

Therapeutic Potential of Matrix Metalloproteinase Inhibition in Breast Cancer

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ABSTRACT

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that cleave nearly all components of the extracellular matrix as well as many other soluble and cell-associated proteins. MMPs have been implicated in normal physiological processes, including development, and in the acquisition and progression of the malignant phenotype. Disappointing results from a series of clinical trials testing small molecule, broad spectrum MMP inhibitors as cancer therapeutics led to a re-evaluation of how MMPs function in the tumor microenvironment, and ongoing research continues to reveal that these proteins play complex roles in cancer development and progression. It is now clear that effective targeting of MMPs for therapeutic benefit will require selective inhibition of specific MMPs. Here, we provide an overview of the MMP family and its biological regulators, the tissue inhibitors of metalloproteinases (TIMPs). We then summarize recent research from model systems that elucidate how specific MMPs drive the malignant phenotype of breast cancer cells, including acquisition of cancer stem cell features and induction of the epithelial–mesenchymal transition, and we also outline clinical studies that implicate specific MMPs in breast cancer outcomes. We conclude by discussing ongoing strategies for development of inhibitors with therapeutic potential that are capable of selectively targeting the MMPs most responsible for tumor promotion, with special consideration of the potential of biologics including antibodies and engineered proteins based on the TIMP scaffold. *J. Cell. Biochem.* 118: 3531–3548, 2017. © 2017 The Authors. *Journal of Cellular Biochemistry* Published by Wiley Periodicals, Inc. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

KEY WORDS: MATRIX METALLOPROTEINASES; TISSUE INHIBITORS OF METALLOPROTEINASES; BREAST CANCER; TUMOR PROGRESSION; EPITHELIAL MESENCHYMAL TRANSITION; MMP INHIBITORS; CANCER BIOMARKERS; TUMOR MICROENVIRONMENT

STRUCTURE AND FUNCTION OF THE MATRIX METALLOPROTEINASE FAMILY

MMPs are a large family of zinc-dependent endopeptidases found in all kingdoms except protozoa (MEROPS database: <http://merops.sanger.ac.uk/>) [Rawlings et al., 2012]. Humans express 23 MMPs. These enzymes possess a modular domain structure (Fig. 1A), the minimal form of which consists of a signal peptide for extracellular localization, a prodomain that inhibits the zymogen form of the enzyme until its removal by a separate, activating protease, and a conserved catalytic domain. This most simplified domain organization is found in MMP-7 and -26; additional modules found in other MMPs facilitate localization, association with multiprotein complexes, or selectivity for specific protein substrates. Most

MMPs are soluble proteins, although MMP-14, -15, -16, and -24 are directly tethered to the cell membrane through C-terminal transmembrane domains, MMP-17 and -25 are localized to the cell membrane via C-terminal glycosylphosphatidylinositol (GPI) anchors, and MMP-23 via an N-terminal type II transmembrane domain. Recent NMR studies demonstrate the ability of soluble MMP-7 and MMP-12 to bind directly to membrane bilayers, which may prove to be a new general mechanism by which these and other soluble MMPs are directed toward pericellular proteolytic activities [Koppiseti et al., 2014; Prior et al., 2015]. Beyond the minimal domain architecture, many MMPs also contain hemopexin-like (PEX) domains, which assist in localizing MMPs to the cell membrane via interactions with other cell-surface molecules [Piccard et al., 2007; Murphy and Nagase, 2011; Bauvois, 2012].

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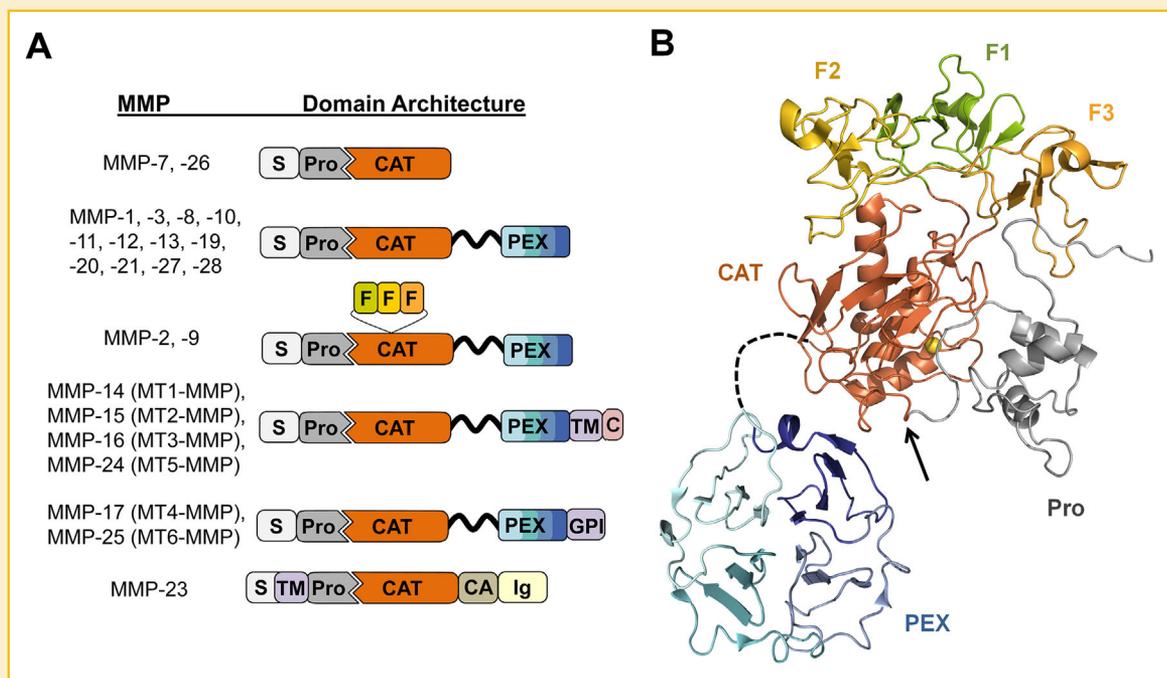


Fig. 1. MMP domain structure and protein fold. (A) The domain organization of each human MMP is illustrated schematically; S, signal peptide; Pro, propeptide; CAT, catalytic domain; F, fibronectin type II repeats; PEX, hemopexin domain; TM, transmembrane domain; GPI, glycosylphosphatidylinositol membrane anchor; C, cytoplasmic domain; CA, cysteine array; Ig, immunoglobulin-like domain. The flexible, variable length linker between CAT and PEX is shown as a black ribbon. (B) The representative 3D protein fold of proMMP-2 is illustrated; individual domains are colored as in panel A. The flexible linker between CAT and PEX domains, shown as a black dashed line, varies in length among MMPs. The prodomain (gray) inhibits activity by coordinating the catalytic zinc (yellow sphere) and blocking access to substrates. Activation requires proteolysis within the loop indicated by the black arrow, leading to dissociation of the prodomain. Figure was generated with PyMOL (Schrodinger, LLC) from coordinates of PDB ID: 1GXD [Morgunova et al., 2002].

The PEX adaptor modules also mediate interactions with other soluble proteins, controlling distinct patterns of localization and substrate specificity [Piccard et al., 2007; Sela-Passwell et al., 2010]. The three fibronectin type II repeats in MMP-2 and MMP-9 further assist in recognition of specific extracellular matrix substrates, including elastin and denatured collagen [Murphy et al., 1994; Steffensen et al., 1995; Shipley et al., 1996; Mikhailova et al., 2012]. Finally, MMP-23 has several unusual modules, including the unique cysteine array domain that has homology to potassium channel blocking toxins and may modulate ion channel activity [Rangaraju et al., 2010], and an immunoglobulin-like domain that has been implicated in protein-protein interactions that affect localization or substrate recognition, a function similar to the PEX domains found in other MMPs [Galea et al., 2014].

DETERMINANTS OF CATALYTIC ACTIVITY AND SUBSTRATE SPECIFICITY

The MMP catalytic domain is highly conserved among members of the family, and contains key features of the larger metzincin metallopeptidase clan, including the conserved HExxHxxGxxH motif which functions to coordinate the catalytic zinc ion. The MMP catalytic mechanism involves activation of a water molecule by the zinc ion and a conserved Glu residue for nucleophilic attack on the target peptide bond [Tallant et al., 2010; Cerda-Costa and

Gomis-Ruth, 2014]. Prior to enzyme activation, the prodomain of the MMP blocks access to the active site cleft (Fig. 1B), usually (with the exception of MMP-26) through interaction of the “cysteine switch” motif containing a conserved PRCGxPD with the catalytic zinc. Activation of the MMP involves disruption of the cysteine switch interaction and cleavage of the prodomain by an activating protease [Rosenblum et al., 2007a; Tallant et al., 2010]. MMP-26, which uniquely possesses a mutated and nonfunctional cysteine switch motif, is also activated proteolytically, although the mechanism by which its zymogen retains latency is not clearly understood [Marchenko et al., 2002]. Many of the MMPs, and all of the transmembrane MMPs, can be activated by furin in the secretory pathway of the cell, while others can be activated once localized to the extracellular space by serine proteases or other MMPs [Ra and Parks, 2007; Tallant et al., 2010].

The MMP active site is located within a broad, shallow cleft that is capable of accommodating a peptide substrate in extended conformation, with the target peptide bond positioned for cleavage adjacent to the catalytic zinc. Binding subsites in the catalytic cleft provide a limited degree of substrate sequence specificity [Tallant et al., 2010]; relative to many other proteases, MMPs have less defined intrinsic specificity for the primary sequence of the cleavage site, with critical determinants of specificity located at exosites further removed, both on the surface of the catalytic domain and on

adjacent accessory domains [Palmier et al., 2010; Arnold et al., 2011; Robichaud et al., 2011; Mikhailova et al., 2012; Stura et al., 2013]. Beyond enhancing molecular recognition of specific substrates, accessory domains may also help to promote catalysis, as in the case of MMP-1 collagenolysis, where cooperative reorganization between the catalytic and PEX domains after substrate binding leads to a deformation of the collagen triple helical structure necessary for proteolysis [Bertini et al., 2011; Manka et al., 2012]. Similar interdomain flexibility is present in MMP-9 and MMP-12, and may be a general property of MMPs with PEX domains [Rosenblum et al., 2007b; Bertini et al., 2008, 2009]. Such independent domain mobility may facilitate unique MMP functions, as in the case of MMP-9, where the particularly long and flexible linker domain may allow the catalytic domain access to complex pericellular substrate networks while the PEX domain maintains cell surface localization [Rosenblum et al., 2007b]. A better understanding of the roles of domains, recognition sites, and their cooperative interactions can help to identify new strategies for therapeutic targeting of MMPs; using this understanding, it may be feasible to selectively block a subset of MMP activities responsible for specific protumorigenic functions.

REGULATION BY TISSUE INHIBITORS OF METALLOPROTEINASES

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous protein protease inhibitors that regulate MMPs along with several other families of zinc-dependent metalloproteinases, forming very tight 1:1 stoichiometric inhibitory complexes. Upon secretion, TIMPs can interact with a variety of membrane-anchored or secreted MMPs (Fig. 2) [Jackson et al., 2017]. Structurally, TIMPs are comprised of two domains that pack side-by-side, each stabilized by three internal disulfide bonds [Gomis-Ruth et al., 1997; Batra and Radisky, 2014]. The N-terminal domain is sometimes referred to as the “inhibitory domain” due to the fact that as an isolated recombinant domain it remains capable of MMP inhibition [Murphy et al., 1991]; however, in MMP complexes with intact TIMPs the interaction is more extensive, spanning both domains [Batra et al., 2013; Batra and Radisky, 2014], resulting in stronger inhibition by nearly an order of magnitude in several cases that have been examined [Huang et al., 1996]. The four human TIMPs possess broad, overlapping specificity and each is capable of inhibiting most MMPs, although with a spectrum of affinities spanning many orders of magnitude, from 0.6 fM for the association of TIMP-2 with MMP-2 [Hutton et al., 1998], to the high nanomolar range for the relatively poor inhibition of MMP-14 and MMP-15 by TIMP-1 (Table I). The strongest of these interactions, including those of TIMP-2/MMP-2 and TIMP-1/MMP-9, involve not only the MMP catalytic domain but additional interactions with the PEX domain [O’Connell et al., 1994; Butler et al., 1999a].

The primary interaction of a TIMP with an MMP catalytic domain utilizes a conserved core epitope centered around the N-terminal strand of the TIMP, which coordinates to the active site zinc of the MMP, blocking the substrate-binding cleft (Fig. 3A and B). This interaction also involves adjacent EF and C-connector loops of the TIMP N-terminal domain, which are tethered to the N-terminal strand by disulfide bonds (Fig. 3B). Additional loops flanking the

core epitope, including the AB loop of the N-terminal domain and the multiple turn and GH loops of the C-terminal domain, can form adventitious contacts with exosites on the surface of the MMP catalytic domain (Fig. 3A) [Batra et al., 2013; Batra and Radisky, 2014]. Whereas the core epitope interaction with different MMPs is highly structurally conserved, the residues involved in peripheral interactions are much less conserved among both TIMPs and MMPs, resulting in considerable variation in the extent and nature of exosite interactions in different TIMP/MMP pairs. These regions of sequence and structural variability contribute to the wide variation in TIMP/MMP binding constants [Williamson et al., 2001; Batra et al., 2013], which span many orders of magnitude (Table I). Additional determinants of differential affinity include subtle packing alterations at the intermolecular interface with the core epitope [Lee et al., 2003; Batra et al., 2012a].

Beyond the primary inhibitory interaction of TIMPs with MMP catalytic domains, additional, noninhibitory interactions can occur between select TIMP/MMP pairs. The C-terminal domains of TIMP-2, TIMP-3, or TIMP-4 can bind to the PEX domain of MMP-2 or pro-MMP-2, while the C-terminal domains of TIMP-1 or TIMP-3 can associate with MMP-9 or pro-MMP-9 (Fig. 2) [Brew and Nagase, 2010]. The interaction of pro-MMP-2 with TIMP-2 has been structurally characterized [Morgunova et al., 2002] and shown to be important in MMP-2 activation by MT1-MMP (Fig. 2) [Strongin et al., 1995]. This role is unique to TIMP-2, as TIMP-3 or TIMP-4 do not participate in MMP-2 activation but may serve as negative regulators of the process [Bigg et al., 2001; Hernandez-Barrantes et al., 2001; English et al., 2006]. The pro-MMP-9/TIMP-1 complex has been less well characterized structurally, but biochemical data support the significance of the pro-MMP-9 PEX domain and the TIMP-1 C-terminal domain in this interaction [Murphy et al., 1991; O’Connell et al., 1994]. Several studies have implicated pro-MMP-9/TIMP-1 complex formation as a mechanism that protects the secreted proenzyme from activation by other MMPs both in vitro and in vivo (Fig. 2) [Goldberg et al., 1992; Ogata et al., 1995; Ardi et al., 2007, 2009]. Finally, in addition to their role in MMP inhibition, TIMPs are also multifunctional proteins with emerging pleiotropic activities mediated through MMP-independent protein–protein interactions with other binding partners. These alternative cell signaling activities and their implications in cancer have been reviewed elsewhere [Chirco et al., 2006; Stetler-Stevenson, 2008; Brew and Nagase, 2010], and will not be described here in detail.

TUMORIGENIC PROCESSES ACTIVATED BY MMPS IN BREAST CANCER

MMPs are so named because they are capable of cleaving nearly every component of the extracellular matrix (ECM); through matrix degradation they directly facilitate cancer invasion and metastasis by clearing pathways for invading cancer cells and for ingressing vasculature to provide oxygen and nutrients for the growing tumor [Kessenbrock et al., 2010]. MMPs also play key roles in the generation of disruptive fibrotic stroma, both through degradation of the native ECM and through MMP-induced activation of stromal fibroblasts, and further can stimulate tumor-promoting metabolic

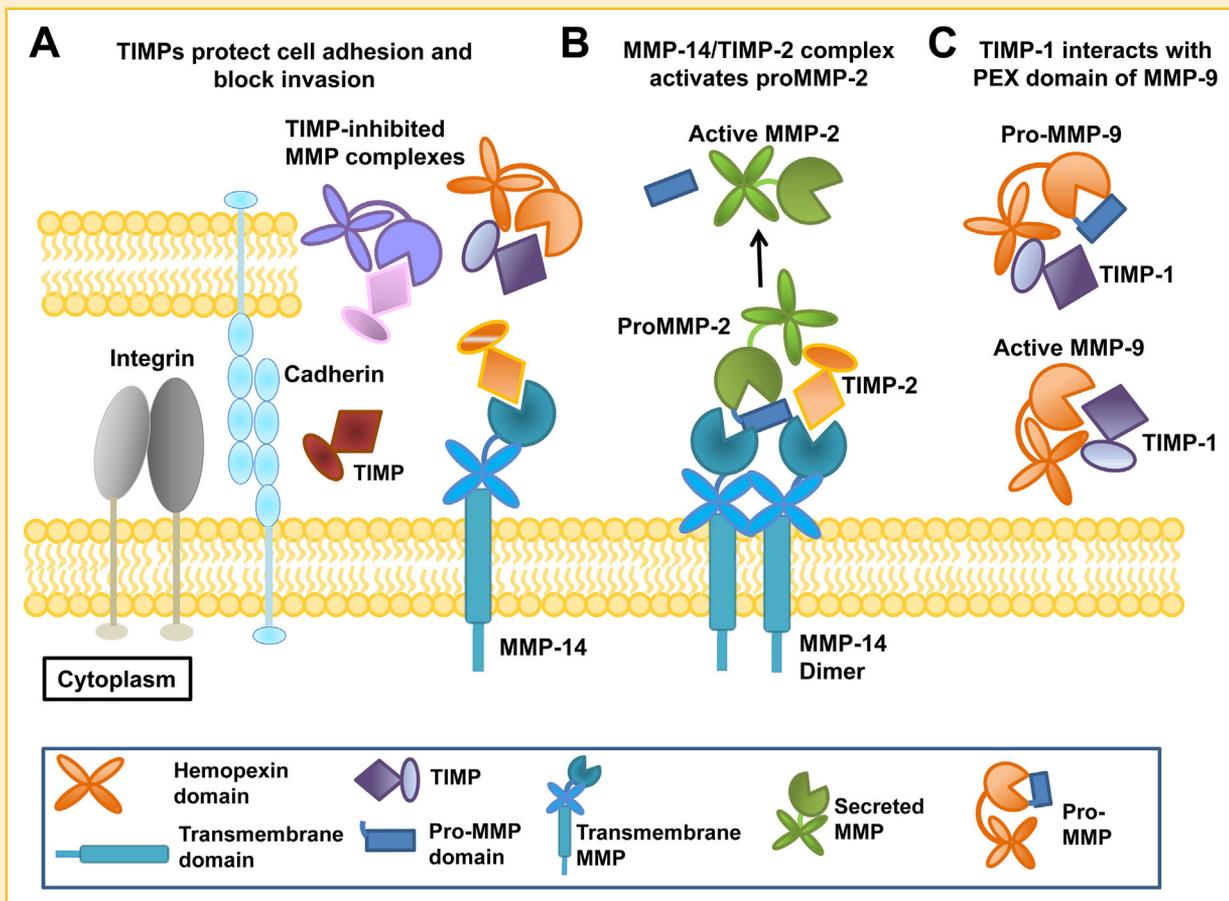


Fig. 2. TIMPs regulate MMP function and activation. (A) Soluble and extracellular matrix-associated TIMPs form inhibitory interactions with the catalytic domains of extracellular and transmembrane MMPs, protecting cell adhesion and blocking invasion. (B) The dimeric form of transmembrane MMP-14 forms a receptor complex with TIMP-2 that is responsible for MMP-2 activation; proMMP-2 is bound via its C-terminal PEX domain, allowing proteolysis of the prodomain by the uninhibited MMP-14 subunit. (C) TIMP-1 forms C-terminal domain interactions with the PEX domain of proMMP-9 or MMP-9, protecting proMMP-9 from activation and quenching activity after activation.

switches through interaction with adipocytes [Kessenbrock et al., 2010]. MMPs additionally target receptors on the surface of the tumor cells themselves, activating pathways that promote proliferation or suppress apoptosis [Gialeli et al., 2011]. MMPs can also indirectly drive tumor initiation and progression, as disruption of the ECM can activate cellular processes that cause DNA damage and stimulate genomic instability [Radisky and Bissell, 2006]. Finally, MMPs can directly induce the epithelial-mesenchymal transition (EMT) in target epithelial cells; EMT is a developmental process that is associated with resistance to apoptosis and increased invasiveness, and has been associated with development of a cancer stem cell phenotype [Radisky and Radisky, 2010; Nistico et al., 2012]. Here, we will describe some of the more recently discovered cancer-associated processes in which MMPs have been implicated.

REGULATION OF PROLIFERATION AND APOPTOSIS

Expansion of tumor mass is associated with increased proliferation and decreased apoptosis. One of the most well-studied regulators of cellular proliferation and apoptosis in the tumor microenvironment is TGF β , which has cytostatic and apoptotic function in most nonmalignant/normal cells, but which can also stimulate proliferation and suppress

apoptosis through canonical and noncanonical pathways in many transformed or malignant cells [Massague, 2012]. TGF β is generated and secreted as a component of an inactive complex; MMPs can cleave the TGF β precursor or other complex components to generate the active cytokine [Yu and Stamenkovic, 2000; Dallas et al., 2002; Mu et al., 2002; Annes et al., 2003; Tatti et al., 2008], and can also modify the ECM to facilitate nonproteolytic activation of TGF β [Cichon and Radisky, 2014a]. MMPs and members of the related ADAM (a disintegrin and metalloproteinase) family can trigger the epidermal growth factor (EGF) receptor by shedding of cell membrane-associated ligands, such as HB-EGF, TGF β , and amphiregulin [Kessenbrock et al., 2010; Gialeli et al., 2011]. MMPs can also proteolytically inactivate the cell death receptor Fas, leading to inhibition of the intrinsic apoptosis pathway which is involved in chemotherapeutic cell killing [Mitsiades et al., 2001; Crawford et al., 2002]; specifically inhibiting the MMP-Fas interaction is thus an important avenue for development of novel therapeutics [Villa-Morales and Fernandez-Piqueras, 2012].

PROMOTION OF INVASION AND METASTASIS

In addition to the well-characterized functions of MMPs to facilitate cancer cell invasion through degradation of ECM, several MMPs

TABLE I. Reported Apparent K_i Values for TIMP–Peptidase Interactions

Enzyme	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MMP-1	100 pM ¹ 250 pM ² 2.7 nM ^{1,a}			650 pM ³ 2.6 nM ^{3,a}
MMP-2	140 pM ² 10 nM ⁴	0.6 fM ⁵ 7 nM ⁴	<2 pM ⁶	4 pM ³
MMP-3	240 pM ² 250 pM ^{2,a} 110 pM ^{7,a}	550 pM ^{7,a}		1.5 nM ³ 1.2 nM ^{3,a}
MMP-7	370 pM ⁸			
MMP-9	<50 pM ⁹ 9 nM ⁴	<50 pM ⁹ 43 nM ⁴		
MMP-10	1.1 nM ^{7,a}	5.8 nM ^{7,a}		
MMP-14	178 nM ^{10,b}	70 pM ^{11,a}	160 pM ^{11,a}	340 pM ^{11,a}
MMP-15	100 nM ^{12,a}			
MMP-16		170 pM ^{11,a}	8.2 pM ^{11,a}	340 pM ^{11,a}
MMP-17	~10 nM ^{13,a}	~10 nM ^{13,a}	~10 nM ^{13,a}	~10 nM ^{13,a}
MMP-19	57.6 nM ^{14,a}	5 pM ^{14,a}	5 pM ^{14,a}	5 pM ^{14,a}
MMP-25	200 pM ^{15,a}	2 nM ^{15,a}		

Sources: ¹Taylor et al. [1996]; ²Huang et al. [1996]; ³Troeberg et al. [2002]; ⁴Olson et al. [1997]; ⁵Hutton et al. [1998]; ⁶Lee et al. [2001]; ⁷Batra et al. [2012a]; ⁸O'Shea et al. [1992]; ⁹O'Connell et al. [1994]; ¹⁰Lee et al. [2003]; ¹¹Zhao et al. [2004]; ¹²Morrison et al. [2001]; ¹³Amour et al. [2002]; ¹⁴Stracke et al. [2000]; ¹⁵Sun et al. [2007].

^aReflects binding of truncated MMP catalytic domain.

^bReflects binding of truncated N-terminal TIMP domain.

have been implicated as direct activators of invasion through breakdown of cell-cell interactions and activation of cell motility and migration processes. As an example of this phenomenon, MMPs cleave the Wnt/planar cell polarity protein-tyrosine kinase-7 (PTK7), stimulating cancer cell invasion and metastasis [Golubkov et al., 2010, 2014; Golubkov and Strongin, 2012]. Many MMPs can also directly activate aspects of the EMT program in epithelial cancer cells [Lamouille et al., 2014]. MMP-induced EMT has been observed in kidney [Cheng and Lovett, 2003; Cheng et al., 2006; Zheng et al., 2009; Tan et al., 2010], ovary [Cowden Dahl et al., 2008], lens [West-Mays and Pino, 2007], lung [Illman et al., 2006; Yamashita et al., 2011; Stallings-Mann et al., 2012], pancreas [Mehner et al., 2014a], and prostate [Cao et al., 2008] cells, although the process of MMP-induced EMT has been best characterized in mammary epithelial cells [Radisky and Radisky, 2010; Foroni et al., 2012]. MMP-3, the most upregulated MMP family member during postlactational involution of the breast [Radisky and Hartmann, 2009], was found to induce spontaneous mammary tumors when expressed in transgenic mice by a milk protein promoter [Sympson et al., 1995; Sternlicht et al., 1999, 2000]. Dissection of this process revealed that MMP-3 directly activates EMT [Lochter et al., 1997a,b] by activating alternative splice pathways which result in the expression of Rac1b [Radisky et al., 2005; Pelisch et al., 2012; Cichon et al., 2015]. Rac1b is an activated splice variant with unique tumor-promoting activities [Orlichenko et al., 2010], which stimulates EMT by increasing levels

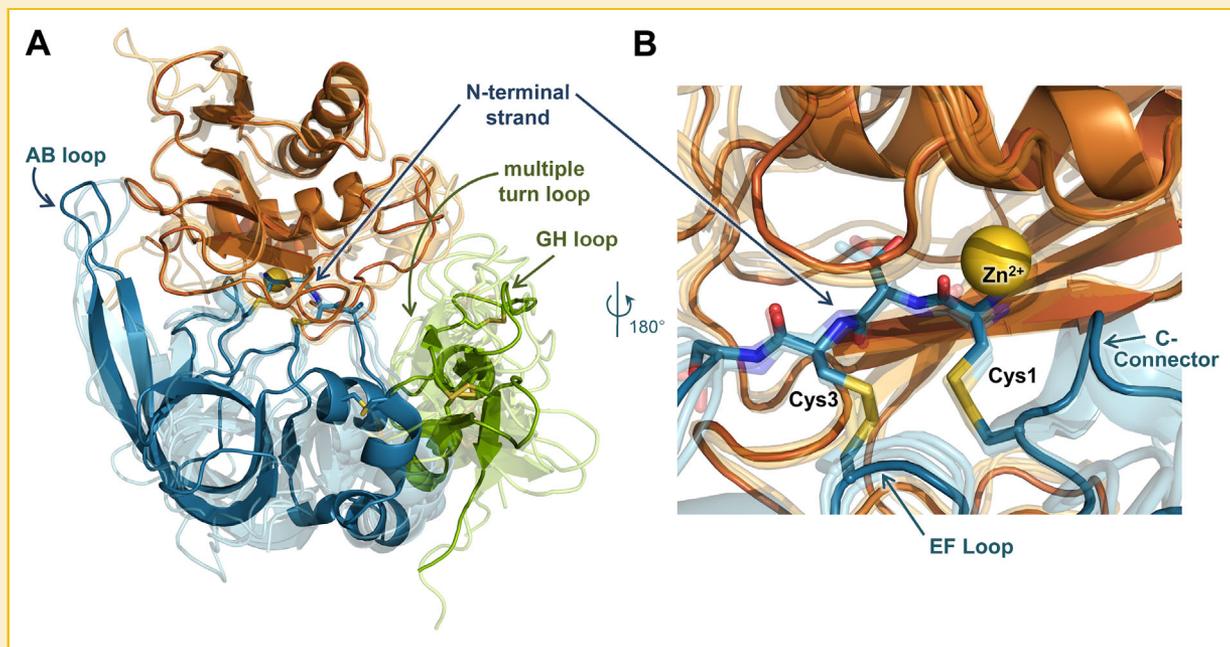


Fig. 3. MMP/TIMP interfaces feature a conserved core interaction and diverse peripheral interactions. (A) The structure of the representative complex of MMP-10_{CAT} (orange) with TIMP-2 (N-terminal domain blue, C-terminal domain green) (PDB ID: 4ILW) [Batra et al., 2013] is shown; the catalytic zinc ion is rendered as a yellow sphere. Four additional MMP_{CAT}/TIMP structures (semi-transparent) are superposed to highlight regions of structural conservation versus diversity: MMP-10_{CAT}/TIMP-1 (PDB ID: 3V96) [Batra et al., 2012a], MMP-3_{CAT}/TIMP-1 (PDB ID: 1UEA) [Gomis-Ruth et al., 1997], MMP-14_{CAT}/TIMP-2 (PDB ID: 1BQQ) [Fernandez-Catalan et al., 1998], and MMP-13_{CAT}/TIMP-2 (PDB ID: 2E2D) [Maskos et al., 2007]. Diverse interactions with different MMPs are formed by peripheral TIMP epitopes of the AB, GH, and multiple turn loops. (B) Closer view of the superposed complexes reveals conserved positioning and interactions of the TIMP core epitope, including N-terminal residues 1–4, the EF loop, and C-connector loop. The amine terminus of residue Cys-1 coordinates directly to the catalytic zinc ion. Figure was generated using PyMOL (Schrodinger, LLC).

of cellular reactive oxygen species [Radisky et al., 2005; Nelson et al., 2008; Cichon and Radisky, 2014b], through a process that depends upon cell–ECM interactions [Lee et al., 2012; Chen et al., 2013a]. Thus, MMPs can stimulate invasion through both extrinsic (ECM-targeting) and intrinsic (cell phenotype alteration) processes.

MMP-induced EMT can also facilitate increased tumorigenicity through induction and regulation of stem cell characteristics. MMPs play a variety of roles in physiological functions of stem cells, including tissue morphogenesis and regeneration; in the mammary gland, MMP-3 regulates Wnt signaling and consequent stem cell expansion via a specific capacity to bind and inactivate the noncanonical Wnt ligand Wnt5b [Kessenbrock et al., 2013]. More recently, MMPs have been identified in cancer stem/progenitor cells (CSCs) as well [Kessenbrock et al., 2015]. Similar to normal stem cells, CSCs have low proliferation rates and resistance to apoptosis, characteristics that confer increased resistance to cytotoxic therapies and tumor recurrence; consequently, identification of CSCs within tumors and development of therapies that specifically target CSCs have become an important area of research [Singh and Settleman, 2010]. An emerging body of research has emphasized that activation of the EMT program, even in an incomplete or dysregulated fashion, can stimulate CSC characteristics [Singh and Settleman, 2010]. Targeting MMP-induced EMT, therefore, is a promising new approach for reducing CSC-associated malignancy.

CLINICAL STUDIES IDENTIFY SPECIFIC MMPs AS MOST LIKELY THERAPEUTIC TARGETS IN BREAST CANCER

While MMPs clearly play prominent roles in shaping the tumor microenvironment and driving cancer progression and metastasis as summarized above, early efforts to target these proteases therapeutically in clinical trials of more than 50 MMP inhibitors led to widely disappointing results [Coussens et al., 2002; Vandenbroucke and Libert, 2014]. A trial of broad spectrum MMP inhibitor marimastat in advanced stage breast cancer patients found no benefit to progression-free survival [Sparano et al., 2004], while phase II trials in early stage breast cancer patients found further exploration of the approach to be unfeasible in this population due to issues of toxicity [Miller et al., 2002, 2004]. In the wake of these trials, consensus has emerged that lack of inhibitor specificity was one of the critical problems with the approach. This is because not all MMPs confer exclusively tumor promoting effects, and some have even been shown to provide a primarily protective function [Lopez-Otin and Matrisian, 2007; Martin and Matrisian, 2007; Decock et al., 2011]. MMP-8 provides one of the more clear-cut examples of this phenomenon. In mouse models of breast cancer, high expression of MMP-8 suppresses metastasis, while MMP-8 silencing or knockout promotes tumor progression and metastasis [Montel et al., 2004; Decock et al., 2015]. Importantly, the net antitumor effect of MMP-8 is corroborated by the association of MMP-8 tumor expression with longer relapse-free survival in breast cancer patients [Gutiérrez-Fernández et al., 2008], and by the association of a high expression promoter variant of the MMP-8 gene with more favorable prognosis [Decock et al., 2007].

For other MMPs, categorization as friend or foe is not as straightforward. Mounting studies reveal that depending on the model system, the animal genetic background, or other experimental factors, an MMP may exert a tumor-promoting, tumor-inhibitory, or null effect. One example of this phenomenon is found in MMP-9. Early studies with MMP-9 knockout mice in a pancreatic carcinogenesis model identified MMP-9 as a critical component of an angiogenic switch, driving angiogenesis and tumor progression through release of vascular endothelial growth factor (VEGF) [Bergers et al., 2000]. Proangiogenic, tumor promoting functions of MMP-9 have subsequently been confirmed in many other models [Deryugina and Quigley, 2010], yet, some studies have also implicated MMP-9 in the proteolytic generation of angiogenesis inhibitors angiostatin (a fragment of plasminogen) [Pozzi et al., 2000], tumstatin (a fragment of collagen IV) [Hamano et al., 2003], and endostatin (a fragment of collagen XVIII) [Bendrik et al., 2008]. A recent study examined the effect of MMP-9 knockout on tumorigenesis in several genetically engineered mouse models; the absence of MMP-9 delayed tumorigenesis in the C3(1)-Tag model of basal-like triple-negative breast cancer, but had no comparable effect on tumorigenesis in the (MMTV)-Neu model of luminal breast cancer [Park et al., 2015].

The impact of MMP-9 on metastasis can also differ depending on the model. In an orthotopic mouse model of human breast cancer, silencing of MMP-9 expression in tumor cells blocked metastasis, demonstrating a critical role for tumor cell-produced MMP-9 in metastasis [Mehner et al., 2014b]. On the other hand, a study examining genetic ablation of MMP-9 in the MMTV-PyVT mouse mammary tumor model demonstrated that host-derived (immune cell) MMP-9 could exert a pro-metastatic influence under some circumstances, depending on the genetic background of the mouse strain used [Martin et al., 2008].

In light of such inconsistent or paradoxical findings from diverse experimental models, each with its own strengths and limitations, how can we best evaluate the likelihood that a given MMP represents a significant and promising therapeutic target in human breast cancer? We argue that evidence from clinical studies examining associations of specific MMPs with poor prognosis should be used as a framework to guide interpretation of experimental results, and to prioritize MMP candidates for development of therapeutic strategies. Among the large MMP family, relatively few have shown consistent association with poor outcomes across multiple cohorts, narrowing the field. Here, we highlight clinical findings that implicate several MMPs as potential therapeutic targets.

MMP-9

Consistent with the demonstrated functional roles of MMP-9 in tumor angiogenesis, invasion, and metastasis [Wang et al., 2010; Bauvois, 2012; Deryugina et al., 2014; Mehner et al., 2014b], in clinical studies of breast cancer patients MMP-9 has been consistently associated with cancer progression and poorer outcomes. Analyses of transcriptional microarray data have shown MMP-9 to be one of only a few MMPs consistently prognostic for poor survival in several large cohorts of breast cancer patients [van de Vijver et al., 2002; McGowan and Duffy, 2008; Dufour et al., 2011; Curtis et al., 2012; Roy and Walsh, 2014]. MMP-9 is also the only MMP gene to be included among the 70 genes in the Rosetta poor

prognosis breast cancer signature [van 't Veer et al., 2002], on which the Mammaprint clinical prognostic assay is based (Agendia, Inc., Irvine, CA). At the protein expression level, tissue microarray analyses of primary breast tumors have found MMP-9 total immunostaining to be associated with shorter relapse-free survival, and individually scored MMP-9 staining of tumor cells, stromal fibroblasts, and mononuclear inflammatory cells were also each significantly associated with distant metastasis and poorer relapse-free survival [Gonzalez et al., 2007; Vizoso et al., 2007]. In other immunohistochemical studies of large breast cancer cohorts, MMP-9 expression specifically in stromal cells was found to be most strongly prognostic for both recurrence and poor survival [Pellikainen et al., 2004; Mylona et al., 2007]. The significance of MMP-9 expression in tumor cells, especially in early stages of cancer, is less clear; in studies examining primarily node negative breast cancers, tumor cell MMP-9 was prognostic for shorter relapse-free survival in one cohort [Li et al., 2004] but associated with better survival in others [Scorilas et al., 2001; Pellikainen et al., 2004]. Serum levels of MMP-9 have also been found to be elevated in breast cancer patients and significantly associated with poor outcomes in numerous studies [Wu et al., 2008; Radisky and Radisky, 2015]. The absence of such prognostic associations for circulating MMP-9/TIMP-1 complexes [Thorsen et al., 2013] suggests that the presence of free, catalytically active MMP-9 may be most relevant for predicting cancer progression, consistent with experimental studies demonstrating that TIMP-free MMP-9 is required for tumor angiogenesis and tumor cell intravasation [Bekes et al., 2011]. The consensus of clinical studies supports consideration of MMP-9 as a potential therapeutic target in breast cancer, particularly for specific subsets of patients with aggressive disease. MMP-9 is most highly expressed in the aggressive basal-like triple negative and HER2-positive breast cancer molecular subtypes [Mehner et al., 2014b; Yousef et al., 2014]. Furthermore, several studies have found MMP-9 protein expression to be significantly prognostic for shorter time to progression and poorer overall survival specifically in basal-like or triple negative breast cancer patients [Gonzalez et al., 2009; Zhao et al., 2013]. Given the limited therapeutic options for these patients, MMP-9 may offer an attractive target for antimetastatic therapies.

MMP-11

MMP-11, also known as stromelysin-2, is another MMP that repeatedly appears in the clinical literature in association with poor breast cancer outcomes. At the transcriptional level, it was associated with poor survival in a microarray dataset representing primary tumors from 1500 breast cancer patients [Curtis et al., 2012; Roy and Walsh, 2014]. MMP-11 is part of the 21 gene signature in the Oncotype DX clinical assay (Genomic Health, Inc., Redwood City, CA), originally developed to predict recurrence of breast cancer after tamoxifen treatment [Paik et al., 2004]. It is also included among the 50 genes of the PAM50 profile used to predict breast cancer intrinsic molecular subtypes and risk of recurrence [Parker et al., 2009]. At the protein level, MMP-11 is found to be expressed by many cell types in the tumor microenvironment, and generally predicts poor prognosis irrespective of localization. In women with invasive ductal breast cancers, total immunostaining scores for MMP-11, like MMP-9, were shown to be significantly prognostic for shorter relapse-free

survival, as were specific expression of MMP-11 by fibroblasts or mononuclear inflammatory cells [Gonzalez et al., 2007; Vizoso et al., 2007]. In a different cohort of 192 patients, MMP-11 staining both in tumor cells and in stromal fibroblast-like cells was found to be significantly associated with poor overall survival [Min et al., 2013]. Yet another study found overall tumor MMP-11 staining to be significantly associated with advanced tumor stage and lymph node metastasis [Cheng et al., 2010]. Further investigations have identified a subset of poor prognosis breast tumors characterized by infiltration of MMP-11-expressing mononuclear inflammatory cells, revealing a distinct profile of associated inflammatory cytokines [Gonzalez et al., 2009; Eiro et al., 2012]. Expression of MMP-11 by endothelial cells in tumor blood vessels has also been found to be associated with shorter relapse-free survival and overall survival [Cid et al., 2016]. In one study, MMP-11 was significantly more strongly expressed by stromal fibroblast-like cells in tumors of the aggressive basal-like and HER2-positive subtypes [Min et al., 2013]. For the basal-like subtype specifically, overall MMP-11 expression, fibroblast, or mononuclear inflammatory cell MMP-11 expression were each individually associated with poorer relapse-free survival [Gonzalez et al., 2009]. Taken together with experimental studies implicating MMP-11 in survival and early tumor invasion of breast cancer cells [Lefebvre et al., 1992; Masson et al., 1998; Boulay et al., 2001; Wu et al., 2001; Takeuchi et al., 2011], the available data suggest that MMP-11 may offer a viable molecular target especially for basal-like or triple negative breast cancers, where its targeting may be of greatest utility in earlier stages of progression.

TRANSMEMBRANE MMPS: MMP-14 AND MMP-15

Consistent with the role of transmembrane MMPs as a key mediators of cancer cell invasion and migration [Koshikawa et al., 2000; Hotary et al., 2003, 2006; Shiomi and Okada, 2003; Sabeh et al., 2004; Cao et al., 2005; Ota et al., 2009; Zarrabi et al., 2011], clinical studies have found both MMP-14 and MMP-15 expression to signal poor prognosis, especially at the transcriptional level. In a transcriptional microarray dataset comprised of primary breast tumors from 295 patients [van de Vijver et al., 2002], high expression of MMP-14 and MMP-15 were each associated with poorer overall survival [McGowan and Duffy, 2008]. MMP-15 expression was associated with higher grade tumors, while MMP-14 was the only MMP to remain a prognosticator of poor survival in multivariate analysis, independent of tumor size, grade, lymph node status, and ER status [McGowan and Duffy, 2008]. MMP-15 was similarly prognostic for poor overall survival in analysis of another transcriptional microarray dataset of 1500 breast cancers [Curtis et al., 2012; Roy and Walsh, 2014]. In a study employing mRNA in situ hybridization to examine MMP-14 expression in 539 breast cancers, MMP-14 was found almost exclusively in stromal cells, and was associated with poor overall survival in univariate analyses as well as multivariate analyses after adjustment for tumor size and lymph node involvement [Tetu et al., 2006]. Studies examining protein expression of MMP-14 by immunohistochemistry have observed expression in cancer cells as well as stromal cells to be common [Mylona et al., 2007; Vizoso et al., 2007]; however only MMP-14 expression by mononuclear inflammatory cells was

significantly associated with a higher rate of distant metastasis in one study [Vizoso et al., 2007]. Several studies have suggested that MMP-14, similarly to MMP-9 and MMP-11, may be particularly relevant as a target in basal-like or triple negative breast cancers. In a study combining patient tumor analyses with experimental investigations, tumor staining for MMP-14 was significantly associated with blood vessel invasion of tumor cells specifically in the subset of triple negative breast cancers, consistent with the demonstration in a mouse orthotopic model that MMP-14 downregulation in tumor cells reduces blood vessel invasion and spontaneous metastasis [Perentes et al., 2011]. Another recent integrative study evaluated MMP-14 transcript expression by qRT/PCR in a cohort of 458 breast cancers patients, finding expression to be associated with high grade, with the triple negative subtype, and with shorter metastasis-free survival [Lodillinsky et al., 2016]. Immunostaining of tissue microarrays for MMP-14 likewise confirmed associations with grade, triple negative subtype, and with progression from ductal carcinoma in situ to invasive cancer. Consistent with these clinical data, the authors found that knockdown of MMP-14 expression in tumor cells impaired invasion in multiple intraductal xenograft models of basal-like breast cancer [Lodillinsky et al., 2016]. These studies help to solidify MMP-14 as a target of potential interest for treating this aggressive breast cancer subtype. Based on transcriptional associations with breast cancer outcome and functional studies of invasion in cell culture models, MMP-15 may also be of interest as a therapeutic target. However, investigations of MMP-15 protein expression and its prognostic significance in breast cancer, along with functional investigations of MMP-15 in mouse models of breast cancer, are lacking and remain an important area for future investigation.

DEVELOPING MMP INHIBITORS FOR THERAPEUTIC APPLICATIONS

As discussed above, several MMPs are strongly implicated as promising targets for breast cancer therapy, yet MMP inhibitors evaluated in clinical trials to date have failed, causing major musculoskeletal toxicity and failing to improve clinical outcomes [Coussens et al., 2002; Vandenbroucke and Libert, 2014]. The musculoskeletal toxicity was thought to derive from inhibition of MMP-1, ADAM family metalloproteases, and/or metalloenzymes outside of the MMP and ADAM families, and in some cases could be avoided by inhibitors with restricted selectivity toward a subset of MMPs [Maquoi et al., 2004; Fingleton, 2008; Vandenbroucke and Libert, 2014]. The poor efficacy of broad spectrum inhibitors was likely caused in part by their inhibition of cancer-protective MMPs such as MMP-8 along with cancer-promoting enzymes. Thus, it is anticipated that development of highly selective inhibitors for the best MMP drug targets could solve both problems, minimizing drug toxicity while sparing enzymes that protect the host from tumor progression.

DEVELOPING MORE SELECTIVE SMALL MOLECULE MMP INHIBITORS

Small molecule MMP inhibitors have typically targeted the catalytic domain active site, binding in the variable S1' pocket of MMPs and

chelating the catalytic zinc ion. Because MMPs have similar active site structures, many such inhibitors have limited ability to discriminate among various MMPs, and development of improved selectivity has proved challenging [Overall and Kleinfeld, 2006; Devel et al., 2010; Jacobsen et al., 2010; Cathcart et al., 2015]. Nevertheless, progress has been made in the development of selective inhibitors for a few MMPs. For instance, optimizing S1' pocket interactions as well as recruiting phosphinate or P2' glutamate as alternatives to more traditional zinc-chelating groups have resulted in very selective inhibitors of MMP-12 [Devel et al., 2006, 2010, 2012]. Other studies have focused on MMP-13, where optimizing interactions with the unusually deep and branched S1' pocket has resulted in potent and selective inhibitors based on a fused pyrimidine zinc binding group [Nara et al., 2017], or lacking interactions with the zinc altogether [Engel et al., 2005; Nara et al., 2014; Hugenberg et al., 2017]. A recent biochemical and biophysical study demonstrated that the zinc-binding group can make surprising contributions to inhibitor selectivity, arguing for drug development strategies that would more widely diversify this functionality in combinatorial approaches [Rouanet-Mehouas et al., 2017].

A unique approach for limiting MMP inhibitor toxicity was reported recently, in a study that aimed to achieve selectivity not only for the target enzyme but also through targeted drug delivery. Although MMP-2 has not generally been considered a primary drug target in cancer due in part to its ubiquitous presence and involvement in many physiological processes, the importance of this enzyme in progression of bone metastatic breast cancer renders it a target of interest in this specific setting [Tauro et al., 2017]. Bisphosphonic-based inhibitors selective for MMP-2 over the similar MMP-9 were developed, which due to the affinity of bisphosphonate for hydroxyapatite were efficiently localized to the bone microenvironment, where they were shown to inhibit MMP-2 and to prevent breast cancer growth and associated bone destruction in a mouse model [Tauro et al., 2017].

Other approaches for developing selectivity aim to target MMP exosites beyond the active site, on catalytic domain or accessory domain surfaces, with the rationale that these sites are less conserved among MMPs. A set of branched amphiphilic polymers was used to probe MMP-12 and -14 catalytic domains for identification of hidden allosteric regulatory sites; these polymers compromised catalytic activity by interacting with flexible surfaces of MMP-12 and -14, altering protein dynamics [Udi et al., 2013]. Comparable but unique allosteric regulatory sites were computationally predicted to exist in other MMP catalytic domains, making these sites potential targets for developing novel therapeutic agents [Udi et al., 2013]. Exosites of accessory domains can also be effective targets for developing MMP inhibitors with greater selectivity. Triple helical peptides (THPs) that mimic collagen substrates of MMPs have been developed as efficient inhibitors that simultaneously target multiple domains of MMPs [Ndinguri et al., 2012]. THP substrate and phosphinate transition state analogues showed high affinity and selectivity toward MMP-2 and -9 [Lauer-Fields et al., 2007], and could be designed for selective inhibition of proteolytic activity toward specific substrates, by targeting fibronectin domain exosite interactions important for the recognition of those substrates [Lauer-Fields et al., 2008]. More recently, heterotrimeric THPs were

synthesized using click chemistry as highly selective binders to MMP-13 and -14, with complete selectivity between MMP-1 and -14 [Bhowmick et al., 2015]. Other peptide-based and small molecule inhibitors have succeeded in blocking MMP-9-dependent cellular migration and metastasis by targeting exosites on the PEX domain that are important for homodimerization and binding to cell surface receptor CD44 [Dufour et al., 2010, 2011]. Similarly, a small molecule targeting the MMP-14 PEX domain inhibited MMP-14 dimerization as well as tumor growth and collagen degradation in an orthotopic mammary tumor model [Remacle et al., 2012].

DEVELOPING ANTIBODY THERAPEUTICS THAT BLOCK MMP FUNCTIONALITY

Many of the preceding examples have achieved selectivity by extending interactions with MMP targets beyond the immediate active site, to encompass broader interaction surfaces mimicking the protein-protein interfaces of natural substrates and effectors. While successful at enhancing selectivity, these larger, often peptide-based inhibitors suffer from stability issues that may limit drug development. An alternative therapeutic approach takes advantage of the naturally broader interaction surface, high affinity and selectivity offered by proteins themselves. Protein-based MMP inhibitors such as antibodies can offer superior potential for higher selectivity and reduced toxicity, and importantly, antibodies represent a class of drugs for which a path to clinical translation is well established.

Mouse monoclonal antibodies raised against human MMPs have demonstrated the advantageous selectivity of these agents toward individual MMPs. For example, REGA-3G12, a function blocking antibody raised against human MMP-9, recognizes a surface epitope of the MMP-9 catalytic domain with a K_d of 2.1 nM and with high selectivity [Paemen et al., 1995; Martens et al., 2007]. Several studies have explored different approaches to develop function-blocking antibodies directed against specific functions of MMP-14, a target of interest in many types of cancers including breast cancer [Pahwa et al., 2014]. By targeting a loop on the MMP-14 catalytic domain far from the active site but important for interaction with TIMP-2, a mouse monoclonal antibody inhibited proMMP-2 activation and lymphangiogenesis, while other catalytic functions were unaffected [Ingvarsen et al., 2013; Shiryaev et al., 2013]. Another mouse monoclonal antibody, raised against a cyclic peptide recapitulating a different surface loop of the MMP-14 catalytic domain, inhibited collagenolytic activity via an allosteric mechanism, with little impact on MMP-14 dimerization or proMMP-2 activation [Gálvez et al., 2001; Udi et al., 2015]. At present it is not yet clear whether superior therapeutic results are likely to be achieved by drugs selectively targeting only a subset of MMP-14 activities, but these reagents can be used to explore this question. In another unique approach, MMP neutralizing antibodies were generated in mice immunized with a synthetic MMP active site mimic. Surprisingly, the antibodies produced using this method targeted MMP-2 and -9 preferentially, and blocked catalytic activity with a mechanism similar to inhibition by natural TIMPs [Sela-Passwell et al., 2012].

Novel directed evolution and high-throughput screening approaches have been applied to engineer human antibodies with high affinity and selectivity toward MMP-14 for potential therapeutic applications. A phage display approach was used to develop a fully

human monoclonal antibody targeting the catalytic domain of MMP-14; this antibody inhibited tumor growth, metastasis, and angiogenesis in an orthotopic xenograft model of breast cancer [Devy et al., 2009]. In a different study, single chain antibodies selectively targeting the MMP-14 catalytic domain exclusive of the active site were selected from a naïve library and affinity matured using mutagenesis and phage display. Although not inhibitory of general catalytic activity, the antibodies potently blocked cell surface activities of MMP-14 including collagenolysis and proMMP-2 activation, and inhibited tumor growth and metastasis in an orthotopic xenograft model of breast cancer [Botkjaer et al., 2016]. Recently, a systematic approach using next-generation sequencing was applied for in-depth analysis of a Fab library enriched for MMP-14 binding using phage display [Lopez et al., 2017]. Interestingly, highly potent and selective Fabs against MMP-14 were found using next-generation sequencing which could not be identified by ELISA. Another recent study generated synthetic Fabs with high selectivity toward MMP-14 by incorporating a previously identified cyclic MMP-14 inhibitory peptide (peptide G) [Suojanen et al., 2009] into the complementary determining region CDR-H3 of a human antibody Fab library [Nam et al., 2017]. A selective binder Fab was isolated using phage panning that possessed more than 1000-fold enhancement in MMP-14 inhibition relative to the inhibitory peptide alone [Nam et al., 2017]. Another novel approach toward human Fab library construction incorporated an extended CDR-H3 length to mimic the convex shape normally found in camelid single chain antibodies, with the rationale that a convex paratope is more amenable to recognition of recessed protease active sites [Nam et al., 2016]. An MMP-14 function-blocking antibody selected from this library was shown to inhibit MMP-14 mediated collagenolysis, cellular invasion, and metastasis in a mouse model of melanoma [Remacle et al., 2017]. Engineering antibodies by directed evolution, using cell display platforms and high-throughput screening methods to improve affinity, selectivity, and physicochemical characteristics, has developed as a fertile area of research [Boder et al., 2012; Doerner et al., 2014], and these approaches are anticipated to lead to yet greater advances toward selective MMP inhibitors with therapeutic potential.

IMPROVING TIMP SELECTIVITY FOR POWERFUL THERAPEUTIC MMP INHIBITION

An alternative to antibodies as scaffolds for engineering therapeutic protein inhibitors of MMPs is presented by the natural human TIMPs [Nagase and Brew, 2003; Radisky and Radisky, 2015]. The TIMPs have essential anticancer functions, as evidenced by the observation that simultaneous knockout of the four TIMPs conferred powerful cancer-promoting properties upon fibroblasts [Shimoda et al., 2014]. Preclinical studies suggest that TIMP-based therapeutics are likely to be well tolerated; for example, systemic gene transfer of each of the TIMPs into mice in a large number of studies has caused no TIMP-related toxicity [Brand, 2002], and recombinant TIMP-1 has been used to treat mice at doses up to 50 mg/kg without reported toxicity [Schultz et al., 1988; Alvarez et al., 1990]. Studies of TIMP gene transfer or treatment with recombinant TIMP proteins have in most models shown antitumor responses for TIMP-1, -2, and -3, whereas systemic gene transfer of TIMP-4 increased tumor incidence and

growth in an orthotopic breast cancer model [Jiang et al., 2001; Brand, 2002]. However, despite promising early results in multiple animal models, it may be unlikely that the native TIMPs will advance as therapeutics, given that they show very limited discrimination among different MMP targets, but instead have evolved to accommodate “multispecific” recognition across the spectrum of MMPs [Sharabi et al., 2014]. Additionally, the natural TIMPs have been found to possess alternative, MMP-independent activities [Chirco et al., 2006; Stetler-Stevenson, 2008; Brew and Nagase, 2010; Jackson et al., 2017] that may be undesirable in MMP-targeted therapeutics. On the other hand, at about 21 kDa in size and with a compact globular protein fold, the TIMPs, like single chain antibodies, could offer versatile scaffolds for protein engineering of altered binding functionality, so as to achieve exquisite selectivity toward individual MMPs for therapeutic applications.

Intriguingly, structural examination reveals similarities between the protein surfaces used for molecular recognition by antibodies and TIMPs (Fig. 4). The antigen-binding variable fragment (Fv) of an antibody is comprised of two domains, one from the heavy chain and one from the light chain; in single chain Fvs these two domains are linked in a single protein. Each Fv domain contains three hypervariable complementarity determining regions (CDRs), flexible loops through which the antibody binds to its antigen (Fig. 4A). Similarly, TIMPs are also comprised of two domains which pack side-by-side, with each domain contributing multiple flexible loops to the MMP-binding surface (Fig. 4B). The six contact regions of a TIMP present a broad binding surface, creating a TIMP/MMP interface area nearly double that of typical contact regions between antibodies and protein antigens. This large intermolecular surface area, involving ~20 TIMP amino acid residues, offers significant opportunity for mutational fine-tuning to modulate selectivity [Sharabi et al., 2014]. Importantly, high-throughput screening approaches used for *in vitro* affinity maturation of therapeutic monoclonal antibodies can also be harnessed for the directed evolution of highly selective TIMPs, as described below.

Various studies employing targeted site-directed mutagenesis, focusing primarily on the isolated N-terminal TIMP domains, have demonstrated that multiple TIMP residues located in the N-terminal strand, AB-loop, C-connector loop, and EF-loop are capable of modulating selectivity [Butler et al., 1999b; Meng et al., 1999; Williamson et al., 2001; Lee et al., 2003, 2004; Wei et al., 2003; Hamze et al., 2007]. For example, a combination of three mutations in the TIMP-1 N-terminal domain, initially a poor inhibitor of MMP-14, improved affinity toward MMP-14 by ~100-fold [Lee et al., 2003], resulting in a molecule capable of blocking collagenase activity and shedding of CD44 in cell culture models of breast cancer and fibrosarcoma [Lee et al., 2010]. While the effect of C-terminal domain mutations of full-length TIMPs have not yet been explored, it is likely that these too may help to fine-tune selectivity. However, given the large number of residues involved in the TIMP-MMP binding interaction, a challenge comes in identifying the best combination of mutations to optimize a TIMP for selectivity toward an individual MMP. The use of structural knowledge to predict the best mutations is made problematic by the high degree of backbone flexibility evidenced in some regions of the TIMP interface [Wu et al., 2000; Grossman et al., 2010; Batra et al., 2013; Batra and Radisky, 2014], and by the complex and unpredictable nature of entropic contributions to MMP-TIMP binding energies [Arumugam et al., 2003; Wu et al., 2011; Zou et al., 2016]. Fortunately, we are not limited to approaches strictly dependent on the ability to predict advantageous mutations, as directed evolution approaches have now shown substantial promise for development of selective TIMPs.

An early proof-of-principle for combinatorial screening as a route to TIMP selectivity was demonstrated by phage display of a TIMP-2 library, incorporating diversity into the N-terminal strand, AB-loop, and C-connector loop, to evolve variants selective for MMP-1 in preference to MMP-3 [Bahudhanapati et al., 2011]. More recent efforts have employed the yeast surface display platform to evolve variants of the N-terminal domain of TIMP-2 with high affinity and selectivity toward MMP-14 [Arkadash et al., 2017]. This recent study

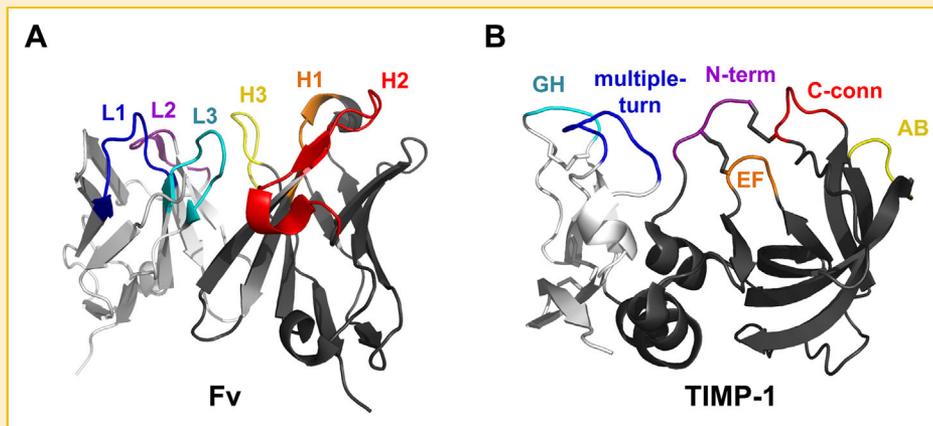


Fig. 4. Similarities between antibody Fv and TIMP binding interfaces. (A) An Fv binds to an antigen through three loops of the heavy chain (dark gray): complementarity-determining regions (CDRs) H1 (orange), H2 (red), and H3 (yellow), and three loops of the light chain (light gray): CDRs L1 (blue), L2 (purple), and L3 (cyan). Coordinates are from PDB ID: 1YQV [Cohen et al., 2005]. (B) TIMPs, like antibodies, recognize the MMP target using a broad interface comprised of multiple loops spread across two domains, including segments of the N-terminal domain (dark gray): N-terminus (purple), AB loop (yellow), C-connector loop (red), and EF loop (orange), and segments of the C-terminal domain (light gray): GH loop (cyan) and multiple-turn loop (blue). Coordinates are from PDB ID: 1UEA [Gomis-Ruth et al., 1997]. Figure was generated using PyMOL (Schrodinger, LLC).

combined the strengths of structure-based design with directed evolution, by using computational predictions of the most promising residues for optimization to guide design of the yeast surface display library. The best variant identified showed 900-fold improvement in MMP-14 affinity with an inhibitory constant of 0.9 pM, greatly enhanced selectivity, and improvements in binding to MMP-14 on the cell surface and inhibition of breast cancer cell invasion [Arkadash et al., 2017]. The encouraging results from these studies point to the utility of integrating structural and computational insights with directed evolution approaches [Rosenfeld et al., 2016], and we anticipate that this could be a general path forward for developing TIMP-based drugs selectively targeting the different MMPs most strongly implicated as therapeutic targets in breast cancer. Keeping in mind the MMP-independent activities of the natural TIMPs [Chirco et al., 2006; Stetler-Stevenson, 2008; Brew and Nagase, 2010], an additional important aspect of developing TIMP-based drugs will be to better define the TIMP epitopes responsible for some of these activities, and to remove unwanted off-target activities through protein engineering. Directed evolution strategies incorporating negative selection may prove to be useful for these efforts as well. A final challenge, given the short serum half-life of unmodified recombinant TIMPs in vivo [Batra et al., 2012b], will be to develop formulations and delivery methods capable of achieving sustained therapeutic concentrations. Promising approaches reported to date include PEGylation [Batra et al., 2012b; Chen et al., 2013b], fusion to serum albumin [Kang et al., 2007; Lee et al., 2011], nanoparticle delivery [Chaturvedi et al., 2012, 2014], and encapsulation in peptide hydrogels [Chowdhury et al., 2017] to enhance recombinant TIMP availability, stability and efficacy in vivo.

PERSPECTIVES

The investigation of MMPs as cancer mediators and therapeutics has now spanned multiple decades, with periods of collective advances and intervening setbacks. The considerable enthusiasm following studies that implicated MMPs in so many different cancer-associated processes prompted a series of inhibitor-based clinical trials that, in hindsight, did not account for the incredible complexity of this protein family and its roles in diverse physiological processes. Now, with the development of a much more sophisticated understanding of how individual MMPs act in different cancers and at distinct stages of cancer progression, in combination with newly developed methods for discovery and refinement of highly selective inhibitors, we are poised for a renaissance of therapeutically valuable MMP inhibition in breast cancer. These approaches will prove especially relevant for triple negative/basal subtype cancers, in which specific MMPs have been shown to play critical roles, and for which targeted therapeutics have been slow to develop.

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