The proteolytic activity of MT4-MMP is required for its pro-angiogenic and pro-metastatic promoting effects

Lorin HOST[1], Alexandra PAYE[1], Benoit Detry[1], Silvia Blacher[1], Carine Munaut[1], Jean Michel Foidart[1], Motoharu Seiki[2], Nor Eddine SOUNNI[1,*] and Agnès NOEL[1,*]

Laboratory of Tumor and Developmental Biology, Groupe Interdisciplinaire de [1]Génoprotéomique Appliquée-Cancer (GIGA-Cancer), University of Liege, B-4000 Liège, Belgium; [2] Division of Cancer Cell Research, Institute of Medical Science, the University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan

*Contributed equally

Running title: MT4-MMP proteolytic activity triggers tumor malignancy

Keywords: MT4-MMP; tumor host interface; angiogenic switch; metastasis

Correspondence:

Dr. Nor Eddine SOUNNI e-mail: nesounni@ulg.ac.be

Laboratory of Tumor and Developmental Biology

University of Liège, Tour de Pathologie (B23)

Sart-Tilman, B-4000 Liège, BELGIUM

Abstract

MT4-MMP expression in breast adenocarcinoma stimulates tumor growth and metastatic spreading to the lung. However whether these pro-tumorigenic and pro-metastatic effects of MT4-MMP are related to a proteolytic action is not known yet. Through site directed mutagenesis MT4-MMP has been inactivated in cancer cells through Glutamic acid 249 substitution by Alanine in the active site. Active MT4-MMP triggered an angiogenic switch at day 7 after tumor implantation and drastically accelerated subcutaneous tumor growth as well as lung colonization in RAG -/- mice. All these effects were abrogated upon MT4-MMP inactivation. In sharp contrast to most MMPs being primarily of stromal origin, we provide evidence that tumor-derived MT4-MMP, but not host-derived MT4-MMP contributes to angiogenesis. A genetic approach using MT4-MMP-deficient mice revealed that the status of MT4-MMP produced by host cells did not affect the angiogenic response. Despite of this tumor intrinsic feature, to exert its tumor promoting effect, MT4-MMP requires a permissive microenvironment. Indeed, tumor-derived MT4-MMP failed to circumvent the lack of an host angio-promoting factor such as plasminogen activator inhibitor (PAI-1). Overall, our study demonstrates the key contribution of MT4-MMP catalytic activity in the tumor compartment, at the interface with host cells. It identifies MT4-MMP as a key intrinsic tumor cell determinant that contributes to the elaboration of a permissive microenvironment for metastatic dissemination.

Introduction

Tumor growth and metastatic dissemination are associated with an important tissue remodeling that requires different proteases. Matrix metalloproteases (MMPs) are zinc-dependent endopeptidases involved in physiological and pathological extracellular matrix remodeling and in cell function regulation [1]. Their roles in tumor growth, angiogenesis and metastasis are now well documented [1]. This protease family includes secreted/soluble (MMP) or membrane type-MMPs (MT-MMP) that are anchored to the cell surface through transmembrane domain or glycosylphosphatidylinositol (GPI) linker [2]. The most studied cell surface-associated MMP is MT1-MMP that has been originally identified as an MMP2 activator [3], that regulates different cell functions including the glycolytic activity of tumor cells. It can also directly induce intracellular responses by controlling signal transduction through interaction with cell surface receptors [4]. The MT1-MMP driven intracellular signaling regulation seems to be, dependent or independent on its catalytic functions. In this context, it is worth mentioning that MT1-MMP participates in ERK1/2 pathway activation during cell migration in a non-catalytic manner after TIMP-2 binding [5-7].

Membrane-type 4 MMP (MT4-MMP, also known as MMP-17) is a member of the GPI-anchored MT-MMP subgroup that is structurally and functionally distant from other MT-MMPs. MT4-MMP was originally cloned form a human carcinoma cDNA library [8, 9] and its mRNA expression was detected in brain, spleen, uterus, ovary and leukocytes in both human and mousse tissues [9-14]. Recently, several reports have provided information on MT4-MMP function in physiological conditions. MT4-MMP deficient mice develop normal and show no abnormalities compared to wild-type mice [14], suggesting that MT4-MMP is not requested for normal development in survival. However, a recent study reported that Mt4-mmp-/- mice have abnormal diminished thirst that could be a result of dysfunction in thirst regulation in the

brain [15]. MT4-MMP seems to contribute to the control of cartilage aggrecan integrity in vitro and in vivo by its capacity to activate ADAMTS-4 through C-terminal domain processing [16]. The ability of MT4-MMP to form a complex with ADAMTS4 recently evidenced by co-immunoprecipitation further support this concept [17]. Accordingly, genetic deletion of MT4-MMP protects mice against IL-1β-induced loss of articular glycosaminoglycans, reinforcing the role of MT4-MMP in agreccan proteolysis *in vivo* [18]. On the focus of the present study is the role of MT4-MMP's catalytic activity in tumor progression.

Previously, we demonstrated that MT4-MMP is localized in cancer cells, but not in stromal compartment of human breast cancer [19]. MT4-MMP overexpression accelerates the *in vivo* growth and promotes the metastatic dissemination of breast cancer cells through tumor vessel architecture alteration [19, 20]. In line with these data, MT4-MMP plays a significant role in hypoxia-mediated metastasis and is also an important prognostic indicator in patients with head and neck cancer [21]. In comparison with the abundant information currently available on other MT-MMPs, MT4-MMP mechanisms of action in cancer remain to be established. In contrast to MT1-MMP, MT4-MMP does not cleave collagens, it is a poor pro-MMP2 activator and has a restricted substrate repertoire including pro-TNF-α, α2-macroglobulin [22], low density lipoprotein receptor related protein (LRP)[23] and aggrecanase (ADAMTS-4) [18].

Whether the pro-metastatic effect of MT4-MMP relies on its catalytic activity is still not yet known. In this report, we addressed this important issue by evaluating the effect of overexpression of MT4-MMP catalytically-inactive mutant, on *in vitro* and *in vivo* angiogenesis, tumor growth and lung metastases. We investigated MT4-MMP functions in physiological and pathological angiogenesis in a non permissive environment. We provide evidence that MT4-MMP triggers angiogenic switch through its catalytic activity in a permissive host environment favorable for tumor progression.

Materials and methods

Cell Culture

Human breast cancer MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and HEK-293T cells were purchased from Thermo Scientific-Open Biosystems (Wilmington, DE). Cells were grown as described previously [19].

Inactivation of the MT4-MMP and cell transfection

Inert MT4-MMP was produced by site-directed mutagenesis in the highly conserved domain HExxHxxGxxH through point mutation of Glutamic acid at position 249 with Glu249 with Alanin [24-26]. The primers used to introduce the mutation were designed as follows: Forward, 5'-GCAGTGGCTGTCCACGCGTTTGGCCACGCCATTGGG-3'; reverse, 5'-CCCAATGGCGTGGCCAAACGCGTGGACAGCCACTGC-3' (Eurogentec, Seraing, Belgium). The mutation and amplification of mutated vector was performed with QuikChange® Site-Directed Mutagenesis Kit (Stratagene, USA) according to manufacturer's instructions. Parental MDA-MB-231 and HEK-293T cells were stably transfected by electroporation (250 V, 960 AF) with pcDNA3-neo vector containing only the neomycin resistance gene (control plasmid, CTR) or with the same plasmid carrying the full-length active (MT4) or inert (MT4-E249A) human MT4-MMP cDNA as described previously [19].

Preparation of proteins cell extracts, conditioned media and total RNAs

Conditioned media were prepared by incubating subconfluent cells in 100-mm diameter Petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) containing 5 ml of serum-free DMEM for 48 hours. Conditioned media were harvested, clarified and concentrated 20-fold by centrifugation, using Centricon® YM-30 (Millipore, Chicago, IL). Total protein cell extracts were prepared as described previously [19]. Total RNAs were extracted from cell

monolayer using High Pure RNA isolation kit (Roche) or from tumor tissue using High Pure RNA tissue kit (Roche Diagnostic Applied Science, Mannheim, Germany).

RT-PCR analysis

RT-PCR was performed as described previously [19]. Specific primers for MT4-MMP used were: forward, 5-AAGGAGACAGGTACTGGGTGTTC-3; reverse, 5-TCGCCATCCAGCACTTTCCAGTA-3 (Eurogentec, Seraing, Belgium). Thirty-five cycles of amplification were run for 15 seconds at 94°C, 20 seconds at 68°C, and 30 seconds, at 72°C. For detection of MT1-MMP expression, twenty-two cycles of amplification were performed using the following specific primers: forward, *5-GCATACCCAATGCCCATTGGCCA-3;* reverse*, 5-CCATTGGGCATCCAGAAGAGAGC-3.*

Western blot analysis

Samples were separated as described previously [19]. Membranes were blocked with casein 1%/PBS/tween-20 0.1% and incubated overnight, at 4°C with anti-human MT4-MMP antibody (1/1000 dilution) (M3684; Sigma, St. Louis, MO) or anti-human MT1-MMP antibody (1/1000) (MAB1767, clone 3G4.2, Millipore- Chemicon) followed by incubation with a secondary horseradish peroxidase (HRP)–conjugated anti-rabbit antibody (Cell Signaling Technology, Danvers, MA). Signals were detected using an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, MA). For loading control membranes were then stripped and reincubated with an anti-actin antibody ((A2066; Sigma).

Cell surface biotinylation

MDA-MB231 cells stably expressing wild type (MT4) or inactive MT4-MMP (MT4-E249A) were incubated 100 mm dish and cell surface biotinylated with the cell impermeable *EZ*link-sulfo-NHS-biotin (Pierce) as described previously [6] . The cells were then lysed with 0.5 ml/well of cold lysis buffer on ice. After centrifugation, 500 µg of total protein were

incubated with streptavidin-agarose beads (Sigma) overnight at 4°C. The precipitated samples were analyzed by western blotting with the MT4-MMP and MT1-MMP antibodies.

Immunofluorescence

Cells were incubated on cover slip in 24-well culture plate at $5x10^4$cells/well. After incubation for 48h, cells were washed with PBS and fixed for 10 min in 4% PFA at 37°C. Cells were either permeabilized or not after incubation for 1 min at room temperature with 0.1% Triton X-100/PBS. Slides were blocked in in PBS containing 3% bovine serum albumin (BSA) solution and incubated with anti-MT4-MMP antibody (1/50) for 1h at room temperature. After washing, cells were incubated with a secondary swine anti-rabbit FITC-conjugated antibody (1/100) (DakoCytomation, Carpinteria, CA, USA) for 1h and mounted with mounting media containing DAPI for nucleus detection (Vectaschield+Dapi, H1200; Vector Laboratories, Burlingame, CA, USA). Fluorescence microscopy analysis was realized using 40-fold magnification.

Flow cytometry

Cells were detached with PBS/EDTA 2.5 mM, counted and $5x10^5$ cells/ml in suspension were incubated with anti-MT4-MMP antibody (1/50) in incubation buffer (PBS/1% BSA) for 20 minutes at 4°C. After washing, cells were incubated with a secondary (1/125) swine anti-rabbit FITC-conjugated antibody (Dako) for 30 minutes, at 4°C. After washing steps, stained cells were incubated for 10 min with 10 µl of Via-probe®. Non-permeabilized cells were analyzed by flow cytometry using $1x10^4$ total events (FACS canto II, BD biosciences).

In vitro BrdU proliferation assay

In vitro proliferation growth rate was assessed using BrdU kit (11647229001; Roche Applied Sciences GmbH, Mannheinm, Germany) according to manufacturer's

recommendations. The assay was conducted after cells incubation (10^5 cells) in 96-well plate for 24, 48 and 72 hours in DMEM medium containing 10 % serum.

In vivo tumorigenicity

Subconfluent MDA-MB-231 cells (5 10^6 cells/ml) were mixed with an equal volume of matrigel and cell suspension (10^6 cells in 400 µl) was injected subcutaneously (s.c.) into recombination activating gene-1 (*RAG1*$^{-/-}$) immunodeficient mice at both flanks (5 or 6 mice per transfected cells in 3 separated assays) as described previously [27, 28]. For some experiments, cells were s.c. injected in double transgenic mice *PAI-1*$^{-/-}$*/RAG1*$^{-/-}$ mice or their control *PAI-1*$^{+/+}$*/RAG1*$^{-/-}$ mice previously generated [29]. Tumor growth was assessed as described previously [19].

Immunohistochemistry

Slides were autoclaved for 11 min at 126°C in Target Retrieval Solution (DAKO, S1699, Glostrup, Denmark) or incubated for 20 minutes at 37°C in trypsin 0,025% (DAKO, S2012) for Ki67 and von Willebrand Factor (vWF), respectively. Endogenous peroxidases were subsequently blocked by 3% H_2O_2/H_2O for 20 minutes, and nonspecific binding was prevented by incubation in PBS/bovine serum albumin 10% (Fraction V, Acros Organics, NJ) for 1 hour. Lung and tumor sections (6 µm thick) were incubated with a mouse monoclonal anti-human-Ki-67 antibody (1/100) (clone MIB-1, M7240; DAKO) or rabbit polyclonal anti-von Willebrand Factor (vWF) (1/200) (DAKO, A0082) at room temperature, for 1 hour, respectively. Slides were then incubated with a HRP-conjugated secondary antibody and counterstained as described previously [19]. For proliferation index determination, plug sections were double stained with anti-human-Ki-67 and anti-vimentin antibodies. Slides stained with Ki-67 antibody were colored with Permanent Red (K0640, Dako). Co-immunostaining with vimentin was first performed by blocking endogenous peroxydases and incubation with anti-vimentin antibody (1:750, diluted in Tris-HCl / Normal Goat Serum) overnight at 4°C,

followed by the Post Antibody Blocking (Immunologic, Duiven, Nederlands) for 15 min. Finally, poly-HRP-goat anti Mouse/Rabbit/Rat IgG was added for 30 min on the slides which were revealed with Vector DAB (SK-4100, Vector Laboratories, Burlingame, CA, USA).

For quantitative measurement of human Ki-67 staining related to human vimentin staining, a specific automatic computer-assisted image analysis was applied on sections by using Application Software (3.2). In RGB images, human vimentin appeared in red and Ki-67 in brown colors. After separation of those components, red and brown images were binarized automatically and these binarized images were used to determine the colocalization of vimentin and ki-67 using a logical operator (AND). The colocalization area density for each condition was finally determined.

Matrigel plug assays

Mt4-mmp-/- mice (previously generated by Motoharu Seiki's laboratory, University of Tokyo, Japan) [14] were subjected to matrigel plug assay. Matrigel (500 µl) supplemented with 25µl/ml of bFGF (R&D, Minneapolis, USA) and 25000 units of heparin (LEO pharma, Zaventem, Belgium) was s.c. injected in both flanks of mice *Mt4-mmp*-/- mice. Plugs were resected after 7 days prior to quantification of hemoglobin content using Drabkin's reagent according to the manufacturer's instructions (Sigma-Aldrich, St Louis, USA). For some experiments, we have adapted matrigel plug assay through substitution of bFGF and heparin with cells (10^6) transfected with active (MT4) or inert MT4-MMP cDNA (MT4-E249A). Cells mixed in matrigel were injected s.c. in *RAG1*$^{-/-}$ mice and 7 days later, hemoglobin concentration was measured with Drabkin's reagent. For both assays, the amount of hemoglobin was normalized to the weight of lyophilized matrigel plug containing or not tumor cells. The data are from three independent experiments.

Aortic ring assay

Aortic explants from *Mt4-mmp*$^{-/-}$ mice (n=6) or *Mt4-mmp* $^{+/+}$ mice (n=6) were incubated for 6 days at 37°C according to the procedure previously described [30]. Images were captured on Zeiss microscope at day 6. A total number of 18 explants were tested by using three aortic explants of each mouse. Computerized quantification of vessel density and branching was performed as previously described [31].

Corneal whole mounts

Corneal lymphangiogenesis was assessed in *Mt4-mmp* $^{-/-}$ (n=6; number of cornea=12) and *Mt4-mmp* $^{+/+}$ mice (n=6; number of cornea=12) induced by thermal cauterization of the central cornea with an ophthalmic cautery (OPTEMP II V, Alcon Surgical, FortWorth, USA). Seven days after cauterization, corneas were resected and blood vessels were highlighted after over-night incubation with rat anti-mouse CD31 monoclonal antibody (Pharmingen, 01951D). Corneas were washed with PBS and incubated 2 hours incubation at room temperature with Alexa Fluor 488 conjugated rabbit anti-goat antibody (Molecular Probes). Computerized quantification of vessel density was performed according to Blacher et al. [32]. Results are expressed as normalized vessel density (Ni) plotted as function of the distance from the external border of the cornea.

Statistical analysis

We assessed statistical differences between different experimental groups using Mann-Whitney test. *P*-value < 0.05 (*) was considered as significant. Statistical analyses were carried out using the Prism 4.0 software (GraphPad, San Diego, CA). * = $p < 0.05$; ** = $p < 0.01$.

Results

The inactivation of MT4-MMP activity does not influence its shedding from the cell surface

To test the importance of the MT4-MMP catalytic activity on cell based system *in vitro* and on tumor malignancy *in vivo*, we have generated a mutated inactive form of the enzyme. Using a similar approach previously described to generate inactive MT1-MMP (MT1-E240A) [33], the highly conserved Glutamic acid-249 (E249) was replaced by Alanine in the catalytic domain of MT4-MMP by site-directed mutagenesis. Human breast adenocarcioma MDA-MB231 cells were stably transfected with an empty vector (pcDNA3neo or "CTR") or a vector containing either the active full length MT4-MMP cDNA (named herein "MT4") [19] or mutant inactive cDNA ("MT4-E249A"). MT4-MMP mRNA expression was analyzed by RT-PCR and protein production was assessed by western blotting performed on total cell lysates (Figure 1A). No MT4-MMP expression was detected in control cells, whereas mRNA and proteins MT4-MMP and MT4-E249A were found in cells transfected with vector containing MT4-MMP cDNA mutated or not (Figure 1A). MT4-MMP being a GPI-anchored membrane type MMP, its distribution at the cell surface and in the cytoplasm was examined by immunofluorescence on adherent non-permeabilized and permeabilized cells, respectively (Figure 1B). Both cells transfected with MT4-MMP or MT4-E249A form showed a specific cell surface and cytoplasmic staining, whereas no staining was detected in control cells. FACS analysis on non-permeabilized cells revealed that the percentage of MT4-MMP captured by flow cytometry ranged from 15 to 20% in both MT4-E249A and MT4-MMP expressing cells (Figure 1C). Because an important amount of the enzyme could be shed into the medium, degraded or internalized in the cell, data from flow cytometry analysis does not reflect the whole levels of MT4-MMP in cells.

To assess cell surface-associated MT4-MMP, cells were surface biotinylated. Biotin-labeled proteins, including MT4-MMP, were precipitated using streptavidin-agarose beads.

The levels of MT4-MMP in the precipitates were determined by Western blotting (Figure 1D). MT4-MMP was not detected in control cells. In cells over-expressing MT4-MMP, the amount of membrane-associated MT4-MMP was significantly higher for the intact than for the mutated MT4-E249A form. These data are in line with those obtained with total cell lysates and through flow cytometry analysis. Since mRNA levels were similar in all cell types, differences at protein levels on the cell surface could rely on posttranslational regulation such as for instances protein shedding or internalization. We checked that MT1-MMP expression levels are not affected at the cell surface of MT4-MMP, MT4-E249A and control cells (Figure 1D). The previous demonstration of MT4-MMP shedding from MDCK and CHO cells [16, 34] prompted us to evaluate the levels of soluble MT4-MMP in medium conditioned by transfected cells (Figure 1E). Whereas no MT4-MMP species was detected in control cells, significant amounts of a soluble MT4-MMP form of around 50 kDa were found in medium conditioned by both active and inactive mutant MT4-MMP expressing cells (Figure 1E). Consistently with a previous study [34], these data suggest that MT4-MMP did not shed itself from the cell surface through its catalytic activity. However, a drastic difference was observed in soluble MT4-MMP processing following its release from the surface of cells expressing active or inactive enzymatic form. Indeed, a 37 kDa fragment and a doublet of degradation products were abundantly detected in the media of cells overexpressing active MT4-MMP, but not in that of cells overexpressing inactive MT4-E249A (Figure 1E). This 37 kDa MT4-MMP species is thus likely generated by active MT4-MMP autocleavage that could be a new regulatory mechanism of the proteolytic activity of this MT-MMP. To check whether MT1-MMP expression is affected in cell transfected with MT4-MMP, and whether this protease may be responsible for MT4-MMP shedding and processing, we have analyzed the level of MT1-MMP by RT-PCR and western blotting on total cell lysates (Figure 1A). MT1-MMP mRNA expression was not affected significantly by the expression of MT4-MMP

or its mutant inactive form. However, a slight increase in MT1-MMP at protein level was detected in MT4-E249A cells, suggesting that MT1-MMP is not responsible for the generation of the 37 kDa form of MT4-MMP.

The in vivo pro-tumorigenic and pro-metastatic effects of MT4-MMP are dependent on its catalytic activity

To investigate the effect of MT4-MMP catalytic activity on *in vitro* proliferation, we first characterized and evaluated the proliferative potential of transfected cells cultured on plastic dish through BrDU incorporation (Figure 2A). No difference was seen in the proliferation rate of the different cells generated. In agreement with our previous observations, MT4-MMP expression did affect neither cell proliferation nor pro-MMP2 activation *in vitro* [19].

To determine whether MT4-MMP catalytic activity is required for *in vivo* tumor growth and metastatic dissemination into lungs, cells were s.c. injected into *RAG1*$^{-/-}$ mice and tumor volumes were measured for 45 days. The production of active MT4-MMP form stimulated drastically tumor growth (Figure 2B). Interestingly, MT4-E249A form was inefficient in promoting tumor growth revealing that the pro-tumorigenic effect of this MMP is dependent to its catalytic activity. In fact, MT4-E249 tumors were smaller than either MT4 or control tumors, suggesting a possible suppressor effect of the mutant inactive form of MT4-MMP. The expression of MT4-MMP and MT1-MMP were measured in tumors at day 45 by RT-PCR analysis (Figure 2C). As expected, MT4-MMP mRNAs were detected at similar levels in both MT4 and MT4-E249A tumors at day 45, but not in control tumors. MT1-MMP mRNA expression was equivalent in all tumors suggesting that the tumor promoting effect of active MT4-MMP is not associated with changes in MT1-MMP expression *in vivo*. The impact on metastasis was next evaluated by immunohistochemical

analysis of lungs and lymph nodes samples (Figure 2D). The inactivation of MT4-MMP catalytic function reduced lung metastasis to similar level of control cells (Figure 2D). However, lymph node metastases were not affected by control cells or cells expressing either active or inactive form of MT4-MMP (data not shown). These results are in line with our previous report [20], confirming that this protease induces preferentially hematogenous metastatic dissemination rather than lymphatic spreading. All together, our data demonstrate that MT4-MMP-driven metastatic effects depend on its proteolytic activity and that targeting the active site of the enzyme would be of interest to limit tumor metastasis of breast carcinoma expressing MT4-MMP.

The proteolytic activity of MT4-MMP is required to trigger the tumor angiogenic switch in a permissive tumor environment.

Our previous finding demonstrated that MT4-MMP expressing tumors have large and bloody vessels [20]. This prompted us to investigate the onset of angiogenic response induced by the different tumor cells generated. With this aim, we have used a modified matrigel plug assay in which matrigel supplemented with cancer cells were s.c. injected into *RAG1*$^{-/-}$ mice. After 7 days, hemoglobin content was determined in whole plugs (Figure 3A, lower panel). MDA-MB231 cells overexpressing active MT4-MMP induced an early angiogenic response at least twice higher than that induced by control cells. In sharp contrast, in the absence of enzyme activity, the pro-angiogenic effect of MT4-MMP was similar to control tumors. Vessel properties and architecture were analyzed by immunohistochemistry staining with von Willebrand Factor on matrigel plug sections at day 7 (Figure 3A, top panel). Accordingly, an increase in vessels density is shown in MT4 tumors but not in control or MT4-E249A tumors. The large vessels detected in MT4 tumors show the presence of red blood cells without hemorrhage or sign of leakage, excluding any interference with hemoglobin measurement in

the matrigel plugs. To check whether the angiogenic effect of MT4-MMP expressing cells might be ascribed to an effect on proliferation, plugs collected at day 7 were double-immunostained with human Ki-67 for proliferating cells and human vimentin for cancer cells (Figure 3B). A computerized method based on image binerization was used to determine the density of proliferative cancer cells (Ki-67 and human vimentin positive). No significant difference of proliferation rate was observed in matrigel plugs containing the different cell types (Figure 3B). In addition, cell density was similar in all plugs (cell number per field of view = 309±45 for MT4-MMP plugs, 297±47 for MT4-E249A plugs and 302±53 for control plugs. These data demonstrate that the angiogenic switch induced early at day 7 by MT4-MMP does not rely on a modulation of cell proliferation and cell density in the plugs.

Moreover, another cell line HEK-293T stably transfected with MT4-MMP, MT4-E249A or control cells showed the same results as MDA-MB231 cells (Figure 3C and D). MT4-MMP expression in HEK-293T cells induced an early angiogenic switch, whereas the inactive form and control cells were unable to induce tumor angiogenesis *in vivo* (Figure 3C). This MT4-MMP-driven angiogenic switch can contribute to the fast growth of tumors, as well as to their metastatic dissemination. The pro-angiogenic effect of MT4-MMP might require appropriate interactions occurring between host cells and tumor cells expressing the enzyme at their cell surface. To evaluate the importance of tumor-host interface in MT4-MMP functions, cells expressing active or inactive MT4-MMP were s.c. implanted into an host environment poorly permissive to angiogenesis. With this aim, we used immunodeficient RAG-1-/- mice in which Plasminogen-Activator Inhibitor-1 (PAI-1) gene has been deleted [29]. There is a large body of evidences demonstrating that PAI-1 is an important host determinant regulating the angiogenic switch in early steps of cancer progression [35]. As anticipated, active MT4-MMP significantly accelerated the growth of tumors induced by cell injection into *PAI-1*$^{+/+}$ */RAG1*$^{-/-}$ mice (Figure 4). In sharp contrast, slowly growing tumors were observed in *PAI-*

1$^{+/+}$ */RAG1*$^{-/-}$ mice xenografted with cells expressing inactive MT4-MMP (MT4-E249A) (Figure 4). At different time points, tumor volumes were reduced in PAI$^{-/-}$/RAG$^{-/-}$ mice inoculated either with MT4-MMP or control tumors compared to PAI$^{+/+}$/RAG$^{-/-}$ mice. Both wild type and PAI-1 deficient mice developed small tumors after inoculation of cells expressing inactive mutant form MT4-E249A. There was no significant difference in tumor volumes between MT4, MT4-E249A and control cells in PAI-1$^{-/-}$/RAG1$^{-/-}$ mice. Thus, MT4-MMP-driven tumor promotion was completely abolished in the absence of host PAI-1. These data suggest that MT4-MMP is an important factor for cancer malignancy where the microenvironment is permissive for tumor development.

MT4-MMP is a selective tumor cell determinant of cancer progression

We next wonder whether MT4-MMP produced by host cells could also affect angiogenesis and tumor growth *in vivo*. We have explored the angiogenic responses elicited in the absence of host MT4-MMP by applying several *in vitro* and *in vivo* models into recently generated *Mt4-mmp*$^{-/-}$ and *Mt4-mmp*$^{+/+}$ mice [14] (Figure 5). Despite MT4-MMP production by vascular cells in the aorta [14], in the aortic ring assay (Figure 5A), no difference was found in vessel sprouting or branching from explants isolated either from *Mt4-mmp*$^{-/-}$ or *Mt4-mmp*$^{+/+}$ mice, computerized quantification of vessel density did not show any difference (Figure 5B). Similarly, identical angiogenesis was evidenced in the matrigel plug assay applied to mutant and WT mice (Figure 5C). These data suggest that MT4-MMP expression by host cells did not affect angiogenesis *in vivo*. We next extended our exploration to angiogenic process associated with tissue injury repair. In the corneal assay induced by thermal cauterization, blood vessels sprouting from the limb and migrating into the cornea center were visualized by immunostaining of whole mounted cornea with an anti CD31 antibody (Figure 5D). Similar decrease of vessel density from corneal periphery to the center of lesion was seen in the two

mice genotypes (Figure 5E). All together, these data demonstrate that MT4-MMP expression in host cells did not affect angiogenesis.

Discussion

Although MT4-MMP has been shown to induce tumor growth and metastasis, the extent to which the effect is mediated through its proteolytic activity has not been examined yet. In this study, we evaluated the pro-angiogenic, the pro-metastatic and pro-tumorigenic effects of active site defective MT4-MMP mutant. We first generated stable adenocacinoma cells expressing the inactive form of MT4-MMP and characterized its production, processing and shedding *in vitro*. The shedding of MT4-MMP from cell surface was not inhibited by inactivating mutation of the enzyme, demonstrating that MT4-MMP did not shed itself from cell surface. However, the form shed in the conditioned media was cleaved and generated a stable 37 kDa form in MT4-MMP expressing cells but not in cells producing the inert enzymatic form. These data suggest the presence of an autoproteolytic activity of the enzyme, and this provides a new regulatory mechanism of MT4-MMP activity. Similarly, in the case of MT1-MMP, a 44 kDa fragment has been reported to result from autolytic shedding of the catalytic domain. This inactive 44 kDa MT1-MMP fragment accumulating on the cell surface does not interfere with pro-MMP2 activation [36], but regulates collagenolytic activity and cell invasion [37]. In the case of MT4-MMP, further investigations are required to determine whether soluble 37 kDa fragment could display specific functions.

MMPs are involved in angiogenesis through different mechanisms including at least perivascular matrix remodeling, release of angiogenic factors from the ECM or cleavage of angiogenic inhibitors [2]. MT4-MMP has been linked to tumor growth and metastasis in several cancer models. It is induced by hypoxia through HIF1-α and activation of SLUG, a well known transcription factor implicated in epithelial-mesenchymal transition (EMT) that promotes cancer cell malignant capacity [21]. Another mechanism of MT4-MMP relays on the induction of vessel enlargement and vessel destabilization that could promote extravasation and metastasis [20]. Herein, by using functional assays, we demonstrate that MT4-MMP-

mediated malignancy relied at least on the induction of an early angiogenic switch in both MDA-MB231 and HEK-293T tumors that drives the fast growth of tumors and metastatic dissemination to the lung. Of interest is our finding that all these malignant phenotypes were abrogated in the absence of catalytic activity of the enzyme *in vivo*. Intriguingly, MT4-E249A expressing tumors grew more slowly than control tumors, suggesting a suppressor effect of this enzymatically inactive form. Although, MT4-MMP was not detected by RT-PCR and western blot analysis in MDA-MB231 cells, the presence of a very thin band after cell surface protein enrichment in figure 1D does not exclude the presence of very low amount of endogenous MT4-MMP which could be inhibited by MT4-E249A form through a competition for substrate. However, one cannot exclude the possibility that MT4-MMP forms heterodimers with a yet unknown membrane molecule as suggested in a recent study [38]. Thus, the inactive MT4-MMP mutant could interfere with such a complex formation resulting in an unexpected modulation of tumor growth. Further studies are required in vitro and in vivo to address this interesting issue. Indeed, although non-proteolytic function of membrane type-MMP (MT1-MMP) are emerging [6, 7], the proteolytic activity of MT4-MMP is required *in vivo* for its pro-angiogenic and pro-metastatic effects.

In contrast to most MMPs being primarily of stromal origin, we provide evidence that tumor-derived MT4-MMP, but not host-derived MT4-MMP contributes to angiogenesis. It identifies MT4-MMP as a key intrinsic tumor cell determinant that contributes to the elaboration of a permissive microenvironment for metastatic dissemination. These data clearly demonstrate that tumor-derived active MT4-MMP cannot overcome the absence of an angiogenic host determinant such as PAI-1. It was worth addressing this issue since our previous work demonstrated that the angiogenic effect of PAI-1 on human cervical and skin carcinoma cells was dependent to their intrinsic invasive potential [29]. Indeed, the lack of PAI-1 significantly reduced the incidence of subcutaneous tumors derived from poorly aggressive

HaCaT II-4 cells, but not the incidence of tumors derived from the more aggressive HaCaT A5-RT3 cells [29]. The fact that MT4-MMP expression promotes tumor growth in the presence of PAI-1 suggests that this enzyme endows tumor cells with some malignant features in a permissive tissue environment, but is not *per se* sufficient to convert a tumor cells into a fully competent malignant cells [39].

While most MMPs are produced by stromal cells including fibroblasts, endothelial cells and inflammatory cells, only a few MMPs are selectively expressed by cancer cells [40]. A proteolytic-dependent regulation of the angiogenic switch has been attributed to several host-derived MMPs [41, 42]. For instances, MMP9 secreted by host macrophages or neutrophils as a key regulators of tumor angiogenesis [43]. On the other hand, MT1-MMP produced by endothelial cells stimulated angiogenesis *in vitro* [44], and its deletion in *Mt1-mmp*$^{-/-}$ mice impaired angiogenesis, delayed skeletal development [45] and increased steady-state vascular leakage *in vivo* [46]. The concept that cancer cell-specific proteinase contributes to angiogenesis within the tumor stroma is supported by a study reporting the capacity of MMP7 to cleave connective tissue growth factor (CTGF), a VEGF sequester [47]. MT4-MMP is among the very few MMPs selectively produced in the tumor compartment, as demonstrated in human breast cancer samples [19]. To determine whether host-derived MT4-MMP could contribute to angiogenesis, we applied to *Mt4-mmp-/-* mice three powerful models of angiogenesis: the aortic ring assay, the matrigel plus assay and, the corneal assay [32, 39, 48]. Our data show that MT4-MMP expression in host cells did not affect angiogenesis in none of these models. This is in line with the absence of apparent phenotype in *Mt4-mmp*$^{-/-}$ mice [14]. In sharp contrast, cancer-derived MT4-MMP expression in cancer cells regulated the tumor angiogenic switch and promoted tumor growth and hematogenous metastases. Therefore, the localization of MT4-MMP in tumor cells is a key determinant for cancer progression. As a possible underlying mechanism, this MT4-MMP tumor promoting effect is likely mediated by

interference with tumor vessels. Indeed, we previously shed light on the capacity of this membrane type protease to induce the detachment of pericytes from endothelial cells leading to vessel destabilization and enlargement [4, 20]. The present demonstration that cancer-derived MT4-MMP is not sufficient to promote cancer progression further supports its key role at the tumor-vessel interface. MT4-MMP appears therefore as a key target deserving further investigation and worth targeting through antibody-based therapy [49] or chemical compounds [50].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from the FP7-HEALTH-2007-A Proposal No. 201279 "MICROENVIMET", the Fonds de la Recherche Scientifique - FNRS (F.R.S.-FNRS, Belgium), the Foundation against Cancer (foundation of public interest, Belgium), the Centre Anticancéreux près l'Université de Liège, the Fonds Léon Fredericq (University of Liège), the Direction Générale Opérationnelle de l'Economie, de l'Emploi et de la Recherche from the S.P.W. (Région Wallonne, Belgium), the Interuniversity Attraction Poles Programme - Belgian Science Policy (Brussels, Belgium).

References

1. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010;141:52-67.

2. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161-74.

3. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 1994;370:61-5.

4. Sounni NE, Paye A, Host L, Noël A. MT-MMPs as regulators of vessel stability associated with angiogenesis. Front. Pharmacol. 2011;2:111.

5. Sounni NE, Roghi C, Chabottaux V, Janssen M, Munaut C, Maquoi E, Galvez BG, Gilles C, Frankenne F, Murphy G, Foidart JM, Noel A. Up-regulation of vascular endothelial growth factor-A by active membrane-type 1 matrix metalloproteinase through activation of Src-tyrosine kinases. J Biol Chem 2004;279:13564-74.

6. Sounni NE, Rozanov DV, Remacle AG, Golubkov VS, Noel A, Strongin AY. Timp-2 binding with cellular MT1-MMP stimulates invasion-promoting MEK/ERK signaling in cancer cells. Int J Cancer 2010;126:1067-78.

7. D'Alessio S, Ferrari G, Cinnante K, Scheerer W, Galloway AC, Roses DF, Rozanov DV, Remacle AG, Oh ES, Shiryaev SA, Strongin AY, Pintucci G, et al. Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism. J Biol Chem 2008;283:87-99.

8. Puente XS, Pendas AM, Llano E, Velasco G, Lopez-Otin C. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. Cancer Res 1996;56:944-9.

9. Kajita M, Kinoh H, Ito N, Takamura A, Itoh Y, Okada A, Sato H, Seiki M. Human membrane type-4 matrix metalloproteinase (MT4-MMP) is encoded by a novel major transcript: isolation of complementary DNA clones for human and mouse mt4-mmp transcripts. FEBS Lett 1999;457:353-6.

10. Gauthier MC, Racine C, Ferland C, Flamand N, Chakir J, Tremblay GM, Laviolette M. Expression of membrane type-4 matrix metalloproteinase (metalloproteinase-17) by human eosinophils. Int J Biochem Cell Biol 2003;35:1667-73.

11. Funatsu N, Inoue T, Nakamura S. Gene expression analysis of the late embryonic mouse cerebral cortex using DNA microarray: identification of several region- and layer-specific genes. Cereb Cortex 2004;14:1031-44.

12. Nuttall RK, Sampieri CL, Pennington CJ, Gill SE, Schultz GA, Edwards DR. Expression analysis of the entire MMP and TIMP gene families during mouse tissue development. FEBS Lett 2004;563:129-34.

13. Plaisier M, Koolwijk P, Hanemaaijer R, Verwey RA, van der Weiden RM, Risse EK, Jungerius C, Helmerhorst FM, van Hinsbergh VW. Membrane-type matrix metalloproteinases and vascularization in human endometrium during the menstrual cycle. Mol Hum Reprod 2006;12:11-8.

14. Rikimaru A, Komori K, Sakamoto T, Ichise H, Yoshida N, Yana I, Seiki M. Establishment of an MT4-MMP-deficient mouse strain representing an efficient tracking system for MT4-MMP/MMP-17 expression in vivo using beta-galactosidase. Genes Cells 2007;12:1091-100.

15. Srichai MB, Colleta H, Gewin L, Matrisian L, Abel TW, Koshikawa N, Seiki M, Pozzi A, Harris RC, Zent R. Membrane-type 4 matrix metalloproteinase (MT4-MMP) modulates water homeostasis in mice. PLoS One 2011;6:e17099.

16. Patwari P, Gao G, Lee JH, Grodzinsky AJ, Sandy JD. Analysis of ADAMTS4 and MT4-MMP indicates that both are involved in aggrecanolysis in interleukin-1-treated bovine cartilage. Osteoarthritis Cartilage 2005;13:269-77.

17. Ariyoshi W, Takahashi N, Hida D, Knudson CB, Knudson W. Mechanisms involved in enhancement of the expression and function of aggrecanases by hyaluronan oligosaccharides. Arthritis Rheum 2011; Sep 8. doi: 10.1002/art.33329.

18. Clements KM, Flannelly JK, Tart J, Brockbank SM, Wardale J, Freeth J, Parker AE, Newham P. Matrix metalloproteinase 17 is necessary for cartilage aggrecan degradation in an inflammatory environment. Ann Rheum Dis 2011;70:683-9.

19. Chabottaux V, Sounni NE, Pennington CJ, English WR, van den Brule F, Blacher S, Gilles C, Munaut C, Maquoi E, Lopez-Otin C, Murphy G, Edwards DR, et al. Membrane-type 4 matrix metalloproteinase promotes breast cancer growth and metastases. Cancer Res 2006;66:5165-72.

20. Chabottaux V, Ricaud S, Host L, Blacher S, Paye A, Thiry M, Garofalakis A, Pestourie C, Gombert K, Bruyere F, Lewandowsky D, Tavitian B, et al. Membrane-Type 4 Matrix Metalloproteinase (MT4-MMP) induces lung metastasis by alteration of primary breast tumor vascular architecture. J Cell Mol Med 2009; 13(9B):4002-13.

21. Huang CH, Yang WH, Chang SY, Tai SK, Tzeng CH, Kao JY, Wu KJ, Yang MH. Regulation of membrane-type 4 matrix metalloproteinase by SLUG contributes to hypoxia-mediated metastasis. Neoplasia 2009;11:1371-82.

22. English WR, Puente XS, Freije JM, Knauper V, Amour A, Merryweather A, Lopez-Otin C, Murphy G. Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2. J Biol Chem 2000;275:14046-55.

23. Rozanov DV, Hahn-Dantona E, Strickland DK, Strongin AY. The low density lipoprotein receptor-related protein LRP is regulated by membrane type-1 matrix metalloproteinase (MT1-MMP) proteolysis in malignant cells. J Biol Chem 2004;279:4260-8.

24. Noel A, Boulay A, Kebers F, Kannan R, Hajitou A, Calberg-Bacq CM, Basset P, Rio MC, Foidart JM. Demonstration in vivo that stromelysin-3 functions through its proteolytic activity. Oncogene 2000;19:1605-12.

25. Rozanov DV, Deryugina EI, Ratnikov BI, Monosov EZ, Marchenko GN, Quigley JP, Strongin AY. Mutation analysis of membrane type-1 matrix metalloproteinase (MT1-MMP). The role of the cytoplasmic tail Cys(574), the active site Glu(240), and furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. J Biol Chem 2001;276:25705-14.

26. Valtanen H, Lehti K, Lohi J, Keski-Oja J. Expression and purification of soluble and inactive mutant forms of membrane type 1 matrix metalloproteinase. Protein Expr Purif 2000;19:66-73.

27. Sounni NE, Devy L, Hajitou A, Frankenne F, Munaut C, Gilles C, Deroanne C, Thompson EW, Foidart JM, Noel A. MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. Faseb J 2002;16:555-64.

28. Noel A, De Pauw-Gillet MC, Purnell G, Nusgens B, Lapiere CM, Foidart JM. Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts. Br J Cancer 1993;68:909-15.

29. Maillard C, Jost M, Romer MU, Brunner N, Houard X, Lejeune A, Munaut C, Bajou K, Melen L, Dano K, Carmeliet P, Fusenig NE, et al. Host plasminogen activator inhibitor-1 promotes human skin carcinoma progression in a stage-dependent manner. Neoplasia 2005;7:57-66.

30. Masson VV, Devy L, Grignet-Debrus C, Bernt S, Bajou K, Blacher S, Roland G, Chang Y, Fong T, Carmeliet P, Foidart JM, Noel A. Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. Biol Proced Online 2002;4:24-31.

31. Blacher S, Devy L, Burbridge MF, Roland G, Tucker G, Noel A, Foidart JM. Improved quantification of angiogenesis in the rat aortic ring assay. Angiogenesis 2001;4:133-42.

32. Blacher S, Detry B, Bruyere F, Foidart JM, Noel A. Additional parameters for the morphometry of angiogenesis and lymphangiogenesis in corneal flat mounts. Exp Eye Res 2009;89:274-6.

33. Labrecque L, Nyalendo C, Langlois S, Durocher Y, Roghi C, Murphy G, Gingras D, Beliveau R. Src-mediated tyrosine phosphorylation of caveolin-1 induces its association with membrane type 1 matrix metalloproteinase. J Biol Chem 2004;279:52132-40.

34. Itoh Y, Kajita M, Kinoh H, Mori H, Okada A, Seiki M. Membrane type 4 matrix metalloproteinase (MT4-MMP, MMP-17) is a glycosylphosphatidylinositol-anchored proteinase. J Biol Chem 1999;274:34260-6.

35. Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 1998;4:923-8.

36. Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE, Roberts CR. Domain interactions in the gelatinase A.TIMP-2.MT1-MMP activation complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. J Biol Chem 2000;275:39497-506.

37. Tam EM, Wu YI, Butler GS, Stack MS, Overall CM. Collagen binding properties of the membrane type-1 matrix metalloproteinase (MT1-MMP) hemopexin C domain. The ectodomain of the 44-kDa autocatalytic product of MT1-MMP inhibits cell invasion by disrupting native type I collagen cleavage. J Biol Chem 2002;277:39005-14.

38. Sohail A, Marco M, Zhao H, Shi Q, Merriman S, Mobashery S, Fridman R. Characterization of the dimerization interface of membrane type 4 (MT4)-Matrix Metalloproteinase. J Biol Chem 2011;286:33178-89.

39. Berndt S, Bruyer F, Jost M, Noel A. In vitro and In vivo Models of Angiogenesis to Dissect MMP Function. The Cancer Degradome - Proteases and Cancer Biology - Book 2008; Chapter 16:305.

40. Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. Semin Cell Dev Biol 2008;19:52-60.

41. Kruger A, Kates RE, Edwards DR. Avoiding spam in the proteolytic internet: Future strategies for anti-metastatic MMP inhibition. Biochim Biophys Acta 2009.

42. Decock J, Thirkettle S, Wagstaff L, Edwards DR. Matrix metalloproteinases: protective roles in cancer. J Cell Mol Med 2011; 15(6):1254-65.

43. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737-44.

44. Stratman AN, Saunders WB, Sacharidou A, Koh W, Fisher KE, Zawieja DC, Davis MJ, Davis GE. Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices. Blood 2009;114:237-47.

45. Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. Proc Natl Acad Sci U S A 2000;97:4052-7.

46. Sounni NE, Dehne K, van Kempen L, Egeblad M, Affara NI, Cuevas I, Wiesen J, Junankar S, Korets L, Lee J, Shen J, Morrison CJ, et al. Stromal regulation of vessel stability by MMP14 and TGFbeta. Dis Model Mech 2010;3:317-32.

47. Ito TK, Ishii G, Chiba H, Ochiai A. The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells. Oncogene 2007;26:7194-203.

48. El Hour M, Moncada-Pazos A, Blacher S, Masset A, Cal S, Berndt S, Detilleux J, Host L, Obaya AJ, Maillard C, Foidart JM, Ectors F, et al. Higher sensitivity of Adamts12-deficient mice to tumor growth and angiogenesis. Oncogene 2010;29:3025-32.

49. Devy L, Huang L, Naa L, Yanamandra N, Pieters H, Frans N, Chang E, Tao Q, Vanhove M, Lejeune A, van Gool R, Sexton DJ, et al. Selective inhibition of matrix metalloproteinase-14 blocks tumor growth, invasion, and angiogenesis. Cancer Res 2009;69:1517-26.

50. Scott CJ, Taggart CC. Biologic protease inhibitors as novel therapeutic agents. Biochimie 2010;92:1681-8.

Figures legend

Figure 1. **Expression and characterization of inactive form of MT4-MMP in MDA-MB231 cells.** MDA-MB-231 cells stably transfected with empty vector (CTR), MT4-MMP cDNA [19] or inactive MT4-MMP cDNA (MT4-E249A). A. RT-PCR analysis of MT4-MMP, MT1-MMP mRNA and 28S rRNA (loading control) were performed as described previously [19]. Total cell lysates were analyzed by western blotting with the anti-human MT4-MMP, anti-MT1-MMP antibody and anti-β-actin antibody for loading control. Each sample is loaded in duplicate lane for RT-PCR and western blot analyses. B. Immunofluorescent detection of MT4-MMP on non-permeabilized (top panel) and permeabilized (lower panel) cells cultured on coverslips using a fluorescent microscope at 40-fold magnification. C. FACS analysis of cells stained with anti-MT4-MMP antibody. D. Detection of MT4-MMP and MT1-MMP levels at the cell surface on biotin labeled plasma membrane samples by western blotting to MT4-MMP. E. Detection of soluble form of MT4-MMP shed in cell culture media. Data are representative of at least two independent experiments.

Figure 2. **Inactivation of MT4-MMP in breast carcinoma cells inhibits *in vivo* tumor growth and metastasis**. A. Cell proliferation assay of cells cultured on plastic dishes using BrdU kit. B. *In vivo* tumor growth curves of cells transfected with MT4-MMP (MT4), inactive MT4-MMP (MT4-E249A) and control cells (CTR) Tumor cells (10^6) were s.c. injected in *RAG1* $^{-/-}$ mice and tumor were measured for 45 days as described in materials and methods. C. MT4-MMP and MT1-MMP expression levels in tumors at day 45, analyzed by RT-PCR using human specific primers as described in materials and methods. D. Quantification of lung metastases after immunostaining using human-Ki67 antibody. Columns are means of tumor cells counted in 20 microscopic fields of a lung section (magnification 20-fold) (n = 5). Data are those of three independent experiments (n = 5). Bars

are standard errors. Statistical analysis was performed using Mann-Whitney test with Prism 5.04 software (GraphPad, San Diego, CA). *P-value:* * $p < 0,05$; ** $p < 0,01$.

Figure 3. **MT4-MMP catalytic activity triggers early angiogenic switch**. A. Matrigel containing MDA-MB231 cells expressing MT4-MMP or its inactive form (MT4-E249A) and control cells were s.c. injected into *RAG1*$^{-/-}$ mice. Immunohistochemistry detection of vessels on matrigel plug sections at day 7 after von Willebrand Factor staining (top panel). Hemoglobin content was determined after 7 days (left graph below pictures). Values were normalized to the weight of lyophilized tumors. Data are those of three independent experiments (n = 5). B Immunohistochemistry detection of proliferating cells in matrigel plug sections at day 7 after double immunostaining with human Ki-67 antibody (red staining) and human vimentin antibody (brown staining) (top panel). Proliferation index was determining the ratio between, the number of proliferating cells (ki-67 positive, brown color) and total cancer cells (vimentin positive, red color) (right graph below pictures). C. Matrigel containing HEK-293T cells expressing MT4-MMP (MT4-HECK) or its inactive mutant form (MT4-E249A-HEK) and control cells (CTR-HEK) were s.c. injected into *RAG1*$^{-/-}$ mice. Hemoglobin content was determined after 7 days. Values were normalized to the weight of lyophilized tumors. Data are those of three independent experiments (n = 5). Bars are standard errors. D. MT4-MMP expression by RT-PCR and western blot analysis on HEK-293T cells stably transfected with MT4-MMP, MT4-E249A cDNA and control vector. Statistical analysis was performed using Mann-Whitney test with Prism 5.04 software (GraphPad, San Diego, CA). *P-value:* * $p < 0,05$; ** $p < 0,01$.

Figure 4. **Tumor growth of cells expressing MT4-MMP, inactive mutant form MT4-E249A and control cells in the absence of PAI-1**. *RAG1-/-* mice deficient for PAI-1 were

generated by intercrossing *PAI-1*$^{-/-}$ mice with *RAG1*$^{-/-}$ mice [29]. Cells were s.c. injected into both flanks of *PAI-1*$^{-/-}$*/RAG* $^{-/-}$ mice or *PAI-1*$^{+/+}$*/RAG* $^{-/-}$ mice (n = 5). Tumors were measured at day 26, 33 and 41, and mice were sacrificed at day 41. The data are those of three independent experiments. Bars are standard errors. Statistical analyses were performed using Mann-Whitney test with GraphPad Software (GraphPad, San Diego, CA). *P-value:* * $p <$ 0,05; ** $p < 0,01$

Figure 5. **Effect of MT4-MMP produced by host cells on angiogenesis.** A. Aorta ring assay. Mice aortic explants from *Mt4-mmp*-/- mice were incubated for 6 days at 37°C. Similar angiogenic response was observed in WT mice and *Mt4-mmp*-/- mice. B. Quantification of normalized vessel density (Ni) plotted as function of the distance from the external border of the aorta ring. Matrigel plug assay. C. Matrigel supplied with recombinant bFGF was s.c. injected in both flanks of mice. Plugs were resected after 7 days prior to quantification of hemoglobin content with Drabkin's reagent as described in Figure 2. D. Corneal assay. 7 days after thermal cauterization corneas were resected and whole mounted. Blood vessels were highlighted after staining with mouse CD31 and detected with Alexa Fluor 488-conjugated secondary antibody and fluorescent microscopy analysis. E. Computerized quantification of normalized vessel density (Ni) plotted as a function of distance from the external border of the cornea [32].

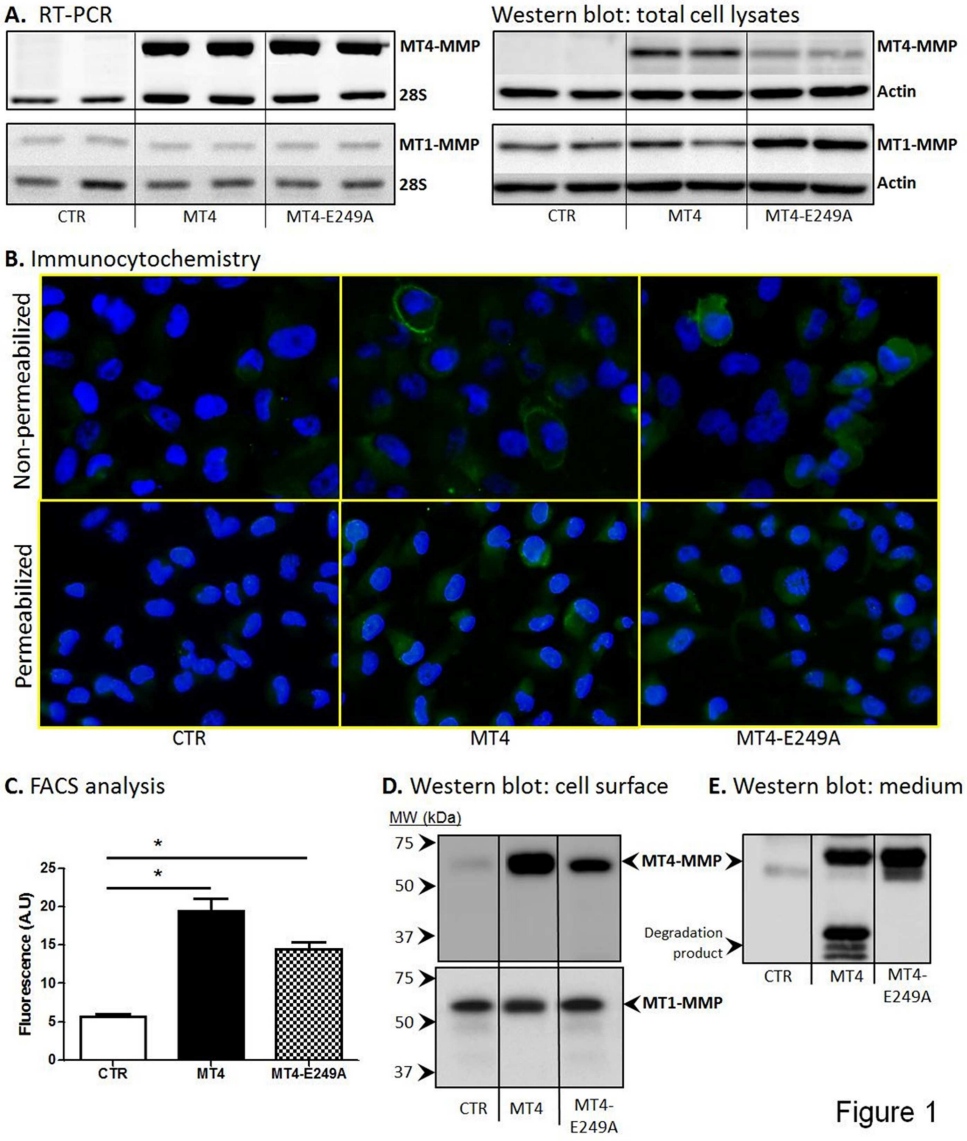

Figure 1

color
235x291mm (300 x 300 DPI)

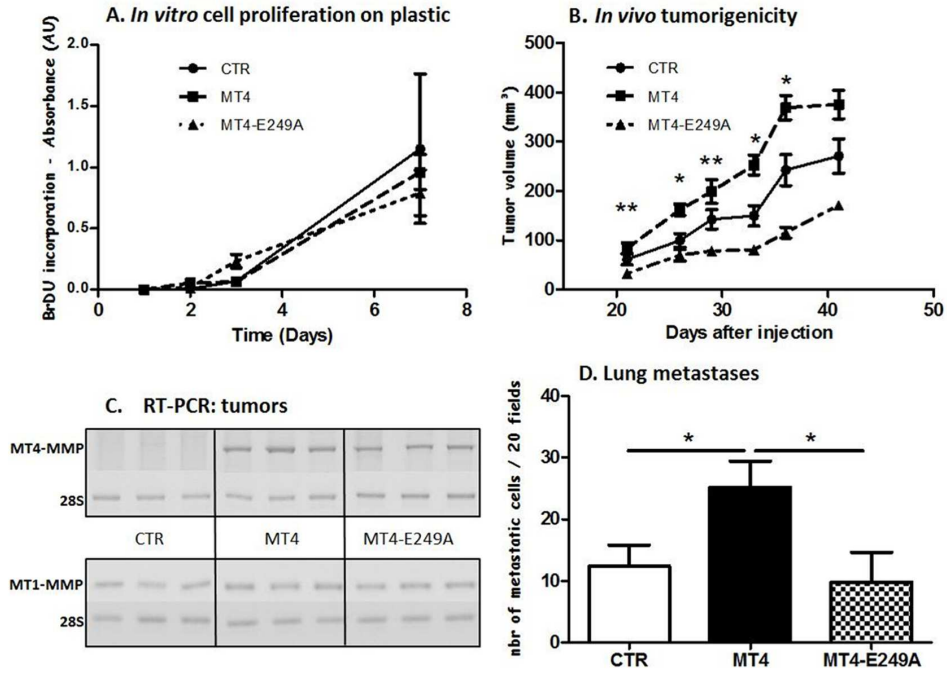

Figure 2

150x118mm (300 x 300 DPI)

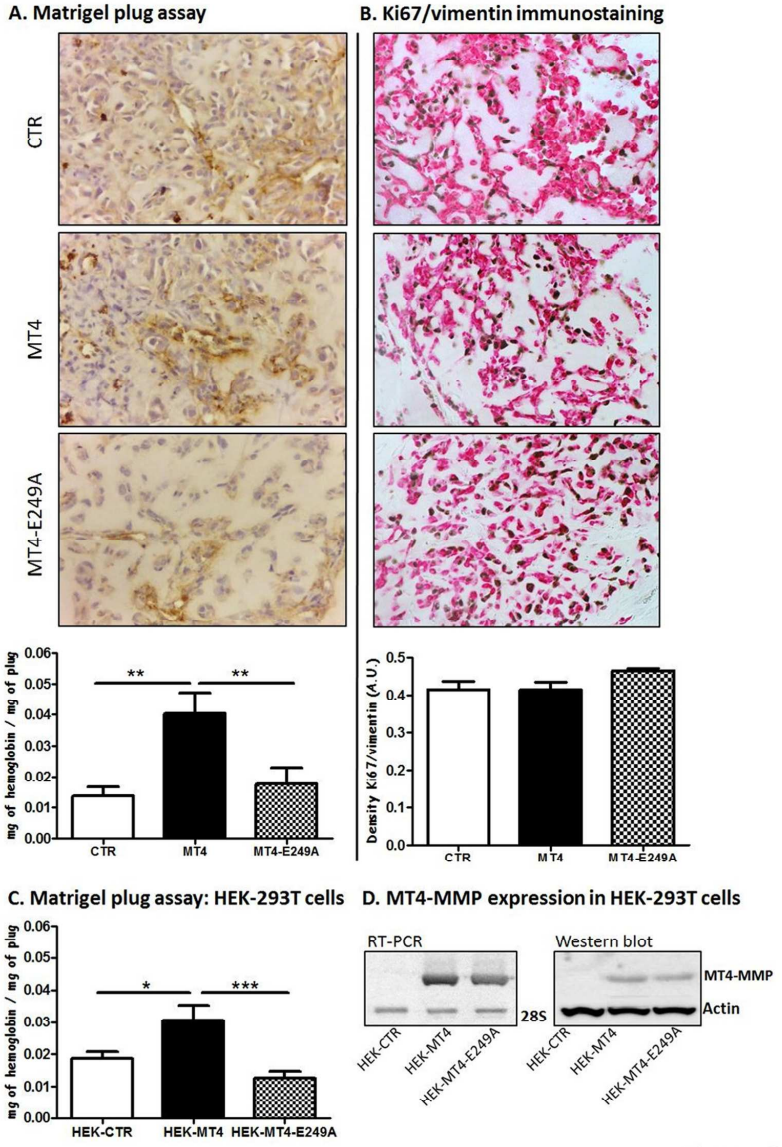

Figure 3

color
275x397mm (300 x 300 DPI)

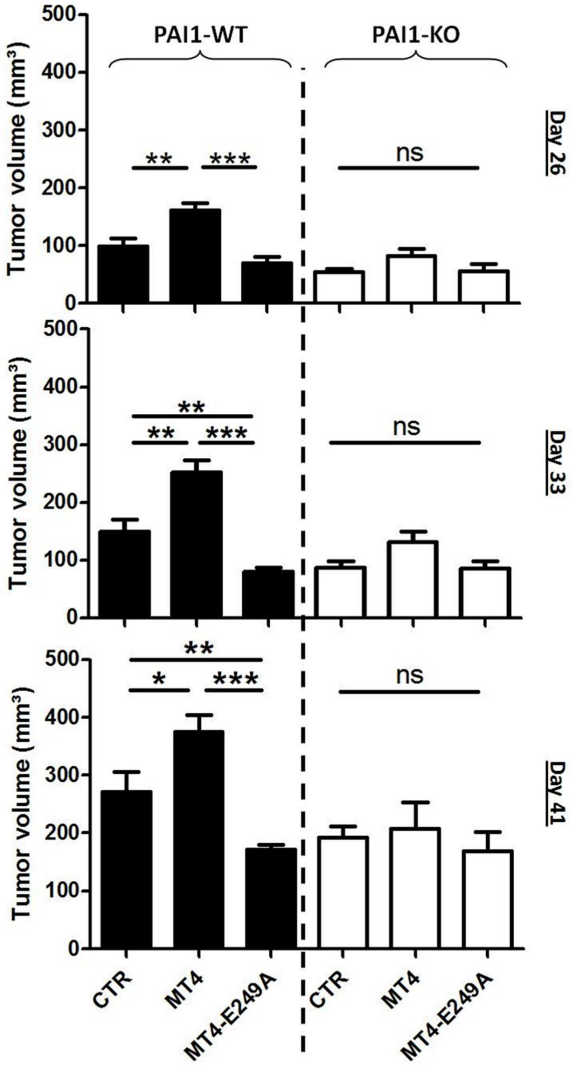

Figure 4

257x434mm (300 x 300 DPI)

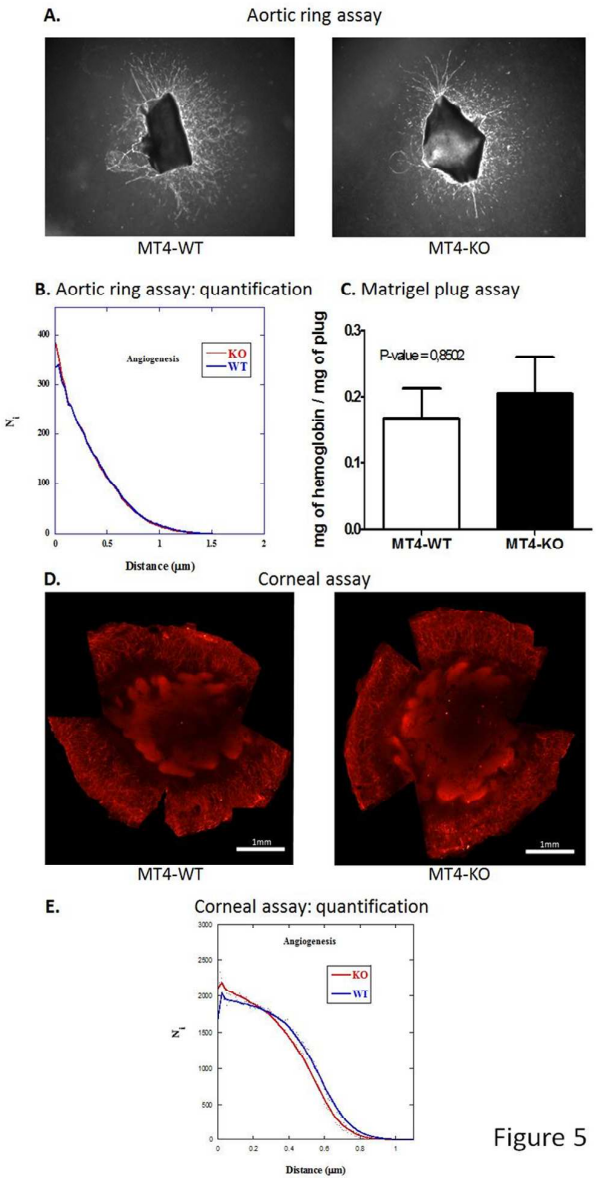

Figure 5

275x503mm (300 x 300 DPI)