



Published in final edited form as:

Trends Cell Biol. 2017 January ; 27(1): 12–25. doi:10.1016/j.tcb.2016.08.003.

Divide or Conquer: Cell cycle regulation of invasive behavior

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Abstract

Cell invasion through the basement membrane (BM) occurs during normal embryonic development and is a fundamental feature of cancer metastasis. The underlying cellular and genetic machinery required for invasion has been difficult to identify, due to a lack of adequate *in vivo* models to accurately examine invasion in single cells at subcellular resolution. Recent evidence has documented a functional link between cell cycle arrest and invasive activity. While cancer progression is traditionally thought of as a disease of uncontrolled cell proliferation, cancer cell dissemination, a critical aspect of metastasis, may require a switch from a proliferative to an invasive state. Here we review evidence that BM invasion requires cell cycle arrest and discuss the implications of this concept with regards to limiting the lethality associated with cancer metastasis.

Keywords

cell invasion; cell cycle arrest

Linking Cell Invasion and Cell Cycle Regulation

The basement membrane (BM, see Glossary), or basal lamina, is a specialized form of extracellular matrix (see Glossary) and a metazoan innovation [1, 2] that likely helped support the evolution of the three-dimensional body plan [3, 4]. Structurally composed of polymeric laminin and cross-linked type IV collagen networks, the BM is a thin, dense, sheet-like material that provides structural support for epithelial and endothelial tissues and functions as a barrier limiting cellular movement [5]. However, specific cell types, notably those involved in embryogenesis and cancer, have evolved the ability to actively breach or cross BM barriers by adopting an invasive phenotype [5, 6] (Fig. 1).

Cell invasion is a morphogenetic behavior that results in the penetration of tissue barriers including the BM and, in vertebrates, the interstitial type I collagen from the stroma [7, 8]. Acquisition of invasive behavior requires both dynamic restructuring of the actin and microtubule cytoskeleton, along with changes in transcriptional and epigenetic states [9-11].

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Cells can invade individually or collectively by maintaining cell-cell adhesions, led by highly protrusive “leader cells” [12, 13]. Invasive cells adopt either a mesenchymal or amoeboid morphology. Molecularly, mesenchymal and amoeboid invasion are defined quite differently, based on reliance of proteolytic versus ROCK/actomyosin-dependent mechanisms, respectively [13-15]. While these different invasive behaviors are usually segregated in the cancer literature, it is becoming more apparent that many invasive cells, particularly during cancer progression, are quite plastic and can adopt different morphogenetic programs based on their local environment [13, 14, 16]. For those cells that utilize a mesenchymal invasion program, the switch from epithelial to mesenchymal cell morphology is often referred to as epithelial to mesenchymal transition (EMT, see glossary), and occurs across a range of cell types throughout organismal development [17]. While defining EMT during cancer progression is more challenging [18], similar transitions between cell morphology have been documented [19]. For the purpose of this review, we refer to this morphogenetic switch in cancer as EMT.

At a cell biological level, invasion requires adhesion to and degradation of extracellular matrix components [8, 20]. While, in many systems, BM invasion is often associated with migration through the stroma, it is important to note that cells require unique genetic control mechanisms to remove the BM, independent of the genetic networks that regulate cell migration [6, 21]. The molecular and genetic mechanisms underpinning invasive cellular behavior have been challenging to elucidate. This is largely due to the difficulty of modeling this dynamic, complex behavior *in vitro* using artificial substrates. Fortunately, recent insights from traditional model systems including *C. elegans* [10, 11, 22], *Drosophila* [23], zebrafish [24], chick [25], and mouse [26-28] have begun to illuminate how cells breach BM *in vivo*.

C. elegans anchor cell (AC) invasion (see Glossary) into the vulval epithelium during nematode larval development has proved particularly useful in decoupling invasion and migration to examine invasive cellular behavior [29] (Fig. 2A). The AC, a specialized somatic gonadal cell, initiates uterine-vulval attachment by invading through the BMs separating these developing tissues [29]. As the non-motile AC maintains adhesion to neighboring uterine cells, examination of this invasive event permits separation of invasion from migratory behavior. Furthermore, researchers can visualize *C. elegans* AC invasion through a fluorescently labelled BM using live cell imaging [30].

Recent data from *C. elegans* AC invasion have linked cell cycle control with BM invasion [9], suggesting that invasive behavior may be functionally coupled to the proliferative states of various cell types. Specifically, the AC must be in the G1/G0 phase of the cell cycle in order to invade [9]. However, it is unclear whether G1/G0 cell cycle arrest (see Glossary) represents a general principle of all invading cells. Here, we review the potential conservation of cell cycle arrest in the invasive cascade across Metazoa, in normal and pathological states. Whether metastatic invasive cells also require discrete cell cycle control is an open question with important implications for future therapeutics designed to regulate invasive behavior during pathogenic processes.

Cell cycle regulation of invasion during development

Invasive behavior is a critical component of metazoan development. This section reviews literature that suggests that the acquisition of invasive behavior during development is specifically regulated in a cell cycle-dependent fashion. During mammalian embryo implantation (Fig. 1A), cytotrophoblasts, the first embryonic cell type to exhibit highly specialized functions, differentiate into extravillous trophoblasts, which then invade into the uterine lining, as the first step of placentation [31]. This differentiation event is regulated by several transcription factors [32] that control the expression of downstream effectors of trophoblast invasion, including adhesion molecules [33] and MMPs [34] and is required for the adoption of the invasive phenotype. To differentiate, extravillous trophoblasts exit the cell cycle in the G1 phase and upregulate cyclin dependent kinase inhibitors (CKIs, see Glossary) such as p16^{INK4a}, p21^{CIP1} and p27^{KIP1} [35]. Whether cell cycle arrest is required for these trophoblast cells to adopt an invasive phenotype is currently unknown.

EMT is often associated with invasiveness and appears to be regulated in a cell cycle-dependent fashion [36-40]. EMT-associated cell behaviors in development and cancer progression demonstrate a strong association between loss of proliferation through downregulation of mitotic cyclin/CDK activity and upregulation of CKIs [36, 40] (Fig. 3 and Table 1). In some animals, gastrulation proceeds through EMT-initiated cellular movements that include endomesodermal cells adopting an invasive phenotype and passing through a BM. In sea urchin (*Lytechinus variegatus*) gastrulation, primary mesenchyme cells cross the BM (Fig. 1B) and divide only after invading into the blastocoel [41, 42]. Similarly, during chick gastrulation, cells undergo an EMT associated with BM removal [25]. Whether invasive gastrulation movements such as these in urchins and chick, require cell cycle arrest is currently unknown.

During vertebrate development, neural crest cells, a population of specialized migratory cells, give rise to melanocytes, craniofacial structures, including cartilage and bone, as well as smooth muscle, and peripheral and enteric neurons and glia. Neural crest cells undergo an EMT-like behavior as they delaminate (see Glossary), crossing the nascent BM that lies over the dorsal portion of the neural tube [43] (Fig. 1C). Trunk neural crest appears to delaminate at the G1/S phase transition [44], while the delamination of the cranial neural crest does not appear to be cell cycle-dependent [45]. Live-cell imaging with FUCCI has revealed that the majority of cranial neural crest cells following delamination are quiescent during their initial migration and show altered cell cycle dynamics dependent on their destination, with some cells rapidly proliferating and others exiting the cell cycle [46]. Unfortunately, the BM has never been visualized during live neural crest migration, making it difficult to draw conclusions related to cell cycle state as individual neural crest cells cross BM barriers.

Development and Cancer: two sides of the same coin

Cancer cells hijack developmental regulatory programs and signaling pathways to execute the suite of behaviors required for metastasis. Thus, the same morphogenetic cell biological behaviors and molecular cues that are required for developmental processes such as gastrulation and neural crest delamination during embryogenesis are also utilized by tumor

cells to proliferate, communicate with the surrounding microenvironment, and adopt an invasive phenotype [47]. For processes like cell invasion, which are challenging to study in the complex *in vivo* environments where they occur, insights gained from the study of simple developmental systems such as *C. elegans* AC invasion have been helpful in elucidating general principles underlying invasive behavior.

The single AC normally exists in a post-mitotic, cell-cycle arrested state [9], where, in response to extracellular cues, F-actin and actin regulators are recruited to the basolateral surface of the AC, generating dynamic, F-actin rich, protrusive, membrane-associated, punctate structures called invadopodia (Fig. 2A) [11]. Through coordination by netrin signaling, a single invadopodium breaches the underlying BM, connecting the uterine and vulval tissues [11, 29, 30, 48]. Intrinsically, AC invasion is under the control of the conserved AP-1 transcription factor, *fos-1a*, which regulates the activity of the matrix metalloproteinase (MMP), *zmp-1* [22]. Loss of *fos-1a* results in the failure of the AC to breach the BM.

What evidence exist that cancer cells and the *C. elegans* AC share conserved genetic programs mediating invasive behavior? First, human orthologs of pro-invasive genes that function during AC invasion (*i.e.*, AP-1/Fos, EVI1, Netrin1 and integrins) have been shown to regulate invasion in mammalian cells [49-52]. Second, RNAi screens in *C. elegans* have identified novel pro-invasive genes (*i.e.*, NLK and the CCT complex), which, when depleted in both breast and colon cancer cell lines resulted in inhibition of invasion [10]. Third, the AC utilizes invadopodia to breach the underlying BM [11, 53, 54] (Fig. 2A). Based on data from cancer cell lines and a wealth of *in vitro* experimental data, invadopodia have been implicated in invasive behavior, but their significance *in vivo* has been controversial due to the difficulties associated with resolving subcellular structures with adequate temporal resolution in complex environments where invasion occurs in vertebrates. However, recent data from *C. elegans* demonstrates a functional requirement for G1/G0 cell cycle arrest in the acquisition of an invasive phenotype *in vivo* [9].

A targeted RNA interference (RNAi) screen identified the conserved NR2E1 class nuclear hormone receptor transcription factor, *nhr-67/tlx*, as a novel regulator of AC invasion. Loss of *nhr-67/tlx* results in the normally post-mitotic AC entering the cell cycle, proliferating, and failing to invade (Fig. 2B). Live-cell imaging revealed that mitotic ACs do not localize invadopodia, nor do they express pro-invasive genes, including MMPs and F-actin regulators, suggesting that the entire invasion program is altered in cycling ACs. In support of the G1 phase of the cell cycle being critical for invasive activity, AC-specific expression of the cyclin-dependent kinase inhibitor, *cki-1* (p21^{CIP1}/p27^{KIP1} homolog), induces G1/G0 arrest in *nhr-67*-deficient ACs, and restores invadopodia formation and MMP expression, thereby rescuing invasion (Fig. 2C) [9].

If the dichotomy between invasion and proliferation were solely based on the incompatibility of cell invasive behavior and active cell division, then one could imagine that a pause in any phase of the cell cycle prior to mitosis could be permissive to invasive activity. Induced cell cycle arrest, however, in the S or G2 phase fails to rescue the invasive activity of mitotic *nhr-67*-deficient ACs, implicating the G1 phase of the cell cycle as critical

for the acquisition of an invasive phenotype [9]. Taken together, data from mammalian embryo placentation and EMT-like behaviors during gastrulation and neural crest delamination indicate that cell cycle arrest may be important for acquisition of an invasive phenotype. These data are strongly supported by genetic and live-cell imaging data from *C. elegans* AC invasion that clearly defines a relationship between G1/G0 cell cycle arrest and invasion [9]. The following section will highlight the cancer biology literature that also suggests that cell cycle regulation may be required during metastatic progression.

Cell cycle regulation of BM invasion during cancer metastasis

Metastatic processes, particularly cell invasion, remain poorly understood *in vivo* [55], due to the difficulty of studying complex biological processes occurring deep within organisms. BM invasion is required at multiple steps in the course of metastasis: at the primary tumor, during intra- and extravasation of blood vessels, and at new tissue compartments, where they can form secondary tumors [5, 6, 8, 20] (Fig. 1D). *In vitro* models of invasion using native BM, such as rat peritoneal BM [56] have not yet been used to assess the cell cycle state of invasive cells. Other biological membranes, such as gelatin [57] exist, but the most prevalent model used for invasion assays is Matrigel, an extracellular matrix-like secretion of EHS sarcoma cells [58]. While Matrigel contains many of the same proteins as BM (*e.g.* laminin, type IV collagen and nidogen), cellular invasion through Matrigel does not necessarily correlate to invasiveness *in vivo* [59]. Specifically, Matrigel has been shown to be more permissive to invasion and lacks the intricate network of covalently crosslinked type IV collagen found in endogenous BM [5, 8, 59, 60]. Thus, the relationship between BM invasion and cell cycle state during cancer metastasis is poorly understood.

As cancer is primarily characterized by uncontrolled cell proliferation [61], it may initially seem counterintuitive that there exists a switch between cell proliferation and BM invasion. Nonetheless, there is mounting evidence that such a switch exists. Recent theoretical work using *in silico* metabolic modeling predicts that cancer cells that are able to switch to a less proliferative state when presented with physical barriers and fewer metabolic resources are more likely to not only survive but to spread more efficiently to distant sites as compared to more proliferative tumor cells [62]. Importantly, this *in silico* model of invasive versus proliferative cancer cell behavior is based on experimental data, and provides a framework for others to test the interplay between proliferation and invasion that likely occurs in many cancers. Here, we review the potential link between cell cycle arrest and invasive behavior. Due to the evolving nature of the field, we have based our assessment of the cell cycle state of cancer cells during invasion on multiple experimental methodologies (Table 1). The strongest experimental evidence stems from *in vivo* histopathological data. We have also included studies that utilize matrix invasion assays coupled with careful analysis of cell cycle state. As simultaneous studies of cell cycle and BM invasion remain challenging in living specimens, we have discussed work using measures of metastatic potential as a proxy for invasiveness. Much of the available data at present remains descriptive and correlative, thus, our ability to make definitive statements about the cell cycle state of tumor cells actively invading BM is limited by the available cancer literature.

Evidence of a switch between proliferation and invasion stems from studies performed in a wide variety of cancer types (Table 1). For cancers of epithelial origin, the ability of cancer cells to undergo EMT has long been associated with invasiveness and other metastatic characteristics (reviewed in [19]). In epithelial cell lines, as in developmental systems, the developmental bHLH transcription factors Snail and Slug have been shown to induce an EMT-like state [63]. Upon Snail-mediated induction of EMT in mammalian embryogenesis and cancer, cell cycle progression is impaired and the cell cycle is arrested in G1/G0 through increased expression of p21^{CIP1} [36]. In melanoma, the bHLHe32 transcription factor MITF controls the switch between proliferative and invasive states [64]. Tumor samples with high MITF expression revealed enhanced invasiveness but decreased tumor size and growth rate [65, 66]. In basal cell carcinomas, immunohistochemistry of tumors has shown that the invasive cell population is nonproliferative and expresses markers associated with G1/G0 cell cycle arrest [67]. Also, epidermoid carcinoma cell lines undergo EMT and reduce of Cyclin D1 levels, thereby enhancing invasiveness *in vitro* [68].

Cell cycle arrest may also be required for invasive behavior in lung cancer where pro-metastatic cell cycle arrest is mediated by the expression of p21^{CIP1} and modulated by the T-box transcription factor, Brachyury [69], which also drives EMT in many contexts during embryonic development [70]. Reuse of developmental transcription factors in cancer is common [61]: in osteosarcomas, the transcription factor RUNX1 drives the expression of MMP9, a pro-metastatic gene, and is specifically expressed in the G1 phase of the cell cycle [71]. Additionally, hepatocellular carcinoma cells invade during the G1 phase of the cell cycle [72]. Thus, it appears that EMT-like behavior from multiple cancer types may be linked to G1/G0 cell cycle arrest.

Perhaps, the strongest association between invasive cells and the G1/G0 cell cycle state is seen in breast cancer metastasis [73-80]. Recent, correlative evidence from polyoma virus middle T oncogene (MMTV-PyMT) organoid culture showed that proliferation is not required for the acquisition of an invasive leader cell phenotype, delineated by the presence of cytokeratin (K14)+ cells, which are found at the invasive front in all major subtypes of human breast cancer [12]. Additionally, microarray analysis of invasive mouse xenograft tumor cells isolated by invasion into microneedles uncovered gene expression associated with the G1/G0 phase of the cell cycle (*i.e.*, p21^{CIP1}), as well as an upregulation of genes associated with cell invasive behavior (*i.e.*, β -catenin, and F-actin regulators such as *cdc42*) [73-75]. There also exists strong *in vivo* evidence for a proliferative to invasive switch, where direct perturbations of the cell cycle in mouse models of breast cancer revealed that the loss of p21^{CIP1} decreases the ability of breast cancer cells to metastasize [76]. Specifically, p21^{CIP1} null pyMT mammary tumors were hyperproliferative but less invasive both *in vitro* and *in vivo* [76]. As p21^{CIP1} functions to regulate the activity of Cyclin E (Fig. 3), human breast cancer cell lines expressing a constitutively active Cyclin E construct were also hyperproliferative and 10-fold less invasive than control tumors in a mouse xenograft model [76].

Clinically, this also appears to be the case, as primary tumors show an inverse relationship between levels of G1 and S phase cyclins (Cyclin D1 and E, Fig. 3) and the infiltration or invasiveness of the tumor [76-78]. Together, these data suggest that manipulation of CKIs

and/or their target cyclins could limit invasive activity during breast cancer progression. Changes in the CKI/Cyclin/CDK axis may not be unique to breast cancer, as histopathological studies have revealed that the invasive fronts of colonic adenocarcinomas do not express the proliferative marker, Ki-67 [81-84] but do express p16^{INK4a} [85, 86] (Fig. 3), indicating that invasive cells are disproportionately arrested in G1/G0. Additionally, it was recently shown in a squamous cell carcinoma mouse model that TGF β signaling positively regulates transcription of p21^{CIP1}, leading to slower cycling of the invasive stem cells and increased resistance to chemotherapy [87]. Traditionally, CKIs (p21^{CIP1}/p27^{KIP1}/p16^{INK4a}) are classified as tumor suppressors, as they limit cellular growth [88-90]. However, since manipulating the CKI/Cyclin/CDK axis, which is required for cell cycle entry and exit, has profound effects on the invasive capacity of tumor cells, this raises the possibility that at least some “tumor suppressors” might function as activators of metastasis (Fig. 3).

The alternative hypothesis: invasion and proliferation are not exclusive behaviors

Contrary to the hypothesis posed above, the majority of cancer literature assumes a positive correlation between cell proliferation and cell invasive activity. However, in most cases where this relationship is examined, the positive correlation obtained is based on *in vitro* assays, which do not simultaneously assess BM integrity and cell cycle state. In the case of *in vivo*, or histopathological work, often, population level effects rather than single-cell state changes are assessed. Indeed two studies in oral squamous cell carcinoma indicated that proliferation is linked with increased invasion, based on the Ki-67 index at the invasive fronts of patient biopsies [91, 92]. Similarly, a recent *in vivo* study using intravital imaging of an HCT116 colorectal cancer cell line, with a FUCCI cell cycle biosensor, showed that that majority of cells infiltrating the stroma were primarily in the S/G2/M phases of the cell cycle [93]. This is in direct contrast to a study using invasive gastric adenocarcinoma cells in a gelfoam based invasion assay, where cells were observed to be predominantly in the G1/G0 phase of the cell cycle [57].

One plausible explanation for how enhanced proliferation and invasion may be linked, as proposed in the studies mentioned above, is that highly proliferative tumors could initiate invasive behavior non-cell autonomously by recruiting stromal cells to facilitate dissemination and intravasation. Indeed, cancer associated fibroblasts (CAFs) and tumor associated macrophages are well known to mediate metastasis [94-96]. In this scenario, the cell cycle state of metastatic tumor cells would be irrelevant if tumor associated immune cells were to mediate BM invasion of the cancer cells as well as subsequent stromal infiltration. This could also serve as an alternative explanation for the results shown in squamous cell carcinoma [91, 92] and colorectal cancer cell lines [93], where CAFs have been shown to be critical in facilitating metastasis [96, 97]. For example, *in vitro* live cell imaging through matrix has shown that SCC cells can either follow directly behind CAFs or utilize tracks made by CAFs during their collective invasion, but are unable to invade without CAF assistance [96]. Although increased proliferation is a characteristic of CAFs, the cell cycle state of individual CAFs or macrophages during tumor dissemination and intravasation is currently unknown. Since cancer does not represent a single disease, but rather a myriad of many different disorders [61], it is possible that some cancers develop the

ability to invade BM and proliferate simultaneously, either through the cooption of the host cells' invasive abilities, or through the acquisition of invasion on their own as the result of currently unknown genetic and/or epigenetic mechanisms. Regardless, these conflicts in the literature highlight the importance that should be placed on the development of new models to directly assess BM invasion and cell cycle state in both cancer cells and the surrounding microenvironment at the onset of metastatic behavior.

Therapeutic implications of cell cycle regulation of invasive behavior

Traditional antineoplastic chemotherapeutics kill rapidly dividing cells. However, since invasive cells appear to exist in quiescent G1/G0 arrest (Table 1), these invasive, metastatic cell populations remain when the bulk of the tumor is killed by classical antineoplastics. For example, sublethal irradiation, which blocks the G1/S phase checkpoint, increases the metastatic potential of gliomas [98]. Further research is necessary to determine if cell cycle arrest triggered by sublethal doses of DNA damaging antineoplastic treatments can drive metastatic behaviors as well.

In 2015, the first antineoplastic chemotherapeutic drug to directly target the cell cycle, Palbociclib (PD-0332991) was approved by the FDA for use in breast cancer treatment [99] and is currently being used in clinical trials to target other cancers [100]. Palbociclib and other drugs are inhibitors of CDK4/6, the G1/S phase transition checkpoint [101] (Fig. 3). Experimental inhibition of the G1/S phase transition through genetic mechanisms such as overexpression of p21^{CIP1} [102, 103] or high levels of p16^{INK4a} [85] have led to increased metastatic characteristics. As Palbociclib similarly blocks cell cycle progression at the G1/S phase transition, this raises the possibility that it may also drive invasive behavior. Notably, in pancreatic cancer cell lines, Palbociclib is sufficient to induce EMT and drive an increase in Matrigel invasion [104]. Therefore, while antineoplastic chemotherapeutic drugs targeting the G1 phase of the cell cycle show great promise, more work must be done to ensure that treatment regimens do not inadvertently drive metastatic progression by facilitating invasive cell behavior by inducing G1/G0 cell cycle arrest.

Concluding Remarks

We have reviewed literature that demonstrates that a broad array of cancers switch between invasive and proliferative states, with evidence ranging from correlative Matrigel invasion assays to histopathological studies of primary tumor samples (Table 1). Together, these data argue that cell cycle arrest may be a requirement for the acquisition of invasive activity. Given recent functional data from a developmental invasion event in *C. elegans*, we suggest that G1/G0 phase cell cycle arrest may be required broadly to properly execute invasive behavior.

In spite of this mounting evidence, our mechanistic understanding of the relationship between cell cycle control and cell invasion remains limited due to a lack of tools to accurately visualize BM *in vitro* or *in vivo* while assaying cell cycle state (see Outstanding Questions). Future use of microfluidics to analyze cells at single resolution *in vitro* [105] paired with advanced imaging modalities, including light sheet microscopy [106] and

dynamic cell cycle biosensors [107, 108] will hopefully provide a more accurate assessment of cell cycle state during invasion. These same advanced imaging techniques, particularly light sheet [109] and two photon microscopy [110], allow for long term vital imaging [27, 111-113] at cellular and subcellular resolution *in vivo*. Going forward, better *in vivo* models are needed. Genome editing combined with improved microscopy should allow simultaneous visualization of labeled BM and invasive cells at single cell resolution. These new models could be paired with functional cell cycle perturbations, across multiple cancer cell types, revealing if the many disparate observations illustrated here represent a deeply conserved evolutionary principle underlying cell invasive behavior between organisms that last shared a common ancestor over 500 million years ago.

Acknowledgements

The authors would like to thank Mathieu Boissan, R. Antonio Herrera and Taylor Medwig for their feedback, as well as the two anonymous reviewers, whose comments greatly improved the manuscript. We also thank Taylor Medwig for confocal micrographs used in Figure 1. D.Q.M. is supported by the Carol M. Baldwin Foundation for Breast Cancer Research and by the National Institute of Health, National Cancer Institute R00-CA154870.

Glossary

Basement Membrane

A dense highly crosslinked sheet of polymeric laminin and type IV collagen forming the substrate for endo- and epithelia and providing a barrier function for most cells

Basement Membrane Invasion

The process by which cells remove basement membrane allowing contact between cell layers or passage through the basement membrane

C. elegans anchor cell invasion

An *in vivo* model system used to examine the process of basement membrane invasion, as a specialized somatic gonad cell, the anchor cell (AC), breaches the underlying basement membrane to initiate uterine-vulval contact, allowing worms to passage eggs to the external environment

Cyclin dependent kinase inhibitors (CKIs)

A family of conserved eukaryotic proteins (p16^{INK4a}, p21^{CIP1}, p27^{KIP1} in vertebrates) that inhibit the activity of G1/S phase cyclins (Cyclins D and E) and cyclin dependent kinases (CDK4/6 and CDK2)

Delamination

The process by which cells leave an epithelium to migrate elsewhere, or form a new epithelial layer. This process is often coupled with epithelial to mesenchymal transition (EMT)

Epithelial to mesenchymal transition (EMT)

The morphogenetic process by which cells switch from an epithelial to mesenchymal morphology occurring during development and cancer metastasis

Extracellular Matrix

The scaffolding of proteins supporting and surrounding metazoan cells

G1/G0 cell cycle arrest

A quiescent cellular state that occurs following mitosis, where a cell either temporarily pauses, prior to entering S phase (G1) or permanently arrests (G0). As these cellular states can be difficult to distinguish, we will refer to this arrest point as G1/G0 arrest for the purposes of this review

Invasion

The morphogenetic process by which cells penetrate the basement membrane and, in vertebrates, remodel the extracellular matrix-derived stroma

Migration

The process by which cells move from place to place

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Outstanding Questions

- Can individual cells switch between invasive and proliferative states and if so, what are the autonomous and environmental signals that dictate the ability to transition between these states?
- Why is the G1/G0 state associated with cell invasive behavior? This review highlights the many cases in different cancers that show correlation between quiescence in G1/G0 and increased invasiveness.
- How can we ensure that cancer therapeutics which promote G1/G0 cell cycle arrest do not inadvertently select for invasive cellular behavior?
- Can we create better *in vivo* models that will allow for single cell visual analyses paired with cell cycle perturbations and live imaging of basement membrane invasion? This will allow for further exploration of this dichotomy between proliferative and invasive cellular states.

Trends

Cell invasive behavior is critical during development and is dysregulated in disease states, including cancer metastasis.

The ability to adopt an invasive phenotype and breach a mechanical barrier such as the basement membrane may be regulated in a cell cycle-dependent fashion. This underlies a dichotomy between cell proliferation and cell invasion.

Invasion occurs primarily in a G1/G0 cell cycle-arrested state, and expression of pro-invasive genes driving EMT and F-actin cytoskeletal reorganization are associated with this cell cycle state.

Changes in the activity of cyclin dependent kinase inhibitors (CKIs) and their target cyclins and cyclin dependent kinases (CDKs) not only mediate the decision to enter or exit the cell cycle, but may be critical to acquiring an invasive phenotype.

Therapeutics which cause G1/G0 arrest, such as palbociclib, show great promise, but further research is required to ensure that these drugs do not inadvertently drive metastatic cancer progression.

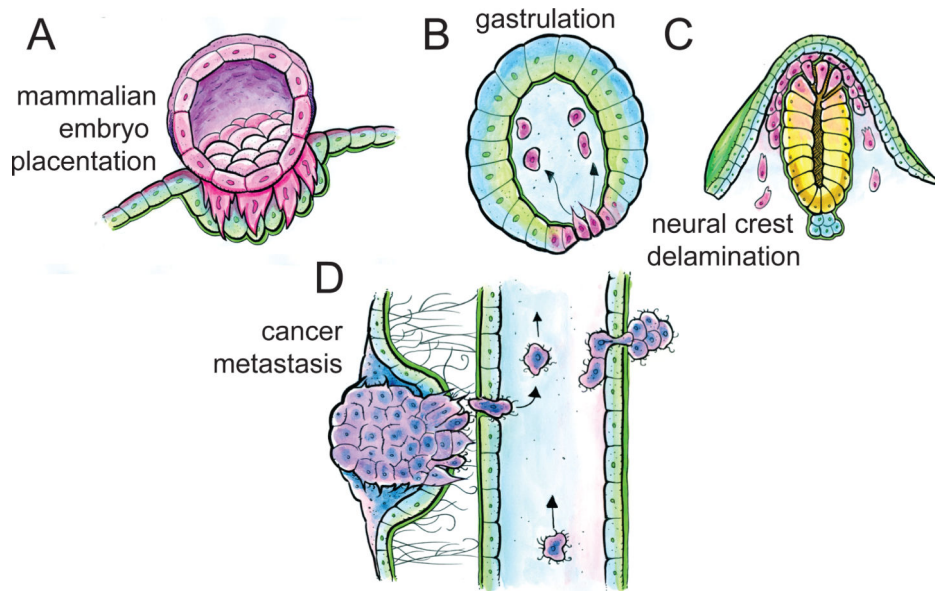


Figure 1. Cell invasion occurs during development and cancer metastasis

(A-C). During development, cells acquire invasive phenotypes to facilitate mammalian embryo placentation (A), gastrulation in many organisms (B), and in neural crest delamination (C). (D) During cancer metastasis, cancer cells are invasive at multiple steps, including escape from the primary tumor, intra- and extravasation from the bloodstream, and establishment of a secondary tumor at a distant site.

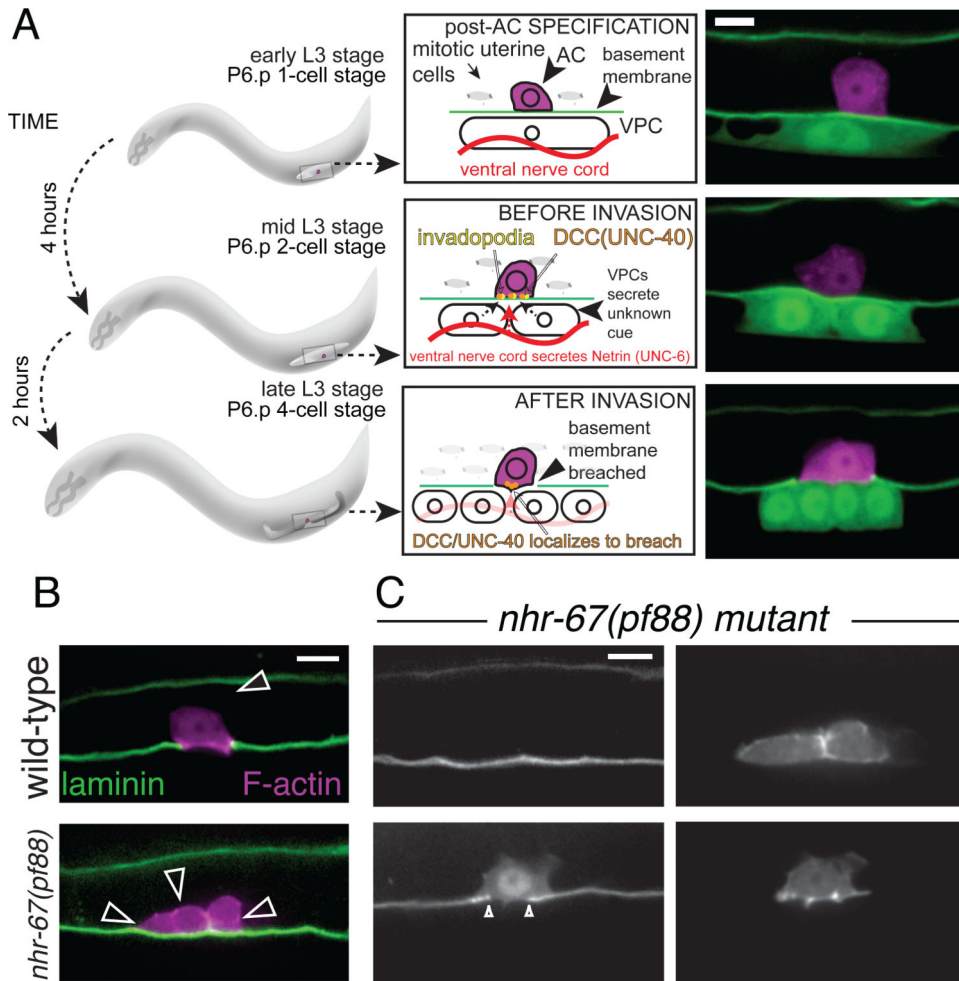


Figure 2. *C. elegans* anchor cell (AC) invasion into the vulval epithelium is a tractable *in vivo* model to examine invasion at single cell resolution in real time

(A) During the third larval stage of *C. elegans* development, the AC invades in a highly stereotyped fashion. Shortly after the AC is specified (top), the invasive AC localizes invadopodia along the basolateral surface in response to extracellular cues (netrin, red, from the ventral nerve cord, and an unknown cue from the vulval cells) from the microenvironment [11] (middle). Next, the AC breaches the BM, contacting the vulval precursor cells (VPCs) and initiating the uterine-vulval connection (bottom). Spinning disc confocal images depict the AC (magenta, expressing *zmp-1*>mCherry) and BM, visualized by laminin::GFP (green), and 1° VPCs (green, expressing *egl-17*>GFP). (B) A single transcription factor, the nuclear hormone receptor, *nhr-67/tlx*, mediates AC invasion by maintaining the AC in a G1 cell cycle arrested state (top, left). Loss of *nhr-67/tlx* results in mitotic ACs that fail to invade (bottom). (C) Induced expression of *cki-1* restores G1/G0 arrest and rescues invasion (middle) [9]. Scale bar, 5 μ m. Images in (C) from [9].

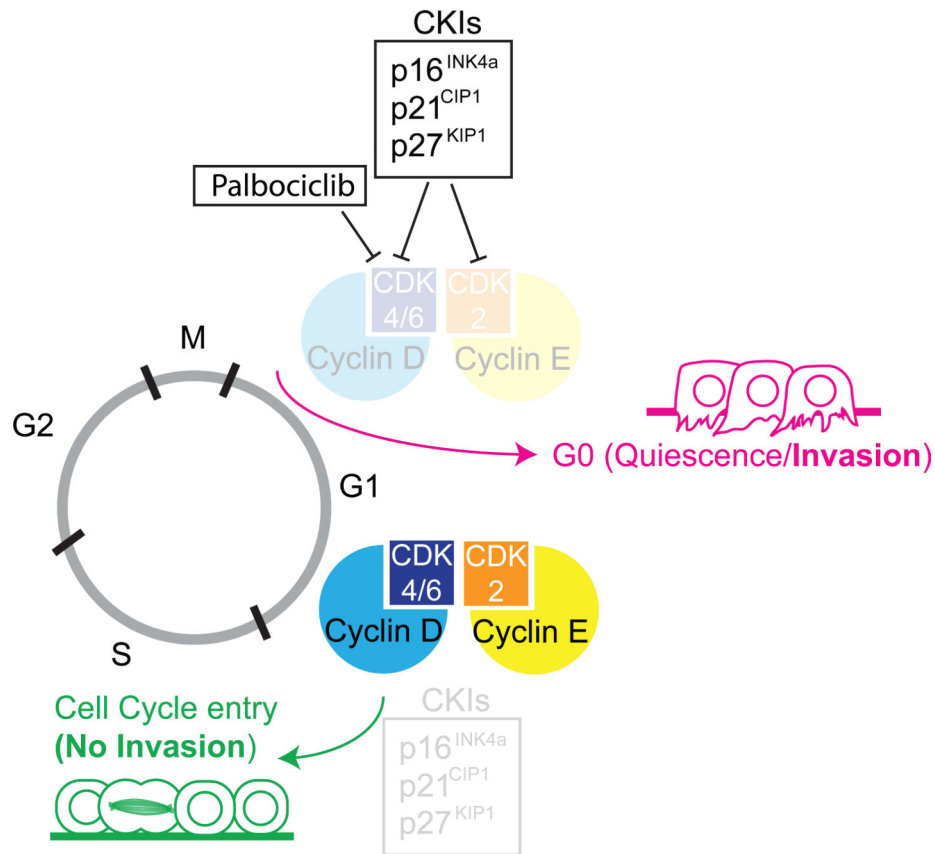


Figure 3. Cell cycle state and invasive activity

The activity of cyclin-dependent kinase inhibitors (CKIs: p21^{CIP1}/p27^{KIP1}/p16^{INK4a}) inhibit G1/S phase cyclins and cyclin dependent kinases (Cyclin D/CDK4/6 and Cyclin E/CDK2), inducing G1/G0 cell cycle arrest and promote quiescence and invasion. Reduced activity of CKIs results in increased levels of G1/S phase cyclins and CDKs, promoting cell cycle entry, preventing invasive behavior. New cancer therapeutics such as Palbociclib limit tumor growth by inducing G1/G0 cell cycle arrest by directly inhibiting CDK4/6 activity.

Table 1

Evidence for cell cycle regulation of cancer cell invasion.

Organ of Origin	Cancer Subtype	<i>In vivo</i> Assays	<i>In vitro</i> Assays	Findings	Ref
	Melanoma	IHC of tumors for MITF and p27 ^{KIP1} , tumor formation assays using SKMel28 cells.	Matrigel invasion assays performed on cell lines generated for this paper	Cell cycle arrest is associated with metastatic potential	[64]
		Histopathology of patient samples and Mouse xenografts of melanoma cell lines for MITF and Ki67.	Gene expression profiling and western blots performed on primary melanoma cells.	Invasive cells spend more time in G1 and there is a switch between proliferation and invasion mediated by transcriptional changes.	[65]
		RNA-seq and IHC of individual human melanoma cells.	-	A sub-population of malignant melanoma cells show a non-cycling and chemoresistant signature based on transcriptome profile.	[66]
Epithelial	Multiple Types	In situ hybridization for Snail in mouse embryos, along with staining for cell death and BrdU incorporation.	Caspase 3 activity assay and western blotting of MDCK and MCA3D cells.	Proliferation and metastatic behaviors are not necessarily linked.	[36]
	Basal Cell Carcinoma	IHC of patient tumor samples for Ki67 and p16 ^{INK4a} .	-	Invasive cells are nonproliferative, express p16 ^{INK4a} , and likely are in G1/G0.	[67]
	Epidermoid Carcinoma	-	Matrigel invasion, FACS, expression reporters, IF, ChIP of A431 cells.	After undergoing EMT, A431 cells, repress the cell cycle by blocking Cyclin D and become more invasive.	[68]
	Squamous Cell Carcinoma	Primary mouse keratinocytes, mouse xenograft and inducible SCC mice. Lineage tracing and metastases probed in vivo using immunofluorescence. FACS and RNA-seq performed on tumor cells.	Primary Keratinocytes and tumor cells: IHC, immunofluorescence.	TGFβ suppresses proliferation and promotes invasion in squamous cell carcinoma stem cells, through regulation of p21 ^{CIP1} , leading to chemotherapeutic resistance.	[87]
Others	Gastric Adenocarcinoma	-	Live cell imaging of MKN45 cells expressing FUCCI invading into a gelfoam based histoculture prep.	Invading cancer cells are predominantly in G0/G1.	[57]
	Lung	Mouse xenografts of H460 cells, quantifying tumor growth and brachyury expression by IHC.	Matrigel invasion, IF, ChIP, FACS sorting and western blots on A549 and H460 cells.	Brachyury blocks the cell cycle by repressing p21 ^{CIP1} rendering cancer cells less sensitive to chemotherapy.	[69]
	Hepatocellular Carcinoma	-	AH130 cells: FACS sorting, cell cycle synchronization and in vitro invasion assays.	Cells invade in the G1 phase of the cell cycle.	[72]

Organ of Origin	Cancer Subtype	<i>In vivo</i> Assays	<i>In vitro</i> Assays	Findings	Ref
Breast		Microarray analysis of cells collected by invasion into microneedles or chemotaxis, and intravital imaging of MTLn3 cells as rat xenograft or PyMT mouse tumor model.	-	Invasive cells upregulate genes associated with cell cycle arrest (i.e., p21 ^{CIP1} , p16 ^{INK4a}), and downregulate those associated with proliferation.	[73-75]
		Quantification of spontaneous metastases in PyMT tumor and mouse xenograft models.	In vitro assays including matrigel invasion and immunofluorescence (e.g. p21), using MCF-7, MCF10A and MDA-MB-231 cells.	Loss of p21 ^{CIP1} or over-expression of Cyclin E prevent metastasis by preventing state-switching between invasion and proliferation.	[76]
		IHC of primary tumors for Ki67 and Cyclin D1 and Cyclin E.	FACS sorting, Matrigel invasion and live cell imaging of MDA-MB-231/435/468 cells	Decreased Cyclin D1/E makes cells more invasive, increased Cyclin D1/E makes cells more proliferative. In patient tissue samples, more Cyclin D1/E correlates to less invasion, and vice versa.	[77,78]
		Quantification of spontaneous and experimental metastases from MDA-MB-231 xenograft tumors in mice.	-	Cell proliferation and invasive behavior show an inverse correlative relationship, mediated by Arg/Abl2 and CSFR1 via TGFβ signaling.	[79,80]
Colon	Carcinoma	IHC of patient samples for Ki67, p16 ^{INK4a} .	-	The invasive fronts of colon cancers are non-proliferative and express p16 ^{INK4a} . Cell cycle exit appears to be required for invasive behavior.	[81-84,86]
	Colorectal	IHC of patient samples	ChIP on DLD-1, HCT-116, LS174T and SW480 Cells.	The proportion of cells expressing p16 ^{INK4a} at the invasive front of the tumor inversely correlates to long-term patient survival.	[85]
Pancreatic		-	Matrigel invasion and gelatin degradation by secreted media performed on gamma irradiated Panc-1, Suit-2 and Hs766T cells.	Gamma irradiated cells with lower proliferative ability exhibit increased invasive potential.	[98]
		-	Microarray analysis, FACS and IF on AsPC-1, PANC-1 and COLO-357 cells.	The CDK4/6 inhibitor Palbociclib increases EMT and invasiveness.	[104]

Note On Abbreviations. IHC: immunohistochemistry, ChIP: Chromatin Immunoprecipitation, IF: Immunofluorescence, FACS: Fluorescence-activated cell sorting, PyMT: Polyoma middle T, BrdU: Bromodeoxyuridine.