Evolution of Developmental Control Mechanisms

Lineage analysis of micromere 4d, a super-phylotypic cell for Lophotrochozoa, in the leech *Helobdella* and the sludgeworm *Tubifex*

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ARTICLE INFO

ABSTRACT

The super-phylum Lophotrochozoa contains the plurality of extant animal phyla and exhibits a corresponding diversity of adult body plans. Moreover, in contrast to Ecdysozoa and Deuterostomia, most lophotrochozoans exhibit a conserved pattern of stereotyped early divisions called spiral cleavage. In particular, bilateral mesoderm in most lophotrochozoan species arises from the progeny of micromere 4d, which is assumed to be homologous with a similar cell in the embryo of the ancestral lophotrochozoan, more than 650 million years ago. Thus, distinguishing the conserved and diversified features of cell fates in the 4d lineage among modern spiralians is required to understand how lophotrochozoan diversity has evolved by changes in developmental processes. Here we analyze cell fates for the early progeny of the bilateral daughters (M teloblasts) of micromere 4d in the leech *Helobdella* sp. Austin, a clitellate annelid. We show that the first six progeny of the M teloblasts (em1–em6) contribute five different sets of progeny to non-segmental mesoderm, mainly in the head and in the lining of the digestive tract. The latter feature, associated with cells em1 and em2 in *Helobdella*, is seen with the M teloblast lineage in a second clitellate species, the sludgeworm *Tubifex tubifex* and, on the basis of previously published work, in the initial progeny of the M teloblast homologs in molluscan species, suggesting that it may be an ancestral feature of lophotrochozoan development.

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Introduction

A central question in developmental biology is that of how changes in developmental processes underlie the diversification of body plans evident in extant animals. For addressing this question, spiralian taxa (Mollusca, Annelida, Platyhelminthes, Nemertea, and Entoprocta and others) provide species with homologous cells in their early embryos that lead to a remarkably diverse set of adult body plans. They share a characteristic pattern of early embryonic cell divisions (spiral cleavage) that is now regarded as an ancestral character of the super-phylum Lophotrochozoa (Dunn et al., 2008; Hejnol et al., 2009). In spiral cleavage, the second embryonic axis is established by specifying one quadrant of the embryo as the unique "D quadrant" (by cell interactions in equal cleavers or by the segregation of determinants in unequal cleavers (Freeman and Lundelius, 1992). Micromere 4d, arising within the D quadrant at sixth cleavage, typically divides equally to form left and right precursors of bilaterally symmetric mesoderm (but see Meyer et al., 2010), and thus provides an example of inter-phyletic homology at the single cell level that has no known parallel in the other metazoan super-phyla.

In the leech *Helobdella*, a clitellate annelid, micromere 4d is designated proteloblast DM″; its bilateral division gives rise to two large stem cells (M teloblasts), whose iterated divisions yield precursors (m blast cells) of the segmental mesoderm (Fernández and Stent, 1980; Zackson, 1982; Weisblat and Shankland, 1985; Bissen and Weisblat, 1989). Beyond this segmental contribution, early progeny of the M teloblasts also contribute to the unsegmented prostomium at the anterior (Anderson, 1973; Zackson, 1982; Gleizer and Stent, 1993). This contribution is of particular interest for comparative studies because it arises relatively early on in the 4d lineage and thus might be expected to show greater conservation across species. The prostomial contribution of the M lineage was poorly defined in these early studies, however, due in part to technical limitations.

Knowledge of the early mesodermal lineages is also necessary for understanding segmentation in leeches and allied taxa. In vertebrates and insects, segments are formed by creating boundaries within fields of initially equipotent cells. In clitellate annelids by contrast, segments represent the extensive interdigitation of spatially stereotyped clones arising from cells in five longitudinal arrays of m, n, o, p and q blast cells; the blast cells arise from a teloblastic posterior growth zone (see Weisblat and Shankland, 1985; Wedeen and Shankland, 1997 for further details). Leech segments may be defined either in terms of the septa arising from the mesodermal hemisomites or in terms of the

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ganglionic repeats within the ventral nerve cord, which straddle the septa; hence the boundaries of neural and mesodermal segments are out of phase with one another; the first purely segmental mesodermal hemisomite is the one that straddles the first two segmental ganglia (R1 and R2). Here, we employ the neural definition of segment boundaries in keeping with most current workers and because the ganglia are more reliably observed throughout development.

The interdigitation of serially homologous clones means that segments at the anterior end of the animal do not receive the complement of cells that would normally be contributed by yet more anterior blast cell clones. This interdigitation is most pronounced for the m blast cell clones, whose definitive progeny span 3 segments in the mid-body of the animal (Weisblat and Shankland, 1985). Does the embryo compensate for the lack of the normal mesodermal complement in the anteriormost segments, and if so, how?

Here, using high-resolution cell lineage tracing techniques, we have studied the early progeny of the M teloblasts in greater detail. We show that, prior to initiating the production of purely segmental m blast cells (sm cells), each M teloblast produces six early mesodermal cells (em cells), which contribute wholly or in part to non-segmental mesoderm. As previously described, all sm cells undergo identical stereotyped early divisions and give rise to homologous sets of pattern elements whose position along the anterior/posterior axis is determined by the birth order of their blast cell of origin (Fig. 1; Weisblat and Shankland, 1985; Gleizer and Stent, 1993). In contrast, the six em cells fall into five groups that differ from each other and from standard sm cells in their early division patterns (with the exception of em6 whose early divisions are indistinguishable from sm cells); each em cell type contributes a distinct component to the later embryo. In addition, we show that em5 and

em6 give rise to hybrid clones, contributing cell types to the first two segments that in midbody segments would be provided by the interdigitation of more anterior m clones.

A parallel re-examination of the 4d lineage in the oligochaete *Tubifex* reveals that in this annelid, too, cell 4d contributes to anterior non-segmental tissue. These two worms have different foregut morphologies and thus distinct anterior contributions from 4d. These differences further illustrate the principle that changes in the developmental program of the 4d lineage are associated with the diversity of spiralian body plans.

Materials and methods

Embryos

Embryos of *Helobdella* sp. (Austin; Hau) collected from Austin, Texas, were obtained from a laboratory breeding colony. Embryos were cultured in HL saline and maintained at 23 °C as previously described (Song et al., 2002). Staging and cell nomenclature are as defined previously for *H. robusta* (Weisblat and Huang, 2001) however there are species specific differences in the cell cycle rates between *H. robusta* and the species used in this study *H.* sp. (Zhang and Weisblat, 2005; Gonsalves and Weisblat, 2007). Embryos of *Tubifex tubifex* were collected as previously described in (Shimizu, 1982).

Plasmid injection, mRNA synthesis, and mRNA injection

pEF-H2B:GFP plasmid (Gline et al., 2009) was injected at a concentration of 96 ng/μl with 3 mg/ml fixable tetramethylrhodamine dextran (RDA; Molecular Probes, Eugene, OR). *h2bGFP* mRNA was
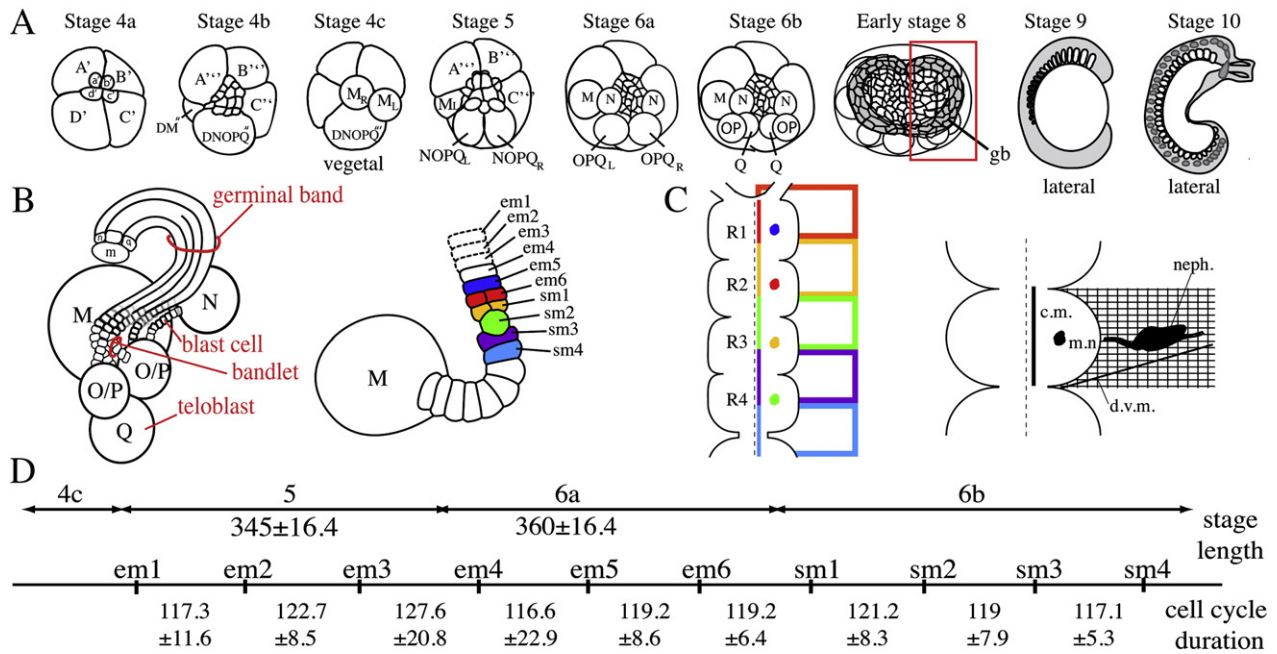
Fig. 1. Mesoderm development in the leech *Helobdella*. A. Representations of selected developmental stages (animal pole views unless otherwise indicated; see text for details). B. Left: schematic showing the relationships of teloblasts, blast cells, bandlets, and germinal band on the right side of an early stage 8 embryo, corresponding to the boxed section in panel (A). Right: schematic showing an M teloblast and its descendant column of em and sm cells, roughly 34 h after the division of DM″; em1–3 are depicted with dashed outlines because the timing and orientation of their first mitoses are unknown; em4 (black outline) has not yet divided at this time, nor has em5 (blue), but em6 (red) and sm1 (yellow) have each undergone bilateral divisions; sm2 (green) is shown rounding up for mitosis while sm3 (purple) and sm4 (turquoise) have not yet divided. C. Left: distribution of em and sm clones across segments R1–R4 (color coded as in B; cells em1–em4 do not contribute to segmental mesoderm). Shown are ganglia R1–R4 (black contours): dashed line marks the midline; colored lines next to the midline indicate muscle cells within the nerve cord; colored circles indicate clusters of M-derived neurons; open boxes, partially obscured by the ganglia, depict hemi-somite boundaries. Right: schematic modified from (Weisblat and Huang, 2001) depicting the mesodermal progeny (elements of 3 sm clones) associated with a typical midbody segment; c.m., connective muscle, m.n., M-derived neurons, d.v.m. dorsoventral muscle, neph. nephridium, hatched lines represent body wall muscles. D. Six cells are born from each M teloblast prior to stage 6b. Durations (in minutes) of relevant developmental stages and M teloblast cell cycles, compiled from time-lapse movies of embryos (Supplemental Movie 1). Cell cycles and stage lengths were calculated and averaged from a total of 13 experiments. Anterior is up in this and all subsequent figures unless otherwise noted.