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Maternal expression of a NANOS homolog is required for early development of the leech *Helobdella robusta*

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Abstract

The gene *nanos* (*nos*) is a maternal posterior group gene required for normal development of abdominal segments and the germ line in *Drosophila*. Expression of *nos*-related genes is associated with the germ line in a broad variety of other taxa, including the leech *Helobdella robusta*, where zygotically expressed *Hro-nos* appears to be associated with primordial germ cells. The function of maternally inherited *Hro-nos* transcripts remains to be determined, however. Here, the function of maternal *Hro-nos* is examined using an antisense morpholino (MO) knockdown strategy, as confirmed by immunostaining and western blot analysis. HRO-NOS knockdown embryos exhibit abnormalities in the distribution of micromeres during cleavage. Subsequently, their germinal bands are positioned abnormally with respect to the embryonic midline and the micromere cap, epiboly fails, and the HRO-NOS knockdown embryos die. This lethality can be rescued by injection of mRNA encoding an eGFP::HRO-NOS fusion protein. HRO-NOS knockdown embryos make their normal complements of mesodermal and ectodermal teloblasts, and the progeny of these teloblasts segregate into distinct mesodermal and ectodermal layers. These results suggest that maternal *Hro-nos* is required for embryonic development. However, contrary to previous suggestions, maternal inherited *Hro-nos* does not appear necessary for ectoderm specification.

Keywords: Leech; Annelid; Spiralian; *nanos*; D-quadrant specification; Maternal inheritants; Lophotrochozoa; Spiral cleavage; Micromeres

Introduction

nanos (*nos*) was first identified as a maternally inherited posterior group gene in the fruit fly *Drosophila melanogaster* (Nusslein-Volhard et al., 1987; Lehmann and Nusslein-Volhard, 1991). Most of the maternal *nos* transcripts are diffusely distributed throughout the zygote and are not translated (Bergsten and Gavis, 1999). A small fraction of the transcripts are localized and translated at the posterior pole (Gavis and Lehmann, 1994; Bergsten and Gavis, 1999; Irish et al., 1989; Forrest and Gavis, 2003), giving rise to a NOS protein gradient that participates in repressing translation of maternally inherited *hunchback* mRNA in the posterior of the embryo (Irish et al., 1989; Wreden et al., 1997). This repression is required for development of the abdominal segments (Irish et al., 1989). *nanos* is also required for normal development of the *Drosophila* germ line (Kobayashi et al., 1996), where it is also thought to participate in repressing transcription (Deshpande et al., 1999, 2005) and translation (Asaoka-Taguchi et al., 1999).

Characterization of a *nos* homolog in *Schistocerca* (Lall et al., 2003) indicates that a dual function of *nos*-related genes, in germ line development and in early embryonic polarity, was ancestral to at least the insects. *nos* homolog transcripts are associated with the germ line in cnidarians (*Hydra*, Mochizuki et al., 2000; *Podocoryne*, Torras et al., 2004; *Nematostella*, Extavour et al., 2005), nematodes (Subramaniam and Seydoux, 1999; Kraemer et al., 1999), and vertebrates (*Xenopus*, Mosquera et al., 1993; MacArthur et al., 1999; zebrafish Koprunner et al., 2001; mouse, Tsuda et al., 2003), but there is no evidence for involvement of maternal *nos* homologs in patterning the early embryos in any of these taxa, with the possible exception of cnidarians. In the sea anemone *Nematostella vectensis*, one of two *nos*-class genes is present as a maternal transcript (Extavour et al., 2005) that either persists or

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is selectively re-expressed in prospective endoderm during gastrulation (Extavour et al., 2005).

In the glossiphoniid leech *Helobdella robusta* (phylum Annelida), a *nanos* ortholog (*Hro-nos*) is also expressed both maternally and zygotically (Pilon and Weisblat, 1997; Kang et al., 2002). Could these observations mean that a maternally expressed *nanos*-class gene functioned in patterning the embryo of the protostome ancestor (the results from nematodes notwithstanding), or are they an example of convergent evolution? Zygotic expression of *Hro-nos* is eventually restricted to presumptive primordial germ cells (Kang et al., 2002), but the developmental significance of the maternally inherited *Hro-nos* transcripts remains to be determined. Elucidating the function of maternal *Hro-nos* should contribute to distinguishing between these possibilities.

As with other clitellate annelids, *Helobdella* oocytes are fertilized internally but arrest in metaphase I of meiosis until after zygote deposition. Between polar body formation and first cleavage, cytoplasmic rearrangements form domains of yolk-deficient cytoplasm (teloplasm) at the animal and vegetal poles of the zygote (Astrow et al., 1989; Holton et al., 1994; Fig. 1A). During cleavage, teloplasm is segregated to the D quadrant and thence to the segmentation stem cells (teloblasts) that constitute the posterior growth zone of the clitellate embryo (Weisblat and Huang, 2001; Fig. 1A). *Hro-nos* mRNA is abundant in the oocyte; its localization and translation in the early embryo have been analyzed by northern and western blots, in situ hybridization, and immunostaining on intact embryos and dissected blastomeres (Pilon and Weisblat, 1997; Kang et al., 2002).

Hro-nos transcripts become localized to the teloplasm in the zygote and are equally distributed between the animal and vegetal hemispheres (Kang et al., 2002). Transcript levels decline gradually during cleavage (Pilon and Weisblat, 1997). Translation appears to be delayed until fertilization; HRO-NOS protein is first detected in the 2-cell stage. It should be noted that we cannot exclude the possibility that *Hro-nos* is being transcribed zygotically during cleavage. But since *Hro-nos* transcript levels are decreasing continuously from the levels present in the oocyte (Pilon and Weisblat, 1997), it is most likely that this early HRO-NOS expression represents translation primarily if not exclusively from maternal transcripts.

HRO-NOS expression peaks at fourth cleavage as macromere D′ cleaves to form proteloblasts DM and DNOPQ, precursors of segmental mesoderm and ectoderm, respectively (Fig. 1). At this stage, HRO-NOS is more highly expressed in the ectodermal precursor DNOPQ than in the mesodermal precursor DM (Pilon and Weisblat, 1997). This difference in HRO-NOS protein levels correlates with the distribution of maternal *Hro-nos* mRNA, which is also more abundant in DNOPQ than in DM (Kang et al., 2002). This could represent either differential localization or differential stabilization, or both, because the overall levels of *Hro-nos* are decaying during cleavage (Pilon and Weisblat, 1997). In the context of previous embryological studies on the specification of ectoderm in leech (Nelson and Weisblat, 1991, 1992), these observations led to the proposal that translation of maternal *Hro-nos* may function in specifying DNOPQ as ectoderm, which would represent a novel function for *nanos*-class genes (Pilon and Weisblat, 1997).

To test this hypothesis, we have examined the developmental function of maternal *Hro-nos* mRNA, using an antisense morpholino oligomer (AS MO) to knock down HRO-NOS expression. HRO-NOS knockdown embryos formed the normal complements of mesodermal and ectodermal teloblasts, contrary to the hypothesis that maternal expression of HRO-NOS is a critical factor in specifying the different fates of mesodermal (DM) and ectodermal (DNOPQ) precursors. Instead, embryos with reduced HRO-NOS expression arrested in development near the onset of epiboly. This developmental arrest was preceded by disorganization of the micromere cap during

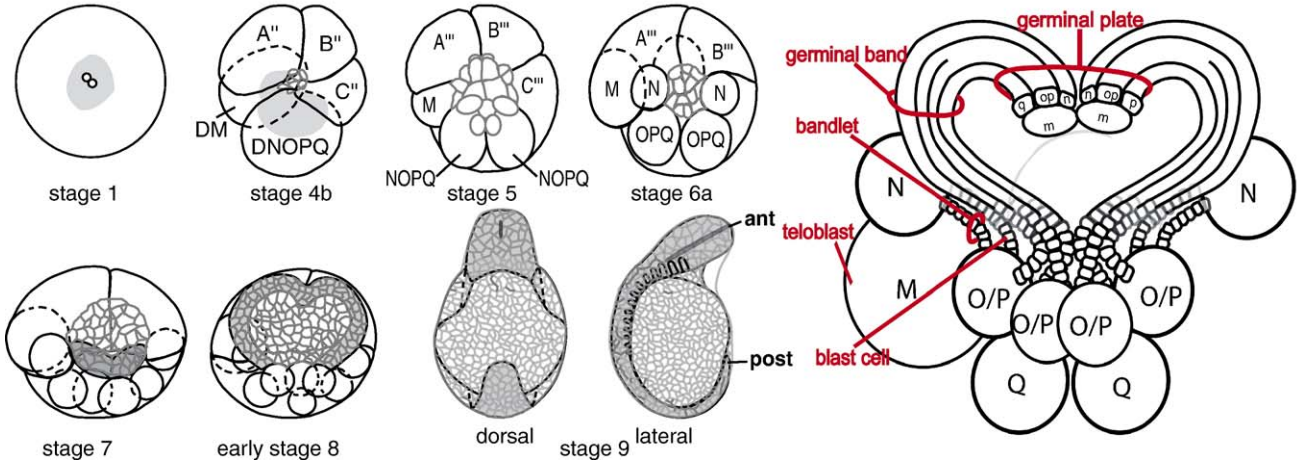

Fig. 1. Relevant stages of *H. robusta* development. Drawings depict animal pole/dorsal views (anterior up) at all stages, plus lateral views (anterior to left) of lateral selected stages. See text for details. Polar bodies are depicted by small circles at early stage 1. Teloplasm is depicted by shading at late stages 1 and 4b. Micromeres and their derivatives are outlined in gray in stages 4b–9. Germinal bands and germinal plate are indicated by gray shading under the micromere-derived epithelium in stages 7–9. The relationship of the teloblasts and their derivatives at early stage 8 (animal/dorsal view) is shown in greater detail in the drawing at right. Abbreviations: ant, anterior; post, posterior.