Chapter 6
Breaching and Opening Basement Membrane Barriers: The Anchor Cell Leads the Way

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Abstract Metastasis is initiated in epithelial-derived tumors when cells at the tumor front breach the epithelial basement membrane (BM). Invasion through BMs is thought to be one of the most rate-limiting steps in cancer progression and thus is a therapeutically attractive target for halting tumor spread. Despite intense interest, it has been challenging to experimentally determine how invasive cells breach and clear BM barriers, which has hindered efforts to block metastasis. Here we discuss how an experimentally tractable developmental invasion event, anchor cell (AC) invasion in the model system *C. elegans*, is offering powerful new insights into the fundamental mechanisms that invasive cells use to breach BM barriers and how cells at the breach site widen BM gaps through a new mechanism called BM sliding. Finally, we cover studies demonstrating that AC invasion can also be used as a new paradigm to examine how alterations in the tumor microenvironment impinge on cell invasive behavior.

6.1 Introduction

Basement membranes (BMs) are thin, dense, sheet-like forms of extracellular matrix that underlie all epithelia and surround blood vessels, muscles, adipocytes, and Schwann cells (Halfter et al. 2015; Yurchenco 2011). Two polymeric protein networks shape BM structure: a cell-associated assembly of laminin molecules and a polymer of type IV collagen proteins. The independent laminin and type IV collagen networks are thought to be connected through other BM proteins, including the glycoprotein nidogen and the heparan sulfate proteoglycans perlecan and agrin (Behrens et al. 2012; Fox et al. 1991; Hohenester and Yurchenco 2013). BMs provide tissues with mechanical and barrier support and harbor growth factors, differentiation signals, and polarity cues (Halfter et al. 2015; Poschl et al. 2004; Yurchenco 2011). The type IV collagen network of BMs is covalently cross-linked through multiple distinct bonds, which helps impart the mechanical and barrier properties to BMs (Fidler et al. 2014; Khoshnoodi et al. 2008; Vanacore et al.

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2009). Transmission electron microscopy studies have indicated that most BMs are approximately 50–100 nm thick; however, more recent atomic force microscopy measurements that allow for the retention of water have revealed that BMs are twofold thicker (Candiello et al. 2007; Halfter et al. 2015). The genes that encode BM proteins are ancient and emerged concurrently with animal multicellularity (Hynes 2012; Ozbek et al. 2010), suggesting that BMs were required to construct and organize tissues. An important implication from its early origins is that the mechanisms that cells use to build, maintain, and remodel BMs are likely evolutionarily conserved between animals (Sherwood 2015).

The network of laminin, type IV collagen, and associated glycoproteins that make up BMs creates a dense meshwork with a pore size ranging from roughly 10 to 130 nm, a diameter far too small for a cell to traverse without removing this barrier (Abrams et al. 2000, 2003; Rowe and Weiss 2008; Wolf et al. 2013; Yurchenco and Ruben 1987). Yet cells repeatedly cross through BMs to enter new tissues during development and normal physiological functions in a process called cell invasion or BM transmigration. For example, BM barriers are crossed during the numerous epithelial-to-mesenchymal transitions that allow cell dispersal and organ formation in development (Cheung et al. 2005; Kelley et al. 2014; Nakaya et al. 2008; Yang and Weinberg 2008). BMs are also traversed during leukocyte trafficking across vascular BMs, and endothelial cells cross the vascular BM to form new blood vessels (Seano et al. 2014). The presence of BMs thus creates a paradox in that they are crucial in providing tissue support and barrier functions; however, they must be repeatedly removed and traversed by cells during development and normal organ function. A solution to this problem has been solved in vascular BMs by the formation of specialized regions with preformed openings as well as reduced BM regions that allow leukocyte trafficking (Baluk et al. 2007; Pflicke and Sixt 2009; Voisin et al. 2009, 2010; Wang et al. 2006). In most cases of BM crossing, however, de novo BM openings must be created to facilitate BM transmigration (Kelley et al. 2014). Epithelial-derived cancers, which account for 90% of cancer-related deaths, also make openings in BMs during tumor progression (Frei 1962; Rowe and Weiss 2008). BM breaching initiates metastasis and is associated with poor patient prognosis (Barsky et al. 1983; Hagedorn and Sherwood 2011). BM invasion is thought to be one of the most rate-limiting aspects of metastasis and an attractive therapeutic target in cancer (Christofori 2006; Madsen and Sahai 2010; Nguyen et al. 2009; Steeg 2003). Thus, understanding how cells transmigrate this barrier is of great interest in order to develop new treatment strategies to curb metastasis.

Cell invasion events are often stochastic and occur deep in tissues in vertebrates. As a result, cell invasive behavior is difficult to visualize with microscopy approaches (Beerling et al. 2011). Furthermore, revealing mechanisms underlying invasion require genetic manipulation of the invading cell, the BM, and the tissue the cell is invading—a prospect that is currently prohibitively time-consuming and expensive in vertebrates. Thus, progress in defining the cellular and molecular regulators in BM cell invasion has been made largely in in vitro and sophisticated ex vivo assays using reconstituted matrices or isolated BMs (Rowe and Weiss 2008;

Schoumacher et al. 2010, 2013). These studies have identified key invasive cellular structures and molecular components associated with invasion; however, these assays do not faithfully recapitulate the in vivo cellular environment nor mimic the physiological relevant composition, cross-linking, or stiffness of cell-associated BMs (Even-Ram and Yamada 2005; Lokman et al. 2012; Rowe and Weiss 2008; Schoumacher et al. 2010, 2013; Wu et al. 2012). Thus, our knowledge of the mechanisms that facilitate the breaching and removal of BM barriers is incomplete.

The anchor cell (AC) is a specialized uterine cell in *Caenorhabditis elegans* that invades through the BM separating the uterine and vulval tissue in a highly stereotyped manner during a 90-min window (Sherwood and Sternberg 2003). AC invasion initiates the attachment of the uterine and vulval tissue during development—a connection necessary for mating and egg-laying in the adult worm. AC invasion is the first in vivo animal model that combines genetic analysis with live-cell subcellular resolution of cell-BM interactions during invasion and BM remodeling (Fig. 6.1; Hagedorn and Sherwood 2011). Many features make this an outstanding model for examining cell invasion. The predictability of this invasion event and its amenability to rapid screening make it ideal for identifying genes that control invasion (Hagedorn et al. 2009; Matus et al. 2010; Schindler and Sherwood 2011; Wang et al. 2014a; Ziel et al. 2009a, b). The structure of the *C. elegans* BM is conserved with that of vertebrates, and most components—including the major structural components laminin and type IV collagen—have been functionally tagged with GFP and GFP derivatives to allow a dynamic visualization of invasion (Fidler et al. 2014; Fitzgerald and Schwarzbauer 1998; Hagedorn et al. 2009; Hesselson et al. 2004; Kramer 2005). Further, CRISPR-mediated tagging, optical highlighting, and photo-bleaching methods allow BM components, AC-expressed proteins, and cell membrane components to be followed dynamically during invasion (Hagedorn et al. 2014; Ihara et al. 2011; Matus et al. 2014; Morrissey et al. 2014). In addition, cell-specific RNAi, cell-specific protein degradation, and temporally controlled cell-specific expression techniques help determine the underlying roles of proteins and genes in invasion and BM remodeling (Armenti et al. 2014; Hagedorn et al. 2009). Finally, methods for quantitative 4D live-cell imaging of cell-BM interactions have been developed to elucidate the cellular and subcellular mechanisms that drive BM transmigration (Hagedorn et al. 2013; Wang et al. 2014b).

This chapter focuses on AC invasion and BM hole widening after invasion during uterine-vulval attachment in the worm and how these findings have advanced our fundamental understanding of similar events in cancer. First we will review the mechanisms that regulate AC invasion, covering the transcriptional programs that prime the AC to invade and the dynamics of invasive structures that breach and clear BM barriers. Then we will discuss how the gap in the BM is widened after AC invasion to allow additional uterine and vulval cells to directly attach through a newly characterized mechanism called BM sliding. Finally, we will analyze AC invasion as a model for examining how extrinsic molecules in the microenvironment impact cell invasion, focusing on the matricellular protein

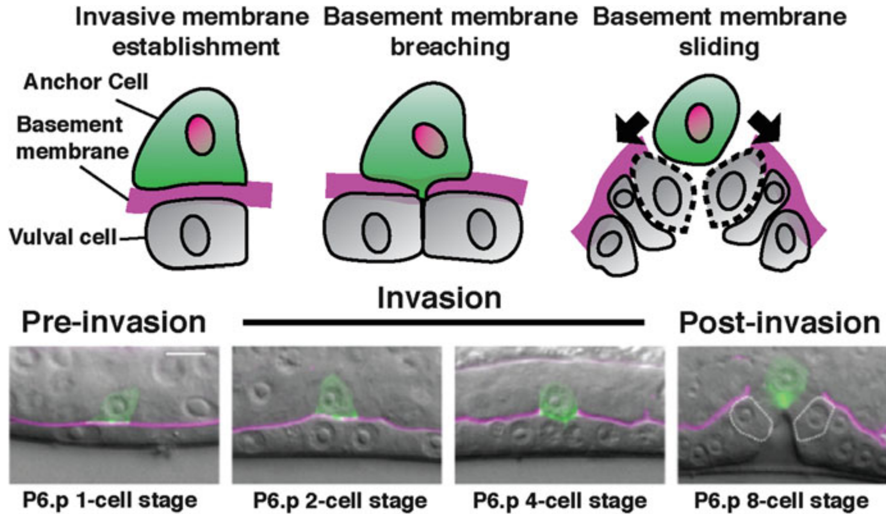

Fig. 6.1 Anchor cell (AC) invasion and basement membrane (BM) remodeling. *Top panel*: A schematic diagram highlighting the major developmental steps of AC invasion and BM remodeling during uterine-vulval attachment in *Caenorhabditis elegans* from *left* to *right*. The *dashed outline* on the *right panel* (and *panel below*) indicates the vulD cell, where the BM stops sliding. *Bottom panel*: Micrographs of AC invasion in vivo with the AC expressing a fluorescent reporter in *green* and the BM labeled with a fluorescent reporter shown in *magenta*. Invasion progresses from *left* to *right* with BM breaching occurring in the *middle panels*. AC invasion occurs in tight synchrony with the division of the underlying P6.p vulval cell and can be staged by the number of P6.p descendants (i.e., P6.p 1-cell stage, 2-cell, 4-cell, and 8-cell stages). *Scale bar* 5 μm (Image reproduced from Hagedorn and Sherwood (2011) with permission from Elsevier)

SPARC, which is overexpressed in many metastatic cancers and promotes invasive behavior.

6.2 Breaching the BM De Novo: AC Invasion

6.2.1 Transcription Factors Promoting Invasion

Cell invasion is a highly specialized process and depends on extensive changes in gene expression, such as the upregulation of actin regulators and matrix metalloproteinases, i.e., MMPs (Kelley et al. 2014; Murphy and Courtneidge 2011; Page-McCaw et al. 2007; Wang et al. 2004). The transcriptional networks that endow cells with the ability to breach BMs, however, are largely unclear. Identifying the regulatory networks that program invasiveness is crucial, as they may be the most promising therapeutic targets to broadly inhibit invasion (Ell and Kang 2013). The stereotyped nature of AC invasion and the ability to conduct large-

scale screens and characterize genes are allowing researchers to elucidate the transcriptional mechanisms that program invasiveness in cells.

The AC is first specified during the late L2 larval stage through a LIN-12/LAG-2 (Notch/Delta)-mediated lateral signaling interaction with a neighboring ventral uterine (VU) cell. In this interaction the AC upregulates the Notch ligand gene *lag-2* and is specified by the absence of Notch activation (Greenwald 2005). After the AC/VU fate decision, the AC expresses specific genes that are involved in invasion, including the protocadherin *cdh-3*, the integrin *pat-3*, and the RhoG GTPase *mig-2* (Hagedorn et al. 2009; Sherwood and Sternberg 2003; Ziel et al. 2009a). Several transcription factors, including the helix-loop-helix factor HLH-2 (vertebrate E protein) and the nuclear hormone receptor NHR-67 (vertebrate TLX), play roles in pro-AC competency, AC/VU Notch-mediated interactions (HLH-2 and NHR-67), and later pro-invasive differentiation (Karp and Greenwald 2004; Schindler and Sherwood 2011; Verghese et al. 2011). These transcription factors are expressed in the AC throughout its development and may belong to a dynamic transcriptional network that controls distinct transcriptional targets at different stages that direct both early specification and later pro-invasive differentiation of the AC (Schindler and Sherwood 2011).

Shortly after the AC's initial specification, the *nhr-67* (TLX) gene becomes upregulated and induces G1 cell-cycle arrest in the AC (Fig. 6.2; Matus et al. 2015). G1 cell-cycle arrest in development is strongly associated with cellular differentiation programs (Gonzales et al. 2015; Mummery et al. 1987; Ruijtenberg and van den Heuvel 2015). Strikingly, G1 cell-cycle arrest is similarly required for the AC to adopt features of an invasive cell (Matus et al. 2015). These characteristics include the expression of pro-invasive genes, such as the *C. elegans* MMPs *zmp-1, zmp-3*, and *zmp-6* and actin regulators, including the formin *exc-6* and the Ena/VASP ortholog *unc*-34 (Matus et al. 2015). G1 arrest is also required for the formation of invadopodia-specialized F-actin-rich subcellular protrusions that breach BMs. Consistent with this being a general feature of invasive cells, invasive ability is correlated with decreased cell proliferation in many cancer cell lines, tumor models, and human cancers, as well as with developmental invasion events (Gil-Henn et al. 2013; Hoek et al. 2008; Vega et al. 2004; Wang et al. 2004; Yano et al. 2014). As most chemotherapies target dividing cells (Yano et al. 2014), an important implication of these findings is that invasive cells may be more resistant to antiproliferative chemotherapy, leaving these cells unaffected and able to reenter the cell cycle at a later time.

The molecular mechanisms that connect G1 cell-cycle arrest with cellular differentiation are thought to involve cell-cycle-dependent alterations in chromatin that may permit the transcriptional activation of differentiation genes (Ma et al. 2015). Consistent with this idea, the conserved histone deacetylase, *hda-1*, a chromatin remodeling protein, is upregulated in the AC after G1 arrest and promotes pro-invasive gene expression and invadopodia formation (Matus et al. 2015). The zinc finger protein MEP-1, a component of the nucleosome remodeling NuRD complex that requires HDAC activity for its function, is also a crucial regulator of AC pro-invasive differentiation and might also be activated by G1 arrest (Leight

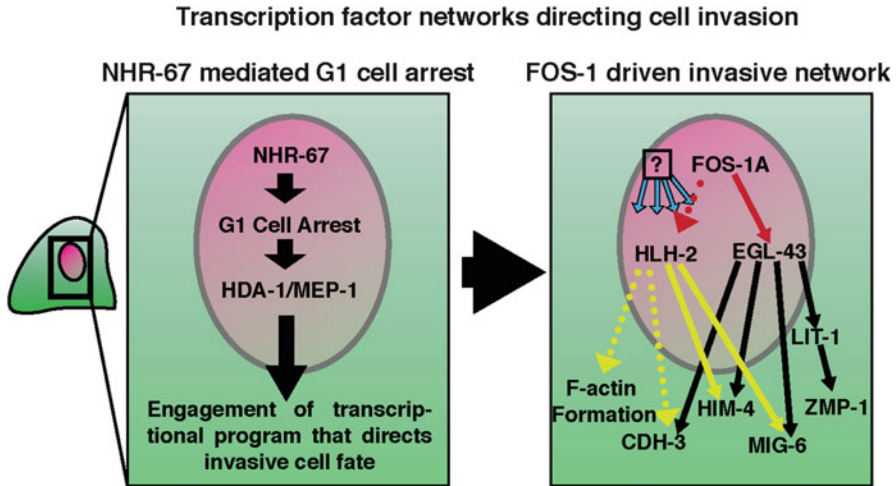

Fig. 6.2 Transcriptional networks programming AC invasiveness. *Left*: After AC specification, the transcription factor (TF) NHR-67 (vertebrate TLX) induces G1 arrest in the AC, which leads to chromatin remodeling through HDA-1 (HDAC) and MEP-1(NuRD complex) that allows pro-invasive gene expression and invasive cell fate differentiation. *Right*: G1 cell-cycle arrest promotes FOS-1A (c-Fos proto-oncogene) expression, which regulates the expression of other TFs such as HLH-2 (E protein) and EGL-43 (EVI1), which in turn direct the expression of a number of pro-invasive genes encoding the proteins CDH-3 (protocadherin), HIM-4 (hemicentin), MIG-6 (papilin), and ZMP-1 (MMP), as well as proteins that generate F-actin. The *solid arrows* indicate strong regulation, and *dashed arrows* indicate partial regulation. The "*question mark*" represents TFs that remain to be identified, as FOS-1A is not required for the expression of many pro-invasive genes in the AC

et al. 2015; Matus et al. 2010; Zhang et al. 1998). After the AC has entered G1 cell-cycle arrest, a transcription factor network involving the bZIP transcription factor FOS-1A, HLH-2 (vertebrate E proteins), and the zinc finger protein EGL-43 (an ortholog of the vertebrate oncogene EVI1) directs pro-invasive gene expression (Fig. 6.2; Rimann and Hajnal 2007; Schindler and Sherwood 2011; Sherwood et al. 2005). How G1 cell-cycle arrest activates this network is unclear but may involve transcriptional upregulation, as HDA-1 and MEP-1 promote *fos-1a* gene upregulation in the AC (Matus et al. 2010). Within this network, the FOS-1A protein regulates *egl-43* (EVI1) expression and promotes increased expression of the *hlh-2* gene (Rimann and Hajnal 2007; Schindler and Sherwood 2011). The HLH-2 protein is required for the expression of several downstream invasion effector genes, including those encoding the extracellular matrix proteins MIG-6 (papilin) and hemicentin. HLH-2 also increases the expression of the *cdh-3* (protocadherin) gene (Schindler and Sherwood 2011). In addition, the loss of HLH-2 disrupts actin formation at the invasive membrane of the AC, which is necessary for invasion to occur (Schindler and Sherwood 2011). FOS-1A, either directly or indirectly, is required for AC expression of the MMP *zmp-1*, the matrix protein hemicentin, and increased *cdh-3* expression (Hwang et al. 2007; Sherwood

et al. 2005). Interestingly, EGL-43 negatively regulates expression of the gene encoding the adhesion protein MIG-10 (lamellipodin), which is a positive target of FOS-1A (Wang et al. 2014a, c). This may establish a system where a circuit of transcription factors maintain basal levels of MIG-10 protein, as over- or underexpression of the *mig-10* gene negatively impacts invasion. EGL-43 also drives *lit-1* (Nemo-like kinase), which is upstream of *zmp-1* expression (Matus et al. 2010).

The vertebrate orthologs of the FOS-1A, EGL-43, and HLH-2 proteins are strongly associated with promoting invasion in normal development and in numerous epithelial-derived cancers (reviewed in Ozanne et al. 2006; Rimann and Hajnal 2007; Sherwood et al. 2005; Young and Colburn 2006), suggesting this regulatory network is conserved. Other transcription factors will certainly be added to the AC invasive fate network, as many AC-enriched genes that promote invasion, such as *cdc-37* (Hsp90 co-chaperone), *mig-2* (RhoG), and *pat-3* (integrin), are not regulated by these transcription factors (Matus et al. 2010; Schindler and Sherwood 2011; Shekarabi et al. 2005).

6.2.2 Establishing the Invasive Cell Membrane

Downstream of transcriptional changes, the AC establishes a unique plasma membrane domain at the cell-BM interface termed the invasive cell membrane (Fig. 6.3a). The heterodimeric transmembrane integrin receptor αINA-1/βPAT-3 and the transmembrane UNC-6 (netrin) receptor UNC-40 (vertebrate DCC) play important roles in invasive membrane establishment and BM adhesion (Hagedorn et al. 2009, 2013; Wang et al. 2014b; Ziel et al. 2009a; Ziel and Sherwood 2010). The *C. elegans* αINA-1/βPAT-3 integrin is most similar to vertebrate BM-laminin-binding integrins (Baum and Garriga 1997). INA-1/PAT-3 expression is upregulated in the AC at the time of AC specification, and the INA-1/PAT-3 heterodimer localizes to the invasive cell membrane. The targeted loss of integrin activity in the AC, however, only slightly reduces AC-BM attachment (Hagedorn et al. 2009). UNC-40 (DCC) may also help adhere the AC to the BM, as loss of UNC-40 or its ligand UNC-6 (netrin) results in weak AC-BM adhesion defects (Ziel et al. 2009a). MIG-10, an ortholog of mammalian lamellipodin, also participates in AC-BM adhesion although its precise role is unclear (Wang et al. 2014c). As the BM adhesion receptor dystroglycan is also expressed in the AC, a number of BM adhesion receptors may play redundant roles contributing to AC-BM attachment (Johnson et al. 2006).

While INA-1/PAT-3 (integrin) plays a minor role in AC-BM adhesion, it is crucial in establishing the invasive cell membrane and promoting cell invasion. The invasive membrane is enriched in actin regulators, F-actin, and the phosphoinositide PI(4,5)P_2 and is the site of polarized secretion and active membrane trafficking (Fig. 6.3a; Hagedorn et al. 2009, 2014; Ziel et al. 2009a). In response to ligand binding, integrins become activated then cluster and recruit

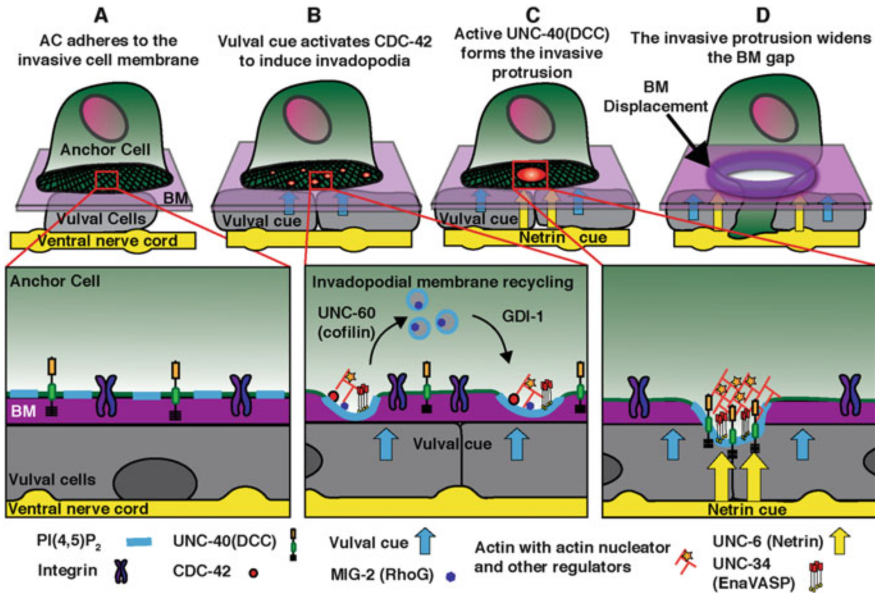

Fig. 6.3 AC breaching and clearing the BM. (**a**) The AC adheres to the BM with integrin, UNC-40 (DCC), and likely other adhesion receptors and establishes the invasive cell membrane—a membrane domain that is rich in F-actin and actin regulators, signaling molecules, vesicle trafficking, and invadopodia. (**b**) Just prior to invasion, the vulval cells secrete an unidentified diffusible "vulval cue" that activates CDC-42 and promotes the seeding of robust F-actin-rich invadopodia that breach the BM. Invadopodia are formed with an F-actin core and a specialized invadopodial membrane that is actively recycled through the endolysosome. Recycling is mediated by UNC-60A (cofilin) and GDI-1 (Rab GDP disassociation inhibitor). UNC-60A (cofilin) also disassembles F-actin at invadopodia to promote rapid invadopodia turnover. (**c**) The UNC-40 (DCC) receptor traffics to the BM breach site, where in response to UNC-6 (netrin) secreted from the ventral nerve cord is activated and recruits its effectors Ena/VASP and MIG-2 (RhoG) to build the invasive protrusion. (**d**) The invasive protrusion displaces the BM physically, and BM proteins accumulate at the edges of the BM opening

adaptor and signaling proteins that mediate F-actin formation and membrane trafficking to the cell surface (Cluzel et al. 2005; Wickstrom and Fassler 2011). Consistent with a key signaling function for integrin at the invasive membrane, a loss of INA-1/PAT-3 in the AC strongly disrupts the recruitment of all known F-actin regulators that localize to this domain and reduces or eliminates F-actin formation. Integrin is also required for the localized BM accumulation of hemicentin, an extracellular matrix protein that promotes invasion (Hagedorn et al. 2009; Morrissey et al. 2014). UNC-40(DCC) also plays a role in establishing the invasive cell membrane. UNC-40 is polarized to the invasive cell membrane by its ligand UNC-6 (netrin), which is secreted from the ventral nerve cord and accumulates in the BM under the AC (Ziel et al. 2009a). UNC-40(DCC) recruits its effectors MIG-2 (RhoG), CED-10 (Rac), UNC-115 (an actin regulator), and

UNC-34 (Ena/VASP), which build the invasive protrusion that crosses the BM (Wang et al. 2014a).

6.2.3 AC Invadopodia and the Invasive Protrusion: Breaching and Clearing the BM

The basally localized invasive cell membrane is an F-actin-rich domain that supports polarized secretion, BM adhesion, and invadopodia formation. Invadopodia are F-actin-rich membrane protrusions that form at sites of matrix removal and have been hypothesized to be the cellular drill bits of invasive cells, mediating invasion through BM barriers (Lohmer et al. 2014). Though studied in vitro for over 30 years, invadopodia function and even existence in vivo have been difficult to establish because visualizing cell invasion in native tissue environments remains challenging (Chen 1989; David-Pfeuty and Singer 1980; Linder et al. 2011; Murphy and Courtneidge 2011).

The development of 4D live-cell imaging approaches during AC invasion revealed that at least 3 h prior to invasion, numerous small (~1.0 μm), protrusive, F-actin-rich invadopodia form and turn over rapidly (~45 s lifetimes) along the AC's invasive cell membrane (Hagedorn et al. 2013). Similar to invadopodia in cancer cell lines observed in vitro, AC invadopodia are dependent on integrin activity and contain numerous actin regulators, including the ADF/cofilin ortholog UNC-60A, the Rho GTPases CDC-42 and MIG-2, and the Rac GTPase CED-10, the N-WASP ortholog WSP-1, and the Ena/VASP ortholog UNC-34 (Fig. 6.3b; Destaing et al. 2011; Hagedorn et al. 2013, 2014; Lohmer et al. 2016). CDC-42 and WSP-1 seed new invadopodia, and UNC-60A (cofilin) disassembles these structures. The function of other actin regulators in AC invadopodia is not yet clear, but the role of such regulators in the invadopodia of cancer cell lines has been studied in vitro (Bergman et al. 2014; Moshfegh et al. 2014).

The activity of these invadopodia in the AC appears tightly regulated. While hundreds of invadopodia form and turn over hours before BM breaching, usually only one or two invadopodia penetrate the BM during a highly stereotyped ~20-min period in the early-to-mid L3 larval stage (Hagedorn et al. 2013). The timing of invadopodia BM breaching is controlled by a diffusible cue from underlying vulval cells that activates CDC-42 in the AC. Activated CDC-42 is found at the invadopodium that breaches the BM and may promote more robust and protrusive invadopodia through WSP-1-directed F-actin generation and perhaps the recruitment of additional components such as proteases that "arm" invadopodia for BM penetration (Lohmer et al. 2016; Poincloux et al. 2009).

In addition to confirming the existence and regulation of invadopodia in vivo, studies in the AC have also revealed new key molecular and structural features of invadopodia. One of the most notable discoveries is that of a unique, actively recycled invadopodial membrane. During invadopodia formation, a specialized

membrane containing the phospholipid PI(4,5)P_2 and membrane-associated Rac and RhoG GTPases is rapidly recycled through the endolysosome during invadopodia assembly and disassembly (Fig. 6.3b; Hagedorn et al. 2014). Through genetic screens, several regulators of invadopodial membrane trafficking have been identified, including UNC-60A (cofilin) and GDI-1 (Rab GDP dissociation factor). UNC-60A appears to regulate the exocytosis of the invadopodial membrane at sites of nascent invadopodia and may promote trafficking through the endolysosomal system (Hagedorn et al. 2014). In the absence of UNC-60A, the invadopodial membrane is trapped in static internal vesicles within the AC and fails to traffic to invadopodia at the invasive cell membrane. GDI-1 is crucial for targeting the invadopodial membrane selectively to the basal invasive membrane. The loss of GDI-1 results in the invadopodial membrane being inappropriately trafficked to apical and lateral plasma membrane domains in the cell (Fig. 6.3b; Hagedorn et al. 2014; Lohmer et al. 2016). Although more difficult to examine because of the slower dynamics of invadopodial turnover in cell culture, similar recycling occurs in several cancer cell lines, suggesting that the invadopodial membrane is likely a shared aspect of invadopodia construction (Artym et al. 2011; Monteiro et al. 2013; Poincloux et al. 2009; Williams and Coppolino 2011). The invadopodial membrane may be required for the concentrated delivery of membrane-associated proteases and to provide a source of membrane addition for protrusive activity (Frittoli et al. 2014; Poincloux et al. 2009; Trimble and Grinstein 2015). The unique composition of the invadopodial membrane might also serve as an organizing platform for the recruitment of actin regulators, adhesion, and signaling proteins that direct invadopodia construction (Moshfegh et al. 2014; Yamaguchi and Oikawa 2010).

During BM breaching by an invadopodium, the netrin receptor UNC-40 (DCC) is trafficked to the breach site, although the mechanism of recruitment remains elusive (Morrissey et al. 2013). UNC-40 (DCC) at the BM breach is activated by its ligand UNC-6 (netrin) and, in response to activation, recruits effectors such as UNC-34 (Ena/VASP) and MIG-2 (RhoG) that promote focused F-actin formation to build a single large invasive protrusion that clears an opening in the BM (Fig. 6.3c, d; Hagedorn et al. 2013). In the absence of UNC-6 (netrin) or UNC-40 (DCC), a large protrusion never forms, and the clearing of an opening in the BM necessary for invasion is perturbed. Type IV collagen degradation products and proteases are expressed near sites of invasion in vivo and in vitro (Page-McCaw et al. 2007; Rowe and Weiss 2008). Thus, it has been generally assumed that BM is degraded and dissolved during invasion, which has led to extensive clinical trials to target MMPs in metastatic cancers. These clinical trials, however, were universally unsuccessful for reasons that remain unknown (Dufour and Overall 2013; Overall and Kleifeld 2006). Optical highlighting experiments using a photo-convertible form of laminin and type IV collagen in *C. elegans* have surprisingly revealed that the BM breached by the AC is physically displaced by the invasive protrusion (Fig. 6.3d; Hagedorn et al. 2013). While these results do not rule out a role for proteases in weakening BM, they indicate that invasion requires physical forces to clear a path through this barrier. This may help explain why clinical trials targeting

MMPs have been ineffective—invasive cells may invade independent of BM proteolysis.

The BM invasion program in the AC is likely conserved, as invadopodia have been observed in numerous invasive cancer cell types (Hoshino et al. 2013) and in invasive vertebrate embryonic and endothelial cells (Patel et al. 2012; Seano et al. 2014). Further, single invasive protrusions have been seen when tumor cells transmigrate BMs in ex vivo BM invasion assays (Hotary et al. 2006; Leong et al. 2014; Schoumacher et al. 2010). Notably, the *netrin-1* ligand is highly expressed and associated with invasion in the most aggressive metastatic cancers, including medulloblastoma, glioblastoma, malignant melanoma, and pancreatic adenocarcinoma (Akino et al. 2014; Dumartin et al. 2010; Kaufmann et al. 2009; Shimizu et al. 2012). Thus, the netrin pathway may guide invasive cells across BM barriers in highly metastatic cancers and be a particularly attractive target for cancer therapeutics.

6.3 Post AC Invasion: BM Sliding Further Opens the Breach

Shortly after the AC completes invasion, the opening in the BM enlarges beyond the boundaries of the AC (Fig. 6.4). This expansion of the BM gap allows additional uterine and vulval cells to directly attach and form the mature uterine-vulval connection (Schindler and Sherwood 2013). Large openings in BMs occur frequently in development, such as during vertebrate and invertebrate gastrulation, mouse distal visceral endoderm formation, and *Drosophila* imaginal disk eversion (Hiramatsu et al. 2013; Nakaya et al. 2008; Saunders and McClay 2014; Srivastava et al. 2007). Large gaps in BMs have been known for decades to occur in cancer at the tumor front, and these openings are thought to be crucial in allowing cancer cells to metastasize (Barsky et al. 1983; Frei 1962; Gabbert et al. 1985; Kobayashi et al. 1995; Rowe and Weiss 2008). It has been suggested that these large BM openings are created by proteolytic degradation based on type IV collagen degradation and protease expression (Hotary et al. 2006; Page-McCaw et al. 2007; Rowe and Weiss 2008; Sameni et al. 2009). Reduced BM synthesis and alterations in composition have also been proposed as mechanisms that may result in the loss of BM (Flug and Kopf-Maier 1995; Frei 1962; Spaderna et al. 2006).

Although it has been postulated that proteases are main drivers of BM loss, experiments in *C. elegans* did not reveal any proteases that are required for BM gap enlargement and furthermore showed that a reduction in BM deposition was not involved (Ihara et al. 2011). Instead, using optical highlighting of BM techniques, it was observed that the BM moves, sliding over the vulval and uterine cells to expand the BM opening. The force that drives BM sliding appears to be the rapid growth and division of the vulval cells (Fig. 6.4). An examination of BM remodeling during uterine-vulval attachment in 20 other species of nematodes (Dieterich et al. 2008;

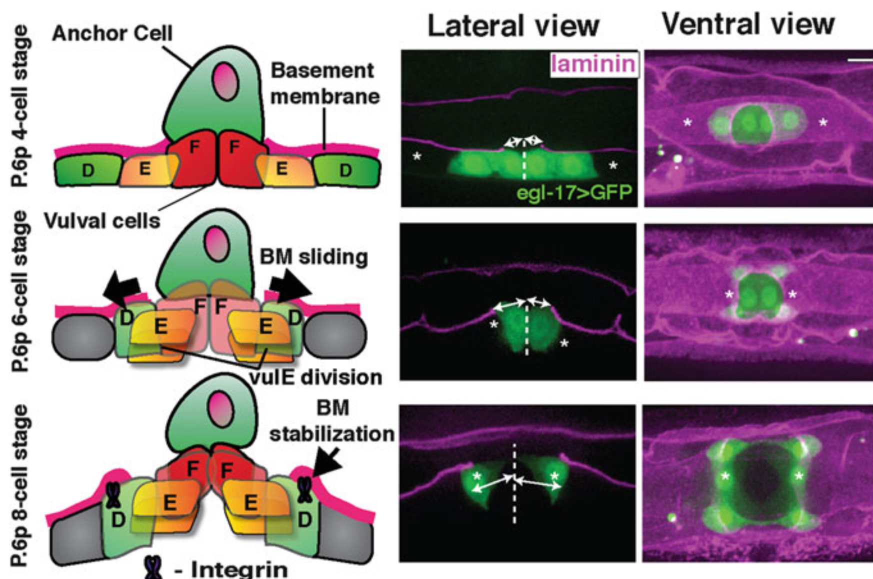

Fig. 6.4 BM sliding. *Top panel*: After the AC has invaded (P6.p 4-cell stage), the vulval cells begin to invaginate, grow, and divide, and the BM gap widens through BM sliding. The images on the right show lateral and ventral fluorescent images of the BM (laminin::mCherry) and the central vulval cells (vulE and vulF cells) expressing a cytoplasmic GFP driven by the *egl-17* promoter. The *dashed line* indicates the vulval midline, and "*asterisks*" marks the center of the vulD cell. *Middle panel*: By the P6.p 6-cell stage, the BM gap expands as it slides away from the AC and over the vulE cells, which have recently divided. *Bottom panel*: At the P6.p 8-cell stage, the vulF cells have divided, and the BM has expanded fully. The edge of the BM gap is stabilized by integrin upregulation on the vulD cells (note: the *egl-17* promoter drives expression in vulD cells at this time). *Scale bar* 5 μm (Reproduced from Matus et al. (2014) with permission from *Nature Communications*)

Matus et al. 2014) showed that a single AC initiates BM breach in all species with the subsequent BM gap stabilizing on vulD cells, the only vulval cell type that does not divide during vulval invagination (Fig. 6.4; Kiontke et al. 2007; Matus et al. 2014). These observations strongly suggest that cell divisions may promote BM sliding and nondividing cells might prevent it. Supporting this notion, live-cell imaging of BM sliding in *C. elegans* revealed that the centrally located vulE and vulF cells lose their attachment to the BM during their division and allow the BM to slide over these cells. Furthermore, experimentally halting the vulE and F cell divisions prevents BM sliding, while stimulating cell division in the vulD cells results in BM gaps moving beyond the dividing D cells. These observations in *C. elegans* and related nematodes indicate that cell division is a powerful regulator of BM sliding that has been maintained for hundreds of millions of years.

In addition to the controlled cell-cycle exit of vulD, increased integrin adhesion also plays an important role in stabilizing the position of the BM gap. Shortly after BM sliding stops on the vulD cell, the integrin adhesion receptor INA-1/PAT3

concentrates at the BM border (Fig. 6.4). Specific loss of integrin in the vulD cell results in further BM gap sliding, indicating that INA-1/PAT-3 (integrin) stabilizes the position of the BM gap border. The concentration of integrin is directed at least in part by increased levels of laminin at the edge of the BM gap, an increase caused by the AC as it pushes the BM aside during invasion (Hagedorn et al. 2013).

BM sliding may be a common morphogenetic mechanism to mediate tissue formation. For example, BM sliding has been observed during salivary gland growth in vertebrates and may allow bud expansion while restricting growth at the duct (Harunaga et al. 2014). Cell-BM shifts also occur to maintain tissues, as BM labeling and pulse chase experiments revealed that intestinal epithelial cells derived from the stem cell crypt slide along the BM toward the villus tips during differentiation to renew the gut epithelium (Clevers 2013; Trier et al. 1990). Additionally, as large gaps in the BM at tumor fronts have long been observed in cancer (Barsky et al. 1983; Frei 1962; Gabbert et al. 1985; Kobayashi et al. 1995; Rowe and Weiss 2008) and the loss of BM contact stimulates tumor cell invasion, it is possible that BM sliding plays a role in promoting the spread of cancer (Nguyen-Ngoc et al. 2012).

6.4 AC Invasion and SPARC: A Model for the Tumor Microenvironment

The tumor microenvironment is composed of the cells (e.g., fibroblasts, endothelial cells, and immune cells) and extracellular matrix of the surrounding tumor tissue and plays dynamic roles in tumor cell progression and metastasis (Joyce and Pollard 2009; Quail and Joyce 2013). AC invasion is regulated by numerous cues from the surrounding tissues, including integrin-BM interactions, an UNC-6 (netrin) chemotactic cue, and a diffusible cue that activates CDC-42 to promote invadopodia formation (Hagedorn and Sherwood 2011; Lohmer et al. 2016; Wang et al. 2014b). Notably, all of these molecules are key mediators of invasion and metastasis in cancer cells (Ko et al. 2014; Seguin et al. 2015; Stengel and Zheng 2011). This suggests that many of the environmental cues that control tumor invasion are conserved. AC invasion in *C. elegans* thus offers a potentially powerful in vivo experimental model to study the tumor microenvironment by altering the environment surrounding the AC and determining how these changes affect invasive behavior.

One example of an extracellular matrix protein misregulated in most tumor environments is the collagen-binding matricellular glycoprotein SPARC (Podhajcer et al. 2008). The overexpression of SPARC is strongly associated with tumor metastasis and poor prognosis in many aggressive cancers, including glioblastoma, pancreatic ductal carcinoma, breast ductal carcinoma, clear-cell renal cell carcinoma, melanoma, and prostate carcinoma (reviewed in Arnold and Brekken 2009; Nagaraju et al. 2014). Data from mouse models have confirmed that the overexpression of

SPARC increases metastatic potential (Minn et al. 2005; Ting et al. 2014) and in vitro assays support a role for increased SPARC in cancer invasion and migration in many different cancers (Briggs et al. 2002; Golembieski et al. 1999; Jacob et al. 1999; Kato et al. 1998; Ledda et al. 1997; Ting et al. 2014). SPARC has been implicated in regulating diverse cellular processes, including cell-matrix adhesion, growth factor activity, MMP expression, and extracellular matrix assembly and disassembly (Aguilera et al. 2014; Arnold and Brekken 2009; Barker et al. 2005; Harris et al. 2011; McClung et al. 2007; Sage et al. 1989; Shi et al. 2007). The numerous functions of SPARC and the challenge of studying invasion through BMs in vivo have made it difficult to determine how SPARC promotes invasive behavior.

In *C. elegans* the SPARC protein is made and secreted primarily by body wall muscles and accumulates in most BMs (Fitzgerald and Schwarzbauer 1998). To understand how the overexpression of SPARC may promote cell invasion, transgenic worms were generated expressing SPARC at two- to fivefold higher than normal levels (Morrissey et al. 2016). Elevated levels of SPARC did not alter the normal AC invasion program; however, increased SPARC fully restored AC invasion in mutants regulating diverse aspects of invasion—FOS-1A (breaching the BM/matrix, metalloproteinases), integrin (invadopodia and adhesion), vulval cue (CDC-42/invadopodia), and netrin (invasive protrusion; Fig. 6.5; Morrissey et al. 2016). These genetic interactions indicate that elevated SPARC is broadly pro-invasive and can compensate for the loss of multiple distinct pathways that promote invasion (Wang and Sherwood 2011).

How does SPARC so potently promote invasion in such a broad manner? Among the many functions of SPARC, likely the most ancient is its direct binding to collagens, where SPARC acts as a collagen chaperone that mediates the solubility and transport of collagens from sites of secretion to deposition (Martinek et al. 2002, 2007, 2008; Shahab et al. 2015). Work in the worm strongly implicates the misregulation of this collagen chaperone activity as underlying its pro-invasive function (Morrissey et al. 2016). First, the collagen-binding pocket of SPARC is essential for SPARC's pro-invasive functions (Sasaki et al. 1998). In addition, increased levels of SPARC dramatically decrease the levels and deposition rate of type IV collagen into the BM. Increased levels of SPARC delivered into the extracellular milieu from neurons (where neither SPARC nor type IV collagen are usually expressed) also promote invasion and decrease BM collagen. Finally, RNAi-mediated reduction of type IV collagen, a key structural and barrier component of BMs, recapitulates the broad pro-invasive functions of SPARC. Together these results suggest that elevated levels of SPARC may increase collagen solubility to such an extent that it inhibits collagen deposition into BMs. The decrease of collagen in BMs likely reduces the barrier properties of BMs, thus allowing even poorly invasive ACs to invade through these BMs (Fig. 6.6).

The observations of SPARC in the worm may account for the broad pro-invasive function of SPARC in numerous types of cancer. Further, these studies could explain the finding that SPARC overexpression by either the tumor or the surrounding tissue promotes metastatic progression (Barth et al. 2005; Iacobuzio-Donahue et al. 2002; Kato et al. 1998; Rich et al. 2003; Rodriguez-Jimenez et al. 2007; Sato

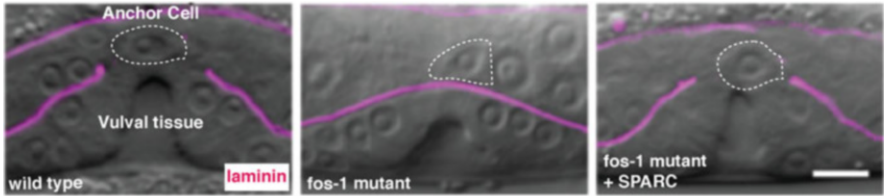

Fig. 6.5 Overexpression of SPARC promotes AC invasion. *Top panel*: A wild-type AC that has successfully invaded by the P6.p 8-cell stage. BM visualized by fluorescently labeled laminin, whose levels are not altered by SPARC. *Middle panel*: AC invasion has been blocked by a mutation in *fos-1*, a major regulator of invasion. *Bottom panel*: The overexpression of SPARC restores the ability of the AC to invade in the *fos-1* mutant. *Scale bar* 5 μm (Figure reproduced from Morrissey et al. (2014) with permission)

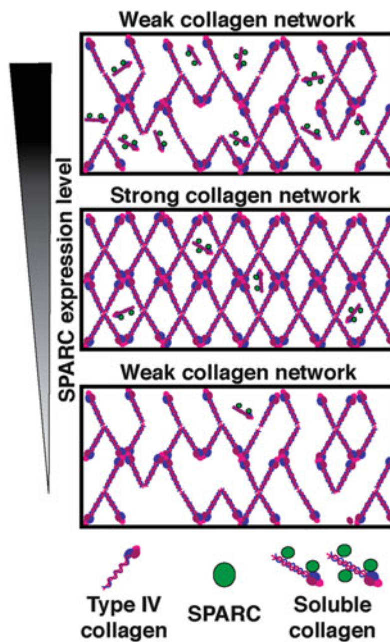

Fig. 6.6 The levels of SPARC expression regulate the BM type IV collagen lattice. A schematic diagram depicting SPARC function. SPARC is a collagen chaperone that maintains extracellular collagen solubility. *Bottom panel*: When SPARC levels are low, there is not enough soluble type IV collagen to build a strong collagen BM lattice. *Middle panel*: At normal levels, SPARC facilitates efficient type IV collagen solubility to allow the transit of collagen from sites of secretion to assembly locations for strong lattice construction. *Top panel*: When overexpressed, SPARC maintains collagen solubility in the extracellular fluid and inhibits efficient collagen deposition in type IV collagen BM networks, resulting in a weak lattice

et al. 2003; Schultz et al. 2002) as secretion of SPARC from any tissue in the vicinity would reduce type IV collagen levels in the BM surrounding the growing tumor or tumor vasculature. The findings of SPARC function in the worm are also consistent with observations in a mouse model of breast cancer metastasis, indicating that SPARC is not sufficient to drive metastasis but dramatically enhances tumor invasion (Minn et al. 2005). Given the complexity of the tumor-stromal interactions, understanding how alterations in the tumor microenvironment regulate invasive behavior is a daunting task. These studies with SPARC illustrate how AC invasion is not only a model to elucidate the normal invasion program but is also emerging as an effective experimental paradigm to determine how alterations in the tumor microenvironment regulate invasion and metastasis.

6.5 Summary and Perspective

Studies of AC invasion and uterine-vulval attachment in the worm have advanced our understanding of the transcriptional networks, signaling pathways, cellular dynamics, and extracellular matrix removal mechanisms that allow invasive cells to transmigrate and widen BM gaps. Notably, these studies have uncovered important new mechanisms underlying the invasion and BM removal that have acute relevance to therapeutic strategies used to block invasive behavior in cancer, such as a requirement for G1 cell-cycle arrest in invasive cells and the ability of cells to remove BM barriers by physical displacement. Further, AC invasion is emerging as a promising model to study how alterations in the tumor microenvironment affect cell invasive behavior. Yet, many important questions in understanding how cells transmigrate BM barriers remain, including the role of proteases in cell invasion and elucidating the complete transcriptional network that programs invasiveness. Furthermore, there is clear evidence that biomechanical properties of tissues and interstitial type I collagen matrices play important roles in cell invasion in cell culture and in tumor invasion (Kai et al. 2016). We do not yet understand what role, if any, BM stiffness plays during BM invasion or how the BM's physical properties might be altered during invasion. With the recent development of single-cell profiling techniques, CRISPR-Cas9 genome editing, FRET-based force sensors, and sensitized screening in the worm (Dickinson et al. 2013; Gayrard and Borghi 2016; Spencer et al. 2014), AC invasion is poised to remain at the leading edge of understanding how cells transmigrate and remove BM barriers.

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