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Developmental significance of D quadrant micromeres 2d and 4d in the oligochaete annelid *Tubifex tubifex*

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ABSTRACT The annelid *Tubifex tubifex* is a cosmopolitan freshwater oligochaete and a member of the Spiralia, a large group of invertebrate phyla displaying spiral development. Because its developing eggs are easily obtained in the laboratory, this animal has long been used as material for developmental studies, especially spiralian embryology. In spiralian embryos, it has long been known that one blastomere at the four-cell stage, the D cell, and its direct descendants play an important role in axial pattern formation. Various studies have suggested that the D quadrant functions as the organizer of the embryonic axes in molluscs and annelids, and it has recently been demonstrated that the D quadrant micromeres, 2d[11] and 4d, which had been transplanted to an ectopic position in an otherwise intact embryo induce a secondary embryonic axis to give rise to the formation of duplicated heads and/or tails. That 2d and 4d play a pivotal role in *Tubifex* embryonic development was first suggested from the classic cell-ablation experiments carried out in the early 1920s, and this has been confirmed by the recent cell-ablation/restoration experiments using cell-labeling with lineage tracers. These studies have also shown that in the operated embryos, none of the remaining cells can replace the missing 2d and 4d and that both 2d and 4d are determined as ectodermal and mesodermal precursors, respectively, at the time of their birth. The anteroposterior polarity of these micromeres is also specified at the time of their birth, suggesting that nascent 2d and 4d are specified in their axial properties as well as in cell fate decision.

KEY WORDS: *D quadrant, 2d, 4d, cell lineage, embryonic axis, axial organizer, anteroposterior polarity, Tubifex tubifex*

Introduction

"That a single cell can carry the total heritage of the complex adult, that it can in the course of a few days or weeks give rise to a mollusc or a man, is one of the great marvels of nature."

E. B. Wilson (1925)

The annelid *Tubifex tubifex* is a freshwater oligochaete, which is a member of "Spiralia" (Schleip, 1929), a clade of invertebrate phyla showing spiral cleavage during their early stages of development. Since the first publication of its embryonic cell lineages (Penners, 1922), this animal has long been serving as a model organism to study spiralian embryology. Here, we review the results of our recent experiments as well as Penners' classic experiments on embryos of *Tubifex tubifex*.

The Spiralia comprises around seven animal phyla, including Annelida, Mollusca, Sipuncula, Nemertini, Platyhelminthes, Entoprocta, and Gnathostomulida, which exhibit high diversity of adult body plans (Nielsen, 2001). Descriptive and experimental studies on spiralian embryology, which have been done since the publication of *Nereis* cell lineage by Wilson (1892), have revealed that in contrast to the diversity of adult body forms, spiralians share a highly conserved early developmental program, especially in cell division pattern, D quadrant blastomere specification, the formation of 4d micromere referred to as the mesentoblast, and the ability to organize an embryo of the D quadrant and its descendants.

In spiralian embryos, typically, the first two cleavages generate four macromeres named A to D; each macromere corresponds to roughly one quadrant of the embryo (see Fig. 1C), and the D cell marks the later dorsal side of the embryo. At the third cleavage,

Abbreviations used in this paper: GB, germ band; PGC, primordial germ cell.

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each cell divides usually unequally to give an animal micromere and a vegetal macromere: 1A and 1a, 1B and 1b, etc. The macromeres then generate further quartets of micromeres via a coordinated set of cleavage (see Wall, 1990, for details).

During embryogenesis, one of the macromeres (generated at the four cell stage) and its descendants become determined as dorsoposterior quadrant of the embryo, which gives rise to the establishment of the future dorsoventral axis. This process has been referred to as D quadrant specification; it occurs, via cytoplasmic localization in unequal cleavers such as *Tubifex* and *Ilyanassa* at the time of the second cleavage, and, via cell interactions in equal cleavers such as *Lymnaea* and *Patella* around the time of the 5th cleavage (Freeman and Lundelius, 1992).

In many spiralian species, mesoderm arises from two distinct regions of the embryo, the second and third quartet micromeres (collectively called ectomesoderm) and the fourth micromere (4d) of the D quadrant; the latter cell is referred to as the mesentoblast because it contributes to both mesodermal and endodermal tissues (Lyons *et al*., 2012). The formation of the mesentoblast (i.e., 4d micromere) from the D quadrant, which is a conserved feature of spiralian development (Lambert, 2008; Lyons *et al*., 2012), is a consequence of D quadrant specification. In contrast, the particular micromeres that give rise to both mesoderm and ectoderm (i.e., ectomesoderm) vary among species.

Another consequence of D quadrant specification is the emergence of cells that possess the ability to organize an embryo. In not a few spiralian species so far studied, the D quadrant and/or its descendants (viz., 2d and 4d) have been demonstrated to function as embryonic organizers, which are defined as embryonic cells or group of cells that has axial patterning effects on other cells of the embryo through an inductive signal, i.e., the organizing activity acts on cells that are not part of the embryonic organizer (Lambert, 2008; Amiel *et al*., 2013).

In this paper, we focus on the developmental potency of the D quadrant and its descendant cells in the *Tubifex* embryo. As described below, *Tubifex* is an unequal cleaver, and D quadrant specification takes place at the 4-cell stage, probably via cytoplasmic localization. As in other spiralians, the fourth micromere (4d) of the D quadrant generates mesodermal fate in the *Tubifex* embryo. However, it should be mentioned here that the D quadrant micromeres (especially 2d and 4d) of the *Tubifex* embryo are different from those in other spiralians in two respects. First, 4d micromere of the *Tubifex* embryo is not a mesentoblast but an immediate precursor of teloblasts that generate only mesoderm but not endoderm. Second, 2d and 4d are categorized as "micromeres", but both cells are as large as macromeres (see Fig. 1F) while other D quadrant micromeres (i.e., 1d and 3d) are as small as other quadrant micromeres (see Fig. 1D). This large size of 2d and 4d (and their equivalence) appears to be a conserved characteristics among clitellate annelids (i.e., oligochaetes + leeches; Anderson, 1973).

A brief historical note on experimental embryology of *Tubifex*

Tubifex tubifex (Annelida; Clitellata; Oligochaeta; Tubificidae) is a cosmopolitan freshwater oligochaete distributed on all six continents, excluding Antarctica (Shimizu, 1982). This animal is readily available to most investigators and can be collected in abundance. Furthermore, developing eggs are easily obtained in the laboratory. For these reasons, *Tubifex* has long been used as material for developmental studies.

In the early 1920s, German embryologist Andreas Penners published the results of his observations on normal embryos of *Tubifex*

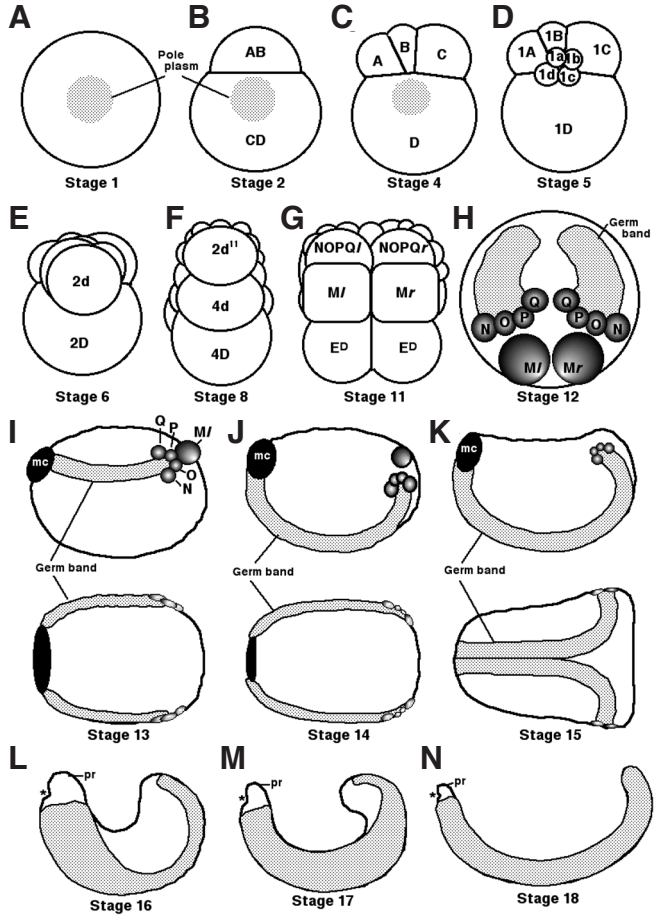

Fig. 1. Summary of Tubifex development. *Diagrammatic illustration of selected stages of embryonic development.* **(A-D)** *Animal pole views of embryos at stages 1-cell (A), 2-cell (B), 4-cell (C) and 8-cell (D).* **(E-F)** *Embryos at stages 10-cell (E) and 22-cell (F). Posterior view with dorsal to the top. Three cells (2d^{11}, 4d and 4D) of the D cell line come to lay in the future midline.* **(G)** *Stage 11 embryo with ectoteloblast precursors (NOPQl, NOPQr), mesoteloblasts (Ml, Mr) and endodermal precursors (E^{D}).* **(H)** *Stage 12 embryo at the completion of teloblastogenesis. Dorsal view with anterior to the top.* **(I-K)** *Left side views (upper) plus ventral views (lower) of embryos (with anterior to the left) at stages 13-15 undergoing gastrulation that consists of ventralward movement of elongating germ bands (shaded) and spreading of micromere-derived epithelial cells (not depicted here) over the endoderm. The germ band 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 (I). Along with their elongation, the germ bands on both sides of the embryo gradually curve round toward the ventral midline (J) and finally coalesce with each other along the ventral midline (K).* **(L-N)** *Left side views of embryos (with anterior to the left) undergoing body elongation, which begins in the anteriormost region of the embryo (L), continues in an anterior-to-posterior fashion (M), and completes in the caudal end at stage 18 (N). Body elongation is accompanied by formation of segmental ectoderm, which is accomplished by dorsalward expansion of germ bands (shaded).*

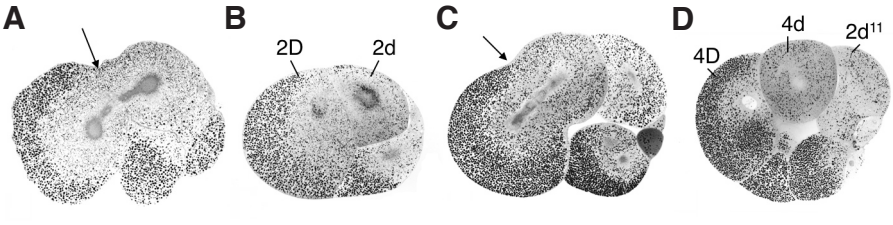

Fig. 2. Sagittal sections of embryos undergoing early cleavages. *Thick sections of Epon-embedded embryos were stained with toluidine blue and prepared for light microscopy. Anterior is to the right and dorsal is to the top.* **(A)** *An embryo at the fourth division forming 2d and 2D. The arrow indicates the cleavage furrow on dividing 1D cell.* **(B)** *An embryo shortly after the fourth division. Note the yolk-deficient cytoplasm (i.e. pole plasm) in nascent 2d and 2D.* **(C)** *An embryo at the sixth division forming 4d and 4D. The arrow indicates the cleavage furrow on 3D.* **(D)** *An embryo shortly after the sixth division. The yolk-deficient cytoplasm in 3D has been segregated into 4d. Scale bar: 100 μm.*

rivulorum (Penners, 1922, 1924a). In these studies, he followed the prospective fate of the four lineages of the embryo (viz., the A, B, C and D quadrants) during embryogenesis and suggested that the pole plasms (yolk-deficient cytoplasm segregated to both poles of the 1-cell embryo) which are specifically inherited by the D quadrant micromeres 2d and 4d of late cleavage stages play an important role in embryonic differentiation. The importance of pole plasm-bearing blastomeres (i.e., 2d and 4d) for *Tubifex* development was verified by his cell-ablation experiments (Penners, 1926), though it was not examined whether the pole plasm itself plays a role in embryonic differentiation. A thorough review of the Penners' experiments will be presented in the following sections.

Since then, the relationship between the pole plasms and the determinate development in the *Tubifex* embryo has been investigated by many students in various countries such as Italy, Germany, Japan, and Switzerland (see Shimizu, 1982 for references). From the late 1970s on, Shimizu and colleagues investigated the mechanisms underlying cleavage pattern formation and pole plasm localization (see Shimizu, 1982, 1990; Shimizu *et al*., 1998 for reviews). From the 1990s on, Shimizu and colleagues have undertaken cell lineage analyses of pattern formation in the *Tubifex* embryo using cell-labeling techniques with lineage tracers and classic embryological techniques such as cell ablation and transplantation (see Shimizu *et al*., 2001; Shimizu and Nakamoto, 2001; Nakamoto *et al*., 2006 for reviews). These studies have characterized thoroughly the developmental processes of 2d and 4d (a thorough review of these studies will be presented in the following sections). As described below, Nakamoto *et al.,* (2011) have recently succeeded in inducing secondary axis formation by transplantation of D quadrant micromeres 2d[11](a descendant of 2d) and 4d.

Normal development in *Tubifex tubifex*

A brief review of *Tubifex* development is presented here as a background for the observations described below (for details, see Penners, 1922, 1924a; Shimizu, 1982; Shimizu *et al*., 2001; Shimizu and Nakamoto, 2001; Nakamoto *et al*., 2006). *Tubifex* fertilized eggs, which are oviposited at metaphase of the first meiosis, undergo polar body formation twice and then enter the first mitosis. Before the first cleavage, yolk-deficient cytoplasm called pole plasm accumulates at both poles of the egg (Fig. 1A). The early development of *Tubifex* consists of a stereotyped sequence of cell divisions. The first cleavage of the *Tubifex* egg is unequal and meridional, and produces a smaller AB-cell and a larger CD-cell (Fig. 1B). The second cleavage is also meridional and yields cells A, B, C and D: the CD-cell divides into a smaller C-cell and a larger D-cell while the AB-cell separates into cells A and B of various sizes (Fig. 1C). From the third cleavage on, the quadrants A, B and C repeat unequal divisions three times, and the D quadrant four times, producing micromeres at the animal side and macromeres at the vegetal side (Fig. 1D-F). The quadrants A, B and C then divide equally at the sixth cleavage, followed by the D quadrant at the seventh cleavage; the resulting yolky macromeres are endodermal cells, and these repeat equal divisions thereafter. During early cleavages, the pole plasm is inherited by the D lineage cells; it is finally partitioned into the second (2d) and fourth (4d) micromeres (Fig. 2). Then, 2d divides unequally into a larger 2d[1] and a smaller 2d[2]; 2d[1] divides into a larger 2d[11] and a smaller 2d[12]. At the 22-cell stage, 2d[11], 4d and 4D (sister cell of 4d) all come to

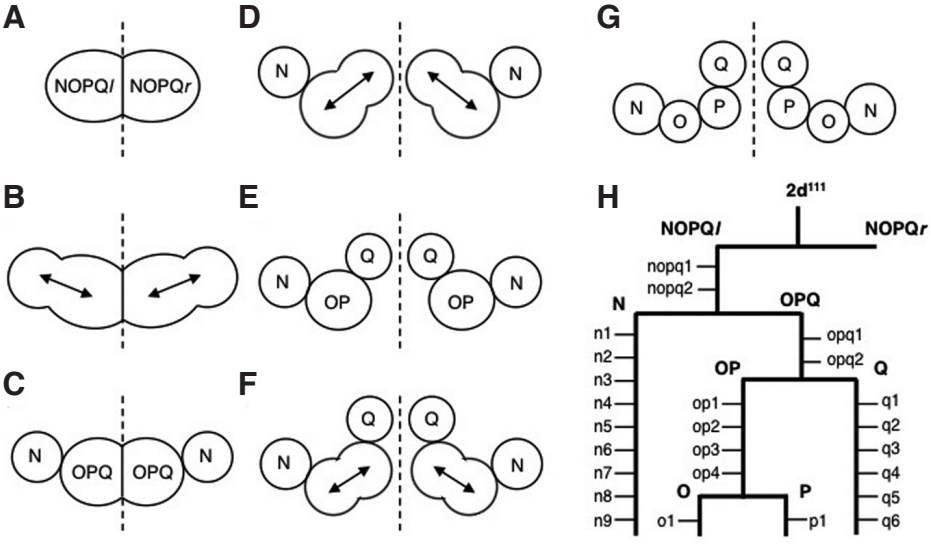

Fig. 3. Ectodermal teloblastogenesis. (A-G) *Spatiotemporal aspects of ectoteloblast formation. Broken lines indicate the dormal midline. Anterior is to the top. Arrows indicate the direction of cell division.* **(H)** *Cell lineage diagram showing the production of ectoteloblasts (N,O,P,Q) on the left 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). Adapted from Nakamoto* et al., *(2004).*

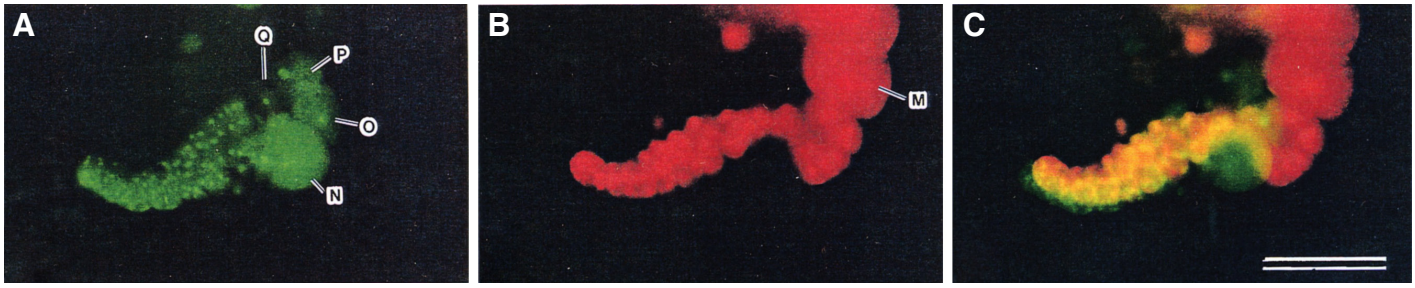

Fig. 4. Fluorescence micrographs showing overlap of mesodermal and ectodermal germ bands. *A 4d cell of a 22-cell embryo was injected with Texas Red dextran (TRD) and, 3 h later, the left NOPQ of the same embryo was injected with fluorescein dextran (FLD). After a 36-h culture in the darkness, the embryo was fixed and photographed by epifluorescence microscopy using filter cassettes for fluorescein* **(A)** *and rhodamine* **(B)**. *All panels show dorsal views of the same field at the same magnification.* **(C)** *A merged image of (A) and (B). Anterior is to the left.* **(A)** *Distribution of FLD. The ectodermal germ band generated by the posteriorly located teloblasts (N, O, P and Q) inherit FLD. Bright dots are nuclei in blast cells and teloblasts.* **(B)** *Distribution of TRD in the teloblasts (M) and the germ bands extending therefrom.* **(C)** *The ectodermal germ band (green) is superimposed on the mesodermal germ band (red). Regions of overlap are yellow. Note that mesodermal blast cells (red) located in the vicinity of the M teloblast are not overlain by the ectodermal germ band. Scale bar: 200 μm. Adapted from Goto* et al., *(1999b).*

lay in the future midline of the embryo (Fig. 1F). Then, 4d divides equally to yield the left and right mesoteloblasts, M*l* and M*r*; $2d^{111}$ (derived from $2d^{11}$) divides into a bilateral pair of ectoteloblast precursors, NOPQ*l* and NOPQ*r*; and 4D divides equally yielding endodermal precursors E^D (Fig. 1G). Ectoteloblasts N, O, P and Q arise from an invariable sequence of divisions of cell NOPQ on both sides of the embryo (Figs. 1H and 3; see Nakamoto *et al.*, 2004 for details). When teloblastogenesis is complete (stage 12), ectoteloblasts which are present in front of the M teloblasts, are organized in the order of Q, P, O, and N along the dorsoventral axis on either side of the embryo (Fig. 3).

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). 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 germ band (GB), while the bandlet from the M teloblast becomes a mesodermal GB that underlies the ectodermal GB (Fig. 4). The GBs are initially located on the dorsal side of the embryo (Fig. 1I). Along with their elongation, they gradually curve round toward the ventral midline (Fig. 1J) and finally coalesce with each other along the ventral midline (Fig. 1K). The coalescence is soon followed by dorsalward expansion of the GBs. The edges of the expanding GBs on both sides of the embryo finally meet along the dorsal midline to enclose the elongated cluster of yolky endodermal cells (Fig. 1L-N). Concurrently with this enclosure, the embryo becomes elongated in an anterior-to-posterior progression, and curved with ventral convexity (Fig. 1 L-N). Enclosed portions of the embryo begin to exhibit peristaltic movements. Embryogenesis is judged to complete when the expanding GBs have enclosed the posterior end of the embryo, which then exhibits movement throughout its length (Fig. 1N).

D quadrant micromeres, 2d and 4d, serve as essential "organ-forming blastomeres": Penners' classic experiments

During the course of his pioneering study on *Tubifex* embryology (Penners, 1922, 1924a), German embryologist Andreas Penners found, amongst the abnormal embryos, so-called double embryos that belong to the so-called Janus type of embryo showing Duplicitas cruciate (Fig. 5A; Penners, 1924b). While inspecting living embryos, he also came across younger embryos that each exhibited 16 large cells on the surface which resembled normal ectoteloblasts in their size and arrangement manner (Fig. 5B). Such embryos developed into double embryos. Based on these observations, Penners (1924b) speculated that blastomeres equivalent to normal 2d and 4d are produced in duplication and each of these blastomeres generates teloblasts, which then produce GBs, giving rise to duplicated embryonic axes.

Under natural conditions, embryos like those described above are extremely rare. Penners (1924c) searched for the protocols (culture condition etc) that are expected to increase the frequency of double embryo occurrence, and found that double embryos are obtained when cocoons containing 1-cell embryos have been brought from a low temperature (10°C) to a higher temperature (15-20°C) and also when the water is lacking in oxygen. Observing such treated embryos, he found that embryos that appear to have experienced the first or second equal cleavage generate blastomeres equivalent to 2d and 4d in duplication (Fig. 5C), which then produce teloblasts and germ bands in duplication. Based on these observations, Penners came to conclusion that double embryos are ascribable to the generation of 2d and 4d in duplication in each embryo (Penners, 1924c).

On the basis of his observations on double embryos, Penners (1924c) speculated that 2d and 4d play an essential role in *Tubifex* development to serve as an organizing center. To verify this notion, Penners set out to examine the developmental capacity of isolated groups of blastomeres. For this purpose, Penners (1926) carried out a set of cell-ablation experiments in which given cells were irradiated (and killed) with a narrow ray of ultraviolet light. The embryos were operated on in the intact cocoons and allowed to develop therein. Penners clearly demonstrated that the isolated cells show a determinate cleavage pattern carrying out a prescribed number of divisions. However, when A-, B-, and C-cells of 4-cell embryos are all killed, the remaining D-cell produces a small, but perfectly proportioned animal. On the other hand, any combination of A-, B-, and C-cells produces only endoderm and an epithelial sheet of ectoderm (see Figs. 5D and 6B). Evidently, only the D-cell descendants are capable of establishing the axial polarity of the *Tubifex* embryo after they divide and differentiate into the total

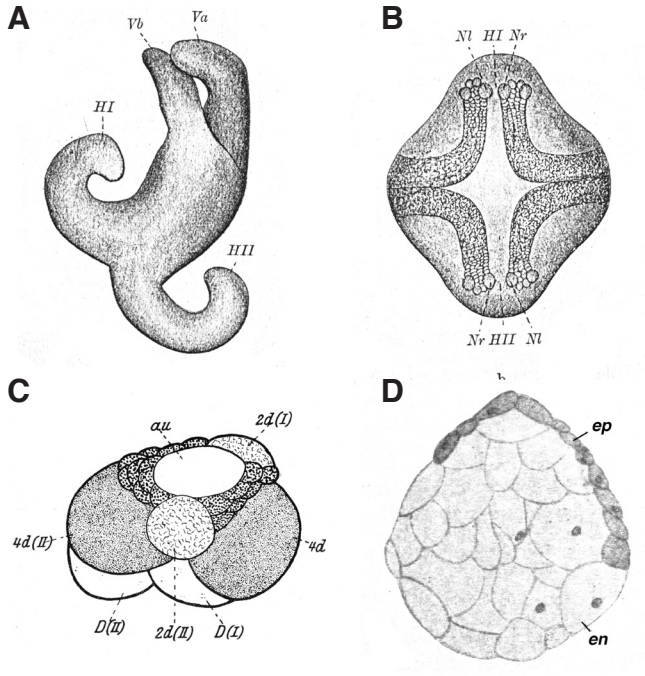

Fig. 5. Andreas Penners' classic experiments on Tubifex embryos. (A) *A twinned* Tubifex *embryo of Janus type with duplicated heads (Va, Vb) and tails (HI, HII).* **(B)** *An embryo (younger than that shown in A) exhibiting 16 ectoteloblast-like cells at the presumptive posterior ends (HI, HII). N teloblast-like cells are labeled* Nl *and* Nr. **(C)** *Duplication of 2d-like and 4d-like cells in an embryo that was brought from a low temperature to a higher temperature (for details, see text). au, bladder-like protrusion.* **(D)** *This ball of endodermal (en) cells (covered with an epithelial (ep) sheet of cells) developed from an embryo from which both 2d and 4d had been ablated with UV irradiation. A and B, adapted from Penners (1924b); C, adapted from Penners (1924c); D, adapted from Penners (1926).*

range of cell types found in the normal organism; furthermore, it is the only blastomere that produces cells responsible for the establishment of embryonic axes such as the anteroposterior and dorsoventral axes.

To obtain an insight into the developmental potential of cleavage-stage cells, Penners (1926) performed another set of cell-ablation experiments in which either of 1D, 2d, 2D, 4d, $2d^{111}$+4d, or 4D were eliminated by irradiation. He found that ectodermal teloblasts and GBs are formed only when 2d is present (even in the absence of 4d) and that mesodermal teloblasts and GBs are formed only when 4d is present (even in the absence of 2d). When both $2d^{111}$ and 4d were ablated simultaneously, the resulting embryos developed into endodermal balls covered with ectodermal epithelium (Fig. 5D). These results suggest that in *Tubifex*, developmental potential to generate ectodermal and mesodermal GBs are specifically segregated to 2d and 4d, respectively, but not to the remaining cells. Penners (1926) described that $2d^{111}$ and 4d play a central role in growth, determination and differentiation in the *Tubifex* embryo. Regarding his results, Penners emphasizes the significance of the pole plasm in *Tubifex*, and he interpreted the pole plasm as an organ-forming materials. It has been shown that the pole plasm segregation during the early cleavage depends on actin cytoskeleton (see Shimizu, 1995, 1999 for reviews). Since these Penners' classic works, the molecular nature of the pole plasm has been an open question for a long time.

Re-examination of developmental capacity of early blastomeres in the *Tubifex* embryo

The previously mentioned Penners' cell ablation experiments not only suggest the importance of 2d and 4d in *Tubifex* development, but also raise a possibility that none of the blastomeres other than 2d and 4d can replace the missing 2d and 4d cells. Is this assumption really correct? But we need to notice that in the Penners' experiments, cells that had been killed by UV irradiation remained attached to the undamaged part of the embryo and may still induce a signal to the surrounding cells. The assumptions mentioned above that are coming from this last experiment may be discussed in regard to previous classical embryology experiments performed in the frog embryo. Using the frog embryo, Wilhelm Roux performed his famous experiment in which one cell of a frog embryo at the two-cell stage that had been pricked (and damaged) with a heated needle remained attached to the undamaged sister cell. The resulting embryo developed into a half-embryo but not into a half-sized whole embryo (Roux, 1888). However, it was later demonstrated that each cell of a two-cell stage frog embryo is able to develop into a whole embryo if it is separated from its sister cell (McClendon, 1910; Schmidt, 1933). It is apparent that cells of the two-cell stage frog embryo have the capacity to regulate their development and that if the living cell of the operated embryo remains attached to the damaged sister cell it could not regulate

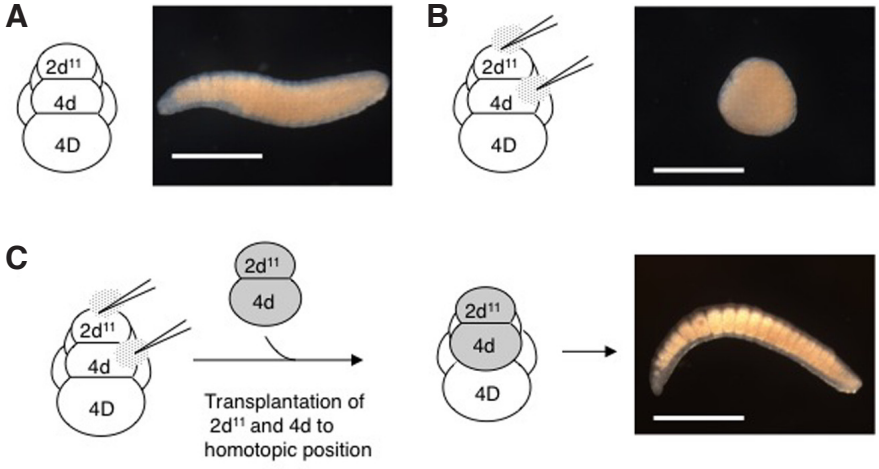

Fig. 6. Ablation and transplantation experiments with D quadrant micromeres. (A) *Normal development of* Tubifex tubifex. *Right-hand panel shows a 9-day-old embryo which is segmented and elongated along the anteroposterior (A/P) axis. Anterior is to the left.* **(B)** *Ablation of* $2d^{111}$ *and 4d with a fine glass needle. The embryo shown was incubated for 9 days before fixation. It developed into a rounded cell mass with no embryonic axis.* **(C)** *Homotopic transplantation of* $2d^{111}$ *and 4d.* $2d^{111}$ *and 4d of the host embryo were ablated and the same set of micromeres from a donor embryo were transplanted to the positions of* $2d^{111}$ *and 4d. Right-hand panel shows a representative 9-day-old embryo with a restored embryonic axis and that developed normally. Scale bars: 500 μm. After Nakamoto* et al., *(2011).*

its development. We wondered if a similar situation was present in the Penners' experiments in which damaged (or killed) cells were left in their positions in embryos for a while after operation. So we decided to follow the development in operated embryos from which damaged (or dead) cells were completely removed. For this purpose, 22-cell stage embryos were taken out of cocoons and vitelline membranes on an agar bed, and $2d^{11}$ and 4d were ablated with fine glass needles, and discarded by means of pipets; the resulting operated embryos were cultured for 9 days in the culture medium containing antibiotics (Nakamoto *et al.*, 2011). The operated embryos developed into rounded cell masses that failed to exhibit any morphological sign of axial development (Fig. 6B); this phenotype was essentially the same as that obtained in the Penners' experiment (Fig. 5D). This result supports the notion that axial pattern formation in *Tubifex* early development proceeds normally only when both $2d^{11}$ and 4d are present in the embryo and that any of the embryonic cells other than $2d^{11}$ and 4d is unable to play the part of these micromeres.

We also found that when $2d^{11}$ and 4d (co-isolated from a donor embryo) were transplanted to the position of $2d^{11}$ and 4d of 22-cell stage embryos (which had been deprived of $2d^{11}$ and 4d), such reconstituted embryos "restored" embryonic axis formation and developed into juveniles of normal morphology (Fig. 6C; Nakamoto *et al.*, 2011). These results from the ablation/restoration experiment reinforce the notion that the D quadrant micromeres, $2d^{11}$ and 4d, play a pivotal role in axial pattern formation in the *Tubifex* embryonic development including axial patterning (Penners, 1926).

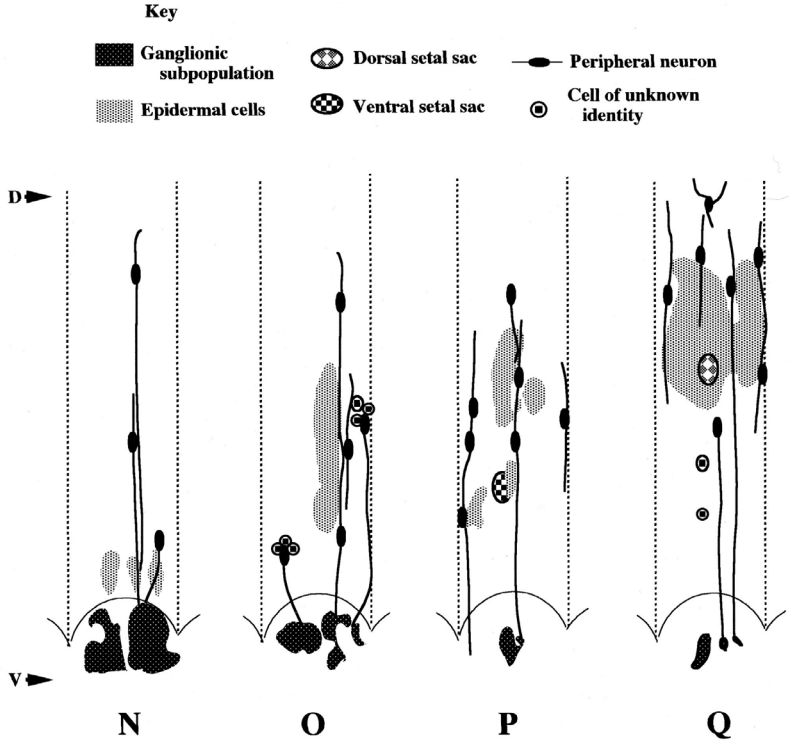

Fig. 7. Diagrammatic summary of cellular contributions of the teloblasts N, O, P and Q to a mid body segment of Tubifex. *For each pattern, the left half of one segment is shown, with the ventral midline (V) and ganglion (shown in outline) to the bottom, dorsal midline (D) to the top, and anterior to the left. Dashed lines indicate segmental boundaries. Adapted from Goto* et al., *(1999a).*

2d and 4d are the exclusive sources of ectodermal and mesodermal segmental tissues

The 2d cell lineage

The second micromere 2d undergoes three unequal cell divisions before dividing equally into a pair of NOPQ proteloblasts. This division pattern apparently characterizes the 2d cell lineage. This division pattern was retained even if a nascent 2d was transplanted to the ventral side of a stage 11 embryo (from which one endodermal cell had been removed; unpublished result). This may suggest that 2d is specified as a precursor of proteloblasts as early as the time of its birth.

As described before, ectodermal teloblastogenesis (see Fig. 3) in the *Tubifex* embryo is a spatiotemporally regulated process that gives rise to four bilateral pairs of ectoteloblasts (N, O, P, and Q) that assume distinct fates (Fig. 7). The differences between ectoteloblasts are also manifested in the expression pattern of *hunchback* protein (T-hb): In *Tubifex* embryos (at stage 14) undergoing gastrulation, T-hb expression in the ectoteloblasts appears to occur transiently at intervals that are shortest in the P teloblast, longest in the N and Q teloblasts, and intermediate in the O teloblast (Shimizu and Savage, 2002). Cell ablation experiments (Arai *et al.*, 2001) have shown that fates of teloblasts N, P and Q 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 (Fig. 8A). In the absence of this signal, the O lineage assumes the P fate. At present, nothing is known about the molecular mechanisms for this fate specification. In this regard, it is interesting to note that BMP5-8 signaling has been shown to be involved in fate specification in the ectodermal teloblast lineages in the leech embryo (Kuo and Weisblat, 2011). In the clitellate leech *Helobdella*, O/P teloblast pairs are "equivalence group" and they have the potential to follow either O or P fate. The O/P blast cells assume P fate if they interact with a bandlet derived from the Q teloblast (referred to as q-bandlet); otherwise, they follow O fate (Fig. 8B; Shankland and Weisblat, 1984; Huang and Weisblat, 1996). It has been shown that BMP5-8 signaling derived from the q-bandlet specifies the P fate of the neighboring O/P bandlet, and this signaling upregulates the expression of Gremlin (BMP antagonist) in the p-bandlet (Kuo and Weisblat, 2011). Gremlin derived from the p-bandlet specifies the O fate in another O/P bandlet (Kuo and Weisblat, 2011). It remains to be explored whether similar molecules are involved in fate specification in the *Tubifex* ectoteloblast lineages.

Ectodermal teloblastogenesis in *Tubifex* is followed by formation and elongation of the ectodermal GB, a cell sheet consisting of four bandlets of blast cells derived from ectoteloblasts N, O, P and Q (Figs. 8A and 9B). As the GB elongates, it undergoes "segmentation" that is a process of separation of 50-μm-wide blocks of cells from the initially continuous cell sheet (Fig. 9A; Nakamoto *et al.*, 2000). The formation of ectodermal segments begins with formation of fissures, first on the ventral side and then on the dorsal side of the GB (Fig. 9B); the unification of these fissures gives rise to separation of a 50-μm-wide block of ~30 cells from the ectodermal GB (Fig. 9 A,B).

As development proceeds, an initially linear array of blast cells in each ectodermal bandlet gradually changes its shape; its contour becomes indented in a lineage-specific manner (Fig. 9 C,D). These morphogenetic changes result in the formation of distinct cell clumps, which are separated from the bandlet to serve as segmental elements. Ectodermal segmentation is complete when the separated segmental elements space themselves at regular intervals along the anteroposterior axis. It should be noted that the generation of segmental elements in each cell bandlet occurs autonomously and that the ensuing alignment of separated segmental elements is dependent on the underlying mesoderm (Nakamoto *et al.*, 2000).

The 4d cell lineage

As described before, segmental organization arises sequentially in the anterior-to-posterior direction along the longitudinal axis of the mesodermal GB, a coherent column of primary m blast cells that are produced from the M teloblast (Fig. 10A). Shortly after its birth, each primary blast cell undergoes a spatiotemporally stereotyped sequence of cell divisions to generate three classes of cells (in terms of cell size), which together give rise to a distinct cell cluster, which becomes a mesodermal segment (Fig. 10B). The emergence of the three classes of cells in terms of cell size is the first sign of morphological manifestation of dorsoventral polarity in the mesoderm. That is, as Fig. 10B shows, middle-sized cells are formed, for the first time, on the ventral side of a cell cluster located at a distance of 10 cluster blocks from the M teloblast. Each cluster is composed of descendants of a single primary blast cell; there is no intermingling of cells between adjacent clusters (Goto *et al.*, 1999b; Kato *et al.*, 2013). Relatively small-sized cells in each cluster become localized at its periphery, and they form coelomic walls including an intersegmental septum to establish individuality of segments. Cell clusters thus generated come to be arranged in a chain running along the anteroposterior axis. Thus, the metameric segmentation in the mesoderm arises from an initially simple organization (i.e., a linear series) of primary m blast cells that serve as segmental founder cells (Fig. 10A; Goto *et al.,* 1999b).

Although mesodermal segments of the *Tubifex* embryo are homologous, they are regionally differentiated along the anteroposterior axis. Among mesodermal organs, for instance, nephridia and

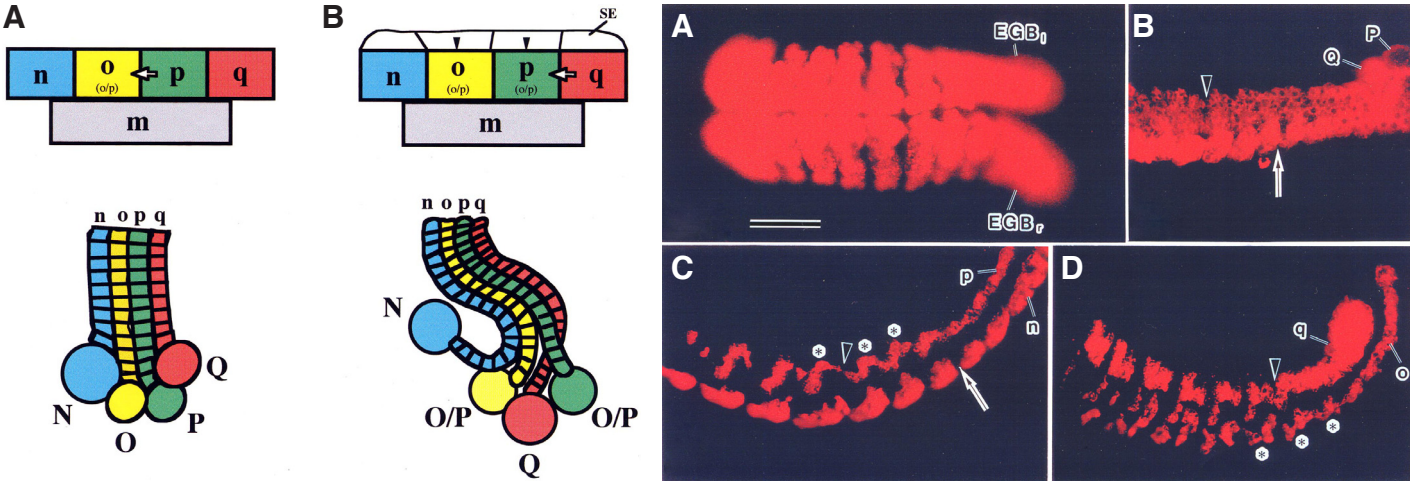

Fig. 8 (Left). Comparison of cell interactions for patterning of the ectodermal germ band in Tubifex (A) and Helobdella (B). *In each panel, a cross section of a left germ band (including m bandlet) is shown in the upper part; the early stage of germ band formation is presented in the lower part. Dorsal is to 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 (open arrow) emanating from the p bandlet, to assume the O fate. 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. 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). 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). After Arai* et al., *(2001).*

Fig. 9 (right). Segmentation in ectodermal GBs. *2d*^{111} *cell (A), left NOPQ (B) or individual teloblasts (C, D) were injected with a lipophilic tracer DiI and allowed to develop for 3 days before fixation. Wholemount preparations were viewed from the ventral side (A) or left side (B-D). In all panels, anterior is to the left; in (B-D), dorsal is to the top.* **(A)** *Both the left and right GBs (EGBl and EGBr, respectively) are labeled with DiI. Both GBs have coalesced with each other along the ventral midline in the anterior and mid regions of the embryo. Only the mid region of the embryo is in focus here. Note that GBs are divided into 50-μm -wide blocks of labeled cells by intersegmental furrows, which are recognized as non-fluorescent transverse stripes.* **(B)** *The posterior portion of the left GB is shown. P and Q teloblasts are seen, but N and O teloblasts are out of the field. The arrow indicates the site where a fissure becomes evident in the ventralmost bandlet (i.e., n bandlet). The arrowhead indicates fissures at the dorsal side of the GB.* **(C)** *Fluorescent n and p bandlets in the left GB. These bandlets were derived from left N and P teloblast that had been injected simultaneously with DiI shortly after the birth of the P teloblast. Asterisks indicate S-shaped segmental elements (SEs) in the P lineage. The arrow and arrowhead indicate the sites where separation of an SE from the bandlet has taken place. Note that the separation of an SE in the P lineage lags behind that in the N lineage by three segments.* **(D)** *Fluorescent o and q bandlets in the left GB. These bandlets were derived from O and Q teloblasts that had been injected simultaneously with DiI shortly after the birth of the O teloblast. Asterisks indicate W-shaped SEs of the O lineage. The arrowhead indicates the boundary between two consecutive SEs. Scale bars: 100 μm (A,B); 80 μm (C,D). Adapted from Nakamoto* et al., *(2000).*

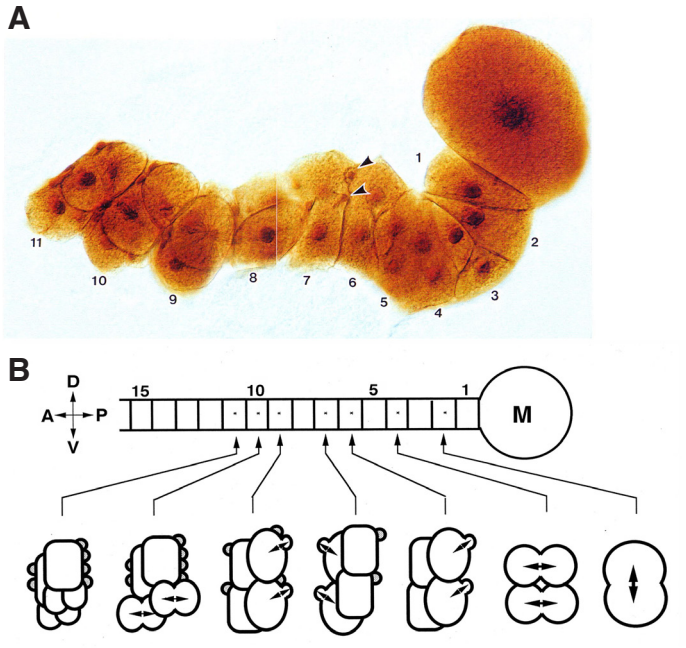

Fig. 10. Organization of blast cells in the mesodermal germ band (GB). **(A)** *A left M teloblast of a* Tubifex *embryo was injected with horseradish peroxidase (HRP) 15 h after their birth and allowed to develop for 24 h before fixation. HRP-containing cells were visualized histochemically according to the method described in Goto* et al., *(1999a). Anterior is to the left and dorsal is to the top. The HRP-labeled portion of the GB is comprised of 11 cell clusters, including primary blast cells located at positions 1 and 2. In this preparation, unlabeled clusters are present in front of this labeled portion of the GB, though they are invisible here. Arrows indicate tiny cells that have been produced by cells located in the cluster at position 8. Note that tiny cells form a thin layer at the boundary between the clusters at positions 10 and 11. Clusters at positions 4 and 5 are out of focus here due to the presence of overlying ectodermal teloblasts (O and P), which are invisible in this preparation. Adapted from Shimizu and Nakamoto (2001).* **(B)** *Schematic summary of pattern and sequence of divisions in mesodermal blast cells. Inequality and direction of divisions are reflected by position and orientation of mitotic spindles in dividing cells. The M teloblast is illustrated to the right of the figure. Each block in the germ band represents a cell cluster. Arrows indicate the approximate position, along the germ band, where each division occurs. A, anterior; D, dorsal; P, posterior; V, ventral. Adapted from Goto* et al., *(1999b).*

genital primordia (i.e., primordial germ cells or PGCs) are localized in segments VII-VIII and X-XI, respectively. As demonstrated by cell-ablation experiments, in normal *Tubifex* embryos, nephridia and PGCs arise specifically from the 7th and 8th and the 10th and 11th primary m blast cells, respectively (Kitamura and Shimizu, 2000; Oyama and Shimizu, 2007). Recent cell-transplantation experiments have shown that fates of primary m blast cells of the *Tubifex* embryo are determined according to the genealogical position in the M teloblast lineage (Kitamura and Shimizu, 2000; Kato *et al.*, 2013). That is to say, the segment-specific occurrence of nephridia and PGCs is only because m blast cells that have already been committed to the segment-specific fates come to take up the positions corresponding to those segments, but not because m blast cells that have been situated in these segments are committed to take on segment-specific fates. It is at the time of their birth that primary m blast cells acquire specific identities.

Evidence that $2d^{11}$ and 4d can function as an embryonic axial organizer

If one interpretes the aforementioned Penners' results simply, what occurs in embryos that are to develop into the twined embryos could be described as follows. When *Tubifex* embryos divide equally at the time of first or second cleavage, two sets of cells equivalent to normal 2d and 4d are eventually formed. (These cells are referred to as $2d^{eq}$ and $4d^{eq}$.) Each set of $2d^{eq}$ and $4d^{eq}$ generate four "bilateral" pairs of ectoteloblasts and a "bilateral" pair of mesoteloblasts, respectively. These teloblasts cooperatively form embryonic axes by generating GBs therefrom. Since there exist two sets of axis-forming cells in each embryo, the resulting embryos finally develop into embryos with duplicated embryonic axes, viz. Janus twins (see Fig. 5A). If this interpretation is correct, it is not implausible to envisage that 2d and 4d have the ability to induce an embryonic axis when placed in ectopic positions. So we decided to examine whether 2d and 4d that have been transplanted to an ectopic position could form a secondary embryonic axis as the transplanted dorsal blastopore lip of newt embryo can do (Spemann and Mangold, 1924).

Secondary axis formation by transplantation of $2d^{11}$ and 4d

The aforementioned ablation/restoration experiments (Fig. 6C) suggests that the D quadrant micromeres $2d^{11}$ and 4d play a significant role in *Tubifex* development, but it does not necessarily verify a view that D quadrant micromeres can function as the organizer for the embryonic axis, since in this experiment transplanted cells were placed to their "original" positions (see Fig. 6C). In this regard, one of the most convincing ways to test if the D-quadrant micromeres function as an organizer could be to examine their ability to induce an embryonic axis when transplanted to an ectopic position in a recipient embryo. The quantity of the embryonic tissues manipulated during these experiments may be the only reason why the resulting embryo where so affected. Then, in order to verify if the D quadrant micromeres, $2d^{11}$ and 4d, may function as an embryonic organizer by specifying cell fate, orchestrating morphogenesis and axial patterning, we examined the ability of $2d^{11}$ and 4d cells together in the induction of a secondary axis in *Tubifex* embryo. Similarly to the protocol used during the famous Spemann-Mangold organizer experiment, we transplanted both, $2d^{11}$ and 4d, to an ectopic position in a host embryo. Therefore, we transplanted $2d^{11}$ and 4d (that had been co-isolated from a donor embryo at stage 8) to the ventral region of a recipient embryo (at stage 11, i.e., about 6 hr from stage 8) from which one endodermal cell had been ablated (Nakamoto *et al.*, 2011). This allows us to use the position of the ablated endodermal cell as mold for transplanted cells. (We used older embryos as recipients only because it was easier to prepare such a mold using stage 11 embryos than using stage 8 embryos.) The resulting chimeric recombinant embryos had pairs of NOPQ proteloblasts and M teloblasts (immediate progeny of $2d^{111}$ and 4d) on the dorsal side and the transplanted cells $2d^{11}$ and 4d on the ventral side (Fig. 11A). The embryos were incubated for 9 days and examined for secondary axis formation. We found that most of the reconstituted embryos formed secondary head and/or tail (Fig. 11 A,C). This result shows that the transplanted $2d^{11}$ and 4d have the ability to form a secondary embryonic axis.

The ability of $2d^{11}$ and 4d to form an embryonic axis was further verified by another transplantation experiment. $2d^{11}$ and 4d (co-

isolated from a donor embryo) were transplanted to the ventral side of a recipient embryo that had been deprived of the same set of micromeres (Fig. 11B). Most of the reconstituted embryos elongated to a significant extent and had either head or tail. In addition, some of the embryos formed distinct head, tail, and clearly segmented endoderm. Their overall morphology was similar to that of an intact embryo (compare Fig. 11B with Fig. 6A). These results suggest that the embryonic axis and endodermal segmentation are partially rescued by transplanted $2d^{11}$ and 4d.

Origin of neuroectoderm and mesoderm along the secondary axis

To determine the origin of cells comprising the secondary axis, we analyzed the cell fates of transplanted D quadrant micromeres (Nakamoto *et al.*, 2011). Either $2d^{11}$ or 4d of donor embryos were labeled with fluorescent tracers and they were co-isolated and transplanted to the ventral region of a normal recipient embryo as described before (see Fig. 11A). The reconstituted embryos were incubated for 7 days and the distribution patterns of labeled cells were analyzed. This cell lineage tracing demonstrated that neuroectoderm and mesoderm along the secondary axis were derived from the transplanted cells $2d^{11}$ and 4d, respectively. The descendants of transplanted $2d^{11}$ differentiated into ganglia, peripheral neurons, setal sacs and epidermis along the secondary axis (Fig. 11C). The descendants of transplanted 4d contributed to the mesoderm of the secondary axis (Fig. 11 D,E). These distribution patterns were comparable to those in normal development (Goto *et al.*, 1999a; Goto *et al.*, 1999b). To examine whether the endoderm along the secondary axis was derived from the host embryo, endodermal macromeres (E^D, see Fig. 1G) of host embryo were labeled with

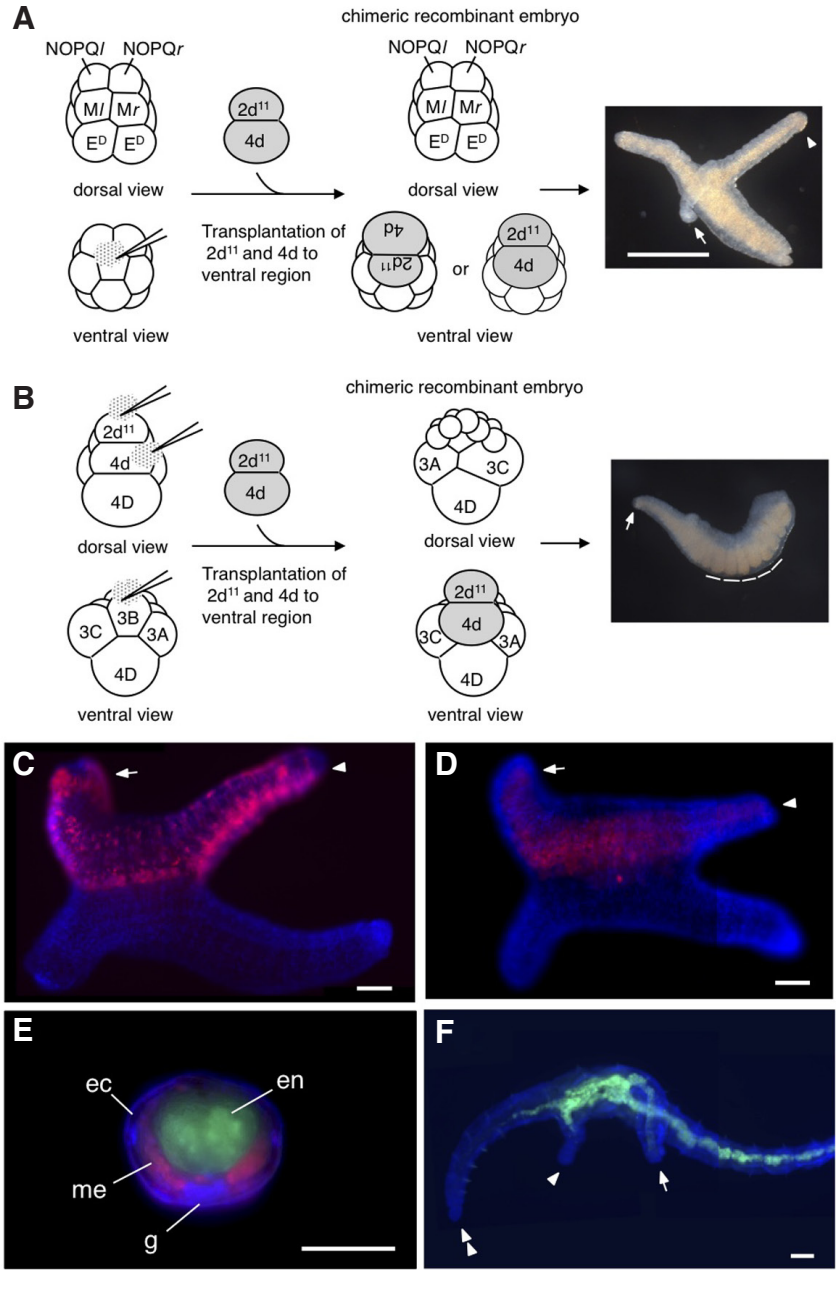

Fig. 11. Ablation and transplantation experiments with D quadrant micromeres. (A) *Transplantation of the D quadrant micromeres to the ventral region of a host embryo. $2d^{11}$ and 4d were co-isolated from a donor embryo (at stage 8) and transplanted to the vegetal region of a recipient embryo (at stage 8) from which one endodermal cell had been ablated (see ventral view). The donor micromeres were transplanted in two different orientations. In one orientation, the transplanted micromeres maintained the A/P polarity of the host, whereas in the other the A/P polarity of the transplant was reversed. The resulting chimeric recombinant embryo was incubated for 9 days and a representative embryo is shown. The arrow and arrowhead indicate secondary head and tail, respectively.* **(B)** *Transplantation of the D quadrant micromeres to a recipient embryo from which the D quadrant micromeres had been ablated. The 3B cell of the recipient embryo had been ablated from the recipient embryo to make the mold for transplantation (see ventral view). The prospective A/P axis of the transplanted D quadrant micromeres ($2d^{11}$ and 4d) ran parallel to that of the host embryo. The resulting chimeric recombinant embryo was incubated for 9 days and a representative embryo is shown. Note that the endoderm is clearly segmented (dashed lines). The arrow indicates the anterior; dorsal is to the top. (C-F) Neuroectoderm and mesoderm along the secondary axis are derived from transplanted $2d^{11}$ and 4d, respectively. The reconstituted embryos were stained with DAPI to visualize nuclei.* **(C)** *Cell fate of transplanted $2d^{11}$. The DiI-labeled descendants of transplanted $2d^{11}$ are confined to the ectoderm along the secondary axis. Arrowhead and arrow indicate secondary head and tail, respectively.* **(D)** *The descendants of transplanted 4d differentiate into the segmented mesoderm along the secondary axis. Arrowhead and arrow indicate secondary head and tail, respectively.* **(E)** *Cross section of anterior region of a secondary axis. The transplanted 4d was labeled with Rhodamine dextran and endodermal macromeres of the host embryo were labeled with Oregon green dextran. The descendants of transplanted 4d differentiate to mesodermal layer (me) underlying the ectoderm (ec; labeled with DAPI). The descendants of host macromeres contribute to the endoderm (en) of the secondary axis. g, ganglion.* **(F)** *Cell fate of the host endodermal macromeres. The descendants of host macromeres, which were labeled with Oregon green dextran, contribute to the gut tissue of the secondary axis. Arrowhead and arrow indicate secondary head and tail, respectively. Double arrowheads indicate the head of the primary axis (i.e., host embryo). Scale bars: 500 μM (A,B); 100 μM (C-F). After Nakamota* et al. *(2011).*

a fluorescent tracer. We observed that descendants of the host macromeres contributed to the endoderm and gut of the secondary axis (Fig. 11F). Thus, transplanted micromeres recruit endoderm from the host embryo and induce the secondary gut formation. Importantly, the D quadrant micromeres rescued the endodermal segmentation when transplanted to the ectopic position of the host embryo from which endogenous D quadrant micromeres were ablated. This suggests that the D quadrant micromeres regulate segmental patterning and morphogenesis of the endoderm.

At present, it is not known whether the secondary brain (supraesophageal ganglion) is formed by the D quadrant micromere transplantation. Previous cell lineage analysis has shown that the descendants of $2d^{111}$ do not contribute to the supraesophageal ganglion (Goto *et al*., 1999a). Recent cell lineage studies on micromeres other than 2d and 4d have shown that at least descendants of micromeres 1c and 1d contribute cells to the supraesophageal ganglion (Miho Moriya, unpublished results). Most of the remaining micromeres do not appear to contribute to the brain (fate of 3d still remains to be explored). If a brain is formed at the anterior end of the secondary anteroposterior axis, cells comprising the brain might be derived from micromeres other than 1c and 1d. In the future studies, the first thing to be done would be to examine whether a morphologically identifiable brain is actually formed along the secondary axis.

Acquisition of axial polarity information in the 2d and 4d cells

Anteroposterior polarity

In the aforementioned transplantation experiments, the behavior of transplanted cells was not followed in individual reconstituted embryos. Judging from the organization of the resulting secondary embryos along the anteroposterior axis, however, it appears that teloblasts produced from the transplanted $2d^{11}$ and 4d generate GBs along their presumptive anteroposterior polarity. That is to say, even when they are subjected to such severe procedures as isolation and transplantation, $2d^{11}$ and 4d seem to retain their original anteroposterior polarity. It is apparent that the anteroposterior polarity in $2d^{11}$ and 4d are specified as late as the time when 4d is born and when 2d undergoes cell division twice (giving rise to $2d^{11}$). In the aforementioned transplantation experiments, $2d^{11}$ and 4d were allowed to be in contact with each other during transplantation as well as isolation. Therefore, it is possible that contact between these two cells mediates the retention of their anteroposterior polarity.

To find out when 2d and 4d acquire their anteroposterior polarity information, we have performed some preliminary cell-transplantation experiments as follows: presumptive posterior ends of nascent 2d and 4d were stained with Nile red crystal at stages 6 and 8, respectively (see Fig. 1E and F). These cells were isolated and immediately transplanted to stage 11 embryos which had been deprived of all of M teloblasts and NOPQ cells (see Fig. 1G). Isolated cells were placed to the dorsal region of a host embryo (i.e., the position where M teloblasts and NOPQ cells had been present), and they were oriented with an eyelash so that their presumptive anteroposterior axis was opposite to that of the host embryo. When transplanted cells were intergrated into the host embryos, they resumed cell divisions (see Nakamoto *et al*., 2004). We found that the transplanted cells underwent cell divisions in a pattern that accords with their "original" presumptive polarity but not with that of the host embryos. The transplanted 2d cells, which had undergone three unequal divisions (forming smaller cells equivalent to $2d^{2}$, $2d^{12}$, and $2d^{112}$), divided equally to form a bilateral pair of large cells equivalent to normal NOPQ proteloblasts. These two large cells were then found to divide unequally producing smaller cells toward the "original" presumptive anterior end of the transplanted 2d cell (i.e., toward the posterior end of the host embryo). It has been known that NOPQ proteloblasts in normal embryo undergo two unequal divisions producing smaller daughter cells toward the anterior end of the embryo (Arai, 2000). Unlike the transplanted 2d cells, transplanted 4d cells divided directly into a bilateral pair of large cells (equivalent to normal M teloblasts), which then underwent a series of unequal divisions producing smaller daughter cells toward the "original" presumptive anterior end of the transplanted 4d cell. These results suggest that in 4d, anteroposterior polarity is specified as early as the time of its birth. At present, it cannot be determined when anteroposterior polarity is specified in the 2d cell. However, it seems likely that at the time of its birth, the 2d cell acquires some kind of information that gives rise to specification of anteroposterior polarity.

It is not known whether the anteroposterior polarity specification occurs via cytoplasmic inheritance (i.e., cell-autonomously) or cell interactions with surrounding cells. In this regard, it is interesting to note that cortical F-actin in the D cell of the 4-cell embryo is distributed most densely at the animal pole and thins out toward the equator (Shimizu, 1995). If such polarized cortical organization is carried over to subsequent stages, it should be inherited by large micromeres 2d and 4d. It is envisaged that polarized cortical actin could serve as a cue for specification of anteroposterior polarity.

Dorsoventral polarity

In normal embryos, dorsoventral polarity in the 2d lineage is specified by cellular interactions between sister cells NOPQ which are resulted from equal division of $2d^{111}$ (daughter cell of $2d^{11}$; Nakamoto *et al*., 2004). It is natural to think that in the aforementioned transplantation experiment, $2d^{11}$ (transplanted together with 4d) produces a pair of cells equivalent to normal NOPQ, which interact with each other to specify dorsoventral polarity in a similar manner to intact embryo.

At present, nothing is known about how dorsoventral polarity is specified in the 4d lineage, except that dorsoventral polarity is manifested morphologically when a primary m blast cell undergoes cell division three times (Fig. 10B; Goto *et al*., 1999b). Dorsoventral polarity in the 4d lineage could be specified in M teloblasts shortly after their emergence from 4d or in nascent primary m blast cells shortly after their birth. Of course, at present, it is equally possible that dorsoventral polarity is already specified in 4d itself and inherited by its daughter cells M teloblasts. Regardless of timing and places of dorsoventral polarity specification, it is unlikely that this dorsoventral polarity specification in the 4d lineage depends on endodermal cells or the 2d lineage.

To examine whether the evolutionally conserved signaling pathways for the dorsovental patterning are also involved in the specification of dorsoventral polarity in the *Tubifex* embryo, we characterized expression patterns of orthologue of *dorsal* gene (*Ttu-dl*) (Matsuo *et al*., 2005). During the early cleavage stages, *Ttu-dl* mRNA is detected in most of the blastomeres; however, strong expression is observed in 2d and 4d cells. In the gastrulation

stage, ectodermal bandlets exhibit different expression levels: The ventralmost bandlet (N lineage) exhibits the highest level of *Ttu-dl*, with the lowest level in the two middle bandlets (O and P lineages) and an intermediate level in the dorsalmost bandlet (Q lineage). These lineage-specific expression patterns indicate a possibility that *Ttu-dl* is involved in the patterning along the dorsovental axis. Further functional analyses will be required to clarify this issue.

Embryonic axial organizers in other spiralians

It has long been suggested that the D quadrant of spiralian embryos has an organizing activity that gives rise to establishment of the future dorsoventral axis (Freeman and Lundelius, 1992; Lambert, 2008). There have been known only a few spiralian organisms in which cells that function as embryonic axial organizers are experimentally identified: polychaete annelid *Capitella teleta*, and gastropod molluscs *Crepidula fornicata*, *Ilyanassa obsolete*, *Lymnaea stagnalis* and *Patella vulgate*. Interestingly, cells serving as organizers are 3D macromere in *Ilyanassa* (Clement, 1962), *Patella* (Damen and Dictus, 1996) and *Lymnaea* (Martindale, 1986), 4d micromere in *Crepidula* (Henry *et al*., 2006), and 2d micromere in *Capitella* (Amiel *et al*., 2013). In *Ilyanassa*, MAPK (mitogen activated protein kinases) have been shown to be required in dorsovental axis formation (Lambert and Nagy, 2001). Also in *Patella*, MAPK activation in 3D is required for the establishment of bilateral symmetric cleavage pattern (Lartillot *et al*., 2002). In contrast, the embryonic organizer in *Capitella* and *Crepidula* has been suggested to function independently of MAPK signaling (Henry and Kimberly, 2008; Amiel *et al*., 2013). In *Tubifex*, MAPK activation has not been detected in the D quadrant micromeres (unpublished observation). These findings suggest that embryonic organizers in spiralians are considerably diverse not only in the timing of their birth but also in the mode of executing organizing activity.

In *Ilyanassa* and *Capitella*, it has been demonstrated that organizing activity is executed via cell-cell interactions that give rise to cell fate specification in the surrounding tissues (Lambert and Nagy, 2001; Amiel *et al*., 2013). This apparently contrasts with the aforementioned *Tubifex* embryo in which 2d and 4d serve as mother cells of teloblasts that directly form embryonic axes without affecting fate specification in the surrounding tissues. In *Tubifex*, it remains to be explored whether the ectodermal and/or mesodermal GB affect cell fate specification in the endodermal cells. In this regard, it is intriguing to examine whether 3D of the *Ilyanassa* embryo and 2d of the *Capitella* embryo are able to induce secondary axis when transplanted to ectopic positions of the recipient embryos. The production of double embryos by equalization of unequal first cleavage has long been reported in many spiralians including molluscs (Guerrier, 1970b; Guerrier *et al*., 1978; Render, 1989) and polychaete annelids (Titlebaum, 1928; Tyler, 1939; Novikoff, 1940; Guerrier, 1970a; Render, 1983; Dorresteijn *et al*., 1987; Henry and Martindale, 1987). This may suggest that in these animals, duplication of cells with organizing activity leads to the duplication of embryonic axes in individual embryos as in the case of *Tubifex*.

Concluding remarks

In this review, we have described the ability of the D quadrant micromeres, $2d^{11}$ and 4d, in organizing a secondary embryo in *Tubifex*. This remarkable developmental potential seems to be unique to these specific D quadrant micromeres. One of the most significant features these micromeres exhibit is their capability to produce teloblasts that act as embryonic stem cells in the *Tubifex* embryo. To finalize the body plan of *Tubifex tubifex*, two morphogenetic events, viz. body elongation and segmentation, are required to occur. The formation of the GBs is essential for these events to proceed normally, and it solely depends on the presence of teloblasts. Thus, it is no exaggeration to say that in the *Tubifex* embryo, nothing will happen in the absence of teloblasts, hence 2d and 4d micromeres.

How do these micromeres acquire such developmental potentials? This is just the question Penners (1926) asked; he postulated the relationship between the developmental potentials of 2d and 4d and pole plasm localization. In a previous review (Shimizu, 1982), we mentioned similarly that the most important problem in *Tubifex* development is the relationship between the localization of pole plasm and the determination of cells. This problem remains to be explored, however. To date, there have been available few clues to probe into this relationship. In this regard, it would be intriguing to note that in leech embryos, not only teloplasm (i.e., yolk-deficient cytoplasm corresponding to pole plasm in the *Tubifex* embryo) but also animal cortical regions are involved in the ectodermal fate decision (Nelson and Weisblat, 1992). The same authors have also reported that in leech, teloplasm-containing cells adopt the mesodermal fate (as a ground state) if they lack in the animal pole cortex. At present, it is not known whether similar fate decision mechanisms operate in *Tubifex* embryos. However, it should be promising to explore the mechanisms for cell fate determination in the *Tubifex* embryo focusing on the animal pole cortex as well as the pole plasm itself in the future causal analytic studies.

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