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Evolutionary changes of developmental mechanisms in the absence of cell lineage alterations during vulva formation in the Diplogastridae (Nematoda)

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SUMMARY

The origin of novelty is one of the least understood evolutionary phenomena. One approach to study evolutionary novelty comes from developmental biology. During developmental cell fate specification of the nematode *Pristionchus pacificus* (Diplogastridae), five cell fates can be distinguished within a group of twelve ventral epidermal cells. The differentiation pattern of individual cells includes programmed cell death, cell fusion and vulval differentiation after induction by the gonad. A cell lineage comparison among species of seven different genera of the Diplogastridae indicates that the differentiation pattern of ventral epidermal cells is highly conserved. Despite this morphological conservation, cell ablation experiments indicate many independent alterations of underlying mechanisms of cell fate specification. Cell fusion and individual cell competence change during evolution as well as the differentiation property in response to inductive signaling. These results suggest that developmental mechanisms, some of which are redundantly involved in vulval fate specification of the genetic model organism *Caenorhabditis elegans*, can evolve without concomitant morphological change.

Key words: vulva development, evolution, nematode, novelty, *Pristionchus*

INTRODUCTION

The basis for evolutionary change in morphology is the modification of ontogeny. Developmental processes differ between species by the number, potency and connectivity of cells involved in a particular process. Thus, evolutionary changes of cell fate specification are basic to alterations underlying morphological diversification. A comparative analysis of development can reveal evolutionary changes of cell fate specification and might provide insights into the acquisition of evolutionary novelty, one of the least understood evolutionary phenomena (for a review see Sommer et al., 1994).

The combination of cellular, genetic and molecular tools have provided a detailed understanding of several developmental processes in the free-living nematode *Caenorhabditis elegans*. One aspect of development that is tractable for comparative analysis is the generation of the vulva, the egg-laying structure of nematode females or hermaphrodites (Sommer and Sternberg, 1996b). The vulva is a derivative of the ventral epidermis, which consists of 12 epidermal cells called P1.p-P12.p (Fig. 1A). In *Caenorhabditis*, the six central epidermal cells P(3-8).p form a so-called equivalence group or competence group for vulva formation, as all cells have the potential to participate in vulva development (Fig. 1B). Pattern formation within the equivalence group begins with induction of three VPCs by the gonadal anchor cell early in the third larval stage (L3) (Kimble, 1981). As a result, the cell fate pattern 3°-3°-2°-1°-2°-3° is established (Fig. 1B) (Sulston and White, 1980; Sternberg and Horvitz, 1986). P6.p has the 1° fate and generates 8 progeny, P5.p and P7.p have the 2° fate and generate 7 progeny each. The progeny of P(5-7).p form the wild-type vulva. The other VPCs, P3.p, P4.p and P8.p, divide once and fuse with the epidermis during the L3 stage. Much is known about the molecular processes underlying vulval development in *Caenorhabditis*. At least three intercellular signals - an inductive signal, a lateral signal, and a negative signal - are involved in this pattern formation process (for reviews see Katz and Sternberg, 1996; Sundaram and Han, 1996).

The VPCs can replace one another after cell ablation. After ablation of P6.p, both P5.p or P7.p can adopt the 1° fate (Sulston and White, 1980; Sternberg and Horvitz, 1986). After ablation of P(5-7).p, the three cells that form the vulva in an intact worm, a normal vulva can be formed by P(3,4,8).p with the pattern 2°-1°-2°. After ablation of five of the six VPCs, the remaining cell can adopt the 1° fate. The epidermal cells outside the vulva eqivalence group, P(1,2).p and P(9-11).p fuse with the epidermis during the L1 stage (Fig. 1B). P12.p is a special cell, it divides once more in the L1 stage and forms part of the rectum, called hyp12 (Fig. 1B) (Sulston and Horvitz, 1977).

Comparative studies of vulval development have been initiated by cell lineage analysis and cell ablation experiments in species of different families of nematodes (Sommer and Sternberg, 1994, 1995, 1996a). This approach was extended to the genetic level in the newly described species *Pristionchus pacificus* (Sommer et al., 1996). Several aspects of cell fate specification during vulval development differ between *Caenorhabditis* and *Pristionchus*. Two of the six cells that correspond to the *Caenorhabditis* vulval equivalence group die by programmed cell death in *Pristionchus*. Another cell fuses with

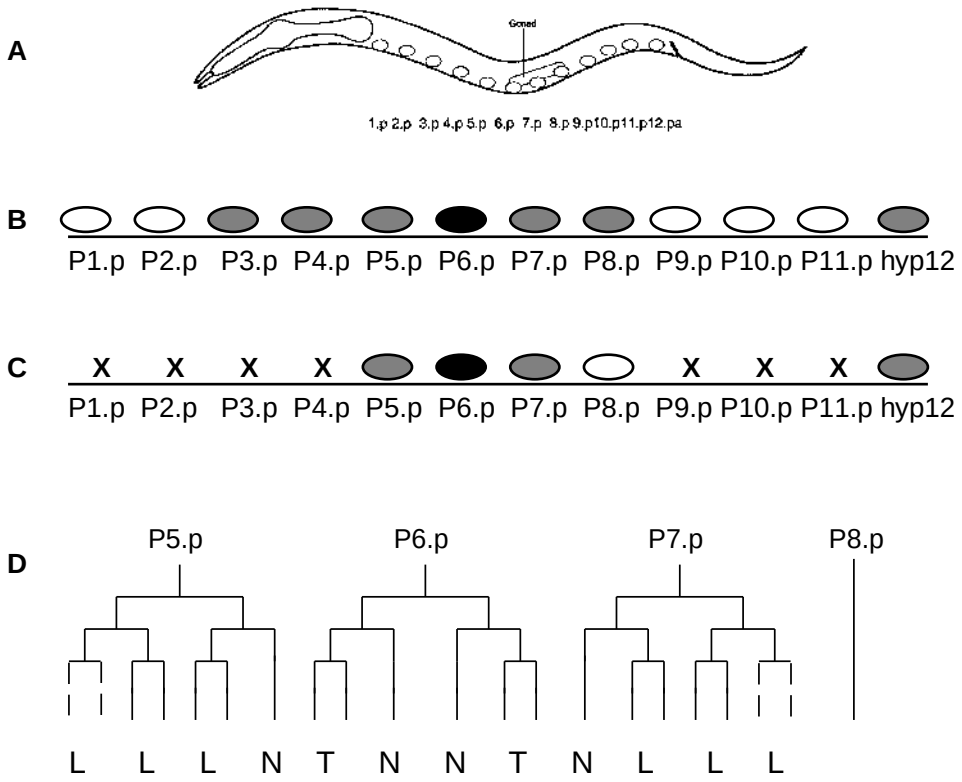

Fig. 1. Schematic summary of the position and the cell fate of ventral epidermal cells and the vulva cell lineage in the Diplogastridae and in *C. elegans*. (A) In the L1 stage in *C. elegans*, the twelve epidermal cells are equally distributed in the region between the pharynx and the rectum. (B) Differentiation pattern of ventral epidermal cells in *C. elegans*. P(1,2).p and P(9-11).p fuse with the hypodermal syncytium hyp7 in the L1 stage (white circle). P12.pa forms hyp12 and is part of the rectum (horizontal hatched circle). P(5-7).p form the vulva; P6.p adopts the 1° fate (black circle), P(5,7).p adopt the 2° fate (vertical hatched circles) and P(3,4,8).p adopt the 3° fate (speckled circles). (C) Differentiation pattern of ventral epidermal cells in the Diplogastridae. P(1-4).p and P(9-11).p undergo programmed cell death (X). The descendant of P12.p forms hyp12 (horizontal hatched circle). P(5-7).p form the vulva; P6.p adopts the 1° fate (black circle), P(5,7).p adopt the 2° fate (vertical hatched circles) and P8.p fuses with the hypodermis (white circle). (D) Canonical vulval cell lineage in the Diplogastridae. Dashed lines represent the observed variation of P5.paa and P7.ppp division in *Pseudodiplogasteroides* and *Diplogasteroides*; see text for details. There is no time scale given, as timing differs among species. In fast developing species, such as *Pristionchus*, all vulval cell divisions occur within 5 hours, whereas they can take up to 8 hours in slower developing species like *Aduncospiculum*. T, transverse division; L, longitudinal division; N, no division of the Pn.p granddaughters according to the definition of Sternberg and Horvitz (1986).

the epidermis early in development, making it incompetent to form vulval tissue. A third evolutionary change of cell fate exists, as another cell has only limited developmental potential and can adopt the 2° but not the 1° fate. In *Caenorhabditis*, all six vulval precursor cells (VPCs) are multipotent, i.e. they can adopt all three cell fates. Thus, in a group of six cells, three evolutionary alterations of cell fate specification occurred between the two species. Initial genetic work in *Pristionchus* suggests that one can study evolutionary changes of cell fate specification at the genetic level. Mutants that alter cell fate specification of those cells that differ in their developmental potential between *Caenorhabditis* and *Pristionchus* have been isolated (Sommer and Sternberg, 1996a; Eizinger, Schlak and R. J. S., unpublished observations).

Caenorhabditis belongs to the family Rhabditidae (Goodey, 1963; Sudhaus, 1976, Andrassy, 1984) whereas *Pristionchus* is a member of the family Diplogastridae (Goodey, 1963) or Neodiplogastridae (Andrassy, 1984), respectively. *Pristionchus* was the first member of the suborder Diplogastrina to be analyzed with respect to vulval development. Systematic description of the Diplogastrina is limited to the monographic work by Goodey (1963) and Andrassy (1984), both of which are based on comparative morphological studies. Recently, morphological revisions of particular subgroups were initiated (Sudhaus and Rehfeld, 1990; Kiontke and Sudhaus, 1996). All authors give slightly different phylogenetic relationships for the major taxa within the suborder. Table 1 summarizes and compares the taxonomic relationship of the species analyzed in this paper with respect to the taxonomy of Goodey and Andrassy. With the information currently available, it is

Table 1. Taxonomic relationship of analyzed species, based on the monographic descriptions of Andrassy (A) and Goodey (B)

(A) ANDRASSY, 1984:	
Subordo Diplogastrina:	
Familia: Cylindrocorporidae:	*Goodeyus ulmi*
Familia: Pseudodiplogasteroididae:	*Pseudodiplogasteroides* sp.
Familia: Diplogasteroididae:	*Diplogasteroides* sp.
Familia:Diplogastridae:	*Diplogaster maupasi*
	Aduncospiculum halicti
Familia: Neodiplogastridae:	*Pristionchus pacificus*
	Koerneria sp.
(B) GOODEY, 1963:	
Suborder Rhabditina:	
Superfamily Diplogasteroidea:	
Family: Diplogasteridae:	*Diplogaster maupasi*
	Koerneria sp.
	Pristionchus pacificus
	Diplogasteroides sp.
	Aduncospiculum halicti
Family: Cylindrocorporidae:	*Goodeyus ulmi*
Superfamily Pseudodiplogasteroidoidea:	*Pseudodiplogasteroides* sp.

Aduncospiculum halicti is not listed in these monographic descriptions. The given taxonomic position was proposed by W. Sudhaus (personal communication). Note, that this list does not infer phylogenetic relationship among species.

impossible to propose a phylogram with more detailed information about the species considered. However, morphological and molecular analysis of some of these species is ongoing (W. Sudhaus and K. Thomas, personal communication).

To determine if the three observed changes of cell fate specification between *Pristionchus* and *Caenorhabditis* evolved independently, species of six additional genera of the Diplogastridae were studied. Here, I report comparative studies of cell lineage and pattern formation during vulval development in the species *Diplogaster maupasi*, *Koerneria* sp., *Diplogasteroides* sp., *Pseudodiplogasteroides* sp., *Aduncospiculum halicti* and *Goodeyus ulmi*. All species display an identical differentiation pattern of ventral epidermal cells as revealed by cell lineage analysis. Despite these morphological similarities, cell ablation experiments suggest differences in the underlying pattern formation processes. In four species, the cell that has limited developmental potential in *Pristionchus* can adopt either vulval cell fates. In two species, the cell that is incompetent in *Pristionchus* is competent in a manner similar to the corresponding cell in *Caenorhabditis*. Furthermore, in two species, some VPCs show differentiation properties in the absence of gonadal inductive signal. The finding that the pattern of cell competence varies among species indicates that developmental mechanisms can change during evolution while differentiation pattern remain invariant.

MATERIALS AND METHODS

Strains

The strains used in this study were kindly provided by J. Baldwin (Riverside, USA), L. Carta (Pasadena, USA), P. De Ley (Gent, Belgium) and W. Sudhaus (Berlin, Germany). In the following text, I will refer to the name of the genus to describe a particular species. The full scientific names of the analyzed species are as follows: *Pristionchus pacificus* (PS 312), *Diplogaster maupasi* (RS 104), *Diplogasteroides* sp. (RS 125), *Aduncospiculum halicti* (RS 112), *Koerneria sp.* (RS 113), *Goodeyus ulmi* (RS 100) *and Pseudodiplogasteroides sp.* (RS 103). All strains are cultured at the Max-Planck Institute, Tübingen. *Pristionchus, Diplogaster, Diplogasteroides* and *Pseudodiplogasteroides* are hermaphroditic, the others are male-female species. All species were grown on lawns of *E.coli* OP50 at 20°C as described for *Caenorhabditis* (Brenner, 1974). Stocks were maintained by crossing several males and females or by handling the hermaphrodites as described for *Caenorhabditis* (Wood, 1988).

Cell lineage

For cell lineage observation, the technique of Sulston and Horvitz (1977), modified by Sternberg and Horvitz (1981), was used. Nematodes were observed using a Zeiss microscope with a Plan 100 objective and Nomarski interference contrast optics. Cell lineages were determined by continuous observation of nuclei as they divided.

Laser microsurgery

Ablation experiments were done according to the method of Sulston and White (1980), using the microbeam system described by Avery and Horvitz (1987). Animals were picked into 2.5 µl of S Basal and placed on a pad of 5% agar in water containing 10 µM sodium azide as anaesthetic. In Tables 2-8, 'early' refers to experiments where cell ablation was performed in the first few hours after hatching. 'Late' refers to experiments where cell ablation was performed in the late L1 stage or early L2 stage.

Cell position and nomenclature

In all free-living nematodes examined, 12 ectodermal P-cells migrate into the ventral cord region. These cells are named P1-P12 from anterior to posterior according to the *Caenorhabditis* nomenclature (Fig. 1A) (Sulston and Horvitz, 1977). After an asymmetric cell division in the first larval stage (L1), the posterior daughters of the P cells, P1.p-P12.p (Pn.p) become ectoblasts; the anterior daughters (Pn.a) become neuroblasts. As cell divisions occur, the progeny of the neuroblasts intercalate with the epidermal cells (Pn.p). In the late L1 stage, adjacent epidermal cells are separated from one another by at least 5 differentiated neurons. By Nomarski observation nuclei of similar epidermal cells appear at identical positions with respect to the gonad and other morphological structures in all analyzed species. The nucleus of P6.p is originally located anterior of the gonad, whereas the nucleus of P7.p is located immediately posterior of the gonad (Fig. 2A).

A subset of Pn.p cells in the central body region are the vulva precursor cells (VPCs) (Fig. 1B). In the third larval stage (L3), the epidermal cells enlarge and start to divide. Using Nomarski optics, one can distinguish by lineage and morphology cells that adopt an epidermal or a vulval fate. VPCs that adopt a vulval fate differ in the number of progeny, the orientation of axes of the third cell division and whether an attachment to the gonadal anchor cell (AC) is established. To distinguish the different lineages, we use the terminology 1°, 2°, 3°, hybrid and S. The 1° cell type forms vulval tissue and some of the progeny attach to the AC. This differs from the 2° cell type that also forms vulval tissue but does not have an AC contact. The hybrid cell fate is characterized by variable cell lineages. In hybrid cells, an AC contact is missing or variable between individuals (Sommer and Sternberg, 1994). VPCs that adopt an epidermal fate in intact animals, but that were competent to adopt vulval fate, are considered to adopt the 3° cell fate. S refers to non-specialized epidermal Pn.p cells that fuse with the surrounding hypodermis early in development, causing the cell to be incompetent to form vulval tissue. Vulval cell lineages are described in the following text using the described criteria. Cell division terminology (T, L, N) is used according to Sulston and Horvitz (1977), Sternberg and Horvitz (1986) and Sommer and Sternberg (1994, 1996a) and is based on the phenotype of the third round of cell division. 'T' refers to a transverse division; 'L', to a longitudinal division; and 'N' refers to no division of the Pn.p granddaughters, according to the definition of Sternberg and Horvitz (1986).

Ablation experiments can reveal a hierarchy of cell fates. The primary cell type is the one that is replaced by a secondary or tertiary cell after it is destroyed. A secondary cell is one that replaces a primary or is replaced by a tertiary cell. A tertiary cell is one that replaces a primary or secondary cell (Kimble and Hirsh, 1979). The first sign of replacement after cell ablation is the migration of the nucleus of the replacing cell to the original position of the nucleus of the cell that has been destroyed. In cell ablation tables, the fate 'S' designates Pn.p cells that do not form part of the equivalence group in the way the term is used for *Caenorhabditis*. Note, however, that until cell fusion occurs, the corresponding cells might have the potential to replace other cells after individual VPCs were destroyed. The fate 'D' designates VPCs that form vulva-like structures in the absence of gonadal signal, according to the definition of Sommer and Sternberg (1996a).

RESULTS

Pristionchus pacificus

In *P. pacificus*, the 12 epidermal cells, P1.p-P12.p, are generated by an asymmetric cell division of the ectoblasts P1-P12, as described for *Caenorhabditis.* During late embryogenesis seven epidermal cells, P(1-4).p and P(9-11).p, undergo programmed cell death (Sommer and Sternberg, 1996a). Thus, only five epidermal cells are present in *Pristionchus* larvae;

Table 2. Cell ablation experiments in *Pristionchus pacificus*

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	S	
B	Z(1,4)$^-$		3°	3°	3°	S	10/10*
C	P(6-8).p$^-$	late	1°	X	X	X	8/11*
	P(6-8).p$^-$	late	2°	X	X	X	3/11*
D	P7.p$^-$	early	2°	1°	X	2°	3/6
	P7.p$^-$	early	2°	1°	X	S	3/6
E	P7.p$^-$	late	2°	1°	X	S	6/6
F	P(5,6).p$^-$	late	X	X	2°	S	6/8*
	P(5,6).p$^-$	late	X	X	3°	S	2/8*
	P(5,6).p$^-$	early	X	X	2°/hybrid	S	8/8
G	P(5-7).p$^-$	early	X	X	X	S	22/22

*Data from Sommer and Sternberg, 1996a.

Cells were ablated in the L1 stage unless otherwise noted; for details see Materials and Methods. Each line in the table represents one type of experiment. Nomenclature is described in Materials and Methods. Vulval development is gonad dependent, because after ablation of the gonad primordium all VPCs adopted the 3° fate (B). P8.p is competent to adopt a vulval fate only after ablation of P7.p in the first few hours of larval development (D,E). P7.p is limited in its developmental potential and can only adopt the 2° fate (F), whereas P5.p is multipotent and can adopt the 1° fate (C).

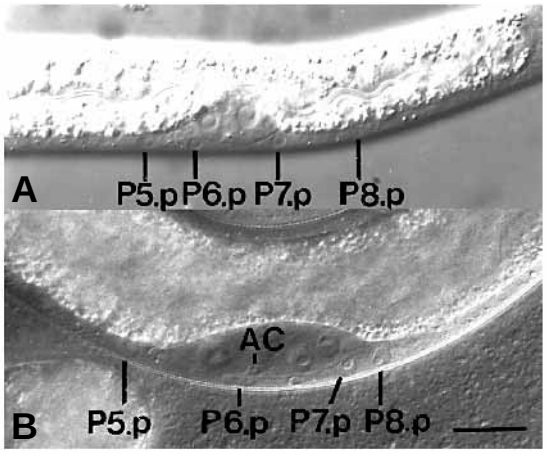

Fig. 2. Nomarski photomicrographs of a *Pristionchus* hermaphrodite and a *Koerneria* female at different developmental stages. Anterior is to the left. P(5-8).p are shown for both species, demonstrating the different morphological appearance of the nucleus of P8.p with respect to the more anterior cells. (A) Late L1 stage of *Pristionchus*. The nucleus of P8.p is smaller than the nucleus of P(5-7).p. (B) L3 stage of *Koerneria*. The AC is already formed. The nucleus of P8.p is very close to the nucleus of P7.p (the nucleus of P8.p in *Pristionchus* is always far more posterior) and is morphologically similar. P5.p is not clearly visible in this plane of focus. Scale bar is 20 μm.

four, P(5-8).p, located in the central body region, and one, P12.p, in the posterior region (Fig. 1C). As in *Caenorhabditis*, P12.p divides during the L1 stage to form hyp12 (Fig. 1C) (Sommer and Sternberg, 1996a). The programmed cell death of P(1-4,9-11).p limits the number of potential VPCs to the four central epidermal cells.

Cell ablation studies have indicated that the developmental potential of P(5-8).p is non-equivalent. After ablation of individual epidermal cells, only P5.p and P6.p can adopt the 1° fate (Table 2C-E) (Sommer and Sternberg, 1996a). In contrast, an isolated P7.p generated 2°, 3° or hybrid fates, indicating an intrinsic difference of P7.p (Table 2F) (Sommer and Sternberg, 1996a). However, after ablation of P(5,6).p, the migration of P7.p towards the AC is delayed, so that one cannot rule out the possibility that the limited competence of P7.p is due to its distance from the AC at the time of induction. Vulval development in *Pristionchus* depends on a gonadal signal. After ablation of Z(1,4), the precursors of the somatic gonad, no vulva formed and all cells adopted the 3° cell fate (Fig. 3A) (Table 2B).

Ablation experiments indicated that P8.p is incompetent to adopt a vulval fate. After ablation of P7.p in the mid or late L1 stage, P8.p fused with the epidermis without further cell divisions (Table 2E) (Sommer and Sternberg, 1996a). These results are consistent with morphological differences among the ventral epidermal cells. As soon as the epidermal cells enlarge in the L1 stage, the nucleus of P8.p is morphologically distinct and smaller than the nucleus of P(5-7).p (Fig. 2A).

To test if the competence of P8.p is already determined early in the L1 stage, the cell ablation experiments were performed as early as possible after hatching. The cell division pattern of the neuroblasts P6.a and P7.a were used as landmarks to stage larval animals. If P7.p was ablated when P7.a or P7.aa were present, P8.p adopted the 2° cell fate in 3 of 6 animals (Table 2D). In the other 3 animals, P8.p fused with the epidermis. If P7.p was ablated at later stages, P8.p fused with the epidermis in 6 of 6 animals (Table 2E). Thus, the determination process of P8.p is ongoing at hatching. Until approximately 5 hours after hatching the wild-type fate of P8.p can be altered. Interestingly, if P(5-7).p were ablated in the early L1 stage, P8.p fused with the hypodermis in all 22 ablated animals (Table 2G).

To test if also the intrinsic difference of P7.p is determined during the same time period, the corresponding ablation experiments were repeated at earlier stages. After ablation of P(5,6).p early after hatching, P7.p adopted the 2° or the hybrid fate in 8 of 8 animals, as in the original ablation experiments (Table 2F). This result suggests that the molecular events leading to the different cell fate specification of P8.p and P7.p are uncoupled in the early L1 stage.

Vulval cell lineage conservation

The comparison of vulva development was initiated by cell lineage analysis in *Diplogaster*, *Koerneria*, *Diplogasteroides*, *Pseudodiplogasteroides*, *Aduncospiculum* and *Goodeyus*. As in *Pristionchus*, only five ventral epidermal cells are present after hatching in all analyzed species. These five cells are P(5-8).p and P12.p, respectively (Fig. 1C). The four anterior cells, P(1-4).p, and the three posterior cells, P(9-11).p, are absent in L1 animals of all species. Embryonic cell lineages were determined in *Diplogaster*, *Goodeyus* and *Pseudodiplogasteroides* and the programmed cell death of these cells was observed.

The vulva of all analyzed species is formed in the central body region by P(5-7).p (Fig. 1D). Cell lineages of all three cells are highly conserved among these species. In four species (*Diplogaster*, *Koerneria*, *Aduncospiculum* and *Goodeyus*), P6.p generates six progeny and P5.p and P7.p generate seven progeny each. In *Pseudodiplogasteroides* the division of

Table 3. Cell ablation experiments in *Diplogaster maupasi*

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	S	
B	Z(1,4)$^-$		3°/D	3°/D	3°/D	S	8/9*
	Z(1,4)$^-$		3°	3°	3°	S	1/9
C	P7.p$^-$	early	2°	1°	X	2°	5/7
	P7.p$^-$	early	2°	1°	X	S	2/7
	P7.p$^-$	late	2°	1°	X	S	6/6
D	P(5,6,8).p$^-$	late	X	X	2°/hybrid	X	3/3
	P(5,6).p$^-$	late	X	X	2°/hybrid	S	4/4
E	P(6-8).p$^-$		1°	X	X	X	2/2
	P(6,7).p$^-$		1°	X	X	S	1/1
F	P(5,7,8).p$^-$		X	1°	X	X	3/3

*Variable, only one or two VPCs were induced after ablation, see text for details.

For nomenclature see Materials and Methods. D, cells, which differentiated and formed vulva-like structures in the absence of gonadal signal. Each line in the table represents one type of experiment. After ablation of the gonad primordium all three VPCs can differentiate to form an irregular vulva-like structure or they can adopt the 3° fate (B). P7.p is limited in its developmental potential and can only adopt the 2° fate (D), whereas P5.p and P6.p are multipotent and can adopt the 1° fate (E,F).

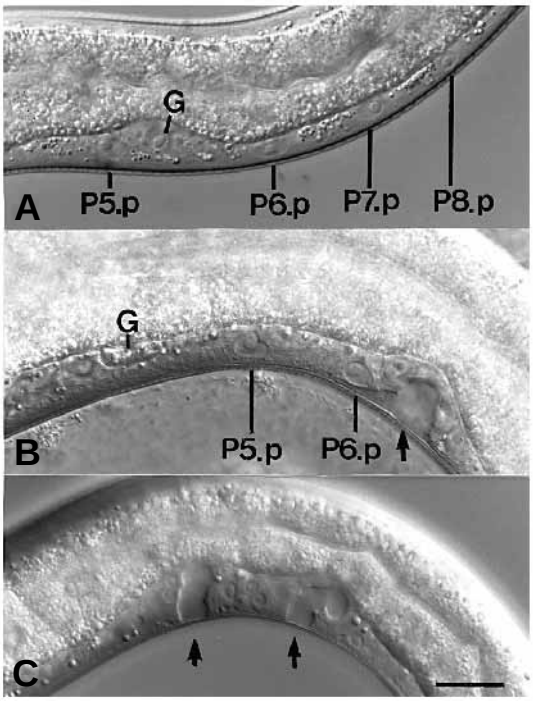

Fig. 3. Nomarski photomicrographs of Z(1,4)-ablated animals of *Pristionchus* (A), *Diplogaster* (B) and *Goodeyus* (C). (A) Early-L4 stage of a *Pristionchus* hermaphrodite. No cell divisions of the VPCs have occured. G, Germ-line nuclei (Z2 and Z3) that do survive but do not divide after ablation of Z(1,4) in some animals. (B) Mid-L4 stage of a *Diplogaster* hermaphrodite. No uterus and no other gonadal derivatives are formed. G, debris of gonad ablation in the central body region. A vulva-like structure is formed by the progeny of P7.p (arrow). Most of the seven progeny formed by P7.p are not visible in this plane of focus. P5.p and P6.p adopted the 3° fate. P8.p is not visible. In this animal, the VPCs have migrated to a more posterior position, a behavior frequently seen after gonad ablation. (C) Mid-L4 stage of a *Goodeyus* female. No uterus and no other gonadal derivatives are formed. Two vulva-like structures are formed by the progeny of P5.p and P6.p (arrows). 14 nuclei were formed by P(5,6).p, some of which are visible in this plane of focus. VPCs have migrated to a more posterior position. P7.p and P8.p are not visible in this plane of focus. Scale bar is 20 μm.

P5.paa and P7.ppp is variable, so that animals with six or seven progeny from P5.p and P7.p were observed. The only consistent alteration to this highly conserved lineage pattern exists in *Diplogasteroides*, where P5.paa and P7.ppp do not divide so that only six progeny are formed by P5.p and P7.p (Fig. 1D). P8.p does not divide in any of the analyzed species. Thus, the cellular composition and the differentiation pattern of ventral epidermal cells, including the cells that give rise to the formation of the vulva, are highly conserved in all members of the Diplogastridae studied. Therefore, the following sections on individual species will focus on cell ablation experiments that give information on the competence of individual ventral epidermal cells.

Diplogaster maupasi

Based on morphological studies, *Diplogaster* is one of the closest relatives to *Pristionchus* of all species studied in this report (W. Sudhaus, personal communication). Nonetheless, a large difference between *Pristionchus* and *Diplogaster* was observed after ablation of the gonad. If Z(1,4) were ablated in *Diplogaster*, no gonadal structures were present but individual VPCs formed hybrid lineages in 8 of 9 animals (Table 3B; Fig. 3B). Without an AC, the structures formed were highly irregular. In 4 of 8 animals, one VPC differentiated, in the other 4 animals, two VPCs differentiated. In the latter, descendants of each VPC formed independent invaginations. Thus, although cell divisions and differentiation of VPCs was observed in the absence of the gonad, the organization of vulva morphogenesis in wild type is dependent on the AC. In contrast to this 'partial gonad-independent' differentiation in *Diplogaster*, no differentiation was observed in *Pristionchus* after the ablation of Z(1,4) (Fig. 3A; Table 2).

Cell ablation of individual VPCs gives a pattern of cell fates that is identical to *Pristionchus*. P8.p can adopt vulval fate only after VPC ablation early in the L1 stage. If P7.p was ablated early in development, P8.p adopted the 2° fate in 5 of 7 animals (Table 3C). If P7.p was ablated later on, P8.p fused with the hypodermis in 6 of 6 animals (Table 3C). P7.p is limited in its developmental potential. After ablation of P(5,6).p or P(5,6,8).p, P7.p generated only hybrid or 2° fates in 7 of 7 animals (Table 3D). However, a single P5.p after ablation of P(6-8).p (Table 3E) or a single P6.p after ablation of P(5,7,8).p, could adopt the 1° fate (Table 3F).

Koerneria sp.

Vulval development in *Koerneria* depends on a gonadal signal. After ablation of Z(1,4), no vulva formed and all cells adopted the 3° cell fate (Table 4B). Further cell ablation experiments in *Koerneria* revealed differences in cell competence of ventral epidermal cells with respect to *Pristionchus*. After ablation of P7.p, P8.p adopted the 2° fate and formed part of the vulva in 3 of 4 animals (Table 4C). After ablation of P(5,6).p, P8.p was induced in 6 of 7 animals (Table 4D). In both experiments, cells were ablated in the mid or late L1 stage, indicating that the competence of P8.p is not limited to an early time period.

Table 4. Cell ablation experiments in *Koerneria* sp.

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	3°	
B	Z(1,4)$^-$		3°	3°	3°	3°	3/3
C	P7.p$^-$	late	2°	1°	X	2°	3/4
	P7.p$^-$	late	2°	1°	X	3°	1/4
D	P(5,6).p$^-$	late	X	X	1°	2°	4/7
	P(5,6).p$^-$	late	X	X	hybrid	2°	1/7
	P(5,6).p$^-$	late	X	X	hybrid	hybrid	1/7
	P(5,6).p$^-$	late	X	X	1°	3°	1/7
E	P(6,7).p$^-$	late	1°	X	X	3°	2/2
F	P(5-7).p$^-$	late	X	X	X	3°	7/7

For nomenclature see Materials and Methods. Each line in the table represents one type of experiment. Vulval development is gonad dependent, because after ablation of the gonad primordium all VPCs have the 3° fate (B). P8.p is competent to adopt vulval fate after the ablation of individual VPCs (C,D). P7.p can adopt the 1° fate (D), as can P5.p (E).

Table 5. Cell ablation experiments in *Diplogasteroides* sp.

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	3°	
B	Z(1,4)$^-$		3°	3°	3°	3°	4/4
C	P6.p$^-$	late	1°	X	2°	3°	2/3
	P6.p$^-$	late	2°	X	2°	3°	1/3
D	P7.p$^-$	early	2°	1°	X	2°	5/6
	P7.p$^-$	early	2°	1°	X	3°	1/6
	P7.p$^-$	late	2°	1°	X	2°	4/6
	P7.p$^-$	late	2°	1°	X	3°	2/6
E	P(5,6).p$^-$	late	X	X	1°	2°	2/4
	P(5,6).p$^-$	late	X	X	1°	3°	2/4
F	P(5-7).p$^-$	late	X	X	X	3°	3/3

For nomenclature see Materials and Methods. Each line in the table represents one type of experiment. Vulval development is gonad dependent, because after ablation of the gonad primordium all VPCs adopt the 3° fate (B). P8.p is competent to adopt vulval fate after ablation of individual VPCs (C,D). P7.p can adopt the 1° fate (E), as can P5.p (C).

Table 6. Cell ablation experiments in *Pseudodiplogasteroides* sp.

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	S	
B	Z(1,4)$^-$		3°	3°	3°	S	6/6
C	P7.p$^-$	early	2°	1°	X	S	7/11
	P7.p$^-$	early	3°	1°	X	S	1/11
	P7.p$^-$	early	2°	1°	X	2°	3/11
	P7.p$^-$	late	2°	1°	X	S	9/9
D	P(5,6).p$^-$	early	X	X	1°	S	7/7
E	P6.p$^-$	early	1°	X	2°	S	3/4
	P6.p$^-$	early	hybrid	X	2°	S	1/4

For nomenclature see Materials and Methods. Each line in the table represents one type of experiment. Vulval development is gonad dependent, because after ablation of the gonad primordium all VPCs adopt the 3° fate (B). P8.p is competent to adopt a vulval fate only after ablation of P7.p in the first few hours of larval development (C). P7.p can adopt the 1° fate (D), as can P5.p (E).

The developmental potential of P8.p in *Koerneria* resembles that of P8.p in *Caenorhabditis*. Therefore, the wild-type fate of P8.p should be designated 3°, in agreement with the nomenclature of *Caenorhabditis* (Sulston and White, 1980; Sternberg and Horvitz, 1986). This result is consistent with the observation that P8.p appears morphologically similar to the other VPCs before induction occurs (Fig. 2B). Also, the nucleus of P8.p in *Koerneria* has a more anterior position than the nucleus of P8.p in *Pristionchus* (Fig. 2).

The competence of P7.p in *Koerneria* also differs with respect to P7.p in *Pristionchus*. After ablation of P(5,6).p, P7.p adopted the 1° fate in 5 of 7 animals and the hybrid fate in 2 of 7 animals (Table 4D). After ablation of P(6,7).p, P5.p also adopted the 1° fate (Table 4E). This results suggests that all VPCs have the same developmental potential, as P(5-7).p can adopt the 1° fate. It remains unclear, however, if P8.p can adopt 1° fate. After ablation of P(5-7).p, P8.p adopted the 3° fate in all 7 of 7 animals (Table 4F). One possible interpretation is that P8.p might be too far away from the source of the inductive signal when induction occurs. A similar situation has been observed in *Pelodera* of the Rhabditidae (Sommer and Sternberg, 1995).

Diplogasteroides sp.

Vulval development in *Diplogasteroides* is induced by a gonadal signal. After ablation of Z(1,4), the ventral epidermal cells do not divide and no vulva is formed (Table 5B). The competence pattern of individual epidermal cells in *Diplogasteroides* resembles that observed in *Koerneria*. P8.p can adopt a vulval fate. After ablation of P7.p early or late in the L1 stage, P8.p adopted the 2° fate in 9 of 12 animals (Table 5D). Therefore, the fate of P8.p in wild-type animals can be defined as 3°. After individual VPC ablation, P7.p and P5.p could adopt the 1° fate (Table 5C and E).

Pseudodiplogasteroides sp.

According to both taxonomies (Goodey, 1963; Andrassy, 1984), *Pseudodiplogasteroides* is only distantly related to most other species studied. Vulval development in *Pseudodiplogasteroides* is induced by the gonad, as no vulva was formed after the ablation of Z(1,4) (Table 6B). Further ablation experiments indicate that cell competence of P7.p and P8.p has evolved independently from one another with respect to *Pristionchus*. After ablation of P7.p immediately after hatching, P8.p was induced in 3 of 11 animals (Table 6C). If P7.p was ablated at later stages, P8.p fused with the epidermis in 9 of 9 animals (Table 6C). This result suggests that P8.p might become incompetent during the same time period as was observed in *Pristionchus*. However, after ablation of P(5,6).p in the early L1 stage, P7.p adopted the 1° fate in 7 of 7 animals (Table 6D). As P5.p adopted the 1° fate after ablation of P6.p (Table 6E), all three VPCs of *Pseudodiplogasteroides* can adopt the 1° fate. These results indicate that evolutionary changes in the cell fate specification mechanisms of P7.p and P8.p can occur independently. The developmental potential of P8.p is similar between *Pristionchus* and *Pseudodiplogasteroides*, whereas the developmental potential of P7.p differs between species.

Aduncospiculum halicti

Cell ablation experiments in *Aduncospiculum* could only be carried out in the late L1 or L2 stage, as epidermal cells are

Table 7. Cell ablation experiments in *Aduncospiculum halicti*

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	S	
B	Z(1,4)⁻		3°	3°	3°	S	5/5
C	P7.p⁻	late	2°	1°	X	S	4/4
D	P(5,6).p⁻	late	X	X	1°	S	4/5
	P(5,6).p⁻	late	X	X	hybrid	S	1/5
E	P6.p⁻	late	1°	X	2°	S	3/4
	P6.p⁻	late	hybrid	X	2°	S	1/4

For nomenclature see Materials and Methods. Each line in the table represents one type of experiment. Note, that cell ablation experiments in this species could only be carried out in the early L2 stage as cells are indistinguishable from surrounding neurons prior to this time. Vulval development is gonad dependent because after ablation of the gonad primordium, all VPCs adopt the 3° fate (B). P8.p is incompetent to adopt a vulval fate after ablation of individual VPCs (C,D). P7.p can adopt the 1° fate (D), as can P5.p (E).

Table 8. Cell ablation experiments in *Goodeyus ulmi*

			P5.p	P6.p	P7.p	P8.p	
A	Wild type		2°	1°	2°	S	
B	Z(1,4)⁻		D	D	3°	S	6/6
C	P7.p⁻	early	2°	1°	X	2°	5/9
	P7.p⁻	early	2°	1°	X	S	3/9
	P7.p⁻	early	2°	1°	X	hybrid	1/9
	P7.p⁻	late	2°	1°	X	S	3/3
D	P(5,6).p⁻	late	X	X	2°/hybrid	S	5/6
	P(5,6).p⁻	late	X	X	3°	S	1/6
E	P6.p⁻	late	1°	X	2°	S	2/3
	P6.p⁻	late	2°	X	2°	S	1/3
F	P(5-7).p⁻	early	X	X	X	S	4/4
	P(5-7).p⁻	late	X	X	X	S	2/2

For nomenclature see Materials and Methods. D, cells that differentiated and formed vulva-like structures in the absence of gonadal signal. Each line in the table represents one type of experiment. After ablation of the gonad primordium P5.p and P6.p can differentiate to form an irregular vulva-like structure, whereas P7.p adopts the 3° fate (B). P8.p is competent to adopt a vulval fate only after ablation of P7.p in the first few hours of larval development (C). P7.p is limited in its developmental potential and can only adopt the 2° fate (D), whereas P5.p is multipotent and can adopt the 1° fate (E).

indistinguishable from the surrounding differentiated neurons prior to this time. With this limitation, ablation experiments give results similar to those described for *Pseudodiplogasteroides* (Tables 6, 7). The vulva is induced by the gonad: after ablation of Z(1,4), all VPCs adopted the 3° fate (Table 7B). After ablation of P7.p, P8.p never adopted a vulval fate (Table 7C). P5.p and P7.p can adopt the 1° fate after P6.p and P(5,6).p ablation (Table 7D and E).

Goodeyus ulmi

Goodeyus belongs to a separate phylogenetic taxon, and might be as distantly related to most other analyzed species as *Pseudodiplogasteroides*. A new pattern was observed after ablation of the somatic gonad primordium (Table 8B). P7.p and P8.p fused with the hypodermis, whereas the two anterior cells P(5,6).p differentiated in an irregular fashion (Fig. 3C). As in *Diplogaster*, the lineages formed were that of 2° or hybrid fates, but in *Goodeyus* only the two anterior cells P(5,6).p showed this behavior.

The competence of individual epidermal cells resembles that of *Pristionchus* in many respects. After ablation of P7.p early in the L1 stage, P8.p adopted the 2° fate in 5 of 9 animals (Table 8C). In contrast, if P7.p was ablated later on, P8.p did not form part of the vulva in 3 of 3 animals (Table 8C). After ablation of P(5-7).p, an isolated P8.p fused with the hypodermis (Table 8F). P7.p is limited in its developmental potential. After ablation of P(5,6).p, P7.p adopted the 2° or hybrid cell fate in 5 of 6 animals and the 3° fate in 1 of 6 animals (Table 8D), as described for *Pristionchus* and *Diplogaster*. P5.p adopted the 1° fate after ablation of P6.p (Table 8E).

DISCUSSION

I have analyzed vulval development in members of seven different genera of the family Diplogastridae. In all analyzed species, the differentiation pattern of ventral epidermal cells, including those cells that form the vulva, are highly conserved. Despite this morphological conservation, cell ablation experiments indicate alterations of underlying mechanisms of cell fate specification. The competence pattern of P7.p and P8.p differs among species. The pattern observed in *Pristionchus* (limited competence of P7.p; competence of P8.p restricted to an early time period) was only seen in *Diplogaster* and *Goodeyus* (Tables 3, 8). However, in these two species, some VPCs are able to differentiate in the absence of a gonadal signal.

The observed differences of cell competence among species could be due to minor changes in cell position. However, this seems unlikely as the nuclei of P(5-8).p are located in similar positions with respect to the developing gonad and other morphological structures in all analyzed species. In addition, adjacent epidermal cells are separated from one another by at least five differentiated neurons during early larval stages. Therefore, it seems more likely that the observed differences are due to changes in developmental mechanisms other than cell position.

Table 9 summarizes the developmental potential and cell competence of the six central epidermal cells P(3-8).p in a comparison with *Caenorhabditis*. Based on the limited phylogenetic understanding of the Diplogastridae, it is impossible to draw conclusions as to the plesiomorphy or apomorphy of individual characters. However, the presented data indicate that the competence of P7.p and P8.p can evolve partially independent within the Diplogastridae. In *Pseudodiplogasteroides*, P7.p can adopt the 1° fate which is different from *Pristionchus*, whereas P8.p is only competent to adopt vulval fate during an early time period, resembling the situation seen in *Pristionchus*. Results in *Aduncospiculum* are similar but remain restricted based to the fact that ablation experiments can just be performed at late stages. I draw five conclusions with respect to developmental cell fate specification of ventral epidermal cells in this family.

(1) Conserved differentiation pattern of ventral epidermal cells

The differentiation pattern of all 12 ventral epidermal cells is highly conserved among all species studied. Thus, no mor-

Table 9. Comparison of pattern characteristics of the analyzed Diplogastridae to *Caenorhabditis*

Species	Ind.	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
Caenorhabditis elegans	+	3°/2°/1°	3°/2°/1°	2°/1°	1°	2°/1°	3°/2°/1°
Pristionchus pacificus	+	PCD	PCD	2°/1°	1°	2°/hybrid	S
Diplogaster maupasi	(–)	PCD	PCD	2°/1°	1°	2°/hybrid	S
Pseudodiplogasteroides sp.	+	PCD	PCD	2°/1°	1°	**2°/1°**	S
Aduncospiculum halicti	+	PCD	PCD	2°/1°	1°	**2°/1°**	S
Koerneria sp.	+	PCD	PCD	2°/1°	1°	**2°/1°**	**3°/2°**
Goodeyus ulmi	(–)	PCD	PCD	2°/1°	1°	2°/hybrid	S
Diplogasteroides sp.	+	PCD	PCD	2°/1°	1°	**2°/1°**	**3°/2°**

PCD = programmed cell death.

Summary of numerical aspects of vulval pattern formation in the analyzed species in comparison to *Caenorhabditis elegans.* Data are from species described in this paper plus *Caenorhabditis* (Sulston and White, 1980; Sternberg and Horvitz, 1986). The observed fates in wild type and after cell ablation are given. Ind, gonad dependence of differentiation properties: +, no differentiation after ablation of gonad precursors; (–), partial gonad independent differentiation of VPCs. Changes of individual species with respect to *Pristionchus* are indicated in bold.

phological alterations at the cellular level were introduced during the evolution of many branches of the Diplogastridae. This result differs from the observations made by comparing vulva development among the Rhabditidae, the family that *C. elegans* belongs to. Within the Rhabditidae, analysis of cell lineage and pattern formation in members of several genera indicated changes at many levels (Sommer and Sternberg, 1995). The vulva is formed by P(5-7).p in all rhabditid species studied; P6.p adopts the 1° fate and P(5,7).p adopt the 2° fate. However, the cells with the 2° fate generate between four and seven progeny in a species-specific manner. The 3° lineages of P(4,8).p vary between two and six progeny in different species. Thus, the type and pattern of evolutionary alterations of vulval development differs between families of free-living nematodes.

(2) Ancestry of programmed cell death

Only five ventral epidermal cells are present in larval stages of all species of the Diplogastridae studied to date. In all species where embryonic cell lineages were analyzed, the programmed cell death of P(1-4).p and P(9-11).p was observed. These results indicate, that the pattern of five ventral epidermal cells might be ancestral within the Diplogastridae. This differs from the Rhabditidae, where the 'five-cell pattern' was observed in the genus *Poikilolaimus* only and is thought to be a derived character (Sommer and Sternberg, 1996a,b). As long as the phylogenetic relationship between the Rhabditidae and the Diplogastridae is unresolved, it remains open as to whether the twelve- or the five-cell pattern is ancestral. However, it was speculated that the cell fate pattern observed in *Pristionchus* might be derived from the one in *Caenorhabditis* (Sommer and Sternberg, 1996a).

(3) P7.p competence differs among species

Of the three evolutionary changes of cell fate specification observed between *Caenorhabditis* and *Pristionchus*, the competence of P7.p is the most variable character. In most species of the Diplogastridae, P7.p can adopt the 1° fate after cell ablation experiments. In *Pristionchus, Diplogaster* and *Goodeyus*, however, the developmental potential of P7.p is limited, as it can only adopt the 2° fate. *Goodeyus* seems to be phylogenetically unrelated to *Pristionchus* and *Diplogaster*. Therefore, one could speculate that independent evolutionary changes of P7.p fate specification have occurred within the family.

(4) Competence of P8.p differs among species

P8.p is a member of the vulval equivalence group in *Caenorhabditis*. In contrast, P8.p is incompetent to adopt a vulval fate in *Pristionchus*, except for an early time period immediately after hatching. In most analyzed species, P8.p is incompetent to replace P7.p as in *Pristionchus*. However, in *Koerneria* and *Diplogasteroides*, P8.p is competent to adopt a vulval fate, resembling the situation in *Caenorhabditis*. As for P7.p, one could speculate that independent evolutionary changes of P8.p fate specification have occurred within the family.

The evolutionary change in P8.p fate specification could be considered a case of heterochrony in cell fusion. By changing the timing of this developmental decision, a different competence pattern is created. An unfused P8.p cell has the developmental competence to participate in organ differentiation later in development.

(5) Gonad dependence versus gonad independence

The most striking evolutionary alteration was observed in the 'partial' gonad independence of VPC differentiation (Fig. 3). In *Diplogaster*, all three VPCs can differentiate in the absence of a gonadal signal. However, not all cells do so in every ablated animal and, if more than one cell differentiates in the same animal, independent invaginations are formed by individual precursor cells. Thus, the produced structures are highly irregular and do not resemble functional vulvae. In *Goodeyus*, gonad-independent differentiation was only seen for P5.p and P6.p.

Although the formed structures are irregular in form in both species, the general phenomenon of gonad-independent differentiation could be of evolutionary importance. In *Mesorhabditis* and *Teratorhabditis* of the Rhabditidae, the vulva forms in the posterior body region and is AC independent (Sommer and Sternberg, 1994). In these species, the VPCs migrate posteriorly from the central body region during the L2 stage and start vulva differentiation before the AC reaches the differentiating VPCs. Altogether, three changes occur in *Mesorhabditis*, VPC migration, delayed AC contact to VPCs and gonad-independent vulva differentiation. Which of these changes is the evolutionary original alteration? The finding of 'partial' gonad-independent differentiation of individual VPCs in *Diplogaster* and *Goodeyus* suggests that gonad independence might be a prerequirement for the subsequent formation of a posterior vulva.

Changes of developmental mechanisms in the absence of cell lineage alterations

The presented data for the Diplogastridae indicate that developmental mechanisms and differentiation patterns can evolve differently during the evolution of the nematode vulva. Developmental mechanisms, some of which are redundantly involved in vulval fate specification in *Caenorhabditis*, might be able to evolve without concomitant morphological change. The potential uncoupling of morphological and molecular evolution is known for a long time. King and Wilson (1975) described 'evolution at two levels' in humans and chimpanzees, arguing that based on the similarity of macromolecules in the two taxa, only regulatory mutations could account for their biological (morphological) differences.

Evolution, considered at the morphological level, does not appear to occur at a constant rate. The recognition of stasis as an important pattern within the history of species, caused Eldredge and Gould (1972) to propose 'punctuated equilibrium' as a meaningful addition to evolutionary theory. Change, if it occurs, is rapid. In the special case of vulval development, the observed uncoupling of the developmental mechanisms and the differentiation patterns resembles the situation seen during evolutionary stasis. Changes of developmental mechanisms underlying vulval development can accumulate without immediate cellular and morphological consequences. Only later, the introduced variation might allow evolutionary novelty. As diversity in developmental mechanisms is morphologically invisible, an evolutionary scenario like the one described would have a punctuated appearance at the macroscopic level. Thus, novelty in evolution might arise in a punctuated fashion after substantial accumulation of molecular changes.

A major task is to understand the genetic changes that underlie such alterations and the evolutionary mechanisms of fixation of those changes in individual evolutionary lineages. At the genetic level, changes might be tolerated most efficiently in those aspects of development that are redundantly specified by alternative signaling mechanisms, so that changes are initially neutral. Without a selective advantage at the macroscopic level, genetic drift (Kimura, 1979) or molecular drive (Dover, 1986) could equally account as evolutionary mechanisms of fixation. However, the latter is unlikely as it applies mainly to changes in the structure and function of multigene families.

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