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Anchor Cell Invasion into the Vulval Epithelium in *C. elegans*

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Summary

An understanding of cell-invasive behavior has been limited by the lack of in vivo models where this activity can be clearly visualized and manipulated. We show that a single cell in the *Caenorhabditis elegans* gonad, the anchor cell (AC), initiates uterine-vulval contact through a cell invasion event. Using genetic analysis, laser ablations, and cell-specific markers, we demonstrate that AC invasion is predominantly stimulated by the 1° vulval lineage cells, which generate a diffusible signal that promotes AC invasive behavior toward these cells and further targets invasive processes between the two central 1° vulval lineage cells. We also show that AC invasion is regulated by the AC response to this cue, as well as a vulval-independent mechanism that weakly drives invasion. These studies dissect the regulatory mechanisms that underlie a simple cell-invasive behavior in vivo, and introduce AC invasion as a model for understanding key checkpoints controlling cell invasion.

Introduction

The regulation of cells associating with, removing, and then traveling through basement membranes, an activity known as cell-invasive behavior (Stetler-Stevenson et al., 1993), plays critical roles in blastocyst implantation, angiogenesis, and organogenesis (Cross et al., 1994; Pepper, 1997; Affolter et al., 2003). Loss of control over invasive activity is also associated with numerous human pathologies, including metastatic cancer (Folkman and Shing, 1992; Cross et al., 1994; Hanahan and Weinberg, 2000). The regulatory mechanisms that control invasive behavior, however, have been difficult to elucidate (Hanahan and Weinberg, 2000), in part because of the challenge of dissecting cell-invasive activity in complex in vivo environments (Condeelis et al., 2001; Keely, 2001).

Formation of the uterine-vulval connection in *Caenorhabditis elegans* provides a simple, visually accessible model for examining mechanisms underlying the connection of independently developing tissues. While much is known about cell fate specification of the cells that participate in uterine-vulval attachment (Newman et al., 1995, 2000; Chang et al., 1999; Hanna-Rose and Han, 1999; Wang and Sternberg, 2000; Palmer et al., 2002) and about the formation of the mature connection (Newman and Sternberg, 1996; Newman et al., 1999; Vogel and Hedgecock, 2001; Hanna-Rose and Han, 2002), the initial morphogenetic mechanisms that bring the uterine and vulval tissues together is not known. Prior to attachment, uterine and vulval cells are separated by a basement membrane covering each tissue (White et al., 1976; Kramer, 1994), such that the uterine and vulval cells shift in position relative to one another during movement of the animal (Sulston and White, 1980). These membranes present a barrier for the proper alignment and attachment of cells forming the uterine-vulval connection. Nomarski optics and electron micrograph studies indicate that a single specialized cell in the gonad, the AC, is the first uterine cell to contact the vulval cells; specifically, the descendants of the 1°-fated vulval precursor cell (VPC) P6.p (Sulston and White, 1980; Sharma-Kishore et al., 1999). The mature uterine-vulval connection ultimately forms around the AC, which plays a key role in directing the specification of the cells that form the connection (Newman and Sternberg, 1996). These observations, however, suggest the AC may also have a morphogenetic role in initiating uterine-vulval attachment. Consistent with this possibility, laser ablation of the AC just prior to and during initial contact with the P6.p descendants results in failure to make a uterine-vulval connection (Kimble, 1981; Wang and Sternberg, 2000).

We have visualized AC behavior in the early stages of attachment using AC-specific and basement membrane markers. Prior to uterine-vulval connection, the basement membranes between the AC and vulval cells are intact and the AC is closely associated with the gonadal basement membrane. During the mid-to-late L3 stage, however, both basement membranes are lost precisely at the site of contact with the AC. The basolateral portion of the AC then crosses through this gap and attaches to and then inserts between the two central descendants of the 1°-fated P6.p cell. These observations demonstrate that the AC establishes contact with the vulval cells through a cell invasion event.

Using genetic mutations, targeted laser ablations, and cell-specific markers, we have begun to dissect the regulatory mechanisms that control AC invasion. We find that the underlying 1°-fated vulval cells play a critical role in stimulating AC invasion at the mid-to-late L3 stage and that a less potent vulval-independent mechanism also drives invasion. We show that isolated 1° vulval cells generate a diffusible signal that promotes AC invasion toward these cells and that this or an additional cue further directs invasion between the central cells of this lineage. We also demonstrate that the competence of the AC to respond to the vulval invasion signal is regulated. Our findings reveal a set of regulatory mechanisms that control a simple cell invasion event in vivo and offer an experimental paradigm for examining the distinct steps that guide a regulated cell-invasive behavior.

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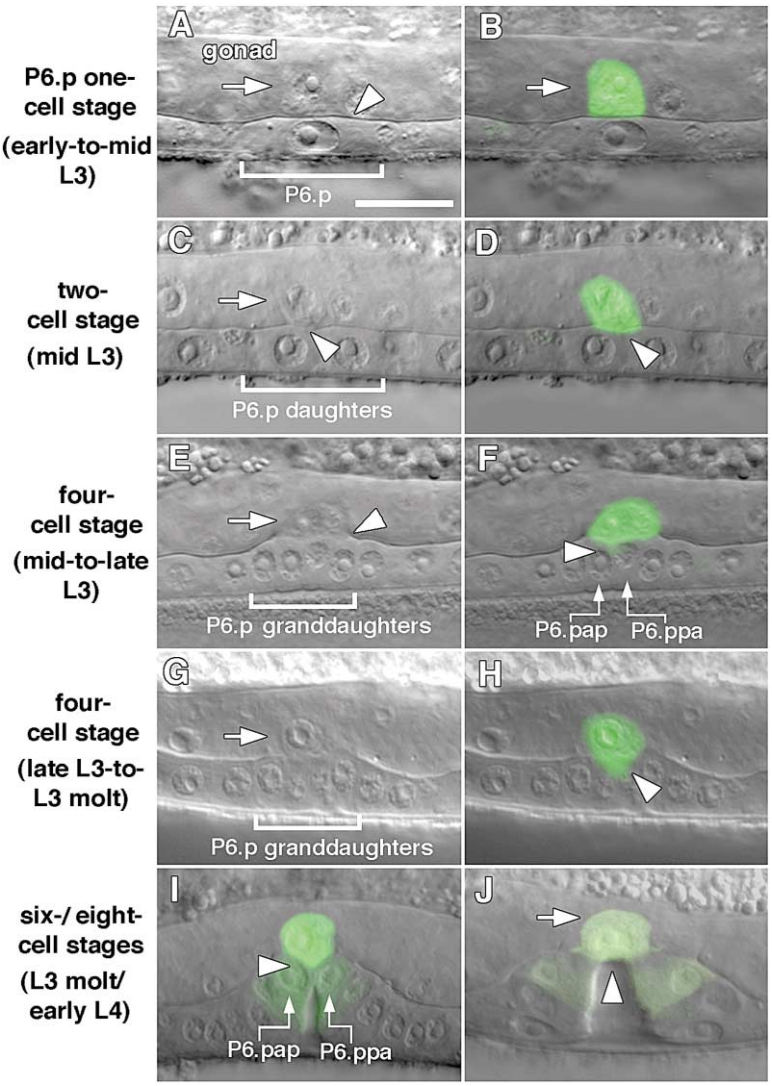

Figure 1. AC Behavior During Formation of the Uterine-Vulval Connection

All panels are viewed with Nomarski optics; (B), (D), (F), (H), (I), (J) are overlaid with the GFP fluorescent signal from the *cdh-3*::GFP expression in the AC. Anterior is to the left, ventral is down, and the scale bar is 10 μm for this and all subsequent figures.

(A and B) At the P6.p one-cell stage, the AC (arrow) is directly over the P6.p cell (bracket denotes P6.p nucleus, which is located at the center of the cell) and does not cross the putative basement membranes that separate the gonad and VPCs (arrowhead in [A]).

(C and D) At the late P6.p two-cell stage, the basement membranes under the AC (arrowhead in [C]) now appear interrupted over the P6.p daughters, and the basolateral portion of the AC crosses the basement membrane at this site (arrowhead, [D]).

(E and F) After the P6.p granddaughters have formed (P6.p four-cell stage), the putative basement membrane region is interrupted (arrowhead, [E]) over the central granddaughters at the site of AC contact. GFP fluorescence further reveals the extension of a fine process from the AC between the P6.pap and P6.ppa cells (arrowhead, [F]).

(G and H) The AC (arrow, [G]) remains over the P6.p granddaughters as they invaginate, and the AC invasive process broadens (arrowhead, [H]).

(I) The basolateral portion of the AC continues to invade (arrowhead) between the central P6.pap and P6.ppa cells after the outer P6.p granddaughters have divided transversely (P6.p six-cell stage) and are no longer within the plane of focus. Some vulval cells also begin to express *cdh-3*::GFP at this stage (arrows).

(J) By the P6.p eight-cell stage, the AC has completed invasion in this animal and moved through the inner P6.p granddaughters to the apex of the vulva (arrowhead). The P6.pap and P6.ppa cells have divided transversely and moved out of the plane of focus. The apical end of the AC remains within the uterus (arrow).

Results

The AC Initiates Contact with the Vulval Cells through a Cell-Invasive Behavior

To examine AC behavior during uterine-vulval attachment, the AC was visualized in animals containing a *cdh-3*::green fluorescent protein (GFP) promoter fusion transgene (*syIs50*; Inoue et al., 2002). *cdh-3* is a cadherin family member with no apparent function in the AC, but its promoter drives high levels of GFP expression there from the L2 molt until the early L4 larval stage (Pettitt et al., 1996). AC behavior over time was classified with respect to the number of divisions that the 1°-fated VPC, P6.p, had undergone. At the early-to-mid L3, the AC was positioned dorsal to (over) the P6.p cell (P6.p one-cell stage), and there was no apparent contact between the two cells (20/20 animals; Figures 1A and 1B); as seen under Nomarski optics, a distinct line, likely representing the juxtaposed gonadal and ventral epidermal basement membranes, separated the AC and P6.p cell. After division of the P6.p cell (P6.p two-cell stage), the AC remained over the daughter P6.pa and P6.pp cells and in most cases did not attach to these cells (16/21 animals), as evidenced by independent sliding of uterine and vulval tissues alongside one another. Occasionally, however, a process from the basolateral side of the AC interrupted the distinct line separating the uterine and vulval precursor cells and attached to the underlying P6.p daughter cells (5/21 animals; Figures 1C and 1D), impeding the independent movement of uterine and vulval cells. At the mid-to-late L3 when the P6.p granddaughters form (P6.p four-cell stage), the AC attached to the central P6.pap and P6.ppa cells, correlating with a well-defined break in the putative basement membrane (21/21 animals; Figures 1E and 1F). Furthermore, the AC extended a narrow process between these cells, which broadened after invagination of the P6.p granddaughters (21/21 animals; Figures 1G and 1H). Analysis of ten

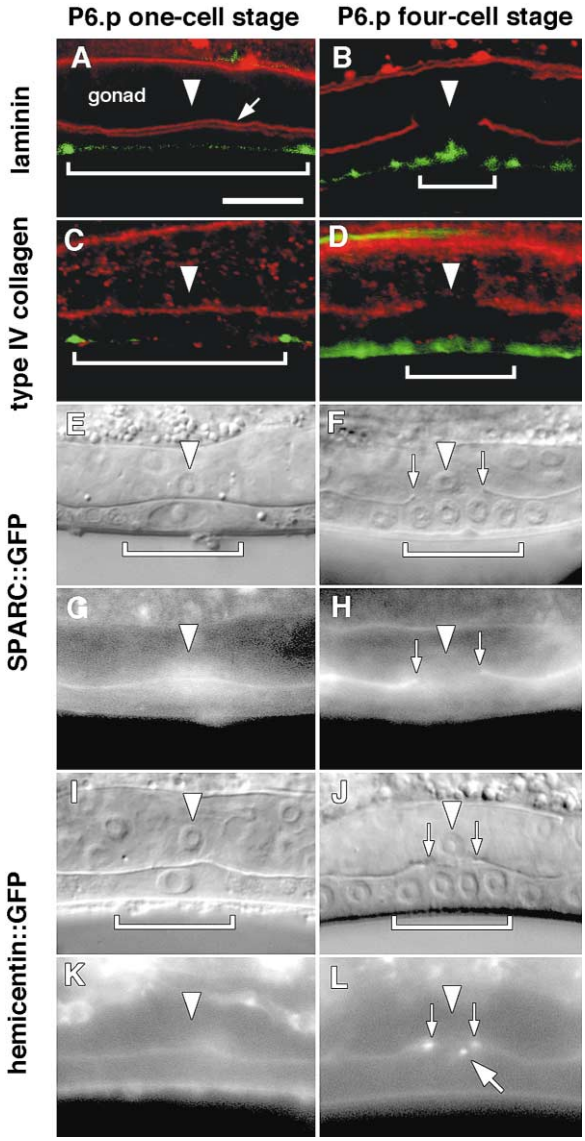

Figure 2. Major Components of the Basement Membrane Are Lost Specifically under the AC during Attachment

(A) At the P6.p one-cell stage, laminin staining (arrow, red) is continuous between the P6.p cell (bracket, cell borders labeled in green with an antibody specific to the apical adherens protein AJM-1) and the AC, which is positioned over the center of the P6.p cell (arrowhead denotes inferred location; see Figures 1A and 1B). The gonadal and ventral epidermal basement membranes in this animal have separated, revealing continuous laminin staining along both.

(B) At the P6.p four-cell stage, laminin is lost over the central P6.p granddaughters (bracket), corresponding with the site of AC contact (arrowhead; see Figures 1E and 1F).

(C and D) Similarly, type IV collagen (red) is intact between the P6.p cell (bracket) and the AC (arrowhead) at the P6.p one-cell stage (C), but lost at this location (D) at the P6.p four-cell stage.

(E–H) Nomarski images (E and F) and GFP fluorescence (G and H) of SPARC::GFP transgenic worms show that SPARC::GFP under the AC (arrowhead) is intact at the P6.p one-cell stage (bracket, nucleus), but is specifically lost at the site of AC contact (arrows mark borders of AC attachment) at the P6.p four-cell stage.

(I–L) Nomarski images (I and J) and GFP fluorescence (K and L) of hemicentin::GFP transgenic worms reveal that hemicentin::GFP is intact under the AC (arrowhead) at the P6.p one-cell stage (bracket, nucleus), but is lost at the site of AC contact (small arrows denote borders of attachment) at the P6.p four-cell stage. Large aggregates of hemicentin::GFP (large arrow in [L]) were often observed during clearing of hemicentin::GFP under the AC.

animals visualized from the early P6.p two-cell through the four-cell stage revealed that the AC attached within a 1 hr window between the late P6.p two-cell and early four-cell stages. During the L3 molt when the P6.paa and P6.ppp cells divide (P6.p descendants distal to the AC; P6.p six-cell stage), the basolateral portion of the AC continued to extend between the innermost cells, P6.pap and P6.ppa (21/21 animals; Figure 1I). At the early L4 when the P6.pap and P6.ppa cells divide (P6.p eight cell stage), the AC completed invasion through the vulval cells to the apex of the invaginating vulva in most animals (15/21 animals; Figure 1J). Shortly after this time, the AC fuses with daughters of the neighboring uterine π cells to form the multinucleate utse cell, which participates in establishing the mature uterine-vulval connection (Newman et al., 1996).

A basement membrane covers the gonad and ventral epidermis (White et al., 1976; Kramer, 1994). To determine if changes occur in basement membrane distribution at the site of AC contact with the P6.p descendants, the localization of laminin and type IV collagen was analyzed in fixed worms using specific antibodies, and GFP fluorescence was examined in animals expressing GFP translational fusion genes to SPARC (*syEx564*; Fitzgerald and Schwarzbauer, 1998) and hemicentin (*rhIs23*; Vogel and Hedgecock, 2001). At the P6.p one-cell stage, laminin, type IV collagen, SPARC::GFP, and hemicentin::GFP were each present in an uninterrupted line between the AC and P6.p cell (20/20 animals for each protein; Figure 2, left panels), demonstrating that the AC and P6.p cells were in contact with an intact basement membrane lying between them. In contrast to the P6.p one-cell stage, at the P6.p four-cell stage, laminin, type IV collagen, SPARC::GFP, and hemicentin::GFP were all absent specifically at the site of AC contact with the P6.pap and P6.ppa cells (20/20 animals for each protein; Figure 2, right panels). These simultaneous observations of AC behavior and basement membrane composition show that the AC initiates contact with the vulval cells through a stepwise cell-invasive behavior: (1) the AC is initially in contact with the gonadal basement membrane, (2) the gonadal and ventral epidermal basement membranes are then specifically lost under the AC, and finally (3), the basolateral portion of the AC crosses through the hole in the basement membranes and penetrates between central 1°-fated vulval cells. The initiation of AC invasion within a precise 1 hr window at the mid-to-late L3 and targeting between the innermost 1° vulval cells further indicate that AC invasion is temporally and spatially regulated.

The Vulval Cells Facilitate AC Invasion

To determine if AC invasion is regulated by surrounding cells, we first ablated neighboring gonadal cells in *cdh-3*::GFP transgenic animals. These ablations were carried out such that all other gonadal cells were killed by the

time of AC specification at the L2 molt (see Experimental Procedures). Ten of 11 isolated ACs invaded normally into the underlying P6.p descendants, strongly suggesting that gonadal cells neighboring the AC do not regulate AC invasion.

We next tested whether the underlying vulval cells regulate AC invasion. The entire vulva is derived from a single 1°- and two 2°-fated VPCs, which give rise to lineages of eight and seven cells, respectively, with distinctive division and gene expression patterns (Sulston and Horvitz, 1977; Inoue et al., 2002). Specification of these VPCs to a vulva fate is dependent on LIN-3, a protein similar to mammalian epidermal growth factor, which is produced by the AC during the late L2 to early L3 larval stage. High levels of LIN-3 activate a receptor tyrosine pathway in the nearest VPC, P6.p, specifying it to adopt a 1° fate. Subsequently, the neighboring VPCs, P5.p and P7.p, are specified by the activity of the receptor LIN-12, a Notch homolog, presumably because P6.p expresses a LIN-12 ligand (reviewed in Greenwald, 1997). Without the inductive LIN-3 signal from the AC, all VPCs divide once and then adopt a nonvulval 3° fate and contribute to the external epithelium that covers the animal (Sulston and White, 1980; Kimble, 1981). We thus examined AC behavior in vulvaless *lin-3*(*n1059*)/*lin-3*(*n378*) animals (Liu et al., 1999) containing the *cdh-3*::GFP transgene to determine if induced VPCs or their descendants regulate AC invasion. Because these animals lack vulval cells, the age of the AC was determined by tracking neighboring ventral uterine cell (VU) divisions and the degree of reflection in gonad arm development (Kimble and Hirsh, 1979; Newman et al., 1996). In no case did the AC invade during the early-to-mid L3 without LIN-3 induced VPCs (equivalent of P6.p one-cell stage; 30/30 animals), indicating that in wild-type animals, induced VPCs do not inhibit precocious invasion. Indeed, only 20% of ACs invaded into the underlying epidermis in vulvaless animals at the appropriate time for invasion at the mid-to-late L3 (6/30 animals; Figures 3A and 3B), suggesting that induced vulval cells stimulate invasion. The lack of invasion was not due to reduction in LIN-3, as VPC removal by laser ablation at the L1 molt similarly resulted in only 24% of ACs invading (6/25 animals). Examination of laminin, hemicentin::GFP, and SPARC::GFP expression in vulvaless animals confirmed that the basement membrane remained intact under ACs that failed to invade (≥20 animals for each protein; Figures 3C and 3D).

AC invasion was also followed later in development in vulvaless animals. ACs that invaded at the mid-to-late L3 stage in most cases maintained invasion through the early L4 (9/11 animals). Also, there was no significant increase in the percentage of ACs that had invaded from the late L3 to the early L4 stages (20% and 23% invasion, respectively; 30 animals examined for each stage; $p = 1$, Fisher's exact test), suggesting that invasion is not simply delayed. Consistent with a loss of invasive ability, most ACs that failed to invade either detached or were detaching from the gonadal basement membrane (15/23 animals; Figures 3E and 3F). Taken together, these experiments demonstrate that the vulval cells play a predominant role in stimulating AC invasion at the mid-to-late L3 stage and that there exists a concurrent, but less robust, vulval-independent mechanism that drives AC invasion (Figures 3G and 3H).

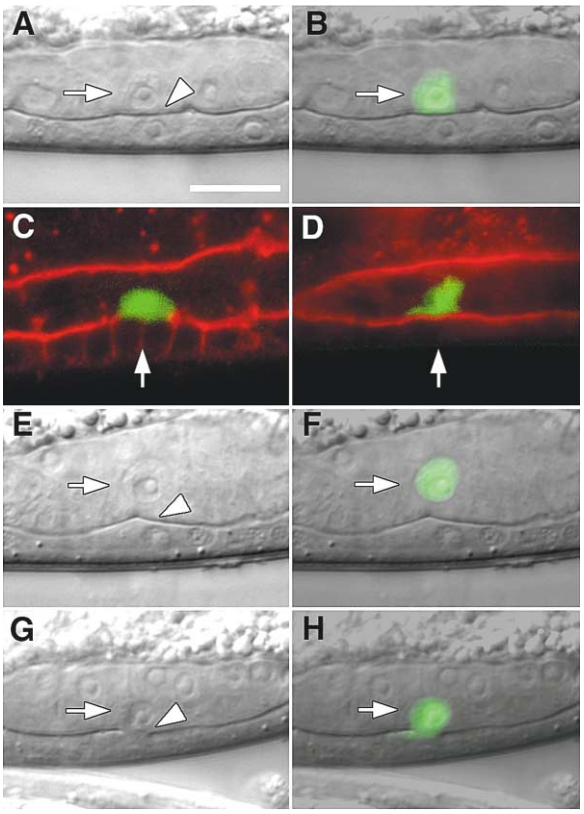

Figure 3. The Vulval Cells Play a Predominant Role in Stimulating AC Invasion

(A and B) A Nomarski image and *cdh-3*::GFP overlay, respectively, show an AC (arrow) in a vulvaless *lin-3*(*n1059*)/*lin-3*(*n378*) animal at the mid-to-late L3 stage that has not invaded into the underlying epidermis. Note the intact basement membrane under the AC (arrowhead).

(C) Laminin staining (red) and fixed GFP fluorescence (green) in a *cdh-3*::GFP transgenic wild-type animal reveal a loss of laminin directly under the AC (arrow) at the mid-to-late L3.

(D) In contrast, laminin remains intact under the AC in a similarly staged vulvaless animal where the AC has failed to invade (arrow).

(E and F) A Nomarski image and a *cdh-3*::GFP overlay of an early L4 vulvaless animal shows an AC (arrow) that has detached from the basement membrane (arrowhead) and has been displaced dorsally in the gonad.

(G and H) In approximately 20% of vulvaless animals, the basolateral portion of the AC still crosses the basement membrane (arrowhead) and invades into the underlying nonvulval epidermis.

The 1° but Not the 2° Vulval Lineage Promotes AC Invasion

To determine which induced vulval cells are required for stimulating AC invasion, we first examined whether isolated 1° lineages were capable of promoting invasion. All VPCs except for P6.p were ablated at the L1 molt in animals carrying an *egl-17*::GFP transgene (*ayIs4*). *egl-17* encodes a fibroblast growth factor with no apparent role in AC invasion, and its promoter drives expression of GFP specifically in 1°-fated VPCs and their descendants (Burdine et al., 1997, 1998). All isolated P6.p descendants expressed *egl-17*::GFP at the mid-to-late L3 and

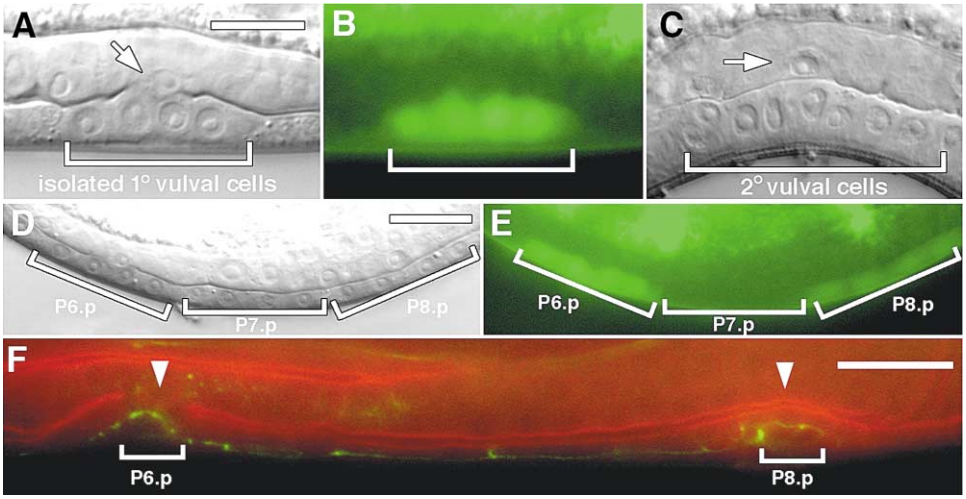

Figure 4. The 1° Vulval Lineage Promotes AC Invasion, but Not through the Direct Removal of the Basement Membrane

(A and B) An animal with isolated P6.p descendants at the four-cell stage. The AC (arrow) invades normally into the isolated P6.p descendants (bracket), which express the 1° fate marker *egl-17*::GFP ([B], bracket).

(C) An AC (arrow) overlying 2° vulval cells in a *lin-10*(*e1439*); *lin-12*(*n137*)/*lin-12*(*n137n720*) mutant does not invade into the underlying 2° vulval cells.

(D and E) A Nomarski image (D) and *egl-17*::GFP expression (E) in a *lin-15*(*e1763*) mutant demonstrates that the P8.p descendants express the 1° fate marker *egl-17*::GFP. P8.p 1° fate specification appears slightly delayed in *lin-15* mutants, thus levels of *egl-17*::GFP expression lag that of P6.p descendants.

(F) Expression of laminin (red) in a *lin-15*(*e1763*) L3 molt animal reveals that laminin expression (arrowheads) remains intact over the 1°-fated P8.p descendants (stained with anti-AJM-1 staining in green), but is lost over the P6.p descendants where the AC is located.

stimulated AC invasion between the P6.pap and P6.ppa cells normally (30/30 animals; Figures 4A and 4B).

We next examined whether 2° vulval cells are capable of stimulating AC invasion using *lin-10(e1439); lin-12*(*n137*)/*lin-12*(*n137n720*) animals, in which the VPCs adopt only 2° fates (Sternberg and Horvitz, 1989). Similar to AC invasion in vulvaless animals, invasion occurred in 20% of late L3 to early L4 animals containing only 2° vulval cells (4/20 animals; Figure 4C). These experiments demonstrate that the 2° lineage does not account for the stimulation in AC invasion seen with vulval cells and that the 1° lineage alone is responsible.

The 1° Lineage Does Not Stimulate AC Invasion by Removing the Basement Membrane

Disruption of the basement membrane has been shown to stimulate invasive cellular behavior in *C. elegans* germ cells, which are normally noninvasive (Huang et al., 2003). To determine if 1° vulval cells stimulate AC invasion by directly removing the basement membrane, we examined the basement membrane in *lin-15(e1763)* mutants, which produce ectopic 1° lineages that lack an overlying AC. While only P5.p, P6.p, and P7.p form vulval cells in wild-type animals, outlying P3.p, P4.p, and P8.p ectoblasts also have this potential (Sulston and White, 1980; Sternberg and Horvitz, 1986). These VPCs distal to the AC normally adopt a nonvulval epidermal fate because the *lin-15* gene suppresses vulval development in the absence of LIN-3 (Ferguson and Horvitz, 1989; Huang et al., 1994). In *lin-15* mutants, cell division patterns have indicated that in most cases P8.p adopts a 1° fate (Sternberg, 1988). Confirming this observation, we found that almost all P8.p granddaughter cells in *lin-15(e1763)* hermaphrodites expressed the early 1° fate marker *egl-17*::GFP (22/23 animals; Figures 4D and 4E; see also Burdine et al., 1998). We thus examined laminin, type IV collagen, SPARC::GFP, and hemicentin::GFP localization dorsal to 1°-fated P8.p descendants in *lin-15* mutants and found all four basement membrane proteins intact in late L3 to early L4 animals (20/20 animals per protein; Figure 4F). Therefore, the 1° vulval cells do not stimulate invasion by directly disrupting the basement membrane.

The 1° Vulval Lineage Stimulates Invasive Behavior with a Diffusible Cue

Human breast cancer cell metastasis appears to be targeted to certain organs through secreted chemokines that stimulate cell-invasive behavior (Müller et al., 2001). To examine whether 1° vulval cells similarly promote AC invasion with a diffusible cue, we ablated all VPCs during the early-to-mid L2 except for the P8.p cell. Under these conditions, the isolated P8.p cell apparently receives LIN-3 signal and often takes on the 1° fate (as judged by cell division patterns), and the descendants of this cell move toward the AC (Sulston and White, 1980; Sternberg and Horvitz, 1986). Consistent with this finding, the descendants of 54 of the 60 P8.p cells isolated by laser ablation expressed the 1° fate marker *egl-17*::CFP (cyan fluorescent protein, *syIs59*; Inoue et al., 2002) and moved toward or under the AC by the early L4. To determine the response of the AC to isolated P8.p cells, we examined AC behavior in L3 to early L4 animals containing both *egl-17*::CFP and an integrated *zmp-1*::YFP (yellow fluorescent protein; *syIs77*) transgene. *zmp-1* encodes a zinc metalloproteinase with no apparent function in the AC, but its promoter drives expression of YFP in the AC (Inoue et al., 2002). As with

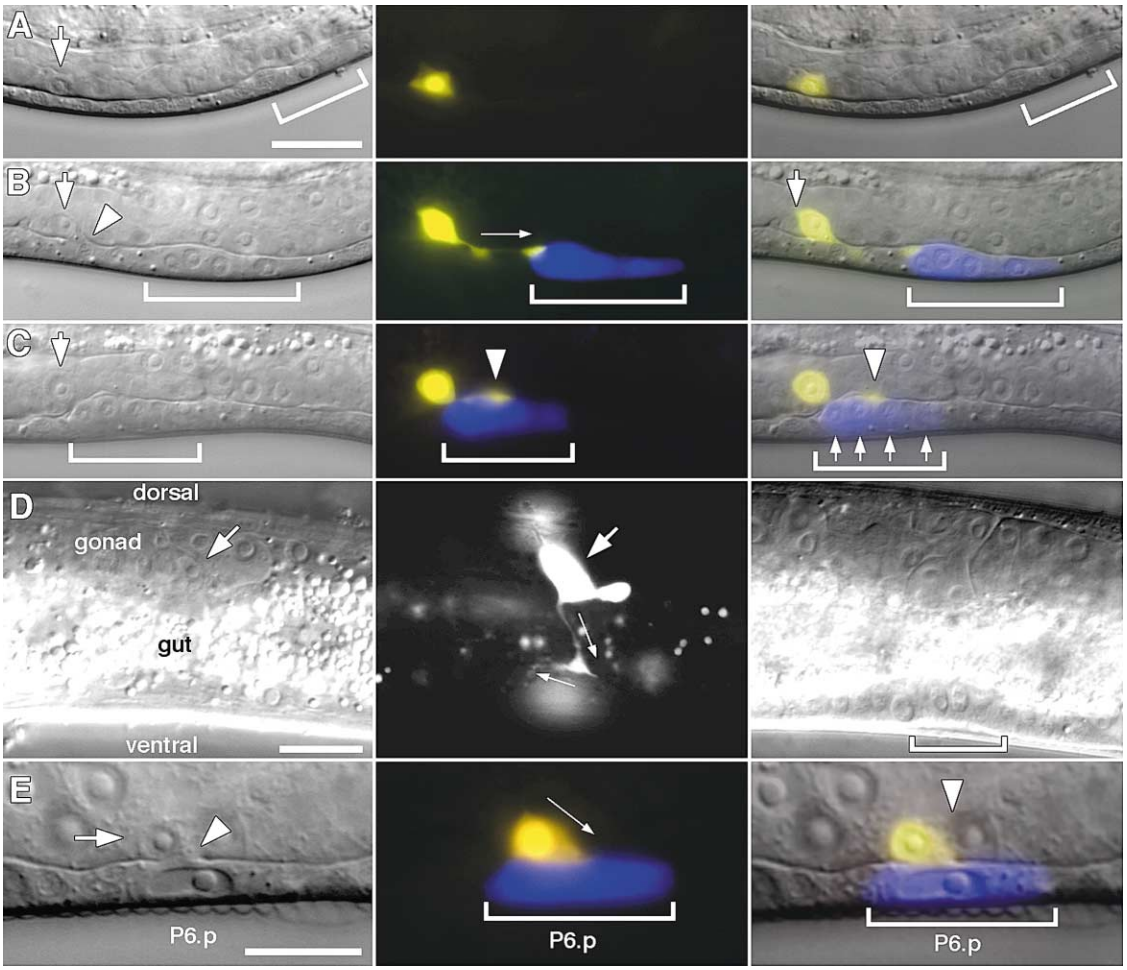

Figure 5. 1°-Fated VPCs and Their Descendants Stimulate AC Invasion through a Diffusible Cue

(A) A Nomarski image (left panel), *egl-17*::CFP and *zmp-1*::YFP expression (center), and an overlay image (right) show an isolated P8.p cell (bracket) that does not yet express *egl-17*::CFP and elicits no response from the *zmp-1*::YFP-expressing AC (arrow) in an early-to-mid L3 larva.

(B) A similar series of images shows an isolated AC (arrow, left panel) whose apical end appears anchored (arrow, right) but whose basolateral portion directs a cell-invasive process that crosses the basement membrane (arrowhead, left), extends (small arrow, center), and makes contact with the 1°-fated *egl-17*::CFP-expressing P8.p descendants (bracket) at the four-cell stage.

(C) An isolated AC (arrow, left panel) that has targeted a cell-invasive process (arrowhead in center, right) between the central 1°-fated descendants of the P8.p cell (small arrows, right).

(D) A Nomarski image (left panel) and *cdh-3*::GFP expression (center) along the lateral side of a *dig-1* mutant animal with a dorsally displaced gonad. The AC (large arrows) extends a fine process (small arrows in the center panel show direction) that travels over the gut and then turns at the ventral side toward the medial region of the animal. A medial Nomarski section (right panel) of the same animal shows that the AC process is directed toward 1° vulval cells that have begun to invaginate (bracket).

(E) A Nomarski image (left panel), *egl-17*::CFP and *zmp-1*::YFP expression (center), and an overlay image (right) show a hydroxyurea-treated larva at the normal time for AC invasion at the mid-to-late L3. P6.p cell division has been blocked, but the cell still expresses the 1° fate marker *egl-17*::CFP (bracket, center). The AC (arrow, left) contacts (arrowhead, left) and directs invasion toward the center of the P6.p cell (small arrow, center; arrowhead, right).

AC invasion in wild-type animals, no AC response to the isolated P8.p cell was observed prior to P8.p division (13/13 animals; Figure 5A). At the P8.p two-, four-, and six- to eight-cell stages, however, the majority of ACs directed cellular extensions from as far as 25 μm away toward *egl-17*::CFP-expressing P8.p progeny (79%, 11/14 animals; 95%, 18/19 animals; and 100%, 4/4 animals, respectively), which were never observed extending similar processes to the AC. Furthermore, AC extensions directed toward 1°-fated P8.p descendants often crossed the gonadal and ventral epidermal basement membranes prior to reaching these cells (45%, 5/11 animals; 83%, 15/18 animals; and 75%, 3/4 animals, respectively; Figure 5B). Similar to normal invasion, hemicentin::GFP localization in animals with isolated P8.p descendants indicated that the basement membrane was only lost directly bordering AC processes that crossed into the ventral epidermis (19/19 animals). Thus, a diffusible signal(s) generated by the 1° vulval cells both stimulates AC invasive behavior and directs invasion toward these cells. No invasive AC processes were directed toward P8.p descendants that failed to

Table 1. The AC Extends Cellular Processes toward 1° Vulval Cells in *dig-1* Mutants with Dorsally Displaced Gonads

	Number (Percentage) of ACs Responding to VPCs or Their Descendants	
VPC Development (Larval Stage)[a]	No Response	Extension of Cellular Process toward VPCs or Their Descendants
One-cell (early L3)	22 (100%)	0
Two-cell (mid L3)	26 (90%)	3 (10%)
Four-cell (late L3 to early L4)	22 (67%)	11 (33%)
Six- to eight-cell (L3 molt to early L4)	25 (66%)	13 (34%)
No vulval induction (L3 molt to early L4)	19 (100%)	0

[a] In dorsal gonads, the AC induces 1° and 2° lineages in the VPCs directly below the AC in most animals despite the distance between the cells (the distance from the AC around the gut and to the VPC descendants was calculated to be $\geq$30 μm, based on the circumference of the inner body wall of the animal where the AC and VPCs lie). At the four- to eight-cell stage, 1° fate was determined by scoring the detachment of cells from the cuticle and symmetry of invagination (Katz et al., 1995). It is possible that some one-cell and two-cell stage VPC descendants were not induced to a 1° fate, although this number should be low, as it has been shown that 75% of *dig-1* animals with dorsal gonads induce 1°-fated VPCs (Thomas et al., 1990). In *dig-1* mutants, approximately 10% of animals have no vulval induction, and these were scored at the L3 molt to early L4 stage. In general, VPC induction in *dig-1* mutants appeared delayed approximately 2 hr compared to wild-type.

express *egl-17*::CFP (6/6 animals), consistent with the idea that this diffusible invasion cue is dependent on 1°-fated vulval cells.

Because both undivided P8.p cells and P8.p descendants that were not induced to a 1° fate did not move toward the AC, the average distance from the AC was significantly greater than in animals with 1°-fated P8.p descendants (24.0 $\pm$ 0.18 μm versus 15.2 $\pm$ 0.08 μm, respectively; $p < 0.0001$, Student's t test). To determine whether lack of AC response to these cells was due to the increased distance, AC invasion was also viewed in *dig-1* mutants with dorsally displaced gonads (Thomas et al., 1990). The AC-to-VPC distance is at a fixed and greater average distance in *dig-1* mutants than in isolated P8.p cells that fail to move toward the AC (Table 1). ACs sent cellular processes only toward induced 1°-fated VPC descendants (Figure 5D; Table 1), confirming the invasion cue is specific to the 1° lineage.

In the P8.p isolation experiments, AC extensions that reached P8.p descendants further demonstrated that a signal(s) from the 1° lineage directs invasion between the central 1°-fated vulval cells. Of 28 animals in which the 1°-fated P8.p descendants did not move directly under the AC, 75% of ACs targeted invasive cellular processes between the centrally located 1° vulval cells by the early L4 (21/28 animals; Figure 5C). Notably, while AC cell bodies were often displaced in the direction of the basolateral-derived process directed toward 1°-fated P8.p descendants, the apical end of the AC was not shifted and appeared attached to neighboring uterine cells (for example, see Figure 5B). Taken together, these experiments demonstrate that: the 1° VPC descendants generate a diffusible cue(s) that stimulates a directed invasion response from the basolateral side of the AC; and this same cue or an additional signal(s) targets invasion between the centrally located 1° vulval cells.

Cell Division in 1°-Fated VPCs Is Not Required to Promote or Target AC Invasion

As we have shown, the AC invasion response is directed only toward 1° VPCs after VPC division. Hydroxyurea (HU) treatment at the L2 molt through the mid-to-late L3 arrests the VPCs in S phase, but P6.p still undergoes early steps in execution of the 1° fate (Ambros, 1999). To determine if the AC invasion signal generated by the 1° vulval lineage is linked to cell division, cell division was blocked with HU, and animals were analyzed just prior to and after the normal time for AC invasion. While no invasion was observed in animals at the early-to-mid L3 (20/20 animals), at the mid-to-late L3, 95% of ACs attached to the undivided 1° fated P6.p cells (19/20 animals; Figure 5E). Only 13% of ACs invaded in vulvaless HU-treated animals at this same time (2/16 animals), demonstrating that the 1°-fated P6.p cell was predominantly stimulating invasion. Moreover, in six of eight animals in which the AC and 1°-fated P6.p were not properly aligned, the AC directed invasion toward the center of the P6.p cell (Figure 5E). We infer that generation of the invasion cue is not linked to cell division, but rather to the time after 1° fate specification and that targeting of invasion does not rely on division of 1°-fated VPCs.

The AC's Ability to Respond to the 1° Vulval Cell Invasion Cue Is Regulated

We next wanted to determine when the AC is capable of invading in response to the 1° vulval cell-derived signal. Cell ablation experiments have indicated that the presumptive AC becomes committed to an AC fate during the L2 molt, approximately 7 hr prior to AC invasion at the mid-to-late L3 (Kimble, 1981). To determine when the AC first becomes capable of responding to the invasion cue, we examined invasion in *lin-28*(*n719*) animals, in which AC and gonad development are normal, but the VPCs are induced precociously at the L2 rather than the L3 stage (Ambros and Horvitz, 1984; Euling and Ambros, 1996). Examination of 29 *lin-28* mutant animals at 2 hr time intervals from the late L2 through the early L4 stage revealed that no ACs invaded into the 1°-fated P6.p descendants (all P6.p four- to six-cell stage) during the L2 molt, the time of AC fate commitment (Figure 6A). Later in development, however, 83% of ACs initiated invasion into the P6.p descendants (all at P6.p eight-cell stage) near the time of first division of the neighboring VU cells (24/29 ACs; Figure 6B). This time corresponds to 3–4 hr before the normal time of invasion (Kimble and Hirsh, 1979). In the remaining five animals, the AC failed to invade even by the early L4, possibly because the AC invasion signal from older P6.p eight-cell stage vulval

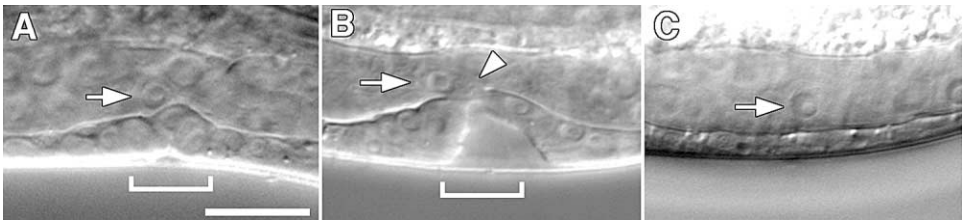

Figure 6. AC Competence to Respond to the 1° Vulval Cell-Derived Invasion Cue Is Regulated

(A) A *lin-28* mutant at the L2 molt. The recently specified AC (arrow) does not invade into the precociously induced P6.p descendants (bracket, dividing P6.p four-to-six-cell stage; compare to wild-type in Figure 1E).
(B) During the early-to-mid L3 stage, however, the AC (arrow) invades (arrowhead) into the 1°-fated vulval cells (bracket, P6.p eight-cell stage).
(C) In a vulvaless *lin-28* mutant at the same stage as (B), the AC (arrow) does not invade into the underlying epidermis.

cells is diminished. In vulvaless *lin-28* animals, no ACs invaded precociously (22/22 animals; Figure 6C), demonstrating that invasive behavior in *lin-28* mutants at this time is stimulated exclusively by the induced vulval cells. Furthermore, 16% of ACs invaded at the mid-to-late L3 stage (4/25 animals), suggesting that the vulval-independent mechanism that drives AC invasion is not affected by loss of *lin-28*. These experiments show that AC invasion is also regulated by the ability of the AC to respond to the 1° vulval cell-derived invasion signal.

Discussion

The AC Initiates Uterine-Vulval Connection through a Cell Invasion Event

The uterine and vulval cells in *C. elegans* are initially separated by distinct basement membranes, preventing the alignment and attachment of cells that form the uterine-vulval connection. We demonstrate that a specialized ventral uterine cell in the gonad, the AC, has a morphogenetic role in establishing this connection. In the early-to-mid L3, the AC is in contact with the underlying gonadal basement membrane. By the mid-to-late L3, however, there is a loss of gonadal and ventral epidermal basement membranes specifically below the AC, at which time the basolateral portion of the AC moves through this gap and inserts between the central 1° VPC descendants, P6.pap and P6.ppa. This targeted cell-invasive behavior initiates uterine-vulval attachment and likely helps to set the alignment of uterine and vulval cells that ultimately form the mature connection around the AC (Newman and Sternberg, 1996). Invasion may also be required to ensure direct cell membrane contact of the AC and the central cells of the 1° vulval lineage to properly pattern these cells, as the AC conveys a putative short-range signal at the time of invasion (Wang and Sternberg, 2000). The ability to directly visualize AC invasion and manipulate the cellular environment surrounding the AC allowed us to dissect how this spatially and temporally precise cell invasive behavior is regulated.

The 1° Vulval Cells Play a Key Role in Stimulating and Targeting AC Invasion

When all of the VPCs were removed, only 20% of ACs invaded into the underlying epidermis. The presence of only the centrally located 1°-fated vulval cells under the AC fully restored normal invasion, while flanking 2°-fated vulval cells alone under the AC did not promote invasion. Our data show that 1° vulval cells stimulate a targeted invasive response from the AC through the generation of a diffusible attractive signal. When 1° vulval cells were placed at a distance from the AC, either through dorsal displacement in *dig-1* mutants or by isolation and conversion of the P8.p cell to a 1° fate, the ACs directed invasive processes toward the descendants of these cells. Little is known about the regulatory mechanisms that control cell invasion through basement membranes during development. One possibility is that a single cue both stimulates and targets invasion. Consistent with this notion, human breast cancer cells are stimulated by and target invasive activity in vitro toward either the CCL21 or CXCL12 chemokine ligands, and blocking the receptor for the CXCL12 ligand in vivo significantly inhibits its metastasis to organs expressing CXCL12 (Müller et al., 2001).

Since AC invasion occurred at the appropriate time in approximately 20% of animals without induced VPCs, another mechanism (either an AC cell-autonomous invasion program or an unidentified extrinsic signal from nonvulval cells) also exists to help drive AC invasion at the mid-to-late L3. Together, both the 1° lineage-generated cue and the vulval-independent mechanism may act to ensure spatial and temporal accuracy of AC invasion so that a functional uterine-vulval connection is formed.

The P8.p isolation experiments also indicated that the 1° lineage provides targeting information that guides invasion specifically between the central 1°-fated VPC descendants; most AC invasive processes that reached isolated 1°-fated P8.p descendants further targeted invasion between the cell membranes of the central cells. This precise targeting likely helps secure the proper alignment of uterine and vulval cells that form the mature connection around the AC. The same signal that initiates and directs invasion toward the 1° vulval cells may also guide invasion between the central 1° lineage cells. Alternatively, an additional and possibly membrane-tethered cue(s) may participate. During *Drosophila* development, the RP3 motoneuron similarly targets a cellular extension (growth cone) between two cells (muscles 6 and 7), and this targeting is thought to involve both secreted and membrane-tethered cues (Chiba and Rose, 1998). Notably, blocking division in the 1°-fated P6.p cell did not abolish stimulation or targeting of invasion toward the center of this cell, demonstrating that

the invasion promoting and targeting mechanism is not dependent on the presence of multiple cells but is tied to the time after 1° fate specification.

The Ability of the AC to Respond to the 1° Vulval Cell Invasion Cue

The lack of AC invasion at the L2 molt in *lin-28* mutants with precocious vulval development suggests that while the AC is committed to its fate at this time (Kimble, 1981), it is not yet able to carry out cell-invasive behavior in response to the 1° vulval cell-generated cue. It is not until 3–4 hr after the L2 molt that the AC appears capable of invading into the 1° vulval cells. This delay in invasive ability may represent an active mechanism to coordinate AC invasion with 1° vulval cell development so that the uterine and vulval cells are brought together at the appropriate time to form a connection. Consistent with this notion, the timing of human blastocyst implantation is critical for successful pregnancy and appears to be regulated in part by the expression of the adhesion receptor L-Selectin, which is expressed on the trophoectoderm just prior to implantation (Cross et al., 1994; Genbacev et al., 2003). Active regulation of the ability of cells to invade may be a common mechanism to further control cell-invasive behavior in developmental processes where the timing of this activity is crucial.

The AC Displays Both Epithelial and Mesenchymal Properties

AC behavior during wild-type invasion and in response to isolated P8.p cells showed that only the basolateral portion of the AC extended invasive cellular processes toward and between 1° vulval cells, while the most apical end remained attached to neighboring uterine cells. The AC is thus polarized; the basolateral portion of the AC has migratory mesenchymal-like properties, and the apical end has cell-cell adhesion epithelial characteristics. Many invasive cells appear to undergo partial epithelial-to-mesenchymal transitions. For example, carcinoma cells often invade en masse rather than as single cells and are attached by cell-cell contacts at one end, while extending lamellae from the other (Nabeshima et al., 1999). In addition, endothelial cells that initiate vessel outgrowth maintain cell-cell junctions with neighboring endothelial cells while extending cellular protrusions that are devoid of basement membrane (Paku and Paweletz, 1991). It will thus be important to determine whether genes that have been associated with regulating partial epithelial to mesenchymal transitions facilitate AC invasion (e.g., Nabeshima et al., 1999; Rørth, 2002; Montell, 2003).

AC invasion: A Simple In Vivo Model for Examining Regulated Cell-Invasive Behavior

Our studies implicate multiple regulatory steps in controlling AC invasion. These include (1) factors promoting the ability of the AC to invade in response to the invasion cue(s); (2) production of the cue(s) by 1° vulval cells and components mediating targeting to the central cells of this lineage; (3) factors involved in the vulval cell-independent pathway that promotes AC invasion; (4) components mediating the localized removal of basement membranes under the AC; and (5) factors promoting the mesenchymal-like invasive cellular protrusions from the AC. At present, the genetic regulatory networks that control cellular invasion remain poorly understood (Hanahan and Weinberg, 2000). Our dissection of the AC invasion process provides the assays with which to identify genes that regulate and execute the distinct steps of invasion.

Experimental Procedures

Worm Handling and Strains

C. elegans strains were reared and viewed at 20°C as in Brenner (1974). The genes, alleles, and integrated transgenes used in this work were: *lin-10*(*e1439*)*I*, *lin-3*(*n1059*)*IV*, *lin-3*(*n378*)*IV*, *lin-15*(*e1763*)*X* (Ferguson and Horvitz, 1985); *lin-28*(*n719*)*I* (Ambros and Horvitz, 1984); *lin-12*(*n137*)*III*, *lin-12*(*n137n720*)*III* (Greenwald, 1985); *dig-1*(*n1321*)*III* (Thomas et al., 1990); *dpy-19*(*e1259*)*III*, *unc-32*(*e189*)*III*, *unc-31*(e169)*IV*, *unc-24*(*e138*)*IV*, *dpy-20*(*e1282*)*IV* (Brenner, 1974); *let-59*(*s49*)*IV*, *unc-22*(*s7*)*IV* (Moerman and Baillie, 1981); *rhIs23*(hemicentin::GFP)*III* (Vogel and Hedgecock, 2001); *ayIs4*(*egl-17*::GFP)*I* (Burdine et al., 1998); *syIs77*(*zmp-1*::YFP)*II*; *syIs50*(*cdh-3*::GFP)*X*; *syIs59*(*egl-17*::CFP)*X*; *syIs57*(*cdh-3*::CFP)*X* (Inoue et al., 2002).

The complete strain compositions for *lin-3*(*n1059*)/*lin-3*(*n378*) and *lin-10*(*e1439*); *lin-12*(*n137*)/*lin-12*(*n137n720*) animals were *lin-3*(*n378*) *let-59*(*s49*) *unc-22*(*s7*) *unc-31*(*e169*)/ *lin-3*(*n1059*) *unc-24*(*e138*) *dpy-20*(*e1282*); *syIs50* (strain PS3391) and *lin-10*(*e1439*); *dpy-19*(*e1259*) *lin-12*(*n137*)/ *unc-32*(*e189*) *lin-12*(*n137n720*); *syIs57* (strain PS3996), respectively.

The SPARC::GFP transgene *syEx564* was created as previously described (Fitzgerald and Schwarzbauer, 1998).

Cell Ablations

Laser-directed cell ablations were performed as previously described (Bargmann and Avery, 1995). Removal of gonadal cells surrounding the AC was performed through a two-step ablation process. First, the Z2, Z3, and Z4 cells were ablated from the gonadal primordium in early L1 animals, leaving the Z1 cell (Kimble and Hirsh, 1979). When the AC was specified by one of the Z1 descendants (Z1.ppp) at the L2 molt (Kimble, 1981), the remaining gonadal cells were then ablated. Pn.p ablations to create vulvaless animals and isolated P6.p cells were performed at the L1 molt.

Immunolocalization, Microscopy, and Image Analysis

Staged L3 and early L4 worms were obtained from starved hatchlings as described previously (Lewis and Fleming, 1995). Animals were fixed for AJM-1 and collagen type IV colocalization using a sequential methanol/acetone fixation as described (Graham et al., 1997). AJM-1 was stained with the MH27 monoclonal antibody at 1:1500. Affinity-purified rabbit polyclonal antibodies to *C. elegans* type IV collagen (anti-LET-2) were used at 1:1000. Animals were fixed for AJM-1 and laminin colocalization using methanol as described (Maloof and Kenyon, 1998). Affinity-purified rabbit polyclonal antibodies to laminin (anti-laminin αB) were used at 1:400. Cy3- and Cy2-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) were used for double labeling. For preserving GFP fluorescence and staining for laminin, larvae were fixed as described (Finney and Ruvkun, 1990), except that 2% paraformaldehyde was used and larvae were fixed for 2 hr on ice. Double stained or labeled images were obtained by sequential sectioning of stained larvae on a Zeiss 510 confocal laser scanning microscope, and these images were overlaid using Adobe Photoshop 5.0.

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