective way to ensure early protection of the genome from somatic mutations during the life history of multicellular animals (Extavour, 2007). There are two major modes for the specification of PGCs (Extavour and Akam, 2003). In some animals, PGCs are specified by the localization of maternal cytoplasmic determinants into specific blastomeres during early development (preformation); however, in other species, PGCs are determined later in development by inductive signals from neighboring tissues (epigenesis). Comparative studies based on both morphological criteria and conserved germ cell-specific markers, such as the *Vasa* and *Nanos* gene products (Ewen-Campen et al., 2010), have provided new insights into the evolution of germ cell specification mechanisms (Brown and Swalla, 2007; Brown et al., 2009; Chang et al., 2002; Dill and Seaver, 2008; Extavour and Akam, 2003; Extavour et al., 2005; Extavour, 2007; Fabioux et al., 2004; Ikenishi and Tanaka, 2000; Ozhan-Kizil et al., 2009; Pfister et al., 2008; Rebscher et al., 2007; Rosner et al., 2009; Shirae-Kurabayashi et al., 2006; Swartz et al., 2008; Voronina et al., 2008; Yoon et al., 1997); however, many important questions remain unanswered: for example, how did mechanisms for PGC specification evolve during metazoan radiation; and can comparative studies reveal the ancestral/derived modes of PGC specification in specific animal groups?

In studying the evolution of germ cell specification mechanisms, we focused on cephalochordates (commonly known as amphioxus or lancelets) because of their basal phylogenetic position within the chordates (Bourlat et al., 2006; Putnam et al., 2008). The mode of germ cell specification in amphioxus is controversial. The earliest recognizable PGCs are found in clusters at the ventral extremities of the somites in late larvae (Nieuwkoop and Sutasurya, 1979; Ruppert, 1997). Due to their segmental distribution and rather late origin during development, it has been argued that PGCs are formed epigenetically in amphioxus (Extavour, 2007; Nieuwkoop and Sutasurya, 1979).

However, an electron microscopic study identified a region of subcortical cytoplasm near the vegetal pole of amphioxus eggs that contains whorls of endoplasmic reticulum (ER) with associated electron-dense particles and mitochondria (Holland and Holland, 1992). Based on morphological comparisons, this vegetal pole plasm was suggested to be the amphioxus equivalent of the germ plasm (Holland and Holland, 1992); however, the fate of this pole plasm was not analyzed beyond the mid-blastula stage, and its precise molecular components were not determined. In this study, we demonstrate that

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the gene products of the germ cell-specific markers *Vasa* and *Nanos* co-localize with the vegetal pole plasm during amphioxus development, and the cells containing these markers display typical characters of PGCs. In addition, blastomere separation experiments show that this localized pole plasm is required for the formation of putative PGCs in amphioxus embryos. Thus, we propose that these cells are the presumptive amphioxus PGCs. These results provide strong evidence for the preformation mode of germ cell determination in amphioxus.

Materials and methods

Cloning of Bf-Vasa and Bf-Nanos

A BLAST search using zebrafish Vasa (NP_571132) as the query against the assembled *Branchiostoma floridae* draft genome (Putnam et al., 2008) led to the identification of two predicted gene models (estExt_GenewiseH_1.C_5230018 and e_gw.58.215.1) coding for a Vasa-like protein. Detailed analysis of the sequence and synteny structure around these two gene models confirmed that they represent two different haplotypes of the same *Vasa* locus in the amphioxus genome. We used the better annotated gene model (estExt_GenewiseH_ 1. C_5230018) to search the amphioxus cDNA/EST database (Yu et al., 2008b) (http://amphioxus.icob.sinica.edu.tw/) and identified one cDNA cluster (00124) representing *Vasa* cDNA. Sequencing the longest cDNA clone (bfne054i20, 3165 bp in length) from this cluster indicated that it contained most of the coding sequence of *Vasa*, along with its 3′UTR; however, part of the 5′ coding sequence and the 5′UTR were missing. We, therefore, performed 5′RACE to obtain the full-length cDNA for *Vasa* using an outer gene-specific primer (5′-AATTCTCGGGTGTCGGGATT-3′) and the 5′RACE Outer Primer provided in the FirstChoice RLM-RACE Kit (Ambion) with cDNA prepared from unfertilized eggs. Next, we performed nested PCR using the inner gene-specific primer (5′-TCCTCTCCGCATCTGAAACA-3′) and the 5′RACE Inner Primer. We obtained a 946-bp cDNA fragment that overlapped with the cDNA clone bfne054i20. Assembly of the isolated cDNA clone and the 5′RACE fragment revealed a cDNA sequence of 3939 bp with a putative open reading frame encoding a protein of 785 amino acids. We designated the assembled cDNA *Bf-Vasa* (NCBI GenBank accession number HM004550). A similar BLAST search using human NANOS1 (NP_955631) as the query led to the identification of one predicted amphioxus gene model (fgenesh2_pg.scaffold_142000014) for amphioxus *Nanos*. Subsequently, we identified the corresponding cDNA cluster (01416) in the amphioxus cDNA/EST database. The longest cDNA clone (bfad001o17) from this cluster was 2684 bp, with an open reading frame encoding a putative Nanos protein of 294 amino acids, and we designated this sequence *Bf-Nanos* (NCBI GenBank accession number HM004549). These orthology assignments were further confirmed by sequence alignments and/or molecular phylogenetic analysis of the translated amino acid sequences from the amphioxus *Vasa* and *Nanos* cDNA sequences (Suppl. Figs. S1–S3). The translated amino acid sequences were aligned using the Clustal X program (Jeanmougin et al., 1998), and the alignment was confirmed manually. After removing gaps, the verified alignments were used to construct phylogenetic trees with the MEGA program, version 4.0, based on the neighbor-joining method (Tamura et al., 2007). Bootstrap support values were calculated by 1000 pseudoreplications.

Quantitative PCR (Q-PCR) analysis

Messenger RNA from each embryonic stage was isolated using the RNeasy Micro kit (Qiagen) and then reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) as previously described (Yu and Holland, 2009c). The resulting cDNA was used as the template for Q-PCR analysis. The Q-PCR analysis was performed on a Roche LightCycler 480 machine using the LightCycler 480 SYBR Green I Master system (Roche). The expression levels of *Vasa* and *Nanos* were normalized to the 18S rRNA level at each specific stage. The sequences of Q-PCR primers were as follows: BfVasaQF, 5′-CCAATACCATGCCCAAGACT-3′ and BfVasaQR, 5′-ACAAACCAAGTGCCTTCACC-3′ for *Bf-Vasa*; BfNanosQF, 5′-CCCTGTCCTT-ATGGCCTACA-3′ and BfNanosQR, 5′-GTTGGGTAGCTGGTTGGTGT-3′ for *Bf-Nanos*; Bf18SQF, 5′-CCTGCGGCTTAATTTGACTC-3′ and Bf18SQR, 5′-AACTAAGAACGGCCATGCAC-3′ for 18S rRNA.

In situ hybridization

We used a primer matching the vector sequence adjacent to the 3′ end of the cDNA insert with a T7 promoter sequence added to its 5′ end as a reverse primer (pDONR222-T7-Reverse, 5′-TAATACGACTCACTA-TAGGGAGGGGATATCAGCTGGATG-3′) and a primer matching the vector sequence adjacent to the 5′ end of the cDNA insert with an SP6 promoter sequence added as a forward primer (pDONR222-SP6-Forward, 5′-ATTTAGGTGACACTATAGAAGACGGCCAGTCTTAAGCTC-3′) for PCR amplification of the full-length cDNA for riboprobe synthesis. The following PCR protocol was used: 2 min 94 °C; 5 cycles of 30 s 94 °C, 30 s 45 °C, 3 min 68 °C; 25 cycles of 30 s 94 °C, 30 s 58 °C, 3 min 68 °C; 1 cycle of 7 min 68 °C. DIG-labeled *Bf-Vasa* antisense riboprobes and fluorescein-labeled *Bf-Nanos* antisense riboprobes were synthesized using T7 RNA polymerase. Control sense riboprobes were synthesized by SP6 RNA polymerase. The procedure for *in situ* hybridization in amphioxus embryos was performed as previously described (Yu et al., 2008a; Yu and Holland, 2009b). For color detection, alkaline phosphatase-conjugated anti-DIG or anti-fluorescein antibodies were used (Roche). Alkaline phosphatase reaction products were visualized with NBT-BCIP (dark-purple color for DIG-labeled probes) or Fast Red tablets (Sigma) (red color for fluorescein-labeled probes). For double fluorescent *in situ* hybridization, we used anti-DIG-POD and anti-fluorescein-POD antibodies (Roche) to detect the anti-sense riboprobes and then used the TSA Plus Cyanine 3 & Fluorescein system (PerkinElmer) to amplify the fluorescent signal. In some cases, DAPI (Invitrogen, 1 μg/ml in PBST) was used for nuclear staining, and the plasma membrane stain CellMask Orange (Invitrogen, 2.5 μg/ml in PBST) was used to mark the general outline of the cells. The samples were visualized and photographed using either a Zeiss Axio Imager.A1 microscope with DIC optics or a Leica TCS-SP5-AOBS confocal microscope. The TEM methods used were as described previously (Holland and Holland, 1992).

Antibody generation and immunoblot

A polyclonal antibody was produced using the COOH-terminal region of the BfVasa protein (amino acid 271 to the stop sequence) for rabbit immunization. The recombinant BfVasa protein was expressed using the pET system (Novagen). After the initial immunization, the rabbit was boosted and bled in two-week intervals. Batches of antisera were checked for antibody titer by western blots. For western blot analysis, 5 μg of amphioxus proteins from each developmental stage was resolved by SDS-PAGE, and the proteins were then transferred onto a PVDF membrane. The membrane was probed with primary antibodies (anti-BfVasa serum 1:50,000, or an anti-alpha-tubulin antibody, Sigma, 1:50,000), detected with an HRP-conjugated goat anti-rabbit secondary antibody (Jackson, 1:50,000), and developed with the SuperSignal West Dura Extended Duration Substrate (Pierce).

Antibody staining

For BfVasa protein staining, samples were fixed in 4% PFA-MOPS-EGTA fixation buffer (Yu and Holland, 2009a) for 1 h at room temperature or overnight at 4 °C, and samples were then stored in 70% ethanol (EtOH) at −20 °C until use. Fixed samples were rehydrated in a stepwise fashion (50% EtOH/PBS; 30% EtOH/PBS; and PBST washes three times). After rehydration, samples were permeabilized in 5 μg proteinase K/ml PBST for 3–5 min. Proteinase K