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Specification of ectodermal teloblast lineages in embryos of the oligochaete annelid *Tubifex*: involvement of novel cell-cell interactions

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SUMMARY

In embryos of clitellate annelids (i.e. oligochaetes and leeches), four ectodermal teloblasts (ectoteloblasts N, O, P and Q) are generated on either side through a stereotyped sequence of cell divisions of a proteloblast, NOPQ. The four ectoteloblasts assume distinct fates and produce bandlets of smaller progeny cells, which join together to form an ectodermal germ band. The pattern of the germ band, with respect to the ventrodorsal order of the bandlets, has been highly preserved in clitellate annelids. We show that specification of ectoteloblast lineages in the oligochaete annelid *Tubifex* involves cell interaction networks distinct from those in leeches. Cell ablation experiments have shown that fates of teloblasts N, P and Q in *Tubifex* embryos are determined rigidly as early as their birth. In contrast, the O teloblast and its progeny are initially pluripotent and their fate becomes restricted to the O fate through an inductive signal emanating from the P lineage. In the absence of this signal, the O lineage assumes the P fate. These results differ significantly from those obtained in embryos of the leech *Helobdella*, suggesting the diversity of patterning mechanisms that give rise to germ bands with similar morphological pattern.

Key words: Teloblast, Cell interactions, Germ band, Annelid, *Tubifex*

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 (Anderson, 1973; Devries, 1973a; Fernandez and Olea, 1982; Shimizu, 1982; Irvine and Martindale, 1996). Teloblasts, which are derived from micromeres of the D quadrant, repeatedly undergo extremely unequal divisions 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. From previous descriptive and cell ablation studies (Whitman, 1878; Penners, 1924; Penners, 1926; Devries, 1973a; Devries, 1973b), 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). (Note that the precursors of the M and NOPQ in leech embryos have been designated as DM and DNOPQ, respectively; see Stent et al., 1982.) Recent cell lineage analyses of teloblasts have shown that developmental fates of the four ectoteloblasts are not only different from those of the mesoteloblast but also distinct among themselves (Weisblat et al., 1980; Weisblat et al., 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); however, nothing is known about the causal relationship between developmental fates of teloblasts and spatiotemporal aspects of their emergence. The only thing that is known about specification of ectoteloblast lineages in clitellate annelids is that in the leech *Helobdella*, bandlets derived from the O and P teloblasts are initially equipotent and can be differentiated from each other according to their position within the GB (Weisblat and Blair, 1984; Zackson, 1984). Recently, it has also been suggested that the cell fate determination in this equivalence group occurs through inductive signals from another teloblast lineage and the

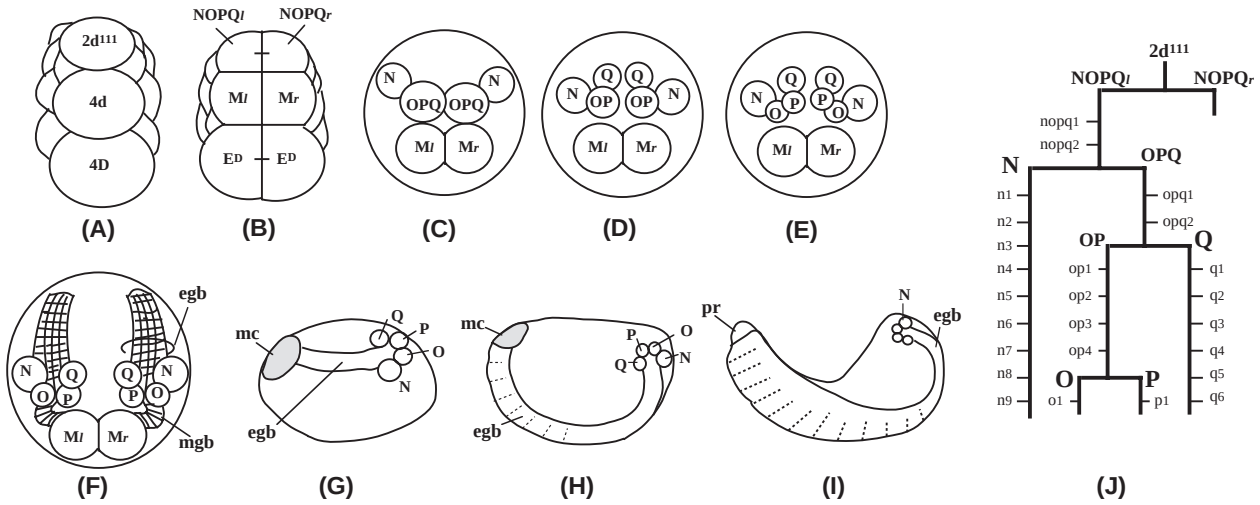


Fig. 1. Summary of *Tubifex* development. (A-I) Selected stages of embryonic development. (A,B) Posterior view with dorsal towards the top; (C-F) dorsal view with anterior towards the top; (G-I) side view with anterior towards the left and dorsal towards the top. (A) A 25-cell stage embryo. Cells $2d^{111}$, 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-E) 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. 1J.). N teloblasts are born first (C), Q teloblasts next (D), and then O and P teloblasts (E). (F) 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. (G-I) Morphogenesis of the ectodermal germ band. Embryos are shown at 2.5 (G), 4(H) and 6 (I) 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 (G). 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 (H). The coalescence is soon followed by dorsalwards expansion of the edge of the germ band (I). (J) 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).

micromere-derived epithelium (Ho and Weisblat, 1987; Huang and Weisblat, 1996). It remains to be determined whether this network of cell interactions is widespread in clitellate annelids.

The present study was undertaken to gain an insight into the mechanisms that underlie specification of ectoteloblast lineages in the oligochaete annelid *Tubifex*. The objectives of this study were to determine the timing of specification of ectoteloblast lineages and to determine whether specification of each lineage depends on external cues. For this purpose, we used embryological techniques such as cell ablation in combination with labeling of specific blastomeres with lineage tracers. Our results show that teloblasts N, P and Q are specified to express the N, P and Q fates, respectively, as early as their birth, and that 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 suggest that it is unlikely that sister teloblasts O and P in *Tubifex* embryos constitute an equivalence group such as that seen in the leech embryo.

MATERIALS AND METHODS

Embryos

Embryos of the freshwater oligochaete *Tubifex hattai* were obtained as previously described (Shimizu, 1982) and cultured at 22°C. For the experiments, embryos were all freed from cocoons in the culture medium (Shimizu, 1982). To sterilize their surface, cocoons were treated with 0.02% chloramine T (Wako Pure Chemicals, Osaka, Japan) for 3 minutes and washed thoroughly in three changes of the culture medium. The culture medium used in cell-ablation experiments was autoclaved and, shortly before use, antibiotics (penicillin G and streptomycin, 20 units/ml each) were added. Unless otherwise stated, all experiments were carried out at room temperature (20-22°C).

Microinjection of lineage tracer DiI

To label ectoteloblasts and their progeny cells, ectoteloblasts or their precursors (cells NOPQ, OPQ and OP; see Fig. 1B-D) were pressure-injected with DiI (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes). DiI was dissolved in ethanol at 100 mg/ml and stored at room temperature. Before use, an aliquot of this solution was diluted 20 times in safflower oil (Kitamura and Shimizu, 2000). Ectoteloblasts or their precursors were injected with oil droplets containing DiI by means of micropipettes. DiI-injected embryos were kept in darkness.

Blastomere ablation

Embryos without vitelline membranes were placed on 2% agar in the culture medium. Blastomeres were killed by making a wound on their surface with fine forceps. Within a minute, the yolk mass of these cells began to coagulate. The coagulating cells were removed by pulling them away from the remainder of the embryo. The operated embryos were allowed to develop in culture medium containing antibiotics, which was renewed daily.

Preparation of embryos for observation

DiI-labeled embryos were fixed with 3.5% formaldehyde in phosphate buffer (40.5 mM Na_2HPO_4, 9.5 mM NaH_2PO_4, pH 7.4) for 1 hour and mounted in phosphate buffer for observation. Images were collected on a Molecular Dynamics Sarastro-2000 confocal laser-scanning microscope. Some specimens were viewed under a Zeiss Axioskop epifluorescence microscope.

RESULTS

Summary of early development of *Tubifex*

A brief review of the early development in *Tubifex* is presented here as a background for the observations described below (for details, see Shimizu, 1982; Goto et al., 1999a; Goto et al., 1999b). Precursors of teloblasts are traced back to the second (2d) and fourth (4d) micromeres of the D quadrant. At the 25-cell stage, $2d^{111}$, 4d and 4D (sister cell of 4d) all come to lie in the future midline of the embryo (Fig. 1A). 4d divides equally to yield the left and right mesoteloblasts (M*l* and M*r*); $2d^{111}$ divides into a bilateral pair of ectoteloblast precursors, NOPQ*l* and NOPQ*r*; and 4D divides equally yielding endodermal precursors E^D (Fig. 1B). Ectoteloblasts N, O, P and Q arise from an invariable sequence of divisions of cell NOPQ on both sides of the embryo (Fig. 1J; Goto et al., 1999b). NOPQ on either side of the embryo undergoes unequal divisions twice after its birth and then divides into a smaller N teloblast and a larger cell, OPQ (Fig. 1C). Similarly, after producing small cells twice, OPQ divides into a smaller Q teloblast and a larger cell, OP (Fig. 1D). Finally OP undergoes unequal division four times after its birth and then cleaves almost equally, yielding the third-born ectoteloblasts O and P (Fig. 1E), at which point teloblastogenesis is complete.

After their birth, each of the teloblasts thus produced divides repeatedly, at 2.5 hour intervals (at 22°C), to give rise to small cells called primary blast cells, which are arranged into a coherent column (i.e. a bandlet; Fig. 1F). Within each bandlet, primary blast cells and their descendants are arranged in the order of their birth. Bandlets from N, O, P and Q teloblasts on each side of the embryo join together to form an ectodermal GB, while the bandlet from the M teloblast becomes a mesodermal GB that underlies the ectodermal GB (Fig. 1F; Goto et al., 1999a). The GBs are initially located on the dorsal side of the embryo (Fig. 1G). Along with their elongation, they gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (Fig. 1H). The coalescence is soon followed by dorsalwards expansion of GBs. The edges of the expanding GBs on both sides of the embryo finally meet along the dorsal midline to enclose the yolky endodermal tube (Fig. 1I; Goto et al., 1999a; Goto et al., 1999b).

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 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. 2; Goto et al., 1999b).

Fates of 'solitary' ectodermal bandlets

Our previous study has shown that a bandlet derived from a 'solitary' O teloblast (resulting from removal of all of its ipsilateral sister teloblasts) exhibits early morphogenetic features (e.g., shape of bandlets) characteristic to the P lineage rather than the O lineage, while bandlets derived form 'solitary' N, P and Q teloblasts are very similar to the respective bandlets in intact embryos (Nakamoto et al., 2000). In this study, we have extended this observation to more advanced developmental stages when cells are terminally differentiated. To do this, we labeled one of the four ectoteloblasts with DiI and ablated the other three ipsilateral ectoteloblasts (or their precursors) simultaneously. After 5 days culture, we examined the composition and distribution of labeled cells descending from 'solitary' teloblasts. In nearly all of the operated embryos, bandlets derived from 'solitary' teloblasts were found to have elongated along the anteroposterior axis in a normal fashion; the dorsalward migration of blast cell progeny also occurred to the same extent

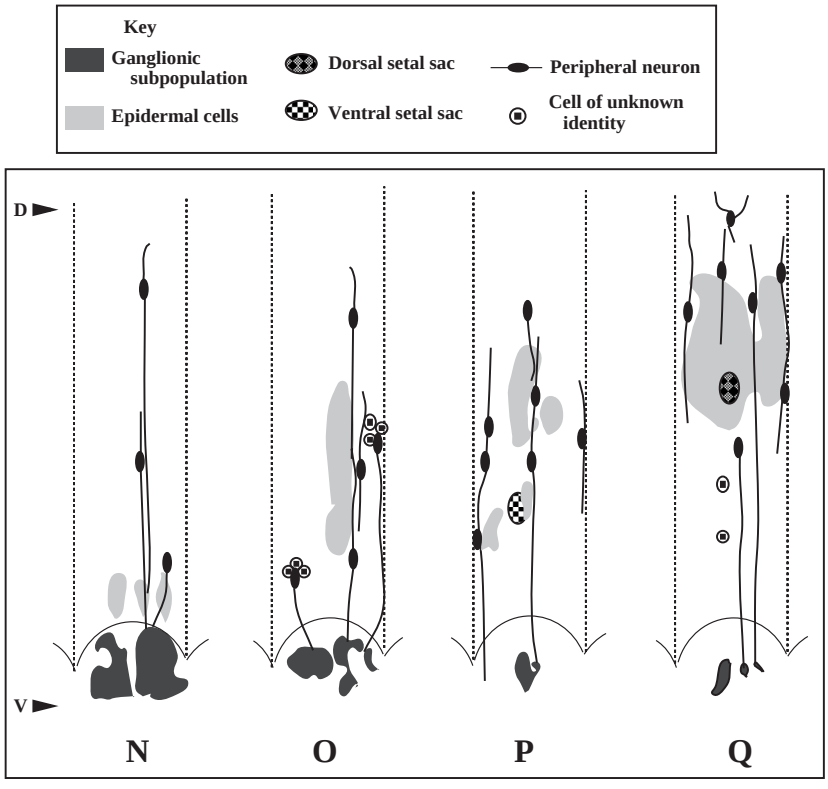


Fig. 2. Cellular contributions of the teloblasts N, O, P and Q to a mid body segment of *Tubifex*. For each pattern, the left half of one segment is shown, with the ventral midline (V) and ganglion (shown in outline) towards the bottom, dorsal midline (D) towards the top and anterior towards the left. Broken lines indicate segmental boundaries.

as that in intact embryos. This allowed us to assess fates of 'solitary' teloblasts on the basis of the distribution pattern of differentiated cells. More than 15 embryos were examined for each lineage.

Fig. 3A,B shows 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. 3E-H).

In contrast, organization and composition of cells derived from 'solitary' O teloblasts are distinct from those in normal o bandlets. As Fig. 3D shows, 'solitary' o bandlets apparently exhibited a P pattern rather than an O pattern of progeny cells (also see Fig. 3C). This result suggests that 'solitary' o bandlets adopt the P fate rather than the O fate.

In the foregoing experiments, only the left GB was operated on and the contralateral (right) GB remained intact in each embryo. This raises the possibility that the fate of the 'solitary' bandlets on the left side could be affected by the intact right GB when they aligned themselves along the ventral midline (Fig. 1H). To examine this possibility, we followed the fate of 'solitary' left bandlets in embryos that had been subjected to bilateral ablations of ectoteloblasts. To do this, we ablated right NOPQ (i.e. exclusive source of the right ectodermal GB; see Fig. 1B) and three of the four left ectoteloblasts, leaving a single (DiI-labeled) ectoteloblast in each embryo. After 5 days culture, the operated embryos (five to seven 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 (Fig. 4A,C,D) and that o bandlets followed the P fate rather than the O fate (Fig. 4B). These results are apparently the same as those obtained in the unilateral ablation experiments. Thus, we 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

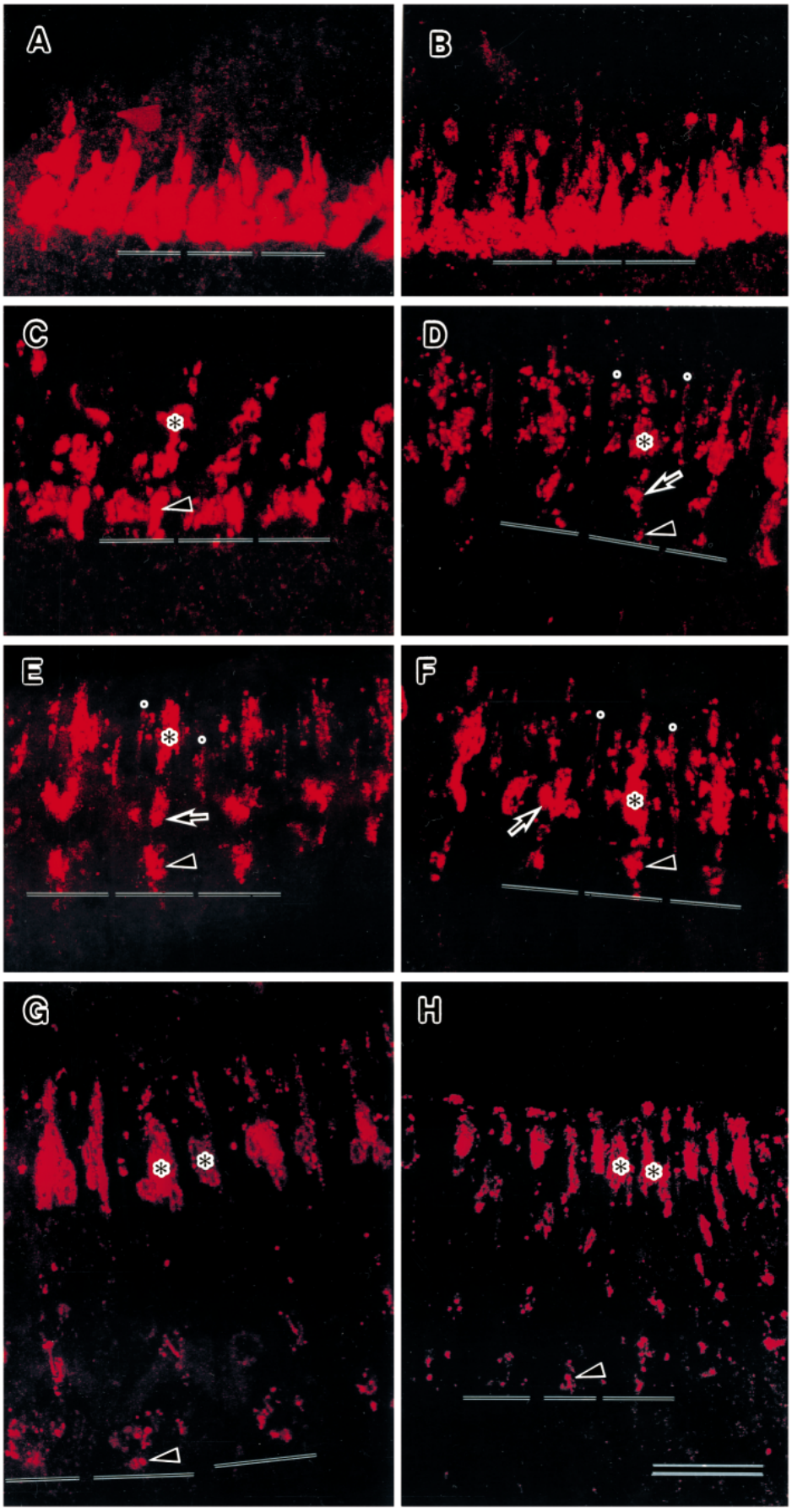
The aforementioned results suggest the possibility that in intact GB, o bandlets are induced to assume O fate by interactions with other bandlets. To test this possibility and to find out which bandlet acts as such an inducer, we ablated teloblasts in various combinations, leaving an O teloblast plus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

The results are summarized in Table 1. It was only when p bandlets survived that o bandlets assumed the O fate (Fig. 5B). Neither n nor q bandlets were effective at all in this respect (Fig. 5A); even when both n and q bandlets coexisted with o bandlets, they failed to induce o bandlets to assume the O fate (Fig. 5C). It is unlikely that this failure resulted from separation of o bandlets from n and/or q bandlets in operated embryos, as bandlets in a GB from which one bandlet had been deleted were found to be aligned tightly with each other (not shown). These results suggest that p bandlets exclusively serve as an inducer of O fate in o bandlets.

Fig. 3. 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,G) were allowed to develop without any further treatment after DiI injection. In experimental embryos (B,D,F,H), 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 towards the left and dorsal is towards 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. 1C). 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. 1E). 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 in D), 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. 3E). (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. 1E). 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. (G) Control q bandlet. Two large clusters of epidermal cells (asterisks) are located near the dorsal midline in each segment. Along the ventral midline only a few central neurons (arrowhead) are seen. (H) 'Solitary' q bandlet. Shortly after its birth, the Q teloblast was injected with DiI and simultaneously ipsilateral N teloblast and OP proteloblast were ablated (see Fig. 1D). As in controls, clusters of epidermal cells (asterisks) are located near the dorsal midline, and a few central neurons (arrowhead) are seen along the ventral midline. Scale bar: 100 μm.

Interaction between O and P lineages occurs prior to first division of primary blast cells

In normal *Tubifex* embryos, differences between the O and P



lineages are manifested as early as the time of the first division of primary blast cells. Primary o blast cells enter first mitosis at a distance of 7 cells from the parent teloblast and undergo equal division (Fig. 6A); in contrast, primary p blast cells undergo unequal division at a distance of 5 cells (Fig. 6C). These differences suggest the possibility that the induction of the O lineage by the P lineage occurs prior to first mitosis of primary o blast cells. This possibility was verified by our observation that primary blast cells derived from 'solitary' O teloblasts entered first mitosis at a distance of 5 cells from the parent teloblast and underwent unequal division (Fig. 6B). This division pattern is evidently a P pattern, not an O pattern. Thus, it is likely that in intact GB, inductive signals from the P lineage also determine the pattern of first division in primary o blast cells.

P teloblast may be specified to assume the P fate at 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. 1J), 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. After 5 days culture, we examined the distribution of labeled cells.

The results are summarized in Table 2. Irrespective of the presence of any other teloblasts, p bandlets assumed the P fate (Figs 4C, 5D). 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 their birth.

Table 1. Effects of neighboring bandlets on the fate of O-derived bandlets

Combination of teloblasts	Teloblast(s) ablated	Number of embryos	Number of 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

Left O teloblasts were injected with DiI and simultaneously other ipsilateral teloblasts were ablated in combinations as indicated. After 5 days culture, embryos were examined for the distribution of progeny of DiI-labeled O teloblasts.

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

Combination of teloblasts	Teloblast(s) ablated	Number of embryos	Number of 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

Left P teloblasts were injected with DiI and simultaneously other ipsilateral teloblasts were ablated in combinations as indicated. After 5 days culture, embryos were examined for the distribution of progeny of DiI-labeled P teloblasts.

DISCUSSION

During *Tubifex* embryogenesis, NOPQ on either side of the embryo generates four ectoteloblasts (N, O, P and Q) through a stereotyped sequence of cell division. These teloblasts are strictly regulated not only in the order of their emergence but also in their position along the embryonic axes. Furthermore, these four teloblasts assume distinct fates (Goto et al., 1999b). The present study shows that the fates of any of the N, P and Q teloblasts are not affected by ablation of the other three teloblasts at birth. This suggests that these teloblasts are committed to their respective fates at their birth.

At present, nothing is known about either the mechanisms for cell fate determination in teloblasts or the way in which different teloblasts acquire distinct developmental fates. One possibility is that fates of teloblasts are determined according to positional cues along the embryonic axes. Alternatively, fates of teloblasts could be determined by intrinsic factors that are asymmetrically segregated during teloblastogenesis. Additionally, it is possible that the commitment of a teloblast is due to inductive signals coming from its sister cell at their birth. It is therefore interesting to note that left NOPQ that has been transplanted to the right side of another embryo (from which right M and NOPQ had been ablated) undergoes a sequence of cell division that is identical to that in left NOPQ of intact embryos; a first-born teloblast, which is located dorsalmost on the right side of such a reconstituted embryo, assumes the N fate, and a second-born teloblast, located ventralmost, expresses the Q fate (A. A., unpublished). In intact embryos, the N teloblast is born first and located ventralmost, and the Q teloblast, which is born next, is located dorsalmost (Fig. 1E). Thus, these results suggest it is unlikely that cell fates of teloblasts are determined according to the positional cues along the embryo's dorsoventral axis. Rather, it seems more likely that NOPQ is already polarized at its birth and that cell fate decision occurs along this polarity during teloblastogenesis.

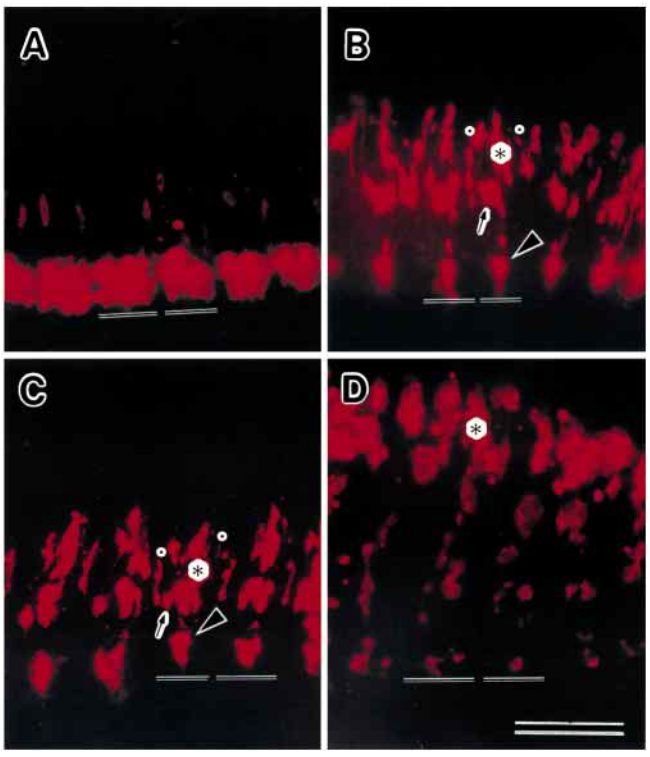


Fig. 4. Fate of 'solitary' ectodermal bandlets in the absence of both ipsilateral and contralateral bandlets. In each experimental embryo from which right NOPQ had been ablated, one of the left ectoteloblasts was injected with DiI shortly after its birth, and the other three ipsilateral ectoteloblasts were all ablated. Experimental embryos were allowed to develop for 5 days before fixation and observed in whole mounts. In all panels, anterior is towards the left and dorsal is towards the top. In each panel, two horizontal lines indicate the position of two consecutive segments and the approximate level of the ventral midline. (A) 'Solitary' n bandlet exhibits N pattern of distribution of progeny cells. In each segment, a large proportion of labeled cells are organized in a cluster that is reminiscent of a hemiganglion. Peripheral neurons are also seen in the ventral region. (B) 'Solitary' o bandlet exhibits P pattern of distribution of progeny cells. Central neurons are organized in a single cluster (arrowhead) located in the mid region of each segment. Peripheral neurons (dots), a cluster of deep cells (arrow) and a cluster of epidermal cells (asterisk) all show the distribution pattern characteristic of the P lineage. (C) 'Solitary' p bandlet. Progeny cells are distributed in a pattern comparable to that in intact p bandlets (see Fig. 3E). The arrow indicates a cluster of deep cells (i.e. the ventral setal sac), the arrowhead indicates a cluster of central neurons and the dots indicate peripheral neurons at the anterior and posterior margins of the segment. (D) 'Solitary' q bandlet exhibits Q pattern of distribution of progeny cells. Clusters of epidermal cells (asterisk) are located near the dorsal midline, and a few central neurons are seen along the ventral midline. Scale bar: 100 μm.

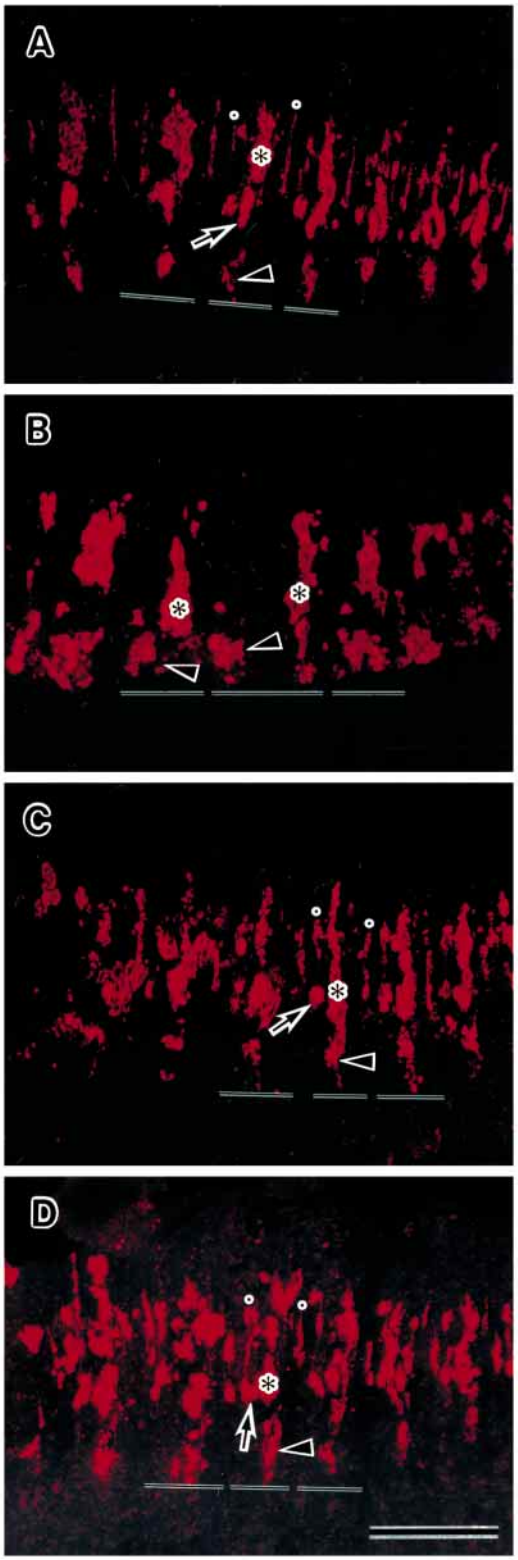


Fig. 5. (A-C) Fate of o bandlets in the presence of various neighboring bandlets. Left O teloblasts were injected with DiI shortly after birth, and simultaneously ipsilateral teloblasts, P+Q (A), N+Q (B) or P(C), were ablated. Operated embryos were allowed to develop for 5 days before fixation and were observed in whole mounts. In all panels, anterior is towards the left and dorsal is towards the top. In each panel, three horizontal lines indicate the position of three consecutive segments and the approximate level of the ventral midline. (A) An o bandlet in the presence of the n bandlet (but in the absence of p and q bandlets). DiI-labeled cells exhibit a distribution pattern characteristic of the P fate. The arrow indicates a cluster of deep cells (i.e. the ventral setal sac), the arrowhead indicates central neurons in the ganglion, and the dots indicate peripheral neurons at the anterior and posterior margins of the segment. Epidermal cells (asterisk) are organized in a large cluster in the mid zone of each segment. (B) An o bandlet in the presence of the p bandlet (but in the absence of n and q bandlets). DiI-labeled cells exhibit a distribution pattern characteristic of the O fate. Relatively large clusters of central neurons (arrowheads) are located in the ganglion. Epidermis (asterisks) and peripheral neurons are localized in the posterior half of each segment. (C) An o bandlet in the presence of the n and q bandlets (but in the absence of the p bandlet). DiI-labeled cells exhibit a distribution pattern characteristic of the P fate. The arrow indicates the ventral setal sac, the arrowhead indicates central neurons in the ganglion and the asterisk indicates the epidermis. Peripheral neurons (dots) are seen at both the anterior and posterior margins of each segment. (D) Fate of the p bandlet in the presence of the n and o bandlets (but in the absence of the q bandlet). A left P teloblast was injected with DiI and simultaneously ipsilateral Q teloblast was ablated. Anterior is towards the left and dorsal is towards the top. Three horizontal lines are added to indicate the position of three consecutive segments and the approximate level of the ventral midline. Judging from the distribution of central neurons (arrowhead), peripheral neurons (dots), epidermal cells (asterisk) and the ventral setal sac (arrow), this bandlet expresses the P fate. Scale bar: 100 μm.

Developmental plasticity in o blast cells

Of the four ectoteloblast lineages, the O lineage is the only one that is affected by ablation of the other lineages (i.e. bandlets). The present study showed that 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. Thus, it is reasonable to assume that O teloblasts are pluripotent.

It appears that in intact GBs, pluripotent o blast cells are induced, by interactions with a p bandlet, to assume the O fate prior to their entry into first mitosis. In intact embryos, the O and P teloblasts lie next to each other, and primary o blast cells come to be in contact with primary p blast cells during their birth (Fig. 7A). It is conceivable that primary o blast cells are induced to assume the O fate as early as their birth.

Sister teloblasts O and P: 'equivalence group'?

The O and P teloblasts are sister blastomeres resulting from the equal division of an OP proteloblast. As discussed 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. On the basis of 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.

Comparison with other annelids

The aforementioned situation of *Tubifex* O and P teloblasts is

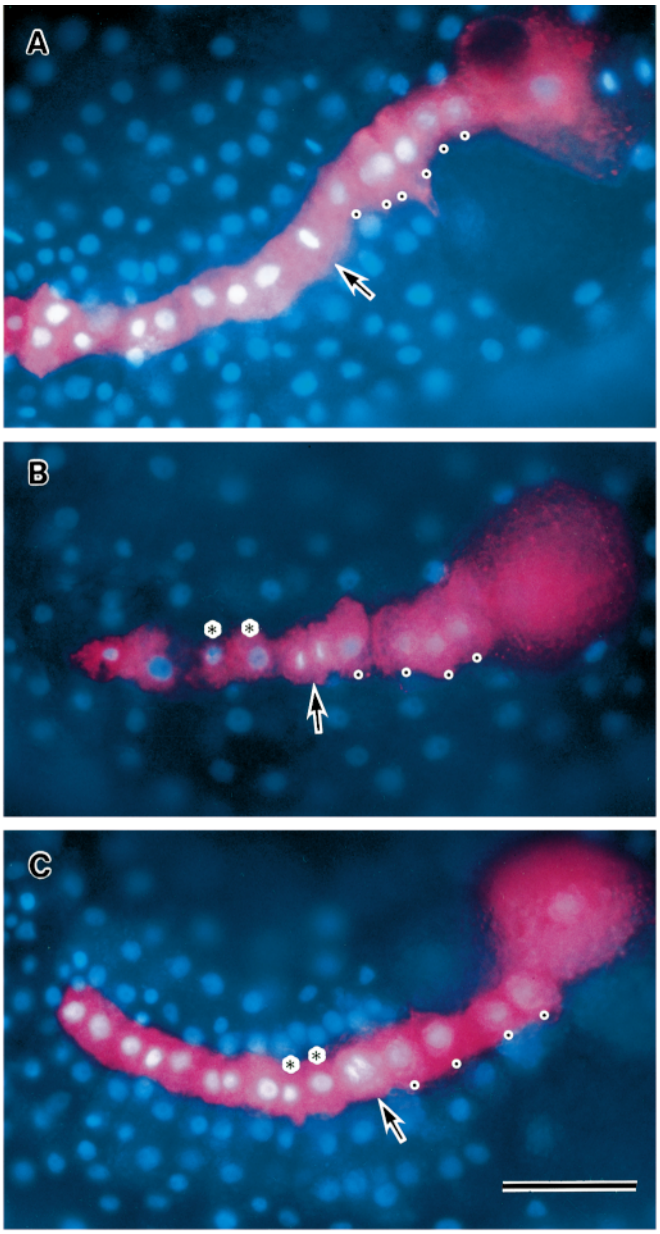


Fig. 6. Pattern of first mitosis in primary o and p blast cells. Shortly after their birth, left O (A,B) or P (C) teloblasts were injected with DiI and in some embryos, ipsilateral teloblasts other than a DiI-labeled one were simultaneously ablated. These embryos were fixed after 30-36 hours and stained with Hoechst 33342 to visualize nuclei. Left germ bands were dissected out and observed by epifluorescence microscopy. Double exposure fluorescence micrographs are shown. DiI fluorescence is red and Hoechst dye fluorescence is blue; regions of overlap are white. In all panels, anterior is towards the left and dorsal is towards the top. (A) An o bandlet in an intact embryo. Six primary blast cells (indicated by dots) are seen between the O teloblast (upper right) and the dividing blast cell (arrow). (B) An o bandlet derived from a 'solitary' O teloblast. Four primary blast cells (dots) are seen between the teloblast and the dividing blast cell (arrow). The asterisks indicate two cells of different sizes, which result from unequal first division. (C) A p bandlet in an intact embryo. Four primary blast cells (dots) are seen between the P teloblast (upper right) and the dividing blast cell (arrow). Note two cells of different sizes (asterisks) located anteriorly to the dividing cell. Scale bar: 100 μm.

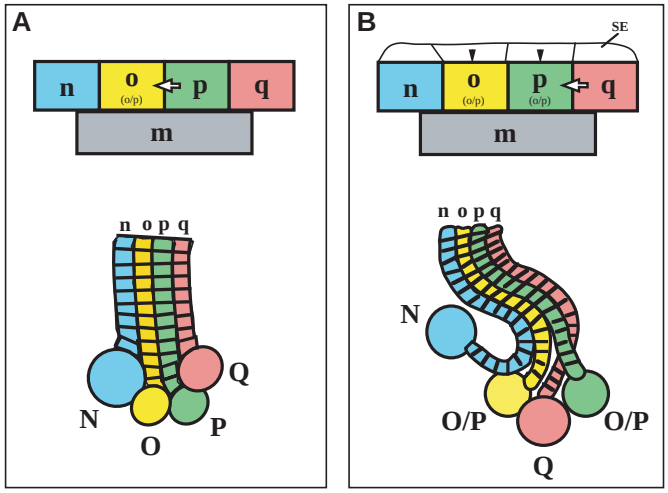


Fig. 7. Comparison of cell interactions for patterning of the ectodermal germ band in *Tubifex* (A) and *Helobdella* (B). In each panel, a cross section of a left germ band (including m bandlet) is shown in the upper part; the early stage of germ band formation is presented in the lower part. Dorsal is towards the right and ventral is to the left. (A) The o bandlet in the *Tubifex* embryo is initially pluripotent (as indicated by 'o/p') and it is induced, by a signal (arrow) emanating from the p bandlet, to assume the O fate. This induction presumably occurs as early as the birth of primary blast cells from the parent O teloblast. In contrast, teloblasts N, P and Q are determined autonomously at their birth. (B) In *Helobdella*, the third-born teloblasts (corresponding to the O and P teloblasts in *Tubifex*) have been designated as O/P teloblasts because of their equal developmental potential (Weisblat and Blair, 1984). Their undetermined progeny cells are therefore designated as o/p blast cells and bandlets. It should be noted that the ventrodorsal order of O/P teloblasts is not necessarily reflected in the ventrodorsal order of their descendant bandlets in the germ band; this is because in one third of leech embryos, o/p bandlets cross each other before they enter the germ band. The bandlets derived from the O/P teloblasts are initially equipotent and differentiate from each other through an inductive signal (open arrow) from the q bandlet (Huang and Weisblat, 1996). An o/p bandlet that happens to lie next to the q bandlet is induced to assume the P fate (hence it becomes the p bandlet); the other o/p bandlet lying next to the n bandlet follows the (primary) O fate, owing to the lack of an inductive signal (hence becoming the o bandlet). Thus, the location of both o/p bandlets between the ventralmost n and the dorsalmost q bandlets leads autonomously to the ventrodorsal order of bandlets (n-o-p-q) that is identical to that in *Tubifex*. Though less characterized, some kinds of signals (arrowheads), originating from the squamous epithelium (SE), may play a role in specifying o/p bandlets (Ho and Weisblat, 1987).

in sharp contrast to that of O/P teloblast pairs in the leech *Helobdella*, which are thought to constitute a so-called 'equivalence group' (Weisblat and Blair, 1984; Zackson, 1984). Leech O/P teloblasts are homologs of *Tubifex* O and P teloblasts, in that O/P teloblasts are third-born teloblasts that result from the equal division of an OP proteloblast (Fernandez and Olea, 1982; Sandig and Dohle, 1988). Unlike the O and P teloblasts in *Tubifex*, however, sister O/P teloblasts in *Helobdella* are both pluripotent and have the potential to follow either an O or P fate. Blast cells derived from either O/P teloblast assume the secondary (P) fate if they interact with a bandlet derived from the Q teloblast; otherwise o/p blast cells

express the primary (O) fate (Huang and Weisblat, 1996; Fig. 7B). More importantly, an o/p bandlet is unable to induce another o/p bandlet of the same GB to assume the secondary fate (Huang and Weisblat, 1996). Furthermore, in *Helobdella*, it has also been suggested that the micromere-derived epithelium that overlies the GB plays a role in fate decision in o/p bandlets (Ho and Weisblat, 1987). This is not the case for *Tubifex*, however, as the ectodermal GB in embryos of this animal is not overlain by an epithelium during its migration toward the ventral midline (see Fig. 1G,H; A. A., unpublished). Thus, *Tubifex* and *Helobdella* involve distinct cell interaction networks in patterning the ectodermal GB. It would be of interest to investigate whether the molecular natures of inductive interactions (between o and p in *Tubifex* and o/p and q in *Helobdella*) are also different in these animals.

In relation to these differences in the mechanisms for specification of ectoteloblast lineages, it is noteworthy that *Tubifex* and *Helobdella* are also different in the mode of bandlet formation. In *Tubifex*, the four ectoteloblasts on either side of the embryo are arranged in a row running along the dorsoventral axis, and they are closely associated with each other (Fig. 7A). Blast cells produced from each teloblast are located on the surface of the embryo and they are integrated into a GB shortly after their birth. There is a strict correspondence in the ventrodorsal order of the ectoteloblasts and their descendant bandlets (Fig. 7A). In *Helobdella*, the four ectoteloblasts are arranged in an invariable pattern, but as a result of extensive changes in their relative positions during teloblastogenesis, their final positions do not necessarily reflect their birth order (Fig. 7B). The Q teloblast is always located between O/P teloblasts; the N teloblast is located at a distance from the other teloblasts (Fernandez and Stent, 1980). As a result of such an irregular location of teloblasts, bandlets are initially ‘solitary’; in about one third of embryos, two bandlets derived from an O/P pair cross each other before they are integrated in the GB (Fernandez and Stent, 1980; Zackson, 1984; Weisblat and Blair, 1984), suggesting the spontaneous transposition of o/p bandlets in *Helobdella* embryos.

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. Similar evolutionary changes in cell interaction networks that can produce structures with similar morphological pattern have also been reported in vulval development of nematodes (Sommer and Sternberg, 1994; Sommer et al., 1994).

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