Review Open Access

Barbara Breznik, Helena Motaln and Tamara Lah Turnšek*

Proteases and cytokines as mediators of interactions between cancer and stromal cells in tumours

DOI 10.1515/hsz-2016-0283
Received September 12, 2016; accepted December 14, 2016; previously published online December 20, 2016

Abstract: Proteolytic enzymes are highly relevant in different processes of cancer progression. Their interplay with other signalling molecules such as cytokines represents important regulation of multicellular cross-talk. In this review, we discuss protease regulation mechanisms of cytokine signalling in various types of cancer. Additionally, we highlight the reverse whereby cytokines have an impact on protease expression in an autocrine and paracrine manner, representing complex feedback mechanisms among multiple members of these two protein families. The relevance of the protease-cytokine axis is illustrated in glioblastoma, where interactions between normal mesenchymal stem cells and cancer cells play an important role in this very malignant form of brain cancer.

Keywords: cellular cross-talk; glioblastoma; invasion; mesenchymal stem cells; protease-cytokine signalling.

Introduction

Proteases (also termed proteolytic enzymes, proteinases and peptidases) irreversibly catalyse the hydrolysis of peptide bonds by attacking the carbonyl group of the peptide bond. Each protease is assigned to a family on the basis of similarities in the primary structure, and families that are homologous are grouped together in clans. A clan comprises proteases with the same catalytic mechanism, based on the active site amino acid, i.e. aspartic, cysteine, glutamic, metallo, serine, threonine, but there are also proteases where these are unknown or mixed in addition to the asparagine peptide lyases (Rawlings et al., 2016). Proteases are deposited in the comprehensive MEROPS database, where on June 2016 over 4400 protease genes were described (http://merops.sanger.ac.uk/about/merops.shtml). Intracellular and secreted proteases are responsible for both total protein degradation and limited proteolysis that control various key physiological processes such as cell cycle progression, cell death, tissue remodelling, homeostasis, wound healing and immune responses (López-Otín and Bond, 2008).

To preserve homeostasis under normal physiological conditions, protease activity is tightly regulated at different levels, from genetic and epigenetic factors controlling gene expression and protein biosynthesis, and post-translational modifications that affect trafficking and compartmentalisation of proteins, to zymogen activation. Ultimately, activity is also controlled by the abundance of selective endogenous protease inhibitors (Lah et al., 2006; Turk et al., 2012). Collectively, the complex network and interactions between proteases, their inhibitors and substrates is called the degradome (López-Otín and Overall, 2002), the functionality of which is dependent on cellular context and tissue physiology. Furthermore, proteases are involved in a highly organised network of proteolytic events and a hierarchical cascade of steps, which Turk et al. (2012) described as protease signalling, where the initial proteolytic step does not cause complete substrate degradation, but rather its activation, thus initiating a chain of reactions that lead to specific biological effects.

In humans, 1208 and 1857 known and putative proteases and protease inhibitor genes have been identified, respectively (Rawlings et al., 2016), representing ~7% of the genome (Verbovšek et al., 2014, 2015). When

***Corresponding author: Tamara Lah Turnšek,** Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia; Jožef Stefan International Postgraduate School, Jamova 39, SI-1000 Ljubljana, Slovenia; and Department of Biochemistry, Faculty of Chemistry and Chemical Engineering, University of Ljubljana, Večna pot 113, SI-1000 Ljubljana, Slovenia, e-mail: Tamara.Lah@nib.si
Barbara Breznik: Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia; and Jožef Stefan International Postgraduate School, Jamova 39, SI-1000 Ljubljana, Slovenia
Helena Motaln: Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

homeostasis is challenged, the protease-inhibitor-substrate balances may become disrupted, resulting in altered protease signalling. This may be either the cause or the consequence of a diseased state, as is the case in various inflammatory conditions, neurodegenerative and cardiovascular diseases, viral infections, atherosclerosis and osteoporosis. In cancer, the altered protease signalling is termed the "cancer degradome" (López-Otín and Overall, 2002). Protease signalling is coupled to other types of cellular signalling, altogether being involved in various biological and pathological processes. Typical examples in cancer are the interplay between kinases and proteases (López-Otín and Hunter, 2010), and between proteases and cytokines (Mason and Joyce, 2011) that modulate angiogenesis, extracellular matrix (ECM) composition and integrity, cancer cell invasion and other signalling pathways in the tumour microenvironment (TME). In tumour progression, the deregulation of protease homeostasis plays a highly relevant role (Lah et al., 2006; López-Otín and Bond, 2008; Verbovšek et al., 2014, 2015), where cancer and stromal cells contribute to a complex proteolytic network within the TME (Mason and Joyce, 2011), having both pro- and anti-tumorigenic effects (López-Otín and Matrisian, 2007).

The role of various protease classes in cancer

The most extensively studied class of proteases in cancer are matrix metalloproteases (MMPs), which are the zinc-dependent endopeptidases, classified on the basis of protein-domain structure and sequence homology into the four main subclasses: collagenases (MMP-1, MMP-8 and MMP-13), MMPs of the stromelysin subclass (MMP-3, MMP-10, MMP-11, MMP-7 and MMP-26), gelatinases (MMP-2 and MMP-9) and membrane-type MMPs, termed MT1-MMP to MT6-MMP, alternatively classified as MMP14-17, MMP-24, and MMP-25 (Cathcart et al., 2015; Rawlings et al., 2016). These MMPs directly degrade ECM proteins, but differ in substrate specificity. MMPs are counteracted by their endogenous tissue inhibitors TIMPs (1–4), nearly all of them playing a distinct role in cancer progression (Yamamoto et al., 2015). Besides the MMP activity regulation by TIMPs, extracellular MMP activity is also regulated by their binding to ECM proteins and cell surface molecules, such as integrins (β1 subunit) and tetraspanins and by endocytotic trafficking (Yamamoto et al., 2015). A related protease family consists of a disintegrin and metalloprotease with thrombospondin motifs (ADAMTSs), which mostly act in the pericellular milieu. These proteases can be secreted by cancer and stromal cells and may contribute to modifying the TME by multiple mechanisms, related to both oncogenic and tumour-protective functions (Cal and López-Otín, 2015).

Another frequently investigated class of proteases in cancer progression are the serine proteases, subdivided with respect to their substrate specificity as trypsin-like and chymotrysin-like serine endopeptidases (Rawlings et al., 2016). Among these serine endopeptidases, the plasminogen/plasmin activator system and kallikreins have received most attention in oncology. Urokinase-type plasminogen activator (uPA), its receptor (uPAR) and serpin inhibitors PAI-1 and -2 comprise the cancer membrane degradome system, which activates plasminogen to plasmin, a feedback activator of PAs and various MMPs that then degrade ECM proteins (Lah et al., 2006; Mason and Joyce, 2011). In concert with cysteine cathepsins, uPA/uPAR signalling induces activation of a proteolytic cascade, which facilitates cancer cell invasion. Besides proteolytic activity, the uPA/uPAR system has important proteolyisis independent functions in cancer cell invasion, as plasma membrane linked uPAR interacts directly with integrins and vitronectin, thereby mediating cell adhesion and migration (Rao Malla et al., 2013). Because expression of uPA, uPAR and PAI-1 is increased in many types of cancers, they were among the first proteins that were proposed as prognostic biomarkers for cancer patients, in particular for breast cancer (Schrohl et al., 2003; Lah et al., 2008).

Human tissue kallikreins (hKs) comprise the family of 15 homologous secreted serine proteases with trypsin or chymotrypsin-like activities and are aberrantly expressed in different types of cancers, as recently reviewed (Avgenis and Scorilas, 2016). The best known is hK3 (prostate-specific antigen, PSA) used as a biomarker for detection and prognosis of prostate cancer, although its relevance is questionable, as it is also highly increased in inflammation that is not related to neoplasms (Diamandis 2012). Despite the fact that roles of distinct hKs in cancer are not completely understood, studies indicates that hK-mediated pericellular proteolysis may be important in cancer cell invasion, angiogenesis and growth in particular due to modulating the activities of various cytokines, growth factors and other proteases (Avgenis and Scorilas, 2016). As mentioned above (López-Otín and Matrisian, 2007), not all proteases are tumour promoting and, for example, Strojnik et al. (2009) found downregulation of hK6 in glioma progression, although expression of this kallikrein in tumours had no impact on patients' survival.

Originally recognised by their intralysosomal activity, cysteine proteases such as papain-like cathepsins and caspases are now known to be active in other subcellular compartments as well extracellularly, and cysteine proteases and caspases are implicated in cancer development and progression. Human cysteine cathepsins comprise an 11-member family: cathepsins B, C, F, H, K, L/V, O, S, Z, X and W (Kos et al., 2015; Rawlings et al., 2016). Although initially associated with increased metabolism and lysosomal protein degradation in cancer cells, diverse and distinct other functions of cathepsins have later been revealed, for example, their involvement in resistance development to therapeutics (Olson and Joyce, 2015). The expression pattern of cathepsins in different types of cancer is rather diverse and again, not all cathepsins are pro-tumorigenic in all cancers. For example, Reinheckel et al. (2012) demonstrated that cathepsin L possesses tumour suppressor anti-proliferative activity in the Rip1-Tag2 mouse model, but enhances tumorigenesis in APC^{min} and K14-HPV16 mice. We found that in glioblastoma cathepsin L upregulation has an anti-apoptotic effect by increasing caspase 3 synthesis (Kenig et al., 2011), supporting earlier reports that cathepsin L facilitates drug resistance (Lankelma et al., 2010).

Caspases are the most relevant proteases in initiation and execution of apoptosis and altered apoptotic signalling pathways are one of the hallmarks of cancer (Hanahan and Weinberg, 2011). The caspase cascade is initialised by caspases 2, -8, -9 and -10, which in various pathways directly or indirectly activate effector caspases -3, -6 and -7 triggering apoptosis (Mason and Joyce, 2011). Downregulation of caspases can lead to impaired apoptosis, higher cancer cell survival and therapy resistance. For example, progression of some types of cancer, including glioblastoma, has been associated with a lack of caspase-8 expression. On the other hand, caspase-8 was found to be upregulated in other types of cancers and promotes malignancy probably by its other non-apoptotic functions such as inducing NF-κB signalling, altering endosomal trafficking, regulating autophagy and enhancing cellular adhesion and migration (Stupack 2013).

The interplay between mitochondrial and lysosomal compartments in cell death has been established as well as a distinct role of lysosomal cathepsins in apoptosis (Droga-Mazovec et al., 2008). Cysteine cathepsins B, L, S and K cleave Bid and Bcl-2 homologues, which can when released from damaged lysosomes not only affect mitochondrial membrane integrity, but also enhance caspase activity through degradation of the caspase endogenous inhibitor XIAP. Therefore, the cellular compartment and context of lysosomal protease activity dictates the substrate targets and pro- versus anti-tumorigenic functions.

Proteases in stromal cells

An additional complexity of the role of proteases in cancer is related to their expression and regulation in tumour-associated stromal cells, including endothelial cells, fibroblasts, immune cells and mesenchymal stem cells (Hanahan and Weinberg, 2011). As active participants in TME, these stromal cell support cancer cell proliferation, invasion, metastasis and drug resistance (Bougnaud et al., 2016). However, the expression pattern of proteases in stromal cells and their processing may differ from that of cancer cells, and thus may have a different impact on tumour progression (Olson and Joyce, 2015). For example, Gocheva et al. (2010) observed that cathepsins B and S secreted from tumour-associated macrophages (TAMs) in a Rip1-Tag2 pancreatic neuroendocrine tumour (PanNET) mouse model are predominantly responsible for pancreatic tumour growth, angiogenesis and invasion *in vivo*. In contrast, cathepsin L expressed by cancer cells has a pancreatic neuroendocrine tumour promoting role. This indicates that there are different and even opposite effects of proteases expressed by cancer and stromal cells, depending on the type of cancer. In other words, the heterogeneity and plasticity of the tumour in response to cancer cell evolution may differentially affect the protease web, depending on the cellular context. As a consequence, selective pressure resulting from these cellular cross-talks is also pushing cancer cells to compensation of one type of protease, when this protease is not expressed or inhibited, by the activation of other protease(s), as was suggested by Akkari et al. (2016). It was demonstrated that high amounts of cathepsin X are present in TAMs as a functional compensation of cathepsin B and cathepsin S depletion in RT2 PanNET mouse model (cathepsin $B^{-/-}S^{-/-}$ RT2 mice). Interestingly, this compensation occurs in a tumour stage-dependent manner, empasizing the plasticity of the TME.

These phenomena may be a reason that protease inhibitors, such as MMP inhibitors, failed in clinical trials as anti-cancer agents, because resistance to specific protease inhibitors may be due to compensation by non-targeted types of protease(s) (Cathcart et al., 2015). For example, enhanced invasion of MMP-9-deficient tumours is associated with homing of cathepsin B-expressing leukocytes to invasive tumour fronts, facilitating invasion (Shchors et al., 2013). Finally, inefficacy of protease

inhibitors may be due to non-proteolytic roles of proteases such as their interaction with other proteins (Kessenbrock et al., 2015). For example, TAM-derived and cancer cell-derived pro-form of cathepsin X promotes cancer cell adhesion and migration through binding to the RGD domain of integrins on cancer cell membranes (Kos et al., 2015). Furthermore, inhibitors that target the hemopexin domain (HPX) in MMPs, which is located at the C-terminal of most MMP members and is crucial for protein-protein interactions, block tumour growth (Kessenbrock et al., 2015). Taken together, it means that the cellular context, the redundancy and selectivity of proteases make therapeutic targeting of proteases in cancer difficult.

Protease signalling: cytokines as protease substrates

Cytokines are a heterogeneous group of small soluble signalling molecules comprising chemokines, interleukins (ILs), interferons (IFNs) and some growth factors such as TGF-β (Seruga et al., 2008). Cytokines exert their function via binding to their membrane-associated receptors. Each group of cytokines binds to corresponding receptors, classified as type I and II cytokine receptors, immunoglobulin, TNF, TGF-β and chemokine receptors (Borish and Steinke, 2003). Cytokines are secreted by both cancer cells and stromal cells, and act as means of communication among these cells. Cytokines can act in autocrine or paracrine manners to exert their functions such as regulating cell proliferation in the TME. Additionally, cytokines enhance cancer cell invasion and tumour angiogenesis by inducing the expression of invasion-associated proteolytic enzymes (Kenig et al., 2010; Sevenich and Joyce, 2014) as is discussed in the next chapter.

Chemotactic cytokines – chemokines play a crucial role by regulating recruitment of immune cells to tumours. The chemokine subfamily is classified according to the position of the first cysteines at the NH_2-terminus of the protein into four groups: CXC, CC, C and CX3C. Chemokine receptors are G-protein-coupled receptors and bind to more than one type of chemokine. These chemokine receptors are grouped into four classes depending on the type of binding of the chemokine: CXCR, CC, CX3CR1, XCR1. The chemokine receptors CXCR4, CXCR5, CXCR6, CCR6, CCR9 and CX3CR1 bind only one ligand (Borish and Steinke, 2003). During cancer progression the protease network interferes with chemokine signalling and thus affects recruitment and proliferation of immune cells to the tumours, where they can have either pro- or anti-inflammatory and even immunosuppressive activity. However, there is a high redundancy among chemokines with respect to target cell selectivity and in many cases it is difficult to ascribe a distinct role to a single chemokine (Wolf et al., 2008).

Proteases affect cytokine expression by different signalling pathways and affect their activity post-translationally by proteolytic processing. For example, cathepsin S functions as transcriptional mediator of the pro-inflammatory chemokine CCL2/MCP-1 *via* type I transmembrane glycoprotein CD74 in cancer cells. Within endosomes, cathepsin S is the key protease responsible for the cleavage of CD74 and liberation of its intracellular domain (CD74-ICD), being then translocated into the nucleus, where it interacts with NFκB, that in turn regulates the expression of CCL2/MCP-1, among others. Accordingly, downregulation of cathepsin S expression in cancer cells reduces macrophage recruitment to the tumour site and correlates with reduced tumour growth in mice (Wilkinson et al., 2015). Hu et al. (2014) demonstrated that activation of the uPA-uPAR axis in cancer cells increases gene expression and protein secretion of IL-4 via ERK1/2 signalling and TGF-β by a thus far unknown mechanism. Both cytokines are responsible for increased mobilisation and polarisation of macrophages towards the M2 status, which promotes tumour progression.

Proteases selectively cleave cytokines leading either to activation or inactivation of cytokine function or altered receptor specificity (Wolf et al., 2008). Some cytokines thus require proteolytic activation to exert their function. For example, both MMP-8, -9, and cathepsin L process IL-8 into an up to 30-fold more active conformation after N-terminal cleavage (Van Den Steen et al., 2003). Moreover, a positive feedback loop is created as IL-8 induces MMP-8 and -9 release from inflammatory cells, which contributes to ECM degradation. Additionally, elevated levels of IL-8 in glioma cells trigger the epithelial-mesenchymal transition (EMT) and enhances their invasiveness (Zhang et al., 2015). Other examples are ADAM-17 and MMPs, such as MMP-2, -9 and -14, that convert the inactive stromal macrophage and T cell membrane-bound precursor of TNF-α into the soluble pro-inflammatory cytokine. Active TNF-α then increases infiltration of tumour-promoting macrophages into the tumour. TNF-α also increases cancer cell proliferation *via* the transcription factor NF-κB (Kessenbrock et al., 2010).

On the other hand, proteases may inactivate cytokines and alter their receptor binding, impairing cancer progression. For example, MMPs, such as MMP-1, -2, -3, -9, -13 and -14, cleave CXCL12/SDF-1α that results in loss of its ability to bind to its receptor CXCR4 (Manicone and McGuire, 2008).

This specific MMP activity inhibits local chemotaxis of endothelial cells of existing vessels and prevents tumour angiogenesis (Yamada et al., 2015). Reduced chemotactic activity of tumours towards lymphocytes, expressing CXCR4 receptors, was found to be caused by cleavage of its ligand CCL22, by the tumour-derived serine protease CD26/dipeptidyl-peptidase IV (Mortier et al., 2016).

Proteases regulate cytokine bioavailability by proteolytic processing of ECM proteins, that are the essential cytokine binding partners. By limited proteolytic processing of the ECM, active cytokines are liberated. Extracellular MMP-2 and membrane-associated MMP-14 indirectly activate TGF-β by releasing its binding to latent TGF-β-binding protein 1 (LTBP-1), which is an ECM component (Kessenbrock et al., 2010). TGF-β signalling in combination with cathepsin B activity in direct co-cultures of human melanoma cells and fibroblasts *in vitro* activates the fibroblasts in a similar manner as stromal fibroblasts are activated *in vivo*, thereby inducing cancer cell invasion (Yin et al., 2012). In addition, proteases orchestrate recruitment of leukocytes and promote tumour-associated inflammation by modulating cell surface proteoglycans. For example, MMP-7 shedding of heparin sulphate proteoglycan syndecan-1 releases the CXCL1-syndecan-1 complex from the cell surface to generate a chemokine gradient for recruitment of tumour-promoting neutrophils into the tumour (Wolf et al., 2008).

Proteases are also key players in the formation of metastatic and cancer stem cell niches. For example, MMP-9 is involved in pre-metastatic niche formation by cleaving the cytokine c-KitL (soluble Kit ligand, stem cell ligand), which releases c-KitL from the surface of stromal cells such as endothelial cells and fibroblasts at secondary tumour sites distant to the primary tumour. This cytokine then recruits hematopoietic progenitor cells from the bone marrow into the niche to create a metastasis-supporting TME, which sustains metastatic cell growth (Kessenbrock et al., 2010). An example of proteases that contribute to cancer stem cell niche formation are MMPs and cathepsin K. The latter has been associated with periarteriolar stem cell niches where it co-localises with CXCL12/SDF-1α (Hira et al., 2015). This chemokine attracts glioblastoma cells into niches, where the cells become glioblastoma stem-like cells. It is hypothesised that focal activation and N-terminal SDF-1α cleavage by cathepsin K inactivates this chemokine, which possibly induces release of stem cells from the niche and promotion of glioblastoma stem-like cell invasion as well as their differentiation (Verbovšek et al., 2015).

Taken together, proteases affect cytokine signalling in various ways; proteases directly activate or inactivate cytokines by proteolytic cleavage or liberate cytokines from the ECM. As a consequence, cytokines (a) affect recruitment and proliferation of immune cells, (b) impact proliferation and migration of cancer cells, and (c) sustain metastatic niches and cancer stem cell niches and thus affect tumour cell homing. Proteases, released from both, cancer cells and stromal cells are responsible for the fine tuning of cytokine function in the complex TME, as shown in Figure 1.

Cytokine signalling affects protease activity

Cytokines, as major mediators of paracrine cell signalling, are potent regulators of protease activity, virtually affecting all types of cells in the TME (Figure 1). Pro-inflammatory cytokines stimulate expression, secretion and activation of MMPs in cancer cells, as well as in various stromal cell types (Sevenich and Joyce, 2014). Stromal cells secrete cytokines, which induce the expression of MMPs in cancer cells and thus promote their invasion, as was illustrated by Kenig et al. (2010) in an *in vitro* glioma and endothelial cell co-culture. Endothelial cell-derived SDF-1α upregulates the expression of MMP-9 together with cathepsins B and S in glioblastoma cells that enhance invasiveness. Similar observations were made *in vivo* by Wang et al. (2013) when co-injecting endothelial and hepatocellular carcinoma cells into nude mice and found an increased tumorigenicity of the carcinoma cells. This was associated with expression of MMP-2 and -9 in hepatocellular carcinoma cells, induced by the cellular cross-talk *via* secreted CCL2/MCP-1, IL-8 and IL-16 (Wang et al., 2013).

A vast body of literature describes infiltrating inflammatory cells, such as macrophages, dendritic cells, mast cells, as well as neutrophils and B and T lymphocytes that produce a variety of pro-inflammatory cytokines, inducing MMP activity in the TME. For example, macrophage-derived IL-1β induces the expression of MMP-1, -3, -10 and -14 in metastatic renal cell carcinoma *via* a mechanism dependent on activation of the transcription factor CCAAT-enhancer binding protein β (CEBPβ) by IL-1β (Petrella and Vincenti, 2012). On the other hand, cancer cells upregulate by secretion of soluble cytokines the activity of MMPs in stromal cells. For example, IL-6 is one of the major inflammatory cytokine in cancer progression. IL-6, produced by breast cancer cells induces expression and secretion of MMP-2 and -9 and cathepsin B from tumour-associated monocytes *in vitro* (Mohamed et al., 2010).

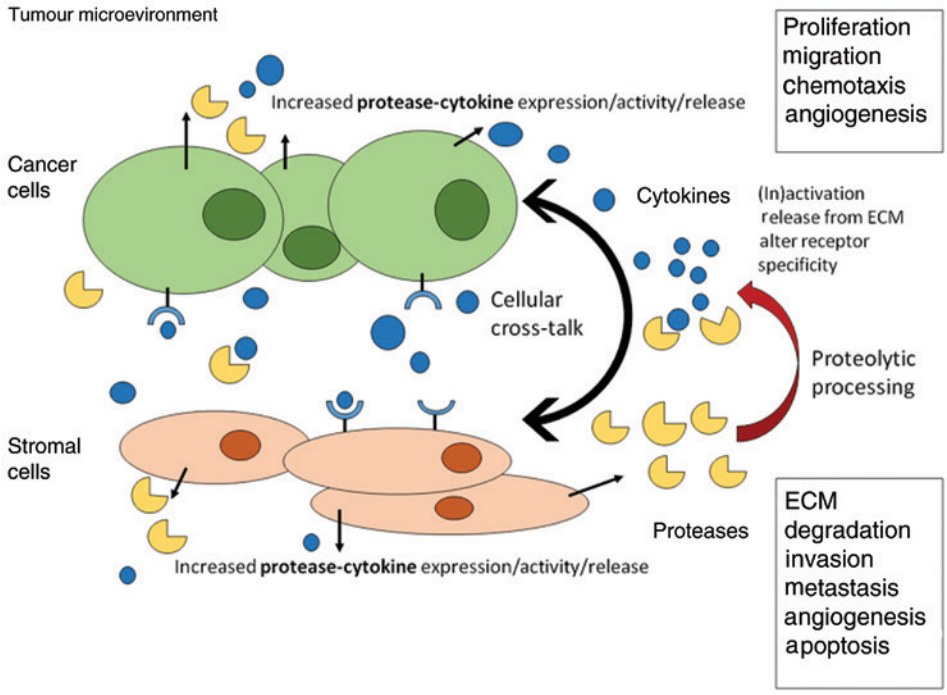

Figure 1: Proteases and cytokines as mediators of cellular cross-talk in the tumour microenvironment that promotes tumour progression. Schematic presentation of interactions between cancer and stromal cells within the tumour microenvironment, that are mediated by the protease-cytokine interplay. Cytokines induce protease expression and activity in stromal and cancer cells as well as induce their release, whereas proteases can also induce cytokine expression. Proteases by proteolytic processing alter cytokine activity, their receptor specificity and/or their bioavailability that may lead to altered cancer cell and stromal cell proliferation and migration, recruitment of other cell types such as immune cells, fibroblasts and mesenchymal stem cells to the tumour site and angiogenesis of the tumour. On the other hand, increased activity of proteases in tumour stroma affects ECM degradation, invasion, metastasis, angiogenesis and apoptosis.

Fibroblasts are an important component of the TME and are a source of cytokines. For example, a dynamic interplay between colon cancer cells and fibroblasts *via* activated TGB-β signalling leads to activation of serine protease uPA, its inhibitor PAI-1 and some types of MMPs, as well as the autocrine TGF-β loop in normal fibroblasts, transforming them in cancer-associated fibroblasts (CAFs), playing an important role in various types of cancer (Hawinkels et al., 2014).

Cathepsins in the TME may originate from various cell types (Mohamed et al., 2010; Mason and Joyce, 2011). Pro-inflammatory IL-4 upregulates cathepsin B and S expression in TAMs that enhances pancreatic cancer cell invasion *in vivo* and *in vitro*, and promotes pancreatic cancer growth as well as angiogenesis in a PanNET mouse model (Gocheva et al., 2010). Increased levels of cathepsin K in fibroblasts enhances invasiveness of squamous cancer cells in fibroblast/cancer cells co-cultures *in vitro* by cancer cell derived IL-1α (Xie et al., 2011).

Cytokine-regulated activity of cathepsin L (Lankelma et al., 2010; Kenig et al., 2011) and cathepsin B (Bruchard et al., 2013) has been reported to be important in the development of chemo-resistance. It has been also shown (Tuomela et al., 2013) that cancer cells take DNA from dead cancer cells, killed by chemo-therapy, inducing expression of Toll-like receptor TLR9. This was mediated by MMP-13 activation and downregulation of TIMP-3 expression.

Taken together, cytokines that are secreted into the TME from endothelial cells, immune cells and fibroblasts affect the expression of all classes of proteases in an autocrine and paracrine manner usually to promote tumour progression.

Case study: interactions between mesenchymal stem cells and glioblastoma

An additional component of the TME are mesenchymal stem cells (MSCs), although their exact role in brain tumour and progression is still not fully understood (Barcellos-de-Souza et al., 2013). Tumour-secreted cytokines and growth factors such as TGF-β, VEGF and IL-8 (Motaln et al., 2012)

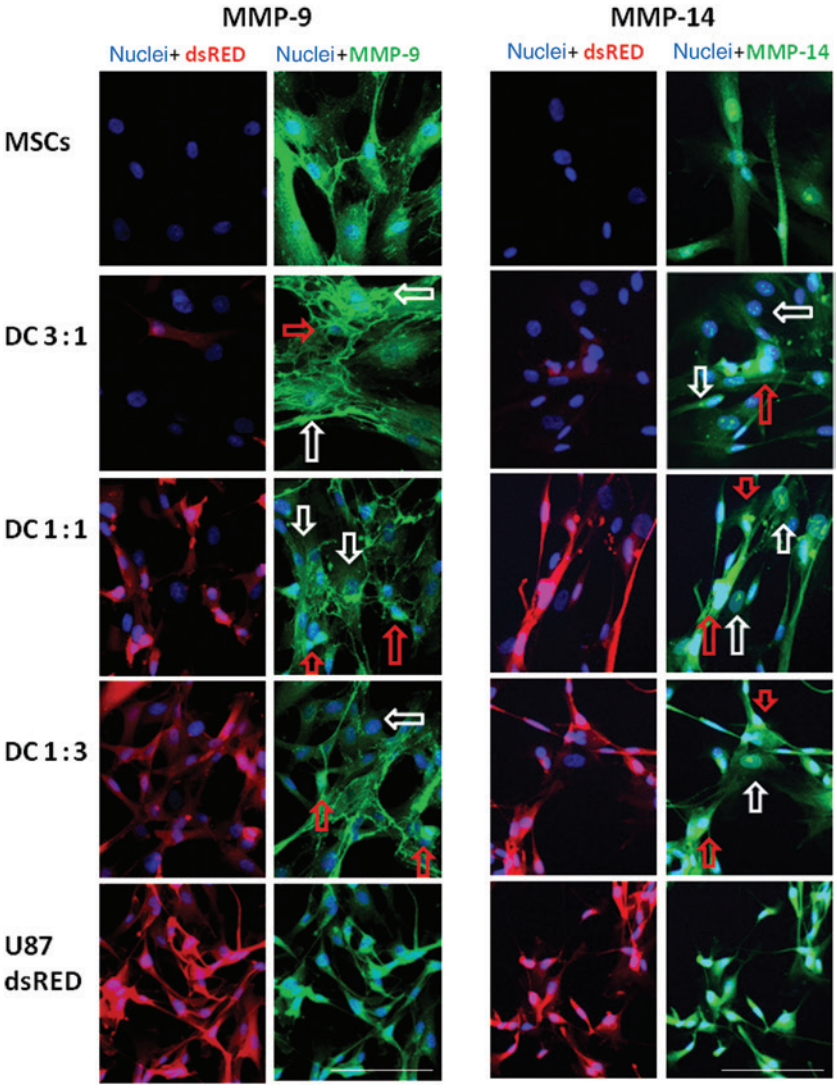

Figure 2: Localization of MMP-9 and MMP-14 in MSCs, U87dsRED cells and their direct co-cultures (DC).
After 3 days of direct co-culturing in three different MSC-U87dsRED ratios (DC 1 : 3, 1 : 1 and 3 : 1), immunocytochemistry was performed. Cells were fixed using 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and incubated with anti-MMP-9 (1 : 100 dilution) or anti-MMP-14 (1 : 200) primary antibody (both Abcam, Cambridge, UK), followed by incubation with secondary antibody conjugated with Alexa Fluor 488 (green; Applied Biosystems, Carlsbad, CA, USA). Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Life technologies, Carlsbad, MO, USA) and analysed by inverted fluorescence microscope Nikon (Tokyo, Japan; 200× original magnification; scale bar = 100 μm). Images taken from DAPI and dsRED (blue nuclei, red U87 cells) and DAPI and MMP-9/MMP-14 (blue nuclei, green antibody) were merged, respectively. White arrows indicate protease expression in MSCs and red arrows in U87dsRED cells.

mediate tropism and homing of MSCs from bone marrow or endogenous brain tissue niche to the brain tumour glioblastoma (Schichor et al., 2012). Due to their high expression of cytokines and their receptors, MSCs play an important immunomodulatory role in TME. Furthermore, we showed that MSCs in the presence of glioblastoma cells overexpress and secrete a range of cytokines and chemokines such as TGF-β, IL-1α and β, IL-6, IL-8, CCL2, CCL13, CCL20, CCL26, CXCL1-2, CXCL6, CXCL12, in particular CCL2/MCP1 (Motaln et al., 2012; Motaln and Turnsek, 2015). Increased levels of inflammatory cytokines enhance in autocrine manner expression of proteases such as MMP-2, -9 and -14 in MSCs that promote MSC motility towards the tumour (Ries et al., 2007). Ho et al. (2009) showed that MMP-1 is crucial for MSC migration toward glioma *in vivo*, as well as *in vitro*. MMP-1 cleaves G-protein-coupled receptor PAR1 on MSC surface, triggering intracellular signalling, which promotes MSC glioma tropism. Gutova et al. (2008) confirmed that MSC tropism is also mediated through uPA and uPAR overexpression in GBM cells, besides increased levels of cytokines IL-6, IL-8 and CCL2/MCP-1 in conditioned medium of glioblastoma cells.

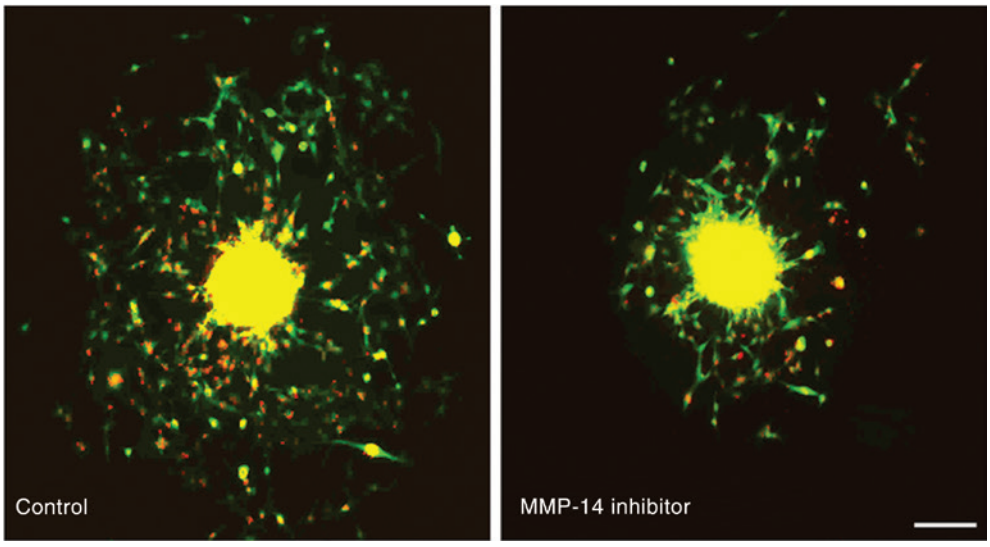

Figure 3: Invasion of DiI-labelled MSCs (red) and U373 eGFP cells (green) out of mixed spheroids upon treatment with a selective inhibitor of MMP-14.
For monitoring of invasion of MSCs and GBM cells out of MSC/GBM mixed spheroids, MSCs were labelled prior to spheroid formation with the fluorescent dye Vybrant DiI (Molecular Probes, Eugene, OR, USA). For mixed spheroid formation, DiI-MSCs and U373 eGFP cells were mixed in a 1 : 1 ratio and seeded in medium containing 4% methylcellulose (Sigma-Aldrich) in U-bottomed 96-well plates (BD Biosciences, San Jose, CA, USA), which were centrifuged at 850 *g* for 90 min, and then incubated overnight. Generated MSC/GBM mixed spheroids were transferred in laminin-coated wells and treated with MMP-14 inhibitor NSC405020 (Tocris Bioscience, Bristol, UK) in a final non-cytotoxic concentration 10 μM, diluted in 0.1% DMSO, or with control (0.1% DMSO). Cell invasion was monitored using an inverted fluorescence microscope with NIS elements software (Nikon; 40× original magnification; scale bar = 200 μm) after 72 h. Images taken using either TRITC (red DiI-MSCs) or FITC (green U373 eGFP cells) philtres were merged, respectively.

Glioblastoma-associated MSCs also affect endothelial cells and immune cells in stroma, thus increasing intra-tumoural protease activity, which facilitates tumour progression. Macrophages, which express high levels of cathepsins B, L and S are attracted to tumours by cytokines such as IL-6 and CCL2/MCP-1 that are secreted by activated MSCs (Akkari et al., 2016). Furthermore, the MSCs promote macrophage polarisation into the tumour promoting M2 type, that is associated with immunosuppressive effects (Jia et al., 2016).

MSCs may enhance cancer cell invasion in a paracrine manner *via* protease activty regulation, as Swamydas et al. (2013) showed *in vitro*. MSCs in mammary cancer cells activate MMP-9, -13 and -14 through chemokines CCL-5 and CCL-9. Therefore, we were interested whether protease expression and activity is also affected by direct cross-talk between bone marrow-derived MSCs and glioblastoma cells. To analyse the cellular origin of invasion-related proteases MMP-9 and -14 in direct co-cultures of bone marrow-derived MSCs and glioblastoma cells and to distinguish between the two cell types in the direct co-culture set up, we used U87 cells, which stably expressed dsRED fluorescent protein (U87 dsRED). Our preliminary results of immunocytochemical analysis of MSC/U87 direct co-culture shows that both MSCs and U87 cells are important sources of proteases such as MMP-9 and -14 (Figure 2). This indicates that MSC-derived MMPs also contributed to the complex proteolytic signalling in the glioblastoma TME, possibly regulating ECM degradation and enabling glioblastoma cell invasion into surrounding healthy brain parenchyma. Furthermore, our unpublished findings indicate that direct cross-talk between MSCs and GBM cells in 3D-spheroids indeed enhances U373 cell invasion *via* MMP-14 upregulation, as a selective MMP-14 protease inhibitor decreases both MSCs and U373 cell invasion out of MSC/U373 mixed spheroids (Figure 3). As secretion of CCL2/MCP-1, which is an activator of MMPs via ERK 1/2signalling (Yang et al., 2016), is increased in direct MSC and glioblastoma co-cultures (Motaln and Turnsek, 2015), it is possible that paracrine signalling through CCL2 enhances MMP activity and drives glioblastoma cell invasion.

Conclusions

Proteases are key regulators of cancer progression and metastasis, and proteases in cancer cells as well as in stromal cells, such as fibroblasts, MSCs, immune cells and endothelial cells, are all part of the TME. These

non-neoplastic cells actively contribute to the proliferative and invasive behaviour of cancer cells by secretion of proteases and cytokines. As proteases and cytokines exhibit both tumour-promoting and tumour-suppressive functions, it is important to develop therapeutic strategies that target tumour-promoting functions and restore those that suppress tumorigenesis.

Acknowledgements: We are grateful to Dr. Belinda Parker, Latrobe Institute for Molecular Science, La Trobe University, Melbourne, Australia, for reviewing the manuscript and for her valuable comments. We thank Mrs. Monika Golob for technical assistance with the immunocytochemical analysis. This work was supported by the ARRS project J1-4274, ARRS Programme P1-0245 (all to T.T.L.) and the ARRS Young Researcher grant (to B.B.).

References

Akkari, L., Gocheva, V., Quick, M.L., Kester, J.C., Spencer, A.K., Garfall, A.L., Bowman, R.L., and Joyce, J.A. (2016). Combined deletion of cathepsin protease family members reveals compensatory mechanisms in cancer. Genes Dev. *30*, 220–232.

Avgenis, M. and Scorilas, A. (2016). Kallikrein-related peptidases (KLKs) as emerging therapeutic targets: focus on prostate cancer and skin pathologies. Expert Opin. Ther. Targets *20*, 801–818.

Barcellos-de-Souza, P., Gori, V., Bambi, F., and Chiarugi, P. (2013). Tumor microenvironment: bone marrow-mesenchymal stem cells as key players. Biochim. Biophys. Acta *1836*, 321–335.

Borish, L.C. and Steinke, J.W. (2003). Cytokines and chemokines. J. Allergy Clin. Immunol. *111*, S460–S475.

Bougnaud, S., Golebiewska, A., Oudin, A., Keunen, O., Harter, P.N., Mäder, L., Azuaje, F., Fritah, S., Stieber, D., Kaoma, T., et al. (2016). Molecular crosstalk between tumour and brain parenchyma instructs histopathological features in glioblastoma. Oncotarget, *7*, 31955–31971.

Bruchard, M., Mignot, G., Derangère, V., Chalmin, F., Chevriaux, A., Végran, F., Boireau, W., Simon, B., Ryffel, B., Connat, J.L., et al. (2013). Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. Nat. Med. *19*, 57–64.

Cal, S. and López-Otín, C. (2015). ADAMTS proteases and cancer. Matrix Biol. *44–46*, 77–85.

Cathcart, J., Pulkoski-Gross, A., and Cao, J. (2015). Targeting matrix metalloproteinases in cancer: bringing new life to old ideas. Genes Dis. *2*, 26–34.

Diamandis, E.P. (2012). The failure of protein cancer biomarkers to reach the clinic: why, and what can be done to address the problem? BMC Med. *10*, 87.

Droga-Mazovec, G., Bojic, L., Petelin, A., Ivanova, S., Romih, R., Repnik, U., Salvesen, G.S., Stoka, V., Turk, V., and Turk, B. (2008). Cysteine cathepsins trigger caspase-dependent cell death through cleavage of bid and antiapoptotic Bcl-2 homologues. J. Biol. Chem. *283*, 19140–19150.

Gocheva, V., Wang, H.W., Gadea, B.B., Shree, T., Hunter, K.E., Garfall, A.L., Berman, T., and Joyce, J.A. (2010). IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. Genes Dev. *24*, 241–255.

Gutova, M., Najbauer, J., Frank, R.T., Kendall, S.E., Gevorgyan, A., Metz, M.Z., Guevorkian, M., Edmiston, M., Zhao, D., Glackin, C.A., et al. (2008). Urokinase plasminogen activator and urokinase plasminogen activator receptor mediate human stem cell tropism to malignant solid tumors. Stem Cells *26*, 1406–1413.

Hanahan, D. and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Hawinkels, L.J., Paauwe, M., Verspaget, H.W., Wiercinska, E., van der Zon, J.M., van der Ploeg, K., Koelink, P.J., Lindeman, J.H., Mesker, W., ten Dijke, P., et al. (2014). Interaction with colon cancer cells hyperactivates TGF-β signaling in cancer-associated fibroblasts. Oncogene *33*, 97–107.

Hira, V.V., Ploegmaker, S.K.J., Grevers, F., Verbovšek, U., Silvestre-Roig, C., Aronica, E., Tigchelaar, W., Turnšek, T.L., Molenaar, R.J., and Van Noorden, C.J. (2015). CD133$^+$ and Nestin$^+$ glioma stem-like cells reside around CD31$^+$ arterioles in niches that express SDF-1α, CXCR4, osteopontin and cathepsin K. J. Histochem. Cytochem. *63*, 481–493.

Ho, I.A., Chan, K.Y., Ng, W.H., Guo, C.M., Hui, K.M., Cheang, P., and Lam, P.Y. (2009). Matrix metalloproteinase 1 is necessary for the migration of human bone marrow-derived mesenchymal stem cells toward human glioma. Stem Cells *27*, 1366–1375.

Hu, J., Jo, M., Eastman, B.M., Gilder, A.S., Bui, J.D., and Gonias, S.L. (2014). uPAR induces expression of transforming growth factor β and interleukin-4 in cancer cells to promote tumor-permissive conditioning of macrophages. Am. J. Pathol. *184*, 3384–3393.

Jia, X.H., Feng, G.W., Wang, Z.L., Du, Y., Shen, C., Hui, H., Peng, D., Li, Z.J., Kong, D.L., and Tian, J. (2016). Activation of mesenchymal stem cells by macrophages promotes tumor progression through immune suppressive effects. Oncotarget *7*, 20934–20944.

Kenig, S., Alonso, M.B., Mueller, M.M., and Lah, T.T. (2010). Glioblastoma and endothelial cells cross-talk, mediated by SDF-1, enhances tumour invasion and endothelial proliferation by increasing expression of cathepsins B, S, and MMP-9. Cancer Lett. *289*, 53–61.

Kenig, S., Frangež, R., Pucer, A., and Lah, T. (2011). Inhibition of cathepsin L lowers the apoptotic threshold of glioblastoma cells by up-regulating p53 and transcription of caspases 3 and 7. Apoptosis *16*, 671–682.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell *141*, 52–67.

Kessenbrock, K., Wang, C.Y., and Werb, Z. (2015). Matrix metalloproteinases in stem cell regulation and cancer. Matrix Biol. *44–46*, 184–190.

Kos, J., Vižin, T., Fonović, U.P., and Pišlar, A. (2015). Intracellular signaling by cathepsin X: molecular mechanisms and diagnostic and therapeutic opportunities in cancer. Semin. Cancer Biol. *31*, 76–83.

Lah, T.T., Durán Alonso, M.B., and Van Noorden, C.J. (2006). Antiprotease therapy in cancer: hot or not? Exp. Opin. Biol. Ther. *6*, 257–279.

Lah, T.T., Obermajer, N., Duran Alonso, M.B., Kos, J. (2008). Cystein cathepsins and cystatins as caner biomarkers. In: The Cancer Degradome: Proteases and Cancer Biology, D. Edwards et al.,

eds. (New York: Springer Science and Business Media), pp. 575–613.

Lankelma, J.M., Voorend, D.M., Barwari, T., Koetsveld, J., Van der Spek, A.H., De Porto, A.P., Van Rooijen, G., and Van Noorden, C.J. (2010). Cathepsin L, target in cancer treatment? Life Sci. *86*, 225–233.

López-Otín, C. and Bond, J.S. (2008). Proteases: multifunctional enzymes in life and disease. J. Biol. Chem. *283*, 30433–30437.

López-Otín, C. and Hunter, T. (2010). The regulatory crosstalk between kinases and proteases in cancer. Nat. Rev. Cancer *10*, 278–92.

López-Otín, C. and Matrisian, L.M. (2007). Emerging roles of proteases in tumour suppression. Nat. Rev. Cancer *7*, 800–808.

López-Otín, C. and Overall, C.M. (2002). Protease degradomics: a new challenge for proteomics. Nat. Rev. Mol. Cell Biol. *3*, 509–519.

Manicone, A.M. and McGuire, J.K. (2008). Matrix metalloproteinases as modulators of inflammation. Semin. Cell Dev. Biol. *19*, 34–41.

Mason, S.D. and Joyce, J.A. (2011). Proteolytic networks in cancer. Trends Cell Biol. *21*, 228–237.

Mohamed, M.M., Cavallo-Medved, D., Rudy, D., Anbalagan, A., Moin, K., and Sloane, B.F. (2010). Interleukin-6 increases expression and secretion of cathepsin B by breast tumor-associated monocytes. Cell. Physiol. Biochem. *25*, 315–324.

Mortier, A., Gouwy, M., Van Damme, J., Proost, P., and Struyf, S. (2016). CD26/dipeptidylpeptidase IV-chemokine interactions: double-edged regulation of inflammation and tumor biology. J. Leukoc. Biol. *99*, 955–969.

Motaln, H. and Turnsek, T.L. (2015). Cytokines play a key role in communication between mesenchymal stem cells and brain cancer cells. Protein Pept. Lett. *22*, 322–331.

Motaln, H., Gruden, K., Hren, M., Schichor, C., Primon, M., Rotter, A., and Lah, T.T. (2012). Human mesenchymal stem cells exploit the immune response mediating chemokines to impact the phenotype of glioblastoma. Cell Transplant. *21*, 1529–1545.

Olson, O.C. and Joyce, J.A. (2015). Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. Nat. Rev. Cancer *15*, 712–729.

Petrella, B.L. and Vincenti, M.P. (2012). Interleukin-1β mediates metalloproteinase-dependent renal cell carcinoma tumor cell invasion through the activation of CCAAT enhancer binding protein β. Cancer Med. *1*, 17–27.

Rao Malla, R., Gopinath, S., Alapati, K., Gorantla, B., Gondi, CS., and Rao, J.S. (2013). Knockdown of cathepsin B and uPAR inhibits CD151 and α3β1 integrin-mediated cell adhesion and invasion in glioma. Mol. Carcinog. *52*, 777–790.

Rawlings, N.D., Barrett, A.J., and Finn, R. (2016). Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. *44*, D343–D3450.

Reinheckel, T., Peters, C., Krüger, A., Turk, B., and Vasiljeva, O. (2012). Differential impact of cysteine cathepsins on genetic mouse models of *de novo* carcinogenesis: cathepsin B as emerging therapeutic target. Front. Pharmacol. *3*, 133.

Ries, C., Egea, V., Karow, M., Kolb, H., Jochum, M., and Neth, P. (2007). MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. Blood *109*, 4055–4063.

Schichor, C., Albrecht, V., Korte, B., Buchner, A., Riesenberg, R., Mysliwietz, J., Paron, I., Motaln, H., Turnšek, T.L., Jürchott, K., et al. (2012). Mesenchymal stem cells and glioma cells form a structural as well as a functional syncytium *in vitro*. Exp. Neurol. *234*, 208–219.

Schrohl, A.S., Holten-Andersen, M., Sweep, F., Schmitt, M., Harbeck, N., Foekens, J., Brünner, N.; European Organisation for Research and Treatment of Cancer (EORTC) Receptor and Biomarker Group. (2003). Tumor markers: from laboratory to clinical utility. Mol. Cell. Proteomics *2*, 378–387.

Seruga, B., Zhang, H., Bernstein, L.J., and Tannock, I.F. (2008). Cytokines and their relationship to the symptoms and outcome of cancer. Nat. Rev. Cancer *8*, 887–899.

Sevenich, L. and Joyce J.A. (2014). Pericellular proteolysis in cancer. Genes Dev. *28*, 2331–2347.

Shchors, K., Nozawa, H., Xu, J., Rostker, F., Swigart-Brown, L., Evan, G., and Hanahan, D. (2013). Increased invasiveness of MMP-9-deficient tumors in two mouse models of neuroendocrine tumorigenesis. Oncogene *32*, 502–513.

Strojnik, T., Kavalar, R., Zajc, I., Diamandis, E.P., Oikonomopoulou, K., and Lah, T.T. (2009). Prognostic impact of CD68 and kallikrein 6 in human glioma. Anticancer Res. *29*, 3269–3279.

Stupack, D.G. (2013). Caspase-8 as a therapeutic target in cancer. Cancer Lett. *332*, 133–140.

Swamydas, M., Ricci, K., Rego, S.L., and Dréau, D. (2013). Mesenchymal stem cell-derived CCL-9 and CCL-5 promote mammary tumor cell invasion and the activation of matrix metalloproteinases. Cell Adh. Migr. *7*, 315–324.

Tuomela, J., Sandholm, J., Kaakinen, M., Patel, A., Kauppila, J. H., Ilvesaro, J., Chen, D., Harris, K.W., Graves, D., and Selander, K.S. (2013). DNA from dead cancer cells induces TLR9-mediated invasion and inflammation in living cancer cells. Breast Cancer Res. Treat. *142*, 477–487.

Turk, B., Turk, D., and Turk, V. (2012). Protease signalling: the cutting edge. EMBO J. *31*, 1630–1643.

Van Den Steen, P.E., Wuyts, A., Husson, S.J., Proost, P., Van Damme, J., and Opdenakker, G. (2003). Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. Eur. J. Biochem. *270*, 3739–3749.

Verbovšek, U., Motaln, H., Rotter, A., Atai, N.A., Gruden, K., Van Noorden, C.J., and Lah, T.T. (2014). Expression analysis of all protease genes reveals cathepsin K to be overexpressed in glioblastoma. PLoS One *9*, e111819.

Verbovšek, U., Van Noorden, C.J., and Lah, T.T. (2015). Complexity of cancer protease biology: cathepsin K expression and function in cancer progression. Semin. Cancer Biol. *35*, 71–84.

Wang, Y.H., Dong, Y.Y., Wang, W.M., Xie, X.Y., Wang, Z.M., Chen, R.X., Chen, J., Gao, D.M., Cui, J.F., and Ren, Z.G. (2013). Vascular endothelial cells facilitated HCC invasion and metastasis through the Akt and NF-κB pathways induced by paracrine cytokines. J. Exp. Clin. Cancer Res. *32*, 51.

Wilkinson, R.D., Magorrian, S.M., Williams, R., Young, A., Small, D.M., Scott, C.J., and Burden, R.E. (2015). CCL2 is transcriptionally controlled by the lysosomal protease cathepsin S in a CD74-dependent manner. Oncotarget *6*, 29725–29739.

Wolf, M., Albrecht, S., and Märki, C. (2008). Proteolytic processing of chemokines: implications in physiological and pathological conditions. Int. J. Biochem. Cell Biol. *40*, 1185–1198.

Xie, L., Moroi, Y., Hayashida, S., Tsuji, G., Takeuchi, S., Shan, B., Nakahara, T., Uchi, H., Takahara, M., and Furue, M. (2011). Cathepsin K-upregulation in fibroblasts promotes matrigel invasive ability of squamous cell carcinoma cells via tumor-derived IL-1α. J. Dermatol. Sci. *61*, 45–50.

Yamada, K., Maishi, N., Akiyama, K., Towfik Alam, M., Ohga, N., Kawamoto, T., Shindoh, M., Takahashi, N., Kamiyama, T., Hida, Y., et al. (2015). CXCL12-CXCR7 axis is important for tumor endothelial cell angiogenic property. Int. J. Cancer *137*, 2825–2836.

Yamamoto, K., Murphy, G., and Troeberg, L. (2015). Extracellular regulation of metalloproteinases. Matrix Biol. *44–46*, 255–263.

Yang, J., Lv, X., Chen, J., Xie, C., Xia, W., Jiang, C., Zeng, T., Ye, Y., Ke, L., Yu, Y., et al. (2016). CCL2-CCR2 axis promotes metastasis of nasopharyngeal carcinoma by activating ERK1/2-MMP2/9 pathway. Oncotarget *7*, 15632–15647.

Yin, M., Soikkeli, J., Jahkola, T., Virolainen, S., Saksela, O., and Hölttä, E. (2012). TGF-β signaling, activated stromal fibroblasts, and cysteine cathepsins B and L drive the invasive growth of human melanoma cells. Am. J. Pathol. *181,* 2202–2216.

Zhang, B., Shi, L., Lu, S., Sun, X., Liu, Y., Li, H., Wang, X., Zhao, C., Zhang, H., and Wang, Y. (2015). Autocrine IL-8 promotes F-actin polymerization and mediate mesenchymal transition via ELMO1-NF-κB-Snail signaling in glioma. Cancer Biol. Ther. *16*, 898–911.