Review

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The multifaceted roles of tumor-associated proteases and harnessing their activity for prodrug activation

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Abstract: The role of proteases in cancer was originally thought to be limited to the breakdown of basement membranes and extracellular matrix (ECM), thereby promoting cancer cell invasion into surrounding normal tissues. It is now well understood that proteases play a much more complicated role in all stages of cancer progression and that not only tumor cells, but also stromal cells are an important source of proteases in the tumor microenvironment. Among all the proteolytic enzymes potentially associated with cancer, some proteases have taken on heightened importance due to their significant up-regulation and ability to participate at multiple stages of cancer progression and metastasis. In this review, we discuss some of the advances in understanding of the roles of several key proteases from different classes in the development and progression of cancer and the potential to leverage their upregulated activity for the development of novel targeted treatment strategies.

Keywords: cancer; prodrug; protease; proteolysis; tumor microenvironment.

Introduction: upregulated protease activity is a hallmark of cancer

Progression, invasion and metastasis of cancer result from several interdependent processes in which proteolytic enzymes are implicated (Liotta and Kohn, 2001; Mason and Joyce, 2011; Dudani et al., 2018), and it has been recognized for many years that protease activity is required for maintenance of the transformed phenotype (Turk, 2006). The role of proteases in cancer was originally thought to be limited to the breakdown of basement membranes and extracellular matrix (ECM), thereby promoting cancer cell invasion into surrounding normal tissues. It is now well understood that proteases play a much more complicated role in all stages of cancer progression and that not only tumor cells, but also stromal cells are an important source of proteases in the tumor microenvironment (Figure 1) (Sevenich and Joyce, 2014). The complexity of proteolytic systems is impressive, as evidenced by the finding that more than 500 genes encoding proteases or protease-like proteins are present in the human genome (Puente et al., 2003) with multiple nodes of interaction (Mason and Joyce, 2011). However, among all the proteolytic enzymes potentially associated with cancer, some proteases have taken on heightened importance due to their significant up-regulation and ability to participate at multiple stages of cancer progression and metastasis. Several novel technologies have been developed that leverage these proteases to make more effective approaches for tumor diagnosis and treatment (Dudani et al., 2018).

Protease activity is tightly regulated

In contrast to post-translational modifications such as phosphorylation, proteolysis is an irreversible event that is regulated by multiple mechanisms (Figure 2). Most proteases are translated as inactive precursors called zymogens that require the removal of a pro-domain for enzymatic activity. In addition, proteases and their cognate inhibitors are often spatially co-localized, setting up a competitive process between the generation of an active protease and inhibition of proteolytic activity (Figure 2) (Turk, 2006). These regulatory mechanisms suggest that proteolytic activity in healthy tissue is transient and a

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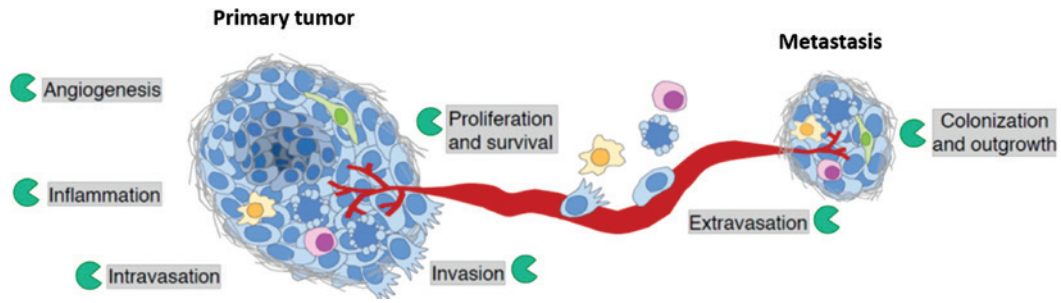

Figure 1: Schematic overview of the rate-limiting steps during primary tumor growth and metastasis that are regulated by pericellular proteases.
Adapted with permission from Sevenich and Joyce (2014).

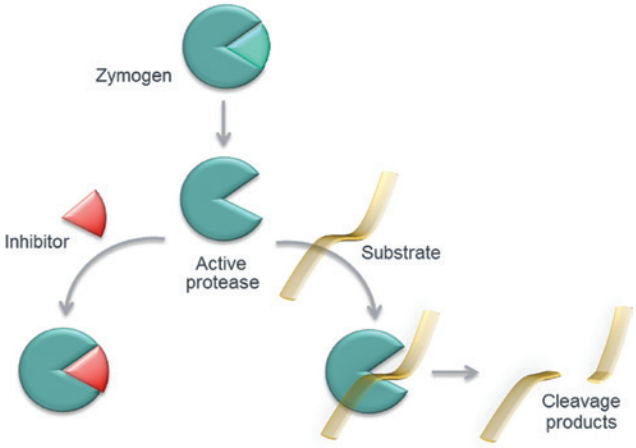

Figure 2: Proteases are highly regulated on the protein level. Expressed as inactive precursors called zymogens, proteases are then activated by multiple mechanisms. Once active, substrate and inhibitor compete for protease binding, and the outcome is defined by the local concentration of inhibitor.

short duration of activity is sufficient to mediate a biological outcome. In contrast, protease activity in tumors is thought to be constitutive, reflecting the underlying requirement of proteolysis to maintain the transformed state. The development of tools to image protease activity *in vivo* in preclinical experiments and more recently in clinical studies has supported this model (Whitley et al., 2016; Yim et al., 2018). For example, *ex vivo* fluorescence imaging of resected soft tissue sarcoma tissue and *in vivo* imaging of breast cancer patients intravenously injected with a probe that is cleavable by tumor-associated protease activity showed that the fluorescence from the tumor was significantly higher than the fluorescence from normal tissue (Whitley et al., 2016). Taken together, these data suggest a model in which proteolytic activity is constitutive in tumors and transient in healthy tissue.

The proteolytic enzymes in the tumor microenvironment implicated in tumor progression and metastasis belong to four major catalytic classes: (1) serine proteases (Murray et al., 2016; Mahmood et al., 2018), (2) cysteine-type lysosomal proteases (Turk et al., 2012; Olson and Joyce, 2015), (3) aspartyl-type lysosomal proteases (Sevenich and Joyce, 2014) and (4) metalloproteinases [soluble and integral membrane matrix metalloproteinases (MMP), adamalysin-related disintegrin and metalloproteinases (ADAMS), and bone morphogenetic protein-1-type proteases] (Kessenbrock et al., 2010; Vadon-Le Goff et al., 2015; Mullooly et al., 2016). Several example proteases are discussed further to highlight the potential of harnessing upregulated tumor-associated protease activity for prodrug activation.

Urokinase plasminogen activator (uPA) in cancer

Urokinase-type plasminogen activator (uPA) is a serine endopeptidase (clan PA, family S1) that is extracellularly localized and involved in the plasminogen activator system. Cleavage of plasminogen by uPA produces active plasmin, which leads to degradation of fibrin and extra-cellular matrix (ECM) as well as activation of growth factors; additionally, uPA can directly activate procollagenase. Plasmin can also degrade the ECM indirectly through activation of numerous matrix metalloproteinase zymogens (Blasi and Carmeliet, 2002). Thus, using a combination of these pathways, uPA regulates tumor progression and metastasis (Mahmood et al., 2018).

In the past two decades, uPA has increasingly been shown to play a role in cancer and is linked with disease progression and a negative outcome in patients (Mahmood et al., 2018). High levels of uPA in many cancer types, including colorectal and breast cancer, have been shown

to be indicative of more aggressive tumors and reduced overall survival (OS) rates. Notably, there is abundant evidence of uPA playing a role in breast cancer, summarized in a review of 18 independent datasets containing a total of 8377 patients (Look et al., 2002). High levels of uPA were found to be indicative of poor OS and poor relapse-free survival. In lymph node-negative patients, high uPA levels are strongly prognostic and correlate with poor disease-free survival (Duffy et al., 1998; Janicke et al., 2001; Look et al., 2002). These data, combined with the growing body of literature regarding the mechanisms by which uPA contributes to cancer progression, has led to evaluation of uPA levels as selection criteria to determine suitability for adjuvant treatment regimes in breast cancer (Harbeck et al., 2004; Duffy et al., 2014). uPA expression and activity has been shown to be directly up-regulated by JAG1/Notch in breast cancer cell lines. These results correlate well with a survey of expression in primary carcinomas of the breast (Shimizu et al., 2011).

In colorectal cancer, high uPA is found in carcinomas compared to either adenomatous polyps or normal mucosa, and these high uPA levels correlate with low OS (Skelly et al., 1997). High levels of uPA in primary colorectal tumors have also been shown to positively correlate with distant metastases and negatively correlate with patient OS (Yang et al., 2000). Increases in uPA expression, protein levels and activity have also been confirmed in seminomas compared to normal tissue (Ulisse et al., 2010).

Recently, imaging of uPA activity in prostate cancer xenograft models has been achieved using a recombinant uPA antibody (U33) labeled with near-infrared fluorophores or radionuclides (Figure 3) (LeBeau et al., 2015). U33 preferentially binds to the active form of the protease and can therefore be used for the detection of protease activity in animal models. In addition, inhibition of uPA in numerous cell lines has been shown to reduce tumor cell invasion (Ossowski et al., 1991; Ertongur et al., 2004). For example, inhibition of uPA activity in CT-26 murine colorectal carcinoma cells completely abolished the increased invasion caused by cytokine stimulation *in vitro* and almost completely ameliorated both LPS- and surgery-induced metastatic tumor growth compared to controls *in vivo* (Killeen et al., 2007). Inhibition of uPA in a rat breast cancer xenograft model was shown to decrease the ability of the tumor to proliferate and invade surrounding tissue; this effect was more pronounced in combination with tamoxifen (Xing et al., 1997). Interestingly, uPA/uPAR have also been shown to be upregulated in tamoxifen-resistant breast cancer cell lines (LeBeau et al., 2013a, 2014).

Inhibiting the binding of uPA to uPAR suppresses angiogenesis, migration and tumor growth both *in vitro* and *in vivo* (Crowley et al., 1993; Min et al., 1996). Antagonistic recombinant antibodies for uPAR were developed and found to inhibit non-small cell lung cancer (NSCLC) invasion by blocking the uPA-uPAR interaction (Duriseti et al., 2010). These uPAR-targeted antibodies, both as a monotherapy and coupled to ^{177}Lu, inhibited tumor growth in a xenograft model of triple-negative breast cancer (LeBeau et al., 2013a). A uPAR-targeted multimodal tracer for pre- and intraoperative imaging in cancer surgery was recently developed and tested in pre-clinical models (Boonstra et al., 2015). Furthermore, clinical PET imaging of uPAR using a peptide-based positron-emission tomography (PET) imaging ligand was performed in patients with breast, prostate and bladder cancer (Persson et al., 2015).

Together, these studies confirm that uPA up-regulation and activity in cancer correlates with increased rates of tumor invasion, metastasis and ultimately, poor survival.

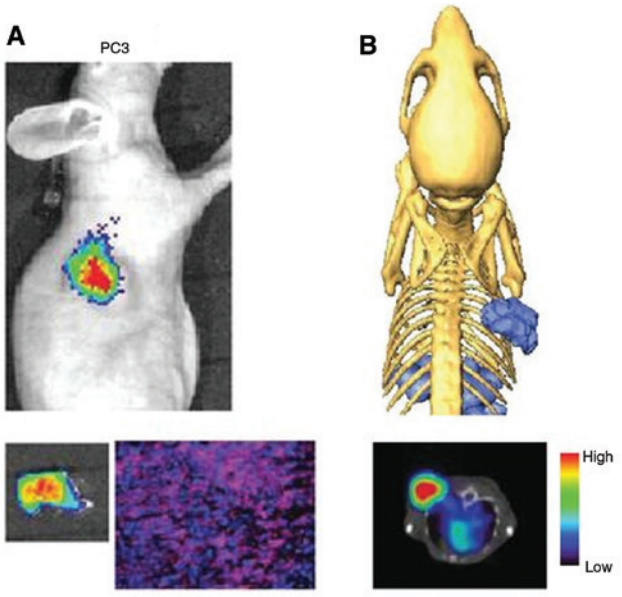

Figure 3: Visualization of uPA activity in a prostate cancer xenograft model with optical and SPECT/CT imaging using a recombinant uPA antibody (U33) that can detect uPA activity.
(A) U33 tumor labeling is detected *in vivo* with NIR optical imaging and *ex vivo* (a, lower panel) in an excised tumor and tumor section.
(B) SPECT/CT imaging demonstrates tumor update of ^{111}In-labeled U33 in the PC3 model with a reconstructed transverse view (b, lower panel) also provided. Adapted with permission from LeBeau et al. (2015).

Serine protease MT-SP1 is proteolytically active in tumors

Type II transmembrane serine proteases (TTSPs) are a family of cell surface proteases that contain an

extracellular C-terminal serine protease domain and are divided into four subfamilies: (1) HAT/DESC, (2) hepsin/TMPRSS, (3) matriptase, and (4) corin. mRNA expression of most family members has been determined in normal human tissue and changes in gene expression of several TTSP members have been documented in human tumors (Szabo and Bugge, 2008; Murray et al., 2016).

MT-SP1, a member of the matriptase subfamily, has a strong link to cancer. Skin-specific overexpression of MT-SP1 causes spontaneous epithelial malignancies in a transgenic mouse model (List et al., 2005). Moreover, impairment of MT-SP1 reduces tumor cell growth and invasion through the decreased activation of hepatocyte growth factor (pro-HGF) and pro-uPA (Suzuki et al., 2004; Forbs et al., 2005). Recently, reduced MT-SP1 expression in a transgenic mouse model was shown to display attenuated mammary tumor growth that was linked to a down-regulation of the HGF/c-Met signaling pathway (Zoratti et al., 2015).

MT-SP1 mRNA expression is strictly epithelial with widespread but not ubiquitous expression in normal tissue (Bhatt et al., 2003). Mouse studies have shown an essential role for MT-SP1 in the oral epithelium, epidermis, hair follicles, and thymic epithelium (List et al., 2002, 2003, 2006b). Expression of MT-SP1 has also been documented in a number of different cancers (List et al., 2006a). As such, a survey of MT-SP1 expression in breast cancer cell lines and 107 primary breast tumors identified a correlation between MT-SP1 expression and HER2 levels (Welman et al., 2012). The cognate inhibitor for MT-SP1, HAI-1, is an important regulator of protease activity. For example, MT-SP1 in normal human skin is in complex with HA1-1, whereas in squamous cell carcinomas, MT-SP1 is in its unassociated form (Bocheva et al., 2009), suggesting that its proteolytic activity might be up-regulated in these cancers. MT-SP1 is also inhibited by anti-thrombin, a plasma inhibitor that is also found in the interstitial matrix of tissues. MT-SP1 is inhibited endogenously by anti-thrombin in normal epithelial cell lines but not in cancer cell lines (Darragh et al., 2010).

Some of the strongest data for up-regulated MT-SP1 activity leverages an antibody called A11 that is both a potent inhibitor of MT-SP1 activity ($K_i \sim$ pM) and can discriminate between the proteolytically active and inactive protease (Darragh et al., 2010). This active site-specific antibody also can be used for staining of active protease in formalin-fixed paraffin-embedded (FFPE) tissue sections. In one study, the A11 antibody did not detect active matriptase in healthy colon tissue, but positive staining for active matriptase was evident in primary and metastatic cancer tissue cores (Figure 4) (LeBeau et al., 2013b). Furthermore, A11 was recently used to demonstrate MT-SP1-specific activation of an EGFR-targeting antibody prodrug *in vivo* in an H292 xenograft model (Wong et al., 2016).

Cysteine cathepsins in cancer

Lysosomal cysteine proteases (cathepsins) belong to the family of papain-like proteolytic enzymes (clan CA, family C1) principally localized intracellularly in the endosomal/lysosomal compartment. Seven of these proteases – cathepsins B (*CTSB*), C (dipeptidyl peptidase I), F, H, L, O and X/Z/P – exhibit ubiquitous but differential expression in mammalian tissues, whereas other papain-like cysteine proteases (i.e. cathepsins J, K, S, V and W) are only expressed by specific cell types (Rawlings and Barrett, 2000; Deussing et al.,

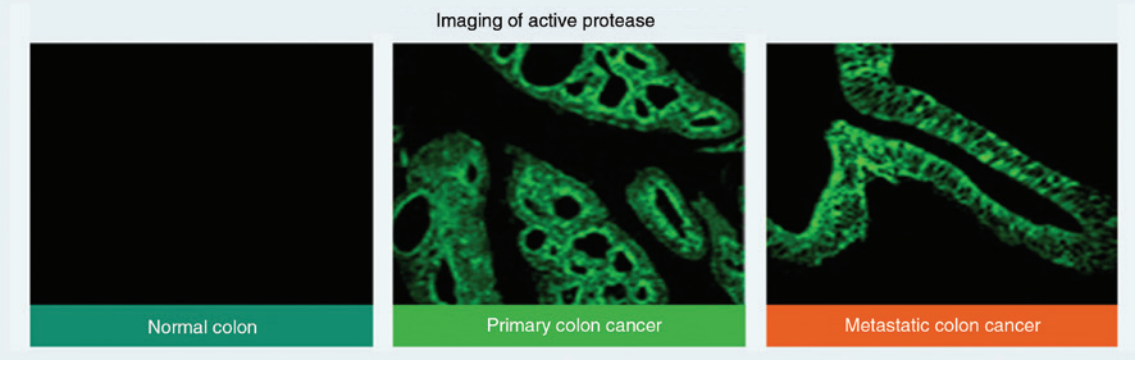

Figure 4: Visualization of MT-SP1 activity in healthy and malignant colon tissue using immunofluorescence staining with a recombinant MT-SP1 antibody (A11) that can detect MT-SP1 activity.
No active matriptase was detected in healthy colon, whereas positive staining for active matriptase was found in a stage II (T3N1M0) primary colon cancer section and a hepatic metastasis section. Adapted with permission from LeBeau et al. (2013b).

2002). Traditionally, lysosomal cysteine proteases are considered to execute nonspecific protein degradation within the lysosome at acidic pH (Barrett, 1992). Yet, there is growing evidence for specific functions of these enzymes and for their function in the extracellular space (Vasiljeva et al., 2007). Elevated cathepsin expression and/or activity has been shown to be associated with cancer progression in many different tumor types (Gabrijelcic et al., 1992; Hirano and Takeuchi, 1994; Kos et al., 1995; Khan et al., 1998; Fernandez et al., 2001; Talieri et al., 2004; Gocheva and Joyce, 2007) and supported by numerous studies in mouse cancer models (Mohamed and Sloane, 2006; Gocheva and Joyce, 2007; Vasiljeva and Turk, 2008). Moreover, the level of cathepsin expression has been found to positively correlate with a poor prognosis for cancer patients and has been suggested to be a prognostic marker (Campo et al., 1994; Lah et al., 2000; Scorilas et al., 2002).

Among the lysosomal cysteine proteases, cathepsin B is the most abundant and the most thoroughly studied. Increased extracellular levels of cathepsin B have been reported in human colorectal, liver and lung cancer cells (Maciewicz et al., 1989; Heidtmann et al., 1997; Koblinski et al., 2002). Notably, the secretion of cathepsin B was shown to occur from cells that do not exhibit an increase in mRNA levels, indicating that its secretion is likely due to altered intracellular trafficking and distribution (Frosch et al., 1999). Another indication that tumor cells secrete cathepsin B is the increased serum level of this protease in patients with hepatocellular and ovarian carcinomas, prostate cancer and melanoma (Gabrijelcic et al., 1992; Kos et al., 1997; Leto et al., 1997; Kos et al., 1998; Miyake et al., 2004; Herszenyi et al., 2008). Cathepsin B has also been found in other body fluids surrounding tumors, such as bronchoalveolar lavage fluid of lung cancer patients or cerebrospinal fluid from patients with leptomeningeal metastasis (Luthgens et al., 1993; Nagai et al., 2003). In addition, the up-regulation of cysteine cathepsins by other cells of the tumor microenvironment, such as macrophages, has been reported (Mohamed and Sloane, 2006; Vasiljeva et al., 2006; Gocheva et al., 2010; Mikhaylov et al., 2011; Akkari et al., 2016).

In addition to being secreted, cathepsin B has been found to be plasma membrane-associated in numerous cancer cell lines (Rozhin et al., 1994), and cathepsin B activity is evident on the surface of living cells in culture (Linebaugh et al., 1999). One mechanism of cathepsin B association with the cell surface is through an interaction with annexin II heterotetramers, directing cathepsin B to caveolae (Cavallo-Medved et al., 2003), which harbor a wide variety of other interdependent proteases (e.g. membrane-bound matrix metalloproteases and serine proteases).

Once localized in the extracellular space, active cathepsins have been shown to be able to degrade the protein components of basement membranes and the interstitial connective matrix including laminin, fibronectin, elastin, tenascin, E-cadherin and various types of collagen (Creemers et al., 1998; Mai et al., 2002). Cathepsin B also indirectly enhances proteolysis by activating precursors of serine proteases and matrix metalloproteases, including pro-uPA, plasminogen or pro-MMPs, to their active forms (Kobayashi et al., 1991, 1993) (Figure 5). In addition, other tumor-associated cysteine cathepsins, cathepsins L and S, have been shown to act as 'sheddases,' cleaving the extracellular domains of adhesion molecules and transmembrane receptors from the surface of cancer cells (Sobotic et al., 2015).

Leveraging the selective extracellular presence of cysteine cathepsins in the tumor microenvironment, several approaches for imaging agents and drug delivery systems targeting cathepsins have been developed recently. Many of them employ various nano-carriers, such as liposomes, nanofibers, ankyrin repeat proteins (DARPins), polyglutamate and dendrimer nanoparticles functionalized with cathepsin binding moieties or

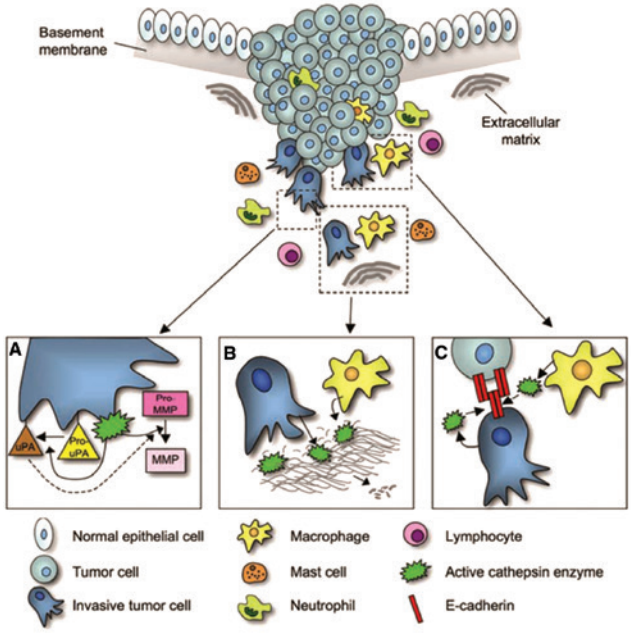

Figure 5: Role of extracellular cathepsins in cancer progression and invasion.
Cathepsin activity mediates (A) proteolytic cascade activation, (B) degradation of the ECM, and (C) inactivation of cell adhesion proteins. Adapted with permission from Gocheva and Joyce (2007).

cleavable substrates (Mikhaylov et al., 2014; Ben-Nun et al., 2015, 2017; Jeong et al., 2015; Kramer et al., 2017a,b).

Together, these data provide strong evidence that the 'lysosomal' cysteine proteases are secreted or translocated to the extracellular milieu in cancer to fulfill specific functions and thus, may be utilized for the targeted delivery of diagnostic and/or therapeutic compounds.

Legumain in cancer

Legumain is a cysteine endopeptidase (clan CD, family C13) and the only asparaginyl endopeptidase in mammals. In normal tissues, legumain is primarily a lysosomal protease, as with cathepsins. However, in cancer, legumain is localized to the cell surface and in membrane-associated vesicles concentrated along the invadopodia of tumor cells (Liu et al., 2003a). Analogous to cathepsins, legumain is synthesized as a pro-enzyme that undergoes autoactivation in the acidic conditions found in the lysosome or tumor microenvironment.

Human legumain was cloned in 1997 (Chen et al., 1997) and shown to process antigens for MHC class II presentation (Manoury et al., 1998; Watts et al., 2005). Expression in normal tissue is highest in the kidney with detectable staining in the liver and spleen. Legumain is expressed in a wide variety of solid tumors, including 100% of breast and 95% of colon tumors tested in one study (Table 1) (Liu et al., 2003a). Legumain staining in IHC also may have prognostic value in breast cancer patients (Gawenda et al., 2007). In colorectal tumors, legumain has been correlated with a poor prognosis (Murthy et al., 2005). Notably, knock-down of legumain in mouse models of cancer resulted in a marked decrease in tumor growth and metastasis (Luo et al., 2006). The differential expression of legumain between tumor and normal tissue was exploited initially in the protease-activated pro-drug legubicin (Liu et al., 2003a) and subsequently in protease-activated etoposide- (Stern et al., 2009) and auristatin-derived prodrugs (Bajjuri et al., 2011), with all three approaches demonstrating efficacy in tumor models.

These studies confirm upregulation of legumain in cancer and support the role of legumain in disease progression. Extracellular localization of legumain, coupled with activation in the acidic conditions of the tumor microenvironment, suggests that its proteolytic activity can be leveraged for therapeutic targeting.

Matrix metalloproteinases (MMPs) in cancer

MMPs are a family of zinc-dependent endopeptidases that play a crucial role in various physiological processes including tissue remodeling and organ development (Page-McCaw et al., 2003), regulation of inflammatory processes (Parks et al., 2004) and in diseases such as cancer (Egeblad and Werb, 2002). The 23 MMPs expressed in humans are categorized by their architectural features. Closely related to the MMPs are the so-called ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) families of metzincin proteinases. ADAMs are membrane-anchored proteases and fulfill a broad spectrum of functions with roles in fertilization, development and cancer (Berndt et al., 2008). The roles of the other metzincin proteinases in cancer have also been extensively reviewed (Murphy, 2008).

MMPs and ADAMs have been found to be upregulated in numerous types of cancer. Moreover, although tumor cells from various tissues can express members of the MMP and ADAM families, the major source of these proteases appears to be from stromal cells infiltrating the tumor (Egeblad and Werb, 2002). Different types of stromal cells produce a specific set of proteases and inhibitors

Table 1: Legumain detection in human tumors (Liu et al., 2003a).

Carcinoma type	Number analyzed	Number positive	Percentage positive	Degree of positivity
Breast carcinoma	43	43	100%	+++
Colon carcinoma	34	32	95%	+++
Lung carcinoma	24	14	58%	+++
Prostate carcinoma	56	42	75%	++++
Ovarian carcinoma	23	17	73%	++
CNS tumors	8	8	100%	++
Lymphoma	14	8	57%	+
Melanoma	12	5	41%	+

(Kessenbrock et al., 2010), which are released into and alter the tumor microenvironment. The cellular source of MMPs can therefore have important consequences on their function and activity (Ardi et al., 2007).

MMPs were initially viewed primarily as regulators of tissue destruction or remodeling but are now well-recognized players in numerous steps of tumor progression and metastasis (Szarvas et al., 2011) and are therefore considered to be multifunctional proteases (Coussens et al., 2002; Egeblad and Werb, 2002; Freije et al., 2003; Hojilla et al., 2003; Decock et al., 2011). The characterization of new MMP substrates as well as the generation of genetically modified animal models with gain or loss of MMP function have been used to demonstrate the relevance of MMP activities in the early stages of cancer development. Proteolytic processing of bioactive molecules by MMPs contributes to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer. For example, growth-factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors are some examples of the diversity of substrates targeted by MMPs (Egeblad and Werb, 2002; Hojilla et al., 2003; Folgueras et al., 2004). In particular, it has become clear that MMPs contribute to angiogenesis by more than just degrading matrix components. They are capable of processing a large array of extracellular and cell-surface regulatory proteins and therefore, contribute both in the onset and in the maintenance of angiogenesis (Berndt et al., 2008).

Numerous protease-activated prodrugs, nanotechnology-based drug delivery systems, gene delivery systems and imaging systems have been engineered by taking advantage of tumor-associated MMPs. For example, several different MMP-activated imaging agents have been developed and successfully validated (McIntyre et al., 2004; Shi et al., 2006; Zhang et al., 2006). Recently, nanoparticle sensors equipped with an MMP9 substrate have been used to non-invasively detect tumor-associated MMP activity in mouse models of ovarian and liver cancer through the release of a fluorescent reporter group in the urine (Kwon et al., 2017).

Although extracellular proteolysis is widely implicated in cancer promotion, MMPs and other proteases exhibit tumor-suppressing effects in several circumstances (Folgueras et al., 2004). These observations, together with the identification of novel anti-tumorigenic functions of MMPs in numerous steps of tumor progression and invasion, might partly explain why broad-spectrum MMP inhibitors failed in phase III clinical trials and have made necessary a reformulation of MMP inhibition strategies for cancer treatment. Whereas multiple protease inhibition strategies have been largely disappointing for cancer therapy, approaches exploiting upregulated proteases for tumor-targeting therapies have demonstrated potential. Among these approaches, the prodrug-based strategy is one of the most extensively researched in the last decade.

Protease-activatable prodrugs

The increased activity of proteases in cancer, coupled with the tight regulation of protease activity in normal tissues, creates an opportunity to design novel protease-activatable therapeutics in which effective therapy is selectively delivered to tumors while minimizing drug toxicity to normal tissues (Choi et al., 2012; Weidle et al., 2014). One of the most common strategies for protease-activatable prodrug design is to conjugate protease-cleavable peptide substrates to chemotherapeutic compounds. This approach has been applied to multiple classes of proteases known to be upregulated in the tumor microenvironment, such as cathepsins (Baurain et al., 1980; Ueki et al., 2013; Zhong et al., 2013), plasmin (Barthel et al., 2012; Chakravarty et al., 1983), matrix metalloproteases (Albright et al., 2005), and legumain (Liu et al., 2003a; Stern et al., 2009), and has resulted in increased efficacy and reduced off-tissue drug accumulation and toxicity *in vivo*.

To create protease-activatable prodrugs with improved pharmacokinetic properties, numerous drug delivery systems have been explored, including serum albumin (Trouet et al., 1982; Mansour et al., 2003), hydrogels (West and Hubbell, 1999; Lutolf et al., 2003), liposomes (Kondo et al., 2004), nanoparticles (Gu et al., 2013; van Rijt et al., 2015; Xu et al., 2015) and other synthetic polymers (Li et al., 1998; Chau et al., 2004). As an example, PK1 (FCE 28068), which consists of N-(2-hydroxypropyl) methacrylamide (HPMAcp) polymer conjugated to doxorubicin via a cathepsin B activation sequence, was found to elicit improved doxorubicin exposure and efficacy compared to doxorubicin alone (Loadman et al., 1999). PK1 and polymers containing cathepsin B or prostate-specific antigen (PSA) activation sequences have been evaluated in the clinic and showed signals of activity and reduced toxicity (Seymour et al., 2009). Paclitaxel poliglumex (PPX), a macromolecular drug conjugate that links paclitaxel with a biodegradable polymer, poly-L-glutamic acid, was designed to release paclitaxel from the polymeric backbone by cathepsin B proteolysis (Chipman et al., 2006). A phase III clinical trial comparing PPX vs docetaxel in the second-line treatment of NSCLC has shown that while PPX and docetaxel produced similar survival results, different

toxicity profiles were observed between the two treatment groups (Chipman et al., 2006).

In addition to small molecules, protease-activated prodrugs have also been designed using cytotoxic proteins or cytotoxic receptor ligands. For example, *in vivo* feasibility has been demonstrated using engineered anthrax toxin that requires uPA and MMP activation for toxin assembly (Liu et al., 2003b, 2005). Recently, initial clinical safety has been demonstrated with protease-activatable cell-penetrating peptides (Unkart et al., 2017). Tumor necrosis factor and CD95L prodrugs have also been developed to induce apoptotic pathways upon uPA and MMP proteolysis (Gerspach et al., 2006; Watermann et al., 2007).

Incorporation of tumor-targeting moieties into protease-activatable prodrug formats is used to enhance tumor-specific activation (Choi et al., 2012). Among the strategies evaluated, antibody-based targeting of drugs (i.e. antibody drug conjugates) has been the most successful to date. The FDA-approved anti-CD30 antibody-drug conjugate (ADC) ADCETRIS® (Brentuximab vedotin or SGN-35) contains a cathepsin-cleavable valine-citrulline linker that utilizes the activity of lysosomal proteases for aurostatin payload release intracellularly (Katz et al., 2011). Non-internalizing, protease-activatable ADCs are also being evaluated for extracellular payload release through the activity of cathepsins in the tumor microenvironment (Gebleux et al., 2017).

A unique approach of targeting therapeutic antibodies has been developed by CytomX Therapeutics by engineering protease-activatable monoclonal antibody prodrugs called Probody™ therapeutics (Desnoyers et al., 2013). The Probody technology utilizes a peptide mask that is attached to the antibody through a protease-cleavable linker and limits the ability of the antibody to bind to its target. The linker can be cleaved in the tumor microenvironment by protease(s) preferentially active in cancer tissue, leading to removal of the mask and target engagement by the released antibody (Desnoyers et al., 2013; Wong et al., 2016; Lin and Sagert, 2018). In this way, Probody therapeutics have the potential to minimize systemic on-target toxicity while maximizing anti-tumor activity. Notably, this platform has been shown to be compatible with multiple antibody-based therapeutic modalities, including naked antibodies to immune targets, antibody drug conjugates, bispecific T cell-engaging antibodies and CAR-based cellular therapy (Weaver et al., 2015; Tipton et al., 2016; Spira et al., 2017; Boustany et al., 2018).

Taken together, proteases clearly play an important and complex role at different stages of tumor progression and metastasis. Harnessing upregulated protease activity in the tumor microenvironment has emerged as a promising and powerful approach for cancer detection and treatment. The use of protease-targeted and protease-activatable prodrug technologies can enable tumor microenvironment-specific therapeutic activity, thereby reducing on-target toxicity in normal tissues and improving the therapeutic index. Continued efforts to uncover upregulated protease activities during tumorigenesis is expected to further expand the diagnostic and therapeutic potential of protease-guided technologies and promote the development of next-generation cancer therapeutics.

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