

Chapter 2

Mouse Models of MMP and TIMP Function

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Abstract

As their name implies, matrix metalloproteinases (MMPs) are thought to be responsible for the turnover of connective tissue proteins, a function that is indeed performed by some family members. However, matrix degradation is possibly not the predominant function of these enzymes. Several studies have demonstrated that MMPs also act on a variety of non-matrix extracellular proteins, such as cytokines, chemokines, receptors, junctional proteins, and antimicrobial peptides, to mediate a wide range of biological processes, such as repair, immunity, and angiogenesis. Our understanding of the many, diverse and, at times, unexpected functions of MMPs largely arose from the use of gene-targeted mice. In this chapter, we discuss the phenotypes of some MMP-deficient and TIMP-null mice and strategies and pitfalls in targeted mutagenesis.

Key words: Knockout, knockdown, substrate identification, conditional, inhibitors, tissue specific, compensation, redundancy.

1. Introduction

Proteolysis is one of the several post-translational mechanisms that regulate protein activity, and it is the principal means of ending a protein's life and recycling its amino acids for reuse. Evolution has provided us with six families of proteinases, defined by the amino acid or the cofactor that catalyzes the nucleophilic attack on the peptide backbone of substrate proteins. Proteinases function both inside and outside of the cell, with serine and metalloproteinases being the most abundant of extracellular proteinases (1). Of the metalloproteinases, matrix metalloproteinases (MMPs) – the subject of this volume – have been long thought

to be responsible for turnover of connective tissue proteins, such as the collagens, elastin, and basement membrane components, functions which some specific MMPs – often to a limited extent – do confer. However, matrix turnover is neither the sole nor the predominant function of MMPs.

Findings from many groups over the past decade or so have shown that MMPs function in a variety of processes, such as immunity, epithelial repair, leukocyte influx and activation, and more (2–6). Over a tenth of our genome codes for proteins with a signal sequence, thus placing them (as well as secreted proteins whose genes do not code for a signal peptide) within the reach of MMPs. That is a lot of potential substrates. Because proteolysis is a common mechanism used to control the activity of extracellular proteins (e.g., the coagulation and complement cascades, activation of latent cytokines, prohormones, neuropeptides, and digestive enzymes, processing of matrix precursors, and more), it is not at all surprising that a large family of proteinases, like the MMPs, shape a variety of physiological processes.

The realization that, as a family, MMPs have evolved pleiotropic functions (in contrast, individual MMPs are limited in their scope of activities and, hence, substrates) came about with the widespread use of reverse genetics. Understanding what an MMP actually does (as opposed to what it can do, i.e., what it can cleave or degrade in a defined, *in vitro* setting) was not achievable until the consequence of enzyme depletion could be studied in genetically modified mice. In this chapter, we discuss how targeted mutagenesis in mice can be used to understand MMP function.

2. Matrix Metalloproteinases (MMPs)

MMPs are a family of zinc-dependant endopeptidases composed of 25 known members, 24 of which are in mammals (3). As stated, MMPs cleave a variety of extracellular substrates, resulting in the release and activation of growth factors from the cell membrane or the extracellular matrix, the shedding of receptors and cell adhesion proteins from the cell surface, the breakdown or modification of connective tissue proteins, and potentially the activation of the zymogen form of other MMPs, among several other functions (5, 7–9). As a consequence of this ability to act on effector proteins, MMP-mediated proteolysis controls a wide range of cell behaviors and responses to environmental insults.

The structural features of MMPs have been thoroughly discussed in several reviews (6, 10–12). The defining features of MMPs – the pro-, catalytic, and hemopexin-like domains – are quite similar among members (10), and the inclusion of other

motifs or the omission of the hemopexin-like domain (as in MMP7 and 26) provides the individual enzymes with their unique catalytic properties. MMPs are often subdivided into subgroups based on structural similarities or substrate preference, the latter classification being largely misguided. There are, however, two motifs that we agree can be used to sub-classify MMPs in a meaningful way. One is the furin-recognition site found at the junction of the pro and catalytic domains in about one-third of MMPs. Pro-MMPs that contain this motif are activated within the secretion pathway. In contrast, we really know little of how the other two-thirds are activated. Identifying the mechanisms controlling how pro-MMPs are activated *in vivo* is an area ripe for investigation. We have recently discussed these concepts in another review (9).

The other clear division among MMPs, and one which impacts function, is between those enzymes with a transmembrane domain (the MT-MMPs) and those without. The MMPs without a transmembrane domain are often called “soluble MMPs”. This term is not used to imply that the MT-MMPs are insoluble, but rather that the non-MT-MMPs are secreted. But what does soluble mean? That the enzymes are floating around aimlessly in search of a substrate? Unlikely. Modeling and biochemical studies of granular serine proteinases released by neutrophils demonstrate that proteinases rapidly lose effective catalytic ability as they diffuse from the cell surface (14). In contrast, at the cell surface, enzymes (and other proteins) can be oligomerized into locally high concentrations. We propose that all “soluble” MMPs are anchored to something (integrins, proteoglycans, lipids, etc.) at or on the cell surface and it is in this compartmentalized state that the proteinases act on their target substrates (3, 9). The MT-MMPs have a built-in means to be compartmentalized at the cell surface. If this anchored-to-the-cell concept is indeed true, then the substrates must also be nearby. Indeed, many of the confirmed physiological substrates of MMPs are membrane proteins themselves.

3. Tissue Inhibitors of Metalloproteinases (TIMPs)

Once activated, MMP catalysis needs to be eventually silenced, and being bound by one of the four tissue inhibitors of metalloproteinases (TIMPs) is considered the principal mechanism of enzyme inactivation (13, 15, 16). However, other inhibitors and inhibitory mechanisms of metalloproteinases have been identified. These include α_2 -macroglobulin, a potent inhibitor of proteinases in tissue fluids, and reversion-inducing cysteine-rich protein with Kazal motifs (RECK), the only known transmem-

brane MMP inhibitor (7, 15, 17). Reactive oxygen species and endocytosis may function in silencing MMPs in vivo (18). Despite the identification of these various MMP inhibitors, there are many gaps in our knowledge of how MMP catalysis is silenced in vivo.

TIMPs inhibit MMPs in a 1:1 inhibitor-to-enzyme ratio (13, 15, 19). This inhibition occurs through interaction of the N-terminal domain of the TIMP molecule, specifically the first four amino acids, with the active site of the MMP (19). TIMPs coordinate the catalytic site Zn^{2+} and bind to the active site in a similar fashion to an MMP substrate (19). Generally, all TIMPs are capable of inhibiting all known MMPs; however, the efficacy of MMP inhibition varies with each TIMP (15, 19). TIMP1, 2, and 4 are secreted (15, 19) (and likely indeed function in a soluble state), while TIMP3 is bound to sulfated glycosaminoglycans in the extracellular matrix (20). Interestingly, TIMP2 is required for MMP2 activation in vivo (21, 22), and other TIMPs have demonstrated functions independent of blocking MMP activity (23, 24).

4. MMP Function: Identifying Substrates

To understand the function of an individual MMP expressed by a specific cell type within a physiological setting – such as organogenesis, repair, inflammation, or tumor progression – we need to determine both the protein substrate upon which the proteinase acts and the consequence of that proteolysis, be it a gain- or loss-of-function processing. Thus, an important goal of current MMP research is the identification of physiological substrates and an understanding of how this proteolysis affects a specific function. Because MMPs do not act on consensus cleavage sites, candidate substrates cannot be selected *in silico*. Identifying MMP substrates has been accomplished using various strategies. Possibly the most common approach has been to incubate an active MMP with a suspected substrate under optimal, defined conditions and assess if the target protein is cleaved or degraded (*see, e.g., Chapters 15, 16, 22, and 24*). However, this approach tells us only what an MMP can do, not what it does do. In a test tube, most MMPs are non-specific and – as for many proteinases – can cleave peptide bonds in proteins they may never see in real life. (Papain is an illustrative example of this concept. This enzyme is routinely used to cleave all sorts of animal proteins in a lab setting, although it is unlikely that papain evolved in papaya to perform such processing events in the living plant.) Although in vitro proteolysis assays are easy and an essential tool for verification (*see below*), most of the proteins identified as MMP substrates *only by this approach* probably are not.

Some groups have successfully used exosite screening and other affinity-based systems to find potential substrates. This approach takes advantage of the ability of the non-catalytic domains of MMPs, typically the hemopexin domain, to bind potential substrates. For example, using exosite screening in a yeast two-hybrid system, Overall and coworkers identified CCL7/MCP3 as a substrate of MMP2 (25), providing among the earliest evidence supporting the concept that MMPs function – either directly or indirectly – in controlling chemokine activity. Over the past decade, proteomics has emerged as the way to find substrates when used to study proteinases, this approach is sometimes referred to as degradomics (26). The basic strategy is to use mass spectrometry to compare the proteome of a control sample to that of sample with altered expression (either loss or gain of function) of a specific MMP. Tryptic peptides that are underrepresented in the presence of an over-expressed MMP or over-represented in a sample from knockout or knockdown tissues or cells would be candidate substrates (*see e.g. Chapter 26*). Such an approach was recently used to identify CD18 as a substrate shed by MMP9 from the surface of macrophages (89).

The use of knockout and transgenic mice, however, dramatically improves the odds of uncovering specific MMP-substrate relationships, and as alluded to above, control over MMP expression is key to proteomics approaches. Genetic manipulation of MMP expression coupled with deductive experimentation has been used to successfully identify substrates in the pre-proteomics era. Close examination of the phenotypes of knockout mice does point to candidates, such as an excess of type I collagen deposition in MMP14 null mice (27) and reduced apoptosis in the prostate glands of castrated MMP7 deficient mice (28). By careful observation of phenotypes, substrates can be found and confirmed.

5. MMP and TIMP-Null and Transgenic Mice

The 2007 Nobel Prize in Medicine was awarded to Mario R. Capecchi, Sir Martin J. Evans, and Oliver Smithies “for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells” (nobel-prize.org). The ability to selectively knock out, knock down, mutate, or over-express specific genes has proved to be a valuable tool in modern biology, and several studies have used mice deficient in specific MMPs to identify *in vivo* substrates.

Several mouse strains have been engineered for many of the MMP and TIMP families using traditional gene-targeting technology (**Tables 2.1, 2.2, and 2.3**). These mouse lines have resulted in identification and verification of biological processes requiring MMPs and TIMPs. The accompanying tables

Table 2.1
Developmental Phenotypes in MMP and TIMP Null Mice

Mouse	Development/Homeostasis				
	Lethal		Tissue Defect		Growth Metabolism
	Embryonic	Postnatal	Transient	Permanent	
Mmp2 ^{-/-}					
Mmp3 ^{-/-}					
Mmp7 ^{-/-}					
Mmp8 ^{-/-}					
Mmp9 ^{-/-}					
Mmp2/9 ^{-/-}					
Mmp10 ^{-/-}					
Mmp11 ^{-/-}					
Mmp12 ^{-/-}					
Mmp13 ^{-/-}					
Mmp14 ^{-/-}					
Mmp2/14 ^{-/-}					
Mmp16 ^{-/-}					
Mmp14/16 ^{-/-}					
² Mmp17 ^{-/-}					
Mmp19 ^{-/-}					
Mmp20 ^{-/-}					
Mmp24 ^{-/-}					
² Mmp25 ^{-/-}					
Mmp28 ^{-/-}					
Mmp7/28 ^{-/-}					
Timp1 ^{-/-}					
Timp2 ^{-/-}					
Timp3 ^{-/-}					
Timp4 ^{-/-}					

Developmental Phenotypes¹

None	Mild	Severe

1. Scoring is based on phenotypes reported.
2. Generated by companies and included in NIH Mouse Genome Informatics Database; however, little information on these mouse lines are provided.

summarize – albeit somewhat subjectively and incompletely – the phenotypes observed for the knockout mice models generated to date. These tables were generated from data of many papers, too many to list here. For detailed information on the phenotypes reported in specific MMP and TIMP knockout mice, we recommend that the original reports be read, and a good starting point are some reviews from the past several years focused on more detailed information on phenotypes (3, 4, 6, 7, 13, 29–33).

The most evident conclusion from this collection of information is that MMPs and TIMPs have a minor, if any, role in development and homeostasis in the unchallenged mouse. A key exception to this generalization is MMP14. Mice lacking this proteinase suffer from extensive bony defects and die some weeks after birth. Although mice deficient in either MMP2, 9, 12, 13, 14, or 20 have permanent tissue defects, most are not severe and

Table 2.2
Predicted Functions of MMPs and TIMPs in Repair and Immunity¹

Mouse	Normal Response to Injury & Infection		
	Immunity Inflammation	Angiogenesis	Tissue Repair
<i>Mmp2</i> ^{-/-}			
<i>Mmp3</i> ^{-/-}			
<i>Mmp7</i> ^{-/-}		NF	
<i>Mmp8</i> ^{-/-}			
<i>Mmp9</i> ^{-/-}	2		
<i>Mmp2/9</i> ^{-/-}			
<i>Mmp10</i> ^{-/-}			
<i>Mmp11</i> ^{-/-}			
<i>Mmp12</i> ^{-/-}			
<i>Mmp13</i> ^{-/-}			2
<i>Mmp14</i> ^{-/-}			
<i>Mmp2/14</i> ^{-/-}			
<i>Mmp16</i> ^{-/-}			
<i>Mmp14/16</i> ^{-/-}			
<i>Mmp17</i> ^{-/-}			
<i>Mmp19</i> ^{-/-}			
<i>Mmp20</i> ^{-/-}			
<i>Mmp24</i> ^{-/-}			
<i>Mmp25</i> ^{-/-}			
<i>Mmp28</i> ^{-/-}			
<i>Mmp7/28</i> ^{-/-}			
<i>Timp1</i> ^{-/-}			
<i>Timp2</i> ^{-/-}			
<i>Timp3</i> ^{-/-}			
<i>Timp4</i> ^{-/-}			

MMP Function in Tissue Responses

	Promotes
	Inhibits
	None Reported
NF	None Found

1. The functions assigned are based on observed phenotypes in null mice and are those predicted that the MMP or TIMP serves when present (i.e., in wildtype mice). Thus, the functions suggested in this table are the opposite of those seen in null mice.

2. Different phenotypes observed with different tissue-specific injury models or knock-out lines.

do not grossly affect fertility, growth, or lifespan. It is interesting to note that three of the TIMP knockout animals have permanent tissue defects, such as spontaneous emphysema in *Timp3*^{-/-} mice. The broader role of TIMPs rather than MMPs in development may be related to there being fewer TIMP family members or, more likely, to the multiple potential targets of TIMP inhibition (i.e., if TIMP1 inhibits several MMPs, its deletion could result in the disruption of several MMP-dependent responses).

Overall, most MMPs appear not to be necessary for the development and maintenance of an independently living animal. The lack of a phenotype in an MMP-null mouse could be attributed to compensation by another proteinase or redundancy by co-expressed backup systems. However, in vivo evidence for compensation among MMPs is lacking (3, 34). But what is meant by compensation? For MMPs, compensation is the activity of one MMP making up for the loss of another. Currently, there is little evidence for increased or compensating MMP activity in MMP knockout mice. Although increased expression of stromelysin-

Table 2.3
Predicted Function in Disease¹

Mouse	Disease		
	Joint & Bone ³	Cancer	Vascular ³
<i>Mmp2</i> ^{-/-}			
<i>Mmp3</i> ^{-/-}			
<i>Mmp7</i> ^{-/-}			NF
<i>Mmp8</i> ^{-/-}			
<i>Mmp9</i> ^{-/-}			
<i>Mmp2/9</i> ^{-/-}			
<i>Mmp10</i> ^{-/-}			
<i>Mmp11</i> ^{-/-}			
<i>Mmp12</i> ^{-/-}			
<i>Mmp13</i> ^{-/-}			
<i>Mmp14</i> ^{-/-}			
<i>Mmp2/14</i> ^{-/-}			
<i>Mmp16</i> ^{-/-}			
<i>Mmp14/16</i> ^{-/-}			
<i>Mmp17</i> ^{-/-}			
<i>Mmp19</i> ^{-/-}		2	
<i>Mmp20</i> ^{-/-}			
<i>Mmp24</i> ^{-/-}			
<i>Mmp25</i> ^{-/-}			
<i>Mmp28</i> ^{-/-}			
<i>Mmp7/28</i> ^{-/-}			
<i>Timp1</i> ^{-/-}		2	
<i>Timp2</i> ^{-/-}			
<i>Timp3</i> ^{-/-}			
<i>Timp4</i> ^{-/-}			

MMP Function in Disease

	Promotes
	Inhibits
	None Reported
NF	None Found

1. The functions assigned are based on observed phenotypes in null mice and are the functions predicted that the MMP or TIMP serves when present (i.e., in wildtype mice). Thus, the functions suggested in this table are the opposite of those seen null mice.
2. Different phenotypes observed with different injury models or knock-out lines.
3. A role in these categories refers to predicted functions in acquired, spontaneous, or induced disease models. Congenital defects, which are listed in Table 2.1, are not included here.

1 (MMP3) and stromelysin-2 (MMP10) is seen in the uteri of some *Mmp7*^{-/-} mice (35), the stromelysins are expressed in compartments distinct from where matrilysin (MMP7) is produced. Because MMP7 functions in mucosal immunity (3), the increased expression of MMP3 and MMP10 may represent an altered host response and not compensation.

Instead of compensation, redundancy in essential biological processes may explain the overall lack of development phenotypes among MMP-null mice. This possibility is suggested by the examination of MMP-substrate interactions in vitro, where multiple MMPs have been demonstrated to cleave the same substrate (29). For example, many MMPs, including MMP2, 3, 7, 11, 12, 14, 19, 25, and 26, can degrade fibronectin in vitro

(29). The efficacy, however, with which these MMPs cleave fibronectin *in vitro* differs among enzymes. If the principal MMP that cleaves fibronectin *in vivo* is deleted (and it is not yet clear if fibronectin turnover is the responsibility of an MMP), it is possible that another MMP may process (or degrade) fibronectin but at a slower rate. In a wild-type animal, this lower affinity MMP would likely not interact with fibronectin, as fibronectin would have already been bound and processed by the higher affinity MMP; however, in the knockout mouse, the substrate is suddenly made available and, as observed *in vitro*, the lower affinity MMP subsequently cleaves fibronectin providing redundancy for the system and leading to a lack of identifiable phenotypes. However, reduced processing of a substrate may not be manifest by an overt phenotype.

Demonstrating compensation or redundancy in developmental models would require generating mice with multiple MMP gene deletions. Triple ADAM (a disintegrin and metalloproteinase domain) null mice, lacking ADAMs 9, 12, and 15, have been generating, yet these animals have no overt phenotype or alterations in litter size or expected genotype ratios (36). Either the individual role of each ADAM is subtle or these enzymes do not function in developmental processes. However, assigning relevance to an enzyme (or any protein, for that matter) by its requirement for development or homeostasis is, of course, limiting. After all, nature did not allow MMPs and TIMPs to expand through evolution just be vestigial genetic baggage.

The generalized lack of developmental phenotypes among most MMP knockout mice is not surprising. Typically, MMPs are not expressed in normal, healthy tissues, or with notable exceptions, their production and activity are at nearly undetectable levels. In contrast, some level of MMP expression is seen in any repair or remodeling process and in any diseased or inflamed tissue. Although the qualitative patterns and quantitative levels of MMPs vary among tissues, diseases, tumors, inflammatory conditions, and cell lines, a reasonably safe generalization is that activated cells, whether in tissues or in culture dish, express MMPs. As seen in **Tables 2.2** and **2.3**, phenotypes are revealed under challenged states (and due to space considerations, we highlight only a few, broad processes). Importantly, phenotypes are seen and are mechanistically distinct among MMP-null mice, and the fact that many roles for specific MMPs and TIMPs have been reported in challenged mice argues against compensation or redundancy within the family. Even though two or more MMPs may be able to cleave or degrade the same proteins *in vitro*, this does not mean that they do so *in vivo*. Overall, it appears that the MMP family expanded and evolved to function in the host response to environmental stress.

As seen in **Tables 2.2** and **2.3**, MMPs can either promote or restrain disease or repair processes and likely do so by affecting multiple and apparently opposing processes by the same cell at the same time (3, 7, 37). For example, in our studies in tissue repair and inflammation, we have determined that MMP7 is required for wound closure and generation of antimicrobial activity (38–40), clearly beneficial functions, but this MMP also promotes neutrophil influx and activation, which can be damaging and lethal (41, 42). Cancer provides another illustrative example of the good and bad effects of MMPs. Numerous studies have shown that most MMPs are expressed in some form of cancer and that MMPs are produced by all cells (cancer cells, interstitial cells, endothelial cells, leukocytes) in the tumor environment (7). However, these studies have been largely descriptive and have not shed much light on function. Thus, it is not clear if an individual MMP made by a given cell type contributes to cancer progression and growth or if it functions as part of a host defense mechanism.

Several studies have shown that specific MMPs function in cancer progression. For example, when crossed with *min/+* (*Apc^{Min}*) mice, which spontaneously develop intestinal tumors, *Mmp7^{-/-}* mice – the first MMP null generated – had a significantly lower tumor load and the tumors that arose were significantly smaller than those in *Mmp7^{+/+} min/+* mice (38). Gelatinase-B (MMP9) is seen in essentially all forms of cancer, where it is primarily produced by neutrophils and macrophages in the inflammatory reaction surrounding the tumor. Although these leukocytes have generally been considered to be part of host-mediated anti-tumor immunity (and hence a beneficial response), Coussens and coworkers, using a multi-stage model of viral-induced tumorigenesis, demonstrated that macrophage-derived MMP9 contributes to the progression of more advanced and aggressive adenocarcinomas (43). Furthermore, studies by Weiss and coworkers established that MT1-MMP (MMP14) is essential for tumor cell survival and growth within an interstitial matrix environment (44, 45). On the other hand, reduced tumor burden is seen in mice lacking MMP3 (46) or MMP8 (47). These studies – and others (7) – demonstrate that specific MMPs function in different aspects of tumorigenesis.

6. Basic Strategies for Targeted Mutagenesis

Over the past 12 years, mice carrying mutations causing loss of function for many of the MMP and TIMP families have been generated using standard targeted mutagenesis strategies and techniques. A vector designed to mutate or delete regions of the gene that code for functional elements is transfected

into embryonic stem (ES) cells. In other words, these vectors lack the sequences to be targeted. For the MMPs, most knockouts were made by deleting the catalytic domain or by targeting upstream exons, resulting in premature stop codons and/or frame-shift mutations. TIMP-null mice were generated by deletion of known or suspected function motifs. One or more exons are usually targeted, and the vector is designed such that a frame shift is inserted into the remaining exons causing generation of new, premature stop codons. With somewhat broad probability (about 1/100 or lower), the vector is incorporated into the targeted allele by homologous recombination. ES cells with the incorporated vector are identified and amplified by positive selection, typically by including a neomycin phosphotransferase cDNA driven by a constitutive promoter, such as the phosphoglycerate kinase (PGK) promoter. The PGK-neomycin cassette is often used to replace the targeted gene elements and is flanked by arms of homologous gene sequence. The homologous arms can be between 1 and 6 kb, and in general, the longer the arms, the greater the probability of homologous recombination. Constructs containing a short and long arm, rather than two long arms, may lead to a slightly decreased incidence of homologous recombination but have the advantage of allowing a PCR genotyping strategy running from the PGK-neomycin cassette to gene sequence outside of the targeting construct. To control for insertion at a nonhomologous locus, targeting vectors can also include an expression cassette for a poison, such as diphtheria toxin, as we used in generating *Mmp28*^{-/-} mice (90). Such negative selection cassettes are placed at either end of the targeting vector, thereby ensuring that they are deleted during homologous (but not non-homologous) recombination.

Selected ES cells are then injected in blastocysts, which requires a skilled technician. Typically, mouse ES cells used to generate MMP and TIMP knockout mice typically originated from 129/SJ strain or close variants and the blastocysts were from pseudo-pregnant C57Bl/6 mice. Chimeric mice are identified by mixed coat color and are interbred to generate germline heterozygotes, which are then bred to produce homozygous nulls. If no homozygous nulls are produced, then silencing the specific gene likely leads to a lethal embryonic defect. If wild-type, heterozygous nulls and homozygous nulls are born in the expected Mendelian ratio (i.e., 1:2:1), which has been the case for all MMP and TIMP knockouts, then one may conclude that the targeted gene is not essential for development. However, as is seen with *Mmp14*^{-/-} and *Mmp20*^{-/-} mice, prominent defects may be seen in specific tissues.

Compared to other congenic strains, the 129/SJ line has marked genetic heterogeneity that may confound interpretation of phenotypes when animals are studied in a mixed background.

In these instances, the inclusion of the right control animals is critical to minimize strain effects. Interbreeding heterozygotic null (+/−) mice should yield litters with Mendelian ratios of genotypes, that is, 25% wild-type (+/+), 50% heterozygous (+/−), and 25% homozygous null (−/−). These littermate wild-type and null offspring are the most appropriate choice for experiments to control for unwanted or unexpected strain effects. The preferred alternative to mixed-strain animals is to backcross heterozygotic null (or viable homozygous nulls) mice upward of ten generations to established congenic mouse lines such as C57Bl/6, BALB/c, and others.

Various optional features can be added to enhance your knockout experience. Targeting vectors can include a reporter gene, most typically β-galactosidase, whose expression is driven by the target gene's promoter. An important advantage of a reporter is that it can be used to assess where and when the target gene is being expressed. A LacZ expression cassette was inserted in *Mmp14*^{−/−} line generated by Seiki and coworkers (48) and the *Mmp16*^{−/−} and *Mmp17*^{−/−} made by Deltagen, Inc. (available through the Mouse Genome Informatics database managed by the Jackson Laboratories; <http://www.informatics.jax.org/>).

A truly powerful optional strategy is the ability to generate conditional knockouts, in which gene deletion is confined to specific cell types, to specific investigator-defined times, or both. Conditional nulls are made by incorporating either or both the Cre/loxP or the Flp/FRT recombination systems. With conditional knockouts, the gene exon sequence is left unaltered but the PGK-Neomycin resistance cassette is flanked by FRT sites and two loxP sites (a 34-bp palindromic sequence with two 13-bp inverted repeats separated by a central 8-bp that defines loxP orientation) are placed in intronic sequence on either side of the exon-intron run to be deleted. With this somewhat complex approach, PCR screening strategies are needed to track each stage of the knockout process. Usually, once the complete targeting construct has been incorporated into the germline to generate the conditional knockout mice, the selectable marker is removed by FLP recombinase activity introduced by breeding with a commercially available germline-specific *Flp* transgenic mouse. This step can also be done at the level of ES by transfection of a recombinase expression construct. Generation of the null animal is accomplished by Cre recombinase activity in vivo that can be introduced by breeding to transgenic germline-specific Cre recombinase animals or animals expressing Cre under the control of a tissue- or cell-specific promoter. Many Cre-expressing mouse lines are available (e.g., from the Jackson Laboratories, <http://jaxmice.jax.org/>).

An advantage of removing the resistance cassette is the reduction of potential “neighborhood” effects on nearby genes caused by the active PGK promoter. Inserting an exogenous strong

promoter, such as PGK, within a targeted locus combined with alternations to native chromatin structure, due to deletion of endogenous sequences and insertion of new DNA, can potentially lead to confounding artifacts. Targeted mutagenesis can influence the expression of neighboring genes, especially if the targeted gene is a member of a cluster of genes, such as the down-regulation of *Cyp2a5* in *Cyp2g1* knockout animals (49), or if a locus control site (50) or other regulatory element is affected. In most reported instances of neighborhood effects, the downstream gene has attenuated expression, although it is not known whether the influence on expression is due to the presence of the PGK-neomycin resistance promoter, the removal of gene regulatory sequence, or changes in chromatin structure surrounding the mutated site. One way to minimize targeting vector promoter effects is to remove the PGK-neomycin resistance cassette after generation of targeted ES cells or in the whole animal by the recombinase strategies discussed here. Neighborhood artifacts are a real issue, and investigators should take the steps to check for and ideally correct this potential problem.

Controlling when the targeted allele is mutated can also be done using inducible Cre systems. For example, Hayashi et al. used a fusion protein of Cre and a mutated form of the ligand-binding domain of the estrogen receptor (Cre-ER) to create tamoxifen-inducible Cre transgenic mice (51). The transgenics are then bred with mice containing the conditional or “floxed” knockout allele, thereby generating animals that express the Cre-ER transgene and that are homozygous for the conditional knockout allele. Alternatively, heterozygous nulls can be bred with floxed mice to generate mice with one functional, yet floxed allele of the gene being targeted. Not only does this approach increase the efficiency of the subsequent recombination step, but it is also particularly useful if one has already generated conventional homologous nulls that turn out to be embryonic lethal or otherwise highly impaired. These mice are treated with tamoxifen to induce Cre expression and generate the null allele. Analysis of Cre recombinase efficiency can be tracked using the Rosa reporter allele *R26R* (52), and past studies have emphasized the need to verify Cre-mediated recombination to generate the null allele. Some recombination was detected in the absence of tamoxifen treatment, attributed to tamoxifen-independent, leaky recombination of Cre. Additionally, while most organs showed efficient tamoxifen-induced Cre activity, the liver resulted in a mosaic pattern, pointing out the need to evaluate the effectiveness of the gene activity manipulation in all organs under study.

A similar strategy can be used with the tetracycline (TET) response elements, resulting in Cre under the control of this element. The TET system relies on two components, which include a tetracycline-controlled transactivator (tTA or rtTA) and

a tTA/rtTA-dependent promoter that controls the expression of the downstream Cre in a tetracycline-dependent manner. Similar problems can potentially occur with this control system as well. Leaky Cre expression or incomplete recombination penetrance in cells of a given tissue or a whole animal must be evaluated, emphasizing the need for a reporter system like the Rosa allele for determination of recombination efficiency.

Some good news is that obtaining new knockout lines is becoming easier. For example, with the initiation of the knockout mouse project and a collaboration of three major funding agencies (the NIH, the European Commission, and Genome Canada and its partners), gene targeting and trapping will be used to complete a library of mutated ES cells or mice. Distribution centers and web-based data dissemination will be established for the biomedical community and all mouse genes may be knocked out and available to researchers in the next 5 years (53).

6.1. Verification

Evidence that a given MMP can cleave a substrate, even coupled with the absence of that cleavage in a knockout animal, is suggestive but not sufficient to prove a direct MMP/substrate relationship. To be more confident of such a conclusion, various verification studies should be done. Ideally, an MMP-substrate relationship would be supported by the following observations: (1) the substrate is cleaved *in vivo* in wild-type but not in MMP-null mice (under the appropriate conditions); (2) *in vivo*, the substrate and the MMP co-localize; (3) the cleavage site (or sites) produced *in vivo* is identical to that produced *in vitro*; and (4) over-expression or add-back of the MMP increases substrate cleavage in wild-type mice or cells and restores cleavage in null models. A further control is to mutate the substrate cleavage site, rendering the protein resistant to MMP proteolysis. In addition, generating knockout or knock-in mutant mice is not the only way to ablate a specific MMP. RNA interference, dominant negative proteins, or blocking antibodies provide other approaches to inhibit the activity of a specific enzyme.

Regarding colocalization, an MMP and a substrate must be in the same microenvironment during periods of proteolysis. Compartmentalization, that is, where and how in the pericellular environment an MMP is released and held, may be the most important step in regulating the specificity of proteolysis (9). Colocalization can be assessed by immunostaining, fractionation, co-immunoprecipitation, or pull-down, among other means. A caveat with immunoprecipitation/pull-down approaches is that a proteinase may only transiently interact with its substrate. However, a catalytically dead proteinase should theoretically stably bind substrate. For MMPs, activity is typically blocked by mutating the glutamate in the catalytic domain (HEXXHXXGXXH) to an alanine.

Gain-of-function approaches include either the seemingly straightforward add-back of exogenous (ideally, activated) proteinase or a variety of over-expression strategies. Addition of exogenous proteinase to restore function has some important caveats. In vivo, the activity of an MMP is constrained by compartmentalization. Endogenous enzymes are often anchored to membrane proteins and accessory factors confining their proteolytic potential to specific substrates within specific pericellular environments (9). Thus, adding excess amounts of a truly soluble enzyme may lead to indiscriminate proteolysis of other proteins, resulting in confounding effects. Still, the ease of this approach – particularly in cell-based models – makes it an attractive option.

There are several ways to over-express an MMP (or any gene), and the use of viruses is well established as a powerful tool to restore function in knockout mice. In such restoration studies, one should include an inactive form of the MMP to control for confounding results due to the infection or the expression system itself. Interestingly, some MMPs may contribute to the dissemination of viral vectors as was observed when an MMP8-expressing virus and an oncolytic virus were co-infected into a tumor, resulting in the spread of the virus throughout the tumor (54). Transgenic mice are a powerful tool for verifying and potentially identifying MMP substrates. For example, creating a mouse that conditionally expresses an MMP in specific cell types or tissues on the same MMP-null background provides a highly convincing approach for evaluating a cleavage target.

It is important to remember that rescue of a substrate cleavage in either of the in vivo models described above only indicates an MMP's role in the cleavage of the potential target, since substrate cleavage may be the result of a series of required steps. To confidently conclude a direct role for an MMP in cleaving its substrate, the sequence at the site of cleavage in a controlled system should be recapitulated in the cleavage site observed in vivo. Nevertheless, the use of only a few of the techniques described herein will often provide sufficient evidence to reliably predict an MMP-substrate relationship.

7. Insight from Knockout Mouse Studies: MMP-TIMP Interactions

As stated, mice lacking MMP7, generated by Carole Wilson, Brigit Hogan, and Lynn Matrisian, were the first line of MMP knockout mice and were also the first used to uncover a novel physiological substrate: pro- α -defensins, a family of antimicrobial peptides (55). Key to this discovery was the observation that

MMP7 is co-packaged with pro- α -defensins in the secretory granules of mouse Paneth cells and the knowledge that these factors had to be cleaved to gain activity. Since then, *Mmp7*^{-/-} mice have been used to identify or at least highly implicate FasL (28), syndecan-1 (41), E-cadherin (40), RANKL (56), latent TNF- α (57), elastin (58), and notch-1 (59) as substrates of MMP7. From the perspective of identifying physiological substrates – via the lack of proteolysis observed in null mice – MMP7 may be the best understood member of the MMP family.

MMPs are regulated at multiple levels including expression, activation, compartmentalization, inhibition, and degradation. Many describe TIMPs as the primary inhibitors of MMPs; however, much of this is based on in vitro data with little information regarding TIMP function in vivo. Despite the fact that TIMPs are effective inhibitors of MMPs in vitro (e.g., the K_i for TIMP1:MMP1 interaction is about 10^{-11} M), no group has – until recently (see below) – demonstrated an interaction between a TIMP and an active MMP in vivo. In fact, in one study of wound fluids, which contain both active MMP1 and TIMP1, all recovered MMP1 was complexed to α_2 -macroglobulin (60). However, phenotypes observed in mice either over-expressing or lacking a TIMP family member do provide support – albeit indirectly – for the in vitro data, at least for TIMP1 and 3.

Data in models of lung injury provided compelling evidence that a specific TIMP can block the activity of a specific MMP in vivo. Re-epithelialization and neutrophil influx are impaired in the injured lungs of *Mmp7*^{-/-} mice (41), whereas these repair processes are accelerated or enhanced in *Timpl*^{-/-} mice (61, 62). These opposite phenotypes suggest that MMP7 may be silenced by TIMP1, and indeed Chen et al. have recovered complexes of active MMP7 bound to TIMP1 in airway wound fluids (62). Furthermore, mice lacking TIMP1 have increased metallo-gelatinolytic activity in their livers and when these mice are bred into *ApoE*^{-/-} mice, increased metallo-collagenolytic activity is observed in atherosclerotic plaques (63–65). In addition, transgenic mice over-expressing TIMP1 under the control of the MMP9 promoter have reduced gelatinolytic activity during cutaneous wound healing (66).

In the absence of TIMP3, increased gelatinolytic activity is seen in a number of tissues, including lung and kidney, and, at least in the developing lung, this increased activity is possibly the result of increased activation and activity of MMP2 (67–70). TIMP3-deficient mice develop a spontaneous enlargement of their alveolar space (67), and such emphysematous-like changes are often attributed to aberrant MMP activity. Indeed, Gill et al. demonstrated increased gelatinase activity in *Timp3*^{-/-} lungs (68) and partially rescued the developmental lung phenotype with a synthetic MMP inhibitor (71). Similarly, Khokha and coworkers

have reported that other phenotypes in *Timp3*^{-/-} mice, such as increased apoptosis during mammary gland involution (72) and spontaneous cardiomyopathy (73), are associated with increased MMP levels and are partially reversed by global non-specific inhibition of metalloproteinase activity. Furthermore, TIMP3 seems to govern TNF- α levels by moderating metalloproteinase activity. TNF- α is primarily released from its cell-bound latent form by ADAM17/TACE (74). Constitutive levels of TNF- α are elevated in *Timp3*^{-/-} mice, as well as the basal activity of ADAM17 (75), providing strong evidence that TIMP3 does function to govern the activity of this metalloproteinase in vivo. However, direct evidence that TIMP3 silences MMPs in vivo remains lacking.

Roles for TIMP2 and TIMP4 in regulating MMP activity, however, are not so clear. In cell-based and defined in vitro models, activation of pro-MMP2 is mediated by the activity of MMP14 and requires the presence of the right amount of TIMP2 (76, 77). Although the role of TIMP2 in pro-MMP2 activation is supported by a lack of zymogen activation in TIMP2 null mice (21, 22, 78), a similar defect in MMP14 knockout mice has not been reported and activation of pro-MMP2 in fibroblasts is not markedly, if at all, affected by deficiency of MMP14 (79). The role of MMP14 in activation of MMP2 is further questioned by a lack of phenocopy between null mouse lines. Whereas MMP2 null mice reveal mild phenotypes, mostly related to neovascularization and inflammation (80–84), MMP14 knockout mice have severe defects in skeletal development and turnover of type I collagen (85–88). Overall, the in vivo data indicate that MMP14 is not required for activation of MMP2 but that TIMP2 is.

8. Summary

The evaluation of MMP and TIMP function in vivo is essential to understanding the role of these complex enzyme families. Knock-out technology provides enormous contributions to biology in general and has notably furthered the understanding of how MMPs and TIMPs function in vivo. The existing mouse models are an important first step in discovering how these proteins relate to physiology and pathophysiology. Future work must be focused on the generation of conditional knockout and inducible transgenic models. Additionally, future experiments using these models to identify MMP substrates or TIMP interactions must include the controls discussed above to confidently conclude novel MMP and TIMP biology.

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