3D/4D Functional Imaging of Tumor-Associated Proteolysis: Impact of Microenvironment

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Abstract

Proteases play causal roles in many aspects of the aggressive phenotype of tumors, yet many of the implicated proteases originate from tumor-associated cells or from responses of tumor cells to interactions with other cells. Therefore, to obtain a comprehensive view of tumor proteases, we need to be able to assess proteolysis in tumors that are interacting with their microenvironment. As this is difficult to do *in vivo*, we have developed functional live-cell optical imaging assays and 3D and 4D (i.e., 3D over time) coculture models. We present here a description of the probes used to measure proteolysis and protease activities, the methods used for imaging and analysis of proteolysis and the 3D and 4D models used in our laboratory. Of course, all assays have limitations; however, we suggest that the techniques discussed here will, with attention to their limitations, be useful as a screen for drugs to target the invasive pheno-type of tumors.

1. Introduction

Recent studies in our laboratory have focused on establishing live-cell assays to image the proteolysis that is associated with the progression of premalignant breast lesions to malignant carcinomas. This chapter will use our studies to illustrate how the interactions of tumor cells with their microenvironment contribute to this proteolysis and how functional imaging assays and 3D/4D coculture models might be used to identify druggable targets and screen therapeutic agents.

1.1. Why image proteolysis rather than protease activity?

Proteolysis or the hydrolytic degradation of proteins occurring as the result of interactions among proteases of more than one catalytic type, that is, proteolytic pathways or networks, has been shown to be critical to malignant progression (e.g., see DeClerck and Laug, 1996; Ellerbroek *et al.*, 1998; Kim *et al.*, 1998; Krol *et al.*, 2003; Muehlenweg *et al.*, 2000; Ramos-DeSimone *et al.*, 1999). Prior research in our laboratory concentrated on one cysteine cathepsin, that is, cathepsin B (for reviews, see Cavallo-Medved and Sloane, 2003; Podgorski and Sloane, 2003; Roshy *et al.*, 2003; Yan and Sloane, 2003). Analysis of any one protease or protease class, however, does not define the "tumor degradome" (Balbin *et al.*, 2003). Therefore, we established an assay to study proteolysis of extracellular matrix substrates encountered by tumor cells as they invade into normal tissues surrounding tumors. This protein-based assay contrasts with those measuring degradation of synthetic substrates that are selective for one protease or one catalytic type of protease. Data generated in our laboratory (Cavallo-Medved *et al.*, 2009; Jedeszko *et al.*, 2009; Li *et al.*, 2008; Podgorski *et al.*, 2009; Sameni *et al.*, 2000, 2001, 2003, 2008, 2009) and other laboratories (Kjoller *et al.*, 2004; Madsen *et al.*, 2011; Wolf *et al.*, 2009) by means of functional proteolysis assays that

employ protein substrates support our contention that meaningful analyses of tumor proteolysis require assessment of the roles played by multiple proteases as well as assessment of how those proteases interact to modulate the activities of other proteases.

1.2. Why image tumor proteolysis?

More than one catalytic type of protease has been implicated in the progression of human tumors (for reviews, see Choong and Nadesapillai, 2003; Fuchs, 2002; Hojilla *et al.*, 2003; Podgorski and Sloane, 2003). Although the preclinical data implicating matrix metalloproteinases (MMPs) in malignant progression were particularly compelling, the clinical trials on MMP inhibitors (MMPIs) did not fulfill the promise of MMPs as therapeutic targets in cancer. There are several possible explanations for this apparent "disconnect" between the preclinical and clinical data; please see Chau *et al.* (2003), Coussens *et al.* (2002), Egeblad and Werb (2002) for insightful and thought-provoking reviews on this topic.

The failures of MMPIs in clinical trials have resulted in allegations that MMPs and, by extrapolation, other proteases are not appropriate therapeutic targets in cancer. Is this true or rather might the failure of the MMPI trials reflect problems in clinical trial design for cytostatic agents and in particular the need to use imaging (Adjei *et al.*, 2009; Ang *et al.*, 2010)? This is the case as among the critical questions is whether the MMPIs actually reached and reduced the activity of their target MMPs *in vivo*. The MMPI trials did not include surrogate endpoints so it is not known whether MMPs were actually inhibited in the patients enrolled in the trials (Chau *et al.*, 2003; Li and Anderson, 2003; McIntyre and Matrisian, 2003). Clinical trials without surrogate endpoints to monitor and confirm the efficacy of the therapeutic strategies being tested should not be viewed as definitive (Adjei *et al.*, 2009; Li and Anderson, 2003; McIntyre and Matrisian, 2003; Seymour *et al.*, 2010). There are other concerns about the MMPI trials. Coussens *et al.* (2002) cite data revealing that the tumors studied in the clinical trials did not necessarily express the particular proteases targeted by the MMPIs. None of the clinical trials with BAY 12-9566 or other MMPIs included patients with breast cancers although this is a cancer for which there had been strong preclinical evidence that MMPs impact progression. For example, Sledge and colleagues (Nozaki *et al.*, 2003) demonstrated efficacy for BAY 12-9566 in an orthotopic model in which human breast tumor cells were implanted in the mammary fat pads of mice. Furthermore, not all MMPs should be inhibited; this is clearly true for MMP-8 (collagenase-2), an MMP expressed by inflammatory neutrophils, which plays a protective role in skin cancer (Balbin *et al.*, 2003). The data on MMP-8 and more recent data on a variety of other MMPs (for reviews, see Lopez-Otin and Matrisian, 2007; Lopez-Otin *et al.*, 2009) support the concept that broad-spectrum MMPIs would have unanticipated side effects and indicate how essential it is "to define precisely the tumor degradome" (Balbin *et al.*, 2003) before using MMPIs or for that matter other protease inhibitors for cancer therapy. In fact, unanticipated side effects did occur in the MMPI clinical trials leading to limitations in the amount of MMPIs that the patients could take and in some patients necessitating MMPI-free holidays (Coussens *et al.*, 2002). How endogenous protease inhibitors factor into the equation is also of relevance. As just one example, TIMP-1 has been shown to promote carcinogenesis of squamous cell carcinoma of the skin, exerting "differential regulation on tissues in a stage-dependent manner" (Rhee *et al.*, 2004). We are still far from having a thorough understanding of the roles of proteases in cancer: this includes understanding the roles of MMPs; the roles of other classes of proteases; the roles of endogenous protease inhibitors, activators and receptors, or binding proteins; that those roles are dynamic and may change during the course of malignant progression; whether the proteases playing critical roles in malignant progression come from tumor, stromal, or inflammatory cells; whether the critical proteases are affected by interactions of the tumor with its

microenvironment; what the relevant substrates are for proteases that play causal roles in malignant progression; etc. Many of these issues are discussed in a large volume on what constitutes the cancer degradome that was published in 2008 (Edwards *et al.*, 2008). Nonetheless, we do not yet know which protease(s) or proteolytic pathway is the most appropriate target for antiprotease therapies or when antiprotease therapies might prove most effective.

2. Assays for Functional Imaging of Proteolysis

The terminology "functional imaging" was originally used to describe imaging methods that assessed changes in physiological processes such as metabolism and blood flow, for example, positron emission tomography (PET) and magnetic resonance imaging. Here, we use this terminology because the live-cell assays that we have developed for imaging proteolysis can be used to quantify changes in proteolytic activity as well as to localize sites at which proteolysis is occurring (Jedeszko *et al.*, 2008). Functional imaging often uses agents or probes; in PET, for example, a glucose analogue fluorodeoxyglucose is used as an F^{18} radioisotopic tracer to detect and localize the high levels of metabolic activity associated with tumors (Mankoff *et al.*, 2007).

2.1. Probes

Probes for imaging proteases have been based on either substrates or inhibitors. A substrate probe can be a signal amplifier as, in the presence of an active target protease, the substrate will be cleaved continuously and thus the signal intensity increased. This property also makes substrate probes sensitive reagents for detecting reductions in protease activity due to protease inhibitors, etc. A potential negative with substrate probes is that the cleavage products may not remain at the site where cleavage occurred and thus would not accurately localize an active protease. This is especially true for extracellular or cell surface proteolysis where substrates and/or cleavage products might diffuse away from the site at which they are generated. Protease probes based on inhibitors that bind covalently to the active site of a protease are more effective in localizing protease activity. On the other hand, they cannot amplify the signal and therefore are less sensitive in detecting proteases that are not highly expressed or reductions in protease activity (Fonovic and Bogyo, 2007).

Whether protease probes are based on a substrate or an inhibitor, proteases are ideal targets for selective, activatable contrast agents. In this case, a fluorescent probe is synthesized in a quenched state in which a nonfluorescent quencher is attached to the probe in close proximity to the reporting fluorophore via a protease-selective peptide linker. The proximity of the fluorophore to the quencher causes transfer of energy from the former to the latter, thus preventing emission of fluorescence. When such a probe encounters an active target protease, the peptide will be cleaved and the quencher released, resulting in emission of a fluorescent signal. Alternatively, probes can be developed utilizing a synthetic (e.g., dendrimer or polylysine—see Marten *et al.*, 2002; McIntyre *et al.*, 2004, respectively) or protein (collagen) backbone to which a large number of reporters are attached via peptide linkers in close proximity to each other (for review see, Sloane *et al.*, 2006). The overabundance of the reporter in close proximity causes self-quenching due to a FRET (Forster Resonance Energy Transfer) effect (Brzostowski *et al.*, 2009). When an active target protease cleaves the reporter off the backbone, fluorescence is emitted. Such probes have been used for both *in vitro* and *in vivo* systems (Brzostowski *et al.*, 2009; McIntyre *et al.*, 2004).

2.1.1. Fluorescently tagged proteins—The primary probes that we use in our laboratory to image proteolysis are quenched fluorescent or dye quenched (DQ) extracellular matrix proteins that are commercially available from Invitrogen, that is, DQ-collagen IV and

DQ-collagen I. Our use of these substrates was featured in their newsletter (Visualizing tumor metastasis: CellTracker™ dyes, DQ™ collagen, and Geltrex™ matrix. BioProbes 60, pp. 32–33, October 2009). We selected the two collagen substrates because type IV collagen is a major component of the basement membrane (Aumailley and Gayraud, 1998) and dissolution of type IV collagen has been shown to be integral to normal developmental processes and an early step in malignant progression (for review, see Liotta and Kohn, 2001). Fibrillar collagen I in the connective tissue through which tumor cells invade is an impediment to cell growth and invasion (Henriet *et al.*, 2000; Sabeh *et al.*, 2009).

By using quenched fluorescent derivatives of type IV and I collagen, we have been able to image and localize proteolysis, that is, a gain-of-function/fluorescence, by live cells (Sameni *et al.*, 2000, 2001, 2003, 2009). This is in contrast to using nonquenched FITC-labeled proteins where one is imaging a loss of fluorescence and has to fix the cells and substrate before imaging (Demchik *et al.*, 1999; Sloane, 1996).

A critical point is that, by virtue of their fluorescent labeling, the DQ-collagens are no longer native proteins. Therefore the ability to cleave these substrates may not be representative of an ability to cleave native forms of these proteins. This is likely more the case for DQ-collagen I as gelatin or denatured collagen I is readily degraded by many proteases. There are collagenase-resistant forms of collagen I in which the cleavage sites in the helical region have been mutated so that they cannot be degraded by true "collagenases" such as MMP-1 (Wu *et al.*, 1990). Studies using collagenase-resistant collagen I have demonstrated that proteolysis of collagen I is required for motility of vascular smooth muscle cells on collagen I (Li *et al.*, 2000) and for invasion of ovarian cancer cells into collagen I (Ellerbroek *et al.*, 2001). Unfortunately, DQ-collagenase-resistant collagen I is not commercially available.

2.1.2. Activity-based probes (ABPs)—We also employ ABPs developed by Bogyo and colleagues for imaging cysteine proteases of the papain family (Greenbaum *et al.*, 2002a,b). These probes are based on the broad-spectrum cysteine protease inhibitor E-64 and bind covalently to the active sites of the enzymes. Therefore, with these probes, one can image active cysteine cathepsins *in situ* in cells and also identify what active protease is being detected by visualizing the fluorescently tagged protease(s) in cell lysates/conditioned media on SDS-PAGE gels. These probes have been used to identify and image cysteine cathepsins in live cells *in vitro* (Blum *et al.*, 2005) and *in vivo* in a transgenic mouse model of pancreatic cancer (Joyce *et al.*, 2004) and a transgenic mouse model of mammary cancer (Vasiljeva *et al.*, 2006). These ABPs are cell permeable and thus allow us to image the intracellular activity of cysteine cathepsins in the lysosomes (Blum *et al.*, 2005). Furthermore, the ABPs are available in both unquenched and quenched versions albeit the selectivity of the ABPs for a given cysteine cathepsin seems to be reduced in the quenched versions (Blum *et al.*, 2005).

2.1.3. Other protease probes—The two other types of protease probes that we are using *in vitro* are fluorogen-activating protein (FAP) protease biosensors developed by Berget and colleagues at Carnegie-Mellon and proteolytic beacons that Matrisian and colleagues have designed and developed for real-time analysis of MMP activity, including activity of individual MMPs like MMP-7 (McIntyre and Matrisian, 2009; McIntyre *et al.*, 2004, 2010; Scherer *et al.*, 2008). The FAPs are engineered proteins based on single chain antibodies (Falco *et al.*, 2009). At present, FAPs to assess the activity on the surface of live cells of MMP-14, -2, -9, and cathepsin K are being tested. The proteolytic beacons are protease substrates, which are built on a nanodendron scaffold and use FRET between two fluorophores linked to a selective peptide substrate (see McIntyre and Matrisian, 2003; Scherer *et al.*, 2008 for review). When in close proximity, the sensor fluorophore is quenched by the reference fluorophore, which also serves to monitor substrate

concentration. The ability of these beacons to monitor how much of a probe is delivered and demonstrate that the probe is delivered is a crucial one. Proteolytic cleavage of the peptide linker results in increased fluorescence of the sensor so that the ratio of sensor to reference can be used as a quantitative measure of proteolytic activity. The design permits a great deal of flexibility: fluorophores can be in either the visible or near infrared range (NIR), protease selectivity can be modulated by alterations in the peptide sequence, clearance kinetics and route can be determined by the size and composition of the dendron backbone, and the multifunctionality of the dendrons allows for optimization of fluorophore concentration and solubility characteristics. Published proteolytic beacons include a visible one that is selective for MMP-7 (McIntyre and Matrisian, 2003; Wadsworth *et al.*, 2010), NIR MMP-7- and MMP-9-selective beacons (McIntyre *et al.*, 2010; Scherer *et al.*, 2008), and a NIR beacon that detects general MMP activity (McIntyre *et al.*, 2010).

2.2. Analysis

The development of new fluorogenic dyes and of confocal, multiphoton, and structured illumination microscopes has made optical imaging the method of choice for direct observations in living systems (for review, see Andrews *et al.*, 2002; Swedlow and Platani, 2002). These state-of-the-art advanced imaging systems allow one to perform 3D and 4D analyses using multiple probes.

For live-cell imaging there is an absolute requirement to perform experiments under physiological conditions. For this reason all of our imaging systems are equipped with environmental chambers (temperature, CO_2, and humidity controlled). This allows us to image live cultures over extended times without removing the cultures from the microscope stage. In addition, we can image the dynamics of proteolysis in real-time as impacted by tumor–stromal interactions. Another common feature of our systems is their motorized, fully automated stages. With this type of stage, we are able to select several areas of interest and program the system to image these areas sequentially at multiple time points and in 3D. The environmental chamber allows us to establish 3D models, place them on the microscope stage and program the microscope to acquire images as the cells attach, migrate, and invade into the surrounding extracellular matrices; we illustrate the association of proteolysis with cell migration and cell– cell interaction in Fig. 10.1. We now routinely label the various types of cells in our cocultures by prestaining with vital cytoplasmic dyes (Fig. 10.1) or transducing with fluorescent proteins (Fig. 10.2) so that they can be readily distinguished from one another when analyzing cell– cell interactions. We obtain optical sections through the entire volume of the fluorescently labeled specimen in real time at various time points. The data are then analyzed with the 3D and 4D image reconstruction software as described below and as illustrated in Fig. 10.2.

The operating software for the imaging systems performs basic quantitative analyses, but is limited in regard to the extensive 3D and 4D quantitation required by the studies described here. Therefore, we use advanced stand-alone image analysis software such as Metamorph™ 7.64 and Volocity™ 5.5 to analyze fluorescence intensities, volumetric areas, surface areas, etc. Volumetric measurements are critical for accurately assessing changes in cell–cell interactions over time in 3D, that is, in 4D. To assess interactions, we label samples for detection of the different cell types, specific target proteins, proteolytic degradation products, etc., as we have described (Jedeszko *et al.*, 2008; Sameni *et al.*, 2003, 2009). Volumetric measurements of the sample components are acquired in multiple channels with each channel representing a single target. Datasets from each time point are then loaded for 4D quantification, analysis, and rendering of the data in 3D for each time point. Importantly, the software allows one to mark specific regions of interests in the 3D image stacks. The software can then quantify each region in 3D as well as quantify changes over time, that is,

in 4D. The compiled images can be presented in many formats, including movies of live events.

3. 3D/4D Models for Analysis of Biological Processes Linked to Proteolysis

The working hypothesis for ongoing studies in our laboratory is that 3D mammary cell-based models will recapitulate the proteolytic mechanisms integral to developmental and neoplastic processes. Using such models to image proteolysis over time (i.e., 4D imaging) should allow us to place proteolysis, including the proteases identified and their interactions, within the context of the signaling pathways and other functions already elucidated by Bissell and her many collaborators as essential to malignant progression of mammary cancer. Those studies have convincingly demonstrated the importance of context to developmental and neoplastic processes in the mammary gland (for review, see Lee *et al.*, 2007; Schmeichel and Bissell, 2003). By growing mammary epithelial cells "within 3D basement membrane-like-matrices," the Bissell laboratory has been able to reproduce signaling pathways and functions (e.g., milk protein production) *in vitro* that are not observed when the same cells are cultured in 2D monolayers. Furthermore, the *in vitro* 3D mammary models have shown the need to match cell types with appropriate extracellular matrices, in this case mammary epithelial cells with basement membrane-like-matrices. When Gudjonsson *et al.* (2002) substituted an interstitial connective tissue matrix protein, that is, collagen I, acini formed, but they were inside-out. Adding normal myoepithelial cells to the collagen I gels resulted in correctly polarized acini, an effect dependent on production by the myoepithelial cells of the -1 chain of the basement membrane protein laminin. There is a wealth of studies by Bissell and her collaborators showing that 3D cell-based assays can be used to study mechanisms for morphogenesis and neoplasia of human breast *in vitro* (for review, see Gudjonsson *et al.*, 2003; Schmeichel and Bissell, 2003). They also have demonstrated that proteases, in particular MMPs, are involved in morphogenesis and neoplasia. Studies by Weiss and his colleagues also implicate MMPs, in this case, MMP-14, or MT1-MMP, which they have shown to degrade collagen I and to be a prerequisite for proliferation of tumor cells in 3D collagen I gels (Hotary *et al.*, 2003). The Brugge laboratory has used 3D monotypic cultures grown in reconstituted basement membrane (rBM) to demonstrate a role for caspase-family cysteine proteases and apoptosis in the formation of lumens in the mammary acini (Debnath *et al.*, 2002; Shaw *et al.*, 2004). They have shown that migration and invasion of the mammary epithelial cells can be induced by coexpression of activated ErbB2 and TGF- (58), but have not analyzed the proteases responsible for the invasion. Further studies based on the Brugge 3D monotypic cultures by Debnath and colleagues have identified a role for lysosomal proteases. They found that autophagy and proteolysis within lysosomes plays both a suppressive and a promotion role that is context dependent (Chen and Debnath, 2010; Roy and Debnath, 2010).

Liotta and Kohn (2001) have suggested that cancer therapies should target the stroma or the tumor–stroma interface and hypothesized that stromal therapy could require lower doses than therapies that target the tumor. The need to target stroma would certainly appear to be true for protease inhibitors, as cells present in the tumor-associated stroma (e.g., fibroblasts, endothelial cells, inflammatory cells, myofibroblasts) are all important sources of proteases (for reviews, see Almholt and Johnsen, 2003; Bogenrieder and Herlyn, 2003; Coussens and Werb, 2001; DeClerck, 2000; Johnsen *et al.*, 1998; van Kempen and Coussens, 2002). For example, MMP-8 is expressed by neutrophils (Balbin *et al.*, 2003); urokinase plasminogen activator (uPA) by myofibroblasts, macrophages, and endothelial cells in ductal breast cancer (Nielsen *et al.*, 2007); MMP-9 by neutrophils, macrophages, and mast cells in a mouse model of squamous cell carcinoma of the skin (Coussens *et al.*, 2000); MMP-3 by subepithelial myofibroblasts in human colon (Bamba *et al.*, 2003); the cysteine proteases cathepsins B, K, L, and S and MMP-7, -9, and -12 by macrophages (Filippov *et al.*, 2003;

Punturieri *et al.*, 2000; Reddy *et al.*, 1995); cathepsin B by endothelial cells from breast, glioma, and prostate (for review, see Keppler *et al.*, 1996); and MMP-2 by endothelial cells (Han *et al.*, 2003). Tumor-associated macrophages in human carcinomas also express high levels of cathepsin B (Campo *et al.*, 1994; Fernandez *et al.*, 2001; McKerrow *et al.*, 2000) and this macrophage cathepsin B enhances malignant progression in mouse transgenic models for mammary carcinoma (Vasiljeva *et al.*, 2006) and pancreatic carcinoma (Gocheva *et al.*, 2010). Stromal cells can also affect tumor proteolysis through the expression of endogenous protease inhibitors. Myofibroblasts, for example, are suggested to be the primary source of plasminogen activator inhibitor-1 (PAI-1) in human breast carcinomas (Offersen *et al.*, 2003). Overall such data indicate that we will not be able to define the "tumor degradome" (Balbin *et al.*, 2003) unless we study proteolysis in the context of tumor cells interacting with their microenvironment, interactions that we contend can be modeled *in vitro* in organotypic cocultures.

Using cocultures, we have established that stromal cells significantly impact tumor proteolysis (Sameni *et al.*, 2003). Degradation of DQ-collagen IV is increased as much as 17-fold in live 3D cocultures of stromal cells (fibroblasts or fibroblasts + macrophages) with breast or colon tumor cells (Sameni *et al.*, 2003). Such findings are pertinent to whether protease inhibitors would be efficacious *in vivo*. Also relevant is that fibroblasts isolated from invasive ductal breast carcinomas, but not normal breast fibroblasts, can recruit infiltration of other stromal cells, in this case blood monocytes, into 3D spheroids (Silzle *et al.*, 2003). Analyzing the contribution of inflammatory components to the "tumor degradome" is important as infiltration of macrophages *in vivo* potentiates malignant progression of tumors, for example, in a transgenic mouse model for mammary carcinoma (Gouon-Evans *et al.*, 2002; Lin *et al.*, 2001).

The composition and density of the extracellular matrix appears to be critical to tumor cell invasion as well as to fibril formation by collagen I. Brugge and colleagues (Seton-Rogers *et al.*, 2004) have reported that breast epithelial cells do not invade in 3D cultures plated on undiluted rBM; however, when the basement membrane was diluted with collagen I (in this case pepsin-solubilized collagen I), the cells did invade. Such observations indicate the need to compare various matrix compositions if we are to reach any definitive conclusions about whether proteolysis is or is not required for tumor invasion in such models. Intriguing work by Weaver and colleagues suggests that increased cross-linking of collagen I promotes tumor invasion *in vitro* and *in vivo* (Levental *et al.*, 2009). This finding seems counterintuitive to a role for proteolysis in tumor invasion, as cross-linked collagen is more resistant to proteolysis (Sabeh *et al.*, 2009). It is the dynamics of collagen remodeling that appear to be critical (Egeblad *et al.*, 2010), supporting a need for not only 3D models but also for studying 3D models over time, that is, in 4D.

4. Live-Cell Imaging of MAME Models: A Screening Tool for Drug Discovery

There is substantial evidence that 3D cultures are predictive of the resistance of tumor cells to cytotoxic therapy and can be used to identify targets and validate potential therapeutic agents (Li *et al.*, 2008, 2010; Nam *et al.*, 2010). We hypothesize that functional imaging of proteolysis by live cells in 3D and 4D can be used as an *in vitro* screen for testing alternative strategies to target the malignant phenotype of tumor cells, using as a readout proteolysis of extracellular matrix proteins. Cell-based 3D models have already been proposed for use in high-throughput screening of drugs (Schmeichel and Bissell, 2003) as well as their use for analyzing dynamic interactions between tumor cells and cellular and noncellular constituents of their microenvironment (Ng and Brugge, 2009). Therefore, we contend that the dimension of time needs to be part of high-throughput screening, including screening of therapeutic strategies to reduce protease activity, whether those strategies are ones that

directly impact activity such as protease inhibitors or ones that target upstream effectors of protease activity.

We have developed a robust preclinical *in vitro* 3D/4D model to recapitulate paracrine interactions between tumor cells and other cells that comprise the tumor microenvironment. We have named these models MAME for *m*ammary *a*rchitecture and *mi*croenvironment *e*ngineering (Sameni *et al.*, 2009). Our MAME models are designed to closely mimic the architecture of normal breast tissue, a need strongly advocated by Weigelt and Bissell (2008), as they provide a readily adaptable system through which to determine the contribution of individual cell types of the tumor microenvironment to the aggressive phenotype of breast cancers. In 4D (3D + time), MAME models in conjunction with live-cell imaging techniques are allowing us to determine the timing as well as the respective contributions of various cell types, proteolytic pathways, signaling pathways, etc., to progression from a preinvasive to an invasive phenotype.

Our MAME tripartite cocultures (Figs. 10.2 and 10.3A) consist of a bottom layer of interstitial type I collagen in which we embed breast fibroblasts, a second layer of rBM on which we plate normal breast epithelial cells, premalignant breast epithelial cells or breast carcinoma cells and a top layer of 2% rBM. Thus, the second and top layers are based on the rBM overlay cultures used by the Brugge laboratory (Debnath and Brugge, 2005; Debnath *et al.*, 2003; Shaw *et al.*, 2004). In order to study proteolysis, we incorporate DQ-collagen I in the collagen I layer and DQ-collagen IV in the second layer of rBM. Other iterations of the MAME models consist of only the second and top layers of rBM in which DQ-collagen IV is incorporated (Fig. 10.3B). The tripartite cocultures recapitulate tumor–tumor microenvironment interactions that occur *in vivo* in human breast tumors as a result of indirect interactions. With the tripartite model we have observed over time the migration of fibroblasts toward the tumor cells, eventually infiltrating into the tumor structures over a period of 7 days. The breast tumor cells also migrate towards the lower layer of fibroblasts, but do so more slowly over a period of 3 weeks. Proteolysis is associated with the migrating tumor cells and fibroblasts, in this case pericellular fluorescent degradation products. In contrast, there is extensive diffuse fluorescence associated with the bottom layer of fibroblasts, indicative of the high protease activity produced by fibroblasts. To date, we have maintained the tripartite MAME models for as long as 24 days and imaged live cultures at intervals over that period. If we use preinvasive breast epithelial cells in the MAME models, we are able to image the progression of those cells to an invasive phenotype, accompanied by an increase in proteolysis. Furthermore, we have demonstrated the ability of a variety of antagonists to reduce the invasive phenotype and the proteolysis (Jedeszko *et al.*, 2009; Sameni *et al.*, 2009). We have used comparable models for analysis of the invasive phenotype of a variety of cancers, including inflammatory breast cancer (Victor *et al.*, in press), and prostate cancer (Hayward and Sloane, unpublished data). Recently, we have used MAME models to demonstrate that pericellular proteolysis is increased by incubating the cultures at a slightly acidic pH comparable to that found in tumors *in vivo* (Rothberg and Sloane, unpublished data).

Through live-cell imaging of MAME models, we are able to both image and quantify the cleavage products of the DQ-collagens (Jedezsko *et al.*, 2008; Sameni *et al.*, 2009). We are of course limited to localizing and quantifying cleavage of the labeled collagens and therefore our findings need to be considered in that context. Despite this limitation, however, we can visualize proteolysis associated with migration of individual cells and cellular structures and invasive protrusions from those structures (Jedeszko *et al.*, 2009) and to do so over long time periods. In the case of individual endothelial cells migrating to form tube-like structures, we have imaged proteolysis over a 20-h period (Cavallo-Medved *et al.*, 2009). That these labeled collagens may be more easily degraded than native collagens is

another caveat that we must consider. Nonetheless, the labeled collagens do allow us to monitor protease activity and in combination with other protease probes such as those discussed above should allow us to identify proteases that participate in progression to an invasive phenotype and proteases that may be read-outs for therapeutic strategies to abrogate that progression.

We are now adapting a WaferGen SmartSlide Microincubation System for real-time monitoring of our MAME models. The system was designed for monolayer culture of cells in six-well plates on the stage of an inverted confocal microscope. Each well can be individually perfused and effluents individually collected for immunochemical and biochemical analysis of secreted proteins without disturbing the integrity of the cultures. Furthermore, we can acquire optical sections of the six wells sequentially at multiple time points. In addition, we can harvest media for immunochemical and biochemical analyses at times corresponding to changes in aggressive phenotype. We will be able to image and assay conditioned media from six cultures at once and at multiple time points, thus increasing the throughput of our live-cell imaging assays of MAME models. Although we are primarily interested in using MAME models for studying proteolysis, the tripartite and other modifications of the MAME models along with microincubation systems such as the Wafergen provide an experimental system in which one can test various cellular and noncellular aspects of the tumor microenvironment as it affects the progression of human breast cancer and other cancers.

Acknowledgments

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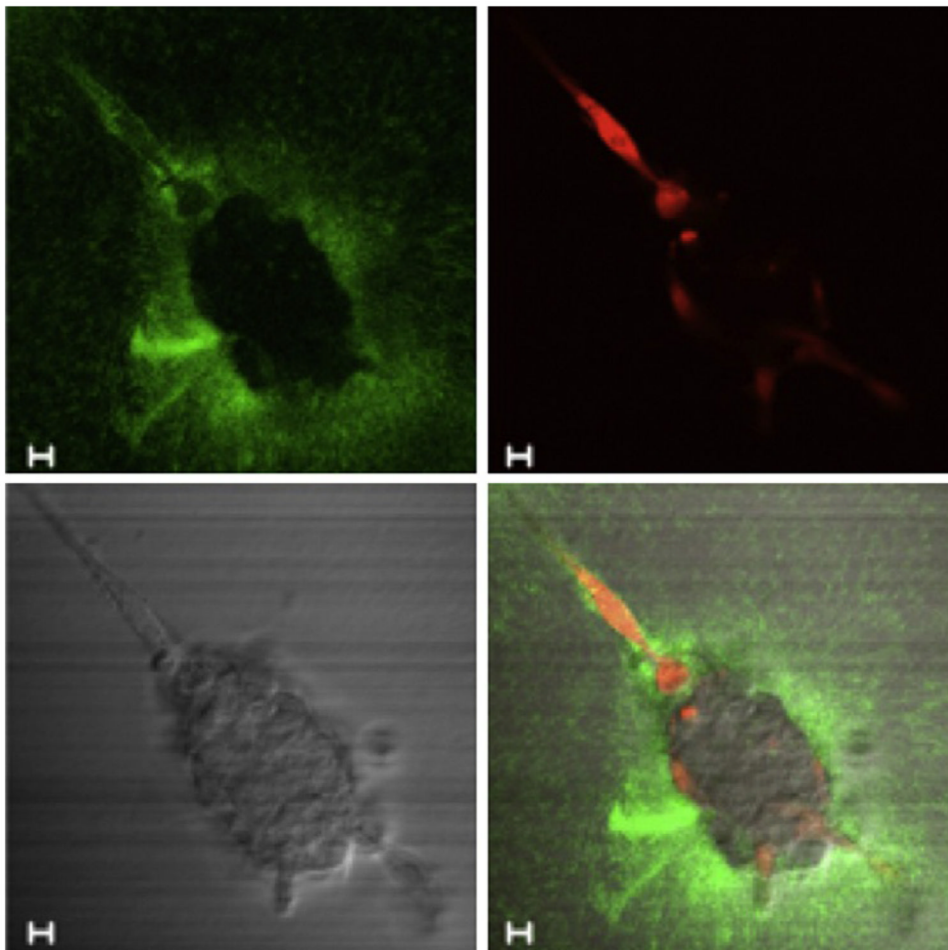
Figure 10.1.
Still images of time-lapse series (90 min total) to follow degradation of DQ-collagen IV (green fluorescence) by carcinoma cells in coculture with fibroblasts. CCD-112CoN colon fibroblasts (red due to prestaining with CellTracker Orange) were cocultured with a spheroid of HCT 116 colon carcinoma cells. In this image taken after an overnight period of coculture, the fibroblasts can be seen to be moving toward and entering the HCT 116 spheroid. Note the pericellular fluorescent cleavage products due to degradation of DQ-collagen IV by the fibroblasts as they infiltrate into the HCT 116 spheroid, which is more readily apparent in video format. The four panels of this figure represent degradation products of DQ-collagen IV (green), fibroblasts (red), spheroid of HCT 116 cells (DIC), and a composite of the other three images. Bar, 10 μm.
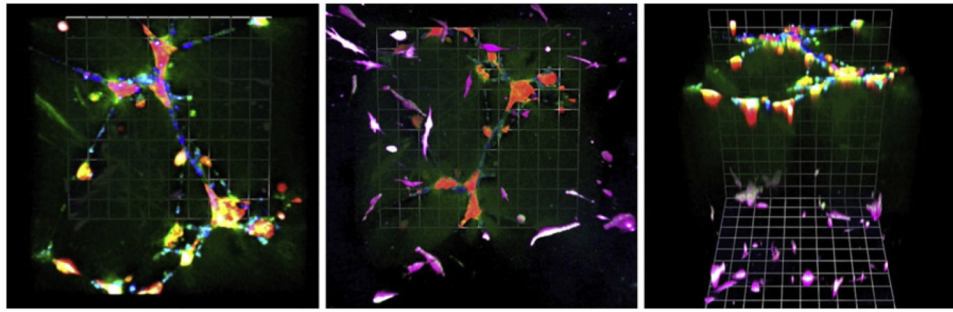
Figure 10.2.
MAME tripartite cocultures of human SUM102 breast carcinoma cells (red; transduced with Lenti-RFP) with human umbilicial vein endothelial cells (HUVEC; blue; stained with CellTrace Far Red) in reconstituted basement membrane above a bottom layer of WS-12Ti human breast tumor-associated fibroblasts (magenta; transduced with Lenti-YFP) in interstitial collagen I (see also Fig. 10.3A). At 2 days of coculture as illustrated here, the SUM102 cells cluster around the branching networks formed by the HUVECs. Degradation products of DQ-collagen IV (green) are apparent around the interacting cells. The fibroblasts, which are primarily in the layer of collagen I, are associated with pericellular degradation products of DQ-collagen I (white). A few fibroblasts, also associated with proteolysis, can be seen to have migrated into the upper layer. The three panels from left to right are 3D reconstructions of the MAME tripartite coculture from the top, the bottom and at a 45° angle. Magnification, 20×.
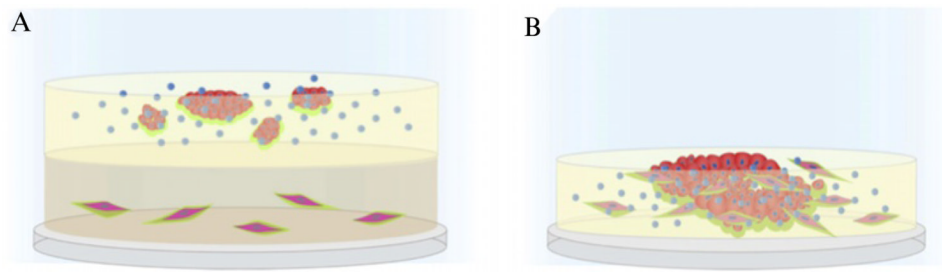
Figure 10.3.
Schematic of MAME tripartite and mixed cocultures of tumor cells, fibroblasts, and macrophages. (A) Coverslips are coated with collagen I containing DQ-collagen I™ and fibroblasts (elongated red cells). A second layer of rBM containing DQ-collagen IV™ is added and tumor cells (round red cells) plated on top along with macrophages (blue). The cultures are then overlaid with a third layer of 2% rBM, which also is included in subsequent changes of media. (B) Coverslips are coated with rBM containing DQ-collagen IV™ and a mixture of fibroblasts, tumor cells, and macrophages plated on top. Cocultures are imaged live to follow changes in morphogenesis and collagen degradation, depicted here in green.