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Review

Proteolytic and non-proteolytic roles of membrane type-1 matrix metalloproteinase in malignancy

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ABSTRACT

This manuscript provides an overview of the dynamic interactions which play an important role in regulating cancer cell functions. We describe and discuss, primarily, those interactions which involve membrane type-1 matrix metalloproteinase (MT1-MMP), its physiological inhibitor tissue inhibitor of metalloproteinases-2 (TIMP-2), furin-like proprotein convertases and the low density lipoprotein-related protein 1 (LRP1) signaling scavenger receptor. The interaction among these cellular proteins controls the efficiency of the activation of MT1-MMP and the unorthodox intracellular signaling which is generated by the catalytically inert complex of MT1-MMP with TIMP-2 and which plays a potentially important role in the migration of cancer cells. Our in-depth understanding of these cellular mechanisms may provide the key to solving the puzzling TIMP-2 paradox. This unsolved paradox arises from the fact that TIMP-2 is a powerful inhibitor of MMPs including MT1-MMP, but at the same time high levels of TIMP-2 positively correlate with an unfavorable prognosis in cancer patients. Solving the TIMP-2 paradox may lead to solving a similar PAI-1 paradox and produce a clearer understanding of the biochemical mechanisms which control the functionality of the urokinase-type plasminogen activator•urokinase receptor•plasminogen activator inhibitor type-1 (uPAR•uPA•PAI-1) system in cancer.

1. Matrix metalloproteinases (MMPs)

Historically, interstitial collagenase (MMP-1) was the first identified member of the now extensive matrix metalloproteinase (MMP) family. MMP-1 was initially discovered in the course of studying collagen remodeling during the metamorphosis of a tadpole into a frog and much later the presence of this enzyme was confirmed in humans [1,2]. Because collagens, especially type I collagen, represent the major structural proteins of all tissues and serve as the main barrier to migrating cells, more than three decades ago an innovative hypothesis was postulated and this hypothesis has now been validated. According to this hypothesis, collagenolytic enzymes including MMP-1 play pivotal roles in multiple physiologic and especially, pathologic processes, which involve both extensive and aberrant collagenolysis. Recent scientific discoveries have vastly expanded our knowledge of MMPs' structures and functions. These discoveries directly implicate a number of the individual MMPs, including MMP-1, in multiple diseases of the cardiovascular, pulmonary, renal, endocrine, gastrointestinal, musculoskeletal, visual, and hematopoietic systems in humans.

MMPs belong to a zinc endopeptidase, metzincin superfamily [3]. This superfamily is distinguished from other proteinases by the presence of a strictly conserved HEXXHXXGXX(H/D) histidine sequence motif. This motif exhibits three histidine residues that chelate the active site zinc and also a canonical methionine residue which is the C-terminal to the conserved histidine sequence. The canonical methionine is part of a tight 1,4-beta-turn that loops the polypeptide chain beneath the catalytic zinc ion, forming a hydrophobic floor to the zinc ion binding site. The metzincin family is normally divided into four sub-families: seralysins, astacins, adamalysins [ADAMs (proteins with a disintegrin and a metalloproteinase domain) and ADAM-TS (ADAM with thrombospondin-like motif)] and MMPs. Although our knowledge of MMP biology is rapidly expanding, we do not as yet understand precisely how these enzymes regulate various cellular functions.

The human MMP family is comprised of 24 currently known zinc-containing enzymes which share several functional domains. MMPs are often referred to by a descriptive name such as gelatinases (MMP-2 and MMP-9) and collagenases [MMP-1, MMP-8, MMP-13, MMP-14/membrane type-1 matrix metalloproteinase (MT1-MMP) and, some what conclusively, MMP-18] and this name is generally based on a preferred substrate. Collagenases are the only known mammalian enzymes capable of degrading triple-helical fibrillar collagen into distinctive 3/4 and 1/4 fragments. An additional and widely accepted MMP numbering system based on the order of discovery is also in use (Fig. 1) [4,5].

MMPs may be described as multifunctional enzymes capable of cleaving the extracellular matrix components (collagens, laminin, fibronectin, vitronectin, aggrecan, enactin, versican, perlecan, tenascin, elastin and many others), growth factors, cytokines and cell surface-associated adhesion and signaling receptors. Because of their

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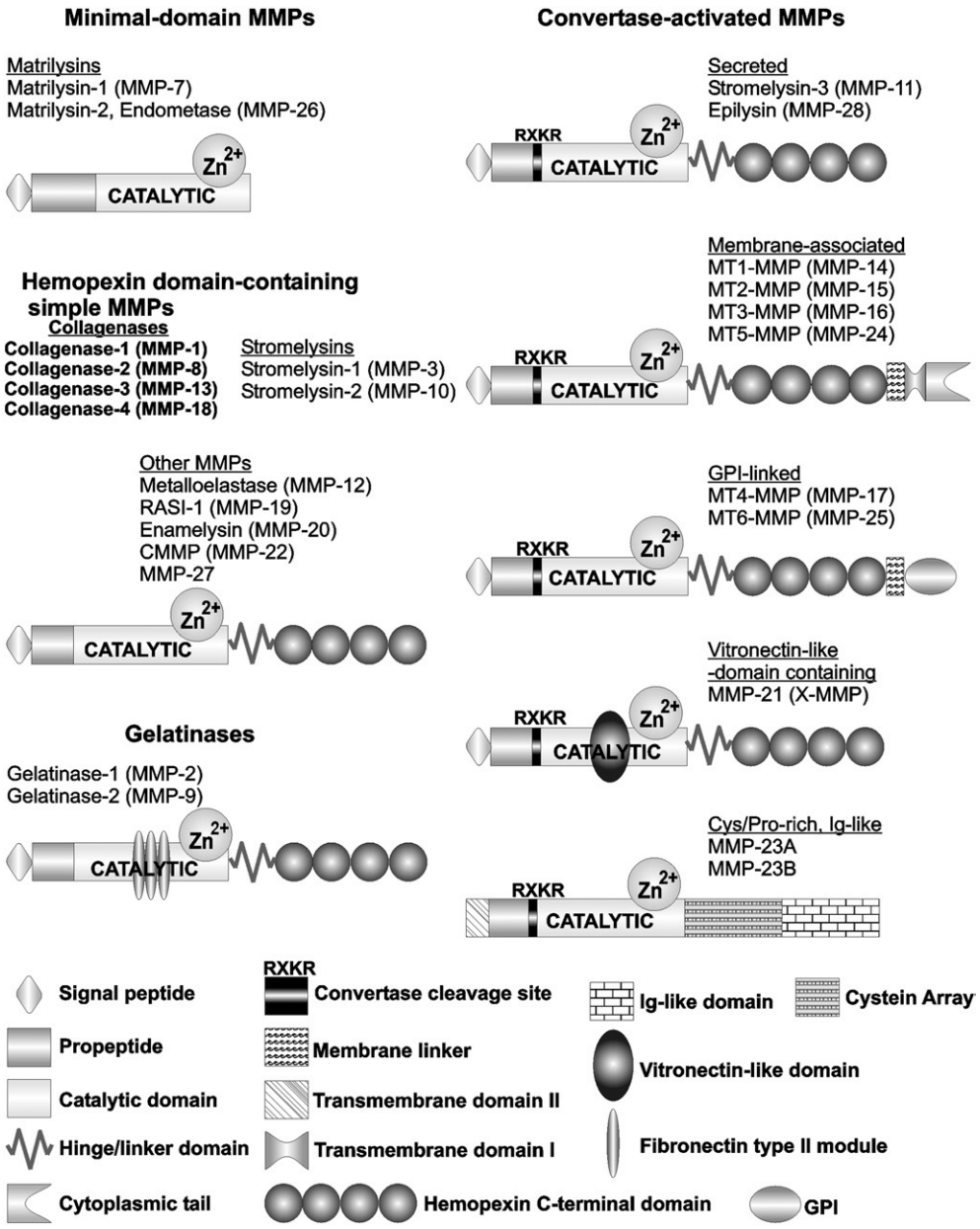

Fig. 1. MMP family. The structure of MMPs is made up of the following domains: 1) a signal peptide; directs MMPs to the secretory or plasma membrane insertion pathway; 2) a prodomain; confers latency to MMPs, 3) a catalytic domain with the active site zinc atom; 4) a hemopexin-like domain; in coordination with the catalytic domain controls the interactions with substrates, and 5) a flexible hinge region; links the catalytic and the hemopexin domain and provides each domain mobility relative to the other. The membrane-type MMPs contain an additional transmembrane domain and a short cytoplasmic tail domain (MT1-MMP/MMP-14, MMP-15, MMP-16 and MMP-24) or a glycosylphosphatidyl inositol linkage (MMP-17 and MMP-25) to be tethered to the cell surface. MMP-2 and MMP-9 contain fibronectin-like type II repeats which assist in collagen substrate binding. A hemopexin domain is absent in MMP-7 and MMP-26. MT-MMPs (in addition to several other MMPs) exhibit the RXKR motif in the C-terminal region of the inhibitory prodomain and, therefore, are activated by furin.

high degrading activity and potentially disastrous effect on the cell microenvironment, cellular MMPs are expressed in small amounts, and their cellular localization and activity are tightly controlled, either positively or negatively, at both the transcriptional and the post-transcriptional levels by cytokines, including interleukins (IL-1, IL-4 and IL-6), growth factors (epidermal growth factor, hepatocyte growth factor and transforming growth factor-β), and tumor necrosis factor-α [6,7]. In a feedback loop, some of these regulatory factors themselves are proteolytically activated or inactivated by the individual MMPs [8].

MMPs are synthesized as latent zymogens. The active site zinc of the MMP catalytic domain is coordinated with the three histidines of the active site and with the cysteine of the "cysteine switch" motif of the N-terminal prodomain [9]. To become functionally potent proteinases, the zymogens of MMPs require proteolytic activation. In the process of this activation, the N-terminal inhibitory prodomain is removed and the catalytic site of the emerging enzyme becomes liberated and exposed. The activation of MMPs may occur both intracellularly and extracellularly [10,11]. MMPs including MMP-11, MMP-28 and several MT-MMPs with the furin-cleavage motif RXK/RR in their propeptide sequence are normally activated in the *trans*-Golgi network by serine proteinases such as furin and certain other members of the proprotein convertase family. Activation of MMPs which are secreted in the extracellular milieu is frequently mediated by serine proteases, including plasmin, by the membrane-type MMPs (e.g., activation of the latent soluble MMP-2 proenzyme by MT1-

MMP) or by other pre-existing active MMPs (e.g., activation of the latent soluble enzymes of MMP-1 and MMP-9 by the soluble MMP-3 enzyme).

With the exception of the activated MMP-7 and MMP-26 enzymes, which are represented by the catalytic domain alone, all other MMPs have a C-terminal hemopexin-like domain. This domain regulates the activity and the specificity of the catalytic domain function. The hemopexin domain is separated from the catalytic domain by a flexible hinge region. Membrane-tethered MMPs are distinguished from soluble MMPs by an additional transmembrane domain and a short cytoplasmic tail (MMP-14/MT1-MMP, MMP-15/MT2-MMP, MMP-16/MT3-MMP and MMP-24/MT5-MMP). In contrast to these four MT-MMPs, MMP-17/MT4-MMP and MMP-25/MT6-MMP are attached to the cell membrane *via* a glycosylphosphatidyl inositol (GPI) anchor (Fig. 1). Historically, MMPs were initially characterized by their extensive ability to degrade extracellular matrix proteins including collagens, laminin, fibronectin, vitronectin, aggrecan, enactin, tenascin, elastin, and proteoglycans. More recently, it has been recognized that MMPs cleave in addition to the extracellular matrix components, many other protein types including cytokines and cell adhesion signaling receptors.

Because the individual MMPs have overlapping substrate cleavage preferences, MMP knockouts and inactivating mutations in individual MMP genes in mice, with the exception of MT1-MMP, do not elicit an easily recognized phenotype and are non-lethal, at least up to the end of the first few weeks after birth, suggesting a functional redundancy among MMP family members. MT1-MMP knockout, however, has a profound effect: MT1-MMP null mice develop dwarfism, extensive bone malformations and die before adulthood, thus supporting an important role of MT1-MMP in collagen type I turnover [12–14]. Mice lacking both MMP-2 and MT1-MMP die immediately after birth of respiratory failure, abnormal blood vessels, and immature muscle fibers reminiscent of central core disease [15].

2. Tissue inhibitors of matrix metalloproteinases (TIMPs)

Once activated, MMPs are normally inhibited by tissue inhibitors of metalloproteinases (TIMPs). Four individual species of TIMPs are known in humans (TIMP-1, -2, -3 and -4) [16] (Fig. 2). The MMP/TIMP balance is believed to be a major factor in the regulation of the net proteolytic activity of the individual MMPs. Structurally, TIMPs contain two domains. The inhibitory N-terminal domain binds non-

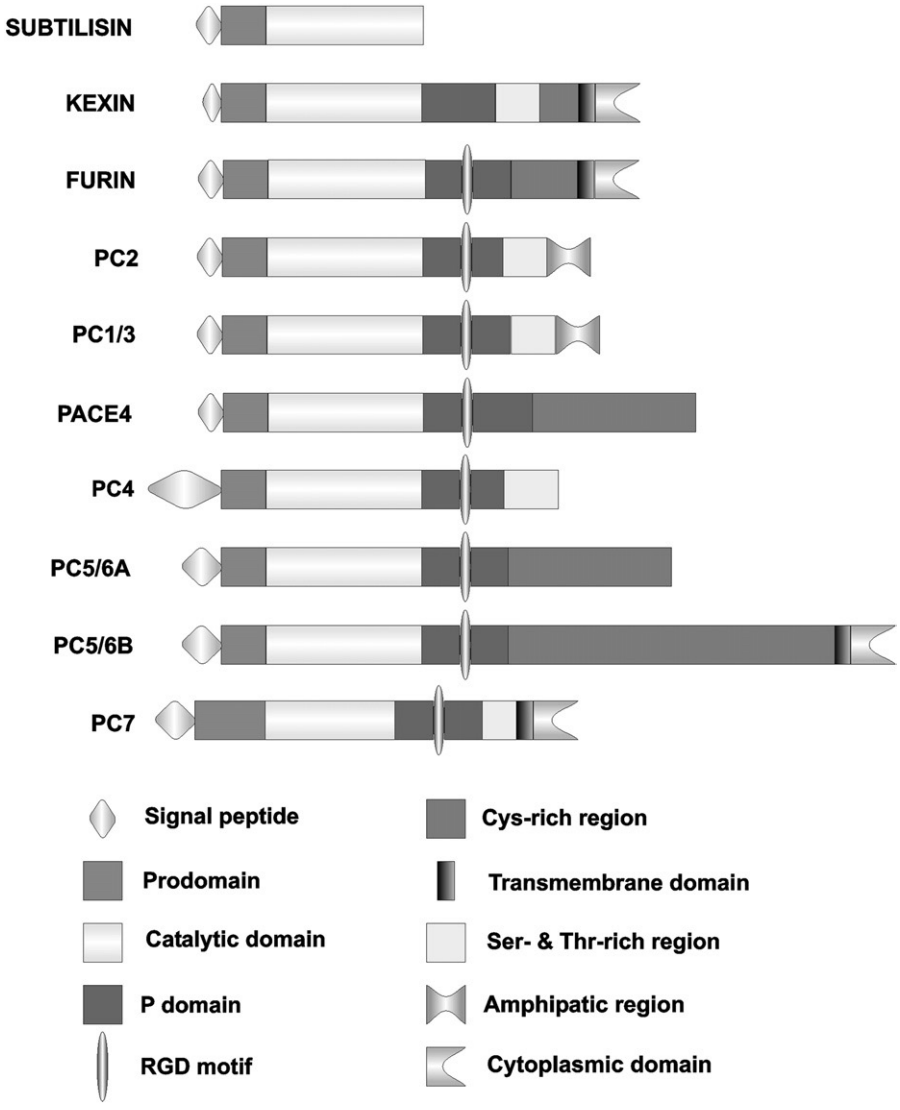

Fig. 2. Modular domain structure of furin and six related proprotein convertases (PCs). The A and B isoforms of PC5/6 are encoded by the same gene. The structure includes (1) the N-terminal signal peptide, which directs proteins into the secretory pathway, (2) a prodomain, which maintains the inactive zymogen state of PCs and which also acts as an intramolecular chaperone for proper folding, (3) a catalytic domain with the active site that exhibits an Asp-His-Ser catalytic triad and an additional Asn, (4) a barrel-like structured P domain that regulates enzyme stability, and (5) a C-terminal domain that contains membrane attachment sequences, a Cys-rich region and intracellular sorting signals.

covalently and stoichiometrically to the active site of the active mature MMPs, blocking access of substrates to the catalytic site. The C-terminal domain of TIMP-1 and TIMP-2 binds to the hemopexin domain of the proenzymes of MMP-9 and MMP-2, respectively. The latter binding is essential for the cell surface activation of MMP-2 by MMP-14/MT1-MMP.

In this well-characterized unconventional activation mechanism, MMP-14/MT1-MMP on the cell surface acts as a receptor for TIMP-2. TIMP-2 binds *via* its N-terminal domain to the active site of MT1-MMP. This binary complex then acts as a receptor for the MMP-2 proenzyme, with the TIMP-2 C-terminal domain binding to the C-terminal hemopexin domain of MMP-2 and with the formation of a trimolecular MT1-MMP–TIMP-2–MMP-2 complex. A TIMP-2-free, second MT1-MMP molecule which is close to the trimolecular complex then cleaves the N-terminal propeptide of the MMP-2 proenzyme, generating an intermediate species. Further proteolysis of the MMP-2 propeptide through an autocatalytic mechanism generates the fully active enzyme of MMP-2 which is then released from the complex.

3. Structure and function of MT1-MMP

MT1-MMP, a prototypic membrane-type MMP, is distinguished from soluble MMPs by a C-terminal transmembrane domain and a cytoplasmic tail [4]. In the human genome, MT1-MMP is encoded by a single copy gene located on chromosome 14. MT1-MMP is widely expressed and its presence has been documented in multiple cell types [17]. Because both the expression and the activity of MT1-MMP are elevated in tumor cells and because high levels of MT1-MMP directly correlate with enhanced cell migration, this proteinase is generally considered pro-invasive and pro-tumorigenic.

Because the prodomain part of MT1-MMP has the furin-cleavage motif, furin is an essential component of the activation pathway that results in the generation of the active, mature cellular MT1-MMP [11,18]. MT1-MMP was originally thought to exhibit a single function as a membrane activator of soluble MMPs, including MMP-2 [19,20] and MMP-13 [21]. Recent data, however, has provided evidence that, in addition, MT1-MMP degrades multiple components of the extracellular matrix and a number of cell adhesion and signaling receptors [22]. MT1-MMP is regulated at the transcriptional and post-transcriptional levels by multifaceted, tightly controlled and well-coordinated mechanisms both as a proteinase and as a membrane protein. These multidimensional mechanisms regulate MT1-MMP spatially and temporally, and they are essential not only for the proper functioning of MT1-MMP alone but also for the performance of the normal multiple biological functions of the entire cell. These mechanisms control the functional activity of cell surface-associated MT1-MMP and they include control of the extent of activation of the MT1-MMP proenzyme by furin, the level of inhibition of MT1-MMP by TIMPs and self-proteolytic inactivation, a homophilic complex formation involving the hemopexin domain and the cytoplasmic tail, the efficiency of trafficking of MT1-MMP through the cell compartment to the plasma membrane, the rate of the internalization of MT1-MMP into the transient compartment inside the cells and, lastly, the extent of the recycling of MT1-MMP back to the plasma membrane [17,22,23].

Internalization *via* clathrin-coated pits and also through caveolae is also recognized as an important mechanism to regulate MT1-MMP activity [24–27]. The “up/down” switch may have been built into the peptide sequence of the MT1-MMP's cytoplasmic tail to regulate the recruitment to the plasma membrane and to target the protease to the invasive front in migrating cells. Transient changes in subcellular compartmentalization of MT1-MMP, which occur in its trafficking and internalization pathways, are, probably, the underlying mechanisms which specifically control the functions of MT1-MMP in malignant cells [28,29].

TIMP-2, TIMP-3 and TIMP-4 are highly potent inhibitors of MT1-MMP. TIMP-1, however, is a very poor inhibitor of MT1-MMP [5,16,30]. Current evidence suggests that the activity of cellular MT1-MMP is short-lived and that the half-life of active, mature MT1-MMP attached to the plasma membrane is approximately 1 h [29,31]. During this time period, active MT1-MMP is either inactivated by TIMPs or autolytically degraded or internalized with only a subsequent partial recycling [32]. Because MT1-MMP, in addition to its role in matrix degradation, is directly involved in the cleavage of cell surface receptors, this short-lived proteinase, exerts a long-lasting effect on cell behavior and functions in cancer cells as the main mediator of proteolytic events on the cell surface. Evidence suggests that the proteolysis of CD44, integrins, tissue transglutaminase, the low density lipoprotein receptor-related protein (LRP1), E-cadherin and related cell-associated adhesion signaling receptors is the important role of MT1-MMP [22]. By cleaving these receptors, the short-lived MT1-MMP has a long-lasting effect on the cell microenvironment and cell behavior. In addition, MT1-MMP, as opposed to the soluble MMPs, is ideally positioned to regulate pericellular proteolysis and the functionality of the neighboring cell receptors, including CD44 [23]. CD44 is the most well-recognized target of MT1-MMP proteolysis in tumor cells [33–37]. MT1-MMP acts as a processing enzyme for CD44, releasing it into the medium as a soluble 70-kD fragment. This processing event stimulates cell motility. In turn, expression of either CD44 or MT1-MMP alone did not significantly stimulate cell motility. Co-expression of MT1-MMP and mutant CD44 lacking the MT1-MMP-processing site did not result in shedding and did not promote cell migration, suggesting that the processing of CD44 by MT1-MMP is critical in the migratory stimulation. MT1-MMP forms a complex with CD44 *via* the hemopexin domain and, as a result, CD44 acts as a linker that connects MT1-MMP to the actin cytoskeleton and plays an important role in directing MT1-MMP to the migration front.

Since the discovery of MT1-MMP in 1994–1995 and the findings showing its role in the activation of MMP-2 there remains an unanswered question: what is the mechanism of the activator's activation? [19,20,38]. Volumes of data generated through the years suggest that MT1-MMP is a key player in tumor cell migration and that MT1-MMP is a likely drug target in multiple pathologies. A precise and complete understanding of the activation and regulation mechanisms of MT1-MMP is required for the design of effective therapies.

4. Proprotein convertases (PCs)

Furin and related proteases are specialized serine endoproteinases, which cleave the multibasic motifs R-X-R/K/X-R and transform proproteins into biologically active proteins and peptides [39,40]. Structurally and functionally, furin resembles its evolutionary precursor: the prohormone-processing kexin of yeast *Saccharomyces cerevisiae*. The enzyme furin, which is encoded by the *fur* gene, was the first and can be considered the prototype of a mammalian subclass of subtilisin-like proteases. The localization of the gene immediately upstream from the FES oncogene (V-FES feline sarcoma viral oncogene homolog) generated the name FUR (for FES upstream region). Furin is similar to other PCs in that it contains a signal peptide, a prodomain, a subtilisin-like catalytic domain, a middle P domain, a cysteine-rich region, a transmembrane anchor, and a cytoplasmic tail (Figs. 1 and 2). Furin and PCs are normally N-glycosylated glycoproteins. Phosphorylation of the cytoplasmic tail is required for the *trans*-Golgi localization of furin which *in vivo* exists as di-, mono- and non-phosphorylated forms.

Furin is currently the most studied enzyme of this protease family. Seven furin-family proteases (furin, PC1/3, PC2, PC4, PC5/6, PC7 and PACE4) have been identified in humans [41]. Isozyme-1 (SKI-1) [42] or Site1 Protease (S1P) (PCSK8) [43] (pyrolysin subfamily) and Neural Apoptosis Regenerative Convertase-1 (NARC-1) or PCSK9 [44] (proteinase K type) which cleave peptide bonds C-terminal to a

non-basic amino acid were later discovered. Furin is ubiquitously expressed and it is primarily localized in the *trans*-Golgi network. A proportion of the furin molecules cycles between the *trans*-Golgi and the cell surface. Because of the overlapping substrate preferences and cell/tissue expression, redundancy exists in the functionality of PCs *in vivo*, although certain distinct functions of the individual members of the family have been demonstrated. For example, furin knockout is lethal in mice [45] attesting to its unique *in vivo* functions. Most of the furin targets, including matrix metalloproteinases such as MT1-MMP, growth factors, and adhesion molecules are essential in the processes of cellular transformation, acquisition of the tumorigenic phenotype, and metastases formation. Furin and other PCs are self-activated and, as a result, they initiate the proteolytic cascade leading to the activation of MT1-MMP and the downstream MMP-2 and MMP-13 [40]. Propeptide cleavage is a prerequisite for the exit of furin molecules out of the endoplasmic reticulum. The second cleavage in the propeptide occurs in the *trans*-Golgi network, which is followed by the release of the propeptide bound to furin and the activation of furin.

5. A "single-step" mechanism of MT1-MMP activation is not completely correct

It is known that the $R^{108}RKR^{111}\downarrow Y^{112}$ motif of the prodomain sequence of the latent MT1-MMP proenzyme is processed by furin and several additional furin-like PCs in the course of the secretion pathway and that this "single-step" processing results in the mature enzyme commencing from N-terminal Y^{112} [18]. It is also well established that the Cys residue of the prodomain cysteine-switch motif maintains the latent status of the proenzyme by chelating the active site zinc. The cysteine-switch sequence itself and the prodomain inhibit MMPs including MT1-MMP. Experimental evidence confirms that the individual prodomain is a potent inhibitor of MT1-MMP ($K_i = 200$ nM).

A "single-step" mechanism suggests that the excised prodomain likely remains associated with the mature proteinase and as a result, inactivates the proteinase. Thus, following cleavage of the ADAM12 prodomain in the *trans*-Golgi by a furin proteinase, the prodomain remains non-covalently associated with the mature molecule [46]. We know, however, that in contrast with ADAM12 the activated MT1-MMP is free from its prodomain and, as a result, the mature enzyme exhibits full proteolytic activity.

6. A two-step mechanism of MT1-MMP activation

To inactivate the excised inhibitory prodomain and to liberate the processed active enzyme species from the prodomain's inhibition, multiple members of the MMP family employ a two-step mechanism. This mechanism involves the cleavage of the prodomain peptide sequence both by an external proteinase and by autocatalysis. Based on these considerations and on the conserved three-helix bundle structure of the MMP prodomains, we now believe that, in addition to a furin-dependent step, there is an additional and previously uncharacterized step in the MT1-MMP activation process [47]. Accordingly, we believe that detecting and establishing the precise nature and the N-terminal sequence of the activation intermediate(s), which are generated as a result of the cleavage in the "bait" region of the prodomain, will lead to a more complete understanding of the activation mechanism of MT1-MMP.

The results of our recent biochemical studies support our hypothesis [47]. In our study, we used synthetic peptides, the recombinant prodomain and the soluble MT1-MMP constructs, the furin-cleavage resistant MT1-MMP mutants and the MT1-MMP mutants with the inactivated bait region cleavage sites. The proteolytic processing of these constructs was analyzed by N-terminal sequencing and MS of the resulting cleavage fragments. Our results provide substantial evidence that supports a two-step mechanism of MT1-MMP activation and prodomain sequence processing. Our results suggest that there is a proteolytic processing of the bait region of the prodomain sequence of MT1-MMP (either at the $P^{47}GD\downarrow L^{50}$ or the $P^{58}QS\downarrow L^{61}$ or at both sites). This event results in the activation intermediate of MT1-MMP, the presence of which we have demonstrated using *in vitro* tests and cell-based assays. In agreement, the processing of the prodomain by furin was impaired in the L50D/L61D mutant in which both cleavage sites of the prodomain bait region were inactivated by mutations. The step-wise activation of MT1-MMP also involves the action of a furin proteinase cleaving the inhibitory prodomain at the $R^{108}RKR^{111}\downarrow Y^{112}$ site, where Y^{112} is the N-terminal residue of the mature MT1-MMP enzyme. This two-step mechanism eventually leads to the degradation of the inhibitory prodomain and the release of the activated, mature MT1-MMP enzyme.

7. TIMP-2 paradox in cancer

The TIMP-2 paradox we want to clarify arises from the fact that TIMP-2 is a powerful inhibitor of the pro-invasive, pro-tumorigenic MT1-MMP activity but, at the same time, high levels of TIMP-2 positively correlate with an unfavorable prognosis in cancer patients [48–52]. In a manner counterintuitive to the conventional thinking, it was recently demonstrated that in addition to extracellular proteolysis, the MT1-MMP•TIMP-2 interactions control cell functions through a non-proteolytic mechanism [53]. TIMP-2 (but not structurally similar TIMP-1) binding to MT1-MMP induces, in a matter of minutes, an intracellular ERK1/2 signaling cascade. The induction of the ERK cascade was observed only at the physiologically-relevant, low concentrations of TIMP-2. Excessively high, abnormal concentrations of TIMP-2 (1–5 μg/ml or 50–250 nM) were used in cell systems previously [54–59] and these concentrations are multi-fold higher than the 10–100 ng/ml (0.5–5 nM) range recorded for tissues and biological fluids [60–63]. As a result, the signaling function of the MT1-MMP•TIMP-2 complex were overlooked and remained unidentified.

8. MT1-MMP•TIMP-2-dependent signaling

Our group jointly with Dr. P. Mignatti (New York University) [53] demonstrated that very low, physiological, concentrations of TIMP-2 stimulate, in a dose-dependent and MT1-MMP-dependent manner, the rapid activation of the downstream Ras-Raf-ERK signaling cascade leading to a burst in cancer cell migration. When compared to the MT1-MMP-deficient breast carcinoma MCF7 control, the proteolytically inert mutant MT1-MMP E240A construct also stimulated the Ras-Raf-ERK signaling cascade and promoted cell migration in the presence of physiological concentrations of TIMP-2. This TIMP-2-dependent stimulation, however, was less efficient than which we observed with the wild-type MT1-MMP construct. In turn, in the presence of TIMP-2 the tailless MT1-MMP that lacked the C-terminal cytoplasmic tail did not stimulate the Ras-Raf-ERK cascade and did not promote cell migration. The C-terminal PEX domain (but not the catalytic domain) of the inert mutant binds TIMP-2 — this important discovery was clearly described in our recent paper [53]. This binding mode is similar to that of the proMMP-2•TIMP-2 complex in which the MMP-2's PEX domain (but not the catalytic domain) binds the inhibitor's C-terminal domain. In contrast with TIMP-2, the binding of GM6001 (a small-molecule active site inhibitor) cannot stimulate the ERK signaling pathway. The Ala-N end-appended TIMP-2 with the inactivated N-terminal inhibitory domain also induces the ERK signaling, suggesting again that binding of the C-terminal domain of TIMP-2 with the PEX domain of MT1-MMP is required for signaling [53]. Overall, the data suggested, for the first time, the presence of the non-proteolytic mechanisms involving MT1-MMP and TIMP-2 in the regulation of cell migration.

9. MT1-MMP•TIMP-2-dependent signaling in cancer cells which naturally express MT1-MMP

To demonstrate that the MT1-MMP•TIMP-2-dependent signaling has physiological relevance, fibrosarcoma HT1080 cells, which express MT1-MMP naturally, were studied in addition to the stably transfected, MT1-MMP-overexpressing cells (Sounni et al., manuscript in preparation). In this study, the expression of MT1-MMP was transcriptionally silenced by using the MT1-MMP siRNA and the generated MT1-MMP-siRNA cells were used as a control. As an additional control, siRNAscr cells, which were transfected with the scrambled siRNA construct were isolated. Immunoblotting confirmed the near complete repression of MT1-MMP in MT1-MMP-siRNA cells. In turn, the scrambled siRNA did not have any significant effect on MT1-MMP. Consistent with the presence of MT1-MMP on cell surfaces, only siRNAscr and parental cells activated MMP-2. Because of the silencing of MT1-MMP, MT1-MMP-siRNA cells did not activate MMP-2. Consistent with the silencing of MT1-MMP, the migration efficiency of MT1-MMP-siRNA cells was reduced when compared with both parental HT1080 and siRNAscr cells. In the absence of the externally added TIMP-2, inhibition of MT1-MMP by GM6001 decreased the migration efficiency of the original HT1080 and siRNAscr cells to the levels we recorded with the untreated MT1-MMP-siRNA cells. In turn, GM6001 did not cause any additional suppression in MT1-MMP-siRNA cells.

The physiological concentrations of TIMP-2 (50–100 ng) were used to stimulate ERK signaling in HT1080 cells. A 15-min co-incubation with TIMP-2 (100 ng/ml) was sufficient to induce ERK1/2 phosphorylation in siRNAscr cells. GM6001 did not reverse the stimulatory effect of TIMP-2 on phosphorylation of ERK1/2 in siRNAscr cells. In contrast with pERK1/2, TIMP-2 did not affect the levels of both pSrc and total Src in both MT1-MMP-siRNA and siRNAscr cells, thus supporting the specific activation of the ERK pathway. As expected, TIMP-2 did not induce any significant activation of ERK1/2 in MT1-MMP-siRNA cells in which the expression of MT1-MMP was transcriptionally silenced. As a result of TIMP-2 binding with cell surface-associated, naturally produced MT1-MMP and the subsequent stimulation of the intracellular signaling, the levels of pMEK1/2 that are directly upstream of ERK1/2, pERK1/2 itself and of the ERK1/2 substrate p-p90RSK transcriptional factor increased in HT1080 cells.

Importantly, the TIMP-2/MT1-MMP-mediated signal generated a significant, 2–3-fold, increase in the levels of pMEK/pERK in the cells, thus suggesting that the primary role of the TIMP-2•MT1-MMP interactions is in the activation of ERK. TIMP-2 did not induce the ERK/MEK pathway in the cells which exhibited a low level of MT1-MMP (e.g., MT1-MMP-deficient breast carcinoma MCF-7 cells). The small-molecule inhibitor GM6001 did not reverse TIMP-2•MT1-MMP-mediated stimulation of the MEK–ERK–p90RSK cascade. In agreement with the known pro-migratory function of activated ERK, TIMP-2•MT1-MMP-dependent stimulation of intracellular signaling promotes cell migration of siRNAscr cells but not MT1-MMP-siRNA cells. U0126 (an inhibitor of MEK/ERK) reversed the stimulatory effect of TIMP-2 on pMEK1/2, pERK1/2 and cell migration in siRNAscr cells, while GM6001 alone or in combination with 100 ng/ml TIMP-2 had no significant effect on MEK and ERK.

10. MT1-MMP's PEX binds TIMP-2 similar to PEX in the MMP-2 proenzyme–TIMP-2 complex

Current data regarding the TIMP-2•MT1-MMP-dependent stimulation of intracellular signaling agree well with the mechanism in which the C-terminal domain of TIMP-2 binds directly with the PEX domain of MT1-MMP. This binding is in addition to the binding of the catalytic domain of MT1-MMP with the N-terminal inhibitory domain of TIMP-2. It appears that the PEX-binding mode of MT1-MMP by TIMP-2 is required for the intracellular signaling mediated by the resulting MT1-MMP•TIMP-2 complex. There is solid evidence that this binding mode takes place in the formation of TIMP-2's complex with proMMP-2 [10,19,64]. Because of the high homology between the PEX domains of MMP-2 and MT1-MMP, it is probable that there is a structural rationale for a similar interaction of TIMP-2 with MT1-MMP.

11. The importance of a proteolysis-independent mechanism in orthotopic breast cancer xenograft growth

The biological significance of the proteolysis-independent mechanisms involving MT1-MMP was recently validated by evaluating the growth of tumor xenografts expressing the wild-type or mutant MT1-MMP constructs [53]. For this purpose MCF-7 cells stably transfected with the wild-type MT1-MMP or mutant MT1-MMP devoid of proteolytic activity (E240A) or cytoplasmic tail (ΔCT), or mock transfected were xenografted into the fad pads of immunodeficient mice. Tumor xenografts expressing the wild-type MT1-MMP or proteolytically inert, E240A MT1-MMP grew to a size that greatly exceeded that of tumors expressing no MT1-MMP or MT1-MMP devoid of the cytoplasmic tail, both of which could not elicit the signaling cascade. At day 130 post-implantation there was an over 20-fold size difference between tumors expressing the wild-type MT1-MMP and tumors derived from mock transfected cells. The volume of tumors expressing proteolytically inactive MT1-MMP was 50% that of wt MT1-MMP-expressing tumors. In contrast, tumors derived from cells expressing the tailless, non-signaling MT1-MMP construct were no different in size from those derived from control, empty vector-transfected, cells.

12. LRP1

Low density lipoprotein-related protein 1 (α2-macroglobulin receptor), also known as CD91, is a cell receptor found in the plasma membrane of human cells involved in receptor-mediated endocytosis. LRP1 is a multifunctional receptor that binds and endocytoses multiple structurally and functionally distinct ligands including MMP-2 and MMP-9 [65–69]. Cellular LRP1 consists of a non-covalently associated 515 kDa extracellular α-chain (LRP-515) and an 85 kDa membrane-spanning β-chain (LRP-85), and it plays a dual role as a multifunctional endocytic receptor and a signaling molecule. The functionality of LRP1 and the subsequent uptake of its ligands by malignant cells are both regulated by MT1-MMP proteolysis of the C-terminal part of the LRP-515 subunit [27,31,67,70]. Because LRP1 is essential for the clearance of multiple ligands, these findings have important implications for many pathophysiological processes including the pericellular proteolysis in neoplastic cells as well as the fate of the soluble, matrix-degrading proteases such as MMP-2 and MMP-9 [65,66,71–74]. LRP1 associates with the MT1-MMP's catalytic domain and is highly susceptible to MT1-MMP proteolysis *in vitro*. Similar to MT1-MMP, related MT2-, MT3- and MT4-MMPs also degrade LRP1. In cells co-expressing LRP1 and active MT1-MMP, the proteolytically competent protease decreases the levels of cellular LRP1 and releases its N-terminal portion in the extracellular milieu while the catalytically inert protease co-precipitates with LRP1. These events implicate MT1-MMP (an activator of MMP-2) in the mechanisms which control the clearance and the fate of MMP-2 and MMP-9 in cells/tissues [67].

It should be mentioned that Alzheimer-related γ-secretase activity that cleaves amyloid precursor protein (APP) also attacks the transmembrane domain of LRP1 [75]. This cleavage releases the cytoplasmic domain fragments of LRP1 which are further translocated to the nucleus and act as transcription modulators [76]. The soluble cytoplasmic tail of LRP1 without a membrane tether is sufficient to promote amyloid beta (Abeta) protein generation [77]. If LRP1 processing occurs like Notch processing [78], the shedding of the ectodomain is an initial requirement, and MT1-MMP may be responsible for initiating this event. In addition, LRP1 is known to

associate with APP [79,80] and, furthermore, LRP1 is genetically associated with Alzheimer's disease [81]. MT1-MMP also cleaves APP and releases its truncated soluble form that is deficient in the inhibitory activity against MMP-2 [82]. If MMP-2 itself cleaves APP, the released APP fragment subsequently inactivates the MMP-2 enzyme in a manner similar to TIMPs [83]. Apparently, there is a general similarity in the functional role that MT1-MMP plays acting against APP and LRP1: in both cases this membrane-tethered protease protects the MMP-2 activity from inactivation by inhibition or, similarly, by internalization. In addition, these combined data suggest that APP, LRP1, MT1-MMP and presenilin γ-secretase, reside and act at the plasma membrane in close proximity with each other and that presenilin γ-secretase is the next likely target of MT1-MMP proteolysis.

13. MT1-MMP•TIMP-2•LRP1?

It is highly likely that MT1-MMP itself cannot signal because its cytoplasmic tail is exceedingly short, because it does not exhibit any unusual sequences which are additional to the internalization signals and because the cytoplasmic tail-binding adaptor proteins have not been reliably identified [84]. It may well be that an interaction of the MT1-MMP•TIMP-2 with a cellular signaling receptor is required to elicit the signaling through the latter.

It is tempting to hypothesize that the low density lipoprotein-related receptor protein-1 (LRP1) may fit this signaling receptor shoes. Active MT1-MMP cleaves LRP1 but if MT1-MMP is inhibited by TIMP-2, the MT1-MMP•TIMP-2 inactive complex binds, but does not cleave, the LRP1 receptor. It is possible that as a result of this binding event, LRP1 signals through its long, heavily phosphorylated cytoplasmic tail. LRP1 signaling is well known to stimulate the ERK/MEK pathway [72,85–89]. The MT1-MMP•TIMP-2•LRP1 complex is then internalized by the cells causing a decay of the signal. Other mechanisms, however, can be involved and these mechanisms can also explain, albeit vaguely, why MT1-MMP•TIMP-2-dependent signaling is not observed at high concentrations of TIMP-2.

In contrast with both the wild-type MT1-MMP and LRP1, which are not continuously associated with the caveolin-enriched lipid rafts [90], the tailless ΔCT MT1-MMP mutant, as we demonstrated earlier [27], is always localized in the lipid rafts and, as a result, this mutant cannot access LRP1 and cause LRP1-dependent ERK signaling. The invasion-promoting, non-proteolytic function of the MT1-MMP•TIMP-2•LRP1 complex results in the activation of ERK and this function is novel, additional and distinct from the proteolytic function of MT1-MMP that requires cleavage of the target proteins.

Intriguingly, given that this hypothesis is correct, the functioning of the MT1-MMP•TIMP-2•LRP1 system appears similar to the urokinase-type plasminogen activator•urokinase receptor•plasminogen activator inhibitor type-1•LRP1 (uPAR•uPA•PAI-1•LRP1) system and to the PAI-1 paradox in cancer [91–93]. It is generally assumed that a pro-malignant effect of the uPA–uPAR system is mediated by increased local proteolysis thus favoring tumor invasion, by a pro-angiogenic effect of this system and also by uPA–uPAR signaling towards the tumor thereby shifting the tumor phenotype to a more malignant one. When tumor patients are analyzed for long term survival, those with high levels of the PAI-1 inhibitor have a much worse prognosis than those with low PAI-1 levels. In a manner similar to MT1-MMP•TIMP-2, this indicates that increased overall proteolysis alone is not responsible for the adverse effects of either the plasminogen-activating system or the MT1-MMP system in tumors. It is clear that additional studies are required to precisely establish the role of LRP1 in the MT1-MMP•TIMP-2-dependent signaling.

If the hypothesis that the activation of the ERK pathway involves the MT1-MMP•TIMP-2•LRP1 axis rather than MT1-MMP•TIMP-2 alone is experimentally validated, these results will serve as a paradigm shift in the current understanding of the MT1-MMP and TIMP-2 functionality and as a foundation for a novel and effective cancer therapy. This therapy will target the signal streaming from the MT1-MMP•TIMP-2•LRP1 complex inside the cells.

The existence of the MT1-MMP•TIMP-2•LRP1 cascade in cancer cells may explain a direct rather than an inverse association of TIMP-2 with a poor prognosis in cancer. Because evidence now suggests that the signaling, non-proteolytic function of MT1-MMP and the MT1-MMP•TIMP-2•LRP1-mediated signaling play a significant role in cancer by stimulating invasion and migration of malignant cells, we strongly believe that follow-on in-depth studies are urgently required.

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