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Novel cell-cell interactions during vulva development in *Pristionchus pacificus*

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SUMMARY

Vulva development differs between *Caenorhabditis elegans* and *Pristionchus pacificus* in several ways. Seven of 12 ventral epidermal cells in *P. pacificus* die of apoptosis, whereas homologous cells in *C. elegans* fuse with the hypodermal syncytium. Vulva induction is a one-step process in *C. elegans*, but requires a continuous interaction between the gonad and the epidermis in *P. pacificus*. Here we describe several novel cell-cell interactions in *P. pacificus*, focusing on the vulva precursor cell P8.p and the mesoblast M. P8.p in *P. pacificus*, unlike its homologous cell in *C. elegans*, is incompetent to respond to gonadal signaling in the absence of other vulva precursor cells, but can respond to lateral signaling from a neighboring vulval precursor. P8.p provides an inhibitory signal that determines the developmental competence of P(5,7).p. This lateral inhibition acts via the mesoblast M and is regulated by the homeotic gene *Ppa-mab-5*. In *Ppa-mab-5* mutants, M is misspecified and provides inductive signaling to the vulval precursor cells, including P8.p. Taken together, vulva development in *P. pacificus* displays novel cell-cell interactions involving the mesoblast M and P8.p. In particular, P8.p represents a new ventral epidermal cell type, which is characterized by novel interactions and a specific response to gonadal signaling.

Key words: *Pristionchus pacificus*, *Caenorhabditis elegans*, Cell-cell interactions, Lateral inhibition, Vulva, *Ppa-mab-5*

INTRODUCTION

Cell-cell interactions are crucial for the development of multicellular organisms. In nematodes such as *Caenorhabditis elegans*, the invariant cell lineage allows cell-cell interactions to be studied at the single-cell level. One well-known example is the interaction between the epidermis and the gonadal anchor cell (AC) during the development of the vulva, the egg-laying structure of nematode females and hermaphrodites. Vulva formation in *C. elegans* has been studied in detail, providing insight into the molecular aspects of signaling pathways during development (for review see Kornfeld, 1997).

Based on the genetic and molecular understanding of *C. elegans*, vulva formation can serve as a model system for the evolutionary analysis of developmental processes. Over the years, vulva development was compared at the cellular level among more than 50 nematode species representing six different families (for review see Sommer, 2000; Félix, 1999). One such species, *Pristionchus pacificus* of the Diplogastridae, has been described as a 'satellite' organism for functional comparative studies, and several cellular, genetic and molecular differences were observed during vulva formation between *P. pacificus* and *C. elegans* (Sommer and Sternberg, 1996; Eizinger et al., 1999).

In general, the nematode vulva is a derivative of the ventral epidermis, consisting of 12 precursor cells that are denoted P(1-12).p from anterior to posterior (Fig. 1A) (Sulston and Horvitz, 1977). In *C. elegans*, each precursor cell adopts one of five different cell fates in a region-specific manner. The non-vulval cells P(1,2,9-11).p in the anterior and posterior body region fuse with the hypodermal syncytium *hyp7* (Fig. 1A). The six central cells, P(3-8).p, are the vulval precursor cells (VPC) and form the 'vulva equivalence group', since all cells have the competence to participate in vulva formation. In wild type, only the three central cells, P(5-7).p, are recruited to form vulval tissue after receiving an inductive signal from the AC (Fig. 1B) (Kimble, 1981). In total, P(3-8).p adopt one of three alternative cell fates. P6.p has a 1° fate and generates eight progeny, some of which contact the AC directly. P(5,7).p have a 2° fate and generate seven progeny each, thereby forming the anterior and posterior part of the structure, respectively. P(3,4,8).p have a 3° fate, divide once and remain epidermal.

It is now understood that at least four different signaling systems are involved in vulva formation, some of which act in a redundant manner. The inductive signal from the AC is a secreted epidermal growth factor-like molecule encoded by the gene *lin-3* (Fig. 1B) (Hill and Sternberg, 1992). This signal is transmitted in P(5-7).p via an EGFR/RAS/MAPK signaling pathway. Work by Katz et al. (1995) indicated that LIN-3 can act as a gradient: under experimental conditions, high levels of LIN-3 induce a 1° fate, intermediate levels induce predominantly a 2° fate whereas low levels result in 3° fates (Katz et al., 1995).

In the intact animal, however, cell-cell interactions among the VPCs are also involved in cell fate specification (Fig. 1B).

Using mosaic analysis, Simske and Kim (1995) showed that P5.p and P7.p can adopt a 2° fate in animals in which P6.p, but not P(5,7).p, express the EGF-receptor *let-23*. Thus, correct vulval patterning is observed in the absence of a functional EGF gradient. P6.p, after receiving a sufficient amount of inductive signal, will signal its two neighbors via the *lin-12*/Notch pathway to adopt a 2° fate (Simske and Kim, 1995). These results indicate that the EGF/RAS/MAPK and the *lin-12/Notch* pathways act in a redundant fashion. The interactions among the VPCs represent an example of 'lateral signaling'.

In addition to inductive and lateral signaling, two redundant negative signaling pathways are involved in vulva formation. Genes within these pathways antagonize EGF/RAS/MAPK signaling and prevent inappropriate vulva differentiation (Fig. 1B). Mutants defective in the negative signaling system display ectopic vulva differentiation by P(3,4,8).p and have a 'multivulva' phenotype. The cloning of two such genes, *lin-35* and *lin-53*, revealed that they encode proteins similar to retinoblastoma (Rb) and its binding protein RbAp48 (Lu and Horvitz, 1998). Therefore, it has been proposed that a protein complex similar to the Rb complex in vertebrates, represses the transcription of genes promoting vulva development.

The final pathway involved in vulva formation in *C. elegans* is the Wnt-pathway. *bar-1*, which encodes a β-catenin/Armadillo-like protein, is required for the activation of Hox genes such as *lin-39* (Eisenmann et al., 1998). LIN-39 specifies the vulva equivalence group early in development and has a second function later in development when it transmits the EGF/RAS/MAPK signal (Maloof and Kenyon, 1998).

Vulva development in *P. pacificus* differs from *C. elegans* in several ways (Fig. 1A). Seven of the twelve ventral epidermal cells die of programmed cell death during late embryogenesis (Sommer and Sternberg, 1996). Among the four surviving cells P(5-8).p in the central body region, P(5-7).p form the vulva with a 2°-1°-2° pattern, similar to *C. elegans* (Fig. 1A). Vulva induction in *P. pacificus* relies on a continuous interaction between the gonad and the underlying epidermis. Induction starts several hours after hatching and continues until the birth of the AC (Sigrist and Sommer, 1999). The homeotic transcription factor *Ppa-lin-39* is involved in the regulation of programmed cell death in the ventral epidermis. In *Ppa-lin-39* mutants, P(5-8).p die of apoptosis, indicating that *Ppa*-LIN-39 provides positional information to the central body region (Eizinger and Sommer, 1997). In contrast to *C. elegans*, however, *Ppa-lin-39* is not used during vulval induction (Sommer et al., 1998).

Here we describe cell ablation experiments in *P. pacificus* wild-type and mutant animals, indicating the existence of several novel cell-cell interactions during vulva development. In particular, the posteriormost VPC, P8.p, which does not form part of the wild-type vulva, provides lateral inhibitory and negative signals. Lateral inhibition by P8.p depends in turn, on an interaction with the mesoblast M. In *Ppa-mab-5* mutants, M is misspecified changing some of the cell-cell interactions during vulva formation. Together, the experiments suggest that P8.p in *P. pacificus* represents a novel cell type, with characters unknown in *C. elegans* or other nematodes.

MATERIALS AND METHODS

Strains and cultures

All experiments were carried out using the laboratory strain *P. pacificus* PS312, which is a derivative of a wild isolate from Pasadena, California, USA (Sommer et al., 1996). Worms are grown on *E. coli* OP50 as described elsewhere (Sommer et al., 1996). We use the J1-J3 nomenclature for larval stages according to Félix et al. (1999).

Cell ablation experiments

Cell ablation experiments were carried out using standard techniques described for *C. elegans* in Epstein and Shakes (1995) and using a 'Laser Science' dye laser of the type described by Avery and Horvitz (1987). Animals were picked into M9 buffer placed on a pad of 5% agar in water containing 10 mM sodium azide as anaesthetic. All ablation experiments were carried out 0-1 hour after hatching of the larvae (20°C). The cell ablation data are calculated as percentage frequency and were statistically tested using the χ^2-test.

Immunofluorescence

Immunofluorescence studies were performed by collecting newly hatched larvae over a 5 hour time interval and subsequent aging of these larvae to the particular time point of interest (20°C). Larvae were fixed and stained as described by Finney and Ruvkun (1990), and observed by immunofluorescence microscopy. Cy3-conjugated goat anti-mouse antibodies (Dianova) were used to detect MH27.

Cell lineage characters and cell fate terminology

The different cell fates of the VPCs are distinguished using the terminology 1°, 2°, 3° and 4° for cell fates, and T (transversal), L (longitudinal), N (non-dividing) and O (oblique) for cell division patterns (Sommer and Sternberg, 1995). During normal development, P6.p has the 1° fate and generates six progeny with the cell division pattern TNNT. The two 'N' cells (P6.pap and P6.ppa), which do not divide (in contrast to *C. elegans*), attach to the AC. P(5,7).p have a 2° fate and generate seven progeny each, with a cell division pattern LLLN (for P5.p). When the vulva invagination is formed, the N cell is in close contact to the AC, unlike in *C. elegans*. After ablation of other VPCs, an isolated 1° and an isolated 2° cell can be distinguished from one another by several cell lineage characteristics. In the intermediate 4-cell stage (after two rounds of cell divisions of a VPC) of a 1° cell, the AC moves between the two central cells P6.pap and P6.ppa. In the final 6-cell stage, the cells are located symmetrically around the AC. In the 4-cell stage of a 2° cell, the AC does not move between the central Pn.pxx cells. When the invagination is formed, the distribution of the seven progeny is asymmetric and variable with respect to the AC. VPCs that remain epidermal in the absence of vulva induction were designated as 3°. The fate of P8.p was designated as 4° based on the finding that this cell loses its competence to form vulval tissue during early larval development.

In addition to the 2° and the 1° fate, we use the fate designation 'D' in *P. pacificus*. 'D' refers to Pn.p cells that differentiate ectopically in mutants or to single Pn.p cells that differentiate after the removal of the somatic gonad (Sommer and Sternberg, 1996; Jungblut and Sommer, 1998; Sigrist and Sommer, 1999). Cells with a 'D'-fate have a 2°-type or hybrid lineage and generate between six and eight progeny.

RESULTS

P8.p fuses with the hypodermis in *Pristionchus pacificus*

The four central Pn.p cells in *P. pacificus*, P(5-8).p, adopt a cell fate pattern 2°-1°-2°-4° from anterior to posterior (Table 1A; Fig. 1A) (Sommer and Sternberg, 1996). P8.p was assigned a '4°' fate, because in contrast to *C. elegans*, *P. pacificus* P8.p loses its competence to form vulval tissue during larval development. P8.p could replace P7.p and had the 2° fate if P7.p was ablated during the first few hours after hatching

Table 1. Cell ablation experiments in *P. pacificus* wild-type animals

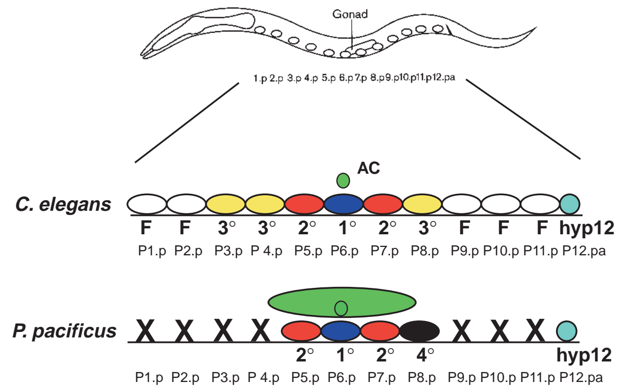
	Cells ablated	P5.p	P6.p	P7.p	P8.p	No. animals (Frequency)
A	Wild-type	2°	1°	2°	4°	many (100%)
B	P7.p early	2°	1°	–	2°	5/10 (50%)*
		2°	1°	–	4°	5/10 (50%)*
	P7.p late	2°	1°	–	4°	6/6 (100%)*
C	P(5-7).p	–	–	–	4°	22/22 (100)*
D	P(6,7).p	2°	–	–	4°	15/17 (88%)
		1°	–	–	4°	1/17 (6%)
		1°	–	–	2°	1/17 (6%)
E	P(6-8).p	1°	–	–	–	15/23 (65%)
		2°	–	–	–	8/23 (35%)
F	P(5,6).p	–	–	2°	4°	17/20 (85%)
		–	–	3°	4°	2/20 (10%)
		–	–	1°	4°	1/20 (5%)
G	P(5,6,8).p	–	–	1°	–	9/17 (53%)
		–	–	2°	–	7/17 (41%)
		–	–	3°	–	1/17 (6%)
H	P(5,7,8).p	–	1°	–	–	5/5 (100%)*
I	P(5,7).p	–	1°	–	2°	5/10 (50%)
		–	1°	–	4°	5/10 (50%)
J	P(6,8).p	2°	–	2°	–	10/20 (50%)
		1°	–	2°	–	7/20 (35%)
		2°	–	1°	–	2/20 (10%)
		1°	–	3°	–	1/20 (5%)
K	P6.p	2°	–	2°	4°	14/17 (82%)
		1°	–	2°	4°	1/17 (6%)
		2°	–	1°	4°	1/17 (6%)
		2°	–	1°	2°	1/17 (6%)
L	P(6,7).p, M	1°	–	–	4°	10/12 (84%)
		1°	–	–	2°	1/12 (8%)
		2°	–	–	4°	1/12 (8%)
M	P(5,6).p, M	–	–	1°	4°	4/8 (50%)
		–	–	2°	4°	4/8 (50%)
N	P(5-7).p, M	–	–	–	4°	9/9 (100%)
O	P8.p	2°	1°	2°	–	12/12 (100%)
P	M	2°	1°	2°	4°	6/6 (100%) (Egl)
Q	Z(1,4)	3°	3°	3°	4°	17/17 (100%)‡
R	Z1, Z4, M	3°	3°	3°	4°	20/21
		D	3°	3°	4°	1/21
S	Z1, Z4, P8.p	3°	3°	3°	–	11/17 (64%)
		3°	D	3°	–	3/17 (18%)
		D	3°	D	–	2/17 (12%)
		3°	D	D	–	1/17 (6%)
T	Z(1,4), M, P8.p	3°	3°	3°	–	16/22
		3°	3°	D	–	2/22
		3°	D	3°	–	3/22
		D	3°	3	–	1/22
U	Z1, Z4 (at 0-1 hours),	3°	3°	3°	–	9/10 (90%)
	P8.p (at 18 hours)	3°	D	3°	–	1/10 (10%)

Data from *Sommer and Sternberg (1996) and ‡Sigrist and Sommer (1999).

(Table 1B; Sommer, 1997). However, if P7.p was ablated later in development, P8.p remained epidermal (Table 1B; Sommer, 1997). In contrast, P8.p in *C. elegans* can replace other VPCs until vulva induction starts (Sternberg and Horvitz, 1986).

Additional experiments indicated that the ability of P8.p to adopt a vulval fate depends on the presence of other VPCs (Sommer, 1997). After ablation of P(5-7).p, the isolated P8.p had an epidermal fate (Table 1C). Again, this result is different from the one obtained in *C. elegans*, where an isolated P8.p had a vulval fate (Sternberg and Horvitz, 1986). Thus, P8.p in *P. pacificus* cannot respond to gonadal signaling as an isolated cell, but can form vulval tissue in response to 'lateral signaling' from P6.p.

A



B

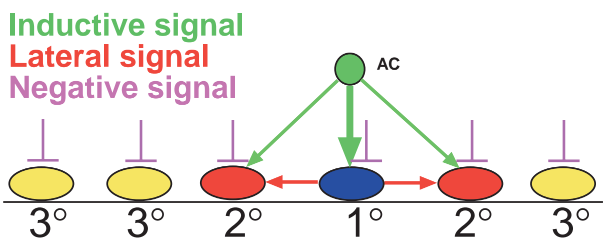


Fig. 1. Evolutionary variations in vulval cell fate specification between *C. elegans* and *P. pacificus* (A) and a schematic summary of vulva development in *C. elegans* (B). (A) During the L1 stage in *C. elegans*, the 12 ventral epidermal cells P(1-12).p are equally distributed between pharynx and rectum. P(1,2,9-11).p fuse with the hypodermal syncytium *hyp7* (F, white ovals). P(3-8).p form the vulva equivalence group and adopt one of three alternative cell fates. P6.p has a 1° fate (blue oval) and P(5,7).p have a 2° fate (red ovals). P(3,4,8).p have a 3° fate and remain epidermal (yellow ovals). The anchor cell (AC, green circle) provides an inductive signal for vulva formation. In *P. pacificus*, P(1-4,9-11).p die by programmed cell death. P6.p and P(5,7).p have a 1° and 2° fate, respectively. P8.p has a 4° fate (black oval) and remains epidermal. Vulva induction is provided by several cells of the somatic gonad (green ellipse). (B) Schematic summary of signaling interactions during vulva formation in *C. elegans*. An inductive EGF-like signal originates from the AC (green arrows). P6.p signals its neighbors to adopt a 2° fate via 'lateral signaling' (red arrows). Negative signaling (pink bars) prevents inappropriate vulva differentiation. See text for details.

To study the differentiation of P8.p in *P. pacificus* in more detail, we used immunofluorescence studies. In *C. elegans*, the non-vulval cells P(1,2,9-11).p and the two daughters of the 3° cells, P(3,4,8).p, fuse with the hypodermal syncytium *hyp7* (Fig. 1A). These cell fusion events can be visualized using the monoclonal antibody MH27, which recognizes an epitope of the JAM-1 protein (Kenyon 1986; Mohler et al., 1998). Unfused cells are labeled, while fused cells remain unlabeled.

MH27 antibody staining in *P. pacificus* showed that P8.p fuses with the hypodermis, 15-20 hours after hatching. In early juvenile stages, 0-5, 5-10 and 10-15 hours after hatching, all four cells are labeled by the MH27 antibody indicating that they are unfused (Fig. 2A). In contrast, 15-20 hours after hatching, animals showed variable staining patterns. In some animals all four cells were labeled, whereas in others only P(5-7).p, but not P8.p, were labeled (Fig. 2B). Thus, P8.p fuses with the hypodermis before the birth of the AC, but after the initiation of gonadal signaling.

P8.p influences the developmental competence of P(5,7).p but not P6.p

Combinatorial ablation studies identified additional cell interactions among the VPCs. After ablation of P(6,7).p, P5.p had a 2° fate in 88% of the animals (Table 1D). In contrast, if P(6-8).p were ablated, an isolated P5.p had a 1° fate in 65% of the animals and a 2° fate in the remaining 35% ($P<0.001$, χ^2-test) (Table 1E). These results suggest that P8.p influences the response of P5.p to gonadal signaling.

P8.p also influences the developmental competence of P7.p. Previous experiments had suggested that the competence of P7.p is limited: after ablation of P(5,6).p, P7.p had a 2° fate in 85% of the animals and a 1° fate only in one out of 20 animals (Table 1F; Fig. 3B) (Sommer and Sternberg, 1996). However, in these experiments only P(5,6).p but not P8.p were ablated. Given the influence of P8.p on P5.p, we analyzed the development of P7.p as an isolated VPC. After ablation of P(5,6,8).p, P7.p had a 1° fate in 53% and a 2° fate in 41% of the animals, indicating that the presence of P8.p also influenced the fate of P7.p ($P<0.004$, χ^2-test) (Table 1G, Fig. 3A). These results indicate that P5.p and P7.p have identical developmental potentials and that their response to gonadal signaling is strongly influenced by P8.p. In the absence of P8.p, both cells can adopt a 1° fate, but in the presence of P8.p, their fate is limited and they adopt predominantly a 2° fate. We refer to this novel type of interaction among the VPCs as ‘lateral inhibition’.

Does P8.p also influence the cell fate decision of P6.p? After ablation of either P(5,7,8).p or P(5,7).p, P6.p had a 1° fate (Table 1H,I). However, P6.p can influence P8.p: in 50% of the animals, P8.p had a 2° fate and formed part of the vulva (Table

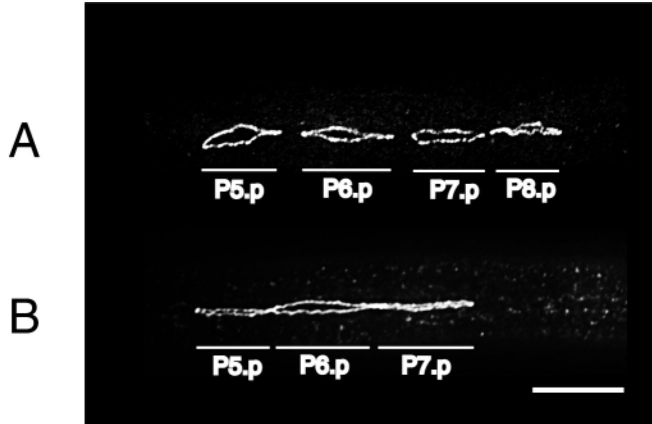


Fig. 2. MH27 antibody staining of the ventral epidermis in *P. pacificus*. (A) 5-10 hours after hatching; all four surviving cells in the central body region are labeled with the MH27 antibody, indicating that P(5-8).p are unfused. (B) 15-20 hours after hatching; P8.p fuses with the hypodermis and is no longer labeled. Scale bar is 10 μm.

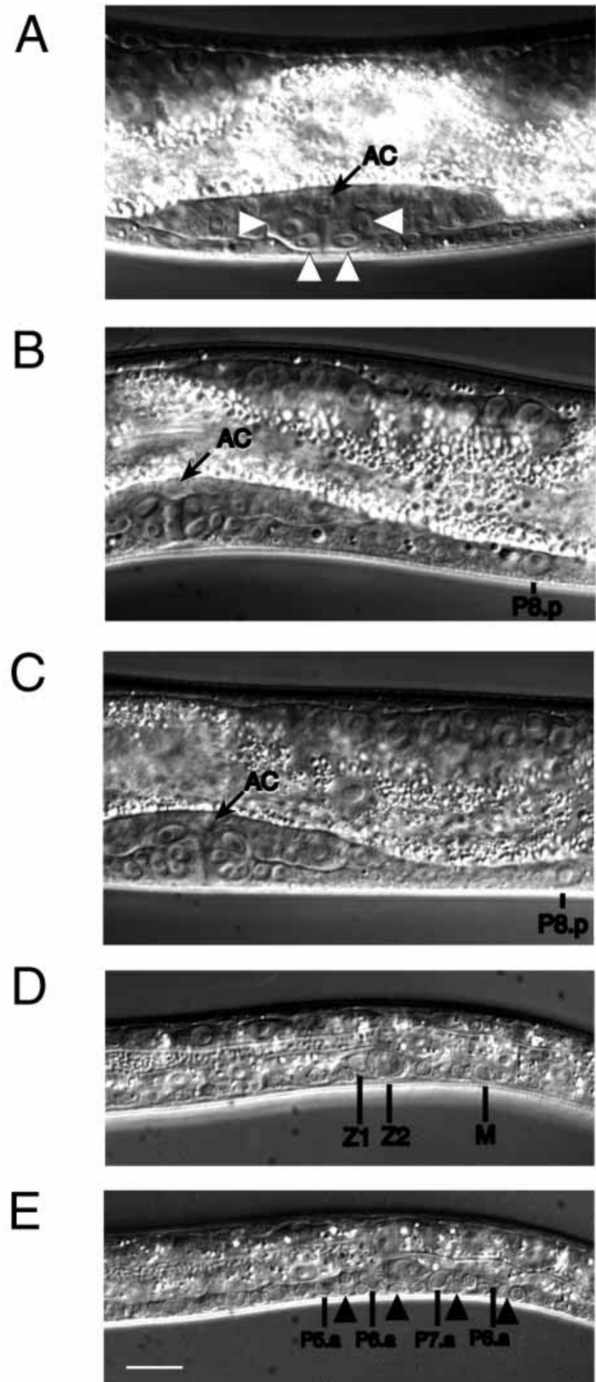


Fig. 3. Nomarski photomicrographs of ablated and unablated wild-type animals. Photographs were taken during the mid-J3 stage (A-C) and 1 hour after hatching (D,E). (A) P7.p adopts a 1° fate after ablation of P(5,6,8).p. The 1° fate is characterized by the symmetric organization of the Pn.pxxx cells around the AC. The final progeny of an isolated 1° cell are in three different focal planes (two cells each). In this picture, two focal planes (with four cells, white arrowheads) have been combined to illustrate the symmetric organization around the AC. (B) P7.p adopts a 2° fate after ablation of P(5,6).p. The 2° fate is characterized by the random distribution of the seven progeny. Six cells are visible in this plane of focus, four cells are towards right, two towards left of the invagination. The AC is not clearly visible in this plane of focus. (C) After ablation of P6.p, P(5,7).p adopt a 2° fate and form one common invagination. (D) A wild-type animal, 0-1 hour after hatching, showing the position of the mesoblast M and Z(1,2) of the gonad primordium. (E) The same animal in a more ventral focal plane, showing the position of the neuroblasts P(5-8).a. The ectoblasts P(5-8).p are indicated by black arrowheads. M is located in the same body region as P8.a and P8.p. Scale bar is 10 μm.

1I). These results indicate that P6.p is not inhibited by P8.p. It can adopt a 1° fate in the presence of P8.p and signals P8.p to adopt a 2° fate.

P8.p influences the fate of P5.p and P7.p in combination

Next, we asked whether P8.p could inhibit P5.p and P7.p simultaneously. After ablation of both P(6,8).p, P5.p and P7.p had a 2° fate in 50% of the animals (Table 1J). In 35% of the animals, P5.p had a 1° and P7.p a 2° fate (Table 1J). These experiments show that P5.p is more likely to adopt a 1° fate than P7.p, indicating a bias between these two VPCs. If only P6.p was ablated, P5.p and P7.p had a 2°-2° pattern in 82% of the animals, which is higher than in P(6,8).p-ablated animals ($P<0.038$, χ^2-test) (Table 1K). In the other 18% of animals, one cell, P5.p or P7.p, had the 1° fate, while the other adopted the 2° fate (Table 1K). Together, these results suggest that P8.p can limit the competence of P5.p and P7.p simultaneously. The 2°-2° pattern was novel in that both cells formed one invagination together, allowing some animals to lay eggs in the absence of a 1° fate (Fig. 3C).

The mesoblast M is necessary for lateral inhibition

The experiments described above indicate that P8.p (i) cannot respond to gonadal signaling as an isolated Pn.p cell, (ii) can respond to lateral signaling from P6.p, (iii) provides a novel type of lateral inhibition to P(5,7).p, and (iv) ultimately fuses with the hypodermis. All these results indicate that P8.p represents a novel cell type in the ventral epidermis of *P. pacificus* that is characterized by several features unknown in other Pn.p cells in *P. pacificus* or *C. elegans*. Are these characters of P8.p established autonomously or by interactions with neighboring cells? One simple hypothesis would be that other cells located in the same body region interact with P8.p during cell fate specification. For reasons that will become apparent later, we investigated a possible interaction between P8.p and the mesoblast M, a cell that in young J1 larvae is located just laterally to P8.p (Fig. 3D,E).

We found that both P8.p and M are required for lateral inhibition of P(5,7).p. As shown above, P8.p inhibits an isolated P5.p from adopting a 1° fate after ablation of P(6,7).p in the presence of M (Table 1D). However, if M was also ablated, P5.p had the 1° fate in 92% of the animals ($P<0.00002$, χ^2-test) (Table 1L). Similarly, in the absence of P(5,6).p, P7.p was more likely to adopt a 1° fate if M was also ablated (Table 1M,F). These results suggest that both P8.p and the mesoblast M are involved in lateral inhibition of P(5,7).p. However, these experiments do not allow us to decide whether the two cells act in parallel or in a linear pathway.

Although M affects the ability of P8.p to inhibit P(5,7).p, it does not affect the fate of P8.p itself. An isolated P8.p cell is unable to adopt a vulval fate even in the absence of M, indicating that the failure of P8.p to respond to gonadal signaling is not due to an inhibition from M (Table 1N).

M is misspecified in *Ppa-mab-5* mutant animals

In order to elucidate molecular aspects of vulval pattern formation, a strategy would be to study mutants that are defective in the specification of P8.p or M. The homeotic gene *Ppa-mab-5*, the homolog of the *Drosophila* gene *Antennapedia*, was previously shown to be involved in P(7,8).p specification (Jungblut and Sommer, 1998). P8.p differentiates ectopically in 80% of *Ppa-mab-5* mutant animals (Table 2A). This differentiation is gonad-independent, since P(7,8).p form vulva-like structures after the ablation of Z(1,4) (Table 2B; Jungblut and Sommer, 1998). We sought to determine whether any of the novel properties of P8.p depend on *Ppa-mab-5*. Therefore, we carried out ablation experiments in the *Ppa-mab-5* mutant background, using the strong ‘reduction-of-function’ allele *tu74* (Jungblut and Sommer, 1998).

Our experiments show that not only P(7,8).p, but also P6.p, can differentiate in the absence of the gonad in *Ppa-mab-5* mutant animals. We ablated Z(1,4) and P(7,8).p in the same animal to determine whether P(5,6).p would differentiate in the absence of the more posterior Pn.p cells. Surprisingly, P6.p formed vulval tissue in four out of seven animals (Table 2C). In these four animals, P6.p had migrated posteriorly prior to differentiation. In the *Ppa-mab-5* mutant, this region is occupied by many ectopic cells that are not found there in wild-type animals. When M was ablated in *Ppa-mab-5* mutant animals, these ectopic cells disappeared (data not shown). We speculate that these cells may result from an ectopic proliferation of the M lineage in *Ppa-mab-5* mutants. In contrast to the four animals with a differentiated P6.p, P6.p remained in the central body region in the three animals in which it had a 3° fate. Therefore, gonad-independent vulva differentiation of VPCs in *Ppa-mab-5* mutant animals might

Table 2. Cell ablation experiments in *Ppa-mab-5(tu74)* mutant animals

	Cells ablated	P5.p	P6.p	P7.p	P8.p	No. animals (Frequency)
A	unablated	2°	1°	2°	D	19/25 (76%)*
		2°	1°	2°	4°	5/25 (20%)*
		3°	2°	1°	D	1/25 (4%)*
B	Z(1, 4)	3°	3°	D	D	12/19 (63%)*
		3°	3°	3°	D	4/19 (21%)*
		3°	3°	D	4°	3/19 (16%)*
C	Z(1,4), P(7,8).p	3°	D	–	–	4/7 (57%)
		3°	3°	–	–	3/7 (43%)
D	M	2°	1°	2°	4°	13/18 (72%)
		2°	1°	2°	D	4/18 (22%)
		2°	1°	D	4°	1/18 (6%)
E	P8.a mock	2°	1°	2°	D	4/6 (66%)
		2°	1°	2°	4°	2/6 (34%)
F	P(5-7).p	–	–	–	D	5/7 (72%)
		–	–	–	2°	1/7 (14%)
		–	–	–	4°	1/7 (14%)
G	P(5-7).p, M	–	–	–	4°	7/14 (50%)
		–	–	–	2°	5/14 (36%)
		–	–	–	D	2/14 (14%)
H	P(6-8).p	2°	–	–	–	5/6 (83%)
		1°	–	–	–	1/6 (17%)
I	P(6,7).p	2°	–	–	D	9/12 (75%)
		2°	–	–	4°	2/12 (16%)
		2°	–	–	1°	1/12 (9%)
J	P(5,6).p	–	–	2°	D	6/9 (67%)
		–	–	1°	D	2/9 (22%)
		–	–	1°	2°	1/9 (11%)
K	P(6-8).p, M	1°	–	–	–	8/13 (62%)
		2°	–	–	–	4/13 (31%)
		3°	–	–	–	1/13 (7%)

Note that in experiment F and G, the difference between a 2° and a D fate refers to the position of the differentiating cells in the animal: a 2° cell differentiates beneath the AC, a D cell differentiates in the posterior region.

*Data from Jungblut and Sommer (1998).

be due to an inappropriate signal from the M cell or its derivatives.

Indeed, our experiments show that the ectopic differentiation of P8.p in *Ppa-mab-5* depends on an induction by the mesoblast M. The differentiation of P8.p decreased from 80% to 22% after the ablation of M in the *Ppa-mab-5* mutant background (P<0.0001, χ^2-test; Table 2A,D). Specifically, P8.p had an epidermal fate in 14 out of 18 animals. As a control, we mock-ablated P8.a and observed no effect on P8.p differentiation (Table 2E). Does M also affect the cell fate of an isolated P8.p? After ablation of P(5-7).p, P8.p had a vulva fate in 86% of the animals (Table 2F). When P(5-7).p and M were simultaneously ablated, P8.p differentiated in only 50% of the animals (Table 2G). Thus, the differentiation of P8.p decreased after ablation of M, but was not completely eliminated. One reason might be that the interaction between M and P8.p begins during embryogenesis, before the time of ablation. Unfortunately, we cannot test this hypothesis, because cells cannot be manipulated by laser microbeam irradiation during late embryonic stages. Taken together, these results suggest that either the M cell or its descendants induce the ectopic differentiation of P8.p in *Ppa-mab-5*.

Lateral inhibition by P8.p acts via M

The experiments described above indicate that the mesoblast M and P8.p are required for lateral inhibition of P(5,7).p in wild-type animals and that M or its descendants induce the ectopic differentiation of P8.p in *Ppa-mab-5* mutants. However, these experiments do not indicate if P8.p and M act in parallel or in a linear pathway during lateral inhibition and if the *Ppa-mab-5* gene plays a role in this process. Since the M lineage is transformed in *Ppa-mab-5* mutant animals, we asked whether its transformation affected lateral inhibition.

Ablation experiments of M and VPCs revealed that M and P8.p act in a linear pathway and that M is the final effector of lateral inhibition. In *Ppa-mab-5* mutant animals, the ablation of P(6-8).p, P(6,7).p or P(5,6).p, respectively, resulted in P5.p and P7.p having predominantly a 2° fate (Table 2H-J). Thus, in *Ppa-mab-5* mutants lateral inhibition occurs irrespective of the presence or absence of P8.p. However, if P(6-8).p and M were simultaneously ablated, P5.p had a 1° fate in 62% of the animals (P<0.045 for the comparison of Table 2H and K, χ^2-test) (Table 2K). These results suggest that, in the *Ppa-mab-5* mutant, M but not P8.p is required for lateral inhibition. Therefore, it is unlikely that both are used in parallel in wild-type animals. Instead, these results suggest that the mesoblast M is the final effector of lateral inhibition and that the activity of M requires a signal from P8.p. Since lateral inhibition occurs in the absence of P8.p in *Ppa-mab-5* mutants, *Ppa*-MAB-5 is most likely a repressor of lateral inhibition acting in the M cell.

Are P8.p and M signaling necessary for vulval pattern formation in *P. pacificus*? No patterning defects were observed after ablation of P8.p or M (Tables 1O,P). Thus, P8.p and M act redundantly with other signaling processes during vulval development.

P8.p provides a negative signal for vulva development

The experiments described above indicate that lateral inhibition by P8.p and M influences the 1° versus 2° cell fate decision of P(5,7).p. Since M induces the gonad-independent differentiation of P(7,8).p in the *Ppa-mab-5* mutant, M could also have inductive properties in wild type. We therefore asked whether M and/or P8.p also play a role during vulval induction in wild-type animals.

When we carried out combinatorial ablation experiments, we found that P8.p provides a negative signal for vulva development. After ablation of Z(1,4), the precursors of the somatic gonad, no vulva differentiation was observed (Table 1Q; Sigrist and Sommer, 1999). Similarly, after ablation of Z(1,4) and M, no vulva differentiation was observed, except for one VPC in one out of 21 animals (Table 1R). In contrast, if Z(1,4) and P8.p were ablated, six out of 17 animals showed gonad-independent vulva differentiation (Table 1S). Specifically, 9 out of 51 VPCs (18%) differentiated in this experiment. After ablation of Z(1,4), P8.p and M together, only 6 out of 66 VPCs (9%) differentiated (Table 1T). Although the reduction from 18% to 9% is statistically insignificant (P<0.169, χ^2-test), the combinatorial ablation of Z(1,4) and P8.p clearly indicates that P8.p provides a negative signal during vulva development.

Does P8.p provide the negative signal before or after its fusion with the hypodermis? To address this question, we ablated Z(1,4) immediately after hatching and P8.p, 18 hours later, at the time of fusion. Only one VPC differentiated in one out of 10 animals (Table 1U). Thus, P8.p provides negative signaling before it fuses with the hypodermis. However, we do not know how early P8.p signals, because P8.p is born during embryogenesis and cannot be ablated during this early time period.

DISCUSSION

P. pacificus P8.p: a novel cell type in the ventral epidermis

The present work describes several novel cell-cell interactions among the VPCs and the mesoblast M during vulva development in *P. pacificus*. Most surprisingly, P8.p, the posteriormost Pn.p cell in the central body region, shows novel developmental properties not associated with any other Pn.p cell in *P. pacificus*, *C. elegans* or any other nematode studied so far. P8.p can respond to lateral signaling by P6.p, but not to gonadal signaling as an isolated VPC. P8.p is involved in lateral inhibition by the mesoblast M, provides negative signaling and, ultimately, fuses with the hypodermis (Table 3). In *C. elegans,* there is no indication for lateral inhibition of the type observed in *P. pacificus* (Sternberg and Horvitz, 1986). Also, any isolated VPC can adopt the 1° cell fate. We conclude that P8.p in *P. pacificus* represents a new cell type in the ventral epidermis, characterized by novel properties that are not found in other Pn.p cells in this or other species.

The mesoblast M as a new element in vulval patterning

We have determined a role for the mesoblast M or its descendants in vulval cell fate specification. It should be noted that the experiments do not directly address the question of whether M or any of its descendants is involved in the observed interactions. Hence, we refer to the role of the M lineage as a function of the 'M cell'. Cell lineage analysis of the M lineage

Table 3. Properties of P8.p in *Pristionchus pacificus*

P8.p characteristics in *P. pacificus*	(Exp. no.)	M Dependence (Exp. no.)	*C. elegans*
1: Unable to respond to gonadal signaling	Table 1C	– (Table 1N)	responds to gonadal signaling
2: Responds to lateral signaling	Table 1B	n.d.	responds to lateral signaling
3: Provides lateral inhibition	Table 1D/E,F/G	+(Table 1L,M)	does not provide lateral inhibition
4: Provides negative signaling	Table 1Q,S	– (Table 1R)	does not provide negative signaling
5: Fuses with hypodermis	Fig. 2	n.d.	fuses with hypodermis after division

will permit the question to be directly addressed by ablating specific descendants of M.

Both P8.p and M are required for lateral inhibition of P(5,7).p. Cell ablation experiments in wild-type animals do not show whether both cells act in parallel or in a linear order, since the removal of both cells alone results in the absence of lateral inhibition. In *Ppa-mab-5* mutants, however, the ablation of M, but not P8.p results in the absence of lateral inhibition, indicating that both cells act in a linear pathway and that the mesoblast M is the final effector. The fact that lateral inhibition is still present in the 'reduction-of-function' mutant *Ppa-mab-5(tu74)* also suggests that *Ppa*-MAB-5 normally acts as a repressor for lateral inhibition. We speculate that P8.p signaling changes the activity of *Ppa*-MAB-5 in M, thereby withdrawing the repression of lateral inhibition (Fig. 4).

Several lines of evidence suggest that the interaction between P8.p and M begins during embryogenesis. Our results demonstrate that the removal of P8.p or M shortly after hatching does not completely eliminate all lateral inhibition of P(5,7).p, since up to 40-50% of the animals are still incapable of adopting the 1° fate. A possible explanation for these results is that the interaction between M and P(5,7).p is a process that begins during embryogenesis, before any ablations are done. If so, the interaction between P8.p and M may also occur during embryogenesis. In addition, we cannot rule out the possibility that not only P8.p, but also its precursor cell P8, interacts with M. All these cells are located in the same body region, so that a direct cell contact is possible (Fig. 3D,E). In *C. elegans*, no interaction has been found between M and P8.p. However, the sex myoblasts, which are derivatives of the M cell, interact with the somatic gonad and some of the progeny of P6.p (Chen and Stern, 1998).

With regard to the timing of the cell-cell interactions, it is important to notice the life cycle differences between *P. pacificus* and *C. elegans*: *P. pacificus* has only three juvenile stages (J1-J3, respectively) instead of the usual four found in other nematodes. Therefore, *P. pacificus* displays major heterochronic changes relative to *C. elegans* (Félix et al., 1999). Although the length of the life cycle is similar in both species, embryogenesis takes much longer in *P. pacificus*. Several developmental events, which occur during the L1 stage of *C. elegans*, in particular in the Pn cells, take place during embryogenesis in *P. pacificus*. It is not unlikely, therefore, that signaling interactions begin early, during the elongated embryogenesis.

A role of M and P8.p in vulval cell fate specification is consistent with the asymmetric organization of the Pn.p cells in *P. pacificus*. In species of the Rhabditidae and the Panagrolaimidae, the vulva equivalence group is patterned symmetrically around the gonad. For example, the typical pattern in rhabditids is 3°-2°-1°-2°-3° (Sommer and Sternberg, 1995). In the Panagrolaimidae, two 1° cells exist with a final vulva pattern being 2°-1°-1°-2° (Félix et al., 2000). In contrast, in *P. pacificus* and all other analyzed species of the Diplogastridae, the pattern is asymmetric: P(1-4,9-11).p die a programmed cell death, and only three of the remaining four cells form the vulva (Sommer, 1997). Thus, the epidermal cell P8.p has no counterpart in the anterior region introducing an asymmetry into the Pn.p system. Therefore, it is interesting to note that an interaction between P8.p and M has only been observed in a species with an asymmetric Pn.p system, thereby introducing a new patterning element into the ventral epidermis.

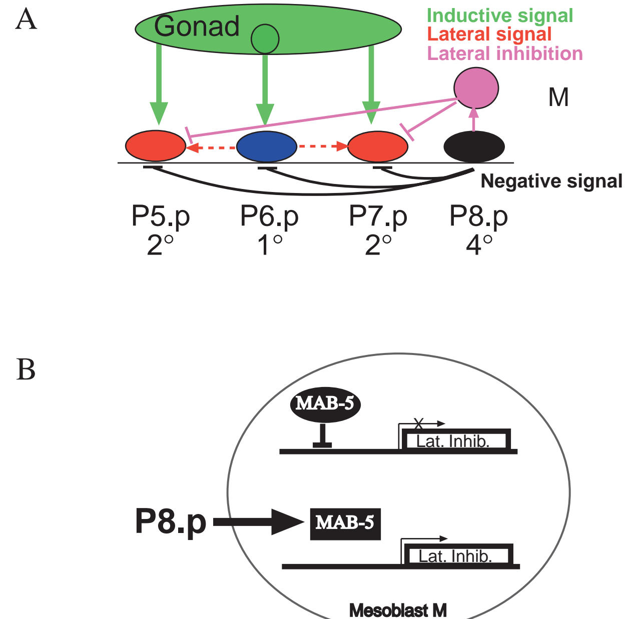


Fig. 4. Model for cell-cell interactions during vulva development in *P. pacificus*. (A) P8.p provides a lateral inhibition to P(5,7).p, mediated by the mesoblast M (pink bars). Lateral inhibition influences the 1° versus 2° cell fate decision of P(5,7).p. P8.p also provides a negative signal (black bars), which influences the vulva versus non-vulval cell fate decision. For clarity, negative signaling is shown here as an interaction between P8.p and P(5-7).p. It is possible that indirect interactions involving other cells could exist. Inductive signaling from the somatic gonad is a continuous process (green arrows). Lateral signaling occurs between P6.p and P8.p (not indicated) and perhaps also between P6.p and P(5,7).p (red dotted arrow). (B) A model for the role of *Ppa-mab-5* in lateral inhibition. Since lateral inhibition can only be prevented by ablating M in the *Ppa-mab-5* mutant, *Ppa*-MAB-5 most likely acts in M as a repressor of 'lateral inhibition' genes. A signal from P8.p modifies *Ppa*-MAB-5 so that repression is withdrawn.

Ppa-mab-5 acts in the M cell

The homeotic gene *mab-5* was shown to be involved in cell fate specification in the posterior body region of *C. elegans*, including specification of P(7,8).p (Kenyon, 1986; Clandinin et al., 1997). The fates of P(7,8).p are also affected in *Ppa-mab-5* mutants, where both cells exhibit gonad independent vulva differentiation (Jungblut and Sommer, 1998). *Ppa-mab-5* mutant animals are multivulva, because of the ectopic differentiation of P8.p, and are also egg-laying defective.

Here, we provide evidence that the defects observed in *Ppa-mab-5* animals result from misspecification of the mesoblast M. A comparison of ablation experiments, carried out in the presence or absence of M, supports the hypothesis that M induces ectopic differentiation of P8.p in *Ppa-mab-5* mutants. Thus, a transformation of the M cell causes the neomorphic phenotype in P8.p. The finding that P8.p differentiation cannot be completely eliminated by the ablation of M suggests that signaling from the M cell starts during embryogenesis.

The misspecification of M may also account for the egg-laying defect of *Ppa-mab-5* mutant animals. Lineage analysis of the M cell in wild type and *Ppa-mab-5* mutants will further address this question (I. Carmi and R. J. S., unpubl. observ.). Unlike *Ppa-mab-5*, *Cel-mab-5* mutants do not exhibit a strong egg-laying defect, suggesting that *mab-5* has a different role in the specification of the M cell in each species.

P8.p provides a negative signal

Combinatorial gonad, P8.p and M ablation experiments suggest that P8.p provides a negative signal for vulva development. However, it remains unknown if cells other than P8.p, also have negative signaling activity. Also, given our current understanding, we cannot conclude if negative signaling by P8.p acts on the source of inductive signaling or directly on the VPCs (Fig. 4A). The analysis of *P. pacificus* vulva-defective mutants might shed light on these issues and might help providing first evidence about the underlying molecular mechanism.

Redundancy among signaling systems

Ultimately, all cell-cell interactions, among the VPCs, the somatic gonad and the mesoblast M, have to be integrated to form a proper vulva. The question therefore arises, whether all of these signaling systems in *P. pacificus* are necessary for the specification of the 2°-1°-2° pattern. Since ablation of P8.p or the mesoblast M results in animals with a normal vulva, lateral inhibition and negative signaling likely represent functions that are redundant with other signaling processes. Thus, it appears that gonadal induction and lateral signaling are sufficient for proper pattern formation. Redundancy has also been documented in *C. elegans* vulva formation. Inductive and lateral signaling act redundantly during the specification of P(5-7).p (Kenyon, 1995), and negative signaling consists of two fully redundant genetic pathways (Thomas, 1993).

Our work does not directly address the question as to how redundancy evolves. One explanation for its existence is that redundancy might provide canalization (Gibson and Wagner, 2000). We speculate however, that once it exists, redundancy may be a step on the way to evolutionary novelty. A pathway or interaction that is initially redundant can, over time, take on new, non-redundant functions, the elimination of which would result in developmental defects. Thus, the existence of redundancy may allow the rapid divergence of developmental mechanisms. One of the future questions will be to identify the evolutionary mechanism of fixation of such redundant developmental processes.

Diverse signaling processes can pattern the nematode vulva

In *C. elegans*, multiple cell-cell interactions act in combination or in a redundant manner to pattern the vulva (Fig. 1B). This and previous studies indicate that in *P. pacificus* gonadal signaling, lateral signaling, lateral inhibition and negative signaling, as well as an interaction between P8.p and M, occur during vulva formation. Some of these interactions resemble the ones found in *C. elegans*, while the others are unique to *P. pacificus* (Fig. 4).

The most parsimonious hypothesis is that signaling events common to both *C. elegans* and *P. pacificus* use the same molecular pathway, i.e., EGF/RAS/MAPK signaling during gonadal induction. This hypothesis could be tested by analyzing *P. pacificus* mutants defective in gonadal signaling. In such mutants, P(5-7).p do not divide and remain epidermal. Four mutants, *Ppa-vul-1* to *Ppa-vul-4*, have been described, none of which is genetically linked to the *Ppa-let-60*/Ras gene (Sigrist and Sommer, 1999) or the *Ppa-mpk-1* gene (C. Sigrist and R. J. S., unpublished data). Furthermore, the homeotic transcription factor *lin-39*, which is a target of EGF/RAS/MAPK signaling and a positive effector of vulva development in *C. elegans*, is dispensable for *P. pacificus* vulva induction (Sommer et al., 1998). Thus, there is no present link between vulva induction in *P. pacificus* and EGF/RAS/MAPK signaling.

Lateral inhibition of P(5,7).p by M and P8.p, is not found in *C. elegans*. Several mechanisms could account for this interaction. One simple hypothesis is that M secretes a signaling molecule that interferes with other cell-cell communications, such as vulva induction. A secreted inhibitory signal could function in one of two different modes. Inhibition might act at the surface of the VPCs preventing recognition of the gonadal signal, or alternatively, the signal might interfere with signal transduction within the VPCs. In other developmental systems, such as *Drosophila* EGF signaling, multiple types of inhibition have been described, some of which interfere with the ligand-receptor interaction during the inductive process (Perrimon and McMahon, 1999). In this context, the finding that P6.p is not inhibited by P8.p and M might be of importance. P6.p differs from P(5,7).p because it is located beneath the center of the gonad. P6.p might receive a higher amount of inductive signal over time making it inert to lateral inhibition.

Evolutionary implications: constant patterns but altered interactions

Previous studies on the evolution of vulva development in nematodes resulted in the surprising finding that the mechanisms of vulva induction vary widely among nematode species. In particular, the one-step induction by the AC found in *C. elegans* is a highly derived character. More common is a multistep interaction between the gonad and the underlying epidermis (Félix and Sternberg, 1997; Sigrist and Sommer, 1999; Félix et al., 2000; for review see Sommer, 2000). In all these cases, several cells of the somatic gonad (instead of the

single AC in *C. elegans*) communicate with the Pn.p cells over a longer time interval. In some nematode families, alterations in gonadal induction occur in the absence of vulval cell lineage alterations (Sommer, 1997; Félix et al., 2000). Thus, it seems that the mechanisms of cell-cell interactions are more flexible and opportunist than the vulval cell lineage patterns.

The work described here also indicates that some of the other cell-cell interactions that contribute to vulva development differ between *P. pacificus* and *C. elegans*. It has been suggested that the evolutionary lineages giving rise to these species are separated for at least 100 million years (Eizinger and Sommer, 1997). Therefore, the comparison between *P. pacificus* and *C. elegans* cannot indicate how the cellular mechanisms of vulva development diverge. To understand the diversification in detail, more closely related species, most probably from the same genus, have to be compared.

To summarize, our work describes that different cellular mechanisms are used to construct the vulvae of *P. pacificus* and *C. elegans*. Despite the multiple changes in signaling processes, no immediate alterations in vulval patterning (2°-1°-2°), vulval cells (P5.p-P7.p) or cell lineages are apparent. Cellular interactions patterning an organ are thus more flexible than the final organ pattern itself.

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