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The Role of Type II Transmembrane Serine Protease Mediated Signaling in Cancer

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Abstract

Pericellular proteases have long been implicated in carcinogenesis. Previous research focused on these proteins, primarily as extracellular matrix (ECM) protein degrading enzymes which allowed cancer cells to breach the basement membrane and invade surrounding tissue. However, recently, there has been a shift in the view of cell surface proteases, including serine proteases, as proteolytic modifiers of particular targets, including growth factors and protease-activated receptors, which are critical for the activation of oncogenic signaling pathways.

Of the 176 human serine proteases currently identified, a subset of 17 known as type II transmembrane serine proteases (TTSPs) [1], many have been shown to be relevant to cancer progression, since they were first identified as a family around the turn of the century. To this end, altered expression of TTSPs appeared as a trademark of several tumor types [2, 3]. However, the substrates and underlying signaling pathways remained unclear. Localization of these proteins to the cell surface places them in the unique position to mediate signal transduction between the cell and its surrounding environment. Many of the TTSPs have already been shown to play key roles in processes such as postnatal development, tissue homeostasis, and tumor progression, which share overlapping molecular mechanisms [2, 4-6].

In this review, we summarize the current knowledge regarding the role of the TTSP family in pro-oncogenic signaling.

Graphical abstract

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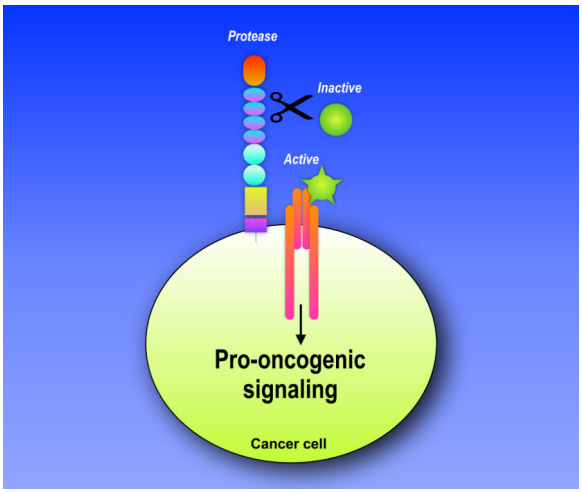

Altered expression of type II transmembrane serine proteases (TTSP) is a feature of several types of tumors. TTSPs are proteolytic modifiers of targets such as growth factors and protease-activated receptors, which are critical for the activation of oncogenic signaling pathways. Localized to the cell surface, TTSPS mediate signal transduction between the cell and its surrounding environment. Numerous studies suggest that TTSPs represent a promising option for therapeutic intervention of cancer.

Keywords

type II transmembrane serine proteases; cancer; matriptase; hepsin; TMPRSS2; TMPRSS4; small molecule inhibitors

Characteristics of TTSP family members

TTSPs have a single pass hydrophobic transmembrane domain near the amino terminus, which separates a short intracellular domain from a larger extracellular portion which contains a variable stem region and a C-terminal serine protease domain that has a histidine, aspartate, and serine triad of residues necessary for catalytic activity [1]. In vertebrates, this family is divided into four subfamilies: 1) matriptase, 2) hepsin/transmembrane protease/serine (TMPRSS), 3) human airway trypsin-like (HAT)/differentially expressed in squamous cell carcinoma (DESC), and 4) corin [1, 2, 7, 8]. All TTSPs are believed to be synthesized as zymogens and require activation by proteolytic cleavage. Many TTSPs, including matriptase, matriptase-2, hepsin, TMPRSS2, TMPRSS3, and TMPRSS4 are capable of auto-activation [1]. While the auto-activation mechanisms are still being elucidated, oligomerization is thought to play a role in this process [1].

Matriptase

Matriptase is among the most studied members of the TTSP family and is expressed in the epithelial compartment in a wide variety of tissues [2, 3, 9-14]. The dysregulation of matriptase is implicated in numerous cancers and associated with poor outcomes. For example, matriptase is shown to be overexpressed in a wide range of epithelial tumors

(carcinomas) including of the breast, ovary, uterus, prostate, colon, cervix, and skin [1, 7, 15-17]. On the other hand some groups have described downregulation of matriptase mRNA in gastric and colorectal carcinomas [18, 19]. An important finding in several cancers is that the ratio of matriptase to its endogenous inhibitors, hepatocyte growth factor activator inhibitor (HAI)-1 and HAI-2, is increased, suggesting that the balance of protease activity can be shifted, leading to unopposed active matriptase, ultimately causing detrimental pro-carcinogenic effects. In normal tissue, the ratio is low, resulting in tightly controlled proteolytic activity [20]. However, the consequences of dysregulated matriptase become apparent in a variety of studies. For example, in a transgenic model of squamous cell carcinoma (SCC) with matriptase expression in the epidermis (K5-matriptase), all animals develop tumors. However, increasing HAI-1 or HAI-2 expression by concomitant transgenic expression abrogate tumor formation [21, 22]. In human colorectal cancer, the ratio of matriptase/HAI-1 mRNA is higher in colorectal cancer adenomas and carcinomas than corresponding tissue from control individuals [18]. During the progression of some cancers, e.g. prostate cancer the matriptase/HAI-1 ratio is observed to increase, leading to more active matriptase on the cell surface in high grade tumors [23]. In 2013, active matriptase was shown to be a functional biomarker for monitoring tumorigenesis in a mouse model of colon cancer [24].

Matriptase was first discovered in breast cancer cell lines and is highly expressed in human breast carcinoma cells [25-30]. Recently, this TTSP was shown to be critically involved in breast cancer progression through activation of the HGF/c-Met signaling axis and therefore, was identified as a potential therapeutic target in this disease (Figure 1) [25, 31].

In Zoratti et al. 2015, it was demonstrated that matriptase initiates the c-Met pathway activation by cleaving and activating hepatocyte growth factor, HGF (also known as scatter factor). HGF is a pleiotropic, paracrine growth factor and key mediator of cell migration, proliferation, survival, motility and morphogenesis in epithelial cells [25, 32-34]. The inactive pro-form of HGF, which is mainly secreted by fibroblasts, binds to the c-Met tyrosine kinase receptor on the surface of breast epithelial cells. Upon proteolytic cleavage by matriptase, pro-HGF is converted to active HGF, which in turn initiates c-Met signaling and activates multiple downstream targets, including the P13/AKT pathway, and the c-Met docking protein, Gab1. Contribution of the matriptase/HGF/c-Met pathway to cancer progression was confirmed in both cell-based assays and mouse models [25]. In the study, the specific function of matriptase in mammary tumorigenesis was probed by crossing a mouse model of invasive ductal mammary carcinoma (MMTV-PymT) with mice expressing less than 25% endogenous matriptase protein (matriptase hypomorphic model). Mouse mammary tumor virus (MMTV)-Polyomavirus middle T (PymT) mice spontaneously develop multifocal mammary carcinomas with tumor progression that mirrors what is seen in human invasive ductal breast carcinomas [35, 36]. Low matriptase expression in the mammary epithelium led to a significant delay in tumor onset, tumor burden (due to decreased cancer cell proliferation), and multiplicity. Additionally, a significant abrogation of tumor progression was observed in the early stages of carcinogenesis. In 2D cell culture of human breast cancer cell lines of diverse origin, silencing of matriptase prevented activation of c-Met, Gab1, and Akt following exposure to pro-HGF suggesting that the mechanism is a general one in human breast cancer. Furthermore, in a 3D cell culture

system, which more accurately mimics the microenvironment *in vivo*, breast cancer cells exposed to pro-HGF developed an invasive morphology (branched structures), while breast cancer cells with matriptase silenced prior to pro-HGF exposure, looked indistinguishable from controls (spheroid structures), which were not stimulated with growth factor [25].

A recent study further explored the matriptase/c-Met pathway in inflammatory breast cancer (IBC) [31]. IBC is a rare and aggressive form of invasive breast cancer, characterized by younger age of onset and lower overall survival compared to other breast cancers. Further complicating treatment is the fact that 20 – 40% of IBC cases are triple-negative breast cancers (TNBC), which excludes hormone therapy and HER2 targeting as treatment options [37]. Importantly, the matriptase/c-Met signaling axis also mediates proliferation and invasion in IBC cell lines, suggesting that targeting this pathway may be a promising new strategy in treating IBC [31].

Proteinase-activated-receptor (PAR)-2 G-protein is implicated in numerous human diseases due to the receptor's ability to induce inflammatory signaling pathways [38-42]. It is expressed by primary keratinocytes and established keratinocyte cell lines [43, 44] where activation can elicit a range of cellular responses, as well as by a variety of leukocyte populations, which infiltrate the skin during pre-neoplastic progression [38]. PAR-2 is also found in activated fibroblasts in inflammatory conditions and in endothelial cells and smooth muscle [45].

Sales et al. 2015 demonstrated that global deletion of PAR-2 from mice blocked the development of SCC of the skin in a transgenic model that expresses matriptase in the epidermis via a keratin-5 promoter (K5-matriptase transgenic mice). In PAR-2 sufficient mice, this model develops spontaneous multistage SCC, with invasive lesions beginning around one year of age [21]. However, mice lacking PAR-2 were histologically indistinguishable from wild-type littermates demonstrating that matriptase-mediated pre-malignant progression is PAR-2 dependent [38].

In a separate model of Ras-dependent SCC in K5-matriptase transgenic mice, induced by exposure to the chemical carcinogen, 7,12-Dimethylbenz[a]anthracene (DMBA), ablation of PAR-2 impeded matriptase-mediated potentiation of SCC, leading to decreased tumor latency [38].

The same group previously showed that in the matriptase-driven SCC model, recruitment of inflammatory cells including mast cells to the underlying dermis [21] precedes SCC development and the newer study builds upon this observation. In cell-based assays, PAR-2 activation by matriptase, caused induction of nuclear factor (NF)κB through the Gαi receptor and the associated release of NFκB-dependent inflammatory cytokines. Importantly, specific deletion of PAR-2 from bone-marrow derived hematopoietic stem cells did not affect matriptase-mediated pre-malignant progression when transplanted to K5-matriptase transgenic mice, suggesting that dysregulated matriptase activates keratinocyte PAR-2 to promote pro-inflammatory and pro-tumorigenic effects [38]. As PAR-2 and matriptase have been shown to be consistently co-expressed in the epithelial compartment of

human SCC [46], this is strong evidence that a matriptase–PAR-2 signaling axis contributes to human disease.

As seen in breast cancer, pro-HGF activation is a critical component through which matriptase exerts promotion of SCC, and matriptase fails to drive tumor progression in mice with specific deletion of c-Met from the epidermal basal cell keratinocyte compartment [47]. In SCC, activation of both the HGF/c-Met and PAR-2 coupled signaling pathway is involved in matriptase-mediated pro-tumorigenic effects. It was shown that the serine-threonine kinase, mTor, is an essential component of matriptase-mediated HGF/c-Met signaling. Furthermore, mTor activation was required for matriptase/c-Met-induced SCC, as K5-matriptase mice treated with the mTor inhibitor, rapamycin, remained tumor-free. [47].

Additionally, it was shown that as with PAR-2, c-Met deficiency in the skin of K5-matriptase mice abrogates the ability of matriptase to potentiate DMBA-induced tumorigenesis. Unlike, PAR-2 deletion, however, loss of c-Met does not prevent inflammatory cell accumulation as a result of dysregulated matriptase in mice. Therefore, it would appear that matriptase induces activation of two separate pro-tumorigenic pathways (c-Met-Akt-mTor and PAR-2-NFκB), both of which are required for SCC promotion. Sales et al. 2015 highlight that an intriguing, unanswered question that arises from these studies is why matriptase-induced inflammatory PAR-2 signaling does not promote malignant progression in the absence of activated c-Met and why, conversely, matriptase-induced c-Met-Akt signaling does not promote malignant progression in the absence of activated PAR-2. These *in vivo* findings were accompanied by cell culture studies where no evidence was found that PAR-2 activation was associated with increased c-Met activity in cell-based assay using HEK293 cells or immortalized keratinocytes [38].

The transcriptional profiling of nearly 2000 human samples, which included normal tissues, cancer cell lines, and cancer tissue, as well as biochemical enzyme characterization found matriptase to be part of a signaling pathway that includes the growth factor, macrophage stimulating protein 1 (MSP-1), and its corresponding receptor, RON (Recepteur d'Origine Nantais) [48]. Cell-based experiments confirmed this interaction: on peritoneal macrophages, cell surface bound matriptase activates MSP-1 into its active form, which then binds to and activates RON-mediated signaling. Activation of RON led to changes in cell morphology as well as downstream biochemical changes, such as modulation of NO production in bone-derived macrophages [48]. While the matriptase/RON interaction has been previously described in macrophages as affecting activation, chemotaxis, and proliferation [49], RON has also received attention for its role in cancer pathogenesis. For example, transgenic mice expressing RON in distal lung epithelial cells under the surfactant protein C promoter, develop multiple lung tumors in which RON was highly expressed and constitutively active [50].

Matriptase has been shown to cleave and activate the pro-form of the urokinase plasminogen activator (uPA) [51-54] and it has been suggested that matriptase promotes progression of cancer through this activation. Thus, it was proposed that inhibition of ovarian cancer cell invasion by RNAi-mediated down-regulation of matriptase occurs through suppression of activation of uPA receptor (uPAR) -bound pro-uPA [55]. In a recent study, matriptase was

not found to be a critical activator of pro-uPA *in vivo* in the MMTV-PymT mouse model of breast cancer [25]. Western blot analysis of whole tissue lysates of mammary tumors from matriptase hypomorphic mice and control, detected uPA in both its pro-form and two-chain active form, with no difference in the pro-uPA/active uPA ratio [25].

The SRC-associated protein CUB-domain-containing protein 1 (CDCP1), also referred to as TRASK and SIMA135, is an integral membrane glycoprotein that has been shown to be upregulated in several kinds of malignancies, including of the breast, colon, and lung [56]. Matriptase and CDCP1 mRNA have correlated expression in many tissue types [48] and matriptase has been shown to both interact with and cleave CDCP1 in *in vitro* experiments [57]. This was corroborated by another study that showed that matriptase was sufficient, but not essential to process CDCP1 in Hela cells and 22Rv1 prostate cancer cells [58]. However, silencing of matriptase in prostate cancer cells lines did not reduce CDCP1 processing [58].

Members of the platelet-derived-growth-factor (PGDF) family are known to induce cell-migration, proliferation, and malignant transformation upon binding to their receptors (PDGF receptor α and β) [59]. In particular, β-PDGFR-mediated signaling is thought to be involved in prostate cancer and PDGF-D as a ligand for β-PDGFR was discovered in prostate cancer cells [60]. The same study showed that in these cells, matriptase activates PDGF-D and can deactivate the resulting growth-factor through further proteolytic cleavage [60].

While this review focuses on the signaling pathways underlying TTSP function and their contribution to cancer pathogenesis, the way in which TTSPs influence the tumor microenvironment should also be considered. The ECM is crucial for the maintenance of tissue homeostasis [61] and a loss of ECM integrity contributes to cancer cell growth and invasion [62, 63]. Cancer cells are known to secrete proteolytic enzymes to degrade ECM and invade surrounding tissue [64]. Recently, TMPRSS2 was shown to activate matriptase (described in the TMPRSS2 section), and in a xenograft model of prostate cancer, the TMPRSS2 protein level was shown to correlate with activated matriptase and degradation of the ECM components, nidogen-1 and laminin β1 [65]. It should be mentioned that direct cleavage of nidogen and laminin β1 by TMPRSS2 was demonstrated whereas no data on the ability of matriptase to do the same was presented [65]. In another study, active matriptase was shown to degrade ECM components fibronectin and laminin, *in vitro* [66].

While there is some evidence for TTSPs influencing ECM integrity either directly through cleaving ECM components, or indirectly through secondary pathways, further exploration in *in vivo* models of cancer is required.

Hepsin

Hepsin is shown to be consistently expressed and upregulated in prostate cancer (reviewed in [67, 68]) and high levels in the tumor are indicative of poor outcome and relapse after radical prostatectomy [67, 68]. A search for genetic factors, which may contribute to prostate cancer susceptibility in men of European origin, identified several single nucleotide polymorphisms (SNPs) in non-coding regions of the *hepsin* gene, however the effect of these

SNPs on *hepsin* expression and activity remains unknown [2, 69, 70]. Similar correlations were found in a population of Korean men [71].

Upregulation of hepsin in the prostate epithelium of mice causes disorganization of the basement membrane (BM) of the prostate in probasin (PB) promoter-driven hepsin transgenic mice. When these animals are crossed into transgenic mice carrying the SV40 large T antigen (Tag) under the control of the large probasin (LPB) promoter, a non-metastatic model of prostate cancer [72], metastasis to distant organs including liver, lung, and bone occurs [73]. The authors suggest that hepsin likely exerts its effects through proteolytic modification of the ECM or through the activation of other proteases.

Hepsin expression has been well-documented in several other types of epithelial cancers, including ovarian, and breast [2, 74-76]. When SKOV3 and C11 human ovarian cancer cells with forced expression of hepsin (through lentiviral transduction) are injected into the flank of female nude mice, tumor growth is promoted. The ability of ectopic hepsin to induce tumor growth is dependent on the catalytic activity of the protein, as mutation of the catalytic triad residues abates tumor progression [75].

Although the presence of hepsin in many tumor types and its ability to promote tumor growth in animal models suggests that its activity may confer oncogenicity, the underlying cellular signaling is still actively being investigated. Several substrates have been identified in cell-free or cell-based assays; these include the pro-forms of HGF and MSP-1 [77-79]. Cleavage by hepsin converts these proteins into their active forms which are then able to activate their cognate receptors, c-Met and RON, respectively [77-79]. Additionally, hepsin cleaves zymogens of other cell-surface associated serine proteases *in vitro*, such as matriptase [40], pro-uPA [80], and prostasin, a glycophosphatidylinositol-anchored serine protease [81]. Prostasin is also known to be a substrate of matriptase [11, 82, 83].

Elevated hepsin levels promote epithelial carcinogenesis potentially though basement membrane (BM) and ECM degradation and remodeling, but the molecular underpinnings are still under study. Deregulated hepsin expression is found to be sufficient to disrupt epithelial integrity in primary mouse mammary epithelial cultures [84]. Hepsin is also reported to be the culprit underlying the defective cell polarity, tight junction positioning, desmosomal integrity as well as BM fragmentation observed in primary epithelial cells and tumors from liver kinase B1 (LKB1) deficient mice crossed with oncogenic c-Myc mice [84].

Tervonen et al. showed that hepsin was overexpressed in more than 40% of breast cancers in tumor microarrays. Hepsin was strongly expressed in 40–50% of luminal A, B, and HER2+ subtypes and up to 60% of TNBCs. Furthermore, hepsin was predominantly expressed as the processed active form, as observed by western blot in five out of seven primary breast cancer samples [74].

Potentiation by hepsin of mammary tumor development was also shown in the transgenic Wap-c-Myc mouse model. In these mice, pregnancy induces activation of the Wap promoter to increase levels of c-Myc expression in the mammary gland which induces mammary carcinomas. Upregulation of endogenous hepsin is not observed in this model and was used to elucidate the effect of transgenic hepsin expression on mammary tumor development.

Transplanted primary mammary epithelial cells were isolated from donor Wap-Myc mice, followed by transplantation of the cells to cleared fat pads of syngeneic wild-type virgin hosts. Lentivirus mediated inducible hepsin expression of the primary cells led to decreased latency of tumor formation. Thus, expression of hepsin decreased latency to 189 days compared to over 260 days for mice expressing catalytically inactive hepsin [74]. These data suggest that hepsin may influence tumor initiation and early progression phases.

HGF/c-Met signaling represents a potential pathway by which hepsin contributes to tumorigenesis. In MCF10A mammary epithelial cells, overexpression of hepsin enables cells to proteolytically activate pro-HGF, leading to activation of the c-Met pathway. Zoratti et al. also described the HGF/c-Met axis as the target of matriptase in a variety of breast cancer cell lines. However, in these lines, matriptase appeared to be the primary activator, since no or minimal residual pro-HGF activity was detected following matriptase silencing. Therefore, perhaps there are different predominant TTSP initiators of c-Met/HGF signaling depending on cell type and context. MCF10A cells differ from the cells examined by Zoratti et al. in that they are not breast cancer cells. Instead they are typically used as a model for normal breast cells. MCF10As were derived from benign proliferative tissue and spontaneously immortalized without defined factors [85, 86]. Additionally, Tervonen et al. explored the effects of overexpressed hepsin which appears to allow for these cells to proteolytically activate pro-HGF, while Zoratti et al. explored the role of silencing endogenous matriptase levels [25]. It was found that the consequences of hepsin overexpression in MCF10A cells include down-modulated demosomal, hemidesmosomal and basal lamina proteins, which damage epithelial cohesion and sites which connect the basal surface of these cells to the basement membrane. It should also be noted that induced hepsin acutely down-modulated its cognate inhibitor, HAI-1 [74]. HAI-1 and HAI-2 have been shown to inhibit hepsin activity in cell-free enzyme inhibition assays [79]. These experiments are consistent with a model of hepsin oncogenic activity, in which increased expression of hepsin and loss of HAI-1 contribute to augmented HGF/c-Met signaling.

TMPRSS2

Like hepsin in prostate cancer, TMPRSS2 and TMPRSS4 may also promote metastasis. TMPRSS2 message is most highly expressed in the epithelium of prostate tissue, with pancreas, kidney, and colon exhibiting strong expression, and lung, small intestine, and liver exhibiting weak expression [87]. *TMPRSS2* gene expression is several fold higher in cancer cells compared to benign cells in human specimens containing cancerous and benign prostate tissue [88]. Additionally, chromosomal rearrangements of the *TMPRSS2* gene have been identified in prostate cancer patients, in which 5′ untranslated region of the gene, containing androgen-responsive elements, becomes fused to coding sequences of transcription factors from the E26 transformation-specific (ETS) family, *ERG, ETV1*, or *ETV4*, thereby making their expression androgen-inducible [89-91]. In fact, *ERG* and *ETV1* are fused with *TMPRSS2* in approximately 50–79% cases of prostate cancer [92] and *TMPRSS2–ETS* fusion is associated with a poor prognosis in localized prostate cancer [93].

TMPRSS2 message is upregulated in androgen-dependent prostate cancer compared to normal prostate epithelium or benign hyperplasia [2, 88, 94], and TMPRSS2 transcription is

regulated by androgenic ligands and the androgen receptor (AR) [94]. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, mice spontaneously develop autochthonous prostate tumors following the onset of puberty [95]. Deletion of *TMPRSS2* in TRAMP mice significantly attenuated metastasis despite increased primary tumor size [96]. This is in line with the emerging view that metastasis and primary tumor growth are controlled by different factors [97] and suggests that TMPRSS2 may be critical in tumor metastatic behavior, specifically. Since the vast majority of prostate cancer associated deaths are due to metastasis, this is of particular importance.

While the relationship between increased gene message or protein expression of TMPRSS2 in prostate cancer aggression is well-established, the signaling underlying its oncogenic role is less clear. In 2005, it was shown that TMPRSS2 is able to cleave and activate PAR-2 on human prostate cancer cells (LNCaP) [98]. A recent study also identified matriptase as a possible substrate of TMPRSS2. Thus, stably transfected TMPRSS2-overexpressing clones from a variety of prostate cancer cell lines showed increased levels of activated matriptase, which formed a 120-kDa complex with HAI-1 and corresponded with reduced levels of latent matriptase (70 kDa) and free HAI-1. These data were further corroborated by the finding that orthotopic grafts of LNCaP cells overexpressing TMPRSS2 increased both active matriptase and metastases, and that this increase is dependent upon TMPRSS2 catalytic activity [15, 65]. High-throughput screens of combinatorial libraries also identified pro-HGF as a substrate and it was confirmed that exogenous pro-HGF activated by incubation with TMPRSS2 was sufficient to activate the c-Met receptor, expressed in DU145 prostate cancer cells. Additionally, LNCaP and C4-2B prostate cancer cell lines which endogenously express TMPRSS2, were shown to be more invasive upon exposure to pro-HGF compared to vehicle or pro-HGF plus an HGF-neutralizing antibody [96].

TMPRSS4

TMPRSS4 was first identified in pancreatic cancer [99]. It is now known to also be overexpressed in ovarian, thyroid, colorectal, breast, cervical, gallbladder, gastric, and liver cancer [100, 101]. However, the mechanism through which levels are increased, for example by gene amplification, chromosomal rearrangement or transcriptional dysregulation is still unclear [100].

Silencing of this TTSP in thyroid cancer cells suppresses proliferation which correlates with decreased cyclin D1 mRNA levels and inhibition of Ser133 phosphorylation of the cyclin D1 transcription factor, CREB [102]. In lung and colon cancer cells TMPRSS14 knock-down impedes migration [103, 104] and invasion through a variety of extracellular matrices and impairs cell proliferation [103], while overexpression enhances migration and invasion in colon cancer [103, 104]. A more recent study reported that TMPRSS4 induces Slug and cyclin D1 through activator protein-1 (AP-1) activation in PC3 cancer cells, leading to invasion and proliferation [105]. Additionally, the authors describe a positive feedback loop between Slug and AP-1, which leads to the induction of cyclin D1 and cell proliferation [105].

Like TMPRSS2, TMPRSS4 appears to enhance metastatic potential, possibly through promoting acquisition of epithelial to mesenchymal transition (EMT) (see [103, 104]). TMPRSS4-overexpression in SW480 (colon cancer) cells causes increased invasion, *in vitro*, and increased metastasis to the liver when injected intrasplenically into nude mice, [103]. While this phenotype is attributed to loss of e-cadherin and EMT, the underlying signaling mechanism has yet to be elucidated. Of the several well-known e-cadherin transcriptional repressors/EMT-inducing transcriptional repressors examined, there was no detectable upregulation of SNAIL, SLUG or TWIST mRNA, although there was concomitant induction of SIP1/ZEB2, which are known to repress e-cadherin, in response to TMPRSS4 overexpression [103]. Studies in lung cancer cell- and animal-based models revealed that TMPRSS4 silencing resulted in a significant reduction in proliferation, clonogenic capacity, and invasion. In addition, a significant impairment of lung colonization and growth was found when mice were tail vein injected with TMPRSS4 silenced lung cancer cells [104].

Further studies in SW480 cells showed that overexpression of TMPRSS4 induces a signaling cascade which includes FAK, ERK1/2, Akt, Src, and Rac1 activation and identified upregulation of integrin α5 as a potential mechanism through which TMPRSS4 induces signaling transduction, invasion, and EMT [106]. However, it is not yet known how the protease regulates integrin expression. In support of a link between TMPRSS4 and integrin α5, transcriptomic profiling studies of TMPRSS4-silenced lung cancer cells, revealed that the gene, MIR205HG coding for the metastasis suppressing micro-RNA, mir-205 increases upon TMPRSS4 knock-down in lung cancer cells and integrin α5 was shown to be a direct target of this micro-RNA [107]. Therefore, a new regulatory pathway was proposed involving TMPRSS4/miR-205/integrin α5.

Although the study of TMPRSS4 has gained momentum in recent years, genetic animal models are necessary to fully unravel the role of the signaling pathways highlighted here. TMPRSS4 null mice were generated in 2015 [108] which are viable, fertile, and did not show any obvious abnormalities. However, at the time this review was written, no groups had reported the use of this model in cancer studies.

TTSPs as novel therapeutic targets in cancer

Although further validation is required in animal models for some of the TTSPs discussed in this review, in general TTSPs represent a viable target for the development of therapeutic agents. Protein-based targeting agents, including antibodies and modified cognate inhibitors, as well as small molecule inhibitors have been shown to effectively block activity of several TTSPs in *in vitro* or *in vivo* settings, and inhibit some aspects of cancer pathogenesis in cell and animal models.

Thus far, early studies targeting matriptase appear to be promising. As proof of principle, matriptase-mediated tumorigenesis was shown to be negated by its cognate inhibitors, HAI-1 and HAI-2. Transgenic expression of either HAI-1 or HAI-2, not only prevents malignant transformation in the SCC model of transgenic mice overexpressing matriptase in the epidermis [21, 22] but importantly, also causes already established tumors to regress demonstrating the power of utilizing this pathway as a means of intervention [22].

A number of small molecule inhibitors targeting matriptase have been described [52, 109-115]. IN-1, which contains a ketobenzothiazole serine trap was designed based on the auto-activation site (RQAR) of matriptase [116]. IN-1 is highly selective for matriptase compared to other related proteases [116]. This compound was shown to efficiently block pro-HGF conversion to active HGF, subsequent activation of c-Met, Gab1, and AKT, and therefore, cell proliferation and invasion in both murine primary mammary carcinoma cells and in human breast cancer cell lines [25]. This same compound was shown to block the aforementioned signaling pathway in human IBC cell lines and inhibit cell proliferation [31].

In 2014, the first substrate-based ketothiazole inhibitors of substrate-based ketothiazole inhibitors of hepatocyte growth factor activator (HGFA), matriptase and hepsin were described. The compounds blocked the conversion of native pro-HGF and pro-MSP by HGFA with equal potency and caused a dose-dependent decrease of c-Met signaling in MDA-MB-231 breast cancer cells [117]. Recently, the optimization of α-ketothiazole inhibitors into selective and potent inhibitors that display sub-nanomolar enzyme inhibition against one, two, or all three of HGFA, matriptase, and hepsin, were described [118]. These inhibitors target activation of pro-HGF and pro-MSP. Further, they blocked pro-HGF-mediated migration of invasive DU145 prostate cancer cells [118]. Around the same time, a study by a different group described the development of a triplex benzamide micromolar inhibitor of matriptase, hepsin, and HGFA, called SRI 2315 [119]. This compound was shown to inhibit fibroblast-induced c-Met activation, EMT, and migration of cancer cells. It also overcame resistance to cetuximab and gefitinib in HGF-producing colon cancer cells as well as averted fibroblast-mediated resistance to epidermal growth factor receptor (EGFR) inhibitors [119].

MCoTI-II, a cyclic microprotein of the squash *Momordica cochinchinensis* trypsin-inhibitor family, is a specific and potent matriptase inhibitor which blocks pro-HGF activation in cell free and cell culture models and was shown to selectively inhibit invasion of matriptase-expressing prostate cancer cells [115]. Additionally, CVS-3983, another selective small molecule matriptase inhibitor was found to suppress the growth of androgen independent human prostate tumor xenografts. [112]

Hypomorphic matriptase mice, which retain <1% of both intestinal [120] and epidermal matriptase message [121] have only mild icthyosis of the skin, which subsides with age [121], and a slight intestinal barrier defect that has no apparent adverse consequences in adult mice [120]. Also, humans with a hypomorphic mutation in the matriptase-encoding *St14* gene resulting in a matriptase mutant protein with 1000-fold lower activity than that of wild-type matriptase, have only a mild and temporary ichthyosis of the skin, brittle hair, but no reported gastrointestinal symptoms [121, 122]. Consequently, drugs which target the matriptase pathway are not anticipated to cause significant side-effects.

Drugs developed against c-Met receptor include several which are currently in phase 2 clinical trials in patients with TNBC, including IBC. Some of these specifically target c-Met only, while others target a combination of c-Met and other kinases: XL184 (cabozantinib), c-Met-vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor, and X-396 and

X-376 (crizotinib), c-Met- anaplastic lymphoma kinase (ALK) inhibitor. Combining targeted therapy to multiple pathways, including TTSPs may prevent resistance to kinase inhibitors [123].

The development and use of selective hepsin inhibitors is still in progress, however a few have been tested in cell-based and animal models. An analysis of small molecule hepsin inhibitors based on benzamidine and benzguanidine arginine mimetics revealed a subset of piperazinyl ureas that had potency and selectivity for hepsin over matriptase and HGFA in enzymatic assays [124]. In 2014, Tang et al. reported the development of a novel, non-toxic, and orally bioavailable small molecule hepsin inhibitor, HepIn-13 [125]. This inhibitor can block pro-HGF conversion to active HGF by hepsin, in an overexpression system in HEK293 cells and displayed minimal inhibition of matriptase (the effect on other related serine proteases was not reported). In the double transgenic line, LPB-Tag/PB-Hepsin mice, which develops metastasis [73], HepIn-13 reduced metastasis to the lungs (42% of control group versus 20% of treatment group).

In a xenograft model of prostate cancer, in which LnCap-34 cells display a hepsin-mediated ability to invade and develop lymph node metastasis, the HAI-1 protein-based hepsin inhibitor; a PEGylated form of the Kunitz domain-1 (KD1-PEG), decreased contralateral prostate invasion and lymph node metastasis by 50% upon daily intraperitoneal administration [126]. The authors cautioned that inhibitory effects of KD1-PEG on other proteases cannot formally be ruled out.

In addition to these inhibitors, studies using antibodies targeting hepsin have also been reported. The monoclonal, humanized, allosteric antibody, hH35 inhibits hepsin enzymatic activity at nanomolar concentrations [127]. Fab25, which was found by screening a Fab phage display library, is another allosteric inhibitor [78], although their effects in either cell- or animal-based systems remain unexplored. Xuan et al. generated monoclonal neutralizing antibodies that inhibited hepsin's proteolytic activity and significantly reduced invasion of DU145 (protaste cancer) and CAOV-3 (ovarian cancer) cells in *in vitro* assays [128].

Although not a specific inhibitor, WX-UK1, which was originally developed against uPA is a micromolar range inhibitor of the hepsin active site. Currently under clinical investigation as an oral pro-drug, WX-671/MESUPRON, for the treatment of pancreatic cancer and metastatic breast cancer [129], this drug was also found to be effective in suppressing hepsin activity in MCF10A hepsin overexpressing cells [74]. Additionally, WX-UK1 demonstrated antitumor activity in a 4T1 orthotopic breast tumor model. 4T1 cells express both zymogen and processed forms of hepsin, and WX-UK1 inhibited the growth of 4T1 tumors in syngeneic mice as efficiently as the standard-of-care drug tamoxifen [74].

It has been shown that *hepsin* is not an essential gene and knockout mice are viable and fertile [130, 131], although they do exhibit hearing loss due to developmental deformities in the cochlea [132], enlarged hepatocytes, and narrowed liver sinusoids [133]. Further, mice treated with the HepIn-13 inhibitor did not develop any apparent deficiencies [125], suggesting that targeting this TTSP would not cause any major detrimental side effects in humans, although more rigorous studies are needed.

Regarding TMPRSS2, a chemical library screen identified bromhexine hydrochloride, an FDA-approved ingredient in mucolytic cough suppressants, as a bioavailable drug suppressing distant metastasis to lung and liver sites in TRAMP mice [96]. Other synthetic inhibitors have been identified, although their mechanism is unclear [15, 134, 135]. Deletion of TMPRSS2 from mice does not appear to affect development or physiological function, suggesting that ablation of TMPRSS2 in cancer may have minimal side effects [136].

For TMPRSS4, a novel series of 2-hydroxydiarylamide derivatives were synthesized and evaluated for inhibiting TMPRSS4 protease activity and suppressing cancer cell invasion. These derivatives exhibited inhibitory activity against the TTSP and correlated with anti-invasive activity against colon cancer cells overexpressing TMPRSS4. However, the selectivity of this inhibitor was not reported [137].

Conclusions

Recent work has established a clear role for TTSPs in cancer pathogenesis in a variety of carcinomas. As the pro-oncogenic signaling is elucidated, recurrent signaling themes have emerged. The HGF/c-Met and PAR-2 signaling pathways link most of the TTSPs discussed in this review and it was recently shown that concurrent activation of both of these pathways by matriptase is required for development of SCC, underscoring the relationship between inflammatory and cell-survival signaling in cancer development [38]. The HGF/c-Met pathway can be activated by matriptase, hepsin and TMPRSS2 and it is likely that there is a degree of functional overlap between the proteases *in vivo* that is influenced by multiple factors including tissue/cancer type as well as genetic and epigenetic determinants. While matriptase and hepsin signaling through the RON receptor has also been described in cell-based experiments, it has yet to be validated in animal models. Studies have also shown that the TTSPs may interact with each other: matriptase, which can auto-activate, may be a substrate of both hepsin and TMPRSS2, and HAI-1/2 are endogenous inhibitors of matriptase and cognate inhibitors of hepsin, suggesting complex interaction between TTSPs and respective downstream signaling cascades. Many TTSPs, especially within the HAT/DESC/HATL and matriptase subfamilies, display a high degree of similarity in both their primary sequence and domain structure. However, it remains to be seen if this translates into overlapping biological functions in tissues with overlapping TTSP expression or with respect to cancer and other diseases.

In sum, TTSPs represent a promising option for therapeutic intervention and numerous studies on the development of inhibitors against TTSP-mediated signaling have been reported, although no specific TTSPs inhibitors are currently in clinical trials. Importantly, based on previous experiments, targeting of matriptase, hepsin, or TMPRSS2 is not anticipated to cause major adverse effects. It is the hope that the TTSP basic science and drug discovery work performed in laboratories across the world, a small fraction of which is described above, will provide a springboard for future clinical applications using TTSPs as targets in cancer intervention.

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Abbreviations

TTSP	type II transmembrane serine protease
TMPRSS	transmembrane protease, serine
HAT	human airway trypsin-like
DESC	differentially expressed in squamous cell carcinoma
HGF	hepatocyte growth factor
SF	scatter factor
HAI-1	hepatocyte growth factor inhibitor-1
HAI-2	hepatocyte growth factor inhibitor-2
SCC	squamous cell carcinoma
MMTV	mouse mammary tumor virus
PymT	polyomavirus middle T
CDCP1	CUB-domain-containing protein 1
PGDF-D	platelet derived growth-factor-D
IBC	inflammatory breast cancer
PAR-2	proteinase-activated-receptor-2
DMBA	dimethylbenzanthracene
K5	keratin-5
NF	nuclear factor
PB	probasin
Tag	T antigen
LPB	large probasin
ECM	extracellular matrix
BM	basement membrane
LKB1	liver kinase B1
E26	transformation-specific

ETS pro-UPA	pro-urokinase type plasminogen activator
AP-1	activator protein-1
AR	androgen receptor
TRAMP	transgenic adenocarcinoma of the mouse prostate
HGFA	hepatocyte growth factor activator
EMT	epithelial to mesenchymal transition
MSP-1	macrophage stimulating protein 1
RON	Recepteur d'Origine Nantais
TNBC	triple-negative breast cancer
ITG	integrin
SNPs	single nucleotide polymorphisms
KD1-PEG	PEGylated form of the Kunitz domain-1
EGFR	epidermal growth factor receptor
VEGFR2	vascular endothelial growth factor receptor 2
ALK	anaplastic lymphoma kinase

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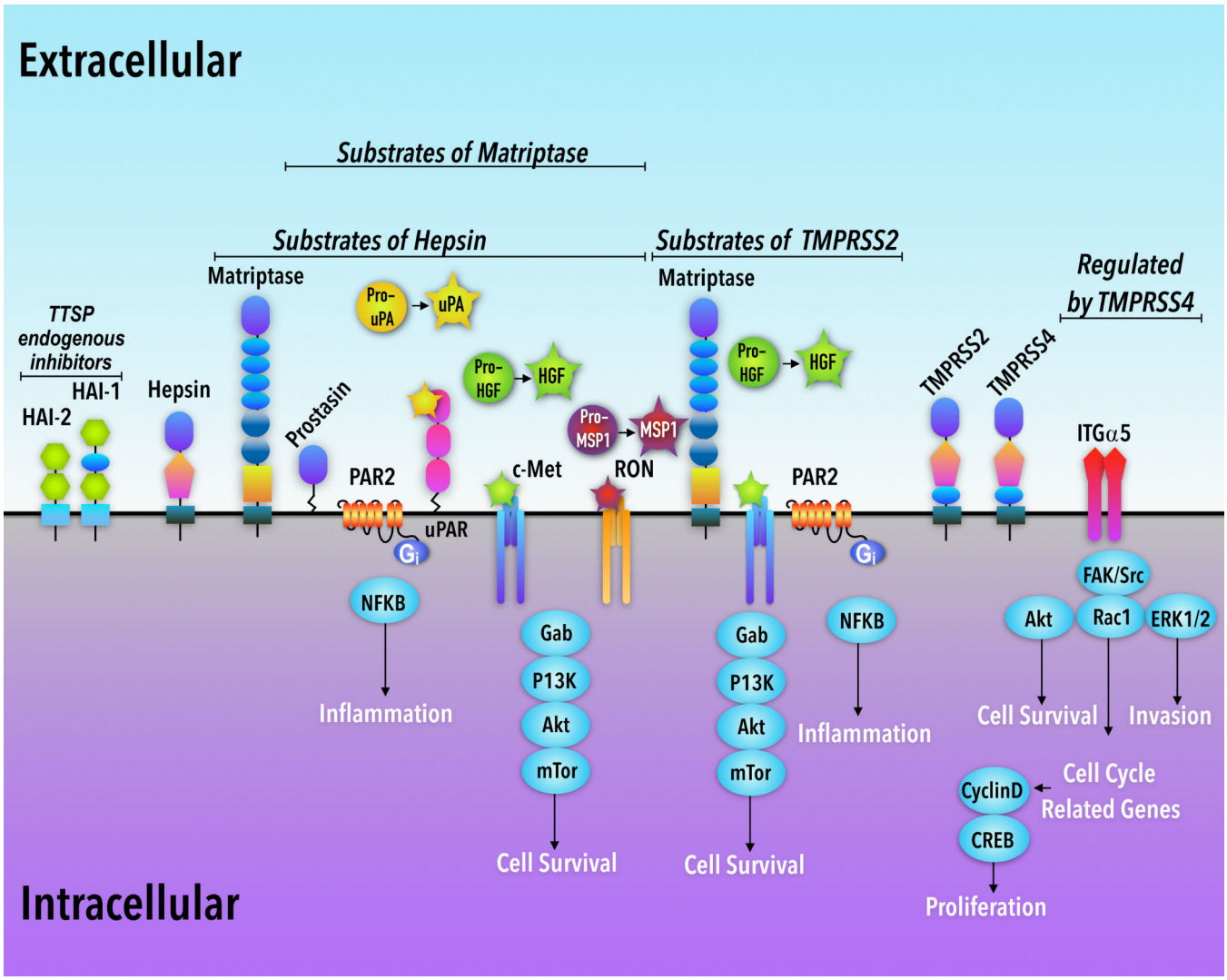

Figure 1. Proposed pro-oncogenic signaling pathways for TTSPs, matriptase, hepsin, TMPRSS2, and TMPRSS4

The HGF/c-Met cell survival pathway is activated by matriptase, hepsin, and TMPRSS2. Similarly, matriptase and hepsin are able to cleave and activate pro-MSP-1, and pro-uPA, and activate RON, and bind to uPAR receptors, respectively. The PAR-2/NFκB pathway is activated by matriptase and TMPRSS2. TMPRSS4 is associated with signaling through the ITG-α5 pathway. Other substrates of matriptase include CDCP1/TRASK/SIMA135 (not shown) and PDGF-D (not shown) and prostasin. Matriptase was also shown to be a substrate of both hepsin and TMPRSS2. HAI-1 and HAI-2 are cell-surface endogenous inhibitors of both matriptase and hepsin