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Nanos functions to maintain the fate of the small micromere lineage in the sea urchin embryo

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ABSTRACT

The translational regulator *nanos* is required for the survival and maintenance of primordial germ cells during embryogenesis. Three *nanos* homologs are present in the genome of the sea urchin *Strongylocentrotus purpuratus*, all of which are expressed with different timing in the small micromere lineage. This lineage is set-aside during embryogenesis and contributes to constructing the adult rudiment. Small micromeres lacking *Sp-nanos1* and *Sp-nanos2* undergo an extra division and are not incorporated into the coelomic pouches. Further, these cells do not accumulate Vasa protein even though they retain *vasa* mRNA. Larvae that develop from *Sp-nanos1* and *2* knockdown embryos initially appear normal, but do not develop adult rudiments; although they are capable of eating, over time they fail to grow and eventually die. We conclude that the acquisition and maintenance of multipotency in the small micromere lineage requires *nanos*, which may function in part by repressing the cell cycle and regulating other multipotency factors such as *vasa*. This work, in combination with other recent results in *Ilyanassa* and *Platynereis dumerilii*, suggests the presence of a conserved molecular program underlying both primordial germ cell and multipotent cell specification and maintenance.

Introduction

Multicellular animals are composed of many specialized cell types, but only the germ cells are capable of passing genetic information on to the next generation. To propagate the species, select cells are set aside during embryogenesis to form the future germline. A conserved set of genes is required to specify and/or maintain the germline during embryogenesis. *Nanos*, one such gene, is required for fertility in *Drosophila*, *Caenorhabditis elegans*, zebrafish, and mice (Kobayashi et al., 1996; Subramaniam and Seydoux, 1999; Koprunner et al., 2001; Tsuda et al., 2003). Germline progenitor cells of the *Drosophila* embryo (pole cells) that lack *nanos* exhibit various defects, including an inability to migrate to the gonad, loss of transcriptional and mitotic quiescence, expression of somatic cell markers, and apoptosis; consequently *nanos*-null pole cells fail to develop into functional germ cells (Kobayashi et al., 1996; Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Hayashi et al., 2004). *Nanos* functions together with *pumilio* to repress the translation of the cell cycle regulator *cyclin B* and the pro-apoptotic gene *head involution defect* (*hid*) in pole cells, thus directly controlling the cell cycle and survival of these cells (Asaoka-Taguchi et al., 1999; Kadyrova et al., 2007). Similarly, loss of *nanos* function in *C. elegans* leads to premature primordial germ cell (PGC) proliferation and in mice its loss results in PGC apoptosis. Thus, *nanos* appears to have conserved functions in animal germlines (Subramaniam and Seydoux, 1999; Suzuki et al., 2008).

In addition to its germline functions, *nanos* is expressed more broadly in multipotent cells. In *Hydra* polyps *nanos* is expressed in multipotent interstitial cells (I-cells), which give rise both to several somatic cell types and to germ cells (Mochizuki et al., 2000). In the polychaete annelid, *Platynereis dumerilii*, and the snail, *Ilyanassa*, both of which develop through a larval stage, multipotent cells of the embryo specifically express *nanos* (Rebscher et al., 2007; Rabinowitz et al., 2008). The *nanos*-positive multipotent cells of *P. dumerilii* give rise to both all of the trunk mesodermal cell types of the adult segments, and to germ cells (Rebscher et al., 2007). *Nanos* is also expressed in the 4d lineage of *Ilyanassa*; this lineage gives rise to adult mesodermal and endodermal tissues (Render, 1997; Rabinowitz et al., 2008). Functional studies in *Ilyanassa* suggest that *nanos* is critical to maintain the fate of the 4d lineage, as the loss of *nanos* function in embryos results in the loss of all 4d-derived adult structures (Rabinowitz et al., 2008).

The majority of the species in the phylum Echinodermata are maximal indirect developers in which embryogenesis culminates with the formation of a free-swimming, feeding larva that supports its developing adult rudiment. At metamorphosis the rudiment will give rise to the juvenile. During embryogenesis in these maximally indirect developing organisms, groups of cells are set aside for use in adult rudiment construction. Unlike the cells that will give rise to the larval structures *per se*, these cells retain proliferative and developmental potential (Peterson et al., 1997). The small micromere lineage of the sea

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urchin embryo, which specifically accumulates *nanos* mRNA, is one such group of set-aside cells (Juliano et al., 2006). During embryogenesis, four small micromeres are formed at the 32-cell stage as a result of two unequal cleavage divisions: a vegetal unequal fourth cleavage division gives rise to a 16-cell embryo with 4 micromeres and a subsequent unequal division of the micromeres results in the formation of 4 small micromeres. In the blastula the small micromeres reside at the vegetal plate where they divide once before being transported through the blastocoel at the tip of the archenteron during gastrulation. The eight small micromere descendants are then partitioned into the left and right coelomic pouches, the site of adult rudiment formation in the pluteus larva. The coelomic pouches are derived from two sources: 60% from a subset of the macromere descendants and 40% from the small micromere descendants (Cameron et al., 1991). During larval formation, the small micromere descendants move into the coelomic pouches from their position at the tip of the archenteron in the gastrula (Pehrson and Cohen, 1986; Tanaka and Dan, 1990). It is clear that the small micromere lineage gives rise solely to adult tissues, but the identity of these tissues has not been experimentally determined (Pehrson and Cohen, 1986; Tanaka and Dan, 1990).

Due to both their slow cell cycle and contribution to adult tissues, it has been suggested that the small micromeres are germline precursors (Tanaka and Dan, 1990). In support of this hypothesis, the conserved germline genes *nanos*, *vasa*, and *piwi* are specifically expressed in the small micromeres (Juliano et al., 2006; Voronina et al., 2008). However, the small micromeres proliferate shortly after they reach the coelomic pouches (Tanaka and Dan, 1990). This would not be expected if the small micromeres were indeed equivalent to PGCs, which typically stay mitotically and/or transcriptionally quiescent until they reach the somatic gonad (Su et al., 1998; Subramaniam and Seydoux, 1999; Seydoux and Braun, 2006; Seki et al., 2007). An alternative hypothesis suggests that the small micromeres are instead multipotent and will give rise to various adult tissues (Ransick et al., 1996). These seemingly contradictory hypotheses can be reconciled when considering recent results pointing to a broader role for conserved germline genes in multipotent cells that give rise to both germ cells and to somatic cells (Mochizuki et al., 2000; Mochizuki et al., 2001; Reddien et al., 2005; Rebscher et al., 2007; Palakodeti et al., 2008; Pfister et al., 2008; Rabinowitz et al., 2008; Swartz et al., 2008). Thus, the small micromere lineage may give rise to both germ and somatic cell types of the sea urchin adult. Here we test the function of *nanos* in the small micromere lineage, a likely multipotent cell population, in order to further understand the potentially ancestral role of this gene in establishing and maintaining multipotential cell populations during embryogenesis.

Materials and methods

Animals

Strongylocentrotus purpuratus were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by either 0.5M KCl injection or by shaking. Eggs were collected in ASW or filtered seawater and sperm was collected dry. To obtain embryos, fertilized eggs were cultured in ASW or filtered seawater at 16 °C. When early stage embryos were required for labeling, fertilization was performed in the presence of 1 mM 3-amino-triazol (3-AT) (Sigma; St. Louis, MO) to inhibit cross-linking of the fertilization envelopes. Before fixing, envelopes were removed by passing the embryos through 80 μM and 64 μM Nitex® mesh. Careful monitoring was required to ensure the integrity of the embryos.

Identification and cloning of Sp-nanos homologs

Three *Sp-nanos* homologs were identified in the *S. purpuratus* genome (spbase.org) by BLAST analysis (Altschul et al., 1990). Full-length genes (complete ORF plus some UTR sequences) were amplified from 24- or 48-h embryonic cDNA by PCR and cloned into pGEMT-EZ (Promega; Madison, WI) for sequencing. Amplification of *Sp-nanos2* required only one round of PCR (Juliano et al., 2006), whereas *Sp-nanos1* and *Sp-nanos3* required 2 rounds of PCR with nested primer sets to amplify full-length sequences. Primers were as follows (listed 5′ to 3′): Sp-Nanos2-F1—TTCTTGACTAGCTCTACGACGTACT; Sp-Nanos2-R1—TCGAGACGAGTAGACCCTACA; Sp-Nanos1-F1 – TAGATCATTCAAGACAAGCTCT; Sp-Nanos1-F2—GGAAGTACATCGCATTTTACAA; Sp-Nanos1-R1—CTAGAAGATCTTAACGGTCG; Sp-Nanos1-R2—TGGGGTTCGATACTGGGATC; Sp-nanos3-F1—GTACACCCGTGTCCGTGAG; Sp-nanos3-F2—CCAATACAACATTAATCTTCAAG; Sp-nanos3-R1—TGTCAAAAACTTTGTGCCAGAA; Sp-nanos3-R2—TACTTCCTACATAGGACGAC. The 3′ and 5′ UTR sequences of the *Sp-nanos* homologs were extended with RACE (Ambion; Austin, TX). Using PAUP, an unrooted neighbor-joining phylogram was made from full-length *nanos* coding sequences collected from NCBI (for accession numbers see Fig. 1); bootstrap replicate values are from 1000 iterations (Swofford, 2002).

Whole mount RNA in situ hybridization (WMISH)

Sequences used to make antisense WMISH probes for *Sp-nanos1*, *Sp-nanos2*, and *Sp-nanos3* were amplified from 48-h embryonic cDNA and cloned into pGEMT-EZ. The *Sp-nanos3* probe template includes the entire ORF plus 650 bases of the 3′UTR; the primer sets used for nested PCR are described above. Two *Sp-nanos2* probes were used: the first is previously described in Juliano et al. (2006) and the second includes 950 bases of the 3′UTR. The primer set used to amplify the *Sp-nanos2* 3′UTR is as follows (listed 5′ to 3′): Sp-Nanos2-F2—TGTAGGGTCTACTCGTCTCGA; Sp-Nanos2-R2—CACCCAGCAATCAGTACTTTC. The *Sp-nanos1* probe template includes the entire ORF plus 130 bases of the 3′UTR; the nested primer sets used for amplification are as follows: forward primers are described above; Sp-nanos1-R3—AGAATGGAGTACTTGCGTAC; Sp-nanos1-R4—ATACACCCAGCAATCAGTAC. The pGEMT-EZ plasmids were linearized using either *Sal*I (T7 transcription) or *Nco*I (SP6 transcription) (Promega; Madison, WI). Antisense DIG-labeled RNA probes were constructed using a DIG RNA labeling kit (Roche; Indianapolis, IN). WMISH experiments were performed as previously described (Minokawa et al., 2004) and the alkaline phosphatase reaction was carried out for 22 h. The *Sp-vasa* probe is described in Juliano et al., 2006. A non-specific DIG-labeled RNA probe complimentary to pSPT 18 (Roche; Indianapolis, IN) was used as a negative control. For uninjected embryos, all steps were carried out in 2 mL screw-top tubes (National Scientific Supply; Claremont, CA). For injected and manipulated embryos, all steps were carried out in 96-well round-bottom PVC plates (ThermoFisher Scientific; Rockford, IL). Samples were imaged on a Zeiss Axiovert 200M microscope equipped with a Zeiss color AxioCam MRc5 camera (Carl Zeiss, Inc.; Thornwood, NY).

Fluorescent RNA in situ hybridization (FISH)

Sp-vasa FISH on mock-injected and *Sp-nanos1* and *2* knockdown embryos was performed as previously described above through the blocking steps. Subsequently, samples were incubated overnight with anti-DIG-POD (Roche; Indianapolis, IN) diluted 1:1500 in blocking buffer II (Minokawa et al., 2004) at room temperature with rotation. Samples were washed 6 times for 1 h in TBST (10 mM Tris-HCl, pH7.4; 0.15M NaCl; 0.1% Tween-20). Signal was detected using the Tyramide Signal Amplification (TSA) kit (Perkin Elmer; Waltham, MA). Samples were washed once with 1X amplification solution and then incubated with cyanine 3 TSA working solution for 15 min. Samples were washed 6 times with TBST, the 5th wash contained a 1:1000 dilution of a 10 mg/mL Hoechst stock solution (Molecular Probes; Carlsbad, CA) for DNA labeling. Z-stacks were acquired for 5 mock-injected and