

REVIEW

Biology of metalloproteinases

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Abstract

Matrix metalloproteinases (MMPs) occupy a central role in embryogenesis and in normal physiological conditions, such as proliferation, cell motility, remodeling, wound healing, angiogenesis, and key reproductive events. MMPs form a multigenic family of proteolytic, zinc-dependent enzymes, with 26 members described until present, displaying multidomain structures and substrate specificities. MMPs are involved in both the turnover and degradation of extracellular matrix (ECM) proteins and in the processing, activation, or deactivation of a variety of soluble factors. They are regulated at the level of transcription, activation of the precursor zymogens, and inhibition mainly by tissue inhibitors of metalloproteinases (TIMPs). Any loss in activity control may result in various diseases. This review provides an update of biological functions of MMPs, facilitating the understanding of the complex pathogenic mechanisms of medical conditions characterized by imbalance between MMP and TIMP expression. The design of potent specific inhibitors for MMPs represents a scientific challenge for the development of new therapies.

Keywords: matrix metalloproteinases (MMPs), protease, tissue inhibitors of metalloproteinases (TIMPs), extracellular matrix (ECM), domain.

☞ Definition and spectrum

Although several terms are currently used for matrix degrading metalloenzymes, matrix metalloproteinases or matrixins, the most common one is that of metalloproteinases (MMPs) [1–4]. MMPs form a multigenic family of proteolytic, zinc-dependent enzymes, functioning at neutral pH [5], firstly secreted in their latent form as proenzymes (inactive zymogens) or pro-MMPs and requiring proteolytic activation [6].

MMPs possess overlapping, but distinct substrate spectra [6]. MMPs activity is closely regulated by their endogenous inhibitors, tissue inhibitors of MMPs (TIMPs). Most of MMPs occupy a central role in embryogenesis and in normal physiological conditions, such as proliferation, cell motility, remodeling, wound healing, angiogenesis, and control key reproductive events, such as ovulation, embryo implantation, uterine, breast, and prostate involution, menstruation, and endometrial proliferation [7–9].

Imbalance between MMP and TIMP expression has been involved in various medical conditions, notably tumor invasion, rheumatoid arthritis, atherosclerosis, aneurysms, nephritis, tissue ulcers, fibrosis and endometriosis [4, 10].

☞ MMPs: general structure and types

MMPs have a characteristic multidomain structure generally consisting of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain [4].

The *signal peptide* directs their secretion from the cell, the *propeptide* is essential for maintaining the pro-MMP in a latent form, the *catalytic domain* contains the highly conserved Zn²⁺-binding site, the proline-rich *hinge region* links the catalytic domain to the C-terminal *hemopexin-like* domain, which determines the substrate specificity of the MMP and mediates the interactions with endogenous inhibitors [11].

To date, 26 different MMPs and 24 of their codifying genes have been identified [4, 5].

On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs, and other MMPs [4, 6, 12] (Table 1).

Table 1 – Groups of MMPs listed together with their codifying genes (adapted from: VISSER, NAGASE H., Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function and biochemistry, Circ Res, 2003, 92(8):827–839) [4]

| Enzyme | MMP | Human chromosome |
|-------------------------------|--------|------------------|
| <i>Collagenases</i> | | |
| Interstitial collagenase; | | |
| collagenase–1 | MMP–1 | 11q22–q23 |
| Neutrophil collagenase; | | |
| collagenase–2 | MMP–8 | 11q21–q22.3 |
| Collagenase–3 | MMP–13 | 11q22.3 |
| <i>Gelatinases</i> | | |
| Gelatinase–A | MMP–2 | 16q13–q21 |
| Gelatinase–B | MMP–9 | 20q11.2–q13.1 |
| <i>Stromelysins</i> | | |
| Stromelysin–1 (progelatinase) | MMP–3 | 11q23 |

| Enzyme | MMP | Human chromosome |
|---|---------------------|------------------|
| Stromelysin-2 | MMP-10 | 11q22.3-q23 |
| Stromelysin-3 | MMP-11 | 22q11.2 |
| <i>Matrilysins</i> | | |
| Matrilysin-1 (uterine matrilysin); Pump-1 | MMP-7 | 11q21-q22 |
| Matrilysin-2 (endometase) | MMP-26 | 11p15.4 |
| <i>Membrane-type MMPs</i> | | |
| Transmembrane | | |
| MT1-MMP | MMP-14 | 14q11-q12 |
| MT2-MMP | MMP-15 | 15q13-q21 |
| MT3-MMP | MMP-16 | 8q21 |
| MT5-MMP | MMP-24 | 20q11.2 |
| GPI anchor | | |
| MT4-MMP | MMP-17 | 12q24.3 |
| MT6-MMP (leukolysin) | MMP-25 | 16p13.3 |
| <i>Others</i> | | |
| Macrophage elastase | MMP-12 | 11q22.2-q22.3 |
| RASI-1 | MMP-19 (MMP-18) | 12q14 |
| Enamelysin | MMP-20 | 11q22.3 |
| XMMP (Xenopus)/Cy-MMP (Cynops) | MMP-21 (MMP-23A) | 10q26.3-1p36.33 |
| Femalysin | MMP-22 (MMP-23B) | 1p36.3 |
| CA-MMP | MMP-23 | 1p36.3 |
| CMMP (Gallus) | MMP-27 | 11q24 |
| Epilysin | MMP-28 | 17q11-17q21.1 |

Collagenases (MMP-1, MMP-8, and MMP-13)

Collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13) are the main secreted neutral proteinases capable of degrading native fibrillar collagens of types I, II, III, V, and XI in the extracellular space. They are composed of three polypeptide chains arranged in a rigid triple helix conformation, rendering them resistant to degradation by proteinases [13].

Collagenases cleave fibrillar collagens at a specific site between Gly775 and Leu:Ile776 of the α chains, generating N-terminal 3:4 and C-terminal 1:4 fragments, which rapidly denature to gelatin at body temperature and become susceptible to degradation by other MMPs, e.g., gelatinases (MMP-2 and MMP-9), MMP-8 and serine proteinases [13].

MMP-1 (interstitial or fibroblast collagenase or collagenase-1) is the major collagenase able to degrade native fibrillar collagens type III, I, and II, at a specific site three-fourths from the N-terminus. MMP-1 is produced by various types of cells, e.g., fibroblasts, keratinocytes, endothelial cells, macrophages, hepatocytes, chondrocytes, and osteoblasts [4].

MMP-8 (collagenase-2) degrades collagen type I, type II (the predominant collagen of cartilage), and nonmatrix proteins such as serpins, bradykinin, angiotensin I, and substance P [13].

MMP-8 is synthesized by polymorphonuclear leukocytes (PMN) during their maturation in bone marrow, in myelocyte stage, stored in the intracellular specific granules, as a latent enzyme (pro-MMP-8, of 85 kDa), and secreted in response to external stimuli. Pro-MMP-8 is rapidly released from activated PMN undergoing degranulation and is activated *via* the

cysteine switch mechanism to yield the active form of the enzyme, of 65 kDa.

Activation may be achieved *in vitro* by organomercurials, serine proteinases, MMP-3, and reactive oxygen species. MMP-8 is involved in wound healing and tissue remodeling [13].

MMP-8 is also expressed by chondrocytes, endothelial cells [14], activated macrophages, smooth muscle cells, and postpartum mouse uterus.

MMP-13 (collagenase-3) cleaves type II collagen more effectively than type I and III collagens and displays stronger gelatinolytic activity than MMP-1 and MMP-8. Both human and mouse MMP-13 cleave type I collagen in the N-terminal nonhelical telopeptide.

The N-telopeptidase activity is required for resorption of type I collagen during fetal development and early postnatal mice life, whereas triple helix activity appears to be required during intense collagen resorption, e.g., involution of postpartum uterus, as well as in normal turnover of collagen in murine skin [15].

Human MMP-13 is highly homologous (86% at the amino acid level) to rat and mouse interstitial collagenases. MMP-13 is characterized by wide substrate specificity and limited expression compared to other MMPs. In addition to fibrillar collagens and gelatin, MMP-13 degrades type IV, IX, X, and XIV collagens, the large tenascin C isoform, fibronectin, laminin, aggrecan core protein, fibrillin-1, and serine proteinase inhibitors. Latent MMP-13 is activated by stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), 72-kDa gelatinase (MMP-2), MT1-MMP (MMP-14), MT2-MMP (MMP-15), trypsin, and plasmin and its activity is inhibited by tissue inhibitor of metalloproteinases, TIMP-1 and TIMP-3, and less potently by TIMP-2. MMP-13 activates latent MMP-2 and 92-kDa gelatinase (MMP-9) [15].

Gelatinases

Gelatinases are MMP-2 and MMP-9; they incorporate three fibronectin type II modules that provide a compact collagen-binding domain [8] and degrade type IV, V, VII, X, XI, and XIV collagens, gelatin, elastin, proteoglycan core proteins, myelin basic protein, fibronectin, fibrillin-1, and precursors of TNF- α and IL-1b. Gelatinases have an important role in ECM degradation and remodeling in various physiological states, such as implantation and wound healing [9].

MMP-2 (gelatinase-A, 72-kDa type IV collagenase) cleaves collagen type IV, the major BM constituent, as well as degraded collagen and some noncollagenous ECM glycoproteins [16].

MMP-2 also degrades native type I collagen. TNF- α and β stimulate MMP-2 production and early conceptus (blastocyst) and IFN- τ repress MMP-2 production. MMP-2 is expressed by various cell types, including fibroblasts, keratinocytes, endothelial cells, chondrocytes, osteoblasts, and monocytes [9].

MMP-9 (gelatinase-B, 92-kDa gelatinase) cleaves N-terminal telopeptide of type I collagen in an acidic environment, playing a role in the remodeling of

collagenous ECM. MMP-9 is produced by normal alveolar macrophages, polymorphonuclear leukocytes, osteoclasts, keratinocytes, and invading trophoblasts [9].

Stromelysins

Stromelysins are MMP-3 (stromelysin-1), MMP-10 (stromelysin-2), and MMP-11 (stromelysin-3), representing enzymes that degrade types IV and IX collagens, laminin, fibronectin, elastin, and proteoglycans [4].

Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) are closely related with respect to structure and substrate specificity. MMP-3 and MMP-10 degrade a wide range of ECM proteins, e.g., type IV, V, IX, and X collagens, proteoglycans, gelatin, fibronectin, laminin, and fibrillin-1. MMP-3 and MMP-10 are expressed by fibroblastic cells and by normal epithelial cells in culture and *in vivo* [4].

Stromelysin-1 (MMP-3) cleaves α 1-proteinase inhibitor, tumor necrosis factor (TNF)- α precursor, myelin basic protein, degrades and inactivates interleukin (IL)-1b, exhibiting a higher proteolytic efficiency than that of MMP-10. MMP-3 activates a number of pro-MMPs, and its action on a partially processed pro-MMP-1 is critical for the generation of fully active MMP-1 [4]. Progesterone is able to suppress MMP-3 and MMP-3 protein secretion *in vitro* [10].

Stromelysin-3 (MMP-11) together with matrilysin (MMP-7) and metalloelastase (MMP-12) are often included in the stromelysin subgroup, although they are structurally less closely related to MMP-3 and MMP-10. MMP-11 degrades serine proteinase inhibitors, α 1-proteinase inhibitor and α 1-antitrypsin. MMP-11 is expressed in uterus, placenta, and involuting mammary gland [4]. The expression of MMP-11 can be suppressed by progesterone treatment in isolated stromal cells *in vitro* [10].

Matrilysins

The matrilysins are characterized by the lack of a hemopexin domain, and are represented by matrilysin-1 (MMP-7) and matrilysin-2 (MMP-26 or endometase). Matrilysins, MMP-9, and MMP-12 are capable of cleaving human plasminogen to produce an angiostatin fragment that is a circulating inhibitor of angiogenesis [5]. Beside ECM components, MMP-7 processes cell surface molecules such as pro- α -defensin, Fas-ligand, pro-tumor necrosis factor (TNF)- α , and E-cadherin [5].

Matrilysin-1 (MMP-7) is secreted as a 28-kDa proenzyme and can be activated through proteolytic removal of a 9-kDa prodomain from the N-terminus [5]. Zymography can separate inactive and active forms, according their molecular weight and recently developed *in situ* zymography using a carboxymethylated transferring as a substrate enables determination of active enzyme *in vivo* [17].

Promatrilysin can be activated by endoproteinases, plasmin, trypsin, and experimentally by incubation with mercurial compounds such as 4-aminophenyl mercuric

acetate (APMA). In addition to a wide range of ECM components, including fibronectin, laminin, nidogen, type IV collagen, and proteoglycans, MMP-7 cleaves β 4 integrin.

Matrilysin also plays a role in the maintenance of innate immunity in lungs, intestine, by proteolytically activating antibacterial peptides such as prodefensins [18]. MMP-7 is expressed by normal polarized glandular epithelial cells in endometrium, mammary gland, parotid, liver, pancreas, prostate, small intestinal crypts, skin, peribronchial glands and conducting airways in the lung, therefore its function may be influenced by its release to either the apical or basolateral compartments, or both [19].

Immunohistochemical analyses have shown matrilysin staining primarily apical and luminal compared with basolateral location within cells [19]. MMP-7 sheds the ectodomain of membrane-bound FasL (mFasL) from cell membranes to generate soluble FasL (sFasL) [5]. Released sFasL increases apoptosis in surrounding cells through the activation of Fas. Also tumor necrosis factor- α (TNF- α) may be cleaved from the cell surface by MMP-7, producing a bioactive cytokine, soluble TNF- α , which may increase apoptosis through binding to the TNF-receptor 1 [5].

Progesterone has been shown to increase stromal cell expression of transforming growth factor (TGF)- β *in vivo*, and this growth factor appears to be required for progesterone suppression of MMP-7 *in vitro* [10].

Matrilysin-2 (MMP-26 or endometase) is the smallest MMP currently known, of 261 amino acids long and contains the signal sequence for secretion, prodomain with an unclassical cysteine-switch for preservation of latency and the catalytic domain with the Zn^{2+} -ion. MMP-26 can undergo autocatalytic activation, a feature exceptional among MMP.

MMP-26 degrades *in vitro* type IV collagen, fibronectin, fibrinogen, vitronectin, denatured collagen types I-IV, α 1-antitrypsin, α 2-macroglobulin, and insulin-like growth factor-binding protein 1 (IGFBP-1) [20].

Additionally, it activates pro-MMP-9 in a specific site to create gelatinase species that are more stably active than when processed by, e.g., MMP-7. TIMP-2 and TIMP-4 can inhibit MMP-26 activity. A significant level of MMP-26 expression in healthy tissues has been found to date by northern analysis in the uterus, kidney, and placenta. Therefore, MMP-26 might be involved in implantation [20].

Membrane-type MMPs (MT-MMPs)

Membrane-type MMPs (MT-MMPs) are transmembrany or anchored proteins, in a total number of six.

Type I transmembrane proteins are: MMP-14 or MT1-MMP, MMP-15 or MT2-MMP, MMP-16 or MT3-MMP, and MMP-24 or MT5-MMP and **Glycosylphosphatidylinositol (GPI) anchored proteins** are: MMP-17 or MT4-MMP and MMP-25 or MT6-MMP [21, 22]. They are almost all (excepting MT4-MMP) capable of activating pro-MMP-2 and to

digest a number of ECM molecules. MT–MMPs contain a *transmembrane domain* of 20 hydrophobic amino acids in the C-terminal end of the hemopexin domain followed by a 24 amino-acid intracellular domain [22, 23].

MT–MMPs contain a cleavage site for furin proteinases between propeptide and catalytic domain, providing the basis for furin-dependent activation of latent MT–MMPs prior to secretion [22, 23].

The furin-like cleavage site is also present in three secreted MMPs (MMP–11, MMP–21 and MMP–28) and in two unusual transmembrane MMPs (MMP–23A and MMP–23B), which are anchored through a N-terminal segment and show identical amino acid sequence, despite being encoded by two distinct human genes [8].

MT1–MMP activates latent MMP–2 and MMP–13 at the cell membrane. MT1–MMP has collagenolytic activity on type I, II, and III collagens. MT1–MMP also cleaves gelatin, fibronectin, laminin–1, vitronectin, cartilage proteoglycans, and fibrillin–1. MT1–MMP also plays an important role in angiogenesis [24]. MT1–MMP is expressed by dermal fibroblasts, and osteoclasts [22].

MT2–MMP activates pro-MMP–2 and pro-MMP–13 and degrades laminin, fibronectin, and tenascin. MT2–MMP is expressed in human placenta, brain, and heart [22].

MT3–MMP activates pro-MMP–2, through formation of a trimolecular complex comprised of MT3–MMP, TIMP–3, and pro-MMP–2 (Zhao H, 2004). MT3–MMP hydrolyzes gelatin, casein, type III collagen, and fibronectin, and can be detected both in membrane-bound and soluble forms. MT3–MMP is expressed in lung, placenta, kidney, ovary, intestine, prostate, spleen, heart, and skeletal muscle [22].

MT4–MMP may have a role in the regulation of cell surface proteins, acting as a TNF- α -converting enzyme, and as a glycosylphosphatidylinositol-anchored enzyme. MT4–MMP high expression in leukocytes in combination with its potential in ectodomain shedding events and ability to degrade fibrinogen and fibrin may indicate a role in inflammatory processes, although its true role remains to be ascertained. MT4–MMP is expressed in the brain, leukocytes, colon, ovary, and testis [26].

MT5–MMP activates latent MMP–2 and is predominantly expressed in brain, kidney, pancreas, and lung. Interestingly, MT5–MMP is also shed from the cell membrane, suggesting that it may function both as membrane-bound and soluble proteinase [21, 22].

MT6–MMP (MMP–25) is a membrane-bound MMP that is specifically expressed in leukocytes, being originally named leukolysin [27]. MT6–MMP is a GPI-anchored proteinase [28]. MT6–MMP can be released from neutrophils by extracellular stimuli (IL8, phorbol-12-myristate-13-acetate) [29].

MT6–MMP mRNA is expressed at high levels in testis, kidney and skeletal muscle [30]. MT6–MMP cleaves collagen type 4, gelatin, fibronectin and fibrin. The enzyme does not cleave laminin–1 and cannot

activate the proform of MMP–9 [31] but activates the proform of MMP–2 [32, 33].

MT6–MMP also cleaves and inactivates α 1-proteinase inhibitor, a known inactivator of destructive serine proteinases at inflammatory sites [34].

Other MMPs

Other MMPs group: MMP–12 (macrophage metalloelastase), MMP–18, MMP–19, MMP–20 (enamelysin), MMP–21, MMP–22, MMP–23 (cysteine array MMP), MMP–27, and MMP–28 (epilysin) [21].

MMP–12 (macrophage metalloelastase) degrades elastin, type IV collagen, type I gelatin, fibronectin, laminin, vitronectin, proteoglycans, myelin basic protein, and α 1-antitrypsin. MMP–12 is expressed in placenta [21].

MMP–18 (MMP–19 precursor) is expressed in a wide variety of normal human tissues, including mammary gland, placenta, lung, pancreas, ovary, small intestine, spleen, thymus, prostate, testis, colon, and heart [35].

MMP–19 (RASI) is expressed in leukocytes and in a wide variety of human tissues specimens (colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart). MMP–19 may be a target of MT1–MMP mediated activation in normal tissues in which both MMPs appear to be coordinately expressed. Recently, MMP–19 effects on proliferation, migration, and adhesion of keratinocytes were described [36, 37].

MMP–20 (enamelysin) was cloned from odontoblasts, has a restricted expression in dental tissues and degrades amelogenin. Recently, enamelysin has been demonstrated in the secretory enamel of developing human teeth and in the immature enamel or amyloid matrix of certain odontogenic tumors [38].

MMP–21 contains a haemopexin-like C-terminal domain and expresses a furin cleavage motif, followed by the catalytic domain representing the active site of the enzyme. Neither the hinge regions nor the transmembrane and cytoplasmic domains are present in the peptide sequence of MMP–21.

The MMP–21 mRNA was identified in fetal brain, kidney and liver. In adults, MMP–21 was found primarily in ovary, kidney, liver, lung, placenta, brain and peripheral blood leukocytes. MMP–21 has an important specific function in embryogenesis, especially in neuronal cells [12].

MMP–22 (MMP–23B or MMP–23 precursor or femalysin) was firstly cloned from chicken fibroblasts, and named CMMP. Sequence comparisons confirmed CMMP was a metalloproteinase, but homology between chicken and human MMPs was too low to assign CMMP as a human ortholog. Several years later CMMP was given the moniker MMP–22. The signal sequence, and lack of a transmembrane domain lead to the assumption that MMP–22 is a secreted MMP, and is speculated that the basic isoelectric point might direct the MMP–22 to interact with ECM [39].

MMP–22 contains no signal peptide or hemopexin-like domain. The enzyme lacks the cysteine switch

motif in the prodomain and the hemopexin domain; instead, it has a cysteine-rich domain followed by an immunoglobulin-like domain. It is proposed to be a type II membrane protein harboring the transmembrane domain in the N-terminal part of the propeptide. Because it has a furin recognition motif in the propeptide, it is cleaved in the Golgi and released as an active enzyme into the extracellular space [39].

MMP-23 is mainly expressed in reproductive tissues [40]. MMP-23 lacks the hemopexin domain and has a novel cysteine array motif and an immunoglobulin-like C2 type fold domain [40].

MMP-27 has all the key domains of a typical MMP and degrades casein. MMP-27 is expressed in normal, intact tissues, such as testis, intestine, lung, and skin, and this pattern of expression suggests that this MMP may serve a role in tissue homeostasis.

It is tempting to speculate that MMP-27 participates in host defense in intact epidermis by processing antimicrobial proteins. Indeed, because it is expressed by basal and suprabasal keratinocytes, released epilysin may not encounter a matrix substrate in intact skin. Thus, though MMP-27 is a member of the matrix metalloproteinases gene family, matrix components are not physiologic substrates for this enzyme [41].

MMP-28 (epilysin) belongs to the MMP-19 subfamily of the MMP superfamily [41]. In contrast to most other soluble MMP, MMP-28 has a furin activation sequence (RRKKR) [42]. MMP-28 mRNA is expressed in human placenta [41].

This suggests a possible role for this novel protease in the tissue remodelling processes in placenta. Recently, it has been suggested that MMP-28 is associated with cell proliferation via restructuring the basement membrane or degrading adhesive proteins between keratinocytes [43].

Three-dimensional (3D) structures of MMPs

The 3D structure of MMPs was indicated by X-ray crystallography and nuclear magnetic resonance (NMR). Prodomain of MMP-2, MMP-3, and MMP-9 consists of three α -helices and connecting loops. The first loop is a protease-sensitive "bait region" and a peptide region after helix 3 lies in the substrate-binding cleft of the catalytic domain. The conserved cysteine switch forms a fourth ligand of the active-site zinc, keeping the zymogen inactive [4].

The polypeptide chain consists of a 5-stranded β -pleated sheet, three α -helices, and connective loops. This proteinase domain contains one catalytic zinc, coordinated by three histidines, one structural zinc, and, generally, three calcium ions. The glutamic acid adjacent to the first histidine is essential for catalysis. Loop region contains a base to support the structure around the catalytic zinc named "Met-turn". The fourth ligand of the catalytic zinc is a water molecule [4].

Three repeats of fibronectin type II domains are inserted between the fifth β -strand and the catalytic site helix, in MMP-2 and MMP-9 [4]. Fibronectin domain consists of two antiparallel β -sheets, connected with a short α -helix and stabilized by two disulfide bonds.

Domains 2 and 3 are flexible, interacting simultaneously with multiple sites in the ECM [4].

Substrate binding into the catalytic site cleft displaces the water molecule from the zinc atom. The peptide hydrolysis is assisted by the carboxyl group of the glutamate, associated with the nucleophilic attack of the water molecule on the carbonyl carbon of the peptide scissile bond [4].

A pocket of the active-site zinc, named the specificity pocket or S1' pocket, accommodates the side chain of the substrate residue, that becomes the new N-terminus after cleavage [4]. Substrate specificity is associated with the S1' pocket sizes.

The hemopexin domains have a 4-bladed β -propeller fold, with a single stabilizing disulfide bond. The hemopexin domains of MMP-9 form an asymmetric homodimer as a result of shifts in blade III and IV structure on dimerization. The hemopexin domain of MMP-9 binds the C-terminal domain of TIMP-1, MMP-9-TIMP-1 complex and the MMP-9 dimer being mutually exclusive, because of an overlap in the TIMP-1-binding site and the dimer interface. The crystal structure of pro-MMP-2-TIMP-2 complex shows that this interaction is through the C-terminal domain of TIMP-2 and blades III and IV of the hemopexin domain, leaving the N-terminal inhibitory domain of TIMP-2 able to interact with other MMPs [4].

β -Propeller domains with a larger number of blades of heterotrimeric G proteins, clathrin, and the α -subunit of integrins often mediate protein-protein interactions. Hemopexin-like domain is important for substrate specificity, being required for proMMP-2 activation and for the dimerization of MT1-MMP and MMP-9 [4].

☞ Regulation of MMP activity

Since MMPs are produced as zymogen forms, expression of the MMPs genes and proteins is not enough for their functioning but activation of MMPs is one of the key steps for their *in vivo* proteinase action [21]. MMP expression is strictly controlled at the levels of transcription, secretion, activation, and inhibition of the activated enzyme. Proenzymes and active forms of MMP are controlled by stoichiometric binding of specific, locally produced tissue inhibitors of metalloproteinases (TIMPs: TIMP-1, TIMP-2, TIMP-3, and TIMP-4) [6].

In vitro, MMPs can be activated by a number of proteases, including plasmin, MMP-3, and MT-MMPs, or by treatment with organomercurial compounds. Most of these enzymes are expressed at low levels in adult tissues, and many are up-regulated during normal and pathological remodeling processes [8].

Most MMPs are not constitutively expressed by cells *in vivo*, but their expression is induced by exogenous signals, e.g., cytokines, growth factors, or altered cell-matrix and cell-cell contacts. However, MMP-8 and MMP-9 are stored in secretory granules of neutrophils and eosinophils and MMP-7 in secretory epithelial cells of exocrine glands.

Expression of MMPs is regulated at the level of transcription, although modulation of MMP mRNA half-life by growth factors and cytokines has also been documented. The proteolytic activity of MMPs is regulated by zymogen activation and inhibition by specific inhibitors, i.e., TIMPs, and by nonspecific proteinase inhibitors, e.g., α 1-proteinase inhibitor and α 2-macroglobulin [8].

Transcriptional regulation

Expression of most MMPs (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13, and MMP-19) is induced at the transcriptional level, e.g., by growth factors and cytokines, hormones, and contact to the ECM.

Accordingly, MMP induction mechanisms appear to be different depending on the characteristics of the diverse cells with ability to produce these enzymes. The promoters of these inducible MMPs contain a conserved AP-1 *cis*-element with respect to the transcription start site. The extracellular stimuli activate the AP-1 transcription factor complexes (dimers composed of members of *Fos* and *Jun* families), which bind to the AP-1-binding site in the promoter and stimulate transcription of the MMP genes.

The promoter regions of the AP-1-responsive MMPs also contain one or multiple polyomavirus enhancer A-binding protein-3 (PEA3) elements, which bind transcription factors of the ETS family and cooperate with the AP-1 element for maximal activation of MMP-1, MMP-3, and MMP-9 promoters. Transcription factor NF- κ B plays a pivotal role in expression of MMP-9 in fibroblasts and vascular smooth muscle cells [8].

The promoter of the MMP-2 gene is relatively unresponsive to stimulation in cultured cells and it lacks not only the adjacent AP-1 and PEA3 elements, but also the classical TATA box. Different types of cells in culture also constitutively express MT1-MMP. The induction of expression of the components of the classical AP-1 dimer, *c-Jun*, and *c-Fos* is mediated by three distinct classes of mitogen-activated protein kinases (MAPKs), i.e., extracellular signal-regulated kinase (ERK), stress-activated protein kinase:Jun N-terminal kinases (SAPK:JNKs), and p38 [44–47].

In general, the ERK1, 2 cascade is activated by mitogenic signals, resulting in phosphorylation of various substrates, including Elk-1, and in subsequent activation of *c-fos* transcription. SAPK:JNKs and p38 are activated by cytokines (TNF, IL-1) and cellular stress, such as UV light, resulting in phosphorylation of *c-Jun* and ATF-2, which then induce *c-jun* transcription. The balance between distinct MAPK pathways is thought to regulate cell growth, differentiation, survival, and death. Distinct MAPKs play an important role in the regulation of MMP expression [8].

TGF- β or retinoids usually repress MMP transcription. However, there are several exceptions to this situation, since some family members such as MMP-11 or MMP-13 can be induced rather than repressed by these factors in diverse cell types [8].

Activation of latent MMPs (Pro-MMPs)

Most MMPs are secreted as latent precursors (zymogens), which are proteolytically activated in the extracellular space. Latent MMPs are retained in the proform by a “cysteine switch” formed by covalent interaction of the conserved cysteine in the propeptide with the catalytic zinc.

Various compounds, e.g., organomercurials, a variety of denaturant agents, can react with cysteine, converting it to a nonbinding form, exposing the catalytic site, and resulting in autocatalytic cleavage of the propeptide, *in vitro*.

The propeptide of most MMPs can also be cleaved by a number of other extracellular proteinases, e.g., plasmin and other MMPs [48].

Stepwise activation mechanism

Chemical agents, such as thiol-modifying agents (4-aminophenylmercuric acetate, HgCl₂, and N-ethylmaleimide), oxidized glutathione, SDS, chaotropic agents, and reactive oxygen can activate by proteinases or *in vitro* mMPs. Low pH and heat treatment can also lead to activation. These agents most likely work through the disturbance of the cysteine–zinc interaction of the “cysteine switch”. Studies of pro-MMP-3 activation with a mercurial compound have indicated that the initial cleavage occurs within the propeptide and that this reaction is intramolecular rather than intermolecular [48].

The subsequent removal of the rest of the propeptide is due to intermolecular reaction of the generated intermediates. Proteolytic activation of MMPs is stepwise in many cases. The initial proteolytic attack occurs at an exposed loop region between the first and the second helices of the propeptide. The cleavage specificity of the bait region is dictated by the sequence found in each MMP.

Once a part of the propeptide is removed, this probably destabilizes the rest of the propeptide, including the cysteine switch-zinc interaction, which allows the intermolecular processing by partially activated MMP intermediates or other active MMPs. Thus, the final step in the activation is conducted by an MMP. *In vivo*, MMP activation requires the participation of other proteases to remove the propeptide domain. In most cases, these activating proteases form part of a proteolytic cascade that take place in the immediate pericellular space [48].

Activation of pro-MMPs by plasmin is a relevant pathway *in vivo*. Plasmin is generated from plasminogen by tissue plasminogen activator bound to fibrin and urokinase plasminogen activator bound to a specific cell surface receptor. Both plasminogen and urokinase plasminogen activator are membrane-associated, thereby creating localized pro-MMP activation and subsequent ECM turnover. Plasmin has been reported to activate pro-MMP-1, pro-MMP-3, pro-MMP-7, pro-MMP-9, pro-MMP-10, and pro-MMP-13. Activated MMPs can participate in processing other MMPs. The stepwise activation system may have evolved to accommodate finer regulatory mechanisms to control destructive

enzymes, inasmuch as TIMPs may interfere with activation by interacting with the intermediate MMP before it is fully activated [48].

Intracellular activation

Most pro-MMPs are secreted from cells and activated extracellularly. Pro-MMP-11 is activated intracellularly by furin. Pro-MMP-11 possesses a furin recognition sequence, KX(R/K)R, at the C-terminal end of the propeptide. Several other MMPs, including the six MT-MMPs, MMP-23, and MMP-28, have a similar basic motif in the propeptide which allows their intracellular activation by furin-like proprotein convertases [22]. Because these proteins are most likely secreted as active enzymes, their gene expression and inhibition by endogenous inhibitors would be critical for the regulation of activity.

Cell surface activation of Pro-MMP-2

Pro-MMP-2 is not readily activated by general proteinases. The main activation of pro-MMP-2 takes place on the cell surface and is mediated by MT-MMPs [22, 33, 49]. This includes MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MT6-MMP. MT4-MMP does not activate pro-MMP-2.

MT1-MMP-mediated activation of pro-MMP-2 has been studied extensively. The unique aspect is that it requires the assistance of TIMP-2. The transmembrane MT1-MMP interacts via its N-terminal domain to the N terminus of TIMP-2, forming a "receptor" onto which pro-MMP-2 (72 kDa) binds. Pro-MMP-2 is initially cleaved to its intermediate form (64 kDa) by an adjacent active MT1-MMP.

The second stage of MMP-2 processing, resulting in its fully active form (62 kDa), involves an autocatalytic event that requires a MMP-2 molecule in *trans*. The cell surface-bound pro-MMP-2 is then activated by an MT1-MMP that is free of TIMP-2. Alternatively, MT1-MMP inhibited by TIMP-2 can act as a "receptor" of pro-MMP-2. This trimolecular MT1-MMP-TIMP-2-proMMP-2 complex is then presented to an adjacent free MT1-MMP for activation [50].

Clustering of MT1-MMP on the cell surface through interactions of the hemopexin domain facilitates the activation process. The maximum enhancement of pro-MMP-2 activation is observed at a TIMP-2/MT1-MMP ratio of 0.05, suggesting that a large number of free MT1-MMP may surround the ternary complex of pro-MMP-2-TIMP-2-MT1-MMP for effective pro-MMP-2 activation. Pro-MMP-2 activation by MT2-MMP is direct and independent of TIMP-2. Interestingly, TIMP-4 binds to the pro-MMP-2 hemopexin domain, and it inhibits MT1-MMP, but it does not result in pro-MMP-2 activation by MT1-MMP. The reason for this is not clear, but it may be due to an incorrect molecular assembly with TIMP-4.

MT1-MMP also activates proMMP-13 on the cell surface; this activation is more efficient in the presence of active MMP-2. The activation of pro-MMP-13 by MT1-MMP is independent of TIMP-2 but requires the C-terminal hemopexin domain of pro-MMP-13 [50].

Alternative MMP activation mechanisms may be based on the formation of a S-nitrosylated derivative with the thiol group of the cysteine switch [51] or by the *in vivo* MMP binding to a ligand or to a substrate [8, 52].

Endogenous inhibitors. Substrate specificity of MMPs

Substrate specificities of MMPs have been studied either by identifying the cleavage sites of protein substrates or by a series of synthetic peptide substrates. In general, MMPs cleave a peptide bond before a residue with a hydrophobic side chain, such as Leu, Ile, Met, Phe, or Tyr [8]. A peptide bond with a charged residue at this position is rarely cleaved, with the cleavage of the X-Lys bond by MMP-12 being an exception.

The hydrophobic residues fit into the S1' specificity pocket, whose size and shape differ considerably among MMPs. In addition to the S1' pocket, other substrate contact sites (subsites) also participate in the substrate specificity of the enzyme.

In some cases, noncatalytic domains influence the enzyme activity, particularly against large extended macromolecules of the ECM. For example, the fibronectin domains of MMP-2 and MMP-9 are important for its activity on type IV collagen, gelatin, and elastin. In collagenase 1 (MMP-1), the loop region just before the catalytic site helix is essential for collagenolytic activity. Furthermore, the hemopexin domain and the hinge between the catalytic and the hemopexin domains also play key roles in collagenolysis [8].

Physiological functions of MMPs

By regulation of ECM proteins and of numerous soluble factors, MMPs are involved in embryogenesis and in several physiological activities, mainly in the reproductive system. Matrilysin, stromelysins, gelatinase A, collagenase-2, and collagenase-3 are up-regulated during postpartum uterus involution [8].

Experimental studies demonstrate that matrilysin, stromelysins and gelatinase A are involved in estrous cycle and are involved, together with collagenase-2 and collagenase-3 in postpartum uterus involution. Experiments using mutant mice deficient in specific MMPs suggest a functional redundancy among MMPs, or between these enzymes and components of the plasminogen system [8, 53, 54].

MMP-9 is responsible for implantation and for endochondral bone formation [8]. MT1-MMP is involved in skeletal and connective tissue development and in angiogenesis [8].

MMPs are involved in tissue remodeling: MMP-2 and MMP-3 regulate mammary gland branching morphogenesis [55], MMP-2 and MMP-9 contribute to adipogenesis [56], and MMP-1 is required for keratinocyte migration [8].

MMP-2 and MMP-9 have synergic effect in angiogenesis [57], being associated with MMP-13,

MT1–MMP, as demonstrated by inhibition by endostatin (endogenous angiogenic inhibitor) [58].

Biological activities generated by MMP-mediated cleavage

A major function of MMPs is thought to be the removal of ECM in tissue resorption. However, the ECM is not simply an extracellular scaffold; it also acts as a reservoir of biologically active molecules, such as growth factors. Some ECM components can express cryptic biological functions on proteolysis. Hence, degradation of ECM components by MMPs can alter cellular behavior and phenotypes [8] (Table 2).

MMP–2–MMP and MT1–MMP–mediated cleavage of the γ_2 chain of laminin 5 exposes a cryptic promigratory site and promotes the migration of normal breast epithelial cells [8].

Cleavage of CD44 by MT1–MMP is associated with cell migration. MMP–2 expressed in the Schwann cells of peripheral nerves degrades chondroitin sulfate proteoglycans and promotes neurite growth. The expanding number of non-ECM proteins that are MMP substrates and exert biological activities adds new dimensions to the complexity of MMPs' role in health and disease[8].

Table 2 – Biological activities of MMPs listed together with their specific substrates (adapted from: VISSE R., NAGASE H., Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function and biochemistry, *Circ Res*, 2003, 92(8):827–839) [4]

| Biological effect | Responsible MMPs | Substrate cleaved |
|--|---|--|
| Keratinocyte migration and reepithelialization | MMP–1, MMP–19 | Type I collagen |
| Osteoclast activation | MMP–9, MMP–13 | Type I collagen |
| Ossification | MMP–9, MMP–14 | Not identified |
| Neurite outgrowth | MMP–2 | Chondroitin sulphate proteoglycan |
| Adipocyte differentiation | MMP–3, MMP–7 | Fibronectin |
| Cell migration | MMP–1, MMP–2, MMP–3, MMP–26 | Fibronectin |
| Cell migration | MT1–MMP | CD44 |
| Mammary epithelial cell apoptosis | MMP–3 | Basement membrane |
| Mammary epithelial alveolar formation | MMP–2, MMP–3 | Basement membrane |
| Epithelial-mesenchymal conversion (mammary epithelial cells) | MMP–3 | E-cadherin |
| Alveolar branching morphogenesis | MMP–3 | Not identified |
| Innate intestinal immunity | MMP–7 | α -defensin (cryptidin) in Paneth cells |
| Mesenchymal cell differentiation with inflammatory phenotype | MMP–2 | Not identified |
| Platelet aggregation | MMP–1 | Not identified |
| Generation of angiostatin-like fragment | MMP–2, MMP–3, MMP–7, MMP–9, MMP–12 | Plasminogen |
| Generation of endostatin-like fragment | MMP–3, MMP–7, MMP–9, MMP–12, MMP–13, MMP–20 | Type XVIII collagen |

| Biological effect | Responsible MMPs | Substrate cleaved |
|--|---|--|
| Generation of neostatin–7 fragment | MMP–7 | Type XVIII collagen |
| Generation of tumstatin fragment | MMP–9 | Type IV collagen |
| Enhanced collagen affinity | MMP–2, MMP–3, MMP–7, MMP–9, MMP–13 | BM–40 (SPARC/osteonectin) |
| Kidney tubulogenesis | MT1–MMP, MMP–2, MMP–3, MMP–9 | Type I collagen |
| Release of bFGF | MMP–3, MMP–13 | Perlecan |
| Increased bioavailability of IGF1 and cell proliferation | MMP–1, MMP–2, MMP–3, MMP–7, MMP–9, MMP–19, MMP–11, MMP–26 | IGFBP–3, IGFBP–5, IGFBP–1 |
| Activation of VEGF | MMP–1, MMP–3, MMP–7, MMP–9, MMP–13 | CTGF (connective tissue growth factor) |
| Epithelial cell migration | MMP–2, MT1–MMP | Laminin 5 γ 2 chain |
| Embryonic implantation | MMP–9 | Not identified |
| Apoptosis (amion epithelial cells) | MMP–1, MMP–3, MMP–8 | Type I collagen |
| Endometrial menstruation | MMP–1, MMP–3, MMP–7, MMP–9 | Collagen |
| Proinflammatory | MMP–1, MMP–3, MMP–9 | Processing IL–1 β from the precursor |
| Proinflammatory | MMP–8 | Type I and II collagen |
| Tumor cell resistance | MMP–9 | ICAM–1 |
| Decreased tumor growth | MMP–2, MMP–11 | Endothelial cells |
| Antiinflammatory | MMP–1, MMP–2, MMP–9 | IL–1 β degradation |
| Antiinflammatory | MMP–1, MMP–2, MMP–3, MMP–13, MMP–14 | Monocyte chemoattractant protein-3 |
| Increased bioavailability of TGF– β | MMP–2, MMP–3, MMP–7 | Decorin |
| Disrupted cell aggregation and increased cell invasion | MMP–3, MMP–7 | E-cadherin |
| Reduced cell adhesion and spreading | MT1–MMP, MT2–MMP, MT3–MMP | Cell surface tissue transglutaminase |
| Fas receptor-mediated apoptosis | MMP–7 | Fas ligand |
| Reduced IL–2 response | MMP–9 | IL–2R α |

Endogenous MMP inhibitors

The activity of MMPs in the pericellular space is strictly controlled by nonspecific inhibitors, e.g., α_2 -macroglobulin and by the specific inhibitors, TIMPs.

Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are the naturally inhibitors of MMP activity [50]. TIMPs (21 to 29 kDa) have an N- and C-terminal domain of 125 and 65 amino acids, respectively, with each containing three conserved disulfide bonds [59]. The N-terminal domain folds as a separate unit and is capable of inhibiting MMPs [8]. NMR first solved the structure of the N-terminal domain of TIMP–2 in 1994. The complete structure of TIMP–1 and of the inhibition mechanism was determined by X-ray crystallographic

studies of the TIMP-1-MMP-3 complex, and soon after, that of the TIMP-2-MT1-MMP complex was determined [59].

The overall shape of the TIMP molecule is like a wedge, which slots into the active-site cleft of a MMP in a manner similar to that of the substrate. The main sites of interaction of TIMP-2 with the catalytic domain are the N-terminal four residues and the CD-loop region adjacent to them. The N-terminal four residues bind in the catalytic site cleft, making backbone contacts similar to those of a substrate.

Residues at 1 and 3 are strictly conserved cysteines that form disulfide bonds in the main body of the protein. Cys1 is instrumental in chelating the active-site zinc with its N-terminal α -amino group and carbonyl group, thereby expelling the water molecule bound to the catalytic zinc [8].

TIMPs contain 12 conserved cysteine residues, which form six disulfide bonds. TIMPs bind to the zinc-binding catalytic site of the MMPs with a 1 : 1 molar ratio.

At present, four members of the TIMP gene family are known: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.

TIMP-1 inhibits the activity of most MMPs, with the exception of MT1-MMP and MMP-2. TIMP-1 and TIMP-2 can bind to the hemopexin domain of latent MMP-9 and MMP-2, respectively. TIMP-1 binds to and inactivates MMP-1, MMP-2, MMP-3 and MMP-9. In addition, TIMP-1 has the capacity to form a complex with pro-MMP-9, thereby blocking the activation of the enzyme [60]. Expression of TIMP-1 in cultured cells is stimulated e.g., by growth factors, cytokines, and phorbol ester.

TIMP-2 inhibits the activity of most MMPs, except MMP-9 [8], but also to the latent form pro MMP-2. The complex between TIMP-1 with any of MMP-1, MMP-2, MMP-3 or MMP-9 leads to protease inactivation. A synthetic MMP inhibitor, BB94 blocks the gelatinolytic activity (measured by *in situ* zymography) [21].

Recent studies have focused on the dual role of TIMP-2 in regulating the processing of pro-MMP-2. A threshold level of TIMP-2 is required in relation to MT1-MMP to construct the trimolecular complex, which still leaves sufficient MT1-MMP uninhibited to cleave pro-MMP-2. At higher concentrations, TIMP-2 prevents MMP-2 processing by inhibiting all free MT1-MMP [61]. Expression of TIMP-2 is constitutive.

TIMP-3 inhibits the activity of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13. Expression of TIMP-3 is induced in response to mitogenic stimulation and during normal cell cycle progression and it is inhibited by TNF- α in fibroblasts. TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix, inhibits MMP-3 [60, 61].

MT1-MMP and MMP-2, TIMP-3 is unique among the TIMP family in that it is bound to the ECM rather than remaining a freely soluble protein and it has a broader inhibitory profile that extends to members of ADAM (a disintegrin and metalloproteinase domain) and ADAM-TS (aggrecanases – ADAMs with

thrombospondin domains) families, proteases that have the potential to control the bioactivity of many growth factors and cytokines [8, 50, 62]. Kinetic studies have indicated that TIMP-3 is a better inhibitor for ADAM-17 and aggrecanases than for MMPs.

TIMP-4, the most recently discovered member of the family is able to associate with and inhibit both MT1-MMP and MMP-2 [50, 63].

TIMP-4 inhibits the activity of MMP-2, MMP-9, and MMP-7. Expression of TIMP-4 *in vivo* is especially abundant in the heart, but it is also expressed at the sites of tissue injury, i.e., dermal wounds and vascular injury.

There is a clear potential for the application of TIMPs as endogenous inhibitors, especially because the results of clinical trials with small molecule inhibitors have been disappointing. However, expressing wild-type TIMPs could have drawbacks because multiple MMPs may be inhibited, and in the case of TIMP-3, ADAMs and ADAMTSs may be inhibited as well. Probably the best route to success will be the development of engineered TIMPs with altered specificity, to allow targeting of specific proteinases.

In addition to metalloproteinase-inhibiting activities, TIMPs have other biological functions. TIMP-1 and TIMP-2 have erythroid-potentiating activity and cell growth-promoting activities. TIMP-1 is expressed in the nucleus of fibroblasts. TIMP-2, but not TIMP-1, inhibits endothelial cell growth induced by basic fibroblast growth factor [64].

TIMP-2 inhibits endothelial cell proliferation *in vitro* and angiogenesis *in vivo* through a MMP-independent mechanism [60].

Likewise, TIMP-3 can also block the binding of VEGF to VEGF receptor-2, thereby inhibiting downstream signaling and angiogenesis [65].

TIMP-3 has proapoptotic activity, possibly through the stabilization of TNF- α cell receptor 1, Fas, and TNF-related apoptosis, inducing ligand receptor-1, as shown for some tumor cells. On the other hand, TIMP-1 and TIMP-2 have antiapoptotic activity [66].

Other inhibitors of MMPs

Several other proteins have been reported to inhibit MMPs. Tissue factor pathway inhibitor-2 is a serine protease inhibitor that inhibits MMPs. A C-terminal fragment of the procollagen C-terminal proteinase enhancer protein has been shown to inhibit MMP-2. The secreted form, membrane-bound β -amyloid precursor protein, has also been reported to inhibit MMP-2.

RECK (reversion-inducing cysteine-rich protein with kazal motifs), a GPI-anchored glycoprotein that downregulates the levels of MMP-9 and active MMP-2 and suppresses angiogenic sprouting, inhibits the proteolytic activity of MMP-2, MMP-9, and MT1-MMP [67, 68]. MMP-2, but not MMP-1, MMP-3, and MMP-9, is inhibited by chlorotoxin, a scorpion toxin [69].

Proteins such as plasma α -macroglobulins are general endopeptidase inhibitors that inhibit most

proteinases by trapping them within the macroglobulin after proteolysis of the bait region of the inhibitor. MMP-1 reacts with α_2 -macroglobulin more readily than it reacts with TIMP-1 in solution [70].

☐ Conclusions and future prospects

MMPs are involved in many biological processes, mainly because of their ability of ECM proteolysis, as an initiator of unrevealed functions. Recent progresses were made in understanding biochemical and structural aspects of MMPs, and their molecular complexes with TIMPs. The design of potent specific inhibitors for MMPs represents a challenge for scientists, not only for gaining insights into the biological roles of MMPs but also for the development of new therapies.

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