Chapter 15
Proteases in Cancer: Significance for Invasion and Metastasis

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15.1 Introduction

There is an extensive body of literature documenting the association of proteases with cancer. Indeed, a search of PubMed for the phrase "proteases in cancer" brings up a list of ~73,000 papers, including >7,200 reviews. Nonetheless, the protease community still has not identified and validated all of the proteases and proteolytic pathways that play causal roles in neoplastic progression, nor determined which proteases would be appropriate therapeutic targets in pre-malignant lesions as compared to end-stage cancers or in any one type of cancer. Furthermore, more than one catalytic type of protease has been implicated in the progression of human tumors, as have interactions among proteases of more than one catalytic type. How vast a repertoire of proteases has been implicated in cancer is evident from a

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comprehensive tome on the many proteases that comprise the cancer degradome (Edwards et al. 2008b).

The initial working hypothesis for those studying proteases in cancer was that invasive processes (local and during metastatic spread) require degradation of extracellular matrices by proteases. The roles of proteases in cancer are now known to be much broader, as will be discussed here. Furthermore, the proteases themselves derive not only from tumor cells, but also from other cells that make up the tumor microenvironment, e.g., fibroblasts, macrophages, mast cells, neutrophils and endothelial cells. A critical factor to remember when considering whether a protease plays a causal role in malignant progression is that a purified protease capable of cleaving a protein substrate *in vitro* may not be the protease or the only protease responsible for degradation of that substrate *in vivo*. Transgenic mice deficient in specific proteases have helped elucidate the *in vivo* functions of proteases, but have also confirmed that there is redundancy and compensation. This along with the large number of proteases in the human genome, the interplay of proteases with one another as well as with their endogenous inhibitors and activators and the complexity of their biological roles [for review, see (Doucet and Overall 2008; Ordonez et al. 2009; Puente et al. 2003; Rawlings et al. 2010)] suggest that we should employ multiple technologies to identify proteases and proteolytic pathways that are associated with the processes of tumor initiation and progression (Fig. 15.1) and that may be targets for therapeutic intervention.

15.2 Metalloproteases

Metalloproteases are a heterogeneous group of proteolytic enzymes that use a metal ion to polarize water and hydrolyze proteins. The MA clan, the largest clan of metalloproteases, binds zinc using a HExxH motif. The ADAM and ADAMTS proteases are included in the adamalysin subfamily of metzincins within the MA clan, and the MMPs are in the matrixin subfamily. These three metalloprotease families have been implicated in a number of human pathologies; their various roles in cancer development and progression are summarized below.

15.2.1 Matrix Metalloproteases

15.2.1.1 The MMP Family

The 23 MMPs expressed in humans all contain the minimal domains of a signal sequence that directs protein secretion, a pro-domain to maintain latency, and a Zn-containing catalytic domain. Two of the MMP family members (MMP-7,26) contain only the minimal domains and the rest possess additional domains that facilitate substrate or inhibitor binding such as the hemopexin and fibronectin-like domains, and/or domains that anchor the enzyme to the cell surface such as the

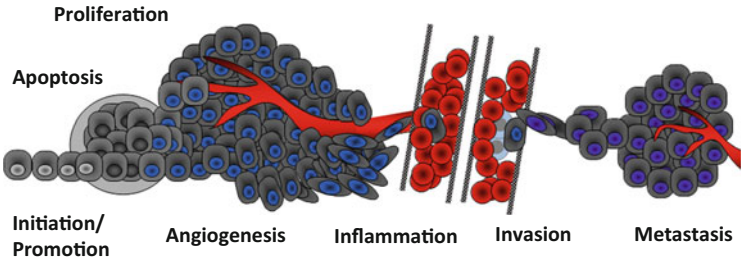

Fig. 15.1 Steps in tumor development, from initiation to metastasis, in which proteases have been shown to participate

transmembrane or GPI anchor domains [see (Brinckerhoff and Matrisian 2002) for review].

"Matrix" metalloproteinases are so named because the prototypic family member, MMP-1/collagenase-1, was initially purified as the enzyme responsible for degradation of fibrillar collagen in the tadpole tail [see (Brinckerhoff and Matrisian 2002) for review]. Several MMP family members possess the rare ability to cleave collagen in its native triple helical structure and are collectively referred to as collagenases (MMP-1,8,13, and the membrane-type MMP-14). The stromelysins (MMP-3,7,10) are MMP family members with greater activity against proteoglycans and glycoproteins such as laminin and fibronectin; gelatinases (MMP-2,9) cleave basement membrane and denatured collagens efficiently; and MMP-12/metalloelastase is particularly potent against elastin. As a result of their combined ability to degrade virtually all components of the extracellular matrix, MMPs have been implicated in diseases characterized by extensive matrix destruction, which includes basement membrane dissolution as benign tumors progress to malignant, invasive cancers.

15.2.1.2 MMPs in Cancer

The enzymatic activities described by Liotta in 1980 as being associated with tumor metastasis (Liotta et al. 1980) were eventually ascribed to MMP-2/gelatinase A and MMP-9/gelatinase B. The ease of identifying these two MMPs, in particular by gelatin zymography, resulted in an abundant literature documenting their expression in cancerous tissues and their association with cancer invasion and metastasis. Our view of the role of MMPs in cancer has, however, greatly expanded over the past decade and is no longer restricted to invasive activity as a result of gelatinase-mediated matrix degradation. Virtually all MMP family members have been detected in tumor tissue, and a causal association for at least half of them has been determined by genetic manipulation of MMP levels. In Fig. 15.2 we illustrate the association of some MMPs with various stages of tumor development. MMP substrates have expanded to include growth factors, cytokines, cell surface receptors and adhesion molecules, and the biological activity of these factors can

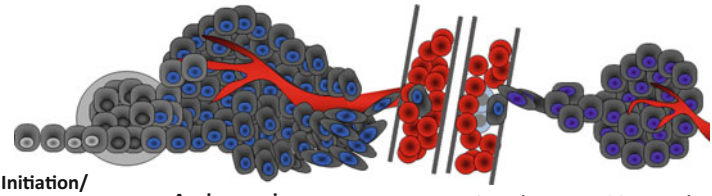

Fig. 15.2 Matrix metalloproteinases in cancer: representative examples of family members demonstrated by *in vivo* experimentation to play roles in the indicated stages of tumor progression

be linked to all stages of cancer progression. MMP cleavage can either activate or inactivate these substrates, influencing fundamental activities of proliferation, apoptosis, angiogenesis and inflammation in addition to invasion and metastasis. The role of MMPs in cancer is discussed in detail in several excellent recent reviews (Fingleton 2006; Kessenbrock et al. 2010), and there is a comprehensive review of biologically relevant MMP substrates (Cauwe et al. 2007).

MMPs in tumor initiation and/or promotion: The earliest steps of tumorigenesis are classically defined as tumor initiation, a mutational event, which is followed by tumor promotion, a process that selects and expands the population of mutated cells. A number of experiments using genetic manipulation of specific MMPs in mouse models of cancer have linked MMPs to these early steps of carcinogenesis, although in general the two processes of initiation and promotion have not been dissected.

The development of benign papillomas and malignant squamous cell carcinomas in the skin of mice treated with a single dose of the carcinogen DMBA (7,12-dimethylbenzanthracene, a tumor initiator) followed by multiple treatments with the phorbol ester tumor promoter TPA (12-0-tetradecanoylphorbal-13-acetate) is a classic model of multistage carcinogenesis. The overexpression of MMP-1 (collagenase-1) in the skin of transgenic mice produces hyperplastic lesions and increases the number of DMBA/TPA-induced tumors (D'Armiento et al. 1995). Using the viral oncogenes of the human papilloma virus HPV16 as initiator and promoter, the ablation of the gelatinase MMP-9 decreases tumor incidence in the skin, but interestingly increases the progression of the resulting tumors to metastasis (Coussens et al. 2000). Mice null for MMP-19, a soluble MMP with potent activity again basement membrane and cartilage, show decreased sensitivity to methylcholanthrene-induced skin fibrosarcomas (Pendas et al. 2004).

In contrast to the documented pro-tumorigenic effects of MMPs-1, 9, and 19, several MMPs have been shown to have protective effects in skin

carcinogenesis models. Genetic ablation of MMP-8 (collagenase-2) increases the number of tumors in male mice undergoing a DMBA/TPA skin carcinogenesis regimen (Balbin et al. 2003), a result that contrasts with the promoting effect of MMP-1 and indicates that, although MMP-1 and MMP-8 are both collagenases, they have opposing effects on early stages of skin tumorigenesis. Genetic ablation of MMP-3 (stromelysin-1) also revealed a protective effect of this MMP on skin carcinogenesis with an increase in tumor growth rate in both the DMBA/TPA model and enhanced growth and progression in mice treated repeatedly with the complete carcinogen MNNG (1-methyl-3-nitrosoguanidine) (McCawley et al. 2004). The protective effect of MMP-3 in skin carcinogenesis was confirmed in transgenic mice overexpressing MMP-3 in skin keratinocytes; these mice demonstrate increased keratinocyte differentiation and decreased carcinogen-induced tumor multiplicity (McCawley et al. 2008). Collectively, these observations reinforce the notion that MMPs are much more sophisticated than simple "bulldozers" that destroy matrix and can act as signaling molecules with specific downstream effects that either enhance or retard individual steps of tumor progression.

Early stages of colorectal cancer are commonly initiated by the loss of function of the APC gene, an event that is recapitulated in the Multiple Intestinal Neoplasia (Min) mouse model of intestinal tumorigenesis. Using genetically deficient mice, a tumor promoting role for MMP-7 (matrilysin) and MMP-9, but not MMP-2, 12, or 19, was identified in the development of Min adenomas (Sinnamon et al. 2008; Wilson et al. 1997). Treatment with the carcinogen azoxymethane followed by the inflammatory agent dextran sodium sulfate results in colon cancer that is associated with colitis. In contrast to its role in Min-mediated colon cancer, MMP-9 plays a suppressive role in colitis-associated colon cancer; MMP9-null mice demonstrate a significant increase in tumor number and size (Garg et al. 2010). This result is surprising in that there are well known pro-inflammatory effects of MMP-9 activity (see below). The effect was traced to MMP-9 cleavage of Notch-1 in the epithelial cells of the intestine and the modulation of cellular apoptosis.

The contributions of MMPs to prostate tumor progression were examined in transgenic mice expressing SV40 large T antigen targeted to prostatic neuroendocrine cells (Littlepage et al. 2010). The results from experiments using mice deficient in the gelatinase MMP-2 suggest an effect of this MMP on prostate cancer cell growth, angiogenesis, and metastasis to the lung. In contrast, mice deficient for MMP-7 and MMP-9 do not exhibit effects on primary tumor growth, although a reduction in angiogenesis is observed in both cases, and MMP-9-null mice also demonstrate reduced invasive foci and perivascular invasion.

The role of MMPs in mammary carcinogenesis has been particularly well studied. The overexpression of both MMP-3 and the membrane-type collagenase MMP-14 in the mammary gland is sufficient to induce mammary adenocarcinoma, suggesting both initiating and promoting roles for these MMPs (Ha et al. 2001; Sternlicht et al. 1999). MMP-7 may play a similar role, although in this study the hyperplastic lesions that develop do not progress to frank adenocarcinoma (Rudolph-Owen et al. 1998). The activity of MMP-3 that results in tumor initiation was traced to the generation of cellular reactive oxygen species that induce DNA

damage and genomic instability (Radisky et al. 2005). In MMTV-Wnt1-driven mammary carcinogenesis, inhibition of MMP activity by the overexpression of a natural MMP inhibitor, TIMP-2, influences both initiation and promotion as measured by the number of tumors and their growth rate; this effect can be attributed to MMPs-2, 3, 9, 13, and/or 14 (Blavier et al. 2006). In MMTV-*neu*-induced carcinogenesis, the overexpression of MMP-7 increases the number and growth rate of mammary tumors (Rudolph-Owen et al. 1998). Despite the effects of MMP overexpression on mammary tumor progression, genetic ablation of MMP-3 or MMP-7 in MMTV-polyoma middle T-induced mammary tumors has no effect on primary tumor development, suggesting that the effect of these MMPs on initiation and promotion are relatively modest and their expression is not required for the activity of a strong oncogene (Martin et al. 2008). Similarly, genetic ablation of MMP-9 and 13 has no effect on the development of MMTV-polyoma middle T-induced primary mammary tumors (Martin et al. 2008; Nielsen et al. 2008). The effect of MMPs on mammary tumors that develop following initiation with the carcinogen DMBA has also been examined. Ablation of MMP-11 (stromelysin-3) from stromal fibroblasts decreases DMBA-induced mammary tumor formation (Masson et al. 1998). The overexpression of MMP-3 in the mammary gland has been reported to decrease tumor incidence following DMBA treatment, a result that challenges the tumor promoting activity of MMP-3, yet may be explained by the elimination of initiated cells through elevated cellular turnover (Witty et al. 1995).

There is abundant evidence that MMPs can alter cellular proliferation and apoptosis, both key components of the process of tumor promotion. The mechanism underlying this activity is often, but not exclusively, associated with the "sheddase" function of MMPs and the release of growth- and apoptosis-factors from the cell surface. The role of MMPs in cell proliferation and apoptosis and the relevant substrates has been extensively reviewed (Fingleton 2006; Kessenbrock et al. 2010). The anti-promoting effect of MMPs can also be related to cell autonomous-effects, but has been particularly associated with modulation of inflammation as a result of either proteolytic processing or cleavage-mediated inactivation of inflammatory cytokines. These functions of MMPs are discussed in more detail below.

15.2.1.3 MMPs and Angiogenesis

The ability of tumor cells to stimulate the production of new blood vessels from existing local vessels or bone marrow progenitor cells is an adaptive mechanism that facilitates tumor growth and provides an avenue for tumor cell dissemination. Angiogenesis is influenced by MMPs through a wide variety of mechanisms. Perhaps the most obvious is facilitating endothelial cell invasion and migration, an activity that has been ascribed to MMP-2 and requires its association with the integrin alphaVbeta3 since inhibitors of this interaction disrupt angiogenesis in the chick chorioallantoic membrane (Brooks et al. 1998). MMP-2-null mice show reduced angiogenesis and reduced lung and melanoma tumor growth (Itoh

et al. 1998). An important mechanism is modulating the availability and signaling of angiogenic factors. MMP-9, but not MMP-2, null mice are impaired in their ability to vascularize tumors in the pancreatic islets induced by the SV40 T-antigen (Bergers et al. 2000). In this model, MMP-9 increases the bioavailability of Vascular Endothelial Cell Growth Factor (VEGF), presumably by releasing it from matrix stores (Bergers et al. 2000), although MMP-9 can also directly cleave VEGF to remove domains responsible for matrix binding (Lee et al. 2005). The basic fibroblast growth factor 2 pathway is activated by neutrophil MMP-9 and induces angiogenesis in a collagen implant assay (Ardi et al. 2009). MMP-9 is also essential for vasculogenesis—the generation of tumor vessels from bone marrow precursors. MMP-9-null mice lack bone marrow-derived endothelial cells in neuroblastoma-associated blood vessels (Jodele et al. 2005), and MMP-9-positive myeloid cells can restore vasculogenesis in tumors transplanted into irradiated tissues that are incapable of angiogenesis (Ahn and Brown 2008). Bone marrow-derived Myeloid-Derived Suppressor Cells (MDSCs) lose their tumor-promoting function and fail to differentiate into tumor-associated endothelial cells in the absence of MMP-9 (Yang et al. 2004). In addition, the recruitment of pericytes and subsequent maturation of tumor blood vessels in a neuroblastoma model is dependent on MMP-9 (Chantrain et al. 2004). Thus, MMP effects on angiogenesis include increasing the concentration of pro-angiogenic factors, delivering signals to critical bone marrow cells, facilitating endothelial cell invasion, and regulating pericyte function to influence vascular stability and permeability.

There are also anti-angiogenic properties of MMPs, in particular related to their ability to generate small inhibitory peptides from higher molecular weight precursors. Angiostatin, a potent suppressor of angiogenesis, is generated from plasminogen most efficiently by MMP-12 (Cornelius et al. 1998), an activity that explains the protective effect of MMP-12 in mouse models of lung cancer (Acuff et al. 2006; Houghton et al. 2006). MMP-9-mediated generation of angiostatin also influences lung tumor growth (Chen et al. 2005). MMP-9-deficient mice can also demonstrate a surprising acceleration in tumor growth, a result that can be attributed to the cleavage of the alpha3 chain of IV collagen to generate tumstatin (Hamano et al. 2003). Endostatin is generated by cleavage of type XVIII collagen by MMPs-3, 7, 9, 13, and 20 (Cauwe et al. 2007; Heljasvaara et al. 2005). Thus, the angiogenic activity of MMPs is balanced by the release of anti-angiogenic factors, an effect that is likely to be part of a normal homeostatic mechanism.

15.2.1.4 MMPs and Inflammation

The multifaceted role of the innate immune response and inflammation in cancer initiation, promotion, and progression has been increasingly apparent (Grivennikov et al. 2010). There are now many examples of MMP effects in *in vivo* models that are mediated by modulation of the inflammatory process [reviewed in (Fingleton 2006; Kessenbrock et al. 2010; Parks et al. 2004)]. The complexity of MMP actions on the inflammatory response is layered upon the complexity of the effect of

inflammation on cancer, so that both pro- and anti-inflammatory activities of MMPs have been documented. For example, in a transgenic mouse study MMP-12 expression directed to lung epithelial cells results in increased inflammatory cell infiltration, emphysema, and the development of bronchioalveolar adenocarcinoma (Qu et al. 2009). MMP-12 is also upregulated in human chronic obstructive pulmonary disease and lung cancer, and is speculated to play a critical role in the transition from emphysema to lung cancer. In contrast, in Helicobacter pylori-induced gastric cancer, the expression of MMP-7 in the gastric epithelial cells has a protective effect on H. pylori-induced inflammation. MMP-7-null mice demonstrate an increase in Th1- and Th17-mediated inflammation and accelerated epithelial cell turnover mediated by increases in both proliferation and apoptosis (Ogden et al. 2010). Similarly, and as discussed previously, MMP-3 and MMP-8 have protective effects in skin carcinogenesis, and in both cases this effect correlates with a reduction in inflammation (Balbin et al. 2003; McCawley et al. 2004). MMP-8 has been implicated in the resolution of acute inflammation, and the loss of MMP-8 results in chronic inflammation that may contribute to its tumor suppressive effects (Gutierrez-Fernandez et al. 2007). Interestingly, loss-of-function mutations in MMP-8 have been reported in melanoma, suggesting that the inability to resolve inflammation may also contribute to melanoma development (Palavalli et al. 2009).

The mechanisms underlying the effects of MMPs on inflammation and innate immunity are diverse, but one that stands out is the influence of MMPs on chemokine gradients that attract inflammatory cells. For example, the CCL monocyte-attracting chemokines, CCL2, -7, -8, and -13, are cleaved by MMPs-1 and -3, which converts them into receptor antagonists and reduces monocyte chemotaxis and the inflammatory phenotype (McQuibban et al. 2002). MMPs-8, -9, and -12 similarly cleave CXCL11, a T lymphocyte chemotactic factor. This first inactivates CXCL11, then reverses the inactivation, but removes the domain responsible for matrix binding and alters its ability to generate a haptotactic gradient (Cox et al. 2008). MMP-7 modulates the neutrophil chemoattractant CXCL1 indirectly through the cleavage of syndecan-1 from cell surfaces, releasing chemotactic CXCL1/syndecan-1 complexes (Li et al. 2002). Many other inflammatory cytokines and chemokines, including Tumor Necrosis Factor-alpha and CXCL12/SDF-1, are processed by MMPs, resulting in a wide range of effects on inflammatory cell infiltration and biological activities [see (Cauwe et al. 2007; Parks et al. 2004) for review].

15.2.1.5 MMPs in Cancer Invasion

Invasion, the migration of tumor cells from a central cluster into surrounding tissue and into lymphatic or blood vessels can occur either at a single cell or multicellular level. The latter, referred to as collective invasion, is dependent on the destruction of fibrillar collagen fibers (Friedl and Wolf 2008). Thus, MMPs with the specialized ability to cleave fibrillar collagens, making distinct ¾ and ¼ fragments in collagen

chains and unraveling the fibrillar structure, are likely to contribute to this process. These include MMP-1, 8, 13, and 14, but of these MMP-14 is implicated as the rate-limiting step in invasion through collagen-containing ECM (Sabeh et al. 2004). In contrast to the other three collagenases, MMP-14 is membrane anchored and (along with the transmembrane serine proteinase seprase) is a critical component of invadopodia, actin-rich protrusions that focalize matrix-degrading activity to cell: substratum contact points [(Weaver 2006) for review]. An additional well-known function of MMP-14 is to activate the secreted gelatinase MMP-2. MMP-2 and MMP-9 are also found associated with invadopodia, perhaps functioning to facilitate further degradation of the denatured collagen/gelatin fragments.

The role of macrophages in assisting tumor cells in invading into surrounding tissue has been elegantly demonstrated by Pollard, Condeelis, and colleagues [(Qian and Pollard 2010) for review]. Macrophages produce a number of proteases that have been associated with their ability to invade tissues, and thus are candidates for facilitating the invasive activity of macrophage-mediated tumor cell invasion. Of the MMPs, MMP-2 and MMP-9 produced by immature myeloid cells are required for the collective invasion of sheets of tumor cells in a mouse model of colon cancer (Kitamura et al. 2007). These same enzymes have been implicated in macrophage infiltration in a model of autoimmune disease (Agrawal et al. 2006) and may thus represent a general mechanism for inflammatory cell infiltration.

15.2.1.6 MMPs in Metastasis

Based on the original concept of MMP activity in cancer, there is extensive documentation in experimental metastasis assays of a role for MMP activity following genetic manipulation or pharmacological inhibition. Since many of these studies were performed in immunodeficient mice, there is reason to focus on authochthonous or syngeneic mouse models of cancer metastasis so the pleiotropic effects of MMPs on the immune system are considered in this multistep process. The MMTV-polyoma middle T model of mammary tumorigenesis has revealed a role for MMP-9 and -14 in spontaneous metastasis to the lung, but no effect of MMPs-3,7, or 13 (Martin et al. 2008; Nielsen et al. 2008; Szabova et al. 2008). MMP-14 deficiency does not eliminate the development of hyperplastic lesions in bigenic mice and in fact seems to enhance their growth following transplantation into cleared mammary fat pads of recipient animals. MMP-14-null glands, however, display a markedly reduced ability to metastasize to the lungs (Szabova et al. 2008). MMP-9 deficiency has no effect on the development of primary tumors, but markedly inhibits lung tumor burden. This effect is only observed in C57Bl/6 mice and not in FVB/N mice, a dependency on genetic background that was confirmed with a small molecule MMP-9 inhibitor (Martin et al. 2008). In the SV40-large T antigen model of prostate cancer, MMP-2, but not MMP-7 or 9, deficiency reduces metastasis to the lung (Littlepage et al. 2010).

The effect of ablating the stromal production of several MMP family members on metastasis has been examined in syngeneic models of experimental metastasis,

which examine primarily the ability of the cells to establish lesions in distant organs. The injection of syngeneic melanoma or lung cells into MMP-2- (Itoh et al. 1998) and MMP-9- (Itoh et al. 1999) null mice results in reduced metastatic foci in the lung following intravenous injection. Syngeneic colon cancer cells injected into MMP11-deficient mice reduce the number of lung foci, but paradoxically favor their growth (Brasse et al. 2010). The injection of syngeneic breast cancer cells into the tibia of MMP-7 mice greatly reduces their ability to establish lesions in the bone microenvironment (Thiolloy et al. 2009). MMP-2 ablation has a similar effect on reducing the establishment and growth of bone lesions, although stromal MMP-9 ablation has no effect on this process (Thiolloy et al. 2009; Thiolloy et al. 2012).

Tumor cells can stimulate bone marrow cells to populate distant organs and prepare a pre-metastatic niche that fosters the establishment and growth of tumor cells in that organ. The gelatinase MMP-9 plays an important role in the formation of this niche. MMP-9 ablation destroys the pre-metastatic niche (Kaplan et al. 2005), an effect likely be attributed to MMP-9 releasing soluble Kit-ligand (Heissig et al. 2002) and/or VEGF (Bergers et al. 2000). There is evidence that it is the disruption of vessels in the metastatic organ that leads to the enhanced intravasation of both bone marrow-derived cells and circulating tumor cells. The stromelysins MMP-3 and MMP-10 play an important role in disrupting blood vessel integrity, and targeted downregulation of these MMPs reduces vessel permeability, reduces infiltration of myeloid cells, and reduces the ability of human breast cancer cells implanted in the mammary fat pad to spontaneously metastasize to the lung (Huang et al. 2009). The production of MMP-2 by myeloid cells that are recruited to the pre-metastatic niche results in collagen cleavage that, in combination with the effect of the collagen crosslinker lysyl oxidase, enhances the invasion and recruitment of additional bone marrow derived cells and metastasizing tumor cells (Erler et al. 2009). Thus, MMPs do contribute to tumor metastasis, but the mechanisms behind this effect are much more diverse than originally envisioned.

15.2.2 ADAM and ADAM-TS Proteases

The ADAM and ADAM-TS families of metalloproteinases are so-named for their multiple domains: A Disintegrin And Metalloprotease (ADAM) with Thrombospondin motif (ADAM-TS). Despite similar structure, proteolytic activity is absent in some family members. These family members are thought to be especially important in cell adhesion events such as sperm-egg fusion (Klein and Bischoff 2011). Altogether in man, there are 22 ADAMs, 12 of which have protease activity, and 19 ADAM-TS proteins. Proteolytically active members of both families have critical physiological roles in diverse processes such as shedding/solubilization of membrane proteins (‘sheddase’ activity), matrix protein processing and degradation and control of coagulation factor activation. Unlike the closely related MMPs, genetic deficiency of several individual ADAMs or

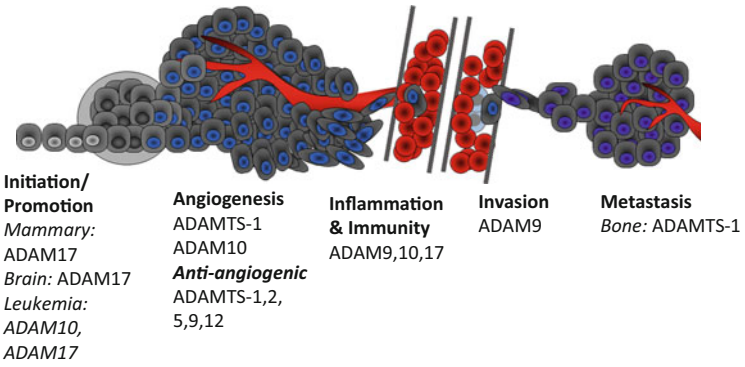

Fig. 15.3 ADAMs and ADAM-TSs in cancer: representative examples of family members demonstrated by *in vitro* and *in vivo* experimentation to play roles in the indicated stages of tumor development and progression

ADAM-TSs in animals or humans has resulted in severe phenotypes including embryonic lethality, underlying their essential roles in normal homeostasis. The reader is referred to several recent articles for an overview of these families of proteases (Apte 2009; Edwards et al. 2008a; Klein and Bischoff 2011). Here, we discuss specific activities that relate to cancer development, progression, treatment or detection. Representative examples are illustrated in Fig. 15.3. More extensive discussion relating to some of these activities can be found in several comprehensive reviews (Edwards et al. 2008a; Klein and Bischoff 2011; Murphy 2008; Turner et al. 2009; Wagstaff et al. 2011).

ADAMs and ADAM-TSs in initiation, promotion and growth of tumors: ADAM-17, also known as TACE or tumor necrosis factor alpha converting enzyme, is a widely expressed sheddase responsible for the regulated cleavage of several important signaling molecules such as TNF-a, L-selectin and various epidermal growth factor (EGF) ligands (Blobel 2005; Edwards et al. 2008a). Studies from the laboratory of Mina Bissell have indicated that increased ADAM17 activity is sufficient to provide an autocrine oncogenic stimulus in a 3D breast cancer model, without mutation of any proto-oncogene (Kenny and Bissell 2007). In these cells, the primary substrate for ADAM17 responsible for tumorigenesis is transforming growth factor alpha, an EGF receptor ligand. A cortical astrocyte cell line, originally developed from normal brain, also can be converted to a malignant tumor line by overexpression of ADAM17 (Katakowski et al. 2009). Again, generation of active EGF receptor ligand is the identified mechanism.

Notch signaling is a critical pathway for development of multiple cell lineages. Interaction of the Notch receptor with ligand (members of the Jagged or Delta families) leads to a confirmation change and subsequent exposure of proteolytic cleavage sites. ADAMs-10 and -17 are both Notch1 cleaving enzymes; however, ligand-induced N1 signaling requires ADAM-10, whereas ligand independent N1 signaling requires ADAM-17 (Bozkulak and Weinmaster 2009). In several tumor types, particularly leukemias, mutations in Notch are associated with disease. Many

such mutations result in constitutive exposure of the S2 ADAM cleavage site thereby removing the requirement of ligand binding for Notch activity (van Tetering et al. 2009) and both ADAM-10 and -17 participate in signaling in leukemias (Bozkulak and Weinmaster 2009). Overexpression of ADAM10 in B cells has a similar result of excessive Notch signaling and a block in B cell differentiation (Gibb et al. 2011).

15.2.2.1 ADAMs and ADAM-TSs in Angiogenesis

Several ADAM-TS proteases have been identified as regulators of tumor angiogenesis. Much of this regulation is negative, likely due to the presence of the thrombospondin motifs, since thrombospondins are known angiogenesis inhibitors (Ribatti 2009). Although originally identified as an angiogenesis inhibitor (Iruela-Arispe et al. 2003), the activity of ADAM-TS1 is more complex as it appears to enhance tumor vasculature development in a fibrosarcoma model. Rather than through classical angiogenesis, this occurs by promotion of tumor cell plasticity toward an endothelial phenotype resulting in a vascular mimicry phenomenon (Casal et al. 2010). ADAM-TS1 has also been shown to lead to accelerated tumor growth *in vivo* through induction of a profound stromal response (Rocks et al. 2008). Recruitment of activated fibroblasts can then enhance classical angiogenesis. ADAM-TS12-null mice, which appear normal and viable, allow the accelerated growth of implanted tumors as compared to wildtype counterparts (El Hour et al. 2010). This is related to enhanced vascularity of the tumors. In complementary *in vitro* assays, addition of ADAM-TS12 to aortic rings suppresses angiogenic outgrowth. As might be expected, this anti-angiogenic function of ADAMTS-12 is independent of its proteolytic activity. Similarly, ADAM-TS2 shows anti-angiogenic activity that is independent of proteolysis, and possibly mediated through binding to the cell surface receptor nucleolin (Dubail et al. 2010). In ADAM-TS5, the anti-angiogenic property has been mapped specifically to only the first of the thrombospondin-like domains (Sharghi-Namini et al. 2008). ADAM-TS9 has been implicated as an anti-angiogenic molecule in head and neck cancers (Lo et al. 2010). Here, the antiangiogenic activity appears related to reduced generation of the pro-angiogenic factors VEGF and MMP9.

ADAM10, in addition to its major role in Notch signaling, is also a sheddase for several other cell surface proteins. One of these, osteoactivin (also known as GPNMB), is highly expressed in triple negative breast cancers, a subtype of breast cancer that is associated with shorter median times to relapse and death. In an effort to determine whether osteoactivin is related to the aggressiveness of this tumor type, Rose et al. (2010) showed that vessel density within tumors is significantly associated with higher osteoactivin levels, that ADAM10 is also expressed and can cleave osteoactivin and finally, that the shed protein can promote endothelial migration.

15.2.2.2 ADAMs and ADAM-TSs in Invasion and Metastasis

ADAM9 expression has been linked to increased invasive and metastatic activity in many cancers. This is a protein with splice variants that appear to have opposite effects in some tumor types. ADAM9-S is a secreted version that can promote migration of breast cancer cells in a manner dependent on its proteolytic activity (Fry and Toker 2010). On the other hand ADAM9-L is a transmembrane protein that suppresses migration of breast cancer cells independent of its proteolytic activity (Fry and Toker 2010). ADAM9 appears to be regulated by oxidative stress, with increased activity resulting in increased invasiveness under conditions of higher stress (Mongaret et al. 2011). One way in which ADAM9 can promote invasion is through increasing the levels of other proteases, e.g., MMPs. Such a mechanism has been reported in melanoma, where ADAM9 mediates binding between tumor cells and adjacent stromal fibroblasts with resulting up-regulation of MMPs-1 and 2. Blocking ADAM9 is sufficient to inhibit invasion in this system (Zigrino et al. 2011).

ADAM-TS1 is one of the molecules that appears to regulate osteolytic bone-metastasis in breast cancer (Lu et al. 2009). The mechanism is related to release of epidermal growth factor receptor (EGFR) ligands such as amphiregulin and HB-EGF from tumor cells that then bind to receptors on osteoblasts, activating a signaling pathway that downregulates osteoprotegerin production and subsequent enhanced osteoclast differentiation.

15.2.2.3 ADAMs and ADAM-TSs in Inflammation and Immunity

The sheddase activity of ADAM proteases is an important regulator of immune system function. As discussed previously, overexpression of ADAM10 in hematopoietic cells can lead to elevated Notch signaling and decreased B-cell development. A further consequence is a switch of lineage from lymphoid to myeloid with a particular increase in the myeloid derived suppressor cell (MDSC) group (Gibb et al. 2011). As MDSCs are considered important drivers of the tumor-associated immunosuppressive phenotype, this consequence of increased ADAM10 could be significantly beneficial for tumor development. Further amplifying this effect is expression of another ADAM, ADAM17, by MDSCs, which enhances tumor immune evasion through shedding of L-selectin from T cells (Hanson et al. 2009). Without cell surface L-selectin, neither helper (CD4+) nor cytotoxic (CD8+) T cells home to lymph nodes for activation or to tumors. Natural killer (NK) cells represent an arm of the immune system that has profound anti-tumor effects, but which is frequently suppressed in cancer. They respond to one of a limited number of surface antigens frequently found on tumor cells, one example of which is MHC class I-related chain A (MICA). In two different studies, Kohga et al. have found that both ADAM9 and ADAM10 can shed MICA from hepato-cellular carcinoma cells, thus preventing NK-mediated clearance (Kohga

et al. 2009, 2010). These authors found that two different anti-cancer drugs, epirubicin and sorafenib, inhibit ADAM10 and ADAM9-mediated MICA shedding, respectively. They thus suggest that a secondary effect of these agents is the boosting of NK cell anti-tumor activity.

In contrast to the aforementioned roles in promoting immune evasion, ADAM17 may also be beneficial for stimulating anti-tumor immunity. In breast, ovarian and prostate cancers, ADAM17 on tumor cells can be processed and presented as an HLA A2 restricted epitope that is not present on normal cells (Sinnathamby et al. 2011). This offers the possibility of ADAM17 as a suitable antigen for a cancer vaccine.

15.2.2.4 ADAMs and ADAM-TSs as Markers or Therapeutic Targets in Cancer

As with many of the other proteases discussed in this chapter, there is a large body of literature that describes correlations between levels of particular ADAM or ADAM-TS proteases and cancer progression. Overviews of these studies can be found in recent excellent review articles (Edwards et al. 2008a; Murphy 2008; Turner et al. 2009; Wagstaff et al. 2011). One particularly exciting study from the laboratory of Marsha Moses uses urinary levels of ADAM12 as a predictive marker of breast cancer risk (Pories et al. 2008). As a truly non-invasive test that can be used to detect early stage disease, this has the potential to really impact treatment decisions for patients.

In addition to potential use as biomarkers for assessing cancer presence, progression or response to therapy, ADAMs and ADAM-TS proteases also represent potential targets for therapeutic development. As is evident from the previous paragraphs, these families of proteases have multiple functions, both pro- and anti-tumorigenic. Therefore, as is the case for inhibitors of MMPs, ADAM or ADAM-TS protease inhibitors will require careful testing to ensure no or minimal blocking of beneficial activities. ADAM17 is considered a particularly appealing target in cancer, mainly because of its roles in EGFR ligand processing (Kenny 2007). ADAM10 is also considered a therapeutic target in cancer, largely due to its Notch cleavage ability as well as EGFR processing (Crawford et al. 2009). Several small molecule inhibitors have been developed for these targets (Kenny and Bissell 2007; Ludwig et al. 2005; Murumkar et al. 2010; Zhou et al. 2006). While such drugs have shown some efficacy in pre-clinical models, particularly when combined with EGFR inhibitors (Kenny and Bissell 2007; Witters et al. 2008; Zhou et al. 2006), they suffer from specificity problems due to the similarities in the protease domain across this family of enzymes. Thus, as is happening with MMP inhibitors, a new approach that is being explored is the targeting of other parts of the ADAM or ADAM-TS molecules using antibodies (Tape et al. 2011).

In some cases, protease activity is of benefit for therapeutic efficacy through processing targets of other drugs. A recently reported example is the monoclonal antibody DN30, which targets c-Met, the hepatocyte growth factor (HGF) receptor.

In order to be efficacious, the antibody must bind to the receptor and induce its shedding from the tumor cell, a process that is dependent on ADAM10 activity (Schelter et al. 2010).

15.3 Serine Proteases

There are 175 predicted serine proteases in humans with the vast majority being secreted (Puente et al. 2005). Secreted proteases play essential roles in a multitude of physiological processes, which include digestion, blood clotting, and immunity. Many studies have defined pivotal roles of secreted serine proteases in pathological conditions including inflammation, cancer progression and metastasis. A subgroup of serine proteases is directly anchored to plasma membranes, a finding that substantially expanded the known repertoire of enzymes that execute proteolytic cleavage reactions within the pericellular and extracellular environments. Although the role of the majority of both secreted and membrane serine proteases in tumorigenesis is still unexplored, important information has been garnered on several proteolytic systems and individual members. The association of selected serine proteases with various stages of tumor development is depicted in Fig. 15.4. In the sections below we will focus on particular serine proteases, which have been reported to be critical players in cancer progression and metastasis.

15.3.1 Secreted Serine Proteases

15.3.1.1 The Plasminogen Activation System

The plasminogen activation (PA) system has been extensively studied in cancer and it has been reported that several of the components play an important role, mainly in metastasis. The proteolytic effector enzyme is plasmin, which has broad substrate specificity and is capable of degrading various extracellular matrix proteins including fibrin, fibronectin and laminin. Plasmin can also indirectly promote matrix degradation through the activation of certain pro-MMPs. In addition, plasmin may be involved in functions unrelated to matrix degradation, e.g., in the activation of growth factor precursors such as transforming growth factor beta (TGF-β). Plasmin is produced in the liver and is present at high concentrations in the plasma (~2 μM). Two mammalian plasminogen activators are known, urokinase type-plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Studies on tPA have mainly focused on its role in thrombolysis whereas the function of uPA has been thoroughly studied in both cancer progression and fibrinolysis. uPA is inhibited by two specific plasminogen activator inhibitors, PAI-1 and PAI-2 belonging to the serpin family of serine protease inhibitors. uPA binds to a cell

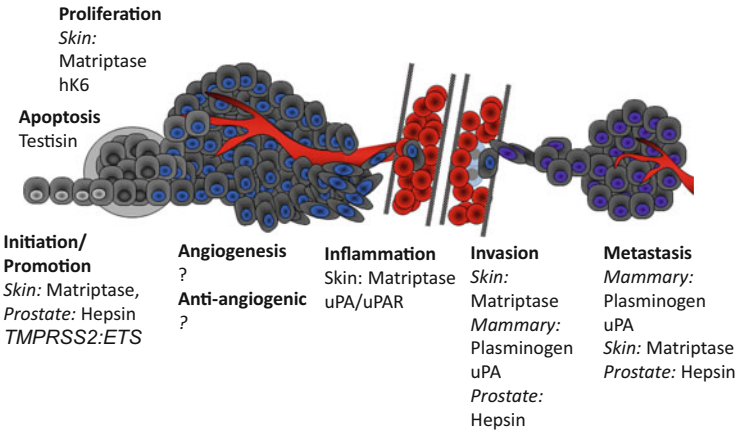

Fig. 15.4 Serine proteases in cancer: representative examples of family members demonstrated by *in vitro* and *in vivo* experimentation to play roles in the indicated stages of tumor progression

surface-bound receptor, uPAR, and the bound form induces plasminogen activation much more rapidly than does fluid-phase uPA (Ellis et al. 1989).

uPA, uPAR and PAI-1 are consistently expressed in the invasive areas of a broad range of carcinomas. As a result, these proteins have been studied in detail for their potential as prognostic markers. uPA was the first protease shown to be a prognostic marker in human malignancy. Duffy et al. showed that patients with breast tumors containing high levels of uPA enzyme activity have a significantly shorter disease-free interval than do patients with low levels (Duffy et al. 1988). Later, high uPA antigen levels were found to correlate with a shortened overall survival in this disease (Duffy 1990). Since then, high levels of uPA, uPAR and PAI-1 have been demonstrated to be predictive of poor patient outcome in many malignancies including cancers of the lung, esophagus, breast, stomach, and colorectum [reviews in (Dass et al. 2008; Duffy 2004; Gandolfo et al. 1996; Han et al. 2005; Schmitt et al. 1997)]. In contrast, high levels of PAI-2 have mainly been associated with decreased tumor growth and metastasis [reviewed in (Croucher et al. 2008)]. Breast cancer is the most frequently studied cancer type in which the prognostic value of PAI-2 expression has been assessed and the majority of studies demonstrate a significant association between PAI-2 expression and prognosis. Specifically, relatively high tumor-associated PAI-2 expression is linked with prolonged survival, decreased metastasis, or decreased tumor size (Croucher et al. 2008). The reason for an apparently paradoxical action of two similar serpins is not fully understood, but it has been proposed that key structural differences control interactions with components of the extracellular matrix and endocytosis–signaling co-receptors.

Carcinomas contain a variety of stromal cells, including fibroblasts, endothelial cells and macrophages, in addition to the malignant epithelial compartment. PA system components are often expressed by stromal cells rather than by epithelial cancer cells, in patterns that are characteristic for each type of cancer (Almholt and

Johnsen 2003; Hewitt and Dano 1996; Johnsen et al. 1998). In ductal mammary carcinoma, uPA is primarily expressed by myofibroblasts, yet is also found in some macrophages and endothelial cells, as well as, in rare instances, in a small subpopulation of cancer cells. PAI-1 is also chiefly expressed by myofibroblasts, whereas uPAR is mainly seen in macrophages (Almholt et al. 2003).

Functional studies using genetic mouse loss-of-function models have provided valuable insights into defining the role of the PA system in cancer progression *in vivo*. Studies of the consequence of uPA deficiency on tumor progression in the MMTV-PymT transgenic breast cancer model revealed that tumor incidence, latency, growth rate and final primary tumor burden are not significantly affected by uPA deficiency. In contrast, metastasis to both lungs and brachial lymph nodes is significantly reduced in uPA null mice as compared to wild-type controls (Almholt et al. 2003). Similar results were observed in plasminogen deficient mice (Bugge et al. 1998) indicating that both uPA and plasminogen are primarily involved in invasion and metastatic dissimination and not in primary tumor growth. In contrast, neither uPAR nor PAI-1 deficiency significantly affects primary tumor growth and lung metastatic burden in the MMTV-PymT model; vascular density is also unaffected by PAI-1 status (Almholt et al. 2003, 2007). Other transgenic cancer models that have been employed to study the function of PA system proteases and inhibitors include the RIP1-Tag2 pancreatic cancer model and the HPV16 skin cancer model. In the RIP1-Tag2 model, induction of angiogenesis and the number of pancreatic islet tumors are not affected by uPA deficiency (Bergers et al. 2000). Recently, it was reported that skin carcinogenesis in K14-transgenic mice deficient for PAI-1 is unimpeded (Masset et al. 2011). In another study focusing on skin, transgenic mice overexpressing either uPA or uPAR in basal epidermis cells and hair follicles have no detectable cutaneous alterations (Zhou et al. 2000). In contrast, bi-transgenic mice overexpressing both uPA and uPAR develop extensive alopecia induced by involution of hair follicles, epidermal thickening and sub-epidermal blisters. The phenotype is due to uPA catalytic activity since combined overexpression of uPAR and uPA that binds to uPAR but is catalytically inactive in the same tissue is not detrimental in another bi-transgenic line (Zhou et al. 2000). These data indicate that combined overexpression of uPA and uPAR acts in synergy to promote pathogenic extracellular proteolysis. It is important to note, however, that no malignant lesions were observed in the bi-transgenice mice.

In sum, there is clear evidence for direct involvement of the PA system, particularly uPA and plasminogen, in cancer invasion and metastasis.

15.3.1.2 Tissue Kallikreins

Human tissue kallikrein (KLK) genes represent the largest contiguous group of proteases within the human genome comprising 15 members. These genes, and their encoded proteins, share a high degree of homology and are expressed in a variety of different tissues. In relation to cancer, the most prominent member of the family is human kallikrein (hK) 3—also known as prostate-specific antigen (PSA).

PSA is the best known cancer biomarker in clinical medicine, being widely used for the early detection as well as management of prostate cancer [review by (Diamandis 1998)]. Several other kallikreins, including hK2 and hK11, are emerging as complementary prostate cancer biomarkers. Along with these kallikreins, several others have been implicated in other types of cancer. For example, hK5, 6, 7, 10, 11, and 14 are emerging biomarkers for ovarian cancer [reviewed in (Paliouras et al. 2007)].

Despite their substantial use in clinical applications, the involvement of the hK family, in the pathophysiology of cancer is just beginning to be understood. Accumulating evidence indicates that the *hK* family is dysregulated in cancer. Numerous studies report aberrant amounts of kallikrein transcripts and/or proteins in cancer cells, particularly adenocarcinomas derived from steroid-hormone-regulated tissues, as compared with their normal, benign and/or pre-malignant tissue counterparts. The most striking example is the concurrent upregulation of 12 *KLK* genes in ovarian carcinoma [reviewed in (Borgono and Diamandis 2004). Recent *in vitro* studies implicate kallikreins in cancer-related processes, including cell-growth regulation, angiogenesis, invasion and metastasis. They have been shown to promote or inhibit neoplastic processes, acting individually on growth factors or growth factor receptors and/or in cascades with other kallikreins and members of other protease families *in vitro* (Borgono and Diamandis 2004; Borgono et al. 2004). For example, several reports indicate that hK2 and hK3 might represent important regulators of the insulin-like growth factor (IGF) axis in prostate carcinogenesis. IGF1 and IGF2 are important mitogenic peptides involved in regulating normal and malignant cellular proliferation, differentiation, apoptosis and transformation. hK4 can activate pro-hK3/PSA and pro-uPA (Takayama et al. 2001) and hK2 can activate pro-uPA and inactivate PAI-1 (Mikolajczyk et al. 1999). Tumor-suppressive actions have been assigned to certain kallikreins. hK3/PSA is able to inhibit the growth of the estrogen-receptor-positive MCF-7 breast cancer cell line by stimulating the conversion of the potent estrogen estradiol to the less potent estrone (Lai et al. 1996). In contrast in prostate cancer, hK3/PSA protein does not affect growth of prostate cancer cells *in vitro* or prostate cancer xenografts *in vivo* (Denmeade et al. 2003).

Only a few studies to date have used genetic animal models to analyze the function of kallikreins in cancer. In a mouse model, both hK3/PSA and hK2 were overexpressed in the prostate by transgenesis. Those double transgenic mice produce more active PSA than single transgenic mice indicating that hK2 is activating the pro-form of hK3/PSA *in vivo*. In a longitudinal evaluation over a 2-year period, no morphologic changes (i.e., no PIN or prostate cancer) can be observed due to hK3/PSA or hK3/PSA plus KLK2 double transgene expression when compared to non-transgenic mice (Williams et al. 2010). Transgenic expression of hK6 in skin keratinocytes of mice causes increased keratinocyte proliferation and migration and decreased levels of E-cadherin protein (Klucky et al. 2007). This observation, combined with the finding that hK6 is up-regulated in squamous skin tumors of human patients, may implicate hK6 in the early events of squamous cell carcinogenesis (Klucky et al. 2007).

Thus far, the *in vivo* role of most kallikreins has not been elucidated. Future studies using loss-of-function or gain-of-function animal models may shed light on their potential roles in tumor growth and metastasis.

15.3.2 Membrane-Anchored Serine Proteases

Over the last decade a novel subgroup of membrane serine proteases has emerged as an important component of the human degradome, garnering significant attention for their roles in tissue homeostasis and in cancer progression and metastasis. The membrane anchored serine proteases are tethered to the cell membrane via three different means: (1) a carboxy terminal glycophosphatidylinositol (GPI) anchor, which is added posttranslationally, (2) a carboxy-terminal transmembrane domain (Type I), or (3) an amino terminal proximal transmembrane domain (Type II transmembrane serine proteases—TTSPs).

The catalytic domain of all membrane-anchored serine proteases belongs to the S1 peptidase family, which includes the prototypic chymotrypsin and trypsin (Rawlings and Barrett 1993). The biochemical properties of the catalytic domain have been studied in great detail [see (Hedstrom 2002; Page and Di Cera 2008) for reviews]. Importantly, all membrane-anchored serine proteases show a strong preference for cleavage after arginine or lysine residues. Membrane-anchored serine proteases are synthesized as catalytically inactive zymogens that are irreversibly converted to active proteases by autocatalytic or heterocatalytic cleavage after an arginine or lysine residue located in a conserved activation motif within the catalytic domain. Regulation of proteolytic activity is often attributed to shedding of the protease from the cell surface upon complex formation with membrane associated or secreted serine protease inhibitors or by internalization followed by degradation within lysosomes [reviewed in (Bugge et al. 2009)].

Although the role of the majority of membrane serine proteases in tumorigenesis is still unexplored, important information has, in recent years, been obtained about several of the members.

15.3.2.1 GPI-Anchored Proteases: Testisin

Testisin (also known as PRSS21) was first described as a candidate tumor suppressor in the testis where it is expressed in premeiotic testicular germ cells but is lost in testicular germ cell tumors (Hooper et al. 1999). Subsequently, it was demonstrated that hypermethylation of the 5′ CpG island of the gene encoding testisin promotes its loss in testicular tumorigenesis (Manton et al. 2005). Testisin expression in normal tissues appears to be highly restricted in both mice and humans and has to date only been detected in testis and in human eosinophils (Hooper et al. 1999, 2000). Interestingly, in a study aiming to identify proteases that were overexpressed in ovarian cancer, the testisin transcript was found to be abundant in ovarian

carcinoma but not detected in the normal ovary. Furthermore, there is a positive correlation between increased testisin mRNA levels and tumor stage (Shigemasa et al. 2000). In cell culture studies, *si*RNA–mediated knockdown of endogenous testisin mRNA and protein expression in an ovarian tumor cell line (CaOv3) leads to increased apoptosis. Conversely, overexpression of testisin in the ovarian cell line SKOV3 causes an increase in subcutaneous tumor growth in severe combined immunodeficient mice (Tang et al. 2005). These data indicate that testisin may be involved in ovarian tumor progression possibly by facilitating anti-apoptotic signals; however, the molecular mechanism and candidate substrates for testisin in this context are yet to be identified.

15.3.2.2 GPI-Anchored Proteases: Prostasin

Prostasin (or PRSS8) is a serine protease with trypsin-like substrate specificity. Prostasin is also known as channel-activating protease (CAP)-1 and is the first of several membrane serine peptidases found to activate the epithelial sodium channel (ENaC) (Vallet et al. 1997). Prostasin was first identified in human seminal fluid, but also is expressed in epithelial cells and ducts of the prostate gland (Yu et al. 1994). Prostasin is also found at lower levels in normal colon, lung, kidney, pancreas, salivary gland, liver, and bronchi (Yu et al. 1994). Later, it was reported that endogenous prostasin is GPI anchored to the cell surface and is secreted from the cell upon cleavage of the GPI anchor by endogenous GPI-specific phospholipase D1 (Chen et al. 2001).

Expression studies in human prostate cancer cell lines and tumors demonstrated that prostasin is expressed in normal human prostate epithelial cells and a non-invasive human prostate cancer cell line, whereas it is not found in invasive human prostate cancer cell lines (Chen et al. 2001). Others found that prostasin mRNA and protein expression is down-regulated in high-grade prostate cancer or hormone-refractory human prostate cancers (Chen et al. 2001; Takahashi et al. 2003). The regulatory mechanisms for prostasin expression and activity are not completely understood. Prostate cell culture experiments indicate that prostasin is regulated at the transcriptional level by DNA methylation and growth factors and at the posttranslational level by the serpin protease nexin-1 (PN-1) (Chen et al. 2004).

Interestingly, similarly to testisin, prostasin is detected at higher levels in ovarian epithelial tumors than in normal ovarian tissue (Costa et al. 2009; Mok et al. 2001). Furthermore, prostasin can be detected in serum from ovarian cancer patients and postoperative prostasin levels are significantly lower than preoperative levels (Mok et al. 2001). These data suggest that prostasin is shed from the cell surface of ovarian cancer cells and may be a candidate serum marker for ovarian cancer.

In bladder cancer, a phenomenon similar to that in prostate cancer is observed. Prostasin is expressed in the normal human urothelium and in a normal human urothelial cell line, but is significantly down-regulated in high-grade transitional

cell carcinoma (TCC) and lost in TCC cell lines. Loss of prostasin expression in the TCC cell lines correlates with loss of or reduced E-cadherin expression, loss of epithelial morphology, and promoter DNA hypermethylation (Chen et al. 2009). In colorectal cancer the mRNA level of prostasin is slightly, but significantly decreased in both mild/moderate dysplasia and severe dysplasia, and in carcinomas as compared to normal tissue from the same individual. The mRNA level of the prostasin inhibitor, PN-1, is more that two-fold elevated in colorectal cancer tissue as compared to healthy individuals and elevated in both mild/moderate dysplasia, severe dysplasia and in colorectal cancer tissue as compared to normal tissue from the same individual (Selzer-Plon et al. 2009). In gastric cancer, prostasin mRNA and protein expression are significantly down-regulated in tumor tissues. In those patients whose tumor expresses prostasin mRNA at reduced levels, a shorter survival is observed (Sakashita et al. 2008). The specific physiologic substrates for prostasin are as yet undefined and the mechanisms whereby the proteolytic activity of prostasin participates in tumor cell biology are unknown. In cell culture based studies, prostasin has, in addition to being involved in ENaC processing, been proposed to mediate proteolytic modification of the epidermal growth factor receptor and regulation of iNOS and cyclin D1 expression by modulating protease-activated receptor-2 signaling (Chen et al. 2008, 2009).

15.3.2.3 Type II Transmembrane Serine Proteases

The type II serine proteases (TTSPs) have garnered significant attention for their roles in epidermal development and homeostasis, iron metabolism and hearing. Defining the role of TTSP's in carcinogenesis and metastasis is still ongoing; however, recent studies have implicated several members of this family as critical players in neoplastic progression. Several TTSPs display aberrant expression in a variety of cancers, often in correlation with poor patient prognosis [reviewed in (Antalis et al. 2011; List et al. 2009; Magee et al. 2001; Netzel-Arnett et al. 2003; Wallrapp et al. 2000; Webb et al. 2011)]. Of particular interest are the proteins hepsin, matriptase and TMPRSS2, which have been linked to epithelial carcinogenesis and metastasis in the prostate and in the skin.

TTSPs: Hepsin

Hepsin/TMPRSS1 is the first TTSP to be cloned and was named based on its original identification in hepatocytes and hepatoma (Leytus et al. 1988; Tsuji et al. 1991). Hepsin has been implicated in oncogenesis due to its overexpression in several human cancers including renal cell carcinoma, hepatocellular carcinoma, breast cancer, endometrial cancer and ovarian cancer. Hepsin has been most extensively studied in prostate cancer where its over-expression is among the most consistent biomarkers for malignant transformation from benign prostate hyperplasia. Additionally, high hepsin expression correlates with a high Gleason

score for prostate tumors and poor clinical outcome with relapse following prostatectomy (Saleem et al. 2006; Stamey et al. 2001; Stephan et al. 2004; Wu and Parry 2007).

The role of hepsin in prostate cancer has been studied experimentally using both genetic mouse models and cell culture-based systems. Transgenic overexpression of hepsin in the prostate epithelium of mice causes disorganization of the basement membrane, promotion of primary prostate cancer progression and metastasis to distant organs (Klezovitch et al. 2004). Interestingly, the prostate of transgenic mice does not display increased cell proliferation, indicating that hepsin may affect cancer cell migration, invasion and metastasis more than tumor growth. In support of this hypothesis, antibodies neutralizing the protease activity of hepsin do not impact cell growth, but do inhibit invasion by prostate and ovarian tumor cells in culture (Xuan et al. 2006). Hepsin may be involved in direct degradation of extracellular matrix proteins and/or activation of other proteases and growth factors or growth factor receptors. Several candidate substrates have been identified including laminin-322 and the pro-forms of uPA and HGF. Nonetheless, the physiologically relevant substrates are, as yet, not known (Herter et al. 2005; Moran et al. 2006; Tripathi et al. 2008).

TTSPs: Matriptase

This protease is among the most extensively studied TTSP with more than 100 published papers characterizing various aspects of matriptase and its role in cancer, including expression profiles, gene-regulation, regulation by cognate inhibitors, identification of proteolytic substrates, and *in vivo* studies using tumor transplantation models or genetically engineered mouse models.

Matriptase expression is deregulated in breast, prostate, endometrial, cervical, colorectal, gastric, and pancreatic carcinoma and in tumors of the lung, liver, and kidney among others [reviewed in (Bugge et al. 2007; List et al. 2009; Webb et al. 2011)]. An increase in matriptase expression correlates with the malignancy of tumors in the breast and prostate. *De novo* expression is found in ovarian and cervical carcinomas and expression levels in these tumors also correlate with histopathological grade. It has been hypothesized that a disturbance of the balance between matriptase and its two cognate inhibitors hepatocyte growth factor activator inhibitor 1 and 2 (HAI-1 and HAI-2) may be critical for cancer progression. In support of this hypothesis, the ratio of matriptase:HAI-1 mRNA is increased in both ovarian and colorectal cancer, and the expression of HAI-1 and HAI-2 is reduced in prostate and endometrial carcinoma when compared with normal tissue (Bergum and List 2010; Nakamura et al. 2011; Oberst et al. 2002; Vogel et al. 2006).

The functional role of matriptase is being studied by manipulating levels of expression or activity *in vitro* and *in vivo*. Inhibition of endogenous matriptase synthesis in cultured colon and prostate carcinoma cells by specific siRNAs significantly reduces invasiveness *in vitro* (Forbs et al. 2005). The same effect is observed after treatment of the cells with synthetic matriptase inhibitors. HGF has been

proposed to be functionally involved since inhibition of matriptase in these cell lines impairs the conversion of pro-HGF into HGF on the cell surface and inhibits cell scattering upon stimulation with pro-HGF. In a human prostate cancer xenograft model, a selective small molecule matriptase inhibitor reduces growth of established tumors. The inhibitory mechanism of tumor expansion is proposed to be through impairment of invasion rather than inhibition of tumor cell proliferation, since matriptase inhibition has no effect on prostate cell growth rate *in vitro*, whereas invasion is significantly reduced (Galkin et al. 2004).

The role of matriptase *in vivo* has been studied in detail in squamous cell carcinogenesis of the skin. Interestingly, matriptase is expressed in all stages of carcinogenesis: hyperplasia, dysplasia, carcinoma *in situ*, invasive lesions and lymph node metastases. Spatial deregulation of matriptase expression is critical for epidermal carcinogenesis. Thus, in normal murine epidermis, matriptase expression is found solely in differentiated, nonproliferating keratinocytes; however, treatment with carcinogens induces *de novo* expression in the proliferating basal layer of cells, where the target cells for carcinogenesis reside (List et al. 2006). The expression of matriptase in the proliferative layer of the epidermis plays a causal role in tumor initiation and promotion in mice in which there is transgenic expression in the basal layer of the epidermis (keratin-5 matriptase mice). These transgenic mice develop spontaneous squamous cell carcinomas, severe dermal inflammation, and lymph node metastases and display dramatically increased susceptibility to carcinogen-induced tumorigenesis (List et al. 2005). Importantly, the oncogenic properties of matriptase are completely abolished in double transgenic mice that also express the inhibitor HAI-1 in the basal layer, providing evidence that a disturbance in the matriptase:HAI-1 balance in the epidermis is causative for carcinogenesis (List et al. 2005). Recently, a critical molecular target for matriptase in epidermal carcinogenesis has been identified using mouse genetic models (Szabo et al. 2011). When keratin-5 matriptase transgenic mice are crossed to mice with conditional epidermal deletion of c-Met, the membrane receptor for pro-HGF/HGF, the matriptase-mediated premalignant and malignant lesions are strongly abrogated, demonstrating that the oncogenic potential of matriptase in mouse epidermis requires c-Met. Pro-HGF binds to c-Met, but requires proteolytic cleavage to activate c-Met and elicit downstream signaling events. To this end, it was demonstrated that elevated levels of matriptase increase processing of pro-HGF, which results in initiation of the c-Met-mTOR signaling pathway to induce proliferation and migration in primary epithelial cells. From a cancer intervention standpoint, it is encouraging that epidermal carcinogenesis is efficiently inhibited by rapamycin, a pharmacological inhibitor of the mTOR pathway (Szabo et al. 2011). Accordingly, matriptase is being actively pursued as a candidate for pharmacological intervention as well as for a biomarker of disease onset and progression.

TMPRSS2

The major role of TMPRSS2 in cancer results from genetic fusion with members of the erythroblast transformation specific (ETS) transcription factors [reviewed in (Clark and Cooper 2009; Shah and Small 2010)]. TMPRSS2-ETS fusions occur via complex chromosomal translocation events that combine the 5′ regulatory region of TMPRSS2 in frame with either the entire or partial coding sequence of ETS genes, resulting in androgen-induced expression of the transcription factor. Four TMPRSS-ETS somatic fusions have been identified, with fusions to ERG (ETS-related gene) being the most prevalent. Both the prevalence and prognostic significance of the TMPRSS2–ERG fusion have been examined in multiple studies with some discrepancy in results. Although TMPRSS2–ERG fusions have typically been reported as prevalent in 40–50 % of prostate tumors, the range has varied by as much as 25–60 % (Barwick et al. 2010). Normal TMPRSS2 expression is localized to the prostate epithelium, and TMPRSS-ETS fusions result in the over-expression of the ETS member proto-oncogene in the prostate (Tomlins et al. 2006, 2007). Furthermore, TMPRSS2-ERG fusion events appear to be induced in both malignant and non-malignant prostate cells through androgen signaling, which can cause topoisomerase II beta-mediated DNA double strand breaks at the regions of genetic fusion (Bastus et al. 2010). ETS transcription factors are known to regulate cell growth, and TMPRSS2-ERG fusions most likely play a role early in prostate cancer development and/or progression. Targeted expression of ERG in luminal prostate epithelial cells of transgenic mice results in initiation of prostate neoplasia observed as the development of focal precancerous PIN lesions (Klezovitch et al. 2008; Tomlins et al. 2008). TMPRSS2-ETS fusion proteins are currently being assessed for both their potential as therapeutic targets and as biomarkers for early detection of disease (Tomlins et al. 2009).

15.3.3 Serine Protease Summary

A wide spectrum of serine proteases is deregulated in cancer, and may contribute to both tumor formation and metastasis through diverse molecular mechanisms. The loss of precise regulation of serine protease expression and/or catalytic activity may contribute to the etiology of several types of cancers. There is a strong impetus to understand the events that lead to deregulation at the gene and protein level, and how this precipitates various stages of tumorigenesis. Additionally, many serine proteases have yet to be assessed for physiological and pathological functions, and the mechanisms by which many characterized members affect tumorigenesis are largely unknown. Progress in these areas will add to our burgeoning knowledge of how proteases are involved in cancer progression and metastasis.

15.4 Cysteine Proteases

The cysteine cathepsins are lysosomal proteases that are part of clan CA, which contains several families of cysteine proteases that are structurally similar to the papaya cysteine protease papain (Rawlings et al. 2010). Other families in clan CA that have been implicated in cancer include a number of cytosolic proteases: calpains [for review, see (Storr et al. 2011)], autophagins (Marino et al. 2007) and a variety of deubiquitinating enzymes or DUBs (Colland 2010; Fang et al. 2010; Harhaj and Dixit 2011; Hymowitz and Wertz 2010; Sacco et al. 2010; Sun 2010), e.g., A20, BAP1, CYLD, USP28. The DUB family [for review, see (Komander et al. 2009; Reyes-Turcu et al. 2009)], which currently has 95 members, is intriguing as it consists of 80 clan CA cysteine proteases and 15 metalloproteases and in addition provides a link to the threonine proteases of the proteasome. Clan CD cysteine proteases (Rawlings et al. 2010), also have been associated with cancer: legumain (Lewen et al. 2008; Luo et al. 2006), a lysosomal protease originally identified in plants, and the cytosolic caspases of the apoptotic cascade (Demon et al. 2009; Ghavami et al. 2009). Here we will focus on cysteine cathepsins and cancer.

15.4.1 Cysteine Cathepsins

There are 11 human cysteine cathepsins: B, C, F, H, L, K, O, S, V, W and X (Rawlings et al. 2010). Their localization within lysosomes and late endosomes, vesicular compartments often referred to as the digestive tract of the cell, may have influenced research on these enzymes and the conclusions that we have reached about the roles they play in cancer. The 11 human cysteine cathepsins degrade a wide array of substrates within lysosomes and late endosomes and are stable and active at the acidic pH found in these vesicular compartments. For reviews on these enzymes see Vasiljeva and Turk (2008) and Reiser et al. (2010). Seven of the cysteine cathepsins are endopeptidases and thus presumed to participate in complete digestion of proteins within the lysosomes. On the other hand, four of the cysteine cathepsins are exopeptidases, cathepsin C is an aminopeptidase and cathepsin X is a carboxypeptidase, whereas cathepsins H and B have both endopeptidase and exopeptidase (aminopeptidase and carboxydipeptidase, respectively) activities. The two activities of cathepsin B depend on the position of an occluding loop that covers the active site pocket. At acidic pH such as in the lysosome the occluding loop limits access to the active site so that cathepsin B acts as a carboxydipeptidase. At neutral pH the occluding loop is displaced so that cathepsin B acts as an endopeptidase. The observations that cathepsin X (Sevenich et al. 2010b; Vasiljeva et al. 2006), a carboxypeptidase, and cathepsin L (Felbor et al. 2002; Stahl et al. 2007), an endopeptidase, are able to compensate for the absence of cathepsin B in mouse models would be consistent with cathepsin B

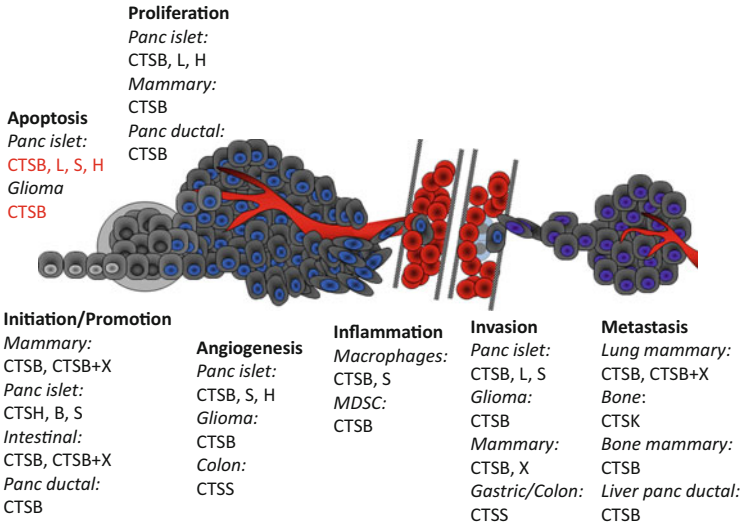

Fig. 15.5 Cysteine cathepsins in cancer: representative examples of family members demonstrated by *in vivo* experimentation to play roles in the indicated stages of tumor progression. An absence of the enzymes listed in *red font* under the heading apoptosis has been shown to induce apoptosis in pancreatic islet tumors and gliomas

exhibiting both exo- and endopeptidase activities *in vivo*. Although cysteine cathepsins normally function intracellularly in lysosomes and late endosomes in a number of developmental and pathological processes, individual cysteine cathepsins have been found to be secreted, associated with the cell membrane, released into the cytoplasm and present in the nucleus [for review, see (Mohamed and Sloane 2006; Reiser et al. 2010; Turk et al. 2012; Vasiljeva and Turk 2008)] (Fig. 15.5). These enzymes have been shown to participate in degradative processes at these extra-lysosomal sites, indicating that their stability and activity can be maintained under conditions not normally found in the lysosome, i.e., at a neutral pH and in a non-reducing environment. Since the roles of cysteine cathepsins in cancer have been previously reviewed (Mohamed and Sloane 2006), we concentrate here on those studies published more recently.

15.4.1.1 Cathepsin B

Among the cysteine cathepsins, cathepsin B has been the one most often linked to cancer. Expression is elevated in a wide variety of human cancers [for review, see (Jedeszko and Sloane 2004; Mohamed and Sloane 2006)]. The mechanisms responsible include amplication of the cathepsin B gene, which is localized on chromosome 8 p22. This region is a novel amplicon that is associated with amplification and overexpression of cathepsin B in Barrett's esophagus, a premalignant lesion that predisposes to esophageal cancer (Hughes et al. 1998). Additional mechanisms

for altered expression include elevated transcription, use of alternative promoters and alternative splicing. These molecular changes lead to increased cathepsin B protein levels and in turn redistribution, secretion and increased activity (Yan and Sloane 2003).

Studies by Joyce and colleagues (Gocheva et al. 2006) in which cathepsin B knockout mice were crossed with a transgenic model for pancreatic islet cell carcinogenesis have established roles for cathepsin B at multiple points in the development of these tumors, i.e., initiation, proliferation, angiogenesis, invasion and apoptosis. The absence of cathepsin B reduces initiation, proliferation, angiogenesis and invasion, but increases apoptosis. Peters, Reinheckel and colleagues (Vasiljeva et al. 2006, 2008) have crossed cathepsin B knockout mice with transgenic models for mammary carcinoma. These studies demonstrated a role for cathepsin B in initiation, proliferation, invasion and metastasis in these tumors, including a role for cathepsin B in macrophages in lung metastases. Interestingly, there was compensation for the loss of cathepsin B in the mammary tumors and specifically for the loss of cathepsin B on the tumor cell surface by a redistribution of cathepsin X to the tumor cell surface (Vasiljeva et al. 2006). As cathepsin X is exclusively a carboxypeptidase, this suggests that the function of cathepsin B on the tumor cell surface is through its carboxypeptidase activity. A single deficiency of cathepsin B exerts partial reciprocal compensation for cathepsin X; double deficiencies of cathepsins B and X revealed additive effects, indicative of the two enzymes contributing to tumor initiation, progression, invasion and metastasis. Compensation by cathepsin X has also been observed in the intestinal polyps that form in mice with mutations in the adenomatous polyposis coli gene (Gounaris et al. 2008). In these mice, the active cathepsins could be used to image the polyps and myeloid derived suppressor cells and macrophages within the polyps. Polyposis and infiltration of pro-inflammatory cells could be reduced by ablation of cathepsin B. A compensatory role for cathepsin B has recently been observed in two mouse models of pancreatic neuroendocrine carcinogenesis (Shchors et al. 2013). In these tumors this is due to infiltration of myeloid derived suppressor cells expressing cathepsin B to the invasive front of the tumors, infiltration of which is associated with an increased invasive phenotype. In a transgenic mouse model of pancreatic ductal adenocarcinoma that was crossed with cathepsin B knockout mice, a role for cathepsin B in tumor initiation, proliferation and progression has been identified (Gopinathan et al. 2012). In these tumors cathepsin L was upregulated, once again showing compensation by one protease for deficiency in another.

Downregulation of cathepsin B by antisense, siRNA and shRNA technologies has established a causal role for this enzyme in a variety of tumors. For a review of the studies by Rao and colleagues in glioma, see (Rao 2003; Vink and Boomsma 2002). A recent study in a murine mammary carcinoma model found that shRNA knockdown of cathepsin B reduces degradation of type I collagen in vitro and bone metastasis in vivo (Withana et al. 2012). Deficiencies in both cathepsins B and X are synergistic, significantly delaying mammary tumor development and inhibiting metastasis (Sevenich et al. 2010b). Studies in which cathepsin B is downregulated in concert with other proteases/protease receptors have shown that cathepsin B is

part of a proteolytic network that involves the plasminogen activator cascade and MMPs. Rao and colleagues have found that downregulation of cathepsin B and uPAR in glioma induces apoptosis (Malla et al. 2010, 2012) and of cathepsin B and MMP9 in meningioma reduces growth in vitro and in vivo and reduces invasion, angiogenesis and regulation of downstream kinase signaling pathways in vitro (Tummalapalli et al. 2007). They reported similar findings when cathepsin B, MMP9 and uPAR were downregulated in prostate carcinoma (Nalla et al. 2010) and in glioma, a finding linked to downregulation of integrins (Veeravalli et al. 2010). Also in glioma specific inhibition of alpha3beta1 integrin and the tetraspanin CD151 occurs as a result of knockdown of cathepsin B and uPAR (Rao Malla et al. 2012). An interplay between integrins and proteases including cathepsin B has also been shown in breast and prostate tumor cells (Sameni et al. 2008). In those studies downregulation of beta1-integrin reduced secretion of cathepsin B and MMP13, expression of MMP14 and degradation of type IV collagen. In gliomas, downregulation of cathepsin B and uPAR decreases proliferation due to G0/G1 arrest and reduced phosphorylation of PI3K/Akt (Gopinath et al. 2010; To et al. 2010) and also inhibits tumor-induced angiogenesis (Malla et al. 2011). Comparable reductions of angiogenesis in meningiomas could be reversed by overexpression of cathepsin B and uPAR, a finding linked to increased FAK phosphorylation (Gupta et al. 2011). A complex signaling network that increases transcription of cathepsin B in association with the invasive phenotype induced by ErbB2 in breast cancer has been identified (Rafn et al. 2012). These studies provide strong evidence that proteases act in pathways that extend beyond a single family of proteases and that those pathways are activated by and also modulate downstream signaling pathways. Furthermore, the roles played by proteases are not constant. In the studies cited above from the laboratories of Joyce and Rao, downregulation of cathepsin B was shown to induce apoptosis, yet in other studies cathepsin B and other lysosomal cysteine cathepsins have been shown to mediate apoptosis [for a review discussing these opposing functions, see Vasiljeva and Turk (2008)].

15.4.1.2 Cathepsin X

In melanomas and mammary carcinomas, the presence of cathepsin X in tumor cells has been associated with malignant progression (Rumpler et al. 2003; Sevenich et al. 2010b; Vasiljeva et al. 2006). As noted above, cathepsin X, which is an exopeptidase, compensates for a deficiency in cathepsin B in mammary carcinoma cells, including becoming associated with the surface of the carcinoma cells (Vasiljeva et al. 2006). Deficiencies in both cathepsins X and B delay development of early and advanced mammary carcinomas and reduce the number and the size of their lung metastases (Sevenich et al. 2010b). In contrast, studies in lung tumors found cathepsin X in macrophages rather than in tumor cells (Kos et al. 2005). Similarly, cathepsin X functions in gastric epithelial cells in the

inflammatory response to *Helicobacter pylori* infection (Bernhardt et al. 2010), which increases the risk for gastric cancer (Leung 2006).

15.4.1.3 Cathepsins L and V

Determining the relationship between cathepsins L and V and cancer is made more difficult by the existence of two similar enzymes in human, i.e., cathepsins L and V (sometimes referred to as L2). Cathepsin V is the human orthologue of mouse cathepsin L (Dennemarker et al. 2010). Reinheckel and colleagues (Sevenich et al. 2010b) postulate that functional differences between the two enzymes are determined by their tissue specific expression patterns, cathepsin V being expressed primarily in thymus, cornea, testis and epidermis (Hagemann et al. 2004). In support of this postulate, either human cathepsin L or human cathepsin V can compensate for a deficiency of mouse cathepsin L in the thymus of cathepsin L knockout mice (Sevenich et al. 2010a). Nonetheless, tissue specific expression patterns do not explain why in a single tissue, i.e., in human cervical carcinomas cathepsin V is upregulated while cathepsin L is downregulated (Vazquez-Ortiz et al. 2005). If both are performing the same function, it seems counterintuitive for expression of one to go up and expression of the other to go down. There is an extensive literature implicating secretion of procathepsin L in malignant transformation, literature that predates the identification of cathepsin V. Indeed the major excreted protein of malignantly transformed murine fibroblasts is procathepsin L (Kane and Gottesman 1990). In agreement with a role for cathepsin L in malignancy, the deletion of cathepsin L in a transgenic model for pancreatic islet cell carcinogenesis reduces proliferation and invasion and increases apoptosis (Gocheva et al. 2006). In contrast in another transgenic model, i.e., a model of multistage squamous carcinogenesis, cathepsin L plays a protective role as the progression of tumors in mice crossed with mice deficient in cathepsin L is enhanced (Dennemarker et al. 2010). A comparable finding was made in regard to a protective role for cathepsin L in chemically-induced skin carcinogenesis (Benavides et al. 2012). These two findings may be indicative of functional differences due to tissue specific expression patterns of mouse cathepsin L and its human orthologue cathepsin V in the epidermis.

Cathepsin L may play roles other than degradation of extracellular matrices such as activation of other enzymes that degrade matrices. For example, cathepsin L has been shown to activate pro-heparanase; heparanase itself degrades heparan sulfate in the extracellular matrix and has pro-metastatic, pro-angiogenic and pro-inflammatory activities (Abboud-Jarrous et al. 2008). There is evidence from several laboratories that a nuclear form of cathepsin L plays a role in cell cycle progression through the processing of CDP/Cux transcription factor (Goulet et al. 2004, 2006, 2007), drug resistance through the cleavage of topoisomerase II alpha (Zheng et al. 2009; Murray et al. 2008), in colon cancer through degradation of histone H1 (Puchi et al. 2010) and in thyroid cancer through the modification of DNA-associated proteins (Tedelind et al. 2010). Nuclear cathepsin L has been

observed in human colorectal tumors in both the tumor cells and stromal cells, increasing in parallel with stage in the tumor cells (Sullivan et al. 2009). The nuclear cathepsin in thyroid cancers has been identified as cathepsin V rather than cathepsin L (Tedelind et al. 2010). Upregulation of cathepsin V is also observed in human breast carcinomas where it has been shown to be one of two protease genes that comprise a predictive invasive signature for these tumors (Paik et al. 2004), in particular for node-negative breast tumors. This invasive signature is part of the gene panel used clinically in the Oncotype DX test to make treatment decisions.

15.4.1.4 Cathepsin H

Cathepsin H is upregulated in early stage, node negative human lung tumors and in lung tumors in a transgenic mouse model (Linnerth et al. 2005). In human tumors, cathepsin H is upregulated in immunological cells surrounding basal cell carcinoma nodules (Frohlich et al. 2004), in transitional cell carcinoma of the bladder (Staack et al. 2004), in brain tumors (Hunter and Moreno 2002) and in hepatomas where it is associated with microvascular invasion and kinase activation (Wu et al. 2011). Deletion of cathepsin H in a mouse model of pancreatic islet carcinoma reduces tumor progression, growth, burden and angiogenesis, thus demonstrating a tumor-promoting role for this enzyme (Gocheva et al. 2010).

15.4.1.5 Cathepsin C

Cathepsin C is highly expressed in several tumors: rat esophageal adenocarcinoma (Cheng et al. 2005), murine pancreatic islet cell carcinoma (Gocheva et al. 2006) and human non-small cell lung carcinoma (Cordes et al. 2009) and Hodgkin lymphoma (Ma et al. 2008). Whether cathepsin C plays a causal role in malignant progression depends on the tumor. Downregulation of cathepsin C in the RIP-Tag model of pancreatic islet cell carcinoma had no effect on tumor formation or progression (Gocheva et al. 2006). Similarly in murine mammary carcinomas in which cathepsin C expression is increased as a result of infiltration of macrophages, deletion of cathepsin C had no effect on any parameters of primary or metastatic tumor development. On the other hand in a transgenic model of multistage squamous carcinogenesis a deficiency of cathepsin C reduces tumor progression (Ruffell et al. in press).

15.4.1.6 Cathepsin K

Cathepsin K is highly expressed in osteoclasts where its predominant role is in bone resorption (Saftig et al. 1998) and therefore many cathepsin K inhibitors have been developed for treatment of osteoporosis (Lewiecki 2011). Indeed 50 patent

applications were filed between 2004 and 2010 (Wijkmans and Gossen 2011). Cathepsin K is viewed as a therapeutic target for the osteolytic metastases associated with a variety of human tumors, e.g., prostate (Podgorski et al. 2009), breast (Tomita et al. 2008), lung (Rapa et al. 2006; Tomita et al. 2008), multiple myeloma (Hecht et al. 2008). The source of cathepsin K in tumors may be tumor cells as in melanomas (Quintanilla-Dieck et al. 2008), stromal fibroblasts and osteoclasts activated by breast and lung tumors (Tomita et al. 2008) and stromal macrophages and fibroblasts in squamous cell carcinomas (Yan et al. 2011). Secretion of cathepsin K by stromal fibroblasts increases invasiveness of breast tumor cells, a paracrine interaction that can be blocked with cathepsin K inhibitors (Kleer et al. 2008). In cathepsin K knockout mice, the progression of prostate tumor bone metastases is reduced as a result of a deficiency of cathepsin K in bone marrow-derived macrophages (Herroon et al. 2013).

15.4.1.7 Cathepsin S

Cathepsin S is highly expressed in a number of tumors. In astrocytomas, the level of expression is highest in grade IV tumors (Flannery et al. 2003) and in glioblastomas is associated with significantly shorter survival (Flannery et al. 2006). In uveal melanomas, cathepsin S expression and activity is increased, the latter in parallel with decreases in expression of cystatin C, an endogenous inhibitor (Paraoan et al. 2009). The source of cathepsin S may vary from tumor to tumor. In a mouse model of prostate carcinoma, cathepsin S is found in tumor-associated macrophages (Lindahl et al. 2009), whereas in human hepatocellular carcinoma (Xu et al. 2009) and gastric carcinoma (Yang et al. 2010), cathepsin S is associated with the tumor cells. Cathepsin S has been shown to participate in tumor progression. Tumor proliferation and angiogenesis are reduced in a mouse model for pancreatic islet carcinoma crossed with cathepsin S deficient mice (Wang et al. 2006). Another study reported reduced tumor formation and angiogenesis and increased apoptosis in the same model crossed with cathepsin S deficient mice (Gocheva et al. 2006). In yet other studies in which cathepsin S was downregulated a causal role for this enzyme in migration and invasion of gastric carcinomas has been established (Yang et al. 2010).

15.4.1.8 Cathepsins F and W

Cathepsins F and W form an evolutionarily related subgroup of the cysteine cathepsins (Wang et al. 1998; Wex et al. 1999). Cathepsin F has a longer propeptide domain than the other cysteine cathepsins and is present at high levels in only a few cancer cell lines (Santamaria et al. 1999), e.g., cervical carcinoma lines (Vazquez-Ortiz et al. 2005). In contrast, cathepsin F expression is low in highly metastatic osteosarcoma lines (Husmann et al. 2008). Cathepsin F is also found in macrophages (Kaakinen et al. 2007; Shi et al. 2000), but to our knowledge has

not been studied in tumor-associated macrophages. Cathepsin W, which is also called lymphopain, is one of a series of genes associated with cytotoxic function that are upregulated in large granular lymphocyte leukemia (Kothapalli et al. 2003).

15.4.1.9 Cathepsin O

Cathepsin O has been identified as a potential marker for metastases of tongue carcinoma.

15.4.1.10 Cysteine Cathepsins as Therapeutic Targets

A broad-spectrum inhibitor of cysteine cathepsins, JPM-OEt, has been shown to be efficacious in a transgenic mouse model for pancreatic islet tumors (Bell-McGuinn et al. 2007). Tumor burden, vascularity and invasion were all reduced by JPM-OEt, findings consistent with cysteine cathepsins being therapeutic targets in this tumor model. A note of caution is needed in regard to how these preclinical findings will translate to clinical use of cysteine cathepsin inhibitors as broad-spectrum inhibitors of MMPs failed in clinical trial despite showing efficacy in preclinical studies in a wide variety of tumor models (see section on MMPs). A potential problem with use of broad-spectrum inhibitors is that some cysteine cathepsins also protect against tumor development just as has been shown for MMPs, e.g., MMP-12 in lung cancer (Acuff et al. 2006; Houghton et al. 2006). Bioavailability of the inhibitors may also impact their effectiveness as has been shown in studies comparing the use of JPM-OEt in mouse models for pancreatic islet tumors and mammary tumors. In the former inhibition of cysteine cathepsins reduced tumor development, yet not in the latter model (Schurigt et al. 2008). Poor bioavailability of the inhibitor in the mammary carcinomas and the lungs, yet not in the liver, kidney or pancreas of the mammary-tumor bearing mice was shown to account for the differences in efficacy in the two preclinical models.

van Noorden and colleagues have reviewed the case for cathepsin L being a therapeutic target and in so doing raise a concern that selective inhibition of cathepsin L will lead to compensatory changes in other cysteine cathepsins (Lankelma et al. 2010). This has been shown in a transgenic mammary carcinoma model in which deficiency in cathepsin B, an enzyme localized on the tumor cell surface (Mai et al. 2000; Sloane et al. 1994), is compensated for not only by increased expression of cathepsin X, but also by an association of cathepsin X with the tumor cell surface (Vasiljeva et al. 2006). van Noorden and colleagues further postulate that combination therapy with a cathepsin L inhibitor and conventional chemotherapy may be a better strategy than just use of a cathepsin L inhibitor (Lankelma et al. 2010). Thus it is of interest that inhibition of cathepsin S enhances the therapeutic effect of chemotherapy in colorectal carcinoma (Burden et al. 2012) and the broad-spectrum inhibitor JPM-OEt used in combination with chemotherapeutic regimens exhibits a greater therapeutic response (tumor regression,

decreased invasion and increased survival) than is observed when only cysteine cathepsins are inhibited. Despite these promising findings with JPM-OEt, this is an irreversible inhibitor and therefore will not be used in patients. More relevant in terms of clinical applicability is that a reversible broad-spectrum cysteine cathepsin inhibitor has also been shown to exhibit effectiveness in the pancreatic islet tumor model (Elie et al. 2010).

Among the cysteine cathepsins, the enzyme most widely acknowledged as an important therapeutic target is cathepsin K due to its critical role in bone resorption by osteoclasts (Onishi et al. 2010; Rachner et al. 2012; Sturge et al. 2011). A number of selective inhibitors for this enzyme have been developed and have been shown to decrease bone resorption by breast tumors as well as bone metastases in preclinical studies (Jensen et al. 2010; Le Gall et al. 2007). Selective inhibition of other cysteine cathepsins has also shown some efficacy in preclinical studies. For example, a highly selective cathepsin B inhibitor suppresses metastasis of mammary tumors to bone (Withana et al. 2012). An inhibitory antibody to cathepsin S inhibits invasion and angiogenesis of colon tumors (Burden et al. 2009; Ward et al. 2010). These studies suggest that cathepsins K, B and S may be therapeutic targets; however, whether selective inhibition of any individual cysteine cathepsin will prove to be of value in the clinic will await further study.

Tumor-associated increases in expression and secretion of cysteine cathepsins occur in both tumor cells and inflammatory cells found at the invasive margins. The elevated expression of cysteine cathepsins in tumor-associated macrophages at both primary and metastatic sites means that targeting these enzymes will require targeting both the tumor microenvironment and the tumor. This was the rationale in part for the development of a novel drug delivery system in which magnetic nanoparticles were incorporated into liposomes, termed ferri-liposomes, along with the broad-spectrum cysteine cathepsin inhibitor JPM-OEt (Mikhaylov et al. 2011). The ferri-liposomes could be targeted to tumor xenografts in mice using an external magnet with their delivery to the tumor site being imaged non-invasively by MRI. How such a delivery system would work for human tumors is less clear. Nonetheless, delivery of JPM-OEt to mouse mammary fatpads containing tumor xenografts significantly reduced tumor size in contrast to the lack of efficacy of JPM-OEt not incorporated in liposomes. Cysteine cathepsins and in particular the nuclear form of cathepsin L has been implicated in drug resistance of tumors (Zheng et al. 2009). Resistance to doxorubicin could be abrogated by a cathepsin L inhibitor and combination therapy with doxorubicin and a cathepsin L inhibitor suppressed the proliferation of drug-resistant tumors in a preclinical mouse model. Doxorubicin resistance as well as resistance to a number of other chemotherapeutics has recently been linked to the cysteine cathepsins B and S in tumor-associated macrophages (Shree et al. 2011). By combining the inhibition of macrophage cysteine cathepsins with chemotherapeutics, there was a significant increase in the chemotherapeutic response at both primary and metastatic tumor sites, indicating the importance of macrophages and cysteine cathepsins at both sites as well as the need to develop therapeutic strategies that target tumors in the context of their microenvironment.

15.5 Threonine Proteases

The threonine proteases are part of a specialized group of enzymes in which the N-terminal amino acid acts as the nucleophile that attacks the peptide bonds of substrates. In mammals, the predominant threonine proteases are constituents of the proteasome complex. The mammalian proteasome is a multi-constituent complex that resembles a stack of rings, with each ring having seven subunits. This is an ancient structure with a simpler version, also possessing threonine protease activity, present in archaebacteria (Coux et al. 1996). Initial naming of the proteasome was based on size and appearance so that the designation '26S proteasome' referring to an estimated sedimentation coefficient is now the accepted term for a complex consisting of both the 20S proteolytic and a single 19S regulatory subunit (Dahlmann 2005). The proteolytic part can be separately referred to as the '20S proteasome'. There are multiple other names used in the literature including 'cylindrin', 'high molecular weight protease', '19s ring-type particles' among others. The reader is referred to the chapter 'Eukaryotic 20S proteasome' in the encyclopedic 'Handbook of Proteolytic Enzymes, 2nd Edition' for a more complete description of the history and biochemistry of the proteasome (Seemuller et al. 2004). One point that is critical for understanding the vast array of proteasome substrates is that there are three enzymatic activities within the subunits of the 20S proteasome. These are referred to as caspase-like, trypsin-like and chymotrypsin-like activities (Adams 2004a). Here we focus on the importance of the proteolytic activity of the proteasome in cancer development and progression.

15.5.1 The Proteasome in Tumor Initiation/Promotion

Since the proteasome is the major regulator of protein stability in the cell, it is unsurprising that its activity can impact tumor development at many levels. A number of cancer-associated proteins, either directly oncogenic or constituents of oncogenic signaling pathways, are regulated by proteasomal degradation. Mutations in these molecules, as occurs in tumorigenesis, often lead to proteins that have significantly altered turnover. An example of an oncogene whose half-life is greatly increased by mutation leading to a prolonged oncogenic signal is c-myc (Bahram et al. 2000; Gavine et al. 1999). In contrast, Smads 2 and 4, components of the TGF-β signal transduction pathway that are often mutated in colorectal cancers, have a much shorter half-life when mutated, thus impeding tumor suppressive signaling by TGF-β (Xu and Attisano 2000). The driving mutation in the majority of colon cancers occurs in the adenomatous polyposis coli (APC) gene or components of a complex involving APC (Voutsadakis 2008). A major function of this APC complex is to target the protein beta-catenin for proteasomal degradation (Easwaran et al. 1999). In the absence of proteasomal degradation, beta-catenin accumulates, translocates to the nucleus and, in partner with DNA binding

transcription factors such as lymphoid enhancer factor-1, effects transcription of oncogenic proteins including c-myc and cyclin D1.

Gastric cancer, although relatively rare in the United States, is the second leading cause of cancer-related mortality worldwide (Jemal et al. 2010). One of the major causes is chronic infection with the bacterium *Helicobacter pylori* (Piazuelo et al. 2010). There are multiple mechanisms by which chronic *H. pylori* infection could result in gastric cancer. One suggestion is via proteasomal-mediated degradation of the cell cycle inhibitor $p27^{kip1}$ in gastric epithelial cells exposed to *H. pylori* (Eguchi et al. 2003). Interestingly, this appears to be a differently regulated proteasomal degradation pathway than that which occurs in normal physiology. Thus, it seems that bacterial infection can alter proteasome activity to result in continuous cycling of infected cells, which may favor development of neoplasias. The existence of an alternatively regulated proteasomal degradation pathway for $p27^{kip1}$ had previously been demonstrated in an elegant mouse model (Malek et al. 2001). Investigators 'knocked-in' a mutant $p27^{kip1}$ in which the regulatory threonine residue is replaced with an alanine and were surprised that effects of this mutation are relatively subtle. The results point to a novel pathway, but the stimulus for this pathway remains elusive. Another mechanism of cell cycle regulation is also linked to the proteasome. Cyclin degradation by proteasomal activity is important in preventing carcinogenic transformation of bronchial epithelial cells, and in determining responsiveness to chemopreventative agents such as all-trans-retinoic acid (Dragnev et al. 2004). In fact, regulation of cyclins and cyclin-dependent kinase inhibitors is a critical function of the proteasome that can be perturbed by proteasome inhibitors in cancer cells (Adams 2004b).

The principal result of therapeutic proteasome inhibitors in cancer therapy is increased apoptosis. This has been attributed to increased stability of several substrates including NF-κB, p53, death receptors and pro-apoptotic Bcl2 family members (Adams 2004b; Drexler 1997; Drexler et al. 2000). Thus, in the absence of inhibition, proteasome activity leads to increased survival of tumor cells (Hu et al. 2004). Another important mechanism of action is increased endoplasmic reticulum (ER) stress associated with the 'unfolded protein response' or UPR (Zhang and Kaufman 2006). There have been several suggestions that proteasome activity is increased in cancer cells as compared to non-transformed cells (An et al. 1998; Testa 2009) and, with the increase in cell cycling discussed above, in addition to increased survival, outgrowth of a cancer is favored.

15.5.2 The Proteasome in Angiogenesis

The process of angiogenesis is complex involving multiple components such as endothelial cell proliferation, migration and tube formation as well as recruitment of supporting mural cells, generation of appropriate matrix and orchestration of an array of signaling molecules to achieve these results. Proteasomal regulation of almost every aspect has been demonstrated using combinations of *in vivo* and

in vitro approaches. Many studies use chemical inhibitors of proteasomal activity, e.g., lactacystin or MG-132 as well as newer inhibitors, to indicate a role for the proteasome (Drexler et al. 2000; Matsuo et al. 2010; Oikawa et al. 1998; Sunwoo et al. 2001). Recently, Chou et al. (2010) dissected an elegant angiogenic signaling pathway present in many tumor types, that is ultimately dependent on the proteasome. They showed that stability of the tumor suppressive protein Von-Hippel Lindau can be regulated by oncogenic Src, ultimately leading to increased levels of VEGF and angiogenesis. The stability and signaling of the two major receptors for VEGF, VEGFR1 and VEGFR2, are also controlled through ubiquitination and proteasomal degradation (Bruns et al. 2010), although there is some disagreement on which is the more sensitive target of the proteasome (Meissner et al. 2009). One possible explanation for the discrepancy is the presence of the ligand VEGF, which can simultaneously promote increased stability of VEGFR1 and increased degradation of VEGFR2, overall leading to enhanced endothelial cell survival (Zhang et al. 2010).

Despite the clear role for altered protein stability in promotion of angiogenesis, inhibition of proteasome activity *in vivo* can actually result in enhanced angiogenesis and increased tumor burden in a mouse model of pancreatic adenocarcinoma (Marten et al. 2008). One possibility is that the effect is related to the proteasome inhibitor used, i.e., bortezomib, as a different type of proteasome inhibitor with more limited activity called argyrin A significantly attenuates angiogenesis in a colon cancer model (Nickeleit et al. 2008). The activity of argyrin against the proteasome is actually more broad-spectrum than bortezomib as it inhibits all three of the enzymatic activities whereas bortezomib targets the chymotrypsin-like activity. Nevertheless, argyrin A appears to have fewer off-target effects (Nickeleit et al. 2008).

15.5.3 The Proteasome in Inflammation

One of the best-known functions of the proteasome is in regulation of the transcription factor nuclear factor kappa B (NFκB) (Amit and Ben-Neriah 2003). While it is increasingly clear that NFκB plays multiple roles in different cell types, it is especially associated with promoting inflammation. Indeed pro-inflammatory cytokines, induced by NFkB signaling, are considered tumor promoters in many types of cancer [reviewed in (Ben-Neriah and Karin 2011; Cortes Sempere et al. 2008; Grivennikov et al. 2010)].

In addition to direct effects on tumor cells, altered proteasomal activity can affect other cells of the tumor microenvironment. These effects may be anti-tumorigenic. For example, *in vitro* analysis of macrophages exposed to the endotoxin lipopolysaccharide (LPS) indicates that proteasomal activity is required for the generation of the pro-inflammatory, classically activated macrophage (Cuschieri et al. 2004). When proteasomal activity is inhibited, LPS-stimulated macrophages have a different cytokine profile that is more anti-inflammatory. Since

alternatively activated, anti-inflammatory macrophages are thought to promote tumor progression in many types of cancer (Qian and Pollard 2010), this study may indicate that proteasomal inhibitors could have unanticipated side-effects in the tumor stroma. Such thinking has led to attempts to specifically alter NFκB signaling in tumor-associated macrophages *in vivo* (Hagemann et al. 2008).

15.5.4 The Proteasome in Drug Resistance

Resistance of cancer cells to chemotherapeutic drugs is a significant clinical problem in oncology. Many mechanisms have been identified that can contribute to this phenomenon including increased expression of efflux pumps like multidrug resistance (MDR)-1, mutation of drug targets, increased expression of anti-apoptotic molecules or up-regulation of alternative signaling pathways (Fodale et al. 2011; McCormick 2011; Wilson et al. 2009). The proteasome is a significant player in drug resistance, partly through controlling the balance of pro and anti-apoptotic signals, but also in other ways (Adams 2004a). One example is that which occurs with the DNA topoisomerase II poisons, a class of drugs that includes etoposide and doxorubicin, which are widely used against many types of cancer. These drugs are often rendered ineffective through proteasomal-mediated degradation of their target topoisomerase, which occurs when stress conditions such as low glucose and hypoxia are encountered (Tsuruo et al. 2003).

Autophagy is a cellular survival process that is thought to be a major contributor to drug resistance, although this is controversial (Maycotte and Thorburn 2011). Autophagy is normally induced by environmental stresses such as nutrient or oxygen deprivation. The degradation of cellular proteins that occurs in autophagy generally involves proteolysis within the lysosomes and this is considered a complementary mechanism to proteasome-mediated degradation. One potential problem with overcoming drug resistance through inhibiting the proteasome is that autophagic survival mechanisms may be enhanced. For this reason, new combinations of proteasome inhibitors and agents that prevent autophagy have been suggested (Wu et al. 2010). Such combinations would be useful in multiple situations where cancer cells are placed under stress, including stress induced by chemo- or radiation therapy, as well as by naturally occurring nutrient limitation.

15.5.5 Proteasome Inhibitors in Cancer Therapy

As is evident from the foregoing paragraphs, proteosomal activity is increased in tumor versus normal cells and contributes to the tumor phenotype in multiple ways. Therefore, it is not surprising that the proteasome should emerge as a primary 'target' for targeted therapies. Bortezomib (Velcade™, Takeda Millennium Pharmaceuticals) is the first of these targeted therapies to be approved (Adams

2004a), gaining fast-track approval status from the Food and Drug Administration in the USA based on especially encouraging results in a Phase II trial in refractory multiple myeloma patients (Chen et al. 2011). Bortezomib is termed a dipeptidyl boronate inhibitor, which describes the chemical moiety that interacts with the active site threonine (Adams 2004a). It particularly binds to the active site threonine in the chymotryptic-like subunit of the 20S proteolytic core. Recent evidence has suggested that bortezomib is not, however, specific for the proteasome, but has off-target effects (McConkey 2008; Testa 2009). While bortezomib is often used in hematological malignancies, such as multiple myeloma, it has not demonstrated significant efficacy in solid tumors. This has been attributed to a number of factors including development of resistance, dose-limiting side-effects and interaction with natural chemicals that interfere with its activity (Chen et al. 2011; McConkey and Zhu 2008). Since resistance to many chemotherapeutic agents, as outlined above, appears related to proteasomal activity, the combination of a proteasome inhibitor with traditional chemotherapy remains an attractive therapeutic strategy (Adams 2004a). Hence, development of newer generation proteasome inhibitors has received much attention. One particularly promising candidate is marizomib (NPI-0052), an irreversible inhibitor that shows stronger effects at lower doses over a longer time (Chauhan et al. 2005; Potts et al. 2011). Given the inevitable development of resistance seen with bortezomib (McConkey and Zhu 2008), one very attractive feature of marizomib is that it is unaffected by transporter proteins such as members of the MDR family (Obaidat et al. 2011). Other possible proteasomal inhibitory drugs include argyrin A (Nickeleit et al. 2008), as well as a number of natural products (Cecarini et al. 2011). Overall, the clear contribution of threonine protease activity to cancer makes this class of enzyme an important and rapidly evolving drug target.

15.6 Conclusions

We are still far from having a thorough understanding of the functions of proteases in cancer: this includes understanding the roles of the proteases discussed in this chapter, 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, etc. In short, we do not yet know which protease(s) is the most appropriate target for anti-protease therapies or when anti-protease therapies might prove most effective. Elevated expression of proteases can be documented at the transcript and protein levels in many tumors, as described here. This elevated expression does not, however, necessarily translate into elevated activity as most proteases are synthesized as inactive proenzymes that require activation. In addition, there may be changes in tumors in the expression of endogenous protease inhibitors that impact protease activity. Thus to assess whether a protease is active and has the

potential of playing a functional role, we need techniques that allow us to assess the activity of that protease, both in cell culture-based assays and in whole organisms. We also need such techniques to assess the efficacy of therapies that alter protease activity. One of the difficulties we face in evaluating why the clinical trials of MMP inhibitors did not meet the promise of the preclinical trials is that the clinical trials did not include any surrogate endpoints to assess whether the activity of the MMPs targeted was actually reduced. For discussions of the implications of these clinical trials, including why imaging is important, the reader is referred to the following articles (Coussens et al. 2002; Egeblad and Werb 2002; Marlowe 2005; Scherer et al. 2008a; Zucker et al. 2000; Chau et al. 2003; Li and Anderson 2003; McIntyre and Matrisian 2003; Moin et al. 2012). At the time of the MMP inhibitor trials, there were few methodologies to image protease activity *in vivo* or for that matter *in vitro*. This is an expanding field with a variety of imaging modalities and probes now available for functional imaging of protease activity *in vivo* [for review, see (auf dem Keller and Schilling 2010; Li and Anderson 2003; Moin et al. 2007; Scherer et al. 2008a)].

For *in vivo* imaging of protease activity, Matrisian and her coworkers have developed optical proteolytic beacons (Scherer et al. 2008b) and MRI contrast agents (Lepage et al. 2007) for MMP-7 and optical proteolytic beacons for MMP-9 (Fig. 15.6) or MMPs in general (McIntyre et al. 2010). These reagents are based on known MMP substrates. A recent study also used an MMP substrate, but in this case a triple helical peptide substrate, to develop near-infrared probes that detect activity of MMP-2 and -9 in vivo in mouse tumors (Akers et al. 2012). An alternative strategy is the use of small molecule inhibitors of MMPs, a strategy being developed for in vivo molecular imaging of active MMPs by SPECT and PET (Wagner et al. 2006).

Activity of serine proteases has been imaged in vivo by optical methods using target-activatable self-assembled gold nanoparticle probes (Mu et al. 2010) and fluorescently-labeled blocking antibodies (Darragh et al. 2010). Bogyo and colleagues have pioneered the use of activity-based probes (ABPs), which are based on small molecule cysteine protease inhibitors that bind covalently to their target enzymes, for *in vivo* optical imaging of the activity of several classes of cysteine proteases, including cysteine cathepsins (Blum et al. 2007) and caspases (Edgington et al. 2009). In a side-by-side comparison (Blum et al. 2009) of inhibitor-based probes and substrate-based probes, which were pioneered by Weissleder and colleagues [see for example (Weissleder et al. 1999)], for cysteine cathepsins, Bogyo and colleagues show that both can be used for *in vivo* imaging; however, the rapid uptake and clearance of ABPs reduces background, thus improving image contrast. For further discussion of the use of PET in conjunction with ABPs and the advantages/disadvantages of substrate-based probes versus ABPs, see auf dem Keller and Schilling (2010). Nonetheless, the potential clinical applicability of substrate-based probes has been established as colonic adenocarcinomas in mice could be detected non-invasively with micro-fiberoptic catheters due to the presence in these tumors of the cysteine protease cathepsin B (Alencar et al. 2007). Multimodal activatable optical (fluorescence) and MRI

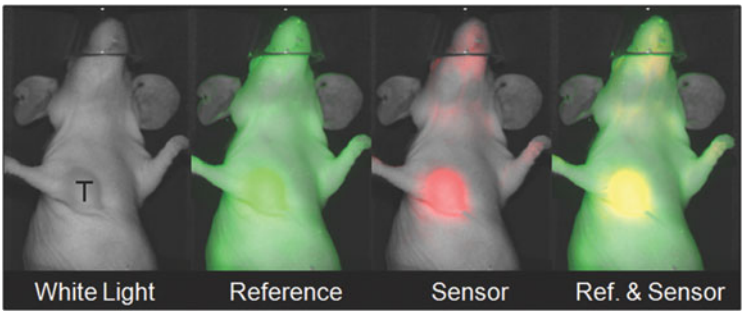

Fig. 15.6 *In vivo* imaging of tumor-associated MMP-9 activity with a *two-color* NIR proteolytic beacon, PB-M9nir. An orthotopic MDA-MB231 tumor, established in a *nu*/*nu* mouse, was imaged using a Pearl® Impulse imaging system (LI-COR Biosciences: White light, 800 nm Reference and 700 nm Sensor) ~4 h post retro-orbital administration of ~2 nmol PB-M9nir (Samuelson, Matrisian and McIntyre, unpublished studies)

probes for caspases have been developed, but there are not yet reports on their use in vivo (Kikuchi 2010). While the aforementioned imaging reagents/methods will be important for defining the roles of proteases *in vivo* and determining the efficacies of therapies that alter protease activities, they will require further validation before moving to clinical use. On the other hand, there are now reports that imaging protease activity during surgery allows one to distinguish tumor margins and improve excision of the tumors. Tsien and colleagues have shown that protease activatable cell penetrating peptides injected i.v., allow one to visualize the invasive margins of tumors in mice and by so doing during tumor removal improves tumor-free and overall survival (Nguyen et al. 2010). These dendrimeric probes detect activities of MMP-2 and -9 by either fluorescence or MRI (Olson et al. 2010). Recently, topical application of quenched fluorescent activity-based probes has been shown to detect active cysteine cathepsins and shown useful for the visualization and resection of gliomas in mice (Cutter et al. 2012). This latter methodology may be better applicable to the clinic as when these authors compared topical and systemic delivery of the probes they showed that topically applied probes were activated within 5 min and differentiated tumor margins from normal tissue. These examples of the successful use of active proteases/protease activity to monitor and improve surgical removal of tumors are particularly exciting developments as they have the potential for rapid translation to the clinic and for making a positive impact on cancer treatment.

Overall, this chapter summarizes the strong rationale for focusing on proteases in cancer. As described above, recent advances in imaging technology will greatly improve our ability to determine which proteolytic activities should be inhibited, as well as provide potential biomarkers for disease presence and response to therapy, including surgery. Continued efforts to develop efficacious inhibitors will hopefully yield targeted-therapies with minimal side-effects, which can be combined with existing drugs or with each other to treat multiple types of cancer.

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