

Traversing the basement membrane in vivo: A diversity of strategies

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The basement membrane is a dense, highly cross-linked, sheet-like extracellular matrix that underlies all epithelia and endothelia in multicellular animals. During development, leukocyte trafficking, and metastatic disease, cells cross the basement membrane to disperse and enter new tissues. Based largely on *in vitro* studies, cells have been thought to use proteases to dissolve and traverse this formidable obstacle. Surprisingly, recent *in vivo* studies have uncovered a remarkably diverse range of cellular- and tissue-level strategies beyond proteolysis that cells use to navigate through the basement membrane. These fascinating and unexpected mechanisms have increased our understanding of how cells cross this matrix barrier in physiological and disease settings.

Introduction

Throughout animal development and normal homeostasis, cells move to construct tissues and to reach distant sites. During these migrations cells confront a variety of barriers, including other cells, cell–cell junctions, and extracellular matrices of different densities and composition. One of the most difficult barriers to navigate through is the basement membrane, a thin, dense and highly cross-linked extracellular matrix (ECM) that underlies all epithelia and endothelia and surrounds muscle, fat, and Schwann cells (Kalluri, 2003; Yurchenco, 2011). The basement membrane is an ancient form of ECM, encoded by a core set of approximately ten highly conserved genes that arose with the emergence of metazoans (Ozbek et al., 2010; Hynes, 2012). These genes encode predominantly large, insoluble secreted proteins. Most notable are heterotrimeric laminin and type IV collagen, which provide a scaffolding that shapes the basement membrane into sheet-like structures between 50 and 100 nm thick along cell surfaces (Hohenester and Yurchenco, 2013).

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Abbreviations used in this paper: CLASP, cytoplasmic linker-associated protein; DCC, deleted in colon cancer; EMT, epithelial-to-mesenchymal transition; LER, low-expression region; MMP, matrix metalloproteinase.

Basement membrane assembly is initiated through the recruitment of laminin by integrin and α -dystroglycan adhesion receptors, as well as sulfated glycolipids (Fässler and Meyer, 1995; Stephens et al., 1995; Henry and Campbell, 1998; McKee et al., 2007). At the cell surface, secreted laminin molecules self-associate, forming a polymerized network. Laminin assembly is thought to seed recruitment of additional basement membrane proteins, including type IV collagen, which also self-polymerizes and forms a second independent network. Type IV collagen has the unique feature of self-associating through intramolecular covalent bonds, providing barrier and mechanical strength properties to basement membranes (Pöschl et al., 2004; Khoshnoodi et al., 2008). The basement membrane component nidogen, and the heparan sulfate proteoglycan perlecan, bind collagen and laminin and are thought to connect the type IV collagen and laminin networks (Hohenester and Yurchenco, 2013). Understanding how cells pass through the basement membrane has been of great interest because of its widespread occurrence in normal development and leukocyte trafficking, its misregulation in cancer and immune disorders, and its necessity for pathogen entry into host tissues (Rowe and Weiss, 2008; Hagedorn and Sherwood, 2011; Singh et al., 2012). Uncovering the mechanisms that cells use to traverse the basement membrane, however, has been hampered by the difficulty of experimentally examining cell–basement membrane interactions during invasion events *in vivo*. As a result, most of our mechanistic understanding of invasion has been derived from *in vitro* studies (Even-Ram and Yamada, 2005; Rowe and Weiss, 2008). Although these studies have identified important molecular players required for invasion through artificial matrices and denuded acellular basement membranes, *in vitro* conditions do not recapitulate the dynamic chemical, mechanical, or cellular environment where cells traverse these barriers. Thus, many important mechanisms underlying basement membrane transit have likely been overlooked. This review highlights recent studies in many model organisms that have revealed unexpected molecular-, cellular-, and tissue-level strategies that cells use to remodel and cross basement membrane barriers. We also discuss future directions and challenges to our understanding of this important biological process.

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Breaching the epithelial basement membrane

Many basement membrane invasion events involve crossing through (or transmigrating) the epithelial basement membrane. These occur during immune cell trafficking, epithelial-to-mesenchymal transitions (EMTs), and collective cell migration (Ratzinger et al., 2002; Micalizzi et al., 2010; Friedl et al., 2012; Nakaya and Sheng, 2013). Where these crossings through epithelial basement membrane have been carefully observed, the basement membrane appears to be specifically lost at the site of transmigration (Cheung et al., 2005; Bort et al., 2006; Nakaya et al., 2008; Gouzi et al., 2011; Ihara et al., 2011; Hiramatsu et al., 2013). Regulating basement membrane openings is not only important in controlling invasion, but also in maintaining tissue integrity and preventing inappropriate cell death (Li et al., 2003; Domogatskaya et al., 2012). Further, loss of the basement membrane might directly stimulate invasive behavior through cues released from the degraded basement membrane or the resultant exposure to the underlying interstitial matrix (Egea et al., 2008; Nguyen-Ngoc et al., 2012). In this section we highlight recent work on basement membrane remodeling events during worm, mouse, fly, and chick development that are beginning to provide insight into how breaches in epithelial basement membranes are initiated, expanded, and regulated.

***Caenorhabditis elegans*: Invadopodia breach and basement membrane slides.** During *C. elegans* larval development, the nascent uterine and vulval tissues are initially separated by juxtaposed gonadal and epidermal basement membranes (Sherwood and Sternberg, 2003). A specialized uterine cell, the anchor cell, initiates uterine–vulval attachment by invading through both basement membranes and intercalating between the underlying vulval cells (Fig. 1, A and B; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003). Recently, the anchor cell has been shown to breach the basement membrane using invadopodia, protrusive F-actin–rich, membrane-associated subcellular structures (Hagedorn et al., 2013). Invadopodia were first identified in transformed fibroblasts and cancer cell lines over 20 years ago and have been studied extensively in cell culture (Chen, 1989; Linder et al., 2011). The physiological significance of these structures, however, had been controversial, due to the inability to clearly detect invadopodia in vivo (Beerling et al., 2011). The invadopodia that form during anchor cell invasion contain actin regulatory proteins that have also been associated with invadopodia in cancer cell lines. Further, invadopodia in both the anchor cell and cancer cell lines are similarly dependent on integrin for their formation (Hagedorn et al., 2009, 2013; Destaing et al., 2010). Thus, studies in the anchor cell have confirmed the physiological relevance of invadopodia and suggest that they are a conserved subcellular structure used by invasive cells to penetrate the basement membrane.

Electron micrographs of tumors and invading cells placed on a denuded basement membrane have shown cells extending single protrusions across this structure, suggesting that invadopodia transform into a single protrusion during invasion (Hotary et al., 2006; Schoumacher et al., 2010). Studies on anchor cell invasion have revealed a molecular mechanism that controls

this switch. Before invasion the anchor cell forms multiple invadopodia. Shortly after an invadopodium breaches the basement membrane, the *C. elegans* orthologue of the netrin receptor DCC (deleted in colon cancer) traffics to the initial breach site, recruits F-actin effectors, and directs the formation of a single large invasive protrusion that crosses the basement membrane and intercalates between the vulval cells (Fig. 1, A and C; Ziel et al., 2009; Hagedorn et al., 2013). By recruiting F-actin effectors away from invadopodia to form the invasive protrusion, the action of DCC leads to the cessation of invadopodia inhibiting additional breaching events. This elegant mechanism provides a morphogenetic switch at the cell–basement membrane interface from invadopodia-driven basement membrane penetration to the formation of an invasive protrusion that guides the cell across a single basement membrane gap.

Based on the presence of type IV collagen degradation products at sites of invasion in vitro and the expression of proteases within invading cells, it has generally been assumed that invading cells dissolve the basement membrane (Overall and Kleinfeld, 2006; Cavallo-Medved et al., 2009; Valastyan and Weinberg, 2011). Work on anchor cell invasion has called this into question, however. The accumulation of basement membrane components and optical highlighting of laminin and type IV collagen revealed that the basement membrane was moved aside by the invasive protrusion of the anchor cell, rather than being dissolved (Fig. 1 D; Hagedorn et al., 2013). Importantly, these observations do not rule out a role for proteases acting on the basement membrane. For example, limited proteolysis might make the basement membrane more pliant for displacement. Further, proteases might be necessary for initial basement membrane breaching. Consistent with a possible role for proteases in anchor cell invasion, mutations in the *C. elegans* Fos transcription factor orthologue, *fos-1a*, result in invadopodia that fail to breach the basement membrane (Sherwood et al., 2005). One of the genes regulated by FOS-1A is *zmp-1*, a member of the matrix metalloproteinase (MMP) family of proteases that is strongly expressed in tumors and implicated in cell invasion (Overall and Kleinfeld, 2006). Animals harboring mutations in *zmp-1*, however, do not have defects in anchor cell invasion, suggesting that one or more of the other five *C. elegans* MMPs encoded in the genome might be required to breach the basement membrane (Sherwood et al., 2005; Altincicek et al., 2010).

After the anchor cell breaches the basement membrane, the gap in the basement membrane widens beyond the boundary of the anchor cell as the underlying vulval cells grow, divide, and invaginate (Fig. 1, A and B). Widening of the gap facilitates direct cell–cell adhesion of vulval and uterine cells that make up the complete uterine–vulval connection during development (Ihara et al., 2011). Although proteolysis and reduced basement membrane synthesis have been postulated to underlie localized loss of large regions of the basement membrane (Rowe and Weiss, 2008), optical highlighting and landmark photobleaching experiments indicated that increased gap formation was not accompanied by changes in basement membrane deposition or degradation (Ihara et al., 2011). Surprisingly, these experiments revealed that the basement membrane widens by sliding over the invaginating and actively dividing vulval cells. Limiting

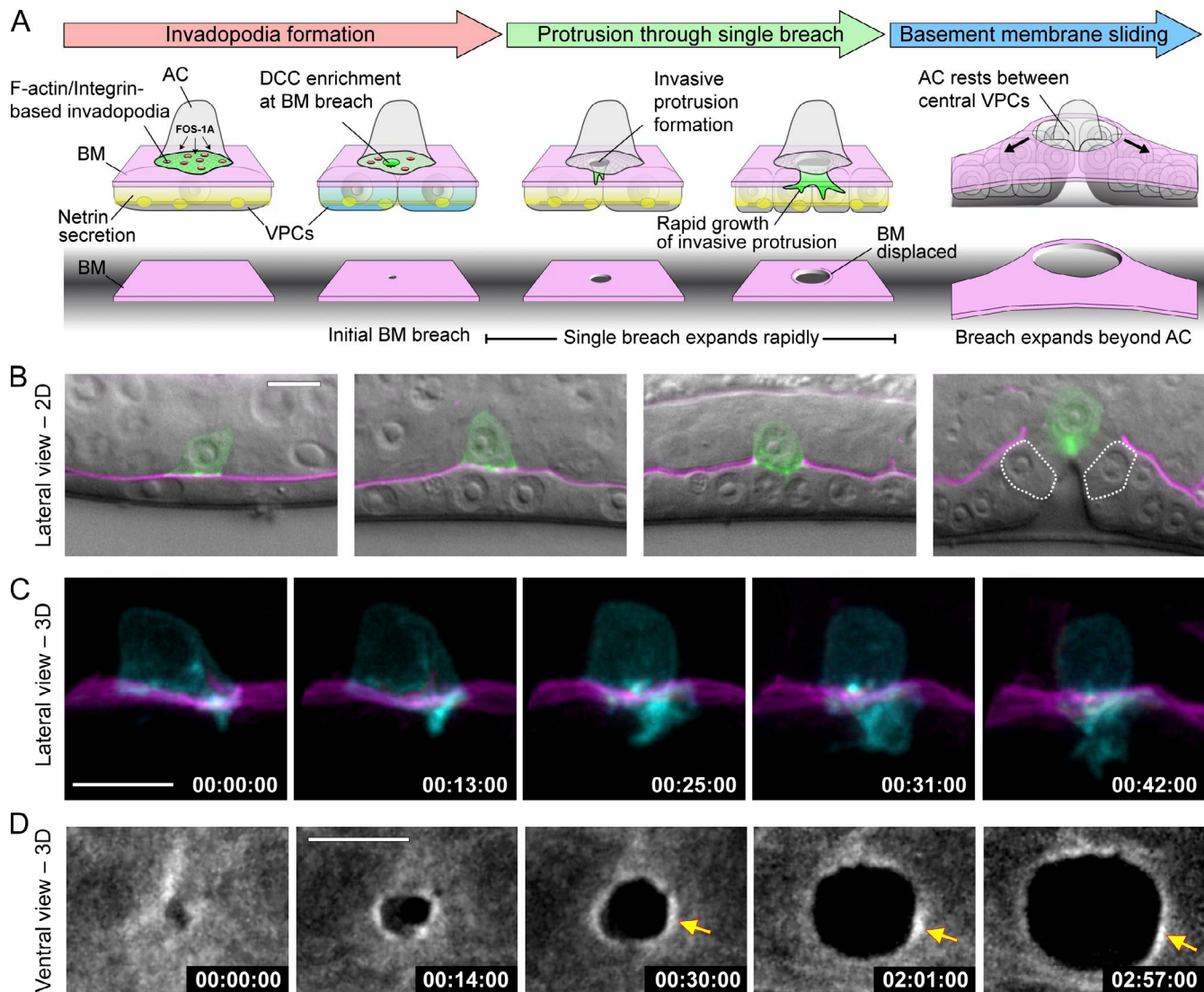


Figure 1. Basement membrane removal during uterine–vulval attachment in *C. elegans*. (A) Schematic diagram of basement membrane remodeling. The gonadal anchor cell (AC) initiates the basement membrane (BM) breach with invadopodia. The DCC (deleted in colon cancer) receptor enriches at the site of breach and through its effectors generates F-actin that builds a large protrusion. This invasive process physically displaces the basement membrane and directs invasion through a single basement membrane gap into the underlying dividing vulval precursor cells (VPCs). The basement membrane gap then expands through basement membrane sliding (black arrows in right panel) by sliding over the underlying VPCs as they invaginate. (B) Confocal imaging over the course of anchor cell (green) invasion from a lateral view with fluorescent images overlaid on a DIC micrograph. The basement membrane (magenta) ultimately stabilizes over a specific vulval cell, named the vulD cell (white dotted lines in the far right image). Image reproduced from Hagedorn and Sherwood (2011) with permission from Elsevier. (C) A lateral-view confocal time series showing the growth of the invasive membrane protrusion of the anchor cell (cyan) as it advances through the basement membrane (magenta). (D) A ventral-view time series shows the expanding hole in the basement membrane that forms during anchor cell invasion. Yellow arrows point to physically displaced basement membrane. C and D reproduced from Hagedorn et al. (2013). Bars, 5 μ m.

invagination and division of vulval cells reduced basement membrane movement. These observations suggest that the growing and moving vulval cells generate tension forces that shift the basement membrane as the vulval cells invaginate. Up-regulation of the integrin heterodimer, INA-1/PAT-3, which is most similar to vertebrate laminin receptors, anchors the basement membrane in place and halts basement membrane sliding at a specific vulval cellular boundary (Fig. 1, A and B). The placement of this basement membrane gap boundary is important in allowing direct cell–cell adhesion of the specific uterine and vulval cells that mediate the mature uterine–vulval connection. Cell proliferation, movement, and growth occur during

many morphogenetic events and in tumors where similar forces might shift basement membrane barriers (Birbeck and Wheatley, 1965; Hanahan and Weinberg, 2011). Thus, movement in basement membrane sheets might be a common strategy to enlarge openings for migrating cells.

Mouse embryogenesis: Mechanical force triggers basement membrane breaches. Tissue and sub-cellular level mechanical forces are emerging as a significant player in morphogenesis, differentiation, and disease (Mammoto and Ingber, 2010; Janmey and Miller, 2011). Mechanical forces have recently been implicated in breaking down and crossing the basement membrane (Hiramatsu et al., 2013). During

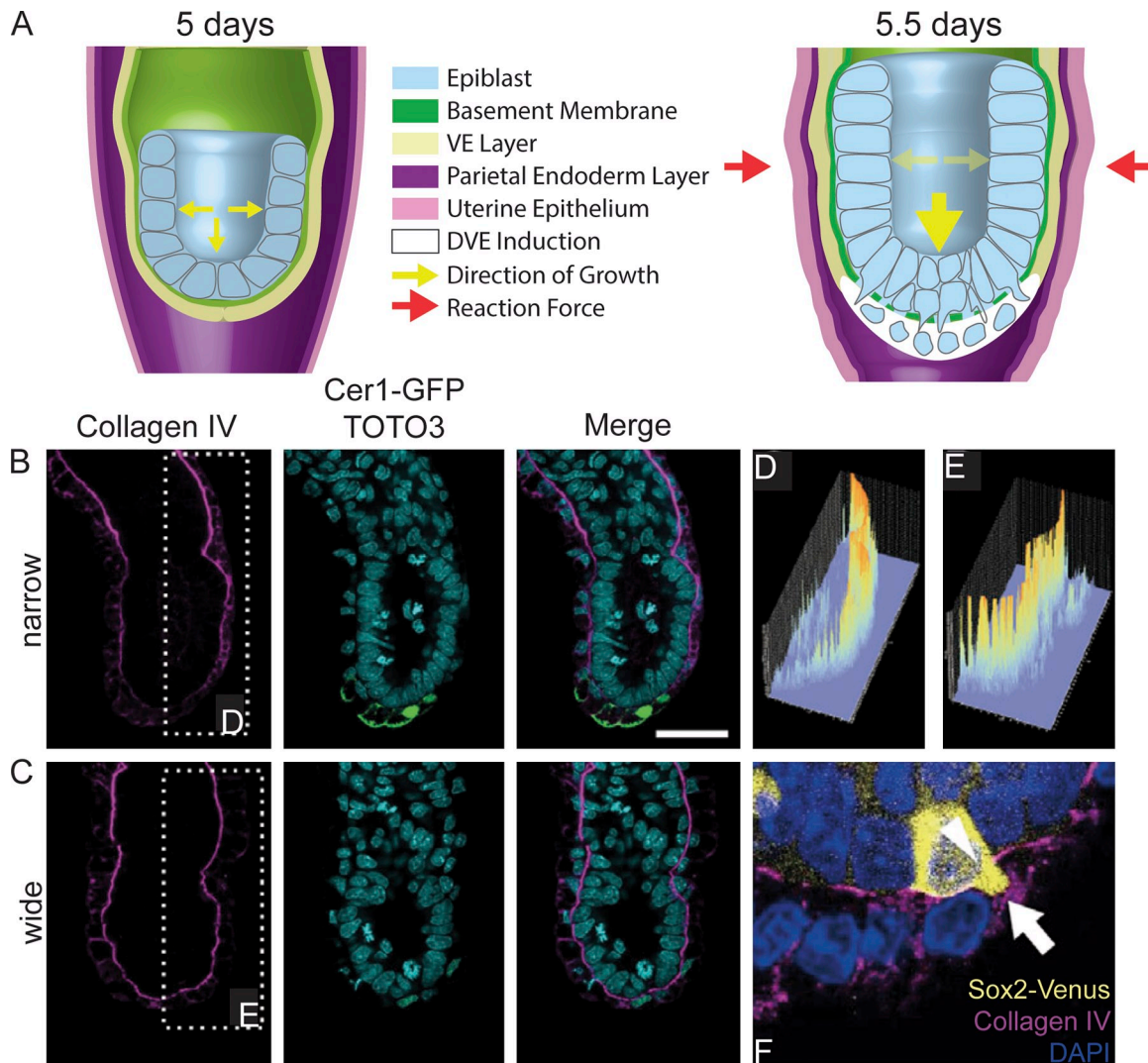


Figure 2. Basement membrane breaching is triggered by mechanical force in mouse embryos. (A) Schematic model of distal visceral endoderm (DVE) formation resulting from constrained growth by the maternal tissue. The growing embryo expands from day 5.0 to 5.5 and becomes restricted laterally (red arrows) by the maternal uterine tissue (uterine epithelium; light pink). Forced to elongate in the proximal–distal axis direction (large yellow arrow), the distal basement membrane (green) breach is triggered by mechanical strain. Epiblast cells (light blue) exit through the basement membrane gaps into the visceral endoderm (VE) layer (yellow), forming the DVE (white). (B and C) Fluorescence microscopy of explanted embryos expressing Cer1 (cerberus-related cytokine 1)-EGFP (a DVE marker, which is induced after the cells breach the basement membrane; green) that were cultured in narrow (growth restricting) and wide (nongrowth restricting) cavity devices and immunostained with anti-collagen IV (labeling the basement membrane; magenta) and TOTO3 (nuclei; green). The basement membrane is breached and newly induced DVE cells transigrate from the VE layer in embryos cultured in the narrow, but not wide, cavities. (D and E) 3D rendering of the intensity of the collagen IV signal in B and C. The loss of collagen signal can be seen at the distal tip of the embryo. (F) Confocal imaging of a cellular protrusion (arrow) forming from Sox2-Venus (epiblast lineage; yellow) into the VE layer through a breach in the basement membrane (arrowhead; anti-collagen IV, magenta). Nuclei are stained with DAPI (blue). Bar, 50 μ m. Images adapted from Hiramatsu et al., (2013) with permission from Elsevier.

post-implantation development in the mouse, the maternal uterine tissue spatially restricts the growing embryo, causing the elongated shape of the egg cylinder (Fig. 2 A). This spatial restriction is thought to increase mechanical stress specifically at the distal tip of the forming embryo, where ruptures in the basement membrane occur (Fig. 2 A). These breaches allow early epiblast cells to migrate through gaps and form the distal visceral endoderm, a group of cells critical to the establishment of the anterior–posterior axis (Rossant and Tam, 2009). To investigate a possible role of mechanical force in generating breaches, a series of sophisticated experiments were performed with micro-fabricated cavities of varying width and shape as well as pressure

applied at the distal end of the embryo with an atomic force microscopy cantilever (Hiramatsu et al., 2013). This work demonstrated that the spatial restriction exerted on the developing mouse embryo and increased mechanical forces at the distal region can generate basement membrane breaches (Fig. 2, B and C). Further, in addition to mechanical pressure, specific thinning of the basement membrane as identified by decreased type IV collagen deposition also appears to contribute to basement membrane breaching (Fig. 2, B–E). Interestingly, key MMPs are not expressed at the distal tip and fluorescent protease reporters were not active, suggesting that MMPs and protease are not required for disrupting the basement membrane.

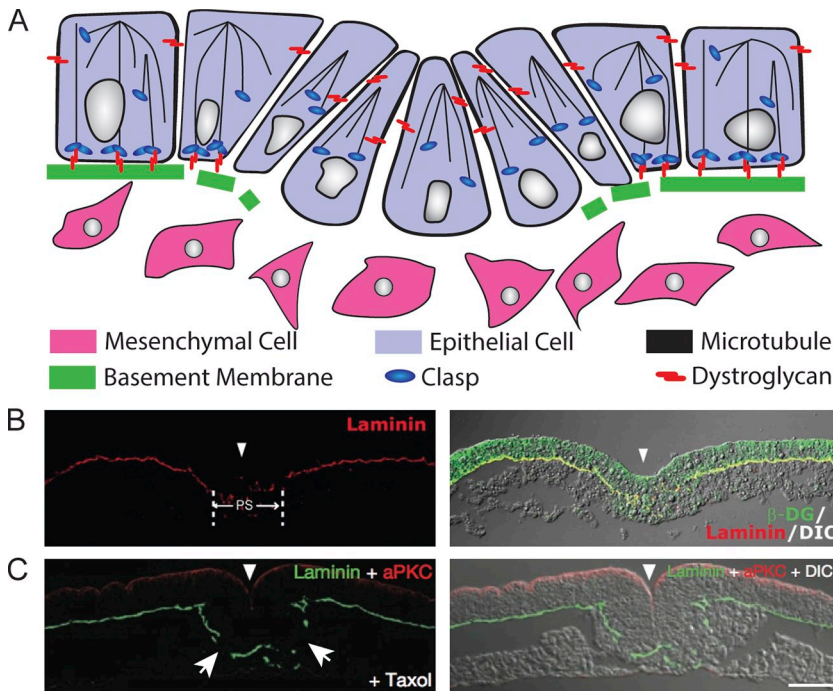


Figure 3. Basement membrane disassembly during EMT in the gastrulating epiblast cells of the chick embryo. (A) Before EMT, the epiblast cells (light purple; lateral cells) interact with the underlying basement membrane (green) through CLASP-mediated (blue) and dystroglycan-mediated (red) cortical anchoring of microtubules (black). At the initiation of EMT, basement membrane is lost under the epiblast cells at the streak midline (epiblast medial cells). This loss is associated with the down-regulation of CLASP proteins leading to the destabilization of microtubules and a loss of dystroglycan localization at the cell–basement membrane interface. The invading epiblast cells de-epithelialize, ingress, and form into mesoderm and endoderm precursors (dark pink) during EMT. (B) Fluorescence microscopy showing that laminin (marking basement membrane; red) and dystroglycan (green) are lost in the primitive streak (PS; arrowheads indicate streak midline) during chick gastrulation EMT. Images adapted from Nakaya et al. (2013). (C) Treatment of embryos with taxol, a microtubule-stabilizing agent, results in retention of the basement membrane (laminin; green) in the medial epiblasts (arrowheads indicate streak midline). Numerous gaps in the basement membrane, however, are still present (arrows). Images adapted from Nakaya et al. (2008) with permission from Macmillan Publishers Ltd.: *Nature Cell Biology*, copyright 2008. Bar, 20 μm .

Together, these studies confirm a role for mechanical force in directing basement membrane breaching.

A key question remaining is how mechanical forces lead to basement membrane breaches. Epiblast cells at the distal region where basement membrane breaks occurred transformed from organized squamous or cuboidal rows to a highly irregular pattern of cell shapes and protrusions, consistent with invasive, migratory behavior (Fig. 2 F). It is unknown if this change in cell organization preceded or followed disruptions in the basement membrane. Thus, mechanical forces could be acting directly on the distal epiblast cells, transforming them into invasive cells. Consistent with this possibility, physical forces generated by unregulated contractions in the intestinal smooth musculature in zebrafish has been shown to trigger basement membrane invasion and formation of invadopodia-like protrusions in neighboring epithelial cells (Seiler et al., 2012). Alternatively, mechanical stresses might directly act on the basement membrane to induce tears or gaps, which then transform the epiblast cells to an invasive state. To further understand the role of mechanical forces, live-cell imaging will be important in deciphering the temporal and spatial dynamics that regulate basement membrane integrity and cell morphology.

Chick gastrulation: Loss of dystroglycan disrupts basement membrane. Large breaches in epidermal basement membranes have been observed during EMT events in sea urchin, chick, and mouse embryos, when epithelial cells lose cell–cell contacts and adopt an invasive, mesenchymal phenotype (Shook and Keller, 2003; Bort et al., 2006; Gouzi et al., 2011; Williams et al., 2012). Studies of chick gastrulation, during which mesodermal and endodermal cells undergo EMT and migrate into the interior of the developing embryo, have begun to reveal new mechanisms that regulate basement membrane clearance. In chick and mouse the first cell-biological sign of EMT during gastrulation is the initiation of basement membrane

breakdown (Nakaya et al., 2008; Williams et al., 2012). Loss of the basement membrane in the chick is regulated by the destabilization of microtubules at the basal cortex of the epithelial cells at the primitive streak (site of gastrulation; Fig. 3 A; Nakaya et al., 2008, 2011). Before the initiation of basement membrane breakdown, the microtubules are positioned along the apico-basal axis with their plus-end oriented toward the basal region of the cell that contacts the basement membrane. Several cytoplasmic linker-associated proteins (CLASPs), microtubule plus-end-tracking proteins, have recently been shown to mediate the stabilization of microtubules in the basal cortex of chick epithelial cells (Nakaya et al., 2013). At the initiation of basement membrane breakdown, CLASP expression is down-regulated in primitive streak cells and basal microtubules are lost. Localized RhoA inactivation is also thought to destabilize the microtubules, but its connection to CLASP activity is unclear. Intriguingly, CLASP directly binds to the dystroglycan receptor, a key laminin- and basement membrane-binding receptor, and supports dystroglycan localization along the basal surface in contact with the basement membrane (Fig. 3 A; Nakaya et al., 2013). During primitive streak formation, when basal CLASP expression is lost, dystroglycan receptor polarization to the basal membrane is disrupted (Fig. 3, A and B). Notably, overexpression of CLASP in primitive streak cells led to retention of dystroglycan and the basement membrane in these cells. In addition, loss of dystroglycan in epithelial cells lateral to the primitive streak led to basement membrane breakdown. These observations lead to an attractive model for basement membrane removal, where localized loss of a key receptor mediating basement membrane maintenance results in disruption of the basement membrane. Integrins may also be involved, but the presence of numerous integrin receptors present in the primitive streak has hindered experimental dissection (Nakaya et al., 2011).

Given the prevalence of EMT in development, it will be important to examine the role of microtubules, CLASP proteins, and dystroglycan (and integrin) in other EMT and invasion events. Importantly, dystroglycan is not generally essential for basement membrane assembly (Li et al., 2003) and its genetic loss does not result in the disruption of most basement membranes (Williamson et al., 1997; Johnson et al., 2006). The requirement for dystroglycan in basement membrane formation and maintenance appears to be limited to specific tissues (Williamson et al., 1997; Michele and Campbell, 2003). Moreover, breaks in the basement membrane were still observed after inappropriate retention of microtubules, CLASP, and dystroglycan in the primitive streak cells undergoing EMT (Fig. 3 C; Nakaya et al., 2008, 2013), suggesting that other mechanisms act in parallel to promote basement membrane removal. Consistent with this notion, loss of the basement membrane in streak cells is also accompanied by reduced expression of basement membrane components and increased expression of MMPs that might contribute to basement membrane loss (Alev et al., 2010; Nakaya et al., 2011, 2013). In addition, it is unknown if invadopodia contribute to breaching the basement membrane during chick gastrulation. It will be important to fully elucidate these other factors, as they might lead to an understanding of how multiple activities coordinately contribute to breaching and removing the basement membrane.

***Drosophila* metamorphosis and tumors: An undefined role for MMPs.** MMPs are up-regulated in most tumors, cleave collagen *in vitro*, and loss of MMPs in cultured cancer cells inhibits invasive behavior through artificial matrices and denuded basement membranes (Liotta et al., 1980; Overall and Kleinfeld, 2006; Rowe and Weiss, 2008). Despite this evidence, studies in mouse models have yet to confirm a role for MMPs in cell invasion through the basement membrane, possibly due to genetic redundancy (Rowe and Weiss, 2008). Further, clinical trials that targeted MMPs in late-stage cancer patients failed to increase survival, for reasons that are unclear (Coussens et al., 2002; Overall and Kleinfeld, 2006).

The *Drosophila* model of MMPs offers a means of uncovering the role of these proteins during invasion through basement membrane. One key advantage of *Drosophila* is the simplicity of the MMP family of proteases. Whereas vertebrate genomes encode ~24 MMPs, *Drosophila* harbors only two, *Mmp1* and *Mmp2* (Page-McCaw et al., 2007). These MMPs, one secreted and one membrane bound, contain the canonical MMP structure but have no direct human MMP orthologues (Page-McCaw, 2008).

Drosophila imaginal discs are formed by two juxtaposed epithelia, an outer squamous peripodial epithelium and stalk (PS) and an inner columnar disc proper epithelium. During metamorphosis the PS epithelium and larval epidermis become apposed along their basal surfaces, positioning their respective basement membranes in direct contact. The PS cells then undergo a partial EMT and invade the larval epidermis, breaking through the juxtaposed PS and larval basement membranes (Pastor-Pareja et al., 2004). This invasive process clears a path through the larval epidermis for disc eversion and formation of adult structures, such as the wing. EMT in the PS cells requires activation of the Jun N-terminal kinase (JNK) pathway, which

up-regulates the expression of *Mmp1* and *Mmp2* in the invasive PS cells (Srivastava et al., 2007). Hypomorphic mutants in *Mmp1* and *Mmp2* lead to maintenance of Viking-GFP, a functional type IV collagen fusion protein, indicating that MMPs promote basement membrane removal during eversion. In addition, ectopic activation of JNK signaling in epithelial cells that normally maintain the basement membrane also led to *Mmp1*-dependent basement membrane loss (Srivastava et al., 2007). These results establish a role for MMPs in promoting basement membrane loss. Whether MMPs remove the basement membrane through bulk dissolution, limited proteolysis, or through indirect mechanisms, however, remains unclear.

Drosophila imaginal discs are also emerging as a powerful model for tumor invasion and dissemination in which mosaic loss of tumor suppressors or overexpression of oncogenes results in invasive epithelial cells (Gonzalez, 2013). In all models examined to date, MMPs appear to be required for dissemination of tumor cells, which are thought to cross basement membranes (Vidal et al., 2006; Beaucher et al., 2007a,b; Page-McCaw, 2008). In both tumor development and disc eversion, however, the interactions between cells and matrix that lead to basement membrane removal have not yet been visualized. MMPs have numerous targets and activities outside of matrix proteolysis and thus they could mediate indirect functions that contribute to basement membrane loss (Overall and Kleinfeld, 2006). Advances in imaging could make *Drosophila* an opportune model to define the specific role of these proteases in basement membrane invasion.

Breaching the endothelial basement membrane

Most blood vessels are composed of two cell populations, an interior thin layer of endothelial cells that form a confluent monolayer and an exterior group of contractile pericytes arranged in a loose net or patch-like manner with gaps between the cells. Both cell types contribute components to a basement membrane that surrounds the endothelium and embeds the pericytes (Hallmann et al., 2005). Leukocytes traffic through the circulatory system during immune surveillance in response to inflammatory signals and cross this basement membrane. Further, metastatic cancer cells also undergo intravasation (entry) and extravasation (exit) from the circulatory system to enable their spread (Madsen and Sahai, 2010). In the process of making additional vessels from preexisting ones (angiogenesis), sprouting epithelial cells have also been suggested to breach the basement membrane (summarized in Fig. 4; Senger and Davis, 2011). This notion remains controversial, however, as there are many reports of endothelial sprouts covered by a basement membrane, suggesting that the basement membrane might be continually remodeled as endothelial sprouts emerge and new vessels extend (Baluk et al., 2003). In this section we discuss vertebrate models (mice and zebrafish) that are revealing a remarkably diverse set of strategies that cells use to break through or avoid the basement membrane during leukocyte trafficking. We also review studies that are providing hints at how cancer cells might traverse this barrier.

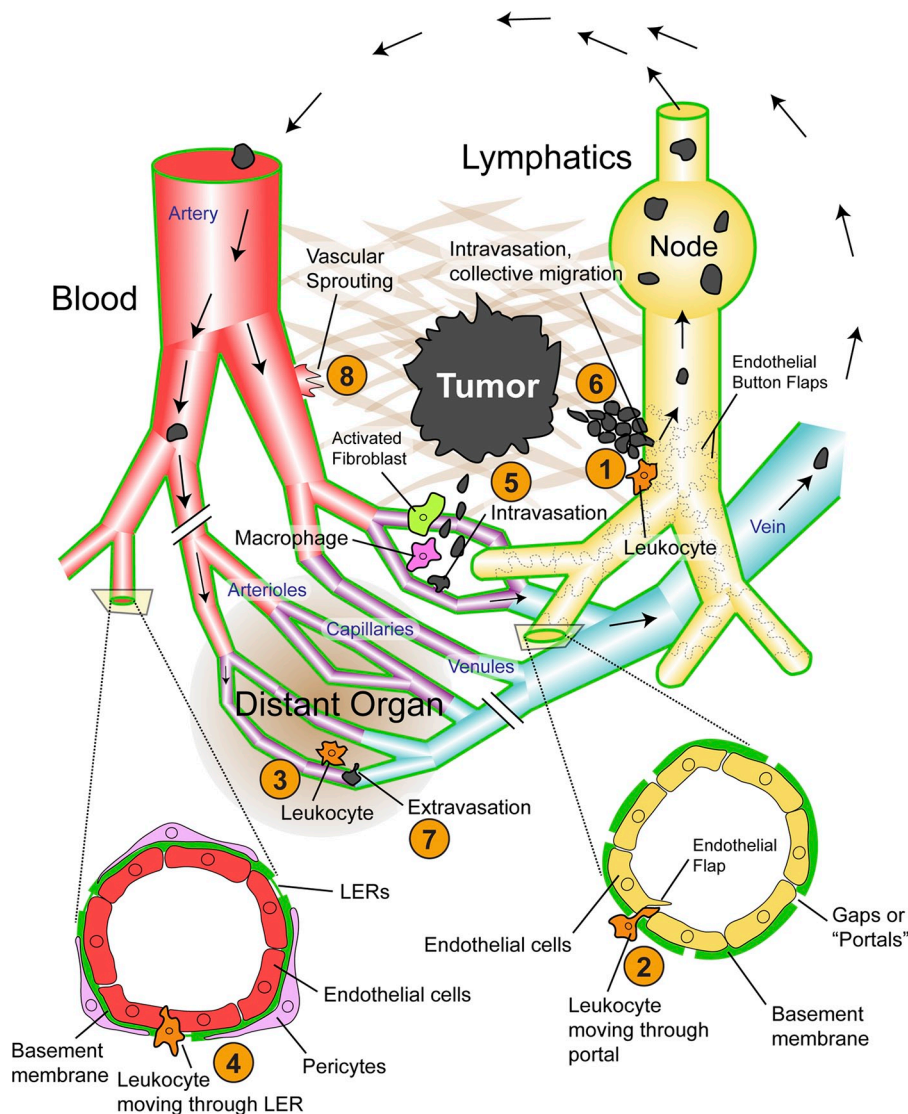


Figure 4. Crossing of vascular endothelial basement membrane. Leukocytes readily traverse vessel walls during immune surveillance by entering the lymph (1) through gaps in the basement membrane (2) and exiting through venules (3) at low expression regions (LERs) of basement membrane in capillary beds (4). Tumor cells migrate away from the primary tumor and intravasate via the blood (5) or lymphatic system (6), perhaps aided by activated fibroblasts and/or macrophages. During metastatic dissemination, circulating tumor cells ultimately become lodged in capillary beds and extravasate to colonize distant organs (7). In addition, activated endothelial cells might break down basement membrane during vascular sprouting at the initiation of angiogenesis (8). See text for additional details.

Leukocytes traffic through preformed exit and entry sites. Leukocytes primarily exit the vasculature in venules, small blood vessels that drain capillary beds (Fig. 4). Electron microscopy studies have suggested that leukocytes easily traverse the thick layer of endothelial cells that line venules but stall when they reach the basement membrane, indicating that leukocytes might use a distinct strategy in crossing this barrier (Thompson et al., 2001; Nourshargh et al., 2010). Recently, an effective model using an *in vivo/ex vivo* assay for venule extravasation in the mouse has been developed. In this assay the tissue surrounding the venules can be experimentally manipulated by inducing inflammatory responses or by injecting inhibitors of specific molecules. After perturbations, the tissue is then fixed and immunostained for basement membrane composition and leukocyte location and morphology (Wang et al., 2006). This approach has identified regions at gaps between pericytes that have reduced expression of basement membrane components (low-expression regions [LERs]; Wang et al., 2006; Voisin et al., 2010). After stimulation by cytokines and several other inflammatory stimuli, neutrophils and monocytes were preferentially localized at LERs (Fig. 4). Further, both populations

of cells generated protrusions before and during localization to LERs, suggesting that they actively seek these sites for crossing the venule basement membrane (Voisin et al., 2009). It is not understood whether these sites require further matrix remodeling to allow leukocyte passage. Monocytes can squeeze through LERs (~1.8 μm wide) without remodeling the basement membrane, whereas neutrophils cause enlargement of the LERs during invasion and are decorated with laminin on their surface (Wang et al., 2006; Voisin et al., 2009, 2010).

Several proteases have been implicated in LER remodeling including MMP8, MMP9, and neutrophil elastase (Young et al., 2007; Lin et al., 2008; Reichel et al., 2008; Voisin et al., 2009). Notably, pharmacological inhibition of neutrophil elastase reduces neutrophil migration through the basement membrane (Voisin et al., 2009). These results suggest that proteolytic cleavage of the basement membrane might be necessary at LER sites. Given off-target effects of inhibitors and lack of leukocyte extravasation phenotypes in mice genetically lacking elastase, the role of proteases remains controversial (Yadav et al., 2003; Wang et al., 2006; Reichel et al., 2008). Further, endothelial cell-associated proteases have also been implicated in generating

chemotactic cues that promote leukocyte migration (Deem and Cook-Mills, 2004; Rowe and Weiss, 2008). A recent study has begun to clarify the cellular characteristics that dictate protease-dependent and -independent invasion (Wolf et al., 2013). Nuclear deformity was found to be the limiting factor allowing cells to invade through small pore sizes without dependence on protease-mediated ECM remodeling. Leukocytes appear able to migrate through narrow gaps because they have the ability to greatly deform their nuclei, owing to the absence of the nuclear envelope structural protein laminin A/C. The emerging model of leukocyte invasion is that cells detect and use these existing LER “gates” in vessels that impose the least structural resistance to invasion. Proteases might play a role in restructuring the basement membrane to enlarge or deform the matrix to facilitate passage. This might explain why neutrophil transmigration of LER sites is often associated with carriage of laminin (Voisin et al., 2009).

Leukocytes primarily enter the vasculature through the peripheral lymph system (Fig. 4). Examination of cells and matrix in this region has revealed a unique organization. This area lacks pericytes (Petrova et al., 2004; Hallmann et al., 2005), and rather than an arrangement of continuous, zipper-like cell–cell junctions, lymphatic endothelial cells have junctional arrangements that allow periodic $\sim 3.0\text{-}\mu\text{m}$ moveable flaps between cells, just above the limits of leukocyte deformability (Baluk et al., 2007). Examination of basement membranes in the peripheral lymph system using whole-mount immunohistology revealed the existence of gaps completely devoid of a basement membrane, which accounted for up to 30% of the total vessel area (Pflücke and Sixt, 2009). Further, using live-cell imaging of an *ex vivo* model, where living explanted mouse ears are fluorescently labeled with antibodies to laminin or type IV collagen, leukocytes (dendritic cells) were observed extending protrusions into these gaps and then entering vasculature through the button-like flaps between endothelial cells (Fig. 4). These observations offer an explanation as to why the genetic loss of integrin function and broad-spectrum inhibitors of ECM proteolysis fail to block leukocyte intravasation (Lämmermann et al., 2008; Pflücke and Sixt, 2009). Thus, analogous to leukocyte exit, leukocyte entry occurs through specialized portals where the basement membrane is absent.

Dynamic interactions of cancer cells at sites of vascular invasion. A large majority of cancers are epithelial in origin. To successfully metastasize, these tumor cells must first breach the underlying epithelial basement membrane, then traverse the stromal ECM and enter the vascular system. Although cancer cells can enter the vasculature through lymphatic vessels, cancer cells primarily use the blood vasculature as an entry route (Madsen and Sahai, 2010; van Zijl et al., 2011). Once in the vasculature, cancer cells exit through extravasation at distant sites (summarized in Fig. 4; Valastyan and Weinberg, 2011). Intravital (optical live-animal) microscopy systems allow for *in vivo* imaging of labeled epithelial tumor cells in mice over several days or weeks (Chishima et al., 1997; Farina et al., 1998; Pittet and Weissleder, 2011). These studies have revealed how tumor cells invade stroma, but have not yet visualized the initiation of the epithelial invasion program through the

basement membrane. This gap in our understanding is largely due to the stochastic nature of these invasions and lack of GFP-tagged basement membrane components in mouse models (Alexander et al., 2013). Although an understanding of tumor cell–basement membrane interaction is lacking, important insights have been made into cellular aspects of tumor cell entry into and exit from the vasculature that suggest diverse mechanisms of crossing the endothelial basement membrane.

In vivo cell tracking by photoconversion is commonly used to indirectly measure intravasation capacity (Kedrin et al., 2008; Gligorijevic et al., 2009; Roussos et al., 2011). In this assay, tumor cells expressing Dendra2 are converted from green to red and then imaged over several hours to measure the rate of cells entering the nearby vasculature (Kedrin et al., 2008). This assay was used to show the requirement of the actin-nucleating protein N-WASP (neural Wiskott-Aldrich syndrome protein) during intravasation of rat adenocarcinoma cells (Gligorijevic et al., 2012). Fixed samples indicated that tumor cells adjacent to blood vessels had immunolocalized N-WASP within membrane extensions that were also enriched with the invadopodia marker cortactin and degraded collagen I. These results suggest that intravasation by adenocarcinoma cells might be mediated by matrix degrading invadopodia. Unfortunately, the resolution required to clearly image invadopodia structures in mouse models is not currently attainable (Beerling et al., 2011).

Elegant *in vivo* studies of tumor cell migration have also indicated that metastatic cancer cells chemotax toward the vasculature and coordinate with macrophages, and possibly even endothelial cells to gain entry into the bloodstream (Fig. 4; Condeelis et al., 2005; Wyckoff et al., 2007; Robinson and Jones, 2009; Roh-Johnson et al., 2013; Zhou et al., 2013). Multiphoton imaging of dual-labeled cancer cells and macrophages revealed that intravasation occurs selectively in regions where perivascular macrophages are present (Wyckoff et al., 2007). It is possible that the macrophages directly remodel the vessel and the cancer cells follow in their tracks. Alternatively, macrophages secrete chemotactic and migration cues including CSF-1, EGF, and TGF- β , which could also act to promote invasion into the vasculature (Wyckoff et al., 2007; Giampieri et al., 2009; Zervantonakis et al., 2012).

Zebrafish xenografts have complemented mouse work and have proven to be an effective model for studying interactions between engrafted tumor cells and the vascular endothelium during intravasation (Stoletov et al., 2007). Stoletov et al. (2007) imaged RFP-tagged breast adenocarcinoma cells for 17 d after injection into the peritoneal cavity of adult fish containing GFP-labeled endothelium. They found that tumors locally invade the body wall and often induce remodeling of the vasculature, which was exacerbated when the injected adenocarcinoma cells overexpressed vascular endothelial growth factor (VEGF). The remodeled vasculature was leaky and displayed large gaps in the endothelial lining in which the cancer cells integrated, a process known as vascular mimicry. Co-expression of VEGF with the Ras homologue gene family member C (RhoC) endowed the adenocarcinoma cells with the ability to extend long protrusions into the VEGF-induced vascular gaps and intravasate. This study represents an unexpected model of

tumor intravasation where cancer cells enter the blood by inducing gaps in the vasculature (likely resulting in gaps the basement membrane), thus avoiding directly breaching the basement membrane.

Xenograft studies in zebrafish and mouse models have also begun to reveal cellular aspects of extravasation (Kienast et al., 2010; Stoletov et al., 2010). For example, multi-photon imaging in mice has shown that circulating melanoma cells passively arrest at vascular branch points and then actively exit blood vessels by dynamically extending and retracting cellular protrusions through the vessel wall (Fig. 4; Kienast et al., 2010). Hours after injection of colon carcinoma cells into the tail vein in mice, tumor cells were seen using electron microscopy extending processes through gaps in the endothelial layer toward the basement membrane (Weis et al., 2004). Similar to intravasation, tumor cells induce remodeling and generate gaps in endothelial cells. In this case, secretion of VEGF induces Src kinase activation in the endothelium and uncouples VE-cadherin junctions. Confocal imaging revealed that extravasation of cancer cells in zebrafish is remarkably similar (Stoletov et al., 2010). Cells arrest at vessel branch points, migrate along the vessel wall with or against blood flow in an integrin-dependent manner, and induce endothelial remodeling to extravasate. Recent *in vitro* 3D microvascular networks have supported these findings, and have shown using live-cell imaging that cancer cells extend filopodial protrusions ($\sim 1 \mu\text{m}$ in width) that breach the endothelial barrier through endothelial cell-cell junctions (Chen et al., 2013). The breach site ultimately expands to $\sim 9 \mu\text{m}$ and thus allows the nucleus to pass. After invasion, there are no signs of junction disruption, and perfusion with dextran revealed no leaks at the transmigration site, suggesting that alterations to the endothelial cell layer are temporary and the integrity of the vessel wall is quickly restored.

Taken together, these studies suggest that holes in the basement membrane created during vascular crossing could be actively made by tumor cells themselves using invadopodia, by tumor-associated fibroblasts (Gaggioli et al., 2007), or macrophages (DeNardo et al., 2009; Dovas et al., 2013). Tumor cells also appear capable of inducing remodeling of the endothelium to induce gaps (Stoletov et al., 2007, 2010; Chen et al., 2013). It seems likely that tumors could also use LER sites or gaps in the basement membrane at lymphatic entry sites (Azzali, 2007). Tumor cells might also use physical cues to cross the basement membrane, such as mechanical stress (Goel et al., 2011) or expanding tumor bulk (Butcher et al., 2009). Finally, the vasculature associated with tumors in some mouse models has abnormal organization, structure, and areas that are devoid of a basement membrane (Jain, 1988; Fukumura et al., 2010; Beerling et al., 2011), suggesting that intravasating tumor cells in these contexts can completely avoid this barrier. What is lacking from these studies is the ability to visualize the basement membrane during tumor cell invasion. The development of fluorescently tagged basement membrane components, similar to *C. elegans* and *Drosophila*, would advance the field, particularly for live-cell imaging. In addition, CLIM (cryosection labeling and intravital microscopy; van Rijnsvoever et al., 2008) and direct immunostaining of matrix components (Kilarski et al., 2013)

would also allow for staining of the basement membrane in areas where cells are actively invading the epithelial or vascular basement membrane.

Summary and perspective

Basement membrane transmigrations occur during development, physiological processes, and disease states. Recent studies on cell transit through the basement membrane highlighted here suggest that in physiological settings cells cross through this structure in many ways. These include actively removing the barrier through breaching with invadopodia, disruptions by down-regulating of adhesion receptors, and physical forces that might break the basement membrane apart. Also, cells use indirect means to cross basement membrane barriers through induction of cellular remodeling that slide or generate gaps in this structure. Further, in the vasculature there has been the apparent evolution of preformed portals in the basement membrane that facilitate leukocyte trafficking. These diverse mechanisms are likely inappropriately activated or used in numerous inflammatory diseases and cancer, providing cells with a rich and varied toolkit to cross through basement membranes and enter new tissues. Several key questions remain in our understanding of cell transit through the basement membrane. Among these are the role and regulation of basement membrane structure and cross-linking during transit events. With at least 20 different basement membrane proteins and no methods for examining cross-linking status at regional sites, it is unknown, but seems highly likely, that basement membrane composition and cross-linking are actively regulated to facilitate transit (Rowe and Weiss, 2008; Halfter et al., 2013). The role of proteases is also a significant open question. There are over 500 proteases encoded in vertebrate genomes and over 200 in *C. elegans*; thus, standard genetic approaches might not be sufficient for analysis of their critical and possibly overlapping functions (Rowe and Weiss, 2008; Ihara et al., 2011). More sophisticated assays to follow proteolysis, cross-linking, composition, and fates of basement membrane components will be central to advancing our understanding of invasion. Finally, real-time analysis of cell-basement membrane interactions in living model organisms is key to further elucidating mechanisms underlying invasion. This will be important in determining if invadopodia-like structures are a required component (and thus a possible therapeutic target in human diseases) of the cellular machinery to make *de novo* breaches in the basement membrane (Rottiers et al., 2009; Seiler et al., 2012; Hagedorn et al., 2013). Given that the basement membrane arose at the dawn of animal multicellularity ~ 600 million years ago, we also expect that many undescribed and fascinating mechanisms have evolved for transit through this structure that await discovery through simply watching cells interact with the basement membrane.

We thank A. Schindler and M. Morrissey for helpful comments.

This work was supported by grant F32 GM103148 (to L.C. Kelley), The Pew Scholars Program in the Biomedical Sciences, and National Institutes of Health grants GM079320 and GM100083 (to D.R. Sherwood).

The authors declare no competing financial interests.

Submitted: 27 November 2013

Accepted: 14 January 2014

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