Generation of bilateral symmetry in the ectoderm of the *Tubifex* embryo: involvement of cell-cell interactions

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Abstract

In embryos of the oligochaete annelid *Tubifex*, most ectodermal tissues are derived from four bilateral pairs of embryonic stem cells called teloblasts (ectoteloblasts N, O, P and Q). Ectoteloblasts are generated on both left and right sides of the embryo through an invariable sequence of cell divisions of a proteloblast, NOPQ, and they are positioned in a mirror symmetric pattern relative to the embryonic midline. This mirror symmetry of ectoteloblast arrangement gives rise to the generation of bilateral symmetry in the ectoderm. Here we review results of our recent experiments on *Tubifex tubifex* that were designed to gain an insight into the mechanisms underlying the generation of the bilaterally symmetric organization of ectoteloblasts. Cell transplantation experiments have shown that nascent NOPQ cells can be polarized according to positional information residing in the embryo. If a left NOPQ cell is transplanted to the right side of a host embryo, it exhibits polarity comparable to that of right NOPQ cells. It has also been shown that contact between NOPQ cells serves as an external cue for their polarization. Another series of cell transplantation experiments have suggested that the competence of NOPQ cells to respond to external cues becomes undetectable shortly before the production of the first teloblast (N) from the NOPQ cell. Another series of experiments utilizing cell ablation techniques have shown that teloblasts N, P and Q are specified to express the N, P and Q fates, respectively, as early as their birth. In contrast, the O teloblast and its progeny are initially pluripotent and their fate becomes restricted through inductive signals emanating from its sister P lineage. On the basis of these findings, we have proposed a model for polarization of ectodermal teloblastogenesis in the *Tubifex* embryo.

Introduction

Embryogenesis in clitellate annelids (i.e., oligochaetes and leeches) is characterized by the generation of five bilateral pairs of embryonic stem cells called teloblasts early in development (Shimizu and Nakamoto, 2001). Teloblasts, which are derived from micromeres of the D quadrant, undergo extremely unequal divisions repeatedly to produce a coherent column (bandlet) of smaller daughter cells (referred to as primary blast cells). Four of the five bandlets on each side of the embryo join together to form an ectodermal germ band (GB), while the remaining bandlet becomes a mesodermal GB. Based on results of previous descriptive and cell ablation studies (Whitman, 1878; Penners, 1924, 1926; Devries, 1973a, b), it has been suggested that teloblasts (and their progenies) play a pivotal role in clitellate annelid development. In fact, teloblasts are the only source of ectodermal and mesodermal segmental tissues; none of the non-teloblastic cells can replace missing teloblasts in this respect. Furthermore, morphogenetic events such as body elongation and segmentation depend solely on the presence of teloblasts and their progeny (Blair, 1982; Wedeen and Shankland, 1997; Goto et al., 1999a; Shain et al., 2000; Kitamura and Shimizu, 2000; Nakamoto et al., 2000).

Ectodermal teloblasts (ectoteloblasts N, O, P and Q) on either side of the embryo are produced through an invariable sequence of cell division of a proteloblast, NOPQ, that is derived from the second micromere 2d; a bilateral pair of mesodermal teloblasts (mesoteloblasts M) results directly from equal division of the fourth micromere 4d (Fig. 1A, B). Recent cell lineage analyses of teloblasts have shown that developmental fates of the four ectoteloblasts

are not only different from that of the mesoteloblast but also distinct among themselves (Weisblat et al., 1980, 1984; Storey, 1989; Goto et al., 1999b). At present, it is not clear how and when these teloblasts (and their progeny) acquire distinct developmental fates. As has been well documented, teloblasts N-Q emerge at different positions along the embryonic axes and at different times (Fernandez and Olea, 1982; Shimizu, 1982; Sandig and Dohle, 1988). In the oligochaete *Tubifex*, the N teloblast is generated first and located ventralmost, and the Q teloblast, which is generated next, is located dorsalmost; finally the O and P teloblasts are generated by almost equal division of their precursor cell, OP (Fig. 2). There is no doubt that this spatial arrangement of ectoteloblasts gives rise to the generation of bilateral symmetry in the ectoderm. At present, however, nothing is known about the causal relationship between developmental fates of teloblasts and spatiotemporal aspects of their emergence.

In this paper we review results of our recent experiments on embryos of *Tubifex tubifex* that were designed to gain an insight into the mechanisms underlying the generation of the bilaterally symmetric organization of ectoteloblasts. In the first series of experiments, we addressed mechanisms for polarization of NOPQ. For this purpose, we transplaned left NOPQ cells to various ectopic positions of host embryos and examined the polarity of transplanted NOPQ cells (Nakamoto et al., 2003). The second series of experiments was carried out to determine how and when ectoteloblasts are specified. We studied this issue by ablating specific ectoteloblasts from embryos (Arai et al., 2001). Experimental procedures for cell ablation, transplantation, labeling and culture have been described in detail in our previous papers (Kitamura and Shimizu, 2000; Nakamoto et al., 2000).

Specification of polarity of the ectoteloblast precursor cell NOPQ

Ectoteloblasts N, O, P and Q arise from an invariable sequence of divisions of the cell NOPQ on both sides of the embryo (Fig. 1D; Goto et al., 1999b). NOPQ on either side of the embryo undergoes unequal divisions twice after its birth (Fig. 2H) and then divides into a smaller N teloblast and a larger cell, OPQ (Fig. 2B, C). Similarly, after producing small cells twice, OPQ divides into a smaller Q teloblast and a larger cell, OP (Fig. 2D, E). Finally OP undergoes unequal division four times after its birth (Fig. 2H) and then cleaves almost equally, yielding the third-born ectoteloblasts O and P (Fig. 2F, G), at which point teloblastogenesis is complete.

As mentioned before, NOPQ on either side of an intact embryo divides into an N teloblast located distal from and an OPQ located proximal to the dorsal midline (i.e., the contact plane between sister NOPQ cells; see Fig. 2C). We regard this configuration of the two cells (i.e., N and OPQ) as a manifestation of polarity that NOPQ has at the time of its division into these two cells. Similarly, in the following cell transplantation experiments, we assessed polarity of transplanted NOPQ cells according to fates that two large cells (equivalent to N and OPQ in intact embryos) descending from the transplanted NOPQ take on.

We found that when oriented in an appropriate direction in host embryos, transplanted NOPQ underwent cell division in a normal fashion to produce two large cells (which correspond to N and OPQ in intact embryos) at about 8 hrs after its birth (see Fig. 2H). In this paper, we refer to these two large cells as a Dst-cell and a Pxm-cell (Fig. 3). The Pxm-cell is a cell that inherits the surface of NOPQ originally facing (proximal to) the midline, and

the Dst-cell is a cell containing the opposite surface of NOPQ (Fig. 3). It should be noted here that the two large cells resulting from transplanted NOPQ are designated Dst- or Pxm-cells according to their original orientations relative to the midline of a donor embryo (that is, irrespective of their positions relative to the midline of host embryos; Fig. 3).

In intact embryos, the Pxm-cell is always an N teloblast and the Dst-cell is an OPQ proteloblast. Subseqeuntly, the N teloblast generates a single bandlet of n-blast cells (called the N pattern or N fate in this paper). In contrast, the proteloblast OPQ gives rise to the production of three teloblasts and hence three bandlets of blast cells (called the OPQ pattern or OPQ fate in this paper).

Development of nascent NOPQ cells transplanted to ectopic positions

To determine whether NOPQ cells are already polarized at the time of their birth, we isolated a left NOPQ (NOPQ*l*) shortly after its birth and transplanted it to the position of the right NOPQ (NOPQ*r*) of the host embryo (from which NOPQ*r* had been ablated; Fig. 4B). The transplanted cell was oriented in the host embryo so that its anteroposterior polarity remained unchanged. In a preliminary experiment, we found that the progeny of transplanted NOPQ*l* is confined to the right side of a 7-day-old operated embryo and that its distribution pattern is quite similar to that of authentic NOPQ*r* in intact embryos. This indicates that if transplanted to the right side of the embryo, nascent NOPQ*l* adopts the "right side fate".

To verify that NOPQ*l* transplanted to the right side of the embryo acquires the same polarity as that of NOPQ*r*, we followed the fate of Dis- and Pxm- cells resulting from the division of a transplanted NOPQ*l* (see Fig. 3).

When either Dst-cells (*n* = 8) or Pxm-cells (*n* = 13) derived from transplanted NOPQ*l* were labeled with DiI and their development was followed, it was found that Dst-cells (7 cases) individually gave rise to three bandlets (see Fig. 5B). (In one case, the number of bandlets was unclear.) On the other hand, Pxm-cells (12 cases) each gave rise to a single bandlet (see Fig. 5A); in no case did Pxm cells produce three bandlets. These findings suggest that Dst- and Pxm- cells descending from NOPQ*l* placed on the right side adopt the OPQ and N fates, respectively. This notion was confirmed by the distribution pattern of the progenies of Dst- and Pxm-cells derived from transplanted NOPQ*l*. In all cases examined (*n* = 5), Dst-cells contributed cells to the dorsal epidermis, dorsal and ventral setal sacs, and ventral ganglia, suggesting that Dst-cells followed the OPQ fate rather than the N fate. Furthermore, progenies of Pxm-cells (7/7 cases) contributed cells mainly to ventral ganglia. In no cases did the progenies differentiate to setal sacs, which are known to differentiate from the OPQ cell (Goto et al., 1999b).

In a control experiment, an isolated NOPQ*l* was transplanted to the position of NOPQ*l* of a host embryo (from which NOPQ*l* had been ablated). Dst- and PXm-cells derived from the transplanted NOPQ*l* were found to exhibit N and OPQ patterns of cell division and cell fate, respectively.

On the basis of these observations, it is reasonable to conclude that when transplanted to the right side of the embryo, a nascent NOPQ*l* exhibits features of the authentic NOPQ*r* and that NOPQ*l* is initially plastic in terms of division pattern and fates.

To determine whether a transplanted nascent NOPQ*r* also adopts the "left side fate" of the host embryo, we transplanted a nascent NOPQ*r* to the left side (i.e., the position of NOPQ*l*) of a host embryo (from which NOPQ*l* had

been ablated) and followed the division pattern of Pxm-cells derived from the transplanted NOPQ*r*. In most cases (11/15), Pxm-cells generated a single bandlet (that is, in the N pattern); there were no cases in which three bandlets were formed. These results suggest that a nascent NOPQ*r* is not endowed with fixed polarity and that it is able to adopt the "left side fate" of the host embryo if placed to the left side of the embryo.

The dorso-ventral polarity of NOPQ is gradually determined prior to its division into cells N and OPQ

The above-described experiments show that NOPQ is initially plastic in terms of division pattern and fates. It is natural to assume that the polarity along the dorso-ventral axis is specified in NOPQ at some time after its birth. We next tried to determine the time when the polarity of NOPQ is fixed. For this purpose, we isolated NOPQ*l* at three different stages [i.e., shortly after its birth (NOPQ *early*), shortly after production of nopq1 (NOPQ *mid*) and shortly after production of nopq2 (NOPQ*late*); see Fig. 2H] and transplanted it to the position of NOPQ*r* of a host embryo at the stage of NOPQ*early* (from which NOPQ*r* had been ablated). Either Dst- or Pxm-cells derived from the transplanted cells were labeled and their fates were followed as described above.

The results are summarized in Fig. 6. When NOPQ*early* was transplanted, nearly all of the resulting Dst-cells (7/8 cases) exhibited the division pattern of an OPQ cell (Fig. 6A). Similarly, Dst-cells (5/7 cases) derived from NOPQ*mid* individually generated three bandlets. It is notable that Dst-cells at these stages never gave rise to a single bandlet. In contrast, most Dst-cells derived from NOPQ*late* individually produced only one bandlet

(11 out of 16 cases; see Fig. 5A); there were no cases in which Dst-cells gave rise to three bandlets.

Pxm-cells derived from NOPQ*early* (12/13 cases) generated a single bandlet (i.e., in the N pattern). As development proceeded, however, the frequency of cases in which Pxm-cells gave rise to single bandlets decreased. Conversely, the frequency of cases in which single Pxm-cells gave rise to two or more bandlets significantly increased (Fig.6B). These results suggest that as NOPQ*l* undergoes cell division twice to produce blast cells nopq (see Fig. 2H), it gradually loses its flexibility to adopt the "right side fate" of the host embryo.

It should be noted, however, that about 25% of the Pxm-cells derived from NOPQ*late* exhibited the N pattern (Fig. 6B), whereas none of the Dst-cells took on the OPQ fate (Fig. 6A). This difference suggests that the distal portion of NOPQ*late* is already specified rigidly, while the remaining (proximal) portion of the cell still retains the flexibility to adopt the "right side fate" to some extent.

Contact between NOPQ cells enables them to become polarized

In the transplantation experiments described above, transplanted NOPQ cells were always placed in contact with NOPQ cells of the host embryos at the midline (Fig. 4B). Under such conditions, the Pxm-cells, which were located distal to the site of contact between the transplanted and host NOPQ cells, were always found to assume the N fate. This suggests that contact between NOPQ cells enables them to become polarized. To test this possibility, we transplanted an isolated NOPQ*l* to the distal side (i.e., on the leftmost surface) of NOPQ*l* of a host embryo from which NOPQ*r* had been ablated (Fig. 4C) and

followed the cells descending from the host NOPQ*l*.

At about 8 hrs after transplantation, the host NOPQ*l* divided, along the dorsoventral axis, into two large cells (i.e., Dst- and Pxm- cells) in a normal fashion. When Pxm-cells derived from the host NOPQ*l* were followed, nearly all of the Pxm-cells (9/11 cases) that generated cell bandlets successfully were found with single bandlets (see Fig. 5A); in no cases did these cells gave rise to three bandlets. This suggests that these Pxm-cells assume the N fate. Given that the Pxm-cells in intact embryos assume the OPQ fate (see Fig. 3), it is apparent that the fate of NOPQ-derived cells depends on their distance from the site of contact between NOPQ cells rather than their position along the embryonic axis. If located distal to the site of contact between NOPQ cells, cells resulting from the division of the NOPQ were always found to assume the N fate.

Development of "solitary" NOPQ cells placed in various positions

In previous studies, we found that even if an NOPQ cell is deleted, the remaining sister NOPQ cell, which remains in the position where it has been, undergoes a series of divisions in a normal (or scheduled) fashion and finally generates a full set of ectoteloblasts, which are arranged, along the dorsoventral axis, in the same order as those in intact embryos (Nakamoto et al., 2000; Arai et al., 2001). These observations suggest the existence of some kind of positional cues other than NOPQ cells that are involved in polarization of NOPQ cells. To investigate this issue, we examined the mode of development of NOPQ*l* transplanted to various positions of host embryos from which both NOPQ*l* and NOPQ*r* had been ablated (Fig. 4D-F). In this study, we focused on the fates of Pxm-cells descending from transplanted

NOPQ*/*.

The results are summarized in Table 1. When NOPQ*/* was transplanted to the left side of a host embryo (Fig. 4E), the resulting Pxm-cells gave rise to three bandlets (viz., in the OPQ pattern). In contrast, Pxm-cells derived from NOPQ*/* transplanted to the right side (Fig. 4D) individually produced a single bandlet (viz., in the N pattern). This suggests that NOPQ*/* transplanted to the left side and the right side adopt the "left side fate" and "right side fate", respectively, in the absence of host NOPQ cells

In this connection, it was interesting to learn how NOPQ*/* placed centrally (i.e., on the dorsal midline of the host embryo) reacts to the position (Fig. 4F). Pxm-cells of some such reconstituted embryos generated one bandlet (2/8 cases) and others generated three bandlets (2/8 cases). These results reconfirm the previous notion that a nascent NOPQ cell is plastic in terms of division pattern and cell fate. At present, it is not known why Pxm-cells descending from NOPQ*/* placed centrally assume two different fates. Considering the close relationship between the positions where transplanted cells are placed and the fates they follow, however, it is conceivable that NOPQ*/* placed centrally would shift to the left or right during their development to adopt the left or right side fate.

Specification of ectodermal teloblast lineages

To determine the extent to which specification of ectoteloblast lineages depends on external cues, we followed the development of ectoteloblasts that had been forced to be “solitary”. In this study, we assessed the fates of operated ectoteloblast lineages according to compositions and spatial distribution of terminally differentiated cells descending from these

ectoteloblasts. In intact embryos, each ectoteloblast makes a topographically characteristic contribution to the ectodermal tissues, which exhibit a segmentally repeated distribution pattern (Fig. 7; Goto et al., 1999b).

Fates of "solitary" ectodermal bandlets

Our previous study showed that a bandlet derived from a "solitary" O teloblast (resulting from removal of all of its ipsilateral sister teloblasts) exhibited early morphogenetic features (e.g., shapes of bandlets) characteristic to the P lineage rather than the O lineage, while bandlets derived form "solitary" N, P and Q teloblasts were very similar to the respective bandlets in intact embryos (Nakamoto et al., 2000). To extend this observation to more advanced developmental stages when cells are terminally differentiated, we labeled one of the four ectoteloblasts with DiI and ablated the other three ipsilateral ectoteloblasts (or their precursors) simultaneously, and after 5-day culture, we examined the composition and distribution of labeled cells descending from "solitary" teloblasts.

Fig. 8A, B show the organization of labeled cells derived from an intact and a "solitary" N teloblast, respectively. These two cases are indistinguishable from each other in that nearly all of the labeled cells were located in the ventral region of the embryo and occupied each hemiganglion. Similarly, labeled cells derived from "solitary" P and Q teloblasts are organized in a pattern comparable with that in intact P and Q lineages, respectively (Fig. 8E, F; not shown for the Q lineage).

In contrast, organization and composition of cells derived from "solitary" O teloblasts are distinct from those in normal o bandlets. As Fig. 8D shows, "solitary" o bandlets apparently exhibited a P pattern rather than an

O pattern of progeny cells (also see Fig. 8C). This result suggests that "solitary" o bandlets adopt the P fate rather than the O fate.

In another series of experiments, we followed the fate of "solitary" left bandlets in embryos that had been subjected to bilateral ablations of ectoteloblasts. To do this, we ablated the right NOPQ (i.e. exclusive source of the right ectodermal GB; see Fig. 1E) and three of the four left ectoteloblasts, leaving a single (DiI-labeled) ectoteloblast in each embryo. After 5-day culture, the operated embryos (5-7 for each lineage) were examined for the composition and distribution of labeled cells. We found that even after bilateral ablations of ectoteloblasts, "solitary" n, p and q bandlets exhibited N, P and Q patterns of distribution of progeny cells, respectively, and that o bandlets followed the P fate rather than the O fate. These results are the same as those obtained in the unilateral ablation experiments. The results therefore suggest that the presence of contralateral GBs does not influence the fate decision of "solitary" bandlets. In the following experiments, we used embryos in which right GBs were intact.

O fate in o bandlets is induced by interaction with p bandlets

The above-described results indicate the possibility that in intact GB, o bandlets are induced to assume O fate by interactions with other bandlets. To test this possibility and to determine which bandlet acts as such an inducer, we ablated teloblasts in various combinations, leaving an O teloblast plus one or two other teloblasts in each embryo and followed the fates of the progenies of O teloblasts.

The results are summarized in Table 2. It was only when p bandlets survived that o bandlets assumed the O fate. Neither n nor q bandlets were

effective at all in this respect; even when both n and q bandlets coexisted with o bandlets, they failed to induce o bandlets to assume the O fate. It is unlikely that this failure resulted from separation of o bandlets from n and/or q bandlets in operated embryos, since bandlets in a GB from which one bandlet had been deleted were found to be aligned tightly with each other. These results suggest that p bandlets exclusively serve as an inducer of O fate in o bandlets.

P teloblast may be specified to assume the P fate at the time of its birth

Unlike O teloblasts, fates of N, P and Q teloblasts do not appear to be affected by neighboring bandlets. This suggests that these three teloblasts are specified as early as their birth. Considering the fact that the O teloblast is the sister of the P teloblast (see Fig. 2F, G), however, it is also possible that, like the O teloblast, the P teloblast is pluripotent and can express fates other than the P fate in an appropriate environment. To distinguish these possibilities, we labeled P teloblasts with DiI shortly after birth and simultaneously ablated other teloblasts in various combinations, leaving a labeled P teloblast plus one or two other teloblasts in each embryo, and after 5-day culture, we examined the distribution of labeled cells.

The results are summarized in Table 3. Irrespective of the presence of any other teloblasts, p bandlets assumed the P fate. The p bandlets did not show any sign of N, O or Q pattern of progeny cells in any of the combinations with other teloblasts. These results suggest that p bandlets are unlikely to receive inductive or inhibitory signals from neighboring bandlets. Thus, it is more likely that P teloblasts are specified to assume the P fate at the time of their birth.

Taken together, the results suggest that the N, P and Q teloblasts of the *Tubifex* embryo are committed to their respective fates at the time of their birth. In contrast, it is reasonable to assume that O teloblasts are pluripotent. As found in the above-described experiments, o bandlets assume the O fate in the presence of p bandlets; otherwise they express the P fate. Apparently, for the O lineage, the P fate is the primary fate and the O fate is the secondary fate.

The O and P teloblasts are sister blastomeres resulting from the equal division of an OP proteloblast. As stated above, the O teloblast is pluripotent and its progeny cells are able to respond to inductive signals from the neighboring p bandlet. In contrast, P teloblasts do not appear to be affected by the O teloblast. Furthermore, P teloblasts assumed only the P fate under any of the experimental conditions that included ablation of teloblasts in all possible combinations. Based on these observations, we suggest that sister teloblasts O and P in the *Tubifex* embryo are not equivalent but are distinct from each other in their developmental potency. It appears that the OP proteloblast undergoes an asymmetric division giving rise to two equal-sized teloblasts.

Cellular basis for bilaterally symmetric organization of the ectoderm

The bilaterally symmetric organization of the ectoderm in *Tubifex* is traced back to mirror symmetry of ectodermal germ bands (GBs) and ectoteloblasts. The right and left GBs, which coalesce with each other along the ventral midline, contribute ectodermal cells, respectively, to the right and left halves of the embryo. During gastrulation, ectodermal cells originating from the ventrally-located GB migrate toward the dorsal midline; during this process,

these cells do not cross over the midline (Goto et al., 1999b). Organization of bandlets comprising the left and right GBs exhibits mirror symmetry. Apparently this mirror symmetry gives rise to mirror symmetry of the ectoderm, and it is a direct consequence of the bilateral arrangement of ectoteloblasts, which are organized in the order of N, O, P and Q along the ventrodorsal axis on either side of the embryo.

In the present study, we examined the possibility of the involvement of cell-cell interactions in the generation of mirror symmetry in the ectodermal teloblastogenesis. The results obtained suggest that cell-cell interactions are involved at least in two stages during teloblastogenesis, viz., prior to the division of NOPQ into N and OPQ and shortly after the division of OP into O and P. NOPQ cells, which do not initially have a rigidly fixed polarity, become polarized through external cues, including signals emanating from their sister NOPQ cells (Fig. 9B). It is conceivable that during this polarization process, some kind of a gradient is generated in NOPQ cells (Fig. 9C); a Dxt-cell descending from an NOPQ cell might be determined as an N teloblast, and a Pxm-cell might be determined as an OPQ cell (Fig. 9D). The OPQ cell subsequently undergoes "polarized" cell divisions so that it produces a dorsalmost cell that is determined as a Q teloblast. Apparently the OPQ cell acquires polarized properties. In view of the fact that OPQ cells remain in contact with each other shortly after their birth (Fig. 9D), it seems possible that these cells undergo the second round of the polarization process mediated by the contact between themselves. However, this point remains to be studied experimentally.

At present, nothing is known about the ground (default) state of nascent NOPQ cells. Upon its birth, the NOPQ cell may be determined as a

precursor of ectoteloblasts. However, it is unclear whether nascent NOPQ cells already possess polarized properties that foreshadow the polarity of teloblastogenesis. Therefore, it remains to be determined whether the external cues endow NOPQ cells with de novo polarity or strengthen (or stabilize) the preexisting polarity of NOPQ cells.

Comparison with other clitellate annelids

As mentioned before, in *Tubifex*, a pair of ectoteloblast precursor NOPQ cells are derived from the 2d cell and make contact with each other along the midline after their birth. Each NOPQ, which is associated with an anterior arc of micromeres, gives rise to four ectoteloblasts. Similar cellular configurations of NOPQ and sequence of ectodermal teloblastogenesis have been reported in a wide variety of clitellate annelids, including enchytraeid *Lumbricillus lineatus* (Penners, 1930), tubificid *Peloscolex benedeni* (Penners, 1929), lumbricid *Eisenia foetida* (Devries, 1973a), branchiobdellid *Bdellodrilus philadephicus* (Tannreuther, 1915), and leeches *Glossiphonia complanata*, *Theromyzon rude* and *Helobdella stagnalis* (Whitman, 1878; Fernandez and Olea, 1982). Given that leeches and branchiobdellidans form a monophyletic group of oligochaetes (Siddal et al., 2001), such a mode of ectodermal teloblastogenesis as seen in *Tubifex* is an ancestral form of oligochaete embryogenesis and has been preserved widely in the Clitellata. At present, however, it is not known whether these cell-cell interactions in annelids such as those demonstrated in *Tubifex* are involved in the generation of bilateral symmetry in the organization of the ectoderm. As discussed later, it is equally possible that evolutionary changes in cell interaction networks produce embryonic structures with similar morphological pattern. In this connection, it

is interesting to note that the lumbriculid *Rhynchelmis* and naids *Stylaria* and *Chaetogaster* exhibit developmental patterns distinct from those in other clitellates. *Rhynchelmis* embryos have four bilateral pairs of ectoteloblasts, of which one originates from 2d and the remaining three from 3d (Svetlov, 1923). *Stylaria* and *Chaetogaster* form the segmental ectoderm without production of ectoteloblasts; these two oligochaetes also differ from each other in that the source of the segmental ectoderm is 2d for *Stylaria* and 1d for *Chaetogaster* (Svetlov, 1926; Davydov, 1942). Given that these oligochaetes have a bilaterally symmetric body plan as other clitellates do, these exceptional cases suggest it unlikely that the bilaterally symmetric organization of 2d-derived ectoteloblasts is solely responsible for the generation of bilateral symmetry in the ectoderm. It will be of interest to determine, in future studies, the extent to which mechanisms for generation of bilateral symmetry in the ectoderm are diverse in the Clitellata.

As stated before, the N, P and Q teloblasts in the *Tubifex* embryo are committed to their respective fates at the time of their birth. In contrast, the O teloblast is pluripotent at the time of its birth and is induced to assume the O fate by the P teloblast lineage; otherwise it expresses the P fate. As to the mode of specification of ectodermal teloblast lineages, the only thing that is known in other clitellate annelids is that in the leech *Helobdella*, bandlets derived from a pair of O/P teloblasts (equivalent to the O and P teloblasts in oligochaetes) are initially equipotent and differentiate from each other according to their position within the GB (Weisblat and Blair, 1984; Zackson, 1984). Recently, Huang and Weisblat (1996) have suggested that blast cells derived from either O/P teloblast assume the P fate if they interact with a bandlet derived from the Q teloblast and that otherwise o/p blast cells express

the O fate. Thus, it is apparent that distinct cell interaction networks are involved in patterning the ectodermal GB in *Tubifex* and *Helobdella*.

In spite of these differences, however, the resulting (final) pattern of the ectodermal GB is strikingly similar in *Tubifex* and *Helobdella* (Weisblat and Shankland, 1985; Goto et al., 1999b). Thus, we suggest that during their evolutionary isolation, oligochaetes and leeches have preserved an ancestral pattern of the ectodermal GB despite the divergence of cell interaction networks through which this pattern is brought about.

References

Arai, A., Nakamoto, A. and Shimizu, T. 2001. Specification of ectoermal telolablast lineages in embryos of the oligochaete annelid *Tubifex*: involvement of novel cell-cell interactions. Development 128: 1211-1219.

Blair, S. S. 1982. Interactions between mesoderm and ectoderm in segment formation in the embryo of a glossiphodiid leech. Dev. Biol. 89: 389-396.

Davydov, C. 1942. Etude sur l'embryologie des Naididae indochinos. Arch. Zool. exp. gen. Notes et Revue 81: 173-194.

Devries, J. 1973a. La formation et la destinée des feuillets embryonnaires chez le lombricien *Eisenia foetida* (Annélide Oligochete). Arch. Anat. Microsc. 62: 15-38.

Devries, J. 1973b. Détermination précoce du développement embryonnaire chez le lombricien *Eisenia foetida*. *Bull. Soc. Zool. Fra.* **98**, 405-417.

Fernandez, J. and Olea, N. 1982. Embryonic development glossiphoniid leeches. In Developmental Biology of Freshwater Invertebrates (ed. F. W. Harrison and R. R. Cowden), pp. 286-316. New York: Alan R. Liss.

Goto, A., Kitamura, K. and Shimizu, T. 1999a. Cell lineage analysis of pattern

formation in the *Tubifex* embryo. I. Segmentation in the mesoderm. Int. J. Dev. Biol. 43: 317-327.

Goto, A., Kitamura, K., Arai, A. and Shimizu, T. 1999b. Cell fate analysis of teloblasts in the *Tubifex* embryo by intracellular injection of HRP. Dev. Growth & Differ. 41: 703-713.

Ho, R. K. and Weisblat, D. A. 1987. A provisional epithelium in leech embryo: cellular origins and influence on a developmental equivalence group. Dev. Biol. 120: 520-534.

Huang, F. Z. and Weisblat, D. A. 1996. Cell fate determination in an annelid equivalence group. Development 122: 1839-1847.

Irvine, S. M. and Martindale, M. Q. 1996. Cellular and molecular mechanisms of segmentation in annelids. Sem. Cell Dev. Biol. 7: 593-604.

Kitamura, K. and Shimizu, T. 2000. Analyses of segment-specific expression of alkaline phosphatase activity in the mesoderm of the oligochaete annelid *Tubifex*: Implications for specification of segmental identity. Dev. Biol. 219, 214-223.

Nakamoto, A., Arai, A. and Shimizu, T. 2000. Cell lineage analysis of pattern formation in the *Tubifex* embryo. II. Segmentation in the ectoderm. Int. J. Dev. Biol. 44: 797-805.

Nakamoto, A., Arai, A. and Shimizu, T. 2003. Specification of polarity of teloblatogenesis in the *Tubifex* embryo: cellular basis for bilateral symmetry in the ectoderm. (Submitted for publication).

Penners, A. 1924. Über die Entwicklung teilweise abgetöteter Eier von *Tubifex rivulorum*. Verh. Deutsch Zool. Ges. 29: 69-73.

Penners, A. 1926. Experimentelle Untersuchungen zum Determinationsproblem am Keim von *Tubifex rivulorum* Lam. II. Die

Entwicklung teilweise abgetöteter Keime. Z. Wiss. Zool. 127: 1-140.

Penners, A. 1929. Entwicklungsgeschichtliche Untersuchungen an marinen Oligochaten. I. Furchung, Keimstreif, Vordendarm und Urkeimzellen von *Pelescolex benedeni* Udekem. Z. wiss. Zool. 134: 307-344.

Penners, A. 1930. Entwicklungsgeschichtliche Untersuchungen an marinen Oligochaten. II. Furchung, Keimstreif und Keimbahn von *Pachydrilus* (*Lumbricillus*) *lineatus* Mull. Z. wiss. Zool. 137: 55-119.

Sandig, M. and Dohle, W. 1988. The cleavage pattern in the leech *Theromyzon tessulatum* (Hirudinea, Glossiphoniidae). J. Morph. 196: 217-252.

Shain, D. H., Ramirez-Weber, F.-A., Hsu, J. and Weisblat, D. A. 1998. Gangliogenesis in leech: morphogenetic processes leading to segmentation in the central nervous system. Dev. Genes Evol. 208: 28-36.

Shimizu, T. 1982. Development in the freshwater oligochaete *Tubifex*. In Developmental Biology of Freshwater Invertebrates, (ed. F. W. Harrison and R. R. Cowden), pp. 286-316. New York: Alan R. Liss.

Shimizu, T. and Nakamoto, A. 2001. Segmentation in annelids: Cellular and molecular basis for metameric body plan. Zool. Sci. 18: 286-298.

Siddal, M. E., Apakupakul, K., Burreson, E. M., Coates, K., Erseus, C., Gelder, S. R. , Kallersjo M. and Trapido-Rosenthal, H. 2001. Validating Livanow: molecular data agree that leeches, branchiobdellians, and *Acanthobdella peledina* form a monophyletic group of oligochaetes. Mol. Phylogenet. Evol. 21: 346-351.

Storey, K. G. 1989. Cell lineage and pattern formation in the earthworm embryo. Development 107: 519-531.

Svetlov, P. 1923. Sur la segmentation de l'oeuf de *Rhynchelmis limosella* Hoffmstr. Izv. biol. nauchno-issled. Inst. biol. sta. Perm. gosud. Univ. 1: 141-152.

Svetlov, P. 1926. Über die Embryonalentwichlung bei den Naididen. Izv. biol. nauchno-issled. Inst. biol. sta. Perm. gosud. Univ. 4: 359-372.

Tannreuther, G. W. 1915. The embryology of *Bdellodrilus philadelphicus*. J. Morph. 26: 143-216.

Wedeen, C. J. and Shankland, M. 1997. Mesoderm is required for the formation of a segmented endodermal cell layer in the leech *Helobdella*. Dev. Biol. 191: 202-214.

Weisblat, D. A. and Blair, S. S. 1984. Developmental interdeterminacy in embryos of the leech *Helobdella triserialis*. Dev. Biol. 101: 326-335.

Weisblat, D. A., Harper, G., Stent, G. S. and Sawyer, R. T. 1980. Embryonic cell lineages in the nervous system of the glossiphoniid leech *Helobdella triserialis*. Dev. Biol. 76: 58-78.

Weisblat, D. A., Kim, S. Y. and Stent, G. S. 1984. Embryonic origin of cells in the leech *Helobdella striserialis*. Dev. Biol. 104: 65-85.

Weisblat, D. A. and Shankland, M. 1985. Cell lineage and segmentation in the leech. Phil. Trans. R. Soc. Lond. B312: 39-56.

Whitman, C. O. 1878. The embryology of *Clepsine*. *Quart.* J. Microsc. Sci. 18: 215-315.

Zackson, S. L. 1984. Cell lineage, cell-cell interaction, and segment formation in the ectoderm of a glossiphoniid leech embryo. Dev. Biol. 104: 143-160.

Figure legends

Figure 1. Summary of *Tubifex* development. (A, B) posterior view with dorsal to the top; (C-E) dorsal view with anterior to the top; (F-H) side view with anterior to the left and dorsal to the top. (A) A 25-cell stage embryo. Cells $2d^{111}$, 4d and 4D all come to lie in the future midline. (B) After 4d divides bilaterally into left and right mesoteloblasts, M*l* and M*r*, 4D divides into a pair of endodermal precursors (E^{D}). Then $2d^{111}$ cleaves bilaterally, yielding ectoteloblast precursors, NOPQ*l* and NOPQ*r*. (C, D) Sequence of the formation of ectoteloblasts N, O, P and Q. For brevity, only teloblast precursors (OPQ and OP) and teloblasts (N-Q) are depicted (See cell lineage diagram shown in Fig. 2H.). N teloblasts are born first (C), Q teloblasts next, and then O and P teloblasts (D). (E) A 2-day-old embryo following the bilateral division of $2d^{111}$. Only teloblasts and associated structures are depicted. At this stage, a short ectodermal germ band (egb) extending from the teloblasts N, O, P and Q is seen on either side of the embryo. A mesodermal germ band (mgb) extending from the M teloblast is located under the ectodermal germ band. (F-H) Morphogenesis of the ectodermal germ band. Embryos are shown at 2.5 (F), 4 (G) and 6 (H) days following the $2d^{111}$ division. The germ band (egb) is associated, at its anterior end, with an anteriorly located cluster of micromeres (called a micromere cap; mc), and

it is initially located at the dorsal side of the embryo (F). Along with their elongation, the germ bands (egb) on both sides of the embryo gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (G). The coalescence is soon followed by dorsalward expansion of the edge of the germ band (H).

Figure 2. Ectodermal teloblastogenesis. (A-G) Spatiotemporal aspects of ectoteloblast formation. Broken lines indicate the dormal midline. Anterior is to the top. Arrows indicate the direction of cell division. (H) Cell lineage diagram showing the production of ectoteloblasts (N, O, P and Q) on the left side of the embryo. Short horizontal bars added to the vertical thick line indicate the time when small cells (n-q, op, opq, and nopq) are formed. All cell divisions included in this lineage tree occur at 2.5-hour intervals (at 22°C).

Figure 3. Designation of cells Dst and Pxm. The side of an NOPQ cell (NOPQ*l* here) proximal to the donor embryo's midline (broken line) is indicated by a cross, and the opposite side (i.e., distal to the midline) is indicated by a dot. When a transplanted NOPQ cell divides into two large cells, one of them, which has inherited the "distal" surface of NOPQ, is referred to as a Dst-cell, and the other, which has inherited the "proximal" surface, is referred to as a Pxm-cell, irrespective of the position of these cells relative to the midline of host embryos.

Figure 4. Schematic representation of cell transplantation experiments showing positions of transplanted NOPQ*l* (shaded) in host embryos. Cells that have been ablated from host embryos are indicated in parenthesis at the

lower right of each panel.

Figure 5. Patterns of development of cells descending from transplanted NOPQ cells. Fluorescence images are shown. Two large cells (i.e., Dst- and Pxm-cells) resulting from the division of NOPQ cells were labeled with lipophilic dye DiI and fixed for observation after 2-day culture. (A) N pattern with a single cell bandlet extending from a single teloblast (arrowhead). (B) OPQ pattern with three bandlets associated with three teloblasts (arrowheads). (C) A pattern other than N or OPQ. Two teloblast-like cells (arrowheads) are recognizable, but the number of bandlets is uncertain. Scale bar, 20 μm (A-C).

Figure 6. Frequency of developmental patterns of Dst- (A) and Pxm-(B) cells descending from NOPQ*l* transplanted to the right side of host embryos (see Fig. 4B). NOPQ cells to be transplanted were isolated from embryos shortly after its birth (NOPQ *early*), shortly after production of nopq1 (NOPQ *mid*) and shortly after production of nopq2 (NOPQ*late*) (See Fig. 2H.).

Figure 7. Diagrammatic summary of cellular contributions of the teloblasts N, O, P and Q to a mid body segment of *Tubifex*. For each pattern, the left half of one segment is shown, with the ventral midline (VML) and ganglion (shown in outline) to the bottom, dorsal midline (DML) to the top, and anterior to the left. Dashed lines indicate segmental boundaries.

Figure 8. Fate of ectodermal bandlets in the absence of neighboring bandlets. One of the left ectoteloblasts was injected with DiI shortly after its birth.

Control embryos (A, C, E) were allowed to develop without any further treatment after DiI injection. In experimental embryos (B, D, F), ipsilateral ectoteloblasts (or precursors) other than the DiI-injected one were all ablated, so that DiI-labeled ectoteloblasts were forced to be “solitary”. Control and experimental embryos were allowed to develop for 5 days before fixation and observed in whole-mounts. In all panels, the mid region of the embryo is viewed from the side. Anterior is to the left and dorsal is to the top. In each panel, three horizontal lines indicate the position of three consecutive segments and the approximate level of the ventral midline. (A) Control n bandlet. In each segment, a large proportion of labeled cells are localized in the ganglion and they are organized in two large clusters. Cells seen outside the ganglion are epidermal cells and peripheral neurons (not depicted here). (B) “Solitary” n bandlet. Shortly after its birth, the N teloblast was injected with DiI and the cell OPQ was ablated (see Fig. 2C). As in controls, a large proportion of labeled cells are seen in the ganglion located along the ventral midline. Epidermal cells and peripheral neurons are also seen outside the ganglion. (C) Control o bandlet. The arrowhead indicates clusters of central neurons located in the ganglion. Epidermis (asterisk) and peripheral neurons are localized in the posterior half of each segment. (D) “Solitary” o bandlet. Shortly after its birth, the O teloblast was injected with DiI and simultaneously ipsilateral teloblasts (N, P and Q) were ablated (see Fig. 2G). Unlike controls, only a few central neurons (arrowhead) are detected in the region of the ganglion. Peripheral neurons (dots) are located at both the anterior and posterior margins of each segment. The asterisk indicates a centrally located cluster of epidermal cells. In addition, a cluster of deep cells (arrow), which is identified as the ventral setal sac in the normal P lineage, is also seen

between the ganglion and the epidermis. This distribution pattern is reminiscent of the P pattern (see Fig. 8E). (E) Control p bandlet. Three clusters of labeled cells are located in the mid region of each segment. The ventralmost cluster (arrowhead) is located in the ganglion; the dorsalmost cluster (asterisk) is epidermis plus a few peripheral neurons; and the intermediate cluster (arrow) consists of deep cells (i.e., the ventral setal sac) and a few epidermal cells. Peripheral neurons (dots) are also seen at both the anterior and posterior margins of each segment. (F) “Solitary” p bandlet. Shortly after its birth, the P teloblast was injected with DiI and simultaneously ipsilateral teloblasts (N, O and Q) were ablated (see Fig. 2G). As in controls, a large cluster of epidermal cells (asterisk) and a small cluster of central neurons (arrowhead) are located in the mid region of each segment. Peripheral neurons (dots) are also seen at both the anterior and posterior margins of each segment. A cluster of deep cells (arrow) is present in each segment. Scale bar, 100 μm (A-H).

Figure 9. Model for patterning of ectodermal teloblastogenesis in the *Tubifex* embryo. Filled arrowheads (B) indicate the interactions between NOPQ cells that polarize themselves. Open arrowheads (D) indicate tentative interactions between OPQ cells that are responsible for further polarization of OPQ cells. An inductive signal from the P teloblast is indicated by a filled arrowhead (E).

Table 1. Division pattern of Pxm-cells derived from transplanted NOPQ*l*

Position of transplanted NOPQ*l* [a]	No. of embryos [b]	No. Pxm-cells		
		N pattern	OPQ pattern	Other
Right	13	11	0	2
Left	6	0	5	1
Center	8	2	2	4

[a] *Position of transplanted NOPQl in reconstituted embryos are schematically illustrated in Fig.4 D-F.*

[b] *Both of NOPQl and NOPQr were ablated from host embryos*.

Table 2. Effects of neighboring bandlets on the fate of O-derived bandlets

Combination of Teloblasts[a]	Teloblast(s) ablated	No. of embryos	No. O-derived bandlets		
			O fate	P fate	N or Q fate
O	N, P, Q	15	0	15	0
O+N	P, Q	8	0	8	0
O+P	N, Q	8	8	0	0
O+Q	N, P	6	0	6	0
O+N+Q	P	5	0	5	0

[a] *Left O teloblasts were injected with DiI and simultaneously other ipsilateral teloblasts were ablated in combinations as indicated.*

Table 3. Effects of neighboring bandlets on the fate of P-derived bandlets

Combination of Teloblasts[a]	Teloblast(s) ablated	No. of embryos	No. O-derived bandlets	
			P fate	N, O or Q fate
P	N, O, Q	20	20	0
P+N	O, Q	7	7	0
P+O	N, Q	8	8	0
P+Q	N, O	5	5	0
P+N+O	Q	10	10	0
P+N+Q	O	6	6	0

[a] *Left P teloblasts were injected with DiI and simultaneously other ipsilateral teloblasts were ablated in combinations as indicated.*