FUNCTIONAL STUDIES OF MATRIX METALLOPROTEINASES (MMP14, 15, 16) IN ANIMAL AND CELL CULTURE MODELS

DISSERTATION

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Abbreviations

µ.....micro (10^{-6})

x g.....g-force

°C.....grad Celsius

aa.....amino acid

A .....adenine

Amp .....ampicillin

APS.....ammoniumperoxodisulfate

bp.....base pairs

BSA .....bovine serum albumin

C .....cytosine

cDNA.....complementary deoxyribonucleic acid

CHO.....Chinese Hamster Ovarian

CMV .....cytomegalovirus

DaK.....dalton

DMEM.....Dulbecco's Modified Eagle Medium

DMSO.....dimethylsulfoxide

DNA .....deoxyribonucleic acid

DTT .....dithiothreitol

EDTA .....ethylendiamintetraacetic acid

EGFP .....enhanced green fluorescent protein

FCS.....fetal calf serum

g.....gram

G .....guanosine

GAG .....glycosaminoglycans

h.....hour

IgG .....Immunoglobulin G

kb.....kilo base pairs

l.....liter

LB .....Luria Bertani

m.....milli (10^{-3})

min.....minute

mRNA.....messenger ribonucleic acid

PAGE.....polyacrylamide gel electrophoresis

PBS .....Phosphate Buffered Saline

PCR.....polymerase chain reaction
RNA.....ribonucleic acid
T.....thymine
TABS .....(N-tris(Hydroxymethyl)methyl-3-aminopropane-sufonic acid
TE .....tris-EDTA
TEMED .....N,N,N',N'-tetraethylenamine
Tet.....tetracycline
Tm.....melting temperature
Tris.....tris(-hydroxymethyl)-aminomethane
U .....unit (enzymatic)
V .....volt
v/v.....volume per volume
Vol.....volume
w/v.....weight per volume
rpm.....rounds per minute
RT .....room temperature
S.....second
SDS.....sodium dodecyl sulfate

I. Introduction

I-1. Extracellular matrix

Many of the cells in multicellular organisms are embedded in extracellular matrix (ECM) which is composed of fibrous proteins, polysaccharides and adhesion proteins (Ruoslahti et al, 1994). However, in different tissues and organs the types of the extracellular matrix always vary. For example, tendon contains a high proportion of fibrous proteins, whereas cartilage contains a high concentration of polysaccharides (Cooper, 1997). The major structural proteins of the extracellular matrix are collagens consisting of at least 19 different members. (Brown and Timpl, 1995). Type I collagen is the most abundant type of collagen in interstitial stroma. Type IV collagen is a network-forming collagen that forms basal lamina (Kuhn, 1995). Besides collagens, connective tissue also contains elastin which participates in inhalation and exhalation movement in the lung (Adams, 2002).

In the polysaccharides, glycosaminoglycans (GAGs) are highly negatively charged and can bind positively charged ions and water molecules. These bindings make the extracellular matrix steady (Ernst et al., 1995). GAGs also link the core proteins to form proteoglycans which play an important role in cell adhesion (Kreis and Vale, 1999).

Adhesion proteins including fibronectin, laminin, entactin or nidogen as well as vitronectin contribute to link the matrix together or attach the cells to surrounded components of the matrix (Aplin et al., 1999). Fibronectin as the principal adhesion protein of connective tissues has sites for binding to proteoglycans, collagen (types I, II, III) and cell (Potts and Campbell, 1996). Laminin is the major structural component in the basal lamina, which has binding sites for cell surface receptors, type IV collagen, perlecan and agrin. In addition, laminin is tightly associated with other adhesion proteins, called entactin or nidogen (Colognato and Yurchenco, 2000). Entactin, a sulphated glycoprotein, is found in all basement membranes and also binds to collagen IV. As a result of these multiple interactions, laminin, entactin, type IV collagen and perlecan form crosslinked networks in the basal lamina (Kreis and Vale, 1999). Vitronectin, a plasma glycoprotein that circulates in the blood and presents in the ECM of many tissues, is a major ligand for the avß3 or avß5 integrin on adhesive cells (Schvartz et al., 1999). It also binds heparin, collagen, osteonectin and associates other important protein such as trans-glutaminase (Factor XIIIa), cAMP-dependent protein kinase, and tyrosyl protein sulfotransferase (Preissner and Seiffert, 1998). Extracellular binding of vitronectin to

plasminogen activator inhibitor-1 (PAI-1) and plasminogen stabilizes the inhibitor and thus affects tissue plasminogen activator-mediated plasmin formation (Ayad et al., 1998).

The major cell surface receptors responsible for the attachment of cells to the extracellular matrix are integrins. The integrins are a family of transmembrane proteins consisting of two subunits, designated α and β, which contain binding sites for collagen, fibronectin and laminin (Stupack and Cheresh, 2002). Integrins also serve as anchors for the cytoskeleton, which links the cytoskeleton to the extracellular matrix, thereby stabilizing the cell-matrix adhesion (Martin et al., 2002).

The ECM provides an essential framework for cells growth, migration and differentiation. Not only this, but the ECM is able to help cells to obtain the message or information from outside environments, which is essential for morphogenesis controlling, cell proliferation, cell migration, tissue repair and cell death (Werb, 1997). In this communication process, ECM proteolysis (degradation or activation) is believed to a key event, which includes remodel of the ECM, cleaning of excess ECM components, ECM assembly, and release of bioactive fragments and growth factors (Alexander and Werb, 1998). For instance, the release of important fragments and growth factors makes cells rapidly and irreversibly to respond the changes in the cellular microenvironment during growth and development such as neovascularization, tissue repair and wound healing (Chang and Werb, 2001). It is worth noting that the ECM is not the only target of this pericellular proteolysis. The cell surface proteins such as receptors and other transmembrane proteins are also involved in above processes (Sternlicht and Werb, 2001).

Matrix metalloproteinases (MMPs) and serine proteases such as thrombin, tissue plasminogen activator (tPA), urokinase (uPA) and plasmin are associated in this proteolytic cascades (Van den Steen et al., 2001). Furthermore, adamalysin-related membrane proteinases (ADAMs), and bone morphogenetic protein 1 (BMP1)/tolloid (tld) family also play a role in the degradation of ECM or other cell proteins, thereby freeing cells or releasing latent growth/angiogenesis factors from ECM during cancer cell invasion and tumor progression (Werb, 1997).

I-2. Matrix metalloproteinases (MMPs) and their multiple functions

I-2.1. MMPs

MMPs, also referred to as matrixins, are a family of 28 endopeptidases (Lohi et al., 2001). The MMP family is encoded by different genes and is subdivided into four groups: the minimal MMPs which contain the pre-, pro- and catalytic domains (matrilysin/MMP-7), the hemopexin MMPs which contain an additional terminal domain similar to hemopexin and vitronectin (collagenases: MMP-1, -8, -3 and -18; stromelysins: MMP-3, -10, -11 and metalloelastase MMP-12), the fibronectin MMPs (gelatinase A/MMP-2, gelatinase B/MMP-9), and the transmembrane type MMPs (MT-MMPs) (Alexander and Werb, 1998; Nagase and Woessner, 1999).

MMPs have the potential ability to cleave and degrade the various ECM proteins which include the structural components of mature extra cellular matrix such as collagens and the major component of provisional matrix such as fibrin(ogen) (Quaranta, 2000). Furthermore, the substrate repertoire of MMPs can be extended to nonmatrix substrates containing chemokines, growth factors, growth factor receptors, adhesion molecules and apoptotic mediators (Overall, 2002). For example, gelatinase B is responsible for the posttranslational processing of various cytokines, converting pro-IL-1 ß into active IL-1ß (Opdenakker et al., 2001; Vaday et al., 2001).

The MMPs have been implicated in many normal biological processes, such as embryonic development, organ morphogenesis, blastocyst implantation, trophoblast invasion, ovulation, cervical dilatation, endometrial cycling, hair follicle cycling, bone remodelling, wound healing, angiogenesis, tissue remodelling, reproduction, apoptosis, postpartum uterine, breast and prostate involution (McCawley and Matrisian 2001). There is convincing evidence showing that the matrixins are involved in a lot of pathological processes, such as rheumatoid arthritis, cardiovascular disease, nephritis, neurological disease, breakdown of blood grain barrier, periodotal disease, skin ulceration, gastric ulcer, corneal ulceration, liver fibrosis, emphysema, fibrotic lung disease, macular degeneration, amyotrophic lateral sclerosis, Alzheimer's disease, vasculitic neuropathy and chronic inflammatory demelinating polyneuropathy (CIDP), Guillain-Barre syndrome (GBS), inflammatory myopathies and stroke, cancer, tumor invasion and metastasis (Parks and Mecham, 1998; Leppert et al., 2001).

Because of their multiple and crucial roles, activities of the MMPs must be tightly controlled and regulated. The regulation of MMPs activities occurs at many stages including activation of transcription, translation and secretion of latent enzyme, proenzyme activation (processing) and inhibition of enzymatic activity (Sternlicht and Werb, 2001). All currently known members of the MMPs are synthesized in a latent or inactive form since an intramolecular complex between the single cysteine residue in their propeptide domain and the essential zinc atom in their catalytic domain blocks the active site which is essential for catalysis (Van Wart and Birkedal-Hansen, 1990). Therefore, the MMPs can be activated by the dissociation of the cysteine residue from the complex, which is referred to as the "cysteine-switch" mechanism of activation (Van Wart and Birkedal-Hansen, 1990). Moreover, it has been known that most MMPs can be inhibited or regulated by their natural tissue inhibitors such as TIMP-1 and TIMP-2 (Woessner and Nagase, 2000). Importantly, if the regulation of MMPs is out of control, it causes a number of diseases such as rheumatoid arthritis, osteoarthritis, and degradation of the mylein-basic protein in neuroinflammatory diseases (Hernandez-Barrantes et al., 2002). Furthermore, MMPs are known to be over-expressed in many tumor tissues rather than in normal tissues. The high expressions and activities of MMPs have been associated with tumor invasion, metastasis earlier stages of the tumor progression, non-invasive tumors, tumor-associated angiogenesis and malignant conversion (Yana and Seiki, 2002).

I-2.2. Functions of MMPs

I-2.2.1. MMPs in would healing

Wound healing is a complex process characterized by hemostasis, re-epithelialization, granulation tissue formation, neoangiogenesis and remodelling of the ECM (Vaalamo et al., 1999). During this process, keratinocytes, endothelial cells, fibroblasts and inflammatory cells, which interact with each other or with ECM, will proliferate and/or migrate rapidly to the site of injury via a controlled degradation of the provisional matrix to produce new extracellular matrix and release growth factors, subsequently remodeling the newly formed tissues (Steffensen et al., 2001). Two classes of molecules cooperate closely to contribute this process, including matrix-degrading and -processing enzymes such as serine protease and MMPs, and the matrix adhesion and signalling receptors such as integrins.

Expression of several MMPs and integrins is found to be up-regulated in wound repair. For instance, six MMP genes including stromelysin 1 (MMP-3), stromelysin 3 (MMP-11),

collagenase 3 (MMP-13), gelatinase A (MMP-2), gelatinase B (MMP-9) and MT1-MMP are highly expressed during rat skin wound healing (Okada et al., 1997). An up-regulation of a2ß1 complex, av integrin subunit and ß1 integrin subunit is observed in granulation tissue and in dermal scar (Noszczyk et al., 2002). However, mice expressing human MMP-1 exhibit a 2-3 day delay compared to wild type mice due to an incomplete re-epithelialization, i.e., epithelium does not migrate sufficiently to cover the wound bed and keratinocytes do not contact each other in the transgenic wound (Colandrea et al., 1998). Thus, the activation or expression of MMPs should be tightly controlled in wound healing, and the natural inhibitors of MMPs, TIMPs, play a role in regulation of activation of MMPs. It is agreement that there is a delicate equilibrium between metalloproteinases and their inhibitors, and the imbalance caused by metalloproteinase over-expression finally affects wound closure (Vaalamo et al., 1999). On the other hand, the expression time of certain MMPs is also crucial to the wound repair. In vivo, MMP-1 expression peaks in migrating basal keratinocytes at 12-24 h after wounding at the wound edge, then gradually decreases and becomes undetectable at day 9 when healing is complete (Inoue et al., 1995). It is noted that cytokines and growth factors also play an important role in the above wound repair processes (Gharaee-Kermani et al., 2001).

I-2.2.2. MMPs in cell migration, invasion and cancer metastasis

There are three major steps for normal cell migration, tumor cell invasion and cancer metastasis including cell attachment to the ECM, localized degradation of the ECM and cell migration through the digested barrier (Yana and Seiki, 2002). All these processes depend on cooperation between adhesive and proteolytic events.

Through ligation with outside important components of the ECM, integrins, a family of cell adhesion proteins, trigger a variety of intracellular signaling events, including the activation of Ras, MAP kinases, focal adhesion kinase (FAK), Src, Rac/Rho/cdc42 GTPases, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) (Dans et al., 2001; Deryugina et al., 2002; Chellaiah et al., 2000; Rabinovitz et al., 1999; Gambaletta et al., 2000), which mediate the cytoskeletal rearrangements that finally affect forward protrusion and rear retraction during cell migration (O'Connor et al., 2000). Integrins promote continuous cell migration (cycles of adhesion and detachment) through integrin adhesion cascades (integrin cross-talk) in terms of a cyclic turnover from tethering adhesion proteins (like $\alpha 4\beta 1$) to firm adhesion proteins integrins (like ß2 and ß3) (Worthylake and Burridge 2001). In this process, early

adhesive interactions stimulate further integrin adhesion. Reciprocally, engagement of integrins late in the adhesion sequence negatively feedback to promote detachment of the upstream adhesions. For example, α4β1 clustering increases β2 integrin clustering (Chan et al., 2000). Consequently, the ß2 and ß3 integrins downregulate adhesion of a4ß1 to VCAM or fibronectin (Porter and Hogg, 1997; Imhof et al., 1997). Furthermore, avß3 engagement decreases the adhesiveness of ß2 integrins for ICAM and increase the migration of endothelial cells (Weerasinghe et al., 1998).

Based on abundant documents correlating invasive phenomena and the presence of MMPs, there is general agreement that MMPs are important in the execution of migration and invasion through the matrix. For example, the invasion of human malignant melanoma cells into ECM is associated with the accumulation of activation of MMPs at sites of ECM degradation (Nakahara et al., 1997). MMPs, including MT1-MMP, can degrade ECM and cell surface receptor molecules and have an essential function in malignancy (Egeblad and Werb, 2002). There is evidence that MMP-2, MMP-9 and MT1-MMP are implicated in glioma malignancy (Friedberg et al., 1998; Fillmore et al., 2001). It has also been shown that integrins regulate the expression of MMPs in cell migration and cancer invasion events. For example, avß3 produces the active form of metalloproteinase MMP-2 and leads to elevated mRNA levels of MT1-MMP and TIMP-2 in human melanoma metastasis (Felding-Habermann et al., 2002). Moreover, integrin avß6-associated ERK2 mediates MMP-9 secretion in colon cancer cells (Gu et al., 2002). On the other hand, certain MMPs such as MMP-2 are able to localize on the cell surface and participate in localized matrix degradation via binding to integrins or other receptors (Brooks et al., 1998). Interestingly, MMPs may as an integrin convertase respond to tumor cell migration. For instance, MT1-MMP processed pro-αv and triggered outside-in signal transduction for breast carcinoma cell migration (Deryugina et al., 2002). Through proteolytically degrading the existing matrix molecules the MMPs may induce changes in cell behavior and function from a state of rest to migration. Furthermore, MMPs may contribute to form new structures such as tubules, alveoli and acini, thereby reshaping tissues and organs during cells migration and cancer invasion (Quaranta, 2000).

I-2.2.3. MMPs in angiogenesis or vasculogenesis

Angiogenesis represents a controlled cellular invasive process that requires the functional activity of a wide variety of molecules, including growth factors, ECM proteins, adhesion

receptors and proteolytic enzymes. The coordination and regulation of these proteins lead to endothelial cell proliferation, matrix remodelling, cellular migration/invasion and differentiation (Oh et al, 2001).

Interaction of endothelial cells and the ECM mediated by integrins transfers positional message from the outside environment to the intracellular signalling machinery. As noted earlier, integrin ligation induces the activation of Ras, MAP kinase, FAK, Src, Rac/Rho/cdc42 GTPases, PKC and PI3K. In addition, integrin ligation increases intracellular pH and calcium levels, inositol lipid synthesis, cyclin synthesis and the expression of immediate early genes, thereby promoting endothelial cell proliferation and differentiation (Eliceiri and Cheresh, 2001). Many of the signalling pathways and effectors initiated by integrin ligation are also activated after growth-factor stimulation, suggesting that integrin and growth-factor-mediated cellular responses may synergize and coordinate. For example, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) can stimulate endothelial cell proliferation and migration, which are associated with integrin-mediated adhesion (Song et al., 2001; Borges et al., 2000; Liu et al., 2001).

Thus, in response to angiogenic stimuli such as growth factors (transforming growth factor-β, VEGF, thrombospondin) and the interaction with the ECM, endothelial cells that localize on the existing microvessels degrade their basement membrane by MMPs and serine proteases, migrate through the degraded region and further break down the interstitial stroma (Chang and Werb, 2001). Simultaneously, rear cells at the base of the existing vessel proliferate, differentiate and align to form a lumen. The newly formed hollow sprouts to form a capillary as a new blood vessel (Nguyen et al., 2001). For example, angiogenesis and atherogenesis induced by transmyocardial laser revascularization correlate with high expressions of MMP-1, -2, TIMP-1, -2 and urokinase-type plasminogen activator (u-PA) (Li et al., 2003). Furthermore, MT1-MMP or MMP-2 deficient mice have defects in experimental neovascularization, respectively (Zhou et al., 2000; Cornelius et al., 1998). There is new evident that MT-MMPs but not soluble MMPs are required in macro- and microvascular endothelial tubulogenesis (Lafleur et al., 2002). However, MMP activity may be involved in downregulating angiogenesis as well. For instance, plasminogen degradation to produce the anti-angiogenic fragment angiostatin can be mediated by MMP-12 in vitro (Cornelius et al., 1998).

I-2.2.4. Role of MMPs in tumor progression and possible clinical therapy

I-2.2.4.1. MMPs in tumor progression

It is agreement that MMPs contribute to tumor progression. The evidence shows that highly invasive ovarian cancer cell lines produce MMP-2, MMP-1, MT1-MMP and uPA (Nishikawa et al. 2000). Furthermore, MMP-1, MMP-2 and MMP-9 are detected in many kinds of tumors (Schutz et al., 2002; Franchi et al., 2002; Bachmeier et al., 2001). It is also found that overexpression of MMP-1 in mouse skin increases tumor incidence after chemical induction (D'Armiento et al., 1995). Moreover, overexpression of MMP-3 or MT1-MMP in mammary epithelium results in the development of hyperplasia and benign and malignant carcinomas (Sternlicht et al., 1999; Ha et al., 2001). Importantly, MMPs have effect on early stages of tumor progression through their cellular functions such as proliferation and survival. For instance, overexpression of human MMP-7 (mytrilysin) in the mammary epithelium of transgenic mice contributes to early-stage mammary tumorigenesis (Rudolph-Owen et al., 1998). In a syngeneic tumorigenesis mice model, MMP-11(stromelysin-3)-increased tumorigenesis does not result from increased neoangiogenesis or cancer cell proliferation but from decreased cancer cell death through apoptosis and necrosis, suggesting that the cellular function of MMP-11 benefits cancer cell survival in the stromal environment (Boulay et al., 2001).

Deleting a single MMP may attenuate tumor progression in several mouse models. For example, MMP-11-null mice demonstrate a decreased tumorigenesis in response to the 7, 12-dimethylbenzanthracene induction (Masson et al., 1998). Tumor-induced angiogenesis is suppressed in MMP-2-deficient mice and metastasis of melanoma cells or Lewis lung carcinoma cells is suppressed in MMP-9-deficient mice, respectively (Itoh et al., 1998; Itoh et al, 1999a). However, the matrix degradation is the result of the collaborated effects of multiple proteases from different levels, thus targeting an individual may be not enough to eliminate tumor invasion and metastasis.

I-2.2.4.2 Clinical cancer treatment

Because MMPs are massively up-regulated in malignant tissues and have the ability to degrade all components of the extracellular matrix, and the suppression of certain MMPs has significant effects in tumor models as mentioned earlier, synthetic metalloproteinase inhibitors (MPIs) are rapidly developed and studied in human clinical trials. Until now, the results of these trials are very disappointing. Certainly, the comparison of therapeutic benefit

mostly at late stages of cancer minimizes the effect of MMP inhibition (Coussens et al., 2002). The new survey of MMP expression in human tumor tissue sections reveals that MMPs are largely produced by reactive stromal cells which are recruited to the neoplastic environment (Sternlicht and Werb, 2001). Furthermore, TIMPs also have stimulating effect on the activation of MMPs (Jiang et al., 2002), maybe contributing to the poor clinical results of MPIs in tumor therapy. With extensive knowledge of the contribution of MMPs to the progression of specific cancer types and stages, the anti-MMP therapies would be greatly improved (Coussens et al., 2002).

I-2.2.5. MMPs in multiple sclerosis

Multiple sclerosis (MS) is regarded as a chronic autoimmune disorder of the central nervous system leading to progressive dysfunction in motor, sensory and vegetative systems, and eventually causing disability in patients (Rosenberg, 2002). The evidence shows that MMPs are involved in recurrent focal blood-brain barrier (BBB) damage, perivascular lymphocyte infiltration and patchy degradation of myelin, the formation of gliotic lesions (plaques) and the axonal disruption (Christopher et al., 1999). For example, immunohistochemistry of brain tissue of MS displays that the expression of MMP-1, -2, -3, -7, -9 is increased in the vicinity of plaques, stressing that MMP overproduction may be a key factor for the development of neuronal loss in MS (Leppert et al, 2001).

I-3. Membrane type of MMPs (MT-MMPs)

To date six MT-MMPs have been identified. MT1-, MT2-, MT3-, and MT5-MMP are localized on the plasma membrane via their transmembrane domains (Sato et al., 1994; Will et al., 1995; Takino et al., 1995; Pei., 1999). MT4 and MT6 are anchored to the cell surface via a GPI anchor (Itoh et al., 1999b; Kojima et al., 2000). With the exception of MT4-MMP, the other MT-MMPs have high sequence homology (82.5% between MT1-MMP and MT2-MMP, 66% between MT1-MMP and MT3-MMP, 53% between MT1-MMP and MT5-MMP, 39% between MT1-MMP and MT6-MMP, while only 29% between MT1-MMP and MT4-MMP). Moreover, the localizations of genomic sequences for MT-MMPs are various comparing that of MMPs. MT-MMP is at human chromosome 14q11 (MT1-MMP), 16q13 (MT2-MMP), 8q21 (MT3-MMP), 12q24 (MT4-MMP), 20q11.2 (MT5-MMP) and 16p13.3 (MT6-MMP), respectively. In contrast, all secreted MMPs locate at 11q22 (Mattei et al., 1997; Kinoh et al., 1999; Llano et al., 1999; Velasco et al., 2000).

I-3.1. MT1-MMP (MMP-14)

MT1-MMP is widely expressed in most tissues in different species of animals as well as humans. For example, MT1-MMP mRNA is detectable in lung, liver, testis, kidney, muscle, spleen and brain but not heart in rat tissue (Shofuda et al., 1997). High level of MT1-MMP RNA is detected during rat skin wound healing (Okada et al., 1997). It is also found in brain stem and spinal cord of wobbler mutant mice affected by progressive neurodegeneration and astrogliosis (Rathke-Hartlieb et al., 2000). In humans, MT1-MMP is mainly expressed in mesenchymal tissues of embryos such as bone, muscle and fibroblastic tissues (Sato, 1994). It is also found in human microglial cells of white matter (Yamada et al., 1995) and fibroblastic cells of colon and breast (Yana and Seiki, 2002).

MT1-MMP has been considered to involve in the breakdown of various ECM components including collagens Type I, II, and III, laminins 1 and 5, entactin/nidogen, vitronectin, fibronectin, fibrin and proteoglycans (Sternlicht and Werb, 2001). However, matrix breakdown may not be the primary function of MT1-MMP in tumor cells. It also activates other MMPs on the cell surface including pro-MMP-2 (Sato et al., 1994) and pro-MMP-13 (Knauper et al., 1996). MT1-MMP is directly associated with endoproteolytic modifications of cell surface receptors, including CD44, tissue transglutaminase and integrin $\alpha_v\beta_3$ (Kajita et al., 2001; Belkin et al., 2001; Mu et al., 2002).

I-3.1.1 Functions of distinct domains to MT1-MMP

I-3.1.1.1. Functions of C-terminal domain to MT1-MMP

I-3.1.1.1.1. Cytoplamic domain in internalization and endocytosis

MT1-MMP is internalized from cell surface depending on its cytoplasmic tail via clathrin-coated vesicles and a dynamin-dependent process (Jiang et al., 2001a). Another independent experiment further confirms that the binding of cytoplasmic tail (LLY^{573} sequence) of MT1-MMP with a component of clathrin-coated pits, mu2 subunit of adaptor protein 2, is crucial in this internalization (Uekita et al, 2001). In contrast, cytoplasmic deletion mutants of MT1-MMP fail to promote cell migration and invasion though a net proteolytic activity is not affected (Uekita et al, 2001). Maybe, continuous internalization of MT1-MMP at cell edge substitutes inactivated MT1-MMP molecules by newly synthesized ones during cell migration. This also indicates that the proteolytic activity of MT1-MMP is important but not sufficient for invasion, whereas the continuous change (turnover) of MT1-MMP at the

migration edge by internalization may be a dominant element for proper enzyme function during cell migration and invasion (Yana and Seiki, 2002). The internalization offers a mechanism to regulate MT1-MMP activity on the cell surface where MT1-MMP is removed and subsequently degraded.

I-3.1.1.1.2. Cytoplamic domain in oligomerization

In MCF7 breast carcinoma cells MT1-MMP is covalently linked as oligomers (dimer and/or multimers) (Rozanov et al, 2001). The MT1-MMP oligomers are also found in human fibrosarcoma cells (HT1080) in which the enzyme is naturally expressed. Lehti et al (2002) demonstrated that MT1-MMP was expressed as 200-240 kDa oligomeric complexes on the cell surface, which contained active 60 kDa and autocatalytically processed 43 kDa species in both HT-1080 cells and SV40-transformed lung fibroblasts. The further study exhibits that the multimeric complexes of MT1-MMP is formed by the interactions of its hemopexin or transmembrane/cytoplasmic domain, perhaps by the Cys574 of the enzyme's cytoplasmic tail (Lehti et al, 2000; Rozanov et al, 2001). The oligomerization facilitates MT1-MMP autocatalytic inactivation or pro-MMP-2 activation through either limiting the access of proteinase inhibitors or concentrating proteolytic activity on cell surface (Lehti et al., 2002). Therefore, MT1-MMP oligomerization is crucial for cell invasion and migration.

Thus, the cytoplasmic domain of MT1-MMP is critical for MT1-MMP to localize at the migration edge. It is important for promotion of cell invasion, migration and locomotion by controlling targeting and degradation/turnover of MT1-MMP through internalization or formation of oligomeric complexes. It is essential for activatition of gelatinase A in invasive cancer cells. It is also conceivable that the cytoplasmic domain of MT-MMPs may play a role in cellular signalling and cell/ECM communication or associate with other events via binding or release of another protein, phosphorylation or other modifications within the cytoplasmic tail.

I-3.1.1.2. Function of transmembrane domain

Transmembrane domain (TM) of MT1-MMP is essential in the pro-gelatinase A activation by MT1-MMP because pro-gelatinase A activation function of MT1-MMP is abolished by truncation of the TM domain and recovered by fusing the MT1-MMP mutant with the TM domain of interleukin 2 receptor alpha-chain (Cao et al, 1995). However, Pei et al (1996)

demonstrated that deletion of the TM domain of MT1-MMP could also activate the pro-MMP-2 through incubating purified MT1-MMP mutant and recombinant gelatinase A. Surprisingly, although deleting the TM domain the mutant MT1-MMP are still retained on the surface of cells, indicating an additional factor may involve in keeping MT1-MMP on the cell surface (Cao et al, 1995).

I-3.1.1.3. Function of propeptide domain of MT1-MMP

The sequences within the propeptide domain or between the propeptide and the catalytic domain of MT-MMPs contain a furin-recognition motif RRK(P)R. Furin readily cleaves soluble MT1-MMP lacking the transmembrane domain (Pei and Weiss, 1996); however, COS-1 cells cotransfected with wild type MT1-MMP cDNA and furin cDNA only express a 63 kDa form protein (latent enzyme) (Cao et al., 1996). Although Yana et al., (2000) detected at least three forms of MT1-MMP including 63 kDa, 58 kDa and 43 kDa in MT1-MMP transfected COS-1 cells, the inhibition of furin activity with furin inhibitor did not affect the pro-gelatinase A activation. Furthermore, transfected COS-1 cells containing a deletion of the N-terminal propeptide domain of MT1-MMP or a chimeric construction (substitution of the propeptide domain of MT1-MMP with that of collagenase 3) neither bind ^{125}I-labeled TIMP-2 of the cell surface nor initiate the activation of progelatinase A in COS-1 cells (Cao et al, 1998). Most interestingly, co-expression of both the mature sequence MT1-MMP and the propeptide of MT1-MMP in COS-1 cells results in reconstitution of MT1-MMP functions including the binding of ^{125}I-labeled TIMP-2 and subsequent activation of progelatinase A, suggesting that propeptide domain of MT1-MMP plays an essential role in TIMP-2 binding and activation of pro-gelatinase A (Cao et al, 2000). The recent data display that the propeptide domain of MT1-MMP functioning perhaps as an intramolecular chaperone is involved in protein folding and trafficking to cell surface, in which a conserved sequence, ^{42}YGYL^{45}, is important for the activation of pro-MMP-2 and the binding of TIMP-2 to the cell surface and is crucial for intramolecular chaperone function and for MT1-MMP-induced migrating of COS cells (Pavlaki et al, 2002).

I-3.1.2. Regulation of MT1-MMP

I-3.1.2.1. Regulated by non-self components

MT1-MMP is synthesized as a zymogen. Because of the existence of two sets of basic motifs (^{86}KAMRRPR and ^{108}RRKR) in the propeptide region of MT1-MMP, proprotein convertases potentially process and activate the MT1-MMP (Yana et al., 2000). It is also shown that furin-

dependent processing pathways such as the activation of intrinsic MT1-MMP in HT1080 cells (Maquoi et al., 1998) as well as furin-independent MT1-MMP processing pathways such as the activation of MT1-MMP in MCF7 breast carcinoma cells coexist (Rozanov et al, 2001). A matrixin-like proteinase sensitive to hydroxamates could be involved in a furin-independent activation of MT1-MMP in the breast carcinoma cells (Rozanov et al, 2001). Thus, MT-MMPs can be activated either intracellularly or on the cell surface via a dependent or an independent proprotein convertase pathway (Yana et al., 2000; Nagase et al., 1999; Cao et al., 1996).

In vitro, ConA is required for elevated MT1-MMP expression and trafficking or clustering of the MT1-MMP at the surface of TIMP-2 free skin fibroblast cells, resulting in the efficient activation of MMP-2 (Morrison et al, 2001). Moreover, ConA regulates MT1-MMP activity, and induces pro-MMP-2 activation in HT1080 cells by shifting endogenous MT1-MMP from intracellular compartments to cell surface through cytoplasmic domain-dependent trafficking (Jiang et al., 2001a).

Fibronectin and its peptide fragments derived from the central cell binding domain promote MMP-2 activation of endogenous MMP-2 by MT1-MMP and up-regulate the expression of MT1-MMP in HT1080 fibrosarcoma cells, indicating that signals may be involved via fibronectin binding to integrin a5ß1 receptors (Stanton et al., 1998). Furthermore, Type IV collagen down- regulated the expression of MT1-MMP in the medial smooth muscle cells (SMC) (Shofuda et al., 1998).

Besides the regualtion by proprotein convertase, ConA and ECM protein, the activity of MT1-MMP can also be regulated by a major component of endothelial tight junctions, claudin. Claudin-1, claudin-2, claudin-3 and claudin-5 promote pro-MMP-2 activation mediated by all MT-MMPs (MT1-6-MMPs) or MT1-MMP mutants lacking the transmembrane domain in a TIMP-2 independent pathway in human embryonic kidney 293T cells (Miyamori et al, 2001). Further study reveals that the ectodomain of claudin is crucial for this stimulatory effect, which interacts with MT-MMPs and recruits pro-MMP-2 on the cell surface to achieve elevated focal concentrations and consequently enhances activation of pro-MMP-2 (Miyamori et al, 2001).

Remarkably, RECK (a membrane-anchored glycoprotein) inhibits activation of MT1-MMP and blocks activity of MMP-2 in vitro (Oh et al., 2001). Comparing the secreted TIMPs, the membrane-anchored RECK can concentrate on the plasma membrane and effectively regulate local proteolytic events which always carry on at the cell surface (Welm et al., 2002). Through negatively regulates MMP-9, MMP-2 and MT1-MMP. RECK may play a key role in inhibiting tumor invasion, angiogenesis and metastasis (Oh et al., 2001).

I-3.1.2.2 Autocatalytic processing (self-regulation)

MT1-MMP is also regulated by itself. Active MT1-MMP is able to process into an inactive 43 kDa form and the process is inhibited by recombinant TIMP-2 in fibrosarcoma cells (HT1080) (Maquoi et al, 2000). In human Bowes melanoma cells active 60 kDa MT1-MMP is autocatalyticly cleaved to the inactive 43 kDa membrane form and to a soluble 20 kDa fragment containing the catalytic center (Lehti, et al., 2000). Furthermore, a soluble 56 kDa form of MT1-MMP is generated by autoproteolytic processing or shedding in human Bowes melanoma and HT1080 cells (Rozanov et al, 2001). Recently, Toth and his colleagues (2002) found a complex pattern of shedding through analysing various cell types. Besides active MT1-MMP undergoes autocatalytic processing on the cell surface and leads to formation of the inactive 43-44 kDa fragment and release of the entire catalytic domain, another two major fragments of 50 and 18 kDa and two minor species of 56 and 31-35 kDa including autocatalytic (18 kDa) and non-autocatalytic (56, 50, and 31-35 kDa) also exist in several cell types (Toth et al., 2002).

MMP-2 is not involved in this MT1-MMP inactivation (Toth et al., 2002). However, the autocatalytic cleavage of endogenous MT1-MMP to the 43 kDa species is regulated by the transmembrane and cytoplasmic domains of MT1-MMP through formation oligomeric complexes on the cell surface, in which MT1-MMP can inactivate its neighbor MT1-MMP (Lehti et al., 2002). Interestingly, such inactive fragment (43 kDa) may compete with the formation of homoligomers of intact MT1-MMP molecules and eventually inhibit pro-MMP-2 activation (Lethi et al., 1998). Moreover, fibronectin contributes to an increased level of a truncated 45 kDa form (Stanton et al., 1998). This implicates that a self-regulatory mechanism terminates MT1-MMP activity on the cell surface by disrupting enzyme integrity at a vital structural site. Thus, ectodomain shedding regulates the pericellular and extracellular activities of MT1-MMP through a delicate balance of active and inactive enzyme-soluble fragments.

I-3.1.3. Functions of MT1-MMP

I-3.1.3.1. Activation of pro-MMP-2 by MT1-MMP

Pro-MMP-2 is cleaved at Asn^{37}-leu^{38} by MT1-MMP and TIMP-2 is required for MT1-MMP-dependent activation of pro-MMP-2 on cell surface (Will et al., 1996). In a typical model of activation of pro-MMP-2 by MT1-MMP an active form of MT1-MMP firstly binds TIMP-2 through its catalytic domain, tethering soluble TIMP-2 to cell surface. This TIMP-2-MT1-MMP complex subsequently as a receptor allows pro-MMP-2 to form a ternary complex with TIMP-2-MT1-MMP through the interaction of its carboxyl-terminal domain and the carboxyl terminal domain of TIMP-2. Propeptide of pro-MMP-2 is cleaved by an adjacent TIMP-2-free MT1-MMP, thereby generating an activated intermediate form. This MMP-2 intermediate form is further processed to a fully activated form by an autocatalytic event (Will et al., 1995; Sato et al., 1996; Zucker et al., 1998; Lehti et al., 1998; Bigg et al, 2001). Afterwards, MT1-MMP is downregulated by itself processsing. When TIMP-2 exists in excess relative to MT1-MMP, these activation processes are blocked (Strongin et al., 1995; Lehti et al., 2000). It has recently been shown that only a minor proportion of MT1-MMP molecules form ternary complexes with TIMP-2 and MMP-2 and that the half-life of such complexes is relatively short in HT1080 and CCL-137 embryonic lung cells (Lehti et al., 2002). The new model is that tetramers of MT1-MMP are involved in its proteolytic events, in which the interaction through hemopexin domains bring adjacent MT1-MMP molecules into close contact on the cell surface, thus enabling pro-MMP-2 activation. Moreover, the interaction through the cytoplasmic tail regulates enzyme targeting to the sites of activity and to its inactivation and removal from the cell surface (Lehti et al., 2000).

I-3.1.3.2. Proteolytic activity of MT1-MMP towards ECM molecules

During tissue-invasive events, migrating cells need to penetrate type I collagen-rich interstitial tissues by providing proteolytic enzymes. Hotary et al. (2000) remarkably demonstrated that only MT1-MMP and MT2-MMP, but neither MT3-MMP nor soluble MMPs such as collagenases (MMP-1, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -11), or matrilysin (MMP-7) were able to directly confer invasion-incompetent cells with the ability to penetrate type I collagen matrices, indicating the potential function of these two MT-MMPs in interstitial tissues. Koike et al. (2002) confirmed that MT1-MMP, but not secreted MMPs (MMPs-1, -2, -9, and -13), influenced the migration of human microvascular endothelial cells in 3-dimensional collagen gels.

Cross-linked fibrin is deposited in tissues surrounding wounds, inflammatory sites or tumors and serves as a supporting substratum for trafficking cells and a structural barrier to invasion. MT1-, 2-, 3-MMP but not MT4-MMP display potential fibrin-invasive activity, which is independent of the plasminogen activator-plasminogen axis (Hotary et al., 2002). Moreover, MT-MMPs including MT1-MMP but not soluble MMPs are required for the VEGF/FGF-2-stimulated tubulogenesis of macro- and microvascular endothelial cells in a 3D fibrin matrix (Lafleur et al., 2002). Thus, MT1-MMP shows proteolytic activity towards ECM molecules, which are independent of MMP-2 or MMP-13.

I-3.1.3.3. Functions of MT1-MMP to adhesion receptors

MT-MMPs, in addition to the breakdown of the ECM, may be engaged in proteolysis of adhesion receptors on tumor cell surfaces. MT1-MMP processes pro-αv through generating a 115 kDa heavy chain with the truncated C terminus and a 25 kDa light chain commencing from the N-terminal Leu^{892} in breast carcinoma MCF7 cells (Ratnikov et al, 2002). The processing of pro-α_v by MT1-MMP does not affect the ability of $\alpha_v\beta_3$ integrin to efficiently bind the RGD ligand (Ratnikov et al, 2002), stressing that MT1-MMP may act as an integrin convertase in migrating tumor cells. Further studies displays that MT1-MMP cleavage of pro-α_v in cells facilitates outside-in signal transduction through enhancing tyrosine phosphorylations of focal adhesion kinase (FAK) for cell adhesion and migration on vitronectin (Deryugina et al., 2002). Moreover, through the binding of latent cytokine TGF-β and αvβ8, an integrin expressed by normal epithelial and neuronal cells in vivo, MT1-MMP facilitates the release of active TGF-β1, which leads to growth inhibition in epithelial and lung carcinoma cell (Mu et al., 2002). Regulation of integrin maturation and functionality may be an important role of MT1-MMP in tumor cells.

MT1-MMP proteolytically degrades cell surface tissue transglutaminase (tTG) at the leading edge of motile cancer cells such as glioma and fibrosarcoma. The degration of tTG alters the pattern of cell matrix recognition from the binding to fibronectin (Fn) to a more efficient interaction with collagens or other ECM proteins, thus leading to suppression of cell adhesion and migration on Fn as well as stimulation of cell migration on collagen matrices (Belkin et al., 2001). Therefore, MT1-MMP may serve as both positive and negative regulator of cell motility depending on the structure and composition of the ECM in high metastatic potential of tumors.

CD44 is a cell adhesion molecule involving in cell–cell and cell–matrix interactions. Through its NH2-terminal globular domain CD44 binds hyaluronic acid (HA), type I collagen, fibronectin, fibrin, laminin and chondroitin sulfate and plays roles in many important physiological and pathological processes such as lymphocyte homing, T cell activation, wound healing, angiogenesis and metastatic spread of cancer cells (Isacke and Yarwood, 2002). MT1-MMP processes CD44H and stimulates cell motility in pancreatic tumor cells through its detachment from the ECM or its C-terminal crosslink with signal transductions (Kajita et al., 2001). Recent data show that MT1-MMP forms a complex with CD44H via its hemopexin-like (PEX) domain and localizes at lamellipodia through the cytoplasmic tail of CD44H. This suggests that MT1-MMP connects to the actin cytoskeleton and localizes to the migration front via CD44H (Mori et al., 2002).

I-3.1.3.4. Physiological and pathological functions of MT1-MMP

MT1-MMP mRNA is expressed at a high level in various cancer tissues compared to corresponding normal tissues or benign tumors (Gilles et al, 1997; Nakamura et al., 1999; Ellenrieder et al., 2000). On the other hand, MT1-MMP is a physiological activator of pro-MMP-2, an important MMP involved in malignancy. MT1-MMP expression correlates with the activation of MMP-2 in various human malignant tumors, including melanomas, gliomas, lung, colon, breast, gastric, head and neck, lung and cervical carcinomas (Gilles et al, 1997; Nakamura et al., 1999; Ellenrieder et al., 2000; Okada et al, 1995; Yamamoto et al, 1996; Lafleur et al., 2002; Shimada et al., 2000). It has been proven that pro-MMP-2 is activated at the stromal cell surface by MT1-MMP and this event contributes to the restoration of connective tissue during rat skin wound healing (Okada et al., 1997). However, regardless of MMP-2 expression levels, overexpressing MT1-MMP in human breast adenocarcinoma MCF7 cells leads to enhanced invasiveness in vitro, rapid development of highly vascularized tumors and enhanced blood vessel sprouting (Sounni et al., 2002). This suggests that MT1-MMP but not of MMP-2 plays a critical role in the promotion of tumor growth and angiogenesis.

In vivo, the development of MT1-MMP-deficient mice is retarded and the mutant mice die early (Holmbeck et al., 1999; Zhou et al., 2000). MT1-MMP deficiency causes skeletal dysplasia, arthritis, severe osteopenia, dwarfism and soft tissue disorders due to loss of a collagenolytic activity (Holmbeck et al., 1999), suggesting that MT1-MMP plays an essential role in the formation and maintenance of skeletal tissues and angiogenesis. In contrast, over-

expression of MT1-MMP induces mammary gland abnormalities and adenocacinoma, resulting in lymphocytic infiltration, fibrosis, hyperplasia, alveolar structure disruption, dysplasia and adenocarcinoma (Ha et al., 2001). This shows that MT1-MMP induces remodeling of the extracellular matrix and tumor formation in the mammary glands of transgenic mice.

In summary, MT1-MMP appears to play a role in ECM remodelling through activation of pro-MMP-2 and pro-MMP-13 (collagenase 3) or direct cleavage of some ECM macromolecules such as type I collagen and fibronectin. Besides ECM, MT1-MMP can modify some cell surface receptors, thus being involved in a variety of physiological or pathological events. In vivo, MT1-MMP plays a pivotal function in connective tissue metabolism and modeling of the soft connective tissue matrix by resident cells. It is essential for the development and maintenance of the hard tissues of the skeleton. MT1-MMP is overexpressed in the mammary glands. Finally, MT1-MMP can be regulated by autoactivation or other proteins such as furin and claudin.

I-3.2. MT2-MMP (MMP-15)

MT2-MMP was originally isolated from a human lung cDNA library and is expressed in a wide variety of normal tissues and organs (Will et al, 1995). In rat tissues, MT2-MMP mRNA is most abundant in the lung and detectable in the brain, liver, skeletal muscle and kidney, but undetectable in the heart, spleen and testis (Shofuda et al., 1997). Although the catalytic domain of MT2-MMP (Kolkenbrock et al, 1997) or COS-1 cells transfected by mouse MT2-MMP activate pro-MMP-2 (Tanaka et al, 1997), the results regarding the activation of pro-MMP-2 by human MT2-MMP are conflicting (Tanaka et al, 1997; Miyamori et al., 2000). In a TIMP-2-free cell line derived from a *Timp*2-/- mouse, transfected cells expressing human MT2-MMP efficiently activate both endogenous and exogenous pro-MMP-2 in the absence of TIMP-2 and Con A (Morrison et al, 2001). This activation is inhibited by TIMP-2, TIMP-4 and MMP-2 hemopexin C domain (PEX) (Morrison et al, 2001). Like MT1-MMP, MT2-MMP forms a ternary complex with the progelatinase A/TIMP-2/MT2-MMP, the tethering pro-MMP-2 is then activated by excess MT2-MMP (Will et al, 1995). Elevated levels of MT2-MMP have been reported in human glioblastomas, breast or urothelial carcinomas and injured liver (Ueno et al, 1997; Kitagawa et al, 1998; Lampert et al, 1998), but the frequency is lower than that of MT1-MMP.

I-3.3. MT3-MMP (MMP-16)

MT3-MMP was first isolated from a human placenta cDNA library and consists of 604 amino acids (Takino et al., 1995). It is expressed in human microglial cells, central nervous system, grey and white matter of brain, placenta, heart, Alzheimer brain tissues and some carcinoma cell lines such as bladder carcinoma T24 and larynx carcinoma Hep2 cells, but is not detected in human lung, kidney, liver, spleen and muscle (Takino et al., 1995; Shofuda et al. 1997; Yoshiyama et al., 1998). In rat tissues, MT3-MMP mRNA is detected at high levels in the lung, brain and rat vascular smooth muscle cells, only weakly expressed in the spleen and liver, but undetectable in the heart, skeletal muscle and kidney (Shofuda et al., 1997).

Soluble MT3-MMP is able to cleave type III collagen and fibronectin (Matsmoto et al., 1997). In addition, a truncated form of MT3-MMP lacking the transmembrane and intracytoplasmic domain digests cartilage proteoglycan, gelatine, vitronectin, laminin-1, a1-proteinase inhibitor and a 2-macroglobulin but not type I collagen (Shimada et al, 1999). Furthermore, MT3-MMP has much stronger casein-degrading activity (Shofuda et al, 2001). However, a stable transfected MT3-MMP in Madin-Darby canine kidney (MDCK) cells shows very weak invasion in collagen gels (Kang et al, 2000).

In transfected COS cells human MT3-MMP induces the activation of pro-gelatinase A, but the ability of human MT3-MMP to process the pro-MMP-2 is weaker compared to MT1-MMP (Takino et al., 1995). They also found that the human MT3-MMP can not process pro-MMP-2 in HT-1080 cells. Interestingly, the transfection of rat MT3-MMP without transmembrane domain does not efficiently convert progelatinase A to its mature form (Shofuda et al., 1997). However, a soluble type of human MT3-MMP is able to activate pro-MMP-2 and this activity is inhibited by TIMP-2 (Matsumoto et al., 1997), indicating that the difference between species rather than the transmembrane domain may determine the activity of MT3-MMP in COS cells.

The activity of MT3-MMP can be regulated in various levels or mechanisms. Expression of MT3-MMP mRNA is strongly increased by platelet-derived growth factor (PDGF) and fibronectin (Shofuda et al., 1998). Rat MT3-MMP without transmembrane domain or human full-length MT3-MMP is rapidly degraded after maturation in COS-7 transfected cells (Shofuda et al., 1997). MT3-MMP and MT1-MMP are also found to be rapidly degraded in rat smooth muscle cells (SMCs) due to autodegradation or by other BB94 sensitive

proteinases (Shofuda et al, 2001). In addition, the maturation of MT3-MMP in COS cells appears three smaller fragments (52, 33, and 30 kDa, respectively) (Takino et al., 1995).

MT3-MMP plays an important role in many cases. MT3-MMP associates with melanoma chondroitin sulfate proteoglycan (MCSP) in a vertical growth phase melanoma cell line through mediation of chondroitin sulfate glycosaminoglycan (CS) (Iida et al., 2001). The formation of a complex between MT3-MMP and MCSP may contribute in promoting melanoma invasion and proteolysis. Interestingly, when MT3-MMP and MT1-MMP are coexpressed, MT1-MMP degradation is enhanced in baboon SMCs, suggesting that MT3-MMP may down-regulate MT1-MMP and play a important role in the matrix remodelling of blood vessels (Shofuda et al., 1997; Shofuda et al, 2001). Gelatinase A may produce secretory amyloid precursor protein (APP) on the plasma membrane and degrade soluble ß-amyloid protein (BAP) in the extracellular matrix. Because gelatinase A and its major activators, MT1-, 3-MMP, are found in white matter microglial cells, it has been speculated that MT1-, 3-MMP may play a role in APP physiological degradation and BAP aggregation (Yoshiyama et al., 1998).

However, it is interesting to know whether MT3-MMP has functions and substrate specificities different from the other MT-MMPs. The differences in the pro-MMP-2 activation and tissue distribution suggest that MT3-MMP and MT1-MMP play different roles in physiological and pathological events.

I-3.4. MT4-MMP (MMP-17)

MT4-MMP was first isolated from a human breast carcinoma and is mainly expressed in brain, leukocytes, colon, ovary and testis in human tissues (Puente et al., 1996). MT4-MMP shows the least sequence homology with other MT-MMPs. Interestingly, mouse full-length MT4-MMP expressed on the surface of COS-7 cells does not show ability to activate pro-MMP-2, but also the recombinant mMT4-MMP catalytic domain is unable to activate pro-MMP-2 (Kajita et al, 1999). However, mouse MT4-MMP can hydrolyze hydrofibrinogen and fibrin and shows a tumor necrosis factor-alpha convertase activity (English et al, 2000). With [H^3]ethanolamine and phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, MT4-MMP is proved to be a GPI-anchored protein (Itoh et al., 1999b). GPI type MT-MMP will utilize the GPI-moiety to localize in microdomain structures of the plasma membrane. Moreover, MT4-MMP appears to be shed from the cell surface by the action of an

endogenous metalloproteinase in COS-1 cells (Itoh et al., 1999b). The MT4-MMP may play a role in inflammation and tumoral process (English et al, 2000).

I-3.5. MT5-MMP (MMP-24)

MT5-MMP was first isolated from mouse brain (Pei, 1999a) and is predominantly expressed in brain, kidney, pancreas, lung, astrocytomas and glioblastomas from humans (Llano et al., 1999). Mouse full-length or human catalytic domain of MT5-MMP shows the activity to process progelatinase A in MDCK cells and COS cells, respectively. MT5-MMP tends to shed from cell surface as soluble proteinases in 46 kDa and 44 kDa forms and this shedding is performed by furin-like convertases (Wang et al, 2001). MT5-MMP may contribute to facilitate tumor progression, neuronal development and might be associated with Alzheimer disease (Sekine-Aizawa et al., 2001) and diabetes (Romanic et al., 2001).

I-3.6. MT6-MMP (MMP-25)

MT6-MMP was first identified from leukocytes (Pei, 1999b). Besides leukocytes, MT6-MMP is also expressed in, lung, spleen, colon carcinoma cells as well as in some neoplastic astrocytomas or glioblastomas (Velasco, 2000). Controversial results are reported on the activation of human MT6-MMP to process the pro-MMP-2. Although human MT6-MMP activates progelatinase A in transfected COS-7 cells (Velasco, 2000), human MT6-MMP does not activated pro-MMP-2 in transfected COS-1 or CHO-K1 cells even if it is expressed at the cell surface (Kojima et al., 2000). MT6-MMP is the second of GPI-anchored protein in the MT-MMPs family and may contribute to facilitate tumor progression (Kojima et al., 2000).

I-4. Tissue inhibitors of MMPs (TIMPs)

TIMPs are natural inhibitors of the matrix metalloproteinases, which exhibit multiple biochemical and physiological/biological functions including inhibition of active MMPs, pro-MMP activation, cell growth promotion, matrix binding and turnover, inhibition of angiogenesis, tumor metastasis and the induction of apoptosis (Bigg, 2001).

I-4.1. TIMP-1

TIMP-1 is expressed in the ovary and uterus of females (Nothnick, 2001). TIMP-1 does not bind pro-MMP-2 (Howard et al, 1991). Furthermore, MT1-, MT2-, MT3-, and MT5-MMP are insensitive to TIMP-1 (Goss et al., 1998). Interestingly, forced TIMP-1 expression in

transgenic mice has tumor-promoting activity in animal model (Goss et al., 1998). Certainly, there is an MMP/inhibitor balance in maintaining tissue homeostasis.

I-4.2. TIMP-2

TIMP-2 is a 22 kDa protein and widely expressed in the human body (Bigg et al., 1997). It has been shown that TIMP-2 plays a dual biological role in the activation of MMP-2 i.e. promoting and inhibiting the process of pro-MMP-2 mediated by MT-MMPs (Kinoshita et al, 1998; Caterina et al, 2000; Hernandez-Barrantes et al., 2000). Since TIMP-4 posses a close homologue of TIMP-2, therefore it binds to pro-MMP-2 and even inhibits MT1-MMP autocatalytic processing (Bigg et al, 2001). Unlike TIMP-2, TIMP-4 does not promote the pro-MMP-2 activation (Hernandez-Barrantes et al., 2001). Furthermore, when coexpressed with TIMP-2, TIMP-4 competitively reduces this activation (Wang et al., 2000). These differences between TIMP-2 and other TIMPs are likely to result from its structural features. One feature that may allow TIMP-2 to participate in pro-MMP-2 activation is in its C-terminal domain containing a 10-residue-long negatively charged C-terminal tail. This C-terminal tail may enable TIMP-2 to form salt bridges with pro-MMP-2, which are required to form a pro-MMP-2/TIMP-2 complex (Wang et al., 2000). New evidence shows that two amino acis (Glu^{192} and Asp^{193}) in the C-terminal tail of TIMP-2 is crucial for the binding to pro-MMP-2 (Kai et al., 2002). A second structural feature is at the N- terminal residues particularly within the unique long N-terminal A-B b-hairpin loop of TIMP-2 which may be required for specific interactions with MT1-MMP (Williamson et al., 2001). These or other structural differences between TIMP-2 and other TIMPs may cause them to associate with MMPs very differently and may thus prevent other TIMPs from replacing TIMP-2 as a co-activator of pro-MMP-2.

I-4.3. TIMP-3

TIMP-3 gene is expressed in many tissues and highly expressed in the placenta (Apte et al., 1994). Like TIMP-1 and TIMP-2, TIMP-3 inhibits MMP-1, MMP-2, MMP-7 and MMP-9 (Brew et al., 2000). It is thought that the inhibition of TIMP-3 and TIMP-1 is similar since the TIMP-3 and TIMP-1 genes are high homologous (Apte et al., 1996). However, TIMP-3 binds to the hemopexin domain of pro-MMP-2 and inhibits the catalytic domains of MT1-MMP as well as MT2-MMP similarly to TIMP-2 and TIMP-4 (Butler et al, 1999). TIMP-3 also inhibits the activity of ADAMTS4 (aggrecanase-1) which plays a key role in the degradation of aggrecan in arthritides (Hashimoto et al., 2001). Furthermore, the identification of TIMP-3

mutations in Sorsby's fundus dystrophy has highlighted the functional importance of TIMP-3. At least four mutations, including Ser156Cys, Gly167Cys, Tyr168Cys, and Ser181Cys in TIMP-3, are found in Sorsby's fundus dystrophy that is a rare autosomal dominant macular disorder disease with age of onset usually in the fourth decade and with unusual clinical features (Felbor et al., 1995; Felbor et al., 1996; Felbor et al., 1997)

I-4.4. TIMP-4

Like other members of the TIMP family, TIMP-4 protein is encoded by five exons (Olson et al., 1998). Only adult heart shows abundant TIMP-4, whereas very low levels are detected in kidney, placenta, colon and testis. Moreover no transcripts are detected in liver, brain, lung, thymus and spleen in humans (Greene et al., 1996). Pathologically, TIMP-4 is detected in infiltrating breast carcinoma cells in tumor tissues (Dollery et al., 1999). It has been proven that TIMP-4 inhibits MMP-1, -3, -7, and MMP-9 (Liu et al., 1997). Notably, the residue 2 (Ser^2) in the inhibitory domain of TIMP-4 plays a role in MMP-TIMP interaction (Stratmann et al., 2001). It further blocks the concanavalin A-induced cellular activation of progelatinase A and interferes with the invasion potential of the cells in vitro (Morrison et al, 2001). TIMP-4 is unable to promote pro-MMP-2 activation by either MT1-MMP or MT2-MMP and does not substitute for TIMP-2 in this process (Wang et al., 1997). The regulation of gelatinase A activity by TIMP-4 occurs through binding to the hemopexin domain of MMP-2 in a manner similar to that of TIMP-2 (Bigg et al, 2001). Besides the inhibitor functions to soluble MMPs TIMP-4 also inhibits MT1-MMP and MT2-MMP (Bigg et al., 1997; Morrison et al, 2001).

TIMP-4 shows inhibitory ability to metalloproteinase in human breast cancer cells (Greene et al., 1996), in primary tumor and in lung metastasis (Wang et al., 1997). TIMP-4 causes a significant reduction in invasion of rat vascular smooth muscle cells (Dollery et al, 1999). More complexly, human TIMP-4 can stimulate tumorigenesis of human breast cancer cells in nude mice due to up-regulation of Bcl-2 or Bcl-xL, suggesting antiapoptotic activity in breast cancer cells might cause a tumor-promoting effect of TIMP-4 (Jiang et al., 2001b).

I-5. Tc regulatory systems

Functions of mammalian genes can be defined by their actions in dominant gain-of-function experiments in transgenic animals. This gain-of-function is always hoped to be regulated by a "genetic switch" which allows the control of target gene activities quantitatively and reversibly in a temporal and spatial manner.

The Tc-regulatory system fits this requirements, in which the gene expression is tightly regulated and can be switched between the "off and on" and "more and less" expression states by the controller of transactivators/promoter and effector (tetracycline or doxycycline).

The Tc regulatory systems are composed of two sets of complementary mode vectors, tTA and rtTA. By fusing a mutant bacterial Tet repressor (TetR) with the activating domain VP16 from herpes simplex virus, Tc-controlled transactivator protein (tTA) will bind to relevant tet operator (tetO) sequence and activate the responsive promoter only in the absence of Tc. The presence of the effector will lead to a conformational change in the TetR moiety, thereby preventing the transactivator from binding to the target gene, consequently abolishing the tTA-dependent transcriptional activation (Furth et al., 1994).

The reverse Tc-controlled transactivator (rtTA) is obtained by an exchange of four amino acids in the tTA, therefore its DNA-binding behaviour is reversed. rtTA will bind to tetO only in the presence of certain Tc or Tc derivatives with subsequent activation of transcription. In the absence of the effector, the transactivator does not recognize its specific DNA target sequence (tetO) so that transcriptional activation of interest gene will not occur (Kistner et al., 1996). There are three elements in Tc regulatory systems: promoter, reporter unit, and effector. The human cytomegalovirus (hCMV) minimal promoter is chosen because it is expressed in a broad spectrum of tissues in transgenic animals. For specific applications or when tissue-specific expression is required, specific promoters will be considered. Recently, several minimal promoters were developed for this goal. CaMKIIalpha promoter was specifically used in neurones of the forebrain (Winder et al., 1998), CC10 promoter in human lung (Ray et al., 1997) and PLAP in mouse liver (Kistner et al., 1996).

The luciferase or the β-galactosidase genes can be additionally used for reporting the expression and regulation in this system. The effector, Tc or Tc's derivatives such as Dox-HCl, is applied for inducing or blocking the expression of the respective target genes.

II. Objective

It has been known, MT1-, 2-, 3-, 5-MMP have a transmembrane (TM) domain and MT4-, 6-MMP contain a GPI anchor, both of which are supposed to localize these proteins on cell surface. However, a truncated MT1-MMP that is deleted its TM domain still retains on the surface of transfected COS cells (Cao et al, 1995). Moreover, human MT2-MMP is not detected on COS cell surface, indicating an unidentified component of structure or a mechanism may contribute to transport MT-MMPs, particularly MT2-MMP, onto the cell plasma membrane.

MMP-2, particularly active MMP-2, has been regarded as a central player for angiogenesis, cellular tumor invasion as well as metastases in both animal model and clinic samples. One of important functions for MT-MMPs is to process or activate pro-MMP-2 into an active form. Although a model for manifesting the activation of pro-MMP-2 by MT1-MMP is put forward and widely accepted, however, controversial results were reported on the ability of MT2-, -3, -4, -6-MMP to process the pro-MMP-2 (Tanaka et al, 1997; Miyamori et al., 2000; Shofuda et al., 1997; Matsumoto et al., 1997; English et al, 2000; Velasco et al, 2000; Kojima et al., 2000). Thus, an unclear mechanism does exist for MT-MMPs to perform its activity in the processing of pro-MMP-2. In contrary to a hot study of MT1-MMP, rare data is available to MT3-MMP and MT2-MMP so far. To explore possible mechanism of localization and activity of human MT2-MMP and mouse MT3-MMP, a series of chimeric constructs and mutants were generated. After transfection of COS and HEK cells by MT1-, 2-, 3-MMP and relevant mutant constructs, influence of various domains on localization, detergent solubility and activity of these enzymes to process pro-MMP-2 were defined.

To investigate function of MT3-MMP in biological processes, after cloning a full-length of mouse MT3-MMP, stable tetracycline regulatory transgenic mice were screened and established by genomic PCR. Expression and regulation in organs of double transgenic mice were analyzed by RT-PCR, β-galactosidase assay, immunohistochemistry and Western blot. Phenotypes of transgenic mice were observed and examined by histochemistry. Biological functions of MT1-, 3-MMP particularly in later stage of development were assessed.

III. Materials and Methods

III-1. Study of MT3-MMP in vivo

III-1.1. cDNA cloning of murine MT3-MMP

Total RNA from C57/BL6 mice whole brain was obtained using the High Pure RNA Tissue Kit (Roche, Mannheim) and reverse transcribed using oligo(dT) (Amersham Pharmacia Biotech, Heidelberg) and SuperScript^{TM} II (RnaseH$^-$) reverse transcriptase (Life Technologies, Karlsruhe). Amplification was performed with oligonucleotides corresponding to consensus sequences in human and rat MT3-MMP PBMMP16-K*pn*I 5' GGTACCGTTCACTATGATCTTACTC-3' and PBMMP16-X*ba*I 5'-TCTAGACTCAAGTT ACCACAAACT -3' using Elongase (Life Technologies). The amplified 1892 bp full-length DNA fragment was digested with K*pn* I and *Xba* I and cloned into pBluescript (Stratagene). Finally, the full-length mouse MT3-MMP was inserted into pcDNA3 (Invitrogen) and pBI-G (Clontech), respectively.

III-1.2. Small-scale isolation of plasmid by CTAB

A single colony from a fresh LB-Amp plate was picked into 3 ml LB Amp medium and incubated overnight. After full-speed centrifugation for 2 min, the bacterial pellet was mixed with 400 µl of STET buffer and 8 µl of lysozyme (50 mg/ml), and lysed for 10 min RT. The lysate was boiled for 45 seconds and centrifuged at full-speed for 10 min. The pellet was removed by a tip. The supernatant was added to 16 µl of CTAB buffer containing 5% (w/v) CTAB and 0.5 M NaCl. Following another centrifugation, the pellet was dissolved in 150 µl of 1.2 M NaCl. Then 750 µl of absolute ethanol was added and incubated for 30 min at –70^o. After centrifugation, the pellet was washed with 70% ethanol and dissolved in an appropriate volume of TE buffer.

III-1.3. Preparation of plasmid DNA for oocytes injection

III-1.3.1. Maxi purification of plasmid DNA

DH5α bacteria were transformed with pBI-G-MT3-MMP and streaked on a LB plate supplemented with ampicillin (Amp). A single colony from the above plate was picked into 5 ml of LB-Amp medium and further incubated until an OD_{600} was ~0.5 at 37^{0}C. Afterwards, 250 ml of LB was inoculated and shaken (225 rpm) for 16 h at 37 ^{0}C. The cultured bacteria were chilled on ice and pelleted at 6.000 x g for 20 min. The bacterial pellet was resuspended in 10 ml solution I (50 mM Tris.Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and then was kept on ice for 5 min after adding 20 ml solution II (200 mM NaOH, 1% SDS). Then 10

ml solution III (5.0 M potassium acetate, pH 5.5) was added and mixed well. The mixture was incubated on ice for 15 min and centrifuged for 10 min at 20.000 x g at 4°C using a JA-14 rotor. Supernatant was filtrated through a sterile folding whatman paper into a 250 ml bottle. The bottle was filled with 0.6 volume of isopropanol and incubated for 15 min at RT. After centrifugation (15.000 x g, 10 min, 4°C), the DNA pellet was washed with 70% ethanol, air-dried for 10 min and re-dissolved in TE. For oocyte injection, the plasmid DNA was resuspended in 3.75 ml of TE. Afterwards, 4.5 g CsCl and 0.75 ml ethidium bromide (0.5µg/ml) were added and the DNA solution was filled into a VTI 65.2 tube (Beckman Coulter). The solution was centrifuged (55.000 x g, 6 h, 20°C) and the DNA was removed into a 15 ml tube with a 1 ml syringe. After extraction with n-butanol, 1 volume of 1 M ammonium acetate and 2 volume of 95% ethanol were added. Following pre-precipitation overnight at –20°C, the DNA was recovered at 4°C in corex glass tubes with a JA-20 rotor for 5 min at 15.000 x g. After a wash with 70% ethanol, the dried DNA pellet was dissolved in 200 µl TE pH7.4.

III-1.3.2. Examination of pBI-G-MT3-MMP construct by restriction enzyme digestion

1 µg DNA was mixed with 0.2 µl BSA (100x), 2 µl Buffer H (10x) and 1 unit *Pst*I (Roche) for1 h at 37°C in a total volume of 20 µl. The 1.89 kb and 7.7 kb fragments were the expected sizes after digestion. The second check was performed with 1 µg DNA, 0.2 µl BSA (100x), 2 µl Buffer B (10x), 1 unit *Hind* III (Roche) and 1 unit *Eco* RV for1 h at 37°C in a total volume of 20 µl. The expected sizes were 2.73 kb and 6.87 kb. The third check was first done with 1 µg DNA, 0.2 µl BSA (100x), 2 µl Buffer B (10x) and 1 unit *Hind* III for1 h at 37°C in a total volume of 20 µl. Then 0.2 µl of 3 M sodium acetate and 40 µl of 100% ethanol were added. The reaction mixture was incubated for 1 h at –70°C. After centrifugation and wash with 70% ethanol, the pellet was dissolved in a total volume of 20 µl mixture containing 0.2 µl BSA (100x), 2 µl Buffer H (10x) and 1 unit *Not* I (Roche). After incubation for1 h at 37°C, the reaction mixtures were electrophoresed in 1.2% agarose (Sigma) gels. The expected sizes were 842 bp and 8.64 kb.

III-1.3.3. Linearization of plasmid (pBI-G-MT3-MMP)

6 µg of CsCl purified pBI-G-MT3-MMP was digested with 6.5 µl of NE buffer and 2.5 µl of *Ase*I (New England Biolabs) for 2 h at 37°C. The reaction mixture was electrophoresed in a low melting agarose gel and was purified by gel extraction kit (Qiagen).

III-1.3.4. DNA concentration determination

Concentration of linear pBI-G-MT3-MMP plasmid was determined by λDNA/E*co*91I (B*st* EII) marker (MBI Fermentas). In detail, 3 µl of 0.5 µg/µl λDNA/E*co*91I (B*st* EII) marker was mixed with 3 µl of 6 x DNA loading buffer and 12 µl of injection buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 7.4, 70 µM spermidine and 30 µM spermine) in a total volume of 18 µl. Afterwards, 1 to 5 µl of the marker or linear CsCl purified plasmid were loaded in a 1% agarose gel, respectively. The gel was run for 1.30 h at a constant voltage of 72 V. After staining with ethidium bromide, the concentration was defined through a comparison of DNA width and brightness under UV-light. Meanwhile, a concentration was measured by a UV-spectrophotometer at 260 nm (A_{260}) and 280 nm (A_{280}) as a reference.

III-1.4. Generation of transgenic mice

pBI-G plasmid containing a full-length of mouse MT3-MMP was injected into CD1 mouse oocytes at a concentration of 5 ng per µl by using conventional microinjection (Volker C. Schmidt, Prof. Jockusch group, Bielefeld). Founder lines were checked by genomic PCR and the positive transgenic mice were designated “response transgenic mice”. For reproduction, the founder mice were mated with nontransgenic CD1 mice. CR4 regulatory transgenic mice that produce rtTA controlled by P_{hCMV} were kindly provided by Prof. Bujard (ZMBH, Heidelberg). GFAP regulatory transgenic mice were a generous gift from Dr. Kirchhoff (Max Planck Inst Expt Med, Göttingen). Double transgenic mice were generated by breeding responsive and regulatory transgenic mice.

III-1.5. Induction of MT3-MMP expression in double transgenic mice

0.2-4 mg/ml of Doxycycline hydrochloride (MBI Fermentas) was dissolved in 5% sucrose. The container was covered by aluminum paper to protect from the light. For keeping the Dox-HCl fresh, the Dox-CHl water was renewed every three days.

III-1.6. Genomic PCR for screening transgenic offspring

Tails of mice were cut and digested in 300 µl digestion buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.045% Nonidet P40, 0.045% Tween 20 and 20 µl of 1mg/ml proteinase K). After 12-18 h incubation at 50^{o}C, the proteinase K (Roche) was inactivated at 95^{o}C for 10 min. The reaction contents were centrifuged at 13.000 x g for 10 min. rtTA-encoding transgene was identified by using a pair of primers, in which rtTA 5’ recognized to the 821-841 kb and rtTa3’ to the 1271-1293 kb sequence fragment in ptet-on or ptet-off vectors. The

amplification products were 472 bp, respectively. Mouse MT3-MMP gene was identified with different pairs of primers (appendix D). PCR was performed with 2.5 µl of 10x PCR buffer (Qiagen), 5 µM of each dNTPs (Amersham PharmaciaBiotech, Heidelberg), 0.625 unit of *Taq* DNA polymerase (Qiagen), 2.5 pmole of each prime and 2.5 µl tail-digested buffer in a total volume of 25 µl.

The PCR was performed with the following program:

..... 1) Initial denaturing..... 94°C..... 3 min
..... 2) Denaturing..... 94°C..... 45 sec
..... 3) Annealing..... Tm-4°C..... 45 sec
..... 4) Extension..... 72°C..... 45 sec/ 300-500 bp or 1 min/1 kb DNA
..... 5) Termination..... 72°C..... 7 min
..... 6) Cooling..... 10°C
..... 7) No. of cycles 30

III-1.7. Isolation and assay of mouse fibroblasts

III-1.7.1. Culture of mouse ear fibroblasts

After an ear of mouse was cut into small pieces with a pair of scissors, the pieces were put into a falcon tube containing 3 x Penicillin/Streptomycin and 10% FCS in 5 ml of RPMI 1640 for several hours. The pieces were removed into a 3.5 cm tissue culture dish including 1 mg/ml of Collagenase Type Ia (Sigma) and 2 ml RPMI 1640/FCS, and were incubated at 37^{o}C for overnight. Disconnected cells were obtained by pipetting for several times and harvested through centrifugation (1.000 x g, 5 min, TM). The cell pellet were resuspended in 3 ml of RPMI with 1 x Gentamycin and 1 x Amphotericin B and the cells were cultured in 3.5 cm dishes until 80% confluent.

III-1.7.2. Preparation of mouse embryonic fibroblasts

Embryos were transferred into sterile Betaisoodnna/PBS solution. Uterus, heart, liver and head were discarded from the embryos. Following a brief wash with PBS, the embryos were cut into small pieces with a scalpel. Afterwards, 30 ml of trypsin/EDTA was added. The solution was stirred at room temperature for 30 min and was then stopped by adding 30 ml of FCS. After 5 minutes, supernatant was transferred to a clean tube and briefly spun (1000 rpm, 5 mi, 10^{o}C). The pellet cells were resuspended in DMEM medium containing 10% FCS, 1% nonessential amino acids and 2 mM L-glutamine, and were transferred into a T-150 flask.

After incubation overnight in CO_2 incubator, cellular debris was removed and the flask was returned back into an incubator. The cells were split (1:3) into 10 cm gelatin-coated dishes every 3 days.

III-1.7.3. Assay of b-galactosidase activity

Fibroblasts of double transgenic mice were seeded on coverlips and rinsed 3 x with PBS buffer. After fixation in 1% Glutardiadehyde, 1 mM $MgCl_2$ and 0.1 M PB buffer (pH 7.0) for 15 min, the fibroblasts were permeated with ethanol at –20°C for 5 min. Afterwards, the fibroblast cells were stained in X-gal staining buffer (containing 0.2% X-gal, 150 mM NaCl, 1mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$ x 3 H_2O, 3.3 mM $K_3Fe(CN)_6$ and 10 mM PB buffer, pH 7.0) for 30 min at 37°C, and then the coverlips were subsequently mounted with 70% glycerol. The fibroblasts were finally analyzed using an inverted microscope.

III-1.8. Detection of MT3-MMP expression by RT-PCR

III-1.8.1. Isolation of total RNA from culture cells and mouse tissues or organs

Cultured cells were treated with trypsin, pelleted by centrifugation (300 x g, 5 min) and then dissolved in 350 ml of lysis buffer (10 mM Tris-Cl pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$, 0.5% NP 40 and 10% β-Mercaptoethanol) for 1 min. After vortex, 1 volume of 70% ethanol was added and the lysate was applied to an RNeasy mini spin column (Qiagen). After centrifugation (8.000 x g, 15 sec), 700 µl of wash buffer I (40% ethanol, 10 mM Tris-Cl pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$ and 0.5% NP 40) was pipetted onto the column and briefly centrifuged for 15 sec. The column was washed two times with 500 µl of wash buffer II (90% ethanol, 10 mM Tris-Cl pH 7.4, 0.15 M NaCl and 1 mM $MgCl_2$), and was centrifuged for 15 sec at 8.000 x g and then 1 min at 14.000 x g, respectively. The column was conveyed into a 1.5 ml sterile tube, and 50 µl of RNase-free water was added onto the RNeasy membrane. Finally, the RNA was eluted for 1 min at 8.000 x g of centrifugation and was stored at –70°C.

To isolate total RNA from animal tissues or organs, 20-30 mg of tissues or organs were placed into a mortar containing liquid nitrogen, and were ground into a fine powder using a pestle. The powder was transferred into a 1.5 ml tube. Afterwards, 600 µl of lysis buffer was added and the lysate was homogenized by passing through a 20-G needle. The homogenized lysate was pelleted for 3 min at maximum speed of centrifugation. Supernatant was transferred onto an RNeasy mini spin column. The next steps were as described above. Finally, the RNA was dissolved in 150-200 µl of RNase-free water. Concentration and purity

of RNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a UV-spectrophotometer. If amount of RNA is low due to the discrepancy of organs or occasionally due to operation, the RNA was incubated with 10% 3 M potassium acetate and 1 volume of ethanol for 30 min at –70°C. After centrifugation at maximum speed for 20 min at 4°C, the RNA pellet was dissolved in an appropriate small volume of water.

III-1.8.2. Reverse transcription of RNA

1 µg RNA from cells or organs was mixed with 1 µl of oligo (dT) 12-18 (500 µg/ml), heated at 70°C for 5 min, and then quickly chilled on ice. Reaction mixture was prepared from 2 µl of 10 mM dNTPs, 2 µl of 0.1 M DTT, 4 µl of 5x first-strand buffer and 1 µl of M-MLV reverse transcriptase (200 U/µl) (GibcoBRL, life technology) in a total volume of 20 µl. The reaction content was incubated for 50 min at 37°C and was inactivated by heating at 70°C for 15 min.

The following PCR was performed as described in III-1.6. except for using 2.0 µl of the above treverse transcripted content as template.

III-1.9. Preparation of organs and cryosection

Spleen, kidney, heart, testis, lung, liver, tongue, muscle, gut, bone marrow, pancreas, cerebrum, cerebellum and spinal cord were taken from different kinds of mice including wild type mice, single transgenic mice and double transgenic mice. The huge number of organs were marked clearly and stored in –70°C. For preparation of cryosections, each organ was fixed on a small cork with Tissue-Tek® O.C.T.™ Compound (Sakura, Japan) and was frozen in propane cooled by liquid nitrogen. The cryosections were cut using a cryostat (Reichert-Jung, Leica, Bensheim, Germany) and was placed on SuperFrost® Plus microscope slides (Menzel-Gläse, Germany).

III-1.10. β-galatosidase assay in organ cryosection (modified from Furth et al., 1994)

10 µm cryosections from different organs were fixed in 2% paraformaldehyde and 0.02% glutaraldehyde in PBS for 1 h at 4°C, and were washed 3 times by PBS. After staining with X-gal solution (0.1% X-gal, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% nonidet P-40, 5mM potassium hexacyano ferrate (II) and 5 mM potassium hexacyano ferrate (III)), the cryosections were washed 3x with PBS and 2x with water, and were counter-stained with

nuclear fast red (Sigma) for 5 min. After 3x washing with water, the cryosections were mounted with Mowiol 488.

III-1.11. Histochemical staining procedures (modified from EBScience)

Cryosections of tissues on slides were stained in Grill's hematoxylin (III-3.2) for 15 min and were washed with tap water for 10 min. The cryosections were decolorized with acid alcohol (4 ml of 25% HCl and 100 ml of 70% ethanol) in a few seconds and then were rinsed with tap water. After the blue stain with bicarbonate buffer (1% MgSO4 and 0.066% sodium bicarbonate), the cryosections were rinsed again. The cryosections were placed in 70% ethanol for 3 min and were subsequently stained in eosin for 2 min. Following three washing steps with 95% ethanol, the cryosections were cleared by absolute ethanol.

III-1.12. Immunohistochemistry (modified from Nolte et al., 2001)

10 µm cryosections were set on a slide and were fixated by 2% Paraforaldehyde and 0.25% Glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Following 3x wash with PBS, the cryosections were placed in 0.2% Glycin/PBS for 20 min. After 3x wash by PBS, the cryosections were permeated with 0.1% Triton-X-100/PB for 20 min. The cryosections were blocked with 0.5% bovine serum albumin in 0.01% Triton-X-100/PBS for 1 h at 37°C, and were then incubated with 10 µg/ml of rabbit anti-MT3-MMP antibodies in 0.5% BSA/0.01% Triton-X-100/PBS for 6 h at 4°C. Following 3x wash with PBS, the cryosections were incubated for 2 h at room temperature with goat anti rabbit Cy2–conjugated antibodies (1:500, Jackson Immuno Research) alone or together with mouse anti-smooth muscle a actin-conjugated Cy3 antibodies (1:400, Sigma) for a double staining, respectively. Finally, the cryosections were washed 3x with PBS, mounted with Mowiol 488, and then analyzed by immunofluoresence microscopy.

III-1.13. Preparation of membrane proteins from tissues or organs (modified from Strongin et al., 1993)

0.5-0.6 mg of each organ was collected and was powdered by a set of mortar and pestle with liquid nitrogen, and then 3.5 ml STM buffer (25 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM CaCl2 100 µM ZnCl2 and protease inhibitor) containing 8.5% sucrose was added. Following homogenization using Douce homogenizer, the content was briefly pelleted at 3.000 x g for 30 min at 4°C. The supernatant was centrifuged at 100.000 x g for 1 h in a SV 40 rotor, and was designed 'intracellular membrane protein'. The pellets were resuspended in 3 ml STM

buffer and were laid on a discontinuous sucrose gradient (8.7%, 30% and 50% sucrose). After centrifugation at 100.000 x g for 2 h at 4°C, plasma membrane-enriched fraction was collected and continually centrifuged for 1.30 h (100.000 x g, 4°C). Finally the plasma membrane fractions were resuspended in an appropriate volume of STM and were stored at –70°C.

III-1.14. Quantitation of protein

A series of standard BSA and BC assay reagents (Uptima, France) were prepared according to the manual (Uptima). 50 µl of duplicate samples and the standards were pipetted into 1.5 ml tubes, and 1 ml of BC assay was added and mixed well. Following a 30 min incubation at 37°C for, the tubes were cooled to a room temperature, and the optical absorbance (OD) was measured at 562 nm against the blank (dissolving buffer + BC assay reagent). For Bradford protein assays, 0-11 µg of BSA and samples were mixed with 150 mM NaCl to a total volume of 150 µl and were mixed with 850 µl of Bradford reagent. After a 2 min incubation at room temperature, OD of the Bradford mixtures was measured at 585 nm. A standard curve was plotted, and protein concentrations of the samples were interpolated from ODs.

III-2. Localization and activity of MT-MMPs in vitro

III-2.1. Construction of MT-MMPs mutants and chimeric constructs

Chimeric MT2xFcRII and FcRIIxMT2 were constructed by introducing a S*ma* I site behind the second immunoglobulin domain of human Fc?RIIa mutant FcRIIa-C62. The following mutagenic primers were used to generate a S*ma* I site in FcRIIa-C62: 5'-CCCGGGGT TTCCTGTGCAGTG-3' and 5'CCCGGGTACACGCTGTTCTCA-3'. A S*ma* I site within the hemopexin-like domain of MT2-MMP was used to generate in frame fusions with either the C-terminal or N-terminal part of FcRII-C62. The MT2-MMP$^{S183/D185}$ mutant was generated by site directed mutagenesis (MT2muta5 5'-GGA GGT GTC CTA TGA CGA CAT C-3'; MT2muta3 5'-GGT CAG TGC TGG AGA AGG TC-3'; MT2mutb5 5'-GGT ACG AGT GAA AGC CAA CC-3'; MT2mutb3 5'-GTC GTC ATA GGA CAC CTC CTG -3'), and then fused into an internal A*ge* I site of human cDNA MT2-MMP. Full-length FLAG-tagged MT2-MMP was generated by adding the FLAG-epitope (DYKDDDDK) at its C-terminus by PCR (Forward 5'-GAC TAC AAG GAC GAC GAT GAC AAG –3'; Reverse 5'- CTA TCA GAC CCA CTC CTG CAG CGA GAC TAC AAG GAC GAC GAT GAC AAG). All constructs were further cloned into pcDNA3, respectively (Invitrogen).

Chimeric MT3xMT1-MMP (MT31) and MT3xMT2-MMP (MT32) were obtained by inserting a *BamH*I/*Xba*I fragment of MT1-MMPor MT2-MMP, which includes the complete of transmembrane/cytoplamic domain and a small part of hemopexin-like domain, into a B*amH* I/*Xba* I digested pBluescript II SK which contained cDNA fragment corresponding to aa 1-482 mouse MT3-MMP. Then the MT32 and MT31/pBluescript II SK chimeric constructs were cut with *Xba*I/K*pn*I and were inserted into pcDNA3. The construction of MT1xMT3-MMP (MT13) was followed the same way except that a B*amH*I/*Xba*I fragment of MT3-MMP was introduced into a B*amH*I/*Xba*I-MT1-MMP(1-458 aa)-pBluescript II SK. The MT13 was cloned into pcDNA3. All constructs were finally verified by DNA sequencing.

A full-length cDNA clone for human MT1-MMP was kindly provided by Dr. H. Tschesche (Bielefeld). Full-length human cDNA of MT2-MMP cDNA was a generous gift of Dr. H. Will (In Vitek GmbH, Berlin). The full-length cDNAs of MT1-, 2-MMP were cloned into pcDNA3, respectively.

III-2.2. General molecular biology methods (Sambrook et al., 1989)

III-2.2.1. Bacterial culture and store

Bacteria transformed by plasmid were selected on LB plates with antibiotics (Amp) for 16-24 hr. For mini cultures, one colony was picked and 5 ml LB medium was inoculated with an overnight shake (225 rpm) at 37°C. The mini culture was then used for a large scale of cultures to further prepare plasmid DNA or freeze glycerol stocks. For storage of bacteria, an OD from the cultures was measured with a photometer at 600 nm. When the OD_{600} reached 0.8, 500 µl bacterial culture was mixed with 500 µl 80% glycerol and added into 1.5 ml tubes. These stock solutions were subsequently stored at –70°C.

III-2.2.2. Preparation of competent cells (CaCl2 method)

Bacterial cells such as DH5 α from frozen stocks were streaked onto SOB plates and were incubated for 16 h at 37°C. A single colony was picked into 1 ml of SOB containing 20 mM $MgSO_4$ for 3-4 h incubation, later the culture content was dispersed into 100 ml of fresh medium, and was grown at 37°C until an OD_{650} reached 0.2 to 0.3. The culture was cooled down on ice for at least 15 min. The following steps were done at 4°C. The cells were harvested by a 5 min centrifuge at 5.000 x g, and the supernatant were discarded. The bacterial pellets were resuspended thoroughly in 20 ml of ice-cold transfomation buffer TFB (III-3.2) and the resuspended cells were stored on ice for 10 min. Following the centrifugation

for 10 min at 5.000 x g, the pellets were resuspended in 4 ml of ice-cold TFB and then in 140 µl of DnD solution (III-3.2). The resuspended cells were stored on the ice for 15 min. An additional 140 µl of DnD solution was added, mixed and stored on ice for 15 min. The suspension was aliquoted in 50 –200 µl and was stored at –70°C.

III-2.2.3. Transformation of competent bacteria

Competent bacteria were thawed on ice. 20 ng of ligated DNA or 2 ng of purified plasmid-DNA was added to 100 µl competent cells in a cold 1.5 ml tube. The content was mixed carefully and kept on ice for 20 min. The bacteria were shocked at 42°C for 90 sec, and then 0.8 ml antibiotic-free LB medium was added. The bacteria and medium were warmed to 37°C for 2 min and were transferred to a shaking incubator at 37°C for 45 min at 225 rpm. A selection of transformed bacteria was done by plating 100 µl of the bacterial suspension on agar plates containing the respective antibiotic. After 16-24 h, one of the colonies was selected and expanded in LB medium containing antibiotics for further DNA preparation.

III-2.2.4. CIP reaction

To prevent a re-ligation of vectors, 5' phosphate groups from vector or DNA fragments were removed by Calf-intestinal-phosphatase (CIP). 2.5 ng of vector/DNA fragments were dephosphorylated at 37^{o}C for 30 min in 100 µl of reaction consisting of 1x CIP buffer and 1µl of phosphatase. To inactivate the enzyme, 5 mM EDTA was then added to the reaction and incubated at 65^{o}C for 15 min. The vector/DNA fragments were purified by phenol and ethanol precipitation before ligation reaction.

III-2.2.5. Cohesive-end ligation

Plasmid DNA or DNA fragments were prepared by cutting with suitable restriction enzymes. 1:3 molar ratio of vector: insert DNA fragments together with 1 µl of T4 ligase (Roche) were incubated in 1x Ligation Buffer in a total volume of 20 µl overnight at 16^{o}C. Finally, the mixture was heated at 65^{o}C for 10 min to inactivate the enzyme.

III-2.3. Cellular biology methods

III-2.3.1. Cell culture

COS-1 (ACC63), COS-7 (ACC 60), and HEK293 (ACC 305) cells were obtained from the German Collection of Microorganisms and Cell Culture DSMZ (Braunschweig, Germany). HepG2 (HB-8065) cells were purchased from the American Type Culture Collection. COS-

M6 cells were kindly provided by Dr. B. Seed (Boston). COS-7 tet-off and CHO AA8 tet-off cell lines were purchased from Clontech. All cells were cultured in DMEM containing 10% FCS.

III-2.3.2. COS cell transfection (DEAE Dextran or FuGEN6)

1 or 2 days prior to transfection, the COS cells were split in 1:3 in 10 cm dishes so that cells were 50-75% confluent on the day of transfection. 400 µl of 10 mg/ml DEAE Dextran (Mw 500.000, Sigma) in PBS was mixed well with 5 ml of warm DMEM containing 10% Nu-serum (Gibcor BRL) and 100 µM Chloroquine, 10 µg plasmid DNA was added and mixed gently. Following aspiration of the old medium from the culture plate, the DEAE Dextran/DNA mixture was added, and the cells were incubated for 3.5 h in a tissue culture incubator. Then, the mixture was discarded and 10% of DMSO was gently added in PBS for 2 min. The DMSO/PBS mixture was immediately removed and 10 ml of DMEM/10% FCS was added. After 16-24 h, the cells were detached with trypsin treatment and transferred into 10 cm dishes. RNA isolations were done 48 h post transfection. In contrast, protein extracts were prepared 72 h after transfection. For zymography assay the COS cells were transiently transfected with FuGEN6 (Roche). In detail, 1.3 x 10^5 COS cells were seeded per well in 6-well plate, and incubated at 37°C in a CO_2 incubator until the confluence was 50-80%. A DNA/FuGEN6 complex was made by adding 3 µl FuGEN6 Reagent in an appropriate volume of serum free-DMEM medium. After gentle tap, 1 µg DNA solution was added. After mixing, the complex was dropped into 1.9 ml DMEM, and then the transfected cells were returned into the incubator.

III-2.3.3. HEK293 cell transfection (calcium phosphate method)

10 µg of plasmid DNA was mixed with 50 µl of 2.8 M $CaCl_2$ and millipore H_2O in a total volume of 500 µl, then 500 µl of 2x BBS [50 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 0.75 mM Na_2HPO_4 and 0.75 mM NaH_2PO_4, pH 6.95] was added in drops by vortexing. Following incubation for 20 min at room temperature, 1 ml of the mixture was dropped onto the 60-70% confluent HEK293 cells. After 48 h, the expression of target genes was checked by immunofluorescence microscopy or Western blot. In the presence of 400 µg/ml of G418, stable HEK293 cells clones were selected.

III-2.3.4. Triton extraction of MT-MMPs

After 3 days, stable or transient transfection cells were washed three times with PBS in 10 cm dishes and then were detached with a scraper. Following a 5 min centrifugation at 300 x g, the supernatant was aspirated, and 100 µl of PBS/1%Triton X-100 extraction buffer with protease inhibitor (complete mini, Roche) or 100 µl of RIPA buffer (0.1% SDS, 10 mM Tris-HCl pH 7.4, 1% Triton X-100, 158 mM NaCl, 5 mM EDTA, 1% sodium desoxycholate, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 mM NaF and complete mini protease inhibitor) was added. The cell/Triton or cell/RIPA mixture was strongly shaken for 1 h at 4°C and centrifuged at 12.000 x g for 10 min. The protein concentrations in the supernatants were determined with the BC Assay or Bradford test. The supernatant was added with 100 µl 2x Laemmli buffer containing 5 % of β-Mercaptoethanol (Sigma) and was heated for 5 min at 95°C, respectively. The pellets were washed 3 X with PBS/1%Triton X-100 extraction buffer or RIPA buffer, and was resuspended in 200 µl of β-mercaptoethanol/1x Laemmli buffer. After shaking for 30 min at room temperature, the pellet was heated for 5 min at 95°C. All samples were stored at –20°C.

III-2.3.5. Preparation of whole cell lysates

Cells were scraped off in 5 ml of PBS and were pelleted by centrifugation at 300 x g. The cells were resuspended in 150 µl of 1x Laemmli buffer containing 5 % β-Mercaptoethanol. The lysates were cleared by centrifugation at 12.000 x g for 2 min at 4°C and were incubated for 5 min at 95°C.

III-2.3.6. Preparation of crude plasma membrane fractions from transfected cells (modified from Belien et al, 1999)

Cells were harvested in 5 ml PBS. Following 5 min centrifugation at 300 x g, the cells were resuspended in 3.5 ml of 8.7% sucrose and passed through a 20-G needle with 5 ml syringe at least 80 times. The homogenized lysate was laid on the surface of 9 ml of 38% sucrose in a SW 40 tube and centrifuged at 33.000 x g for 1 h at 4°C. The plasma membrane enriched fractions were transferred into a new SW 55 tube with a syringe. The tube was filled with 5 ml of 8.7% sucrose. After centrifugation at 40.000 x g for 1 h at 4°C, the pellet was resuspended in an appropriate volume of assay buffer (5 mM Tris/HCl, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$ and 100 µm $ZnCl_2$) and the protein concentration was measured by BC assay or Bradford test.

III-2.3.7. Immunofluorescence microscopy

For immunofluorescence studies, transiently transfected COS cells were grown on multiwell slides and analyzed 72 h post transfection. HEK293 cells were grown on poly-D-lysine (0.01% (w/v), Sigma) coated multiwell slides, and were washed with PBS/0.5 mM $CaCl_2$ prior to fixation. Cells were fixed with 2% (w/v) paraformaldehyde in PBS, washed with PBS containing 100 mM glycine and either permeabilized with PBS/0.2 (w/v) Triton X-100 or directly incubated with primary antibodies for 1 h at 4°C. Antibody dilutions were: mAb 113-5B7, 40 µg/ml; anti-MT2-MMP-HR, 15 µg/ml; anti-MT2-MMP-HD, 15 µg/ml; anti-MT3-MMP-HR, 10 µg/ml; anti-FcRII mAb II3A6, 5 µg/ml; and anti-octapeptide epitope tag rabbit IgG (anti-FLAG-tag), 2.5 µg/ml. After extensive washing the samples were incubated with Cy2 or TRITC or Cy3-labeled goat anti-rabbit or goat-anti mouse antibodies (1:400, Dianova GmbH). Following extensive washing cells were mounted on glass slides with Mowiol 4-88 (Hoechst). The slides were examined on an Olympus BX50 fluorescence microscope and the images were collected using 40x objective and a Sony progressive 3CCD camera.

III-2.3.8. Metabolic labeling and Endo H treatment of cells (This work was done in collaborated with Dr. Gatsios, Aachen)

COS-7 cells were transiently transfected with pcDNA3-human MT2-MMP. 48 h after transfection and 30 min prior to radioactive labeling the culture medium was changed into serum-free medium minus methionine. The cells were then pulse-labelled for 10 min with 250 µCi/ml Trans^{35}S-methionine (ICN), washed twice in PBS and then incubated for the indicated times in unlabeled growth medium containing methionine (chase). MT2-MMP was immunoprecipitated from the cell pellet and supernatant with antibodies against the catalytic domain (anti-MT2-PCD) (2µg/ml) and visualised by fluorography.

MT2-MMP transfected COS-7 were metabolically labelled for 15 min and immunoprecipitated with antibodies against the catalytic domain (anti-MT2-PCD) (2µg/ml). Supernatant and pellet were treated with EndoH. The Endo H digestion of 15 min ^{35}S-labeled cells was carried out essentially as recommended in the manual (Roche).

III-2.3.9. Zymography

III-2.3.9.1. Preparation of pro-MMP-2

Insect S2 cells were transfected with pAc5.1/V5-His A, B, C (Invitrogen) containing pro-MMP-2 cDNA (kindly provided by Dr. Will, In Vitek GmbH, Berlin) and were cultured in

serum free Schneider's Drosophila modified medium (Gibrco) at 23°C. After 3 days, the medium was harvested and used for zymography.

III-2.3.9.2. Gelatine zymography

48 hours after transiently transfected COS cells were washed with three times PBS and replaced with serum-free DMEM containing pro-MMP-2 in 35 cm culture dish. After 24h, aliquots of the medium were mixed with an equal volume of 2xSDS sample buffer, and resolved on 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatine (Sigma-Aldrich) under nonreducing condition. The SDS-gel was rinsed twice in 2.5% Triton-X-100 for 30 min at room temperature and then incubated in developing solution (50 mM Tris-HCl, 100 mM $CaCl_2$, 1 µM $ZnCl_2$, 1% Triton-X-100, 0.02% NaN_3, pH 6.8) for 18-24 h. The gel was stained with Coomassie blue and dried with DryEase™ Mini-cellophane (Novel Experimental Technology, Company).

III-2.4. Biochemistry methods

III-2.4.1. SDS-PAGE

The resolving and stacking gels of 7.5 % and 10% SDS-PAGE were prepared as followed:

Resolving	**7.5%**	**10%**	**Stacking**
30% Acrylamid/0.8% Bis	1.88 ml	2.50ml	30% Acrylamid/0.8% Bis 420 µl
H_2O	2.60 ml	1.99ml	H_2O 750 µl
1 M Tris-HCl, pH 8.8	2.81 ml	2.81ml	0.25 M Tris-HCl, pH 6.8 1.25 ml
5% SDS	150 µl	150 µl	5% SDS 50 µl
TEMED	4 µl	4 µl	TEMED 3 µl
10% Ammonium persulfate	46 µl	46 µl	10% Ammonium persulfate 30 µl
Total volume	7.5 ml	7.5 ml	Total volume 2.5 ml

An equal amount of protein extract was loaded per well. The gel was run at constant current of 10 mA, and then at 20 mA when the bromphenol blue line entered the separation gel. After notching at a corner, the gel was used for silver staining or Western blot or zymography.

III-2.4.2. Silver staining

SDS-gels were immersed in 45% (v/v) methanol and 10% acetic acid solution for 15 min, and soaked in Farmer's Reducer containing 0.03 M $K_3Fe(CN)_6$ and 0.032 M $Na_2S_2O_3$ for 2 min. Following a complete wash with Millipore water, 0.1% $AgNO_3$ was added for 20 min. After 2 x washing with Millipore water and 2.5% Na_2CO_3, the gel was developed with 2.5% Na_2CO_3 containing 0.15% formaldehyde until the bands appeared. The development was stopped by placing the gel in 10% acetic acid. The gels were dried with DryEase^{TM} Mini-cellophane.

III-2.4.3. Western blot

PVDF membranes (Millipore) were treated with 100% methanol for 15 sec and then with Millipore water for 2 min. The PVDF membrane, SDS-gel and whatman paper were incubated in transfer buffer (25 mM Tris-HCl, pH 8.5, 0.192 M glycin and 20% methanol), and assembled in a semi-dry transfer chamber. Proteins were transferred at a constant current of 150 mA for 1.5h. Membranes were stained in a Ponceau-S solution (Sigma) for 1 min. After taking a photo, the membranes were destained with PBST (0.05% Tween 20 /PBS) buffer or 0.1 N NaOH, and blocked with 5% fat-free milk/PBS for 1 h at room temperature. Afterwards, the primary antibody was added at the appropriate dilution (antibody dilutions were: mAb 113-5B7, 2 µg/ml; anti-MT2-MMP-HR, 4 µg/ml; anti-MT2MMP-HD, 4 µg/ml; anti-MT2-MMP-PD, 10?µg/ml; anti-MT3-MMP-HR, 5 µg/ml; anti-MT3-MMP-PD, 10?µg/ml; anti-FcRII mAb II3A6, 5 µg/ml) for 2 h at RT. After washing with PBST, the respective peroxidase labeled secondary antibody (Jackson Immuno Research) was applied for 30 min at RT (1:10.000 dilution). The blot was developed with lumi-light western blotting substrate (Roche).

For a purpose of reuse, the transferred membranes was incubated for 30 min at 70^{o}C in stripping solution (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol), and then washed with PBST until no smell of β-mercaptoethanol. Soon after, the blot was performed according to a standard Western blot protocol.

III-2.4.4. Immunoprecipitation

For immunoprecipitation of MT-MMPs, transfected cells were lysed with 1 ml of lysis buffer (1% (w/v) Triton X-100, 0.4% (w/v) Na-desoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.25 mM PMSF, 5 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin) for 30 min at 4°C. The lysate was cleared by centrifugation (12.000 x g; 15 min; 4°C). Insoluble material

was solubilised in 100 µl SDS-buffer (1% SDS, 50 mM Tris- HCl, pH 8.8 and 5 mM EDTA) for 10 min at 95 °C. DNA was sheared by passage through a 22 gauge needle. Then the lysate was diluted by adding 900 µl lysis buffer. Supernatant was adjusted to 0.1% (w/v) SDS before immunoprecipitation. Afterwards, anti-MT2-PCD (2µg/ml) was added and allowed to bind for 18 h at 4°C. The immune-complexes were bound to protein A-Sepharose (5 mg/ml in lysis buffer) for 1 h at 4°C. After centrifugation, the sepharose beads were washed three times with lysis buffer. The samples were boiled in gel electrophoresis sample buffer, and the precipitated proteins were separated on 10% SDS-polyacrylamide gels. The gel was fixed in 40% methanol and 10% acetic acid for 30 min, soaked in water for additional 30 min and treated with 1 M Na-salicylate solution for 30 min. Immunoprecipitated proteins were visualized by autoradiography.

III-2.4.5. Processing of pro-MMP-2 by APMA

1 M APMA stock solution was diluted to 2.5 mM with TTC buffer (50 mM Tris-HCl, pH7.5, 0.05% Triton X-100, and 5 mM $CaCl_2$). Purified pro-MMP-2 (Roche) or culture supernatant from pro-MMP-2 transfected insect cells was incubated with 7.5 µl of 2.5 mM APMA for 30 min at 37^{o}C, and then the reaction was stopped by dipping into liquid nitrogen and stored at –70^{o}C immediately.

III-3. Reagents

III-3.1. Antibodies

Fc anti-RII-specific mouse mAb IIA15 and II3A6 were produced in our laboratory (Rathke-Hartlieb et al., 2000). Anti c-myc antibody 9E10 was purchased from Roche. Anti-octapeptide epitope tag rabbit IgG (anti-FLAG-tag) was obtained from Zytomed (Berlin, Germany). Rabbit polyclonal antibodies reactive for MT1-MMP, MT2-MMP and MT3-MMP were raised against the following peptides: CDGNEDTVAMLRGEM (anti-MT1-MMP-HD), PQPSRHMSTMRSAQILAS (anti-MT2-MMP-PD), PQPLTSYGLGIPYDRIDT (anti-MT2-MMP-HD), RPGRPDHRPPRPPQPPPG (anti-MT2-MMP-HR), HRSIPPPADPRKNDRPK (anti-MT3-MMP-HR) and PTDPRMSVLRSAETMQSA (anti-MT3-MMP-PD). Peptides MT2-MMP-HD and MT2-MMP-HR were coupled to Epoxy-activated Sepharose 6B (AmershamPharmaciaBiotech) and used to purify the respective antiserum. All other antibodies were purified with Protein A Sepharose (Sigma). Cy2-, Cy3 and horseradish-peroxidase-labelled goat anti-rabbit and anti-mouse antibodies were obtained from Dianova GmbH (Hamburg, Germany). TRITC-labelled goat anti-rabbit IgG was purchased from

Sigma. MT1-MMP specific mAb 113-5B7 (anti-HD) was purchased from Fuji Chemicals (Japan). MT2-MMP specific antiserum reactive for a recombinant protein containing a part of the propeptide and whole catalytic domain (anti-MT2-PCD) has been described, previously (Will et al., 1995).

III-3.2. Buffers and solution

STET-Puffer

8 % (w/v) Sucrose, 50 mM EDTA, 0.1% (w/v) Triton X-100, 50 mM Tris-HCl pH 8.0. The solution was autoclaved.

DnD solution

Dithiothereitol ..... 1.53 g
DMSO ..... 9 ml
1M potassium acetate pH7.5 .....100 µl
Millipore water was added until a final volume is 10 ml.

TFB

1M MES pH 6.3 .....10 ml
$MnCl_2.4H_2O$ .....8.91 g
$CaCl_2.2H_2O$ ..... 1.47 g
KCl ..... 7.49 g
Hexaminecobalt chloride..... 0.80 g

Mowiol 488

Glycerin (P.A) ..... 6.0 g
Mowiol 4-88 (Hoechst) .....2.4 g
For 1 h in RT and shake
Water .....6 ml
2 h in RT and shake
0.2 M Tris-HCl pH 8.5 ..... 12 ml
The mixture was incubated for 10 min at 50°C, and then centrifuged at 5.000 x g for 15 min. The aliquots were stored at –20°C.

Laemmli-buffer (1x)

62 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerin, 0.01% Bromophenol blue.

Gill's hematoxylin (total 200 ml)

Hematoxylin	1.2	g
Sodium iodate	0.12	g
Aluminum surfate	17.56	g
Ethylene glycol	50	ml
Glacial acetic acid	12	ml
Distilled water	138	ml

Eosin B (total 200 ml)

0.3 g of eosin was dissolved in 100 ml H_2O and 100 ml absolute ethanol. Finally, 1 drop of acetic acid was added.

Bradford reagent

100 mg of Coomassie Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml 85% (w/v) phosphoric acid. The Millipore water was added until the final volume is 1 liter.

IV. Results and Conclusions

IV-1. Functional studies of MT3-MMP in vivo

IV-1.1. Establishment and analyses of tet regulatory MT3-MMP in vivo

IV-1.1.1. Construction and examination of pBI-GMT3-MMP plasmid

IV-1.1.1.1. Cloning of mouse MT3-MMP cDNA and construction of responsive pBI-G-MT3-MMP plasmid

To study the role of MT3-MMP in vivo, total RNA was isolated from a C57/BL6 mouse and reverse transcribed by using the method described in the 'Materials and Methods'. Restriction sites for *Kpn*I (GGTACC) and *Xba*I (TCTAGA) were introduced at both ends of MT3-MMP cDNA. Four independent clones were sequenced and compared with the DNA sequences in the Genbank database using the BLASTN program. 98% homology was found between our MT3-MMP cDNA (accession number AF282844) and a present murine cDNA (Genbank accession number AB021228). A comparison of deduced amino acid sequences from AB021228 and from our mouse MT3-MMP revealed a total of three amino acid substitutions i.e. Arg^{17} for His^{17}, Val^{19} for Gly^{19} and Arg^{196} for Ser^{196}. To know whether the amino acid substitutions popularly exist in mice or only specially exhibit in our clones, a further amplification was performed with the RNA from FVB mice. Sequence results showed that these substitutions also existed in FVB MT3-MMP clones so as to confirm that the discrepancy within the above sequences was due to a species variation. The deduced amino acids from mouse, rat and human are listed in figure 1. A total of 14 residues are different between human MT3-MMP (accession number D85511) and our mouse MT3-MMP, in which Thr^{5} of human was substituted for Ala^{5} of mouse, Thr^{8} for Ser^{8}, His^{17} for Arg^{17}, Gly^{19} for Val^{19}, His^{114} for Asn^{114}, Lys^{150} for Arg^{150}, Arg^{196} for Ser^{196}, Ile^{312} for Val^{312}, Lys^{319} for Arg^{319}, Asn^{320} for His^{320}, Ser^{432} for Asn^{432}, Val^{479} for Ile^{479}, Val^{539} for Asp^{539} and His^{543} for Leu^{543}, respectively. Only four amino acids of rat MT3-MMP were replaced in mouse i.e. Gly^{19} was substituted for Val^{19}, Phe^{23} for Leu^{23}, Lys^{319} for Arg^{319} and Asn^{320} for His^{320}. The 1892 bp of the amplified full-length DNA fragment of MT3-MMP was digested with *Kpn*I and *Xba*I and cloned into pBluescript KS first, then it was cut with *Eco*RI and *Xba*I, and finally inserted into pcDNA3 expression vector.

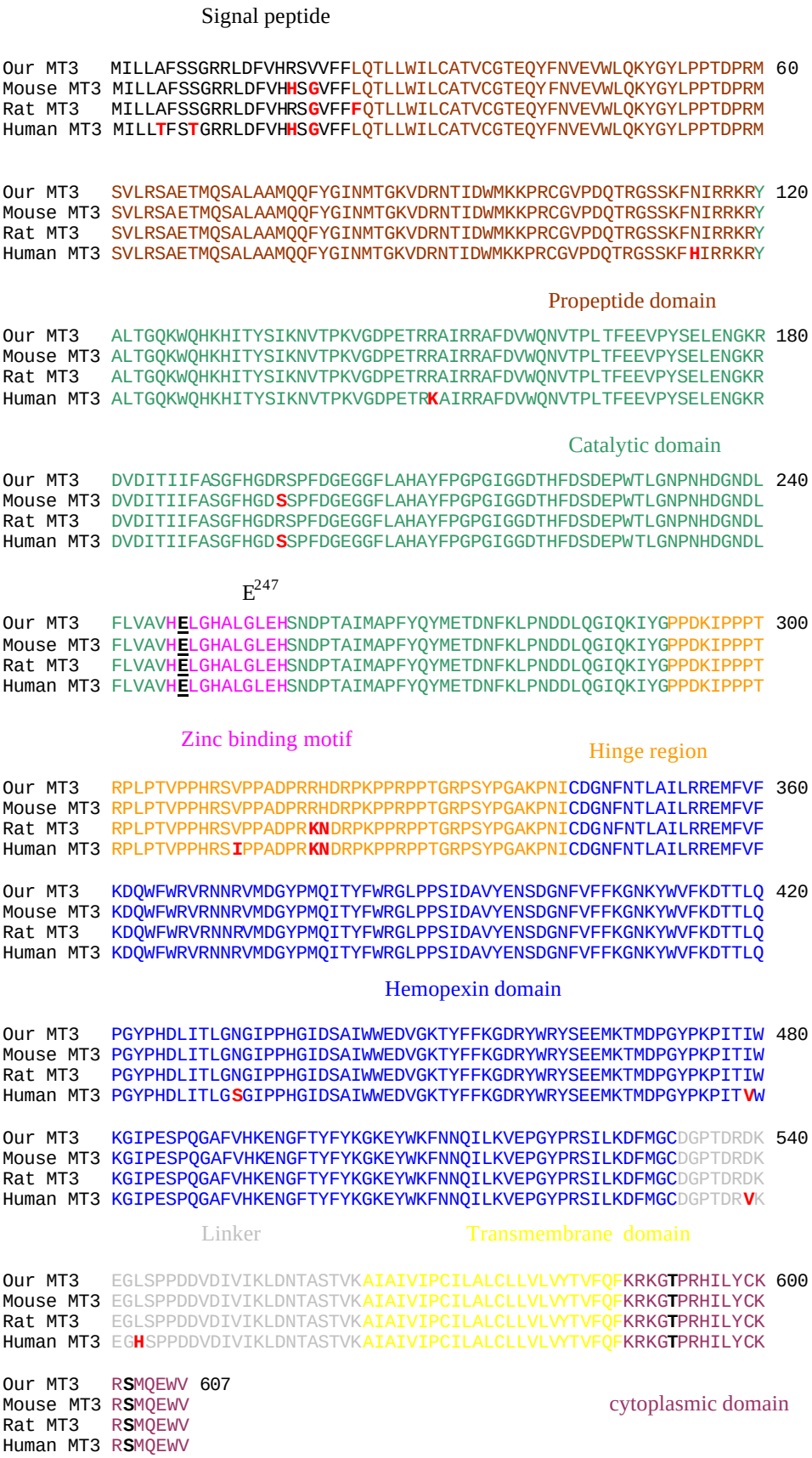


Figure 1: Amino acid sequence comparison of mouse MT3-MMP with other species

The different amino acids are highlighted in red colour.

IV-1.1.1.2. Analysis of the pBI-G-MT3-MMP construct by restriction enzymes

pBI-G-MT3-MMP plasmids were purified and analyzed with the different combinations of restriction enzymes (Figure 2). Given to the molecular mass of pBI-G-MT3-MMP to be 9.5 kb, the digestion with H*ind*III and E*co*RV showed a large fragment of 6.87 kb and a small one of about 2.7 kb (Figure 2, lane 2). Consistently, H*ind*III and N*ot*I cut the plasmid into 8.6 kb and 840 bp fragments (Figure 2, lane 3). Both digested results were identical to the expected results.

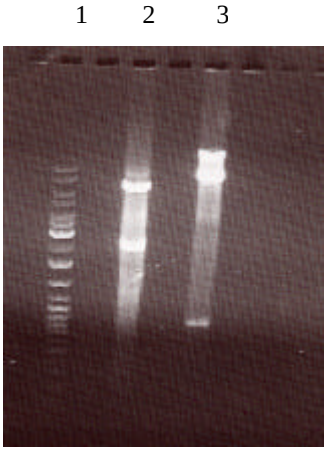


Figure 2: Restriction digestion of pBI-G-MT3-MMP plasmid

Purified pBI-G-MT3-MMP was examined by H*ind*III and E*co*RV (lane 2), or H*ind*III and N*ot*I (lane 3) digestion. Lane 1: 1 kb DNA marker (GeneRuler^{TM}).

IV-1.1.1.3. Purification, linearization and quantitation of pBI-G-MT3-MMP

A high purity plasmid is necessary for the further generation of transgenic mice. In order to achieve that aim, pBI-G-MT3-MMP was purified twice by using CsCl gradient ultra-centrifugation in the presence of ethidium bromide. The concentration of the plasmid is very crucial in oocytes microinjection. The absorbance measure of low amounts of DNA with a UV spectrophotometer always produces significant errors in DNA quantitation. Therefore, we compared the pBI-G-MT3-MMP plasmid with a set of volume λDNA/E*co*91I (B*st*EII) markers in an agarose electrophoresis. One microliter of the mixture has 14.49 ng of 8.453 kb fragment and 12.41 ng of 7.242 kb fragment. In the agarose electrophoresis gel, the amount of pBI-G-MT3-MMP was much more similar to that of 7.242 bp fragment (Figure 3, arrow head), so we judged that the concentration of linear target plasmid was 12.41 ng per microliter. The sequencing of pBI-G MT3-MMP clones was done and confirmed that all selected clones were correct and contained the full-length of MT3-MMP cDNA (Appendix A).

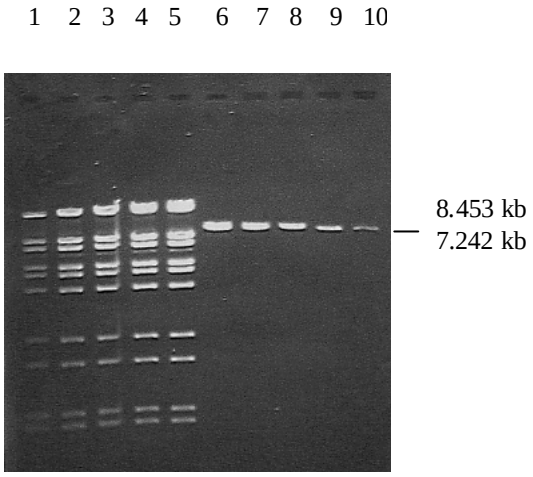


Figure 3: Quantification of linearized and CsCl-purified pBI-G-MT3-MMP plasmid

The linearized and CsCl-purified pBI-G-MT3-MMP plasmid was quantified with λDNA/E*co*91I (B*st*EII) DNA maker (MBI Fermentas). 3 µl of 0.5 µg/µl λDNA/E*co*91I (B*st*EII) marker was mixed with 3 µl of 6 x DNA loading buffer and 12 µl of injection buffer. Lanes 1-5: 1µl to 5 µl of mixed marker were loaded, respectively. Lanes 6-10: 1µl to 5 µl of pBI-G-MT3-MMP plasmid were loaded onto a 1 % agarose gel, respectively.

IV-1.1.2. Assessment of the tet regulatory system in tet-off cell lines

IV-1.1.2.1. β-galactosidase assay of the reporter gene

To prove whether the construct of pBI-G-MT3-MMP works in a tet regulatory system, COS-7 tet-off and CHO AA8 tet-off cell lines were transiently transfected with the DEAE Dextran method with pBI-G-MT3-MMP, or with empty pBI-G plasmid as a control. The tet-off cells contain a regulatory vector (pTet-Off) and constitutively express a regulatory element tTA that activates the transcription of target gene in the absence of doxycycline. The transfected cells were used to detect the β-galactosidase activity (Figure 4). As shown in the pBI-G plasmid map (Appendix B), one Tet-responsive element (TRE), which consists of seven copies of the 42-bp tet operator sequence (tetO) in the regulatory unit, tightly tethers with two minimal CMV promoters (PminCMV) which lack the enhancer of the complete CMV promoter in reverse orientations. Therefore, the tet-off cells transfected with pBI-G-MT3-MMP cDNA should express both the transgenic MT3-MMP and the reporter Lac Z gene in the absence of effector (tetracycline). Indeed, 20-25% of $3x10^5$ cells showed the β-galactosidase activity without tetracycline treatment (Figure 4A); in contrast, no positive blue stained cell was found in a same number of observed cells on the fourth day post transfection in the presence of 0.1 µg/ml tetracycline (Figure 4B). The transfection efficiency with DEAE Dextran was grossly estimated in parallel transfections with the green fluorescent protein (GFP-pcDNA3) in three independent experiments. The ratio of green colored (GFP) cells was consistent with that of β-galactosidase positive cells, indicating that most of the transfected

tet-off cells expressed the reporter Lac Z gene under the conditions. The β-galactosidase assay clearly suggested that the regulatory unit was strongly responding to the tetracycline-controlled transactivator (rTA) without tetracycline and the expression was tightly controlled by tetracycline in the Lac Z reporter gene inserted orientation in the p-BI-G-MT3-MMP construct.

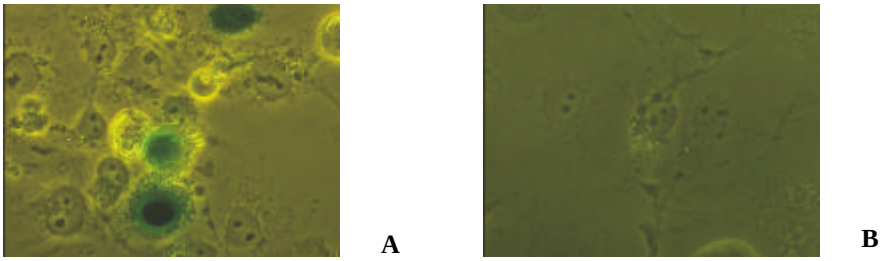


Figure 4: b**-galactosidase activity assay in transfected COS-7 tet off cells**

COS-7 tet off cells transfected with pBI-G-MT3-MMP were treated with (B) or without doxycycline (A). β-galactosidase activity assay was performed as described in "Materials and Methods" and analysed by phase contrast microscopy (400x).

IV-1.1.2.2. Examination of transgenic MT3-MMP expression on RNA level

To examine the response and control ability in the MT3-MMP inserted orientation in the pBI-G-MT3-MMP construct, COS-7 and CHO AA8 tet-off cells transfected with pBI-G-MT3-MMP or empty pBI-G plasmid were collected and RNA was isolated to perform RT-PCRs with rtTA/tTA primers and MT3-MMP primers, respectively (Figure 5). The tTA primers specifically bind to the DNA fragments corresponding to the tet repressor (TetR) and the C-terminal activation domain of VP16 protein of Herpes simplex virus in pTet-Off vector, respectively. The amplification products of RT-PCR in figure 5A show no significant difference from lane 1 to lane 4, suggesting that tTA was constitutively expressed in the tet-off cells regardless of the presence or absence of tetracycline. The detection of MT3-MMP expression was first carried out with MT3 primers, one of which binds to the cDNA fragment corresponding to the catalytic domain of MT3-MMP and another binds to the cDNA fragment corresponding to the cytoplasmic domain. In the absence of tetracycline (Figure 5B, lanes 1 and 2), MT3-MMP expression was much stronger than in the presence of tetracycline (Figure 5B, lanes 3 and 4). The results were confirmed with another pair of primers, rtMT3, the 5' end of which bound to the DNA fragment corresponding to the C-terminal hemopexin-like

domain of MT3-MMP and the 3' end primer specifically annealed to the region of the SV 40 poly A which is close to the MT3-MMP gene in the pBI-G plasmid. When a cycle number of RT-PCR amplification was 25, the expression of MT3-MMP in the presence of tetracycline was close to nothing (Figure 5C, lanes 6 and 8). However, after increasing the cycle number to 30 (Figure 5B, lanes 3 and 4), the amplification products were apparently raised, suggesting that MT3-MMP had some leakage in the tet-off cell lines. In contrast, the expression of MT3-MMP in the absence of tetracycline was very significant (Figure 5C, lanes 5 and 7).

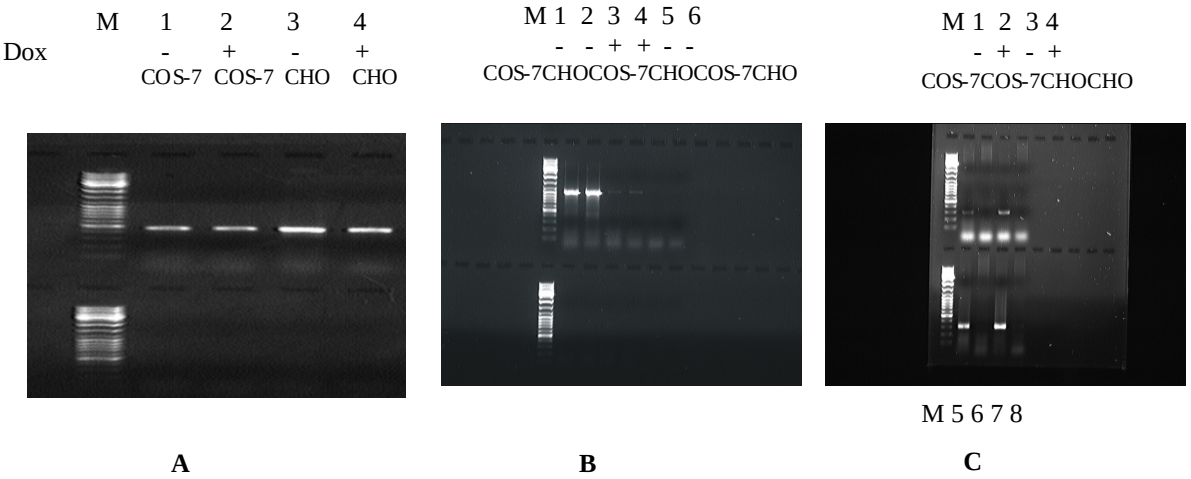


Figure 5: RT-PCR to detect MT3-MMP expression in tet off cells transfected with pBI-G-MT3-MMP

COS-7 (lanes 1-2 of A; lanes 1, 3 of B; lanes 1-2, 5-6 of C) or CHO tet-off (lanes 3-4 of A; lanes 2, 4 of B; lanes 3-4, 7-8 of C) cells were transiently transfected with pBI-G-MT3-MMP and treated with or without Dox-HCl. RNA from the above transfections or untransfected cells (lanes 5-6 of B) was isolated and RT-PCRs were carried out with rtTA primers (A; expected size 470 bp), MT3 primers (B; expected size 1kb) and rtMT3 primers (C; expected size 300 bp). M: DNA ladder mixer marker (GeneRuler^{TM}). Upper half of A or B: RT-PCR. Lower half of A or B: PCR without prior RT as controls. B: 30 cycles. Upper half of C: 20 cycles. Lower half of C: 25 cycles.

IV-1.1.2.3. Examination of MT3-MMP on the protein level

RT-PCR results only indicate whether an inserted interest gene is transcribed or not, but it does not prove whether the interest gene is translated. To detect the expression of MT3-MMP on the protein level, COS-7 and CHO AA8 tet off cells transiently transfected with pBI-G-MT3-MMP or empty pBI-G plasmid were harvested, lysed and probed with rabbit anti-HR-MT3-MMP antibodies that specifically recognize a peptide fragment of the hinge region

(amino acid 305 to 320 in MT3-MMP; see Appendix C) in Western blots. Lanes 3 and 5 of figure 6A show that no MT3 -MMP protein was detected in the presence of tetracycline in both tet-off cell lines. In contrast, without tetracycline the MT3-MMP protein levels were significantly enhanced as shown in lanes 4 and 6 (Figure 6A). In lanes 1 and 2 of figure 6A, the MT3-MMP proteins were not seen in empty vector transfected cells, stressing that the expression of MT3-MMP proteins was from the transgene rather than from the endogenous gene. Taken together, the results from β-galactosidase assays, RT-PCR and Western blots show that the pBI-G-MT3-MMP plasmid could express the reporter gene (Lac Z) and MT3-MMP in reverse orientations in the absence of tetracycline. In contrast, the expressions was tightly controlled or kept 'silent' in the presence of tetracycline in tet-off cell lines.

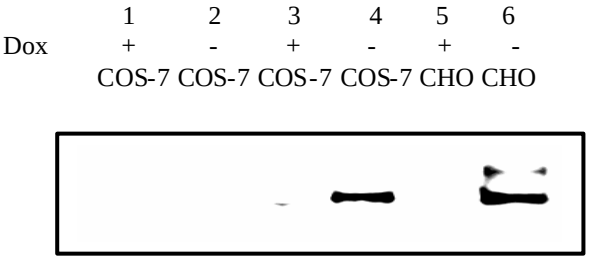


Figure 6A: MT3-MMP detected by Western blot analysis

COS-7 (lanes 1-4) or CHO (lanes 5-6) tet off cells were transiently transfected with empty pBI-G vector (lanes 1-2) or pBI-G-MT3-MMP (lanes 3-6) and treated with or without Dox-HCl. Protein extracts from the above transfections were probed with rabbit anti-HR-MT3-MMP antibodies and goat anti-rabbit antibodies labelled by peroxidase.

To analyze the localization of MT3-MMP proteins, immunofluorescence microscopic studies were performed with the COS-7 tet-off cells transiently transfected with the pBI-G-MT3-MMP, or the empty pBI-G plasmid as a mock. In addition to the anti-HR-MT3 antibodies, MT3-PD antibodies that bind a peptide from aa 54 to 71 amino acids of the propeptide domain (Appendix C) were used to probe for the proform of MT3-MMP. The data clearly demonstrated that without tetracycline treatment (Figure 6B, I) MT3-MMP is mainly located in intracellular organelles such as the endoplasmic reticulum and Golgi apparatus which are close to the cell nucleus, whereas a few molecules were distributed in the whole cells in a permeable staining. Interestingly, the MT3-MMP proteins could not be detected by the PD antibodies, indicating that the propeptide domain of MT3-MMP might be promptly removed

in the trans-Golgi network. The untransfected cells and transfected cells in the presence of tetracycline did not show any signals (Figure 6B, II), suggesting that the expression of MT3-MMP was responding to the tet responsive transactivator and was tightly controlled. In a word, the construct of pBI-G-MT3-MMP worked well in the cell models studied.

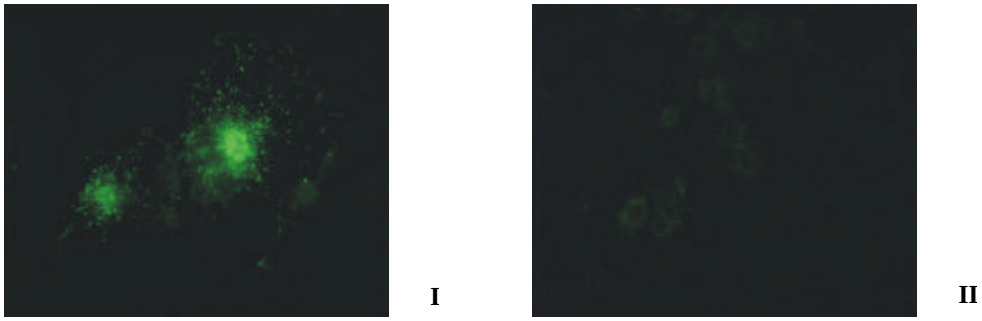


Figure 6B: Immunofluorescence microscopy of COS-7 cells transfected with pBI-G-MT3-MMP
COS-7 tet off cells were transfected with pBI-G-MT3-MMP and treated with (II) or without Dox-HCl (I). After permeabilizing with 0.2% Triton X-100/PBS, the transfected cells were probed with rabbit anti-HR-MT3 antibodies and Cy2-conjugated goat anti rabbit IgG. MT3-MMP was detected in the endoplasmic reticulum and Golgi apparatus in the absence of Dox-HCl (I) but not in the presence of Dox-HCl (II).

IV-1.1.3. Generation of pBIG-MT3-MMP/rtTA transgenic mice

IV-1.1.3.1. Generation of MT3-MMP responsive transgenic mice

To examine whether the founder mice derived from injected fertilized eggs contained the pBI-G-MT3-MMP plasmid, genomic PCR was performed with a pair of pBI-G-MT3 primers, the 5' end of which binds to the multiple cloning site (MCS) region of pBI-G plasmid, whereas the 3' end primer anneals to the DNA fragment corresponding to the N-terminal catalytic domain of MT3-MMP. The pBI-G-MT3 primers were specifically designed to discriminate exogenous (transgenic) MT3-MMP from the endogenous one in the mouse model since its 5' end primer only recognizes pBI-G-MT3-MMP rather than endo-MT3-MMP. However, the amplification products from these primers only revealed very weak bands even though PCR conditions had gradually modified such as decrease of annealing temperature, increase of cycle numbers, or concentration change of dNTPs and magnesium chloride. Because of the unsatisfied results from the pBI-GMT3 primers, MT3 primers were applied, which recognized a sequence located in catalytic domain and cytoplasmic domain respectively as shown in

appendix D. Although the exact number of exons of mouse MT3-MMP in the chromosome is unclear, but it is not preposterous to imagine that there should be some introns located between catalytic domain and cytoplasmic domain through referring the information from mouse MT1-MMP, in which a total of ten exons are separated by nine introns (Zhou et al., 2000). Therefore, MT3 primers can be used to detect pBI-G-MT3-MMP in genomic PCR. A mixture of primers, one of which is the 5' end primer of the pBI-G-MT3 primers that specifically recognizes the MCS region of pBI-G-MT3-MMP and another is the 3' end primer of the MT3 primers that recognizes the cDNA fragment corresponding to the cytoplasmic domain, were employed to confirm the PCR results from the MT3 primers. Consequently, genomic DNA from each founder mouse was analyzed with different pairs of primers. Only those founders, which clearly showed the expected amplification products in the all three PCRs, were finally judged to be positive. Moreover, the PCRs with actin primers were used to indicate whether the genomic DNA was successfully isolated. In the first batch of founders, one female out of six CD1 mice was defined to be a transgenic (or positive) mouse in terms of carrying the pBI-G-MT3-MMP transgene. In the second batch, one female out of two C57BL/6 founders and one female out of two CD1 founders were integrated with the MT3-MMP transgene in their chromosome. Totally, two female CD1 mice and one female C57BL/6 mouse were affirmed to carry the pBI-G-MT3-MMP plasmid as a transgene.

Through breeding the CD1 transgenic founders with CD1 wild type mice, two responsive transgenic mouse lines carrying pBI-G-MT3-MMP were established and designated 3001 and 3002. Offspring from the transgenic mouse lines were checked with the MT3 primers first and then confirmed by a pair of primers, rtMT3, which can distinguish the MT3-MMP transgene from the endogenous one (Appendix D). These two pairs of primers (MT3 or rtMT3) were both suitable and alternately used to detect the MT3-MMP transgene in the responsive transgenic mice or in double transgenic mice that will be discussed in IV-1.3.2. A low inheritance of the MT3-MMP transgene in F1 offspring was found, in which 16.6% was to 3001 and 15.3% to 3002, respectively (Table 1). However, the percentage of transmitting the transgene significantly arose to 76%-79% in F2 and then reached to more than 90% in the F3 generation in the both transgenic mouse lines.

Table 1: Inheritance of MT3-MMP transgene in single response transgenic mice

Type	F1	F2	F3	F4	F5
3001	2/12	31/39	10/10	8/9	11/11
3002	2/13	35/46	23/25	18/19	13/13
%	15.3-16.6	76.0-79.4	92-100	88-94.7	100

The offspring carrying the MT3-MMP transgene were bred in order to obtain homozygous responsive transgenic mice. Simultaneously, some of them were mated with regulatory transgenic mice to achieve double transgenic mice that carried both the regulatory element (rtTA) and the responsive construct (pBI-G-MT3-MMP).

IV-1.1.3.2. Generation of MT3-MMP/rtTA double transgenic mice

Three kinds of regulatory transgenic mice including CR4, GFAP and ETGD were applied and all of them contained a pTet-On vector that expressed the reverse tetracycline-controlled transactivator (rtTA). The differences among them is that CR4 mice contain a wide-spectrum CMV promoter upstream of the rtTA gene in NMR1 mice, whereas GFAP and ETGD mice carried a human glial fibrillary acidic protein (GFAP) promoter that selectively expressed rtTA in glial cells of the brain. Moreover, ETGD mice contained enhanced green fluorescent protein (EGFP) as indicator protein. The rtTA can activate the transcription of the MT3-MMP transgene in the presence of doxycycline in double transgenic mice carrying both pTet-on and pBI-G-MT3-MMP. The double transgenic mice were selected by genomic PCR with the rtMT3 and the rtTA primers. Heterozygous double transgenic offspring were bred with each other to get more study materials and to achieve homozygous double transgenic mice. The inheritance of double transgenes (MT3-MMP and rtTA) is listed in table 2. In general, a low percentage (17-38%) was showed in the F1 offspring except CD4x3002 that perhaps applied a homozygous CR4 in the first round mating. The inheritance of both transgenes was increased in the following generations.

Table 2: Inheritance of MT3-MMP and rtTA transgenes in double transgenic mice

Type	F1	F2	F3	F4	F5
CR4x3001	9/25=36%	17/27=62.9%	43/54=79.6%	9/12=75%	16/21=76%
GFAPx3001	13/34=38.2%	20/37=54%	-	-	-
ETGDx3001	15/38=39.4%	32/51=62.7%	-	-	-
CR4x3002	26/49=53%	20/62=32.2%	11/25=44%	11/11=100%	-
GFAPx3002	1/3=33.3%	9/27=33.3%	-	-	-
ETGDx3002	4/23=17.3%	-	-	-	-

The female founder of C57BL/6 was crossed with C57BL/6 wild type mice. Unfortunately, all offspring were killed by her during the suckling period. Finally, the founder died after mating with a CR4 mouse. Therefore, this transgenic mouse line was lost.

IV-1.1.3.3. Cultivation of fibroblasts and detection of b-galactosidase activity

To avoid false positive results from the genomic PCR during the selection of transgenic mice, ear fibroblasts derived from two double transgenic mice (CR4x3001.3 and CR3x3001.5) were isolated and propagated. Ear fibroblasts from a single transgenic CR4 mouse were used as a control. Activity of β-galactosidase was tested with X-gal in the presence or absence of the tetracycline derivative, doxycycline hydrochloride (Dox-HCl) (Figure 7). The fibroblasts from the CR4 single transgenic mouse did not show any blue staining no matter whether or not doxycycline was added previously (Figure 7A-7B). As expected, many CR4x3001.3 cells (~10-15%) derived from double transgenic mice displayed the galactosidase activity in the presence of Dox-HCl (Figure 7C); in contrast, blue colored cells were rarely found (only one or two out of 10^4) without Dox-HCl treatment (Figure 7D). The fibroblasts of CR4x3001.5 produced the similar pattern as shown in CR4x3001.3.

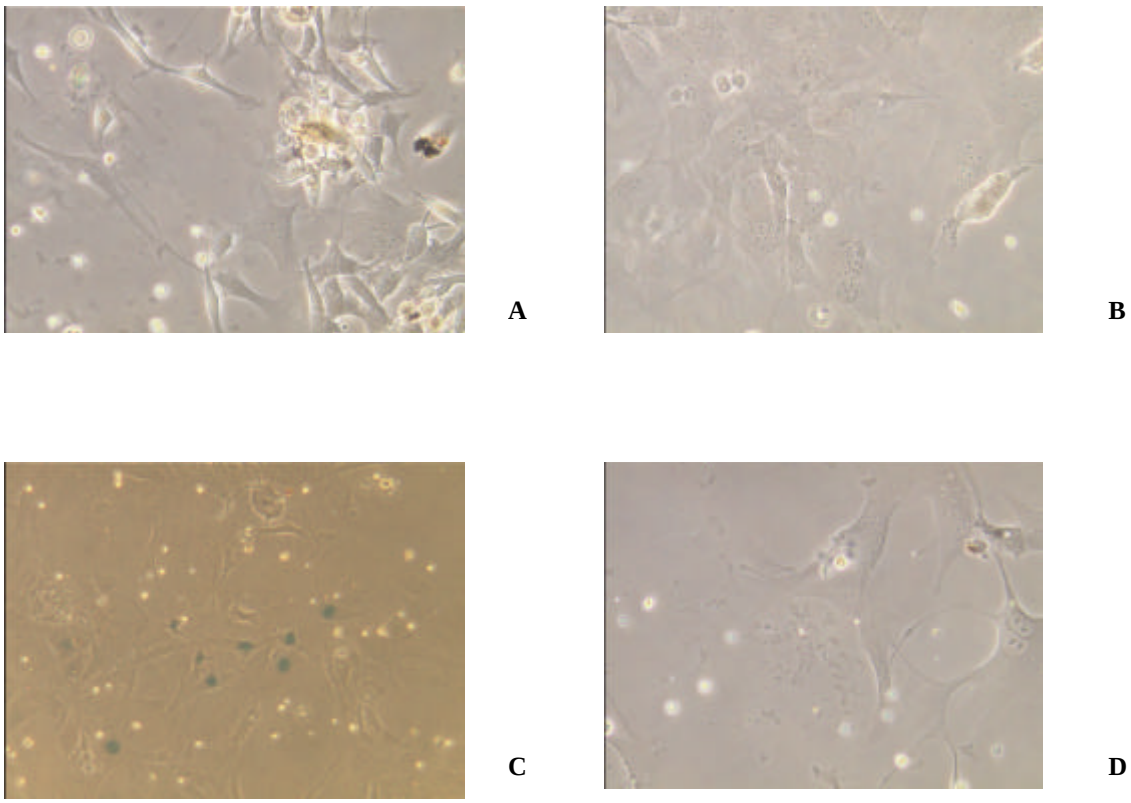


Figure 7: b-galactosidase activity assay in ear fibroblasts of CR4 transgenic mice

Ear fibroblasts were isolated from CR4 control mice (A and B) or CR4x3001 double transgenic mice (C and D) and treated with (A and C) or without (B and D) Dox-HCl. β-galactosidase activity assays were performed in the above cells and observed by phase contrast microscopy (200x for C; 400x for A, B and D). The results show that the reporter gene Lac Z is expressed only in the presence of Dox-HCl.

Theses results demonstrated that, 1) The selection of transgenic mice by genomic PCR with MT3, rtMT3 and rtTA primers in the mouse offspring was successful. 2) The reverse tet-controlled transactivator (rtTA) regulated the expression of the reporter gene (Lac Z) when doxycycline was provided in vitro.

IV-1.1.4. Induction of MT3-MMP expression in double transgenic mice

To define the relationship between expression of transgenes (MT3-MMP and Lac Z) and effector (Dox-HCl) and to trace the physiological changes while exogenous MT3-MMP (i.e. transgenic MT3-MMP or Tg-MT3-MMP) proteins were expressed at different induction conditions, Dox-HCl was given in various doses and time points to double transgenic mice. In the CR4x3001 mouse line, forty double transgenic mice were fed with 1, 2, and 4 mg/ml of Dox-HCl in the Millipore water. These doses of doxycycline were maintained at different time points from one week to four months. In some cases, the mice were fed with doxycycline throughout their whole life (Table 3).

Table 3: Doses and times for inducing the expression of transgenes with Dox-HCl

Type of mouse		CR4x3001															
Time of fed Dox		1 week		2 weeks		3 weeks		4 weeks		5 weeks		8 weeks		4 months		Whole life	
Sex		m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f
Mouse numbers	1mg/ml (+) dox			2													
	2 mg/ml (++) dox	3		2	1	4	3	3	3	2		3	4		1	3	2
	4 mg/ml (+++) dox									1	3						
Total numbers		3		4	1	4	3	3	3	3	3	3	4		1	3	2

m, male. f, female. dox, doxycycline

In other double transgenic mouse lines, eleven (seven males and four females) of CR4x3002, four (two males and two females) of GFAPx3001, five (one male and four males) and two females of GFAPx3002 were fed with doxycycline (2 or 4 mg/ml) and maintained for two time points as described in table 4.

Table 4: Doses and times for inducing the expression of transgenes with Dox-HCl (continue)

Type		GFAPx3001				ETGDx3001		CR4x3002				GFAPx3002	
Time		4 weeks		5 weeks		4 weeks	5 weeks	2 weeks		5 weeks		5 weeks	
sex		m	f	m	f	m	f	m	f	m	f	m	f
Mouse numbers	2 mg/ml	2				1		1	2				
	4 mg/ml				2		4	3		3	2		2
Total numbers		2			2	1	4	4	2	3	2		2

m, male. f, female.

After induction with Dox-HCl, the mice were decapitated and their different organs were used for further RT-PCRs, immunofluorescence microscopy and Western blot analyses to detect expression of transgenes or to define the relationship between the expression of transgenes and effector, Dox-HCl, in the following chapters (IV-1.1.5).

IV-1.1.5. Analysis of expression and regulation of transgenes in induced double transgenic mice

IV-1.1.5.1. Detection of reverse tet-controlled transactivator (rtTA) in organs

rtTA primers was tested in COS-7 tet-off cell line with RT-PCR (Figure 8A). RT-PCRs from nontransgenic mice, Daudi cells or water were used as controls. In lanes 3-5, 6-8 and 10 of figure 8A, RT-PCR amplification products from the COS-7 tet-off cells displayed an expected 470 bp fragment no matter whether the cells were transfected with pBI-G, pBI-G-MT3 or pcDNA3-MT3-MMP. In contrast, the result from lane 11 indicates no genomic DNA contamination in the RNA preparation. Furthermore, no signal was visible in the RT-PCRs amplification products from water, wild type CD1 mice or Daudi cells (Figure 8A, lanes C, 2 and 12). All these results show that, 1) The rtTA primers were specific to indicate the expression of rtTA. 2) rtTA was constitutively expressed in the tet-off cells.

With the specific and sensitive rtTA primers, RNAs isolated from different organs of mice that carried the pTet-On regulatory vector were used for RT-PCR. Weak bands were seen in many organs such as pancreas, lung, thymus and heart in the CR4x3002 mouse line (Figure 8B, lanes 7-12). In contrast, no rtTA was expressed in the 3002 single response transgenic mouse line (Figure 8B, lanes 1-6). In addition, rtTA was also detected in kidney, testis and ear (data not shown). These results were identical to our previous knowledge that the rtTA gene

in mice was widely and constitutively expressed in many organs under the control of the CMV promoter.

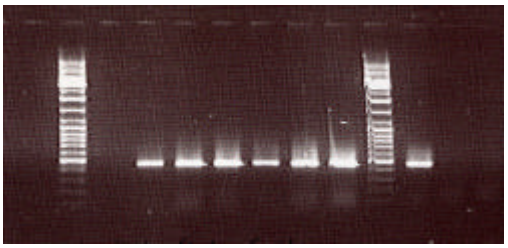


Figure 8A: RT-PCR to detect rtTA expression in transfected COS-7 tet off cells

RNA was isolated from COS7 tet-off cells transiently transfected with pBI-G-MT3-MMP (lanes 3-5; 0.5, 1 and 2 µl of cDNA), pBI-G (lanes 6-8; 0.5, 1 and 2 µl of cDNA) or pcDNA3-MT3-MMP (lane 10; 2 µl of cDNA), wild type CD1 mice (lane 2; 2µl of cDNA) and Daudi cells (lane 12; 2 µl of cDNA). RT-PCRs were carried out with rtTA primers. Lane C: PCR with water was used as a control. Lanes 1 and 9 are DNA marker. Lane 11: PCR using 2 µl of RNA from COS7 tet-off cells transfected with pBI-G-MT3-MMP to check whether the RNA was contaminated by genomic DNA. The expected amplification products are 470 bp. cDNA: RNA reverse-transcripted content as the template in RT-PCR. The results show that rtTA primers are suitable to indicate the expression of rtTA. rtTA was constitutively expressed in tet-off cells.

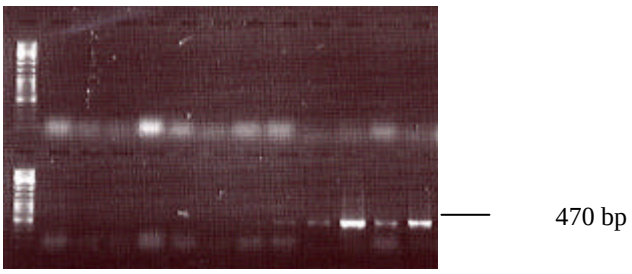


Figure 8B: RT-PCR detection of rtTA in 3002 single response mice or CR4x3002 double transgenic mice

RNA was isolated from 3002 single response transgenic mice (lanes 1-6) or CR4x3002 double transgenic mice (lanes 7-12) and RT-PCRs were carried out with rtTA primers. A weak expression of rtTA was found in liver, pancreas, lung, thymus, kidney and heart in the double transgenic mice (lanes 7-12) but not in the single response transgenic mice (lanes 1-6). M: DNA marker. Upper half of the gel: PCR without prior RT as controls. Lower half of the gel: RT-PCR.

To elucidate whether the rtTA was expressed in brain, total RNAs isolated from brains of double transgenic mice (GFAPx3001 and CR4x3001) were used for RT-PCR with the rtTA primers. As expected, rtTA was weakly expressed in spinal cord, cerebellum and particularly in cerebrum in the GFAP double transgenic mice which contained a glial cell specific promoter (GFAP) upstream of rtTA gene (Figure 9, lanes 1-3). Surprisingly, the rtTA was also detected in cerebellum and cerebellum of the CR4x3001 double transgenic mice at the same level as the GFAP mice (Figure 9, lanes 4-6). The PCR without prior reserve transcription confirmed that the amplification products were not due to the contamination of genomic DNA (Figure 9, lower half of the gel).

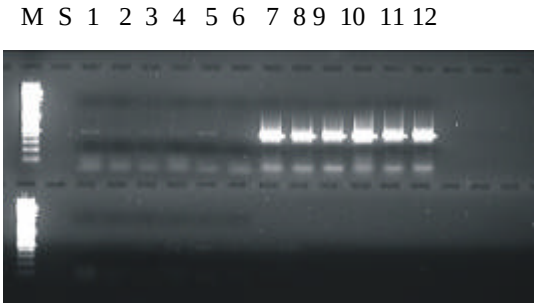


Figure 9: RT-PCR detection of rtTA in brain of Dox-HCl treated GFAP and CR4 double transgenic mice

RNA was isolated from GFAPx3001 mice (lanes 1-3, 7-9 and 13-15) or CR4x3001 mice (lanes 4-6, 10-12 and 16-18) fed with 2mg/ml Dox-HCl. RT-PCRs were carried out with rtTA primers (lanes 1-6), or actin primers (lanes 7-12) as controls. M: DNA marker. S: space lane. Upper half of the gel: RT-PCR. Lower half of the gel: PCR without prior RT as controls. rtTA was detected in cerebrum (lanes 1 and 4), cerebellum (lanes 2 and 5) and spinal cord (lanes 3 and 6) in the both mouse lines.

Table 5: Expression of rtTA in CR4 double transgenic mice

organs	kidney	lung	thymus	heart	kidney	testis	ear	cbl	crb
rtTA	+	+	+	+	+	+	+	+	+

Cbl, cerebellum; cbr, cerebrum

These results suggested that the rtTA was constitutively expressed in most organs in CR4 single regulatory mice and double transgenic, and also in brain of GFAP transgenic mice.

IV-1.1.5.2. Expression of MT3-MMP transgene in brain

To find out whether exogenous MT3-MMP (i.e. Tg-MT3-MMP) was expressed in the brain, RNAs from CR4x3001 and GFAPx3001 transgenic mice were analyzed by RT-PCRs with MT3, MT3new and rtMT3 primers. The CR4x3001 mice were fed with 2 mg/ml Dox-HCl for two weeks and GFAPx3001 mice were fed for 4 weeks with the same concentration of Dox-HCl. The MT3 primers were able to probe both endogenous and Tg-MT3-MMP. In contrast, the rtMT3 and MT3new primers only recognized Tg-MT3-MMP (Appendix D primers). From lane 1 to lane 6 of figure 10, MT3-MMP was detected in both mouse lines. In contrast, only CR4x3001 brain but not GFAPx3001 expressed Tg-MT3-MMP (Figure 10, lanes 16-18 and 28-30). The same experiment was done in the CR4x3001.66, CR4x3002.12 and CRx3002.13 mice that were fed with 2 mg/ml Dox-HCl for 2 weeks. As showed in lanes 7-8 and 19-20 of figure 11, expression of Tg-MT3-MMP was detected only in cerebrum and cerebellum of the CR4x3001 mouse. Amplification products with the actin primers showed that the isolation and reverse transcription of RNAs were successful except spleen of bone marrow of CRx3002.12 and CR4x3001.66 (Figure 11, lanes 25-36). In order to clarify that the difference between the above results was not due to the mouse gender, the opposite sex of mice from CR4x3001 and CR4x3002 were selected. Therefore, four female CR4x3001 and three male CR4x3002 mice were fed with 2 mg/ml Dox-HCl for 2 weeks. Consistent results were obtained, suggesting that CR4x3001 rather than CR4x3002 mice were efficiently regulated and rapidly responding to the Dox-HCl.

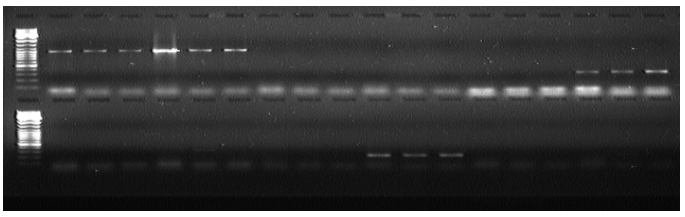


Figure 10: RT-PCR detection of Tg-MT3-MMP in brain of Dox-HCl treated GFAP and CR4 double transgenic mice

RNA was isolated from GFAPx3001mice (lanes 1-3, 7-9, 13-15, 19-21, 24-27 and 31-33) or CR4x3001 mice (lanes 4-6, 10-12, 16-18, 22-23, 28-30 and 34-36) which were fed with 2 mg/ml dox, and RT-PCRs were carried out with MT3 primers (lanes 1-12), rtMT3 primers (lanes 13-24) or $MT3_{new}$ primers (lanes 25-36). Transgenic MT3-MMP was detected in cerebrum (lanes 16 and 28), cerebellum (lanes 17 and 29) and spinal cord (lanes 18 and 30) in the CR4x3001 mice but not in the GFAPx3001 mice. M: DNA marker. Lanes 1-6, 13-18 and 24-30: RT-PCR. Lanes 7-12, 19-23 and 31-36: PCR without prior RT as controls.

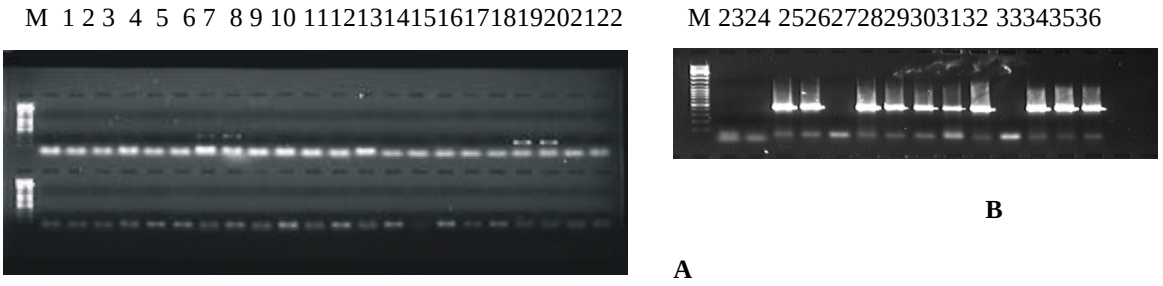


Figure 11: RT-PCR detection of Tg-MT3-MMP in brain of 3001 single response mice or CR4 double transgenic mice

RNA was isolated from CRx3002.12 (lanes 1-3, 13-15 and 25-27), CR4x3002.13 (lanes 4-6, 16-18 and 28-30), CR4x3001.66 (lanes 7-9, 19-21 and 31-33) or 3001 mice (lanes 10-12, 22-24 and 34-36) which were fed with 2 mg/ml dox for two weeks, respectively. RT-PCRs were carried out with rtMT3 primers (lanes 1-12), $MT3_{new}$ primers (lanes 13-24) or actin primers (lanes 25-36). Transgenic MT3-MMP was detected in cerebrum (lanes 7 and 19) and cerebellum (lanes 8 and 20) but not in spleen (lanes 9 and 21) in the CR4x3001 mice. MT3-MMP was not found in cerebrum (lanes 1, 4, 10, 13, 16 and 22), cerebellum (lanes 2, 5, 11, 14, 17 and 23), bone marrow (lanes 3, 15 and 18) or spinal cord (lanes 6, 12 and 24) in the CR4x3002 or 3001 mice. Upper half of the gel A: RT-PCR. Lower half of the gel A: PCR without prior RT as controls.

A high concentration of doxycycline (4 mg/ml) was fed for 5 weeks in four CR4x3001, three CR4x3002, two GFAPx3001, two GFAPx3002 and four ETGDx3001 mice. The mice were decapitated and then brains RNAs were isolated and used for RT-PCR. The RT-PCR results show that the two GFAPx3001 mice but not GFAPx3002 weakly expressed Tg-MT3-MMP under these strong induction conditions. Out of four ETGDx3001, only two of them displayed weak amplification products of Tg-MT3-MMP. All three CR4x3002 weakly expressed Tg-MT3-MMP in brain and spinal cord, but very strongly expressed in testis (data not shown). An extensive expression was found in three of four CRx3001 mice after treatment with this high concentration of Dox-HCl (Figure 12A, lanes 5-7 and 13-15).

Isolation and reverse transcription of the RNAs from above mice were indicated with the actin primers. No contamination of genomic DNA was found in the RNAs by doing PCR without reverse transcription in the similar condition.

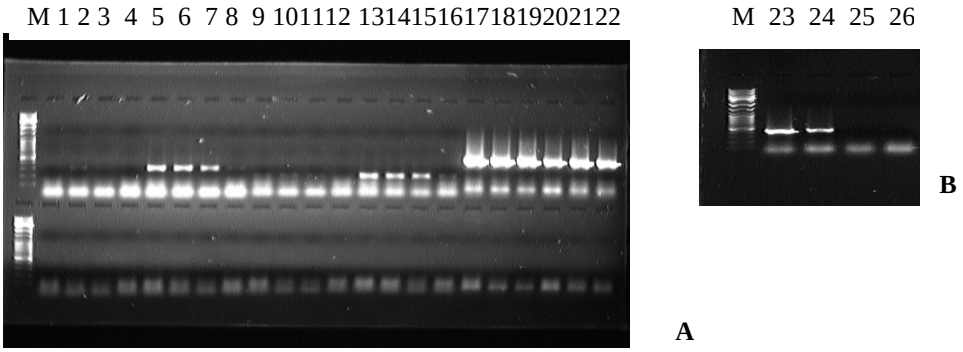


Figure 12: RT-PCR detection of Tg-MT3-MMP in brain of ETGD and CR4 double transgenic mice at high concentrations of doxycycline induction

RNA was isolated from ETGDx3001 transgenic mice (lanes 1-4, 9-12 and 17-20) or CR4x3001 transgenic mice (lanes 5-8, 13-16 and 21-24) that were fed with Dox-HCl (4 mg/ml) for 5 weeks. RT-PCRs were carried out with rtMT3 primers (lanes 1-8), $MT3_{new}$ primers (lanes 9-16) or actin primers (lanes 17-24). Transgenic MT3-MMP was detected in cerebrum (lanes 1, 5, 9 and 13), cerebellum (lanes 2, 6, 10 and 14), spinal cord (lanes 3, 7, 11 and 15) and weakly in pancreas (lanes 4, 8, 12 and 16) in the CR4x3001 double transgenic mice but not in the ETGDx3001 double transgenic mice. Upper half of the gel A and lanes 23-24 of B: RT-PCR. Lower half of the gel A and lanes 25-26 of B: PCR without prior RT as controls.

Table 6: Expression of MT3-MMP in different transgenic mouse lines

line	CR4x3001			CR4x3002			GFAPx3001		
	Crb	Cbl	Sp	Crb	Cbl	Sp	Crb	Cbl	Sp
2 mg/ml	+	+	+	-	-	-	-	-	-
4 mg/ml	+	+	+	weak	weak	weak	weak	weak	weak

Crb, cerebrum; Cbl, cerebellum; Sp, spinal cord. +, RT-PCR positive; -,RT-PCR negative. Weak, weak expression of MT3-MMP.

Taken together, the expression of Tg-MT3-MMP induced by doxycycline indicated a dose-dependent manner in brain of double transgenic mice. CR4 double transgenic mice, particularly CR4x3001 mouse line, showed a more efficient response to Doc-HCl than GFAP double transgenic mice.

IV-1.1.5.3. Distribution of MT3-MMP in transgenic mice

RNAs of different organs from two CR4 double transgenic mouse lines (CR4x3001 and CR4x3002) fed with Dox-HCl were isolated and RT-PCRs were performed with various primers (rtMT3, $MT3_{new}$ or actin primers). As shown in lanes 1-8 of figure 13A, Tg-MT3-MMP was weakly expressed in most organs such as heart, kidney, tongue, lung, thymus, stomach, colon and pancreas, but highly expressed in testis (Figure 13B, lane 8) in both CMV promoter-regulated mouse lines. The Tg-MT3-MMP was also detected in skin and connective tissue in CR4x3001 mice (Figure 13C, lanes 3 and 4). Endo-MT3-MMP was found in cerebellum, cerebrum, spinal cord, heart, kidney, lung, testis and thymus in $MT3^{-}/rtTA^{+}$ mice that only carried a regulatory transgene (rtTA) (Figure 13 D, lanes 1-5; Figure13 E, lanes 7, 10 and 11). The $MT3^{-}/rtTA^{+}$ mice were obtained by crossing CD1 and NMR1 mice respectively, so they shared the similar genetic background as CR4 double transgenic mice and were used as negative control mice.

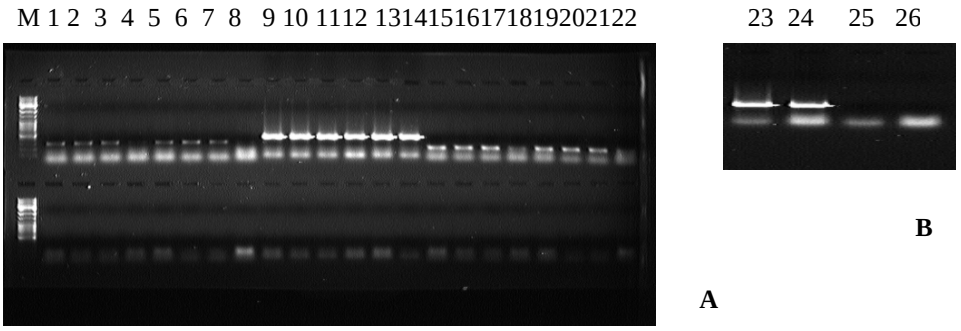


Figure 13A: RT-PCR to detect Tg-MT3-MMP expression in different organs of Dox-HCl-treated CR4x3001 double transgenic mice

RNA was isolated from CR4x3001 double transgenic mice fed with Dox-HCl (2 mg/ml) for two weeks. RT-PCRs were carried out with rtMT3 primers (lanes 1-8), $MT3_{new}$ primers (lanes 15-22) or actin primers (lanes 9-14 and 23-24). Tg-MT3-MMP was detected in heart (lanes 1 and 15), kidney (lanes 2 and 16), tongue (lanes 3 and 17), thymus (lanes 5 and 19), stomach (lanes 6 and 20) and colon (lanes 7 and 21). A weak expression was found in pancreas (lanes 4 and 18) and lung (lanes 8 and 22). Upper half of the gel A and lanes 23-24 of B: RT-PCR. Lower half of the gel A and lane 25-26 of B: PCR without prior RT as controls.

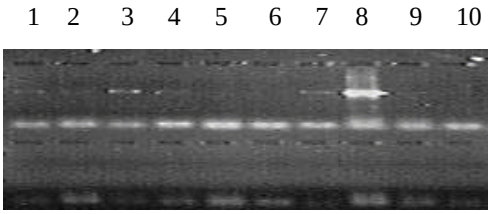


Figure 13B: RT-PCR to detect high Tg-MT3-MMP expression in the testis of Dox-HCl treated CR4x3001 transgenic mice

RNA was isolated from CR4x3001 transgenic mice that were fed with Dox-HCl (2 mg/ml) for two weeks. Tg-MT3-MMP was detected in heat (lane 1), kidney (lane 2), tongue (lane 3), lung (lane 4) and thymus (lane 7). A weak expression of Tg-MT3-MMP was found in pancreas (lane 5) and stomach (lane 9) but undetectable in liver (lane 6) or spleen (lane 10). A high expression of Tg-MT3-MMP was found in testis (lane 8). Upper half of the gel: RT-PCR. Lower half of the gel: PCR without prior RT as controls.

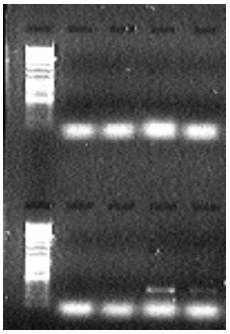


Figure 13C: RT-PCR to detect Tg-MT3-MMP expression in kin and connective issues of Dox-HCl treated CR4x3001 transgenic mice

RNA was isolated from negative control mice (lanes 1-2) or CR4x3001 transgenic mice fed with 2 mg/ml Dox-HCl (lanes 3-4) for two weeks and RT-PCRs were carried out with $MT3_{new}$ primers. Tg-MT3-MMP was detected in skin (lane 3) and connective tissue (lane 4) in the Dox-HCl-induced double transgenic mice but not in the negative control mice (lanes 1 and 2). Lower half of the gel: RT-PCR. Upper half of the gel: PCR without prior RT as controls. M: DNA markers.

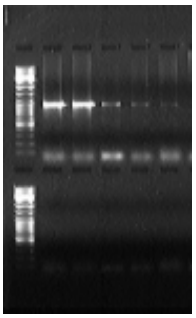


Figure 13D: RT-PCR to detect endogenous MT3-MMP in different organs of $MT3^{-}/rtTA^{+}$ mice

RNA was isolated from $MT3^{-}/rtTA^{+}$ mice and RT-PCRs were carried out with MT3 primers. Endogenous MT3-MMP was detected in cerebrum (lane 1), cerebellum (lane 2), spinal cord (lane 3), heart (lane 4), and kidney (lane 5). M: DNA marker. The PCR cycles: 35. Upper half of the gel: RT−PCR. Lower half of the gel: PCR as without prior RT controls.

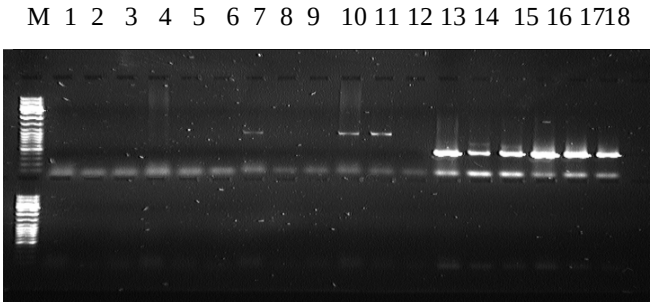


Figure 13E: RT-PCR to detect endogenous MT3-MMP in lung, testis and thymus of MT3^{-}/rtTA^{+} mice

RNA was isolated from MT3^{-}/rtTA^{+} mice and RT-PCRs were carried out with rtMT3 primers (lanes 1-6), MT3 primers (lanes 7-12) or actin primers (lanes 13-18). Endogenous MT3-MMP was detected in lung (lane 7), testis (lane 10) and thymus (lane 11), but not in spleen (lane 8), liver (lane 9) or connective tissue (lane 12). As expected, no signal was found in the RT-PCR with rtMT3 primers. M: DNA marker. Upper half of the gel: RT–PCR. Lower half of the gel: PCR without prior RT as controls.

Expression of Tg-MT3-MMP in testis and pancreas were assessed in single transgenic mouse lines such as CR4 only carrying a regulatory vector (pTet-On) or 3001 that only contained the responsive vector (pBI-G-MT3-MMP), and also in double transgenic mouse line such as CR4x3001 with or without doxycycline treatment. No significant difference was found in the expressions of Tg-MT3-MMP in testis of CR4x3001 double transgenic mice that were treated with or without Dox-HCl, even if the cycle number of RT-PCR was reduced from 30 to 25 (Figure 14A, lanes 6, 8, 20 and 22; Figure 14B, lanes 3-4 and 7-8). However, amplification signal of testis in CR4x3001 double transgenic mice was stronger than single transgenic mice (3001) (Figure 14A, lanes 4, 6, 18 and 20). Furthermore, Tg-MT3-MMP was not detected in the CR4 mice (Figure 14A, lane 2; Figure 14B, lanes 1 and 5). Only weak Tg-MT3-MMP was detected in pancreas (Figure 14A, lanes 7 and 21) in the CR4x3001 mice that were fed with doxycycline. RT-PCRs with actin primers were used as controls.

Table 7: Distribution of MT3-MMP in Dox-HCl-induced CR4x3001 or MT3^{-}/rtTA^{+} mice

	Expression of Tg-MT3-MMP	Expression of endo-MT3-MMP
organs	Heart, kidney, tongue, lung, thymus, stomach, colon, pancreas, testis, skin, connective tissue, brain, spinal cord	Cerebellum, cerebrum, spinal cord, heart, kidney, lung, testis, thymus

In brief, transgenic MT3-MMP (i.e. Tg-MT3-MMP) was expressed in most organs in CR4 double transgenic mice and endogenous MT3-MMP was found in brain, spinal cord, heart, kidney, lung, testis and thymus by RT-PCR. A leakage of transgenic MT3-MMP expression was exhibited in some organs such as testis.

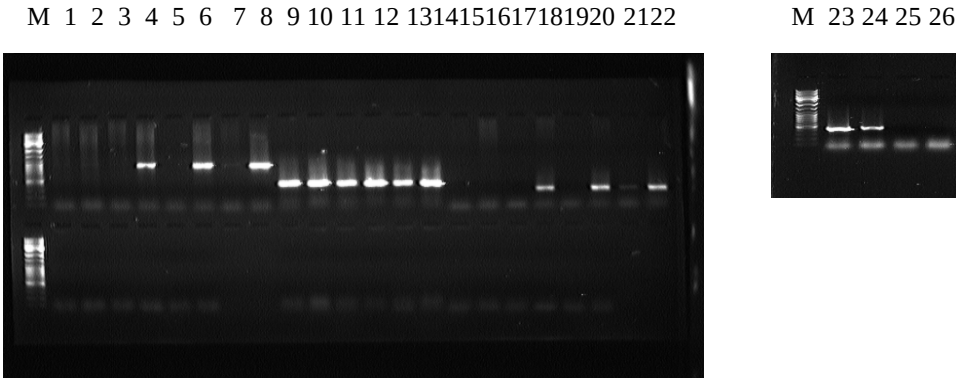


Figure 14A: **RT-PCR to detect Tg-MT3-MMP expression in the testis**

RNA was isolated from CR4 negative control mice (lanes 1-2, 9-10 and 17-18), 3001 mice carrying pBI-G-MT3-MMP plasmid (lanes 3-4, 11-12 and 19-20), CR4x3001 double transgenic mice without Dox-HCl (lanes 5-6, 13-14 and 21-22) or CR4x3001 double transgenic mice treated with 2mg/ml Dox-HCl for two weeks (lanes 7-8, 15-16 and 23-24). RT-PCRs were carried out with MT3 primers (lanes 1-8), actin primers (lanes 9-14 and 23-26) or rtMT3 primers (lanes 15-22). The cycle number of PCR is 30. Transgenic MT3-MMP was detected in testis in the 3001 mice (lanes 4 and 18), CR4x3001 mice without Dox-HCl treatment (lanes 6 and 20) and CR4x3001 mice with Dox-HCl treatment (lanes 8 and 22) but not in the CR4 mice (lanes 2 and 16). A weak signal was detected in pancreas in the CR4x3001 mice after Dox-HCl treatment (lanes 7 and 21) but not in the CR4 mice (lanes 1 and 15), 3001 mice (lanes 3 and 17) or CR4x3001 mice without Dox-HCl treatment (lanes 5 and 19). Upper half of the gel and lanes 23-24: cDNA-PCR. Lower half of the gel and lanes 25-26: PCR without prior RT as controls.

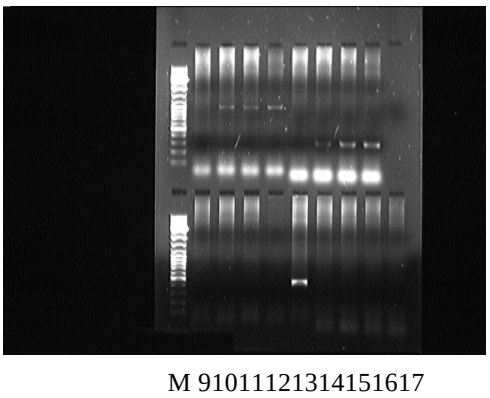


Figure 14B: RT-PCR to detect transgenic MT3-MMP in the testis at low PCR cycles

RNA was isolated from CR4 mice (lanes 1, 5, 9, 13 and 14), 3001 mice (lanes 2, 6, 10 and 15), CR4x3001 double transgenic mice without Dox-HCl treatment (lanes 3, 7, 11 and 16) and CR4x3001 double transgenic mice treated with 2 mg/ml Dox-HCl for two weeks (lanes 4, 8, 12, and 17). RT-PCRs were carried out with MT3 primers (lanes1-4 and 9-12), rtMT3 primers (lanes 5-8 and 14-17) and actin primers (lane 13), respectively. Lanes 1-8: 25 cycles; Lanes 9-17: 20 cycles.

IV-1.1.5.4. Reporter gene assay in organs of transgenic mice

No β-galactosidase activity was detected in cerebrum, cerebellum, pancreas, gut or muscle in GFAPx3001 (Table 8C) and ETGDx3001 (Table 8D) mice, although the mice were fed with doxycycline (2mg/ml) for 4 weeks. Only kidney, tongue and heart but not cerebrum, spleen, thymus, lung, liver or muscle were found to show the typical blue staining in Dox-HCl-induced CR4x3002 mice (2mg/ml for 2 weeks) (Table 8E and 8F; Figure 15). In contrast, kidney, tongue, heart and muscle but not cerebrum, cerebellum, gut, thymus, pancreas, lung, spleen, testis or liver displayed the typical blue dots in Dox-HCl-induced (2mg/ml for 2 weeks) CRx3001 mice (Table 8H and 8I; Figure 15). As listed in the following tables (Table 8A and 8B), no β-galactosidase activity was found in organs of single transgenic mouse lines such as CR4 or 3001. The β-galactosidase activity was also not detected in CR4x3001 mice that were not treated with Dox-HCl (Table 8G). In the CR4x3001 mouse line, three mice (No8, 10 and 50) were fed with doxycycline 2 mg/ml for 1 week. No blue staining cells were found in cerebrum, cerebellum, muscle and tongue in these three mice, suggesting that the expression of reporter gene Lac Z was dependent on the induction time in the presence of Dox-HCl.

Furthermore, whole brains from two CR4x3001 (No3 and 66) mice and two CR4x3002 (No12 and 13) mice fed with 2mg/ml doxycycline for 2 weeks were used for cryosections (20 µm) in sagittal direction. The cryosections were stained with X-gal solution and no blue staining was found. However, in four CR4x3001 mice (No13, 35, 36 and 46) mice that were fed with 2 mg/ml doxycycline for 3 weeks, the blue cells were found in tongue, heart, muscle and kidney, but also in the white matter of the cerebellum, microglial cells and the central part of the brain (Figure 15). Again, the β-galactosidase activity could not be detected in liver, lung, testis, spleen, pancreas and thymus in these four CR4x3001 mice. Interestingly, the tumor tissue of CR4x3001.3 showed no blue staining.

Briefly, the reporter Lac Z gene was detected in certain organs such as heart, kidney, muscle, tongue and occasionally in brain of Dox-HCl-induced CR4 double transgenic mice. The expression of the reporter gene was regulated by doxycycline in a time-dependent manner.

Table 8: b-galactosidase assays in different organs

Table 8A

CR4	cerebrum	heart	testis	thymus	spleen	gut	tongue	pancreas	muscle
Activity of β-galactosidase	-	-	-	-	-	-	-	-	-

Table 8B

3001.36	tongue	muscle	pancreas	gut	kidney
Activity of β-galactosidase	-	-	-	-	-

Table 8C

GFAPx3001.5 (2mg/ml dox for 4 weeks)	cerebrum	cerebellum	pancreas	gut	muscle
Activity of β-galactosidase	-	-	-	-	-

Table 8D

ETGDx3001.8 (2mg/ml dox for 4 weeks)	cerebrum	cerebellum	pancreas	gut	muscle
Activity of β-galactosidase	-	-	-	-	-

Table 8E

CR4x3002.12 (2mg/ml dox for 2 weeks)	tongue	kidney	spleen	heart	muscle
Activity of β-galactosidase	+	+	-	+	-

Table 8F

CR4x3002.13 (2mg/ml dox for 2 weeks)	crb	cbl	heart	thymus	spleen	tongue	lung	muscle	liver	kidney
Activity of β-galactosidase	-	-	+	-	-	+	-	-	-	+

Table 8G

CR4x3001.26 (-dox)	cerebellum	tongue	pancreas	gut	muscle
Activity of β-galactosidase	-	-	-	-	-

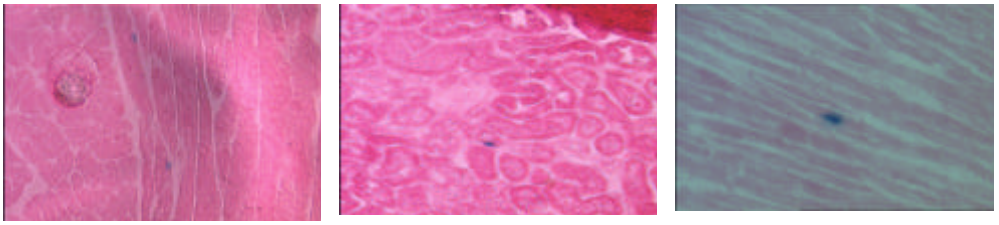
Table 8H

CR4x3001.17 (2 mg/ml dox for 2 weeks)	crb	cbl	kidney	heart	testis	liver	gut	pan	muscle	tongue	lung	spleen
Activity of β-galactosidase	-	-	+	+	-	-	-	-	+	+	-	-

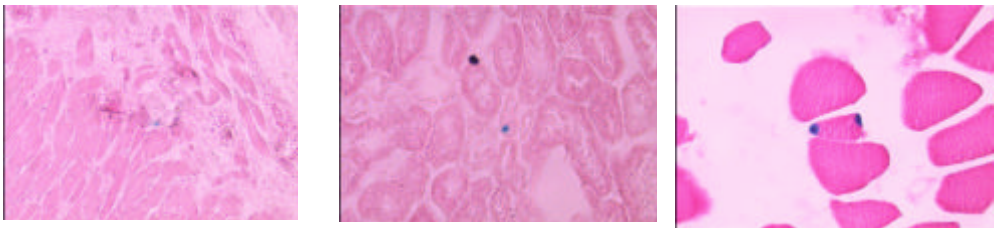
Table 8I

CR4x3001.66 (2 mg/ml dox for 2 weeks)	tongue	kidney	muscle	spleen	liver	lung
Activity of β-galactosidase	+	+	+	-	-	-

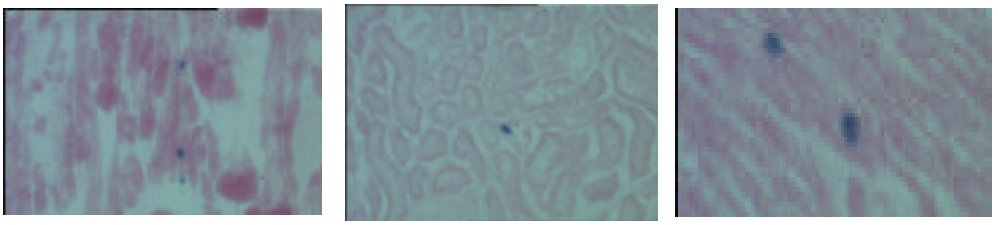
-dox, without doxycycline treatment. +dox, with doxycycline treatment. +, β-galactosidase activity positive. -, β-galactosidase activity negative. Crb: cerebrum; Cbl: cerebellum; Pan: pancreas



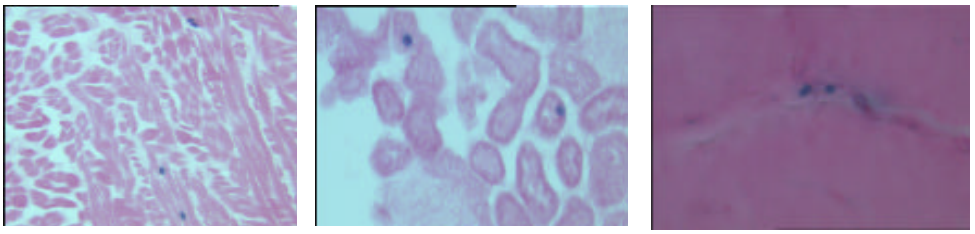
Tongue of CRx3001.17 Kidney of CR4x300.17 Heart of CRx3001.17



Tong of CR4x3001.66 Heart of CR4x3001.66 Muscle of CR4x3001.66



Tongue of CR4x3002.12 Kidney of CR4 x3002.12 Heart of CR4x3002.12



Tongue of CR4x3002.13 Kidney of CR4x3002.13 Brain of CR4 x3001.46

Figure 15: b-galactosidase activity assay in organs of Dox-HCl-induced CR4 double transgenic mice

β-galactosidase activity assays were performed as described in "Materials and Methods" and analysed by phase contrast microscopy (400x).

IV-1.1.5.5. Localization of MT3-MMP in vivo

From the above RT-PCR and β-galactosidase activity assays in various organs and mouse lines, CRx3001 line displayed fast and efficient response to doxycycline, in which several organs such as brain, kidney, tongue, heart and tongue were confirmed to express both transgenes (MT3-MMP and Lac Z) except testis which only showed extremely high expression of transgenic MT3-MMP in RT-PCR. Depending on these results, brain, kidney, tongue, heart, tongue and testis from CR4x3001 mouse line were selected to perform immunofluorescence microscopy (Figure 16).

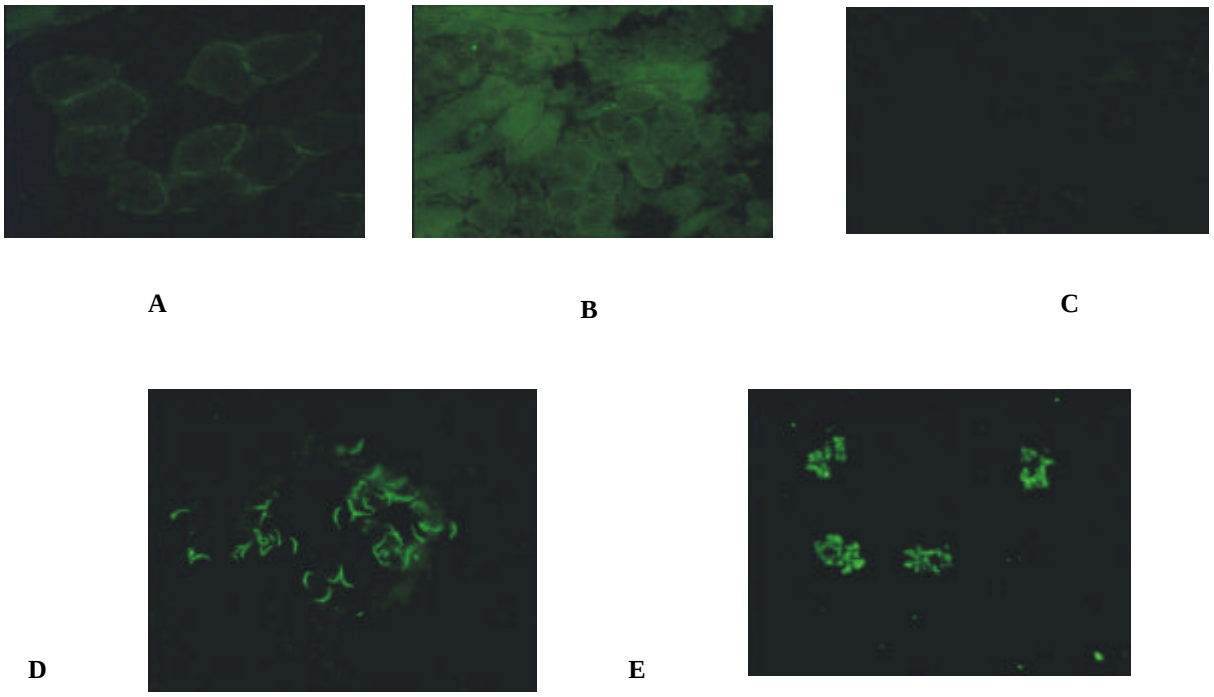


Figure 16: Detection of MT3-MMP proteins in Dox-HCl-induced CR4x3001 double transgenic mice with immunofluorescence microscopy

Cryosections were prepared from muscle (A), tongue (B), brain (C), testis (D) and kidney (E) and probed with rabbit anti-MT3-HR antibodies and Cy2-conjugated goat anti rabbit IgG antibodies. A-D: 400x. E: 200x.

MT3-MMP proteins were detected at the edges of cells in muscle and tongue (Figure 16A-16B), but were not found in brain (Figure 16C) from CR4x3001 mice under induction conditions in several independent experiments. In contrast, clear and strong expression of

MT3-MMP was found in the acrosome of testis and glomeruli of kidney (Figure 16D-16E) in the above mice. Test experiments of MT3 antibodies were performed in kidney of Dox-HCl-treated CR4x3001 mice with pre-immune serum and Cy2-conjugated goat anti rabbit IgG antibodies (Figure 17C), or only Cy2-conjugated goat anti rabbit IgG antibodies (Figure 17B). Only very weak signals were found in the figure 17B and 17C, suggesting that the MT3-MMP proteins were specifically recognized by the anti-HR-MT3 antibodies. MT3-MMP expression extent was compared among Dox-HCl-treated CR4x3001 and MT3–/rtTA+ negative control mice, which had the similar species background. The expression of MT3-MMP in Dox-HCl-treated CR4x3001 mice (Figure 18A and 18D) was stronger than the negative control (Figure 18C and 18F). However, it was very difficult to discriminate the expression extent in double transgenic mice under uninduced and induced conditions.

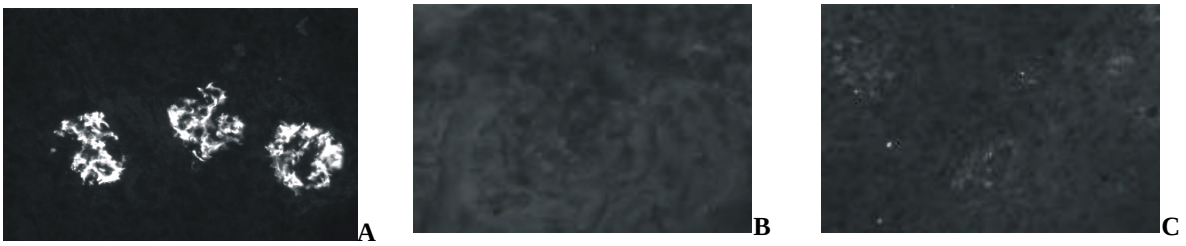


Figure 17: Anti-HR-MT3 antibodies test in the kidney of Dox-HCl - induced CR4x3001 double transgenic mice

Cryosections of kidney from Dox-HCl induced CR4x3001 double transgenic mice were probed with rabbit anti-HR-MT3 antibodies and Cy2-conjugated goat anti rabbit IgG antibodies (A), Cy2-conjugated goat anti rabbit IgG antibodies alone (B) or pre-immune serum and Cy2-conjugated goat anti rabbit IgG antibodies (C). Amplification is 200x.

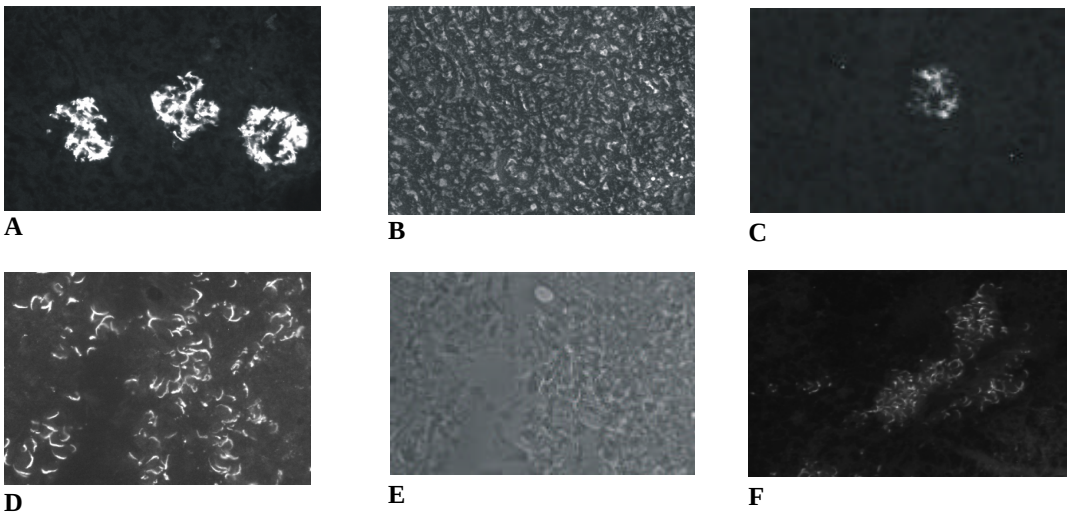


Figure 18: Expression of MT3-MMP in the kidney and testis of Dox-HCl-induced CR4x3001 double transgenic mice compared to negative control mice

Cryosections of kidney (A-C; 200x) or testis (D-F; 400x) from induced CR4x3001 transgenic mice (A, B, E and F) or negative control mice (D and G) were probed with anti-MT3-HR antibodies and Cy2-conjugated goat anti rabbit IgG antibodies. A, C and D, F: immunofluorescence microscopy. B and E: phase contrast photo.

To know whether MT3-MMP proteins were expressed and localized in smooth muscle cells (SMC) in vivo, cryosections were obtained from day 12 and day 14.5 embryos, and from heart, gut, stomach, kidney, testis and liver in ages of two-weeks, two-months and six-months in both negative control mice and Dox-HCl-treated double transgenic mice. Smooth muscle a actin was used as a marker protein to indicate SMCs. No colocalization was found between MT3-MMP and SM a-actin in all above organs (Figure 19).

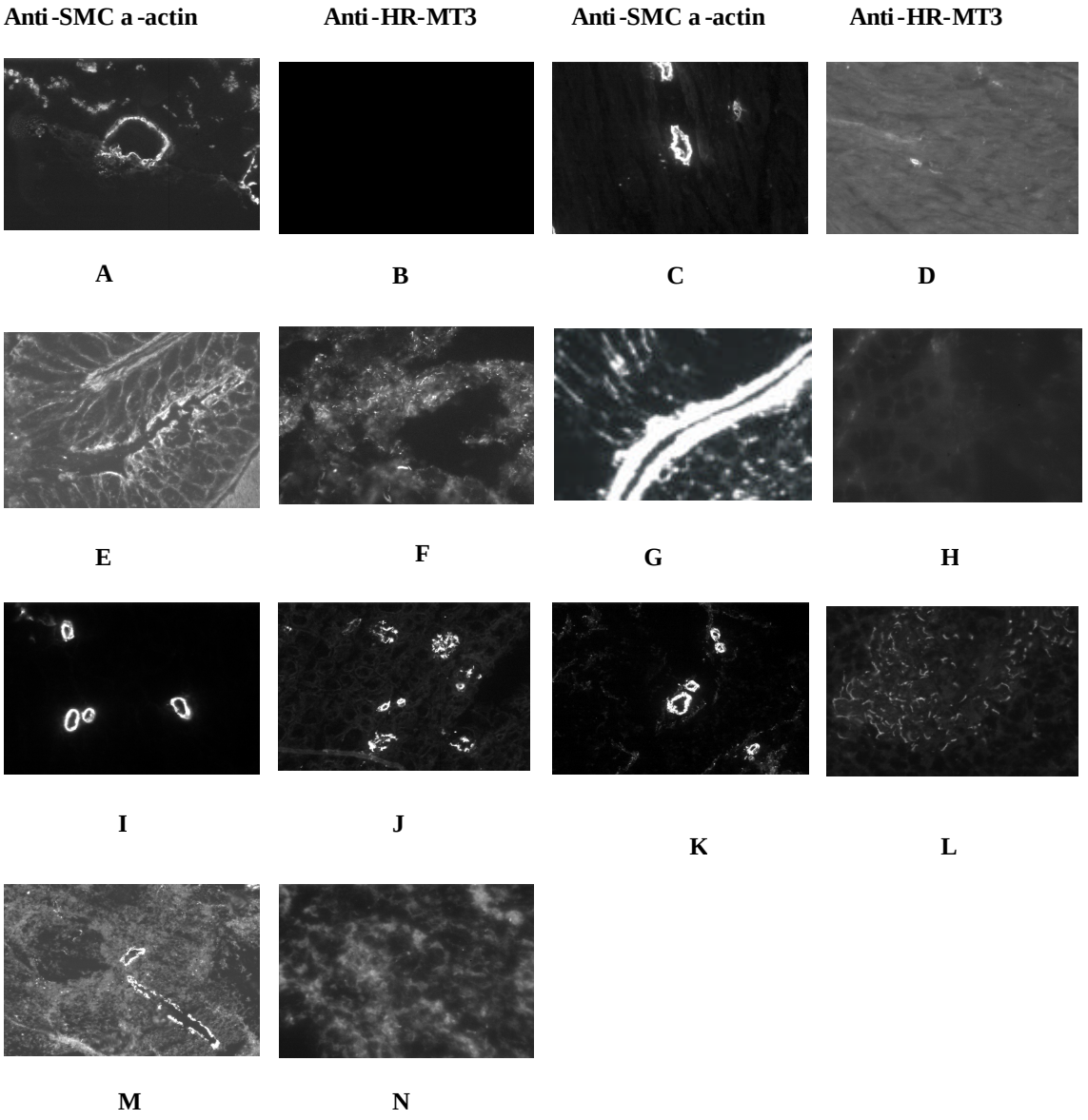


Figure 19: Localization of Tg-MT3-MMP in smooth muscle

Cryosections of a day 14.5 embryo (A-B), heart (C-D), gut (G-H), stomach (G-H), kidney (I-J), testis (K-L) or liver (M-N) from induced CR4x3001 transgenic mice were probed with cy3-conjugated mouse anti-smooth muscle a actin antibodies, rabbit anti-HR-MT3 antibodies and Cy2-conjugated goat anti rabbit IgG antibodies and viewed using either a cy3 filter (B, D, F, H, J, Land N) or cy2 filter (A, C, E, G, I, K and M) set.

IV-1.1.5.6. Detection and analysis of MT3-MMP with Western blot and zymography

Kidney, testis and brain of doxycycline-treated CR4x3001 mice, which have been proved to express Tg-MT3-MMP with RT-PCR, were collected and used to extract plasma membrane factions or intracellular membrane factions using discontinuous sucrose gradient ultracentrifugation. The same kinds of tissues from negative control mice ($MT3^{-}/rtTA^{+}$) which had the identical species background but did not carried MT3-MMP transgene were also employed to extract plasma membrane factions or intracellular membrane factions as controls. The extracts were quantified and probed with rabbit anti-HR-MR3 antibodies and goat anti rabbit IgG antibodies labeled with horseradish peroxidase in Western blots. The intracellular membrane extracts of various organs in both CRx3001 and $MT3^{-}/rtTA^{+}$ mouse lines did not show any signals in Western blots (Figure 20, lanes 1 and 2). In contrast, MT3-MMP proteins were detected in the plasma membrane extracts of kidney in both mouse lines that displayed a distinct pattern (Figure 20, lanes 3 and 4). The control mouse ($MT3^{-}/rtTA^{+}$) only showed a 50-52 kDa size of MT3-MMP proteins in the plasma membrane extracts of kidney (Figure 20, lane 4). Two sizes of MT3-MMP proteins were detected in the kidney plasma membrane extracts of Dox-HCl-treated double transgenic mice, one of which is 50-52 kDa that was similar to the control mice and another was 63 kDa (Figure 20, lane 3). The latter (63 kDa) was close to the active form of MT3-MMP proteins and the former (50-52 kDa) remained to be defined. No significant difference was detected in testis or brain between negative controls and double transgenic mice treated by Dox-HCl. Although increased amount of extracts and another solution buffer which contained high concentration of SDS were applied in the above three organs (kidney, brain, testis), a similar results were obtained. 20 µg or 40 µg of the membrane extracts from the above three organs were analyzed by gelatin zymography. Very weak bands (72 kDa and 92 kDa) were seen only in extracts of kidney but not in testis or brain (data not shown). Whole protein extracts from kidney, testis, brain and lung were collected from the negative control or Dox-HCl-treated double transgenic

mice and resolved in gelatin zymography gel. Consistently, no significant bands were found in kidney, testis and brain. In contrast, a huge amount of 92 kDa (gelatinase B), a large amount of 72 kDa (pro-gelatinase A) and a little amount of 62 kDa (active-gelatinase A) were observed in lung. However, no significant difference was present between the Dox-HCl-treated double transgenic mouse and negative control mouse (Figure 21, lanes 1 and 2).

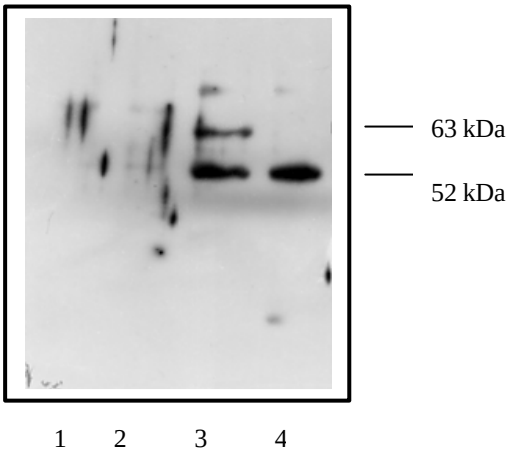


Figure 20: Detection of MT3-MMP with Western blot

Intracellular membrane protein extracts (lanes 1-2) or plasma membrane protein extracts (lanes: 3-4) of Dox-HCl-induced CR4x3001 transgenic mice (Lanes 1 and 3) or negative control mice (lanes 2 and 4) were probed with rabbit anti-HR-MT3 antibodies and goat anti rabbit antibodies labeled with horseradish peroxidase.

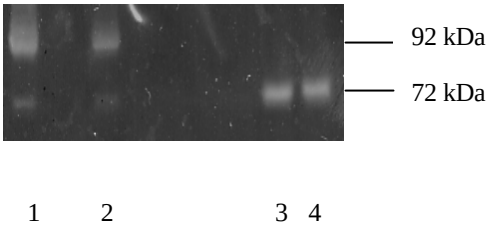


Figure 21: Gelatine zymography with lung extracts

Lung extracts (lane 1) from Dox-HCl-induced CR4x3001 transgenic mice or negative control mice (lane 2) were analyzed by gelatine zymography. The conditioned medium containing pro-MMP-2 was used as control (lanes 3 and 4). The upper band (Mr 92 kDa) is gelatinase B. The down band (Mr 72 kDa) is pro-MMP-2. The weak Mr 65 kDa band (lanes 1 and 2) can be seen in the original gel.

IV-1.1.5.7. Histochemical staining

Cryosections of kidney, testis, brain, spinal cord, spleen, heart, stomach, liver, gut and embryo were stained in Harris and Mayers hematoxylin or Grill's hematoxylin. No significant difference was found between CR4x3001 mice treated with Dox-HCl and negative control

mice ($MT3^{-}/rtTA^{+}$) in the studied organs through hundreds slide analyses. Some cryosections from the double transgenic mice are shown in figure 22.

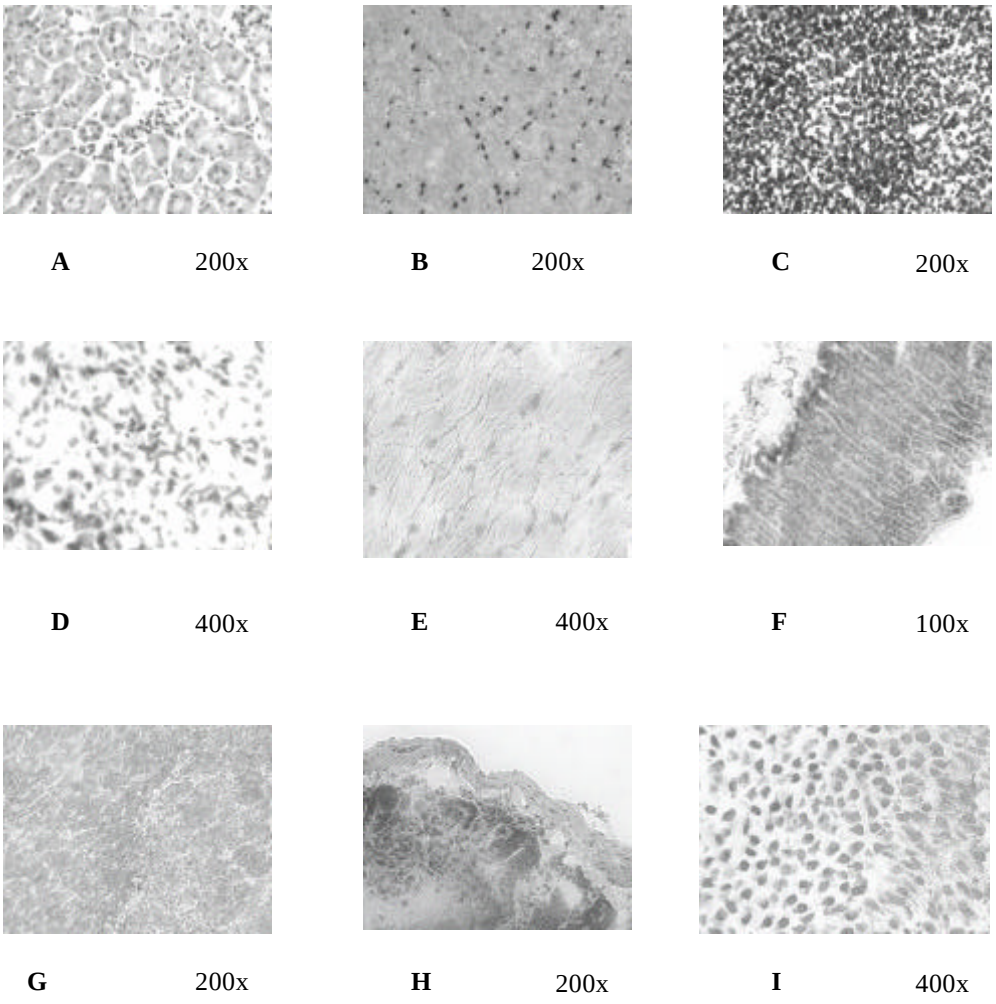


Figure 22: Histochemical staining of various organs of Dox-HCl-induced CR4x3001 double transgenic mice

A: kidney; B: spinal cord; C: spleen; D: testis; E heart: F: stomach; G liver; H: gut; I: embryo.

IV-1.2. Phenotypes (physiology and pathology) observed in single and double transgenic mice

IV-1.2.1. General observation

To study the impact of Tg-MT3-MMP and Dox-HCl at different ages, forty-seven of CR4x3001 double transgenic mice were selected and studied at a young age (six to eight weeks), a middle age (two to six months) and an old age (six to 12 months). Sex and numbers of mice are mentioned in the table 9.

Table 9: Age of CR4x3001 mice fed with Dox-HCl and age of fed mice which were sacrified

Type	Age of feeding Dox-HCl										Age of sacrified											
Time	≤6 weeks		6-8 weeks		2-3 months		3-6 months		6-10 months		≤6 weeks		6-8 weeks		2-3 months		3-6 months		6-10 months		≥12 months	
Sex	m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f	m	f
Total No.	5	5	5	2	2	3	5	3	9	8	2	4	2	2	7	3	7	3	8	8	1	

M: male; F: female.

There were no significant differences between wild type, single and double transgenic mice. Most of them showed healthy growth, reproduction and fertility with or without doxycycline treatment in their life. When transgenic mice became old, a huge amount of fat was accumulated in the abdomen of CR4 MT3-MMP double transgenic mice. Occasionally, a stomach tumor was found in CR4x3001 double transgenic mice treated by Dox-HCl. Moreover, one of the 3001 mouse line and one of 3002 mouse line from single responsive transgenic mice were found to develop tumor in muscle without feeding doxycycline.

IV-1.2.2 Analysis of an unusual phenotype

IV-1.2.2.1. Tumor found in a Dox-HCl-treated CR4x3001 mouse

A 0.2 cm diameter round tumor was found nearby the stomach in a CR4x3001 mouse after a two-week treatment with 1 mg/ml doxycycline. The small bulk (tumor) was collected and its RNA was isolated to perform RT-PCR with the rtTA and rtMT3 primers. In addition, the actin primers were used as controls. Very weak Tg-MT3-MMP was detected in the tumor tissue (Figure 23, lane 4). RT-PCR from gut and epidermis did not show any signals in the same experiment (Figure 23, lanes 2-3, 5-6 and 8-9). More interestingly, expression of the reverse tet-controlled transactivator (rtTA) was extremely high in the tumor tissue (Figure 23, lane 13) where expression extent was comparable to the housekeeping gene actin (Figure 23, lanes 10-12). In contrast, the rtTA was expressed normally (i.e. very low) in gut and epidermis (Figure 23, lanes 14 and 15).

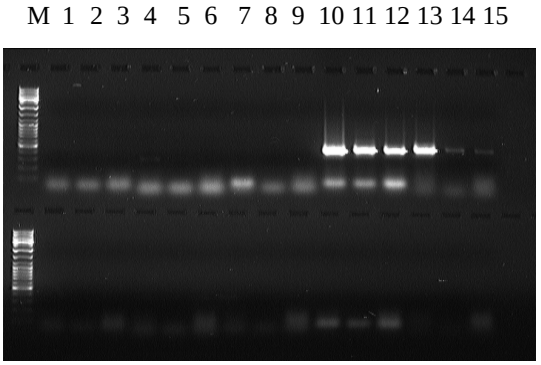


Figure 23: RT-PCR assay in tumor organs of a CR4x3001 double transgenic mouse treated by Dox-HCl

RNA was isolated from tumor (lanes 1, 4, 9 and 13), epidermis (lanes 2, 5, 10 and 14) or gut (lanes 3, 6, 11 and 15) of a Dox-HCl-induced CR4x3001 transgenic mouse and RT-PCRs were carried out with MT3 primers (lanes 1-3), rtMT3 primers (lanes: 4-6), $MT3_{new}$ primer (lanes 7-9), actin primers (lanes: 10-12) or rtTA primers (lanes 13-15). Upper half of the gel: RT-PCR. Lower half of the gel: PCR without prior RT as controls.

IV-1.2.2.2. Tumor found in single transgenic mice

One female of the 3001 mouse line that only carried the responsive vector was found to have a tumor in muscle of the left leg between bone of hip and knee joint, which was 0.8 cm diameter. In contrast, other organs in this mouse looked like normal. The tumor, connective tissue around tumor, normal muscle, normal skin, cerebrum, kidney and pancreas were collected and their RNAs were isolated and carried out RT-PCRs with the rtMT3 primers. Expression of Tg-MT3-MMP was not found in inside and outside of the tumor tissue, tumor skin, normal skin, cerebrum, kidney and pancreas (Figure 24A, lanes 1-8). The actin amplification products indicated that the RNAs were successfully reverse transcribed. The results were reproduced in a female mouse from 3002 mouse line that had a back tumor. So, the tumor occasionally happened in single responsive transgenic mice was not due to over-expression of MT3-MMP in the affected cells.

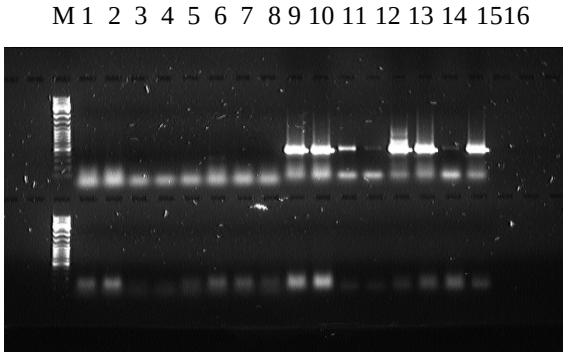


Figure 24A: RT-PCR assay in a single transgenic mouse carrying a tumor

RNA was isolated from inside part of tumor (lanes 1, 9), outside part of tumor (lanes 2, 10), skin of tumor (lanes 3, 11), normal skin (lanes 4, 12), muscle tissue of tumor (lanes 5, 13), cerebrum (lanes 6, 14), pancreas (lanes 7, 15) or kidney (lanes 8, 16). RT-PCRs were carried out with rtMT3 primers (lanes 1-8) or actin primers (lanes 9-16). Upper half of the gel: RT-PCR. Lower half of the gel: PCR without prior RT as controls.

Cryosections from surrounding region (Figure 24B, I, III and V) or malignant region (Figure 24B, II, IV and VI) of the muscle tumor were stained as described in 'Materials and Methods' and shown by phase contrast microscopy at different amplification times.

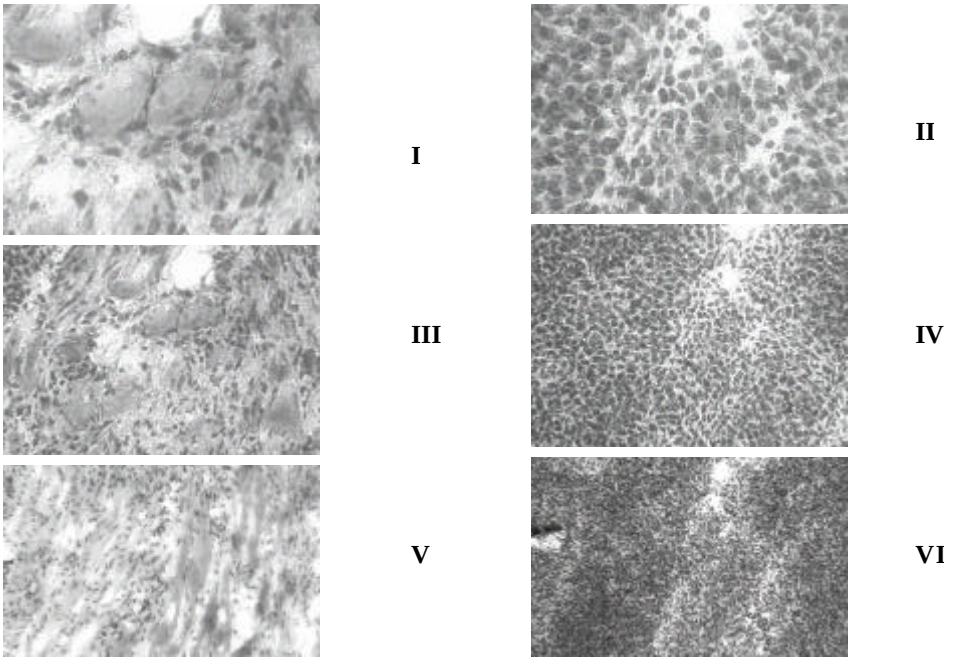


Figure 24B: Histochemical staining of the muscle tumor in the single transgenic mouse

Cryosections from surrounding region of tumor (I, III, V) or malignant region of tumor (II, IV, VI) of the single transgenic mouse having a muscle tumor were stained as mentioned in 'Materials and Methods' and observed by phase contrast microscopy. I-II: 400 x; III-IV: 200 x; V-VI: 100 x.

IV-1.3. Establishment of immortal fibroblasts from mouse embryos

Fibroblasts from embryos of constitutively transgenic MT1-MMP mice were separated and cultivated. In the first two weeks, cells grew fast, and were then kept at a constant number for two-three months. Finally, the cell number significantly increased and cells became immortal fibroblasts. The immortal fibroblasts look a little bit smaller than normal ones. The fibroblast cell lines were checked by genomic PCR. RT-PCR results showed that transgenic MT1-MMP was constitutively expressed in the immortal fibroblasts. The MT1-MMP immortal fibroblasts can be used for the futher analyses to define the biological functions of MT1-MMP such as migration and invasion.

In summary 1:

1. Regulatory MT3-MMP transgenic mice were established.

2. Tg-MT3-MMP was expressed in most organs; in contrast, the reporter gene was only expressed in certain types of organs such as heart, kidney, muscle, tongue and occasionally in brain in Dox-HCl-induced double transgenic mice.

3. Expression of transgenes mediated by Dox-HCl was a dose and time-dependent although a leakage or uncontrolled expression was found in some organs such as testis.

4. Different double transgenic lines exhibited distinct responsive patterns. CRx3001 displayed faster and more efficient response to Dox-HCl than other mouse lines.

5. MT3-MMP proteins did not colocalize with SMC a-actin in the studied organs. More of the active form of MT3-MMP was detected in the kidney of CR4x3001 double transgenic mice treated with Dox-HCl compared to negative control mice.

6. In general, there were no significant differences between wild type, single and double transgenic mice at the later stages of development.

IV-2. Localization and activity of MT-MMPs in vitro

IV-2.1. Characterization of MT1, -2, -3-MMPs

IV-2.1.1. Solubility of MT-MMPs

COS cells transiently transfected with MT1-, 2- or 3-MMP-pcDNA3 plasmids were lysed with 1% Triton X-100/PBS. Then the lysates were centrifuged and separated into supernatant (Triton soluble fractions) and pellet (Triton insoluble fractions). Both fractions were probed with specific anti-MT1-, 2- or 3-MMP antibodies in Western blots, accordingly. As shown in figures 25A.I, 25A.II and 25A.III, MT2-MMP and MT3-MMP were Triton X-100 insoluble proteins. In contrast, MT1-MMP displayed Triton solubility. After replacing the 1% Triton X-100/PBS buffer with RIPA buffer which contained 0.1% SDS and 1% Triton X-100, MT3-MMP was only partially soluble and most of it remained insoluble (Figure 25A, IV). All COS cells (COS-1, COS-7 and COS-M6) showed similar results when they were transiently transfected with MT1- or 2- or 3-MMP-pcDNA3 plasmids. The insolubility of MT2-MMP was also confirmed in human embryonic kidney (HEK 293) cells that were stably transfected with MT2-MMP cDNA, and in untransfected human hepatocellular cells (Hep G2) (Figure 25B).

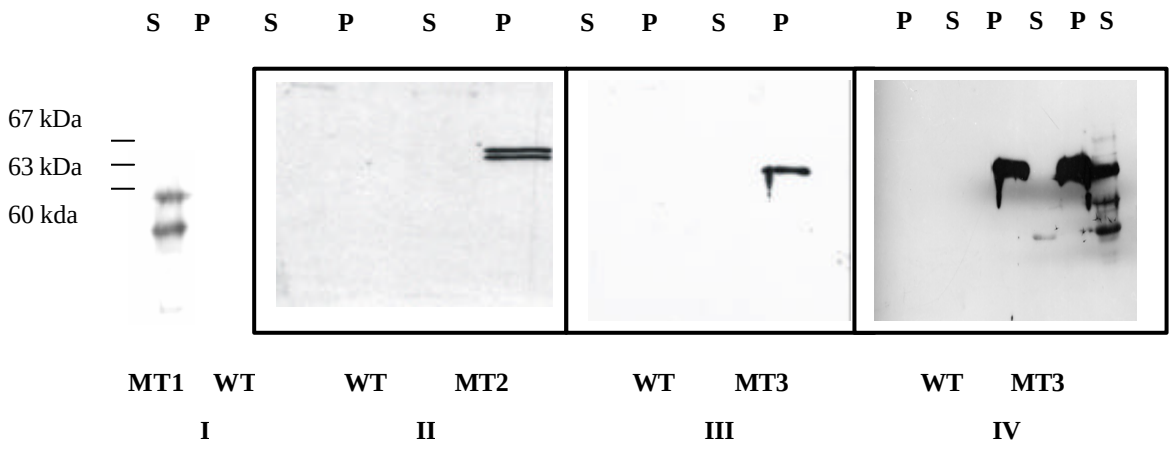


Figure 25A: Solubility of MT1-, 2-, 3-MMP proteins in transiently transfected COS cells.

Proteins from untransfected COS cells (WT), COS cells transfected with pcDNA3-human-MT1-MMP (MT1), COS cells transfected with pcDNA3-human-MT2-MMP (MT2) or COS cells transfected with pcDNA3-mouse-MT3-MMP (MT3) were extracted with Triton X-100 buffer (I-III) or RIPA buffer (IV), and probed with anti MT1-MMP mAb 113-5B7 antibodies (I), anti-HR-MT2 antibodies (II) or anti-HR-MT3 antibodies (III and IV), and appropriate secondary antibodies labeled with horseradish peroxidase (I-IV). S: Supernatant. P: Pellet.

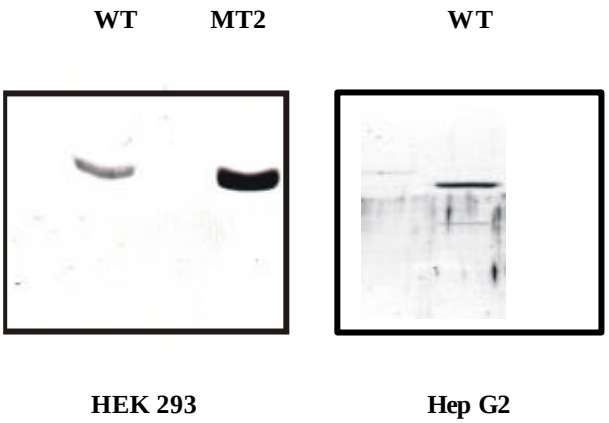


Figure 25B: Triton X-100 insolubility of human MT2-MMP in HEK 293 and HepG2 cells

Protein extracts from HEK 293 cells stably transfected with pcDNA3-MT2-MMP (MT2), untransfected HEK 293 cells (WT) or untransfected Hep G2 cells (WT) were probed with rabbit anti-HR-MT2 antibodies and goat anti rabbit antibodies labeled with horseradish peroxidase. S: Supernatant (i.e. Triton X-100 soluble extracts). P: Pellet (i.e. Triton X-100 insoluble extracts).

IV-2.1.2 Localization of MT1, 2-, 3-MMPs in COS cells

COS cells transiently transfected with human MT1-, human MT2- or mouse MT3-MMP-pcDNA3 plasmids were seeded on the multi-well slides and were probed with anti-MT1- or 2- or 3-MMP primary antibodies, respectively. Then the slides were analyzed by immuofluorescence microscopy (Figure 26A). MT1-MMP and MT3-MMP proteins were observed both on the plasma membrane (Figure 26A, I and VII) and in the intracellular (Figure 26A, II and VIII) of cells. In contrast, MT2-MMP was only detected intracellularly (Figure 26A, III-VI). It was easy to detect MT1-MMP proteins intracellularly (Figure 26A, II), but the signal from plasma membrane MT1-MMP protein was weak (Figure 26A, I). These results were reproduced at least five times independently.

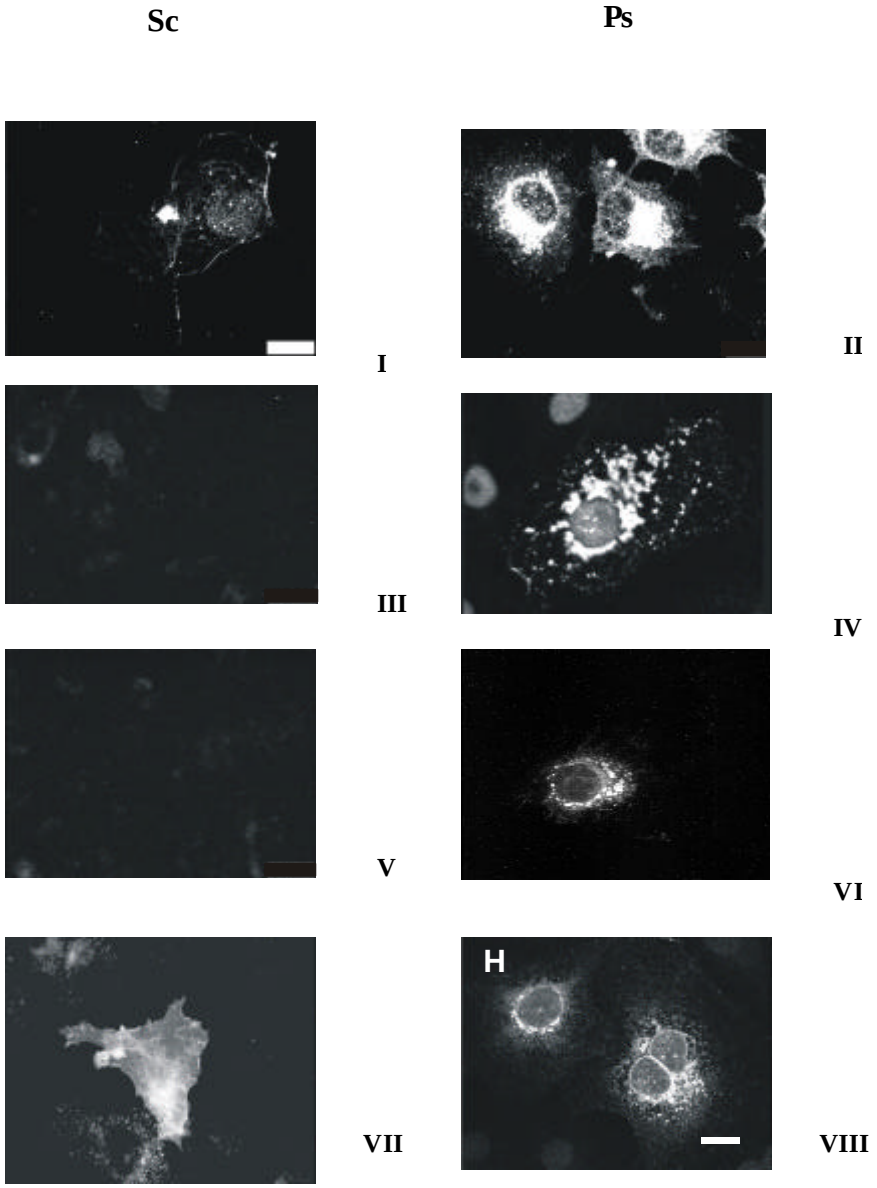


Figure 26A: Localization of MT1-3-MMPs in transiently transfected COS cells

COS cells transiently transfected with pcDNA3-human MT1-MMP plasmid (I and II), pcDNA3-human MT2-MMP plasmid (III and IV), pcDNA3-human MT2-MMP-flag plasmid (V and VI) or pcDNA3-mouse MT3-MMP plasmid (VII and VIII) were probed with anti-MT1 mAb 113-5B7 antibodies (I and II), anti-HD-MT2 antibodies (III and IV), anti-flag antibodies (V and VI) or anti-HR-MT3 antibodies (VII and VIII) and appropriate Cy2-conjugated secondary antibodies and examined by immuofluorescence microscopy. I, III, V and VII: surface cell staining (Sc). II, IV, VII and VIII: permeable staining (Ps).

The surface display of MT3-MMP (Figure 26A, VII) was confirmed by Western blot of plasma membrane extracts from COS cells transiently transfected with pcDNA3-MT3-MMP plasmid. MT3-MMP was detected in plasma membrane extracts of pcDNA3-MT3 transiently transfected cells (Figure 26B, lane 1), but not in other plasma membrane fractions of COS cells transiently transfected with pcDNA3 empty vector, pBI-G empty vector, or pBI-G-MT3-MMP plasmid (Figure 26B, lanes 2-4).

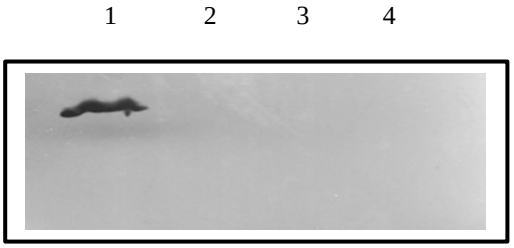


Figure 26B: Detection of MT3-MMP in membrane fractions of transiently transfected COS cells

Plasma membrane proteins were extracted from COS cells transfected with pcDNA3-MT3-MMP (lane 1), pcDNA3 empty vector (Lane 2), pBI-G empty vector (lane 3) or pBI-G-MT3-MMP plasmid (lane 4) and probed with rabbit anti anti-HR-MT3 antibodies (III) and goat anti rabbit antibodies labeled with horseradish peroxidase.

The location of MT2- MMP was also observed in untransfected HEK cells or HEK cells transfected by MT2-flag, in which a DYDDDDDEK flag was inserted at the end of human MT2-MMP (Appendix E and Figure 27). The immuofluorescence microscopy results of figure 27A-27B show that the expression of MT2-MMP proteins was detected on the plasma membrane of HEK cells by using two kinds of MT2-MMP antibodies (anti-MT2-MMP-HR specific recognized the hinge region and anti-MT2-MMP-HD recognized the hemopexin domain of MT2-MMP), respectively. In figure 27D, the location of MT2-MMP proteins on HEK cells was confirmed with anti-flag antibodies.

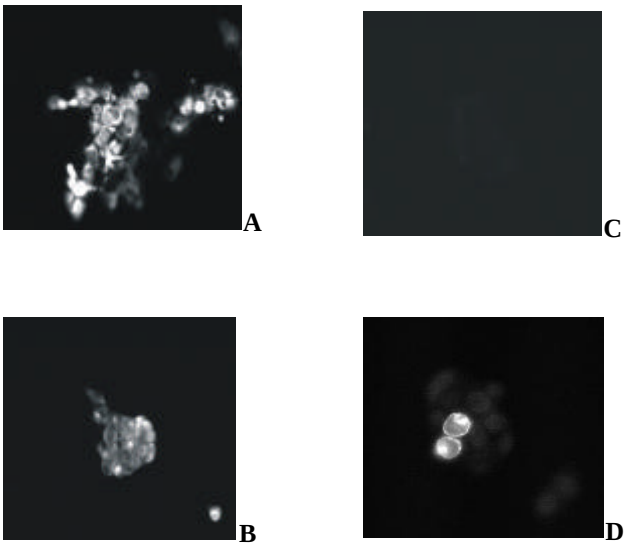


Figure 27: Localization of MT2-MMP in HEK 293 cells

Wild type HEK 293 cells (A, B, C) or HEK 293 cells transfected with pcDNA3-MT2-flag-MMP plasmid (D) were probed with anti-HR-MT2 antibodies (A), anti-HD-MT2 antibodies (B), pre-immune serum (C) or anti-flag antibodies (D) and appropriate cy2-conjugated secondary antibodies and examined by immuofluorescence microscopy.

IV-2.1.3 Biosynthesis and glycosylation assays of MT2-MMP

The biosynthesis of MT2-MMP was traced with ^{35}S labeled methionine in COS cells by pulse-chase experiment. The majority of MT2-MMP remained as Triton X-100 soluble protein shortly after pulse-labeling and then it distributed to the insoluble protein at later time points (Figure 28). After 90 min the majority of MT2-MMP was Triton X-100 insoluble. Interestingly, after treatment with Endo H to remove the high-mannose-type N-linked carbohydrate chains, the upper band was significantly reduced with comparison to untreated MT2-MMP proteins, suggesting that MT2-MMP was glycosylated in transfected COS cells, mostly possibility in Asn^{150} (Figure 29).

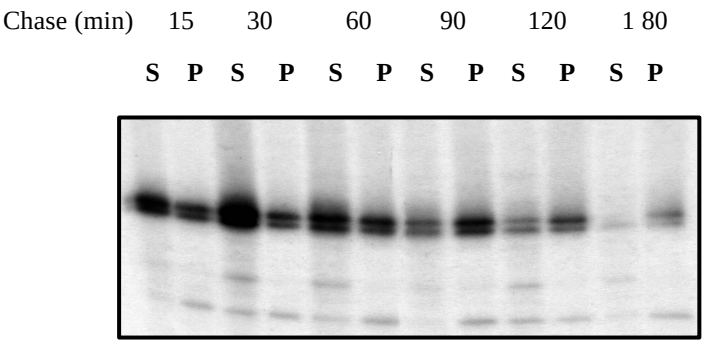


Figure 28: Metabolic labeling MT2-MMP

COS-7 cells transiently transfected with pcDNA3-human MT2-MMP were pulse labeled for 10 min and either directly lysed in Triton X-100 containing buffer or chased for the indicated times. MT2-MMP was immunoprecipitated from the cell pellet (p) and supernatant (s) with antibodies against the catalytic domain (anti-MT2-PCD) and visualized by autoradiography. This experiment was done in collaboration with Dr. Gatsios, Aachen.

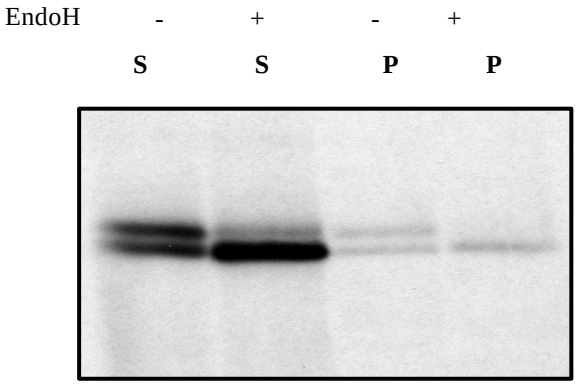


Figure 29: Endo H treatment

Transfected COS-7 cell were metabolically labeled for 15 min and immunoprecipitated with antibodies against the catalytic domain (anti-MT2-PCD). Supernatant (S) and pellet (P) were treated with or without EndoH. This experiment was done in collaboration with Dr. Gatsios, Aachen.

IV-2.2. Localization of chimeric MT2-MMP constructs

Chimeric constructs of MT2-MMP were generated according to 'Materials and Methods'. Like wild type human MT2-MMP (Figure 30A-30B), mutant MT2SD protein (Appendix E) substituting Pro183 and Glu185 with Ser183 and Asp185 in human MT2-MMP were detected intracellularly but not on the cell plasma membrane (Figure 30C-30D). Western blot analyses show that the mutant protein was successfully transfected and expressed in the COS cells. In

contrast, MT2xFcRII chimeric protein, in which the transmembrane and cytoplasmic domains of human MT2-MMP were replaced by the corresponding part of IgG Fc? receptor II (FcRII), was displayed on the cell plasma membrane (Figure 30E-30F). MT2xFcRII protein could be seen either on the central part of the cell surface (Figure 30F) or at the edge of cell surface (Figure 30E).

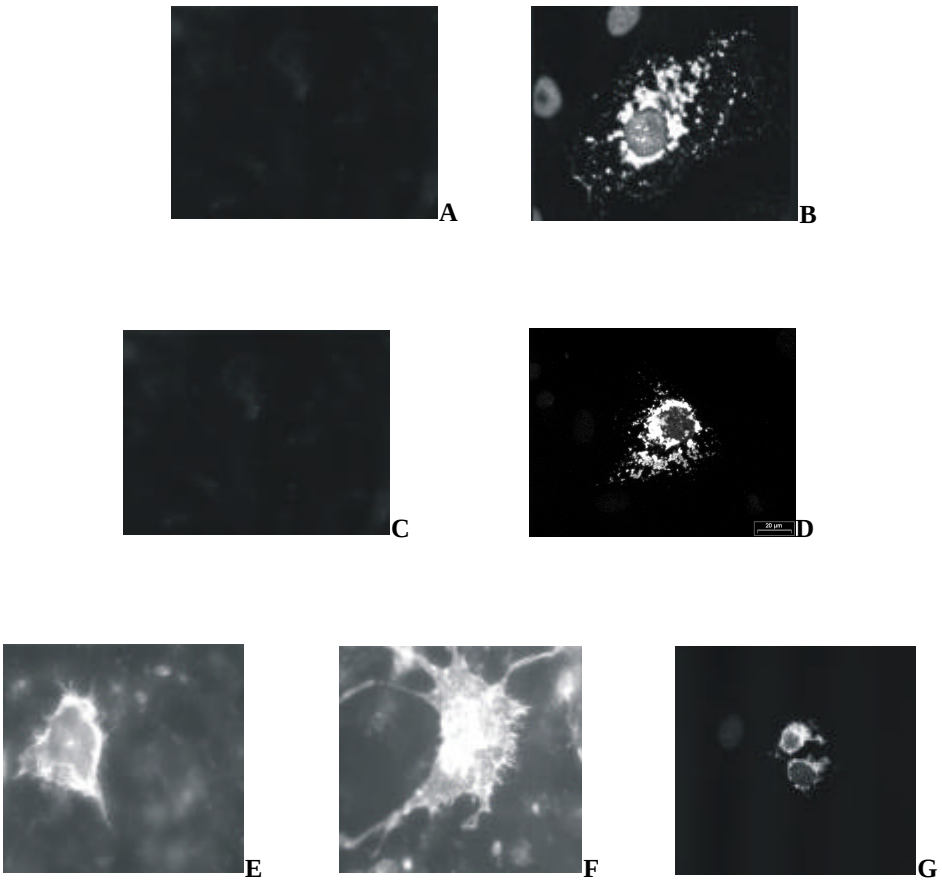


Figure 30: Localization of chimeric MT2-MMP constructs in transfected COS cells

COS cells transiently transfected with pcDNA3-MT2-MMP (A-B), a two-site mutant of MT2-MMP-pcDNA3 (C-D) or MT2xFcRII-pcDNA3 (E-G) were probed with rabbit anti-HR-MT2-MMP antibodies and Cy2-conjugated goat anti rabbit antibodies and examined by immuofluorescence microscopy. A, C, E and F: surface staining. B, D and G: intracellular staining in permeabilized cells.

IV-2.3 Activity assay of chimeric MT2-MMP constructs

It has been proven that COS-1 cells do not produce endogenous MT1-MMP by Northern blot analyses by several groups. Therefore, the COS-1 cells were transiently transfected with pcDNA3-MT2-MMP, pcDNA3-MT2 chimeric constructs (including MT2xFcRII, MTSD, FcRIIxMT2 and MT2F; see Figure 31A), empty pcDNA3 (as a mock) and pcDNA3-MT1-MMP (as a positive control) plasmids, respectively. After three days, old media were replaced by a serum-free DMEM containing pro-MMP-2, and then the media were collected and used for gelatin zymography (Figure 31B). FcRIIxMT2 is a reverse chimeric construct of MT2xFcRII (Figure 31A). Like the empty vector, the FcRIIxMT2 proteins did not show any activation of pro-MMP-2. The MT2SD, MT2-flag and wild type MT2-MMP displayed very weak activities to process proform MMP-2. In contrast, MT2xFcRII showed a strong activity to process the pro-MMP-2 at a level comparable to the human MT1-MMP. The Western blots proved that all these MT2 chimeric constructs were successfully expressed (Figure 31C, I-III) at comparable levels (Figure 31C, V). The MT2 chimeric constructs were only detected by antibodies against the hemopexin domain of MT2-MMP, but not by anti-MT2-MMP-PD antibodies (Figure 31C, IV) which specifically recognize the propeptide domain of MT2-MMP (Appendix C).

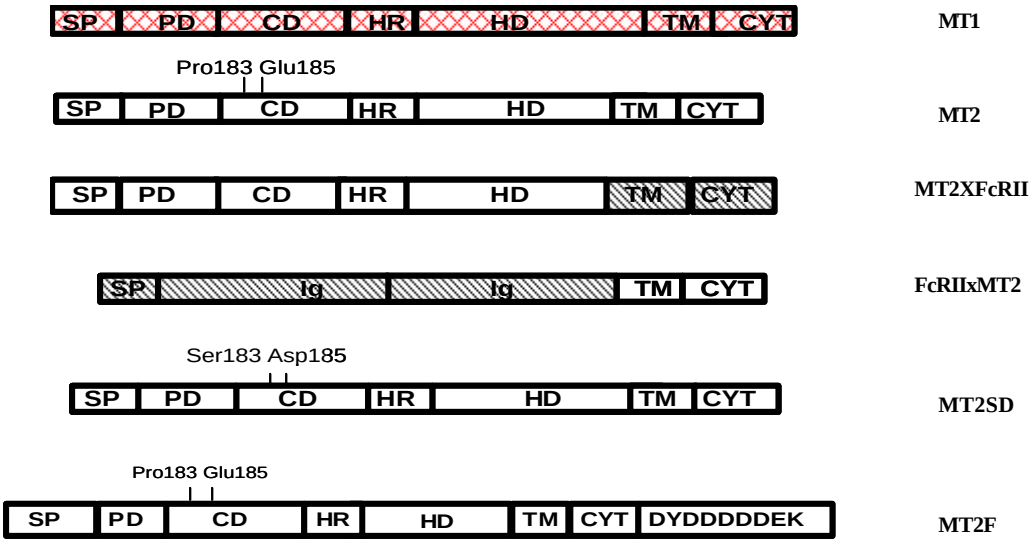


Figure 31A: Schematic diagram of chimeric MT2-MMP constructs

MT1-MMP (MT1). MT2-MMP (MT2).

FcRII. MT2F: The flag fragment is inserted in the C-terminal tail of MT2-MMP

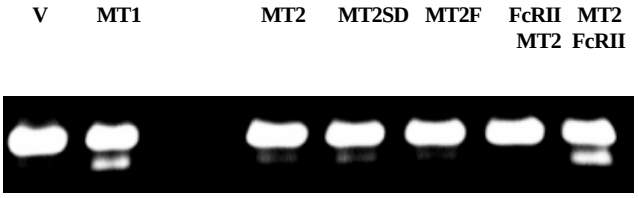


Figure 31B: Activity of chimeric MT2-MMP constructs in transfected COS cells

COS cells transiently transfected with pcDNA3-MT1-MMP (MT1), pcDNA3-MT2-MMP (MT2), the two-site MT2-MMP mutant (MT2SD)-pcDNA3, MT2-flag (MT2F)-pcDNA3, chimeric FcRIIxMT2-pcDNA3, chimeric MT2xFcRII-pcDNA3 or empty pcDNA3 vector were incubated with conditioned medium containing pro-MMP2 and the supernatants were used for gelatine zymography assay.

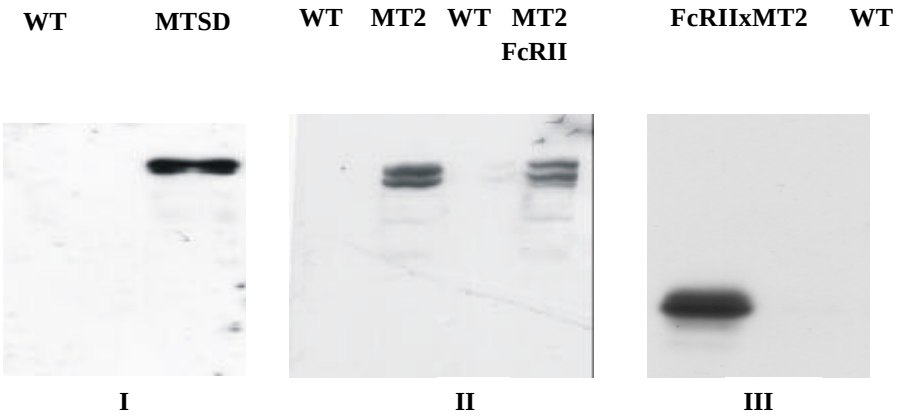


Figure 31C: Detection of chimeric MT2-MMP constructs in transfected COS cells by Western blot

COS cells were transiently transfected with pcDNA3- MT1-MMP (MT1), pcDNA3-MT2-MMP (MT2), two-site MT2-MMP mutant (MT2SD)-pcDNA3, MT2-flag-pcDNA3 (MT2F), chimeric FcRIIxMT2-pcDNA3, chimeric MT2xFcRII-pcDNA3 or empty pcDNA3 vector. Protein extracts from the above transfections were probed with rabbit anti-HD-MT2-MMP antibodies (I, II, V), anti-FcRII antibodies (III) or anti-PD-MT2-MMP antibodies (IV) and goat anti rabbit antibodies labeled with horseradish peroxidase.

IV-2.4 Localization of chimeric MT3-MMP constructs

A series of MT3 chimeric constructs were made (Appendix E). The immunofluorescence microscopy results clearly show that wild type MT3-MMP (Figure 32A and 32B) and all MT3 chimeric constructs including MT31- (Figure 32C and 32D), MT32- (Figure 32E and 32F) and MT13-MMP (Figure 32G and 32H) were expressed both intracellularly (Figure 32B, 32D, 32F and 32H) and on the plasma membrane (Figure 32A, 32C, 32E and 32G) in transiently transfected COS cells.

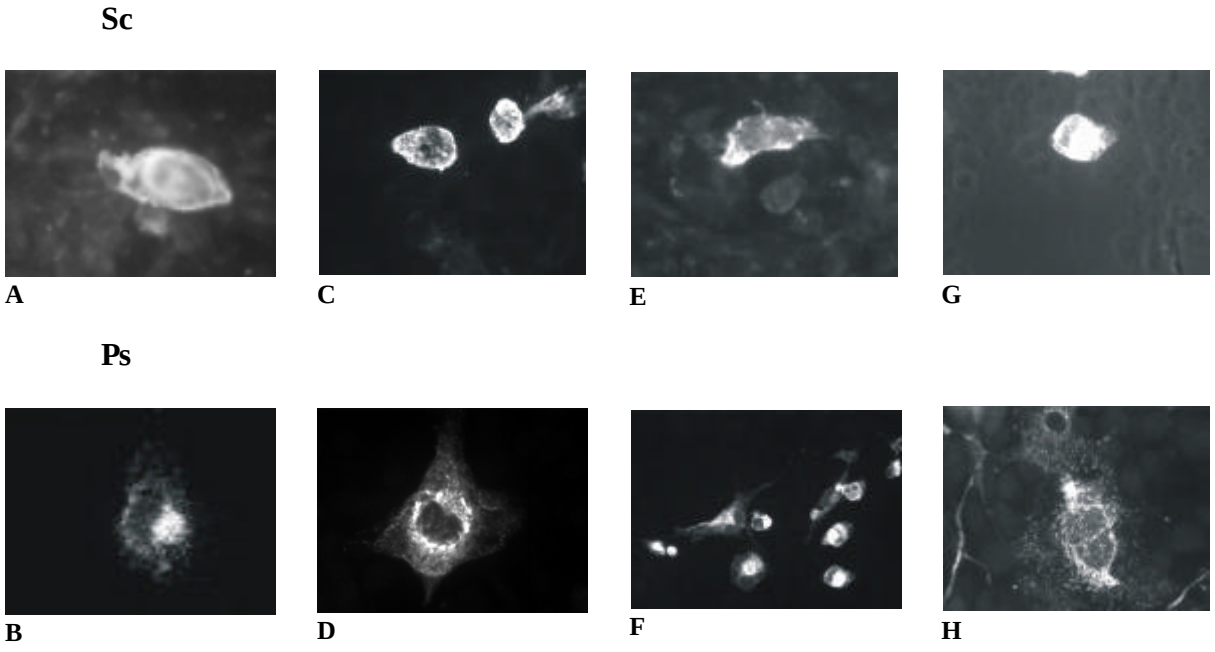


Figure 32: Localization of chimeric MT3-MMP constructs in COS cells

COS cells transiently transfected with pcDNA3-MT3-MMP (A-B), pcDNA3-MT31-MMP (C-D), pcDNA3-MT32-MMP (E-G) or pcDNA-MT13-MMP plasmids (G-H) were probed with rabbit anti-HR-MT3-MMP antibodies and Cy2-conjugated goat anti rabbit IgG antibodies and examined by immuofluorescence microscopy. Sc: surface staining (A, C, E and G). Ps: permeabilized cells (B, D, G and F).

IV-2.5 Activity assay of MT3-MMP constructs

The activities of MT3 chimeric constructs were tested by gelatin zymography (Figure 33B). Human MT1-MMP or chimeric construct MT13 showed very strong activity to process pro-MMP-2 to the mature form. In contrast, the mouse MT3-MMP, chimeric MT31 and chimeric MT32 showed very weak activities to process pro-MMP-2. The auto-activation of pro-MMP-2 by 4-aminophenyl mercuric acetate (APMA) clearly shows that the band of Mr 72 kDa (upper) was pro-MMP-2 and the band of Mr 65 kDa (down) was the active-form of MMP-2 (Figure 33C). The Western blots with antibodies against the hinge region of MT3-MMP showed the same level of expression in each transfection (Figure 33D-33F). However, after striping with β-mecaptoethanol, the same blots were not recognized by antibodies against propeptide domain of MT3-MMP (anti-MT3-MMP-PD). In order to verify that the different results in the Western blots with anti-HR-MT3 or anti-PD-MT3 were due to the different antibodies, two blots loaded with identical samples were probed by anti-hinge region or anti-propeptide domain MT3-MMP antibodies, respectively. Consistently, the same results were reproduced in three independent experiments (Figure 33G and 33H), suggesting that the MT3-MMP was completely processed into the active form in transfected COS cells.

To confirm whether mouse MT3-MMP processed the pro-MMP-2, COS cells were transiently co-transfected with pcDNA3-MT3-MMP and pcDNA3-pro-MMP-2. After two days post transfection, the medium was collected and tested by gelatine zymography (Figure 34A). No active form of MMP-2 was found in the cotransfected COS cells. On the other hand, the plasma membrane extracts of COS cells transiently transfected with pcDNA3-MT3-MMP, which were described in IV-2.1.2, were mixed with different sources of pro-MMP-2. These pro-MMP-2 sources included serum free DMEM medium from pcDNA3-pro-MMP-2 transfected COS cells, serum free Schneider's Drosophila medium from insect S2 cells transfected with pAc5.1/V5-His A,B,C-pro-MMP-2, and pro-MMP-2 purchased from Roche. After incubation, the pro-MMP-2 and the plasma membrane extracts were examined in the gelatin zymography. No difference was found between pro-MMP-2 alone and pro-MMP-2 mixed with MT3-MMP plasma membrane fractions (Figure 34B and 34C), suggesting that mouse MT3-MMP did not efficiently process pro-MMP-2 in COS cells.

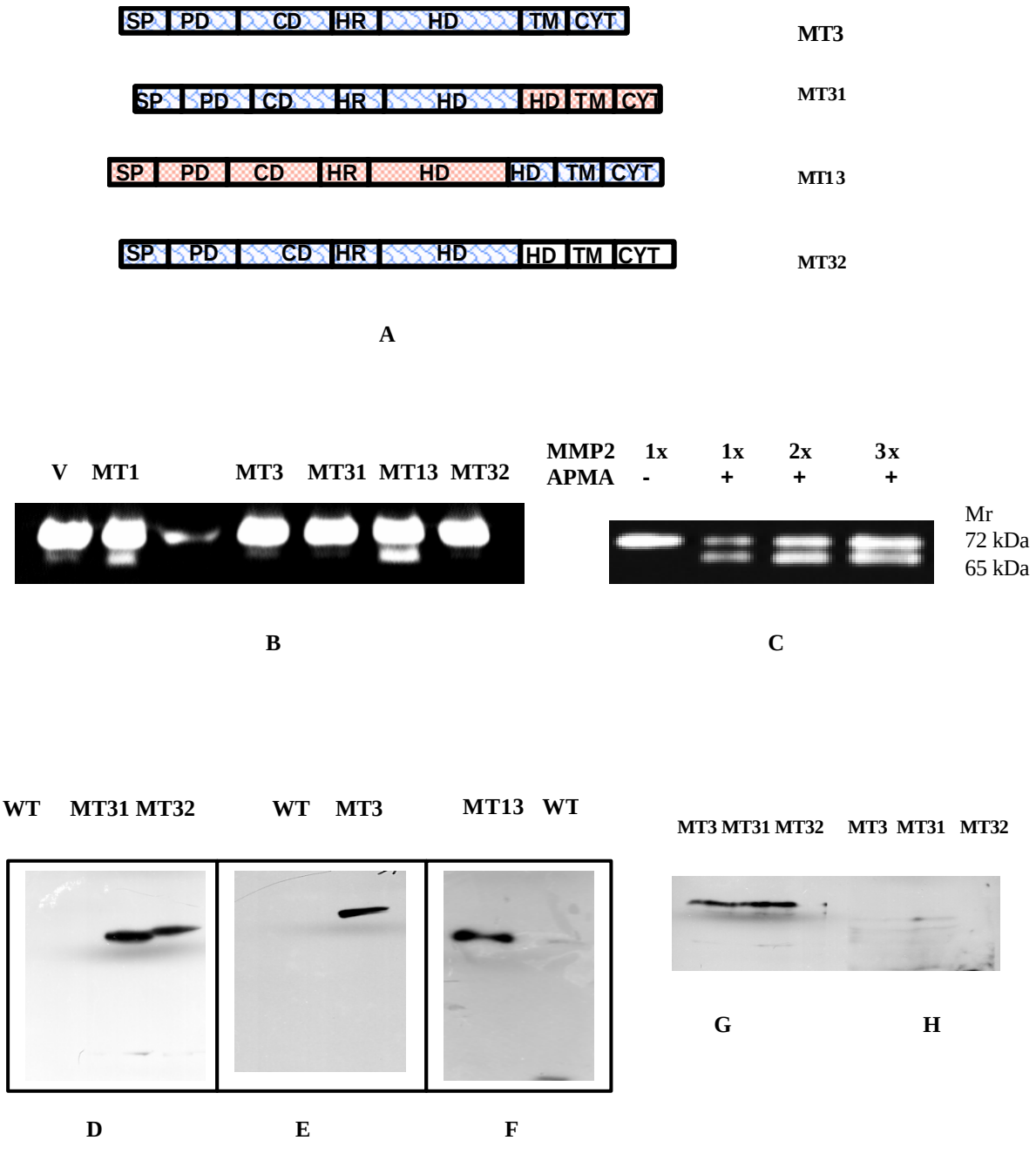


Figure 33: Activity of chimeric MT3-MMP constructs in transfected COS cells

COS cells were transiently transfected with pcDNA3-MT1-MMP (MT1), pcDNA3-MT3-MMP (MT3), chimeric MT31-MMP-pcDNA3, chimeric MT32-MMP-pcDNA3, chimeric MT13-MMP-pcDNA3 or empty pcDNA3 vector and incubated with conditioned medium containing pro-MMP2. The supernatants were analyzed by gelatine zymography (B). pro-MMP-2 was incubated with 4-aminophenyl mercuric acetate (APMA) as controls (C). Protein extracts from the above transfections were probed with rabbit anti-HR-MT3 (D, E, F and G) or anti-PD-MT3 antibodies (H) and goat anti rabbit IgG antibodies labeled with horseradish peroxidase. The schematic diagram of MT3-MMP chimeric constructs is shown in A.

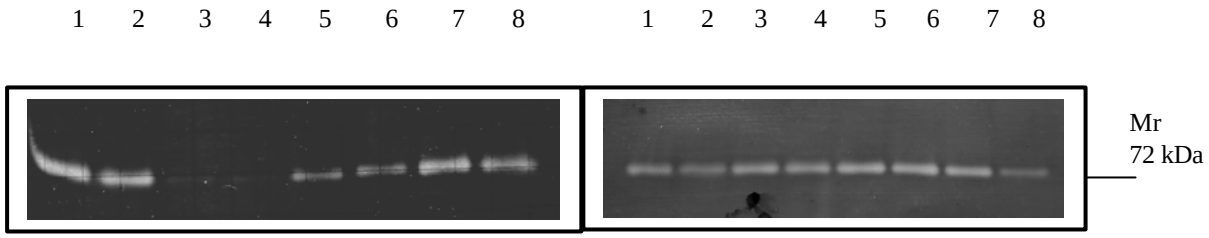


Figure 34A: Activity assay of MT3-MMP by gelatine zymography using cotransfected COS cells

Conditioned media from COS cells transiently transfected with pcDNA3-pro-MMP-2 plasmid (lanes 1-2), pcDNA3-MT3-MMP plasmid (lanes 3-4) or co-transfected with pcNDA3-MT3-MMP and pcDNA3-pro-MMP-2 plasmids (lanes 5-8) were analyzed by gelatine zymography assay.

Figure 34B: Activity assay of MT3-MMP by gelatine zymography using membrane fractions of pcDNA3-MT3-MMP transfected COS cells

Lanes 1-3: Conditioned media from COS cells transiently transfected with pcDNA3-pro-MMP-2 plasmid. Lanes 4-5: Conditioned media from the COS cells transiently transfected with pcDNA3-pro-MMP-2 plasmid was treated with Con A. Lanes 6-8: Conditioned media from COS cells transiently transfected with pcDNA3-pro-MMP-2 plasmid was treated with membrane extracts of COS cells transfected with pcDNA3-MT3-MMP. Lanes 3, 4, 5, 6 and 7: 24 h conditioned media. Lanes 2 and 8: 48 h conditioned media. Lane 1: 72 h conditioned medium.

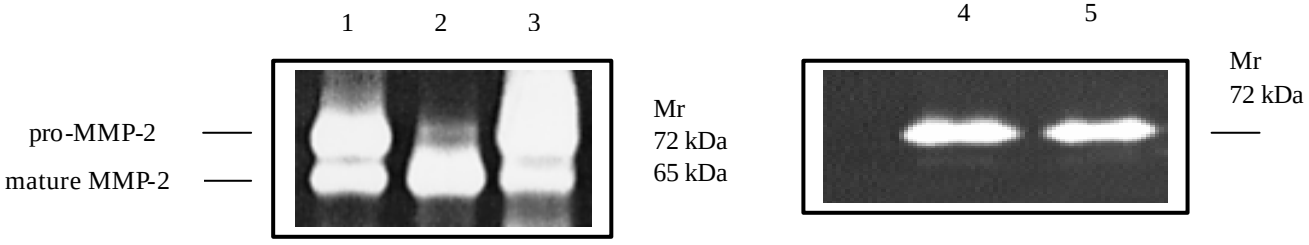


Figure 34C: Activity assay of MT3-MMP by gelatine zymography using different sources of pro-MMP-2

Lane 1: Commercial pro-MMP-2 (c-pro-MMP-2). Lane 2: c-pro-MMP-2 was incubated with APAMA. Lane 3: c-pro-MMP-2 was incubated with membrane extracts of COS cells transiently transfected with pcDNA3-MT3-MMP plasmid. Lane 4: Conditioned medium from insect cells transfected with pAc5.1/V5-His A, B, C-pro-MMP-2. Lane 5: Conditioned medium from insect cells transfected with pAc5.1/V5-His A, B, C-pro-MMP-2 was incubated with membrane extracts of COS cells transiently transfected with pcDNA3-MT3-MMP plasmid.

Localization, Triton X-100 solubility and activity of chimeric constructs of MT2-MMP and MT3-MMP are listed in the following tables (Table 10A and 10B).

Table 10A: Localization, Triton solubility and activity of MT2 chimeric constructs

Type	MT2-MMP	MT2xFcRII	MT2SD	MT2-flag	FcRIIxMT2
Localization Surface (+)	-	+	-	-	-
Triton X-100 solubility Insoluble (-)	-	-	-	-	+
Activity to process pro-form MMP-2	-	+	-	-	-

Table 10B: Localization, Triton solubility and activity of MT3 chimeric constructs

Type	MT3-MMP	MT3xMT1	MT3xMT2	MT1xMT3
Localization	+	+	+	+
Triton X-100 solubility	-	-	-	
Activity of process pro-form MMP-2	-	-	-	+

In summary 2:

1. Unlike human MT1-MMP which is Triton X-100 soluble, mouse MT3-MMP and human MT2-MMP expressed in COS cells are Triton X-100 insoluble.

2. Mouse MT3-MMP and human MT1-MMP was localized on the cell plasma membrane. In contrast, human MT2-MMP was not detectable on the cell surface but only intracellular.

3. Human MT2-MMP did not activate the pro-MMP-2 in the COS cells because its cytoplasmic and transmembrane domains prevented the transport of the enzyme to the cell surface.

4. Mouse MT3-MMP could not efficiently activate pro-MMP-2. The ectodomain of mouse MT3-MMP may play an important role in this event.

V. Discussion

V-1. Expression and localization of MT3-MMP in vivo

V-1.1. Sequence comparison of mouse MT3-MMP with MT3-MMP from other species

The deduced amino acid sequence of our mouse MT3-MMP has 99.5% homology to mouse MT3-MMP currently in Genbank (AB021228), 99.3% homologous to rat MT3-MMP (D85509), 97.7% homologous to human brain MT3-MMP (D85511) or human placenta MT3-MMP (NM005941). Comparing to our mouse MT3-MMP, three amino acid residues were different in the Genbank mouse MT3-MMP, two of which are in the signal peptide and one in the catalytic domain. A total of four amino acids were replaced in the Genbank rat MT3-MMP compared to our mouse MT3-MMP i.e. one amino acid is in the signal peptide, one in the propeptide and two in the hinge region. A total of fourteen residues were substituted in the Genbank human MT3-MMP compared to our mouse MT3-MMP, four of which are in the signal peptide, one in the propeptide, two in the catalytic domain, three in the hinge region, two in the hemopexin domain and two in the linker region prior to the transmembrane domain. Notably, the difference and substitution in the catalytic domain of our mouse MT3-MMP do not occur at the site E^{247} of the zinc binding motif, which is proved to be a key residue to maintain the activity of MT-MMPs since the replacement of E^{240} by A^{240} in MT1-MMP completely abolishes the processing of the pro-MMP-2 by MT1-MMP (Yana et al., 2000). Most interestingly, there were two putative phosphoryl sites in the cytoplasmic tail of MT3-MMP ($VY^{582}T^{583}VFQFKRKG\underline{\mathbf{T}}^{592}PRHILY^{598}CKR\underline{\mathbf{S}}^{602}MQEWV^{607}$). Among of them, one threonine (T^{598}) and one serine (S^{602}) are in cytoplasmic domain, which can be potentially phosphorylated by threonine or serine kinases (NetPhos 2.0 Server, Technical University of Denmark).

V-1.2. Establishment and analyses of transgenic mice

V-1.2.1. Examination of the pBI-G-MT3-MMP construct in tet regulatory cell lines

The construct of pBI-G-MT3-MMP and its response to tet-controlled transactivator (tTA) were examined in tet-off stable cell lines by different approaches. In the absence of tetracycline, tet off cells transfected with pBI-G-MT3-MMP displayed β-galactosidase activity in 20-25% of the cells. In contrast, no positive cells were observed with tetracycline treatment, indicating that the Lac Z reporter gene was tightly regulated in the tet system and the pBI-G-MT3-MMP plasmid worked well in the Lac Z orientation. Expression and regulation of MT3-MMP transgene gene were assayed with specific primers (e.g. rtMT3) by RT-PCR and specific antibodies (e.g. anti-HR-MT3) by Western-blot and immuno-

fluorescence microscopy. All data demonstrated that the construction of pBIG-MT3-MMP was successful and was strictly controlled by the effectors, tetracycline or doxycycline.

V-1.2.2. Screening of transgenes in mice

It is a good idea to prepare a pair of primers and test them in mammalian cells before they are used for transgenic animals. However, since an animal model is much more complex than mammalian cell lines, a good test in cell line does not mean the primers really work in the animal. For example, the pBI-G-MT3 primer designed for transgene selection displayed weak and many unspecific signals in mice although it had been proven to work very well in COS-7 tet off transiently transfected cells. Thus, designing several sets of primers in advance and directly testing them in the same background of mice is necessary for transgenic animal studies.

Genomic PCR with MT3-MMP and rtTA specific primers only demonstrated whether the mice carried pBI-G-MT3-MMP or pTet-on regulatory plasmid, but it could not show whether the transgenes (MT3-MMP and rtTA) were intact or whether the integrated transgenes were able to be efficiently expressed. Furthermore, because the PCR technique is very sensitive, it is easy to produce false positive results due to any sources of contaminations. To make sure that selection of transgenic mice by our genomic PCR was correct and reliable fibroblasts of ear from double transgenic mice were cultivated and incubated in doxycycline medium. The fibroblasts from CMV promoter-regulated double transgenic mice showed blue staining (10-15%) (Figure 7), suggesting that the genomic PCR selection was successful. No positive cells from GFAP promoter-regulated double transgenic mice were detected in assay of β-galactosidase activity. Thus, the expression of the tet-controlled transactivator was also tightly regulated by relevant promoters.

V-1.2.3. CMV and GFAP promoters for MT3-MMP expression

The choice of promoters is essential for expression of the transgenes in specific-selected or broad tissues. It has been known that glial fibrillary acidic protein (GFAP) is expressed mainly in mature astroglial cells but can also be detected in peripheral nervous system Schwann cells and enteric glia of the autonomous nervous system (Nolte et al., 2001). Thus, the GFAP has become the most commonly used marker for astrocytes. A transgenic mouse line, which expressed the reverse tet-controlled transactivator (rtTA) under the control of the human GFAP promoter, was developed by Kirchhoff and his colleagues. Through breeding

MT3-MMP response transgenic mice with GFAP promoter controlled rtTA mice, double transgenic mice containing both MT3-MMP and rtTA transgenes are expected to express transgenic MT3-MMP (i.e. Tg-MT3-MMP) protein in astrocytes although it is reported that a marker protein (EGFP) does not completely overlap with the GFAP protein in immunostaining (Nolte et al., 2001). By regulation of tetracycline, the physiological or pathological functions of MT3-MMP in astroglial cells of brain can be observed in the GFAP double transgenic mice. In contrast, the CMV promoter can mediate transgene to widely express in tissues of animal. Indeed, the rtTA was expressed in kidney, heart, thymus, pancreas, testis, ear and lung in CR4 single regulatory transgenic mice (containing pTet-on vector) or double transgenic mice (containing both pTet-on and pBI-G-MT3-MMP vectors) in our experiments. This finding is consistent with the results from other groups. Kistner and his colleagues (1996) detected expression of transgenes in kidney, heart, thymus, pancreas, muscle, tongue, skin and stomach. These results confirm that under the control of the CMV promoter, the transactivator rtTA is expressed in most tissues and organs in CR4 single or double transgenic mice (Prof. Bujard, Heidelberg).

It is reported that the CMV promoter as a broad-spectrum promoter is found to mediate the expression of rtTA in most organs (thigh muscle, tongue, kidney, pancreas, stomach, gut, heart and thymus) but does not work well in some organs such as liver and brain due to an activity spectrum of transactivator (Kistner et al., 1996; Mansuy et al., 1998). To achieve expression of rtTA in certain organs, a tissue specific promoter such as CaMKIIa was used successfully to express the transactivator protein in mouse forebrain (Mansuy et al., 1998). P_{LAP} is used in the liver (Kistner et al., 1996).

Surprisingly, after crossing of CR4 (rtTA) mice with pBIG-MT3-MMP mice (including P_{minCMV} promoter and Lac Z reporter gene), MT3-MMP was expressed in cerebrum, cerebellum and spinal cord by RT- PCR with Dox-HCl induction in our experiments. In order to confirm whether the expression under the control of Pmin CMV was real in brain, ß-galactosidase assay of cryosections was performed in Dox-HCl induced CR4 double transgenic mice. The blue staining was found in the brain of four transgenic mice, which have the same expression pattern as shown in the tongue, thigh muscle and in kidney. However, the staining pattern was completely different from that reported by Mansuy and his colleagues (1998). By using the CaMKIIa promoter, the Lac Z reporter gene was extensively expressed in the brain (Mansuy et al., 1998).

The pBI-G vector contains a minimal promoter sequence derived from human cytomegalovirus immediate-early promoter (minPhCMV IE). Tet-on vector contains rtTA unit which is also controlled by a promoter of the human cytomegalovirus immediate early genes (PhCMV IE) (Kistner et al. 1996; Tet systems user manual, Clontech). Obvious difference between the minimal CMV promoter in Ptet-1 and the normal CMV in rtTA unit is that the former keeps in silence without binding of rtTA in the absence of effector substances (Dox-HCl). In contrast, the latter always stays in an active state. However, both of them come from one cognate human CMV IE, therefore they should have similar tissue preference or activity spectrum. They can activate and regulate the transgenes in most of cell types or tissues as long as they are integrated in a proper chromosomal locus. This hypothesis was supported by our experiment results, in which the expression of rtTA was clearly detected in most organs of transgenic mice, particularly in cerebrum, cerebellum and spinal cord with RT-PCR. A possibility may exist that the expression of Lac Z or MT3-MMP in brain is due to an un-regulated integration in the response transgenic mouse chromosome. However, the rtTA was constitutively expressed in brain of CR4 double transgenic mice, and the expression of reporter gene Lac Z was doxycycline-dependent, suggesting that the expression of target genes in brain was tightly regulated. Moreover, the double transgenic mouse lines are stably transmitting both transgenes (rtTA and MT3-MMP) in reproduction and development and are now close to become the homozygotes.

V-1.2.4. Induction of transgenes expression

So far, several methods are in use to offer the effector (Dox-HCl) in tet regulatory system animals including oral feeding (water or solid food), injection (intramuscular or intradermic or intravenous or abdomen) and implant subcutaneously by pellet. A limitation for watering Dox-HCl is that a full activation of response plasmid via rtTA is achieved only at 1-2 µg/ml of Dox-HCl. This concentration cannot be readily obtained in some organs, e.g., in the brain of mice. To obtain proper concentration of Dox-HCl in brain, 2-4 mg/ml Dox-HCl were applied in our induction protocol. RT-PCR results (Figure 9) showed that the rtTA was certainly expressed in the cerebrum, cerebellum and spinal cord. Recently, a new mutant rtTA was generated by Urlinger and his colleagues (2000), in which the sensitivity of rtTA to Dox-HCl was increased to 10 folds. When the Dox-HCl was 0.1µg/ml, the transgene is fully activated. On the other hand, selection of regulatory vectors (pTet-on or pTet off) depends on several factors. If the over-expressed interest protein is very harmful to the mice, rtTA (pTet-on) vector is the first choice because it is supposed to remain in silence without effector (Dox-

HCl). Otherwise, tTA (pTet off) vector is preferred because it is easy to induce the expression of transgene and the phenotype of physiology or pathology of transgenic mice is promptly observed in a constitute condition (without feeding the Dox-HCl), thereby saving the time and money.

V-1.2.5. Discrimination of transgenic MT3-MMP from endogenous MT3-MMP

To add an epitope peptide (flag) cDNA at the end of interest gene is a general strategy for detecting and tracing expression of the interest gene in cell biology and transgenic animal. C-terminus or N-terminus of target protein is optimal site for inserting the epitope flag. In the construction of pBIG-MT3-MMP, the case became more complex since the C-terminal amino acid (valine^{582}) in the cytoplasmic domain of MT1-MMP is very crucial to its activation and plasma membrane transportation. A mutation of Val^{582} to Gly^{582} in MT1-MMP is retained in the endoplasmic reticulum (Urena et al., 1999). To avoid possible damage or loss of the crucial Val^{607} in transgenic MT3-MMP (i.e Tg-MT3-MMP), the addition of flag or tag peptides in the C-terminus of MT3-MMP was not adopted. With regard to that the MT-MMPs are zymogens, which may be activated intracellularly via the cleavage of its propeptide domain (Yana et al., 2000), insertion of a flag or tag in the N-terminal propeptide domain in Tg-MT3-MMP was obviously not accepted. Thus, no flag or tag or foreign peptides cDNA was added in the pBI-G-MT3-MMP construct. This caused a potential difficulty at the beginning i.e. how to discriminate the Tg-MT3-MMP from the endogenous one in the transgenic mouse? Certainly, RT-PCR is a suitable approach depending on the specific designed rtMT3 primers, one of which (primer) binds the DNA corresponding to the C-terminal hemopexin domain of MT3-MMP and another specifically binds the pBIG plasmid. Indeed, the RT-PCR by using RNA from negative control mice did not show any signal with this pair of primers. In contrast, the double transgenic mice treated by Dox-HCl significantly showed the expected products of amplification. However, it is very difficult to judge the Tg-MT3-MMP proteins from the endogenous ones with specific antibodies. A new idea is to insert the flag epitope at the downstream of the furin cleavage motif in MT-MMPs as it does in MT6-MMP or MT1-MMP (Kojima et al., 2000; Yana et al., 2000). The targeting proteins will be detected by both Western blot and immunofluorescence microscopy. Remarkably, the flag does not affect the activity of MT6-MMP or MT1-MMP to process pro-MMP-2. The insertion of flag epitope at the downstream of the furin cleavage motif is a good idea for future establishment of MT-MMPs overexpressing transgenic animal model.

V-1.2.6. Distribution of transgenes

The rat MT3-MMP is reported to be detected in the brain, lung, and liver but not in heart, skeletal muscle, kidney, and testis by Northern blot analysis (Shofuda et al. 1997). The human MT3-MMP has been reported to be found in the brain, placenta, maybe in the heart, but not in lung, liver, skeletal muscle, kidney and pancreas with Northern blot analysis (Takino et al. 1995). Here, endogenous MT3-MMP was detected in brain, spinal cord, heart, kidney, lung, testis and thymus in a $MT3^{-}/rtTA^{+}$ mouse that only carried the regulatory transactivator (rtTA) and had a similar CD1xNMR1 mouse background.

The expression of Tg-MT3-MMP was found in heart, kidney, tongue, lung, thymus, pancreas, brain, spinal cord, testis, stomach, colon, skin and connective tissue in the CR4 double transgenic mice treated with Dox-HCl. The reporter Lac Z gene was expressed in heart, kidney, muscle, tongue and occasionally in brain in our experiments. Consistently, the β-galactosidase activity is reported to be expressed in thigh muscle, tongue, and embryo including choroid plexus, nasal region, pituitary, pancreas, thymus and neck by other group in the CMV promoter-controlled transgenic mice (Furth et al., 1994). Furth and his colleagues (1994) also found the expression of another reporter gene (Luc) in pancreas, kidney, stomach, thigh muscle, thymus, heart, and tongue. A high luciferase activity was detected in kidney, heart, tongue, thigh muscle, pancreas, thymus, skin, stomach, duodenum and colon using the tet regulatory system (Furth et al., 1994; Kistner et al., 1996).

V-1.2.7. Expressions of transgenes are doxycycline- and time-dependent

Three CR4x3001 mice (No.8, 10, 50) fed with doxycycline for one week at 2 mg/ml did not show β-galactosidase activity in any of the tested organs. However, the Lac Z reporter gene was expressed in tongue, muscle, heart and kidney of two CR4x3001 mice (No.3 and No.66) which were watered for two weeks at the same concentration of Dox-HCl. Furthermore, not only in the above tissues but also in the brain, the positive blue staining cells were seen in four CR4x3001 mice (No.13, .35, .36, and No.43) that were induced for three weeks with the same amount of doxycycline.

Tg-MT3-MMP could not be detected by RT-PCR in cerebrum and cerebellum in CR4x3001 mice that were watered at 2 mg/ml Dox-HCl for 1 week, but was found to be expressed in the mice that were fed with 2 mg/ml of Dox-HCl for two weeks. When the doxycycline was 4 mg/ml, the Tg-MT3-MMP in the brain displayed an increased expression not only in the CR4

double transgenic mice, but also in the GFAP double transgenic mice (Figure 12). A CR4x3001.86 mouse fed with doxcycline for the whole life presented stronger expression of Tg-MT3-MMP and more blue staining cells than CR4x3001.70 that was watered with Dox-HCl for 2 months (data not shown). Taken together, all these data show that the expression of both transgenes is doxycycline-dependent and is regulated by the effector (doxycyclin) in a dose and time dependent manner.

V-1.2.8. MT3-MMP was detected by Western blot in vivo

Since the hinge region is localized between the catalytic domain and the C-terminal hemopexin-like domain of MT-MMPs, the anti-hinge antibodies can detect the full-length proenzyme, active enzyme and the inactive form lacking the catalytic domain. In kidney, the negative control mice only displayed a 50-52 kDa form of MT3-MMP. In contrast, in the Dox-HCl-induced CR4x3001 double transgenic mice two forms of MT3-MMP were detected with Mr 50-52 kDa similar to the control mice, and 63 kDa. The latter corresponds to the active form of MT3-MMP. The former is likely to be an autoproteolytic product that is often seen in the human Bowes melanoma cells and fibrosarcoma cells (HT1080) (Rozanov et al, 2001; Toth et al., 2002). It has also been demonstrated that a 45 kDa truncation product is present in HT1080 or transfected COS-1 cells by MT1-MMP due to autoproteolytic cleavage of mature 60 kDa enzyme (Lehti et al., 1998; Stanton et al., 1998; Toth et al., 2002). However, Yana et al. (2000) believe that the inactive 45 kDa product of MT1-MMP from both COS-1 and HT1080 cells might be caused by other mechanisms rather than autocatalytic degradation. Anyway, it has been widely accepted that the shedding of membrane proteins or membrane-associated proteins is an important regulatory mechanism to either up-regulate or down-regulate cellular functions by releasing membrane-bound growth factors or removing ectodomains of adhesion molecules or receptors.

MT3-MMP protein was not detected in testis or brain in either negative control mice or double transgenic mice treated by Dox-HCl. According to our current knowledge, most methods for MT1-MMP extraction use Triton X-100 or Triton X-114 Tris-HCl buffers (pH 7.5) (Itoh et al, 1997; Yana et al 2000). However, this buffer did not efficiently dissolve the mouse MT3-MMP and human MT2-MMP from transiently transfected COS or from HEK cells. Thus, it is plausible that the extracts from various organs in mice may contain few MT3-MMP proteins due to an insolubility of the mouse MT3-MMP. To solve this problem, a 2.5-3.5% SDS Tris-HCl (pH 7.5) buffer was used to dissolve the protein extracts, but similar

results were found in Western blot, suggesting that most of the MT3-MMP protein remained insoluble in the extracts of testis and brain.

Membrane proteins of kidney, testis or brain were extracted from negative control mice or Dox-HCl-induced double transgenic mice and analyzed by gelatin zymography. No significant activity was detected.

V-1.2.9. SMC and MT3-MMP

It has been reported that cultured rat vascular smooth muscle cells (SMCs) express MT1-MMP, MT2-MMP and MT3-MMP by RT-PCR or Northern blot analyses (Shofuda et al., 1997). Furthermore MT1-MMP and MT3-MMP mRNAs are detected in intimal dedifferentiated SMCs but not medial SMCs in rat balloon-injured carotid arteries (Shofuda et al., 1998).

a-smooth muscle actin as a marker protein of SMCs is detected in medial SMC of aorta, intestinal muscularis or muscularis mucosae of intestinal villi, small blood vessels of testis interstitium (Skalli et al., 1986) and peritubular interstitium of kidney in rat (Barnes et al., 1999). Thus, cryosections obtained from day 12 and day 14.5 embryos, and from heart, gut, stomach, kidney, testis and liver in ages of two-weeks, two-months and six-months in both negative control mice and Dox-HCl-treated double transgenic mice were used for double immunofluoresence staining with cy3-conjugated mouse anti-smooth muscle a actin antibodies and anti-HR-MT3 antibodies/Cy2-conjugated goat anti rabbit IgG antibodies. The signals from MT3-MMP and SMC a-actin staining did not show colocalization in all above organs. With regard to the results that MT3-MMP and MT1-MMP are undetectable with Western blot in rat SMCs (Shofuda et al, 2001) and no MT3-MMP is found in the SMCs in normal rat carotid arteries (Shofuda et al., 1998), it suggests the limitation expression of MT3-MMP in vascular SMCs under normal physiological condition.

It is well known that vascular SMCs can migrate and proliferate under certain pathological conditions such as intimal hyperplasia after arterial injury, vascular grafting and atherosclerosis, and these processes require degradation of vascular basement membrane and ECM surrounding the vascular cells (Kato et al., 2000). It is plausible that MT3-MMP may be highly expressed in SMCs when the vascular cells are in pathological condition. Indeed, recent evidence proves that abundant MT3-MMP expression is observed in SMCs and

macrophage in atherosclerotic arteries (Uzui et al., 2002). Importantly, tumor necrosis factor-α or platelet-derived growth factor may be involved in this MT3-MMP up-regulation process (Uzui et al., 2002; Shofuda et al., 1998).

V-1.2.10. Homozygote and mosaic expression of MT3-MMP

Usually hemizygous mice are enough for expression analyses in overexpressing transgenic animals. However, in some situations, having homozygotes is still essential. For instance, when the level of transgene expression is near threshold of detection, the amount of transgene expression can increase by achieving homozygosity. To check for homozygotes, the transgenic mouse can be crossed with a wild type animal. A 100% inheritance in offspring will be obtained. The failure to transmit the transgene to all offspring would mean that the transgenic mouse is not homozygous. Another possibility is that the transgene locus may be lost during germ cell development.

Ideally, integration of the gene of interest occurs at a single site in the genome during the one-cell stage of embryogenesis. In practice, integration may occur at several different sites within the genome. These sites may be localized closely on the same chromosome or on completely different chromosomes. More complicatedly, the integration process may occur later than the one-cell stage of development, resulting in the generation of a genetically mosaic animal. Assuming that the transgene is not deleted or lost during gametogenesis that induces transmission ration distortion, and integration of the gene of interest occurs at a single site in the one-cell stage, half of the F1 offspring should inherit the transgene. With multiple integration sites, the inheritance of a transgene is greater than 50% in the F1 generation. A mosaic founder animal with a single integration site will transmit the transgene to less than 50% of its offspring, and the exact percentage will be dictated by the stage at which integration occurs and the contribution of the recipient blastomere to the formation of germ cells during development.

The inheritances of F1 in both responsive transgenic mouse lines were less than 50%, probably meaning that the transgenic mice were mosaic. The β-galactosidase analysis of fibroblasts derived from CR4 double transgenic mice further indicated that the integration of the transgenes might have occurred later than the one-cell stage of development when the founders of transgenic mouse were generated. A genetically mosaic mouse will limit the studies of the functions of transgenes. For example, the effect of the mosaic transgenic mouse

will be weaker than that of a typical transgenic mouse. Furthermore, the responsibility to the transactivator (tTA or rtTA) will be discounted since only a few cells in certain organs are really regulated by tet-controlled transactivator under induction of Dox-HCl.

V-1.2.11. Leakage in tet system

Tg-MT3-MMP was expressed in testis in single response transgenic mice and CR4 double transgenic mice without Dox-HCl treatment because of 'leakage' expression (Figure 14A). The 'leakage' expression of transgene is often reported and the extent is various due to an integration site and a copy number of transgene in chromosome of host animal. For example, if the enhancer element is too close the responsive unit (tTA/rtTA), there may cause elevated background. The high background or 'leakage' expression extremely limits its application in tet system. Two ways are usually used to overcome this deficiency. One can establish many transgenic mouse lines, where a low background and high response line will be selected for further research. It has been counted that around 5-15% of integration may occur at silent loci with no basal activity (Freundlieb et al., 1999), meaning that at least 7-20 founders are required in the beginning in order to obtain one ideal (no basal activity) mouse. Furthermore, the prokaryotic portion of rtTA in tet system may contribute to the failure of expressing a stable rtTA in some cell lines or organs. For example, rtTA exhibits some residual affinity to tetO in the absence of Dox-HCl, which also results in some intrinsic background. Thus, the second approach is to modify the existing system by using a repression in tet system or designing a new rtTA. Recently, Freundlieb and his colleagues developed a tetracycline controlled transcriptional silencers (tTS), which bound to the tetO in absence of the effector (doxycycline). Dox-HCl can prevent the binding of tTS and promotes the binding of rtTA, thereby activating the transcription of gene of interest (Freundlieb et al., 1999).

V-2. Localization and activity of MT1, -2, -3-MMPs in vitro

V-2.1. Different abilities of MT-MMPs to process the pro-MMP-2

MT1-MMP has been proved to process pro-MMP-2 into an active form in all cell lines which are currently used, such as green monkey kidney (COS) cells, Madin-Darby canine kidney (MDCK) cells, human melanoma cells (A2058 and WM1341D), fibrosarcoma cells (HT1080), breast carcinoma (MCF7) cells, and TIMP-2 free skin fibroblast cells (Sato et al., 1994; Hotary et al., 2000; Maquoi et al, 2000; Iida et al., 2001; Rozanov et al, 2001; Morrison et al, 2001). However, the abilities of other members of MT-MMP family to activate the pro-MMP-2 are still pending. The controversial results are present in different species of MT-MMP, or in different cell lines, or by using full-length or part of MT-MMPs. Indeed, mouse MT2-MMP but not human MT2-MMP processes pro-MMP-2 in COS-1 cells (Tanaka et al, 1997; Miyamori et al., 2000). Human soluble MT3-MMP and human full-length MT3-MMP, but not rat transmembrane domain truncated MT3-MMP process pro-MMP-2 in COS-7 cells (Shofuda et al., 1997; Matsumoto et al., 1997). Neither Mouse full-length nor recombinant MT4-MMP processes pro-MMP-2 in COS -7 cells (English et al, 2000). Interestingly, human MT6-MMP is reported to activate progelatinase A in transfected COS-7 cells (Velasco et al, 2000). However, it does not activate pro-MMP-2 in transfected COS-1 or CHO-K1 cells although it is expressed on the cell surface (Kojima et al., 2000).

V-2.2. A two-site mutant in human MT2-MMP is not expressed on the cell surface and does not activate the pro-MMP-2

Controversial reports are also found concerning the activity to process pro-MMP-2 by human MT2-MMP in COS cells. COS-1 cells transiently transfected with human MT2-MMP showed a clear activation of proMMP-2 (Hotary et al, 2000). However, Miyamori et al (2000) found that human MT2-MMP did not process pro-MMP-2. The substitution of pro^{183} and glu^{185} with the corresponding mouse residues (Ser^{183} and Asp^{185} of IS-2) in a chimeric human MT2-MMP recovered pro-MMP-2 activation although this chimeric protein still contained 154 amino acids of N-terminal propeptide domain of mouse MT2-MMP (Miyamori et al., 2000). So, they concluded that the IS-2 of human MT2-MMP is crucial for the activation of pro-MMP-2 (Miyamori et al., 2000). Here, we show that human MT2-MMP is not detected at the surface and therefore did not process pro-MMP-2 in COS cells. Moreover, a full-length of human MT2-MMP replacing Pro^{183} and Glu^{185} with Ser^{183} and Asp^{185} also was also not localized on the cell surface and did not activate the pro-MMP-2 in COS-1 or COS-7 cells. This result is compatible with the findings of Hotary et al. (Hotary, 2002). They found that MT1-MMP

retained fibrin-invasive when its IS-2 domain was deleted, suggesting that IS-2 might not be a key element to determine the activities of MT-MMPs.

In contrast to FcRIIxMT2 that did not process the pro-MMP-2, a MT2-MMP chimeric construct, MT2xFcRII, in which the cytoplasmic and transmembrane domain of human MT2-MMP was replaced by the corresponding part of the Fc? receptor, was detected on the cell surface by immunofluorescence microscopy. The gelatin zymography further demonstrated that MT2xFcRII efficiently activated pro-MMP-2 in both COS-1 and COS-7 cells and the level of activation was comparable to that achieved by MT1-MMP. Thus, it is plausible to deduce that the cytoplasmic and transmembrane (TM) domain of human MT2-MMP plays a key role in localizing the enzyme on the cell surface where it can process pro-MMP-2.

V-2.3. Functions of cytoplasmic domain in MT-MMPs's localization and activity

Localization of proteolytic enzymes at the cell surface is very important for cell migration or cancer invasion. It is tempting to hypothesize that through interaction of their C-terminal cytoplasmic tails with intracellular regulatory proteins MT-MMPs are recruited to the leading edge of migrating cells.

Cytoplasmic/TM domains may mediate localization of MT1-MMP onto cell invadopodia and this localization is critical for ECM degradation or cell invasion in human melanoma. A truncated MT1-MMP mutant lacking cytoplasmic/TM domains and a chimeric MT1-MMP containing the interleukin-2 receptor alpha chain (IL-2Ra) cytoplasmic domains/TM do not localize MT1-MMP to invadopodia or exhibit ECM degradation (Nakahara et al., 1997). Reciprocally, chimera of the cytoplasmic domain/TM of MT1-MMP and TIMP-1 leads the TIMP-1 molecule to invadopodia (Nakahara et al., 1997). Certainly, the transmembrane domain of MT-MMPs is an important element for their localization and activities. However, substituting the transmembrane domain of MT1-MMP for a relevant part of the interleukin-2 receptor still retains its activity to process the pro-MMP-2 (Nakahara et al., 1997). Furthermore, a mutant of MT1-MMP lacking the TM domain is transported to the surface of cells (Cao et al, 1995) and can process pro-MMP-2 into its active form (Pei and Weiss, 1996). This indicates that the cytoplasmic domain but not TM may play an important role in the transport of MT1-MMP to the cell surface. The cytoplasmic domain may involve an unidentified docking or transporting system that recruits the MT1-MMP into invadopodial complexes.

It has been shown previously that a C-terminal valine^{582} in the cytoplasmic domain of MT1-MMP affects the transport of the enzyme to the plasma membrane since a mutation of Val^{582} to glycine is retained in endoplasmic reticulum (Urena et al., 1999). Recently, a middle part of the MT1-MMP cytoplasmic domain (Thr^{567}-Ser^{577}) was demonstrated to be essential for the localization of MT1-MMP to the leading edge of migrating cells in human Bowes melanoma cells (Lehti, et al., 2000). Moreover, new evidence showing that the display of MT1-MMP at the leading edge of migrating cells is associated with the receptor of complement components 1q, gC1qR, through the cytoplasmic tail of MT1-MMP (Rozanov et al., 2002). Thus, the C-terminal amino acids of MT-MMPs may play a key role in their transport and localization to the edge of cells.

The cytoplasmic domain is also regarded to play a role in the cell locomotion, migration, invasion and cancer metastasis. Deleting a part of cytoplasmic domain of MT1-MMP causes the failure to stimulate migration of transfected breast carcinoma cells (Rozanov et al, 2001). Another group showed that the cytoplasmic domain of MT1-MMP plays a role in the internalization of MT1-MMP at the cell edge (Uekita et al, 2001). Seemly, through the continuous internalization at the cell edge, TIMP-2 inhibited molecules and processed fragments are substituted with newly synthesized MT1-MMP. MT-MMPs may affect cell locomotion by their proteolytic activities or by triggering the complex signal transduction, perhaps through FAK or Rac/Rho/cdc42 GTPases pathways. The proteolytic activity and signaling trigger depend on the cytoplasmic tails of MT-MMPs. Interestingly, the divergences of amino acids in cytoplasmic domains of MT-MMPs potently fulfill the requirements in the regulation of MT-MMPs.

V-2.4. Human MT2-MMP in the event to activate pro-MMP-2

Activation of pro-MMP-2 by MT-MMPs is a complex process including transport or localization of MT-MMPs on cell surface and tethering of the pro-MMP-2 via a TIMP-2-MT-MMP complex. In a most acceptable model, activation of pro-MMP-2 supposedly requires two adjacent MT-MMP molecules, one of which functions as a receptor, and another as an activator. New evidence suggests that oligomerized (three or four) MT1-MMPs exist on the cell surface (Lehti et al., 2002). Therefore, focal concentration of MT-MMPs at cell membranes is essential to the activation of pro-MMP-2. Moreover, any extent regulations of MT-MMPs on the cell surface will finally determine the pericellular proteolysis of pro-MMP-2. Besides its transport and localization, the activity of MT-MMPs is also regulated by its

expression, synthesis, interaction, zymogen processing and also by its nature inhibitors, TIMPs.

Human MT2-MMP was not efficiently transported and oligomerized on the cell surface; therefore it could not efficiently process the activation of pro-MMP-2. It is well known that COS cells constitutively express TIMP-2 and furin-like proteinases (Miyamori et al., 2000; Yana et al., 2000). Furthermore, human MT2-MMP as well as its chimeric constructs was completely processed into their active forms, which had been proved by using anti-MT2-hemopexin and anti-MT2-propeptide domain antibodies. Most importantly, the chimeric MT2xFcRII, in which the cytoplasmic/transmembrane domain of human MT2-MMP was replaced with the corresponding part of Fc?RII, demonstrated a strong activity at same level as human MT1-MMP. Moreover, this chimeric construct was efficiently transported to the cell surface. Therefore, we conclude that the cytoplasmic and transmembrane domains of human MT2-MMP are important for the transport to the cell plasma membrane and exerting its activity in pro-MMP-2 activation. The cytoplasmic domain of human MT2-MMP might inhibit to target the (pro or active form) enzyme onto special cell-surface structures. The cytoplasmic domain of MT2-MMP might also fail to substitute the inactive MT2-MMP with active or new one. It has been shown that a cytoplasmic deletion mutant of MT1-MMP fails to regulate its transport (Jiang et al. 2001a). Moreover, cytoplasmic deletion mutants of MT1-MMP fail to promote cell migration and invasion although net proteolytic activity is not affected in transiently transfected CHO-K1 cells (Uekita et al, 2001). The cytoplasmic domain of MT2-MMP might also fail to interact with other MT2-MMP molecules, therefore preventing the formation of oligomers such as tetramers on the cell surface. All these failures of human MT2-MMP functions mediated by its cytoplasmic tail lead to intracellular retention and low efficient activation of pro-MMP-2.

Besides certain domains such as cytoplasmic domain might respond to the low efficient activation of pro-MMP-2 by MT-MMPs, TIMP-2 may also play a key role in this event as a receptor, activator or inhibitor. TIMP-2 promotes the activation of pro-MMP-2 by MT-MMPs at a low concentration, but the high amount of TIMP-2 will block this activation (Kinoshita et al, 1998; Caterina et al, 2000). Thus, another possibility to explain the controversial results from Miyamori et al. (2000) and Hotary et al (2000) on the ability of human MT2-MMP to activate MMP-2 may be the variation in the levels of endogenous TIMP-2 in their system (Morrison et al., 2001). But, it can not be ignored that the different reactions of MT-MMPs

due to the varying concentration of TIMP-2 may play a role in the activation of pro-MMP-2 by MT-MMPs. TIMP-2 concentrations in COS cells serving to activate pro-MMP-2 by MT1-MMP may be too high to support the activation of pro-MMP-2 by MT2-MMP.

V-2.5. Mouse MT3-MMP in the event to activate pro-MMP-2

Mouse MT3-MMP and all its chimeric constructs such as MT31-MMP, MT32-MMP and MT13-MMP were certainly displayed on the surface of COS cells. However, only MT13-MMP exhibited the efficient activity to process the pro-MMP-2 in gelatine zymography assay. In contrast, MT3-MMP, MT31-MMP and MT32-MMP showed very weak activity in the processing of pro-MMP-2. Because only the antibodies against MT3-MMP-hinge region but not anti-propeptide domain recognized MT3-MMP as well as its chimeric construct proteins, we judged that all MT3-MMP chimeric constructs and wild type mouse MT3-MMP were processed into the active form very soon after synthesis. Because COS cells has been shown to express an endogenous convertase protein of MT-MMPs, furin. Moreover, the amount of endogenous furin can efficiently process the pro-MT1-MMP in COS cells (Yana et al., 2000). The chimeric construct results suggested that the cytoplasmic domain of mouse MT3-MMP was able to transport ectodomains of both MT1-MMP and MT3-MMPs to the cell surface, and facilitated the activity of ectodomain-MT1-MMP but not MT3-MMP's ectodomains in the processing of pro-MMP-2. Interestingly, the cytoplasmic tail of MT1-MMP could not help the ectodomains of mouse MT3-MMP exerting their activity function although it was able to localize the MT3-MMP ectodomains on the cell surface. The possible explanations are: 1) Mouse MT3-MMP is a weak activator to the pro-MMP-2. 2) The low efficiency of mouse MT3-MMP in the activation of pro-MMP-2 is not due to the transport blockage (which is shown in the human MT2-MMP). In this process the ectodomains of mouse MT3-MMP play an important role. Perhaps, the mouse MT3-MMP prefers a low concentration of TIMP-2 in the processing of pro-MMP-2 due to its ectodomains i.e. the endogenous TIMP-2 in COS cells is too high to inhibit the potential activity of mouse MT3-MMP.

MT3-MMP is not a major activator for the pro-MMP-2 at least in certain cell types. It is completely consistent with results of other group. Human MT3-MMP only processes weakly pro-MMP-2 in COS-1 cells (Takino et al., 1995) and COS-7 cells (Shofude et al., 1997) although it is transported to the plasma membrane. Moreover, human MT3-MMP can not process pro-MMP-2 in human fibrosarcoma HT-1080 cells which constitutively express pro-

MMP-2 (Takino et al., 1995). Interestingly, MDCK cells stably transfected by MT3-MMP show a very weak invasion comparing to MT1-MMP transfects in collagen gels (Hotary et al., 2000; Kang et al., 2000).

It has been known that the current cell lines used for studying the MT1-MMP do contain a certain amount of endogenous TIMP-2 that is high enough to completely process the pro-MMP-2. For example, the level of endogenous TIMP-2 in COS-1 cells is very high (49 ng/ml) and sufficient to promote activation of pro-MMP-2 by MT1-MMP (Miyamori et al, 2001). Thus, another explanation for the low efficient activation of mouse MT3-MMP and human MT2-MMP is that these two MT-MMPs are inhibited by the level of endogenous TIMP-2 in COS cells. Perhaps, different MT-MMPs have various tolerances to the concentration of TIMP-2 in terms of that different MT-MMPs have distinct requirements for TIMP-2. For example, TIMP-2 free skin fibroblast cells transfected by human MT2-MMP display activation of pro-MMP-2 at 0-0.3 nM concentration of exogenous TIMP-2. This activation was completely blocked when the TIMP-2 was raised to 9 nM. In contrast, MT1-MMP starts to exhibit its activity to process pro-MMP-2 when the concentration of TIMP-2 was increased to 0.3 nM, then it reached the peak when TIMP was 9 nM. Finally, its activation was completely blocked at 27 nM TIMP (Morrison et al., 2001). These results strongly imply that MT2-MMP prefers a lower concentration of TIMP-2 in exerting its activity to pro-MMP-2 than MT1-MMP does. Thus, MT-MMPs display very different responses to the TIMP-2.

In this event, the ectodomains of mouse MT3-MMP may play a role in determining its tolerance or sensitivity to TIMP-2. Certainly, the catalytic domain of MT-MMPs is regarded to bind the N-terminal domain of TIMP-2 and may be responsible for its tolerance to TIMP-2. However, depending on the fact that all catalytic domains of MT-MMPs have the ability to cleave the Asn37-Leu38 peptide bond of pro-MMP-2 during processing of pro-MMP-2 in vitro, the conformation of MT3-MMP ectodomains but not catalytic domain alone is more possible to contribute to this event. It also needs to be stressed that a TIMP-2-independent processing of pro-MMP-2 may possibly exist in certain cell lines. It is clear that other TIMPs like TIMP-3 and TIMP-4 do not replace the TIMP-2 in the activation of pro-MMP-2 although both of them have the ability to bind MMP-2 or MT-MMPs and have been demonstrated to be inhibitors to MT-MMPs in vitro (Butler et al, 1999; Bigg et al., 1997; Morrison et al, 2001). However, the members of claudin family may be candidates to facilitate the activation of pro-

MMP-2 by MT-MMPs in TIMP-2-independent pathways. Indeed, claudin-1, claudin-2, claudin-3 and claudin-5 promote pro-MMP-2 processing mediated by MT-MMPs (including MT2-MMP and MT3-MMP) in HEK 293T cells (Miyamori et al., 2001). More interestingly, COS-1 cells express high levels of endogenous claudin-1 (Miyamori et al., 2001).

A very unlikely possibility is that the developed anti-MT3-PD antibodies might not efficiently recognize MT3-MMP due to its spatial conformation i.e. the targeted peptide perhaps was not exposed on the surface of folded MT3-MMP as an epitope. It is a long matter of debate regarding the function of the N-terminal propeptide domain of MT-MMPs. Some people believe that the propeptide domain must be removed before MT-MMPs are transferred to the cell surface. Yana et al. (2000) demonstrated that processing of MT1-MMP by a furin-like convertase is required to tether the enzyme to the cell surface and to process pro-MMP-2 in COS as well as HT1080 cells. However, the intact propeptide domain of MT1-MMP as a chaperon was proved to be required for binding of TIMP-2 at cell surface and subsequent activation of pro-gelatinase A in COS cells (Cao et al., 1998). Moreover, a furin-independent activation pathway of MT1-MMP exists in different cell lines such as breast carcinoma cells (Rozanov et al., 2001) and rabbit dermal fibroblast (Sato et al., 1999). Even if the cleavage of the propeptide domain in MT-MMPs is a prerequisite, since endogenous furin exists in the COS-1 cells, and its amount is enough to process the MT1-MMP automatically (Yana et al., 2000), it is reasonable to imagine that the processing of mouse pro-MT3-MMP was also carried out efficiently in COS-1 cells in our experiments. Therefore, the activation of mouse pro-MT3-MMP is perhaps not a key fact here.

V-2.6. New method to prepare stable pro-MMP-2

There are several approaches to prepare pro-MMP-2 in gelatin zymography assay: 1) Conditioned medium from COS cells transfected with pro-MMP-2 expression vector (Sato et al., 1994; Yana et al., 2000), 2) Recombinant pro-MMP-2 or FCS medium (Kang et al, 2000). Here, the pro-MMP-2 secreted from an insect cell system had been proved to express the target protein stably and efficiently. It could be processed by human MT1-MMP and APMA into a 62 kDa active form.

V-2.7. Biological function of MMP-2, TIMP-2 and MT-MMPs

A typical model is MT-MMPs through MMP-2 and TIMP-2 to degrade ECM proteins in vivo or vitro. It has been well documented that active MMP-2 plays a pivotal role in many important events such as angiogenesis, metastases and tumor growth (Yoshizaki et al., 2002).

However, MMP-2-deficient mice normally develop without any anatomical abnormalities, and are fertile, although minor growth retardation is displayed in the beginning (Itoh et al., 1997; Kato et al., 2001). Furthermore, TIMP-2-deficient mice develop with normal sizes, fertility, unabridged lifespan, normal immune responses and repeatedly produce healthy offspring (Caterina et al., 2000; Osiewicz et al., 1999; Wang et al., 2000). It indicates that both TIMP-2 and MMP-2 are dispensable for normal development, fertility and growth.

In contrast, MT1-MM–deficient mice cause significant phenotype such as craniofacial dysmorphism, arthritis, osteopenia, dwarfism, increase mortality and fibrosis of soft tissues (Holmbeck et al., 1999), suggesting that MT1-MMP play a crucial role in biological development.

All these facts mean that besides TIMP-2, there are other proteins involving in the regulation of MT-MMPs activity in vivo. The recent evidences demonstrated that TIMP-3 or TIMP-4 or RECK certainly down-regulate the activities of MT-MMPs (Bulter et al., 1999; Morrison et al., 2001) and claudin up-regulates the functions of MT-MMPs (Miyamori et al, 2001; Oh et al., 2001).

MT-MMPs can directly degrade ECM proteins to penetrate and remodel the barrier of certain tissues such as blood vessel (Hotary et al., 2000; Quaranta, 2000). Moreover, MT-MMPs not only through MMP-2 but also through other important molecules such as CD44 or β3 integrins or through signal transductions exerts its functions. Interestingly, coexpression of MT1-MMP and MT3-MMP results in shedding of 70 kDa sCD44H into the media in human breast carcinoma cells (Kajita et al., 2001) implying a synergistic effect among MT-MMPs. All those complex regulations and multiple functions make MT-MMPs play a key role in pathology and physiology.

V-2.8. Role of MT-MMPs with diseases

The expression level of MT1-MMP, but not other MT-MMPs, correlates with the activation ratio of pro-MMP-2 in rheumatoid synovium (Yamanaka et al, 2000), human invasive breast carcinomas (Gilles et al, 1997), human invasive papillary thyroid carcinoma (Nakamura et al., 1999), pancreatic cancer tissues (Ellenrieder et al., 2000), human head and neck squamous cell carcinoma (Imanishi et al., 2000), lymph metastasis carcinoma (Shimada et al., 2000), lymphangioleiomyomatos (Matsui et al., 2000), and WKY rats induced with crescentic glomerulonephritis (Hayashi et al., 2000), suggesting that MT1-MMP plays a key role in the above disorders.

MT1-MMP and MT2-MMP, but not other MT-MMPs, are significantly expressed in human urothelial carcinomas (Kitagawa et al., 1998) and in human malignant astrocytic tumors (Nakada et al, 1999) indicating that MT1-MMP and MT2-MMP contribute to the development of human urothelial carcinomas, and astrocytic tumor invasion.

Expression of MT1-MMP and MT3-MMP are significantly high in rheumatoid arthritis synovium (Pap et al., 2000), suggesting that MT1-MMP and MT3-MMP are involved in rheumatoid joint destruction.

MT1-, 2-, 3-MMP are more intensely expressed and consistently colocalizes with MMP-2 in nodular melanoma and metastatic melanoma cells, displaying that these three MT-MMPs and MMP-2 cooperate in invasive and metastatic process of melanoma cells (Ohnishi et al., 2001). Expression of MT1-, 2-, 3-MMP mRNA in kidney carcinomas is significantly higher than in normal parenchyma (Kitagawa et al., 1999), demonstrating that these three MT-MMPs associated with human kidney carcinogenesis.

Particularly, expression of MT3-MMP mRNA in renal cell carcinomas (clear cell subtype) is higher than in granular cell subtype, suggesting that MT3-MMP may play a special role in carcinogenesis of human kidney (Kitagawa et al., 1999). Furthermore, MT3-MMP may correlates to MMP-2 in lymph node metastasis (Konaka et al., 1999).

Taken together, MT1-, 2-, 3-MMP may cooperate and share substrates or targets in some diseases such as melanoma, but also display specificities in some cases such as kidney carcinogenesis.

V-2.9. Conclusion and perspectives for the future

In conclusion, although proteolytic activation of pro-MMP-2 is a common function of all MT-MMPs, the activities of some MT-MMPs are still controversial. MT1-MMP appears to be the predominant activator of pro-MMP-2 in many systems (in vivo and in vitro). The cytoplasmic tail of MT2-MMP was crucial for the activation of pro-MMP-2. In contrast, mouse MT3-MMP displayed a different mechanism to regulate its activity in pro-MMP-2 processing.

MT4-, 6-MMP are GPI-anchored proteins and human MT2-MMP and mouse MT3-MMP are detergent insoluble proteins. All these proteins may localize in detergent-resistant membrane fractions (DRMs) or 'lipid rafts' (Ikezawa 2002; Lee et al., 2003). Aggregation of glycosphingolipids and cholesterol is thought to contribute to the formation of microdomains in cell membranes that are distinct from the more abundant and diffuse glycerolipids. It has been proposed that the lipid rafts or microdomains are involved the complex signal transduction since LAT, substrates of PLC-?, Ras, various G proteins and members of the Src PTK family are enriched in these domains (Samelson, 2002).

MT-MMPs have the capabilities to be involved in signal transduction because they have potential phosphorylation sites in their cytoplasmic domains. For instance, tyrosine T^{567} is localized in cytoplasmic domain of MT1-MMP and could be phosphorylated by Threonine kinase. MT3-MMP has two potential phosphorylation sites in the cytoplasmic domain (T^{592}; S^{602}) and could be phosphorylated by serine or Threonine kinases (Netphos 2.0, Texhnical University of Denmark). It is not difficult to imagine that MT-MMPs may associate with a complex signal transduction for their functions in cell migration and invasion, and it will be very interesting to define this relationship in the future work. The amino acid sequences in the cytoplasmic tail of MT-MMPs are listed as the following.

MT1 GLAVFFFRRHG**T**^{567}PRRLLYCQRSLLDKV 582
MT2 TYALVQMQRKGAPRVLLYCKRSLQEWV 669
MT3 VY^{582}T^{583}VFQFKRKG**T**^{592}PRHILY^{598}CKR**S**^{602}MQEWV 607
MT4 AATMLLLLPPLSPGALWTAAQALTL 605
MT5 VYTIFQFKNKTGPQPVTYYKRPVQEWV 645
MT6 AAQRWPAPIPLLLLPLLVGGVASR 562

Figure 35: The potential phosphorylation sites in the cytoplasmic domain of MT-MMPs are highlighted in the deduced amino acid sequences of MT-MMPs. They can be phosphorylated by threonine or serine kinases.

Summary

Like human MT1-MMP, mouse MT3-MMP was found to be localized on the plasma membrane of transfected COS cells. In contrast, human MT2-MMP was not detected on transfected COS cell surface. Unlike human MT1-MMP which is soluble in Triton X-100 buffer, human MT2-MMP and mouse MT3-MMP displayed insolubility. Most interestingly, mouse MT3-MMP and human MT2-MMP did not efficiently process the activation of pro-MMP-2 in COS cells. Through a series of chimeric construction of MT2-MMP or MT3-MMP, localization and activity of human MT2-MMP as well as mouse MT3-MMP were investigated by immunofluoresecence microscopy and gelatine zymography. The failure of human MT2-MMP to activate pro-MMP-2 is due to its poor localization at COS cell surface. In this event, the cytoplasmic tail of human MT2-MMP plays an important role since the substitution of its cytoplasmic and transmembrane domain with the corresponding part of FcRIIa receptor not only localized human MT2-MMP ectodomains on the cell plasma membrane but also showed the activity to process pro-MMP-2. In contrast, the cytoplasmic tail of mouse MT3-MMP was able to localize either MT1-MMP or MT3-MMP ectodomains onto the cell surface and interestingly, it only facilitated MT1-MMP but not MT3-MMP ectodomains to exert their activities to process pro-MMP-2. The cytoplasmic tail of MT1-MMP failed to facilitate mouse MT3-MMP ectodomain function in the processing of pro-MMP-2 although they were localized on cell surface. This suggests that there is a different mechanism for mouse MT3-MMP to process low efficiently the pro-MMP-2 compared to human MT2-MMP. The ectodomains of mouse MT3-MMP play an important role in this process.

Stable double transgenic mice were established via crossing response transgenic mice carrying full-length mouse MT3-MMP with regulatory transgenic mice containing reverse tetracycline regulatory transactivator (rtTA) under the control of either the CMV or GFAP promoter. Tg-MT3-MMP was found in the kidney, tongue, lung, thymus, stomach, colon, pancreas, testis, skin, connective tissue, cerebrum, cerebellum and spinal cord; in contrast, the reporter (Lac Z) gene was only expressed in certain types of organs such as heart, kidney, muscle, tongue and occasionally in brain in CR4 double transgenic mice under induction condition. The expression of the transgenes (MT3-MMP and Lac Z) displayed a dose and time-dependent type when the mice were treated with Dox-HCl, although a leakage expression was detected in a few organs like testis. Different double transgenic lines showed

distinct responsive abilities. CRx3001 displayed a faster and more efficient response to doxycycline than other mouse lines (such as CR4x3002, GFAPx3001 and GFAPx3002) did. MT3-MMP did not colocalize with SMC a-actin in the studied organs. More active-form MT3-MMP proteins were found in the kidney of CR4x3001 double transgenic mice treated by doxycycline compared to the negative control mice. In general, there were no significant differences among wild type, single and double transgenic mice at the later stages of development, although many fats were found in the abdomen of transgenic mice when they nearly became homozygous. Some unusual phenomena such as tumors were not due to the overexpression of transgenic MT3-MMP.

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Appendixes

Appendix A: Sequence of our mouse full-length MT3-MMP (AF282844, Genbank, NCBI)

```
   1 ttcactatga tcttactcgc attcagctct ggaagaaggt tggatttcgt gcatcgttcg
  61 gtggtgtttt tcttgcaaac cttgctttgg attttatgtg ctacagtctg cggaacggag
 121 cagtatttca atgtggaggt ttggttacaa aaatacggct accttccacc gactgacccc
 181 agaatgtcag tgctgcgctc tgcagagacc atgcagtcag ctctagctgc catgcagcag
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 301 cctcgatgcg gtgtaccaga ccagacaaga ggcagctcta aattcaacat ccgacgaaag
 361 cgctatgcat taactgggca gaagtggcaa cacaagcata tcacttacag tataaagaac
 421 gtaactccaa aagttggaga tcctgagacc cggagagcaa ttcgccgtgc ctttgatgtg
 481 tggcagaatg taactcctct aacgtttgaa gaagttccct acagtgaact agaaaatggc
 541 aaacgtgatg tggatataac catcattttt gcttctggtt tccatggaga cagatccccc
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1741 gtattggttt acactgtgtt ccagttcaag aggaaaggaa caccccgcca catactgtac
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```
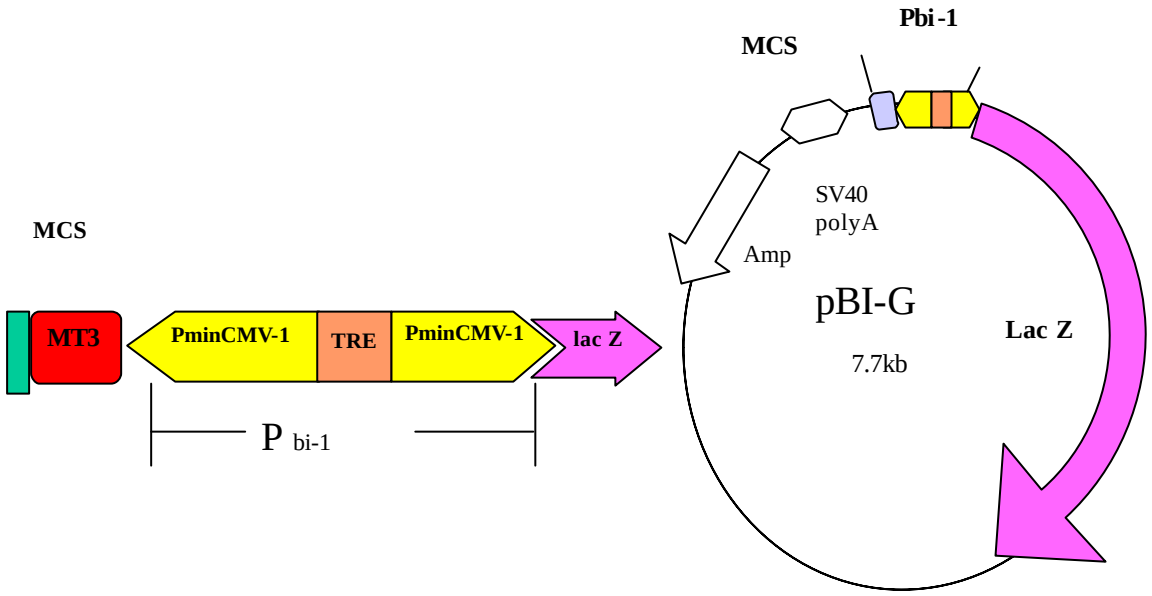
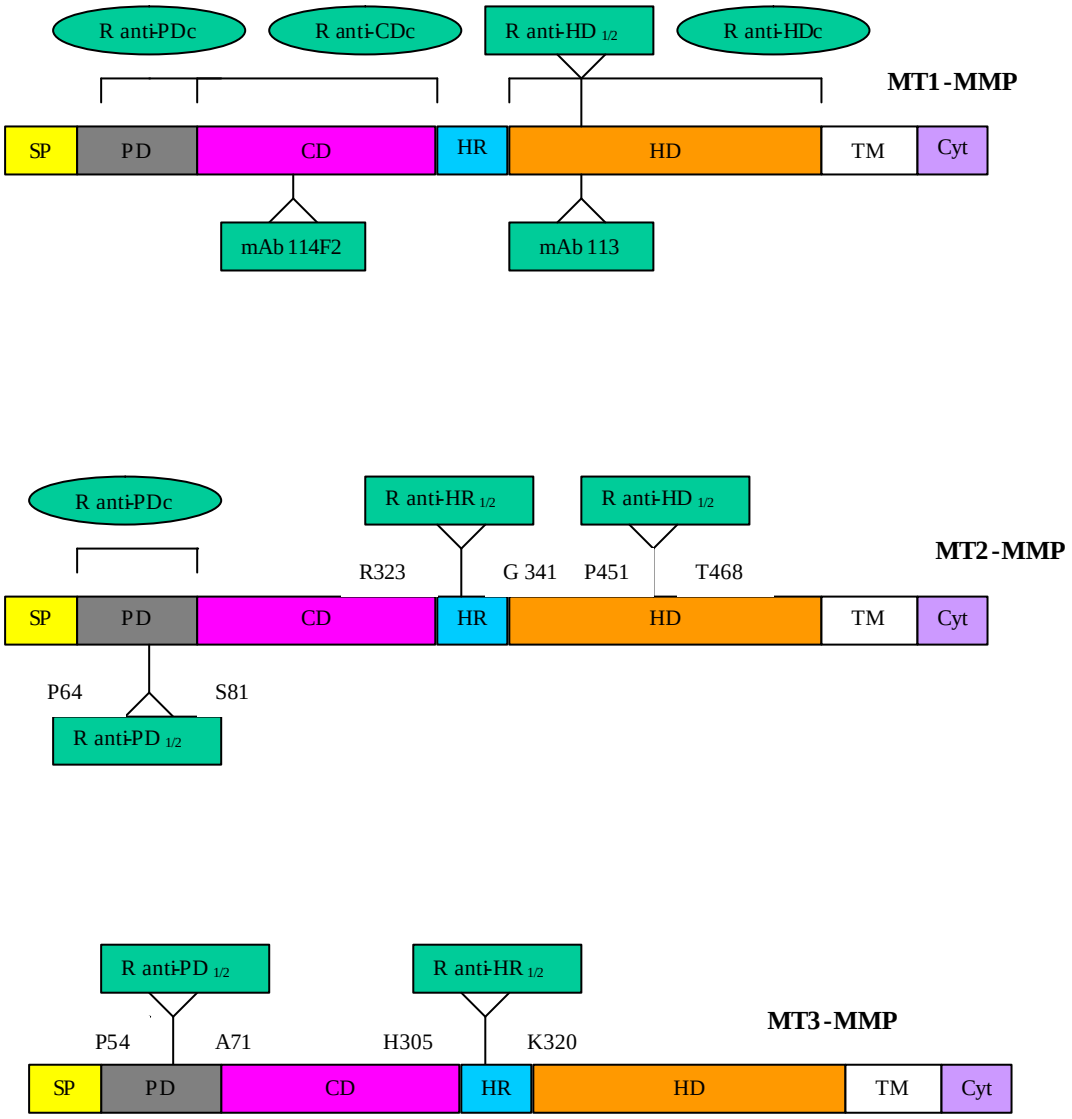

Appendix B: Construct of pBIGMT3-MMP



Appendix C



R anti-PDc: Rabbit anti-propeptide complete domain antibodies.

R anti-CDc: Rabbit anti- complete catalytic domain antibodies.

R anti-HD: Rabbit anti-hemopexin domain antibodies;

R anti-HDc: Rabbit anti- complete hemopexin domain antibodies;

R anti-HR: Rabbit anti-hinge region antibodies;

Appendix D (I)

Primers

Actin primers
Forward 5'-ACC AAC TGG GAC GAC ATG GA-3'
Reverse 5'-GCC ATC TCC TGC TGG AAG TC-3'

FcRIIaxMMP15
SN 5'-TTA ACC CGG GGT TTC CTG TGC AGT G-3'
Sma I

Human MMP14
MMP14 SN 112 5'CTG GCT ACA GCA ATA TGG C-3'
MMP14 ASN 385 5'ACC TTG GGG GTG TAA TTC TGG-3'

Human MMP15 flag primer
Forward 5'-GAC TAC AAG GAC GAC GAT GAC AAG –3'
Reverse 5'- CTA TCA GAC CCA CTC CTG CAG CGA GAC TAC AAG GAC GAC GAT GAC AAG

MT1-MMP Bam HI
5'- CAG GAT CCT CAG CCC CGA AGC-3'

Bam HI

MT1-MMP Hind III
5'-CGT AAA GCT TCC TTC GAA CAT TGG-3'

Hind III

MT2muta5
5'-GGA GGT GTC CTA TGA CGA CAT C-3'

MT2muta3
5'-GGT CAG TGC TGG AGA AGG TC-3'

MT2mutb5
5'-GGT ACG AGT GAA AGC CAA CC-3'

MT2mutb3
5'-GTC GTC ATA GGA CAC CTC CTG -3'

MT2-MMP-seq1 (300)
5'-CAG AGA TGC AGC GCT TCT-3'

MT2-MMP-seq2 (650)
5'-TTT GCC TCT GGC TTC CAC-3'

MT2-MMP-seq3
5'-AAC TTC AAG CTG CCC GAG-3'

MT2-MMP-seq4
5’-AAA GGT GAC CGC TAC TGG-3’

MT2-MMP-seq5
5’-TAC CCC AAG TCC ATC CTG-3’

MMP15xFcRIIa
SN 5’-ATT ACC CGG GTA CAC GCT GTT CTC A-3’

MT3ASN 1778
5’-ATA TTC TAG ATC ACA CCC ACT CTT GCA TAG AG-3’

MT3SN820
5’-GGA AAC AGA CAA CTT CAA GC-3’

MT3-pBI-G
Forward 5’-AAT TCG AGC TCG GTA CCT TC-3’
Reverse 5’-GCC ACT TCT GCC CAG TTA AT-3’

MT3-MMP primers for cloning
PBMMP16-KpnI 5’-GGTACCGTTCACTATGATCTTACTC-3’
PBMMP16-XbaI 5’- TCTAGACTCAAGTTACCACAAACT-3’

MT3SEQ1
5’-CCA GGT TGA AGA GTT GTG TCT-3’

MT3SEQ2
5’-AGG ATG GAT CTT GGA TAA CCA GG-3’

MT3 new 5’
5’-TCA TGT CTG GAT CCT CTA GAA CT-3’

MT3new 3’
5’-ATC TTG GCC TTA TGC CTC CT-3’

MT3 rt5’ 890
5’-ATG GAC CAA CAG ACC GAG AT-3’

MT3rt3’1187
5’ – TCT AGA ACT AGG TCG ACT AGA CTC AAG -3’

MMP16-*Kpn* I
5’-ATT AGG TAC CGT TCA CTA TGA TCT TAC TC-3’

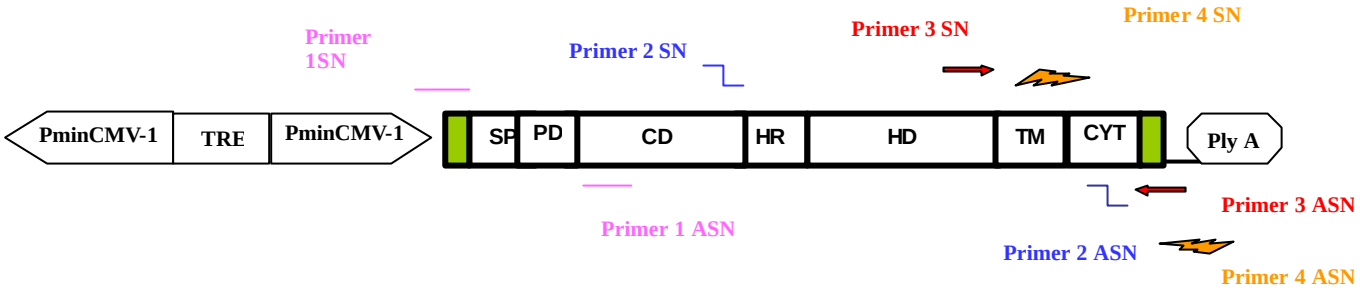
MMP16-*Xba* I
5’-TTA ATC TAG ACT CAA GTT ACC ACA AAC T-3’

rtTA primers

RtTA5’ 5’-AAT GAG GTC GGA ATC GAA GG-3’
RtTA3’ 5’-TAG CTT GTC GTA ATA ATG GCG G-3

Appendix D (II)

Primers for PCR and RT-PCR



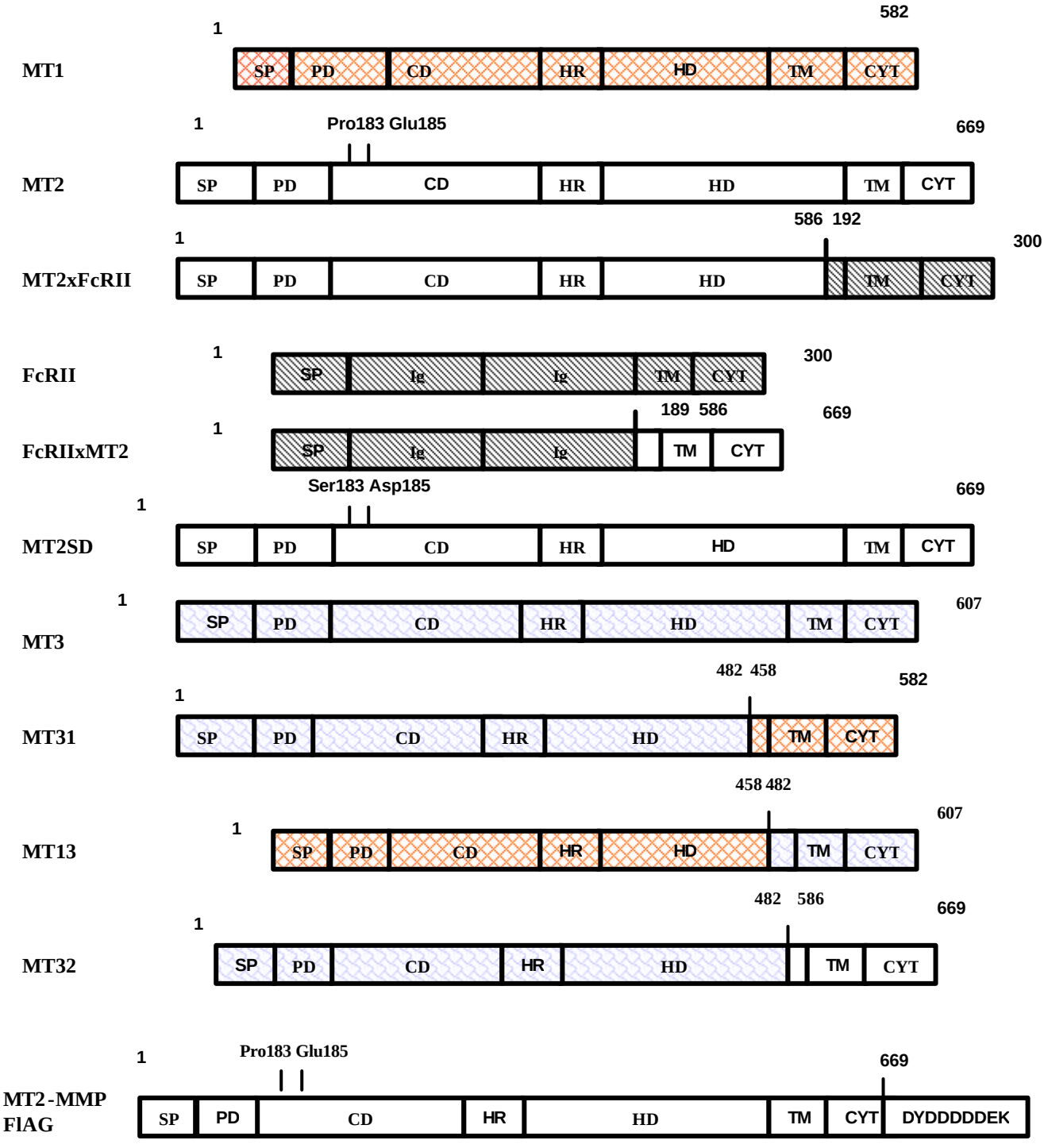
Primer1: expected product: ~410bp pBI-G-MT3

Primer2: expected product: ~950bp MT3

Primer3: expected product: ~300bp rtMT3

Primer4: expected product: ~220bp rtMT3new

Appendix E: schematic diagram of MT2 and MT3 chimerical constructs



Curriculum Vitae

A. Personal Data

Name ..... Hongbin, Li

Date of Birth..... 4 January 1966

Place of Birth..... Ji Nan City, Shan Dong Province, P. R. CHINA

B. Educational Qualifications

High school

Field..... Science

Year 1981-1984

Name and Place of institution..... Shi Yan High School of Shan Dong Province

Degree ..... Bachelor of science (B. Sc.)

Field..... Microbiology

Year 1984-1988

Name and Place of Institution..... Shan Dong University, P.R.CHINA

Degree ..... Master of science (M. Sc.)

Field..... Biochemistry

Year 1997-1999

Name and Place of Institution..... National University of Malaysia

..... (Universiti Kebangsaan Malaysia)

C. Research Experience

7/1988-5/1997 ..... National Vaccine and Serum Institute (NVSI)

..... Beijing, P. R. CHINA

Projects..... 1. Large-scale cuture of *B. tetanus* and high quality

..... purification of *Tetanus* Toxoid

..... 2. Culture, purification and characterization of

..... interferon-α

..... 3. Construction and Purification of Mouse

..... monoclonal antibody (Mab) towards *Japanese*

..... *Encephalitis Virus* (JEV)

..... 4. Construction and expression of single-chain antibody

..... fragment (scFv) towards Carcino Embrynonic

.....antigen (CEA)

7/1997-8/1999 .....National University of Malaysia
.....(Universiti Kebangsaan Malaysia)
Supervisor.....Dr. Sheila Nathan
Project.....Applying phage display technology to construct and
.....express scFv Fab antibodies towards *Burkholderia*
.....*Pseudomallei*

D. Promotion

9/1999-3/2003 .....Department of Biochemistry
.....University of Bielefeld
Supervisor .....Prof. Dr. J. Frey
Project.....Functional studies of matrix metalloproteinases
.....(MMP14, 15, 16) in animal and cell culture models