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Biological role of matrix metalloproteinases: a critical balance

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ABSTRACT: Matrix metalloproteinases (MMPs) are members of the metzincin group of proteases which share the conserved zinc-binding motif in their catalytic active site. It was originally thought that their main function is to degrade the various components of the extracellular matrix (ECM), yet recent studies have led us to appreciate their significance as regulators of extracellular tissue signalling networks. Due to the broad spectrum of their substrate specificity, MMPs contribute to the homeostasis of many tissues and participate in several physiological processes, such as bone remodelling, angiogenesis, immunity and wound healing. MMP activity is tightly controlled at the level of transcription, pro-peptide activation and inhibition by tissue inhibitors of MMPs. Dysregulated MMP activity leads to pathological conditions such as arthritis, inflammation and cancer, thus highlighting MMPs as promising therapeutic targets. Analysis of MMP mutant mice has proved to be an essential tool for the identification of novel functions and interactions of single MMP members. Advancing our understanding of the MMP contribution to tissue homeostasis will lead us to identify causal relationships between their dysregulation and the development of disease pathologies, thus guiding us to successful MMP-directed therapies.

KEYWORDS: Collagen, degradation, extracellular matrix, immunity, substrate, tissue inhibitor of metalloproteinase

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The matrixins or matrix metalloproteinases (MMPs) are members of the large metzincin superfamily like the astacins, serralysins, reprolysins, and adamalysins or disintegrin metalloproteinases (ADAMs). In the classical view, MMPs are collectively capable of degrading all components of the extracellular matrix (ECM) and basement membrane, restricting their functions to tissue remodelling and maintenance. However recent substrate identification studies reveal that MMPs are regulating the release or activation of chemokines, cytokines, growth factors, antibiotic peptides, and other bioactive molecules thus participating in physiological processes such as innate and adaptive immunity, inflammation, angiogenesis, bone remodelling, and neurite growth.

High sequence similarity to MMP catalytic domains is found in almost all kingdoms of life. At least 25 different vertebrate MMPs have been characterised up to now and 24 different MMPs are found in humans, including the two identical forms for MMP-23, encoded by two distinct genes, *i.e. MMP23A* and *MMP23B*. The diversity of the current mammalian MMP gene families is derived particularly from an extensive gene tandem duplication and exon shuffling during evolution in the tetrapod lineages. Taking this into account, some of the actual MMP members are most likely derivatives from a single gene resulting in a MMP gene cluster, whose organisation is preserved from amphibians to mammals. The cluster in the human genome is located at chromosome 11q22 and contains MMP-1, -3, -7, -8, -10, -12, -13, -20 and -27. In contrast, most of the other human MMP genes are located on different chromosomes, resulting in a total of 10 distinct chromosomes for all 24 human MMP genes [1].

Although the activity of MMPs has been shown to be essential in cell biological processes and many fundamental physiological events involving tissue

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remodelling, such as angiogenesis, bone development, wound healing and mammary involution [2], the increasing interest in MMP function mainly stems from their role in several pathological conditions, such as cancer or chronic inflammatory diseases [3].

This review provides an overview of the basic biological functions of these endopeptidases, paying special attention to novel findings on their complex regulation and broad substrate spectrum which has been recently significantly expanded through high-throughput degradomic screens. We further discuss recent information on *MMP* mutant mouse models which shed light on the functional relevance of these enzymes to multiple physiological and pathological processes.

REGULATION OF MMPS

Due to their widespread substrate spectrum, MMPs are integrated as important regulators for tissue homeostasis and immunity in the network of multidirectional communication within tissues and cells. Since uncontrolled MMP activity can easily become destructive and lead to breakdown of homeostasis, their activity has to be tightly regulated.

As shown in figure 1, the catalytic activity of MMPs is strongly controlled at four different levels: 1) gene expression with transcriptional and post-transcriptional regulation; 2) extracellular localisation and tissue or cell type of MMP release, termed compartmentalisation; 3) pro-enzyme activation by removal of the pro-domain; and 4) inhibition by specific inhibitors, *i.e.* tissue inhibitors of matrix metalloproteinases (TIMPs), and by non-specific proteinase inhibitors, *e.g.* α_2-macroglobulin. Once active, MMPs can modulate the global proteolytic potential in the extracellular milieu through zymogen (MMP pro-form) activation and inhibitor degradation or inactivation of other proteases [4, 5].

Modulation of MMP gene expression

Although MMP gene expression is primarily regulated at the transcriptional level, post-transcriptional control of mRNA stability by cytokines, nitric oxide or micro-RNA (miRNA) has been recently described as a significant contributing mechanism.

Despite the low expression of most MMPs under quiescent conditions, their transcription is tightly and individually regulated. No single cytokine, chemokine, oncogene or growth factor has been identified that is exclusively responsible for the overexpression of MMPs in special tumours, although tumour necrosis factor (TNF)-α and interleukin (IL)-1 are often implicated. The signal-transduction pathways that modulate MMP promoter activities are also diverse. Several of the MMP promoters share several cis-elements in their promoter regions, consistent with observations that some MMPs are co-regulated by various inductive stimuli, like growth factors and cytokines, and may also be co-repressed by glucocorticoid hormones and retinoids [6].

It is remarkable that promoters of functionally related MMPs such as MMP-2/MMP-9 (gelatinase) or MMP-1/MMP-8 (collagenase) are clearly distinct, pointing to different ways of activation. Based on their cis-element composition, MMP promoters can be categorised into three groups [7]. The first group represents most MMP promoters and contains a TATA box and an AP-1-binding site close to their transcription start and is very often combined with an upstream PEA3-binding site, for the control of MMP transcription by several cytokines and growth factors, like epidermal growth factor, keratinocyte growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor, TNF-α, and transforming growth factor (TGF)-β [8]. The MMP promoters in the second group (MMP-8, -11 and -21) also contain a TATA box, but lack a proximal AP-1 site. The regulation of these promoters is relatively simple and distinct from the first group promoters. The last group of promoters (including MMP-2, -14 and -28) does not harbour a TATA box, and expectedly, transcription from these promoters starts at multiple sites. Moreover, expression of MMPs in this latter group is mainly determined by the ubiquitous Sp-1 family of transcription factors, which bind to a proximal GC box. Expression of these MMPs in the main part is constitutive, with only modest sensitivity to induction by growth factors or cytokines [9].

The transcriptional control of MMPs is most likely additionally influenced by epigenetic mechanisms like DNA methylation and/or chromatin remodelling with histone acetylation. DNA methylation of cytosines within CpG islands in the promoter region is usually associated with a repressive chromatin state and inhibition of gene expression. Therefore, hypomethylation of MMP promoters can lead to increased enzyme expression in cancer, as observed for MMP-9 in lymphoma cells and for MMP-3 in colon cancer cells [7] or other inflammatory disorders like osteoarthritis, where chondrocytes expressed increased levels of MMP-3, MMP-9 and MMP-13 [10]. Chromatin remodelling is connected with reversible acetylation of histones (mainly H3 and H4) through histone acetyl transferases and leaves the chromatin in a more relaxed state, which is generally associated with gene activation. Epigenetic chromatin remodelling has been demonstrated for IL-1β-induced MMP-1 and MMP-13 expression with changes in histone acetylation states and increased binding of the AP-1 proteins. Conversely, repression of MMP-1 and MMP-13 expression by nuclear hormone receptors retinoid X receptor (RXR) involves deacetylation at this site, perhaps accompanied by decreased AP-1 binding [11]. However, further studies are necessary to understand the underlying epigenetic mechanisms that control MMP gene expression.

Post-transcriptional gene regulation can be driven by cytosolic mRNA stability, which is mediated *via* trans-acting RNA-binding proteins that interact with multiple AU-rich elements (ARE) mostly located in their 3′ untranslated regions (UTRs). The mRNA stability can be enhanced by increased binding of Hu protein family factors (*e.g.* HuR) to ARE elements or can decreased by increased binding of destabilising protein factors, like AUF1 to ARE elements, which promotes mRNA degradation by recruiting the exosome machinery. Indeed, the expression of at least MMP-2, MMP-9 and MMP-13 has been shown to be regulated through mRNA stability. As example, IL-1β-induced MMP-9 gene expression in rat renal mesangial cells was enhanced by ATPγS *via* an increased binding of HuR to the AREs in the 3′-UTR of MMP-9 mRNA. In contrast, incubation of the mesangial cells with nitric oxide induced the inhibition of HuR expression which in turn increased the MMP-9 mRNA decay [7, 12].

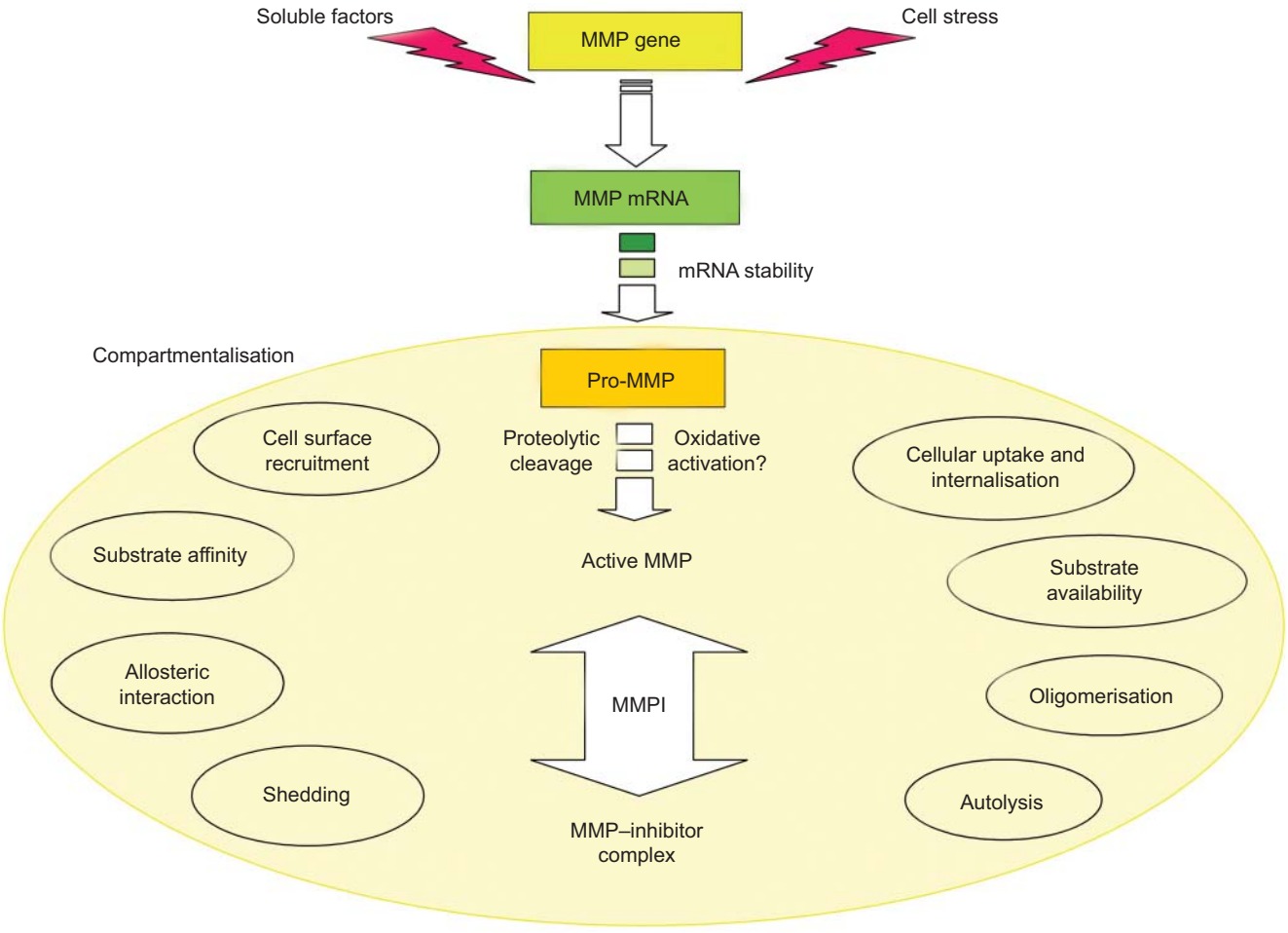


FIGURE 1. Matrix metalloproteinase (MMP) activity is tightly regulated at four different levels: 1) gene expression, mainly by regulating transcription and mRNA stability, 2) compartmentalisation (as light yellow filled eclipse), which regulates efficiency of proteolysis through cell surface recruitment, substrate availability and protein interactions, but also influences 3) pro-enzyme activation and 4) inhibition of proteolysis.

In addition, there is evidence that miRNAs contribute to post-translational regulation of MMP expression. miRNA is an abundant class of short (21–25 nucleotides), noncoding RNA that interferes post-transcriptional gene expression, by causing either translational inhibition or mRNA degradation. Since the number of identified miRNAs has grown rapidly over recent years, many genes of the human genome may be subject to regulation in this fashion. Bioinformatic analysis has revealed that several *MMP* and *TIMP* genes contain miRNA binding sites in their 3′-UTRs, including MMP-2 (miR-29), MMP-14 (miR-24, miR-26 and miR-181), TIMP-2 (miR-30), and TIMP-3 (miR-21, miR-1/206, and miR-181) [12]. So far, a few recent studies have demonstrated that transcripts of MMPs or TIMPs indeed are direct targets of miRNAs, like MMP-2 suppression *via* miR-29b in MBP-1 stimulated prostate cancer cells [13], MMP-13 regulation *via* miR-27b in human osteoarthritis chondrocytes [14], and TIMP-3 suppression through miR-181b in TGF-β-mediated hepatocarcinogenesis [15].

MMP activity regulation starts with compartmentalisation

The regulation of MMP proteolysis specificity starts already early on with their secretion. The extracellular environment and the localisation of MMPs in the pericellular space generally have a strong impact on their inactive pro-form activation and proteolytic efficiency and specificity [5].

Secreted MMPs are often associated to the cell membrane, which focuses their activity to specific substrates in the pericellular space. Examples for cell surface substrate recruitment are binding of MMP-1 to α2β1 integrin, which depends on interaction of α2 integrin with both linker plus haemopexin-like domain of MMP-1 [16], MMP-9 binding to CD44 [17], and binding of MMP-7 (matrilysin-1) to cholesterol sulfate which alters its substrate preference and promotes the degradation of pericellular laminin-332 and fibronectin [18].

Cells use surface receptors, like integrins to inform themselves what protein in the cell periphery has been encountered and consequently, which type of enzyme is needed and where it has to be released. This mechanism has been confirmed *in vivo* with MMP-1 in human cutaneous wounds, where this enzyme was induced in basal keratinocytes just at the moment when the cells were detached from the basement membrane and contacted type I collagen in the underlying dermis [19]. Furthermore, it has been shown that this mechanism depends on the

interaction of integrin α2β1 with type I collagen, which also triggers secretion of the enzyme to the points of cell–matrix contact [20].

Several ways to pro-MMP activation

MMPs are initially synthesised as inactive pro-forms (zymogens) with a pro-domain which has to be removed for activation. The pro-domain harbours a conserved ''cysteine switch'' sequence motif in close proximity to the border zone of the catalytic domain, whose free cysteine residue interacts with the catalytic zinc ion to maintain enzyme latency and prevent binding and cleavage of the substrate [21]. The activation of the MMP zymogen depends on a conformational change in the pro-domain which pulls out the cysteine residue and enables water to interact with the zinc ion in the active site. This event can be initiated by three mechanisms: 1) removal of the pro-domain by direct cleavage of another endoproteinase; 2) allosteric reconformation of the pro-domain; and 3) chemical modification of the free cysteine by reactive oxygen species or nonphysiological agents. Latter events, allosteric control and reduction of the free cysteine will also enable the enzyme to remove its pro-domain by autoproteolysis [5].

11 of the 24 human MMPs, including all membrane bound MMPs, are activated by a well characterised intracellular process *via* pro-protein convertases or furins, as indicated by the conserved furin cleavage site (R-x-R/K-R) in their amino acid sequence before the catalytic domain. Furin is a transmembranous subtilisin-like serine proteinase in the *trans*-Golgi network that is responsible for sorting secretory pathway proteins to their final destinations, including the cell surface and secretory granules. As consequence, all these MMPs members can immediately start their catalytic action when appearing on the cell surface or being secreted in the pericellular environment.

The remaining MMP members are expressed and secreted as inactive pro-forms, which need to be activated. *In vitro*, MMPs can be activated by incubation with single members of either serine proteinases, like plasmin and chymase or other MMPs, like MMP-3 and MMP-14. However the *in vivo* relevant activation mechanism of pro-MMPs is still unclear, since analysis of mutant mice revealed no changes in any pro-MMP activation by knockout of MMP-3 [22], no or little diminished pro-MMP-2 activation by MMP-14 knockout [23], no altered activation of pro-MMP-2 and pro-MMP-9 by plasminogen knockout [24], and partially diminished activation of pro-MMP-2 and pro-MMP-9 by chymase knockout [25]. Therefore, zymogen activation is probably triggered by alternative mechanisms with more than one participant.

Pro-MMP activation is thought to be a stepwise process that takes place in the immediate pericellular space. The first step includes an initial conformation change within the propeptide, which leads to disruption of the cysteine switch–zinc interaction. Subsequently, the pro-domain is removed by intra- or intermolecular processing of partially activated MMP intermediates or other active MMPs [21]. A well documented example for activation with more participants has been shown for pro-MMP-2 activation by cooperative action with TIMP-2 and MMP-14 to build up a cell surface activation complex within a 1:1:1 stoichiometric ratio [26].

An alternative mechanism of zymogen activation is probably initiated by the intrinsic allostery of the MMP molecule. Therefore, domain flexibility of the modular domain organised MMP can contribute *via* promoting long-range conformational transitions induced by protein binding *via* exosites [27]. Evidence for this interaction has been shown by incubation of pro-MMP-9 with β-hematin, the core constituent of haemozoin or malaria pigment, which resulted in autocatalytic cleavage of the pro-domain most likely by allosteric interaction with its haemopexin domain [28]. Another example is the pericellular activation of pro-MMP-7 by the tetraspanin CD151, which is overexpressed in osteoarthritic chondrocytes and most likely leads to increased cartilage destruction by excessive MMP-7 activation [29].

In vitro, incubation of recombinant pro-MMPs with thiol-modifying chemical agents, such as mercurial compounds, SDS, oxidised glutathione and reactive oxygen species (ROS), leads to activation of several MMPs. In humans, reactive oxygen is generated by dedicated enzymes in phagocytic immune cells, like neutrophils and macrophages. The MMP activation by reactive oxygen is driven through preferential oxidation of the thiol–zinc interaction and autocatalytic cleavage, followed by enzyme inactivation with extended exposure by modification of amino acids critical for catalytic activity, as shown *in vitro* for MMP-7 [30]. Therefore, ROS production by phagocytes may regulate the activation and inactivation of MMPs during inflammatory situations, as shown *in vivo* for macrophages which restrain their MMP-12 activity during tissue injury [31]. However, it is still not established that ROS can act as MMP activators/inactivators *in vivo*.

Inhibition of MMPs: finding the right balance

It is an established view that the balance between the production of active enzymes and their inhibition is critical to avoid the conditions of uncontrolled ECM turnover, inflammation, and dysregulated cell growth and migration, which would result in disease. The two major inhibitors of MMPs in body fluids and tissues are α_2-macroglobulin and TIMPs, respectively.

Human α_2-macroglobulin is a broad spectrum proteinase inhibitor of tissue fluids and blood. This homotetrameric macromolecule of 725 kDa inhibits almost all classes of endopeptidases by entrapping the whole enzyme, while these complexes are rapidly cleared by LDL receptor related protein-1 mediated endocytosis [32].

The naturally occurring inhibitors of human MMP activity are four members of the TIMPs. Each TIMP molecule consists of around 190 amino acids composed of two distinct domains, a larger N-terminal and a smaller C-terminal domain, each one stabilised by three conserved disulfide bonds. The N-terminal domain alone can fold independently and is fully functional to inhibit MMPs by chelating their catalytic zinc atom with a 1:1 molar ratio. The function of the C-terminal domain is not fully understood, but it has been shown that it can bind tightly to the haemopexin domain of latent MMPs. It has been described that TIMP-2, or TIMP-3 or TIMP-4 can interact with pro-MMP-2, and TIMP-1 or TIMP-3 with pro-MMP-9 [33]. However, except for the interaction of TIMP-2 with pro-MMP-2,

which is part of the activation mechanism of pro-MMP-2, the biological relevance of these complexes is unknown.

In general all TIMPs are broad spectrum inhibitors of MMPs, but there are differences in their specificity. For example TIMP-1 has been shown to have low inhibitory activity against MMP-19 and membrane bound MMP-14, -16 and -24, while it is more potent for MMP-3 and MMP-7 than TIMP-2 and TIMP-3 [33]. TIMP-2 inhibits the activity of all MMPs, and its expression is constitutive, in contrast to the other TIMP members which are inducible.

TIMP-3 has the ability to inhibit all MMP members (like TIMP-2), but its inhibitory profile further extends to members of the disintegrin and metalloproteinases (ADAMs). Kinetic studies revealed that TIMP-3 is a better inhibitor of TNF-α converting enzyme (TACE or ADAM17) and the aggrecanases ADAMTS-4 and -5 than for MMPs. In addition, TIMP-3 is unique among the TIMP family because it binds tightly to the ECM with its N- and the C-terminal domain to promote MMP inhibition [34], in contrast to the other TIMPs which are soluble free molecules. It seems that TIMP-3 is a more relevant *in vivo* inhibitor than TIMP-2 and -1, since loss of TIMP-3 in mice is associated with pulmonary alveolar enlargement and enhanced apoptosis in mammary gland duct epithelial cells [35], while knockout of TIMP-1 or TIMP-2 in mice does not show any unchallenged abnormalities. TIMP-4, the most recently discovered member of the family shows highest similarity to TIMP-2 and is able to inhibit most of the MMPs.

Several other molecules have been shown to inhibit single or some of the MMPs, like the C-terminal fragment of procollagen C proteinase enhancer, which inhibits MMP-2 [36] or the glycosylphosphatidylinositol (GPI) anchored angiogenesis suppressor glycoprotein RECK, which blocks the activities of MMP-2, -9 and -14 [37].

In addition, and as mentioned before, high concentrations of ROS derived from phagocytic immune cells during inflammatory scenarios are thought to be very potent and rapidly available blockers of massively increased MMP activities [5].

MODULAR DOMAIN ORGANISATION OF MMPs

The MMPs comprise a highly diverse family of enzymes, which share several common properties and domain structures. Based on their domain structure and substrate preference, they are traditionally grouped into: 1) collagenases, including MMP-1/-8/-13; 2) stromelysins, MMP-3 and -10; 3) gelatinases, MMP-2 and -9; 4) matrilysins, MMP-7 and -26; 5) membrane-type MMPs (MT-MMPs); and 6) others. A recently performed phylogenetic grouping, which included the whole genomes of the ascidian *Ciona intestinalis* as closest invertebrate relative of the vertebrates and the zebrafish *Danio rerio*, provided new insights in early stages of the vertebrate evolution of metalloproteinases and suggested six evolutionary subgroups [38].

All MMPs are synthesised as inactive pro-enzymes and, with the exception of the membrane bound MT-MMPs, secreted into the extracellular environment. As shown in figure 2, all MMPs share at least three conserved domain structures, including an N-terminal signal peptide, which is cleaved during transport through the secretory pathway, followed by the pro-domain of about 80 amino acids and a catalytic domain. The two matrilysins MMP-7 and -26 are comprised of these three domains only, thereby representing the minimal-domain family members. The pro-domain possesses a ''cysteine switch'' PRCGxPD consensus sequence. This conserved Cys residue interacts with the zinc ion of the catalytic domain which would otherwise be used for catalysis. The catalytic domain (about 160–170 amino acids) has a high degree of similarity between all MMPs and includes a conserved HExxHxxGxxH catalytic zinc binding motif.

The gelatinases MMP-2 and -9 harbour three uniquely incorporated fibronectin type II modules in their catalytic domain. These serve as a compact collagen-binding domain with high affinity for the alpha 1-chain of collagen I and induce a substantial unwinding of the collagen triple helix. In contrast, the substrate recognition and specificity of collagenases MMP-1, -8, -13 and MT-1 MMP is provided by the C-terminal haemopexin domain. However, binding of the haemopexin domain does not induce conformational changes to the fibrillar collagen molecule but leads to the recognition of a specific cleavage site of the alpha chain generating N-terminal three-quarter and C-terminal one-quarter fragments, which rapidly denature to gelatine at body temperature. In addition to substrate recognition, the haemopexin domain has also been shown to be important for the interaction with TIMPs. The flexible linker region is localised between the catalytic and the haemopexin domain and can participate in the enzymatic cleavage process by positioning the catalytic domain to the substrate [39].

Once secreted, pro-MMPs can be activated by cleavage of the pro-peptide. More than one-third of all discovered human MMPs can be activated by pro-protein convertases or furins. These MMPs contain a furin consensus sequence between their pro- and catalytic domain (fig. 2) which is cleaved during their *trans*-Golgi passage. In contrast to the secreted proteases, the four membrane-type MMPs (MMP-14, -15, -16 and -24) are inserted into the plasma membrane by means of their transmembrane domain and the MMPs -17 and -25 are inserted in the plasma membrane by a GPI anchor. The transmembrane type MMPs (MMP-14, -15, -16 and -24) contain an additional cytosolic domain. Since most attention has been focussed on the enzymatic properties of MT-MMPs, the function of this domain has not been investigated in detail; however, it seems to play a major role in the process of clathrin-dependent internalisation of MT-MMPs, where it might interact with clathrin cages [40].

MMP SUBSTRATES

MMPs are multifunctional proteases

MMPs have been traditionally associated with remodelling and degradation of the ECM but new substrate classes have emerged and MMPs are now considered as multifunctional proteases. A comprehensive list of MMP substrates and cleavage sites can be found in online resources such as MEROPS [41]. Here, we will cover the main substrate classes and provide selected examples.

The ECM has a complex and dynamic composition. Collectively, MMPs are able to cleave every ECM component [42]. However, different ECM components are susceptible to proteolysis by

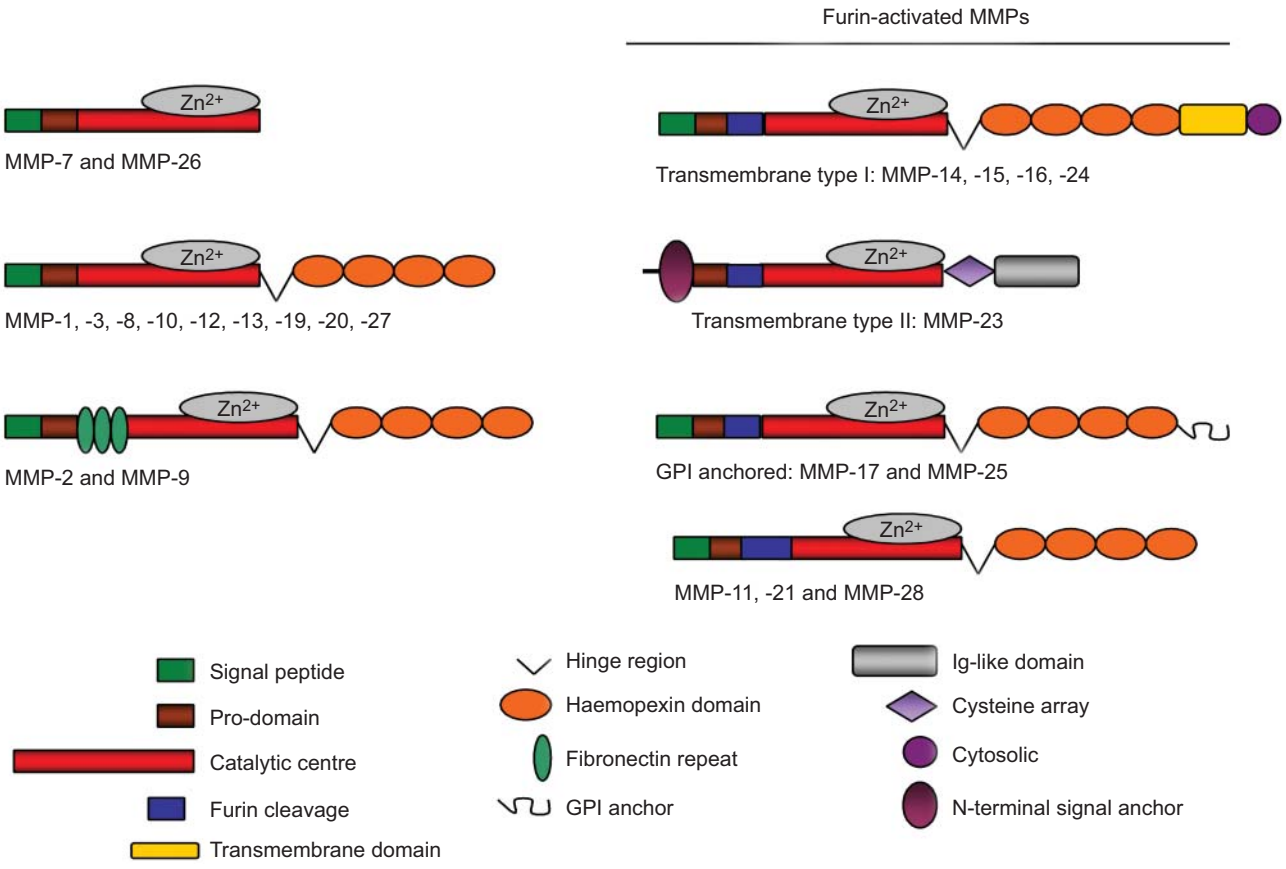


FIGURE 2. Domain structure of matrix metalloproteinase (MMP) groups. All human MMPs share at least the signal peptide, the pro-domain and the catalytic domain. The pro-domain contains the cysteine switch sequence which complexes the catalytic Zn^{2+} in the zymogen form. The catalytic domain harbours the conserved catalytic sequence. The gelatinases MMP-2 and MMP-9 uniquely contain three fibronectin repeats within their catalytic domain. Some MMPs have a furin recognition site before the catalytic domain, which allows intracellular activation of the zymogen by furin. Except for MMP-7 and -26, all MMPs have a flexible proline-rich hinge region and a haemopexin-like C-terminal domain, which function in substrate recognition. The membrane bound MMP-14, -15, -16, and -24 contain in addition a transmembrane and cytosolic domain, while MMP-17 and -25 are integrated in the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Ig: immunoglobulin.

different MMPs and not all ECM components are cleaved by every MMP. Fibrillar collagens such as collagen I, II and III are cleaved predominantly by collagenases (MMP-1, MMP-8, MMP-13), MMP-2 and MT1-MMP [2, 43]. Proteolysis occurs at a specific site three-quarters from the N-terminus. The cleavage fragments rapidly denature and form gelatin that can be degraded by numerous other proteases, such as the gelatinases MMP-2 and MMP-9. The basement membrane component collagen IV is susceptible to proteolysis by gelatinases, stromelysins, matrilysins and some other MMPs, such as macrophage metalloelastase [44], while hemidesmosomal collagen XVII is degraded by gelatinases [45]. ECM components such as laminin, fibronectin, elastin or aggrecan can be cleaved by most, if not all, MMPs. Cell adhesion molecules are also MMP substrates, including E-cadherin cleavage by matrilysin and stromelysin-1 and MT-1 MMP mediated processing of CD44 and pro-α_v integrin [46].

MMP processing of ECM components can yield bioactive fragments. For example, MMP-2 and MMP-9 expose a cryptic epitope within collagen IV that promotes angiogenesis while anti-angiogenic factors such as endostatin can be formed from collagen XVIII. Bioactive cleavage products are also produced from ECM protein, such as perlecan, laminin or fibronectin [47].

In addition to ''shaping'' bioactive cleavage products, ECM degradation releases non-covalently bound growth factors and cytokines and thereby increases their bioavailability. Examples include release of VEGF and TGF-β. VEGF binds non-covalently to heparan sulfate proteoglycans with release upon ECM proteolysis [2, 48]. TGF-β is maintained in a latent state by binding to the latency-associated peptide (LAP). LAP in turn is covalently bound to the fibrillin protein latent TGF-β binding protein (LTBP). ECM degradation releases the latent complexes and dissociation of the TGF-β–LAP complexes increases TGF-β availability. Moreover, LAP is a substrate of MMP-2, -9, -13 and -14, and LTBP can be cleaved by MMP-7 [42]. Similarly, MMPs contribute to cytokine and growth factor bioavailability by proteolysis of soluble binding proteins, such as insulin-like growth factor-binding proteins [49, 50] and pleiotrophin, a VEGF masking protein [51].

MMPs participate in cleavage and domain release of cell surface and membrane-spanning proteins. Shedding is performed by membrane-type MMPs, MMPs that are bound to

membrane receptors (such as a subpopulation of MMP-2 and MMP-9) [43] and soluble MMPs. Important functions of shedding include release of bioactive protein domains and receptor processing, hence altering cellular responsiveness to growth factors and cytokines. The MT1-MMP ''sheddome'' has been profiled in cell-based proteomics [52, 53]. Validation experiments confirmed MT1-MMP shedding of proteins such as fibronectin, death receptor-6, and cysteine-rich motor neuron 1 protein. Further MT1-MMP shedding events include cleavage of syndecan, CD44, semaphorin 4D and betaglycan [46].

Proteomic substrate profiling of MMP-2 revealed shedding of substrates [49, 51] such as the fractalkine chemokine domain. Shedding substrates for soluble MMP-7 include membrane-bound Fas ligand [54]. Some membrane proteins are susceptible to cleavage by several MMPs, such as shedding of urokinase plasminogen-activator receptor by MMP-3, -12, -19 and -25 [46].

MMPs are of outstanding importance in the site-specific cleavage of growth factors and cytokines. Through processing of these signalling molecules, MMPs interfere with cellular communication. For example, MMP-2 converts monocyte chemoattractant protein (MCP)-3 into a receptor-blocking antagonist by removal of four N-terminal residues [55]. Similarly, various MCP-type chemokines are susceptible to MMP processing [56]. Importantly, chemokine processing by MMPs is selective: MMPs differ in processing of chemokines and chemokines differ in cleavage susceptibility [56, 57]. In some cases, chemokine cleavage sites differ in an MMP-specific manner [57]. The list of signalling-related MMP cleavage events is constantly growing and cytokine processing has now been recognised as pivotal MMP function *in vivo* [46]. Further examples include processing stromal cell-derived factor 1, IL-8, IL-1β, connective tissue growth factor and TNF [46].

Proteases and protease inhibitors form an important class of MMP substrates. Through zymogen activation and inhibitor degradation, MMPs potentiate the global proteolytic potential in the extracellular milieu. At the same time, MMPs proteolytically inactivate other proteases. The functional crosstalk between different proteases has now been established as a key regulatory component for the fine-tuning of cellular proteolysis [46]. This is illustrated by MT1-MMP mediated activation of MMP-2 [26]. MMP-2 in turn was found to cleave cystatin C and secretory leukocyte protease inhibitor as well as various cathepsin proteases and bone morphogenetic protein-1 [49–51].

In summary, MMPs are multifunctional proteases that: 1) proteolyse ECM components with subsequent release of bioactive fragments and proteins; 2) participate in membrane shedding; 3) play an important role in chemokine processing; and 4) alter the activity status of other proteases. Novel proteomic techniques are now starting to provide a comprehensive portrayal of MMP substrate degradomes (see below). Since knowledge of protease substrates is key to understand protease biology, this development is essential to better understand MMP involvement in (patho)physiology. A comprehensive classification of MMP substrates according to their (patho)physiological implication is provided by RODRIGUEZ *et al.* [46] and includes cleavage events with relation to bone and ECM remodelling, angiogenesis, cell migration, cellular invasion and metastasis, cell proliferation, tumour growth, apoptosis and inflammation, as well as innate immunity and wound healing.

MMPs have subtle differences in substrate specificity

Protease specificity is fundamentally guided by enzyme-substrate interactions in an elongated active site cleft. The Schechter and Berger nomenclature designates substrate binding pockets N-terminal to the scissile peptide bond as S1, S2, S3, *etc.* and the corresponding substrate residues as P1, P2, P3, *etc.* Binding pockets and substrate residues C-terminal to the scissile peptide bond are named S1′, S2′, S3′, *etc.* and P1′, P2′, P3′, respectively. There are few methods for unbiased profiling of both prime and non-prime specificity [58]. Most MMP specificity information stems from peptide library approaches [59–61] with proteome-derived peptide libraries having yielded >1,200 peptidic cleavage sequences for MMP-2.

MMPs share a general specificity profile with only subtle differences. MMP specificity determinants extend from P3–P3′ and are composed of subsite preferences rather than strict cleavage site motifs. Mixed specificities are observed in some subsites. MMP specificity is primarily guided by a preference for aliphatic amino acids, in particular leucine, in P1′. MMPs -2, -3, -9 and -14 also display a P1′ preference for aromatic residues and are considered to have a deeper S1′ pocket [61, 62]. A preference for proline in P3 constitutes the secondary MMP specificity determinant. However, MMPs also prefer medium sized aliphatic residues (valine, isoleucine) in P3 [60, 61]. Further subsite preferences include a slight preference for small residues such as alanine, in P2 by MMP-2 while MMPs -1, -3, -7, -9 and -14 are reported to prefer aliphatic and aromatic residues in P2. In P1, MMPs prefer small residues such as alanine, glycine and serine. In P2′, MMPs have a mixed preference for basic and large aliphatic residues. In P3′, MMPs -1, -2 and -9 prefer small residues.

These specificity profiles are based on oligopeptide substrates and do not necessarily translate into physiological substrates. Most potential cleavage sites in folded proteins are structurally shielded and selection of *in vivo* substrates involves recognition of substrates by exosites outside the active site cleft (more on exosites below). Hence, prediction of protein substrates solely based on sequence resemblance to MMP specificity profiles can be misleading [60]. Nevertheless, MMP specificity profiles based on protein substrates share the features of peptide-based specificity profiles, hence validating MMP specificity determination [50].

Exosite recognition of protein substrates

While MMPs share a global active site specificity profile, there are marked differences in their protein substrate repertoires. For example, unbiased proteomic determination of the MMP-2 and -9 substrate degradomes revealed limited overlap [63]. This observation stresses the importance of substrate recognition outside of the active site cleft through protein areas and domains termed exosites. Many MMPs are multidomain proteins and whole-domain exosites are important contributors to MMP *in vivo* proteolysis. Exosite interactions mediate cleavage of native collagen fibres. Collagenolysis is a three-step process involving collagen binding, unwinding and cleavage. For collagenases 1–3 and MT1-MMP, the haemopexin domain

binds native collagen [53, 64]. However, this is not the case for MMP-2. Here, the triple fibronectin type II repeat in the catalytic domain forms an alternative collagen binding domain [64]. Collagen binding domains also participate in collagen unwinding. In addition to facilitating collagenolysis, collagen binding directs MMPs to the ECM and thereby influences the spatial distribution of MMPs. This is an indirect contribution to MMP *in vivo* substrate repertoires [64]. The MMP-2 haemopexin participates in the recognition of further, non-collageneous substrates such as certain chemokines [55]. In fact, usage of the MMP-2 haemopexin domain as a bait molecule to identify potential interactors in a yeast two hybrid screen led to the groundbreaking identification of the chemokine MCP-3 as an MMP-2 substrate [55].

System-wide identification of MMP substrates

Protease substrate determination has long been a serial process in which individual proteins were tested for cleavage susceptibility. While fascinating insights into protease biology are based on this approach, it lacks the throughput to fully apprehend *in vivo* proteolysis. Proteomic strategies have now emerged with the ability to characterise cell-contextual proteolysis on a system-wide scale. Generally, these approaches fall in two categories: global quantitative proteomics and direct identification of protease cleavage sites. Global quantitative proteomics determine altered protein abundance in biological systems with different proteolytic activity. In cell- or animal-based systems, this often includes overexpression, depletion or inhibition of the protease under investigation and comparison to a native, untreated control sample. As secreted proteases, MMPs can alter protein abundance in many ways. Degradation reduces protein abundance. Shedding decreases protein abundance on the cell surface but increases ectodomain concentration in the cellular microenvironment. Lastly, limited processing, such as removal of few terminal amino acids, might not be reflected as a quantitative change and hence remains unnoticed by global quantitative proteomics. Tandem mass spectrometry has been established as the method of choice for protein identification in these approaches due to its outstanding analytical power, sensitivity and throughput. Proteome separation and relative quantification has been historically realised with two-dimensional gel electrophoresis. These are now increasingly replaced by liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based shotgun proteomics with greatly increased proteome coverage. Here, relative quantification is typically achieved by stable isotope labelling, although label-free quantification is increasingly gaining attention [58]. MMP-2 substrates such as cystatin C and fractalkine have been identified in cell-conditioned medium by LC–MS/MS-based proteomics screen employing commercially available ICAT or iTRAQ labels [49, 51]. BUTLER and OVERALL [65] present an overview of proteomically identified MMP substrates.

Global quantitative proteomics fail to identify protease cleavage sites and cannot characterise subtle proteome alterations caused by limited proteolysis. To fully characterise proteolysis on a system-wide level, proteomic strategies have been invented that selectively analyse protein N- or C-termini [58, 66]. In combination with quantitative comparison of systems lacking or overexpressing a protease under investigation, substrate candidates can be identified and distinguished from background proteolysis (fig. 3). ''Terminal'' proteomic strategies rely either on positive selection of protein termini or depletion of internal peptides (negative selection), with negative selection strategies also identifying modified (*e.g.* cyclised or acetylated) N-termini [50, 67]. Terminal amine isotopic labelling of substrates (TAILS) is a novel integrated platform for the system-wide identification of protease substrates by quantitative N-terminome analysis [50]. TAILS relies on a highly efficient polymer-based negative selection strategy. So far, MMP cell-contextual cleavage events have been exclusively analysed using TAILS and numerous novel substrates and cleavage sites have been revealed [50, 63, 67]. For MMP-2 this includes proteins substrates such as extracellular matrix protein 1, biglycan, and macrophage migration inhibitory factor. As outlined above, MMPs possess broad active site specificity. In contrast to proteases such as caspases, MMP cleavage sites cannot be recognised by consensus motifs. A statistics-based platform has been established to carefully discern MMP-2 cleavage sites from background proteolysis in TAILS experiments [67]. Proteome-wide identification of protein C-termini has only recently been established [66].

Proteome-wide substrate and cleavage site identification will open new routes to our understanding of protease biology by comprehensively mapping protease substrates in an unbiased manner. However, candidate substrates require further validation, especially in the case of MMPs. Since these are multifunctional proteases, altered MMP activity can result in various secondary effects. Substrate candidates are typically validated by *in vitro* cleavage assays together with detection of temporal and spatial colocalisation of protease and substrate/product in cell or animal model systems [55]. At the same time, an expanding number of degradomic analyses for a multitude of proteases and model systems will soon lay the foundation for a powerful meta-analysis that discriminates downstream effects and distinguishes protease-specific substrate profiles.

PHYSIOLOGICAL FUNCTIONS OF MMPs

Learning from MMP knockout mice

Analysis of genetic knockout mutants offers the opportunity to identify essential MMP functions and so far 16 MMP knockout mutants have been analysed. However, the analysis of single MMP null mutants revealed surprisingly benign phenotypes with all MMP-knockout mice surviving at least the first three postnatal weeks (table 1), indicating proteolytic redundancy, enzymatic compensation, and adaptive development. Therefore, most of the so far described knockout models only show significant defects in regulating tissue response to environmental challenges, such as wounding, infection and inflammation.

In contrast to other MMP knockout mice, *MMP-20* knockout mice have a striking phenotype even without an environmental challenge. As MMP-20 is expressed during the early through middle stages of enamel development in dental tissue, null mice indeed show severe and profound tooth phenotype, in that they cannot process amelogenin properly and they develop an altered enamel matrix and rod pattern [92].

MMP-19 deficient mice, conversely, are a representative example of MMP-driven dysregulated response to environmental challenges. Even though they are indistinguishable

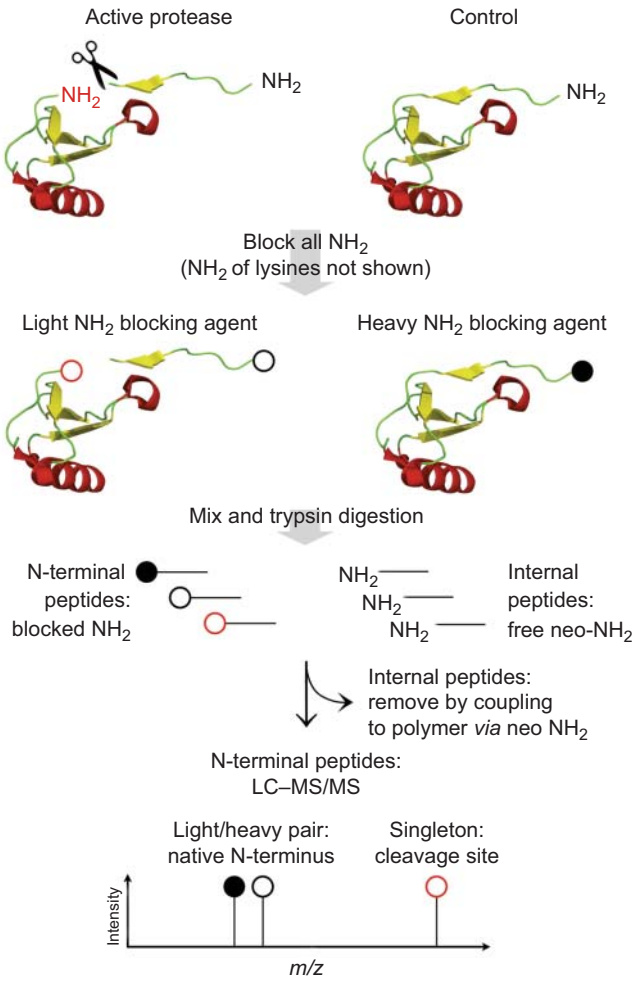


FIGURE 3. Identification of *in vivo* cleavage sites by terminal amine isotopic labelling of substrates. This substrate degradomic strategy focuses on protein N-termini and compares a control sample with a protease-treated sample in order to distinguish induced cleavage events from background proteolysis and native protein termini. Samples are harvested and primary amines, including protein N-termini and lysine side chains are chemically protected, *e.g.* by reductive methylation. Stable isotopes are introduced in this step to allow for relative quantisation by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Samples are then mixed and trypsin digested. Internal and C-terminal peptides possess free neo N-termini whereas N-terminal peptides have chemically protected N-termini. Internal and C-terminal peptides are coupled to a high molecular weight polymer *via* their free NH_2 groups. N-terminal peptides remain uncoupled and are separated from the polymer–peptide conjugate by ultrafiltration. In the following LC–MS/MS analysis, N-termini that appear independently of the protease activity under investigation occur as light/heavy isotope pairs. N-termini from cleavage sites that are based on the protease activity under investigation occur as singletons in the isotopic form used to label the ''active protease'' sample state. *m/z*: mass/charge ratio.

from wild-type mice they develop a diet-induced obesity due to adipocyte hypertrophy and exhibit decreased susceptibility to skin tumours induced by chemical carcinogens [91].

Since MMPs share many substrates *in vitro* it is most likely that genetic redundancy also exists *in vivo*. The fact that MMP double knockout mutants display severe phenotypes supports this notion. One striking example of redundancy is represented by the *MMP-2/MT1-MMP* double null mice that die immediately after birth due to respiratory failure and developmental vascular defects resulting in abnormal vessel formation and immature muscle fibres [96]. These defects result from additive pathology compared to the single knockout, thus providing a genetic proof for developmental redundancy.

A similar phenomenon has been observed in *MMP-2/MMP-9* double null mice that do not show an overt phenotype. However, using a laser-induced injury, LAMBERT *et al.* [97] have demonstrated an amplified vascular defect when compared to the single null mutants. This observation has been independently confirmed by tumour invasion and vascularisation studies [95].

MMPs trigger bone growth and modelling

The formation of the skeleton in humans or mice depends on two major ossification processes which require extensive matrix remodelling. While endochondrial ossification is needed for most bones in the body, intramembranous ossification is essential for flat bones like the skull or mandibles. Up to now, five knockout models have been described that show bone growth phenotypes, namely: MMP-2, MMP-9, MMP-13, MT1-MMP and MT3-MMP. Even though all five knockout models display bone phenotypes the mechanism by which they influence bone development differs remarkably due to their cell type-specific expression in murine bones [35].

Mice devoid of gelatinase A (*MMP-2*) showed growth delay and skull deformities, due to intramembranous ossification defects [70], in contrast to the other MMP knockout mice that developed endochondrial ossification defects.

Gelatinase B (*MMP-9*) null mutants are characterised by delayed long bone growth and development due to impaired vascular invasion in skeletal growth plates. In wild-type mice, MMP-9 generates a gradient of angiogenic signals most probably through the release of those from the ECM, thereby guiding the newly formed vessels with a chemokine-dependent mechanism. Although this phenotype is marked during their growth phase, adult mice show only about 10% shortening in the long bones, again indicating that other MMPs may compensate.

As mentioned before, the expression of both proteases MMP-9 and MMP-13 is mediated by RUNX-2, suggesting a similar bone phenotype in *MMP-13* knockout mice. Indeed, the inactivation of MMP-13 in mice led to abnormal skeletal growth plate development and delayed exit of chondrocytes from the growth plate [86]. Therefore, the deletion of both MMP-9 and MMP-13 leads to severely impaired endochondral bone formation, diminished ECM remodelling, prolonged chondrocyte survival, delayed vascular recruitment and defective trabecular bone formation, thus resulting in drastically shortened bones [86].

Among all single MMP knockout models, only *MT1-MMP* null mice show a severe spontaneous phenotype. These mice exhibit skeletal defects including craniofacial dysmorphism, arthritis, osteopenia, dwarfism, and fibrosis of soft tissues due to ablation of a collagenolytic activity that is essential for modeling of skeletal and extraskeletal connective tissues. These findings reveal an important function of MT1-MMP in

TABLE 1 Phenotypes of matrix metalloproteinase (MMP) knockout mice

MMP	Phenotype	Ref.
MMP-2	No overt phenotype, reduced body size	
	Reduced neovasculation and tumour progression	[68]
	Decreased primary ductal invasion in the mammary gland	[69]
	Decreased bone mineralisation, joint erosion, defects in osteoblast and osteoclast growth	[70]
MMP-3	No overt phenotype	[71]
	Impaired contact dermatitis, impaired onset of T-cell proliferation	[72]
	Defect in wound contraction	[73]
	Altered secondary branching morphogenesis in the mammary gland	[69]
MMP-7	No overt phenotype	
	Impaired tracheal wound repair	[74]
	Innate immunity defects	[75]
	Defective prostate involution	[54]
MMP-8	More susceptible to develop skin cancer, impaired wound healing in skin	
	Altered inflammatory response in wounds, delay of neutrophil infiltration	
	Altered TGF-β signalling	[76]
MMP-9	Impaired primary angiogenesis in bone growth plates	[77]
	Resistant to bullous pemphigoid	[78]
	Contact dermatitis: delayed resolution	[72]
	Impaired vascular remodelling	[79]
	Delayed healing of bone fractures	[80]
MMP-10	No overt phenotype	
	Pathological induced phenotype: pulmonary inflammation and mortality	[81]
MMP-11	No overt phenotype	
	Decreased chemical-induced mutagenesis	[82]
MMP-12	No overt phenotype	[83]
	Impaired macrophage proteolysis	[83]
	Resistant to cigarette smoke-induced emphysema	[84]
MMP-13	No overt phenotype	
	Induction of MMP-8 expression in *MMP13-/-* wounds, no difference in wound healing	[85]
	Bone remodelling defects	[86]
MMP-14 (MT1-MMP)	Premature death; skeletal defects and dwarfism	
	Normal at birth but develop multiple abnormalities (defect in remodelling of the connective tissue, increased bone resorption and defective secondary ossification centres) and die by 3–12 weeks	[87]
	Angiogenesis defect and defects in lung and submandibular gland	[88]
MMP-16 (MT3-MMP)	Growth retardation	[89]
MMP-17 (MT4-MMP)	No overt phenotype	[90]
MMP-19	Obesity and decreased skin carcinogenesis	[91]
MMP-20	Defects in tooth enamel	[92]
MMP-24	Abnormal response to sciatic nerve injury	[93]
MMP-28	No overt phenotype; elevated macrophage recruitment in lung	[94]
MMP-2/MMP-9	Impaired tumour invasion and angiogenesis	[95]
MMP-2/MMP-14	Die immediately after birth with respiratory failure, abnormal vessels and immature muscle fibres	[96]
MMP-9/MMP-13	Shortened bones	[86]
MMP-14/MMP-16	Die one day after birth due to cleft palate	[89]

TGF: transforming growth factor.

connective tissue metabolism during development. Furthermore, it illustrates that modelling of soft connective tissue matrix is essential for the maintenance of the hard tissues of the skeleton [87].

The analysis of *MT3-MMP* (*MMP-16*)-deficient mice reveals a novel mechanism of ECM remodelling which is prerequisite for proper function of mesenchymal cells. In the absence of MT3-MMP, mice display growth inhibition due to decreased viability of mesenchymal cells in skeletal tissues. The inhibition of mesenchymal cell proliferation and migration was caused by the lack of cleavage of high-density fibrillar collagen [89]. The physiological significance of MT3-MMP (MMP-16) was further verified in mice that are double deficient for MT1-MMP and MT3-MMP. Double deficiency transcends the combined effects of the individual single deficiencies and leads to severe embryonic defects in palatogenesis and bone formation, which are incompatible with life. These defects are

directly tied to loss of indispensable collagenolytic activities required in collagen-rich mesenchymal tissues for ECM remodelling and cell proliferation during embryogenesis [89].

MMPs in angiogenesis and vascular development

Besides the previously mentioned angiogenic phenotype of *MMP-9* null mice during long bone growth, *MMP-2* and *MT1-MMP* knockout mice also show modifications in angiogenesis, especially under pathological conditions, *e.g.* tumour-induced angiogenic sprouting [68, 88]. This observation implies that absence of single MMPs does not essentially disturb the formation of the embryonic vascular network. However, absence of two MMPs in the *MMP-2/MT1-MMP* double knockout mice results in severe developmental vascular defects and leads to postnatal death.

The mechanism by which MT1-MMP contributes to the sprouting of neovessels is simply through degrading type I collagen. Therefore *MT1-MMP* null mutants fail to invade collagen type I-rich postnatal matrices [98]. In contrast, embryonic ECM has only low collagen type I content, which might explain the fact that no defects were seen in its vascular system. Besides its role in collagen degradation, MT1-MMP is also involved in the process of pro-MMP-2 activation [26]. Active MMP-2 itself can regulate sprouting of newly formed vessels by the release of cytokines and growth factors.

Besides its structural function the ECM serves as a ''sponge'' that binds cytokines and growth factors. The controlled cleavage of these factors by different MMPs can thereby modulate cell signalling events within the cells. Such is the case of ECM-tethered VEGF, which is inactive in complex with connective tissue growth factor (CTGF), but becomes active after proteolytic cleavage of CTGF by MT1-MMP, MMP-1, -3 and -13 [99]. Furthermore, the specific binding pattern of VEGF to ECM components creates a ''carpet'' to guide invading endothelial cells, thereby supporting the process of neoangiogenesis. However, the cleavage of VEGF by MMP-3 and -9 generates a shorter, non-heparin binding active form of VEGF that subsequently induces irregular vessel sprouting.

Further examples for the release of active molecules by proteases are the catalytic release of insulin-like growth factor by MMP-19 or the release of TNF-α by gelatinases [99]. Importantly, MMPs not only promote angiogenesis but also generate anti-angiogenic peptides: MMP-3, -7, -9, -13 and -20, for example, have been shown to generate the anti-angiogenic endostatin by processing type XVIII collagens [47]. This observation is in line with the observed phenotypes in knockout mice, since all of them show defects in vascularisation.

Major functions of MMPs in the immune response and innate immunity

In lung airway epithelial cells only MMP-7 is expressed. In mice, the re-epithelialisation of wounded trachea is entirely suppressed in the absence of MMP-7, demonstrating the requirement of MMP-7 in normal wound repair. In addition, *MMP-7* mutant mice are less resistant to intestinal bacterial infection, partially due to their inability to release the endogenous antibiotic peptide α-defensin from intestinal epithelia, indicating a role of MMP-7 in innate immunity [75].

In a recent study, CHEN *et al.* [100] have shown that MMP-7 in the lung is essential for the chemotatic recruitment of neutrophils. As syndecan-1 sequesters a number of cytokines, its proteolytical release by MMP-7 forms a chemoattractant gradient necessary for neutrophil guidance in damaged lung epithelia. In contrast, neutrophil migration is abolished in *MMP-7* null mutants.

In agreement with the expression pattern of the MMPs -8, -2 and -9 in innate immune cells, such as monocytes, activated macrophages, alveolar macrophages and neutrophils, these enzymes have been linked to pathologies associated with innate immune dysregulation, as we recently showed for macrophage derived MMP-2 and MMP-9 in a monocyte-driven model of autoimmune kidney inflammation [101]. MMP-2 and -9 participate in immune cell recruitment by providing a chemokine gradient of both the CC- (*e.g.* CCL7) and the CXC-motif (*e.g.* CXCL12, CXCL6 and CXCL8) ligands [64, 102], thereby having both pro- and anti-inflammatory effects. The truncation of macrophage-derived CCL7 by gelatinases, for example, results in the formation of peptides that act as receptor antagonists [55], while MMP-9 mediated cleavage activates CXCL6 and CXCL8 and inactivates CXCL1 and -4 [102]. These observations are in line with the resistance of MMP-9 deficient mice to the autoimmune blistering disease bullous pemphigoid (table 1), which is due to reduced neutrophil recruitment [97].

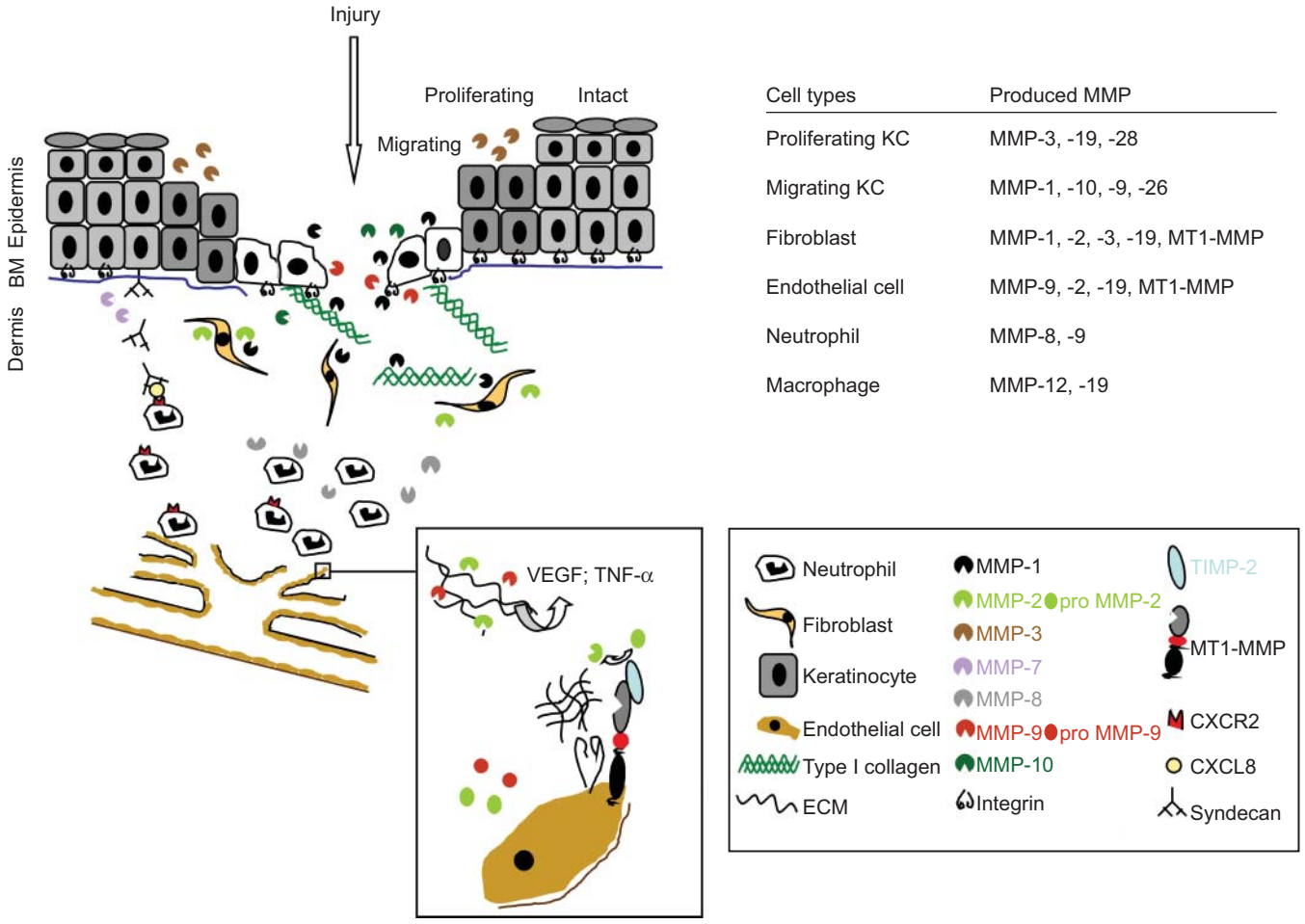
MMPs have essential roles in wound healing and cell migration

In vivo as well as *in vitro* data have demonstrated the importance of many secreted and membrane bound MMPs that contribute either directly or indirectly to the process of wound healing and neovascularisation. Upon injury most, if not all, MMPs are induced and expressed in almost any involved cell types, including mesenchymal, epithelial and immune cells. Thus defining the temporal and spatial patterns of MMP expression has become a major goal in the study of wound healing and cell migration (fig. 4).

The induction of MMP-1 expression by keratinocytes at the wound edge, for example, is mediated by the binding of α2β1 integrin to the dermal collagen type I. This high affinity attachment would tether keratinocytes to the dermis, rendering them unable to migrate. The increase of active MMP-1 leads to the subsequent degradation of dermal collagen type I. Collagen I degradation lowers the affinity of the integrin–collagen binding, thereby allowing the keratinocytes to migrate, and reduces the expression of MMP-1 [20]. This regulatory mechanism demonstrates that cells do not need MMP activity simply to remove matrix barriers.

Another recently identified role of MMP activity in wound healing is the recruitment of immune cells since neutrophil recruitment requires the presence and activity of MMP-7 (see section: Major functions of MMPs in the immune response and innate immunity) and MMP-8 [76].

A vital process in wound healing is neoangiogenesis, which involves migration and proliferation of endothelial cells, and is stimulated by fibrin and fibronectin derived from wound granulation tissue. MMPs induce the release of ECM bound pro-angiogenic factors, including the release of VEGF and TNF-α by MMP-2 and -9 (fig. 4; box with enlarged endothelial cell).



Cell types	Produced MMP
Proliferating KC	MMP-3, -19, -28
Migrating KC	MMP-1, -10, -9, -26
Fibroblast	MMP-1, -2, -3, -19, MT1-MMP
Endothelial cell	MMP-9, -2, -19, MT1-MMP
Neutrophil	MMP-8, -9
Macrophage	MMP-12, -19

FIGURE 4. Distinct functions of matrix metalloproteinases (MMPs) contribute to wound healing and angiogenesis. In healthy skin, basal expression of MMPs is low, but following injury, interaction of many secreted and membrane bound MMPs contribute either directly or indirectly to wound healing. For example, MMP-1 strongly influences keratinocyte migration at the wound edges, while MMP-7 regulates neutrophil recruitment (as shown in the legend on the lower right site). The wound induced neoangiogenesis, a result of migration and proliferation of endothelial cells, is driven by vascular endothelial growth factor (VEGF) and tumour necrosis factor (TNF)-α release through the gelatinases MMP-2 and -9 (as shown in the box with a blow up of a migrating endothelial cell (EC)). BM: basement membrane; KC: keratinocyte; TIMP: tissue inhibitor of matrix metalloproteinase; ECM: extracellular matrix.

Pro-MMP-2 is activated by the well-characterised MT1-MMP/TIMP-2 complex which is expressed on the surface of activated endothelial cells. The co-localisation of MT1-MMP with αvβ3 integrin at the intercellular junctions of endothelial cells suggests an additional regulatory role in cell migration and adhesion.

As illustrated in a simplified cartoon of MMP functions and interactions in figure 4, disclosure of single MMP substrates may not sufficiently explain the *in vivo* MMP function without the knowledge of their complex interactions. More work has to be invested in order to uncover the complex MMP interactions in a context dependent manner, to advance our understanding of the role of MMPs in the regulation of tissue homeostasis.

TISSUE SPECIFICITY OF MMP EXPRESSION

The constitutive physiological MMP expression is normally low, with transiently higher rates due to homeostasis linked matrix remodelling or specific developmental events. However, the strong structural similarities across MMP promoters [7] raise the question of whether and how distinct MMPs are differentially expressed in specific cell types or tissues.

Up to now, MMP-7, -8, -13, -20 and -28 have been found to be expressed in distinct tissues or cell types. Most of them are expressed due to the distinct cell type expression of transcription factors. The prototype for true tissue specific expression is represented by MMP-20, which has been shown to be restricted to dental tissue, most likely driven by tooth-specific transcription factors [103]. In addition, MMP-28 expression seems to be restricted to developing germ cells as its transcription is mediated by the transcription factor Sox-5 [104].

The expression of proteases in hypertrophic chondrocytes and developing cartilage is regulated by the RunT domain factor-2 (RUNX-2), which binds a RUNX-2 site, for example, in the MMP-13 promoter, thereby limiting synthesis of this MMP to bone osteoblasts [11]. In addition, MMP-9 expression is also regulated by RUNX-2, which is in line with its expression pattern in osteoblasts and fits very well with the bone growth phenotype observed in MMP-9 null mice. Nevertheless, bioinformatic analysis revealed RUNX-2 binding sites in an increasing number of MMP promoters, including the promoters of MMP-1, -7, -8 and -13, indicating that most MMPs do

not have real tissue specificity. For example, the gelatinases MMP-2 and -9 have been mainly associated with basement membrane degradation under pathological conditions since they are expressed by reactive epithelial and mesenchymal cells within in the skin. However several observations clearly support an expanded role for these MMPs as they are expressed by a large number of different cell types, including endothelial cells, bone related cells and immune cells [69, 105, 106].

Another obvious example of cell-specific expression is represented by the two interstitial collagenases MMP-1 and -8. Both enzymes share similar substrate specificities in that they both degrade collagens I, II and III. However, their expression pattern is quite different (table 2). MMP-1 is expressed in several types of cells, for example fibroblasts, endothelial cells, chondrocytes and osteoblasts [44], while MMP-8 is mainly secreted by neutrophils [108]. This difference can be explained by the composition of the different cis-elements within their promoters, since MMP-8 promoters lack a proximal AP-1 binding site.

The human MMP-7 promoter encompasses two upstream Tcf/Lef-1 binding sites which mediate transactivation by β-catenin. Therefore, matrilysin (MMP-7) is expressed exclusively in cells of epithelial origin, including epithelial cells of several exocrine glands like mammary, parotid, prostate, pancreas, liver and peribronchial glands [107].

In conclusion, it seems that the expression of only a few MMP members is restricted to specific cell types or tissues under physiological conditions, while the majority is broadly expressed and involved in many essential physiological roles. Future studies on mouse models may reveal disease-specific expression patterns for certain MMP members.

NEW STRATEGIES FOR SELECTIVE MMP TARGETING

Since MMPs are strongly upregulated in cancer and inflammation associated diseases, they have been regarded as attractive therapeutic targets. Thus the design of potent MMP inhibitors has become an area of intense interest over the past 25 yrs. However, the design of selective inhibitors has proved to be extremely difficult since all MMP members share strikingly similar structures around and within their active sites [119]. Consequently, most of the clinical trials using these small-molecule MMP inhibitors have ended with negative results, mostly due to their widespread inhibitory activities. Selective inhibition of distinct MMP members at early disease stages will

TABLE 2 Tissue and cell specificity of matrix metalloproteinase (MMP) expression

MMP	Cell types and organs	Reference
MMP-1 (collagenase 1)	Skin (Fb and KC), EC, chondrocytes, osteoblasts, macrophages, hepatocytes	[44]
MMP-2 (gelatinase A)	Skin (Fb and KC), EC, chondrocytes, osteoblasts, osteocytes, monocytes, alveolar macrophages, polymorphonuclear leukocytes, mammary gland	[69, 105]
MMP-3 (stromelysin 1)	Skin (Fb and basal KC), epithelial cells, mammary gland	[44, 69]
MMP-7 (matrilysin-1)	Glandular epithelial cells in skin, parotid, liver, endometrium, mammary gland, prostate, pancreas, small intestinal crypts, peribronchial glands and conduting airways in the lung	[107]
MMP-8 (collagenase 2)	Primarily by neutrophils	[108]
MMP-9 (gelatinase B)	Skin (Fb and KC), EC, chondrocytes, osteoblasts, osteoclasts, monocytes, alveolar macrophages, polymorphonuclear leukocytes, invading trophoblasts	[105, 106]
MMP-10 (stromelysin 2)	Skin (Fb and KC), epithelial cells	[44]
MMP-11 (stromelysin 3)	Uterus, placenta and involuting mammary gland	[44]
MMP-12 (macrophage elastase)	Macrophages, placenta	[83, 109]
MMP-13 (collagenase 3)	Skin (Fb and KC), osteoblasts, osteocytes	[110]
MMP-14 (MT1-MMP)	Skin (Fb and KC), osteoclasts, osteoblasts, articular cartilage	[111]
MMP-15 (MT2-MMP)	Placenta, brain, heart	[111]
MMP-16 (MT3-MMP)	Lung, kidney, spleen, heart, skeletal muscle, chondrocytes, reproductive tissue (ovary and prostate), placenta, intestine	[111]
MMP-17 (MT4-MMP)	Brain, reproductive tissue (ovary and testis), colon, leukocytes	[112]
MMP-19 (RASI)	Skin (KC), skeletal muscle, liver, lung, kidney, thymus, spleen, brain, heart, reproductive tissue (ovary, testis, prostate), mammary gland, placenta, colon, small intestine, pancreas, leukocytes	[113]
MMP-20 (enamelysin)	Dental tissue	[86]
MMP-21	Fetal: brain, kidney, liver Adult: ovary, kidney, liver, lung, placenta, brain, peripheral blood leukocytes	[114]
MMP-23	Reproductive tissues (ovary, testis, prostate)	[115]
MMP-24 (MT5-MMP)	Brain, kidney, lung, pancreas	[109, 111]
MMP-25 (MT6-MMP)	Skeletal muscle, lung, spleen, testis, kidney	
MMP-26 (matrilysin-2)	Kidney, uterus, placenta	[116]
MMP-27	Bone, kidney, heart	[117]
MMP-28 (epilysin)	Skin (KC), brain, lung heart, kidney, testis, placenta, colon, intestine, pancreas	[118]

Fb: fibroblasts; KC: keratinocytes; EC: endothelial cells.

most likely be much more efficient than the usage of broad-spectrum inhibitors that block the activities of most MMPs and ADAMs, including any as antitargets considered members [4].

One interesting strategy for selective MMP targeting focuses on function-specific enzyme inhibition by the targeting of exosite domains of the MMP molecule, which are involved in macromolecular substrate recognition or cell surface binding on integrins. This has been successfully realised by the generation of blocking peptides or monoclonal antibodies (mAb) that target exosite binding sites of MMPs to their macromolecular substrates, as shown by effective inhibition of MMP-2 collagenolysis through blockage to its collagen binding domain or by the highly MMP-9 specific mAb REGA-3G12, which selectively inhibits the biological activity of MMP-9, and not MMP-2 [27].

Another approach for selective MMP targeting with considerable potential lies in RNA interference technology, in which expression of a target gene is reduced by short double stranded RNA populations that specifically bind target mRNAs and interfere with protein translation and/or promote mRNA degradation. The interfering RNA can be introduced by transient transfection (short interfering (si)RNAs) or by stable integration into the genome of cells (short hairpin RNAs). This technique has been successfully applied in experimental mouse models with MMP-9 directed siRNAs, resulting in suppressed tumour initiation and progression [120], or with MMP-7 targeted siRNA resulting in inhibited metastasis of gastric and colon cancer [121] and seems to be a promising strategy for future therapeutic selective MMP inhibition. However, problems and difficulties with siRNA stability, efficacy of tissue targeting, and off-target effects challenge their potential as therapeutic drugs.

CONCLUSIONS AND FUTURE PERSPECTIVES

MMPs are integrated in the network of multidirectional communication within tissues and cells as important regulators of cell proliferation and differentiation, tissue homeostasis, immune response and several other processes. Since uncontrolled MMP activity can easily become destructive and lead to breakdown of homeostasis, it has been realised that their activity needs to be tightly regulated at different levels, through epigenetic, transcriptional and post-transcriptional control of gene expression, activation of their pro-enzymes, and inhibition of their activity. Many studies have shown that each level of control implicates several alternative regulatory subunits; some of them are highly specific for single MMPs. Furthermore, there is evidence that distinct regulatory patterns of single or small groups of MMPs are associated with different stages of cancer and inflammatory diseases, which can be used for selective MMP targeting at the expression level by RNA interference technology or at the protein level by selective inhibitors and blocking antibodies. However, further investigations on animal models are needed to validate these correlations *in vivo*.

In the past few years it has been established that MMPs are multifunctional proteases with a widespread substrate repertoire. Besides their classical role in ECM degradation with subsequent release of bioactive factors, they participate in membrane shedding, play important roles in chemokine or growth factor processing, and alter the activity status of other proteases. Indeed, the majority of MMP substrates are non-matrix molecules, thus indicating that release of growth factors and cytokines is the predominant function of MMPs. Novel proteomic techniques are now starting to provide a comprehensive portrayal of MMP substrate degradomes and will soon lay the foundation for a powerful meta-analysis that discriminates downstream effects and distinguishes protease-specific substrate profiles. Since knowledge of protease substrates is one of the keys to understanding protease biology, this development is prerequisite to a better understanding of MMP involvement in physiology and pathology.

Analysis of, so far, 16 MMP knockout mutants revealed surprisingly benign spontaneous phenotypes in postnatal skeletal, circulatory and mammary development, indicating proteolytic redundancy and enzymatic compensation. The only exception was the knockout of MMP-14 which causes several abnormalities in skeletal and connective tissue remodelling and angiogenesis, leading to premature death. Furthermore, most of the MMP knockouts have functional phenotypes, in the form of defects in response to environmental challenges, such as acute wounding or bacterial infection. However, the interpretation of mutant mice with loss of enzymes with broad substrate spectra like MMPs is complicated. Due to their activity, MMPs can be involved in many different signalling networks simply by regulating other proteases, both directly and indirectly through cleavage and inactivation of protease inhibitors. Therefore, the MMP knockout mice demonstrate the response of the whole organism as a complex system to the loss of one of its components, and not just the effects of its specific molecule deficiency alone. In this regard the future challenge will be to evaluate MMP mutants as well as transgenic mouse models in more detail.

Most of the MMP members are broadly expressed and involved in numerous essential physiological events, while only a few of them show cell type specificity or tissue restrictions, like MMP-20 for dental tissue. Their constitutive physiological expression is normally low, with transiently higher rates due to homeostatic matrix remodelling or specific developmental events. These short expression peaks underline their stringent regulation under physiological conditions and highlight their important role in tissue homeostasis and development.

The dysregulation of MMP activities, especially in early cancer stages, still raises great interest for MMPs as therapeutic targets, despite the failure of the early clinical trials using broad spectrum MMP inhibitors. The major focus of future research endeavours will lay on the design of therapeutics for selective inhibition of distinct MMP members.

STATEMENT OF INTEREST

None declared.

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