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High-resolution fate map of the snail *Crepidula fornicata*: The origins of ciliary bands, nervous system, and muscular elements

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Abstract

The littorinimorph gastropod *Crepidula fornicata* shows a spiralian cleavage pattern and has been the subject of studies in experimental embryology, cell lineage, and the organization of the larval nervous system. To investigate the contribution of early blastomeres to the veliger larva, we used intracellular cell lineage tracers in combination with high-resolution confocal imaging. This study corroborates many features derived from other spiralian fate maps (such as the origins of the hindgut and mesoderm from the 4d mesentoblast), but also yields new findings, particularly with respect to the origins of internal structures, such as the nervous system and musculature that have never been described in detail. The ectomesoderm in *C. fornicata* is mainly formed by micromeres of the 3rd quartet (principally 3a and 3b), which presumably represents a plesiomorphic condition for molluscs. The larval central nervous system is mainly formed by the micromeres of the 1st and 2nd quartet, of which 1a, 1c, and 1d form the anterior apical ganglion and nerve tracks to the foot and velum, and 2b and 2d form the visceral loop and the mantle cell. Our study shows that both first and second velar ciliary bands are generated by the same cells that form the prototroch in other spiralians and apparently bear no homology to the metatroch found in annelids.

Keywords: Cell lineage; Gastropod; Veliger larva; Spiralia; Ectomesoderm; Ciliary bands; Nervous system; Digestive tract

The Veliger's a lively tar, the liveliest afloat,
A whirling wheel on either side propels his little boat;
But when the danger signal warns his bustling submarine,
He stops the engine, shuts the port, and drops below unseen.
(Garstang, 1951).

Introduction

Knowledge of cell lineage fate maps relating the development of specific blastomeres to the formation of larval/adult structures has proven very useful in understanding embryonic development and the homologies of cells between various groups of organisms. Most theories of bilaterian evolution are based on establishing the ancestral origin of larval/adult structures, such as the ciliary bands (e.g. the "Trochaea" hypothesis), origins/organization of mesoderm, and the organization of the nervous system among a wide range of metazoans, e.g. protostomes and deuterostomes (e.g. Jägersten, 1955; Remane, 1963; Nielsen, 1979; Nielsen and Nørrevang, 1985; Arendt and Nübler-Jung, 1997; Arendt et al., 2001). Establishing the common evolutionary origin of any given structure is facilitated by the study of organisms that display a stereotyped cleavage program in which individual cells can be identified and homologized between a wide range of different taxa. Animals that undergo the spiral cleavage program are among the most amenable to this line of evolutionary inquiry. Taxa that display the spiral cleavage program (e.g. molluscs, annelids, nemerteans, echiurans, sipunculids, platyhelminths) belong to the lophotrochozoan clade, whose phylogenetic relationships are beginning to become resolved (Giribet et al., 2006;

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Passamaneck and Halanych, 2006; Rousset et al., 2006). The highly conserved early developmental program of spiralians allows for direct comparisons of the developmental fate of individually identified, homologous blastomeres (Wilson, 1898; Henry and Martindale, 1999).

This work describes the results of intracellular lineage tracing in the gastropod, *Crepidula fornicata.* While the formation of surface structures are relatively easy to follow and have been reported in several other spiralian taxa (Boyer et al., 1996; Dictus and Damen, 1997; Render, 1997; Henry and Martindale, 1998; Henry et al., 2004; Maslakova et al., 2004; Ackermann et al., 2005), the development of many internal organs such as components of the nervous system, musculature, and digestive tract have been difficult to follow. This study uses three-dimensional reconstruction and confocal imaging to identify the origins of such structures in greater detail. This study not only clears up a number of errors and inconsistencies in an earlier fate map (Conklin, 1897), which preceded the use of cell autonomous lineage tracers, it elucidates the evolution of differences in the fates of particular cells among different spiralian taxa, which have also been studied using modern approaches. These data highlight the importance of determining the range of differences in cell fate maps among distinct clades of spiralian embryos to reveal phylogenetic signatures in these varied developmental programs.

Materials and methods

Embryos of C. fornicata

Gravid adults of *C. fornicata* were collected near Woods Hole, MA, during the months of June and July. Fertilized eggs were obtained by removing them from their egg capsules with watchmaker forceps in gelatin-coated Petri dishes and washed two times with 0.22 μm millipore filtered seawater (MFSW). Although *C. fornicata* takes up to 2 months to hatch under normal conditions, well-formed veliger stages appear approximately 7 days after oviposition at room temperature (21°–23 °C). Developing embryos had to be reared in MFSW containing penicillin (100 U/ml) and streptomycin sulfate (200 μg/ml) to prevent bacterial growth. Embryos and larvae were transferred when necessary to fresh MFSW with antibiotics every 2–3 days.

Lineage tracing

Individual blastomeres of the different stages were pressure injected with Fluoro Ruby dextran and/or Rhodamine Green dextran (Molecular Probes, Eugene, OR) as previously described by (Martindale and Henry, 1995a,b). Due to the asymmetric localization of polar lobe contents (Henry et al., 2006), it was possible to precisely identify each and every cell injected in the developing embryo up through the formation of the fourth quartet of micromeres.

Fixation and documentation

After relaxation with chloral hydrate (5.94 mg/ml) for several minutes, the veliger larvae were fixed in 4% paraformaldehyde in MFSW for 30 min–1 h at room temperature. After fixation, veligers were washed three times with phosphate-buffered saline (PBS: 0.175 mM $NaCl_2$, 8.41 mM Na_2HPO_4, 1.86 mM NaH_2PO_4), incubated for one hour in PBT (PBS+0.2% Triton) followed by incubation in Alexa 488-labeled phalloidin (2.5 μg/100 μl PBT) for 1 h at room temperature (Martindale and Henry, 1995b). Stained larvae were again washed three times in PBS and stained for 15 min in 2 μg/ml DAPI in PBS. For staining of the nervous system fixed veliger larvae were washed three times in PBS and blocked for 1 h in PBT containing 10% normal goat serum. The larvae were incubated in a primary antibody against FMRFamide (ImmunoStar Inc., Hudson, WI) or serotonin (ImmunoStar Inc., Hudson, WI) raised in rabbit at a concentration of 1:250 in PBT+10% normal goat serum at 4 °C overnight. After several washes in PBT+1% BSA, the veligers were incubated with the secondary antibody (Goat-anti rabbit, Alexa 594 conjugated, Molecular Probes, Eugene, OR) at 4 °C overnight (dilution 1:200). Stained larvae were mounted in glycerol and images were taken with a Zeiss Imager.Z1 and an ORCA ER camera (Hamamatsu Inc., Bridgewater, NJ) in different focal planes. Through focus images (Z stacks) were obtained using the Volocity Aquisition software (Improvision, Inc., Coventry, UK) and images volume-rendered using Volocity 4.0.1. Individual preps were examined using a Zeiss 510 confocal microscope to elucidate fine structure that was not clear using standard fluorescent imaging. Images of living larvae were captured using a Zeiss Axioscope microscope equipped with a Zeiss Axiocam and Axiovision software.

Results

Early cleavage and larval morphology of C. fornicata

A time course of the early cleavage divisions is shown in Fig. 1 and the number of specimens examined for each quadrant is summarized in Table 1. The labeling patterns observed were highly reproducible, as is the case in other spiralian embryos (Henry and Martindale, 1999; Nielsen, 2004, 2005). In addition, the asymmetries associated with the localization of the polar lobe contents following the first and second cleavage divisions allowed us to identify specific quadrants at the time of their injection (Henry et al., 2006). These conditions permit the unambiguous assignment of specific blastomere fates and lineage patterns. Larval morphology and the organization of the larval muscular and nervous systems were subsequently examined in the post-torsional veliger larva using confocal microscopy (Fig. 2). Diagrams of basic larval morphology are shown in Figs. 2A–B. The complex muscular system is readily visible using phalloidin staining (Figs. 2C–F). The larval nervous system consists of an apical ganglion with ciliated sensory cells, which may be visualized with anti-FMRFamide or anti-serotonin antibodies (Figs. 2G–H). The apical ganglion

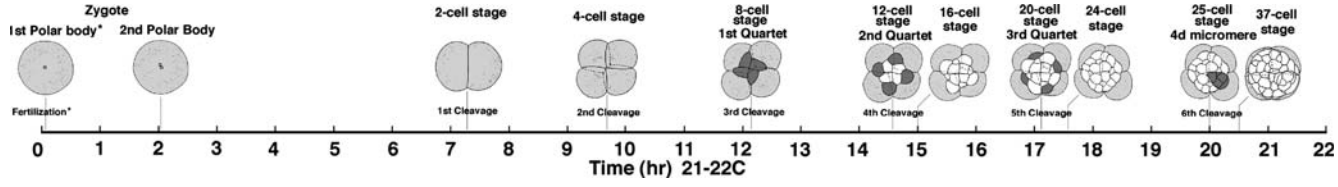

Fig. 1. Time scale of the early development of *C. fornicata.* Developmental time scale from fertilization to the 37-cell stage. Note that the exact time of fertilization is uncertain, as it occurs internally. Times represent the average of numerous collections of embryos. First, second, third quartet micromeres, and 4d are more darkly shaded as they are born.