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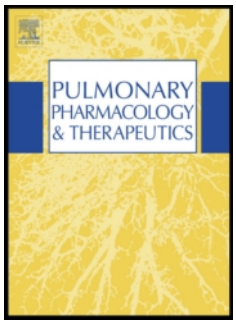

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PII: S1094-5539(09)00036-4
DOI: 10.1016/j.pupt.2009.03.001
Reference: YPUPT 923

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 20 January 2009
Accepted Date: 17 March 2009

Please cite this article as: Garbacki N, Di Valentin E, Piette J, Cataldo D, Crahay Céline, Colige A. Matrix metalloproteinase 12 silencing: a therapeutic approach to treat pathological lung tissue remodeling?, Pulmonary Pharmacology & Therapeutics (2009), doi: 10.1016/j.pupt.2009.03.001

REVIEW

Date of preparation: 20th January 2009

Number of text pages: 26

Number of tables: 0

Number of figures: 7

MATRIX METALLOPROTEINASE 12 SILENCING: A THERAPEUTIC APPROACH TO TREAT PATHOLOGICAL LUNG TISSUE REMODELING?

Nancy Garbacki[a], Emmanuel Di Valentin[b], Jacques Piette[b], Didier Cataldo[c], Céline Crahay[c], Alain Colige[a]

[a] GIGA-Research, Laboratory of Connective Tissues Biology, University of Liège, B-4000 Liège, Belgium.

[b] GIGA-Research, Laboratory of Fundamental Virology and Immunology, University of Liège, B-4000 Liège, Belgium.

[c] GIGA-Research, Laboratory of Biology of Tumours and Development, University of Liège and CHU Liège, B-4000 Liège, Belgium.

[*] Corresponding author:

Prof. Alain Colige

University of Liège, CHU, Laboratory of Connective Tissues Biology

3 avenue de l'Hôpital, B23

B-4000 Liège, Belgium.

Tel: +32 4 3662738

Fax: +32 4 3662457

E-mail address: acolige@ulg.ac.be

[*]

[*] Proofs should be sent to the following address: Prof. Alain Colige, University of Liège, CHU, Laboratory of Connective Tissues Biology, 3 avenue de l'Hôpital, B23, B-4000 Liège, Belgium.

ABSTRACT

Among the large matrix metalloproteinases (MMPs) family, MMP-12, also referred to as macrophage elastase, plays a significant role in chronic pulmonary pathologies characterized by an intense tissue remodeling such as asthma and COPD. This review will summarize knowledge about MMP-12 structure, functions and mechanisms of activation and regulation, including potential MMP-12 modulation by microRNA. As MMP-12 is involved in many tissue remodeling diseases, efforts have been made to develop specific synthetic inhibitors. However, at this time, very few chemical inhibitors have proved to be efficient and specific to a particular MMP. The relevance of silencing MMP-12 by RNA interference is highlighted. The specificity of this approach using siRNA or shRNA and the strategies to deliver these molecules in the lung are discussed.

KEYWORDS

siRNA delivery; RNA interference; MMP-12; asthma; COPD; therapy.

1 TISSUE REMODELING IN LUNG DISEASE

Matrix metalloproteinases (MMPs) are believed to be the main physiological mediators of extracellular matrix macromolecule degradation and to be the initiators of tissue remodeling. These enzymes play a pivotal role in tissue remodeling during physiological processes such as embryonic development, morphogenesis, post natal development and during pathological conditions [1].

MMPs comprise a family of 25 related, yet distinct, zinc-containing enzymes [2]. Collectively, they are able to degrade all extracellular matrix components at neutral pH. MMP-12, also known as macrophage elastase (EC 3.4.24.65) shares common structural domains with other MMPs such as collagenase 1 (MMP-1), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10) and is often classified into the stromelysin-subgroup of MMPs due to its structure and substrate specificity. As several other MMPs (MMP-1, -3, -7, -8, -13 and -20), MMP-12 gene is located on human chromosome 11, at 11q22.3 [2].

MMP-12 is considered to be highly associated with inflammatory diseases implicating macrophage infiltration. The enzyme contributes to macrophage migration through basement membranes, a mandatory process allowing their recruitment to inflammatory sites where they rapidly expand the inflammatory cascade. However, one should bear in mind that MMP-12 expression, at both transcriptional and protein levels, is dependent upon the state of cellular differentiation and is not dectected in monocytes [3], cells from which macrophages derive.

Role of MMP-12 is well documented in lung diseases such as COPD, emphysema and asthma. Its production results from macrophage activation but also from resident cells synthesis (epithelial cells, smooth muscle cells and endothelial cells) along alveolar wall of lungs [4-8]. Elastin represents about 2.5 % (w/w) of the dry weight of the lung and is distributed widely throughout the lungs [9] and is crucial for elasticity and resistance of alveoli and bronchi. Increasing MMP-12 activity results therefore in an important elastin degradation and lung parenchyma disorganization.

COPD is characterized by progressive but not fully reversible airways obstruction that leads to cough, sputum production and dyspnea. The pathogenesis involves chronic airway inflammation, associated with an infiltration of inflammatory cells (macrophages and neutrophils), oxidative stress, parenchymal destruction, recurrent infection and imbalance between proteases and antiproteases activity where an excess of proteolytic enzymes (MMPs, cysteine and serine proteinases) is not counterbalanced by a similar rise of antiproteolytic compounds [10-13]. COPD embraces two separate chronic lung diseases: pulmonary emphysema and chronic bronchitis. Pulmonary emphysema involves destruction of the alveoli in the lungs that results in poor gas exchange capabilities. Cigarette smoking is the major known risk factor of this pathology, contributing to the activation and the recruitment of inflammatory cells to the lung [14, 15] and inducing a chronic MMP-12 production by alveolar macrophages [16] and epithelial cells [4, 17]. MMP-12 plays a pivotal role in the disease evolution as MMP-12 (-/-) knock-out mice were completely protected from development of

emphysema and had impaired recruitment of monocytes/macrophages into lung [18]. Other authors have suggested that this difference was linked to a decrease of dendritic cells migration into tobacco-exposed lungs in MMP-12 (-/-) knock-out mice [19]. Emphysema is also associated with a deficiency of α1-antitrypsin [11] which is, as described before, an inhibitor of pro-MMP-12 release by macrophages. MMP-12 production by macrophages is significantly increased in sputum of COPD patients compared to never or long-time former smokers [12, 14, 20]. This production is suggested to last for some time following smoking cessation, possibly because secretion of MMP-12 leads to a persistent alveolar inflammation [14] before complete depletion from intracellular stores.

Asthma symptoms can be caused by environmental factors such as allergens, pollutants and respiratory infections. Asthma is associated with airway inflammation. During the course of the disease, an airway remodeling usually develops that includes epithelial damage, smooth muscle and mucus gland hyperplasia, bronchial hyper-responsiveness, angiogenesis, collagen deposition and airway wall fibrosis [21]. Mediators released by epithelial cells and invading leukocytes such as cytokines (TNF-α, IL-1β), growth factors (TGF-β, GM-CSF, EGF) and endothelins (ET-1) are largely involved in the inflammation process and airways remodeling [22, 23] by strongly regulating the gene expression pattern and the activation state of many different target cells.

Over the past 15 years, many authors reported the sensitization of mice and rats with various allergens to mimick asthma development allowing to address mechanisms leading to airway remodeling and inflammation [8, 24-27]. The airway wall thickening is correlated to smooth muscle proliferation that leads to bronchial hyper-responsiveness resulting in bronchoconstriction. Smooth muscle cell contraction is calcium-dependent and cytokines such as TNF-α and IL-1β can increase intracellular calcium concentrations in these cells and further exaggerate the pathological process [28]. Few data have been published on the potential role of MMP-12 in asthma. MMP-12 mRNA levels have been shown to increase in the lungs of mice subjected to allergens exposure as compared to sham exposed counterparts [29]. MMP-12 deficient mice display less inflammation than wild type mice [30] and show a less important peribronchial fibrosis when challenged repetitively for 3 months with allergens (Fig. 1).

Patients with chronic asthma are largely resistant to steroid treatment because progressive fibrosis occurs at a subepithelial level. Fibroblasts proliferate, secrete large amount of extracellular matrix component, such as collagen type III and V, laminin, fibronectin and tenascin [22], differentiate into myofibroblasts and finally acquire the ability to contract.

2 BRIEF OVERVIEW OF MMP-12 STRUCTURE AND FUNCTION

Human MMP-12 is translated as a 1.8 kb transcript encoding a 470 amino acid proenzyme that is 64% identical to the mouse protein [31]. Its molecular mass is 54 kDa and comprises three domains (Fig. 2).

A 9 kDa amino-terminal propeptide domain I (following a short signaling peptide) includes a highly conserved cysteine residue where the thiol interacts with the zinc ion in the proenzyme form: this interaction is involved in the maintenance of enzyme latency.

Domain II or catalytic domain (22 kDa) bears the zinc-binding HExxHxxGxxH sequence motif. This motif is conserved in every known mammalian MMP-12 orthologs (Fig. 3). The catalytic domain contains three conserved histidine (H) in the sequence HExxHxxGxxH, which coordinate the zinc ion, necessary for the catalytic integrity of the protease. The glutamate residue (E) within the catalytic motif activates a zinc-bound H_20 molecule providing the nucleophile that cleaves peptide bonds. The conserved glycine residue (G), orientated C-terminal to the second coordinating histidine, allows a sharp turn, permitting the most C-terminal histidine in the triad to associate with the zinc ion.

Haemopexin-like carboxy-terminal or domain III (23 kDa) exhibits sequence homology to vitronectin and haemopexin. This domain determines substrate recognition and/or tissue inhibitor of metalloproteinases (TIMP) binding, and further participates in enzyme localisation in the extracellular matrix compartment as this type of structure is traditionally associated with proteins involved in protein-protein interactions. The haemopexin domain is attached to the catalytic domain by a disordered hinge region, which is suggested to be a flexible junction between the catalytic and the haemopexin domains [2, 11, 32-35].

As a general mechanism, the substrate enters the active site of MMP-12 and interacts with the catalytic zinc ion through carbonyl group of the scissile peptide bond while the peptide group is hydrogen bonded to the carbonyl carbon atom of Ala 182. The peptide substrate is finally hydrolyzed at the peptide bond [36].

One of the most significant role of MMP-12 appears related to the remodeling of the extracellular matrix in tissues, from fetal development to the entire adult life [37]. As suggested by its trivial name (metalloelastase), MMP-12 is clearly the most active MMP against elastin [38] although it can cleave many of the other components of the extracellular matrix such as fibronectin, fibrillin-1, laminin, entactin, type IV collagen fragments, chondroitin sulfate and heparan sulfate proteoglycans and vitronectin [35, 39, 40]. However, MMP-12 can not significantly degrade fibrillar collagen or gelatin [39]. *In vivo*, MMP-12 has the ability to activate other MMPs such as pro-MMP-2 and pro-MMP-3, which, in turn, can activate pro-MMP-1 and pro-MMP-9. This cascade of proteolytic events might explain why MMP-12 exaggerates the cascade of proteolytic processes and leads to the degradation of a wide variety of extracellular matrix proteins, including collagen types I, III, IV and V and gelatin [41].

Extracellular matrix degradation processes are often related to macrophages migration which is largely regulated by MMP-12 expression and activation [42]. Chemotactic activity of the elastin fragment released by MMP-12 [43] and processing of pro-TNF-α to active TNF-α by MMP-12 [44] further emphasize the importance of this protease for macrophage function in various physiological and pathological conditions.

Several other substrates of MMP-12 have been described such as myelin basic protein, α1-antitrypsin [44] and tissue factor pathway inhibitor (TFPI) [45], plasminogen [46, 47] and N-cadherin [48].

Cleavage of TFPI provides for MMP-12 the capacity to interfere with the coagulation pathway in inflammatory diseases and to enhance hemostasis as TFPI inhibits tissue factor (TF) pathway by binding to factor Xa (Fig. 4). The degradation of TFPI is accompanied by considerable loss of anticoagulant and anti-factor Xa activity, leading to thrombosis predisposition [45].

MMP-12 proved to be the most efficient MMP for producing an endogenous inhibition of endothelial cell proliferation from specific degradation of plasminogen [38] therefore designating MMP-12 as one of the regulators of angiogenesis and tumor evolution.

Recently, MMP-12 has been shown to be directly or indirectly involved in N-cadherin cleavage, and so in cell-cell contact disruption. The consequence of this cleavage is the release of β-catenin, that can act as a transcription factor and has central role in human diseases [49]. MMP-12, known as a pro-atherogenic metallopeptidase [50], can contribue to atherosclerotic plaque formation through this mechanism, although it remains to be unambiguously demonstrated.

3 REGULATION OF MMP-12 EXPRESSION AND ACTIVATION IN THE LUNG

Gene expression can be regulated during various steps leading from DNA to mRNA and finally to protein. These regulations can occur at a transcriptional level (control of RNA transcription), at a post-transcriptional level (control of mRNA maturation, stabilization or degradation, splicing, transport and/or localization), at a translational level (control of ribosomal translation of mRNA to protein), at a post-translational level (glycosylation, control of protein activity, degradation, storage, secretion). Regulation can also be achieved through a mechanism of mRNA degradation mainly by RNA interference. MMP-12 gene expression is known to be controlled at both transcriptional and post-translational levels and is strongly suspected to be regulated by microRNA.

3.1 TRANSCRIPTIONAL LEVEL CONTROL

Transcriptional control can occur through cascades leading to the release of various transcription factors that bind to specific conservated sites at the promoter region of the gene.

Promoter region of MMP-12 gene contains at least 6 potential binding sites for sequence specific for transcription factors and one TATA box [51, 52] (Fig. 5). Among them, an AP-1 site spanning the -81 to -75 bp from the start site region and a STAT5 site spanning the -59 to -51 bp region are most probably critical for the induction of MMP-12 promoter activity. Cytokines such as granulocyte-macrophage colony-stimulating factor, GM-CSF [3] or the homodimeric isoform of platelet derived growth factor, PDGF-BB [53] have been shown to induce AP-1 complex following binding to their membrane receptor. GM-CSF receptor, when activated, is also involved in docking and activation

of members of the STAT family of transcription factors STAT1, STAT3 and STAT5 isoforms [54]. Interleukin-1β (IL-1β), is also known to cause spontaneous overexpression of MMP-12 during chronic obstructive pulmonary disease (COPD) or asthma development [55] and one of the pathways induced by IL-1β involves AP-1 activation [56]. Elias research group [57] demonstrated that pulmonary emphysema could be induced in animals through the action of interferon-γ (IFN-γ) and interleukin-13 (IL-13) on MMP-12-dependent pathways. The role of IFN-γ is probably indirect and could be the consequence of IFN-inducible proteins such as the Th1 chemokines CXCL9 and CXCL10 up-regulating MMP-12 production by macrophages [58]. IL-13 is a major inducer of fibrosis in many chronic infectious and autoimmune disorders. However, the mechanism by which it regulates MMP-12 is not fully understood since it requires complex interactions between different cell types, such as inflammatory and mesenchymal cells that are not easily modeled *in vitro* [4]. By monitoring the development of lung inflammation in IL-13 transgenic mice, Lanone and colleagues [59] demonstrated that IL-13 can induce alveolar remodeling, respiratory failure and death, and that upregulation of MMP-2, -9, -13 and -14 by IL-13 is mediated at least partially by a MMP-12-dependent pathway. Other studies also suggested that IL-13 was able to induce transforming growth factor-β1 (TGF-β1) expression in macrophages through the activation of a specific AP-1 complex variant in bleomycin-induced lung fibrosis [60]. TGF-β1, that is often considered as an anti-inflammatory growth factor, inhibits cytokine-mediated induction of MMP-12 mRNA as well as protein and enzymatic activity in chondrosarcoma-derived HTB-94 cells [37]. This finding was confirmed *in vivo* in Fut 8-/- mouse, a model of deficiency in TGF-β1 cascade leading to MMP-12 overexpression [61]. MMP-12 inhibition by TGF-β1 is probably related to the AP-1 site. In response to TGF-β1, Smad3 has been shown to sequester Jun family members (subunits of AP-1 complex) that consequently fail to translocate to the nucleus preventing MMP-12 mRNA upregulation [53]. In light of these data, it has been proposed that IL-13 induces MMP-12 and TGF-β1 expression, leading to a delayed negative feedback from TGF-β1 on MMP-12.

Even if their specific pathways have not yet been completely elucidated, many other factors have been described to induce MMP-12 expression: low molecular fragments of hyaluronan, an extracellular matrix glycosaminoglycan that accumulate at sites of lung inflammation [62], amyloid-β peptide in the microglia through PI3K/Akt pathway [63], VEGF (vascular endothelial growth factor) [53], TNF-α (tumor necrosis factor-α) [37], the substance P, most likely through IL-1β and TNF-α [64], CD40 receptor ligand binding on macrophage cell surface [3] and cigarette smoke [65] as a result of massive production of reactive oxygen species during cigarette combustion. Hydrogen peroxide-dependent pathway involving NADPH oxidase, AP-1 and TNF-α has been pointed out by Lavigne et al. [17] as regulating MMP-12 gene expression. It has also been reported to be induced upon HMG CoA reductase inhibition by statin drugs that alter the synthesis of cholesterol by blocking the conversion of HMG CoA to mevalonate [66]. This observation is correlated with the observations of Curci et al. [50] on the induction of MMP-12 associated with changes in cellular cholesterol metabolism, such as it may occur in the presence of excess cholesterol deposits in atherosclerotic plaques (that in turn inhibit HMG CoA reductase), where MMP-12 is expressed.

3.2 RNA INTERFERENCE

miRNA are an abundant class of short (21 to 25 nucleotides), non-coding RNA that negatively regulate the expression of protein-coding genes at the post-transcriptional level by promoting mRNA degradation or inhibiting mRNA translation.

MMP-12 synthesis is strongly suspected to be regulated by microRNA (miRNA) as miRBase [67], the official miRNA database, presents up to 40 potential effective miRNA for the human sequence and up to 17 for the mouse sequence.

Our laboratory is currently studying pulmonary subepithelial fibrosis resulting from chronic asthma by using a model of ovalbumin sensitization [27]. As a complement to our gene expression study based on high density microarray [27], we are currently evaluating the implication of the RNA interference machinery during the evolution of the disease. Among several regulated miRNA, mmu-miR-672 and mmu-miR-143 were found to be deeply down-regulated (preliminary data). In the miRBase, a database that uses the miRanda algorithm to identify potential binding sites for a given miRNA based on strict complementary of 5'-end seed region, thermodynamic stability and conservation through species, mmu-miR-672 and mmu-miR-143 are reported as valuable candidates for MMP-12 mRNA regulation. At every step of the sensitization model, from short term inflammation to long term fibrosis, an increased MMP-12 expression was observed at both mRNA and protein level. This inverse correlation between MMP-12 mRNA and both miRNA expression suggests that mmu-miR-672 and mmu-miR-143 might act as regulators of both MMP-12 expression and long term asthma progression. mmu-miR-29c was also observed to be down-regulated in a time-dependent way as asthma tends to become chronical (9 times less following a 10 weeks ovalbumin exposure compared to vehicle-treated mice). Since mmu-miR-29c has recently been identified to target genes encoding extracellular matrix proteins (including multiple collagens, fibrillins, elastin and laminin γ1), its down-regulation could take part in the enhanced fibrotic response [68, 69].

3.3 POST-TRANSLATIONAL LEVEL CONTROL

In macrophages, MMP-12 is stored in cytoplasmic vesicles (Fig. 6) and can be promptly secreted as a 54 kDa cytosolic proenzyme. This secretion can immediately follow an appropriate stimulation and lasts for as long as 24 hours before requiring an active protein synthesis.

Plasmin and thrombin are serine protease activators of proteinase activated receptor-1 (PAR-1) that drives the release of MMP-12 from macrophages [70, 71]. The release of large amount of plasmin and thrombin is observed during inflammatory diseases and metastatic progression where tissue remodeling plays a preponderant role [72, 73]. PAR-1 is a G-protein coupled receptor (GPCR) that transduces plasmin or thrombin signals from the extracellular environment across the plasma membrane. GPCR activates several intracellular signaling pathways including the PKC and MAPK pathways that participate to the regulation of secretion of pro-MMP-12 granules [70, 74] (Fig. 4). α1-Antitrypsin, an inhibitor of both plasmin and thrombin, has been shown to prevent the release of MMP-

12 [71]. On the other hand, CXCL9 and CXCL10, by binding to CXCR3 chemokine receptor present on macrophage surface, are able to induce MMP-12 secretion [58, 75].

Once secreted, the 54 kDa MMP-12 proenzyme is activated by disruption of the thiol-zinc ion interaction in domain I and through the loss of the amino-terminal prodomain (domain I and signal peptide), leaving an active 45 kDa enzyme. The activation is completed by the cleavage of the haemopexin domain. At the end of the maturation process, the 22 kDa active form enzyme consists in the catalytic domain alone [11, 33].

Three distinct mechanisms can affect MMP-12 activation:

(1) non-proteolytic modification of the interaction between the free thiol and the histidine-ligated zinc atom by chemical compounds (endogenous or not, e.g. oxidants, electrophiles, heavy metal ions, alkylating agents...) and by allosteric modification [2],

(2) proteolytic cleavage of domain I by another proteinase (e.g. plasmin [70, 76, 77]),

(3) autolysis by chemical or allosteric perturbation (e.g. activation by SDS in an *in vitro* system) of the proenzyme leading to autolytic cleavage [2].

MMP-12 possesses three binding sites for Ca^{2+}, of high, medium and low affinity which is crucial for the structure and the stability of the enzyme and also for its activity. Concentration of calcium is accurately regulated in extracellular environnement but in some particular circumstances, as during inflammation responses, local increase of calcium ions concentration dramatically enhance MMP-12 activity [78].

The activity of MMPs is naturally regulated by a class of natural physiological inhibitors called tissue inhibitors of metalloproteinases (TIMPs). TIMPs inhibit MMPs in a 1:1 inhibitor to enzyme ratio through interaction of the amino-terminal domain of the TIMP molecule with the active site of the MMPs. TIMP-1 inhibits the activity of most MMPs and, among them, of MMP-12 [79].

Macrophages have also been reported to restrain their MMP-12 activity, *in vivo*, by using their own reactive oxygen species produced during inflammatory tissue injury [80].

4 MOST EFFICIENT MMP-12 CHEMICAL INHIBITORS

Since MMP-12 is not only involved in lung tissue remodeling-associated diseases [35, 37, 41, 46, 50, 81-89], substantial efforts have been made to develop MMP-12 synthetic inhibitors. However, inhibiting a specific MMP is a difficult goal because of the high conservation between many MMPs in terms of overall 3D-structure, topology of the catalytic domain and requirement of specific amino-acid residues in the active site [90, 91]. This is why although many MMPs inhibitors have been developed, very few of them have proved to specifically target a particular MMP.

Two major features characterize most of chemical MMPs inhibitors: a zinc chelating ligand and a chemical moiety which binds the substrate recognition site of the enzyme [11]. Inhibitors that have displayed a efficiency *in vitro* are hydroxamic acid, reverse hydroxamic acid, thiol, carboxylic acid,

phosphonic and phosphinic acid [92]. However, since they rely in zinc chelating for activity, a poor selectivity towards MMPs have been observed [93] and disappointing results have been obtained for most compounds except phosphinic acids (reviewed by [11]).

In the present review, only the development of specific MMP-12 inhibitors will be addressed. An extensive review on matrix metalloproteinase inhibitors has recently been written by Hu et al. [94].

Phosphinic peptides are a family of pseudo-peptides where the peptide bond is replaced by a phosphinic acid moiety (reviewed by Dive et al. [93]). Zinc chelating properties of phosphinic group are of weaker potency than hydroxamate group but its chemical structure provides the opportunity to develop more selective inhibitors. The recent developments in phosphinic peptides lead to highly selective MMP-12 inhibitors having the formula p-Br-Ph-(PO_2-CH_2)-Xaa'-Glu-Glu-NH_2. Based upon enzyme activity, the Ki values towards MMP-12 are at minimum 200 fold lower than towards other MMPs. The side chains of the two glutamate residues are in close proximity to the side chains of Thr239 and Lys177 of MMP-12 and can induce a polar interaction between residues[95]. However, these new derivatives have only been characterized in test tube [96] and the next critical step during their development will be to address their potential toxicity and their activity *in vivo*.

Among MMP-12 specific non-peptidic inhibitors, a member of the hydroxamic acid group, AS111793 (2-hydroxy-3-[1-(thiophenyl-oxadiazolyl)-2,2-dimethyl-propylcarbamoyl]-methyl hexanohydroxamic acid), has been tested on mice to investigate its effect on acute airways inflammation induced by cigarette smoke [97]. This inhibitor, orally administrated at doses level of 30 mg/kg, induced a decrease in MMP-12 activity and also resulted in a reduction of the synthesis of both sTNFRSF1A and sTNFRSF1B, the soluble receptors of TNF-α, IL-6 and MIP-1γ (also known as chemokine CCL9) [97].

Ma et al. [98] described a γ-keto carboxylic acid that displays selective inhibition of MMP-2, -9, and -12 with IC50 values between 0.20 and 1.51 μM, and shows protection against porcine pancreatic elastase-induced emphysema in male golden Syrian hamsters. Even if this compound demonstrates capacities to inhibit MMP-12, it is not completely selective.

Recently, a new class of molecular heterocyclic compounds, based on 3-aza-6,8-dioxa-bicyclo[3.2.1]octane skeleton, has been screened and a fairly selective MMP-12 inhibitor (binding affinity of 154 μM, IC50 value of 149 μM) possesses limited activity against other MMPs [99]. This compound could become a guide for future development of MMP-12 inhibitors. Another group of non-peptidic MMP-12 inhibitors that does not interact with the zinc active-site atom is in development. A thiophene template, coupled with a biaryl motif fitting into the S1' pocket of enzyme by mainly hydrophobic interactions, displayed MMP-12 affinities in the nanomolar range [100]. However, none of these compounds have been tested *in vitro* or *in vivo* at this time. Merck Serono is currently testing a MMP-12 inhibitor for the treatment of multiple sclerosis. The last press release available (16th January 2007) reveals the beginning of phase I clinical testing. The case of a natural MMP-12 inhibitor is described by Ando and collegues [101]. Ageladine A is a natural pyrrol-2-aminoimidazole alkaloid reported to inhibit various subtypes of MMPs and, among them, MMP-12. This compound, which can

be chemically synthesized, showed a MMP-12 inhibition at an IC50 of 3.66 µM in test tube [101] but evidences of activity in cell culture or *in vivo* are still lacking.

5 SILENCING MMPS BY RNA INTERFERENCE

5.1 WHY TO CHOOSE SIRNA SILENCING OF MMP-12?

As reviewed above, the pharmacological use of MMPs inhibitors has been largely hampered by their lack of specificity. Moreover, molecules that were tested in clinical trials have failed to prove any beneficial effect while they were associated with a severe side effect consisting in a musculoskeletal syndrome mainly manifesting as pain and immobility of most joints [102]. This may be due to unspecific inhibition of the other MMPs but also of members of other families of metalloproteinases such as ADAM or ADAMTS, enzymes that are also deeply involved in cytokines network regulation and in extracellular matrix turn-over. Off-target metal chelation related to other type of stuctural macromolecules and enzymes would be an alternative or complementary hypothesis. As an additional problem linked to the lack of specificity of inhibitors, different related MMPs can display opposite effects. As an exemple, MMP-9 and MMP-12 have been found to actively participate in the pathological remodeling process during asthma [29, 103] while MMP-8 seems to play an opposite role since its deficiency promotes allergen-induced airway inflammation [104].

RNA interference is the most specific and versatile inhibitory machinery described so far. It includes endogenous non-coding miRNA as described above and 21 to 23 nucleotides double stranded RNA (siRNA). The siRNA can be naturally produced inside the cells from cleavage of long double stranded RNA by an endogenous specific enzyme (DICER). The double stranded nature of siRNA makes it relatively resistant to ribonucleases. They can be also chemically synthesized and delivered into the cell by transfection. Whatever the way they are generated, siRNA are recognized by a specific multi-enzyme complex (RISC), that uses the antisense strand as a guide to recognize a specific target mRNA. If the guiding strand and the targeted sequence are fully anti-complementary, cleavage and degradation of the mRNA usually occur while the presence of mismatch at defined positions can lead to inhibition of translation rather than degradation (for extensive reviews see [105-108]).

There are many marked advantages over other approaches of using siRNA to regulate protein expression or function.

1. Identification of active compounds from chemical libraries or natural extracts requires high-throughput screening, without any guarantee of success. Similarly, development of antibodies that specifically interfere with the function of their target protein (for exemple, the catalytic activity in the case of MMPs) is still a risky project, even when using the more convenient phage-display technology. By contrast, since the determination of the genome sequence of human and many organisms, design of siRNA against any target sequence is now both easy and fast (see below).

2. Due to high specificity of base pairing, siRNA make possible to specifically alter the expression of any protein, even those, such as MMPs, that are members of a highly conserved family. Even more specific use concerns the repression of individual splice variants from a single gene or discrimination between normal and mutated gene product.

3. siRNA are not immunogenic and do not trigger an immune response if their sequence is no longer than 23 nucleotides. Longer sequences can influence cell viability and induce a potent interferon response by a strong up-regulation of the dsRNA receptor, the toll-like receptor 3 [109]. However, as shorter siRNA sequences are most usually used, naked siRNA have the advantage to not elicit immunogenic response compared to protein-related drugs [110].

4. Multiple RNA interference therapies are tested to silence viruses and among them some siRNA-based strategies are currently under clinical trial [111]. Even if not all viral targets are equally suitably or effectively inhibited by siRNA, this strategy could be more potent as vaccine or antiviral drugs by targeting viral RNA genome or those transcripts that encode essential viral factors and that are conserved among virus strains.

5. The most important advantage of siRNA is their ability to inhibit targets that can not be inhibited by conventional chemical synthesized drug or those for which getting selectivity at the protein level is laborious. With siRNA, it theoretically becomes possible to inhibit anything in the proteome by specifically targeting a mRNA sequence. Manipulation of gene expression at mRNA level is more efficient than at protein level because multiple copies of a protein (about 5000) are produced by each mRNA [112]. The siRNA approach is based on preventing a protein production instead of suppressing its activity and, therefore, affords the opportunity to provide greater efficacy in disease control and intervention.

5.2 How to choose siRNA?

The most important features needed to develop high efficient siRNA are potency, specificity and stability. siRNA selection starts with a bioinformatic design based on a combination of general rules [113], Tom Tuschl's rules and rational design [114]. These algorithms predict the siRNA potency, even if, at that time, this technique is perfectible and always necessitates an experimental validation [115]. Even though siRNA can mediate mRNA silencing in a highly specific way, attention must be paid to a potential interference with mRNA sharing partial homology with the target, called off-target genes or to the development of an immunostimulatory effect correlated to interferon response induction. Detailed proteomic analysis is required to identify off-target genes. Off-target and unwanted effects can be minimized by various strategies such as paying attention to nucleotide position in “seed region”, including chemical modifications and delivery methods (reviewed by [105]).

5.3 How to deliver siRNA in the lung?

Transfection reagents used for *in vitro* transfection do not work *in vivo* and relatively inefficient natural siRNA uptake by cell compromises use of this therapeutic. Three directions can be taken to

improve *in vivo* siRNA efficiency: siRNA stabilization, siRNA specific targeting and siRNA viral delivery.

1. Stabilization can be a challenge to take up as naked siRNA are theorically not protected against nuclease digestion. Nevertheless, stabilization does not appear to be necessary in most cases, as rapid excretion seems to occur prior to degradation [116] and double strand RNA are much more resistant to nucleases than single strand RNA. These considerations are supported by long-lasting effect (for weeks) that has been observed in non-dividing cells *in vitro* and *in vivo* [117]. However, if resistance improvement is needed, stabilization can be readily achieved by modifying chemically nucleotides or sugar, or by using protective delivery methods.

Chemical modifications might improve siRNA properties. Thermal stability is increased by introducing a 2'-fluoro, a 2'-O-methyl or a 2′-*O*-methoxyethyl (2'-MOE) on the position 2 of the ribose or by using Locked Nucleic Acid (LNA). Stability regarding nuclease digestion is enhanced by placing these chemically modified bases at the 5' or 3' end of the RNA sequence [118, 119]. siRNA can be complexed with nanoparticles, peptidic or lipidic complexes to promote pharmacokinetics and protection of the siRNA by restricting its access to nucleases. The most common formulations use liposome, lipoplex, polyethylenimine, chitosan nanoparticules, cationic peptides and polymers... Many reviews develop recent sides in these formulations [105, 120, 121].

2. Another strategy for improving the function of siRNA *in vivo* is to conjugate the siRNA to small molecules. Most common non-siRNA parts of the hybrid molecule obtained are polyethylene glycol, transferrin, folate, cholesterol, aptamers, antibodies or sugar that might allow cell type-specific binding and/or induce internalization of the siRNA via receptor mediated-endocytosis [118, 122, 123]. In pulmonary diseases involving MMP-12, targeting endothelial cells can be easily obtained. Nanoparticules coupled to antibodies against endothelial cell adhesion molecules ICAM-1 and PECAM-1 have been shown to be internalized [124, 125]. This observation could be applied to antibodies or aptamers - siRNA conjugates. Targeting macrophages, that are the principal source of MMP-12 production, can be assessed by exploiting the mannose-binding receptor that mediates the non-opsonic phagocytic uptake by macrophages [126] and the endocytosis of soluble glycoconjugates leading to enhanced uptake of ligands [127]. However, other classes of phagocytic-inducing receptors also expressed on macrophage surface can be investigated. Among them are complement receptors (CR3, CR4), lymphocyte function-associated antigen-1 (LFA-1) and Fc fragment of IgG, low affinity II, receptor (FCGR2) [128]. FCGR2B can be of particular interest as it has been demonstrated to be up-regulated in immune cells in acute and chronic mouse asthma models [27, 129-131].

3. Viral vectors, such as adenovirus, lentivirus, retrovirus, Sendai virus or adeno-associated virus, carrying encapsulated siRNA or short hairpin RNA (shRNA) DNA template inserts [110, 132, 133] can be used to induce RNA interference in cells. Viruses envelopes also contribute to protect siRNA against nucleases and provide a carrier stability. After binding to the target cell surface, virus are capable of delivering encapsulated siRNA intracellularly.

Compared to siRNA, shRNA offers advantages in silencing longevity by an efficient transduction of target cell. RNA interference is then initiated by direct expression of the insert as a

single-stranded RNA molecule (shRNA). The transcript is then recognized, processed by the RNA interference machinery and converted into the corresponding siRNA. The duration of siRNA silencing that depends on the rate of cell division would be improved. The next challenge that must be resolved is to overcome the lack of selectivity for the target cell type. The natural tropism of viruses for some cell types may be exploited [123].

In gene therapy, 24.9% of the clinical trials have used adenovirus, 1.2% lentivirus, 21.7% retrovirus and 4.1% adeno-associated virus (2008 data from [134]). However, induction of innate immune responses by viral proteins is still a limitation to the utilization of viral vectors. Adeno-associated virus, a single-stranded DNA parvovirus, is emerging as one of the leading gene therapy vectors owing to its non-pathogenicity and low immunogenicity, stability and the potential to integrate site-specifically without known side-effects [135]. On the other hand, an engineering of artificial viruses to reduce cytotoxicity is still in progress [136].

Local RNA interference (si or shRNA) delivery is preferred to systemic delivery, even if intravenous injection of nanoparticles has been shown to mostly target lung but also spleen and liver [137]. By local pulmonary administration, the retention of particles in the lung, that is dependent on their size and their density, can induce a prolonged drug release [138]. Proper methods for nanoparticles lung delivery are used towards instillation, nebulization and spraying by using dry powder formulations or suspensions (reviewed by [139]) and can be applied to therapeutics by RNA interference.

Regarding pulmonary administration, first conclusive RNA interference tests were made in primates to deliver, by intranasal mode, a SARS virus specific siRNA, resulting in reduced fever, decreased viral load, and reduced alveoli damage [140]. Lentivirus-delivered siRNA has also been used as an approach to inhibit the expression of IL-5 [141] or GATA-3 [142] in ovalbumin-induced murine models of asthma.

Since, some clinical trials are currently going on with siRNA focusing on HIV, C hepatitis, cancer, Alzheimer's disease, age-macular degeneration, diabetic macular edema and respiratory syncitial virus treatment [134]. This last trial has been initiated in 2006 by Alnylam (phase 1) for testing by inhalation nebulized siRNA formulation [143].

5.4 MMP-12 SILENCING

We examined the silencing effect of siRNA targeting MMP-12 (siMMP12) in mouse fibroblasts (L929) transduced by a lentiviral construction to stably express MMP-12 fused to V5 epitope (not published results).

siRNA used in this study was made of two complementary nucleotide strands containing 19 RNA bases followed by two DNA bases (T). The following oligonucleotide sequences were used: 5′-UCACUUACAGGAUCUAUAA-3′ and 5′- UUAUAGAUCCUGUAAGUGATT -3′ (synthesized by Eurogentec, Belgium) and annealed . Calcium phosphate-mediated transfection [144] was performed to achieve a final concentration of 1, 20 and 50 nM (siMMP12) or of 20 nM of siRNA control (siControl), a

randomised sequence with no mRNA target (Negative control from Eurogentec). Cells were collected 48 hours later to be subjected to mRNA and protein analysis of MMP-12 (Fig. 7).

This specific siMMP12 was found to efficiently silence the target MMP-12 mRNA and protein. The control random-sequence siControl caused a slight increase in MMP12 mRNA expression *versus* untreated cells, probably due to phosphate calcium transfection; MMP-12 was not modulated by siControl at the *P-value* cut-off of 0.01. Significant inhibition occured from 1 nM with an inhibition by about 45% rising to a maximum of 90% with the doses of 20 and 50 nM. These effects are strongly correlated to protein concentrations in the cells. Upper doses are not recommanded as non-specific effects on genes regulation has already been described as dependent upon siRNA concentrations [145]. High doses of siRNA administration affect multiple signaling and transcription pathways in addition to a marked influence on protein kinase response and activation of toll-like receptors [146-148]. This is a further reason to choose siRNA demonstrating high inhibitory potency at low concentration.

This experiment demonstrates that, *in vitro*, very low concentrations of siMMP12 can produce significant effects on MMP-12 gene expression. The sequence of this siMMP12 is not homologous to other MMPs sequence and specifically targets, in this point of view, MMP-12 mRNA and, accordingly, inhibits its protein production. Correlated with the fact that siRNA could be stabilized, coupled to macrophage targeting molecules and locally administrated to the lungs with specific delivery methods as described upper, this siRNA can be an interesting candidate to treat lung fibrosis diseases.

Even if our siMMP12 is sequence specific, what about the other MMPs? In the same experiment, the potential effect of the siMMP12 was evaluated on MMPs naturally expressed by this cell line. An equivalent basal expression of MMP-2 and MMP-14 mRNA was detected, similar to those of MMP-12. MMP-14 mRNA expression was not influenced by the siMMP12 treatment while MMP-2 expression was dose-dependently reduced. However, this decrease must not be attributed to a direct effect of siMMP12 on MMP-2 mRNA. MMP-12 has the capacity to activate pro-MMP-2, as described before, and MMP-2 is also involved in fibrosis [149] and particularly in pulmonary fibrosis even if its role remains unclear [31] but nothing is currently known about the influence of MMP-12 on MMP-2 transcription.

6 CONCLUSION

Lung disorders represent a good model for RNA interference therapy development because local administration of siRNA may be easier to achieve than systemic administration. Pulmonary fibrosis, that is characterized by an increase in the expression of extracellular matrix enzyme and protein mRNA and protein such as MMP-12, is not yet successfully treated. Inhibition or degradation of the corresponding mRNA should be a solution to reduce the development of the associated diseases. However, optimizing siRNA *in vivo* delivery is still a challenge to take up but this effort is worthdoing be produced to accelerate the evolution in this novel drug therapy.

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

ACKNOWLEDGEMENTS

We gratefully thank Professor Betty Nusgens (GIGA-Research, Laboratory of Connective Tissues Biology, University of Liège, Belgium) for her helpful suggestions during the preparation of this manuscript.

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Figure 1. Immunohistochemistry of α-smooth muscle actin and collagen type III in lung sections.

Wild type and MMP-12 (-/-) knock-out mice were subjected to exposition to ovalbumine for 10 weeks. Stained areas of α-smooth muscle actin (A and B) and collagen type III (C and D) are more important in lung of wild type mice (A and C) than in lung of MMP-12 (-/-) knock-out mice (B and D).

Figure 2. Domains structure of MMP-12.

MMP-12 protein is divided into three domains: the propeptide domain following a short signaling peptide (SP) includes a highly conserved cysteine residue where the thiol (SH) interacts with the zinc ion in the proenzyme form, the catalytic domain that contains the zinc-binding HExxHxxGxxH sequence motif which coordinates the zinc ion and the haemopexin-like domain attached to the catalytic domain by a disordered hinge region.

Figure 3. Zinc-binding sequence motif conserved in all known mammalian MMP-12 orthologs.

Figure 4. Plasmin and thrombin post-translational regulatory pathway of MMP-12.

Figure 5. Promoter region of human MMP-12 gene and transcriptional control of MMP-12 expression.

PDGF-BB and IL-1β are known to induce MMP-12 transcription through AP-1 while GM-CSF uses both AP-1 and STAT-5. TGF-β negatively regulates MMP-12 transcription through an AP-1 site. Many other factors have been observed to induce MMP-12 transcription but the exact mechanism of action is still unknown.

Figure 6. Immunolocalization of V5 tagged MMP-12 proteins in transduced L929 cells.

L929 cells (murine fibroblasts) stably tranduced with V5 tagged MMP-12 construct were subjected to immunofluorescence assay using a primary mouse anti-V5 antibody (Invitrogen, R960-25) and anti-mouse FITC-conjugated antibody (Dako, F026102). The slides were analyzed under a TCS SP2 Leica confocal microscope. Non-transduced L929 cells were used as controls (not shown).

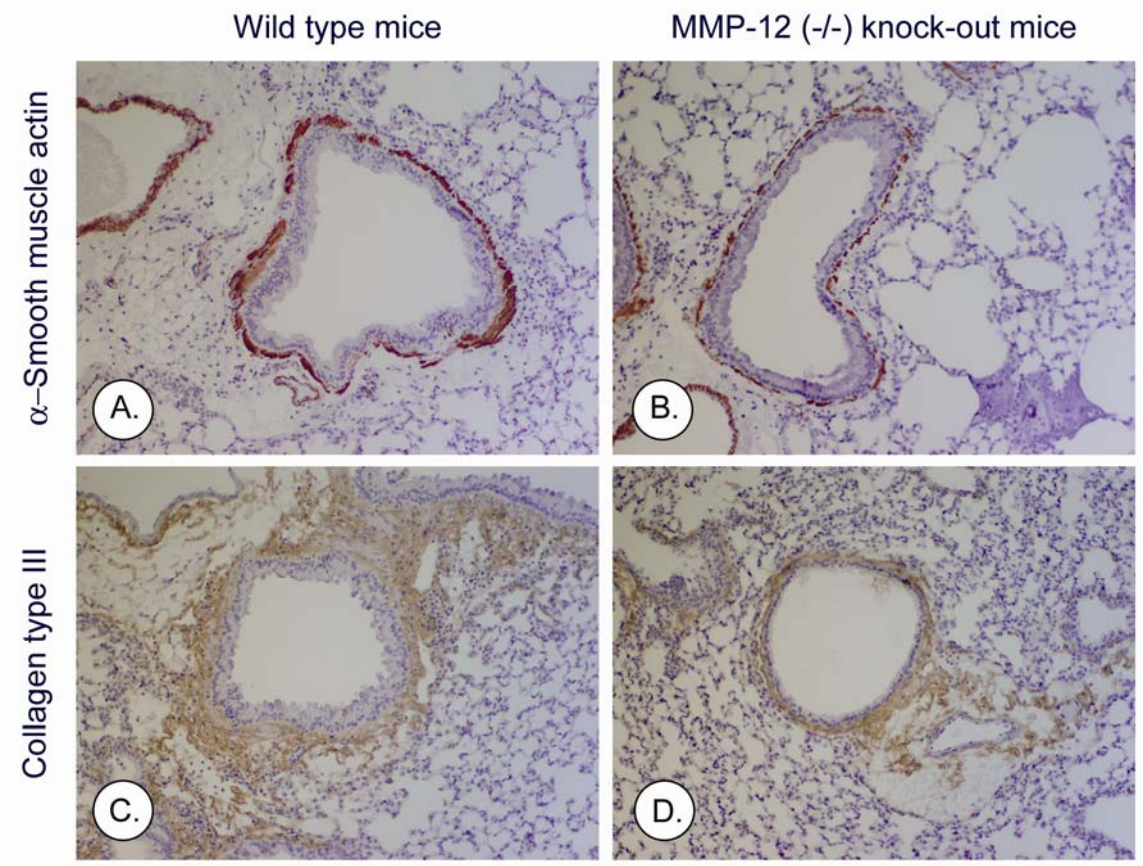
Confocal microscopy shows that the MMP-12 protein is localized in the cytoplasm (green fluorescence) with a particular intensity in a cluster next to the nuclei. The nuclei are counterstained with a blue dye (TO-PRO®-3 iodide (642/661), Molecular Probes).

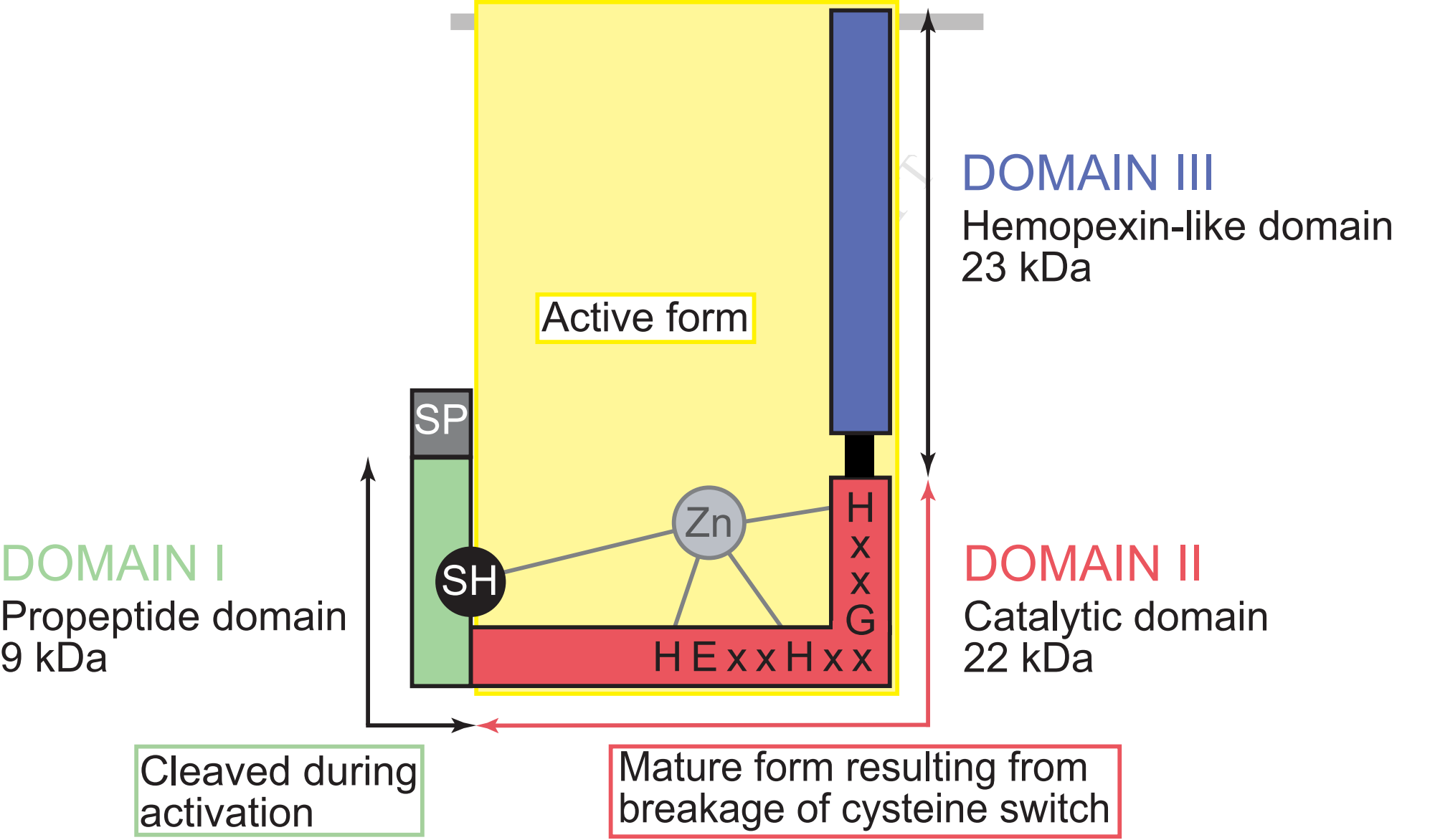
Figure 7. Effect of specific siRNA on MMP-12 mRNA expression and protein production in transduced murine fibroblast.

Briefly, murine fibroblasts L929 were transduced by a lentiviral construction to stably express MMP-12 fused to V5 epitope. Mouse lung MMP-12 mRNA was amplified and reverse transcribed using Easy-A® One-Tube RT-PCR System and then cloned into the pLenti6/V5 Directional Topo Cloning Kit. This vector was co-transfected with psAX2 (Addgene plasmid n°Plasmid 12260) and pVSV-G [150] plasmids into 293FT cells to produce lentivectors as previously described [151] and transduced L929 cell lines.

A. MMP-12 mRNA expression in arbitrary units. L929-MMP12-V5 were treated with 1, 20 and 50 nM siRNA targeting MMP-12 (siMMP12) or 20 nM siRNA Control (siC) which lacks significant sequence homology to the genome and compared to non-treated cells (NT). MMP-12 mRNA quantification was performed after 48 h post-transfection by Real Time PCR on an ABI 7700 instrument and data were analyzed using Sequence Detector software (Applied Biosystems). Results were normalized using 28s mRNA as previously described and *p-values* were calculated using the Graphpad Quickcalcs software (*t test*, www.graphpad.com) [27]. Each value is the mean ± SD of two independent experiments analysed in duplicate each. *P < 0.001 versus non-treated cells (NT).

B. Protein expression of MMP-12 (54 kDa) and GAPDH (32 kDa) in non-treated cells (NT) or in 1, 20 and 50 nM siRNA targeting MMP-12 (siMMP12) or in 20 nM siRNA Control (siC) treated cells was measured by the Western blot analysis.


Wild type mice
MMP-12 (-/-) knock-out mice
α–Smooth muscle actin
Collagen type III
A.
B.
C.
D.


DOMAIN III
Hemopexin-like domain
23 kDa
Active form
SP
Zn
SH
H
x
x
G
H E x x H x x
DOMAIN I
Propeptide domain
9 kDa
DOMAIN II
Catalytic domain
22 kDa
Cleaved during activation
Mature form resulting from breakage of cysteine switch