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Matrix Metalloproteinases: From Molecular Mechanisms to Physiology, Pathophysiology, and Pharmacology[S]

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***Abstract*——The first matrix metalloproteinase (MMP) was discovered in 1962 from the tail of a tadpole by its ability to degrade collagen. As their name suggests, matrix metalloproteinases are proteases capable of remodeling the extracellular matrix. More recently, MMPs have been demonstrated to play numerous additional biologic roles in cell signaling, immune regulation, and transcriptional control, all of which are unrelated to the degradation of the extracellular matrix. In this review, we will present milestones and major discoveries of MMP research, including various clinical trials for the use of MMP inhibitors. We will discuss the reasons behind the failures of most MMP inhibitors for the treatment of cancer and inflammatory diseases. There are still misconceptions about the pathophysiological roles of MMPs and the best strategies to inhibit their detrimental functions. This review aims to discuss MMPs in preclinical models and human pathologies. We will discuss new biochemical tools to track their proteolytic activity in vivo and ex vivo, in addition to future pharmacological alternatives to inhibit their detrimental functions in diseases.**

***Significance Statement*——Matrix metalloproteinases (MMPs) have been implicated in most inflammatory, autoimmune, cancers, and pathogen-mediated diseases. Initially overlooked, MMP contributions can be both beneficial and detrimental in disease progression and resolution. Thousands of MMP substrates have been suggested, and a few hundred have been validated. After more than 60 years of MMP research, there remain intriguing enigmas to solve regarding their biological functions in diseases.**

I. Introduction

Proteolysis is a key posttranslational modification. Every single protein will encounter a protease, at some point in its lifetime, to either remove a signal peptide, cleave a propeptide for activation, process a protein to modify its functions, or initiate ubiquitination and degradation (Fortelny et al., 2014; Kappelhoff et al., 2017). Proteases are fundamental enzymes implicated in all aspects of biology, representing ~3% of the human genome, and are divided into five

ABBREVIATIONS: ABP, activity-based probe; ACPP, activatable cell-penetrating peptide; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; AP, activator protein; BAY 12-9566, tanomastat; BB-94, batimastat; BBB, blood-brain barrier; B-CLL, B cell chronic lymphocytic leukemia; BM, basement membrane; BRET, bioluminescence resonance energy transfer; CD, Crohn's disease; CD*n*, cluster of differentiation *n*; CLP, cecal ligation and perforation; CNS, central nervous system; EAE, autoimmune encephalomyelitis; ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer; FDA, US Food and Drug Administration; Fra, FOS-related antigen; FRET, Förster resonance energy transfer; GI, gastrointestinal; GM6001, ilomastat; GPI, glycosylphosphatidylinositol; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IBD, inflammatory bowel disease; ICU, intensive care unit; IFN, interferon; IL, interleukin; INXN-1001, veledimex; LAP, latency-associated peptide; LPS, lipopolysaccharide; LRP1, low-density lipoprotein receptor–related protein 1; LTBP, latent TGFβ binding protein; mAb, monoclonal antibody; mCD100, membrane-bound CD100; Met, methionine; MMP, matrix metalloproteinase; MPO, myeloperoxidase; MRI, magnetic resonance imaging; MS, multiple sclerosis; MT-MMP, membrane-type matrix metalloproteinase; NF-κB, nuclear factor kappaB; NIR, near-infrared; PAR, protease-activated receptor; PDB, Protein Data Bank; PTM, posttranslational modification; RA, rheumatoid arthritis; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; ROS, reactive oxygen species; sCD100, soluble CD100; SDS, suppressor of defective silencing; SpA, spondyloarthritis; Tat, transactivator of transcription; TGFβ, transforming growth factor-beta; TIMP, tissue inhibitor of metalloproteinase; TNFα, tumor necrosis factor-alpha; UC, ulcerative colitis; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

mechanistic classes of catalysis: metallo, serine, cysteine, aspartic acid, and threonine proteases (Puente et al., 2003; Wang et al., 2021) (Fig. 1); see the MEROPS database (https://www.ebi.ac.uk/merops/) for the lists of all known proteases (Rawlings et al., 2010). Proteases can selectively process, cleave, or degrade all proteins, and our understanding of this web of interactions known as the protease web (Fortelny et al., 2014) remains largely uncharacterized. In this review, we will focus on matrix metalloproteinases (MMPs) and present our current understanding of their biologic functions and roles in diseases, examine biochemical tools to study their functions, and discuss past and future clinical trials of MMP inhibitors. Precisely, we start with a historical perspective of MMPs (section II). Next, we discuss their molecular mechanisms and physiologic roles in cellular processes (section III). We provide examples of how MMPs can be protective or detrimental in various diseases (section IV) and discuss multiple MMP inhibitors (section V). Moreover, we present molecular tools for targeting active MMPs (section VI), and we conclude our review with new perspectives and future directions in the MMP field (section VII).

II. Matrix Metalloproteinases

A. History of Matrix Metalloproteinases

In 1962, J. Frederick Woessner published a landmark paper showing that a protease could degrade collagen in the mammalian uterus (Woessner, 1962). A few months after, Jerome Gross and Charles Lapiere published the first publication describing an MMP by demonstrating that the anuran tadpole underwent collagen turnover during metamorphosis, as shown by collagen degradation in the skin, gills, and gut (Gross and Lapiere, 1962). Four years later, in 1966, the first MMP (MMP1), termed tadpole collagenase at the time, was purified from a tadpole tail fin and back skin (Nagai et al., 1966). Not long after, in the 1970s, MMP2 and MMP3 were identified. MMP2 was first sequenced in 1988 and was first called 72-kDa type IV collagenase/gelatinase A due to its ability to degrade denatured collagen/gelatin (Collier et al., 1988). The laboratories of Guy Salvesen and Hideaki Nagase first purified MMP2 from human rheumatoid synovial fibroblasts in 1990 (Okada et al., 1990). MMP3, originally termed proteoglycanase, was identified due to its lower molecular mass (54 kDa) and its ability to degrade proteoglycan and casein (Galloway et al., 1983). In 1985, the laboratory of Zena Werb first purified MMP3 from rabbit synovial fibroblasts and later called it stromelysin (Chin et al., 1985). The first evidence of the cross-activation of MMPs in a protease web was in 1987 by the activation of proMMP1 (latent MMP1) by MMP3 (Murphy et al., 1987), further demonstrated in 1989 by the laboratory of Gregory Golberg (He et al., 1989). At the time of discovery, MMP4, -5, and -6 were believed to be novel MMPs but were later found to be redundantly identified as MMP1, -2, or -3; therefore, they are not present in the current nomenclature (Overall and Sodek, 1987; Otsuka et al., 1988). As more MMPs were being identified, a single MMP was carrying multiple names; hence, in the late 1980s, the name MMP was first proposed (Okada et al., 1987), and the International Union of Biochemistry and Molecular Biology designated the family with the terminology. By 1991, there were seven known MMPs (MMP1, -2, -3, -7, -8, -9, and -10). After the completion of the human genome project, we now know that the MMP family in humans consists of 23 members, each composed of various domains (Fig. 2).

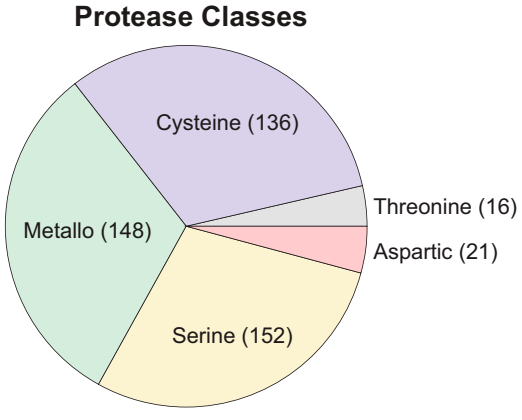

Fig. 1. Types of protease classes. The 473 human proteases are shown by their mechanism of catalysis. There are 152 serine proteases, 148 metalloproteases, 136 cysteine proteases, 21 aspartic proteases, and 16 threonine proteases. For the full list, see the MEROPS database (https://www.ebi.ac.uk/merops/).

B. MMP Domains, Structures, and Mechanism of Action for Proteolysis

MMPs belong to the superfamily of metzincin proteases, which is a family of multidomain zinc-dependent endopeptidases that can be grouped into six families: the astacins, the adamalysins (a disintegrins and metalloproteinases, or ADAMs), and the ADAMs with thrombospondin motif (ADAMTSs), the pappalysins, the serralysins, and the MMPs (Sternlicht and Werb, 2001; Sela-Passwell et al., 2011b; Tokito and Jougasaki, 2016; Wang et al., 2021). The metzincins received this denomination due to a characteristic feature in their structure, a methionine (Met) residue at the active site and the use of zinc in the enzymatic reaction. They share the following conserved domain: HEXXHXXGXXH, where H is histidine, E is glutamic acid, G is glycine, and X is any amino acid. The three histidines bind to zinc in the catalytic site (Maskos, 2005) (Figs. 2 and 3, A and B). The three-dimensional structure of MMPs was elucidated by multiple groups over the years. For example, it has been demonstrated that the active site of this class of proteases has a

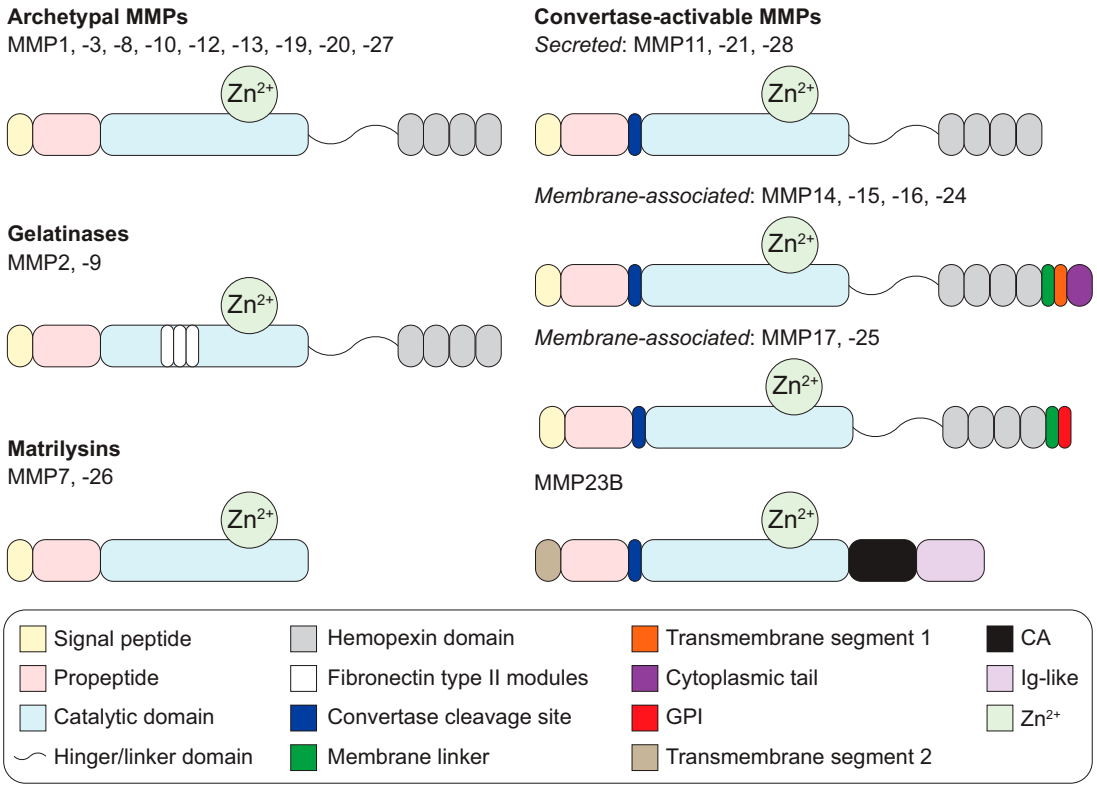

Fig. 2. Domains of human MMPs. Schematic representation of the various domains of the 23 human MMPs. Archetypal MMPs (MMP1, -3, -8, -10, -12, -13, -19, -20, and -27) contain a signal peptide, a propeptide, a catalytic domain that binds a Zn^{2+} residue, and a hemopexin C-terminal domain. Gelatinases (MMP2 and -9) contain a fibronectin type-II modules domain. Matrilysins (MMP7 and -26) contain a signal peptide, a propeptide, and a catalytic domain but lack a hemopexin C-terminal domain. The convertase-activable MMPs (MMP11, -14, -15, -16, -17, -21, -23B, -24, -25, and -28) contain a convertase cleavage site in their propeptide that is cleaved by furin or furin-like proteases. Six MMPs contain a membrane linker and are localized on the cell surface. Additional domains include glycosylphosphatidylinositol (GPI), a transmembrane segment 1 or 2, a cysteine array (CA) and an immunoglobin-like (Ig-like) domain.

deep cavity and that its composition can be quite identical among its members, reaching up to 86% of similarity for MMP3 and -10 (Lovejoy et al., 1994; Laronha and Caldeira, 2020). Although their active sites located in the catalytic domain are highly conserved (Overall and López-Otín, 2002), the 23 members of the MMP family present various domains and exhibit some structural diversity (Fig. 2).

MMPs can be divided into subgroups depending on their linear sequence similarity, domain organization, and substrate specificity. For example, MMP1, -3, -8, -10, -12, -13, -19, -20, and -27 are archetypal MMPs containing a signal peptide, a prodomain, a catalytic domain, and a hemopexin-like C-terminal domain (Fig. 2). MMP2 and -9 were previously called gelatinases A and B due to their ability to cleave denatured collagen or gelatin; they contain a fibronectin type-II modules domain. MMP7 and -26, initially termed matrilysins, lack a hemopexin-like domain. There are six membrane-type matrix metalloproteinases, or MT-MMPs (MT1-MMP or MMP14, MT2-MMP or MMP15, MT3-MMP or MMP16, MT4-MMP or MMP17, MT5-MMP or MMP24, and MT6-MMP or MMP25), that localize to the plasma membrane via a transmembrane segment or a glycosylphosphatidylinositol (GPI) anchor domain (Fig. 2). MMP23 is a unique MMP that contains a cysteine array (CA) and immunoglobulin (Ig)-like domains; there is only limited knowledge in the literature on MMP23, and as such, the precise roles of these unique domains remain elusive (Fig. 2). MMP28 or epilysin is the last member of the MMPs to be identified and characterized, and it contains unique features not observed in other MMPs, such as the presence of threonine within its catalytic sequence (Lohi et al., 2001). Therefore, MMPs contain high structural homology of their catalytic domain despite a lower overall sequence similarity due to the presence of specific domains (Stöcker et al., 1995; Sternlicht and Werb, 2001).

MMP1, -8, -13, and MT1-MMP, which were also termed collagenases in the literature, can cleave fibrillar collagen types I to IV, and also XI, resulting in the generation of two typical fragments: 1/4 C-terminal and 3/4 N-terminal (Visse and Nagase, 2003; Hotary et al., 2003; Nagase et al., 2006; Sabeh et al., 2009). MMP2 and -9 (gelatinases) can also cleave extracellular matrix (ECM) proteins in vitro, including collagens and proteoglycans, resulting in changes in

embryonic growth and development, tissue remodeling, inflammation, and wound healing (Sternlicht and Werb, 2001; Overall and Kleifeld, 2006b; Fallata et al., 2019). They previously received this denomination due to their distinct collagen-binding domain that contains three fibronectin type II repeats, conferring the ability to bind and cleave gelatin (denatured collagen) (Allan et al., 1995) (Figs. 2 and 3). MMP7 and -26 (matrilysins) can also cleave collagen and gelatin in vitro. They are unique due to the lack of a hemopexin domain, which is a characteristic structure found in all other MMPs (Fig. 2). MMP3 and -10 (stromelysins) share the same domain presented by the archetypal MMPs but cannot cleave interstitial collagen. MMP3 and -10 are closely related and share structural characteristics and substrate specificity. In addition, they can cleave numerous substrates and promote the conversion of a proMMP into an active enzyme (Nagase et al., 2006). The MT-MMPs have a prodomain at the C-terminal, which contains a furin-like proprotein convertase site. Therefore, they are activated intracellularly via proteolytic processing of the furin domain, promoting activation of the enzyme that is further directed to the cell surface due to its membrane-anchoring domains (Sohail et al., 2008; Khokha et al., 2013). They can be subdivided into two groups: type I transmembrane proteins (MMP14, -15, -16, and -24) and GPI anchored proteins (MMP17 and -25) (Fig. 2). MMP28 mRNA is typically expressed in epithelial cells of many tissues in both mice and humans, with a high expression in the lungs and skin, as well as in macrophages (Lohi et al., 2001; Rodgers et al., 2009; Gharib et al., 2018). Interestingly, MMP28 appears to contain limited ability to cleave ECM proteins, as very few substrates have been identified to date, but it has been shown to stimulate chemokine expression (Ma et al., 2013; Manicone et al., 2017; Gharib et al., 2018; Long et al., 2018).

MMP transcription is independently regulated, as each cell type, depending on the stimulus, may produce different MMPs and at different levels (Sternlicht and Werb, 2001; Overall and López-Otín, 2002). Most MMPs are not produced or only produced in low amounts under homeostatic conditions; however, this changes dramatically when a cell is stimulated with chemokines, cytokines, or growth factors, which typically results in an elevation of MMP production. Other factors like cell shape or cell stress can also regulate MMP transcription (Kheradmand et al., 1998). Some MMPs are uniquely regulated via specific transcription factors. For example, functional AP-1 site was demonstrated to mediate MMP2 transcription in cardiac cells via the binding of protein c-FOS (FOS)-related antigen (Fra)1-JunB and FosB-JunB heterodimers (Bergman et al., 2003). This regulation is often cell dependent; for example, in glomerular mesangial cells, MMP2 is regulated by the YB1 transcription factor (Mertens et al., 1997). Interestingly, during ischemia-reperfusion injury, elevated levels of FosB and JunB were identified, whereas only JunB was identified under control conditions (Alfonso-Jaume et al., 2006). In another study, ischemia induced the expression and binding of several transcription factors, including c-FOS, c-Jun, JunB, FosB, and Fra2 (Lee et al., 2005). In skeletal muscle, activator protein (AP)1 and repressor element 1 (RE1) binding sites coupled with increased Fra1, Fra2, and AP2 (Liu et al., 2010). Therefore, transcriptional regulation of MMPs appears to be cell selective, tightly regulated, and likely an adaptable process.

Changes in cell signaling via phosphorylation of various kinases such as mitogen-activated protein kinase (MAPK), p38, focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK)1, or protein kinase B (Akt) can induce MMP transcription and translation (Johansson et al., 2000; Dufour et al., 2010; Zarrabi et al., 2011; Madzharova et al., 2019). Despite various extracellular signals and cell signaling pathways shown to regulate MMP expression, the transcription factor AP1 and its binding site are present in the promoter region of most MMPs and appear to regulate their transcription (Pendás et al., 1997). AP1 contains members of the FOS and JUN family of oncoproteins which are likely involved in the regulation of MMPs in cancer cells (Overall and López-Otín, 2002). Transcriptional control of MMPs is complex, and there are multiple other nuclear factors that have been demonstrated to regulate MMPs, including erythroblast transformation-specific (ETS), NF-κB, signal transducers and activators of transcription (STATs), transcription factor 4 (TCF4), CIZ, p53, and core-binding factor alpha 1 (CBFA1) (Bond et al., 1999; Sun et al., 1999; Nakamoto et al., 2000; Crawford et al., 2001). Although most MMPs are regulated by the same transcription factors, some MMPs, like MMP26, have a unique TATA box and a transcriptional site located at 60 and 35 nucleotides upstream of its translation start site (Strongin, 2006). Only MMP12, -26 and -27 contain an unusual poly(A) site located upstream of their promoter, which, for the case of MMP26, abolishes accidental transcription; however, the functional role of this poly(A) site in MMP12 and -27 remains uncharacterized (Strongin, 2006). Overall, MMPs are transcriptionally regulated in multiple ways and tend to be closely associated with an increase in inflammation, immune cell infiltration, and tumorigenesis.

The first MMP 3D structures were published in 1994 by different groups showing the catalytic domain of MMP1 and MMP8 using X-ray crystallography, followed by the determination of the active full-length MMP1 in 1995 (Li et al., 1995). To date, a multitude of new structures have been described for multiple MMPs, using tools

such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. These tools provided the full-length tridimensional structure of a few MMPs, including proMMP1 (Li et al., 1995), proMMP2 (Morgunova, 1999), proMMP7 (Prior et al., 2015), and proMMP12 (Bertini et al., 2008). Based on X-ray crystallography data, MMPs are usually composed of a signal peptide, a prodomain, a catalytic site, a linker or hinge region, a hemopexin domain, and, depending on the MMP, a transmembrane region (Fig. 3A). Signal peptides can be of variable length and are found at the N terminus, targeting the protein for secretion. The prodomain, typically ~80 amino acids, keeps the protease inactive and is proteolytically removed by other proteases once the enzyme has reached the respective site requiring its biologic function. The catalytic site of ~170 amino acids harbors the zinc ion and is composed of five β-sheets and three α-helixes. The linker or hinge region has a variable length, from 14 to 69 amino acids, and links the catalytic domain to the hemopexin-like domain. The hemopexin domain, composed of ~210 amino acids, has a four-blade propeller domain. Six MMPs, the MT-MMPs, have a transmembrane region (Nagase et al., 2006; Cauwe et al., 2007; Laronha and Caldeira, 2020) (Fig. 2). Notable exceptions include MMP7, -23, and -26 lacking the linker peptide and hemopexin domain (Nagase et al., 2006) (Fig. 2). MMP2 and -9 are characterized by three repeats of a fibronectin type-II motif in the metalloprotease domain (Fig. 2).

MMPs are synthesized with a signal peptide located at their N terminus (Fig. 2). This short peptide targets the protein to the secretory pathway before being removed in the endoplasmic reticulum to give rise to the proMMP or zymogen (Sternlicht and Werb, 2001; Maskos, 2005). The prodomain comprises three α-helixes, connecting loops, and a cysteine switch (Sternlicht and Werb, 2001; Visse and Nagase, 2003). The α-helixes assume a nearly perpendicular shape among each other, gaining some flexibility due to the loop (Maskos, 2005). This arrangement promotes an oval structure located close to the catalytic domain and its active site cleft. The first loop, located between helix 1 and helix 2, has a protease-sensitive sequence known as the "bait region." In the case of proMMP1 and proMMP2, it is defined by amino acids EKRRN and SCNLF, respectively (Maskos, 2005; Mannello and Medda, 2012; Laronha and Caldeira, 2020). Cleavage within the bait region can destabilize the prodomain structure in proMMPs (Suzuki et al., 1990; Atkinson et al., 1995). The flexibility that is a feature of this region imposes a challenge for proper elucidation of its structure, which has only been described for proMMP2. There were two factors that played a role in resolving the structure of proMMP2's bait region: 1) a shorter loop with six amino acids fewer when compared with other MMPs and 2) the presence of a disulfide bond that stabilizes the region (Maskos, 2005). In the prodomain of MMP1, a highly conserved sequence follows the three α-helixes and is composed of PRCGXPD (Mannello and Medda, 2012; Laronha and Caldeira, 2020). This region, also termed the cysteine switch, starts at amino acid 90 and ends at the cleavage site located at amino acid 99, marking the beginning of the active enzyme (Maskos, 2005). The cysteine switch rests on top of the substrate-binding site and is so called due to its ability to inhibit the protease's function. Of note, this sequence binds from the P3′ to the P2 at the substrate-binding site, which is the opposite of the actual peptide substrate and which makes five hydrogen bonds with the main chain (Maskos, 2005; Nagase et al., 2006). The Arg91 and the Asp96 form a salt bridge, bending the loop structure placed above a histidine that takes part in the zinc-binding residues. Moreover, the salt bridge is protected from water by the side chains of up to three Tyr/Phe residues located in the intact prodomain (Maskos, 2005). Finally, the sulfhydryl group located on Cys92 interacts with the catalytic zinc ion, working as a fourth ligand and forming a tetrahedral coordination sphere that blocks the enzyme activity (Jacobsen et al., 2010). Cleavage within the bait region allows exposure to other cleavage sites, destabilizing this Cys-Zn^{2+} interaction, allowing the binding of a water molecule to the zinc, and resulting in full activation of the enzyme (Nagase et al., 2006; Jacobsen et al., 2010).

In proMMP1, the prodomain can regulate the entire enzyme configuration because it binds to the hemopexin domain and promotes a locked protein arrangement, which is the opposite of active MMP1 (Li et al., 1995; Jozic et al., 2005). Upon the removal of its prodomain, MMP1 assumes an open configuration that creates a collagen-binding site. In the case of MMP11, -28, and the MT-MMPs, an increase of up to 22 residues can be observed in between the cysteine switch and the N terminus (Marchenko et al., 2002; Maskos, 2005). Cleavage of the furin-like region located in this loop promotes enzyme activation, which takes place in the trans-Golgi network; therefore, these specific MMPs can then reach the cell surface in their active state. Lastly, MMP26 depends on specific processing mechanisms since an Arg/His mutation in the otherwise conserved cysteine motif, PHCGXXD, abolished its function and promoted an alternative activation pathway (Marchenko et al., 2002).

The active site is largely conserved among the different MMPs, presenting as a sphere shape with a diameter of around 40 Å (Tallant et al., 2010). There are two features on the surface of MMPs that are characteristic of the catalytic domain: a pocket that holds the Zn^{2+} ion and a shallow cleft for substrate binding, which further subdivides the region into an upper N-terminal subdomain and a lower C-terminal subdomain (Fig. 3, A and B). The secondary structures that constitute the catalytic domain include five

β-strands (βI to βV), with four parallel strands (βI, βII, βIII, βV) and one antiparallel (βIV), three α-helixes (αA to αC), and eight connecting loops (Tallant et al., 2010) (Fig. 3C). Of note, three loops protrude from the surface of the upper subdomain: the connection between βII and βIII (LβIIβIII), LβIIIβIV, and LβIVβV; these connections are a characteristic feature of MMPs, which separates them from other metzincins (Stöcker and Bode, 1995; Gomis-Rüth, 2003, 2009). Besides the Zn^{2+} ion in the catalytic site, which is required for catalysis, an additional Zn^{2+} ion and three Ca^{2+} ions promote conformational stability for the protein's structure. Starting from the N terminus, the chain goes through βI, αA, βII, and βIII (Maskos, 2005) (Fig. 3, A and C). Next, it enters the LβIIIβIV loop, commonly referred to as the S-loop, which has 16 amino acids and possesses two ion-binding sites (Tallant et al., 2010) (Fig. 3C). Zinc is the first ion housed by this loop, presenting a structural function, tetrahedrally supported by three histidines (His147, His162, and His175, using the proMMP1 sequence), and monodentately supported by an aspartate (Asp149) (Tallant et al., 2010). This chain composition is essential for proper MMP function, as a double mutant presenting no metal ligands is inactive (Tallant et al., 2010; Paladini et al., 2013).

Calcium is the second ion supported by the S-loop, which is coordinated by the side chain of two aspartates and one glutamine (Asp154, Asp177, and Glu180) and three main chain carbonyl oxygens, all of them in a monodentate manner (Maskos, 2005; Tallant et al., 2010). The S-loop connects the chain to the βIV strand, which provides the structure for the active site cleft. Next, the main chain enters the LβIVβV loop that, in combination with LβIIβIII, holds another Ca^{2+} ion (Visse and Nagase, 2003; Tallant et al., 2010; Laronha and Caldeira, 2020). Interestingly, in some structures, a third Ca^{2+} ion is housed in between the LβIαA and LβVαB loops via the carboxylate groups of two acidic groups and two main chain carbonyl groups (Maskos, 2005). After exiting strand βV, the chain goes through the LβVαB loop, which is a source of substrate specificity in the MMP family due to its high variability. This region constitutes the fibronectin type II motif, found in MMP2 and -9 and spanning 177 and 178 residues, respectively (Tallant et al., 2010) (Fig. 3C). Subsequently, the loop connects the polypeptide to helix αB, which also participates in the active site by providing two histidine residues as ligands for the catalytic zinc ion (Tallant et al., 2010) (Fig. 3B). The glycine of the consensus sequence in the helix αB marks the end of the upper N-terminal subdomain. Moreover, this amino acid also

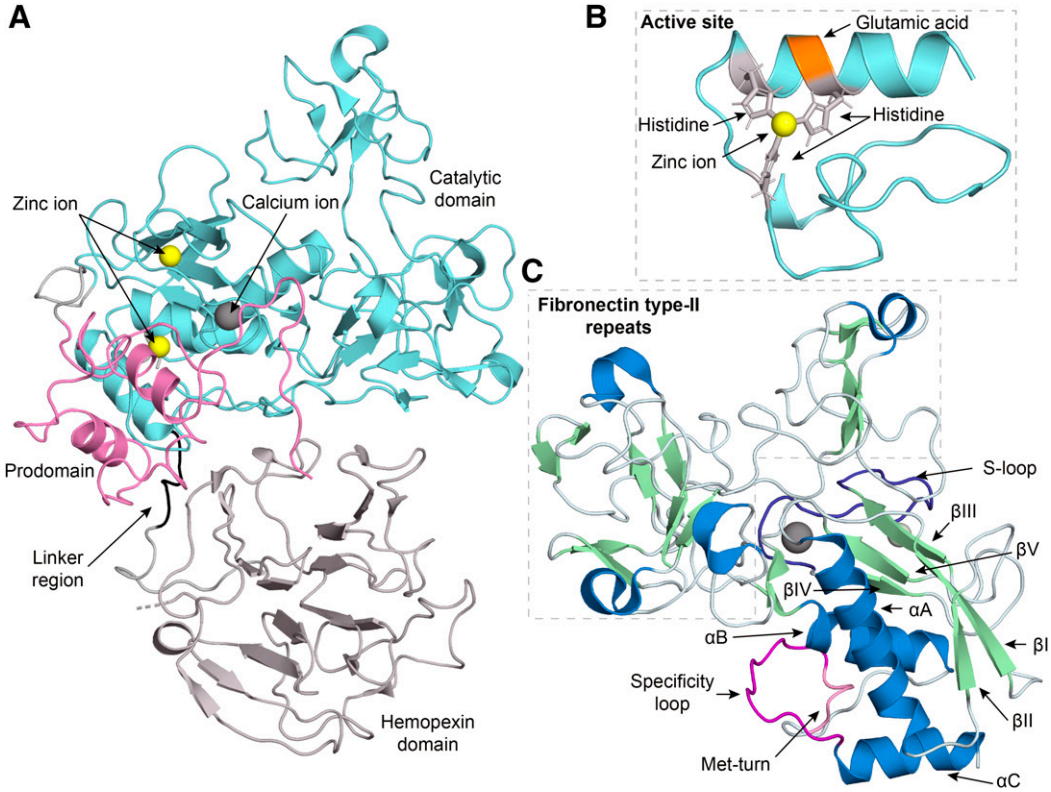

Fig. 3. MMP structure. (A) Ribbon representation of proMMP2 (PDB: 1GXD). MMP domains and inorganic ions are labeled and colored. Prodomain is shown in magenta, catalytic domain in teal, linker region in black, hemopexin domain in gray, zinc ion in yellow, and calcium ion in dark gray. (B) Ribbon representation of MMP1's active site (PDB: 1AYK). The overall structure is colored in teal. Important residues in the zinc interaction are labeled, colored, and represented as sticks. Zinc is shown in yellow, histidine in gray, and glutamic acid in orange. (C) Ribbon representation of proMMP2's catalytic site. Secondary structures are colored, and important segments are also labeled. Calcium is shown in dark gray, zinc in yellow, α-helixes in blue (αA, αB, and αC), β-sheets in green (βI, -II, -III, -IV, -V), S-loop in navy blue, specificity loop in purple, and Met-turn in pink.

allows the chain to perform a sharp turn, closely followed by the third histidine residue that participates in the zinc ion interaction (Tallant et al., 2010) (Fig. 3B). After the third histidine, a short loop precedes a tight 1.4-turn, named "Met-turn" due to a conserved methionine residue (Visse and Nagase, 2003; Maskos, 2005; Tallant et al., 2010; Laronha and Caldeira, 2020). Finally, a specificity loop that is important for substrate specificity connects the Met-turn with the αC helix, the last component of this lower C-terminal subdomain (Fig. 3C).

The catalytic domain is connected to the hemopexin domain via the linker or hinge region (Tallant et al., 2010) (Figs. 2 and 3A). The linker region is a proline-rich sequence of variable length (Laronha and Caldeira, 2020), spanning from 15 to 65 amino acids (Maskos, 2005). It is crucial for the MMP structure since it provides interdomain flexibility (Overall and Butler, 2007; Murphy and Nagase, 2008). Moreover, the hinge region is involved in the hydrolysis of complex substrates by MMP1, -8, and -13 since it requires the coordinated activation of both the catalytic domain and the hemopexin domains. For example, MMP1 and -8 have reduced collagenolytic function when mutations are introduced in the hinge region (Knäuper et al., 1997; Tsukada and Pourmotabbed, 2002). Lastly, the laboratories of Hideaki Nagase (Chung et al., 2004), Chris Overall (Tam et al., 2004), Irit Sagi (Rosenblum et al., 2007), and Ghislain Opdenakker (Van den Steen et al., 2006) have demonstrated that the linker region itself can also contribute in the binding, unwinding, and breakdown of collagen and various other MMP substrates.

The hemopexin-like domain is located at the C terminus of MMP's structure and was named after its similarity with hemopexin, which is a plasma heme-binding and heme-transport protein (Faber et al., 1995; Tallant et al., 2010) (Fig. 3A). The structure of the hemopexin domain can be characterized by the presence of four β-sheets (βI to βIV), or blades, which are named according to their appearance order. Importantly, the β-sheets follow a symmetrical structure that creates a central channel, which resembles a four-bladed propeller structure (Gohlke et al., 1996; Gomis-Rüth et al., 1996; Morgunova, 1999; Cha et al., 2002; Jozic et al., 2005). The β-sheets are connected by short peptide loops and, in combination with short helixes, shape the outer edges of the structure, which is connected and stabilized by disulfide bridges between blade I and blade IV (Maskos, 2005). The hemopexin domain is lacking in three MMPs: MMP7, -23, and -26 (Fig. 2). Interestingly, multiple MMPs that contain a hemopexin domain lose it soon after being activated. One example is MMP12; after losing its hemopexin domain, four amino acids within blade II of its hemopexin domain contain potent antimicrobial activities against both gram-negative and gram-positive bacteria (Houghton et al., 2009). However, in most cases, the hemopexin domain is involved in substrate identification and specificity (Murphy et al., 1992; Dufour et al., 2008, 2010; Zucker et al., 2009; Zarrabi et al., 2011; Manka et al., 2012; Vandooren et al., 2013).

Lastly, there is a total of six binding pockets in MMPs: S1, S2, S3, S1', S2', and S3′ (Rangasamy et al., 2019). The S1, S2, and S3 are located on the right side of the catalytic zinc ion (Rangasamy et al., 2019). Consequently, the S1', S2', and S3′ are located on the left side of the zinc ion. The inhibitors or molecules interacting with the binding pockets are named according to which pocket they interact with, such as P1, P2, P3, P1', P2', and P3′ (Maskos, 2005). Among all of the pockets, the S1' is the most variable and the most important since it determines substrate specificity (Nagase et al., 2006). The pocket cavity, in turn, is defined by the Ω-loop, which is highly hydrophobic (Rangasamy et al., 2019). Alternatively, S2 is the least variable pocket (Laronha and Caldeira, 2020). The remaining pockets, S2, S3′, S1, and S3, sit in between with around the same level of variability (Nagase et al., 2006). The depth of the S1' pocket can be used to subdivide the MMPs since it can be present in a shallow, intermediate, and deep pocket configuration (Eckhard et al., 2016b; Rangasamy et al., 2019). MMPs are activated via various mechanisms to exert their proteolytic functions and need to be tightly regulated by endogenous inhibitors to assure an adequate physiologic balance of their processing and degradative functions.

C. Endogenous Inhibitors and the Tissue Inhibitor of Metalloproteinases

1. Cloning and Identification. The first tissue inhibitor of metalloproteinase (TIMP) was described in 1972 (Bauer et al., 1972). After the initial discovery and subsequent molecular characterization in 1985, TIMP1 was found to be identical to a factor with erythroid-potentiating activity (Gasson et al., 1985). Importantly, due to this initial link between TIMP1 and the physiologic regulation of erythropoiesis, as well as its ability to stimulate cells of the erythroid lineage, TIMPs and MMPs were initially studied separately and not in the same context of protein-protease interactions. In 1990, TIMP2 was cloned and sequenced from an A2058 human melanoma cell cDNA library (Stetler-Stevenson et al., 1990). Importantly, in 1991, TIMP2 was demonstrated to inhibit collagenase activity in a 1:1 molar ratio complex, supporting the role of TIMPs in the inhibition of metalloproteinases (DeClerck et al., 1991). First cloned in chicken in 1992 (Pavloff et al., 1992), the human form of TIMP3 was first cloned in 1994 by multiple groups (Apte et al., 1994; Leco et al., 1994; Silbiger et al., 1994; Uría et al., 1994; Wilde et al., 1994; Jay et al., 2012).

Finally, human TIMP4 cDNA was cloned in 1996 (Greene et al., 1996).

2. Structure and Overall Functions. The four TIMPs are variably glycosylated and have molecular masses between 22 and 28 kDa (Khokha et al., 2013). Mammalian TIMPs exhibit basic similarities in their structure, folding to give a wedge-shaped appearance comprised of two main domains (Masciantonio et al., 2017). The first is an approximately 125 amino acid N-terminal domain, and the second is an approximately 65 amino acid C-terminal domain (Brew et al., 2000). These two domains are connected through six conserved disulfide bonds, and each domain mediates different functions (Brew and Nagase, 2010). The N-terminal domain is critical for inhibiting metalloproteinase activity by forming a ridge that interacts with the catalytic site of metalloproteinases (Jayawardena et al., 2019). This interaction occurs in a 1:1 stoichiometric inhibitor-to-enzyme ratio and blocks the active site Zn^{2+} molecule (Masciantonio et al., 2017; Jayawardena et al., 2019). The C-terminal domain of TIMPs was thought to have a limited role in metalloproteinase inhibition, but instead it plays a role in mediating additional protein-protein interactions such as the interaction with the hemopexin domain of some proMMPs (Brew and Nagase, 2010; Masciantonio et al., 2017). For example, TIMP2, TIMP3, and TIMP4 have the ability to interact with proMMP2, whereas TIMP1 and TIMP3 can interact with proMMP9 (Brew and Nagase, 2010).

3. Localization and Expression. Many mammalian tissues constitutively express the various TIMP family members, and the expression of TIMPs can be induced or inhibited in various situations, including during development, injury, and tissue repair (Masciantonio et al., 2017). Transcriptionally, TIMP levels can be altered by various growth factors, cytokines, and chemokines (Cabral-Pacheco et al., 2020). TIMP1 has been identified in many tissues, including the brain, heart, arteries, colon, kidneys, liver, lungs, bladder, breasts, skin, lymph nodes, ovaries, uterus, prostate, and testes (Cabral-Pacheco et al., 2020). The mRNA and protein levels of TIMP1 are generally elevated in several types of cancers and in settings of inflammation and tissue injury (Cabral-Pacheco et al., 2020). For example, *TIMP1* mRNA expression can be upregulated by proinflammatory cytokines, including tumor necrosis factor-alpha (TNFα) and interleukin-1-beta (IL1β) (Bugno et al., 1999). Moreover, *TIMP1* mRNA and protein levels were found to be increased in the lungs of mice after bleomycin-induced injury (Madtes et al., 2001).

TIMP2 is expressed in tissues such as the lymph nodes, brain, heart, arteries, colon, kidneys, liver, breasts, ovaries, prostate, and testes (Cabral-Pacheco et al., 2020). TIMP2 is generally considered to be constitutively expressed, but its levels can also be modified by stimuli, including hormones, bacterial products, cytokines, and growth factors (Masciantonio et al., 2017). TIMP2 levels can also be altered in response to tissue damage or various disease states. For example, Timp2 mRNA levels were elevated in rats after eccentric exercise-induced muscle damage (Koskinen et al., 2001). Furthermore, TIMP2 was found to be overexpressed in malignant ovarian tissues (Hu et al., 2004).

TIMP3 has been identified in the brain, heart, colon, kidneys, lungs, liver, breasts, ovaries, prostate, and testes (Cabral-Pacheco et al., 2020). The localization of TIMP3 is unique from the other TIMP family members in that it is localized to the extracellular matrix, whereas the other TIMPs are generally considered to be soluble (Stetler-Stevenson, 2008). This interaction with the extracellular matrix has been shown to occur via TIMP3 binding to heparan sulfate and possibly chondroitin sulfate proteoglycans (Yu et al., 2000). Certain growth factors and cytokines can regulate the levels of TIMP3, such as TNFα and transforming growth factor-beta (TGFβ) (Masciantonio et al., 2017). Available TIMP3 levels can also be regulated by low-density lipoprotein receptor–related protein 1 (LRP1), a scavenger receptor that facilitates endocytosis of TIMP3 into the cell (Scilabra et al., 2013). Furthermore, the expression of TIMP3 is frequently altered after infection and injury. For example, Timp3 expression decreased rapidly after bleomycin-induced lung injury, a mouse model of pulmonary fibrosis (Gill et al., 2010). Conversely, TIMP3 was increased in the kidneys of patients with diabetic nephropathy and chronic allograft nephropathy relative to healthy controls (Kassiri et al., 2009).

TIMP4 remains the least studied member of the mammalian TIMPs. TIMP4 has been identified in the brain, heart, kidneys, breasts, uterus, pancreas, colon, ovaries, testes, prostate, and adipose tissue (Cabral-Pacheco et al., 2020). The level of TIMP4 is altered in various types of cancer and is increased in the plasma of patients after acute myocardial infarction (Melendez-Zajgla et al., 2008; Kelly et al., 2010). It is important to mention that the elevation of TIMP1, -2, -3, or -4 expression does not necessarily imply a net beneficial or detrimental pathogenic outcome. Careful validation of MMPs and other proteins linked with TIMPs expression must be evaluated in specific tissues and diseases being investigated.

4. MMP-Dependent TIMP Functions. TIMPs have been found to function through both metalloproteinase-dependent and metalloproteinase-independent mechanisms to regulate cellular functions such as angiogenesis, apoptosis, cell differentiation, growth, and migration (Brew and Nagase, 2010). However, the primary function of TIMPs is thought to be the regulation of metalloproteinase activity (Nagase et al., 2006). The mammalian TIMPs display similar structural properties, so it is not surprising that they share overlapping metalloproteinase inhibition

profiles (Masciantonio et al., 2017). Furthermore, all four TIMPs are collectively able to inhibit the active forms of all metalloproteinases studied to date, with binding constants being in the low picomolar range (Baker et al., 2002). There are, however, some differences in the inhibition profiles of TIMPs and their affinities for different metalloproteinases. For example, TIMP1 does not appear to effectively inhibit several membrane-type MMPs (MT-MMPs), including MT1-MMP, MT3-MMP, and MT5-MMP (Brew and Nagase, 2010). TIMP1 is also a poor inhibitor of MMP19 (Brew and Nagase, 2010). Conversely, TIMP3 has a broad inhibition profile compared with the other TIMPs. In addition to inhibiting MMPs, TIMPs also inhibit several members of the ADAM and ADAMTS families (Brew and Nagase, 2010; Jackson et al., 2017). The in vitro activity of ADAM10 was demonstrated to be inhibited by TIMP1 and -3 but not TIMP2 and -4 (Amour et al., 2000). TIMP3 also inhibits ADAM17 activity resulting in regulation of mitogen-activated protein kinase (MAPK) signaling via extracellular signal-regulated kinase (ERK) and p38 in addition to TGFα in Cα and M2 modified CHO cells, respectively (Xu et al., 2012). In fact, TIMP3 appears to inhibit the greatest number of ADAMs (ADAM10, -12, -17, -28, -33) and ADAMTSs (ADAMTS1, -2, -4, -5) (Jackson et al., 2017). Therefore, when assessing the biologic functions of TIMPs, it is important to also consider ADAM and ADAMTS proteolysis.

TIMP1 has been shown to promote cell survival by reducing apoptosis of hepatic stellate cells. The regulation of cell survival is at least partially MMP-dependent, as a TIMP1 mutant that selectively lacked MMP inhibitory activity had no antiapoptotic effect (Murphy et al., 2002). There is also evidence to suggest that TIMP1 negatively regulates hepatocyte growth factor activity during liver regeneration by an MMP-dependent mechanism (Mohammed et al., 2005). Additionally, TIMP1 can restrict microvascular endothelial cell migration by regulating MMP-mediated disruption of vascular endothelial cadherin (VE-cadherin) at cell-cell junctions (Akahane et al., 2004). TIMP1 has also been found to regulate the process of airway epithelial cell migration by inhibiting MMP7 activity (Chen et al., 2008). Specifically, TIMP1 appears to inhibit the MMP7-dependent cleavage of syndecan-1 from mediating bronchiole epithelial cell migration after injury (Chen et al., 2008, 2009). Syndecans are heparan sulfate glycoproteins found on the surface of adherent cells, and their shedding is associated with increased cell migration (Teng et al., 2012). TIMP2 has similarly been found to restrict syndecan-1 cleavage from the cell surface; however, this cleavage event was mediated by MT1-MMP (Endo et al., 2003). In addition to regulating MT1-MMP, TIMP2 also regulates MMP2 and MMP9 activity (Cabral-Pacheco et al., 2020). After myocardial infarction, *Timp2*$^{-/-}$ mice presented with greater left ventricular dilation, dysfunction, and severe inflammation. This phenotype was associated with impaired MMP2 activation but increased activity of MT1-MMP (Kandalam et al., 2010). Furthermore, TIMP2 was found to inhibit both the invasion and migration of HCT-116 colon carcinoma cells via the regulation of MMP9 (Wang et al., 2019).

Interestingly, TIMP3 is known to promote apoptosis. Through its N-terminal domain, TIMP3 inhibits the shedding of death receptors like Fas from the cell surface, thus promoting activation of an apoptotic signaling pathway (Ahonen et al., 2003). As TIMP3 can be found localized to the ECM, it is unsurprising that TIMP3 also appears to have the ability to impact ECM-cell signaling (Gill et al., 2006). Specifically, TIMP3 has been found to inhibit metalloproteinase-dependent fibronectin degradation during lung development, impairing signaling through focal adhesion kinase (Gill et al., 2006). Similarly, Timp3$^{-/-}$ mice showed enhanced collagen degradation in the peribronchiolar space and disorganized collagen fibrils in the alveolar interstitium that progressed as the mice aged (Leco et al., 2001). This phenotype was proposed to be due to a shift of the MMP/TIMP balance, leading to enhanced metalloproteinase activity and consequent ECM degradation. Importantly, TIMP3 has also been found to promote the normal function of the microvascular endothelial barrier (Arpino et al., 2016). Although the specific metalloproteinase remains unknown, this function of TIMP3 is at least partly mediated through metalloproteinase-mediated disruption of adherens junctions, as treatment with a broad-spectrum metalloproteinase inhibitor rescued both barrier dysfunction and endothelial cell surface localization of vascular endothelial cadherin (Arpino et al., 2016).

TIMP4 was determined to be the primary MMP inhibitor in human platelets and is involved in regulating platelet aggregation and recruitment (Radomski et al., 2002). TIMP4 is known to negatively regulate MT1-MMP activity (Cabral-Pacheco et al., 2020). Specifically, after ischemia-reperfusion injury in Timp4$^{-/-}$ mice, there was a persistent increase in MT1-MMP activity that resulted in exacerbated diastolic dysfunction (Takawale et al., 2014). TIMP4 has also been shown to regulate the activity of MMP2 by inhibiting MT1-MMP (Bigg et al., 2001).

5. TIMPless Mice. To study the biologic functions of the four members in the *Timp* gene family, a quadruple *Timp* knockout termed the TIMPless mouse was generated by the laboratory of Rama Khokha (Shimoda et al., 2014). Genetic removal of the four *Timps* resulted in runted pups with reduced body size and only 25% survival past 10 days postnatal (Shimoda et al., 2014). TIMPless fibroblasts acquired hallmark cancer-associated fibroblast functions via an

increase of α-smooth muscle actin, and exosomes produced by these cells elevated cancer cell motility and cancer stem cell markers. In addition, the proteomes of TIMPless fibroblasts were found to be enriched in ECM proteins and ADAM10. Furthermore, knockdown of ADAM10 in TIMPless fibroblasts was sufficient to abrogate the functions of cancer-associated fibroblasts (Shimoda et al., 2014). This study demonstrated the close interplay between the TIMPs, MMPs, and ADAMs family members and highlighted the difficulties in characterizing a single TIMP's biologic role, as the web of interactions appears to be interdependent among these proteins.

6. Latent MMP Interactions. As their name suggests, TIMPs are primarily recognized for their ability to inhibit metalloproteinases. However, there are instances of TIMPs interacting with latent (inactive) MMPs and facilitating their activation. The best known example of this is the interaction between TIMP2 and proMMP2. First, TIMP2 binds to MT1-MMP on the cell surface, which acts to form a receptor for proMMP2; once proMMP2 is bound to this complex, another free MT1-MMP can cleave the prodomain of proMMP2, converting it to its active form (Sato et al., 1994; Brew et al., 2000; Masciantonio et al., 2017). In *Timp2*$^{-/-}$ mice, proMMP2 activation is impaired, reflecting the unique role of TIMP2 in proMMP2 cell surface activation (Baker et al., 2002). TIMP3 and TIMP4 have also been shown to interact with proMMP2, but both appear to restrict its activation (Brew and Nagase, 2010).

7. Other Endogenous MMP Inhibitors. MMPs have additional endogenous inhibitors, including alpha-2 macroglobulin, a reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tissue factor pathway inhibitor, and MMP prodomains (Sellers et al., 1977; Oh et al., 2001; Iyer et al., 2012). Alpha-2 macroglobulin is a large 720-kDa glycoprotein that is regarded as the inhibitor of several proteases within the plasma (Rehman et al., 2013). The protein contains four identical subunits, each with a 25 amino acid "bait region" that is susceptible to proteolytic cleavage (Rehman et al., 2013; Serifova et al., 2020). Cleavage of the bait region by active proteinases activates alpha-2 macroglobulin, triggering a conformational change that traps the protease (Rehman et al., 2013; Goulas et al., 2017). This interaction is further anchored by the covalent binding of an exposed reactive thioester of activated alpha-2 macroglobulin with accessible lysine residues of the protease (Rehman et al., 2013). For example, Serifova et al. (2020) demonstrated that alpha-2 macroglobulin efficiently traps monomers of MMP9, preventing them from proteolytically cleaving large substrates such as gelatins. Additionally, after alpha-2 macroglobulin activation, receptor-binding domains can be exposed that bind to cell surface receptors such as LRP1, which can stimulate internalization and clearance of the activated alpha-2 macroglobulin/protease complexes from the circulation (Serifova et al., 2020). MMP9 alpha-2 macroglobulin complexes were shown to be removed from the extracellular environment through LRP1-mediated internalization.

RECK is a 110-kDa glycoprotein widely expressed in mammalian tissues; it has been shown to mediate tissue remodeling and inhibit tumor angiogenesis and metastasis (Oh et al., 2001; Alexius-Lindgren et al., 2014). The protein contains serine protease inhibitor–like domains and is the only known MMP inhibitor that is cell membrane–bound, which occurs via a GPI anchor on RECK (Takahashi et al., 1998; Alexius-Lindgren et al., 2014). Previous studies have suggested that RECK negatively regulates MMP2, MMP9, and MT1-MMP (Takahashi et al., 1998; Oh et al., 2001; Takagi et al., 2009). RECK has also been demonstrated to be a physiologic inhibitor of ADAM10, an upstream regulator of Notch signaling that impacts brain development (Muraguchi et al., 2007). RECK is also required for the development of multiple organs; *Reck*$^{-/-}$ mice do not survive past embryonic day 10.5 and present with defects in blood vessel development as well as compromised integrity of collagen fibers and the basal lamina, suggesting that this developmental defect may be due to aberrant metalloproteinase activity (Oh et al., 2001). Therefore, despite a key role in regulating MMP functions, RECK's inhibition of other proteases such as ADAM10 must be taken into consideration when studying its biologic functions.

III. Molecular Mechanisms to Physiologic Roles of Matrix Metalloproteinases

As indicated by their names, MMPs can cleave and degrade ECM proteins. However, the name "matrix metalloproteinase" is causing confusion in the literature in relation to its biologic functions. It is estimated that only ∼31% of their substrates are ECM proteins and ∼69% are non-ECM proteins (Dufour and Overall, 2015) (Fig. 4, as of April 2022; Supplemental Table 1). Therefore, in the recent decade, there has been a reevaluation of their substrates and physiologic roles.

A. Extracellular Matrix Remodeling

The ECM is a noncellular structure that is highly dynamic and remains in close contact with cells either throughout their entire life or at important phases of their development. For this reason, the ECM is present in all tissues and organs of the body, providing structural support as one of its main functions (Hynes, 2009). The ECM has a tridimensional structure characterized by a distinct composition according to each organ; therefore, its composition is crucial since

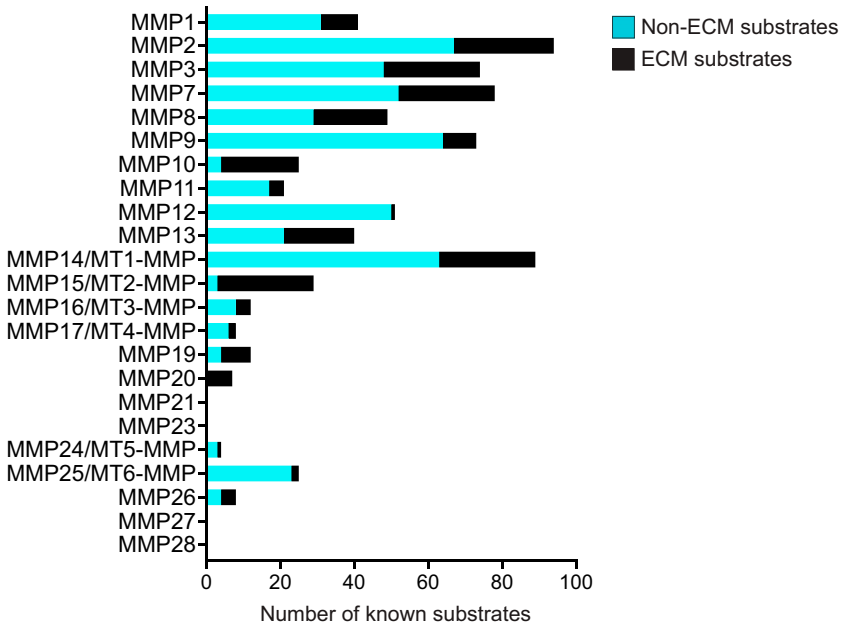

Fig. 4. MMP substrates. Reported ECM (black) and non-ECM (cyan) MMP substrates for all 23 human MMPs as reported by TopFIND (https://topfind.clip.msl.ubc.ca) (Fortelny et al., 2015). TopFIND integrates information from the UniProt knowledgebase (UniProtKB), MEROPS peptidase database, and experimental terminomics studies of eight species, including (*Homo sapiens*, *Mus musculus, Rattus norvegicus, Arabidopsis thaliana, Saccharomyces cerevisiae*, and Escherichia coli).

mutations in genes that encode key components of the ECM can lead to severe tissue defects or even embryonic lethality (Bateman et al., 2009; Järveläinen et al., 2009). For example, gene deletion of fibronectin and collagens are often lethal at the embryonic stage, as shown by multiple loss-of-function studies (Rozario and DeSimone, 2010). In the 1980s, the biochemical studies investigating the ECM composition focused on large extracellular matrices such as those in the bone and cartilage. At the time, the biochemistry of native ECM was challenging due to its intrinsic characteristics, including insolubility and high levels of cross-linkage (Hynes and Naba, 2012). These challenges persist today, but the advancement of biochemical techniques and the availability of complete genome sequences allowed the identification of >300 ECM proteins in mammals (Hynes and Naba, 2012). These proteins constitute the core matrisome, including collagens, proteoglycans, and glycoproteins, as well as ECM-associated proteins that contribute to matrices in different scenarios such as growth factors (Hynes and Naba, 2012).

The ECM can be divided into two main types: the interstitial connective tissue matrix and the basement membrane (BM), which differ according to their localization and composition (Hynes and Naba, 2012). The connective matrix surrounds cells and provides structural support for tissues, whereas the BM is specialized in separating the epithelium from the neighboring stroma (Bonnans et al., 2014). Importantly, the ECM function is not limited to tissue support, integrity, and elasticity. In fact, its structure is constantly being remodeled to sustain tissue homeostasis. ECM remodeling, degradation, or processing result in constant and dynamic changes in cell and tissue behavior (Lu et al., 2011; Cox, 2021). The ECM constitutes ~4% of the human proteome (Cox, 2021) and can regulate basic biologic functions such as cell movement, shape, growth, and survival via cell adhesion, cell-ECM, and cell-cell interactions (Sternlicht and Werb, 2001). Epithelial cells interact with ECM components, which serve as ligands for cell receptors; these interactions result in signaling that controls proliferation, apoptosis, adhesion, migration, survival, or differentiation (Bonnans et al., 2014). Furthermore, many cell types have a major role in remodeling and rebuilding the ECM by degrading, synthesizing, reassembling, and chemically modifying its components. These mechanisms are highly complex and require some fine-tuning to function properly; otherwise, dysregulated ECM composition is associated with a variety of pathologic conditions, especially in response to injuries, and can worsen disease progression (Bonnans et al., 2014). For example, uncontrolled ECM deposition or degradation is linked to fibrosis, osteoarthritis, and cancer (Zhen and Cao, 2014).

ECM remodeling is mainly accomplished by the cleavage of its components, controlling their abundance, composition, and structure. Moreover, the processing of the ECM by proteases can release bioactive molecules, such as growth factors, with a variety of functions. In general, two main pathways are responsible for ECM degradation: an intracellular and an extracellular pathway (Dufour and Overall, 2015). For the intracellular processing, the ECM is degraded in the phagolysosome by lysosomal proteases, whereas the extracellular remodeling is performed by secreted proteases (Dufour and Overall, 2015). One misconception from the literature is that MMPs are the main acting proteases implicated in ECM degradation, especially collagens and proteoglycans. In reality, multiple proteases other than the MMPs regulate ECM

remodeling, such as the ADAMs, ADAMTSs, plasmin, cathepsins, neutrophil elastase, and several others (Brassart-Pasco et al., 2020; Cox, 2021; Martin et al., 2021). In addition, dysregulation or downregulation of protease inhibitors such as the TIMPs, cystatins, or serpins also contribute to changes in ECM remodeling (Cox, 2021). Therefore, ECM degradation is significantly more complex than initially thought and implicates much more than only the MMPs. Although collagen and proteoglycan (ECM) degradation is not carried out by MMPs alone, MMPs do play a major role in this biologic process. For example, thyroid hormones during metamorphosis promote the release of several MMPs that present an active role in remodeling the intestine. Although the exact mechanism is not fully understood, active MMP11 is released by fibroblasts after hormonal stimulation and promotes epithelial cell apoptosis and growth of connective tissues (Patterton et al., 1995; Ishizuya-Oka et al., 2000; Amano et al., 2005; Bonnans et al., 2014). Other MMPs are also upregulated by thyroid hormones, such as MMP2, MMP9, and MT1-MMP, which were shown to be increased in the classic tadpole model (Fujimoto et al., 2007). In contrast to MMP11, MMP2 and MT1-MMP appear to have an important role in ECM remodeling after apoptosis has occurred during the late phases of intestinal morphogenesis (Fujimoto et al., 2007; Hasebe et al., 2007).

MMPs are also important for branching morphogenesis. The ECM at the tip of ducts appears to be thinner than other regions that do not participate in the elongation process, indicating that proteases are likely required to cleave the ECM, allowing proper invasion in the surrounding tissue (Daley and Yamada, 2013). Tan et al. (2014) showed that MMP11 is crucial for mammary gland morphogenesis, and in Mmp11−/− mice, a decrease in branching and downregulation of periductal collagen content occurred. Mmp2−/− and Mmp3−/− mice are also defective in mammary gland branching, showing that these MMPs are implicated and important for proper gland development (Wiseman et al., 2003). MT2-MMP was demonstrated to play a key role in digesting collagen IV in submandibular and mammary glands to sustain the proliferation of tip cells (Rebustini et al., 2009). In lung embryogenesis, Timp3−/− mice exhibited decreased bronchiole branching but enhanced activity of MMPs associated with increased fibronectin degradation compared with wild-type controls (Gill et al., 2006). Although mouse models of various MMP knockouts show defects in ECM remodeling, only the MT1-MMP−/− mice exhibited craniofacial dysmorphism, osteopenia, dwarfism, and fibrosis of soft tissues resulting in death within 50–90 days after birth. Except for MT1-MMP, most MMP knockout mice resulted in fertile, viable, and relatively healthy animals, suggesting that MMPs are not essential for embryogenesis and development (Table 1). Importantly, proteases regulate and modulate the branching process by other mechanisms besides cleavage of the ECM. For example, the hemopexin domain in MMP3 binds to WNT5B, leading to an increase in mammary stem cell numbers, which results in increased branching morphogenesis (Kessenbrock et al., 2013). Taken together, these results demonstrate that MMPs regulate the ECM during branching morphogenesis via proteolytic and nonproteolytic mechanisms.

In the ECM, MMPs are also involved in the degradation and processing of growth factors and their receptors, which actively participate in the wound healing process, including the modulation of angiogenic factors to promote or inhibit angiogenesis (Schultz and Wysocki, 2009). The processing of the diverse reservoir of growth factors located in the ECM can expose regions capable of activating growth factor receptors or release them to the interstitial space (Mott and Werb, 2004). Two examples of growth factors released from the ECM by MMPs include TGFβ and vascular endothelial growth factor (VEGF). TGFβ belongs to the transforming growth factor superfamily, and it is classified as a multifunction cytokine, exerting different tasks on a wide variety of biologic processes (Mott and Werb, 2004). Importantly, after TGFβ is secreted, it is maintained in a latent form by interacting noncovalently and forming a complex with a latency-associated peptide (LAP). The TGFβ-LAP complex remains bound to the ECM via an interaction between LAP and a protein belonging to the fibrillin family, the latent TGFβ binding protein (LTBP) (Mott and Werb, 2004). TGFβ becomes active when released from the complexed form and MMP2, -9, -13, and MT1-MMP can directly activate TGFβ by releasing it from LAP (Yu and Stamenkovic, 2000; Mu et al., 2002). Moreover, MMP2 and -9 can cleave a soluble form of LTBP, whereas only MMP2 can cleave the ECM-bound LTBP (Dallas et al., 2002). These results indicate that MMPs are important for TGFβ activation, either by directly inhibiting the LAP interaction or by releasing the TGFβ complex from the matrix via LTBP cleavage.

MMPs can also activate VEGF, an important regulator of angiogenesis, as it stimulates vascular permeability and vessel growth (Park et al., 1993; Bergers et al., 2000). Heparan sulfate proteoglycan in the ECM can tightly bind specific isoforms of VEGF, reducing its bioavailability (Park et al., 1993). This process can also be reversed by proteolytic cleavage of the ECM, leading to the release of bound VEGF. For example, Bergers et al. (2000) demonstrated that MMP9 is associated with VEGF release and the development of pancreatic neuroendocrine tumors in the RIP-Tag2 insulinoma model, switching the environment from vascular quiescence to active angiogenesis. These studies show the importance of ECM as a rich reservoir of latent growth factors and cytokines,

TABLE 1
MMP knockout mice phenotypes

MMP	Phenotype of MMP Knockout Mouse	Impact on Biologic Functions	References
Mmp1a$^{-/-}$	No obvious abnormalities and both males and females were fertile.	↓Lung tumor growth and angiogenesis using LLC1 lung cancer cell line.	(Fanjul-Fernández et al., 2013, 2018; Foley et al., 2014)
Mmp1b$^{-/-}$	N/A	N/A	N/A
Mmp2$^{-/-}$	Both males and females were fertile. Smaller at birth and ∼15% slower growth rate. Litters from heterozygous crosses yielded ∼85% fewer than expected homozygous *Mmp2*$^{-/-}$ pups.	↑Susceptibility to dextran-sulfate-induced colitis, ↑protection and ↓reduction in hepatocyte apoptosis in TNFα-induced hepatitis, ↑severity of antibody-induced arthritis, ↓white matter sparing and ↓serotonergic fibers in spinal-cord lesions, ↑cardiac allograft survival, delay of neovascularization, ↓atherosclerotic plaques, ↓numbers and proliferation of osteoblasts and osteoclasts, ↑apoptosis.	(Itoh et al., 1997, 2002; Wielockx et al., 2001; Campbell et al., 2005; Samolov et al., 2005; Garg et al., 2006; Hsu et al., 2006; Kuzuya et al., 2006; Martignetti et al., 2014)
Mmp3$^{-/-}$	No obvious abnormalities and both males and females were fertile.	↑Protection and ↓reduction in hepatocyte apoptosis in TNFα-induced hepatitis, ↓immune-complex-induced lung injury and ↓neutrophils, ↑myocardial scar volume post injury, ↓skin-contact hypersensitivity, delayed clearance of bacteria and appearance of $CD4^+$ T lymphocytes into intestinal lamina propria, ↓production of macrophage chemoattractant in disc hernia in vitro.	(Mudgett et al., 1998; Wang et al., 1999; Haro et al., 2000a; Warner et al., 2001; Wielockx et al., 2001; Li et al., 2004; Mukherjee et al., 2005)
Mmp7$^{-/-}$	No obvious abnormalities and both males and females were fertile. Normal lifespan.	↓Release of TNFα from peritoneal macrophage in vitro, ↓transepithelial neutrophil migration in bleomycin-induced lung injury, ↓reepithelization postinjury in trachea, ↓processing of alpha-defensin resulting in ↓innate immunity, ↑corneal neovascularization after injury, ↓epithelial cell apoptosis linked with ↓Fas ligand processing, protected from LPS-induced intestinal permeability and lethality.	(Wilson et al., 1997; Dunsmore et al., 1998; Powell et al., 1999; Wilson, 1999; Haro et al., 2000b; Li et al., 2002; Kure et al., 2003; Vandenbroucke et al., 2014)
Mmp8$^{-/-}$	Normal development, fertile, and no reduction in survival.	↑Incidence of skin tumors, ↓inflammatory cell apoptosis but ↑neutrophils in BAL in an asthma model, ↑protection in TNFα-induced hepatitis and impaired leukocyte influx, ↓neutrophil infiltration toward LPS, ↓mortality and hypothermia in sepsis and renal ischemia/reperfusion.	(Balbín et al., 2003; Gueders et al., 2005; Van Lint et al., 2005; Tester et al., 2007; Vandenbroucke et al., 2012; Fortelny et al., 2014)
Mmp9$^{-/-}$	No gross phenotypic abnormalities, fertile, and normal lifespan. However, they have abnormal development of growth plates in long bones.	↑Protection and ↓reduction in hepatocyte apoptosis in TNFα-induced hepatitis, ↓severity of antibody-induced arthritis, ↓cardiac allograft survival, ↓immune-complex-induced lung injury, prolonged skin-contact hypersensitivity, ↓alveolar bronchiolization after bleomycin treatment, ↑bronchoalveolar lavage cell recruitment post allergic challenge, impaired neutrophil infiltration and ↑ early vascular permeability in a model of zymosan peritonitis, ↓resistance against *Escherichia coli* peritonitis due to ↓leukocyte recruitment, ↓dextran-sulfate-induced colitis, ↑bacterial-induced arthritis but ↓bacterial clearance, ↑brain hemorrhage and injury, ↓remyelination after spinal cord trauma, spontaneous deficient myelination of corpus callosum with fewer oligodendrocytes, ↓experimental autoimmune	(Vu et al., 1998; Dubois et al., 1999; Wang et al., 1999; Betsuyaku et al., 2000; Wielockx et al., 2001; Warner et al., 2001; Itoh et al., 2002; Larsen et al., 2003, 2006; McMillan et al., 2004; Johnson et al., 2004; Tang et al., 2004; Campbell et al., 2005; Heissig et al., 2005; Heymans et al., 2005; Castaneda et al., 2005; Kolaczkowska et al., 2006; Renckens et al., 2006; Calander et al., 2006; Cheung et al., 2008)

(*continued*)

TABLE 1—*Continued*

MMP	Phenotype of MMP Knockout Mouse	Impact on Biologic Functions	References
		encephalomyelitis in young mice, ↓vessel formation in an ischemic limb model, ↓left-ventricle dilation and fibrosis post pressure overload, ↓capillary branching post ischemic insult, ↑myocardial injury and foci of infection when infected with coxsackievirus B3.	
Mmp10$^{-/-}$	No overt defects in fertility, litter size, gross appearance, organ structure, or tissue histology.	Muscles displayed impaired recruitment of endothelial cells, reduced levels of extracellular matrix proteins, diminished collagen deposition, and decreased fiber size muscles displayed impaired recruitment of endothelial cells, reduced levels of extracellular matrix proteins, diminished collagen deposition, and decreased fiber size, muscles display ↓recruitment of endothelial cells, ↓ECM proteins, ↓fiber size, delayed fibrinolysis, ↑collagen deposition in skin wounds, ↑infiltration of macrophages during acute infection.	(Kassim et al., 2007; Orbe et al., 2011; Bobadilla et al., 2014; Rohani et al., 2015; McMahan et al., 2016)
Mmp11$^{-/-}$	Viable, fertile, no behavior differences with wild type littermates.	↑Neointima formation after vascular injury, ↓7,12-dimethylbenzanthracene-induced tumorigenesis, ↑subcutaneous (SC) and gonadal (GON) fat deposits, adipogenesis, adipocyte membrane alteration, ↑dysregulation of metabolism.	(Masson et al., 1998; Lijnen et al., 1999, 2002; Andarawewa et al., 2005; Dali-Youcef et al., 2016)
Mmp12$^{-/-}$	Normal embryonic and postnatal development in the absence of inflammatory stress. Resting hematopoiesis and myelomonocytic development are normal. *Mmp12*$^{-/-}$ mice have a 40% reduction in litter sizes likely due to placental abnormalities.	↓Migration and invasion in macrophages, resistance against cigarette-smoke-induced emphysema, unable to process chemokines and cytokines like IFNα and IFNγ resulting in exacerbation of acute and chronic inflammation, unable to resolve bacterial and virus infections, reduced phagocytosis and hemolysis capacity, dysregulated complement activation, ↓macrophage infiltration in ligament-injury repair, spontaneous deficient myelination of corpus callosum with fewer oligodendrocytes, ↑experimental autoimmune encephalomyelitis severity.	(Shipley et al., 1996; Hautamaki et al., 1997; Weaver et al., 2005; Larsen et al., 2006; Dean et al., 2008; Houghton et al., 2009; Bellac et al., 2014; Marchant et al., 2014; Dufour et al., 2018; Mallia-Milanes et al., 2018)
Mmp13$^{-/-}$	No gross phenotypic abnormalities, fertile, and normal lifespan.	Spontaneous abnormal growth plate and ↑trabecular bone.	(Inada et al., 2004; Stickens et al., 2004)
Mmp14$^{-/-}$/ *Mt1-mmp*$^{-/-}$	Viable but display severe runting, wasting and ↑mortality. Death occurs between 50 and 90 days after birth.	Craniofacial dysmorphism, arthritis, osteopenia, dwarfism, and fibrosis of soft tissues, ↓lung alveolar surface area, retarded lung alveolar development, ↓neovessel formation, poorly differentiated kidney tubular epithelia, lack of molar tooth eruption and root formation, defects in dentin formation and mineralization, ↓metabolic homeostasis, ↓regulation of adipocyte fate determination in the developing mammary gland.	(Holmbeck et al., 1999, 2003; Chun et al., 2004; Koshikawa et al., 2004; Atkinson et al., 2005; Irie et al., 2005; Feinberg et al., 2016; Mori et al., 2016; Xu et al., 2016; Mai et al., 2017)
Mmp15$^{-/-}$/ *Mt2-mmp*$^{-/-}$	Viable, fertile, and live through adulthood.	↑Positive regulators of brown or induced brown (beige) adipocytes production.	(Feinberg et al., 2016)
Mmp16$^{-/-}$/ *Mt3-mmp*$^{-/-}$	Viable, fertile, but displayed retarded growth of the skeleton compared with wild types or heterozygous littermates.	↓Viability of mesenchymal cells in skeletal tissues.	(Shi et al., 2008)

(*continued*)

TABLE 1—*Continued*

MMP	Phenotype of MMP Knockout Mouse	Impact on Biologic Functions	References
Mmp17$^{-/-}$/ *Mt4-mmp*$^{-/-}$	No apparent defects in growth, fertility, and life span.	↑Protection from Il1β-mediated GAG release into synovial fluid in a model of joint inflammation; ↑predisposition to aortic aneurysms; plays a role in embryonic development, brain formation, angiogenesis and limb development.	(Rikimaru et al., 2007; Clements et al., 2011; Martín-Alonso et al., 2015; Blanco et al., 2017)
Mmp19$^{-/-}$	Viable, fertile, and no apparent phenotype under homeostasis.	↑Body weight under high-fat diet, adipocytes hypertrophy, ↓susceptibility to develop skin tumors induced by a model of chemical carcinogens; in a model of contact hypersensitivity, impaired T cell-mediated immune reaction characterized by minimal influx of inflammatory cells, low proliferation of keratinocytes, ↓activated CD8+ T cells in lymph nodes; after an allergen challenge, ↑eosinophilic inflammation and ↑ tenascin protein, ↑lung fibrotic response to bleomycin, ↑protection against hepatic fibrosis, ↑susceptibility to colitis in a dextran sulfate sodium-induced colitis model.	(Pendás et al., 2004; Beck et al., 2008; Gueders et al., 2010; Jirouskova et al., 2012; Yu et al., 2012; Jara et al., 2015; Brauer et al., 2016)
Mmp20$^{-/-}$	Severe tooth defects but mice were viable and had no fertility issues.	Hypomineralization of mantle dentin, defects in processing amelogenin, altered enamel protein, and associated rod pattern.	(Caterina et al., 2002; Beniash et al., 2006)
Mmp21$^{-/-}$	N/A	N/A	N/A
Mmp23$^{-/-}$	N/A	N/A	N/A
Mmp24$^{-/-}$/ *Mt5-mmp*$^{-/-}$	Normal appearance, fertile, and have normal lifespan.	Unable to develop neuropathic pain with mechanical allodynia after a sciatic nerve injury; ↓nerve-fiber sprouting and neural invasion; ↑sensitivity to noxious thermal stimuli under basal conditions; unable to develop thermal hyperalgesia under inflammatory conditions; protected against amyloid pathology, cognitive decline, and inflammation.	(Komori et al., 2004; Folgueras et al., 2009; Baranger et al., 2016)
Mmp25$^{-/-}$/ *Mt6-mmp*$^{-/-}$	Viable, fertile, and no apparent phenotype under homeostasis.	Defective innate immune response via a low sensitivity to bacterial LPS, hypergammaglobulinemia, ↓secretion of proinflammatory molecules, impaired NF-κB activation.	(Soria-Valles et al., 2016)
Mmp26$^{-/-}$	N/A	N/A	N/A
Mmp27$^{-/-}$	N/A	N/A	N/A
Mmp28$^{-/-}$	Viable, fertile, and no apparent phenotype under homeostasis.	↑Macrophage recruitment, ↑macrophage migration, and ↑bacterial clearance into the lung in *Pseudomonas aeruginosa*–treated mice; ↓macrophage polarization, ↓collagen deposition, and few myofibroblasts.	(Manicone et al., 2009, 2017; Ma et al., 2013; Gharib et al., 2014)

capable of modulating the tissue during homeostasis and disease development.

B. Non-ECM MMP Substrates

N-terminomics and proteomics techniques were used to profile hundreds of cleavage sites in proteomes associated with MMP activity and identified numerous MMP substrates unrelated to the ECM (Butler and Overall, 2009; Starr et al., 2012a; Dufour and Overall, 2013, 2015; Bellac et al., 2014; Mallia-Milanes et al., 2018). Data obtained from these techniques have resulted in an expansion and restructuring of our understanding of MMP biology, yet it is still underappreciated in the literature. Bioinformatics tools are essential for handling big data, and multiple programs have been developed to integrate these large datasets, such as MEROPS (Rawlings et al., 2010) and TopFIND (Lange and Overall, 2011; Fortelny et al., 2015). In MEROPS (https://www.ebi.ac.uk/merops/), over 1 million proteases, ~92,000 cleavage sites, and ~6,500 peptidase-inhibitor interactions are included (as of April 2022). In TopFIND (https://topfind.clip.msl.ubc.ca), protein N and C termini, protease substrates, and proteolytic processing are presented from eight different organisms: *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Escherichia coli*, *Caenorhabditis elegans*, and *Danio rerio*. There are hundreds to thousands

of potential MMP substrates, yet only a small number have been extensively validated in vivo, in animal models, or in humans. Apart from ECM and BM substrates, several non-ECM substrates have been identified, including chemokines, cytokines, cell-surface receptors, growth factors, metabolic proteins, and nuclear proteins (for the full list, see Supplemental Table 1) (Cauwe et al., 2007; Butler and Overall, 2009; Cauwe and Opdenakker, 2010; Dufour and Overall, 2013; Chopra et al., 2019; Young et al., 2019).

The tight regulation of MMPs is fundamental to what substrates they cleave, and substrate processing is cell, tissue, and disease dependent. Indeed, changes in the balance between MMP and TIMP ratio have a significant impact on disease progression and resolution of inflammation in both acute and inflammatory conditions. For example, multiple studies have demonstrated a key role of MMPs in regulating the biologic functions of virtually all human chemokines functions by either an activation or inactivation cleavage, resulting in a "go" or "stop" signal via chemokine receptor downstream signaling (McQuibban, 2000; Van den Steen et al., 2003; Dean et al., 2008; Starr et al., 2012b; Young et al., 2019). MMP regulation of chemokines enhances or dampens leukocytes' recruitment to the site of inflammation or pathogen infection. Eight CXCL chemokines have chemoattraction abilities to attract neutrophils, and as an example, two neutrophil MMPs, MMP8 and -9, were demonstrated to cleave and activate interleukin-8 (IL8)/CXCL8 in a feedforward mechanism (Van den Steen et al., 2003; Tester et al., 2007). All of the Glu-Leu-Arg (ELR)+ CXC chemokines, which are proteins able to attract neutrophils to the site of injury or inflammation, are processed by MMP12 released from macrophages, resulting in an inactivation of CXC chemokines signaling (Dean et al., 2008). Other chemokines, such as CCL15 and CCL23, are also processed by multiple MMPs (MMP1, -2, -3, -7, -8, -12, 13, and MT1-MMP), resulting in their activation via a Ca^{2+} increase and an elevation of their chemotactic index (Starr et al., 2012b). MMP1 can cleave the Arg-Ser bond on protease-activated receptor (PAR)1. This substrate is also cleaved by thrombin, and proteolytic processing of PAR promotes its activation, which results, for example, in growth and invasion of breast carcinoma cells (Boire et al., 2005; Nagase et al., 2006). Overall, MMPs can cleave multiple substrates, including ECM and non-ECM related substrates.

C. Intracellular Roles of MMPs

MMPs were first described as secreted proteases capable of cleaving ECM proteins, but we now know that their roles go beyond this initial dogma. A seminal discovery by Dr. Richard Schulz's group in 2002 identified an intracellular form of MMP2 inside cardiac myocytes (Wang et al., 2002). Later work by this group and David Lovett's group demonstrated that there are three intracellular isoforms (splice variants) of MMP2 since its signal peptide inefficiently targets MMP2 to the secretory pathway, thus resulting in ~50% of MMP2 remaining in the cytosol (Ali et al., 2012; Lovett et al., 2012, 2014; Bassiouni et al., 2021). One splice variant of MMP2, identified in the cytosol of cardiomyocytes, lacks 50 amino acids from its N terminus and was termed $MMP2_{NTT50}$ (Ali et al., 2012). Another MMP2 intracellular variant missing 76 amino acids, termed $MMP2_{NTT76}$, was identified after induction of oxidative stress and was shown to activate NF-κB and nuclear factor of activated T cells (NFAT) mitochondrial-nuclear stress signaling (Lovett et al., 2012). MMP3 contains a proline residue that impacts its conformation and could weaken its signal sequence efficiency, resulting in MMP3 localized in the nucleus in human chondrosarcoma-derived chondrocytic HCS-2/8 cells (Eguchi et al., 2008). Additionally, MMP3 was found to be localized in the nucleus of human liver cancer cells (HepG2 cells) and liver myofibroblasts and was demonstrated to induce apoptosis via its catalytic activity (Si-Tayeb et al., 2006) (Fig. 5).

In dopaminergic neurons, intracellular MMP3 was also shown to induce apoptosis (Choi et al., 2008). Further, intracellular MMP10 was also identified within neurons, where it was demonstrated to cleave huntingtin into a toxic fragment, resulting in cell death (Miller et al., 2010). An MMP11 isoform of ~40 kDa was identified inside cancer cells due to an alternative gene promoter (Luo et al., 2002). During viral infection, intracellular MMP12 (in HeLa cells) was demonstrated to mediate NF-κB transcription, resulting in interferon alpha (IFNα) secretion and host protection (Marchant et al., 2014). Nuclear MT1-MMP was shown to modulate inflammation independent of proteolysis via the activation of a phosphoinositide 3-kinase delta (PI3Kδ)/protein kinase B (Akt)/glycogen synthase kinase 3 beta (GSK3β) signaling cascade (Shimizu-Hirota, et al., 2012) (Fig. 5). MMP23 lacks a recognizable signal sequence (Fig. 2) and is therefore suggested to be intracellular, although it has not been validated yet (Velasco et al., 1999). Interestingly, most of the MMP26 produced by cells remains intracellular due to MMP26's unique latency motif (Pro-His-Cys-Gly-Val-Pro-Asp) containing the conserved cysteine that is inactive (Marchenko et al., 2002; Savinov et al., 2006; Strongin, 2006).

Although multiple MMPs have been identified as intracellular proteases, it remains enigmatic as to how some MMPs are first secreted and can reenter the cells at a later stage. Some MMPs have been found to enter the cells by endocytosis, via either clathrin-dependent or clathrin-independent mechanisms (Cauwe and Opdenakker, 2010). Clathrin-dependent mechanisms use cargo proteins that recognize adaptor

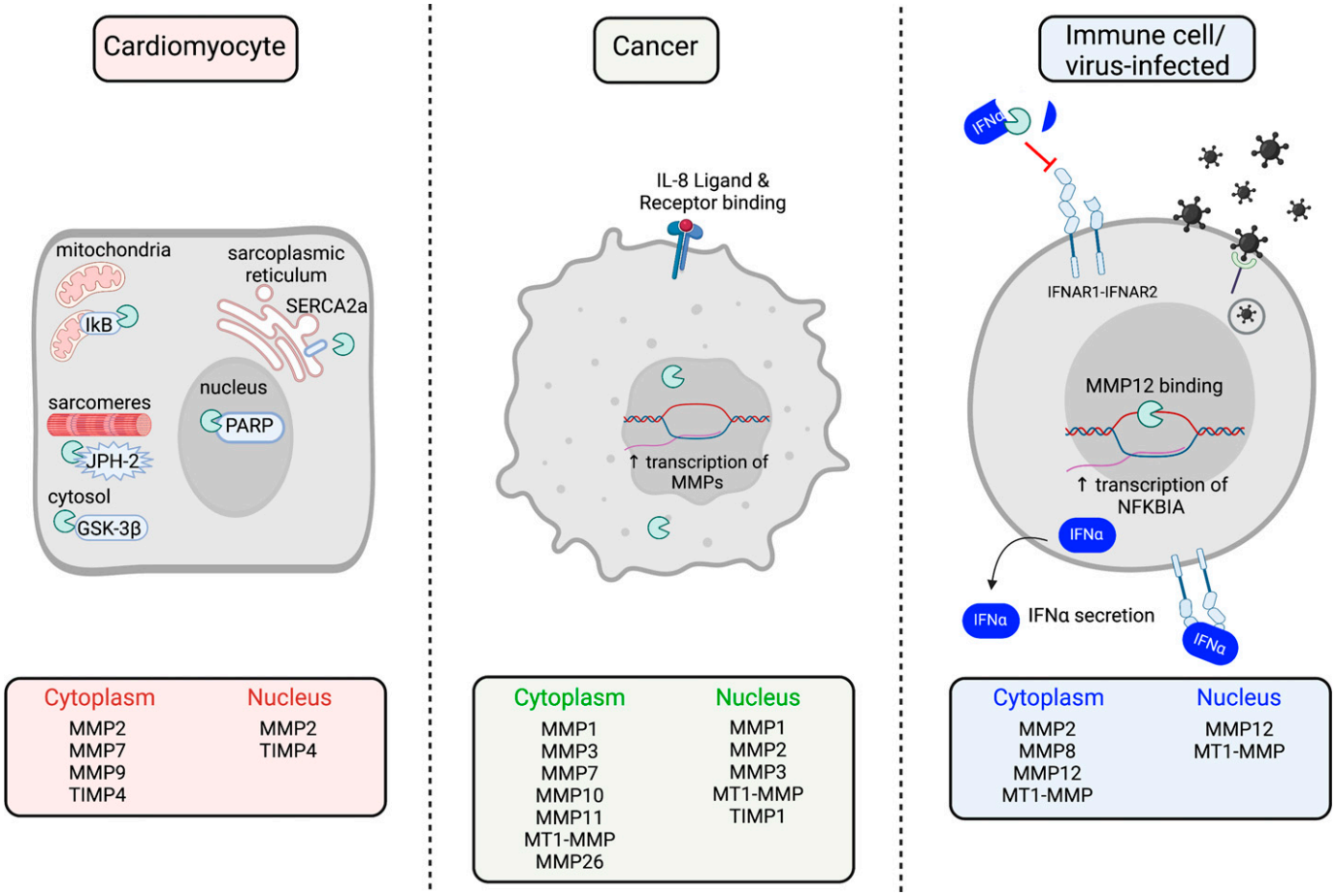

Fig. 5. Intracellular MMPs and localization to the cytoplasm or nucleus in three different cell types. Left: some intracellular MMPs associated with cardiomyocytes. Some examples of known MMP2 interactions within organelles are illustrated. Middle: known intracellular MMPs in cancer cells. One example is the upregulation of MMP2 by IL8-mediated signaling, as observed in melanoma cells. Right: known intracellular MMPs in immune cells or virus-infected cells. An example is illustrated of MMP12 mediated INFα release during viral infection.

proteins, resulting in packaging inside clathrin-coated vesicles subsequently absorbed by the cells (Grant and Donaldson, 2009; Traub, 2009). Clathrin-independent mechanisms are less characterized and function via caveolae or flotillin-dependent manner and can be linked to macropinocytosis and phagocytosis (Swanson, 2008; Grant and Donaldson, 2009). For example, in neurons, MMP7 has been demonstrated to enter the cells via a clathrin-dependent mechanism, implicating the processing of synaptosomal-associated protein of 25 kDa (Szklarczyk et al., 2007). Other MMPs such as MMP2, -9, and -13 have been demonstrated to enter the cells via the low-density lipoprotein-related protein 1 (LRP1) (Yang et al., 2001; Raggatt et al., 2006; Van den Steen et al., 2006). MT1-MMP appears to be internalized via either clathrin-dependent (Jiang et al., 2001) or -independent mechanisms (Remacle et al., 2003). Despite supporting evidence of MMPs entering via these mechanisms, it is still unclear as to how MMPs can exit the endosomes, enter the cytosol, and cleave substrates. Flip-flop mechanisms have been suggested to explain these mechanisms, but further experiments are needed to demonstrate these observations better (Cauwe and Opdenakker, 2010). Additionally, several MMPs contain nuclear localization signals in their sequences, which could potentially explain their entry to the nucleus or, alternatively, could be brought into the nucleus via RNA-binding cargo proteins (Cauwe and Opdenakker, 2010; Xie et al., 2017; Frolova et al., 2020). Further characterization of the intracellular roles of MMPs will likely unravel new substrates and identify novel functions.

D. Oxidative and Nitrosative Stress Activation of MMPs

The production of reactive oxygen species (ROS) is an important mechanism to regulate normal metabolism but is also a key immune response to external and internal stimuli. ROS production includes numerous chemical oxidants such as hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and superoxide anion (O_2•–) (Sies and Jones, 2020; Muri and Kopf, 2021). During phagocytosis, ROS are generated and play important biologic roles. Similarly, free radical nitric oxide (NO•) is an important intra and extracellular

regulatory chemical. When imbalances occur between the levels of O_2•– and NO•, peroxynitrite (ONOO–) can form, resulting in nitrosative stress and increased damage to DNA, proteins, and lipids (Pacher et al., 2007; Brandes et al., 2009). Reactive nitrogen species can result in three different posttranslational modifications: 1) S-nitrosylation, 2) S-glutathiolation, and 3) tyrosine nitration; these can impact the latency-inducing cysteine to Zn^{2+} coordination in MMPs (Ali and Schulz, 2009; Martínez and Andriantsitohaina, 2009).

We now know that MMPs have both extracellular and intracellular functions, as MMPs have been identified in various subcellular compartments, including the cytosol, sarcomere, ER, nucleus, and mitochondria (Bassiouni et al., 2021). However, it is still unclear how the proteolytic activity of intracellular MMPs is regulated. One mechanism that contributes to regulating MMP activity inside the cell is the generation of reactive oxygen and nitrogen moieties (Cauwe and Opdenakker, 2010; Bassiouni et al., 2021). Via the oxidation of their thiol group within the cysteine switch, ROS has been demonstrated to activate MMPs even in the presence of the propeptide (Okamoto et al., 2001; Viappiani et al., 2009). More precisely, peroxynitrite (ONOO–) has been shown to activate some MMPs by cysteinyl S-glutathiolation or S-nitrosylation of cysteines 60 and 102, resulting in a disulfide S-oxide bond, as demonstrated for MMP1, -2, -8, and -9 (Okamoto et al., 2001; Viappiani et al., 2009; Jacob-Ferreira et al., 2013). These types of MMP activation likely occur during an immune response or during stress. For example, the generation of hypochlorous acid from hydrogen peroxide occurs during neutrophil activation via myeloperoxidase activation and has been shown to activate MMP1, -2, -7, -8 and -9 (Weiss et al., 1985; Peppin and Weiss, 1986; Fu et al., 2001; Meli et al., 2003). Another example is oxidative stress, which can occur during myocardial infarction. Specifically, the levels of peroxynitrite increase during myocardial infarction, resulting in MMP2 activation and cardiac troponin I processing (Cheung et al., 2000; Wang et al., 2002). In a feedforward mechanism, inflammatory cytokines have been shown to increase peroxynitrite activating intracellular MMP2 (Ferdinandy et al., 2000; Gao et al., 2003). Another example is the S-nitrosylation of MMP9 in cerebral ischemia, which results in neuronal apoptosis (Gu et al., 2002). Therefore, oxidative and nitrosative stress increases MMP activation and has been linked to pathologic modifications of the biologic functions of MMPs.

IV. Pathophysiology of Matrix Metalloproteinases

A. Cancer

Tumors are heterogeneous, dynamic, and multicellular and closely interact with their microenvironment (Quail and Joyce, 2013; Maley et al., 2017). Dysregulation of signaling networks in cancer impacts metabolism, growth, blood vessel formation, and immune regulation (Maley et al., 2017; Hiam-Galvez et al., 2021). MMPs have been demonstrated to be implicated in most if not all of the dysregulated processes in cancer (Egeblad and Werb, 2002; Piperigkou et al., 2021). For several decades now, MMPs have been studied in the context of cancer progression, mainly for their ability to remodel the BM and to drive the cell invasion program resulting in increased metastasis (Egeblad and Werb, 2002; Overall and Kleifeld, 2006a; Young et al., 2019; Piperigkou et al., 2021). There is a strong relationship between cancer progression, clinical outcome, and upregulation of MMPs; however, what was originally thought about their detrimental roles in cancer has been challenged in the past two decades. The initial concept about MMPs was that broad-spectrum inhibition of their proteolytic activity would reduce ECM remodeling and prevent cell invasion and cancer metastasis. We now know that an increase of a specific MMP does not necessarily imply the promotion of tumor growth or metastasis; at least 10 MMPs have also been demonstrated to have protective roles in cancer (Dufour and Overall, 2013). Therefore, each MMP must be studied carefully, as it likely has cell-, tissue-, or tumor-specific functions.

1. Matrix Remodeling and Cell Invasion. The ECM is a fundamental component of all tissues and organs. Tissue integrity is maintained when there is a sustained equilibrium of ECM turnover (i.e., when ECM production is in balance with its degradation). However, tumors disrupt ECM homeostasis on the biochemical, biologic, and structural levels due to their dynamic nature and dysregulated growth (Cox, 2021). Importantly, the ECM becomes highly dysregulated within tumors and can be both protumorigenic and antitumorigenic. For example, elevated MMP8 expression and activity are associated with good outcomes in oral squamous cell carcinoma and skin cancers (Korpi et al., 2008; Juurikka et al., 2021) but are associated with poor outcomes in ovarian, digestive, and hepatocellular cancers [reviewed by Juurikka et al. (2019); Cox (2021)]. Several MMPs can cleave ECM proteins and selectively release cell surface–bound cytokines, growth factors, and their receptors, thereby impacting overall ECM integrity and tissue turnover [for additional details, see Piperigkou et al. (2021); Shimoda et al. (2021)]. Multiple MMPs have been demonstrated to remodel the ECM, but there is strong evidence that either MT1-MMP, MT2-MMP, or MT3-MMP alone is sufficient to drive ECM transmigration and invasion and could be the predominant MMPs driving ECM remodeling (Rowe and Weiss, 2008, 2009). However, only the *Mt1-mmp*$^{-/-}$ mouse suffers from severe craniofacial dysmorphism, arthritis, osteopenia, dwarfism, and fibrosis of soft tissues associated with type I collagen remodeling deficiency, which results in death within 50–90 days

(Table 1). *Mt2-mmp*$^{-/-}$ and *Mt3-mmp*$^{-/-}$ mice are viable and live through adulthood with minimal defects, suggesting that ECM remodeling during development is likely MT1-MMP dependent (Holmbeck et al., 2004). Furthermore, MT1-MMP, but not MT2-MMP or other MMPs, was demonstrated to be the dominant MMP impacting both branching morphogenesis and carcinoma cell invasion (Hotary et al., 2003; Feinberg et al., 2018).

2. Angiogenesis. Tumors require an adequate blood supply for their high demands of glucose and nutrients; therefore, angiogenesis, the creation of new blood vessels from preexisting vascular networks, is a key process in cancer progression (De Palma et al., 2017). Hypoxia, the low availability of oxygen, is a key aspect of tumor angiogenesis, where hypoxic cancer cells increase their secretion of multiple growth factors, including VEGFA, to increase vessel formation and reach the appropriate oxygen supply (Potente et al., 2011). MMPs have also been closely implicated in regulating angiogenesis in most cancers, and broad-spectrum inhibition using batimastat (BB-94; Fig. 6) resulted in inhibition of the angiogenic switch in premalignant lesions (Bergers, 1999). Sprouting angiogenesis involves ECM remodeling by MMPs and other proteases produced by activated endothelial and immune cells (De Palma et al., 2017).

In tumors, MMPs often impact the vascular BM, which can be irregular, discontinuous, and loosely associated with endothelial cells and pericytes, resulting in an elevation of vascular leakiness and thus an elevation in cancer cell intravasation and metastasis (Egeblad and Werb, 2002; De Palma et al., 2017; Piperigkou et al., 2021). For example, in a pancreatic neuroendocrine tumor, elevated MMP9 cleaves and releases VEGFA from the matrix, resulting in a switch between vascular quiescence to active angiogenesis initiation (Bergers et al., 2000). There is evidence that multiple MMPs (MMP1, -3, -7, -9, -14, -16, and -19) can cleave and regulate VEGF bioavailability and vascularity in cancer (Piperigkou et al., 2021). Processing of ECM components such as collagen IV, XVIII, and perlecan by various MMPs (MMP1, -2, -3, -9, or -13) can initiate the production of antiangiogenic products like tumstatin, endostatin, angiostatin, and endorepellin (Iozzo et al., 2009). Despite multiple MMPs having important biologic functions in the regulation of angiogenesis, individual MMPs also have tissue-, cell-, and tumor-specific functions. For example, Littlepage et al. (2010) demonstrated that MMP2, -7, and -9 have distinct roles in cancer progression and angiogenesis. The authors used a rodent model of spontaneous prostate cancer and metastasis, where transgenic mice express SV40 large T antigen in their prostatic neuroendocrine cells under the control of transcriptional regulatory

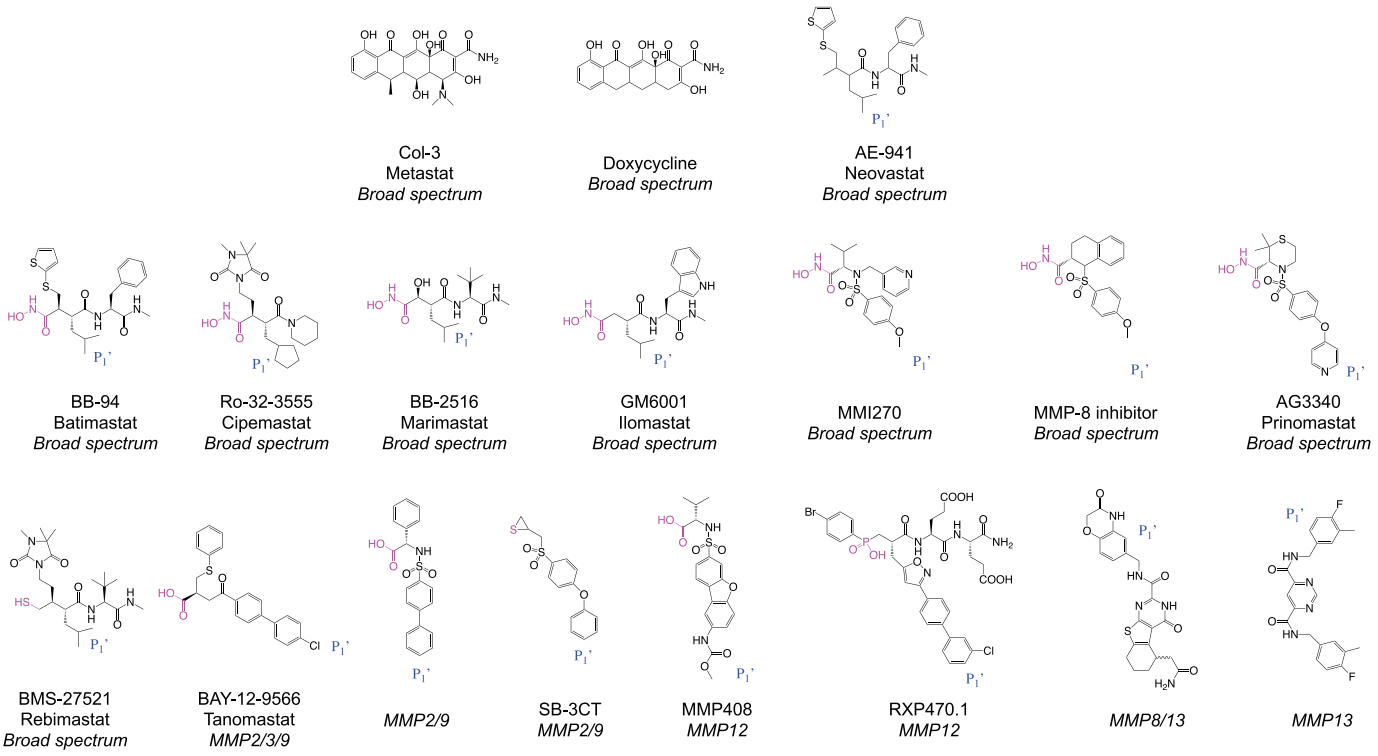

Fig. 6. Structure of MMP inhibitors. The chemical structure of some representative MMP inhibitors from natural sources (row 1), small synthetic hydroxamate-based MMP inhibitors (row 2), and other types of inhibitors with different zinc-binding moieties (row 3): Col-3/metastat, AE-941/neovastat, BB-94/batimastat, BMS-275291/rabimastat, Ro-32-3555/cipemastat, BB-2516/marimastat, GM6001/ilomastat, MMI270/CGS27023A, MMP8 inhibitor, AG3340/prinomastat, BAY 12-9566/tanomastat, MMP408, RXP470.1, SB-3CT, MMP8/13, and MMP13 inhibitor. Key: when known, the zinc-binding moiety is colored in magenta within the inhibitor structure. P_1' position and P_2'/P_3' positions are identified in blue and green, respectively.

elements from the mouse cryptdin-2 gene [Cryptdin-2-T Antigen (CR2-TAg)] (Littlepage et al., 2010). Evaluated after 24 weeks using this model, *Mmp2*$^{-/-}$/*CR2-Tag* mice had reduced tumor burden with fewer lung metastases, increased survival, and decreased blood vessel density (Littlepage et al., 2010). Interestingly, the survival and tumor growth of *Mmp7*$^{-/-}$/*CR2-Tag* or *Mmp9*$^{-/-}$/*CR2-Tag* mice were not impacted, but the *Mmp7*$^{-/-}$/*CR2-Tag* had reduced endothelial area coverage with decreased vessel size and *Mmp9*$^{-/-}$/*CR2-Tag* mice had decreased tumor blood vessel size (Littlepage et al., 2010). In another model, MMP9 was demonstrated to release soluble Kit ligand, resulting in the transfer of endothelial and hematopoietic stem cells from a quiescent to proliferative niche and enabling the bone marrow repopulating cells to a differentiating vascular niche (Heissig et al., 2002). These studies illustrate the heterogeneity of the roles that MMPs exert on tumor angiogenesis.

3. Apoptosis. Evading programmed cell death is key to the survival of cancer cells. Apoptosis can be initiated via extracellular receptors such as the FAS receptors, resulting in a proteolytic cascade by intracellular caspases as well as degradation of additional substrates and nuclear DNA (Kessenbrock et al., 2010; Young et al., 2019). MMPs have been demonstrated to interfere with the induction of apoptosis in cancer cells. For example, MMP7 was shown to cleave Fas ligand in a doxorubicin-induced cell death assay, using SK-N-MC and SW-480 cancer cell lines, and MMP7 had a protective effect by reducing cell death (Mitsiades et al., 2001). In a mouse model of pancreatic acinar-to-ductal metaplasia, MMP7 was demonstrated to accumulate during the metaplastic transition, increasing soluble Fas ligand (Crawford et al., 2002). Additionally, *Mmp7*$^{-/-}$ mice or mice carrying an inactive *FasL* gene demonstrated a significant defect in the development of progressive metaplasia and acinar cell apoptosis (Crawford et al., 2002). When MDA-MD-231 and MDA-MB-435 human breast cancer cells were treated with sodium phenylacetate in the presence of ilomastat (GM6001), a broad-spectrum MMP inhibitor (Fig. 6), apoptosis decreased (Augustin et al., 2009). Under similar conditions, the induction of autophagic vacuoles was also inhibited in MDA-MB-231 cells (Augustin et al., 2009). In patients with B cell chronic lymphocytic leukemia (B-CLL), the interaction of proMMP9 via its hemopexin domain to $\alpha_4\beta_1$ integrin and a 190-kDa variant of cluster of differentiation 44 (CD44v) resulted in an induction of Lyn and signal transducer and activator of transcription 3 (STAT3) phosphorylation signaling as well as prevention of apoptosis (Redondo-Muñoz et al., 2010). Interestingly, the nuclear localization of various MMPs (MMP2, -9, -13) was demonstrated to induce cell death; however, no precise mechanism has yet been demonstrated (Xie et al., 2017). Overall, MMPs are likely implicated in the regulation or inhibition of cell death, but it is still unclear what the precise roles of each MMP are in these processes.

4. Cell Migration. Cancer cells have been demonstrated to have increased motility and migratory capacity over noncancerous cells (Paul et al., 2017). In B-CLL cells, upregulation of MMP9 by the chemokine CXCL12 was demonstrated to enhance cell invasion and transendothelial migration via interaction with $\alpha_4\beta_1$ integrin (Redondo-Muñoz et al., 2006). The chemokine CCL21 was also demonstrated to upregulate MMP9 and enhance cell migration of B-CLL cells via c-c chemokine receptor type 7 (CCR7) (Redondo-Muñoz et al., 2008a). Furthermore, the binding of proMMP9 to $\alpha_4\beta_1$ integrin inhibited B-CLL cell migration, and the proteolytic activity of MMP9 was required; importantly, this effect was not observed in normal B cells (Redondo-Muñoz et al., 2008b). When noncancerous COS-1 cells were transfected with the cDNA encoding for various MMPs, their migration was enhanced, and this was independent of the MMP inhibitor CT1746 or the addition of TIMP1 or TIMP2 (Dufour et al., 2008). This enhancement of cell migration by MMP9 and MT1-MMP was associated with their hemopexin domains and signaling via CD44 and epidermal growth factor receptor (EGFR) (Dufour et al., 2010; Zarrabi et al., 2011). Interestingly, for MMP9, mutations in the outer amino acid sequences of blade I and IV of its hemopexin domain resulted in a reduction of cell migration using uncoated membranes in a transwell chamber assay (Dufour et al., 2010). Using inhibitory peptide sequences mimicking blade I (^{548}SRPQGPFL^{555}) and blade IV (^{689}NQVDQVGY^{696}) of the MMP9 hemopexin domain, HT-1080 and MDA-MB-435 cancer cells were inhibited in a dose-dependent manner (Dufour et al., 2010). Using a similar approach for MT1-MMP and using peptides sequences mimicking blade I (^{349}VMDGYPMP^{356}) and blade IV (^{496}GYPKSALR^{503}), the migration of HT-1080 cancer cells was significantly inhibited (Zarrabi et al., 2011). Interestingly, in a xenograft mouse model using the MDA-MB-435 GFP-labeled cancer cells that can spontaneously metastasize to the lungs, 20 mg/kg of blade I and IV mimicking peptides injected intraperitoneally six times per week significantly reduced the number and size of lung metastases but without any effect on tumor growth (Zarrabi et al., 2011). Additionally, both peptides inhibited new blood vessel formations in a chicken chorioallantoic membrane (CAM) angiogenesis assay (Zarrabi et al., 2011). After a molecular docking study, small molecule inhibitors for MMP9 (Dufour et al., 2011; Das et al., 2020) and MT1-MMP (Remacle et al., 2012) were identified and demonstrated to inhibit cell migration.

There is unequivocal evidence that MMPs are implicated in most if not all aspects of cancer progression and metastasis (Overall and López-Otín, 2002; Kessenbrock et al., 2010; Dufour and Overall, 2013; Das et al., 2020; Piperigkou et al., 2021). However, it is still challenging to determine which role each MMP plays, their synergies with other MMPs, other

proteases, which substrates are cleaved leading to activation or inactivation, and, most importantly, what the net balance is between MMPs' detrimental and beneficial roles in tumors (Overall and Kleifeld, 2006b; Sela-Passwell et al., 2011b; Dufour and Overall, 2013; Young et al., 2019). Therefore, despite over 30 years of investigating the use of MMP inhibitors for the treatment of various cancers, no broad-spectrum or selective MMP inhibitors have yet been approved.

B. Gut and Joint Inflammatory Diseases

The gastrointestinal (GI) tract, encompassing the organs from mouth to anus, breaks down food, absorbs nutrients, and eliminates waste. When homeostasis is lost in the GI tract, there is often an inflammatory response that develops and, if unresolved, can further hinder the gut's ability to perform its functions. Similarly, the joints and musculoskeletal components are susceptible to loss of function once chronic inflammation is initiated or established. Immune cells can secrete proteases and inflammatory components that ultimately result in cartilage and bone destruction. Similar destructive outcomes occur in the GI tract, and loss of epithelial lining results in a leaky gut. The inflammatory pathways that govern gut and joint diseases share common features, including a prevalence of type 3 immunity. Although its very nature remains imprecise, the gut-joint link is evident, with studies being published as early as 1958 (Wilkinson and Bywaters, 1958).

Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions that affect the GI, and they include two distinct manifestations: Crohn's disease (CD) and ulcerative colitis (UC) (Ng et al., 2017). CD and UC differ in the affected parts of the GI, pathophysiology, symptoms, and disease course but are centered on a strong inflammatory component that drives both diseases (Drewes et al., 2020). CD can involve any part of the GI tract, and it is characterized by lesioned areas interposed between normal-appearing mucosa, affecting multiple layers of the tissue and leading to chronic pain, obstructions, and intestinal complications such as fibrotic strictures and fistulae (Drewes et al., 2020). On the other hand, UC involves GFP labeled cancer cells the rectum and colon, with lesions present in a continuous manner and an inflammatory process that is limited to the mucosal surface, leading to erosions and ulcers.

The global prevalence of IBD has been growing for the past 20 years, with Western countries such as Canada projecting an increase of 33.4% from 2015 to 2025 (Coward et al., 2015). Importantly, the disease etiology remains elusive, but numerous studies have proposed MMPs as risk factors for IBD development and progression via proteolytic regulation or modulation of transcription factors (Dufour and Overall, 2015; O'Sullivan et al., 2015). MMPs are upregulated after cell-cell and cell-ECM interactions or as a response mechanism to proinflammatory cytokines widely expressed in IBD (Hu et al., 2007; O'Sullivan et al., 2015). MMPs are produced by multiple cell types, including leukocytes, mesenchymal cells, and epithelial cells. For example, myofibroblasts can produce MMP1, -2, -3, and -9, whereas infiltrating neutrophils and macrophages are an important source of MMP8, -9, -10, and -12 (Andoh et al., 2007; Yoo et al., 2011; Drygiannakis et al., 2013) (Fig. 7). Ulcerated and inflamed colon regions from patients with UC show an increase in MMP1 (Wang and Mao, 2007) and MMP9 (Lakatos et al., 2012), correlating with the inflammation severity. Although the exact mechanism initiating IBD is not yet known, multiple studies point toward the involvement of gut microbes. Moreover, MMPs cleave proteins and peptides that actively control the microbiota. For example, MMP7 can activate procryptdins into their active form, cryptdins, increasing their antimicrobial activity (Wilson, 1999; Weeks et al., 2006) (Fig. 7).

MMPs play a role in the modulation of IBD pathogenesis. Cytokines involved in the inflammatory process developed in the intestine are capable of increasing MMP levels. For example, TNFα and bradykinin can induce MMP3 expression via the signaling cascade containing PKC, PKD1, and MEK (Yoo et al., 2011). Interleukin 17A (IL17A) and IL17F can upregulate the expression of MMP1 and -3 via myofibroblast, with IL17A presenting more potent effects than IL17F (Fig. 7). When combined with IL1β and TNFα, both IL17 cytokines augmented the expression of MMP1 and -3 (Yagi et al., 2007). In UC, TNFα induces expression of MMP1, which damages the colon by remodeling the ECM and leads to additional expression of TNFα in a feedforward mechanism, ultimately culminating in excessive mucosal damage (Wang and Mao, 2007) (Fig. 7). TNFα is also dependent on proteolytic activity since it is synthesized as a membrane-anchored precursor (pro-TNFα) and requires proteolytic processing to release its soluble 17-kDa extracellular domain. The pro-TNFα cleavage is predominantly executed by the protease ADAM17, a member of the larger group of metzincin proteases (Horiuchi et al., 2007) (Fig. 7). Interestingly, studies also indicate protective roles for MMPs in IBD, as Mmp2−/− mice have a severe inflammatory response and higher susceptibility to disease development in a model of mucosal inflammation (Garg et al., 2006) (Fig. 7). Therefore, additional investigation is required to better characterize the protective functions of MMPs in IBD.

The study of MMPs in IBD is not limited to the intestine. A recent study by Majster et al. (2020) demonstrated an elevation of IL6 and MMP10 levels in the saliva of patients with IBD, which correlated positively with the protease expression in the serum. The identification of MMP expression in easily accessible

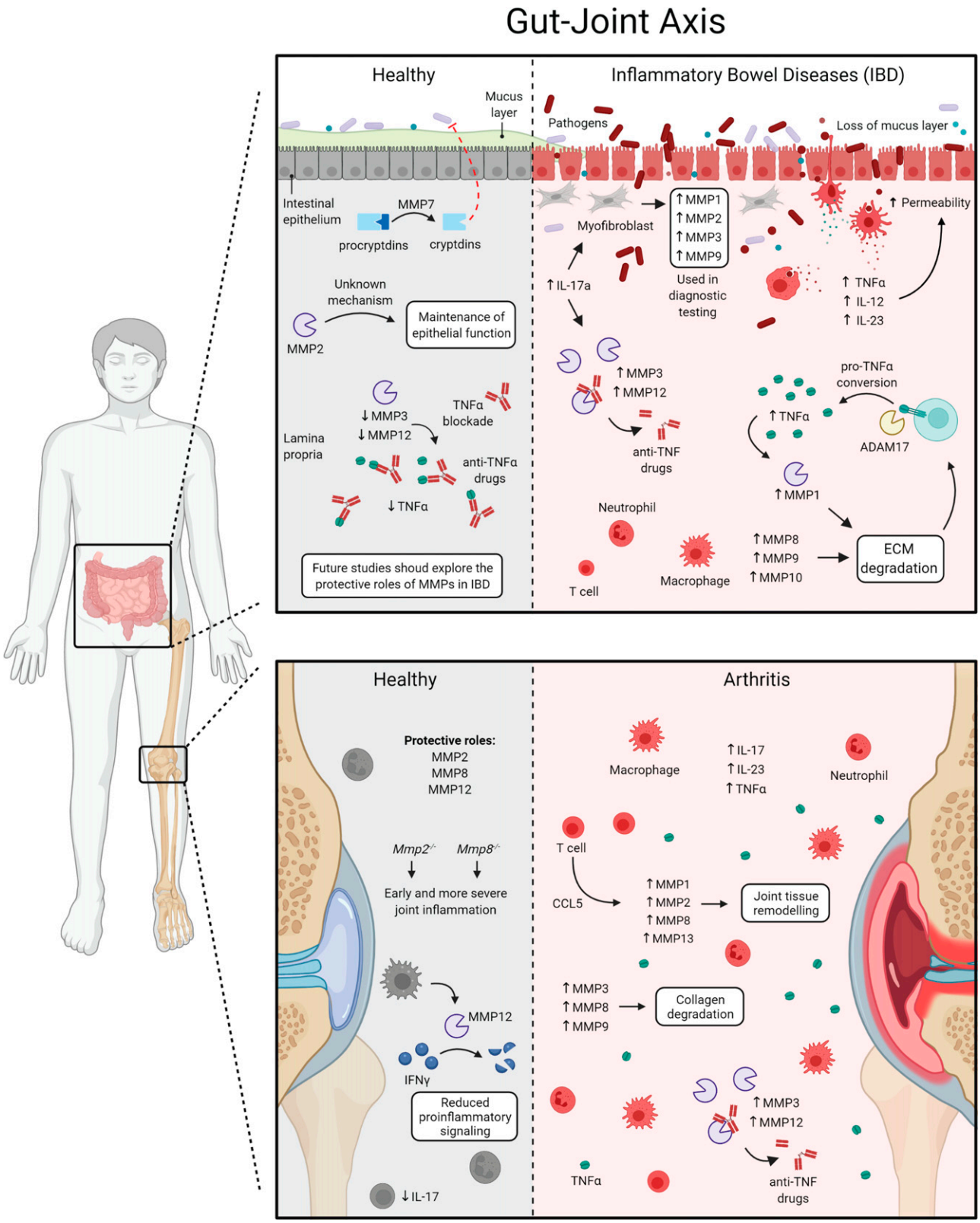

Fig. 7. Matrix metalloproteinase changes in inflammatory bowel disease (IBD) and arthritis. In healthy gut tissue (top left panel), homeostasis is established with a balance of proinflammatory and anti-inflammatory signals. The local immune response can be modulated by multiple factors, including the microbiota. Proteases such as MMP7 can shape the microbiota and gut immunity by aiding the release of antimicrobial peptides. Reduction of proinflammatory signals lowers the expression of MMP3 and -12, setting the stage for successful application of anti-TNFα monoclonal antibodies. MMPs also perform protective roles, as the absence of MMP2 results in gut inflammation, although the exact mechanism remains to be explored. In IBD (top right panel), multiple MMPs are upregulated. For example, increased expression of MMP8, -9, and -10 results in tissue damage, generating damage-associated molecular patterns (DAMPs) that promote further expression of additional MMPs and proinflammatory signals such as TNFα. MMPs are also capable of cleaving anti-TNFα drugs, reducing treatment efficacy. High expression of certain MMPs has been used in diagnostic tests

fluids such as saliva and serum could be exploited to generate diagnostic tools, especially when a significant correlation with disease activity is observed. Currently, there is no single biomarker able to diagnose IBD with high accuracy and sensitivity. However, a panel of 13 blood biomarkers for patients with CD has been developed to improve diagnosis (D'Haens et al., 2020). The panel was evaluated in two cohorts, showing two distinct values, 0.962 and 0.693, for the area under receiver operating characteristic curve (D'Haens et al., 2020). The 13 biomarkers included MMP1, -2, -3, -9, and extracellular matrix metalloproteinase inducer (EMMPRIN) (Fig. 7). Although not yet approved by the US Food and Drug Administration (FDA), the test is commercially available as PROMETHEUS Monitr Crohn's Disease by Prometheus Laboratories.

One of the challenges that patients with IBD face is the development of extraintestinal manifestations, with a prevalence ranging from 6% to 47% (Greuter and Vavricka, 2019). The most common form of extraintestinal manifestations is arthritis present as peripheral arthritis and as spondyloarthritis (SpA) (Greuter and Vavricka, 2019). SpA is a group of inflammatory diseases, including ankylosing spondylitis, psoriatic arthritis, and reactive arthritis (Gracey et al., 2020). Although the precise mechanism is not yet understood, the gut-joint axis is evident, which can be exemplified by type 3 immunity, responsible for governing the immune response in the gut and joint tissue via IL17 and related cytokines (Greuter and Vavricka, 2019). SpA pathogenesis is marked by innate and adaptive immune cells infiltrating the joints, promoting chronic inflammation and tissue remodeling. In SpA, the degradation of collagens appears to be implicated, potentially associated with MMP3, -8, and -9 proteolysis (Moz et al., 2017). MMP3 is elevated in patients with ankylosing spondylitis compared with healthy controls, and it correlates with disease activity and C-reactive protein (CRP) levels, a broad marker of inflammation. Less is known about MMP8 and -9 in SpA, but they also appear to be associated with disease activity (Mattey et al., 2012).

In addition to SpA, studies have suggested an increased risk for the development of rheumatoid arthritis (RA) in patients with IBD. Although the gut-joint axis has already been established, the link between RA and IBD remains debatable. In a systematic review and meta-analysis performed to determine if patients with IBD have a higher risk of developing RA, eight studies were included and revealed a significantly higher risk of RA in patients with IBD (relative risk = 2.59; 95% confidence interval: 1.93–3.48) (Chen et al., 2020). RA is a chronic inflammatory disease that has similarities to IBD and SpA but is generally characterized by the rheumatoid factor, which are autoantibodies to immunoglobulin G (anti-IgG) and anti-citrullinated protein antibodies (Steiner, 2007; Smolen et al., 2018; Zaiss et al., 2021). Importantly, the joint damage promoted by RA can lead to irreversible disability (Zaiss et al., 2021). MMPs contribute to tissue damage in RA and are present starting at the early stages, as evidenced by the high levels identified within the first week after the onset of RA symptoms (Fang et al., 2020). One of the key features in the synovium of patients with RA is the expansion of the intimal lining due to the production of a large number of cytokines and proteases by synoviocytes (Hu et al., 2007; Hueber and McInnes, 2009). MMP1, -8, -13, and MT1-MMP are responsible for the proteolytic cleavage of important tissue components that surround the joint, whereas MMP9 can further degrade type II collagen fragments, generating important immunodominant epitopes (Vankemmelbeke et al., 1998; Van den Steen et al., 2002; Van den Steen et al., 2004; Dufour, 2015). Using monolayers and three-dimensional human RA synovial fibroblasts micromasses, RANTES/CCL5 was demonstrated to induce MMP1 and -13 expression in a dose-dependent manner and the effect was reversed via inhibition of CCL5 (Agere et al., 2017). Although MMPs are commonly associated with ECM degradation and tissue destruction, it is important to note that some MMPs such as MMP2, -8, and -12 have protective roles (Cox et al., 2010; Bellac et al., 2014; Dufour, 2015). For example, MMP12 secreted by macrophages can cleave interferon gamma (IFNγ) at the C terminus (135Glu↓Leu136), and the truncated product of IFNγ loses the region that interacts with the IFN receptor 1 and fails to initiate the proinflammatory signaling pathway (Dufour et al., 2018).

The inflammatory nature of IBD and various forms of arthritis translate to common or overlapping avenues of treatment. For example, therapies targeting TNFα have revolutionized the treatment of IBD, SpA, and RA (Smolen et al., 2018; Friedrich et al., 2019; Gracey et al., 2020). One common approach is the use of anti-TNFα monoclonal antibodies (i.e., infliximab, adalimumab, golimumab, etanercept) that block the cytokine's functions. These inhibitors present different modes of action, as TNFα is not limited to proinflammatory

for Crohn's disease, as the protease levels and cytokines are readily detectable in the blood. The systemic nature of IBD correlates with joint inflammation, where increased expression of multiple MMPs is also observed (lower right panel). High expression of MMPs results in tissue damage and a feed-forward mechanism for further joint inflammation. Remarkably, MMPs also have protective roles, as exemplified by animal models lacking *Mmp2* and *Mmp8* (lower left panel). Lastly, MMP12 can control joint inflammation via IFNγ cleavage, generating a product incapable of promoting downstream signaling of proinflammatory signals.

functions and can modulate cell migration, proliferation, and death (Levin et al., 2016). Unfortunately, the response rate for anti-TNFα therapy remains low to moderate, and ∼40% of patients with IBD present primary or secondary nonresponsiveness to the therapy (Friedrich et al., 2019). Patients with SpA have a higher response rate, with up to 60% of patients responding to the therapy (Gracey et al., 2020). The response rate of patients with RA can be as high as 39% when golimumab is combined with methotrexate (Smolen et al., 2018). Interestingly, MMPs have been linked to the lack of responsiveness toward monoclonal antibody therapy. When tissue homogenates from patients with IBD were incubated with anti-TNFα monoclonal antibodies, MMP3 and -12 were shown to cleave infliximab, adalimumab, and etanercept (Biancheri et al., 2015). Of note, a higher proportion of cleaved monoclonal antibodies is observed in nonresponders to anti-TNFα therapy compared with patients who responded better, which could be partially explained by the higher amounts of MMP3 and -12 that are produced as a response to IL17A, a major player in the gut and joint inflammatory processes (Biancheri et al., 2015). Collectively, these studies show that MMPs have important functions in IBD and arthritis, as they influence disease progression and response to therapy with functions that go beyond the digestion of ECM proteins.

C. Vascular Diseases

Angiogenesis plays a role in several biologic processes, including tumorigenesis, organ regeneration, wound healing, and normal development (De Palma et al., 2017). As MMPs have been demonstrated to regulate angiogenesis, it is not surprising that MMPs play critical roles in vascular diseases such as atherosclerosis, myocardial infarction, hypertension, ischemia, and strokes (Hu et al., 2007). Increased MMP1 levels in the heart were linked to a disruption of structural collagen and associated with cardiomyopathy (Kim et al., 2000). In mice, overexpression of MMP1 in cardiac myocytes showed a decrease in cardiac interstitial collagen along with noticeable systolic and diastolic dysfunction (Kim et al., 2000). Upregulation of circulating MMP2 has been associated with left ventricular remodeling after myocardial infarctions and poor prognostic outcomes (George et al., 2005; Matsunaga et al., 2005). In mice, overexpression of MMP2 in hearts resulted in several physiologic changes that are common with left ventricular remodeling, cardiac mitochondrial disruption, systolic heart failure, myocyte hypertrophy, and troponin I proteolysis (Bergman et al., 2007). Interestingly, an intracellular splice variant of MMP2 has been identified and implicated in the development of diabetic cardiomyopathy (Lee et al., 2019; Bassiouni et al., 2021). This MMP2 splice is missing the 76 amino acids at its N terminus, which includes part of the signal peptide (MMP2-NTT76) and has been observed in cardiomyocytes (Ali et al., 2012; DeCoux et al., 2014; Baghirova et al., 2016; Bassiouni et al., 2021). MMP2 is localized at the sarcomere, whereas MMP2-NTT76 is localized in the mitochondria and in the nucleus (Bassiouni et al., 2021) (Fig. 5). In a rat model of diabetic cardiomyopathy, treatment with doxycycline resulted in a reduction of action potential duration, calcium handling, and cardiac contractile force, suggesting that MMP2-NTT76 could be a contributing factor (Yaras et al., 2008).

After cardiovascular surgery, patients' arteries can develop scar tissue called neointima, leading to a decrease in vascular elasticity and eventual vessel obstruction (Silvestre-Roig et al., 2020). Neointima formation is often caused by the proliferation and migration of smooth muscle cells from the middle layer (tunica media) to the inner layer of the blood vessel (tunica intima), leading to scar tissue (Silvestre-Roig et al., 2020). This scar tissue can lead to a lack of elasticity of the vascular tissue, which can lead to eventual plaque rupture that can obstruct smaller downstream blood vessels. This rupture of atherosclerotic plaques is the leading cause of coronary arterial thrombi in humans, and MMPs have been implicated in this process (Libby and Aikawa, 2002). There are different kinds of plaques that can develop: stable plaques that contain a thick fibrous cap that prevents the lipid core of the plaque from encountering the blood or vulnerable plaques that contain a thick fibrous cap between the plaque and the blood (Fitridge and Thompson, 2012). As the name implies, vulnerable plaques are more likely to rupture and trigger myocardial infarction. Macrophage MMPs have been proposed to be a driving mechanism of this process (Fitridge and Thompson, 2012). In a mouse model of atherosclerosis, treatment with RXP470.1 (Fig. 6), a selective MMP12 inhibitor, significantly reduced atherosclerotic plaque cross-sectional area, by 2-fold (Johnson et al., 2011). In the same model, MMP13 was associated with the rupture of plaques via the degradation of the fibrous cap, as Mmp13−/−/apoE−/− mice displayed reduced collagenolytic activity within their lesions (Deguchi et al., 2005; Quillard et al., 2014). MMP2 and -9 have been demonstrated to have a key role in neointima formation, and inhibition by broad-spectrum MMPs inhibitors (ilomastat or marimastat; Fig. 6) have been shown to reduce arterial constrictive remodeling (Sluijter et al., 2006). Interestingly, Timp1−/− mice have an increased risk for vascular scar tissue (neointima) formation (Lijnen et al., 1999). Mmp11−/− mice have an increase in neointima formation rather than a decrease, suggesting that individual MMP could play distinct roles in angiogenesis (Lijnen et al., 2002). Given the complex role of MMPs in angiogenesis and vascular diseases, the role of individual

MMPs warrants further research and could lead to the identification of new drug targets.

D. Central Nervous System Diseases

MMPs are typically absent from a healthy central nervous system (CNS), and their upregulation may be linked with inflammation, neurologic disorders, or injury (Yong et al., 2001). For example, MMP9 was undetectable in the cerebrospinal fluid (CSF) of healthy individuals but elevated in multiple sclerosis (MS) and other inflammatory neurologic pathologies (Gijbels et al., 1992). During the pathogenesis of several CNS diseases, including multiple sclerosis (MS), bacterial meningitis, gliomas, stroke, and multiple others, CNS resident cells and invading immune cells secrete MMPs that drive blood-brain barrier (BBB) breakdown, permit leukocyte recruitment, and modulate cytokine and chemokine signaling (Leppert et al., 2000; Yong et al., 2001; Meli et al., 2003). Accordingly, many studies have suggested that inhibition of MMPs is neuroprotective, although it has been hard to rule out that some MMPs could limit neuroinflammation or promote tissue repair (Chopra et al., 2019). For example, MMP9 is involved in the remyelination process after a demyelinating injury. In *Mmp9*$^{-/-}$ mice, accumulation of an inhibitory proteoglycan known as NG2 impairs remyelination by interfering with oligodendrocyte precursor cell maturation and differentiation. MMP9 degrades NG2 deposits to facilitate the remyelination and repair response postinjury (Larsen et al., 2003). Additionally, multiple studies suggest that MMP2, -7, and -9 remodel lesioned ischemic and infarct tissue to facilitate angiogenesis, vasculogenesis, and neurogenesis during poststroke recovery (Rempe et al., 2016).

In the postmortem analyses of the brains of patients with MS, elevated levels of MMP2, -3, -7, and -9 were identified as compared with non-MS brains (Cuzner et al., 1996; Anthony et al., 1997). In a rodent model of MS, rats with autoimmune encephalomyelitis (EAE) exhibited increased expression of MMP7, -9, and -12 (Clements et al., 1997). Despite contrasting roles of various MMPs in EAE rodent models and human MS, broad-spectrum MMP inhibitors (GM6001, Ro-9790, or BB1101) alleviate or prevent EAE in rodents (Chandler et al., 1997; Yong et al., 1998). By digesting ECM, increasing the availability of growth factors (Zhao et al., 2006), and aiding the migration of neuronal precursor cells to areas damaged by stroke, MMPs may support tissue remodeling and healing (Lee et al., 2006). Beyond their role in neurodegeneration and stroke, genetic polymorphisms of MMPs (particularly MMP9) have been implicated in neurologic disorders such as schizophrenia, depression, and other neuropsychiatric conditions (Beroun et al., 2019; El Mouhawess et al., 2020). Various mechanisms have been proposed for the involvement of MMPs in neuroinflammatory diseases. When injected into the CNS, MMPs have been demonstrated to induce demyelination; some of the MMP cleavage products of myelin basic protein (MBP) have been shown to induce rodent EAE (Opdenakker and Van Damme, 1994; Chandler et al., 1997). Human MMP9 can also cleave MBP, and one of these peptide fragments is an immunodominant epitope in MS (Opdenakker and Van Damme, 1994).

White matter damage is a major complication in acute and chronic stroke, and MMPs have been demonstrated to play various roles associated with these injuries (Yang and Rosenberg, 2015). MMPs play a role in the acute injury that may lead to the widespread activation of other MMPs that persist in chronic stroke. In chronic injury, secondary damage to the white matter can occur, and MMPs that are expressed within the infiltrated leukocytes and blood vessels may result in disruption of the BBB and the release of toxic byproducts that spread through the white matter (Yang and Rosenberg, 2015). More precisely, in the first phases after stroke, MMPs have been linked to an elevation of cerebral injury, as MMP-mediated alterations often increase BBB leakage, cerebral edema, hemorrhage, and leukocyte infiltration (Yang and Rosenberg, 2015). MMPs have also been associated with damage to myelinated fibers (Rosenberg and Yang, 2007). Although MMPs can be detrimental during the early ischemic phase, they can be beneficial during the recovery phase by digesting the ECM, increasing the availability of growth factors, and aiding the migration of neuronal precursor cells to areas damaged by stroke. (Yong et al., 2001; Rempe et al., 2016; Chopra et al., 2019). Overall, depending on the stage of MS and the timing of poststroke or postneuronal injuries, the role of MMPs can be drastically different.

1. CNS Development and Stem Cell Niche. From the early embryonic stages, MMPs play a role in the structural and functional changes during the development of the CNS. The neural stem cell niche is crucial in providing new neural cells such as the glia and neurons in the postnatal brain (Shan et al., 2018). The ECM architecture and microenvironment directly influence the fate of neural stem cells and impact their regenerative potential. Regulation and remodeling of the ECM are key processes in the development and maturation of stem cell populations during neurogenesis. Both intracellular and extracellular MMP12 have been demonstrated to regulate the generation of the brain's early postnatal ventricular-subventricular zone via ECM remodeling and inactivation of protease inhibitors (Shan et al., 2018). Increased MMP12 expression was correlated with the development of ependymal cells, which line ventricles within the brain (Shan et al., 2018). MMPs have been implicated in other stem cells such as the hematopoietic stem cells [reviewed by Kessenbrock

et al. (2010); Klein et al. (2015)] involved in the induced mobilization process and their ability to remodel the ECM and cleave VEGF, TGFβ, or TNFα; however, their roles in controlling stem cells during neurogenesis still requires in-depth characterization.

2. Breakdown of the Blood-Brain Barrier. Leukocytes infiltrating the CNS parenchyma must transmigrate across multiple layers that comprise the BBB. First, immune cells penetrate the vascular endothelial cell monolayer and the underlying endothelial basement membrane. The process of transendothelial cell migration involves the binding of integrin $\alpha4\beta1$, expressed on leukocytes, to vascular cell adhesion molecule-1 on the endothelial surface of inflamed vessels, which induces MMP2 expression in encephalitogenic T cells and degradation of the subendothelial matrix (Graesser et al., 2000). After extravasation from cerebral blood vessels, leukocytes accumulate in the perivascular space, and inflammatory perivascular cuffs containing these leukocytes are one of the features found in the CNS of patients with MS and other neuroinflammatory conditions (Yong et al., 2001; Lassmann, 2019). In MS brains, perivascular macrophages exhibit an elevated expression of EMMPRIN, an upstream inducer of MMPs (Kaushik et al., 2019). EMMPRIN is also upregulated on peripheral leukocytes before the onset of clinical signs in the EAE mouse model and on infiltrating and resident cells, including microglia within the CNS of symptomatic mice (Agrawal et al., 2011, 2013). Using in situ zymography, Agrawal et al. (2006) previously showed that macrophage-derived MMP2 and -9 were critical for leukocytes to cross the parenchymal basement membrane and infiltrate the CNS parenchyma (Tran et al., 1998). Mechanistically, MMP2 and -9 are able to cleave dystroglycan, a transmembrane receptor that anchors astrocyte endfeet to the parenchymal basement membrane, in turn permitting leukocyte infiltration (Agrawal et al., 2006). Treating EAE mice with an EMMPRIN function-blocking antibody reduces MMP proteolytic activity at the glia limitans, thereby decreasing the infiltration of leukocytes into the CNS parenchyma and reducing disease severity (Agrawal et al., 2011). Overall, the function and localization of MMPs vary across various areas of the brain.

Another example is the elevated expression of myeloperoxidase (MPO) observed in several autoimmune diseases, including MS. Predominantly associated with phagosomes such as neutrophils, macrophages, and dendritic cells, MPO is involved in the creation and maintenance of an alkaline milieu aimed at killing microbes and is also implicated in inflammatory disease progression [reviewed by Arnhold (2020)]. Interestingly, in response to inflammation or microbe infection, the production of ROS via MPO can oxidatively activate proMMPs and inactivate TIMPs (Weiss, 1989; Wang et al., 2007). In active MS, MPO was detected in microglial/macrophage cells within and surrounding CNS lesions (Strzepa et al., 2017). In the EAE mouse model of MS, a magnetic resonance imaging probe (MRI) detecting MPO can reveal lesions with inflammatory monocytes and neutrophils even in the absence of overt BBB breakdown (Pulli et al., 2015). MPO likely contributes to MS pathogenesis through several mechanisms, one of which may involve modulating BBB integrity. For example, MPO inhibition restores BBB integrity (Zhang et al., 2016), and lipopolysaccharide (LPS)-induced BBB dysfunction is significantly lower in $Mpo^{-/-}$ mice compared with wild type (Ullen et al., 2013). Although MMPs have long been implicated to play a direct role in degrading the BBB, MPO may further facilitate BBB damage by oxidizing and inactivating their tissue inhibitors (Wang et al., 2007), thus preventing TIMPs from keeping aberrant MMPs under control. Therefore, beyond tissue expression of MMPs and TIMPs, it is important to consider the effect of posttranslational modifications such as oxidation by MPO on the activity of these proteins (discussed further in Section VII: *Future Perspectives*). Overall, MMPs play both beneficial (angiogenesis, cell survival, myelinogenesis, axonal growth, dampening of inflammation) and detrimental (neuronal death, demyelination, promotion of inflammation, tumorigenesis, disruption of BBB) functions within the CNS (Yong et al., 2001); therefore, we still need to characterize the role of each MMP in specific regions of the CNS and identify which cells produce specific MMPs.

3. Therapeutic Potential of MMP Inhibition in Neurologic Diseases. Despite several disappointing MMP clinical trials, the therapeutic potential of MMP inhibitors for neurologic diseases still exists. In an open-label study, combined treatment of doxycycline (broad-spectrum MMP inhibitor) and interferon beta-1α reduced MMP9 serum levels and brain lesions and improved Expanded Disability Status Scale values in patients with relapsing-remitting multiple sclerosis (Minagar et al., 2008). Tissue plasminogen activator is the only FDA-approved treatment of patients with ischemic stroke; however, the neurotoxic side effects of tissue plasminogen activator are in part mediated by MMP9 upregulation, which can limit its therapeutic potential. Combined therapy with selective MMP9 specific inhibitors that can cross the BBB may be able to address complications caused by tissue plasminogen activator (Tsuji et al., 2005; Gooyit et al., 2012). For MMP-targeted therapies to be effective, the timing of intervention should be carefully considered. In MS, MMP inhibition may be therapeutic during inflammatory phases of the disease, as blocking MMPs may decrease activation of proinflammatory factors and limit infiltration of immune cells into the CNS. On the other hand, promoting MMP activity later in

the disease course may help facilitate tissue repair postinjury by clearing debris and other factors that inhibit remyelination processes within the lesion environment. It is tempting to speculate that MMPs inhibitors would only be useful during acute injury or early in the pathology of neuroinflammatory diseases; however, inhibiting the beneficial effects of MMPs during wound healing may impart more damage (Hsu et al., 2006). Future studies should consider investigating these complex time-dependent functions of MMPs in health and neuroinflammatory diseases.

E. Bacterial and Viral Infections

The host immune response to a pathogen must be carefully coordinated to clear the infection while also sufficiently preserving tissue function. Although some MMPs play a critical role in remodeling the ECM and coordinating the immune response during early infection, uncontrolled MMP activity during late or chronic stages of infection may cause host tissue damage and even death (Chopra et al., 2019). The role of MMPs in bacterial or viral infection has been challenging to characterize, as they play both beneficial and detrimental roles. Early in infection, MMPs facilitate leukocyte recruitment via processing cytokines and chemokines and remodeling the ECM (Houghton et al., 2009; Marchant et al., 2014; Chopra et al., 2019). However, unregulated MMP activity may contribute to immunopathology during chronic phases of the disease. For example, MMPs play a central role in tissue remodeling and fibrosis in patients with chronic infections such as pulmonary tuberculosis. Pharmacological inhibition of phosphodiesterases has shown promise in dampening inflammation and reducing fibrosis, partly by downregulating MMPs in several disease models, including mycobacterium tuberculosis–infected rabbits (Subbian et al., 2016) and chorioamniotic infection that contributes to preterm premature rupture of fetal membranes (Oger et al., 2005).

Severe influenza pneumonia can lead to acute respiratory distress syndrome and death. Doxycycline, a broad-spectrum and unspecific MMP inhibitor, decreased the activity of MMP2 and -9 in a mouse model of virulent H3N2 influenza virus (Ng et al., 2012). Doxycycline treatment decreased the expression of podoplanin (or T1α, a membrane protein of alveolar type I epithelium) and thrombomodulin (an endothelial protein) in the bronchoalveolar lavage fluid. Moreover, it diminished inflammation and protein leakage in the lungs and reduced lung damage measured by histopathologic analyses (Ng et al., 2012). This suggests that inhibiting MMPs may protect against lung injury in severe influenza infection; however, the timepoint of MMP inhibition should be carefully tested, as MMPs may also play a critical role in controlling the early stages of infection. Recruitment of immune cells to the site of infection is essential for the clearance of a pathogen, but infiltrating immune cells may generate excessive inflammatory responses that cause collateral tissue damage. Talmi-Frank et al. (2016) identified MT1-MMP as an ECM-degrading protease expressed by infiltrating myeloid cells in a model of influenza virus. Inhibition of MT1-MMP using a selective allosteric inhibitory antibody preserved lung tissue integrity by maintaining an abundance of collagen type I fibers and laminin during infection. Importantly, anti–MT1-MMP treatment did not modulate the immune response but protected against tissue destruction (Talmi-Frank et al., 2016). Moreover, secondary bacterial infections are the leading cause of mortality in high-risk populations with influenza, and treatment using Tamiflu, an antiviral drug for influenza, does not reduce mortality rates when given postinfection. However, in mice coinfected with influenza virus and *Streptococcus pneumoniae*, preventative or therapeutic combined application of Tamiflu and MT1-MMP inhibition protected mice against sepsis by maintaining basement membrane integrity (Talmi-Frank et al., 2016). This suggests that selectively inhibiting MMPs could be an effective drug target in infection if their inhibition does not impair viral clearance and supports tissue resilience to limit infection-related immunopathology. In response to a primary infection, the integrity of epithelial barriers may be compromised to allow for immune cells to enter tissues. However, failing to resolve this membrane postinfection may leave the tissue susceptible to secondary infections.

MMPs can also cleave transmembrane proteins to release soluble fragments that may exert independent functions. For example, membrane-bound CD100 (mCD100) is abundantly expressed on immune cells such as CD4 T cells, CD8 T cells, and natural killer (NK) cells and is increased upon cell activation. MMP-mediated proteolytic cleavage results in the shedding of a 120-kDa fragment corresponding to the extracellular domain of CD100, known as soluble CD100 (sCD100), which can act as a ligand to modulate the immune response. Altered mCD100/sCD100 ratios have been observed in viral infections such as hepatitis B virus (HBV). Patients with chronic HBV infection have increased mCD100 expression on T cells, decreased serum sCD100 levels, and low serum MMP2 compared with healthy controls, suggesting insufficient cleavage of mCD100 from T cell membranes in patients with chronic HBV. In mice, inhibiting MMP2 and -9 activity during HBV infection using the synthetic peptide CTTHWGFTLC (Koivunen et al., 1999) resulted in decreased serum sCD100 levels and delayed viral clearance (Yang et al., 2019), possibly due to attenuated intrahepatic anti-HBV CD8 T cell responses.

Human immunodeficiency virus (HIV) infection has been associated with increased MMP9 levels in the cerebrospinal fluid (CSF) (Sporer et al., 1998), and it also induces MMP expression in macrophages, causing neurotoxicity within the CNS (Yong et al., 2001). Conant et al. (2004a) showed that HIV-1 protein transactivator of transcription (Tat) increased the release of MMP1 and active MMP2 in mixed human neuron/astrocyte cultures and induced MMP3 and -13 expression in primary murine astrocyte culture (Bozzelli et al., 2019). HIV-1 Tat also increased the expression of MMP9 in the human astrocyte cell line CRT-MG (Ju et al., 2009). Increased MMPs in the brains of patients with HIV infection may be one of the factors contributing to neurologic complications such as HIV-associated encephalitis and dementia. Tat can still be constitutively expressed by HIV-infected cells despite effective antiretroviral therapy; thus, pharmacological inhibition of Tat may be a therapeutic avenue to prevent HIV-associated neurocognitive disorders and reduce viral reservoirs (Sgadari et al., 2019; Joshi et al., 2020). However, depending on the specific MMP and stage of infection, MMPs may play both protective and pathogenic roles. For example, primary human macrophages expressing a Tat sequence derived from a patient with HIV-associated dementia increased MMP2 and -7 release and activation, and supernatants from these cells caused significant neuronal death. Interestingly, this effect is Tat sequence dependent as brain-derived Tat from patients without HIV-associated dementia did not induce the same MMP upregulation. Neurotoxicity was inhibited by anti-MMP2 or -7 antibodies, suggesting that these MMPs are implicated in pathogenesis (Johnston et al., 2001). However, another study suggested that MMP1 can enzymatically degrade Tat and decrease Tat-mediated neurotoxicity as well as HIV transactivation (Rumbaugh et al., 2006). Thus, the protective or pathogenic fate of viral-host interactions is MMP specific. Furthermore, MT1-MMP was demonstrated to be elevated on the neuronal cell surface, resulting in proMMP2 activation from HIV-infected macrophages/microglial cells (Zhang et al., 2003). Upon activation of proMMP2 by MT1-MMP, active MMP2 was demonstrated to switch receptor binding specificity from CXCR4 to CXCR3 via a four amino acid cleavage of the chemokine CXCL12/stromal cell-derived factor 1 (SDF1), resulting in neurotoxicity (McQuibban et al., 2001; Vergote et al., 2006). As an example of the synergy between HIV and bacterial infections, females with bacterial vaginosis present with an altered microbiota, which colonizes the female reproductive tract and increases female susceptibility to HIV infection.

Increased levels and activity of MMPs, particularly MMP1, -10, and -13, are detected in cervicovaginal fluid from women diagnosed with bacterial vaginosis compared with healthy women. In vitro coculture experiments show that endocervical epithelial cells increase the expression of MMPs, particularly MMP7 and -10, in response to challenge with the bacterial vaginosis-associated bacterium *Atopobium vaginae* when compared with *Lactobacillus* species present in healthy vaginal microflora (Cherne et al., 2020). Further, the combination of MMP10 and -13 have been implicated in increasing HIV-1 transmigration through the endocervical epithelium (Cherne et al., 2020). Therefore, the role of MMPs in barrier dysfunction may be one factor that influences one's susceptibility to concomitant or secondary infections. The crosstalk between pathogen-derived factors and host MMPs needs to be further characterized to understand how specific bacteria or viral agents may modulate the host immune response and contribute to pathology.

1. Cytokine and Chemokine Processing. In addition to ECM substrates, MMPs process multiple cytokines and chemokines to drive the recruitment of inflammatory cells and either promote or resolve inflammation at the site of infection (McQuibban et al., 2000; Cauwe et al., 2007; Dean et al., 2008; Starr et al., 2012b; Proost et al., 2017; Young et al., 2019). Importantly, the localization and activation of MMPs is a critical factor that dictates their function. In the antiviral response against coxsackievirus type 3 or respiratory syncytial virus, intracellular MMP12 was demonstrated to mediate NF-κB transcription, resulting in IFNα secretion and host protection (Marchant et al., 2014) (Fig. 5). However, extracellular MMP12 was shown to cleave the IFNα receptor 2 binding site of systemic IFNα to regulate or resolve the antiviral response (Marchant et al., 2014). The selective MMP12 inhibitor RXP470.1 was demonstrated to inhibit extracellular MMP12 from inactivating IFNα but spare the beneficial activity of intracellular MMP12 and, in turn, dramatically increase systemic IFNα levels and decrease viral load in coxsackievirus type B3-infected mice (Marchant et al., 2014) (Fig. 5). Thus, studies testing MMP inhibitors should consider the differential role that intracellular and extracellular MMPs may play in immune responses. Designing specific MMP inhibitors that account for differences between intracellular and extracellular enzyme activity may prove to be more efficacious.

F. Sepsis

Sepsis is defined as a dysregulated host response to an infection leading to organ dysfunction and ultimately organ failure (Singer et al., 2016). Sepsis remains one of the major causes of mortality in intensive care unit (ICU) patients and a leading cause of death from infection (Genga and Russell, 2017). Despite its importance and increasing global prevalence, safe and effective therapeutics for sepsis are lacking. Understanding the pathophysiology behind sepsis is

complicated by the fact that infections can be caused by a broad range of pathogens, including bacteria, viruses, and fungi (Dolin et al., 2019). Several factors have been identified that contribute to the multiple organ dysfunction characteristically observed in patients with sepsis. For example, a prominent feature of sepsis is a strong proinflammatory response resulting in increased levels of circulating leukocytes, cytokines, chemokines, and other mediators of tissue damage (Gyawali et al., 2019). A second prominent feature of sepsis is injury and dysfunction of the microvasculature, leading to extravascular fluid leakage and tissue edema (Arpino et al., 2016).

1. Metalloproteinase Expression in Sepsis. Sepsis has been found to alter the expression of multiple MMPs. For example, a group of ICU patients with severe sepsis presented with plasma levels of MMP3, -7, -8, and -9 that were elevated more than 3-fold compared with healthy controls (Yazdan-Ashoori et al., 2011). Another study found markedly elevated levels of active MMP1 in the plasma of human sepsis patients versus healthy controls (Tressel et al., 2011). Altered expression of TIMPs has also been identified in sepsis. Severe sepsis patients admitted to the ICU were found to have elevated levels of TIMP1, TIMP2, and TIMP4 relative to healthy controls (Yazdan-Ashoori et al., 2011). Conversely, TIMP3 protein and mRNA levels appeared to be decreased in pulmonary endothelial cells after treatment with a mix of clinically relevant septic cytokines (Arpino et al., 2016). Similarly, IL1β and TNFα, two proinflammatory cytokines in sepsis, were determined to synergistically repress the expression of *Timp3* and increase the expression of *Timp1* in mouse brain microvascular endothelial cells (Bugno et al., 1999). Importantly, these changes in the expression of certain MMPs and TIMPs during sepsis have shown to be diagnostic or prognostic, providing useful insights for both researchers and clinicians. For example, several studies have reported that lower levels of MMP9, higher levels of TIMP1, and a higher TIMP1/MMP9 ratio are associated with greater disease severity in patients with sepsis (Lorente et al., 2009; Lorente et al., 2014; Bojic et al., 2018). Moreover, MMP9 and TIMP1 have been associated with circulating cytokine levels, coagulation state, organ dysfunction, and mortality in sepsis (Bojic et al., 2018).

2. Metalloproteinases and Septic Barrier Dysfunction. Septic microvascular barrier dysfunction results from injury and dysfunction of the microvascular endothelial cells, leading to extravascular leakage of protein-rich edema and leukocyte influx into the tissue (Arpino et al., 2016). MMPs have been shown to regulate vascular permeability and barrier function and thus have been examined for their role in the pathophysiology of sepsis. Disruption of microvascular endothelial intercellular junctions, including adherens and tight junctions, occurs during sepsis and contributes to a loss of barrier function (Jayawardena et al., 2019). VE-cadherin, a critical adhesion molecule of adherens junctions, is known to be disrupted during sepsis via mechanisms including VE-cadherin phosphorylation and cleavage by proteases such as MMPs and ADAMs (Jayawardena et al., 2019). Cleavage of VE-cadherin results in the release of soluble VE-cadherin (sVE-cadherin) fragments into the circulation, and plasma levels of sVE-cadherin have been shown to positively correlate with sepsis severity (Zhang et al., 2010). MMP7, which is increased under septic conditions, is known to be capable of cleaving VE-cadherin (Ichikawa et al., 2006; Yazdan-Ashoori et al., 2011). These findings indirectly point to a potential role for MMPs in the breakdown of endothelial intercellular junctions during sepsis.

MMP1 may also contribute to increased vascular permeability during sepsis (Tressel et al., 2011). Specifically, the mouse ortholog of MMP1, MMP1a, was released from endothelial cells of septic mice and cleaved protease-activated receptor 1 (PAR1), resulting in its activation (Tressel et al., 2011). PAR1 activation leads to vascular leakage and leukocyte extravasation as a result of Rho GTPase activation and actin skeleton-dependent contraction of endothelial cells (Vandenbroucke et al., 2011). The administration of an MMP1 inhibitor successfully reduced the increase in lung vascular permeability observed during sepsis, thereby suppressing endothelial barrier disruption (Tressel et al., 2011). The MMP1 inhibitor also suppressed the proinflammatory cytokine response, reduced disseminated intravascular coagulation, and improved survival in septic mice (Tressel et al., 2011). Further investigation into this MMP1/PAR1 axis may uncover a new therapeutic target in sepsis.

The BBB is vital for maintaining normal brain function and can be compromised during sepsis, resulting in cerebral edema and potentially sepsis-associated encephalopathy (Chaudhry and Duggal, 2014). Patients with sepsis-associated encephalopathy present with neurologic dysfunction, altered mental status, and higher mortality rates (Nwafor et al., 2019). MMP2 and -9 may have a role in mediating the increased vascular permeability observed in the brain during sepsis. Using a cecal ligation and perforation (CLP) preclinical model of sepsis, BBB permeability in Wistar rats increased in parallel with levels of MMP2 and -9 in the microvessels of the hippocampus and cortex (Dal-Pizzol et al., 2013). This increase in permeability was successfully reversed by an MMP2 or MMP9 inhibitor (Dal-Pizzol et al., 2013). Although the exact mechanism remains to be determined, MMP2 and MMP9 are both capable of cleaving tight junction proteins, which are crucial in maintaining

BBB integrity and are disrupted during sepsis (Q Li et al., 2009; Feng et al., 2011).

The integrity of the intestinal epithelial barrier can also become compromised during sepsis, with leakage of intestinal components resulting in enhanced systemic inflammation and organ dysfunction (Vandenbroucke et al., 2013). MMP7 and -13 have both been implicated in regulating the permeability of the intestinal epithelial barrier during sepsis (Vandenbroucke et al., 2013, 2014). Specifically, *Mmp13*$^{-/-}$ mice have reduced mortality in both LPS and CLP models of sepsis (Vandenbroucke et al., 2013). This protective effect was partly attributed to the ability of MMP13 to proteolytically cleave and activate membrane-bound TNF into soluble TNF. Soluble TNF is bioactive and can induce disruption of intestinal epithelial tight junctions via caveolin-mediated endocytosis (Vandenbroucke et al., 2013). In another study, *Mmp7*$^{-/-}$ mice were protected against endotoxin-induced increases in intestinal permeability and translocation of bacteria to the mesenteric lymph nodes (Vandenbroucke et al., 2014). Collectively, these studies provide evidence that MMPs are important in mediating intestinal barrier disruption and that inhibiting them may result in protection against sepsis-induced organ dysfunction.

3. Metalloproteinases and the Septic Inflammatory Response. Activation and tissue infiltration of immune cells such as neutrophils are an important element of the host's inflammatory response to sepsis (Rahman et al., 2012). CD40L, a molecule expressed on the surface of platelets, appears to be released into the circulation during abdominal sepsis and subsequently promotes infiltration of neutrophils into the lung, leading to increased tissue edema and damage (Rahman et al., 2009). A recent role has been proposed for MMPs in regulating the shedding of CD40L during sepsis. Administration of GM6001/ilomastat (Fig. 6), a broad-spectrum MMP inhibitor, reduced CD40L shedding from platelets, macrophage-1 antigen (Mac-1) upregulation on neutrophils, and production of CXC chemokines in the lung in a preclinical mouse sepsis model (Rahman et al., 2012). Moreover, it led to decreased neutrophil infiltration and less lung damage. Further studies using *Mmp9*$^{-/-}$ mice determined that MMP9 regulates the shedding of CD40L from platelets and the consequent neutrophil accumulation in the lung during abdominal sepsis (Rahman et al., 2013). Previous work has shown that inhibiting neutrophil recruitment can be protective against septic pulmonary damage (Asaduzzaman et al., 2008). Therefore, targeting the activity of MMP9 may prove to be beneficial in regulating the inflammatory response during sepsis.

MMP8 has also been identified as a critical regulator of the inflammatory response in sepsis. *MMP8* mRNA expression was significantly increased in pediatric patients with sepsis and septic shock versus healthy control patients (Solan et al., 2012). Moreover, the level of MMP8 appeared to correlate with sepsis severity, with increased *MMP8* mRNA expression found in septic shock nonsurvivors compared with survivors (Solan et al., 2012). In a preclinical CLP model of sepsis, *Mmp8*$^{-/-}$ mice had reduced early infiltration of neutrophils into the lung and reduced circulating levels of proinflammatory cytokines IL6 and IL1β (Solan et al., 2012). Furthermore, pharmacological inhibition of MMP8 activity by treating septic mice with (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinolone-3-hydroxamate] significantly reduced mortality (Fig. 6, MMP8 inhibitor) (Solan et al., 2012). In the same study, using an in vitro approach, MMP8 was found to directly activate NF-κB, an important macrophage proinflammatory transcription factor, pointing to a potential mechanism to be further explored (Solan et al., 2012). Collectively, these four lines of evidence suggest that MMP8 plays an important role in modulating the inflammatory response during sepsis and that inhibiting MMP8 activity may be a potential therapeutic strategy.

As TIMPs regulate the activity of MMPs, it is not surprising that they can impact the host's response to sepsis. For example, levels of TIMP3 have been shown to be decreased under septic conditions. (Arpino et al., 2016). In a CLP preclinical model of sepsis, researchers examined the impact of a TIMP3 null mutation on lung structure and function (Martin et al., 2003). *Timp3*$^{-/-}$ septic mice showed an increase in lung compliance accompanied by increased MMP2 and MMP9 activity, as well as decreased levels of collagen and fibronectin (Martin et al., 2003). It was proposed that the observed septic lung abnormalities were enhanced by increased MMP degradation of ECM components. Alveolar macrophages, a major inflammatory cell population in the lung, were determined to be critical for mediating the lung changes in *Timp3*$^{-/-}$ septic mice (Martin et al., 2007). *Timp3*$^{-/-}$ mice depleted of alveolar macrophages were protected from the sepsis-induced alterations in lung mechanics (Martin et al., 2007). Moreover, MMP7 abundance was attenuated after macrophage depletion, suggesting that it may be partially involved in this process (Martin et al., 2007). Overall, these studies demonstrate how changes in TIMP levels during sepsis can alter the MMP/TIMP balance and thus impact the net metalloproteinase activity.

V. Pharmacological Inhibitors of Matrix Metalloproteinases

A. Clinical Trials for Cancer Treatment

The first generation of MMP inhibitors was inspired by mechanistic aspects of peptide hydrolysis. The mechanism by which all MMPs cleave substrates is driven by acid-base catalysis via a Zn^{2+} ion in the

active site (Nagase and Woessner, 1999; Visse and Nagase, 2003; Maskos, 2005). In the 1990s, MMP inhibitors were designed based on the logical, but at the time unproven, concept that cancer cells require MMPs to degrade ECM (predominantly collagens) to facilitate cell migration, invasion, and metastasis. The first generation of MMP inhibitors were peptide and peptide-like compounds mimicking backbone features of P1, P1', and P2' and capable of binding Zn^{2+} ion that resembled collagen (Zucker et al., 2000). The goal was to develop competitive and potent reversible broad-spectrum inhibitors of MMP activity, and the first class of compounds was the hydroxamic acid zinc-binding group (Zucker et al., 2000). The first compound tested in preclinical rodent cancer models and entered into clinical trials was batimastat or BB-94 (developed by British Biotech; Fig. 6), which displays broad specificity in inhibiting multiple MMPs while sparing other proteases. Batimastat was tested on lung colonization and spontaneous metastasis of the HOSP.1P rat mammary carcinoma. Intraperitoneal administration of six doses of 30 mg/kg batimastat inhibited 80% of lung metastases (Eccles et al., 1996). When batimastat was initiated 2 days prior to surgical removal of the tumors, 100% of treated animals survived to 120 days, whereas the control animals all died before day 100 (Eccles et al., 1996). In a mouse model of ovarian cancer, the combination of batimastat and cisplatin resulted in delayed tumor growth and increased the survival time of the mice compared with cisplatin alone (Giavazzi et al., 1998). In the RIP1-Tag2 model of pancreatic islet cell carcinogenesis, batimastat reduced angiogenesis by 49% in a prevention trial and by 83% in an intervention trial with no effect on invasive tumors (Bergers, 1999). Preclinical studies using batimastat for cancer treatment were successful, resulting in human phase I and II clinical trials. However, as batimastat was not soluble and had poor bioavailability, it had to be administered intraperitoneally and intrapleurally to patients, which resulted in sustained plasma concentrations and was found to act as a depot (Nelson et al., 2000). The reported side effects included nausea, fatigue, low-grade fevers, abdominal pain when injected intraperitoneally, and pain at the injection site when injected intrapleurally (Nelson et al., 2000). Thus, clinical trials using batimastat were terminated, and other MMP inhibitors were further tested (Zucker et al., 2000; Overall and Kleifeld, 2006b; Dufour and Overall, 2013).

Marimastat (BB-2516; Fig. 6) was developed after batimastat in an attempt to make the drug more bioavailable and was also a broad-spectrum MMP inhibitor (Nelson et al., 2000). Phase I and II trials in patients with advanced cancers demonstrated that marimastat was well tolerated, and side effects included fatigue and cumulative inflammatory polyarthritis that was reversible when treatments were stopped (Wojtowicz-Praga et al., 1998; Nelson et al., 2000; Zucker et al., 2000).

Prinomastat (AG3340; Fig. 6) was developed by Agouron Pharmaceuticals based on information obtained with X-ray crystallographic analysis of human MMPs and is a picomolar inhibitor of MMPs. Prinomastat was developed to be more specific than previous inhibitors and was aimed to selectively inhibit MMP2, -9, -13, and MT1-MMP (Nelson et al., 2000). In a preclinical model of brain cancer, U87 glioma cells were implanted in severe combined immunodeficiency/nonobese diabetic (SCID/NOD) mice for 3 weeks before being treated with 100 mg/kg of prinomastat or vehicle intraperitoneally (Price et al., 1999). In the prinomastat-treated mice, tumor volume was decreased by 78% compared with the vehicle-treated mice, resulting in less invasive and proliferative tumors and a 2-fold increase in survival time (Price et al., 1999). In an NCI-H460 orthotopic lung cancer model, treatment with prinomastat in combination with carboplatin resulted in longer survival compared with vehicle-treated animal, prinomastat, or carboplatin alone (Liu et al., 2003). When prinomastat was tested in clinical trials, similar side effects to previous MMP inhibitors were identified, such as time- and dose-dependent musculoskeletal stiffness and pain (Nelson et al., 2000). Although there were benefits of combination therapy in preclinical animal models, importantly, a human phase III clinical trial for advanced stage non–small-cell lung cancer that compared standard chemotherapy with or without the addition of prinomastat did not result in increased survival between the two groups (Bissett et al., 2005).

Tanomastat (BAY 12-9566; Fig. 6) was developed by Bayer Corporation and is a nonpeptidic biphenyl inhibitor with a Zn^{2+}-binding carbonyl group and was demonstrated to have a long terminal plasma half-life ranging from 90 to 100 hours (Gatto et al., 1999). Tanomastat is a butanoic acid derivative that could account for distinct selectivity as it cannot inhibit MMP1, -8, and -13 (Nelson et al., 2000). Tanomastat's distinct chemical features could be responsible for different side effects from other MMP inhibitors; there was no musculoskeletal toxicity in the phase I trial in patients with advanced tumors, but new symptoms were identified, including an asymptomatic increase of hepatic enzymes and thrombocytopenia (Nelson et al., 2000). Importantly, patients with metastatic small-cell lung cancer taking tanomastat resulted in a worse disease, suggesting that we lacked a clear understanding of specific MMP functions in cancer (Overall and Kleifeld, 2006a; Dufour and Overall, 2013). MMP inhibitors were also tested in combination with known cancer drugs such as gemcitabine, carboplatin, or paclitaxel, but no additional benefit was clearly demonstrated (Nelson et al., 2000). The

failure of MMP inhibitors in randomized phase III clinical trials has generated numerous updated generations of different inhibitors to directly or indirectly inhibit MMPs (Das et al., 2020).

B. Clinical Trials for Inflammatory Diseases Treatment

MMP inhibitors were also tested in various rodent models of inflammation. For example, in a model of autoimmune inflammatory arthritis in rats using complete Freund's adjuvant, injection of the compound N4-hydroxy-2-(2-methylpropyl)-N1-[2-[[2-(morpholinyl)ethyl]-,[S- (R*,S*)] (GI168) via osmotic minipumps from days 8 to 21 significantly diminished ankle swelling in addition to bone and cartilage destruction (Conway et al., 1995). In another study of adjuvant-induced arthritis, rats were treated for 21 days with 50 mg/kg of tanomastat (BAY 12-9566; Fig. 6) or vehicle (Hamada et al., 2000). The severity of arthritis in this model was determined by measuring an arthritic index indicated by paw volume, urinary pyridinoline, and deoxypyridinoline; by examining joint inflammation; and by microscopic morphometry of articular cartilages. In this adjuvant-induced arthritis model, tanomastat was demonstrated to suppress inflammation and reduce cartilage destruction (Hamada et al., 2000).

Multiple MMPs have been implicated in cardiovascular diseases as having both beneficial and detrimental roles (Bassiouni et al., 2021). For example, myocardial specific overexpression of constitutively active murine MMP2 resulted in cardiac defects, including increased troponin I proteolysis, mitochondrial dysregulation, left ventricular remodeling, and heart failure even in the absence of external stimulation or injury (Wang et al., 2006; Bergman et al., 2007). Using selective MMP12 phosphinic peptide inhibitor RXP470.1 (Fig. 6), atherosclerotic plaques cross-sectional area was diminished by over 50% in both male and female apolipoprotein E knockout mice fed on a Western diet (Johnson et al., 2011). In humans, when batimastat was coated onto drug-eluting stents for the treatment of obstructive coronary artery disease, no clinical benefits were demonstrated, suggesting that a better understanding of the role of individual MMP is required (Sousa et al., 2003).

In the EAE mouse model of MS, ilomastat (GM6001; Fig. 6) was demonstrated to suppress the development of EAE in a dose-dependent manner (Gijbels et al., 1994). Another MMP inhibitor, D-penicillamine, significantly reduced the mortality and morbidity rates in the EAE mouse model (Norga et al., 1995). However, when tested in humans in a double-blind, placebo-controlled pilot trial with D-penicillamine and metacycline, side effects were detected and the trial was stopped (Dubois et al., 1998). Next, minocycline, a tetracycline derivative containing some MMP9-inhibitory activity, was tested and was demonstrated to be highly effective in the EAE model (Popovic et al., 2002). In a human randomized and controlled trial, 100 mg of minocycline taken twice a day was tested to see if it was able to reduce the risk of conversion from a first demyelinating event (also termed clinically isolated syndrome) to MS. In 142 patients with multiple sclerosis, it was demonstrated that minocycline treatment significantly reduced the risk of conversion from a clinically isolated syndrome to MS compared with the placebo groups (Metz et al., 2017). All changes detected on magnetic resonance imaging (MRI) were significant at 6 months but not at 24 months (Metz et al., 2017). Some of the side effects reported in the minocycline-treated group included rash, dizziness, and dental discoloration (Metz et al., 2017). It is important to mention that minocycline is not a selective MMP9 inhibitor but rather results in downregulation of MMP9 activity and likely additional MMPs.

Another approved MMP inhibitor in the clinic is Periostat (doxycycline hyclate) for the treatment of periodontitis (Golub et al., 1990, 2001; Golub and Lee, 2020). Seminal work by Lorne Golub and colleagues (1984) demonstrated that tetracyclines (minocycline) reduced gingival collagenolytic activity in periodontal disease. Clinical studies have shown that doxycycline administered orally, at doses below those needed for antimicrobial efficacy, to adult patients with periodontitis resulted in significantly reduced collagenase activity in gingival crevicular fluid and in extracts of inflamed gingival tissues (Golub et al., 1990, 2001). Seventy-five patients with pathologic levels of periodontal attachment and with positive collagenase activity in gingival crevicular fluid were treated with subantimicrobial-dose doxycycline (Periostat) in a placebo-controlled, double-blind, parallel-group study. Periostat administered at subantimicrobial doses led to improvements in disease parameters (Golub et al., 2001). Additional clinical trial studies for Periostat were conducted and resulted in FDA approval (Caton and Ryan, 2011; Golub, 2011; Gu et al., 2012). Despite a successful approval, there are other effective approaches to treat periodontitis and Periostat is not always a first-line therapy.

C. Potential Reasons for the Failure of MMP Inhibitors

Over the past two decades and since the various clinical trials of MMP inhibitors, multiple lessons have been learned: 1) MMPs can be both drug targets and antitargets depending on cell/tissue localization, type of disease, or stage of the disease; 2) MMP inhibitors are likely to be more effective for short-term dosage as opposed to long-term dosage; and 3) broad-spectrum versus selective inhibition must be carefully examined for a particular disease [reviewed in Overall and Kleifeld (2006b); Dufour and Overall (2013);

Young et al. (2019)]. It has been demonstrated that MMPs have biologic functions other than ECM remodeling: ~31% of their substrates are ECM proteins, and ~69% are non-ECM proteins (Fig. 4; Supplemental Table 1). Therefore, it is likely that the role that MMPs play in diseases is different from what the inhibitors were intended to block during the cancer clinical trials (Dufour and Overall, 2015). Most MMP inhibitors tested were broad spectrum and able to inhibit multiple MMPs in addition to ADAMs and ADAMTSs for some inhibitors. Paradoxically, treating human HT-1080 fibrosarcoma cells with the broad-spectrum MMP inhibitor GI129471 increased MMP9 expression (Maquoi et al., 2002). Additionally, MMP inhibitors can prevent autolytic degradation of MT1-MMP, resulting in an enhancement of proMMP2 activation and MMP2 activity (Toth et al., 2000). Overall, toxicities associated with MMP inhibitors were related to musculoskeletal pain and were assumed to be due to their poor selectivity; however, this has not been fully confirmed yet (Zucker et al., 2000; Skiles et al., 2004; Fingleton, 2008; Devy and Dransfield, 2011). Therefore, it is believed that more selective inhibitors would likely be more effective. In the past decade, several small synthetic inhibitors selectively targeting the MMP active site have been designed with some significant success for MMP2, -9, -12, and -13 (Devel et al., 2010; Mahasenan et al., 2017; Fields, 2019).

Specifically, a significant gain in selectivity was achieved by exploring zinc-binding groups other than the hydroxamate function, as well as other side chains interacting within the MMP specificity loop (S_1' cavity). For example, MMP408 with a carboxylate function and an optimized P_1' side to fit within a deep S_1' cavity (W Li et al., 2009) (Fig. 6) has been reported as a selective inhibitor of MMP12. In the same vein, the phosphinic pseudopeptide displayed a high potency and selectivity for this protease (RXP470.1; Fig. 6) (Devel et al., 2006). In this case, it was demonstrated that the excellent selectivity of this inhibitor mainly relied on three critical features: the presence of two glutamate residues facing the MMPs' S_2' and S_3' subsites (Devel et al., 2006), a long and hydrophobic P_1' side chain inserting within the large S_1' cavity of MMP12 (Czarny et al., 2013), and the chemical nature of the zinc-binding moiety. On this last point, the RXP470.1 analogs with either a hydroxamate or a carboxylate function to chelate the catalytic zinc ion are much less selective for MMP12 than the parent molecule with a phosphinate moiety, highlighting the key role played by the zinc-binding group in subtly tuning inhibitor selectivity (Rouanet-Mehouas et al., 2017). The phosphinate function was also incorporated within the structure of triple-helical peptide mimicking a specific region of collagen (Lauer-Fields et al., 2007), thus resulting in inhibitors displaying inhibition constant (K_i) values in the low nanomolar range for MMP2 and -9. In those two examples, the high potency of phosphinic pseudopeptides was attributed to the capacity of the phosphinate function to reproduce the transition state during peptide bond hydrolysis, making those compounds potential transition-state analogs (Georgiadis and Dive, 2014). In the field of small synthetic compounds, selectivity for a given MMP or a restricted set of MMPs can also be obtained by selectively targeting the S_1' cavity of MMPs. In this respect, two compounds with no zinc-binding moiety were able to target MMP8/13 (Pochetti et al., 2009) (Fig. 6) and MMP13 (Engel et al., 2005) (Fig. 6) with high potency and selectivity. In these cases, the inhibitors bind deeply in the S_1' cavity and extend into an additional side pocket only present in MMP8 and -13.

Many of these inhibitors have been assessed in preclinical models of inflammatory diseases and cancer, but none have been validated in human studies to date. There is also a need to better characterize the role of individual MMPs in a specific disease to establish if a specific MMP is detrimental; currently, at least 10 MMPs have been found to have protective functions and should not be inhibited (Overall and Kleifeld, 2006b; Dufour and Overall, 2013; Brkic et al., 2015; Chopra et al., 2019).

The broad expression of multiple MMPs in various cell types (e.g., epithelial, cancer, immune, fibroblast, and others) could also imply that a local/targeted delivery is likely necessary. Also, the timing of drug delivery is important. MMPs have been shown to have opposite roles in different stages of viral infections. For example, early on, nuclear MMP12 was shown to be protective via its promotion of IFNα but was demonstrated to generate a negative feedback loop by cleaving extracellular IFNα at later timepoints (Marchant et al., 2014). Therefore, treatment with a selective MMP12 inhibitor could result in unintended effects depending on the timing of drug treatment. The clinical use of MMP inhibitors has been more complex than initially thought. Alternatives other than inhibitors that target MMP active sites exist to inhibit and control the biologic functions of MMPs. These options are now being examined in various preclinical models.

D. Alternative Strategies To Inhibit MMPs

The catalytic domains of MMPs are highly conserved, whereas other domains are not present in all MMPs or they are divergent in terms of amino acid similarities. For example, targeting MMPs exosites, the hemopexin domain, or the cytoplasmic tail could result in more selective compounds. Peptides mimicking the amino acid sequences of the hemopexin domains of MMPs have been designed for MMP9 to inhibit cell migration (Dufour et al., 2010), MMP12 to inhibit bacterial functions (Houghton et al., 2009),

and MT1-MMP to inhibit cell migration and invasion (Zarrabi et al., 2011). Small molecule inhibitors targeting the hemopexin domain of MMP9 (Dufour et al., 2011) and MT1-MMP (Remacle et al., 2012) were designed, and both demonstrated antitumor growth activity in murine models of cancer. However, the binding affinity of exosite domains is typically much lower than the catalytic domain; therefore, it creates a new set of challenges for these types of inhibitors (Overall and Kleifeld, 2006a; Dufour and Overall, 2013; Young et al., 2019).

Other than peptidomimetic or small molecule inhibitors, monoclonal antibodies (mAbs) have been designed with the purpose of selectively inhibiting a single MMP. For example, Irit Sagi and collaborators injected a Zn-tripod to elicit an immune response against a synthetic zinc-imidazole motif, resulting in the production of mAbs that recognized MMP9 (Sela-Passwell et al., 2011a). Two of them generated the mAbs suppressors of defective silencing (SDS)3 and SDS4, which inhibited MMP2 and -9 (K_i values were 1 ± 0.01 μM for SDS3 and 0.05 ± 0.006 μM for SDS4) (Sela-Passwell et al., 2011a). In a murine model of dextran sulfate sodium–induced colitis, both prophylactic and therapeutic treatment with SDS3 prevented body weight loss, prevented colon shortening, decreased diarrhea, and improved colonic damage (Sela-Passwell et al., 2011a). Using the Zn-tripod approach, an MMP7 selective antibody (GSM-192) was recently developed and was demonstrated to induce apoptosis and reduce CFPAC-1 cell migration in vitro (Mohan et al., 2021). Using a different approach to generate an antibody-based inhibitor against MT1-MMP called LEM-2/15, mice were immunized using a cyclic peptide of the V-B loop of MT1-MMP comprising amino acid residues 218 to 233 (Udi et al., 2015). The MT1-MMP antibody LEM-2/15 was demonstrated to protect against influenza virus infection and, in combination therapy with oseltamivir (Tamiflu), displayed a synergistic effect and resulted in complete recovery of influenza-infected mice (Talmi-Frank et al., 2016). Another selective MT1-MMP inhibitor, DX-2400, was developed using a human antigen-binding fragment (Fab) displaying phage library (Devy et al., 2009). Using a breast cancer xenograft model with MDA-MD-231 cells, DX-2400 was demonstrated to reduce tumor growth, invasion, and metastasis (Devy et al., 2009). In the intravenous injection of the metastatic model using mouse B16F1 melanoma cells, DX-2400 was shown to diminish the number of lung nodules and reduce metastasis (Devy et al., 2009). Additionally, DX-2400 was demonstrated to reduce tumor growth in the 4T1 and E0771 syngeneic BALB/c mouse model, reducing TGFβ and SMAD2/3 signaling (Ager et al., 2015). Currently, BT1718, a hemopexin-binding MT1-MMP selective inhibitor developed by Bicycle Therapeutics containing a bicyclic peptide, a linker, and a toxin moiety is in clinical trials for the treatment of solid tumors, projected to end in 2022 (ClinicalTrials.gov identifier: NCT03486730). BT1718 was demonstrated to be effective in reducing tumor volume in xenograft mouse models using HT-1080 cells (Harrison et al., 2017).

Recent and ongoing MMP clinical trials include the testing of andecaliximab (GS-5745), an MMP9 humanized monoclonal antibody designed for the treatment of cancer and inflammatory diseases by Gilead Sciences (Marshall et al., 2015). In a phase III study, andecaliximab in combination with modified oxaliplatin, leucovorin, and fluorouracil (mFOLFOX6) were recently tested in patients with gastric or recurrent gastroesophageal junction (GEJ) adenocarcinoma (ClinicalTrials.gov identifier: NCT01803282); however, the addition of andecaliximab to mFOLFOX6 did not improve overall survival of patients (Shah et al., 2021). Importantly, there was no associated toxicity in patients that took andecaliximab (Shah et al., 2018). Andecaliximab has also been evaluated in combination with gemcitabine and nab-paclitaxel in patients with advanced pancreatic adenocarcinoma and was well tolerated (Bendell et al., 2020). There is also an ongoing recruitment of patients for the evaluation of andecaliximab as a treatment of glioblastoma (ClinicalTrials.gov identifier: NCT03631836).

Andecaliximab has also been tested in inflammatory diseases such as RA (ClinicalTrials.gov identifier: NCT02176876), UC (ClinicalTrials.gov identifier: NCT02520284), CD (ClinicalTrials.gov identifier: NCT02405442), and chronic obstructive pulmonary disease (ClinicalTrials.gov identifier: NCT02077465). In patients with RA, andecaliximab was administered as three infusions over 29 days and was generally safe and well tolerated (Gossage et al., 2018). However, only 15 patients were given andecaliximab and three were given the placebo; therefore, studies in larger patient cohorts and also of increased treatment duration are needed to better characterize the efficacy of andecaliximab (Gossage et al., 2018) (ClinicalTrials.gov identifier: NCT02176876).

In a phase II/III study in patients with moderately to severely active UC, subjects were randomized (1:1:1) to receive placebo, 150 mg andecaliximab every 2 weeks, or 150 mg andecaliximab weekly via subcutaneous administration (Sandborn et al., 2018). Although andecaliximab was well tolerated, 8 weeks of treatment with 150 mg andecaliximab did not induce clinical remission or response in patients with UC, and the trial was terminated early (Sandborn et al., 2018) (ClinicalTrials.gov identifier: NCT02520284).

In a phase II study in patients with moderately to severely active CD, subjects were randomized (1:2:2:2) to receive subcutaneous injections of placebo weekly, 150 mg andecaliximab every 2 weeks, 150 mg andecaliximab weekly, or 300 mg andecaliximab weekly (Schreiber et al., 2018). Similar to the patients with UC, andecaliximab was well tolerated in the patients

with CD, but no clinically meaningful symptomatic or endoscopic response was observed, and the trial was terminated due to lack of efficacy (Schreiber et al., 2018) (ClinicalTrials.gov identifier: NCT02405442).

Another interesting strategy is to use the biologic functions of MMPs as a treatment (Gabrielli et al., 2009). For example, the combination of FCX-013 and veledimex is currently being used in clinical trials for the treatment of localized scleroderma, a disease that hardens the skin and connective tissues (Gabrielli et al., 2009) (ClinicalTrials.gov identifier: NCT03740724). This approach uses FCX-013, a human fibroblast genetically modified using a lentivirus vector encoding for MMP1, whose expression is dependent on an agonist. This therapy consists of injecting FCX-013 subcutaneously at the location of the fibrotic lesions. The goal is that the MMP1 producing fibroblasts will break down excess collagen accumulation. In combination with FCX-013, patients will also orally take veledimex (INXN-1001) to induce MMP1 from the injected cells. Veledimex (INXN-1001) is an oral activator and promoter for the gene therapy system (Chiocca et al., 2019). Once the fibrosis is resolved, the patient stops taking veledimex (INXN-1001), which stops MMP1 production. Therefore, the biologic activity of certain MMPs could also potentially be used as a therapeutic to resolve fibrosis in specific diseases (Leong et al., 2021). This approach further supports that a better characterization of the roles of individual MMPs in inflammation and fibrosis could result in the development of novel therapeutics.

Another novel approach to treating cancer could be the use of antibodies derived from patients with cancer as a diagnostic or therapeutic approach. The tumor microenvironment contains antibody-secreting cells that are associated with a favorable prognosis in various cancer types (Zaenker et al., 2016; Helmink et al., 2020; Petitprez et al., 2020). For example, in patients with high-grade serous ovarian carcinoma, it was demonstrated that somatic hypermutations promoted antibody antitumor reactivity against certain surface autoantigens (Mazor et al., 2022). By using antibody-secreting cells within these tumors, cells were mutated and clonally expanded, resulting in the production of tumor-reactive antibodies against MT1-MMP (Mazor et al., 2022). MT1-MMP has been demonstrated to be elevated in ovarian cancer compared with healthy tissues and, using multiple validation ELISA assays, a strong and reproducible antibody reactivity was detected in all tested patients (Mazor et al., 2022). This work suggests a novel approach to the use of patient-derived antibodies either by selectively targeting MT1-MMP or as a drug delivery vehicle to tumors. In addition, this could become an effective strategy for cell-mediated therapy such as chimeric antigen receptor (CAR)-T cell therapy using MT1-MMP as a target.

Finally, a better characterization of the role of individual MMPs could result in novel approaches to inhibit MMP functions or, alternatively, to use the proteolytic activity of MMPs to help treat multiple diseases such as localized scleroderma. Importantly, as MMPs are expressed in various tissues and immune cell types, it is important to analyze and identify local and systemic side effects according to each disease. With many MMPs having beneficial roles, it is key to analyze whether these protective functions will or will not be affected by the specific MMP inhibitor being tested. Furthermore, antibodies, peptidomimetic inhibitors, or small molecule inhibitors are likely to generate different side effects that need to be carefully monitored. Despite decades of MMP research, there have been multiple failed clinical trials for cancer and arthritis but also a few successful ones. For example, Periostat (doxycycline hyclate) was approved to treat periodontitis (Golub et al., 1990, 2001; Golub and Lee, 2020), and minocycline was approved for treating the risk of conversion from a clinically isolated syndrome to MS (Metz et al., 2017). Therefore, many opportunities remain for the use of MMP inhibitors.

VI. Molecular Tools for Targeting Active MMPs

Conventional proteomics approaches aiming at establishing the relation of MMPs to disease states are limited by the fact that they take the total protein amount into account, whereas the protease functional (active) status is often the unique relevant parameter. To determine MMPs' proteolytic activity in biologic samples, two main molecular tools have been developed: 1) substrate-based probes that generate a signal upon proteolytic cleavage and 2) ligand-based probes that bind selectively to MMPs active form and spare inactive and inactivated ones. Within the family of ligand-derived tools, two classes can be further distinguished: 1) MMP inhibitors containing a reporter tag for imaging purposes and 2) affinity-based probes with a reactive motif enabling a permanent attachment of the probe to the target MMP for subsequent analyses. In this section, we will discuss the most significant advancements in those three categories of molecular tools with a special focus on covalent probes, the sole class of molecular probes capable of identifying directly and unambiguously the target MMPs through diverse analytical methods.

A. Substrate-Based Probes for a Cleavage Assay

The design and development of substrate-based probes have been inspired directly from the peptide sequence of natural MMP substrates or from combinatorial libraries of peptides obtained by phage display or chemical approaches (Knapinska and Fields, 2012). In this respect, substrate-based probes generally

consist of a minimum peptide sequence containing six amino acids that extend their side chains from S_3 to S_3' subsites of MMP catalytic cleft (Fig. 8). Within most of the sequences, a proline residue is present in the P_3' position, thus conferring an MMP preference for peptide sequence, and variations of other side chains result in motifs targeting a specific subset of MMPs. A certain selectivity can also be achieved by incorporating additional amino acids on both sides of the minimal P_3-P_3' sequence. By relying on such a scaffold, several types of biosensors have been developed to detect MMP activity (Fig. 8). This mainly includes 1) optical biosensors emitting an optical signal upon proteolytic cleavage, 2) magnetic or photoacoustic biosensors, or 3) sensors leading to the secretion of synthetic biomarkers specifically detected in urine.

Substrates-based probes emitting a fluorescent signal upon proteolytic cleavage are the most widely used optical biosensors and enable the real-time monitoring of MMP proteolytic activity in many different complex proteomes and in vivo (Scherer et al., 2008; Knapinska and Fields, 2012; Hu et al., 2014; Lei et al., 2020). The fluorescence-based biosensors have been designed by relying on fluorescence self-quenching (Bremer et al., 2001), Förster resonance energy transfer (FRET) (Knapinska and Fields, 2012; Hu et al., 2014), or bioluminescence resonance energy transfer (BRET) (Lee and Kim, 2015; Nguyen et al., 2018; Park et al., 2021; Tian et al., 2021). Benefiting from the enzyme turnover, those activatable probes detect active MMPs with high sensitivity and, if the fluorophore possesses an emission wavelength in the near-infrared (NIR) region, this detection can be achieved in vivo within deep tissues. For example, the fluorescence self-quenched probe *MMPsense680* with an organic NIR dye has been used to monitor the activity of MMP2 in tumor tissue (Bremer et al., 2001) and that of MMP2 and -9 in atherosclerosis (Deguchi et al., 2006). Since these seminal reports, this probe has been used in several other preclinical models, including colon cancer (Hensley et al., 2017), colitis (Ding et al., 2014), and bacterial infections (Daghighi et al., 2014). Another example is an NIR probe developed by Tang et al. (2018) whose fluorescence signal was self-quenched upon self-assembly into nanoparticles, which enabled the detection of MMP2 activity in vivo.

Alternatively, several FRET-based activatable probes have been designed and became the standard in the real-time monitoring of MMPs' activity in various biologic matrices and in vivo. In this case, the peptide sequence is flanked by both a fluorescence donor and a fluorescence acceptor/quencher in close proximity. Upon proteolytic cleavage, the distance between the two partners increases, and this irreversible spatial separation induces a ratiometric change in the donor and acceptor emission spectra, resulting in the appearance of a fluorescent signal. Many different types of FRET-base probes incorporating either small organic dyes or nanomaterials as FRET donor/acceptors were designed to target MMPs in complex proteomes [see several reviews for additional information: Garland et al. (2016); Liu et al. (2018); Lei et al. (2020); Oliveira-Silva et al. (2020); Soleimany and Bhatia (2020)]. Despite their high sensitivity and low background to noise in vivo, the limitation of FRET-based probes with organic fluorescent dyes used for long-time imaging is a signal attenuation due to diffusion within the tissues. However, to circumvent these limitations, ultrafast-acting activatable probes enabling the visualization of MMPs expression and inhibition after only 30 minutes have been designed and validated in a tumor-bearing mouse model (Zhu et al., 2011). In this case, the addition of different polyethylene glycol extensions at the C-terminal end of the peptide sequence subtly modulated the activation profiles of probes in vivo.

To limit the diffusion of cleaved fragments tethered to fluorescent emitter away from the protease active site, other effective strategies based on activatable FRET probes connected to cell-penetrating peptides have been developed (Jiang et al., 2004). Specifically, an MMP-cleavable peptide sequence was inserted between complementary polycationic and polyanionic domains. Upon processing by MMP2 or -9, the polycationic entity as a cell-penetrating peptide (CPP) connected to an NIR dye can be released to further bind and enter the cell. Such a selective delivery of fluorescent emitter to MMP-expressing cells by activatable cell-penetrating peptides (ACPPs) has been explored in different mouse models (Chen et al., 2017; Hingorani et al., 2017), notably to detect unstable atherosclerotic plaques within which certain MMPs are overexpressed under their active form (Hua et al., 2015). Interestingly, the ACPP strategy was combined with the MRI technique for identifying plaques at high risk of rupture. The ACPPs have also demonstrated excellent ability to direct fluorescence-guided oncologic surgery in a preclinical model (Metildi et al., 2015) and have been recently assessed in first-in-human (phase I) clinical trials to challenge their capacity to improve intraoperative detection of malignant tissue during breast cancer operations (Unkart et al., 2017). Following an approach similar to ACPPs, Schultz et al. developed an MMP12 selective FRET probe containing a palmitic acid extension installed at the N terminus end of the peptide sequence (Cobos-Correa et al., 2009). Such a lipid tail allowed the fragment containing the NIR dye to anchor onto cell membranes, therefore limiting the diffusion of the fluorescence signal. The use of FRET probes of nanometer size also limits the diffusion fluorescence signal away from the protease active site (Lei et al., 2020). These FRET nanoprobes, exploiting the unique optical

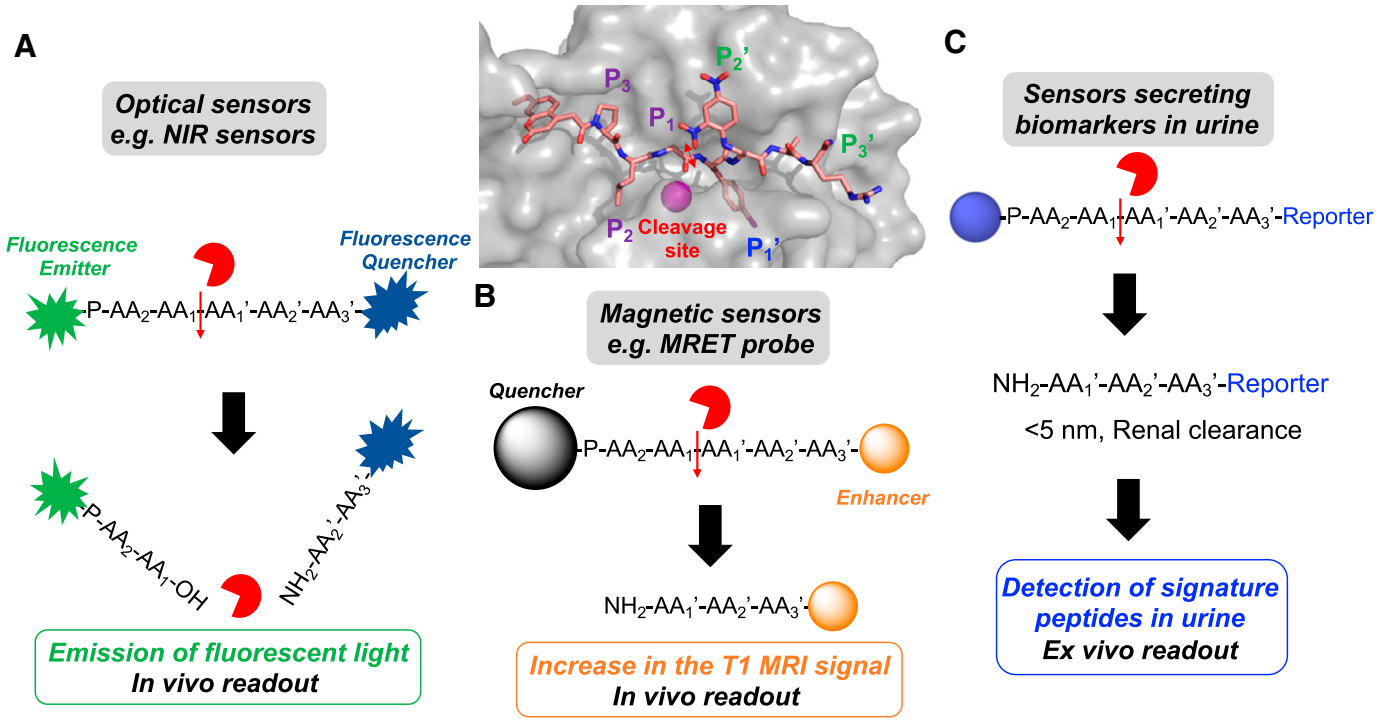

Fig. 8. Design of substrate-based sensors from a minimal peptide sequence of six amino acids incorporating a proline in P_3' position. The peptide sequence corresponds to a fluorogenic substrate crystallized within the catalytic cleft of an inactive mutant of MMP9 (PDB: 4IJI) (Tranchant et al., 2014). From this sequence, different substrate-based probes/sensors have been designed. This notably includes (A) optical sensors (e.g., NIR sensors) with a quencher and emitter of fluorescence, (B) magnetic sensors (e.g., magnetic resonance tuning probe), and (C) synthetic biomarkers, which upon MMP cleavage are secreted and detected in urine. Key: MMP9 (surface), peptide sequence (pale red stick), catalytic zinc ion (magenta ball); unprimed (P_3, P_2, and P_1), P_1' and primed (P_2' and P_3') positions are identified in purple, blue, and green, respectively. The cleavage site is marked with red arrow.

properties of nanomaterials, present several other advantages, including good biocompatibility and stability, high brightness with limited photobleaching, and the possibility to perform multiplexing. In this respect and taking advantage of dopant-dependent multicolor emission of upconversion nanoparticles, FRET nanoprobes with distinct emission wavelengths were designed to measure simultaneously and independently the activity of MMP2 and -7 (Cao et al., 2018).

Due to the extremely low background and high sensitivity of bioluminescent reporters, BRET probes also provide great advantages for imaging MMP activity in vivo (Nguyen et al., 2018; Park et al., 2021; Tian et al., 2021). In this case (and unlike FRET approaches), no external light is required, but an enzyme, often luciferase, as a bioluminescent reporter is essential to emit light upon the appropriate trigger. When the emission spectrum of the bioluminescent reporter overlaps with the excitation spectrum of another fluorophore, the energy transfer results in the emission of a luminescent signal that can be associated with a specific proteolytic activity. Based on this principle, a BRET probe enabling the dynamic monitoring of MT1-MMP activity with high temporal and spatial resolution was validated in a preclinical model of lung cancer (Tian et al., 2021). In this case, a membrane-bound form of luciferase was used, and the MT1-MMP recognition peptide was inserted within the sequence of this enzyme. Upon proteolytic cleavage by MT1-MMP, two luciferase subdomains get in close proximity, which results in the appearance of a high luminescent signal.

As biologic matrices have a negligible magnetic background, several magnetic biosensors for the sensitive detection of MMPs in clinical samples and in vivo have also been developed (Lei et al., 2020). Most of these biosensors exploit the properties of superparamagnetic nanoparticles capable of magnifying the magnetic signal resonance of protons in neighboring water molecules. Upon cleavage by MMPs, those sensors aggregate, which accelerates the dephasing of water protons. This change in the nanoparticles' state can then be detected by monitoring the variation of proton relaxation time, which is associated with MMP proteolytic activity (Harris et al., 2006; Schuerle et al., 2016). Recently and in the same vein of FRET biosensors, a sensor relying on a distance-dependent magnetic resonance tuning (MRET) has been designed; the variation of distance between a paramagnetic enhancer and a superparamagnetic quencher induced the appearance of magnetic resonance imaging signal, linked to the proteolytic activity of MMP2 in vivo (Choi et al., 2017).

Optical-based sensors can also be conjugated with other techniques such as photoacoustic imaging, which

mainly aims at improving imaging resolution. The development of an activatable probe incorporating both a near-infrared dye and a quencher linked through an MMP2 peptide substrate has been done (Yin et al., 2019). Upon cleavage, this probe not only emitted a fluorescence signal but also changed its aggregation state. In this respect, the responsive probe presented a dramatic MMP2 concentration–dependent absorption at around 680 nm, whereas that at around 730 nm was MMP2 concentration independent. These features allowed detection of MMP2 activity via both fluorescence and photoacoustic imaging in vitro and in a model of breast cancer in vivo.

To detect MMPs' activity in vivo, another effective approach involving nanosensors capable of generating synthetic biomarkers upon proteolytic cleavage has been reported (Soleimany and Bhatia, 2020). In this case, and unlike in situ imaging sensors, the peptide fragments resulting from the proteolytic cleavage diffuse away from the protease site, enter circulation, and are then secreted in the urine. Thus, the subsequent detection of those fragments by various analytical techniques such as mass spectrometry (Kwong et al., 2013), ELISA (Dudani et al., 2016), paper lateral flow assay (Warren et al., 2014), or colorimetric test (Loynachan et al., 2019) is indirectly associated to MMPs' proteolytic activity at a pathologic site. Recently, those activity-based nanosensors enabled the profiling of 15 proteases, including five MMPs simultaneously, in a preclinical model of lung cancer (Kirkpatrick et al., 2020).

In all of those substrate-based strategies and mainly due to the highly conserved structural topology between the different MMPs, the peptide sequence is rarely cleaved by a single MMP. To address this issue, an activatable fluorogenic probe with enhanced specificity for MT1-MMP was recently developed (Ji et al., 2020). In this case, the specificity of the FRET probes is increased by tethering the MT1-MMP peptide sequence to an additional recognition element that binds away from the catalytic cleft. A long linker of twelve polyethylene glycol subunits was introduced between these two motifs, resulting in a significant optimization of specificity and selectivity of the fluorogenic probe for MT1-MMP in vivo. Besides their potential lack of selectivity within the MMP family, the ability of substrate-based probes to target MMPs exclusively within the pathologic site is also questionable (Lebel and Lepage, 2014). Indeed, in many pathologic contexts, circulating active MMPs have been reported (Roy et al., 2009; Fontana et al., 2012; Hadler-Olsen et al., 2013; Goncalves et al., 2015; Peeters et al., 2017; Dofara et al., 2020). This may result in substrate-based probes that are partly cleaved in the bloodstream, yielding poor target/nontarget contrast when imaging diseased tissues. Preliminary work in the context of lung cancer detection suggests that a more local delivery of activatable probes, intrapulmonary delivery in this specific case, significantly increases the signal specificity while eliminating off-target activation (Kirkpatrick et al., 2020).

B. Ligand-Based Probes To Bind to Active MMP

By relying on MMP inhibitors scaffold (see also the section on MMP inhibitors), numerous imaging agents have been designed for different types of imaging modalities, including positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging, and optical imaging (Matusiak et al., 2013; Rangasamy et al., 2019). Two types of imaging probes can be distinguished: those possessing an imaging reporter of small size (e.g., a radioisotope such as ^{18}F, ^{11}C, or ^{123}I) that is directly inserted within the structure of the MMP inhibitors, and those incorporating a reporter of comparable size to that of the MMP inhibitor scaffold (e.g., NIR dye, encaged ^{68}Ga or ^{99m}Tc). In this latter case, the imaging reporter is systematically separated from the MMP binding motif by a spacer to limit eventual steric clashes (Faust et al., 2008; Razavian et al., 2010, 2016; Bordenave et al., 2016; Hugenberg et al., 2017; Toczek et al., 2019; Gona et al., 2020). The imaging probes contain a binding motif targeting either a wide range of MMPs or a restrictive set of MMPs. Within this second class of probes, MMP2/9 probes with an SB-3CT (2-[[(4-Phenoxyphenyl)sulfonyl]methyl]thiirane) thiirane scaffold (Hohn et al., 2018), MMP12 probes with either an RXP470.1 phosphinic pseudopeptide (Bordenave et al., 2016; Razavian et al., 2016; Toczek et al., 2019) or a hydroxamate-based scaffold (Gona et al., 2020), and MMP13 probes have been reported (see Fig. 6 for the structure of the motif selectively targeting the MMP active site) (Hugenberg et al., 2017).

All of these MMP inhibitor–derived imaging agents have been assessed in a wide variety of preclinical models, including cancer, atherosclerosis, myocardial infarction, aneurysm, RA, and lung inflammation and, for a rather complete overview of their scope of application, see the review by Rangasamy et al. (2019). In all of those preclinical models, the probes accumulate within pathologic sites overexpressing active MMPs, and the spatiotemporal resolution, penetration depth, and sensitivity are related to the imaging reporter used. Importantly, unlike the substrate-based probes benefiting from the enzyme turnover, the imaging probes bind to MMPs in a stoichiometric manner, with evident consequences on the detection limit. This may be particularly critical in the case of MMPs that are present under their active forms at very low concentrations at a pathogenic site.

Another limitation of the MMP inhibitor–derived probes relies on their structure. Indeed, most MMP inhibitors have been designed to target both the hydrophobic S_1' subsite and the catalytic zinc ion within the MMPs' active site. In this respect, they often

display several aromatic units and negatively charged functions, structural features that favor unspecific binding to abundant plasma proteins (Casalini et al., 2013; Digilio et al., 2014; Bordenave et al., 2016; Razavian et al., 2016). Interestingly, in the series of optical probes with an NIR dye, it has also been shown that the dye itself can drastically modify the in vivo detection of the imaging probes (Razavian et al., 2016). A similar impact of the reporter part has been observed with radiotracers differing in their ^{99m}Tc chelating groups (Toczek et al., 2019). Besides their potential unspecific uptake ascribed to their trends to bind to serum albumin, most probes do not enable the precise documentation of the activation status of individual MMPs. Indeed, most MMP inhibitor–derived tracers are broad spectrum and only provide a general overview of the MMPs' activation pattern. Further, when a selective probe is used, the part of the whole signal corresponding to the probe/MMP complex remains difficult to attribute unambiguously, mainly due to the low concentration of active MMP in vivo.

C. Activity-Based Probes To Crosslink Active MMPs

The concept of activity-based protein profiling (ABPP) emerged at the end of the 1990s, demonstrated by a chemical strategy that utilizes active site-directed covalent probes to profile the functional state of enzymes in complex proteomes and in vivo (Evans and Cravatt, 2006). A typical activity-based probe (ABP) is composed of 1) an electrophilic "warhead" to react with a catalytic nucleophile, 2) a targeting motif that imposes selectivity upon the reactive moiety, and 3) an analytical handle for subsequent visualization and characterization of the resulting covalent adduct. Since MMPs use zinc-activated water molecules rather than a protein-bound nucleophile for catalysis, the ABPs targeting those proteases are systematically composed of a reversible inhibitor to which a photolabile group is attached (Geurink et al., 2011; Garland et al., 2016). Upon photoirradiation, the photolabile group (diazirine, phenyl azido, or benzophenone) is transformed into a reactive intermediate (carbene, nitrenes, or ketyl biradical, respectively) that crosslinks amino acids in close proximity. The resulting covalently modified MMP could be distinguished from unlabeled proteins on SDS-PAGE gel or further captured for identification by mass spectrometry. Based on this principle, three types of MMP-directed ABPs have been designed: those targeting the nonprimed region, those able to crosslink residues within the S'_1 cavity, and ABPs reacting with amino acids present within the S_2' or S_3' primed subsites (Fig. 9). In 2004, two groups independently reported the two first ABPs targeting MMPs in their nonprimed and primed regions, respectively (Chan et al., 2004; Saghatelian et al., 2004). The "left-handed" probe with a C-terminal hydroxamic acid (Fig. 9A) was able to crosslink MMP9 (Chan et al., 2004) but had a relatively low binding affinity toward MMPs, thus limiting its scope of application.

Conversely, the APBs whose structure derived from marimastat (Fig. 9B) were more effective to covalently modify the MMPs' primed subsites. In this respect, ABPs with a benzophenone facing the S_2' subsite could crosslink several MMPs, down to 3 ng/50 fmol of MMP2 spiked into a complex proteome (Saghatelian et al., 2004). The N-terminal succinyl-hydroxamate motif has been further exploited to provide ABPs with a small clickable handle (alkyne tag) for pulldown of active proteases from complex mixtures (Sieber et al., 2006). The goal was to design probes with no bulky reporter groups (e.g., fluorescent) that might adversely affect probe-metalloprotease interactions. Interestingly, the use of those probes on several human cancer cell lines highlighted two major points: the succinyl hydroxamate warhead could target metalloproteases other than MMPs, and no endogenous MMPs could have been detected, suggesting that MMPs are often found entirely in zymogen or TIMP-bound form in cancer cell proteomes. With this type of ABP, endogenous MMP2 was detected in a zebrafish model (Keow et al., 2012). More recently, similar APBs with a cleavable linker were used to characterize active MMPs in an in vitro model of early-stage cartilage degradation in post-traumatic osteoarthritis (Ravindra et al., 2018). In this study, after pulldown, Tris (2-carboxyethyl) phosphine (TCEP)-mediated cleavage, digestion of the released proteases by trypsin, and multidimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using spectral counting, several endogenous MMPs including MMP1, -2, -3, -9, -12, and -13 were detected (Ravindra et al., 2018). Importantly, the authors also showed that a significant fraction of the total secreted MMP was inactive. The marimastat-derived ABPs were also used to implement a metalloprotease activity multiplexed bead-based immunoassay as an effective approach for detecting MMP activity at picomolar range in conditioned cell culture media, peritoneal fluid aspirates, menstrual effluent, and uterine biopsies (Ahrens et al., 2019).

Complementary to ABPs targeting S_2' and S_3' subsites, photoaffinity probes able to react within the hydrophobic S_1' subsite were also developed (David et al., 2007; Bregant et al., 2009; Geurink et al., 2010; Nury et al., 2013b). The first one was reported by the group of Vincent Dive in 2007 (David et al., 2007). This probe contained a phosphinic pseudopeptide scaffold and a P_1' side chain with a phenyl azido moiety as a photolabile group and was shown to covalently react with several MMPs. Importantly, the incorporation of a radioactive isotope (tritium) within its structure not only enabled a high detection threshold by in-gel radio imaging but this also allowed to accurately

quantitate the crosslinking yield for each of the tested MMP. In this series of phenyl azido probes and through further studies, the labeling sites within the S_1' cavity of MMP12 (Dabert-Gay et al., 2008) and MMP3 (Dabert-Gay et al., 2009) were also determined. This enabled a better understanding of the probe's reactivity, which resulted in the conception of the second generation of photoaffinity probes with a diazirine photolabile group (Nury et al., 2013b) (Fig. 9C).

Compared with the phenyl azido probes, such a probe reacted efficiently with a larger set of MMPs and was able to detect endogenous MMP12 in bronchoalveolar lavage fluids (Nury et al., 2013a). More widely, ABPs targeting the S_1' subsite are more effective than those targeting the S_2' and S_3' subsites. This was particularly well illustrated by the work of Geurink et al. (2010), which compared photoaffinity probes with a diazirine moiety in P_1' or P_2' position. Interestingly, the probe with the photolabile group in P_1' crosslinked all of the tested MMPs (MMP1, -2, -3, -7, -8, -9, -10, -11, -12, and -13), whereas the probe with the same group in P_2' only efficiently reacted with MMP9 and -10. This observation has been rationalized by the fact that the P_2' photolabile group was more exposed to solvent than in P_1', which significantly reduced its reactivity toward protein amino acids. Although capable of detecting endogenous MMPs (Ravindra et al., 2018; Ahrens et al., 2019), photoaffinity probes display a major limitation related to their intrinsic reactivity. Indeed, since the photoirradiation step is not straightforwardly achieved in vivo, the use of photoaffinity probes remains restricted to ex vivo samples.

To address this issue, the group of Matt Bogyo designed ABPs with an alpha-chloroacetamide electrophile in P_2' position (Fig. 9D) that can react with a cysteine residue artificially incorporated with the MMPs' catalytic cleft (Morell et al., 2013; Amara et al., 2018). This approach notably enabled monitoring of the activity of MT1-MMP in cells and in vivo but required genetic manipulations incompatible with native conditions. More recently, and inspired by the ligand-directed chemistry developed by the group of Itaru Hamachi (Tamura and Hamachi, 2019), a new type of MMP-directed ABPs has been published (Kaminska et al., 2021). The affinity probe harbors a phosphinic pseudopeptide scaffold and an N-acyl-N-alkyl sulfonamide cleavable electrophile in P_3' (Fig. 9D), which can react selectively with lysine in close proximity within the MMP S_3' region. Through this strategy, MMP12 but also MMP3, -9, and -13 can be covalently tagged and detected by in-gel fluorescence imaging. This "photoactivation-free" strategy was also validated in complex proteomes and on native MMP12 under its active form, paving the way for the development of ABPs capable of reporting the MMP functional status in vivo.

VII. Future Perspectives

MMPs were first identified as ECM-degrading enzymes but were not recognized as multifunctional proteases that influence cell migration, invasion, adhesion, signaling, immune responses, among others. Although it is tempting to inhibit MMPs' proteolytic activities in diseases, we have learned that they also have protective functions and participate in more processes than we initially thought (Sternlicht and Werb, 2001; Overall and López-Otín, 2002; Dufour and Overall, 2013) (Young et al., 2019). Despite MMPs' protective functions, it is still feasible that an effective MMP inhibitor could be used to treat human diseases. There are many areas of MMP research that warrant additional investigation: 1) better characterization of their nonproteolytic roles; 2) identification of their non-ECM substrates; 3) studying the differences between their intracellular and extracellular functions; 4) profiling their posttranslational modifications; 5) measuring their activity in vivo and ex vivo to distinguish between proMMP, inhibitor-bound MMP, and active MMPs; and 6) development of selective activity-based probes (Fig. 10). Genomics, proteomics, and N-terminomics studies have not yet been performed on all human MMPs or MMP-deficient mice; therefore, there are still important basic characterization and discoveries to be made. Here, we identified six areas of future research that will be critical to completing our understanding of MMPs.

A. MMPs and Their Nonproteolytic Roles: Missed in the Myth?

ProMMPs are also capable of impacting key biologic functions such as cell migration (Dufour et al., 2008, 2010; Pavlaki et al., 2011), cell survival (Conant et al., 2004b; Redondo-Muñoz et al., 2010), branching morphogenesis (Correia et al., 2013), bacterial killing (Houghton et al., 2009), and immune regulation (Shimizu-Hirota, et al., 2012; Marchant et al., 2014). The study of the nonproteolytic roles of MMPs has so far revealed unexpected functions and is likely to uncover even more novel roles and possibly generate unique ways to control MMPs' biologic functions. Another therapeutic opportunity could be to develop inhibitors that interfere with proteins that interact with MMPs' exosites or target downstream pathways that are impacted by MMPs' nonproteolytic roles.

B. Non-ECM MMP Substrates

Despite what their name indicates, MMPs can cleave more non-ECM proteins than matrix proteins (~69% vs. ~31%, respectively; Supplemental Table 1). MMPs can cleave cell surface proteins, receptors, chemokines, cytokines, and other nonmatrix proteins,

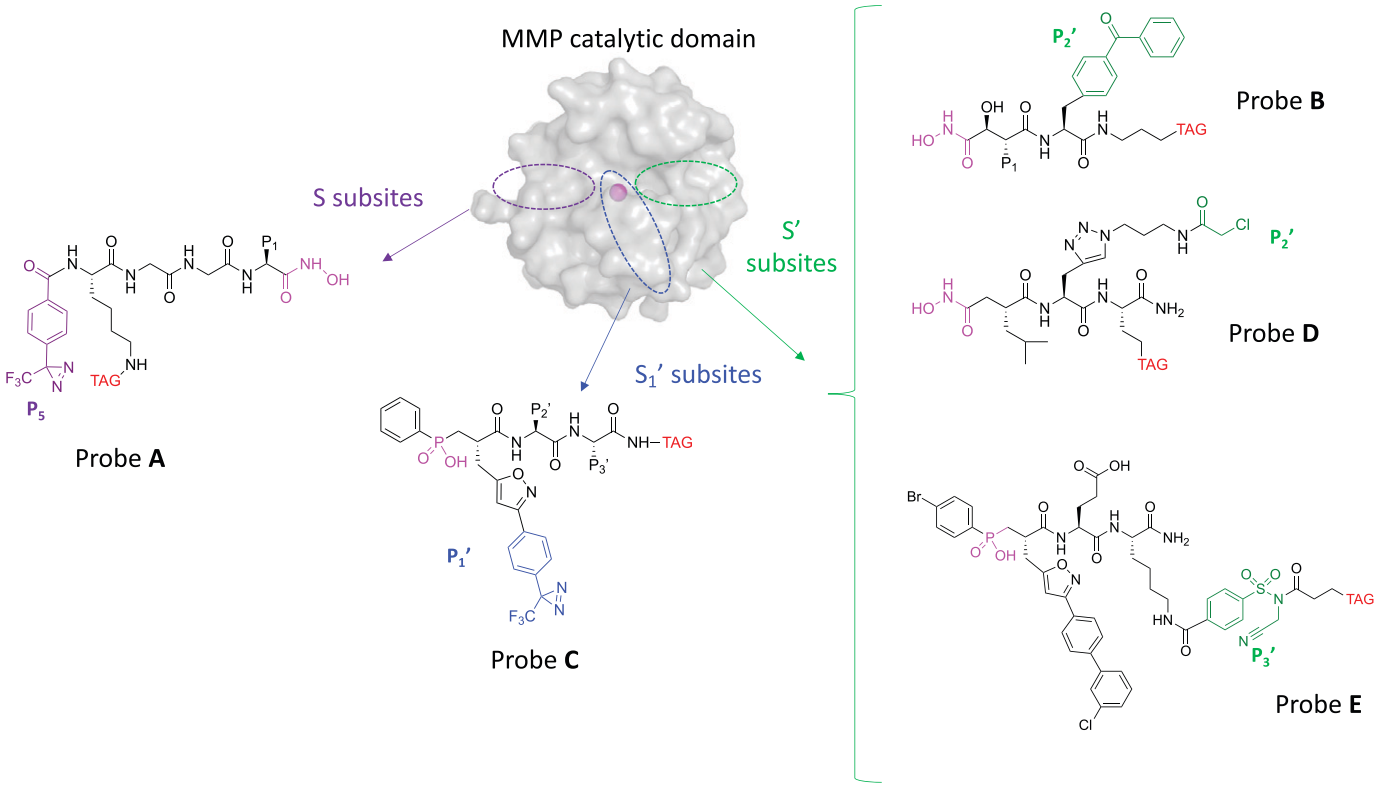

Fig. 9. Activity-based probes (ABPs) directed to the MMP catalytic cleft. From the MMP catalytic domain (PDB: 4IJI) (Tranchant et al., 2014) in surface representation, three regions were distinguished: the S subsites marked in purple, the S_1' subsite in blue, and the primed subsites in green. To each subsite is associated an ABP capable of crosslinking it. Probe A targets the S subsites; probe B, D, and E the S' subsites; and probe C the S_1' subsite. For each probe, the zinc-chelating group is marked in magenta, the photolabile group or reactive electrophile is associated to the color of site within which it reacts, and the analytical tag is in red.

thereby making them more fascinating than initially thought (Butler and Overall, 2009; Dufour and Overall, 2015; Young et al., 2019). N-terminomics approaches have largely contributed to the identification of MMPs non-ECM, and there are still several human and murine MMPs to be characterized and validated in vivo (Butler and Overall, 2009; Bellac et al., 2014; Marchant et al., 2014; Mallia-Milanes et al., 2018).

C. Intracellular versus Extracellular Functions of MMPs

MMPs are secreted proteases, but up to the time of this publication, 11 MMPs have also been demonstrated to have intracellular functions [for additional details, see the review by Bassiouni et al. (2021)]. They can influence immune regulation via DNA interactions (Shimizu-Hirota, et al., 2012; Marchant et al., 2014), oxidative stress via contractile dysfunction (Ali et al., 2011; Fan et al., 2016), mammary epithelial branching morphogenesis (Correia et al., 2013), neuronal signaling (Szklarczyk et al., 2007; Miller et al., 2010) and many others (Bassiouni et al., 2021) (Fig. 5). Intracellular functions of MMPs have been associated with various pathologies, including inflammatory disease, bacterial/viral infections, and cancer; however, there are no specific ways to selectively target intracellular MMPs, and still limited mechanistic information is known on how extracellular MMPs enter inside the cells despite some mechanisms having been proposed (Koppisetti et al., 2014; Bassiouni et al., 2021).

D. Posttranslational Modifications of MMPs

The posttranslational modifications (PTMs) of protease substrates can increase or decrease the rate of proteolysis (Madzharova et al., 2019). In other instances, PTMs can protect protease substrates such as lysine methylation (Wigle et al., 2010), glycosylation (King et al., 2018), or phosphorylation (Dix et al., 2012; Turowec et al., 2014). For example, MMP9 substrate alpha-fetoprotein (*FETA*) was demonstrated to be impacted by polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2) O-glycosylation, and multiple additional MMP9 substrates were identified as being involved in a complex interplay between proteolysis and O-glycosylation (King et al., 2018). Interestingly, MMP9 itself is also N- and O-glycosylated and contains a 64 amino acid residue linker domain implicated in orienting the hemopexin domain for inhibition by TIMP1 and regulating its interaction with cargo receptors (LRP1 and megalin) for internalization (Van den Steen et al., 2006; Rosenblum et al., 2007). Mutant MMP9 lacking the O-glycosylated linker region (OG domain) diminished MMP9's ability to increase cell migration (Dufour et al., 2008). Lack of

glycosylation also impacts the role of MMP9; for example, MMP9 lacking glycosylation at Asn^{38} results in an amino acid–dependent interaction with calreticulin, likely impacting its release toward the secretory pathway (Duellman et al., 2015). MMP9 can also be citrullinated by peptidyl arginine deiminases (PADs), resulting in a higher affinity for gelatin and hyperactivation compared with the uncitrullinated counterpart; citrullinated MMP9 was detected in neutrophil-rich sputum samples of patients with cystic fibrosis, suggesting a key role for PTMs in human disease (Boon et al., 2021). The functions of other MMPs are also modulated by glycosylation (Boon et al., 2016; Madzharova et al., 2019). For example, glycosylated MMP1 (e.g., GalNacβ1,4-(Fucα1,3)GlcNAcβ1,2) has higher levels of interaction with selectin on the cell surface, increasing cell migration (Saarinen et al., 1999; Boon et al., 2016). MMP2, -3, -13, and MT4-MMP also contain several glycosylated sites, yet little is currently known about the impact on their functions.

MMPs have been demonstrated to be phosphorylated, but the biologic significance of this PTM on MMP functions still needs to be characterized. For example, MMP2 contains 29 potential phosphorylation sites, but only five (S32, S160, Y271, T250, and S365) have been validated by mass spectrometry so far (Madzharova et al., 2019). Although little is known, it appears that dephosphorylated MMP2 contains higher enzymatic activity compared with phosphorylated MMP2 due to a conformational change (Sariahmetoglu et al., 2007; Jacob-Ferreira et al., 2013). The phosphorylation of MT1-MMP at nine potential sites also impacts its biologic function, specifically cell migration and invasion (García-Pardo and Opdenakker, 2015). Substitution of MT1-MMP's cytoplasmic domain Thr567 by an alanine residue resulted in an elevation of MT1-MMP levels at the cell surface, diminished internalization, and reduced cell migration and invasion (Williams and Coppolino, 2011). Additionally, several MMPs (MMP1, -12, -13, -14, -16, -24, and -27) can be phosphorylated extracellularly on tyrosine residues located in their hemopexin

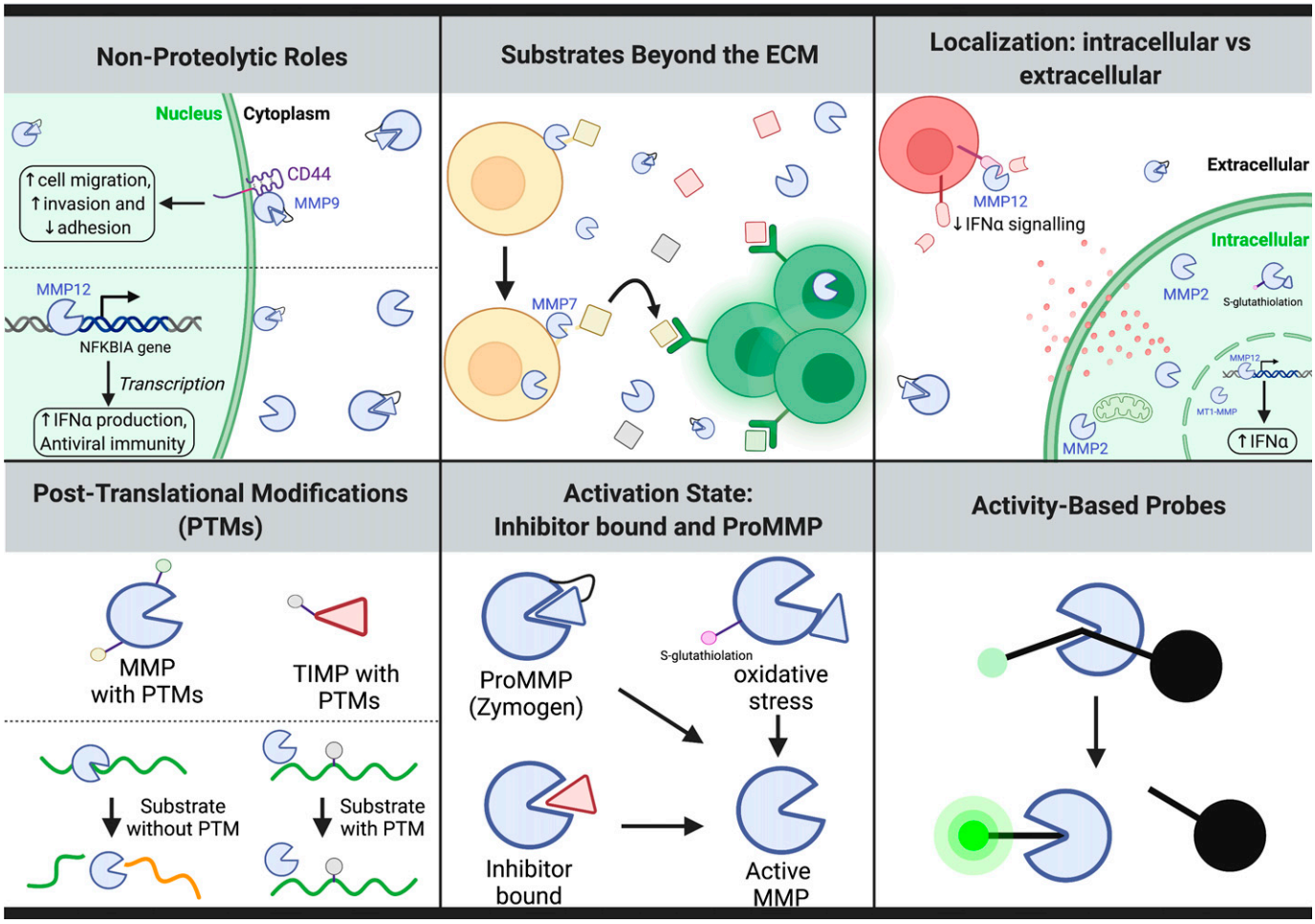

Fig. 10. Topics for MMP research to be studied in the next decade. Schematic representation of six areas of MMP research that warrant further investigation: the nonproteolytic roles of MMPs, their non-ECM substrates, the difference in their intra- and extracellular roles, their posttranslational modifications (PTMs), their activity, and the development of new activity-based probes.

domains by the vertebrate lonesome kinase (VLK) (Bordoli et al., 2014; Tagliabracci et al., 2015).

Furthermore, oxidative and nitrosative stress that increases the activation of MMPs and has been linked to cardiac and neuronal pathologies should be investigated in additional diseases (Cheung et al., 2000; Gu et al., 2002; Wang et al., 2002). Despite numerous examples of how PTMs impact MMPs, there are still multiple PTMs that have not been examined in the context of MMP functions, constituting an important area of future MMP research.

E. Measuring MMP Activity and Diagnostic Applications

As MMPs are present under their active form in several diseases, molecular probes to monitor MMPs activity must be rationally designed with this in mind, and they must be thoroughly validated in different biologic fluids and preclinical models. From the perspective of developing efficient diagnostic tools, their validation in human samples will also be necessary. This will certainly require optimizing not only their specificity but also their capacity to simultaneously detect several MMPs (multiplexing) in many different types of human matrices, this last point being essential for precision diagnostic. To this aim, microfluidic chips and microarray (Lei et al., 2020) allowing automation and high throughput could constitute attractive options for the parallel analysis of multiple samples containing several MMPs. In any case, this will generate a large set of data that will need to be classified and interpreted. In such a scenario, it will become critical not only to provide robust data with a high degree of confidence but also to exploit the resources of artificial intelligence and machine learning for predictive classification (Kirkpatrick et al., 2020). All of those efforts would result in a new generation of activity-based diagnostics suitable for many human pathologies within which active MMPs are dysregulated.

F. The Future of MMP-Directed Molecular Probes

Despite numerous signs of progress in the development of molecular probes for MMPs in the last two decades, some issues and limitations still need to be addressed to determine the MMPs' activation status accurately and unambiguously both in ex vivo biologic fluids and in vivo. In the case of substrate-based probes, identifying a peptide sequence cleaved exclusively by MMPs with no interferences with off-target proteases remains quite challenging. The recent efforts made to document MMPs' endogenous substrates and to identify eventual cooperativity between subsites within the catalytic cleft (Schlage and auf dem Keller, 2015; Eckhard et al., 2016a,b) would facilitate the design of sequences with high selectivity. However, due to the high homology among the different members of the MMP family, identifying substrate sequences capable of discriminating every single MMP seems difficult to achieve. As recently reported for MT1-MMP (Ji et al., 2020), such a limitation can be partly overcome by combining the MMP cleavable sequence with a binding motif interacting away from the catalytic cleft. In principle, and benefiting from recent studies pointing out the presence of discriminating allosteric sites within several MMPs (Sela-Passwell et al., 2010; Udi et al., 2015; Tokmina-Roszyk and Fields, 2018), this strategy should be readily expandable to a larger set of MMPs. A high degree of selectivity for a single MMP can also be obtained when the peptide sequence is installed on nanostructures. In this respect, a recent report showed that the morphology and size of a nanostructure have to be finely tuned to yield a nanosensor with improved selectivity (Son et al., 2019). Specifically, the authors showed that the global charge of MMP (MMP9 in this study) ruled its electrostatic interaction with the nanostructure, stressing the need to take this parameter into account during the development of nanosensors. New selective MMP activatable probes could also be identified using an unbiased activity-profiling strategy with no a priori knowledge of the targeted MMP. This might consist of screening combinatorial substrate library directly on relevant pathologic tissues, as recently validated on substrate-based probes targeting cathepsins (Tholen et al., 2020).

Through the development of the first chemical probes able to covalently modify active MMPs with no photoactivation (Kaminska et al., 2021), a major step has been taken, and the proteomic profiling of active MMPs in vivo should become amenable. However, to limit the background labeling and to expand this approach to a larger set of MMPs, several adjustments in the structure of activity-based probes will probably be necessary. This might require optimizing the structure of the binding motif as well as the positioning of the electrophile warhead within the MMP catalytic groove. In this respect, the phosphinic pseudopeptide scaffold that can project chemical residues on both sides of the MMP cleavage site would turn out to be particularly valuable. In addition, other types of biorthogonal chemistry could be implemented within the MMP catalytic cleft to further improve the probes' specificity and their crosslink efficiency. For instance, recent approaches enabling the chemoselective modification of carboxyl groups in proteins (Martín-Gago et al., 2017; Ma et al., 2020) could be adapted to target the MMP catalytic glutamate, which may result in ABPs capable of crosslinking a very broad set of MMPs.

Beyond the selectivity aspects and having in mind that MMPs under their active form are often present in low amounts in biologic matrices, the sensitivity of the MMP-directed molecular probes could also be improved. This is particularly true in the series of

ligand-based probes that do not benefit from the signal amplification due to enzyme turnover. In this series, a multistep process involving affinity capture, on-bead digestion, and a mass spectrometry analysis remains the standard to reach a high sensitivity of detection (Sieber et al., 2006; Ravindra et al., 2018). However, the recent progress made in mass spectrometry approaches, particularly in the development of derivatization strategies with mass tags to improve the detection of peptides and proteins in complex biologic matrices (Qiao et al., 2014; Sejalon-Cipolla et al., 2021; Zhou et al., 2021), could enable simplifying the analytical procedure. Specifically, by relying on the capacity of ABPs to selectively transfer mass tags to MMPs and by using shotgun proteomics approaches (Zhang et al., 2013), it should be possible to implement a targeted proteomic approach with a minimum of handling steps while maintaining a high sensitivity threshold. Furthermore, this would reduce potential changes coming from variabilities in sample manipulation, thus increasing the robustness of the generated data.

VIII. Conclusions

After several decades of MMP research, there are still many MMPs that have been minimally investigated and remain largely uncharacterized. MMPs have been demonstrated to be implicated in several autoimmune diseases, inflammatory diseases, cancer, and host-microbe interactions. However, a small number of MMP inhibitors are in use in the clinic or effectively treat human diseases. Several questions remain to be answered: 1) Will selective MMP inhibitors be substantially more effective than broad-spectrum inhibitors? 2) Can MMP activity be monitored in vivo or ex vivo and correlated with disease activity? 3) Are there other posttranslational modifications that regulate MMP activity? 4) Will pharmaceutical companies continue to develop MMP inhibitors despite past failures in clinical trials? 5) Are the biologic roles of intracellular MMPs more important than we initially thought? Major gaps left in our understanding of MMP biologic functions under healthy conditions and human diseases are still in place, hindering our ability to answer the proposed questions. This panorama is destined to change in the next decade, as we continue to find additional and unexpected functions that MMPs play. With new technologies come novel ways to characterize MMPs' functions in human diseases, making these proteases even more exciting to study.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: de Almeida, Thode, Eslambolchi, Chopra, Young, Gill, Devel, Dufour.

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