Published in final edited form as:

J Microsc. 2013 September ; 251(3): 250–260. doi:10.1111/jmi.12064.

Extracellular matrix determinants and the regulation of cancer cell invasion stratagems

A.L. WILLIS, **F. SABEH**, **X.-Y. LI**, and **S.J. WEISS**

Division of Molecular Medicine & Genetics, Department of Internal Medicine, and the Life Sciences Institute, University of Michigan, Ann Arbor, Michigan, U.S.A

Summary

During development, wound repair and disease-related processes, such as cancer, normal, or neoplastic cell types traffic through the extracellular matrix (ECM), the complex composite of collagens, elastin, glycoproteins, proteoglycans, and glycosaminoglycans that dictate tissue architecture. Current evidence suggests that tissue-invasive processes may proceed by protease-dependent or protease-independent strategies whose selection is not only governed by the characteristics of the motile cell population, but also by the structural properties of the intervening ECM. Herein, we review the mechanisms by which ECM dimensionality, elasticity, crosslinking, and pore size impact patterns of cell invasion. This summary should prove useful when designing new experimental approaches for interrogating invasion programs as well as identifying potential cellular targets for next-generation therapeutics.

Keywords

Extracellular matrix (ECM); invasion; MT1-MMP

Introduction

In cancer, neoplastic cells inappropriately co-opt normal cell function and escape from their primary locale by engaging invasive machinery that had been ostensibly engineered to control the regulated motility programs operative during growth and development (Rowe & Weiss, 2009; Kessenbrock *et al.*, 2010; Wolf & Friedl, 2011). Tumour progression and invasion are often linked to the up-regulated expression of proteolytic enzymes—generated by both cancer cells and the surrounding tumour microenvironment—that are capable of degrading the major extracellular matrix (ECM) macromolecules that comprise all connective tissues (Rowe & Weiss, 2009; Kessenbrock *et al.*, 2010; Wolf & Friedl, 2011; Lu *et al.*, 2012). To date, multiple proteases have been implicated in the ECM remodelling events associated with cancer, but conflicting results have been reported which question the issue of whether proteolysis is an obligate step in tissue-invasive processes (Rowe & Weiss, 2009; Sabeh *et al.*, 2009; Sabeh *et al.*, 2009b; Kessenbrock *et al.*, 2010; Wolf & Friedl, 2011). Whereas many groups have reported that cancer cells can only traverse the ECM via

Correspondence to: Stephen J. Weiss, M.D., Life Sciences Institute, University of Michigan, 5000 LSI, 210 Washtenaw, Ann Arbor, MI 48109-2216, U.S.A. Tel: 734-764-0030; fax: 734-764-1934; SJWEISS@umich.edu.

the proteolytic dissolution of intervening structural barriers, others have proposed that neoplastic cells can push or squeeze their way through matrix barriers without mobilizing proteases (Wolf *et al.*, 2003, 2007; Sabeh *et al.*, 2004; Sabeh *et al.,* 2009; Madsen & Sahai, 2010; Wolf & Friedl, 2011; Friedl *et al.*, 2012). As such, emerging models of invasion will be reviewed in an effort to help resolve apparent inconsistencies in the field and to outline new experimental approaches that may be applied to outstanding questions.

ECM-based models for proteolytic and nonproteolytic cell invasion and migration

To define the mechanisms that allow cancer cells to infiltrate ECM barriers by protease-dependent or protease-independent processes, a number of *in vitro, ex vivo*, and *in vivo* models have been developed (Rowe & Weiss, 2008, 2009; Ehrbar *et al.*, 2011; Wolf & Friedl, 2011; Friedl *et al.*, 2012; Petrie *et al.*, 2012). In overview, these models have been developed to analyze the mechanisms by which neoplastic cells invade the two major ECM subtypes, that is the basement membrane (BM) and the interstitium (Rowe & Weiss, 2008, 2009; Ehrbar *et al.*, 2011; Wolf & Friedl, 2011; Friedl *et al.* 2012; Petrie *et al.*, 2012). As each construct displays its own unique mix of experimental strengths and weaknesses, insights may only be gleaned from such studies with an appreciation of the limitations inherent in these systems.

Basement membrane

All epithelial layers are subtended by a several hundred nanometer-thick, sheet-like BM, largely comprised of two interlocking networks of heterotrimeric type IV collagen and laminin isoforms which are further interwoven with 25 or more distinct glycoproteins and proteoglycans (Candiello *et al.*, 2007; Rowe & Weiss, 2008; Balasubramani *et al.*, 2010; Yurchenco, 2011). BM constituents are distributed in a tissue-specific fashion that varies with developmental stage (Kabosova *et al.*, 2007; Bai *et al.*, 2009; Candiello *et al.*, 2010). Despite their heterogeneity, the structural and mechanical integrity of BMs is determined primarily by type IV collagen intra-and inter-molecular covalent crosslinks that include disulfide, aldimine and newly characterized, peroxidasin-catalyzed sulfilimine bonds (Rowe & Weiss, 2008; Vanacore *et al.*, 2009; Bhave *et al.*, 2012; Weiss, 2012). Given an effective BM pore diameter ranging from 10 to 90 nm (as assessed by a battery of electron and atomic force microscopic techniques) (Yurchenco & Ruben, 1987; Yurchenco *et al.*, 1992; Abrams *et al.*, 2000) whose size is considerably smaller than the 1–2 μm pores that migrating cells can negotiate, it is difficult to envision a molecular mechanism that enables protease-independent cancer cell invasion across native BMs (Rowe & Weiss, 2008; Wolf *et al.*, 2013). Moreover, cell trafficking through the BM during growth and development—in animal systems ranging from primitive model organisms to higher vertebrates—is tightly associated with proteolytic remodelling (Hotary *et al.*, 2006; Srivastava *et al.*, 2007; Page-McCaw, 2008; Ota *et al.*, 2009; Rebustini *et al.*, 2009; Matus *et al.*, 2010; Yasunaga *et al.*, 2010). From where then has the proposition emerged that proteases need not play a required role in allowing normal or neoplastic cells (of either epithelial or mesenchymal origin) to traverse intact BMs? In large part, the conclusion that cancer cells adopt a protease-independent, amoeboid-like phenotype during BM invasion can be linked to the use of *in vitro* constructs designed to recapitulate native BM structure (Rowe & Weiss, 2008). Despite the fact that surface nanotopography, rigidity, and BM macromolecular composition all

impact cell function, BM 'mimics' have, until recently, been limited to the use of Matrigel, a self-assembling hydrogel derived from tumour cell extracts (Gadea *et al.*, 2007; Sahai *et al.*, 2007; Fackler & Grosse, 2008; Poincloux *et al.*, 2011; Rao *et al.*, 2012; Tilghman *et al.*, 2012). Matrigel, like native BMs, contains type IV collagen and laminin, but the polymeric complex is dominated by laminin (rather than type IV collagen) and is largely devoid of the type IV collagen crosslinks that define the structural properties of native BMs (Rowe & Weiss, 2008). Indeed, unlike native BMs that remain insoluble under harsh extraction conditions, Matrigel hydrogels are readily solubilized by mild chaotropes (Rowe & Weiss, 2008). Furthermore, recent studies demonstrate that the elastic modulus (i.e. rigidity) of Matrigel hydrogels is orders of magnitude less than that of BMs assembled *in vivo* (Candiello *et al.*, 2007; Soofi *et al.*, 2009). Difficulties in interpreting Matrigel-centric experimental designs—at least with regard to invasion—are further compounded by the frequent decision to embed tumour cells within thick, 3-D hydrogels (Gadea *et al.*, 2007; Sahai *et al.*, 2007; Poincloux *et al.*, 2011). Whereas well appreciated that cell behaviour is affected in distinct fashions when cultured under 2-D versus 3-D conditions (Hotary *et al.*, 2003; Yamada & Cukierman, 2007; Grinnell & Petroll, 2010), normal epithelial cells as well as their transformed counterparts only interface a planar, 100–400 nm-thick BM layer in the *in vivo* setting (Candiello *et al.*, 2007; Rowe & Weiss, 2008; Balasubramani *et al.*, 2010). As such, the (patho)physiologic relevance of monitoring cell invasion through a highly elastic hydrogel defined by low concentrations of noncrosslinked type IV collagen remains questionable (Rowe & Weiss, 2008).

To circumvent the limitations associated with BM mimetics, recent studies have begun analyzing the invasion systems mobilized by normal or neoplastic cells as they transmigrate native BMs either under *in vitro* or *in vivo* conditions (Hotary *et al.*, 2006; Srivastava *et al.*, 2007; Page-McCaw, 2008; Rowe & Weiss, 2008; Ota *et al.*, 2009; Rebustini *et al.*, 2009; Rottiers *et al.*, 2009; Matus *et al.*, 2010; Schoumacher *et al.*, 2010; Yasunaga *et al.*, 2010; Hagedorn & Sherwood, 2011). Remarkably, model organisms as well as mammalian cells appear to share complementary BM invasion programs that couple transmigration with ECM proteolysis (Hotary *et al.*, 2006; Srivastava *et al.*, 2007; Page-McCaw, 2008; Rowe & Weiss, 2008; Ota *et al.*, 2009; Rebustini *et al.*, 2009; Rottiers *et al.*, 2009; Matus *et al.*, 2010; Schoumacher *et al.*, 2010; Yasunaga *et al.*, 2010; Hagedorn & Sherwood, 2011; Stevens & Page-McCaw, 2012). In many of these systems, experimental results highlight key roles for membrane-tethered metalloenzymes belonging to the matrix metalloproteinase (MMP) family, that is the so-called the membrane-type MMPs (MT-MMPs) (Hotary *et al.*, 2006; Srivastava *et al.*, 2007; Page-McCaw, 2008; Rowe & Weiss, 2008; Ota *et al.*, 2009; Rebustini *et al.*, 2009; Yasunaga *et al.*, 2010). As this proteinase class has been the subject of recent reviews (Barbolina & Stack, 2008; Poincloux *et al.*, 2009; Rowe & Weiss, 2009; Strongin, 2010), our discussion of these enzymes will be confined to a short summary of their pertinent features. In mammals, six MT-MMPs have been characterized. At least three of the MT-MMPs (i.e. MT1-MMP, MT2-MMP and MT3-MMP) are able to confer recipient cells with the ability to proteolytically remodel native BM structures and trigger transmigration (Hotary *et al.*, 2006; Ota *et al.*, 2009; Rebustini *et al.*, 2009; Riggins *et al.*, 2010). Like all proteolytic enzymes, the MT-MMPs are synthesized as proenzymes, and undergo intercellular activation when their propeptide domains are proteolytically removed during

their transit through the *trans*-Golgi network to the cell surface (Barbolina & Stack, 2008; Poincloux *et al.*, 2009; Rowe & Weiss, 2009; Golubkov *et al.*, 2011). In most cases, MT-MMPs appear to be directed to invadopodia-like structures which allow cells to 'focus' their degradative potential to discrete, subjacent zones that support ECM tunnelling programs while maintaining the structural integrity of the surrounding matrix so as to support propulsive movement (Hotary *et al.*, 2006; Ota *et al.*, 2009; Poincloux *et al.*, 2009; Wang & McNiven, 2012; Yu *et al.*, 2012). Whereas recent studies have emphasized the role of the 20-amino acid long MT1-MMP cytosolic tail in regulating proteolytic and invasive activity (Wu *et al.*, 2005; Wang & McNiven, 2012; Yu *et al.*, 2012), native BM barriers can be transmigrated—*in vitro* as well as *in vivo*—in the absence of this domain (Hotary *et al.*, 2006; Ota *et al.*, 2009). Importantly, MT-MMPs trigger BM remodelling and transmigration programs that function independently of the larger family of secreted MMPs, including the often-cited type IV collagenases, MMP-2 and MMP-9, whose BM-degrading activities remain unproven to date (Rowe & Weiss, 2008).

As Matrigel hydrogels do not recapitulate the structure of native BMs, how is it that many reports have documented the ability of inhibitors directed against secreted *or* membrane-tethered MMPs to block cell invasion through these constructs [e.g., (Ueda *et al.*, 2003; Zaman *et al.*, 2006; Rizki *et al.*, 2008)]? Importantly, even MMPs devoid of BM-degrading activity can indirectly influence invasion programs by (i) processing ECM-tethered growth factors to active intermediates, (ii) hydrolyzing membrane-anchored ligands, (iii) activating/inactivating secreted chemokines, or (iv) triggering motility *independently* of proteolytic activity (Barbolina & Stack, 2008; Kessenbrock *et al.*, 2010; Strongin, 2010; Dufour *et al.*, 2011; auf dem Keller *et al.*, 2013). In this regard, it should be stressed that neither secreted MMPs nor members of any other protease class have been shown to confer expressing cells with the ability to traverse intact BMs (Hotary *et al.*, 2006; Ota *et al.*, 2009). Consistent with an accessory, rather than required role for MMPs in Matrigel invasion, several groups have independently confirmed the ability of multiple cell types to infiltrate these hydrogels in the presence of MMP inhibitors (Even-Ram & Yamada, 2005; Hotary *et al.*, 2006; Rowe & Weiss, 2008; Sodek *et al.*, 2008; Poincloux *et al.*, 2011). As the pore sizes of these hydrogels are estimated to fall within the range of 1–2 μm in diameter (assessed by quick-freeze transmission electron microscopy), MMP-independent invasion mechanisms are most consistent with a model wherein the noncrosslinked components of Matrigel can be mechanically displaced by the migrating cancer cells (Poincloux *et al.*, 2011).

Interstitium

The ECM of connective tissues is dominated by the interstitial collagens (most commonly, type I collagen) that serve as the single-most abundant extracellular proteins found in mammals (Rowe & Weiss, 2009; Grinnell & Petroll, 2010). Coincident with their secretion from fibroblasts, the collagen propeptide domains are proteolytically removed, and a complex auto-polymerization process is initiated (Kadler *et al.*, 2008). Upon lysyl oxidase-mediated crosslinking within the N- and C-terminal nonhelical ends of the secreted and processed collagen molecules (i.e. the telopeptide domains), the fibrils mature into a mechanically reinforced network of fibres and fibre bundles (Eyre *et al.*, 1984; Christiansen *et al.*, 2000). Given the tremendous tissue-to-tissue heterogeneity in interstitial collagen

content and crosslink structure (Eyre *et al.*, 1984; Christiansen *et al.*, 2000; Kadler *et al.*, 2008; Wolf *et al.*, 2009), trafficking cancer cells—as well as recruited stromal cells—would be expected to encounter structurally distinct barriers.

In considering the stratagems that might be deployed at cell-ECM interfaces, a consensus of opinion is building to support a two-body model of invasion. In this model, the size and elasticity of the infiltrating cell's most mechanically rigid sub-cellular compartment, that is the nucleus, dictates the manner in which cells negotiate the fibrillar network of the interstitial matrix (Nakayama *et al.*, 2005; Wolf *et al.*, 2007; Beadle *et al.*, 2008; Fisher *et al.*, 2009; Friedl *et al.*, 2011; Khatau *et al.*, 2012). If the dimensions of a rigid matrix pore are too small to accommodate the nuclear dimensions of the trafficking cell, the barrier will prove impassable unless the matrix is proteolytically remodelled. On the other hand, if the pore size of the matrix is larger than the cell's nuclear dimensions, a physical barrier to cell traffic no longer exists, and invasion proceeds independently of protease-dependent remodelling (Wolf *et al.*, 2013). A more complex invasion scheme is envisioned under those circumstances where the pore size is too small to support passive cell movement, but the barrier might yet be negotiated by either (i) ECM-degrading proteases (i.e. akin to cutting through the bars of a 'cage'), (ii) actomyosin motors and cell adhesion molecules working in concert to mechanically distort the surrounding matrix—in this case, 'bending' the cage bars (Friedrichs *et al.*, 2007) or (iii) intracellular contractile machinery that alters nuclear dimensions, thus permitting invasion to proceed by cell 'squeezing' through a nondeformable matrix cage (Nakayama *et al.*, 2005; Beadle *et al.*, 2008; Friedl *et al.*, 2011; Balzer *et al.*, 2012; Khatau *et al.*, 2012). Whereas portions of these schemes remain somewhat conjectural in nature, recent evidence culled from *in vitro* and *in vivo* systems lends support to these models.

Protease-dependent migration in vitro: covalently crosslinked collagen hydrogels

In vitro studies of cell behaviour in 3-D models of the interstitial matrix have long relied on the use of acid extracts of rat tail tendons – both because of their ability to yield relatively pure solutions of type I collagen as well as the ability to recover full-length type I collagen molecules with intact telopeptide domains (Sabeh *et al.*, 2009). Using these extracts, collagen fibrillogenesis is initiated by manipulating pH, ionic strength and temperature (Raub *et al.*, 2007, 2008). Under standard conditions (i.e. pH ≃7.4, iso-osmotic buffers and 37°C), 2–4 mg/mL solutions of rat tail tendon collagen yield an aldimine-crosslinked network of fibrils with an effective pore diameter in the range of 1–3 μm (Raub *et al.*, 2007, 2008; Mickel *et al.*, 2008). Using these matrices, a number of groups have consistently reported that cancer cells—as well as normal endothelial, fibroblast, bone marrow stromal/stem cells and smooth muscle cells—display tissue-invasive behaviour that is solely dependent on the mobilization of collagenolytic MMPs (primarily MT1-MMP or MT2-MMP, and perhaps, MT3-MMP) (Sabeh *et al.*, 2004; Sodek *et al.*, 2007,2008; Fisher *et al.*, 2009; Rowe & Weiss, 2009; Sabeh *et al.*, 2009; Sabeh *et al.*, 2009b; Stratman *et al.*, 2009; Lu *et al.*, 2010; Sabeh *et al.*, 2010; Rowe *et al.*, 2011). Given our previously described rules of invasion, one presumes that the pores generated in these collagen matrices are sufficiently small and rigid to preclude a protease-independent motile response – a recently confirmed prediction (Wolf *et al.*, 2013). Two exceptions exist, however, to the 'protease required' rule.

First, after tissue-invasive cells have 'tunnelled' through the collagen matrix, patent passageways can be created that allow trailing cells to follow the proteolytic leader by distinct, protease-independent schemes (though some reports have used type I collagen-Matrigel mixtures that may distort the fibrous structure of the network) (Gaggioli *et al.*, 2007; Dewitt *et al.*, 2009; Fisher *et al.*, 2009; Carey *et al.*, 2013). Hence, normal or neoplastic cells can traverse crosslinked collagen matrices without mobilizing proteases, but only if a passageway has already been cleared. Second, unlike all other cancer cell types, myeloid cells (i.e. leukocytes) freely traverse crosslinked hydrogels without proteolyzing the collagen network (Lammermann *et al.*, 2008; Sabeh *et al.*, 2009). Apparently, leukocytes negotiate collagenous barriers by applying two principles unavailable to other cell types; (i) they do not rely heavily on integrins to establish cell-matrix adhesive interactions and (ii) their nuclei are deficient in lamin A/C, a key component of the nuclear scaffold that impacts the rigidity of the organelle (Lammermann *et al.*, 2008; Olins *et al.*, 2009; Friedl *et al.*, 2011; Rowat *et al.*, 2013; Wolf *et al.*, 2013). The unusual ability of leukocytes to rapidly infiltrate tissues is likely a required component of an effective host defense system where microbes must be intercepted quickly. While it is tempting to speculate that cancer cells might adopt a similar phenotype, no neoplastic cell type (save for those of myeloid origin) has yet been shown to display a similar capability. Indeed, though it has been opined that leukocyte-centric schemes for traversing ECM barriers may be relevant to cancer cell invasion schemes (Madsen & Sahai, 2010), side-by-side comparisons—at least *in vitro*—suggest otherwise (Sabeh *et al.*, 2009; Wolf *et al.*, 2013). That is, whereas cancer cell invasion through type I collagen gels in tightly linked to MT1-MMP-dependent collagenolytic activity and the formation of collagen "tunnels" whose generation can be inhibited completely by synthetic MMP inhibitors, human polymorphonuclear leukocytes (PMNs), T-cells or monocytes traverse the same collagen gels without degrading the collagen matrix or leaving discernible tunnels (Fig. 1). Further, whereas aggressive cancer cells require several days to mount an invasion program through dense collagen barriers, human leukocytes, particularly PMNs, complete the bulk of their invasive activity within 4 h via a process that is unaffected by MMP inhibitors or protease inhibitors cocktails (Fig. 1) (Huber & Weiss, 1989; Sabeh *et al.*, 2009). Similarly, though MT1-MMP has been posited to play a direct role in macrophage invasion (Sakamoto & Seiki, 2009), MT1-MMP-null monocytes/macrophages display no major defects in transmigrating tissue barriers *in vitro* or *in vivo* following transfer of MT1-MMP knockout bone marrow into wild-type recipients (Xiong *et al.*, 2009; Shimizu-Hirota *et al.*, 2012). In a more recent study, the conditional knockout of MT1-MMP in mouse monocyte/macrophages resulted in subtle defects in cellular infiltration whose mechanistic underpinnings remain to be determined (Klose *et al.*, 2013). Indeed, highlighting the complexity of assigning specific functions to proteinases in intact cell systems, MT1-MMP unexpectedly regulates macrophage gene expression and immune responses via a novel proteinase-independent mechanism requiring MT1-MMP intracellular trafficking to the nuclear compartment (Shimizu-Hirota *et al.*, 2012).

Protease-independent migration in vitro: pepsin-extracted collagen hydrogels and beyond

As animals age, the intramolecular crosslinks found in type I collagen undergo a complex maturation process that renders the molecules acid-insoluble (Sabeh *et al.*, 2009). For commercial purposes, large quantities of type I collagen can nevertheless be recovered from

animal dermis (primarily bovine in origin) by employing a pepsin-extraction process that hydrolyzes the crosslink-rich telopeptide domains, leaving the tripe-helical domains intact (Sabeh *et al.*, 2009; Kreger *et al.*, 2010; Bailey *et al.*, 2011). Like acid-extracted native collagen, telopeptide domain-free collagen undergoes fibrillogenesis in a pH-, ionic strength- and temperature-dependent fashion. The polymerization process is, however, delayed relative to intact type I collagen, and can yield hydrogels with larger diameter fibrils and consequently, larger pore sizes (Kuznetsova & Leikin, 1999; Olins *et al.*, 2009). The degree to which fibril diameter and pore size are altered remains the subject of some debate as techniques used to judge fibril dimensions, that is primarily confocal reflection microscopy and second harmonic generation, can underestimate fiber density or size while distorting pore size estimates (Sato *et al.*, 2000; Demou *et al.*, 2005; Jawerth *et al.*, 2010; Kreger *et al.*, 2010; Conklin *et al.*, 2011). Nevertheless, reports using pepsin-extracted bovine dermal collagen (previously sold under the name, Vitrogen) or pepsin-treated rat tail collagen, all concur that cancer cells can rapidly infiltrate telopeptide-depleted collagen hydrogels in a protease-independent fashion (Wolf *et al.*, 2003; Sabeh *et al.*, 2004; Sodek *et al.*, 2008; Packard *et al.*, 2009; Sabeh *et al.*, 2009; Wolf *et al.*, 2013). As new studies demonstrate that the pore size of pepsin-extracted collagen hydrogels is sufficiently large to accommodate the nuclear dimensions of invading cancer cells (Wolf *et al.*, 2013), these matrices may well recapitulate the structural characteristics of loosely organized tissues *in vivo* (see further). In pepsinized collagen hydrogels, however, the absence of intramolecular crosslinks may also alter (i) matrix rigidity—and hence, cell behavior, (ii) the ability of collagen fibers to bend and slide past one another with the application of mechanical force, (iii) the ability of cells to transmit mechanical information across large distances via tractional forces, and (iv) the sensitivity of the telopeptide-free fibrils to collagenolytic attack (Woodley *et al.*, 1991; Discher *et al.*, 2005; Perumal *et al.*, 2008; Sander *et al.*, 2009; Winer *et al.*, 2009; Ma *et al.*, 2013). Even so, pepsinized collagen gels can provide a useful model for interrogating the means by which cells invade less-structured matrices though the physiologic relevance of matrix constructs assembled from telopeptide- and crosslink-depleted collagen remains unclear. More ideally, perhaps, native collagen gels may be constructed under conditions where pore size is purposefully varied (Sabeh *et al.*, 2009; Wolf *et al.*, 2013). In fact, collagen fiber diameter and pore size can be tuned by altering the temperature or pH of gelation, though matrix rigidity is also altered under these conditions (Mickel *et al.*, 2008; Raub *et al.*, 2008; Yang *et al.*, 2010). Indeed, native collagen gels can be traversed by neoplastic cells via protease-independent mechanisms if the pore size is sufficiently large (Wolf *et al.*, 2013). Nevertheless, due appreciation of even ‘simple’ polymerization processes is required when formulating type I collagen hydrogels. “Minor” changes in the preparation of collagen gels (e.g. the source of acid-extracted type I collagen, the length of time that neutralized collagen solutions are held at 4°C prior to gelation or the thickness of the cast gel) can all affect the physical properties of the hydrogels as well as the behaviour of embedded cell populations (Jiang *et al.*, 2000; Bailey *et al.*, 2011; Carey *et al.*, 2012; Nguyen-Ngoc & Ewald, 2013). Likewise, as migrating cells move closer to the edges of hydrogels constructed within rigid supports (i.e. glass or plastic containers), abrupt increases in matrix rigidity occur at the gel-support interface via edge effects that can also impact cell phenotype (Rao *et al.*, 2012).

Independent of these cautionary notes, hydrogel pore size is an important determinant of protease-dependent versus proteinase-independent migration schemes (Wolf *et al.*, 2013). Interestingly, even in the absence of ECM barriers that require proteolytic remodelling, marked changes are observed in the motile strategies used by cancer cells to traverse variably sized channel diameters in micro-engineered migration chambers (Rolli *et al.*, 2010; Balzer *et al.*, 2012; Pathak & Kumar, 2012; Tong *et al.*, 2012). Most remarkably, confining carcinoma cells to increasingly smaller channel diameters, Balzer and colleagues demonstrated that motility switches from an integrin-and actomyosin-dependent mechanism to one that relies on microtubule dynamics alone (Balzer *et al.*, 2012).

Devil in the details: Amoeboid-type invasion patterns *in vivo*

Until recently, many groups concluded that cancer cells readily traverse type I collagen barriers by adopting an amoeboid phenotype characterized by its insensitivity to broad-spectrum proteinase inhibitors (for review, see Sabeh *et al.*, 2009). We now know, however, that a wide variety of cancer cell types are absolutely dependent on MT1-MMP when confronting crosslinked type I collagen barriers whose mean pore diameter requires changes in nuclear shape that exceed the maximal deformability of the nuclear envelope (Sabeh *et al.*, 2009; Wolf *et al.*, 2013). Nevertheless, cancer cells can potentially adopt a protease-independent stance when negotiating structural barriers that can either accommodate the semi-deformable nuclear envelope or prove sensitive to mechanical displacement. Presently, however, little is known with regard to the size or structural characteristics of ECM pore sites encountered within the interstitial compartment *in vivo* (Sabeh *et al.*, 2009; Wolf *et al.*, 2009). Keeping in mind the limitations associated with documenting pore size by confocal reflection microscopy or second harmonic generation alone (Conklin *et al.*, 2011), *in vivo* tissue estimates have ranged from 4 to 10 μm^2 “micropores” to 40 to 1000 μm^2 ‘macropores’ (Wolf *et al.*, 2009). These results raise the possibility that collagen constructs assembled *in vitro* may not recapitulate fully the more complex networks assembled *in vivo.* However, it should be noted that defects in vascular smooth muscle cell migration, adipocyte differentiation and stem cell lineage commitment observed in MT1-MMP-targeted mice have been duplicated *in vitro* using dense, acid-extracted type I collagen hydrogels (Filippov *et al.*, 2005; Chun *et al.*, 2006; Tang *et al.*, 2013). Even so, given the more simple composition of type I collagen hydrogels relative to the interstitial matrix *in vivo*, cancer cell migration into tissue explants has also been used as an alternative, albeit, empirical means for assessing the obligate versus disposable roles of proteases during trafficking through ‘authentic’ matrix pores (Sabeh *et al.*, 2004; Nurmenniemi *et al.*, 2009; Sabeh *et al.*, 2009; Booth *et al.*, 2012). When cultured atop acellular human dermal explants, cancer cell invasion has been reported to proceed only in the presence of MT1-MMP-dependent activity (Sabeh *et al.*, 2004). Whereas the preparation of acellular explants potentially distorts the pore architecture of native tissues, live explants have also been used as a platform for assessing the protease-dependent versus protease-independent invasive activity of cancer cells (Sabeh *et al.*, 2009). Under these more *in vivo*-like conditions, cancer cell organoids were inoculated into intact human mammary gland tissues. Though monitoring of invasive activity was limited to a short-term, 3-day culture period, cancer cells failed to infiltrate the surrounding tissues in the absence of MT1-MMP activity (Sabeh *et al.*, 2009). Likewise, MT1-MMP has been shown to support metastatic behaviour of breast cancer cells in mouse

models *in vivo* (Perentes *et al.*, 2011). As little evidence of protease-independent invasive activity has been observed in these scenarios, these results support—at least under these specific conditions—the presence of an interstitial matrix pore size that cancer cells are unable to negotiate without mobilizing proteolytic activity.

Of course, *ex vivo* efforts to directly track patterns of cell invasion fall short of recapitulating the daunting complexities of the *in vivo* environment. Nevertheless, several groups have launched efforts to characterize cancer cell motility *in vivo* and the results have, to varying degrees, been illuminating (Provenzano *et al.*, 2009; Friedl *et al.,* 2012; Weigelin, 2012). Estimates of matrix pore size in normal tissues provide insight into the potential trafficking constraints encountered by invading cells under baseline conditions (Wolf *et al.*, 2009). However, primary tumour sites are frequently associated with the deposition of dense bands of interstitial collagen that increase matrix density and rigidity (Levental *et al.*, 2009; Provenzano *et al.*, 2009). Indeed, in syngeneic mouse models of breast cancer, neoplastic cells interface directly with collagen fibrils that appear to present invading cells with a physical barrier to passive movement (Provenzano *et al.*, 2009). Further, in mouse models of breast cancer, neoplastic cells at the invading front have been observed to contain large quantities of ingested type I collagen, a finding consistent with the proposition that active collagenolysis is integral to the invasion process as cells confront tissue barriers *in vivo* (Curino *et al.*, 2005). Interestingly, the diameter of collagen fibers deposited at neoplastic sites *in vivo* also matches that of self-polymerizing collagen hydrogels prepared from acid-extracted type I collagen under standard conditions (Oldberg *et al.*, 2007). Nevertheless, despite indications that neoplastic cells encounter collagenous barriers *in vivo* that necessitate proteolytic remodelling, other studies have documented the appearance of rapidly migrating cancer cell populations that move with a rapid, amoeboid-like morphology (Madsen & Sahai, 2010). Furthermore, the invasive potential of these cells appears to be insensitive to MMP inhibitors in the *in vivo* setting (Wolf *et al.*, 2003; Madsen & Sahai, 2010). Whereas the observed migration patterns are certainly consistent with cancer cell movement through larger, non-constraining matrix pores, these results alternatively reflect the ability of cancer cells to rapidly migrate through proteolytically precleared tunnels via proteinase-independent processes similar to those observed *in vitro* (Sabeh *et al.*, 2004; Gaggioli *et al.*, 2007; Fisher *et al.*, 2009; Carey *et al.*, 2013). Interestingly, more recent work supports, however, a lower speed of invasion in tumour in the *in vivo* setting, perhaps reflecting requirements for a proteinase-dependent invasion schemes (Weigelin, 2012). Distinguishing between these two possibilities remains difficult and will require determined efforts to track the movement of syngeneic tumours from their primary site of origin.

What more is to be done? A proposition and way forward

In vitro studies allow for the formulation of specific questions and the design of rigorous experimental approaches. However, the testing of even “simple” models will require the melding of expertise in ECM biochemistry, bioengineering, cell biology, enzymology and sophisticated imaging technologies, a mix of skill sets seldom if ever found within a single laboratory group (Morell *et al.*, 2013). Furthermore, by venturing into the *in vivo* setting, additional expertise will be needed in cancer biology and animal modelling as well as the application of specific interventions for silencing proteolytic systems. Attempts to target

MMPs *in vivo*—and this assumes that MMPs play a preeminent role in invasion as opposed to other proteolytic systems—have been confined largely to nonspecific small molecule inhibitors whose efficacy remains the subject of debate (though humanized anticatalytic monoclonal antibodies directed against MT1-MMP have been characterized recently) (Devy *et al.*, 2009). Whereas tissue-specific knockouts are seldom employed for these studies, cooperative interactions between cancer cells and stromal cell populations further complicate efforts to identify a single, monolithic player (Gaggioli *et al.*, 2007; Kessenbrock *et al.*, 2010). Indeed, unpublished efforts ongoing in the Weiss laboratory are focusing on the use of MT1-MMP and MT2-MMP floxed mice to clarify the role of the epithelial-and stromal-derived proteinases in regulating the invasion programs associated with normal mammary gland branching morphogenesis and mammary gland tumorigenesis. Even with all the necessary tools in hand, we cannot overstress the complexities of an ECM comprised of self-polymerizing molecules that can be crosslinked into novel structures by only recently defined processes (Rowe & Weiss, 2008; Vanacore *et al.*, 2009; Bhave *et al.*, 2012; Ilani *et al.*, 2013). Moreover, in terms of understanding mechanisms underlying cancer cell intravasation and extravasation, epithelial BMs likely display characteristics distinct from vascular and lymphatic BMs (Rowe & Weiss, 2008; Pflicke & Sixt, 2009; Rowe & Weiss, 2009), and it is not clear that invasion through the abluminal side of a BM into a vascular/lymphatic network (i.e. intravasation) is equivalent to transmigration from the luminal face of a BM into surrounding stromal tissues (i.e. extravasation). Popular, but perhaps, overly simplistic, cartoon-like depictions of the complex cancer cell-stromal-ECM interface are unlikely to shed new insights into these dynamic environments. Without expending effort to separate dogma from fact, progress in the field will likely be stifled or misguided. New insights into the regulation of the dynamic cancer cell-ECM interface will no doubt require ‘team efforts’ that couple state-of-the-art technologies with scientific rigor. Dividends from these enterprises will accelerate the development of the necessary tools to permit the simultaneous analysis of changes in ECM structure and function as tumour cells infiltrate the 3-D ECM *in vitro* and *in vivo*. The identification and specific targeting of the key proteolytic—or nonproteolytic—systems that drive cancer cell invasion in validated model systems should then allow for the resolution of outstanding questions in the field and pave the way for the intelligent design of new interventions.

Acknowledgments

This work was supported by the NIH (CA88308 and CA71699 to SJ Weiss). We thank K. Wolf (Radboud University) for helpful discussions and comments.

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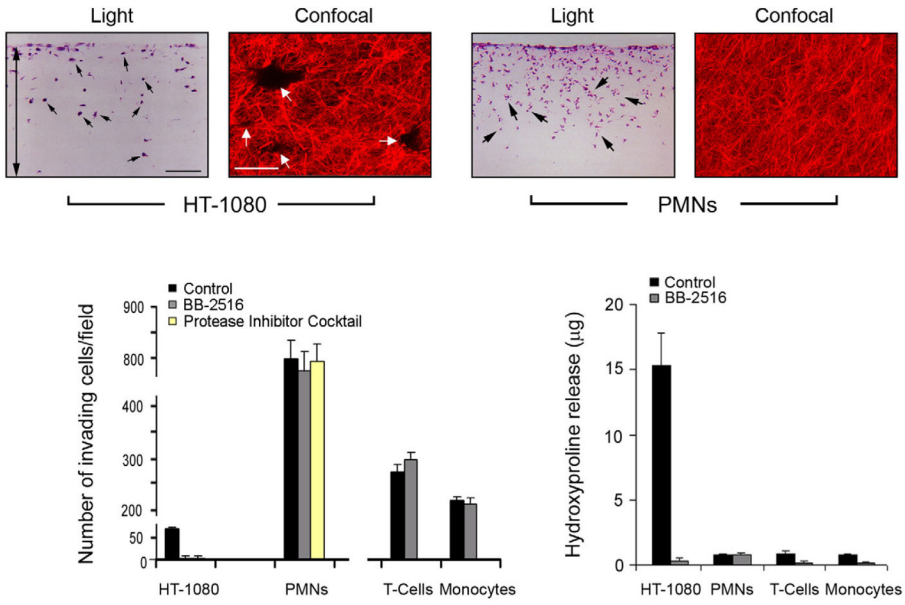

Fig. 1.

Collagen-invasive and degradative activities of cancer cells versus leukocytes. Light micrographs of cross-sections of type I collagen gels (2.2 mg/mL) traversed by HT-1080 or human PMNs prepared as described (Sabeh *et al.*, 2009) for 3 d or 1 d, respectively. Collagen-invasive cells are H&E stained and marked with black arrows. Double-headed arrow marks the boundaries of the underlying collagen gel. Black bar = 100 μm. Laser confocal micrographs of HT-1080 cells cultured atop 3D gels of rhodamine-labeled type I collagen for 3 d demonstrate that invasion is associated with the formation of well-demarcated tunnels (white arrows; white bar = 50 μm). On the other hand, PMNs stimulated with zymosan-activated plasma (Huber and Weiss, 1989) invade rhodamine-labeled collagen gels without perturbing matrix architecture (far right-hand panel). Invasion and collagen-degradative activities of HT-1080, PMNs, T cells, and monocytes were quantified in the absence or presence of BB-2516 (1.5 μM) as described previously (bar graphs, bottom panel) (Sabeh *et al.*, 2004). PMN invasion was also assessed in the presence of the protease inhibitor cocktail prepared as described (Wolf *et al.*, 2003). Results are expressed as mean ± SEM (n = 4). Images and data are reproduced from the original work (Sabeh *et al.*, 2009).