Searching for the stem species of the Bilateria

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ABSTRACT. Some recent molecular phylogenetic studies suggest a regrouping of the bilaterian superphyla into Deuterostomia, Lophotrochozoa (Lophophorata, Spiralia and Gnathifera) and Ecdysozoa (Cycloneuralia as the remaining Aschelminthes, and Arthropoda). In some of these trees Platyhelminthes have a more derived position among the Spiralia. On the other hand, taxa within or close to the Platyhelminthes have been singled out as possible plesiomorphic sister groups to all other Bilateria (Acoela and Xenoturbellida). For both proposals there exists conflicting evidence, both when different molecular features are compared and when molecular and phenotypic characters are used. In this paper we summarise the phenotypic models that have been proposed for the transition between diploblastic and triploblastic organisation (Planula- Phagocytella-Archicoelomate-, Trochaea-, Gallertoid-, Coeloplana-, Colonial- concept). With very few exceptions such models construct a vermiform organism (acoelomate/pseudocoelomate or coelomate) at the base of the Bilateria while the finding of similarities in the genetic regulation of segmentation in vertebrates and arthropods has stimulated the search for larger, more complexly designed ancestors. Because of the possible significance of vermiform organisation for understanding the origin of the Bilateria, we present new data concerning the development and evolution of the complex body wall muscle grid of platyhelminths and new findings on their stem cell system (neoblasts). We show that studying the various features of the development of the body wall in a variety of basal platyhelminths (eg. Acoela, Macrostomida) with cytological and molecular techniques would provide essential data for discussions of the diploblast/triploblast transition, because it is the central element in the biology of vermiform organisms.

KEY WORDS: Evolution of Bilateria, diploblastic/triploblastic transition, Acoela, Xenoturbellida, Platyhelminthes, vermiform organisation, body wall muscle differentiation, neoblasts.

INTRODUCTION

The reconstruction of the ancestral organisation of the Bilateria has regained interest rapidly with the accumulation of molecular data (see lit in PETERSON et al., 2000; PETERSON & DAVIDSON, 2000; ADOUTTE et al., 2000; JENNER, 2000). The recent proposals concerning the evolution of the early Metazoa illustrate the major shift in emphasis that has occured over the last decade in the search for clues for the major transitions in animal body plans. Especially the advances in understanding the evolution of Hox genes, from the bilaterian crown groups such as arthropods and vertebrates down to the level of the sponges, together with 18S rDNA sequences form a new backbone for interpretations of the evolution of the metazoan phenotype.

This paper addresses three points in relation to this topic. We highlight some aspects of body plan evolution and the most recent proposals that Acoela and/or the Xenoturbellida may be the basal-most branches in the Bilateria. Secondly, we give an overview of models, in particular those using the organisation of certain platyhelminth taxa, for the origin of the Bilateria. Finally we give some examples from our own investigations (studies of the platyhelminth body wall musculature and of the neoblast system) which could contribute to an understanding of the origin of the Bilateria.

BODY PLAN EVOLUTION IN LOWER METAZOA AND RECENTLY PROPOSED SISTER GROUPS OF BILATERIA

One of the most significant changes in the phenotype to the level of the Eumetazoa certainly was the appearance of definite epithelial tissue. Steps involved here were specializations of apical junctional complexes to control the

paracellular flow across these covering cell sheaths, as well as the arrangement of the ECM at the base of these sheaths into complex molecular layers, the basal matrices (see RIEGER & WEYRER, 1998 for lit.). A more defined control of two fluid compartments is the result of this evolution: one is surrounded by the basal matrix (the mesogloea of dipoblasts, the primary body cavity, primitive blood vascular systems, and connective tissue fluids of triploblasts), the other one by the apical side of epithelial tissues (the gastrovascular system of diploblasts and the gut, secondary body cavities and duct systems for excretion and for reproduction in the triploblasts).

The most advanced level of eumetazoan organisation is seen in the triploblastic Eumetazoa, the Bilateria. Central for their evolution is the mesodermal tissue developing from the entoderm. This entomesoderm led to the further elaboration of muscle tissues and connective tissue. It occurs in two tissue grades: 1) in the coelomate organisation (the coelom or secondary body cavity) originally as myoepithelial lining, 2) in the acoelomate/pseudocoelomate organisation where muscle tissue is part of the connective tissue. It either fills the space between gut and body wall (acoelomate) or surrounds - in a non-epithelial organisation - fluid-filled compartments derived from the primary body cavity. In the life cycle of such animals, macroscopic adults with coelomic organisation often alternate with mm-sized, acoelomate/pseudocoelomate larvae.

According primarily to molecular studies, the Bilateria have been grouped into three major clades (ADOUTTE et al., 1999; KNOLL & CARROLL, 1999; VALENTINE et al., 1999; GAREY & SCHMIDT-RHEASA, 1998): 1) the Deuterostomia, originally with pseudocoelic larvae and enterocoelous adults, shown by many studies to be most similar to the coelenterate level of organisation (lit in CAMERON et al., 2000), 2) the Lophotrochozoa, which include in addition to the lophophorate phyla the taxon Spiralia and the former aschelminth taxa Rotifera and the Acanthocephala, with the Spiralia very likely representing a monophyletic subunit (BOYER et al., 1998; HENRY et al., 2000). It seems possible that indirect development with a trochophore-like larva is primitive also in the Lophotrochozoa (PETERSEN et al., 2000; PETERSEN & DAVIDSON, 2000). However, ROUSE (2000) concludes that only a lecithotrophic larva and not a planctotrophic trochophore may be plesiomorphic. 3) the Ecdysozoa, direct developers with secondary larvae due to the molting process. They include the Arthropoda and the taxon Cycloneuralia, the remaining aschelminth taxa.

While the concept of the Ecdysozoa is gaining recognition, very little is yet clarified when in comes to deriving segmentation of arthropods and annelids independently or from an unsegmented bilaterian stem species (see KNOLL & CAROLL, 1999; VALENTINE & COLLINS, 1999; JENNER, 2000). In the interpretation of the evolution of the Lophotrochozoa the question whether their common ancestor had an acoelomate/pseudocoelomate design or whether it was a coelomate animal also remains unsolved.

Based on 18S rDNA RUIZ-TRILLO et al. (1999) have singled out one lophotrochozoan taxon, the acoel flatworms, to represent the first branch among all Bilateria. JONDELIUS et al. (this volume) could resolve one major point of critique raised against that data set by showing that the nemertodermatids branch close to the basal branching point of the Acoela. Another recent molecular study using sequences of elongation factor 1-Alpha does not support such an early branching of the Acoela but suggests them to group within the Platyhelminthes (BERNEY et al., 2000).

Phenotypic evidence has led EHLERS & SOPOTT-EHLERS (1997) to suggest a similarly unique basal position for the enigmatic *Xenoturbella bocki* (see also LUNDIN, this volume). There are claims now that this species belongs to the Mollusca and is derived progenetically from protobranch larvae (ISRAELSSON, 1999; NORÉN & JONDELIUS, 1997). *Xenoturbella´s* basiepithelial nervous system and uniquely constructed extracellular matrix (PEDERSEN & PEDERSEN, 1986; 1988) remain an obstacle for morphologists to accept this proposal. In addition, complex character similarities in the epidermal ciliation link *Xenoturbella* and the Acoela (LUNDIN, 1998; this volume). Together with the digestive tract they represent an impressive morphological character sequence that at present can be read only from *Xenoturbella* to the Acoela with the Nemertodermatida as an intermediate taxon (see lit. in LUNDIN, 1998).

A recent cell lineage study shows that the Acoela lack ectomesoderm and that all of the musculature and peripheral parenchyma in the convolutid *Neochildia fusca* is of entomesodermal origin (HENRY et al., 2000). In this feature they are set apart from all other spiralians, but resemble the Ctenophora (MARTINDALE & HENRY, 1999). Ctenophores offer new possible scenarios for the origin of the Bilateria and the description of a fossil ctenophore with 80 comb rows is an unexpected incentive for reconsidering the ctenophores as the plesiomorphic sister taxon of the Bilateria (see MARTINDALE & HENRY, 1998 for lit).

PUBLISHED MODELS FOR THE TRANSITION FROM DIPLOBLASTIC TO TRIPLOBLASTIC ORGANISATION

In the main models proposed for the origin of the diploblastic organisation (Haeckel´s Gastraea-, Bütschli´s Placula-, Lankester´s Planula-, Metschnikoff´s Phagocytella and Gutman´s Gallertoid- hypothesis) original metazoan cell colonies are derived from flagellate unicellular eukaryotes that had developed the characteristic metazoan ECM (see RIEGER & WEYRER, 1998 for lit.). Monociliated cells with one functional cilium and an accessory centriole in a precisely defined position relative to the basal body of the functional cilium represent almost

certainly the plesiomorphic character state of all somatic cells in the early cell colonies.

For the transition from the diploblastic to the triploblastic organisation three groups of models can be distinguished:

The first bilaterians - vermiform organisms with direct development

Vermiform organisation is most often used in reconstructions of the bilaterian stem species. Such organisms have a cylindrical or flattened body profile and use their flexible body wall for locomotion (Fig. 1). The body wall contains helically wound fibers in the cuticle, in the cell web and/or in the basal matrix of the epidermis for retention of shape, and at least two layers of muscles – circular and longitudinal – intimately associated with the basal matrix (RIEGER, 1998). This body wall acts against the extracellular compartments formed by the gut, the pseudocoel and/or the coelom or against intracellular hydrostats of muscle and connective tissue in acoelomates.
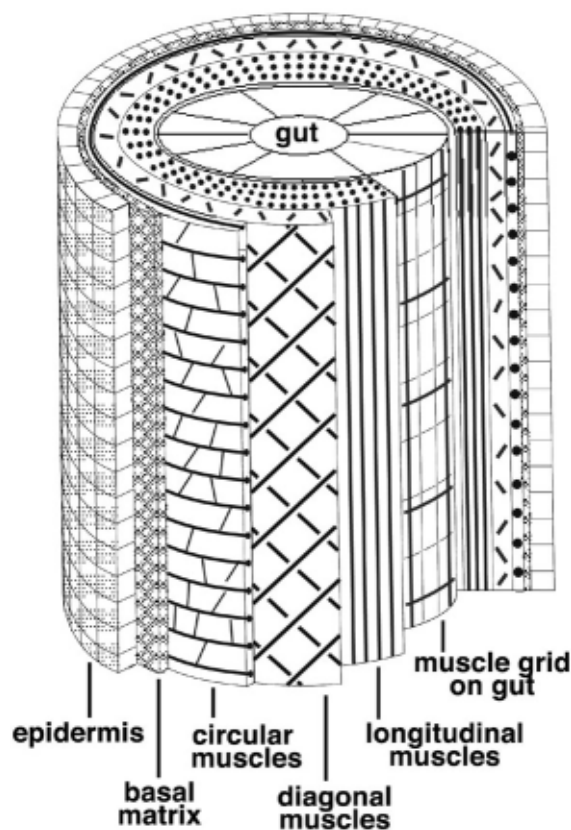

Fig. 1. – Diagram of vermiform body organisation in acoelomates.

Summaries of ideas for the transition at the diplo-/triploblast boundary can be found in REISINGER (1961), SALVINI PLAWEN (1978) and WILLMER (1990). Most widely used are the Planula and Phagocytella (Parenchymula)-concepts where acoels and nemertoder-

matids are model organisms for the original Bilateria. The main differences between the two models are the assumptions concerning the original organisation of the gastrodermis and the muscle tissue: in the Phagocytella hypothesis the gastrodermis is assumed to be a connective tissue including muscles. In the planula hypothesis gastrodermis and muscles are seen as derived from epithelial tissue.

A basiepithelial nerve plexus with first condensations of longitudinal cords is postulated as ancestral in both the Planula and the Phagocytella concept. The epidermal apical organ of the planula would be the structure giving rise to brain differentiations. In immunocytochemical preparations the organisation of the brain of various Acoela appears rather different from all other platyhelminth taxa (REUTER et al., 2001). These findings underscore the unique and possibly basal position of this taxon among living platyhelminths.

Other proposals are the Gastraea-Bilaterogastrea-concept and the Gastraea-Archicoelomate-concept. Both hypotheses propose an enterocoelic entomesoderm, envisioned to have originated from gastric pouches of the coelenterate organisation. Also these concepts assume a vermiform stem species for all Bilateria, however, this organism would have been a coelomate.

Vermiform organisation is as well assumed in two more hypotheses: LANG´s (1884) Coeloplana-concept and the Gallertoid concept by BONIK et al. (1976) and GUTMAN (1981).

The first bilaterians - vermiform organisms with a biphasic life cycle

The Trochaea hypothesis (Gastraea-Trochaea- concept, see lit. in NIELSEN, 1998), going back to the concept of the benthopelagic life cycle of Jägersten, assumes an acoelomate/pseudocoelomate vermiform ancestor for the protostome line of evolution and a vermiform archicoelomate ancestor for the deuterostome line of evolution. The vermiform benthic adults alternate in a life cycle with pseudocoelic pelagic larvae, the latter ones representing the phylogentically older body plan. This hypothesis is supported by the recent proposal resulting largely from developmental studies (lit. in PETERSON et al., 2000, see ROUSE, 2000 for different view).

The biphasic life cycle with a microscopic larva and a macroscopic adult is also central in the proposal for the ancestral metazoans by one of us (RIEGER, 1994). By progenesis several lines of evolution could have lead from the acoelomate and pseudocoelomate larvae or juveniles of an archicoelomate ancestor to adult acoelomates and pseudocoelomates respectively (see lit. in TYLER, 2000). It should be pointed out that any model using a planula at the diploblast/triploblast transition must be seen as considering progenesis: the planula organisation is only found as larval stage, no planula-like adult organism is known.

The first bilaterians - macroscopic organisms with complex body plan

Similarities in the genetic networks specifying segmentation in such distant groups as vertebrates and arthropods has led to the postulation of more complex macroscopic ancestors for the Bilateria (see discussion in KNOLL & CARROLL, 1999; JENNER, 2000). The scaffolding for this idea is seen in certain conserved genes involved in segmentation (see recent review concerning this issue in DAVIS & PATEL, 1999).

The latest attempt in trying to envision a more complex base for the bilaterian stem species has been presented by DEWEL (2000). Frond-like macroscopic colonial diploblasts, similar to organisms in the Ediacara fauna and to extant Pennatulacea serve as models. Colonial organisation is seen as a condition that might have led to a highly compartmented coelomate ancestor.

Considering all evidence, a vermiform bilaterian ancestor in the size of millimeters remains a realistic model. In the case of the Lophotrochozoa the ancestor could be similar to platyhelminths. The position of the Gastrotricha and the Gnathifera (Gnathostomulida, "Rotifera" and Acanthocephala) may be also close to the bilaterian origin. On the other hand, a cm-sized organism similar to *Xenoturbella* may have led to the evolution of archicoelomates in the sense of Jägersten. Progenesis of larvae or juveniles of such ancestors would have led to today's acoelomate and pseudocoelomate phyla (see TYLER, 2000 for lit.).

AVENUES FOR FUTURE RESEARCH

Given the central position of the vermiform organisation in hypotheses about the bilaterian stem species, as much as possible should be known about the development and organisation of organisms of such a design. In the following we report about work in our laboratory on three research topics related to this aspect.

The fate of the coelenterate aboral-oral axis in the Bilateria

The determination and specification of the bilaterian body axes and their role in development have received special attention again in assessing the evolution of the lower Metazoa (eg. MARTINDALE & HENRY, 1998; GOLDSTEIN & FREEMAN, 1997). Both the anterioposterior (A-P) axis and the dorsoventral (D-V) axis have been studied in detail in a variety of species. As far as the origin of these axes from the aboral-oral (A-B) axis of diploblasts is concerned, it is evident that we particularly need more information concerning the origin of the D-V axis.

In Fig. 2 two different processes that could lead from the A-B axis of a diploblast to the condition of a vermiform, bilaterian ancestor are depicted. In process 1) the diploblastic organism undergoes a 90-degree shift in relation to the substrate, subsequently the original mouth/anus moves forward to the ventral side. In process 2) a compression of the A-B axis takes place. Here the apical brain Anlage is moved forward, the mouth remains in the original position. As a result, the A-B axis is parallel with the bilaterian A-P axis in the first case, a new D-V axis must have been specified. In the second case the A-B axis becomes the D-V axis, a new A-P axis must have evolved in the Bilateria.

Such considerations are in line with models for the origin of the Bilateria mentioned above: The Planula-concept is a clear case of process 1 (REISINGER, 1961, fig. 10), whereas the Coeloplana-hypothesis of LANG (1884) is
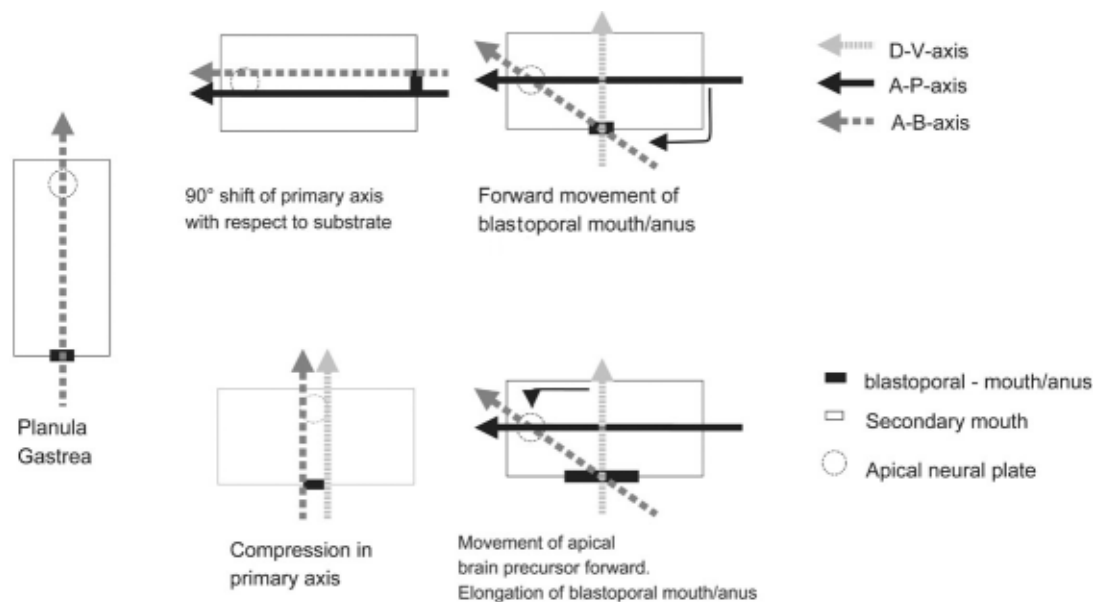

Fig. 2. – Diagram of shifts in body axis at the diploblast/triploblast transition. Upper line, process 1, lower line, process 2, see text for further explanation. Dashed arrow: Aboral-oral axis (A-B), black arrow: anterior-posterior axis (A-P), finely dashed arrow: dorso-ventral axis (D-V).

clearly a case of process 2. During the settling of the Trochaea the mouth remains in the original position and is elongated to form a longitudinal slit (Trochaea-hypothesis: NIELSEN, 1995). The apical plate moves forward, the A-B axis becomes inclined – this behavior and the blastopore in this model are in accordance with model 2.

To study processes that control shifts of the A-B axis we have started to work on the expression of the gene of the Wnt/wingless cascade in *Convolutriloba longifissura* and plan to do this also with *Convoluta pulchra* and *Macrostomum*-species, and the sog/chordin and bmp4/dpp gene family in acoel and macrostomid turbellarians. These genes are part of the genetic network specifying the D-V axis in higher vertebrates and arthropods (see lit. in HOBMAYER et al., in press). Such genes have now also been found in *Hydra*, being expressed in the hypostome in the vicinity of the mouth (HOBMAYER et al., in press). We will trace the expression in the formation of the platyhelminth mouth during embryonic development and during asexual reproduction. We expect that the expression pattern observed in *Hydra* near the mouth will also be found in platyhelminths and will make it possible to follow the separation of the A-B axis from the A-P axis, as depicted in Fig. 2.

In another way axis specificity can also be seen in the muscle development in acoels. Along this route we have studied the formation of the body wall musculature in embryos of *Convoluta pulchra* (LADURNER & RIEGER, 2000) and during asexual reproduction in *Convolutriloba longifissura* (data not shown).

In *Convoluta pulchra,* the cellular orientation relative to the A-P axis is highly specific during the establishment of what we called the original orthogonal muscle grid. Unexpectedly, circular fibers appeared distinctly prior to the longitudinal fibers, and they were oriented along well defined latitudes in embryos at about 50-55% of developmental time (Fig.3A). The A-P axis can be identified from the fiber arrangement because the formation of circular fibers starts closer to the apex of the animal. Since acoels lack any basal matrix of the epidermis, differentiating myocytes may become aligned along differing anterior-posterior densities and/or qualities of receptors in the basolateral epidermal membrane. Ultrastructural data on the cytology of myoblasts actually corroborate this notion (RIEGER, LADURNER, SALVENMOSER, unpublished).

At around 60 % of developmental time from egg laying the longitudinal muscle fibers are visible, forming the primary orthogonal muscle grid (Fig. 3B). As with the original circular fibers, the fibers of the longitudinal layer line up behind each other on defined meridians, in this case parallel to the A-P axis. Differences in this primary muscle grid between the dorsal and ventral side begin to become visible very early, already at about 60 % of developmental time.
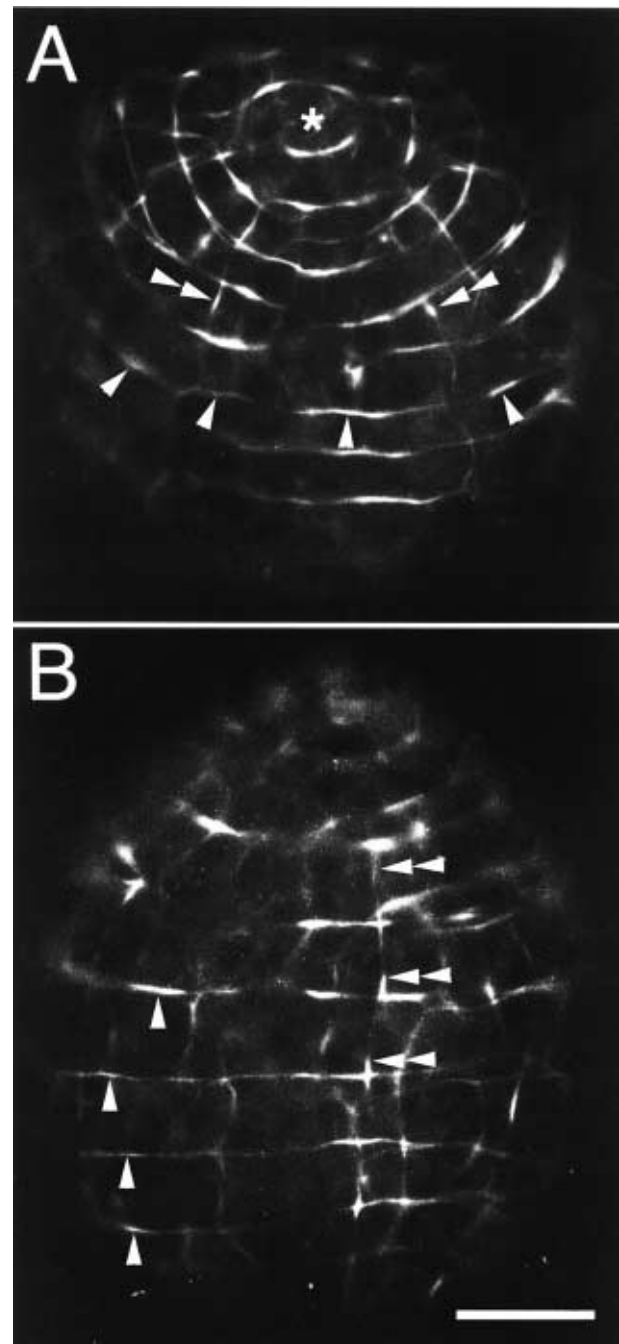

Fig. 3 A, B. – Phalloidin-Rhodamin-stained embryos of *Convoluta pulchra*. A: stage 3-4, about 55% developmental time since egg laying. Note anterior pole of embryo (*), circular muscle fibers aligned on meridians (arrowheads), first longitudinal fibers (double arrowheads). B: stage 4, about 60% developmental time since egg laying. Further development of circular fibers (arrowheads), longitudinal myocytes aligned along meridians (double arrowheads). See also LADURNER & RIEGER (2000). Scale bar: 20 µm.

Transition of the bilayered body wall musculature from two germ layers of diploblasts to one germ layer of triploblasts

The second research topic is concerned with the phylogenetic origin of the complicated adult body wall muscu-

latures of Platyhelminthes (see TYLER & RIEGER, 1999 for lit.). From our ontogenetic studies (LADURNER & RIEGER, 2000) it seems reasonable to assume that an orthogonal pattern of circular and longitudinal muscles may represent indeed the ancestral condition. In the Acoela unexpected variations of fiber orientation in that orthogonal matrix had been shown (see TYLER & RIEGER, 1999 for lit.), although the responsible mechanisms have yet to be specified.

How did such a primary orthogonal body wall muscu-lature evolve from the conditions seen at the coelenterate level? In the body of *Hydra* as in all cnidarians one finds vermiform parts with orthogonal muscle layers (Fig. 4, see LADURNER & RIEGER, 2000 for lit.). The body column, as well as the tentacles, are vermiform as defined above. Two orthogonal layers are present, the circular fibers being more delicate. All fibers are short when compared with fibers in the microturbellarians, which may run almost the entire length or circumference of the body.

Based on present evidence about body wall muscle fibers in platyhelminths, we can propose two different models for the evolution of the body wall musculature of tripoblasts from diplobasts (Fig. 5): a transition from the diploblastic stage with muscle layers in two germ layers (the cnidarian model), and the origin of all muscle layers from one germ layer (the ctenophore model).
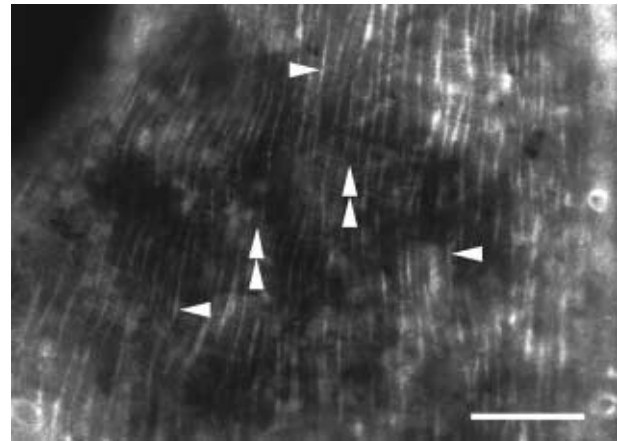

Fig. 4. – Portion of Phalloidin-stained tentacle of *Hydra* sp. showing orthogonal muscle grid. Note longitudinal fibers situ-ated in the epidermis (arrowheads) and circular fibers (double arrowheads) situated in the gastrodermal epithelium. Scale bar: 50 μm.

In both models we assume an important role of the extracellular matrix for the process and start with a ran-dom orientation of the fibers, both in the epidermis and the gastrodermis. In the cnidarian model an inversion of the orientation of epidermal longitudinal and gastroder-mal circular fibers would lead to a situation known from
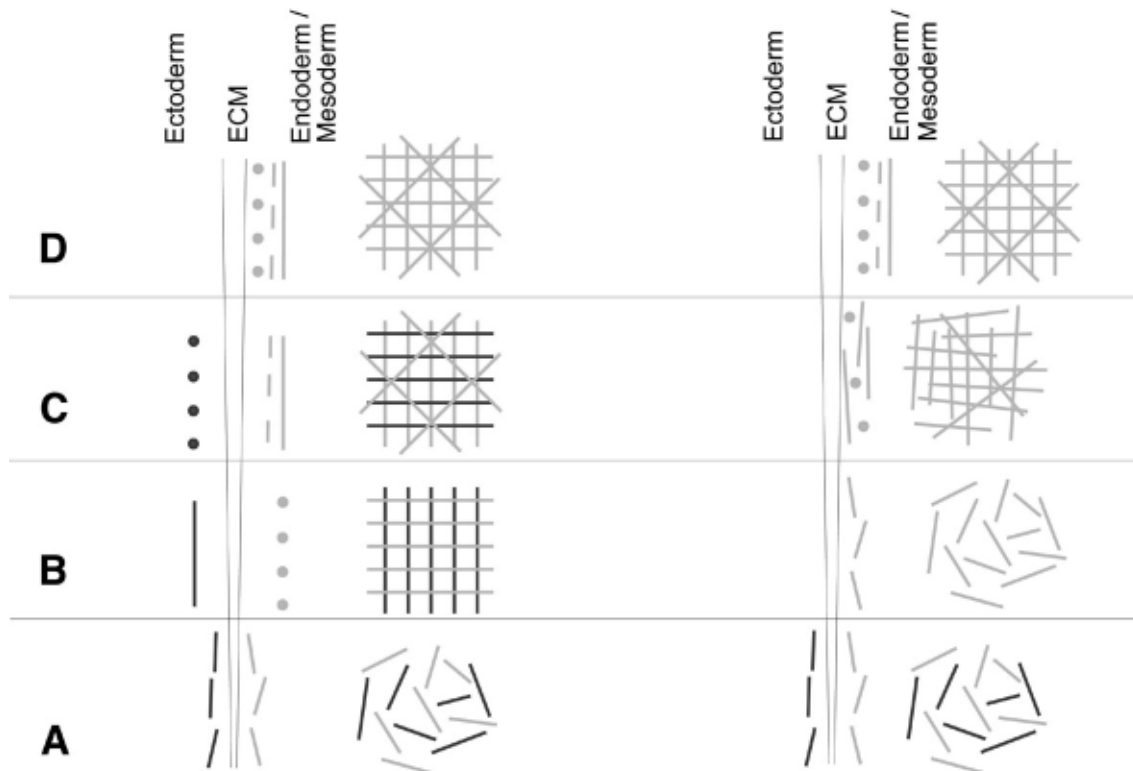

Fig. 5. – Schematic drawing of two possible ways of changes of the body wall muscle grid during the diploblast/triploblast transition (longitudinal sections and head-on views of muscle fibers). Ectodermal muscle fibers in black, entomesodermal muscle fibers in grey. The diagonal fibers are thought to be derived from the longitudinal muscle layer. A-D: evolution from randomly oriented fibers in two germ layers to a muscle grid with outer circular, inner longitudinal and intermediate diagonal fibers in one germ layer. Cnidarian model at left, ctenophore model at right; see text for explanation.

cell lineage experiments in the Müller´s larva of *Hoploplana inquilina* (BOYER et al., 1996; 1998) where the circular muscles, closest to the epidermis, are of ectomesodermal origin while the deeper longitudinal fibers are entomesodermal. A gradual substitution of ectomesodermal muscles by entomesodermal ones is assumed to have occurred in adult spiralians.

Alternatively only the original entodermal muscle grid was retained and became organised later into the orthogonal pattern of the body wall in ancestral bilaterians. This proposal is more in line with the body wall musculature being derived entirely from the entoderm, as has been shown by MARTINDALE & HENRY (1999) for the Ctenophora and by HENRY et al. (2000) for acoels.

The neoblast stem cell system

Investigating the unique neoblast system of the Platyhelminthes with a combination of labelling techniques (eg. BrDU and immunogold-labelling) might reveal another useful set of data for clarifying changes at the diploblast-triploblast transition. As of now (summarized in LADURNER et al., 2000), evidence is increasing that all somatic cells in Platyhelminthes do originate from one cell type. However, more data especially from Acoela, Nemertodermatida and Catenulida are needed before definite conclusions can be drawn (GSCHWENTNER, unpubl. data).

Such a single cell renewal system is not found among the most primitive metazoans, the Parazoa, Placozoa and the coelenterate phyla. What the basal Metazoa seem to suggest is that a combination of the ability of tissue cells to retain mitotic activity and to establish certain stem cell lines is the plesiomorphic condition among the lower Metazoa. Therefore, the neoblast stem cell system may be an autapomorphic trait of the Platyhelminthes.

CONCLUSION

It is of course evident that only the input from many lines of research will lead to further satisfactory progress in reconstructing the body plan of the ancestral bilaterians. As many characters of diploblastic and basal triploblastic animals as possible should be compared, and problematic taxa cannot be disregarded. Contrary to claims in certain molecular studies (ADOUTTE et al., 1999) we think that resolving the original design of the bilaterian body cavity (acoelomate/pseudocoelomate versus coelomate) is still crucial. The free-living platyhelminths are central for answering this question, investigating them with a combination of molecular, cellular, developmental and morphological approaches will be necessary.

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