Dual Origins of Mesoderm in a Basal Spiralian: Cell Lineage Analyses in the Polyclad Turbellarian Hoploplana inquilina

Barbara C. Boyer,* † 1 Jonathan Q. Henry,* ‡ and Mark Q. Martindale* §

*Marine Biological Laboratory, Woods Hole, Massachusetts 02543; †Department of Biology, Union College, Schenectady, New York 12308; ‡Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801; and §Department of Organismal Biology and Anatomy, University of Chicago, Chicago, Illinois 60637

Evolutionary modifications in the origins and organization of the mesoderm represent significant events in the diversification of metazoan body plans. Within the Spiralia, mesoderm comprises ectomesoderm, which typically is generated by derivatives of the second and third quartets of micromeres, and endomesoderm, which is formed from the fourth quartet micromere of the D quadrant (4d). It has been held that endomesoderm generates the majority of adult mesodermal derivatives, while larval mesoderm is formed primarily from ectomesoderm. The evolutionary history of these mesodermal sources could be clarified by examining basal members of the Spiralia such as the polyclad turbellarians, whose embryos exhibit canonical quartet spiral cleavage. Using the fluorescent lineage tracer DiI, we show that larval mesoderm is derived from only two cells, one from the ventral embryonic quadrant (2b, the “mesectoblast” cell), and the other from the dorsal quadrant (4d, the mesentoblast cell). We compare these results with mesodermal origins in other spiralian phyla and conclude that a dual origin of mesoderm is a primitive feature of spiralian development. We also argue that ectomesoderm and endomesoderm should not be considered as the exclusive precursors of larval and adult mesoderm, respectively.


INTRODUCTION

Recent morphological and molecular analyses have demonstrated that the acelomate turbellarian platyhelminthes (free-living flatworms) represent a pivotal extant group in the evolution of metazoan phylum (Hori et al., 1988; Ax, 1995; Valentine et al., 1996). Most phylogenetic analyses indicate that this group shares a common ancestor with the major bilaterian radiations, and with the Spiralia in particular (Fig. 1). The Spiralia constitutes an assemblage of protostome phyla that have a common pattern of early embryonic cell divisions known as spiral cleavage (Fig. 2), and includes free-living platyhelminthes, nemerteans, annelids, molluscs, echiurids, sipunculids, and gnathostomulids. The striking similarities in the cell lineages and cleavage patterns of these groups attest to their close evolutionary relationships.

Within the Turbellaria the polyclads exhibit the plesiomorphic conditions of entolecithy and quartet spiral cleavage, suggesting that they are among the most primitive members of the group (Thomas, 1986; Ax, 1987; Baguñá and Boyer, 1990). Polyclads with indirect development produce a free-swimming, ciliated Müller’s larva (Fig. 3), having distinct anterior–posterior, dorsoventral, and bilateral axial properties. With a solid mass of mesodermally derived mesenchyme, as well as a complex array of circular and longitudinal muscle fibers interposed between the body wall and the gut, the polyclads are classified as acelomates. Much of the larval body, including various symmetry properties, body wall musculature, and specialized cell types (e.g., rhabdites) are retained throughout gradual metamorphosis to the adult worm.

The developmental origin of mesoderm remains an important unsolved problem in metazoan evolution. Radially and biradially symmetrical metazoans, such as the cnidarians and ctenophores (Fig. 1), do not possess a well-defined mesodermal layer. The middle layer, referred to as the mesoglea, is typically composed of gelatinous extracellular
material containing loosely organized cells. For example, muscle cells in Cnidaria are regarded as epitheliomuscle cells, with cell bodies associated with the epidermis or gastrodermis and contractile processes that course through the mesoglea (Hyman, 1940). On the other hand, higher metazoans possess a distinct and well-organized set of mesodermal tissues, including a mesodermally lined coelomic cavity. The flatworms are the most basal bilaterian group with well-organized mesodermal tissues; however, they lack a coelom.

The embryological origin of mesoderm in the spiralians has been attributed to two regionally distinct sources. In "higher" Spiralia (annelids and molluscs) most of the adult mesoderm, referred to as endomesoderm, arises from the fourth quartet of micromeres of the D quadrant (4d), which is called the "mesentoblast" because it also gives rise to some endoderm. A second type of mesoderm, referred to as ectomesoderm, typically is derived from the second and/or third quartets of micromeres, which generate primarily ectodermal fates. Table 1 summarizes previously published reports describing the variety of cells giving rise to mesoderm in the embryos of a number of spiralian species.

The first observation of a double origin of mesoderm was reported in Unio by Lillie (1895), who observed that, in addition to the primary mesoblast (endomesoderm) derived from 4d, the A quadrant cell of the second quartet produced mesenchyme, which he referred to as "larval mesoblast." This mesenchyme gave rise to certain larval structures that disappeared later in development. The term "ectomesoblast" was coined by Wilson (1898a) to describe larval mesenchyme, which he concluded to be a distinctive tissue from that of the definitive "entomesoblast." Wilson (1898b) argued that the ectomesoblast is homologous with the mesenchyme of polyclads, which he regarded as representing the ancestral type, and that endomesoderm was a later development. (Wilson's conclusions were based on his observations of Leptoplana, which suggested that ectomesoderm arises from the second quartet of micromeres and that the fourth quartet is purely endodermal.) Meyer (1901) considered the phylogenetic significance of the two kinds of mesoderm in an extensive review of the evidence and concluded that ectomesoderm is more primitive and that endomesoderm should be regarded as a later derivation originating from the gonad cells.

It has commonly been claimed that larval mesoderm is synonymous with ectomesoderm, and adult mesoderm with endomesoderm, in various spiralian phyla (see Kume and Dan, 1968; Verdonk and van den Biggelaar, 1983). This interpretation has important implications for the phylogenetic origins of mesodermal lineages. One might argue, as did Wilson (1898b) and Meyer (1901), that ectomesoderm is the more ancient source of mesoderm utilized by primitive
FIG. 2. Diagram illustrates the spiral cleavage pattern, specifically of a polyclad turbellarian. The top row depicts an animal pole view, while the bottom row shows a lateral view. At the four-cell stage two cells meet at the animal pole (the A and C blastomeres), which defines the animal cross-furrow, and two cells meet at the vegetal pole (B and D), which defines the vegetal cross-furrow. Each of the first four cells generates a series of micromeres in an alternating orientation from the animal pole. The regularity of orientation of cleavage planes allows individual cells to be named according to a generally accepted nomenclature. The micromeres generated by each quadrant are given lower-case letters corresponding to the macromere that produced them, and a number indicating the quartet to which they belong. Macromeres are also given prefix numbers corresponding to the number of times they have divided but are identified with capital letters. When micromeres divide, they are given a superscript number that indicates whether they are the animal pole daughter cell (designated by the 1) or the vegetal daughter cell (designated by the 2). All drawings are not to scale.
FIG. 3. In many polyclads embryogenesis results in a free-swimming, ciliated “trochophore-like” larva, called a Müller’s larva. In A anterior is up and ventral is facing to the right. This larval form has a pair of lateral ocelli (oc) and an anterior apical tuft (at). Various lobes including the oral hood (oh), left and right lateral lobes (rll), and left and right ventral lateral lobes (lvll and rvll) surround the ventrally located mouth (mo). (B) Ventral view of a 6-day-old Müller’s larva. The body wall contains circular and longitudinal muscle fibers which can be visualized with fluorescent phallacidin dyes that label f-actin.

bilateralians. Endomesoderm might then represent an invention of descendant forms, analogous to “set-aside cells” used in the generation of larger and more complex adult bodies during the subsequent divergence of metazoan animals (Davidson et al., 1995). Endomesoderm in larger metazoans could then be brought into larval stages heterochronically by a process of adultation in order to shorten the metamorphic transition (Gould, 1977; Freeman, 1982; Freeman and Lundelius, 1992). Alternatively, one can argue that both ectomesodermal and endomesodermal lineages were in place during the embryogenesis of bilateral ancestors prior to the radiation of the protostome phyla and both were important in the evolutionary success of descendant forms.

Evidence concerning the origins of the mesoderm in the Spiralia can be obtained by examining development of the polyclad turbellarians, which represent basal spiralian with embryos that exhibit the typical quartet pattern of spiral cleavage. First cleavage in polyclads divides the embryo into two equal-sized blastomeres; second cleavage is unequal producing two smaller blastomeres which are in contact at the animal pole (or animal cross-furrow) and two larger cells that meet at a vegetal pole (or vegetal cross-furrow). Thus, it is possible to distinguish the A and C blastomere pairs (that meet at the animal pole) from B and D (that meet at the vegetal pole), but one cannot differentiate between the two small cells or the two large cells at this early stage of development. Subsequent divisions generate four quartets of micromeres; however, the fourth quartet is unusually large, leaving four very small “macromeres” (Fig. 2, 64-cell stage) that apparently do not contribute to any larval structures (Surface, 1907). Henry et al. (1995) have shown that the first two cleavage planes of the flatworm Hoploplana inquilina are oblique to the plane of bilateral symmetry and blastomeres at the four-cell stage have roughly the same fates as those of the higher Spiralia, in which the A and C quadrants form the left and right sides of the larva and B and D generate dorsal and ventral regions, respectively. This indicates that the cleavage pattern and fates of the four quadrants appear to be highly conserved within the Spiralia (though some exceptions are found in representatives of some phyla, Henry and Martindale, 1994).

There has been considerable controversy as to the origin of the mesoderm in polyclads, including the work of Lang (1884) on Discocoelis in which he attributed the ectoderm
**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Genus (Ref.)</th>
<th>1st quartet</th>
<th>2nd quartet</th>
<th>3rd quartet</th>
<th>4th quartet</th>
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<td>?</td>
<td></td>
<td></td>
<td>4d</td>
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<tr>
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<td>3d</td>
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<td>Dentalium</td>
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<td>2a, 2c</td>
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<td>1a, 1b, 1c, 1d?</td>
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<td>Enoplans</td>
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<tr>
<td>Malacobdella</td>
<td>2a, 2b, 2c, 2d</td>
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Note. *, study was done using modern lineage tracers; —, no known contribution to mesoderm; ?, mesodermal contributions are uncertain. References: 1Crofts (1955), 2Conklin (1897), 3Verdonk and van den Biggelaar (1983), 4Render (unpublished), 5Damen (1994), 6Holmes (1900), 7Anderson (1973), 8Torrey (1903), 9Newby (1940), 10Nusbaum and Oxner (1913), 11Dawydo (1928), 12Hammersten (1918).

to the first quartet of micromeres and mesoderm to the second and third quartets, and Wilson (1898a,b) on Leptoplana who found that mesoderm arose from the second quartet only. The most recent work (Surface, 1907) on Hoploplana (Planocera) inquinilla argues that essentially all mesoderm forms from the 4d cell, with a small contribution from all four of the second quartet micromeres (i.e., mesoderm situated around the blastopore). A reinvestigation of the origins of mesoderm in polyclads is particularly warranted because cell lineage analyses using fluorescent tracers in a variety of spiralian embryos have revealed significant variations with respect to the older literature. For example, differences have been identified in the origins of the posttrochal region in the archeogastropod Patella (Damen, 1994). Such cell lineage analyses have also revealed notable differences in the origins of mesoderm in the nemertean Cerebratulus lacteus (Henry and Martindale, manuscript in preparation). In this study we examine the origins of mesoderm in the polyclad H. inquinilla using a fluorescent lineage tracer.

**MATERIALS AND METHODS**

Adult specimens of the polyclad flatworm, H. inquinilla were extracted from the mantle cavity of the gastropod mollusc Busycan canal culatum, which were obtained from the Aquatic Resources Division, the Marine Biological Laboratory (Woods Hole, MA). Eggs were fertilized in vitro as previously described (Boyer, 1987). Individual blastomeres were surface labeled with Dil (Molecular
Probes Inc., Eugene, OR) dissolved in soybean oil. A 100 mg/ml stock solution of DIL was prepared in ethanol and then diluted 20-fold in Wesson soybean oil. Glass microelectrodes were back-filled with the diluted DIL solution and delivered by pressure with a picospritzer (General Valve, Inc.). A small oil droplet approximately one-sixth the diameter of the cell to be labeled was extruded from the microelectrode and placed on the blastomere membrane. Single cells were labeled from the four-cell stage through the 64-cell stage in different embryos. Labeled embryos were raised at 22–25°C for 5–6 days. Living Müller’s larvae were lightly compressed between a slide and coverslip supported by clay feet and examined with a Zeiss or Olympus fluorescence microscope. Photographs were taken with 400 ISO Ektachrome film. Muscle cells in Müller’s larvae were stained with Bodipy-labeled phalloidin according to the manufacturer’s instructions (Molecular Probes, Inc.; Martindale and Henry, 1995).

RESULTS

Although it is not possible to unambiguously identify each blastomere during development, one can distinguish between vegetal cross-furrow and noncross-furrow macromeres and their descendants. Furthermore, the resulting discrete and consistent patterns of labeled tissue within each quartet define four distinct domains, which correspond to each of the four embryonic quadrants. Thus, the identity of the injected cells could be readily determined by their specific contributions to the formation of larval ectoderm, as well as to other larval cell fates.

Marking of first quartet cells in a total of 64 embryos and third quartet cells in 44 embryos resulted in no labeling of mesodermal derivatives. Figure 4 shows the patterns of labeled second quartet derivatives. When the second quartet cells 2a (Fig. 4E, 15 cases), 2c (Fig. 4G, 20 cases), and 2d (Fig. 4H, 21 cases) were labeled, fluorescence was associated only with ectodermal structures. The marked cells are easily identifiable as cross-furrow or noncross-furrow descendents and the results were consistent from embryo to embryo, allowing for quadrant identification. However, when the 2b blastomere (18 cases) was marked, the elaborate circular musculature of the Müller’s larva was fluorescently labeled (Fig. 4F). The muscles formed concentric rings around the apical tuft, extending into the oral and dorsal lobes. Bands of muscle also ran dorsolaterally around the ventral lateral lobes and lateral lobes.

In order to study the descendants of the fourth quartet micromeres, we first surface labeled their precursors, the third quartet macromeres. Because division of the latter cells results in the formation of four large fourth quartet micromeres, and four tiny macromeres (Fig. 2) that apparently do not contribute to viable cell fates (Surface, 1907), it is reasonable to assume that labeling of the third quartet macromeres and the fourth quartet micromeres will produce very similar results. The embryos in which the noncross-furrow macromeres 3A (Fig. 5E) or 3C (Fig. 5G) were marked (13 cases) produced a small amount of ectodermal labeling, including cilia, associated with the lobes or the stomodeum, as well as masses of endodermal tissue. In 10 of 22 embryos in which 3B or 3D (vegetal cross-furrow macromeres) were labeled, only masses of endodermal tissue and rarely a small patch of ectoderm were fluorescent. These were interpreted to be 3B-labeled embryos (Fig. 5F). However, in the remaining 12 cases (3D), fluorescence was prominent in longitudinal muscle fibers that extend the length of the larva and into the oral and lateral lobes, and in mesenchymal cells widely distributed between the epidermis and gastrodermis (Fig. 5H). Experiments in which the 1D macromere was labeled produced larvae with prominent dorsal ectodermal labeling, as well as the characteristic muscle fibers, indicating that the muscle is produced by the D quadrant. Thus it is clear that when the third quartet macromeres were labeled, fluorescent mesoderm was formed only by D quadrant (3D) derivatives.

When the fourth quartet micromeres from the B or D quadrants were labeled, the same set of longitudinal, diagonal, and oral hood muscles, as well as mesenchyme, were fluorescent in 17 of 40 larvae, representing the derivatives of the 4d cell. No ectodermal or mesodermal cell fates were detected in the remaining 23 cases of labeled cross-furrow micromeres (4b), or as a result of labeling the noncross-furrow micromeres 4a and 4c (18 cases). Due to their extremely small size, fourth quartet macromeres were not labeled in this study.

DISCUSSION

The uniformity of results from the cell labeling experiments indicate that the lineage relationships of H. inquilina are consistent from embryo to embryo. We conclude that the mesoderm in the Müller’s larva is generated from two cells, the second quartet cell from the ventral B quadrant, which we call the “mesectoblast” (2b) and the fourth quartet cell from the dorsal D quadrant, the mesentoblast (4d). These cells give rise to the circular and longitudinal muscles as well as the mesenchyme of the Müller’s larva. The development of this elaborate musculature has been described in the larva of H. inquilina by Reiter et al. (1996).

There is some controversy in the literature as to which blastomere in polyclads actually represents the mesentoblast. Surface (1907) reported that in Planocera (Hoploplana), the 4d cell divides to form an internal blastomere 4d′, which becomes the mesentoblast, and a surface blastomere 4d″ which is entirely entoblastic. This mesentoblast then divides parallel to the plane of bilateral symmetry, as does 4d in annelids and molluscs, to produce the precursors of the mesodermal bands. Although this delayed formation of the mesentoblast was not observed in other polyclad species (see Kato, 1940), the recent work of van den Biggelaar (1996) on Prostheceraeus giesbrechti corroborates the observation of Surface. The acceleration of the time of mesentoblast formation since the divergence of flatworms and higher spiralians from a common ancestor is consistent with the concept of heterochronic changes leading to the appearance of adult structures in larvae as a way of
FIG. 4. Results of labeling second quartet micromeres in 5-day-old Müller's larvae. The larvae in the first and second columns are shown ventral side to the left; those in the third and fourth columns are oriented so that ventral is to the right. Anterior is up in all cases. DIC images of representative examples are shown in the top row, while the bottom row indicates the corresponding fluorescent image. E shows a 2a label, F shows a 2b label, G shows a 2c label, and H shows a 2d label. Note that while all four second-quartet cells make characteristic contributions to epidermal derivatives (ep), only the progeny of the 2b micromere (Fig. 3B, 3F) generates mesoderm in the form of circular muscle fibers (mf). oc, ocelli; oh, oral hood; nf, nerve fibers.

shortening the metamorphic transition (Gould, 1977; Freeman, 1982; Freeman and Lundelius, 1992; van den Biggelaar, 1996).

As indicated in Table 1, precursors of mesoderm in other spiralian are considerably more extensive and varied than in the polyclads. In molluscs, second quartet cells of the A, B, and C quadrants and third quartet cells from all quadrants may form mesodermal structures, and in most species two or three micromeres are ectomesodermal precursors. In annelids, three or four micromeres from the second and/or third quartets are ectomesodermal and across species all quadrants, again, are represented. Interestingly, echiuroids apparently derive mesoderm from the first quartet as well as the third, involving all quadrants, while some nemertean restrict their ectomesoderm to all blastomeres of the second quartet. No clear data are available on mesodermal origins in sipunculids or gnathostomulids. It is important to note, however, that modern lineage-tracing techniques have been used in very few studies (note asterisk in Table 1) and therefore the data must be interpreted with caution. The idea that 2b may represent an ancestral source of ectomesoderm is supported by the cell lineage studies on the spiralian Patella (Damen, 1994), Ilyanassa (Router, manuscript in preparation), and Hoploplana (present study) in which cell-autonomous lineage tracers have been used; in each of these embryos 2b has been shown to generate mesoderm. When more reliable information is available from a more diverse set of taxa it will be possible to analyze mesodermal origins with rigorous phylogenetic methods. Nonetheless, in the ancestral condition, perhaps as represented by present-day polyclads such as H. inquilina, mesodermal precursors may have been more limited than in derived forms, possibly involving only one ectomesodermal blastomere.

If the origins of mesoderm in Hoploplana are indeed representative of the ancestral condition in the Spiralia, then with the evolution of more complex larval and adult body plans (e.g., polychaetes and molluscs), mesodermal contributions apparently changed. In present-day annelids and molluscs, both second and third quartet micromeres from several quadrants may contribute to ectomesoderm (Table 1). These additional mesodermal progenitors may have been recruited initially to increase the complexity of feeding structures in the peristomeal region of planktrophic larvae. This interpretation is supported by the fact that in
planktotrophic polychaete larvae ectomesoderm forms precociously and is involved in the development of feeding structures. On the other hand in polychaetes with larger yolky eggs that produce nonfeeding larvae, ectomesoderm is reduced and the majority of mesoderm is generated from endomesodermal sources (Anderson, 1966).

Changes in ectomesodermal contributions to larval structure also occurred in molluscs. For example, Clement (1960) found that a larval foot retractor muscle may be present in Ilyanassa after removal of 4d, an observation that is corroborated by the lineage-tracing study of Render (manuscript in preparation), who observed fluorescence in the foot retractor muscle when 2b was labeled with lucifer yellow dextran. Furthermore, the musculature of the foot in the gastropod Lymnaea forms in the absence of the 4d lineage (Martindale, 1986), indicating that ectomesodermal contributions from the second and third quartets (Table 1) were recruited to form a novel structure in the molluscan lineage. Thus, subtle modifications of existing cell lineages allowed for the diversification of body plans to meet the rigors of larval existence, akin to the "adaptation in cleavage" idea described by Lillie (1898).

We also argue that ectomesoderm and endomesoderm should not be considered as solely the precursors of larval and adult mesoderm, respectively. Metamorphosis in polyclads (and many other spiralians) is gradual (Lang, 1884; Kato, 1940; Anderson, 1977; Ruppert, 1978) and both ectomesoderm (from 2b) and endomesoderm (from 4d) appear to contribute to the adult mesoderm. Furthermore, Schroeder and Hermans (1975) conclude that in polychaetes both larval and adult mesoderm are derived from 4d. This cell also contributes to larval mesoderm in molluscs, forming some of the larval muscle in Unio (Lillie, 1895), and the larval retractor muscle in four species of gastropods (Crofts, 1955). Render (manuscript in preparation) has found that both 2b and 4d cooperate to produce the larval retractor muscles of the velum and foot as well as larval heart and kidney in Ilyanassa. Thus, there does not appear to be a clear distinction in the sources of larval and adult mesoderm in most of the extant spiralians (Anderson, 1966; Verdonk and van den Biggelaar, 1983).

The increase in overall size and evolution of elongated and segmented body plans in bilaterians occurred through enlargement of the posterior region. Expansion of endomes-
Endomesoderm, therefore, should not be thought of as an of the mode of D quadrant speci®cation in coelomates with spiral zoans and gives rise to the adult mesodermal derivatives. Freeman, G., and Lundelius, J. W. (1992). Evolutionary implications of mesoderm which lines the coelomic cavities of higher metazoans and functioned as a substrate for the refinement of the adult morphology.

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