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Coordinated morphogenesis of epithelia during development of the *Caenorhabditis elegans* uterine–vulval connection

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ABSTRACT **Development of the nematode egg-laying system requires the formation of a connection between the uterine lumen and the developing vulval lumen, thus allowing a passage for eggs and sperm. This relatively simple process serves as a model for certain aspects of organogenesis. Such a connection demands that cells in both tissues become specialized to participate in the connection, and that the specialized cells are brought in register. A single cell, the anchor cell, acts to induce and to organize specialization of the epidermal and uterine epithelia, and registrates these tissues. The inductions act via evolutionarily conserved intercellular signaling pathways. The anchor cell induces the vulva from ventral epithelial cells via the LIN-3 growth factor and LET-23 transmembrane tyrosine kinase. It then induces surrounding uterine intermediate precursors via the receptor LIN-12, a founding member of the Notch family of receptors. Both signaling pathways are used multiple times during development of *Caenorhabditis elegans*. The outcome of the signaling is context-dependent. Both inductions are reciprocated. After the anchor cell has induced the vulva, it stretches toward the induced vulval cells. After the anchor cell has induced specialized uterine intermediate precursor cells, it fuses with a subset of their progeny.**

The *Caenorhabditis elegans* hermaphrodite egg-laying apparatus provides an excellent system to study problems of organogenesis. Since *C. elegans* is internally self-fertilizing, egg laying is not essential, and mutant strains can be propagated (1). In wild-type animals, developing embryos mature in the uterus and are laid through the vulva, a specialization of the external epidermis, through the action of uterine- and vulval-specific muscles and the neurons that innnervate them. The organization of this system involves extensive cell–cell interactions, many of which have been reviewed elsewhere (2).

Here, we focus on the induction, patterning, and differentiation of the relevant uterine and vulval specialization specifically involved in forming a uterine–vulval connection. We describe how a single cell, the anchor cell (AC), coordinates multiple aspects of this process. Having a common inducing source may be an important mechanism in the development of complex systems, as exemplified by the role of the notochord and floor plate in neural development (for review, see ref. 3).

How distinct tissues are connected to make a functional unit is a fundamental question in developmental biology. Well-characterized examples involve invasion of the mesenchyme by an epithelium during tubulogenesis of the lung and kidney (for review, see refs. 4 and 5), which requires proteolysis of the extra-cellular matrix. In the *C. elegans* hermaphrodite, formation of a uterine–vulval connection involves the coordinated morphogenesis of two epithelia (see Figs. 1 and 2). Many aspects of this process (e.g., differentiation of the component cell types, lumen formation, reciprocal signaling, and breakdown of the barrier between the tissues) illustrate common features of organogenesis, and thus its molecular genetic analysis is likely to reveal general mechanisms underlying organogenesis.

FORMATION OF THE UTERINE–VULVAL CONNECTION

The events involved in forming a uterine–vulval connection occur during larval development and can be briefly summarized as follows.

(*i*) Induction and registration. A specialized cell, the AC, first induces the vulva using the ligand LIN-3 (6, 7) and receptor LET-23 (8, 9), and then induces the π cells that will produce the relevant uterine specialization using the receptor LIN-12 (10) Registration is accomplished by having a common inducing source (the AC), ensuring that specialization will be centered identically in both tissues.

(*ii*) Patterning. Formation of a functional uterine–vulval connection involves patterning downstream of the initial inductive event, leading to further differentiation between cell types. This generates the specific vulval progeny (1° cell progeny) and π cell progeny [*ut*erine-*se*am cell (utse) and uterine-vulval-1 cells (uv1)] that will form the connection.

(*iii*) Connection. Connection is dependent on the differentiation of the utse, which forms the thin laminar process that resides between the uterus and the vulva. This process includes fusion of eight π progeny with the AC, thereby moving the bulky AC out of the way and permitting a connection to be formed (11). The utse forms adherens junctions with the uv1 cells that attach to the vulva.

Induction and Registration. The AC is a specialized ventral uterine cell (VU) whose interactions with uterine and epidermal cells create the passageway from the uterus to the outside (the vulva) and connect it to the uterus. Three of these cell–cell interactions use well-characterized signaling proteins (see Fig. 3; Table 1).

Two VU cells (Z1.ppp and Z4.aaa) initially have the potential to become the AC (12, 13). Before the events discussed above, these cells interact using LIN-12, a founding member of the Notch family of receptors (14–16); one cell becomes the AC and laterally inhibits the other cell from also becoming the AC (the AC versus VU decision; Fig. 1*A*).

The cells with the potential to produce vulval tissue [vulval precursor cells (VPCs)] are located in the epidermis ventral to

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Abbreviations: AC, anchor cell; VPC, vulval precursor cell; DU, dorsal uterine precursor cell; VU, ventral uterine precursor cell; utse, uterine-seam cell; uv1, uterine-vulval-1 cell; ut, uterine toroid; L2, L3, L4, second, third, and fourth larval stages.

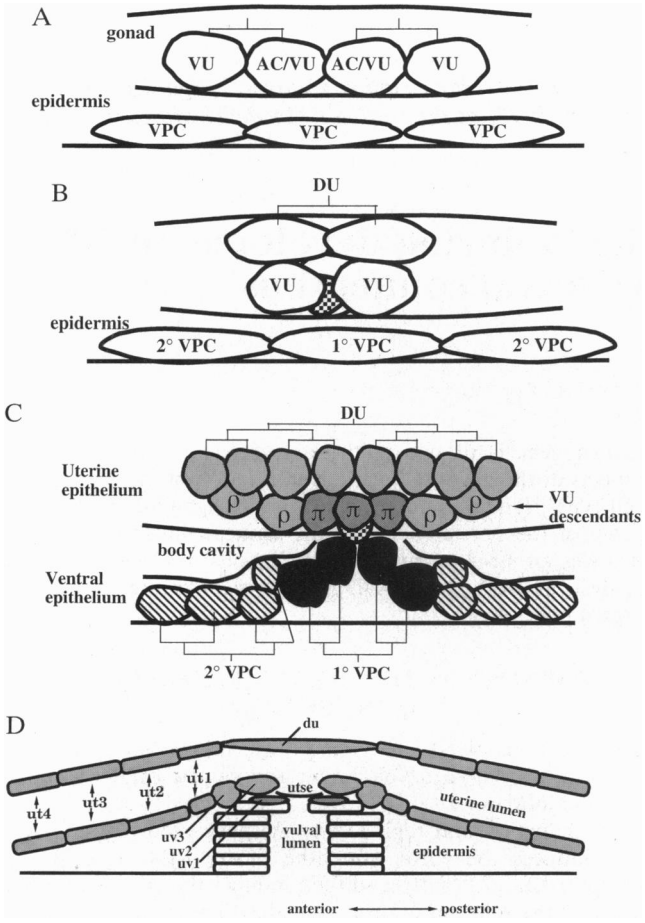

FIG. 1. Uterine and vulval tissues during development. (*A*) Late L2 larval stage, specification of the AC. Two cells have the potential to become the AC; the cell that does not become the AC becomes a VU precursor cell. The sisters of these two cells become VU cells. The VPCs are not yet specified. The LIN-12-mediated lateral inhibitory AC/VU decision occurs during late L2, ≈23 hr after hatching. (*B*) Early L3 stage, the AC (checkered cell) has been specified and is inducing the VPCs to the 1° and 2° fates. (*C*) Late L3 stage, during an intermediate point in the uterine and vulval lineages. The AC (checkered cell) can be seen contacting the surrounding VU intermediate precursor cells (dark grey), which it induces to adopt the π fate. VPCs have been induced and undergone two rounds of cell division (solid cells, 1° VPC progeny; hatched cells, 2° VPC progeny). The AC extends ventrally to contact the innermost VPC progeny. (*D*) L4 stage. A schematic representation of the mature uterine and vulval structures based on the serial electron microscopic reconstructions of J. White and E. Southgate. The cells shown have completed their divisions. Differentiation of uterine and vulval tissue, including lumen formation, has occurred. Uterine cell types include the toroidal ut cells (ut1–ut4), the interfacial uv cells, and the du cell dorsal to the vulva. The thin laminar process of the utse separates the uterine and vulval lumens (open cells, mature vulval cells). The uv1 cells contact the dorsal-most vulval cells and the utse. The uterus is symmetric about the anterior-posterior axis.

the uterus. Initially, the relative positions of the uterus and vulva are not fixed; rather, they slide with respect to one another. Furthermore, the two tissues are separated by an extracellular matrix (ref. 17; L. Carta and P.W.S., unpublished results). The process by which these tissues are aligned with, and ultimately connected to, one another is initiated by two-way communication between the AC and the VPCs (K. Tietze and P.W.S., unpublished results). First, the VPCs move such that the presumptive 1° VPC (P6.p) is always directly ventral to the AC (Fig. 1*B*) and the AC then extends a process toward P6.p and/or its progeny. Ultimately, the barrier between the uterus and the ventral epidermis is broken by the AC such that, while the cell body remains in the uterus, its process extends down to the ventral epidermis, between the P6.p progeny (Fig. 1*C*).

The AC induces three of the six VPCs, P5.p–P7.p, to adopt vulval fates using the LIN-3 growth factor and the LET-23 receptor tyrosine kinase (refs. 6, 7, and 9; see below). LIN-3 is predicted to contain an extracellular epidermal growth factor domain, and this epidermal growth factor domain is sufficient to induce vulval induction in absence of the AC. Thus, this induction can be uncoupled from the physical interactions between the AC and VPCs discussed above, although a close coordination presumably exists between the two *in vivo*.

Approximately 4 hr subsequent to vulval induction, the AC induces a subset of VU intermediate precursor cells (VU cell granddaughters) to adopt the π fate and generate the cells that connect the uterus to the vulva (ref. 10; see below). The receptor LIN-12 is required for π cell induction. A *lin-12–lacZ* reporter construct is expressed in all VU intermediate precursor cells at the appropriate time (18), yet only those cells that are adjacent to (and most likely touching) the AC become π. Thus, the AC appears to signal its immediate neighbors to become π cells, while those cells located more distally become the alternative fate ρ (Fig. 1*C*). This localized effect is consistent with data on Notch/Delta signaling, suggesting that both ligand and receptor are membrane-bound (19).

The above results demonstrate that, in the ventral uterus, LIN-12 mediates first the bidirectional AC versus VU decision and then unidirectional signaling from the AC to adjacent VU grandprogeny. The three cell–cell interactions involving the AC can be summarized as follows: (*i*) AC/VU (lateral inhibitory; *lin-12*-mediated); (*ii*) vulval specification (inductive; *lin-3/let-23*-mediated); (*iii*) π fate specification (inductive; *lin-12*-mediated). Consistent with the results discussed above, a *lin-3*::*lacZ* translational fusion is expressed in the AC at the time of vulval induction and subsequently (through the L4 period; ref. 6). *lag-2*, a gene whose sequence is similar to the *Drosophila* Notch ligands Delta and Serrate (20, 21) is likely the ligand for the AC versus VU decision (22, 23). A *lag-2*::*lacZ* transcriptional fusion is expressed in the AC at the time of the AC/VU decision (23) and through the time of π cell induction (K. Fitzgerald and A.P.N., unpublished results). Thus, *lag-2* may be the ligand for the π cell fate decision as well. Although π cell induction and vulval induction are temporally distinct, *lag-2* and *lin-3* are expressed in the AC during similar time intervals. Several types of mechanisms might determine which signaling program is active. These include the competence of the receiving cells, and subcellular localization and posttranslational regulation of the ligand.

As discussed below, π cells make the uterine cell types (uv1 and utse) that connect to the vulva. Having a common source for induction of the vulva and the π cells ensures registration between the two tissues (Figs. 1*C*, 2*A*, and 3). The vulva is centered directly ventral to the AC, where LIN-3 concentration is presumably highest, and the π cells that produce the connecting uterine cells surround the AC.

Patterning. The cells induced by the AC divide to produce the specialized progeny that form a uterine–vulval connection. Below, we describe the nature of these cell types and the mechanisms by which they are patterned.

Vulval patterning. The 1° VPC progeny (vulE and vulF) connect to the AC. The precise pattern of VPC types, 3°-3°-2°-1°-2°-3°, depends on intercellular signaling. The pattern is established by the action of at least three signaling pathways: (*i*) an inductive signal from the AC mediated by LIN-3 and LET-23, (*ii*) a lateral signal among VPCs mediated by LIN-12, and (*iii*) a negative signal from cells other than the AC and VPCs mediated by LIN-15A, LIN-15B, and related genes.

The inductive signaling pathway from the AC to the VPCs acts via the receptor LET-23, a nematode homolog of the

Table 1. Some genes controlling vulval and uterine development

Gene	Product homologs/domains	Probable biochemical role
lag-2	Delta	Ligand for LIN-12
let-23	EGF-receptor	Cell surface receptor, tyrosine kinase
let-60	Ras	Small GTPase
lin-1	ETS-domain	Transcription factor
lin-3	Epidermal growth factor	Ligand for LET-23
lin-11	LIM domain	Transcription factor
lin-12	Notch	Cell surface receptor
lin-15	None	?
lin-25	None	Possible transcriptional regulator
lin-31	HNF3/forkhead	Transcription factor
lin-45	Raf	Serine/threonine protein kinase
ksr-1	New family	Serine/threonine protein kinase
mek-2	MAP kinase kinase	Protein kinase
mpk-1	MAP kinase	Serine/threonine protein kinase
sem-5	Grb2, Drk	Signaling adaptor
sur-2	None	Possible transcriptional regulator

See text for references.

human epidermal growth factor receptor, a transmembrane protein tyrosine kinase. LET-23 acts via a highly evolutionary conserved signaling pathway using the adaptor SEM-5, an unidentified exchange factor for ras, LET-60 Ras, LIN-45 Raf, LET-357, and MPK-1 (Fig. 3; for reviews, see refs. 24 and 25). The KSR-1 kinase acts in parallel to or downstream of Ras to promote vulval differentiation. The pathway is less well-defined in the nucleus, but several positive and negative acting factors have been cloned: LIN-1, an ETS-domain class transcription factor, and LIN-31, a HNF3/forkhead transcription factor, act to prevent vulval differentiation (26, 27). LIN-25 and SUR-2, both novel proteins, act to promote vulval differentiation (28, 29). It is not known whether the ability of LET-23 to stimulate both 1° and 2° fates branches from the receptor or in the nucleus.

The mode of specification of the 2° VPC fate has not been resolved. The AC and LIN-3 can directly induce 2° VPCs (7, 30, 31), but 2° VPCs also can be induced by a 1° VPC (32, 33). These two modes of 2° specification are likely both used, and together extend the range of conditions (for example, LIN-3 activity levels) in a given animal that will result in a normal 2°-1°-2° pattern of VPC fates.

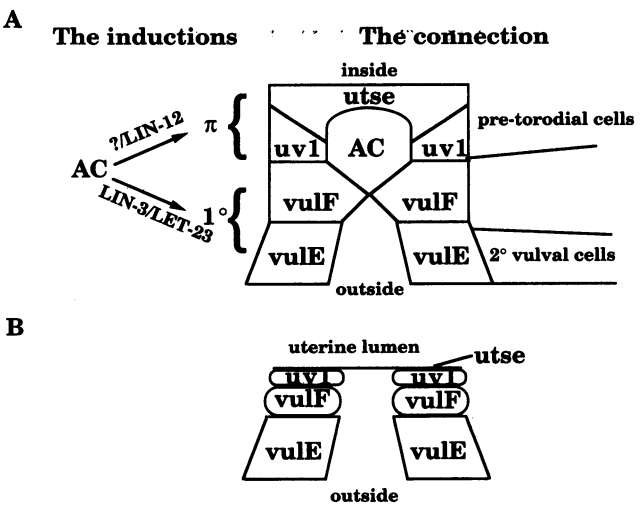

FIG. 2. The role of the AC in formation of the uterine–vulval connection. (*A*) The AC induces vulval cells and the π cells that make the connecting uterine cell types (utse and uv1) using distinct signaling molecules. At the time of induction, the AC resides between the two tissues, impeding contact. (*B*) AC fusion with the utse removes this barrier, allowing a functional connection to be made.

The negative signaling pathway involves a number of proteins including LIN-15A and LIN-15B (34–36). Formally, *lin-15* acts to prevent basal activity of LET-23; in the absence of *lin-15* (which encodes two functionally redundant proteins), all six VPCs are either 1° or 2°, with the pattern controlled by LIN-12-mediated lateral signaling (37, 38). This excessive vulval differentiation depends on LET-23 and downstream components such as LET-60 RAS but not on LIN-3.

Patterning of π cell progeny. π cells generate the two uterine cell types that connect to the vulva: utse and uv1 (Figs. 1*D*, 2, and 4). The utse is a multinucleate cell that forms the thin laminar process of the uterine–vulval interface (see below for further description). The ground state of π progeny is likely to become utse since this tissue is hyperproliferated in a *lin-12* gain-of-function mutant, which has many excess π cells (10).

The π cells (three per side) undergo an asymmetric dorsal-ventral cell division to produce larger dorsal daughters and smaller ventral daughters (Fig. 4*A*). The distal ventral cells, which are born and remain just dorsal to the innermost vulval cells (vulF), become uv1. These cells make adherens junctions with vulF and with the utse, i.e., they literally "connect" the

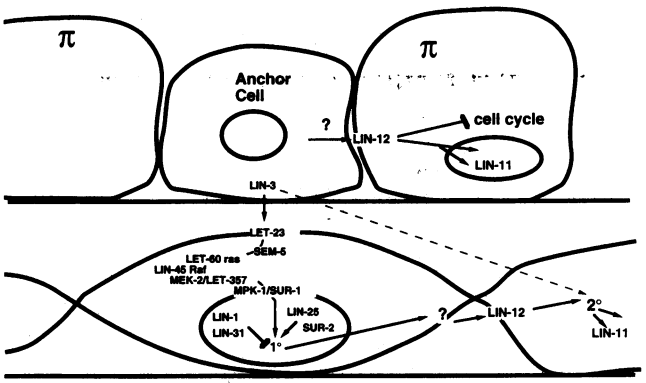

FIG. 3. Genes mediating AC signaling of vulval and π cell fates. The VU-derived cells in contact with the AC respond to LIN-12 by activating transcription of *lin-11*, undergoing one instead of two rounds of cell division, and generating specialized uterine cells (utse and uv1). The ligand for LIN-12 in this induction is not known. The 1° VPC is induced by the growth factor LIN-3 produced by the AC. LIN-3 acts via LET-23 and a Ras-mediated signal transduction pathway, resulting in specification of 1° differentiation. The pathway is complex once the nucleus is reached. LIN-1 and LIN-31 act negatively and LIN-25 and SUR-2 act positively on vulva differentiation. Specification of 2° VPC requires LIN-12, activated by a signal from the 1° VPC or in response to LIN-3 from the AC. One of the characteristics of 2° VPCs is transcription of LIN-11 by some of the 2° progeny.

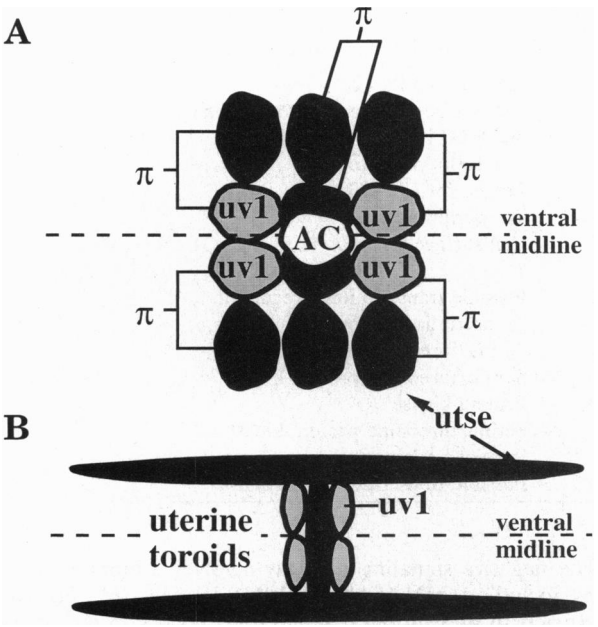

FIG. 4. Patterning and differentiation of π cell progeny, ventral view. (*A*) The ventral distal π daughters become uv1 (light grey), whereas all other π daughters become utse (dark grey). (*B*) Differentiation of the utse involves cell shape changes and growth away from the site of induction.

uterus and vulva. The asymmetric π cell division may contribute to the differentiated fate of the progeny, but cannot be the sole determinant of fate; while all dorsal daughters become utse, only some ventral daughters become uv1 (the proximal daughters become utse).

π cells and vulval 2° cells: Similarities in specification programs? Both π cells and vulval 2° cells generate asymmetric lineages. In addition, both are induced in response to activity of the receptor LIN-12. Three genes required for execution of the vulval 2° cell lineage have been identified: *lin-11*, *lin-17*, and *lin-18*. Mutations in *lin-11* result in a symmetric lineage, while a *lin-11*::*lacZ* reporter construct is expressed in the 2° cell progeny that require it (39–41). *lin-11* is one of the founding members of the LIM domain family of homeodomain-containing transcription factors (42). The *lin-11*::*lacZ* reporter construct is also expressed in the π cells and their progeny, but not in other somatic uterine cells (A.P.N., G. Acton, R. Horvitz, and P.W.S., unpublished results). In both cases, *lin-11*::*lacZ* expression is induced in response to increased activity of the *lin-12* receptor. Since, by this assay, *lin-11* is not transcribed in response to *lin-12* activity downstream of the AC versus VU decision, activation of *lin-11* appears to constitute part of the specific response of π cells and 2° cells to LIN-12 (Fig. 3). In both the π and 2° lineages, LIN-11 functions as only part of the response to LIN-12. LIN-11 appears to be necessary for proper utse differentation but not for inhibition of the π cell cycle (unpublished results). It remains to be determined whether this is part of a more extensive signal transduction cassette common to π and 2° cells and the relationship of *lin-11* expression to their asymmetric lineages.

Lumen Formation and Connection. The developing *C. elegans* egg-laying system provides a model to study tubulogenesis. Here, we discuss first formation of the uterine lumen and then connection between the uterine and the vulval lumens. Formation of the vulval lumen has been examined by J. White (unpublished results) and will not be discussed here. The *C. elegans* hermaphrodite uterus is bilobed with each lobe being composed of four toroidal cells ut1-ut4 (ref. 11; see Fig. 1*D*). Each toroid results from the fusion of the DU and VU descendants at a given anterior-posterior position. Uterine cell fusions and lumen formation occur during mid-L4, a time when the uterus is thus undergoing dramatic morphological changes.

In contrast to the ut cells in the uterine lobes, the uterine epithelium in the central region just dorsal to the vulva is not toroidal. Rather, it is composed of a dorsal uterine cap, the du cell, and eight mononucleate ventral cells, the uv2 and uv3 cells (ref. 11; Fig. 1*D*). This leaves a hole in the bottom through which eggs can be laid. The altered geometry reflects functional constraints.

The utse is a multinucleate cell formed by fusion of eight of the π progeny and the AC. Most of the cell is outside the uterine epithelium (Fig. 4*B*). The utse extends cytoplasmic process laterally away from the site of induction, thus forming an H-shaped cell (Fig. 4*B*). The two sides of the H attach the outside of the uterine epithelium to the lateral epidermis (seam) of the animal and hold it in place, preventing it from everting. Differentiation of the utse is critical to forming a functional uterine–vulval connection; the central portion of the H forms a thin laminar process just dorsal to the vulva that (in contrast to the developing uterine tissue; Fig. 1*C*) is thin enough to be broken when the first egg is laid (Fig. 1*D*).

The utse fuses with the AC, i.e., a subset of the π cell progeny fuse with the very cell that induced them (Fig. 2, compare *A* with *B*). This reciprocal cell–cell interaction illustrates how an inducing cell can be removed when its function is complete. During the time that it is inducing the vulva and the uterine π cells, the AC serves as a barrier and does not permit the other cells in the two tissues to contact one another. This may be important in allowing the cells in the two tissues to differentiate independently. Subsequently, when the AC fuses with the utse, this terminates its distinct identity, including as a barrier. Then, when the utse differentiates to form a thin laminar process dorsal to the vulva, a functional connection is made.

CONCLUSIONS

Formation of a uterine–vulval connection requires coordination of a number of cell types in two different tissues. Several general mechanisms may help to unify this process.

The Multifaceted Role of the AC. The AC induces both the vulva and all the uterine cells that specifically make a connection with it. The AC establishes the initial contact with the vulval cells; genes necessary for this initial connection have been identified and might help elucidate the role of the AC in remodeling the extracellular matrix (R. Palmer and P.W.S., unpublished observations). Ultimately, the AC fuses with a subset of the uterine progeny that it has induced. The critical role of the AC is highlighted by its role in another nematode of the genus *Mesorhabditis* (43, 44); while it is no longer necessary to induce the vulva, it is necessary for the elongation of the gonad toward the vulva. The vulva forms posteriorly in the animal and the AC leads the developing gonad to the site of the vulva.

Use of Common Signaling Proteins in Multiple Contexts. The developing vulva and uterus use two common types of signaling pathways: (*i*) a receptor tyrosine kinase pathway using the ligand LIN-3 and receptor LET-23 and (*ii*) a Notch pathway using the receptor LIN-12.

LIN-12 is used twice in the ventral uterus, first in lateral signaling and then in a unidirectional inductive interaction. LIN-12 is also required to specify the 2° fate in the vulva. Specification of the vulval 2° fate and uterine π fate both result in expression of the predicted transcription factor LIN-11. We speculate that there are common features to their differentiation that are regulated by *lin-11*. In the π cell fate decision, LIN-12 leads to fewer rounds of cell division and to production of specialized progeny and thus is not acting to prevent differentiation as it may be in some instances (21). In other words, the physiological meaning of activated LIN-12 is con-

text-dependent, and might not be uniquely coupled to one cell biological regulatory process.

The LET-23 pathway is also used multiple times during development. In some cases, the same signaling pathways downstream of the receptor are used but with different outcomes; LET-23 induces vulval differentiation in the hermaphrodite and specific neurectoblast fates during male spicule development (45). In another case, stimulation of hermaphrodite fertility, a distinct signaling pathway apparently is used (ref. 46; T. Clandinin, G. Lesa. and P.W.S., unpublished observations). Further study of the specific contexts using both LIN-12 and LET-23 may well help elucidate the determinants of signaling specificity.

Reciprocal Cell–Cell Interactions. Two aspects of the uterine–vulval connections involve reciprocal cell–cell interactions. The first is in relative positioning of the AC and VPCs. The AC induces the VPCs, which divide, and a subset of the progeny signal back to the AC. The second is induction of the π cells by the AC followed by fusion of some π cell progeny (which make utse) with the AC. Such reciprocated interactions may be a generally important mechanism to coordinate the development of complex structures. Where such reciprocal cell interactions are sequential, one outcome of the first signal might be to induce transcription of the second signaling pathway.

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