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Review

Potential of Protein-based Anti-metastatic Therapy with Serpins and Inter α-Trypsin Inhibitors

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Abstract. *In this review we summarize the principles of anti-metastatic therapy with selected serpin family proteins, such as pigment epithelial-derived factor (PEDF) and maspin, as well as inter α-trypsin inhibitor (IαIs) light chains (bikunin) and heavy chains (ITIHs). Case-by-case, antimetastatic activity may be dependent or independent of the protease-inhibitory activity of the corresponding proteins. We discuss the incidence of target deregulation in different tumor entities, mechanisms of deregulation, context-dependent functional issues as well as in vitro and in vivo target validation studies with transfected tumor cells or recombinant protein as anti-metastatic agents. Finally, we comment on possible clinical evaluation of these proteins in adjuvant therapy.*

The vast majority of cancer deaths are due to metastatic disease (1). The sum of all individual steps resulting in tumor metastases is referred to as the metastatic cascade (2-5). Metastasis fomation is a highly inefficient process which starts with increased tumor invasiveness due to epithelial mesenchymal transition (EMT) in the tumor periphery, degradation of the extracellular matrix (ECM), and intravasation and dissemination of individual tumor cells or tumor cell clusters. In the circulation, these tumor cells are protected by platelets and neutrophils and eventually arrive at secondary sites prior to transendothelial migration. The microenvironment in the new organ parenchyma is poorly adapted to colonization with the consequence that tumor cells enter a dormant state which may range from days to several years (6, 7). This state is referred to as minimal residual disease (MRD). In addition, the metastatic microenvironment can be adapted in favour of tumor colonization by bone-marrow-derived cells, resulting in the formation of a prometastatic niche (8, 9). It is believed that only tumor cells with the propensity for tumor initiation, corresponding to a cancer stem cell (CSC) state, are able to act as founders of metastatic colonies (10). Factors influencing metastatic outgrowth are the immune status of the patient, blood supply, composition of the ECM and recruitment of a compatible stromal microenvironment. The metastatic process may begin early or late in primary tumor formation and may require a brief period or decades to complete (11, 12). Multi-organ colonization or tissue-specific colonization are determined by genetic programmes (13). Altogether, the identification and validation of targets suitable for intervention with metastatic disease should be viewed as a high-priority approach in oncology. In this review article we focus on the role of protease-related proteins as potential agents for prevention and treatment of metastatic disease.

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Serpins – General Comments

In humans, serine protease inhibitors (serpins) consist of a gene family of 36 protein-encoding genes and five pseudogenes (14). Most serpins inhibit serine proteases *e.g.* of the chymotrypsin family, a minority inhibits cysteine proteases such as caspases and cathepsins (17, 18). In addition, functions independent of protease inhibition were noted (15, 16). Serpins in general are secreted molecules, but a class of intracellular serpins has also been identified (19). The protease-inhibitory function of serpins is involved in

diverse physiological and pathophysiological processes such as inflammation, coagulation, dementia, tumorigenesis and metastasis (20). On the other hand, serpins functioning as chaperones or being involved in hormone storage and transport are independent of protease inhibition (15, 16). Serpins are composed of 330 to 500 amino acids and their metastable structure is mediated by a highly conserved secondary structure composed of three β-sheets, nine α-helices and a reactive center loop (RCL) which acts as a bait for proteases (21). The RCL forms an extended, exposed structure above the body of the serpin scaffold, and its cleavage and subsequent insertion into target proteases is crucial for effective protease inhibition (22, 23). In the final serpin–protease complex the enzyme is frozen at the acyl-intermediate step of the catalytic cycle and the protease structure is severely distorted in comparison to its native conformation (22, 23). Thus, the enzyme is inhibited irreversibly due to a conformational change which disrupts the active site. This type of mode of action (MOA) is in contrast to competitive inhibition by protease inhibitors that bind to and block access to the active site of the respective protease. Serpins are vulnerable to mutations, protein misfolding, formation of inactive polymers, and can induce organ failure due to accumulation of polymers (14-16).

Metastasis-promoting Serpins

In this review, we focus on the metastasis-inhibitory roles of serpins, however, a brain metastasis-promoting function of serpins in lung- and breast cancer has also been described (24). Cells derived from brain metastases of these tumor types typically express high levels of neuroserpin (NS) and serpin B2, presumably to prevent generation of plasmin which is lethal to tumor cells invading the brain parenchyma (24). Plasmin, which is generated from plasminogen by plasminogen activators urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (25, 26) can mobilize soluble FasL from FasL-expressing astrocytes and thus induce tumor cell death by activating Fas signaling in tumor cells (27). Another substrate of plasmin is L1 cancer cell adhesion molecule (L1CAM) (28-30) which promotes spreading of tumor cells on the abluminal surface of capillaries and their metastatic outgrowth. In lung adenocarcinoma, expression of NS and serpin B2 is associated with brain metastasis both as individual genes or in combination (24). In line with these findings are observations that serpin levels in several types of tumors and blood are associated with poor outcome in several types of cancer (31-33).

Pigment Epithelium-derived Factor (PEDF)

PEDF is a 50-kD glycoprotein, and a non-protease-inhibitory member of the serpin family (34-37). It was discovered as a factor promoting the differentiation of retinal progenitor cells into cells exhibiting a neuronal phenotype and as a promoter of differentiation of retinoblastoma cells (38). PEDF is involved in diverse biological processes such as neurogenesis, neuroprotection, retina protection, inflammation, stem cell renewal, anti-angiogenesis and inhibition of metastasis (34-37). Various functional domains mediating anti-angiogenesis, neurotrophy, cell differentiation, apoptosis, collagen- laminin- and heparin binding as well as multiple phosphorylation sites have been identified (34, 35). In addition, several PEDF receptors, such as PEDF-R, PLXDC1 and PLXDC2 have been described (34, 39). The physiological consequences of PEDF/PEDFRs interaction are context-dependent, with respect to cell type and expression pattern of the corresponding receptor types. Therefore, PEDF/PEDFRs interaction represents a complex system of signaling in relation to physiological functions and also with respect to metastasis.

PEDF has been identified as a suppressor of metastasis with impact on invasion, migration, proliferation, apoptosis and angiogenesis (34-38) by targeting tumor cells as well as endothelial cells. The invasion-promoting functions of matrix metallo-proteinases (MMPs) and their inhibition by PEDF are supported by several observations. For example, in chondrosarcoma cells, PEDF blocks trafficking of membrane-tethered MT1-MMP to the cell surface (39, 40). Also, down-regulation of MT1-MMP by PEDF has been described (41). PEDF can be cleaved by MMP2 and MMP9 and it blocks extravasation of tumor cells (42). Anti-invasion properties of PEDF can also be mediated by mitogen activated protein kinase (MAPK) p38 which together with mitogen activated protein kinase kinases 3 and 6 (MKK3 and MKK6) is activated by interaction of PEDF with a not yet identified receptor (42). Another important function of PEDF is its ability to promote apoptosis of endothelial and tumor cells. PEDF-mediated induction of apoptosis is consistently associated with increased levels of p53 and Bcl-2 associated X protein (Bax), and inhibition of Bcl2 has been observed in prostate- (43), glioma (44), pancreatic (45), osteosarcoma (46) and Wilms tumor-derived cancer cells (47). Furthermore, PEDF is lost during progression of melanoma (48). Pro-apoptotic effects of PEDF can be mediated by cluster of differentation 95/95L (CD95/CD95L) interaction (49), inhibition of FLICE-inhibitory protein (FLIP) (50) or phosphorylation of vascular endothelial growth factor receptor 1 (VEGFR1) by VEGF (51) in tumor and endothelial cells. PEDF induces CD95L, and CD95L-mediated apoptosis relies on p38, mitogen-activated protein kinase 5 (MEK5), and extracellular signal-regulated kinase 5 (ERK5)-mediated induction of peroxisome proliferation-activated receptor γ (PPARγ) which promotes expression of CD95L. The latter can induce apoptosis of endothelial cells and tumor cells by interaction with CD95 (49, 52). Furthermore, PEDF triggers jun N-terminal protein kinase (JNK)-mediated

phosphorylation of nuclear factor of activated T-cells (NFATc2), sequestering it in the cytoplasm and thus preventing NFATc2-mediated expression of apoptosis-inhibitor FLIP, an inhibitor of the cell-death mediator caspase 8 (53). The anti-angiogenic effect of PEDF largely seems to be mediated by modulation of the VEGF pathway (54). The MOA of PEDF is summarized in Figure 1.

In several *in vivo* models the anti-metastatic function of PEDF either with PEDF-transfected cells, recombinant PEDF or PEDF-derived peptides was demonstrated as outlined below. So, PEDF-transfected 4T1 murine breast cancer cells as well as similarly transfected human MDA-MB- 231-BR breast cancer cells displayed significantly reduced outgrowth of experimental brain metastasis after tail vein injection and intracranial implantation (55). The role of PEDF in metastasis formation was also investigated in a murine model of ocular melanoma. Forty percent of primary uveal melanomas metastasize to the liver (56). *In vitro*, B16LS9 melanoma cells treated with PEDF lost the ability to migrate and form tubes, and *in vivo*, tumor growth, number of lymphatic micrometastases and tumor microvessel density were decreased in mice injected with PEDF-overexpressing B16LS9 melanoma cells (56). In addition, a xenograft model of Wilms tumor, a pediatric kidney cancer, was shown to be PEDF-responsive. Thus, intraperitoneal injection of recombinant PEDF inhibited the growth of PK-NEP-1 human Wilms tumor cells by 60%, and no evidence of lung metastases was found in these mice (47). Also, microvessel density and mitotic index of tumor cells were reduced in the treated animals. Finally, osteosarcoma is another PEDF-sensitive tumor in preclinical models (57). Osteosarcoma is a pediatric tumor with the propensity to metastasize to the lungs. Recombinant PEDF inhibited cell proliferation and induced apoptosis in rat UMR 106-01 and human SaOS-2 osteosarcoma cell lines *in vitro* (57). Dose-dependent inhibition of cellular invasion through matrigel, increased adhesion to collagen type I as well as reduced VEGF secretion induced by PEDF was observed in both cell lines (57). Co-administration of PEDF during inoculation with Saos-2 cells reduced primary tumor size by 40% and the number and size of lung metastases by 70% after orthotopic implantation into the bones in mice. Here, recombinant PEDF was only effective as an inhibitor in the orthotopic, but not in ectopic, *i.e.* subcutaneous, setting, suggesting an interaction between PEDF and the tumor microenvironment in bone. Similarly, PEDF-derived peptides were evaluated in the Saos-2-based orthotopic osteosarcoma model by continuous administration through osmotic micro pumps (58). Peptide StVOrth2 (residues 78-102) inhibited tumor growth by 50%, whereas peptide StVOrth3 (residues 90-114) reduced the number and mass of pulmonary nodules by 80% (58). For PEDF-based drug development, the identification of biomarkers indicative for PEDF-mediated metastasis-inhibitory signaling is an essential requirement for identification of PEDF-responsive tumors. Thus, it remains to be shown if in addition to the outlined pediatric tumors other tumor entities are candidates for PEDF-related treatment of metastasis.

Maspin

The mRNA of maspin (serpin B5) was found to be down-regulated in mammary carcinoma cell lines in comparison to non-transformed mammary epithelial cells by differential hybridization (59). In addition, maspin is down-regulated in prostate- and gastric cancer and melanoma, but was found to be overexpressed in pancreatic, colorectal and thyroid cancer in comparison to corresponding non-transformed tissues (60). Accordingly, the expression levels and functions of maspin appear to be strongly context-dependent (61-63). Here we discuss the anti-metastatic functions of maspin. In addition to the differential regulation of its expression, another remarkable feature of maspin is its distribution to different cellular locations with possible compartment-specific functions (60-64). Therefore, maspin can be secreted and associated with the plasma membrane and endocytic vesicles in the cytoplasm, but also nuclear localization has been reported. A further remarkable feature of maspin is the regulation of its expression by epigenetic modification of its gene contributing to context-dependent modulation of its activity (64). Cytoplasmic maspin potentially interacts with and thus modulates components of several distinct pathways while pericellularly located maspin might be responsible for paracrine interaction with the tumor microenvironment. Finally, nuclear maspin has been shown to interact with chromatin and regulate transcriptional programmes (65, 66) (Figure 2).

A property of maspin which affects tumor growth as well as metastasis formation is its ability to inhibit angiogenesis (67). Maspin directly acts on endothelial cells by inhibition of fibroblast-growth factor- and VEGF-induced migration and attenuation of their capability to induce mitogenesis and tube formation *in vitro*. *In vivo*, maspin was found to block neovascularization in the rat cornea pocket model and to inhibit growth of xenograft tumors with a concomittant decrease of microvessel density (67). Several findings indicate a role of maspin as an inhibitor of tumor cell invasion and metastasis formation. For example, a 15-mer oligopeptide mimicking the G-helix of maspin inhibited tumor cell invasion by binding to and and causing the internalilsation of integrin β1 (68, 69). Furthermore, it was shown that the RCL of maspin binds to a cell surface receptor that promotes cell adhesion to type I collagen and fibronectin and contributes to inhibition of tumor metastasis (70). Inhibition of migration of breast cancer cells by maspin is accomplished through inhibition of Ras related C3 botulinum substrate 1 (Rac1) and cell division cycle 42 (cdc42) (71). The chromatin-binding property of maspin

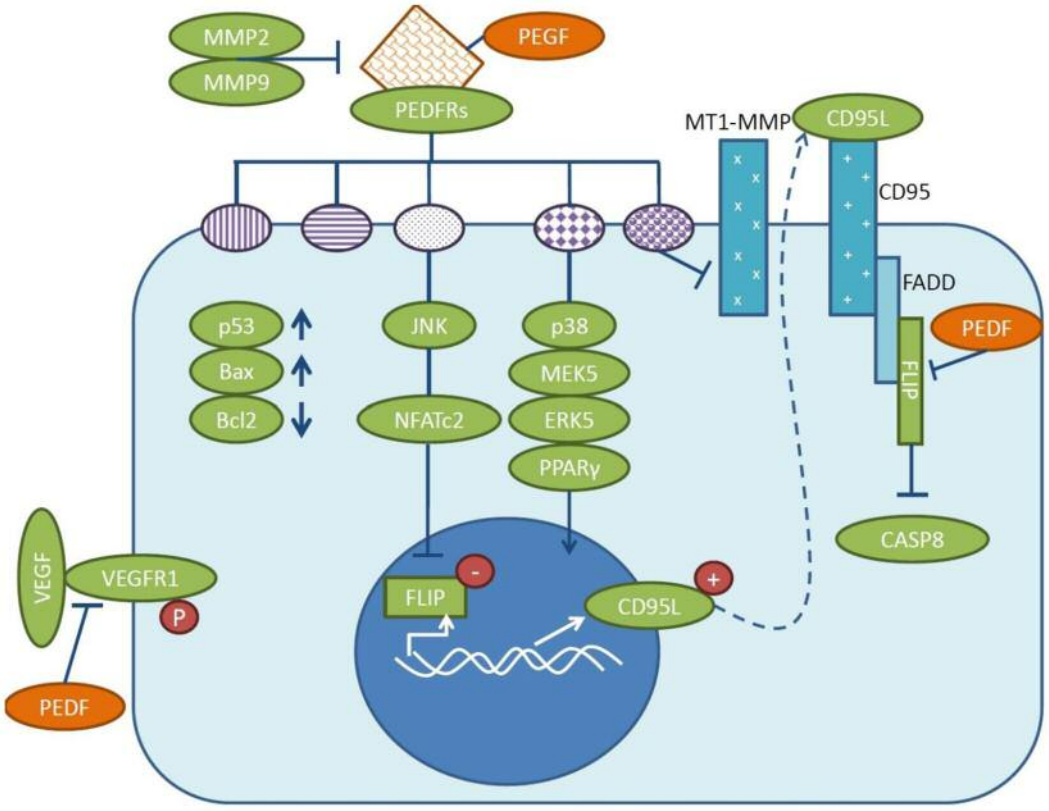

Figure 1. *Anti-metastatic effects of pigment-epithelium derived factor (PEDF). PEDF can bind to several cell surface receptors and activate pro-apoptotic effectors such as CD95L, caspase 8, p53 and Bax. Activation of CD95L by PEDF has been shown in endothelial cells and tumor cells. In addition, PEDF inhibits VEGFR1 signaling in endothelial cells and anti-apoptotic protein Bcl2 in tumor cells. MMP2 and MMP9 inhibit PEDF and MT-MMP1 is inhibited by PEDF. Bax, Bcl2 associated X protein; CD95, cluster of differentiation 95; CD95L, CD95 ligand; ERK5, extracellular signal regulated kinase 5; FLIP, FLICE-inhibitory protein; MEK5, mitogen-activated protein kinase 5; MMP2, 9, matrix metalloproteinases 2 and 9; MT-MMP1, membrane-type matrix metalloproteinase 1; NFATc2, nuclear facor of activated T-cells c2; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1.*

affects cell-matrix interaction by promoting anti-migrational effects based on changes of the transcriptional programme of the corresponding cell (65, 66).

Target validation experiments in breast- and prostate cancer-related preclinical systems for maspin as an anti-metastatic agent. In maspin-transfected MDA-MB 435 cells, reduced invasion in matrigel, decreased growth of xenograft tumors, and inhibition of metastasis formation in lymph nodes was noted (59). Recombinant maspin (r-maspin) inhibited matrigel invasion of MDA-MB-231 and MDA-MB-435 breast cancer and LNCaP, DU145 and PC3 prostate cancer cells. The latter functions were shown to be mediated by maspin acting at the cell surface (63). Similar effects were observed for murine r- maspin in murine breast cancer cell lines CSMLO and CSML100 (72). The anti-metastatic function of maspin is also supported by experiments using transgenic mouse models (73). Double transgenic mice were generated by crossing whey acidic protein (WAP)-SV40 large T and WAP-maspin mice. In these mice reduced growth of breast tumors and corresponding metastases was noted. Further *in vivo* experiments have corroborated the anti-metastatic function of maspin. Mammary fat pad implantation of syngeneic maspin overexpressing TM40D breast cancer cells revealed significant blockage of growth and metastasis formation (74). Another important finding was the demonstration of inhibition of osteolysis, tumor growth, angiogenesis and bone metastasis by maspin in a prostate cancer xenograft model (62). For this purpose, mock- or maspin-transduced DU-145 prostate cancer cells were transferred into fragments of human fetal bone and implanted into immuno-deficient mice (62). The underlying MOA seemed to be based on inhibition of uPA by maspin which also inhibited prostate cancer cell invasion *in vitro* (75). uPA converts plasminogen into plasmin which in turn can

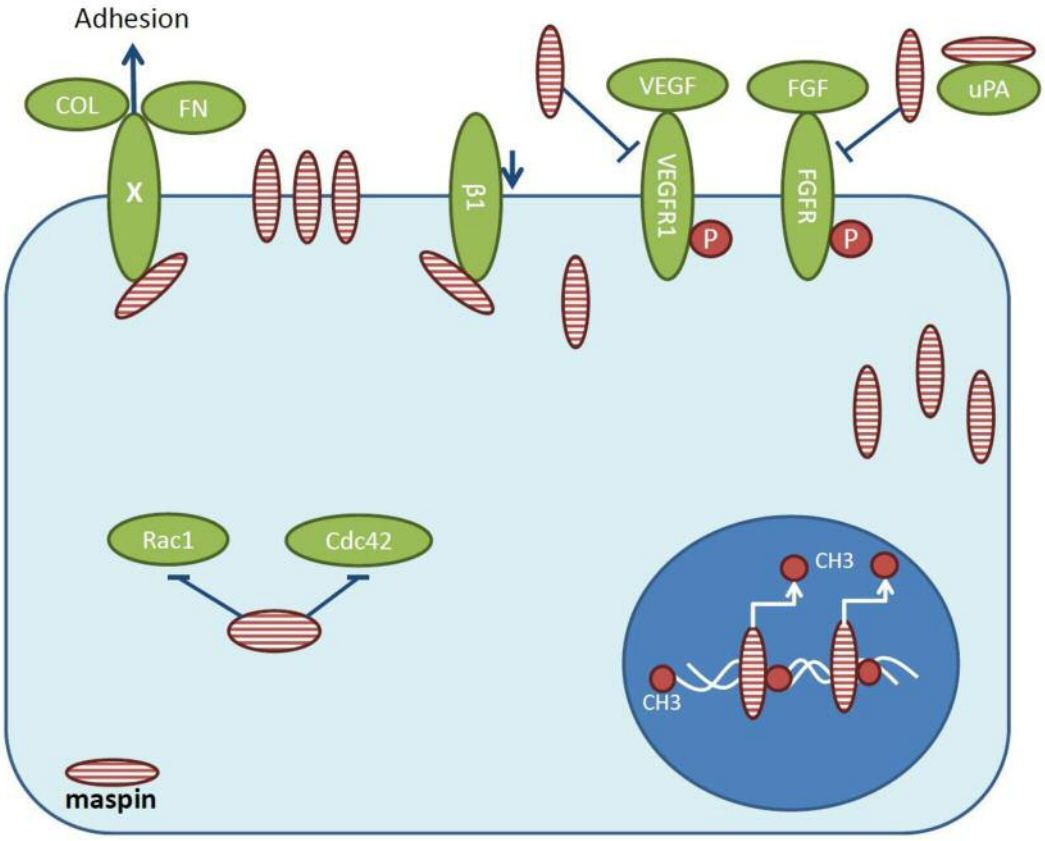

Figure 2. *Anti-metastatic effects of maspin. Maspin can be secreted, localized in the cytoplasm and the nucleus and can associate with the plasma membrane. In endothelial cells, maspin inhibits VEGFR and FGFR signaling. In tumor cells maspin inhibits a not yet identified receptor X which promotes adhesion, promotes internalisation of migration-promoting integrin β1, attenuates migration through inhibition of migration-promoting proteins Rac1 and cdc42 and can initiate an anti-invasive transcriptional program by chromatin modification (methylation) in the nucleus. Extracellularly, maspin can inhibit uPA. β1, Integrin β1; cdc42, cell division cycle 42; CH3, methyl; COL, collagen; FGF, fibroblast growth factor; FGFR1, FGF receptor 1; FN, fibronectin; Rac1, ras-related C3 botulinum substrate 1; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1.*

degrade components of the ECM and activate other zymogen proteases (76, 77). Yeast-derived recombinant maspin (78) inhibited the growth of established MDA-MB-435 xenograft tumors in a dose-dependent manner up to 25%. Low-dose maspin plus paclitaxel resulted in 41% tumor growth inhibition which was superior to maspin or paclitaxel alone (79). The r-maspin was found in cytoplasmic vesicles through multiple endocytic mechanisms of uptake (80, 81). In addition to the general shortcomings of many therapeutic proteins, presently, systematic optimization of the anti-metastatic function of r-maspin is hampered by insufficient knowledge of the functional contribution of membrane- associated, cytoplasmic and nuclear maspin. Even if these issues can be solved, targeting of maspin to a cellular compartment necessary for its anti-metastatic function likely is another problem to be solved. Yet, another open issue is whether the anti-metastatic activity of maspin is dependent on is serpin-related protease-inhibiting function. A study addressing this question found that maspin cannot undergo the stress-relaxed transition typical of proteinase-inhibitory serpins (61). On the other hand, the bone metastasis-inhibitory function of maspin was found to be linked to its proteinase-inhibitory activity. It is possible that these discrepancies are due to differences in the experimental conditions of the corresponding assay systems for assessment of its proteinase-inhibitory activity.

Inter-α Trypsin Inhibitors

Members of the inter-α-trypsin Inhibitor (IαIs) family are composed of a common light (L)- chain and one or two of five heavy (H)-chains (82-86). The L-chain is also known as bikunin (Bk), and is a member of the Kunitz protease inhibitor

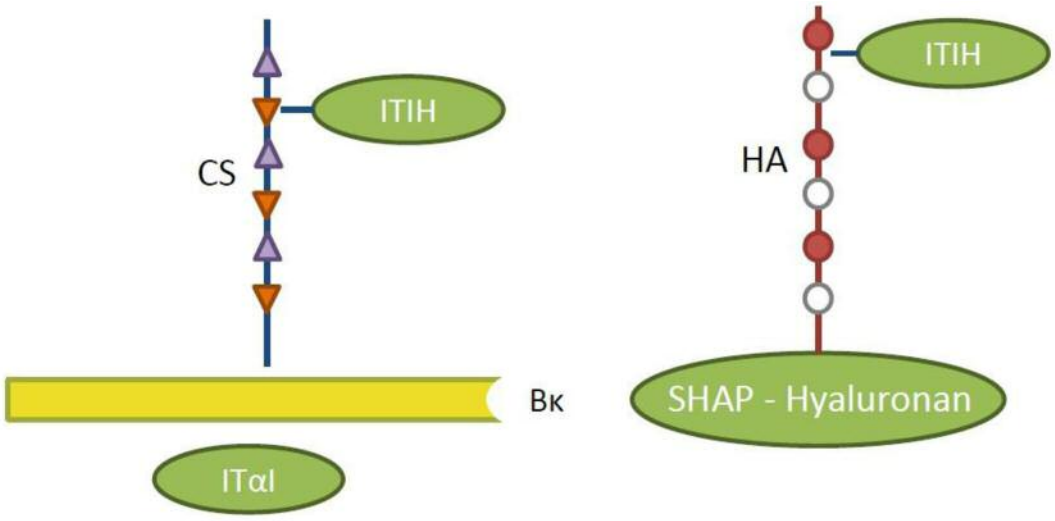

Figure 3. *Composition of inter-α-trypsin inhibitors and serum-derived hyaluronan-associated proteins. ITα1 is composed of a CS backbone (N-acetylgalactosamine and glucuronic acid repeating disacharides), covalently linked to Bk and ITIH chains. SHAP is composed of HA (diglucuronic acid and N-acetyl-D-glucosamine repeating disacharides) covalently bound to ITIHs. CS, Chondroitin sulfate; HA, hyaluronic acid; ITαI, inter-α-trypsin inhibitor; SHAP, serum-derived hyaluronan-associated proteins.*

family. It is a chondroitin-based proteoglycan with a glycan component composed of N-acetylgalactosamine and glucuronic acid as repeating disaccharides. The H-chains are composed of several modules such as vault and von Willebrand type A domains and have the capability to interact with the ECM. Linkage of the H-chains and Bk occurs between the carboxyl group of the C-terminal aspartic acid residue of the corresponding H-chain and the C-6 hydroxyl group of an internal N-acetyl galactosamine residue of the chondroitin sulfate component of Bk. IαI family proteins are mainly synthesized and secreted by the liver and are circulating in the blood at high concentrations between 0.15 and 0.5 mg/ml (84). The inter- α trypsin inhibitor heavy chains (ITIHs) can be covalently bound to locally produced hyaluronic acid (HA), a polymer of dissacharides composed of D-glucuronic acid and N-acetyl-D-glucosamine, *via* a transesterification reaction resulting in the formation of serum-derived hyaluronan-associated proteins (SHAP) (84). Composition of ITαIs and SHAP are shown in Figure 3. ITIHs are involved in a variety of physiological functions such as fertilization, ovulation, inflammation and cancer (82-86).

Bikunin (Bk). Bk is present predominantly in amniotic fluid and urine of healthy individuals and inhibits a broad panel of proteases such as trypsin, chymotrypsin, cell surface-bound plasmin, leukocyte elastase and factor IXa (86). Bk exerts several invasion- and metastasis- inhibitory functions such as inhibition of expression of uPA at both levels of transcription and translation thus interfering with the conversion of plasminogen to plasmin at the cell surface (87). Furthermore, Bk is able to inhibit the dimerization of CD44, resulting in the inhibition of the interaction of CD44 with HA and, consequently, CD44/HA-mediated activation of MAPK signaling (88). In addition to its antimetastatic function Bk has been shown to be involved in the inhibition of formation of urinary stones, inhibition of Ca-channels and as a modulator of inflammation (86, 89). Most of the blood-related Bk is covalently linked to ITIH chains. The MOA of Bk is summarized in Figure 4.

Inter-α trypsin inhibitor heavy chains (ITIHs). ITIHs are frequently down-regulated due to promotor methylation of the corresponding genes in diverse tumor entities (90). ITIH2 was found to be down-regulated in 70% of breast cancers (n=50) and ITIH5 was strongly reduced in 42% (n=217) of invasive breast cancers in comparison to corresponding normal tissues (91, 92). In urothelial cancers (UC) (n=55) loss of ITIH5 expression (61% of cases) correlated with unfavourable prognosis of patients without distant metastases at first diagnosis (93). Most expression-related data are available for ITIH5. Analysis of 385 non-small cell lung carcinomas (NSCLC) found ITIH5 promoter methylation in 47% of samples and correlates with reduced mRNA expression (94). In particular, low ITIH5 mRNA expression was found in magnoid and squamoid NSCLC sub-types which are associated with an unfavorable prognosis (94). In acute myeloloid leukemia (AML), ITIH5 promoter hypermethylation was observed in 15 of 104 (14.4%) of AML samples, however, there was no statistically significant correlation between the methylation status of the ITIH5 promoter and clinical

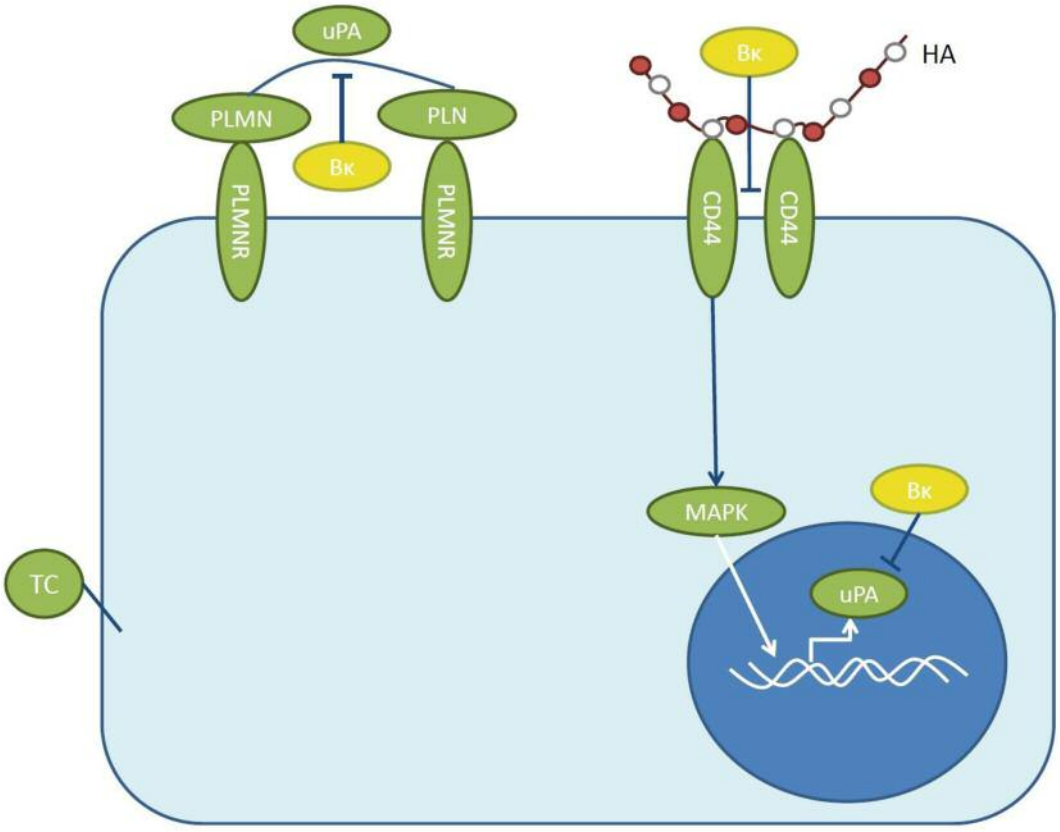

Figure 4. *Anti-metastatic effects of Bikunin (Bk). Bk inhibits PLMNR mediated conversion of PLMN to PLN as well as dimerization of CD44, MAPK activation and transcriptional activation of Bk. Bk, Bikunin; CD44, cluster of differentiation 44; HA, hyaluronic acid; MAPK, mitogen-activated protein kinase; PLMN, plasminogen; PLMNR, plasminogen receptor; PLN, plasmin; uPA, urokinase plasminogen activator.*

diagnostic parameters (95). In addition to these tumor-related findings, a role in inflammation and obesity has been identified for ITIH5 (96-98). ITIH5 is expressed in normal dermal fibroblasts, but not in epidermal keratinocytes and upregulated in inflammatory skin diseases such as psoriasis, atopic dermatitis and allergic contact dermatitis in the areas of immune reactions of the epidermis (96, 97). ITIH5 knock-out mice showed thinning of the epidermis and reduced capability of proliferation of dermal fibroblasts. These findings point to a role of ITIH5 in inflammatory disease and might be might be based on inhibition of degradation of the ECM. However, the functional contribution of ITIH5 in this context has to be investigated in more detail. Furthermore, a correlation between ITIH5 and obesity has been found (98). ITIH5 is expressed in adipocytes and adipose tissue and obese subjects display elevated ITIH5 levels in adipose tissue. Interestingly, *ITIH5* mRNA expression was found to be reduced after diet-induced weight loss. These correlates might point to a possible function of ITIH5 as an adipokine regulating human metabolism. MOA of ITIHs are summarized in Figure 5. Analysis of steady-state RNA levels (TCGA data) in several tumor entities and matching normal tissues revealed consistent down-regulation of ITIH5 mRNA in tumors of the bladder, breast cervix, colon, esophagus and thyroid carcinoma, lung adenocarcinoma, lung squamous carcinoma and tumors of rectum, stomach and the thyroid, whereas ITIH1-4 were only very rarely downregulated in tumor samples (Figure 6), emphasizing the important role of ITIH5 in cancer.

Metastasis-related target validation of Bk and ITIHs. In Bk-deficient knock-out mice a higher frequency of spontaneous lung metastases was observed 3-4 weeks after tumor inoculation in a 3LL Lewis lung cancer model (99). A single dose of *i.p.* injected Bk directly co-administered with tumor cell inoculation caused a significant reducion of number and mass of metastases in $Bk^{-/-}$ mice. In addition, inhibition of MAPK signaling and inhibition of uPA and plasminogen activator inhibitor-1 (PAI-1) expression was observed in 3LL tumor cells (99). This is surprising because the terminal half-life time of Bk ranges between 4-30 min in humans, rats and

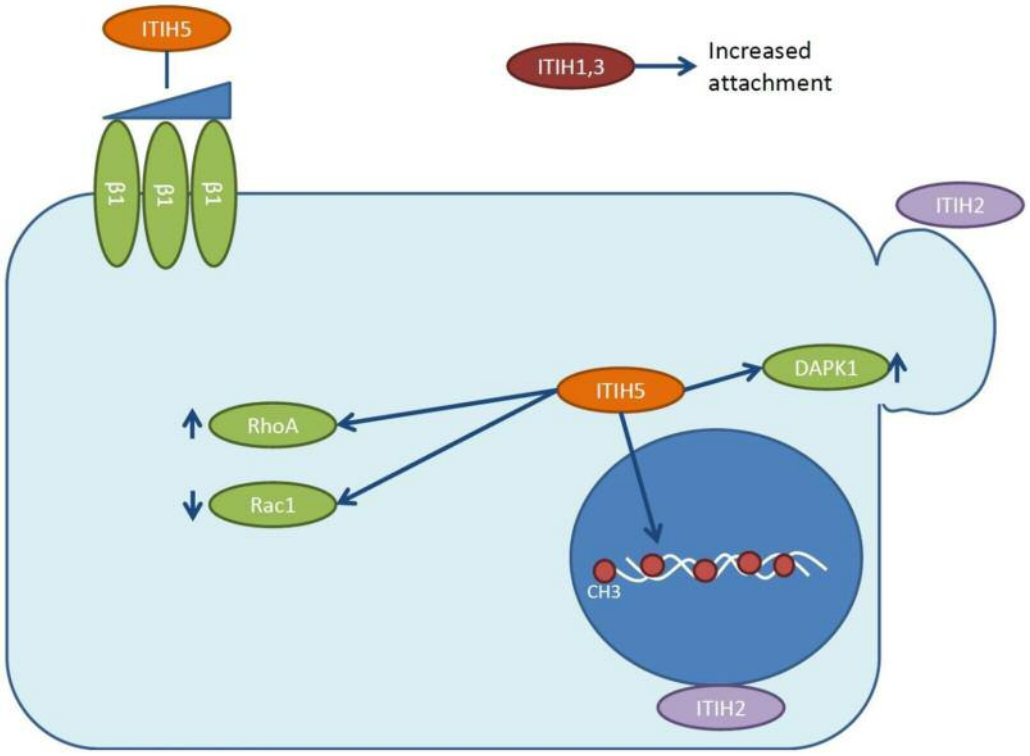

Figure 5. *Anti-metastatic functions of inter-trypsin inhibitor heavy chains (ITIHs). All ITIHs are secreted by tumor cells; in addition, ITIH2 can be located at membrane ruffles and at the periplasmic membrane and ITIH5 can be found in endocytic vesicles in the cytoplasm. ITIH5 increases integrin β1 expression, modulates activity of migration-related GTPases RhoA and Rac1 and epigenetically reprograms the transcriptional program of the tumor cell und supports transcription of tumor suppressor gene DAPK1. CH3, Methyl; DAPK1, death-associated kinase protein 1; ITIH, inter-trypsin inhibitor heavy chains; Rac1, ras-related C3 botulinum toxin substrate 1; Rho A, ras homologue A.*

mice (100). Other studies have investigated the formation of macroscopic lung metastases derived from subcutaneous tumors implanted into the abdominal wall of C57Bl/6 mice which were detectable 21 days after inoculation (101-103). Daily *i.p.* injection of Bk for 7 days and surgical removal of the primary tumors at day 7 after implantation, but not at day 14, followed by Bk treatment as described, resulted in significant reduction of the number of lung metastases. However, the combination of Bk and etoposide led to inhibition of lung colonization also in surgery treated mice at day 14 after implantation (101, 102). In lung carcinoma cell line H460M, Bk decreased proliferation and inhibited growth of *s.c.* injected xenografts and the number of corresponding lung metastases after ectopic expression of Bk (103). Preliminary data for clinical evaluation of Bk in patients with advanced ovarian carcinoma were reported (89). Patients were first debulked surgically, followed by six cycles of chemotherapy, split in two arms, and treated with the above regimen with or without Bk on days 1 to 7 of each 21-day cycle. Five-year survival was improved from 29-44% in the Bk-treated group as compared to the etoposide-only treated group (n=28 and 29, respectively). However, there is no information available that this treatment was approved for the treatment of advanced ovarian carcinoma based on randomized studies with larger patient numbers.

For ITIH1 and ITIH3, metastasis-related target validation experiments were performed using green fluorescent protein-expressing H460M cells which expressed ITIH1, 2 or 3 stably (103). ITIH1 and ITIH3 transfected cells showed increased attachment to matrigel in comparison to mock transfected control cells. Interestingly, tumor growth was not affected by expression of ITIH1,2 or 3. However, ITIH1or -3 transfected cells possessed a reduced propensity to form lung metastases in a spontaneous metastasis model after *s.c.* injection (103). ITIH2 had no impact on metastasis formation in this model.

ITIH2 associated with Bk was isolated as a cell invasion inhibitory factor from C6 astrocytoma cells (104). In addition, U251 glioma cells stably transfected with ITIH2 which did not express H- or L-IαI related chains were used to show that ITIH2 inhibited invasion in three-dimensional collagen gels (104). Finally, ITIH2 was shown to localize to

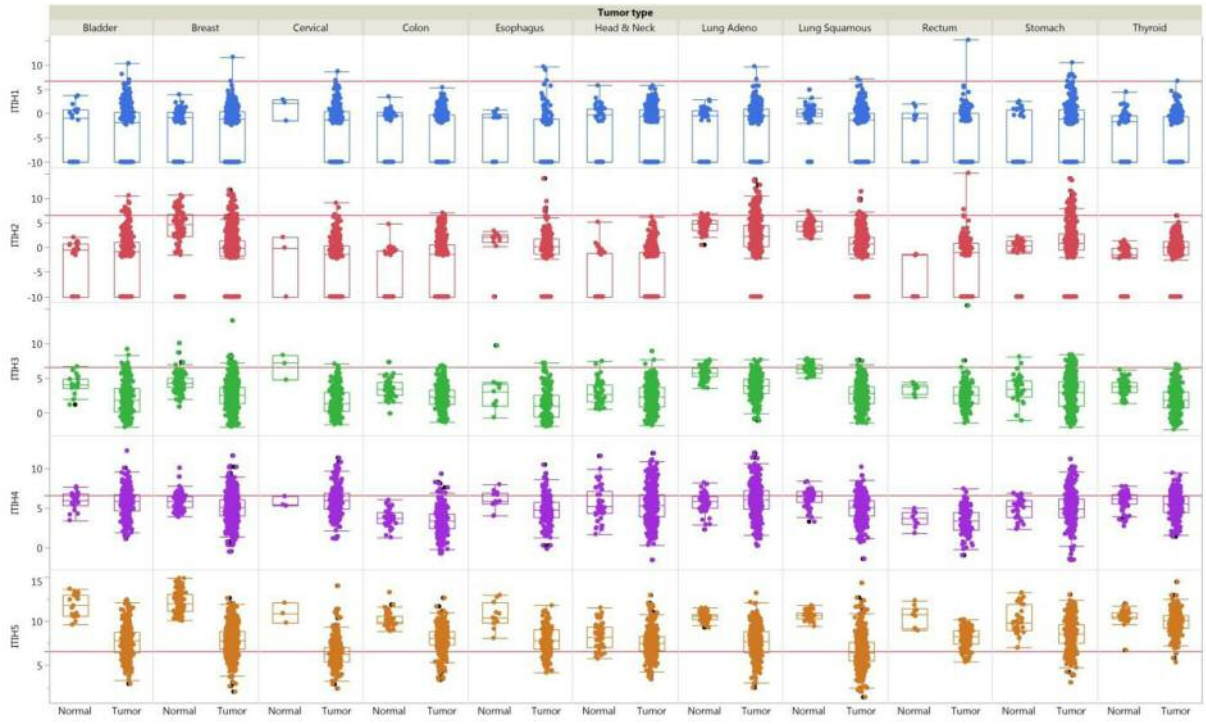

Figure 6. *Expression of ITIH gene family members in tumor and matched normal samples by RNA sequencing of TCGA cohorts. Expression values (y-Axis) are provided as log2 normalized read counts. The red lines indicate a normalized read count of 100 separating very low from higher expression levels. Expression data are shown as box plots where the black line represent the data median, the black rectangle show the upper and lower 25% quartile (therefore, 50% of all data points are included in the black rectangle). All other data points, except for outliers lie within the upper and lower whiskers. Data are presented for the following tumor entities and matched normal tissues: bladder (408/19), breast (1100/112), cervical (306/3), colorectal (457/41), esophagus (185/11), head-and-neck: 522/44, lung adenocarcinoma (517/59), lung squamous cell carcinoma (501/51), rectum (167/10), stomach (415/359 and thyroid (509/59).*

perinuclear regions, lamellipodia and membrane ruffles and to down-regulate AKT signaling in ITIH2-transfected C6-cells. In these cells, ITIH2 inhibited proliferation without causing cell death and increased cell attachment to monolayers of U251 cells (104). Normally, ITIH2 is expressed in normal brain tissue and low-grade CNS tumors, but its expression is lost in high-grade CNS tumors including gliobastoma multiforme further underlining its potential role as an anti-invasive protein (104).

Similarly, ITIH5 transfected MDA-MB-231 breast cancer cells lost the ability to form lung metastases in a mouse model of experimental metastasis (105, 106). In these cells, migration and cell proliferation were impaired, expression of integrin receptors was shifted towards β1 integrin receptor, Rac1 activity was decreased, and Rho activity was increased. The formation of epithelial-like cell clusters was observed in these transfectants, indicating changes in the dynamics of the ECM. Profound changes of the methylation status of histones was seen in the transfected cells, indicating that expression of ITIH5 might have induced epigenetic re-programming. For example, tumor suppressor gene death-associated protein kinase 1 (DAPK1) was a gene upregulated by ITIH5, and its knock-down restored the impaired motility of the ITIH5-transfected MDA-MB-231 cells. In addition, ITIH5 was identified as a suppressor of metastasis formation in pancreatic adenocarcinoma (107). An shRNA library was transfected into non-metastatic S2-028 pancreatic carcinoma cells which were subsequently injected into the spleens of mice. The characterisation of the resulting liver nodules revealed the presence of ITIH-5 specific shRNA suggesting the presence of the transfected cells. Conversely the expression of ITIH5 in metastatic pancreatic carcinoma cell lines inhibited motility and invasion while the reduction of ITIH5 expression using ITIH5 shRNA increased the number and mass of S2-028-derived liver metastases (107). Immuno-histochemistry-based analysis of ITIH5 expression in pancreatic tumor samples demonstrated a positive correlation with survival and invasion/metastasis (107). It remains to be seen whether the observed phenomena can be recapitulated with recombinant ITIH5 or derivatives thereof.

Target validation of ITIHs – critical issues. The terminal half-life of recombinant ITIHs may be short but probably can be improved by applying protein modification technologies (108). As outlined previously, high concentrations of ITIHs can be found in human serum and it remains to be proven that certain ITIHs are expressed at a reduced level in cancer patients as a prerequisite for substitution therapy with recombinant protein. The effect of defined recombinant ITIHs on inhibition of migration and proliferation in a panel of tumor cell lines with known ITIH status would be of interest to generate a strategy for ITIH-based anti-metastatic therapy. Finally, possible redundant as well as differentiating functions of IαI family members remain to be investigated.

Therapeutic Aspects

Most patients presenting in the clinic are diagnosed with disseminated tumor cells in blood or bone marrow (109, 110). An anti-metastatic agent, therefore, should possess both anti-invasive and anti-proliferative properties. Different scenarios of anti-metastatic intervention can be simulated preclinically in experimental and spontaneous metastasis models in the mouse (111-113). In these models, treatment can be varied *e.g.* with regard to time (early or late treatment) or regimen (single agent or combination therapy or treatment after adjunct therapy). In addition, many other variations of the treatment schedule are possible (111-113). In many types of tumors, diagnosis of metastases occurs after varying times of dormancy, a stage in which metastases are probably refractory to agents with anti-proliferative properties due to lack of proliferation (111, 112). Experiments investigating the intervention with drugs at this stage of metastasis are under development (114). Effective treatment of established metastases in preclinical models as well as in patients is impeded by several issues (111-113). Such obstacles are i) physical limitations of drug delivery due to poor tumor vacularization, ii) the blood-brain barrier for treatment of brain metastases, iii) the formation of chemo-preventive niches, iv) the acquisition of tumor stem cell-like characteristics, v) molecularly distinct properties between primary tumor and metastases and vi) heterogeneity of metastases (114-118). Thus, anti-metastatic agents targeting tumor cells, the 'seed' for metastases like *e.g.* with MMP inhibitors, medroxyprogesterone for re-activation of tumor suppressor nm23, cilengitide, a peptidic inhibitor of integrins αvβ3 and αvβ5 or dasatinib, an inhibitor of src kinase, possessed basically no therapeutic efficacy (111). Alternatively, targeting the 'soil', *i.e.* the microenvironment of disseminated tumor cells is another strategy to treat metastatic disease. So, treatment with Denosumab, an antibody direct against receptor activator of NFκB ligand (RANK), which prevents osteoclast-mediated degradation of bone gave rise to significant improvement of skeletal-related events in patients with bone metastasis (119). Other bone-metastasis targeting agents such as bisphosphonates and TGFβ inhibitors are under clinical development (111, 120, 121).

We have described protein-related technical hurdles, target-related issues and clinical issues with an impact for further development of anti-metastatic proteins. The short half-life of the proteins as described such as PEDF, maspin and IαIs can be counteracted by improving the PK profile by protein modification(s) or through delivery technologies such continuous administration of the drug by implanted minipumps (122-126).

Comprehensive analysis of context-dependent functional data and incidence of target expression is a prerequisite for identification of tumor types potentially responding to target-specific anti-metastatic therapy. Another question is whether down-regulation of tumor- or serum levels of the corresponding anti-metastatic protein(s) correlates with the risk of metastasis formation in the tumor types as described above. Furthermore, comparative, functional *in vitro* and *in vivo* experiments using transfected tumor cells and/or recombinant therapeutic proteins might help to elucidate the modulation of anti-metastatic properties depending on the agent under consideration. Also, a differentiation between therapeutic proteins which affect metastases only and those which also have an impact on tumor cell proliferation is important for identifying the most promising combinations for therapy. For the clinical exploration of anti-metastatic proteins in adjuvant settings, patients with evidence of aggressive disease but no identifiyable distant metastases should be selected. Thus, metastasis-preventive trials (adjuvant trials) could be performed with smaller patient numbers and shorter duration times by selection of patients with multiple positive lymph nodes and/or known short-term high incidence of relapse after standard treatment (127-131). For disease recurrence, overall survival (OS) and progression free survival (PFS) could be chosen as appropriate endpoints. These types of suggested trials are markedly different from previous trials in which anti-metastatic drugs were tested in patients with established metastases monitoring shrinkage of metastases (responses) in conjunction with OS and PFS (111, 127).

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