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The role of MMP-9 and its Fibronectin-like domain in tumor growth & invasion: Certainties, Controversies & Discrepancies

DAYER Cynthia

UNIL | Université de Lausanne

Faculté de biologie
et de médecine

Institut de Pathologie

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par

Cynthia DAYER

Biologiste diplômée de l'Université de Lausanne

Jury

Prof. Pedro Romero, Président
Prof. Ivan Stamenkovic, Directeur de thèse
Prof. Anne Angelillo-Scherrer, Expert
Prof. Gerhard Christofori, Expert

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Certainties, Controversies & Discrepancies

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Pedro Romero

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Curriculum Vitae

Cynthia Dayer

Born on the 28 January 1985
Nationality: Swiss
cynthia.dayer@gmail.com

Promenade du Botza 46
1963 Vétroz
VS-CH

004179/7106853

Education

2009-present	**PhD** student in the PhD program "Cancer & Immunology", University of Lausanne, Switzerland
2007-2009	**Master** of Science in Genomics and Experimental Biology, University of Lausanne, Switzerland
2004-2007	**Bachelor** of Science in Biology, University of Lausanne, Switzerland
1999-2004	**Maturity** in option Italian-English, Collège de la Planta, Sion, Switzerland

Research Experiment

2009	**PhD** thesis, CHUV, Institute of Pathology, Professor Ivan Stamenkovic's group. Title: ***Molecular mechanisms involved in tumor-host interactions***
2008	**Master** project, University of Lausanne, Center for Integrative Genomics, Professor Winship Herr's group, supervised by Joëlle Michaud. Duration: 2 semesters. Title: ***Regulation of cell division by the human herpes simplex virus "Host-Cell Factor" (HCF-1)***
2007	**Initiation to master work**, University of Lausanne, Institute of Microbiology of the University Hopital Center, Dr Elena Buetti's group, supervised by Dr Elena Buetti. Duration: 1 semester. Title: ***Analysis of interactions among transcription factors that synergize in the regulation of an oncogenic retrovirus***

Skills

Languages	French (mother tongue), English (fluent), Italian (good knowledge), German (basic)
Computer	MS Office applications and internet use, Bioinformatics database
Laboratory	Primary cell culture, protein chemistry including co-immunoprecipitation, pull-down assays, recombinant protein production, protein purification, yeast two-hybrid assays and mass-spec. analysis; molecular biology, including gene cloning, expression vector construction, sh and siRNA techniques; flow cytometry and immunofluorescence microscopy. Animal experimentation including subcutaneous, orthotopic and intravenous injections of tumor cells

Posters

Dayer C, Le Bitoux MA and Stamenkovic I. *The fibronectin-like domain of MMP-9 provides a means to selectively displace MMP-9 activity from the surface of stromal cell.*
Presented at the ISREC Symposium "Hallmarks and Horizons of Cancer" 2011, Lausanne (Switzerland)

Dayer C and Stamenkovic I. *Dual effect of MMP-9 and its fibronectin-like domain in tumor growth and invasion.*
Presented at the AACR annual meeting 2012, Chicago (USA), FBM day 2012, UNIL Lausanne (Switzerland) and AACR annual meeting 2013, Washington (USA)

Talks

Dayer C. *Dual effect of MMP-9 and its fibronectin-like domain in tumor growth and invasion,* FBM day 2012, UNIL Lausanne, Switzerland

Other skills/Interests

Music	1996-…	Percussionist in a communal band (Fanfare Union of Vétroz, VS)
	2001-2010	Percussionist in a cantonal orchestra (Ensemble de Cuivres Valaisan, VS)
Yoga		

Referees

Prof. Ivan Stamenkovic
Institut de Pathologie CHUV
CH-1011 Lausanne
Ivan.Stamenkovic@chuv.ch

Prof. Winship Herr
Génopode UNIL
CH-1015 Lausanne
Winship.Herr@unil.ch

Dr Michaud Joëlle
Génopode UNIL
CH-1015 Lausanne
Joelle.Michaud@unil.ch

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Summary

Tumors are often compared to wounds that do not heal, where the crosstalk between tumor cells and their surrounding stroma is crucial at all stages of development, from the initial primary growth to metastasis. Similar to wound healing, fibroblasts in the tumor stroma differentiate into myofibroblasts, also referred to as "cancer-associated fibroblasts" (CAFs), primarily, but not exclusively, in response to transforming growth factor-β (TGF-β). Myofibroblasts in turn enhance tumor progression by remodeling the stroma. Among molecules implicated in stroma remodeling, matrix metalloproteinases (MMPs), and MMP-9 in particular, play a prominent role. However, the mechanisms that regulate MMP-9 activation and function remain poorly understood. Recent evidence indicates that tumor cell surface association of MMP-9 is an important event in its activation, and more generally in tumor growth and invasion.

In the present work we address the potential association of MMP-9 activity with cell-surface recruitment to human fibroblasts. We show for the first time that recruitment of MMP-9 to the MRC-5 fibroblast cell surface occurs through the fibronectin-like (FN) domain, shared only by MMP-9 and MMP-2 among all the MMPs. Functional assays suggest that both the pro- and active form of MMP-9 trigger α-smooth muscle actin (αSMA) expression in resting fibroblasts that reflects myofibroblast differentiation, possibly through TGF-β activation. Moreover, the FN domain of MMP-9 inhibits both MMP-9-induced TGF-β activation and αSMA expression by sequestering MMP-9.

Xenograft experiments in NOD/SCID mice using HT1080 fibrosarcoma or MDA-MD231 breast adenocarcinoma cells stably expressing the FN domain of MMP-9 revealed no changes in primary tumor growth. However, in the context of metastasis, expression of the FN domain by these same tumor cells dramatically increased their metastatic proclivity whereas expression of wt MMP-9 either promoted no change or actually reduced the number of metastases. We observed a decrease of an active form of MMP-9 in MDA-MB231 cells overexpressing the FN domain suggesting that the FN domain may inhibit MMP-9 activity in those cells and therefore prevent MMP-9-induced activation of TGF-β, which results in increased invasion. Curiously, xenografts of SW480 colorectal adenocarcinoma cells stably expressing the FN domain of MMP-9 displayed reduced

growth at both the primary (subcutaneous) injection site and the lungs of NOD/SCID mice, in experimental metastasis assays, whilst the same cells overexpressing wt MMP-9 showed enhanced growth and dissemination. Gelatin zymography of conditioned medium revealed that these effects may be due to the FN domain, which displaces MMP-9 from SW480 cell surface.

These observations suggest a dual role of MMP-9 and its FN domain in primary tumor growth and metastasis, underscoring the notion that the effect of MMP-9 on tumor cells may depend on the cell type and highlighting possible protective effects of MMPs in tumor progression.

Résumé

Une tumeur est souvent comparée à une plaie qui ne guérira jamais. En effet, les interactions entre les cellules tumorales et le tissu péritumoral ou stroma, jouent un rôle crucial durant toutes les étapes du développement, de la tumeur primaire à la formation de métastases. De manière similaire aux phénomènes observés lors de la guérison d'une plaie, les fibroblastes du stroma tumoral se différencient en myofibroblastes, ainsi appelés « fibroblastes associés au cancer » (CAF), et ce, principalement en réponse au signal du facteur de croissance transformant-β (TGF-β). Les myofibroblastes promeuvent alors la progression de la tumeur en remodelant le stroma. Parmi les molécules impliquées dans le remodelage du stroma, les métalloprotéinases de la matrice (MMPs), et plus particulièrement MMP-9, jouent un rôle prédominant. Cependant, les mécanismes régulant l'activation et la fonction de MMP-9 restent peu compris. Des études récentes indiquent que l'association de MMP-9 à la surface des cellules tumorales s'avère être un processus important dans son activation, et plus généralement dans la croissance et l'invasion tumorales.

Dans cette étude, nous nous sommes intéressés à la potentielle association entre l'activité de MMP-9 et son recrutement à la surface des fibroblastes humains. Nous avons montré, pour la première fois, que le recrutement de MMP-9 à la surface cellulaire des fibroblastes MRC-5 survient via le domaine fibronectine type II (FN), présent uniquement chez MMP-9 et MMP-2 parmi toutes les MMPs. Des essais fonctionnels suggèrent que tant la pro-forme que la forme active de MMP-9 induisent l'expression de l'α-actine des muscles lisses (αSMA) dans les fibroblastes indifférenciés, reflétant ainsi leur différentiation en myofibroblastes, possiblement via l'activation de TGF-β. De plus, le domaine FN de MMP-9 la séquestre et inhibe ainsi son activité, ce qui engendre une diminution à la fois de l'expression de αSMA et de l'activation de TGF-β.

Des expériences de xénogreffes dans des souris NOD/SCID, utilisant d'une part des cellules d'un fibrosarcome (HT1080) et, d'autre part, celles d'un adénocarcinome mammaire (MDA-MB231), tous deux exprimant le FN domaine de MMP-9, n'ont révélés aucun changement dans la croissance de la tumeur primaire. Néanmoins, dans le

contexte des métastases, l'expression du domaine FN par ces mêmes cellules augmente dramatiquement leur potentiel métastatique et ceci, bien que l'expression de MMP-9 n'induise peu ou pas de changement ou alors réduit le nombre de métastases. Nous avons observé une diminution de l'une des formes actives de MMP-9 dans les cellules MDA-MB231 surexprimant le domaine FN. Ceci suggère que ce domaine pourrait inhiber l'activité de MMP-9 dans ces cellules et de ce fait empêcher l'activation de TGF-β, entraînant ainsi une augmentation de l'invasion. Curieusement, les xénogreffes de cellules d'un adénocarcinome colorectal (SW480) exprimant de manière stable le domaine FN de MMP-9 ont montré une diminution de la croissance de la tumeur primaire ainsi que de la formation de métastases dans les poumons des souris NOD/SCID. Cependant, ces mêmes cellules surexprimant MMP-9 manifestaient une augmentation, à la fois de la croissance de la tumeur primaire, et du nombre de métastases. Une zymographie en gel de gélatine, utilisant le milieu conditionné de ces cellules, a révélé que ces effets pourraient être dû au fait que le domaine FN déplace MMP-9 de la surface des cellules SW480.

Ces observations suggèrent que MMP-9 et son domaine FN peuvent présenter des fonctions divergentes dans le développement de la tumeur primaire, ainsi que dans la formation de métastases en fonction du type cellulaire, soulevant ainsi l'idée d'un potentiel rôle protecteur de MMP-9 face à la progression tumorale.

Introduction

Cancer is the second leading cause of disease related demise worldwide, accounting for 7.6 million deaths in 2008 [*World Health Organization*]. Among cancer patients, metastasis is the principal cause of death [1]. Surgery is the first and major therapeutic strategy to cure cancer, most often in association with chemo- and radiation therapy. However, primary tumors and particularly metastasis are associated with acquisition of resistance to cytotoxic agents that renders the disease incurable with the existing therapeutic arsenal. It is therefore necessary to develop novel and more specific therapeutic strategies to selectively target metastatic cells. One such potentially important approach would be to exploit tumor-host crosstalk events to impair the ability of metastatic cells to survive, grow and invade without affecting the biology of normal cells [1, 2].

Importance of the stroma in tumor development

Tumor progression has been traditionally seen as a multistep process defined by the accumulation of genetic changes, each of which may confer growth and survival advantage required for subsequent dissemination [3]. This cell-centric point of view ignores that cancers are heterogeneous multicellular entities, resembling entire organs, whose growth depends upon reciprocal dynamic interactions between genetically altered transformed cells and the surrounding host tissue microenvironment [4]. Although Paget's "seed and soil" hypothesis dates back to 1889, only in recent years has tumor progression been recognized as the product of an evolving crosstalk between different cell types within the tumor and its surrounding supporting tissue or tumor stroma [5, 6]. The tumor stroma encompasses the extracellular matrix (ECM), diffusible growth factors and cytokines and several non-epithelial cell types, including those that compose the vasculature (endothelial cells, pericytes and smooth muscle cells), the immune system (lymphocytes, macrophages and mast cells) and the supportive and connective tissue (fibroblasts) [7]. All these elements of tumor stroma have been shown to support tumor growth and invasion [4]. Indeed, during epithelial tumor invasion, the breakdown of the basement membrane allows the first and direct contact between tumor cells and stromal cells, which mutually influence each other's behavior and gene expression pattern [8]. A

key feature of tumor-host dynamics is their striking similarity to wound healing; in fact in 1986 Harold Dvorak compared tumors to "wounds that never heal" [9]. Wound healing consists of three overlapping steps: the inflammatory phase that removes bacteria and debris, the proliferative phase characterized by angiogenesis, granulation tissue formation and re-epithelialization, and the remodeling phase in which fibroblasts, and more particularly myofibroblasts, contract and collagen is deposited and cross-linked [10].

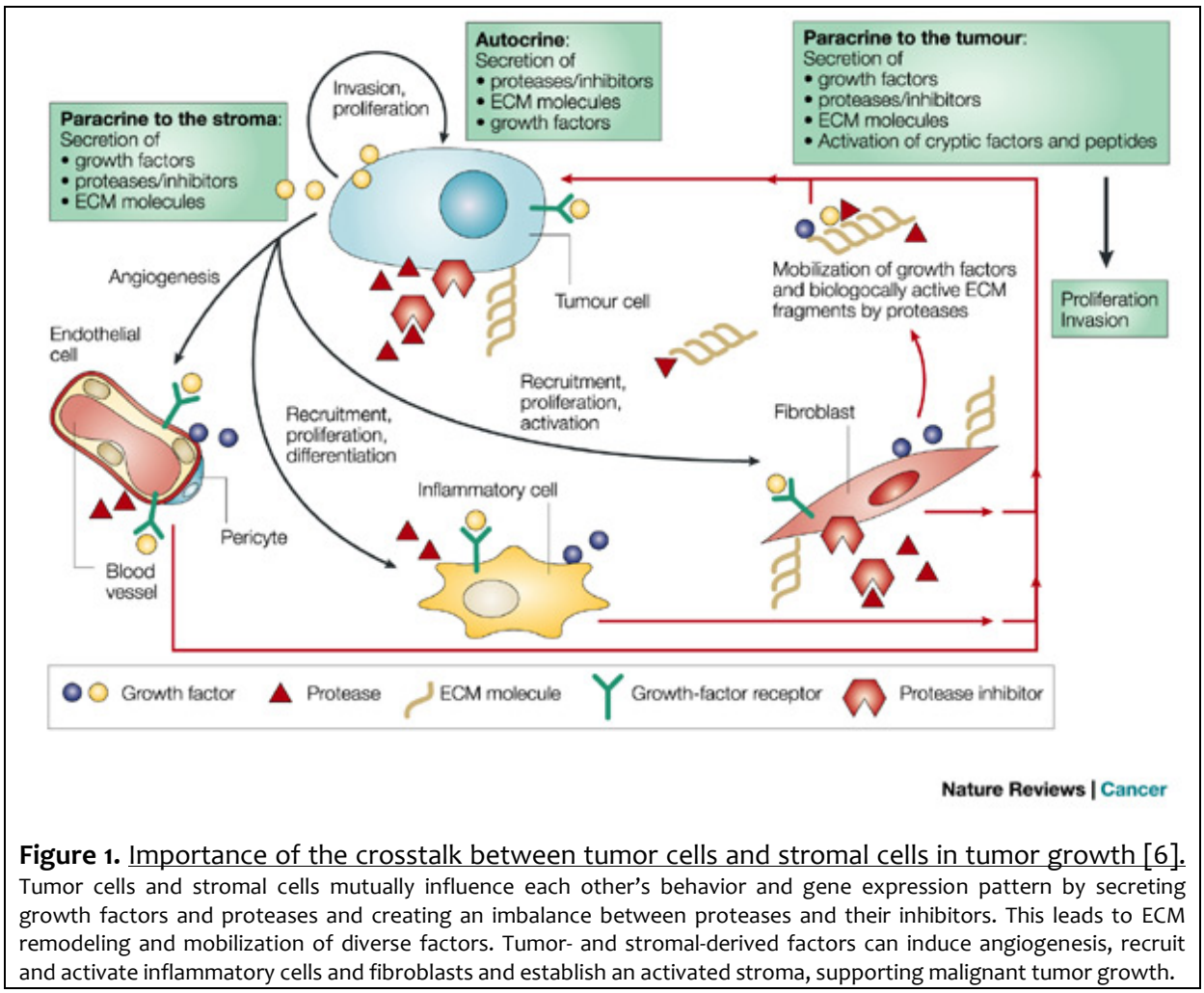


Figure 1. Importance of the crosstalk between tumor cells and stromal cells in tumor growth [6]. Tumor cells and stromal cells mutually influence each other's behavior and gene expression pattern by secreting growth factors and proteases and creating an imbalance between proteases and their inhibitors. This leads to ECM remodeling and mobilization of diverse factors. Tumor- and stromal-derived factors can induce angiogenesis, recruit and activate inflammatory cells and fibroblasts and establish an activated stroma, supporting malignant tumor growth.

Role of fibroblasts and cancer-associated fibroblasts (CAFs) in stroma remodeling

Fibroblasts are the main cells implicated in tissue remodeling associated with injury as well as in the formation of tumor stroma and by regulating ECM turnover they act as prominent modulators of cancer progression. They synthesize constituents of fibrillar ECM (type I, III and V collagen and fibronectin), basement membranes (type IV collagen

and laminin) and growth factors for adjacent epithelial homeostasis [4, 11]. In addition, they are an important source of ECM-degrading proteases, including matrix metalloproteinases (MMPs) [11]. These cells are therefore largely responsible for the deposition of the ECM, regulation of epithelial differentiation and control of inflammation and tissue repair [11]. In the first days following injury, they proliferate, migrate into the wound bed and produce ECM molecules, including collagen and fibronectin and also chemokines and growth factors that attract and activate epithelial cells. Similar to wound healing, the host tissue stroma undergoes major morphological and functional alterations in response to tumor growth, leading to what is commonly referred to as activated stroma, in which fibroblasts differentiate into myofibroblasts also referred to as cancer-associated fibroblasts (CAFs) [6].

Myofibroblasts typically express α-smooth muscle actin (αSMA), vimentin, desmin and fibroblast activation protein (FAP) [4, 6, 11]. In contrast to fibroblasts in wound healing which is a self-limited process, CAFs receive continuous proliferative stimuli from several growth factors including, among others, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), that are secreted by tumor cells and play a role in cancer initiation [6, 11]. In addition, experimental and clinical observations indicate that myofibroblasts, located mainly at the invasion front, can be pro-invasive [12-14]. Indeed, these cells have been shown to secrete various growth factors and cytokines and to induce the expression of serine proteases and MMPs, which promote tumor cell survival, migration, invasion, angiogenesis and lymphangiogenesis and immune and inflammatory cell recruitment [6, 15]. Nevertheless, normal fibroblasts have also been shown to act in host defense against cancer. The crosstalk between cancer- and the surrounding fibroblast stromal-cells, which is mediated, in part, by soluble factors, is essential for the fine tuning of cancer cell invasiveness [16] and targeting cancer associated fibroblasts in combination with chemotherapy has provided promising results toward tumor growth and metastasis control [15].

TGF-β and PDGF are the two key soluble molecules implicated in wound healing. Also secreted by a range of tumor cells, these two growth factors facilitate fibroblast proliferation, migration and production of ECM molecules and are responsible for

fibroblast differentiation into myofibroblast [13]. The role of TGF-β in tumor progression is complex [6, 13, 17]. Indeed, before cancer initiation and during the early stages of carcinogenesis, TGF-β functions as a tumor suppressor by its anti-proliferative and pro-apoptotic effects on target cells. By contrast, during advanced stages of cancer, TGF-β signaling may facilitate epithelial-to-mesenchymal transition (EMT) and promote invasiveness and metastasis [11, 18]. These effects are due, at least in part, to loss of responsiveness by cancer cells to its growth inhibitory effects. TGF-β secreted by tumor cells attracts fibroblasts that differentiate into myofibroblasts [19] whereas PDGF indirectly recruits myofibroblasts by stimulating TGF-β release from macrophages and inducing their proliferation [12, 13, 20]. The dual effect of TGF-β in tumorigenesis and invasion remains to be fully explained and may be both tumor cell and environmental context-dependent.

In response to various stimuli, CAFs induce proliferative signals for adjacent tumor cells by secreting increased amounts of several growth factors and MMPs [11].

MMP-9 : structure, function & roles in cancer

MMPs are zinc-dependent endopeptidases that degrade ECM components, but that are also implicated in the functional regulation of non-ECM molecules such as growth factors and their receptors, cytokines and chemokines, adhesion receptors and cell surface proteoglycans and a variety of enzymes [21, 22]. They thereby control cellular interactions, promote tissue turnover and are regulated by numerous and diverse inhibitors, chief among which are tissue inhibitors of matrix metalloproteinases (TIMPs) [2]. Not surprisingly, elevated expression of many MMPs is associated with tumor progression [23]. Nevertheless, altering their expression levels has shown contradictory effects [24, 25] due to the fact that proteases can be both agonistic and antagonistic for a range of events implicated in tumor progression. This could explain, in part, the failure of MMP inhibitors in clinical trials.

MMP-9, a secreted MMP also known as gelatinase B, has been shown to be a key enzyme in the progression of numerous tumors by playing a pivotal role in tumor cell invasion and angiogenesis [26, 27]. Similar to other MMPs, MMP-9 is synthesized as an inactive zymogen referred to as “proMMP-9”. ProMMP9 is composed of a catalytic domain

containing a fibronectin type-II like (FN) domain or collagen-binding domain, a linker region or hinge domain and a C-terminal domain known as the hemopexin-like (HEX) domain thought to be necessary for MMP inhibitor binding and substrate specificity [2].

The FN domain is found only in MMP-9 and MMP-2 and is composed of three tandem fibronectin type II-like motifs, each of which is characterized by two short antiparallel β-sheets forming a hydrophobic pocket composed of solvent-exposed aromatic residues in the vicinity of the catalytic site. These modules form a collagen-binding domain (CBD), critical for the positioning of substrates for subsequent cleavage [28]. The FN domain of MMP-9 has been shown to bind gelatin [29] but also elastin, native and denatured types I, II, III, IV and V collagens [28, 30]. Each fibronectin type II-like module displays binding specialization, which generated exosites specific for other ligands degraded by the protease [30]. Also, cooperative collagen binding sites between these three modules are certainly present to increase substrate specificity. Thus, they have the potential to localize the enzyme to collagen either in the extracellular matrix or on the cell surface linked to β1-integrins, but their function per se remains unclear [31]. The FN domain of MMP-2 has been studied more deeply and the knowledge gained may provide clues toward understanding of the structure-function relationship of the FN domain of MMP-9. The MMP-2 CBD is required for degradation of denatured type I collagen α-chains, but not for shorter collagen peptide substrates [32]. However, it does not bind native type IV collagen, even though CBD-deletion mutants show reduced native type IV collagen binding and degradation, underscoring the importance of juxtaposed segments or exosites of the catalytic domain in forming the native type IV collagen binding site [31]. Nevertheless, reduction of both gelatinolytic and elastinolytic activity of MMP-2 and MMP-9 occur following deletion of the CBD, confirming its functional importance in binding these substrates. Moreover, MMP-2 CBD can act as a dominant negative for MMP-2-mediated gelatinolysis [33], but also for MMP-2-mediated cleavage of native type I collagen, which inhibits cancer cell migration [34]. In the same way as for MMP-2, deletion of MMP-9 CBD abrogated hydrolysis of type I, V and XI collagens and elastin [35, 36]. On this basis, Xu and coworkers demonstrated that CBDs of MMP-9 and MMP-2 bind the same or closely positioned sites on type I collagen and that they display similar ligand-binding properties to their respective parental enzymes [28]. In addition, the first type II

module of the FN domain of MMP-2 and MMP-9 has been shown to bind long-chain fatty acids, which inhibits both MMP-2- and MMP-9-mediated collagen and elastin degradation [37]. Interestingly, the FN type II-like motifs of MMP-2 compete with MMP-2 binding to native type I collagen-β1-integrin on the fibroblast cell surface and decrease protease activation [38].

The N-terminus of the proform of MMP-9 consists of a propeptide necessary to maintain latency. The cysteine residue at position 99 within the propeptide blocks access to the zinc ion in the active site of the catalytic domain. During a process known as the "cysteine switch", the cysteine loses its coordination with the zinc ion, which triggers the release of the propeptide and results in a fully active enzyme. Activation of MMP-9 can be achieved by autocatalysis, and action of other MMPs (MMP-2 [39, 40]), -3, -7 and -13) or proteases (furin, urokinase, plasmin (but not directly) and trypsin). Interestingly, studies have demonstrated an alternative mechanism for proMMP-9 activation without proteolytic cleavage but via oxidative modification of the cysteine side-chain thiol, which could decrease its ability to serve as an effective ligand to the catalytic zinc ion [41] or via conformational changes induced by binding to substrate [42]. These processes would disengage the propeptide without removing it, leading to an active enzyme. The amino-acid sequence of proMMP-9 contains 3 potential *N*-glycosylation sites (Asn^{38}, Asn^{120} and Asn^{127}) and several *O*-glycosylation sites present in the hinge domain, also named type V collagen-like domain. The role of these carbohydrates still needs to be elucidated and is probably intimately linked to modulation of MMP-9 conformation and function [41, 43, 44].

MMP-9 degrades ECM proteins including decorin, elastin, entactin, fibrillin, fibronectin, laminin, gelatin and collagen types I, IV, V, XI and XVI, but also activates non-ECM molecules, including numerous cytokines exemplified by IGFBP, IL-8, latent TGF-β and TNF-α [31]. MMP-9 expression is low or absent in normal quiescent tissues, but is strongly induced under conditions requiring tissue remodeling such as development, wound healing and tumor invasion. MMP-9 is produced by tumor-associated stromal cells, including endothelial cells, inflammatory cells such as monocytes, macrophages, neutrophils and tumor cells themselves, and is thought to play a key role in both tumor growth and metastasis [2, 26, 45-47]. Indeed, the K14-HPV16 and RIP-Tag mouse models

deficient for MMP-9 display reduced skin and pancreatic carcinogenesis, respectively [26, 27]. In K14-HPV16 mice, this phenotype is rescued by transplanting MMP-9-expressing bone marrow cells to the MMP-9-deficient transgenic mice, thus indicating the importance of inflammatory cell-derived MMP-9 in skin carcinogenesis [26]. Also, MMP-9 appears to promote survival in some tumor types by proteolytic activation of latent TGF-β, which might play a major role in the progression of tumors that are resistant to its growth-inhibitory effects [48]. Moreover, by releasing soluble kit ligand, MMP-9 allows bone marrow repopulating cells to home to a microenvironment that facilitates their differentiation and promotes the reconstitution of the progenitor/stem cell pool [49]. In addition, MMP-9 has been shown to display an immunosuppressive role in cancer by suppressing T cell proliferation through disruption of signaling mediated by IL-2Rα [50]. Importantly, MMP-9 is a functional component of the burst of tumor-associated angiogenesis, known as the angiogenic switch, probably by degrading collagen IV and other ECM components and thus increasing the bioavailability of vascular-endothelial growth factor (VEGF) [2, 27]. Nevertheless, evidence suggests that MMP-9 may also regulate angiogenesis by generating angiogenic inhibitors from substrate cleavage products, including angiostatin from plasminogen [51] and endostatin from basement membrane collagen type XVIII [52], which inhibit endothelial cell proliferation [53]. Moreover, degradation of collagen IVα3 by MMP-9 results in the generation of the monomeric NC1 domain, called tumstatin, which is a potent suppressor of angiogenesis [54].

Secreted MMPs including proMMP-9 bind to the cell surface for both close association with pericellular substrate, regulation of their proteolytic function and protection from natural inhibitors, but the mechanisms enabling their association with the cell membrane remain to be fully elucidated [21, 41, 55]. Nevertheless, shedding of IntraCellular Adhesion Molecule-1 (ICAM-1) has recently been shown to be mediated by MMP-9 and to confer tumor cell resistance to Natural Killer-mediated cytotoxicity [56]. Furthermore, MMP-9 has been shown to use, among others, cell surface hyaluronan receptor CD44 as a docking molecule in TA3 mouse mammary carcinoma and MC melanoma cells. This association stabilizes MMP-9 proteolytic activity at the cell surface in order to degrade collagen IV and to promote invasion [57]. In addition, MMP-9, as well as MMP-2, the other

gelatinase of the MMP family, can cleave and activate latent TGF-β1 and 2 through association with CD44 [58]. Thus, the coordination of CD44, MMP-9 and TGF-β function may provide a physiological mechanism of tissue remodeling that can be adopted by malignant cells to promote tumor growth and invasion. ProMMP-9, and less strongly proMMP-2, concentrates at the cell-ECM interface by binding to the cell surface of various cell lines by a mechanism involving surface-associated α2(IV) chain of collagen IV, a subunit of the basement membrane collagen IV [59]. Finally, low density lipoprotein receptor-related protein (LRP-1) has been shown to be a cell-signaling receptor for MMP-9 that regulates Schwann cell migration in peripheral nervous system (PNS) injury [60]. By contrast, MMP-9 binds to and is negatively regulated by membrane-anchored glycoprotein RECK, which decreases tumor invasion [61, 62]. LRP has also been demonstrated to regulate MMP-9 activity by acting as a functional receptor in order to modulate ECM remodeling [63]. Thus, membrane localization appears to influence MMP-9 activity in a major way even though MMP-9 is naturally secreted [64].

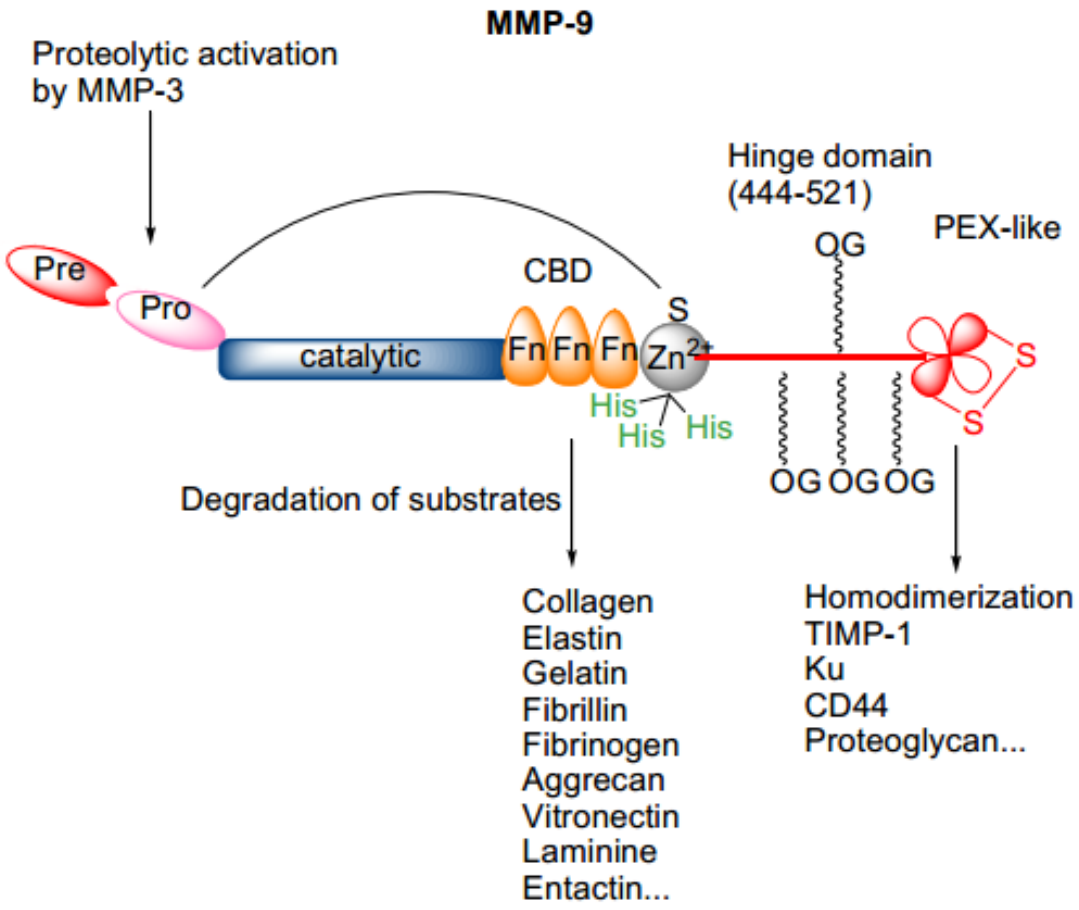


Figure 2. Structure & functions of the different domains of MMP-9 [65]. Pre: signal peptide, Pro: propeptide, FN: FN type II-like motif, CBD: collagen-binding domain, OG: O-glycosylation, PEX-like: hemopexin-like domain. MMP-9 is synthesized as an inactive zymogen, which is activated by proteolytic cleavage (by autocatalysis or other MMPs), triggering the release of the propeptide. The catalytic region contains 3 fibronectin type II-like motifs forming a collagen binding domain necessary for substrate binding. The hinge domain of MMP-9 is highly N- and O-glycosylated and the PEX-like domain is necessary for MMP-9 homodimerization, binding of MMP inhibitors (TIMP-1) and interaction with a variety of cell surface molecules.

Protective roles of MMPs in cancer

MMP regulation and both distinct and overlapping functions in cancer progression are complex and redundant. As mentioned above, certain MMPs, including MMP-9, have revealed discrepant roles in tumor growth, invasion and angiogenesis, exhibiting both pro-tumorigenic as well as beneficial and protective properties at multiple stages of cancer progression, including metastasis [24, 25, 47]. These pro- as well as anti-metastatic roles of MMPs may depend on multiple factors, including the properties of cells that produce them (tumor versus stroma), their pericellular or ECM location, the amounts in which they are secreted, the stage of tumor progression at which their activation or inhibition occurs and the experimental setting. For example, Coussens and coworkers

observed that even if carcinomas in MMP-9-deficient mice were less numerous, the ones that arose were of a more aggressive and higher grade [26]. This suggests that although MMP-9 can enhance tumorigenesis, it may also display inhibitory effects on the progression of these tumors to an invasive state. Indeed, it has been shown to contribute to the early survival and establishment of tumors in the lung and to have little or no effect on subsequent growth [66]. Despite the well described and important role of MMP-9 in promoting angiogenesis, it has also brought to light contrasting functions, particularly by the proteolytic generation of endogenous inhibitors with strong anti-angiogenic properties [47, 67]. Furthermore, a series of experiments performed in integrin α1-null mice, which have increased plasma levels of MMP-9, also highlighted an anti-angiogenic contribution of MMP-9 [68, 69]. Recently, Shchors and coworkers confirmed that in a MMP-9-deficient background, tumors were more invasive due to the homing of cathepsin B expressing CD11b;Gr1-positive cells to invasive fronts [70].

Aim of the thesis

Regulation of MMP-9 activation and activity and its association with the cell surface that appears to be an important mechanism in tumor growth and invasion, remain incompletely understood. The goal of this thesis was therefore primarily to elucidate the potential association of MMP-9 activity with cell-surface recruitment on human fibroblasts, its function and role in these cells and finally its implications in tumor growth and metastasis.

We show for the first time that recruitment of MMP-9 to the MRC5 fibroblast cell surface occurs through the fibronectin-like (FN) domain. Functional assays suggest that the active form of MMP-9 triggers α-smooth muscle actin (αSMA) expression in resting fibroblasts that reflects myofibroblast differentiation, possibly through TGF-β activation. Xenograft experiments in NOD/SCID mice using HT1080 fibrosarcoma or MDA-MD231 breast adenocarcinoma cells stably expressing the FN domain of MMP-9 revealed no changes in primary tumor growth. However, in the context of metastasis, expression of the FN domain by these same tumor cells dramatically increases their metastatic proclivity whereas expression of wt MMP-9 either promotes no change or actually reduces the number of metastases. Curiously, xenografts of SW480 colorectal adenocarcinoma cells

stably expressing the FN domain of MMP-9 display reduced growth at both the primary (subcutaneous) injection site and the lungs of NOD/SCID mice in experimental metastasis assays. This apparent discrepancy is argued to be due to the FN domain that may modulate MMP-9 activity and TGF-β activation in different ways, depending on the tumor cell type. Our observations suggest a dual role of MMP-9 and its FN domain in primary tumor growth and metastasis, highlighting possible protective effects of MMPs in tumor progression and invasion.

Material & Methods

Cell lines

The following cell lines were used: Human Embryonic Kidney (HEK293T), Chinese Hamster Ovary (CHO), osteosarcoma (U2OS), Transformed Mink Lung Epithelial (TMLC), glioblastoma (U251), human skin fibroblasts (HSF), human lung embryonic fibroblasts (MRC-5), fibrosarcoma (HT1080), breast adenocarcinoma (MDA-MB231), colorectal adenocarcinoma (SW480) and breast adenocarcinoma (BT-20) cell lines.

Animals

Female Non-obese diabetic-severe combined immunodeficient mice NOD/SCID mice were obtained from the house breeding of the UNIL mouse facility. Female mice were between 8 and 10 weeks old at the beginning of experiments. Experimental procedures involving mice were approved by the Etat de Vaud, Service Vétérinaire, and authorization n ° 1942.1.

Chemical compounds

4-aminophenylmercuric acetate (APMA) (#164610, Calbiochem), Calcein-AM (#17783, Sigma-Aldrich), Complete Mini-EDTA-free protease inhibitors (#11836170001, Roche), EzBlue Gel Staining Reagent (#G1041, Sigma-Aldrich), FuGene 6 Transfection Reagent (#E2692, Promega), gelatin from porcine skin (#G1890, Sigma), Growth Factor Reduced Matrigel (#356231, BD Biosciences), Protein Deglycosylation Mix (#P603S, BioLabs), SuperSignal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific Pierce), human TGF-β1 (#100-B-001, R&D systems).

Antibodies & beads

The antibodies used in this study were as follow: anti-MMP-9 (#AB19047, Chemicon), anti-αSMA (#A2547, Sigma), anti-tubulin (#CP06, Calbiochem), anti-transferrin receptor (#13-6800, Invitrogen), anti-TGFβ1,2,3 (#MAB1835, R&D Systems), anti-v5 (#R960-25, Invitrogen), donkey anti-mouse Alexa488 (#A21202, Invitrogen), Ni-NTA agarose beads (#30210, Qiagen), Streptavidin-agarose beads (#DAM1467561, Millipore), Anti-v5-agarose

beads (#A7345, Sigma), horseradish peroxidase-(HRP) conjugated sheep anti-mouse (#NA931V, GE healthcare) and goat anti-rabbit (#P0448, DAKO)

Expression constructs

Wild type (wt) proMMP-9 and the different MMP-9 constructs including the catalytic-dead protein containing the E402Q mutation within the catalytic domain, the FN domain composed of the fibronectin type II-like motifs (FN223-389), the hemopexin-homology domain (HEX520-707), the ΔFN or MMP-9Δ223-389 mutant lacking the FN domain, the ΔHEX or MMP-9Δ520-707 mutant lacking the hemopexin-homology domain and CD5 were cloned in pCDM8c and pLIVC vectors, the latter derived from pLVTHM lentiviral vector by the removal of the shRNA cassette and GFP gene and insertion of a PGK-puromycin cassette. All constructs were C-terminally tagged with 6 histidines and v5.

Virus production

60% confluent HEK293T cells in 100 mm dish were transfected with 1.25 µg of pMD2G (envelope plasmid), 3.75 µg of pCMVs (packaging plasmid) and 5 µg of pLIVC (transfer vector) containing MMP-9 or the different mutants using FuGene 6 Transfection Reagent at a ratio 1:3 and incubated at 37°C. Lentiviruses were collected after 48h, passed through 0.45 µm filters and concentrated by ultracentrifugation.

Retroviral infection

Target cells (CHO, U2OS, HT1080, MDA-MB231, SW480, BT-20 and MRC-5) at 40% confluence in 6 wells were washed with PBS and infected in 2 rounds of 8 h of interval with lentiviruses using Polybreene (1:1000) overnight at 37°C. Cells were then washed with PBS and transferred in a 100 mm dish with fresh medium. The day after, cells were selected with puromycin (1 µg/µl for CHO, U2OS, HT1080 and MDA-MB231, 6 µg/µl for SW480, 0.5 µg/µl for SW620, 0.5 µg/µl for BT-20 and 2 µg/µl for MRC-5).

His-tag purification

Stable transfectants of each his-tagged construct were established in U2OS and CHO cells. Purification was performed using the histidine tag and high affinity nickel-beads as

follow: the supernatant of CHO cells provided by the Evitria company (Zürich) was incubated with Ni-NTA agarose beads (2 mL of beads for 1 L of sample), which were then washed with PBS and in Washing solution (5 mM imidazole, 20 mM Tris-HCl pH 7.5 and 200 mM NaCl). Purified proteins were eluted in 20 mM and 200 mM imidazole and fractions were concentrated with Amicon centrifugal filters (Millipore) depending on the molecular weights (50'000 Nominal Molecular Weight Limit (NMWL) for proMMP-9 and ΔFN, and 3'000 NMWL for FN). Protein concentration was determined according to BSA by densitometry using Image J.

ProMMP-9 activation

The activation of proMMP-9 was performed directly on Ni-beads using APMA. 35 mg of APMA was dissolved in 10 mL of 0.1 M NaOH and diluted in TTC reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ and 0.05% Triton X-100) to obtain a 2.5 mM solution. ProMMP-9 bound to Ni-NTA agarose beads was incubated with this solution at 37°C for 3h and eluted as described before.

Recruitment assay

Various tumor cell lines and fibroblasts were incubated overnight at 37°C with filtered conditioned medium from U2OS cells stably expressing recombinant MMP-9, its different mutants or CD5. The target cells that we tested were HEK293T, HT1080, TMLC, U251, U2OS, MDA-MB231, HSF or MRC-5. The day after, cells were lysed using lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100) containing Complete Mini-EDTA-free protease inhibitors. Immunoblotting of conditioned medium (inputs) and cell lysates was performed using anti-v5 antibody and Image J program was used for quantification of the recruitment percentage.

Cell fractionation

Cells grown in 2x150 mm dishes until 60 to 70% confluency were washed and scraped in cold PBS and centrifuge for 5 min at 300xg at 4°C. The membranes were sensitized by resuspending cell pellets with 1 mL of Homogenization Buffer (HB) (250 mM sucrose, 3 mM imidazole, phosphatase and protease inhibitor cocktails, pH 7.4). Post nuclear

supernatant (PNS) was obtained by mechanical disruption of cells with a 22G needle and centrifugation for 10 min at 600xg at 4°C. PNS was subjected to ultracentrifugation for 45 min at 100'000xg at 4°C in order to separate cytosol (supernatant) from membrane (pellet) fractions. Membranes were washed twice with HB and solubilized using lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100) containing Complete Mini-EDTA-free protease inhibitors.

Western Blot

Western Blotting was performed according to standard procedures. The following antibody concentrations were used: anti-v5 (1:5'000), anti-transferrin receptor (1:1'000), anti-SMA (1:5'000), anti-tubulin (1:4'000), anti-MMP-9 (1:5'000), horseradish peroxidase-(HRP) conjugated sheep anti-mouse (1:20'000), goat anti-rabbit (1:20'000). ECL was reveled using SuperSignal West Pico Chemiluminescent Substrate.

Immunofluorescence

MRC-5 fibroblasts were grown on glass coverslips until confluency. Cells were treated with proMMP-9, FN, E402Q, ΔFN and CD5, and were incubated with anti-v5 antibody (1:1'500) for 1h at 4°C, washed with PBS, and further incubated with secondary anti-mouse Alexa488 antibody (1:1'500) for 1h at 4°C. Antibodies were diluted in blocking buffer (PBS-FCS 10%). Cells were then fixed with 4% PFA for 20 min at room temperature, washed with PBS and mounted using Immuno-Mounting. DAPI (Roche) was used to visualize the nuclei. Images were acquired with a Leica SP5 AOBS confocal microscope.

Immunoprecipitation

Confluent MRC-5 cells in 25 cm dish were treated with 2 µg of both wt proMMP-9 and v5-tagged FN or only proMMP-9 overnight at 37°C. MRC-5 were lysed with lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100) and 1 mL of supernatant and 2 mg of cell lysates were pre-cleared with streptavidin-agarose beads for 1h at 4°C and then immunoprecipitated with anti-v5-agarose beads overnight at 4°C. Beads were washed 5 times with lysis buffer and proteins were eluted by boiling the beads for 5 min in sample

buffer. Purified complexes were analyzed by Western blotting using anti-MMP-9 antibody.

Luciferase assay

The Luciferase assay system (#E1501, Promega) was used according to manufacturer's instructions. Briefly, TMLC transfected with the plasminogen activator inhibitor-1 (PA-1) promoter responsive to TGF-β and linked to a luciferase reporter system were plated at $3x10^5$ cells/mL in 24 wells for 6h. MRC-5 conditioned medium collected after 3 days were incubated with TMLC at 37°C for 20h. Cells were then washed with PBS and lysed with 1x Lysis Buffer for 20 min on ice. 20 uL of cell lysates was mixed with 90 uL luciferase substrate. Luminescence was read at 570 nm with Synergy MX luminometer during 2 seconds with autosensitivity.

NOD/SCID mice xenotransplantation

To look at primary tumor growth, MDA-MB231 (10^6 cells), HT1080 ($2x10^5$ cells) or SW480 ($3x10^5$ cells) cells stably expressing proMMP-9 or its FN domain were subcutaneously injected in both flanks of female NOD/SCID mice (5 mice per condition) and primary tumor was weighted after 5 weeks (MDA-MB231), 18 days (HT1080) or 7 weeks (SW480). For experimental metastasis assays, MDA-MB231 ($5x10^5$ cells), HT1080 ($1x10^6$ cells) or SW480 ($2x10^6$ cells) cells stably expressing MMP-9 or its FN domain were injected in the tail vein of female NOD/SCID mice (5 mice per condition) and lung colonization was assessed after 18 days (HT1080), 21 days (SW480) or 35 days (MDA-MB231) by measuring the percentage of lung covered by metastases.

Gelatin zymography

8% polyacrylamide gels were prepared with 0.1% gelatin (1 mg/mL). Cells were starved for 24 to 48h. Supernatants were collected, concentrated with Amicon centrifugal filters 50'000 NLWM and incubated in Zymogram sample buffer (1 g SDS, 0.4 g sucrose, 5 mL 1.5 M Tris-HCl pH 8.8, 10 mg Bromophenol Blue in water to 10 mL) for 10 min at room temperature and loaded on gels. Gels were then incubated in Zymogram Renaturing Buffer (2.5% Triton X-100 in water) for 30 min and equilibrated with Zymogram Developing

Buffer (50 mM Tris-HCl pH 8, 5 mM $CaCl_2$, 0.2% Azide) for 30 min at room temperature and at 37°C overnight. Gels were stained with EzBlue Gel Staining Reagent.

Deglycosylation

SW480 cells expressing MMP-9 or the FN domain were plated in 25 cm dishes and starved for 48h. Conditioned medium was collected, concentrated with Amicon NMWL. Deglycosylation was performed using Protein Deglycosylation Mix in non-denaturing reaction conditions following manufacturer's instructions. Briefly, 40 µl of conditioned medium was incubated with 5 µl of 10x G7 Reaction Buffer and 5 µl of Deglycosylation Enzyme Cocktail at 37°C for 4h. Fetuin was used as deglycosylated control protein. 40 µl of the deglycosylated mix was incubated in zymogram sample buffer and gelatin zymography performed.

Migration & invasion assays

Test cells (MDA-MB231 expressing MMP-9, FN, ΔFN or control cells) were starved overnight in DMEM supplemented with 0.2% BSA. Cells were harvested, washed in PBS, resuspended in serum-free medium containing 0.2% BSA and seeded to 24-well format cell culture inserts with 0.8 µm pore sizes (previously coated with 300 µg/mL of Growth Factor Reduced Matrigel diluted in Coating solution (0.01M Tris-HCl pH 8.0, 0.7% NaCl) for invasion assays). 600 µL of culture medium with or without TGF-β1 (10 ng/µL) and 200 µL of cell suspension were added to each well and insert, respectively, and incubated at 37°C in a 5% CO_2 incubator. After 24h, cell culture medium was replaced with 450 µL of serum free DMEM containing 2 µM of Calcein-AM in each bottom well and incubated for 45 min at 37°C. Fluorescence was measured at an absorbance of 494 nm and an emission of 517 nm in order to determine migration or invasion percentage.

Statistical analysis

Graphs and statistical analysis were carried out using GraphPad Prism 5.0® software. Two-tailed, unpaired t-test was used for lung histomorphometry (lung percentage covered by metastasis), primary tumour weight, MTT assays, adhesion, migration and

invasion assays, comparing two groups. Results represent mean values +/- SEM (standard error of mean) in all graphs.

MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (#G4000, Promega) following manufacturer's instructions. Briefly, 7'000 MDA-MB231 and SW480 cells were plated in 96-well with or without TGF-β1 [10 ng/µl]. After overnight incubation at 37°C, 15 µl of Dye Solution were added to each well and the plate was further incubated for 4h at 37°C. The reaction was stopped by adding 100 µl of Solubilization Solution and incubating for 1h at 37°C. Absorbance was read at 570 nm and 650 nm (reference wavelength) with Synergy MX Monochromator-Based Multi-Mode Microplate Reader.

Adhesion assays

Test cells were resuspended to a final concentration of $5x10^6$ cells/mL in serum-free medium and incubated with 2 µM of Calcein-AM for 30 min at 37°C. Cells were then washed with prewarmed 37°C serum-free medium and incubated on different ECMs containing MMP-9, the FN domain or control at 37°C in 5% CO_2 incubator for 5 to 60 min. Non-adherent cells were washed several times with PBS and then incubated in serum-free medium. Measurement was performed at an absorbance of 494 nm and an emission of 517 nm. Adhesion percentage was calculated by dividing the adjusted fluorescence of adherent cells by the adjusted fluorescence of total cells added to each well and multiplying by 100.

Results

ProMMP-9 is recruited to the fibroblast cell surface

ProMMP-9 recruitment to the tumor cell surface has been shown to be important in promoting invasion and metastasis of at least some tumor cell types. Maintenance, even transient, of MMP-9 on the surface of tumor cells is believed to protect its proteolytic activity from inhibition by natural inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and to provide cells with the ability to degrade the ECM in controlled fashion [41]. One mechanism of MMP-9 recruitment to the tumor cell surface is provided by CD44 [57]. However, it is unclear whether CD44 is the only such mechanism and whether normal stromal cells that play a key role in facilitating tumor invasion, may also possess mechanisms that immobilize MMP-9 on their surface. To address this issue, we compared proMMP-9 recruitment to a variety of tumor cell lines and fibroblasts. We incubated the U2OS osteosarcoma cell line and human fetal lung MRC-5 fibroblasts in conditioned medium from U2OS cells engineered to constitutively express and secrete recombinant v5-tagged proMMP-9 or CD5, used as an unrelated control protein. Following overnight incubation at 37°C, cells were lysed and v5-tagged protein recruitment was assessed by anti-v5 antibody blot analysis. We observed that the proMMP-9 protein but not CD5 was recruited to both cell types, supporting the notion that the observed recruitment is specific to proMMP-9 (*Figure 1A*). Moreover, proMMP-9 was more abundantly recruited to MRC-5 than to U2OS cells.

Next, we tested whether proMMP-9 is preferentially recruited to fibroblasts and in which cell compartment the recruitment occurs. We therefore incubated different tumor cell lines, including HEK293T (Human Embryonic Kidney), HT1080 (fibrosarcoma), TMLC (Transformed Mink Lung Epithelial), U251 (Glioblastoma), U2OS (Osteosarcoma), MDA-MB231 (Breast Adenocarcinoma) and HSF (Human Skin Fibroblasts) as well as MRC-5 (Human fetal Lungs fibroblasts) in U2OS-conditioned medium containing v5-tagged proMMP-9. Following overnight incubation, cell membranes were isolated by cell fractionation and protein recruitment assessed by anti-v5 antibody blot analysis. We observed that proMMP-9 is more strongly recruited to fibroblasts (HSF and MRC-5)

whereas the different tumor cell lines displayed weak or no proMMP-9 recruitment (*Figure 1B*). Moreover, cell fractionation analysis revealed that proMMP-9 recruitment occurs to fibroblast membranes. ProMMP-9 therefore appears to be more abundantly recruited to the fibroblast cell surface than to that of tumor cell lines, bearing in mind that we tested a limited panel of cells and that there may well be tumor cells that behave similarly to fibroblasts with respect to MMP-9 recruitment.

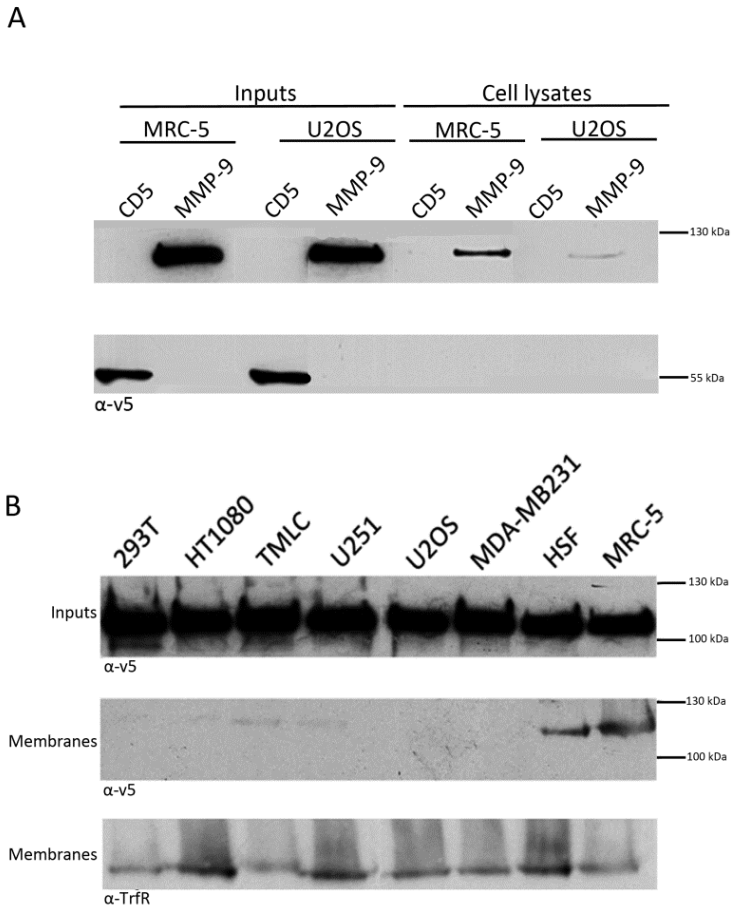


Figure 1. ProMMP-9 is more strongly recruited to the fibroblast than to the tumor cell surface; **A.** ProMMP-9 is selectively recruited to MRC-5 fibroblasts and U2OS osteosarcoma cells. Conditioned medium from U2OS cells constitutively expressing recombinant v5-tagged proMMP-9 or CD5 (*inputs*) and equal amounts of U2OS and MRC-5 cell lysates (*cell lysates*) were loaded. Representative anti-v5 immunoblot of MRC-5 and U2OS cell lysates from 3 independent experiments is shown. **B.** ProMMP-9 is more strongly recruited to the MRC-5 cell surface than to the surface of tested tumor cell lines. U2OS-conditioned medium containing v5-tagged proMMP-9 (*inputs*) and equal amount of membrane fractions (*membranes*) were loaded. Representative anti-v5 immunoblot of membrane preparations from indicated tumor cell lines, HSF and MRC-5 fibroblasts from 3 independent experiments is shown. Transferrin receptor (*TrfR*) was used as a membrane equal loading control.

The FN domain of MMP-9 is necessary and sufficient for its recruitment to the fibroblast cell surface

ProMMP-9 recruitment to tumor cell lines is thought to occur through the hemopexin (HEX) domain [46]. Accordingly, we wanted to determine which domain of MMP-9 mediates the recruitment to the fibroblast cell surface. Thus, we used wild type recombinant proMMP-9 and engineered a series of deletion mutants corresponding to different MMP-9 domains. Each construct was tagged with sequences encoding 6 histidines and the v5 peptide. The mutants included a catalytic-dead protein containing the E402Q mutation within the catalytic domain, ΔFN, lacking the FN domain, ΔHEX, lacking the hemopexin-homology domain, FN, composed of the fibronectin type II-like motifs (FN223-389) and HEX, composed of the hemopexin-homology domain (HEX520-707). All mutants were inserted into the pLIVC retroviral vector and stably expressed in Chinese Hamster Ovary (CHO) cells (*Figure 2*).

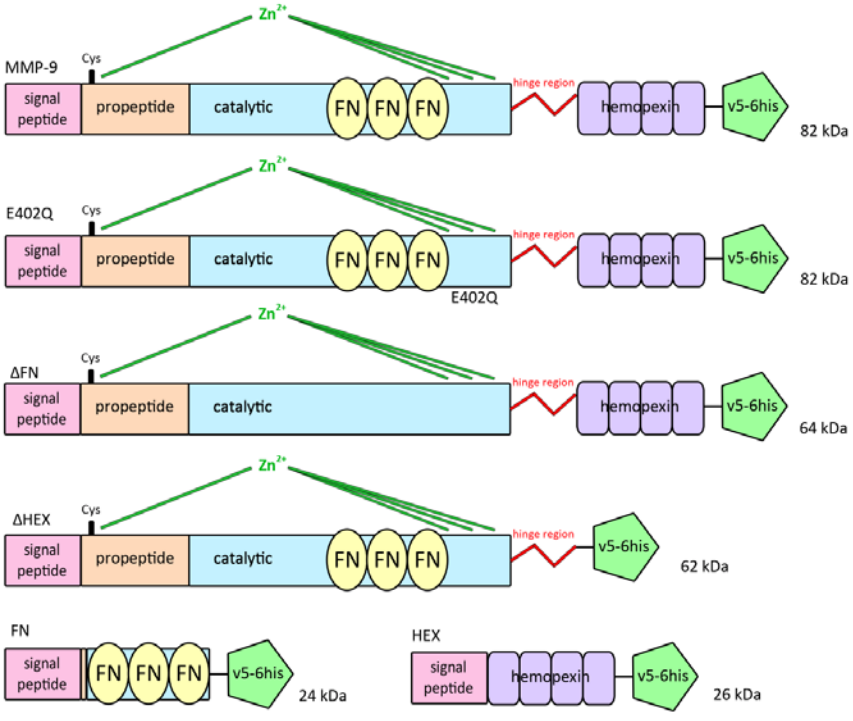


Figure 2. MMP-9 and its different mutants; Schematic vue of wild type proMMP-9, the catalytic-dead protein carrying E402Q mutation within the catalytic domain, ΔFN, lacking the FN domain, ΔHEX, lacking the hemopexin-homology domain, FN, composed of the fibronectin II domain (FN223-389) and HEX, composed of the hemopexin-homology domain (HEX520-707). All constructs were C-terminally tagged with 6 histidines and v5 peptide. All mutants were inserted into the pLIVC retroviral vector and stably expressed in CHO cells.

Each mutant was tested for its ability to be recruited to fibroblasts by incubating MRC-5 cells in the corresponding CHO conditioned medium overnight at 37°C and performing subsequently immunofluorescence and Western blot analysis of intact cells and MRC-5 lysates, respectively. Immunofluorescence analysis using anti-v5 antibody showed that both proMMP-9 and the inactive mutant E402Q were recruited to the MRC-5 cell surface. The FN domain alone was also recruited whereas constructs lacking the FN motifs, including ΔFN and CD5, were not (*Figure 3A*). Anti-v5 antibody blot analysis of cell lysates confirmed the observation that proteins encoded by constructs containing the FN domain (proMMP-9, E402Q, FN and ΔHEX) were recruited to MRC-5 fibroblasts whereas mutants lacking the FN domain (ΔFN and HEX) were not (*Figure 3B*). The observation that ΔFN is weakly detected on the fibroblast cell surface may be explained by the presence of the HEX domain, which is considered to be the "recruitment" domain [46], at least in tumor cells, or some component of its flanking sequences, given that the HEX domain alone showed no recruitment at all to MRC-5 fibroblasts. We also observed that the FN domain appears to display higher affinity for MRC-5 cells than wt proMMP-9. This observation convincingly indicates, for the first time, that the FN domain of MMP-9, which is known to bind gelatin [29], recognizes structures on the fibroblast cell surface.

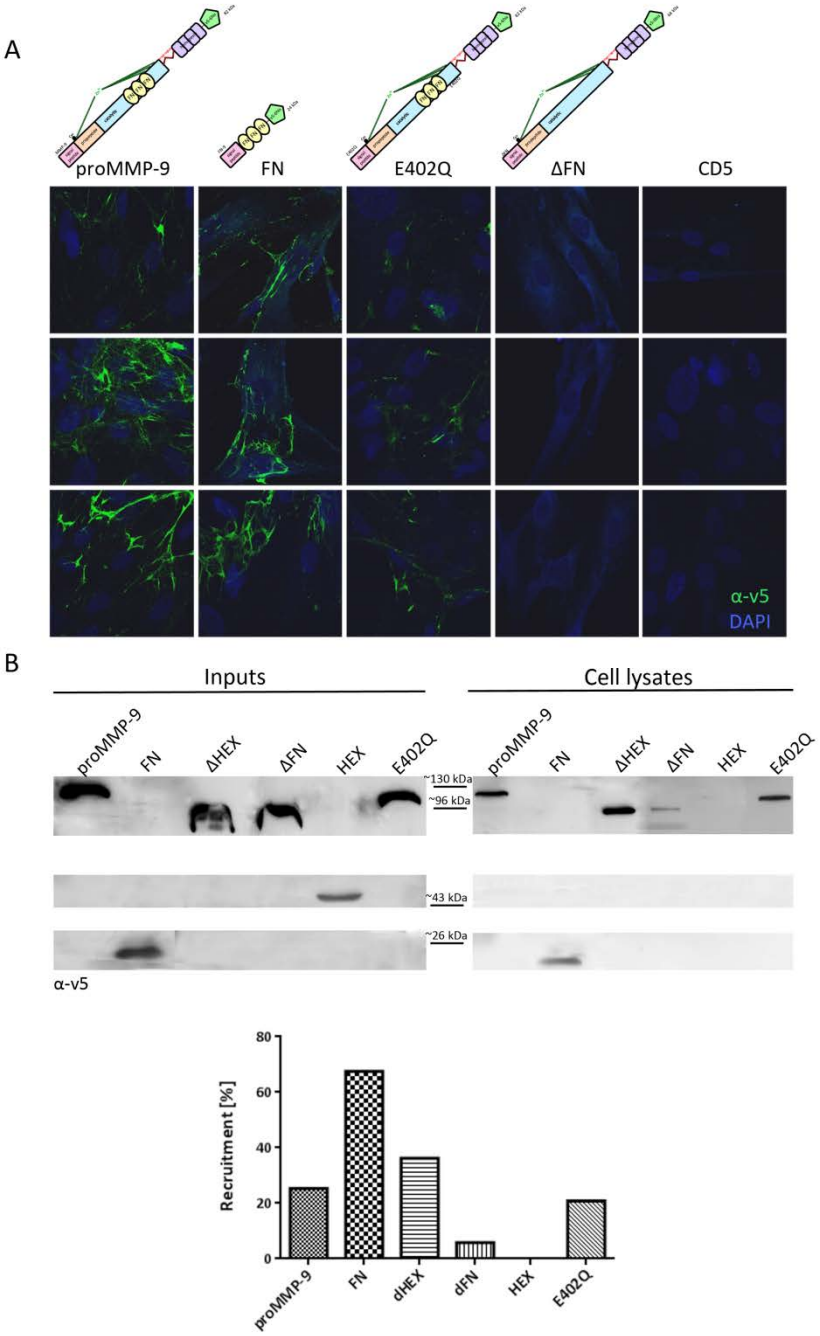


Figure 3. The FN domain of MMP-9 is necessary and sufficient for its recruitment to the fibroblast cell surface; **A.** Conditioned medium from U2OS cells constitutively expressing recombinant v5-tagged proMMP-9 or the different mutants (*proMMP-9, FN, E402Q and ΔFN*) and CD5, used as negative control, were incubated with MRC-5 cells and live anti-v5 antibody immunofluorescence was performed. Note that only the mutants containing the FN domain (proMMP-9, FN and E402Q) are recruited to the MRC-5 cell surface. DAPI (blue) was used to visualize nuclei. **B.** Conditioned medium from U2OS cells constitutively expressing recombinant v5-tagged proMMP-9 or the different mutants (*proMMP-9, FN, ΔHEX, ΔFN, HEX and E402Q (inputs)*) were incubated with MRC-5 cells and equal amount of MRC-5 cell lysates were loaded. Representative anti-v5 immunoblot of MRC-5 cell lysates from 3 independent experiments shows that the mutants containing the FN domain (proMMP-9, E402Q, FN and ΔHEX) are recruited to MRC-5 cell surface whereas those lacking the FN domain (ΔFN and HEX) are not. Recruitment percentage corresponds to the ratio of v5-tagged protein recruited (cell lysate) to the incubated v5-tagged protein (input).

MMP-9 activity promotes latent TGF-β activation and induces αSMA expression in resting fibroblasts

To address the molecular mechanism that underlies MMP-9 recruitment to the fibroblast cell surface, we first determined whether the active form of MMP-9 was also recruited to the MRC-5 cell surface. Thus, recombinant proMMP-9 from conditioned culture medium of stably transfected CHO cells was activated using 4-aminophenylmercuric acetate (APMA) on Ni-beads during the His-tag purification step. Incubation of MRC-5 cells with proMMP-9, the active form of MMP-9 and ΔFN and subsequent anti-v5 antibody blot analysis of cell lysates revealed that both pro- and active MMP-9 are recruited to the fibroblast cell surface (*Figure 5A*). This observation suggests that the proteolytically active form of MMP-9 may be retained at the fibroblast cell surface as a result of the interaction between its FN domain and, as yet unidentified, cell surface ligands.

MMP-9 has been shown to play a key role in tumor growth and invasion by activating latent TGF-β in a functional complex with CD44 at the keratinocyte cell surface [57]. Hence, we asked whether the presence of proMMP-9 and its active form might induce TGF-β activation in MRC-5 conditioned culture medium. Accordingly, we performed a TGF-β functional assay using Transformed Mink Lung Epithelial Cells (TMLC) stably transfected with the plasminogen activator inhibitor-1 (PA-1) promoter which is responsive to active TGF-β and linked to the luciferase reporter gene. We treated MRC-5 fibroblasts for 3 days with purified proMMP-9, its different mutants or TGF-β1 (10 ng/mL) used as positive control. The mutants included: proMMP-9, the active form of MMP-9, the catalytically inactive mutant E402Q and ΔFN (*Figure 4*). The corresponding MRC-5 conditioned media were used for luciferase reporter assays in TMLC. We observed that both proMMP-9 and its active form induce TGF-β activation in resting MRC-5 fibroblasts, whereas the inactive mutants E401Q and ΔFN have no effect on TGF-β activity (*Figure 5B*). This observation confirms that TGF-β activation is specifically induced by MMP-9 activity.

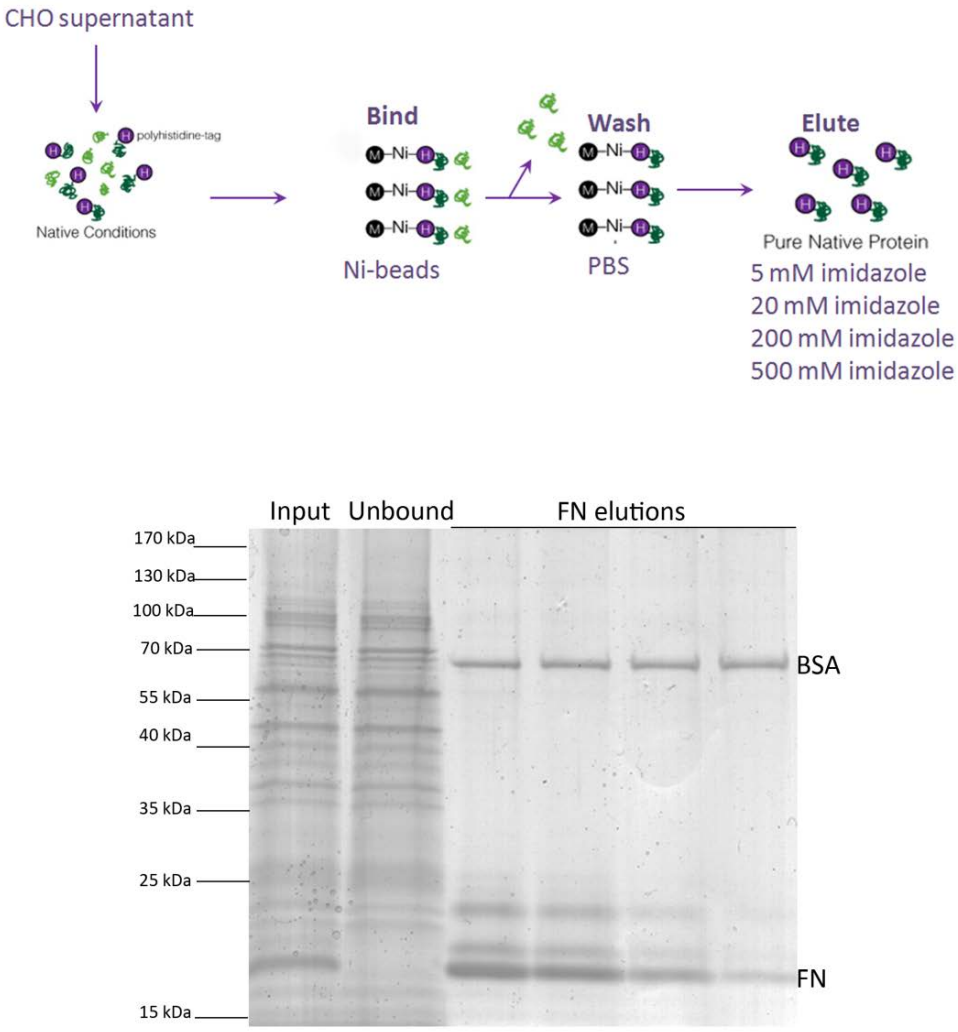


Figure 4. Purification of MMP-9 mutants; **A.** His-tag purification protocol used for proMMP-9 and its different mutants. 1 L of CHO supernatant was incubated with Ni-beads for binding. Beads were washed with PBS and 5 mM imidazole and eluted with increased imidazole concentrations. Purified fractions were concentrated with Amicon filters. **B.** Example of FN-his-purification. The protein concentration was quantified by densitometry relative to BSA concentration with Image J.

TGF-β is an essential factor for the differentiation of fibroblasts into myofibroblasts [19]. Therefore, we addressed the possibility that MMP-9 activity at the surface of MRC-5 cells might induce their differentiation into myofibroblasts. Differentiation was assessed by incubating resting MRC-5 cells for 3 days with purified proMMP-9, the mutants including proMMP-9, the active form of MMP-9, the catalytically inactive mutant E402Q and ΔFN, or TGF-β1 (10 ng/mL). The cells were then lysed and αSMA expression, a well-known myofibroblast marker that is weakly expressed in MRC-5 cells, was assessed. We observed that both pro- and the active forms of MMP-9 led to an increase in αSMA expression in resting MRC-5 fibroblasts (*Figure 5C*). By contrast, E402Q and ΔFN mutants did not induce αSMA expression. These observations support the notion that MMP-9

activity promotes differentiation of fibroblasts into myofibroblasts, as illustrated by the induction of αSMA expression in resting MRC-5 fibroblasts.

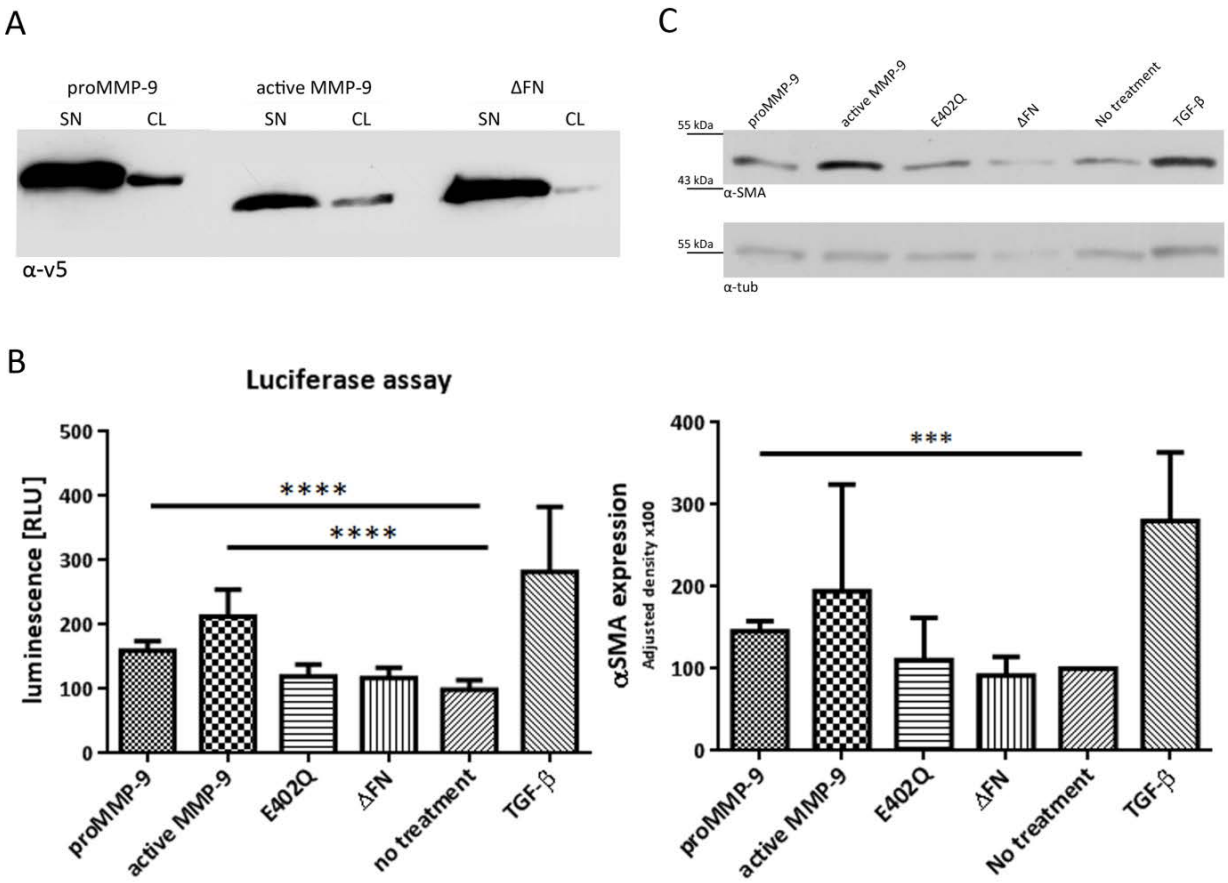


Figure 5. MMP-9 activity promotes latent TGF-β activation and induces αSMA expression in resting fibroblasts; **A.** The active form of MMP-9 is recruited to fibroblast cell surface. Conditioned medium (*SN*) containing 5 ug of purified pro- or active MMP-9 or ΔFN and equal amount of corresponding cell lysates (*CL*) were loaded. Anti-v5 immunoblot was used to show recruitment to the MRC-5 cell surface. **B.** Both pro- and active MMP-9 promote TGF-β activation in resting MRC-5 cells. Conditioned medium from MRC-5 treated for 3 days with 5 ug of proMMP-9, the active form of MMP-9, E402Q, ΔFN or TGF-β1 (10 ng/mL) were collected for luciferase assays using TGF-β-responsive TMLC cells. Luminescence reflecting TGF-β activity was defined by relative light units (RLU). Note the specificity of MMP-9 activity for TGF-β activation. ******** P < 0.0001 **C.** Pro- and active MMP-9 induce αSMA expression in resting MRC-5 cells. MRC-5 were treated for 3 days with proMMP-9, the active form of MMP-9, E402Q, ΔFN or TGF-β1 (10 ng/mL). Representative anti-αSMA immunoblot of equal amount of MRC-5 cell lysates from 4 independent experiments (*upper panel*) is shown. Analysis of αSMA expression from 4 independent experiments (*lower panel*) is shown. ******* P < 0.001

The FN domain behaves as competitive inhibitor of MMP-9 and decreases both TGF-β activation and αSMA expression in resting fibroblasts

Given that the FN domain of MMP-9 is necessary and sufficient for MMP-9 recruitment to the fibroblast cell surface, we investigated whether the FN domain might act as a dominant negative and therefore inhibit MMP-9-induced TGF-β activation in the

supernatant of MRC-5 fibroblasts. We therefore treated MRC-5 cells for 3 days with MMP-9 and the different mutants including, proMMP-9, the FN domain only (*FN*), ΔFN, proMMP-9 with an anti-TGF-β antibody (*proMMP-9: αTGF-β*), proMMP-9 with an excess of the FN domain (*proMMP-9: FN 1:10*) or TGF-β1 (10 ng/mL) as positive control. The corresponding supernatants were collected for luciferase reporter assays. We observed that the FN domain alone (*FN*) and ΔFN have no effect on TGF-β activation (*Figure 6A*). However, an excess of the FN domain in the presence of proMMP-9 (*proMMP-9: FN 1:10*) significantly abrogated TGF-β activation - almost as strongly as an anti-TGF-β antibody (*proMMP-9: αTGF-β*). This suggests that exogenously added FN domain of MMP-9 can inhibit MMP-9 activity, as measured by TGF-β activation at the fibroblast cell surface.

We next asked whether inhibition of TGF-β activation by the FN domain could prevent αSMA expression in resting fibroblasts, which would reflect absence of their differentiation into myofibroblasts. As described above, we treated MRC-5 fibroblasts for 3 days with proMMP-9, the FN domain only (*FN*), ΔFN, proMMP-9 in the presence of anti-TGF-β neutralizing antibody (*proMMP-9: αTGF-β*), proMMP-9 in the presence of an excess of the FN domain (*proMMP-9: FN 1:10*) or positive control TGF-β1 (10 ng/mL). Cells remained viable after the 3 days of treatment and cell lysis was performed to assess αSMA expression. We demonstrated that αSMA expression in MRC-5 treated with the FN domain alone (*FN*) and ΔFN was not different than in untreated MRC-5 (*Figure 6B*). However, the FN domain added in excess in presence of proMMP-9 (*proMMP-9: FN 1:10*) significantly blocked αSMA expression, even more potently than the neutralizing anti-TGF-β antibody (*proMMP-9: αTGF-β*). The fact that MMP-9-induced αSMA expression can be inhibited by neutralizing anti-TGF-β antibody indicates that MMP-9-mediated differentiation of MRC-5 into αSMA-expressing myofibroblasts occurs through the TGF-β pathway. Moreover, abrogation by the FN domain of the ability of proMMP-9 to induce αSMA expression in resting fibroblasts suggests that the FN domain can inhibit MMP-9 activity at the fibroblast cell surface. Indeed, MMP-9 activity at the fibroblast membrane may be needed for TGF-β activation and its downstream signaling effects. These data clearly demonstrate the ability of the FN domain to inhibit MMP-9 activity at the

fibroblast cell surface, as measured by TGF-β activation and αSMA expression.

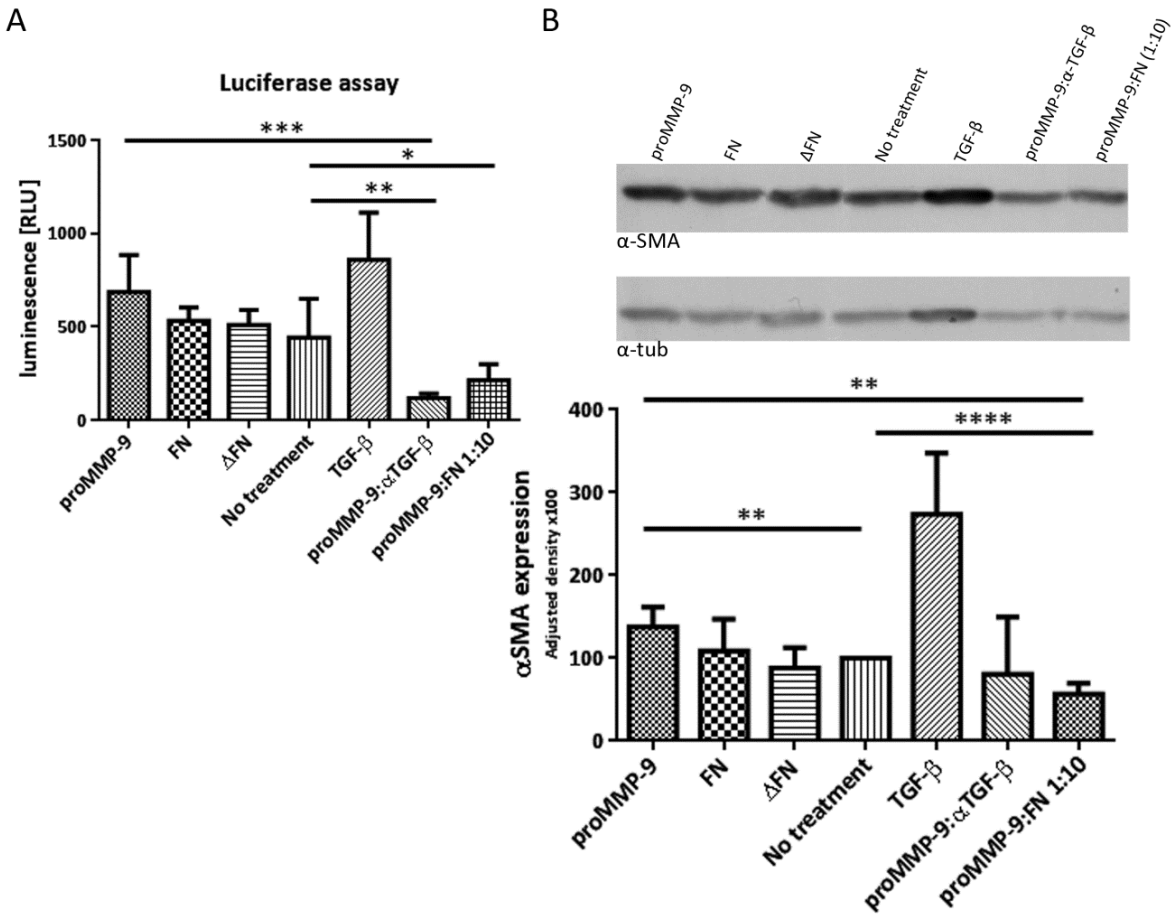


Figure 6. The FN domain behaves as a competitive inhibitor of proMMP-9 blocking both TGF-β activation and αSMA expression in resting fibroblasts; **A.** Recombinant FN domain abrogates MMP-9-induced TGF-β activation in MRC-5 cells. MRC-5 conditioned media incubated for 3 days with proMMP-9, FN, ΔFN, proMMP-9 with an anti-TGF-β antibody (*proMMP-9: αTGF-β*), proMMP-9 with an excess of the FN domain (*proMMP-9: FN 1:10*) or TGF-β1 (10 ng/mL) were applied to luciferase assays using TGF-β-responsive TMLC cells. Luminescence reflecting TGF-β activity was defined by relative light units (RLU). * P < 0.05, ** P < 0.01, *** P < 0.001. **B.** Recombinant FN domain blocks MMP-9-dependent αSMA expression in resting MRC-5. MRC-5 were incubated for 3 days with 1 ug of proMMP-9, the FN domain, ΔFN, proMMP-9 with an anti-TGF-β antibody (*proMMP-9: αTGF-β*), proMMP-9 with an excess of the FN domain (*proMMP-9: FN 1:10*) or TGF-β1 (10 ng/mL). Representative anti-αSMA immunoblot of equal amount of MRC-5 cell lysates from 4 independent experiments (*upper panel*) is shown. Analysis of αSMA expression from 3 independent experiments (*lower panel*) is indicated. * P < 0.05.

The FN domain does not displace proMMP-9 from the fibroblast cell surface but sequesters it in MRC-5 conditioned culture medium

Because the FN domain of MMP-9 inhibits both MMP-9-induced TGF-β activation and αSMA expression, we asked whether it might compete with MMP-9 activity at the fibroblast cell surface. In other words, we addressed the possibility that the molecular mechanism whereby the FN domain inhibits MMP-9 might involve displacement of MMP-

9 activity from the MRC-5 cell surface. To this end, MRC-5 fibroblasts were incubated with purified proMMP-9 and increasing concentrations of the FN domain (1:1, 1:2, 1:5, 1:1 and 1:20) following which the cells were lysed and recruitment assessed. We observed that the FN domain cannot displace proMMP-9 from the fibroblasts, even when it was added in excess (20 times more than proMMP-9). We then confirmed this observation by incubating MRC-5 fibroblasts with MMP-9 and FN at the same ratios and assessing their recruitment in membrane fraction (*Figure 7A*). Our observations suggest that the FN domain inhibits MMP-9-induced-TGF-β activation and αSMA expression by a mechanism other than displacement of proMMP-9 from the cell surface.

Because the FN domain seems to block MMP-9 activity without competing for MRC-5 cell surface ligands, we asked whether it interacts directly with MMP-9 in MRC-5 conditioned medium. To assess interaction between MMP-9 and its FN domain, we incubated MRC-5 fibroblasts with both wt proMMP-9 and v5-tagged FN domain and performed immunoprecipitation using anti-v5 antibody. Anti-MMP-9 antibody blot analysis showed that wt MMP-9 is co-immunoprecipitated with v5-tagged FN domain in MRC-5 conditioned medium (*Figure 7B*). Unfortunately, we were not able to detect interaction at the cell surface due to the insufficient amount of material following cell fractionation. In any case, these observations suggest that the FN domain may inhibit MMP-9 activity by sequestering it in MRC-5 conditioned medium and leave open the possibility that the MMP-9-FN complex may inhibit MMP-9 activity at the cell surface too, as it does not prevent MMP-9 or FN to become anchored to the cell surface.

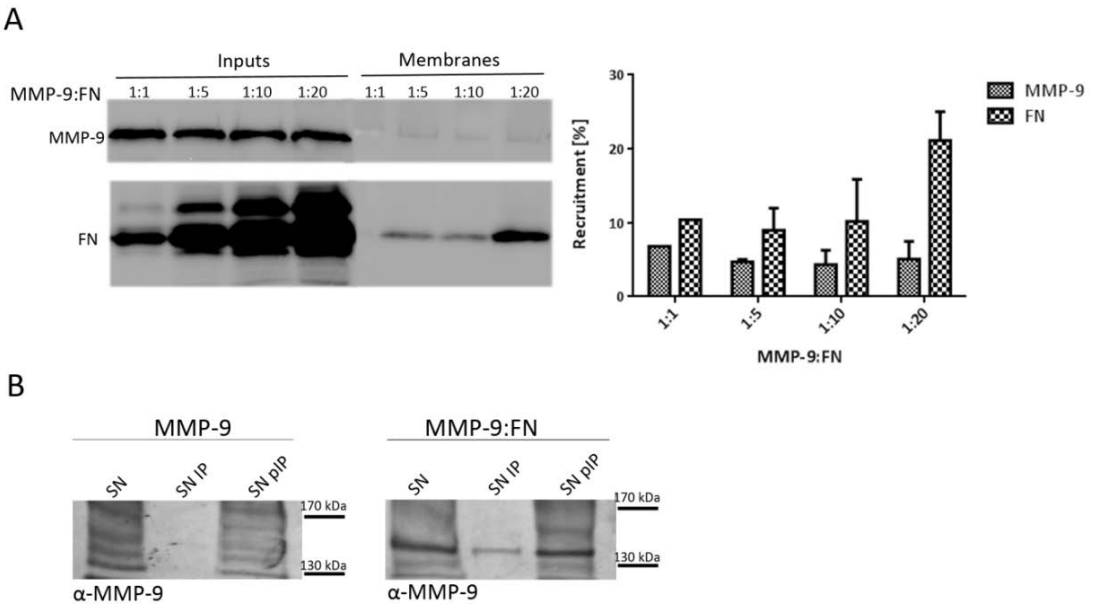


Figure 7. The FN domain does not displace proMMP-9 from the fibroblast cell surface, but sequesters it in MRC-5 conditioned medium; **A.** The FN domain does not displace proMMP-9 from the fibroblast cell surface. Conditioned medium (*inputs*) containing different ratios of MMP-9:FN (1:1, 1:5, 1:10, 1:20) and equal amount of MRC-5 membranes (*membranes*) were loaded. 1 corresponds to 2.3 ug of purified protein. Representative anti-v5 immunoblot (*left panel*) and analysis of recruitment percentage from 2 independent experiments (*right panel*) show the absence of displacement. Recruitment percentage corresponds to the quantity of v5-protein recruited (membrane) compared to the v5-protein incubated (input). **B.** The FN domain interacts with proMMP-9 in MRC-5 conditioned medium. MRC-5 conditioned medium incubated with wt proMMP-9 alone or both wt proMMP-9 and v5-tagged FN domain were used for anti-v5 immunoprecipitation. Representative anti-MMP-9 immunoblot of 3 independent experiments is shown. SN = supernatant, SN IP = immunoprecipitation of the supernatant, SN pIP = post-immunoprecipitation of the supernatant.

The FN domain of MMP-9 shows a dual role in tumor growth and invasion in different tumor cell lines

MMP-9 is a key molecule in tumor growth and invasion in various cancers [26, 27]. It should therefore be interesting to determine whether the FN domain could act as specific MMP-9 inhibitor in *in vivo* situations of tumor growth and invasion. To address this question, we selected different tumor cell lines depending on gelatinase expression and assessed the effect of the FN domain on tumor growth and invasion. We selected the fibrosarcoma HT1080 that highly expresses both MMP-9 and MMP-2, the breast adenocarcinoma MDA-MB231 that displays weak expression of MMP-9 and MMP-2, the colorectal adenocarcinoma SW480 that expresses both MMP-9 and MMP-2 and the breast adenocarcinoma BT-20 that expresses neither MMP-9 nor MMP-2 [71], and stably expressed MMP-9, the FN domain and ΔFN in each cell line. To assess the role of the FN domain in primary tumor growth, xenograft experiments were conducted by

subcutaneously injecting the stable transfectants into NOD/SCID mice. Tumors appeared at different times, according to the baseline tumorigenicity of the different cell lines and mice were sacrificed accordingly - HT1080 fibrosarcoma (at 18 days), MDA-MB231 breast adenocarcinoma (at 5 weeks) and SW480 colorectal adenocarcinoma (at 7 weeks). Neither HT1080 nor MDA-MB231 cells stably expressing the FN domain of MMP-9 revealed any changes in primary tumor growth (*Figure 8A & C*). However, experimental metastasis assays performed by tail vein injection of the stable transfectants, revealed that the expression of the FN domain by these same tumor cells (HT1080 and MDA-MB231) dramatically increases their metastatic proclivity to mouse lungs (*Figure 8B & D*) whereas expression of wt MMP-9 either promotes no change or reduces the number of lung metastases. These observations suggest a potential protective role of MMP-9 in metastasis development, which could be inhibited by the FN domain.

By contrast, xenografts of SW480 colorectal adenocarcinoma cells stably expressing the FN domain of MMP-9 displayed significantly reduced primary tumor growth at the subcutaneous injection site (*Figure 8E*). However, in these cells, overexpression of MMP-9 increased the number of lung metastases in NOD/SCID mice (*Figure 8F*). Nevertheless, overexpression of the FN domain by SW480 cells significantly decreased lung metastasis compared to control cells. It is important to note that no difference in proliferation was observed between cells stably expressing MMP-9 or the different mutants (*data not shown*). The observations described above suggest that the FN domain of MMP-9 is able to inhibit endogenous MMP-9 activity. In this cell type, MMP-9 appears to display pro-invasive properties whereas the FN domain acts as an inhibitor of both engrafted tumor growth and lung colonization by intravenously injected cells.

In BT20 cells, which have no gelatinase expression, neither MMP-9 nor the FN domain affected metastasis (*data not shown*). The fact that the MMP-9 and its FN domain have no effect on a cell line devoid of constitutive gelatinase expression suggests that the effect of the FN domain observed in HT1080, MDA-MB231 and SW480 cells requires endogenous gelatinase activity to be detected. MMP-9 and its FN domain therefore appear to display dual role in tumor growth and invasion in a cell type-specific manner.

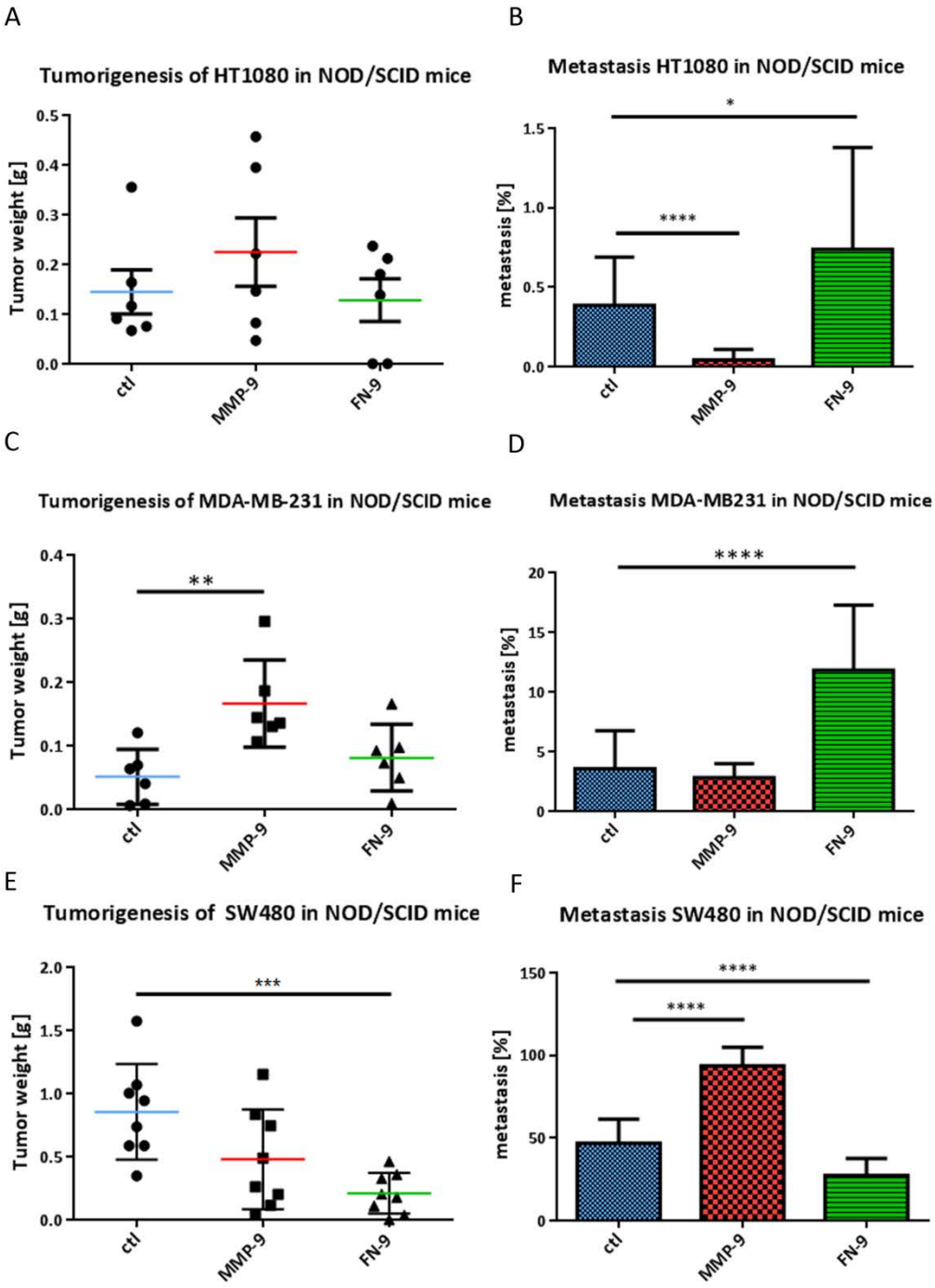


Figure 8. The FN domain of MMP-9 shows a dual role in primary tumor growth and invasion in different tumor cell lines; **A.** The FN domain of MMP-9 has no effect on HT1080 primary tumor growth but **B.** increases lung metastasis in NOD/SCID mice. (A) Primary tumor growth was assessed by subcutaneous injection of $2x10^5$ cells in both flanks of NOD/SCID mice, which were sacrificed after 18 days. Tumor was weighted. n= 2 (B) Experimental metastasis was assessed by tail vein injection of 10^6 cells in NOD/SCID mice. Organs were collected after 18 days and percentage of lung covered metastasis analyzed by H&E staining. n= 2, *P < 0.05, **** P < 0.0001. **C.** The FN domain of MMP-9 has no effect on MDA-MB231 primary tumor but **D.** increases lung metastasis in NOD/SCID mice. (C) NOD/SCID mice were subcutaneously injected with 10^6 cells and sacrificed after 5 weeks for tumor weighting. n= 2, **P < 0.01 (D) For experimental metastasis assay, $5x10^5$ cells were injected in NOD/SCID mice by tail vein and lungs collected after 5 weeks. n= 2, **** P < 0.0001. **E.** The FN domain of MMP-9 decreases both SW480 primary tumor and **F.** lung metastasis in NOD/SCID mice. (E) NOD/SCID mice subcutaneous injection of $3x10^5$ cells was performed and mice sacrificed after 7 weeks for tumor weight assessment. n= 1, *** P < 0.001. (F) Lung metastasis was assessed by injecting in tail vein of NOD/SCID mice $2x10^6$ cells during 21 days. n= 2, **** P < 0.0001.

The FN domain influences MMP-9 activity differently depending on the cell types

Given our previous results showing that the FN domain inhibits MMP-9-induced TGF-β activation *in vitro* (*Figure 6*), we investigated whether the dual, cell type-dependent, effect of MMP-9 and its FN domain on tumor growth and invasion was directly related to a difference in gelatinase expression and activity. We assessed gelatinase expression in conditioned media of the different tumor cell lines used for *in vivo* experiments (HT1080, MDA-MB231 and SW480 cells). Cells were starved in serum-free medium for 48 hours and conditioned media collected, concentrated and analyzed for gelatinase expression and activity by gelatin zymography. No differences were observed in either MMP-9 or MMP-2 activation between HT1080 tumor cell lines expressing the different mutants of MMP-9 (*Figure 9A*). However, we noticed that conditioned medium of MDA-MB231 stably expressing the FN domain of MMP-9 revealed an absence of one of the active forms of MMP-9 compared to MDA-MB231 expressing MMP-9, ΔFN or control cells (*Figure 9B orange arrow*). This suggests that the FN domain of MMP-9 may inhibit MMP-9 activation or activity in MDA-MB231 conditioned medium.

By contrast, the gelatin zymogram of SW480 stably expressing the FN domain of MMP-9 displayed an increase of a 100 kDa band, which corresponds either to an active or a deglycosylated form of MMP-9 (*Figure 9C green arrow*). Moreover, no differences in MMP-2 activity were detected in SW480 conditioned medium. This observation indicates that the FN domain of MMP-9 secreted by SW480 cells affects only MMP-9 activity.

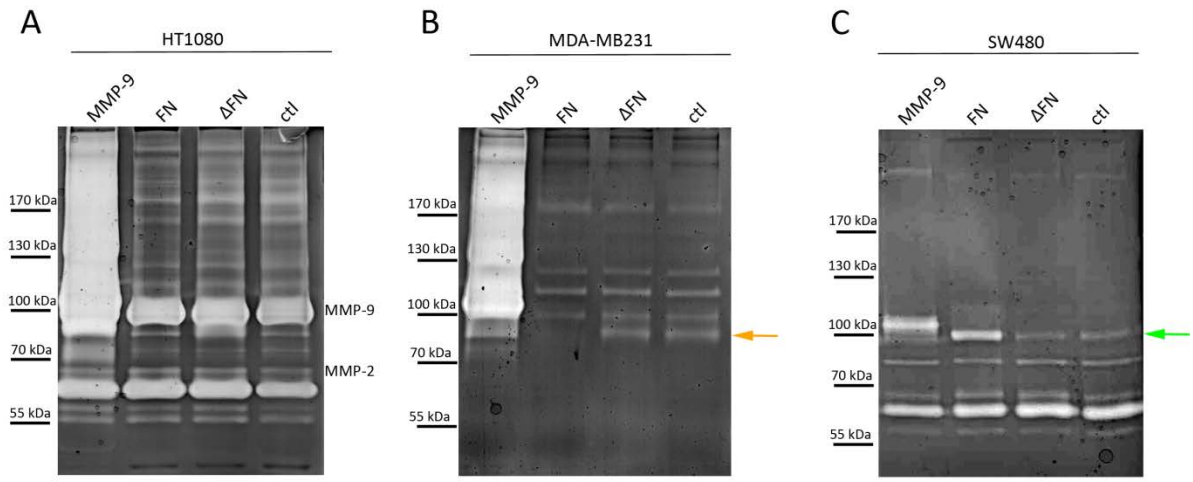


Figure 9. The FN domain influences MMP-9 activity differently depending on the cell types; **A.** Gelatinases expression and activation in HT1080 supernatants. Concentrated conditioned medium from HT1080 overexpressing MMP-9, FN, ΔFN or control cells and starved for 48 hours were loaded on gelatin zymogram. Representative gelatin zymogram of 3 independent experiments is shown. **B.** Gelatinases expression and activation in MDA-MB231 supernatants. Concentrated conditioned medium from MDA-MB231 overexpressing MMP-9, FN, ΔFN or control cells and starved for 48 hours were loaded. Representative gelatin zymogram of 4 independent experiments showed the absence of an active form of MMP-9 *(orange arrow)*. Note that we did not observed MMP-2 expression in those MDA-MB231 cells. **C.** Gelatinases expression and activation in SW480 supernatants. Concentrated conditioned medium from SW480 overexpressing MMP-9, FN, ΔFN or control cells and starved for 48 hours were loaded. Representative gelatin zymogram of 4 independent experiments displayed an increase of an active form of MMP-9 in SW480 expressing the FN domain (*green arrow*).

ProMMP-9 is a highly glycosylated protein containing both N- and O-glycosylation, which play an important role in MMP-9 activation and activity. Accordingly, we asked whether the band observed on the zymogram (*Figure 9C, green arrow*) when analyzing FN-expressing SW480 conditioned medium corresponds to an active or a deglycosylated form of MMP-9. To do so, we used a deglycosylation solution mix that removes both N- and O-linked carbohydrates and compared untreated to deglycosylation mix-treated SW480 conditioned medium. Deglycosylation of both MMP-9 and MMP-2 resulted in the accumulation of a single prominent band on the zymogram for each protease (*Figure 10*). We observed that the 100 kDa MMP-9 protein (*Figure 10 green arrow*) can be deglycosylated, as illustrated by its shift after subjection to the deglycosylation mix. This suggests that the band did not correspond to a deglycosylated form of MMP-9 but more likely to an active form of the gelatinase.

In conclusion, the FN domain of MMP-9 might have opposite effects on MMP-9 activation and/or activity depending on the cell line by which it is produced. Indeed, we observed loss of an active form of MMP-9 in MDA-MB231 conditioned medium versus enhancement

of active gelatinase B in SW480 cell conditioned medium upon overexpression of the FN domain.

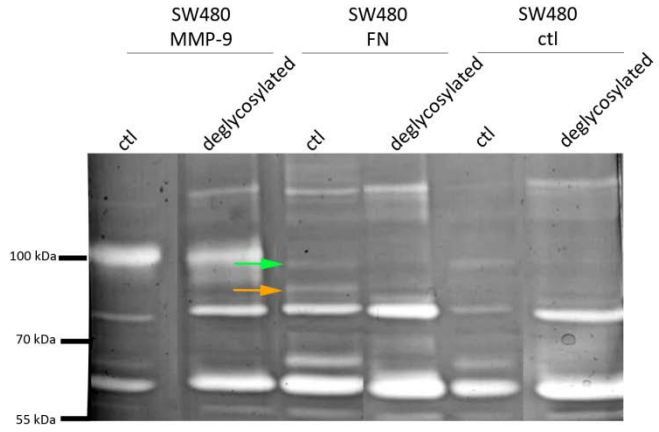


Figure 10. FN-expressing SW480 cells do not secrete a deglycosylated form of MMP-9; Conditioned medium from SW480 cells overexpressing MMP-9 or FN compared to control cells was concentrated with Amicon 50 NMWL filters, then treated at 37°C for 4 hours with Deglycosylation Enzyme Cocktail in non-denaturing conditions and finally analyzed by gelatin zymography. The MMP-9-corresponding band in the supernatant of SW480 cells expressing the FN domain (*green arrow*) shifted in presence of deglycosylation mix, suggesting that this form is an active form and not deglycosylated one. *The orange arrow display the band which is absent in MDA-MB231 cells overexpressing the FN domain and which can also be deglycosylated by the treatment, suggesting also that it corresponds to an active form of the enzyme.*

The FN domain produced by SW480 prevents MMP-9 activity at the cell surface

Because the FN domain of MMP-9 expressed by different tumor cell lines induced either a decrease (MDA-MB231) or an increase of active MMP-9 (SW480) in cell conditioned medium and given that cell surface association is important for MMP-9 activity [41], we asked whether this effect could be due to the fact that the FN domain helps retain or release MMP-9 activity at/from the MDA-MB231 or SW480 cell surface, respectively. We therefore performed cell fractionation using HT1080, MDA-MB231 and SW480 cells stably expressing either MMP-9, the FN domain or control vector and assessed MMP-9 activity at their membrane. By gelatin zymography, we observed that proMMP-9 as well as proMMP-2 appeared both in the cytoplasm and at the membrane of HT1080 cells without any difference among the different conditions. However, active MMP-9 was present only in the cytoplasm and at the membrane of HT1080 cells overexpressing MMP-9 (*Figure 11A orange arrow*), whereas no MMP-9 activity was detected at the membrane of control or

FN domain-expressing cells. This suggests that FN domain expression may not directly affect MMP-9 and MMP-2 expression, secretion, localization and activity in HT1080 cells.

By contrast, we noted MMP-9 activity on the surface of MDA-MB231 cells stably expressing MMP-9 (*Figure 11B orange arrow*), but no activity of the protease at the surface of control cells or FN domain-expressing cells (*Figure 11B*). This observation implies that the absence of an active form of MMP-9 in the conditioned medium of MDA-MB231 expressing the FN domain is not due to the fact that the enzyme activity is retained to the cell membrane. Thus, the FN domain may affect MMP-9 activation or activity through another mechanism, possibly by impairing glycosylation or secretion.

Similarly, SW480 cells overexpressing MMP-9 displayed an increase of MMP-9 activity at their membrane (*Figure 11C green arrow*), whereas SW480 cells expressing the FN domain showed no activity at the cell surface (*Figure 11C*). Therefore, the presence of an increased amount of an active form of MMP-9 in the SW480 cell conditioned medium expressing the FN domain (*Figure 10C*) may be explained by the fact that the FN domain may block or displace MMP-9 activity at SW480 cell membrane, by a mechanism that may be distinct from that observed in MRC-5 fibroblasts.

Consequently, we need to identify other mechanisms whereby the FN domain of MMP-9 expressed by both HT1080 and MDA-MB231 cells increases lung metastasis in immunocompromized mice.

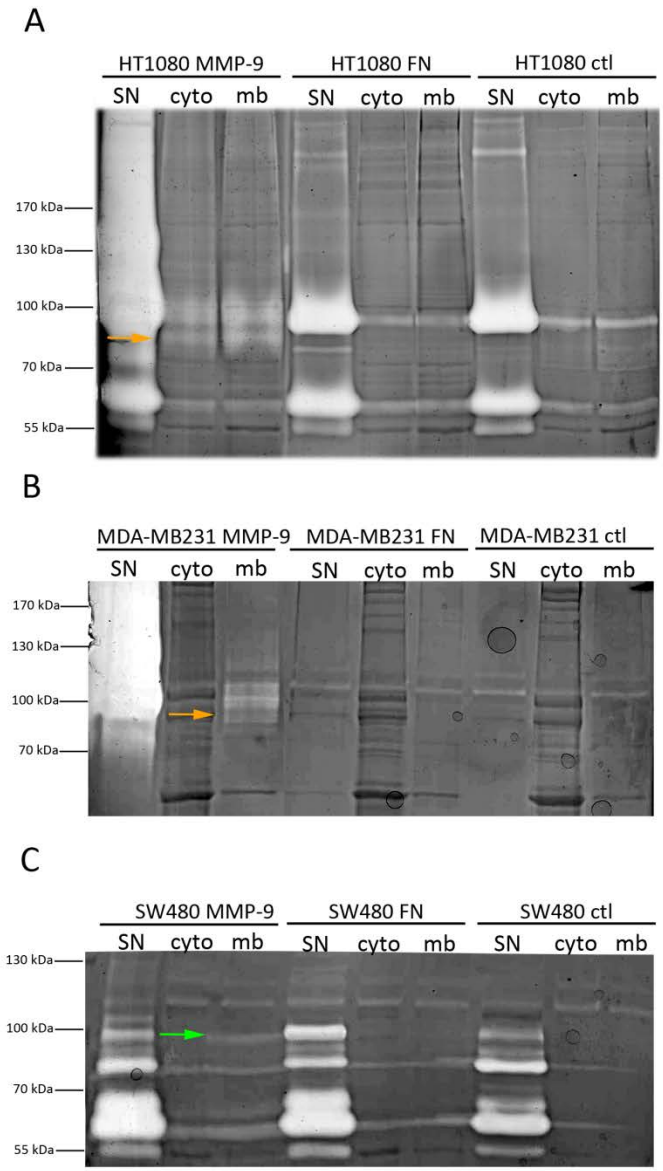


Figure 11. The FN domain produced by both MDA-MB231 and SW480 cells prevents MMP-9 activity at the cell surface; **A.** The FN domain expressed by HT1080 cells does not sequester active MMP-9 at the cell membrane. Conditioned medium of HT1080 cells overexpressing MMP-9 or the FN domain compared to control cells and equal amount of cytosolic and membrane fractions were loaded for gelatin zymography. Representative gelatin zymogram of 2 independent experiments showed activity of MMP-9 expressed by HT1080 cells *(orange arrow)*. **B.** The FN domain expressed by MDA-MB231 cells does not sequester active MMP-9 at the cell membrane. Conditioned medium of MDA-MB231 overexpressing MMP-9 or the FN domain compared to control cells and equal amount of cytosolic and membrane fractions were loaded for gelatin zymography. Representative gelatin zymogram of 2 independent experiments showed activity of MMP-9 expressed by MDA-MB231 cells *(orange arrow)*. **C.** The FN domain expressed by SW480 cells displaces MMP-9 activity from the cell surface. Conditioned medium of SW480 overexpressing MMP-9 or the FN domain compared to control cells and equal amount of cytosolic and membrane fractions were loaded for gelatin zymography. Representative gelatin zymogram of 2 independent experiments showed activity of MMP-9 at SW480 cell surface *(green arrow)*. SN = supernatatant, cyto = cytoplasmic fraction, mb = membrane fraction.

MDA-MB231 cells expressing the FN domain of MMP-9 display increased invasion, which is abolished by the presence of TGF-β

TGF-β is known to act as an inhibitor of proliferation and therefore behaves as a tumor suppressor early in tumor development [18]. Given that the FN domain inhibits MMP-9-induced TGF-β activation *in vitro* (*Figure* 6), we asked whether the increase in lung metastasis number observed in NOD/SCID mice injected with MDA-MB231 cells expressing the FN domain of MMP-9 is related to TGF-β pathway. We started by assessing the proliferation of MDA-MB231 cells expressing MMP-9 or FN compared to control cells in the absence or presence of TGF-β using MTT assay. We observed that TGF-β reduces the proliferation of all cell types and that there were no difference among the different conditions, suggesting that these cells are responsive to TGF-β growth inhibition (*Figure* 12).

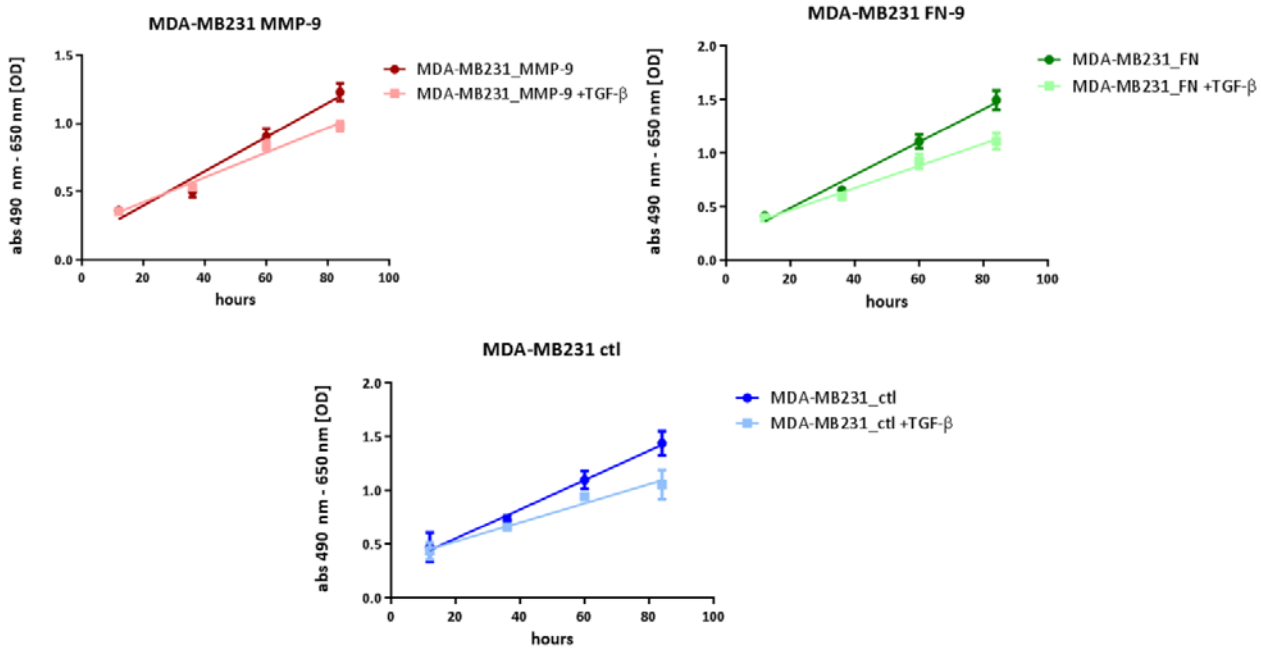


Figure 12. TGF-β decreases proliferation of MDA-MB231 cells; MDA-MB231 cells overexpressing MMP-9 or the FN domain compared to control cells were plated to assess proliferation rate in the absence or presence of TGF-β1 (10 ng/mL) by MTT. Absorbance of formazan was measured at 490 nm after 18h, 36h, 72h and 96h.

Next, we assessed the invasion capacity of MDA-MB231 cells expressing the different mutants of MMP-9 (FN or ΔFN) compared to control cells in the presence of TGF-β. MDA-MB231 expressing MMP-9, FN, ΔFN and control cells were labeled with Calcein-AM and incubated on growth factor reduced matrigel-coated inserts in the absence or presence of TGF-β1 (10 ng/mL) for 24 hours at 37°C. 5 % serum was used as chemoattractant. Invasion was assessed by measuring fluorescence of Calcein-AM. We observed that in the absence of TGF-β, MDA-MB231 ovexpressing the FN domain of MMP-9 display increased invasion compared to control cells (*Figure 13 left*). However, the presence of TGF-β significantly reduced invasion by MDA-MB231 cells expressing the FN domain, and abolished the increased invasiveness triggered by the FN domain (*Figure 13 right*). This finding suggests that the FN domain of MMP-9 may increase MDA-MB231 invasiveness possibly by inhibiting latent TGF-β activation because addition of active TGF-β1 abrogated invasion of MDA-MB231 cells that express the FN domain. This potential mechanism could occur either through MMP-9 inhibition or by direct TGF-β inhibition.

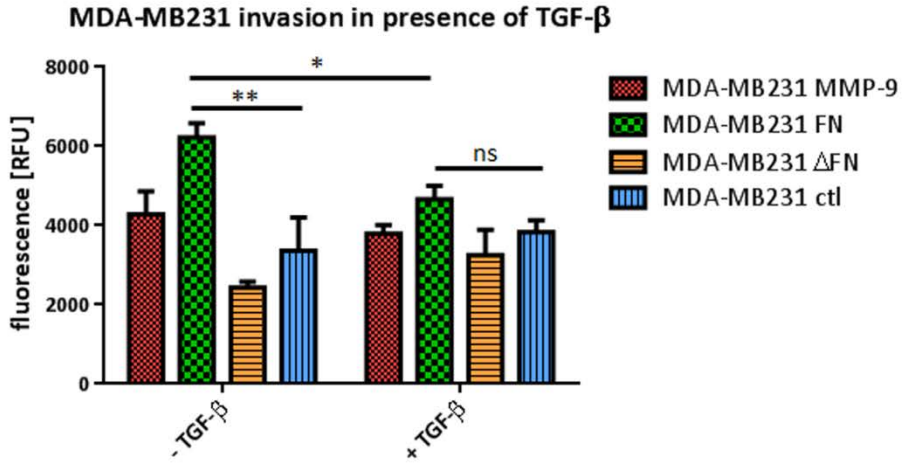


Figure 13. MDA-MB231 cells expressing the FN domain of MMP-9 display increased invasion, which is abolished by the presence of TGF-β; MDA-MB231 expressing MMP-9, FN or ΔFN compared to control cells were labeled with Calcein-AM and incubated on growth factor reduced matrigel-coated inserts for 24 hours in the absence or presence of TGF-β1 (10 ng/µL). Invasion was assessed by measuring Calcein fluorescence at 517 nm. n = 3, *P < 0.05 **P < 0.01 ns= non-significant.

To gain further insight into the molecular mechanism that may explain the higher metastatic proclivity and increased invasiveness of MDA-MB231 cells, we tested adhesion, migration and invasion of MDA-MB231 cells stably expressing the different mutants of MMP-9 in different conditions. We observed that the presence of the FN domain of MMP-9 in the ECM might increase MDA-MB231 adhesion (*Figure 14A*). Moreover, MDA-MB231 cells expressing the FN domain of MMP-9 showed a tendency to migrate more (*Figure 14B*), whereas the presence of the FN domain in the ECM tends to help MDA-MB231 cells to invade (*Figure 14C*). Consequently, the FN domain of MMP-9 may also play a role in the behavior of MDA-MB231 cells independently from MMP-9 activity.

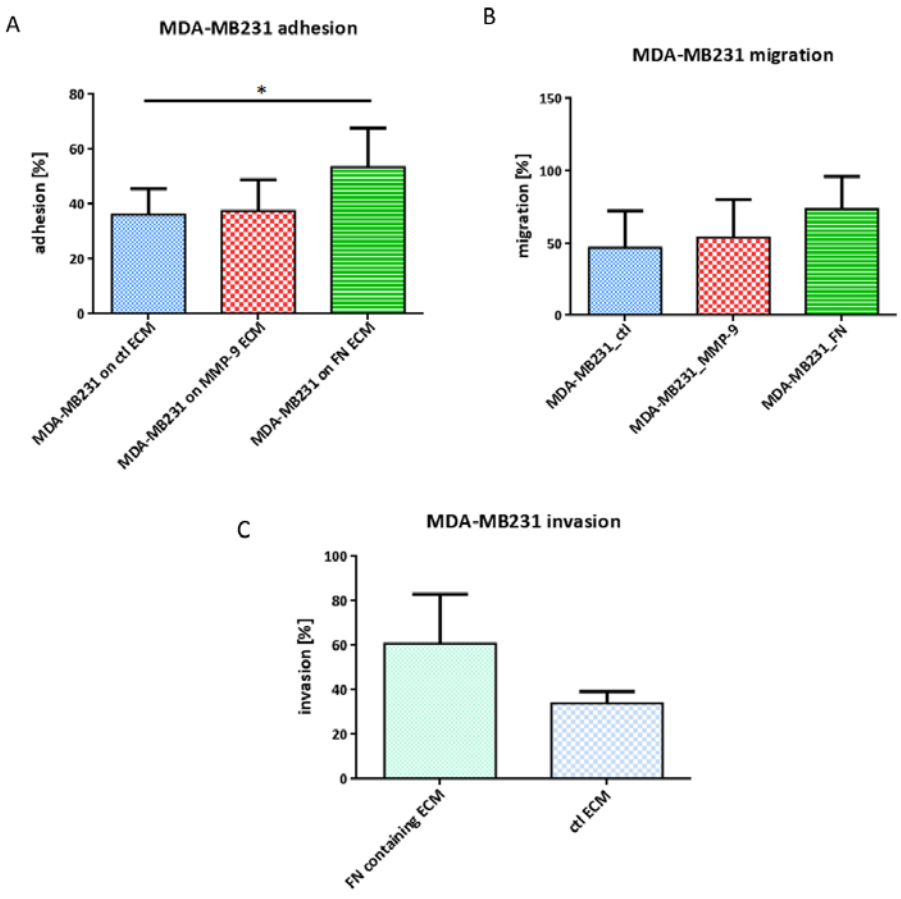
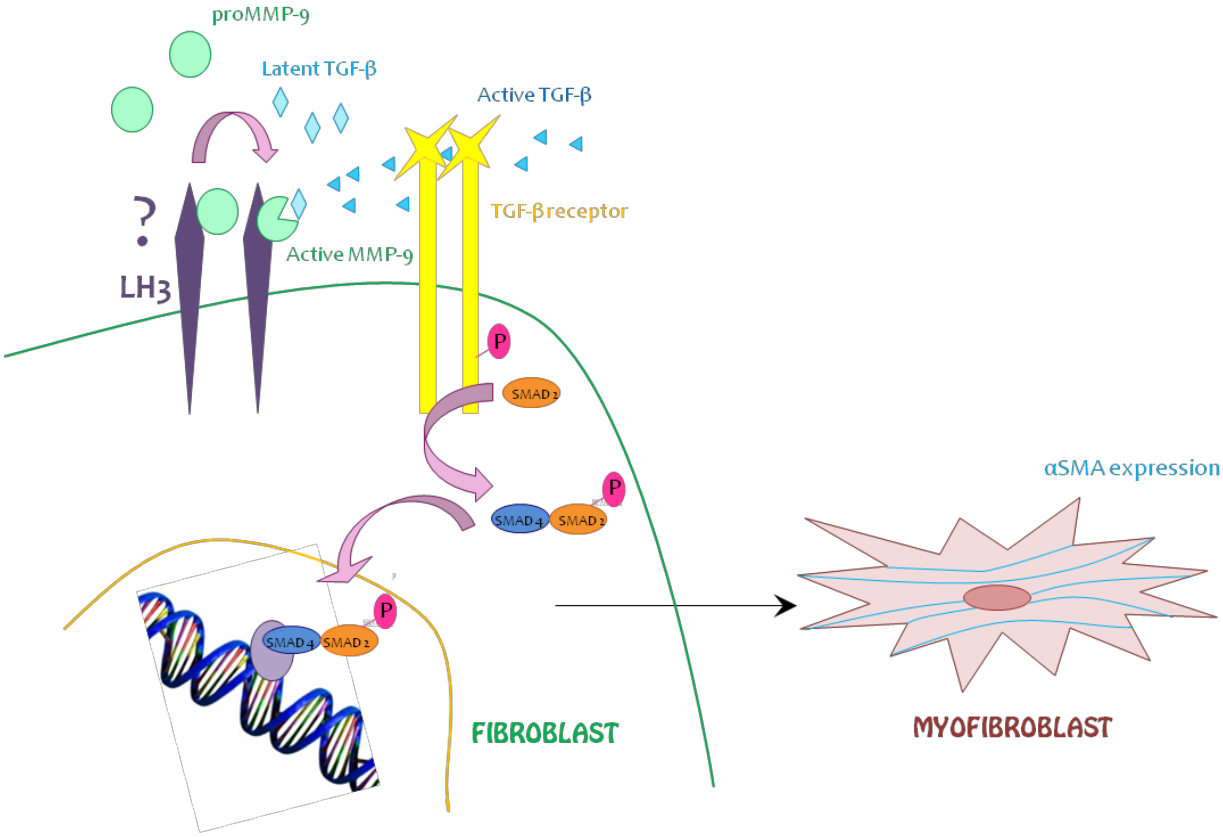


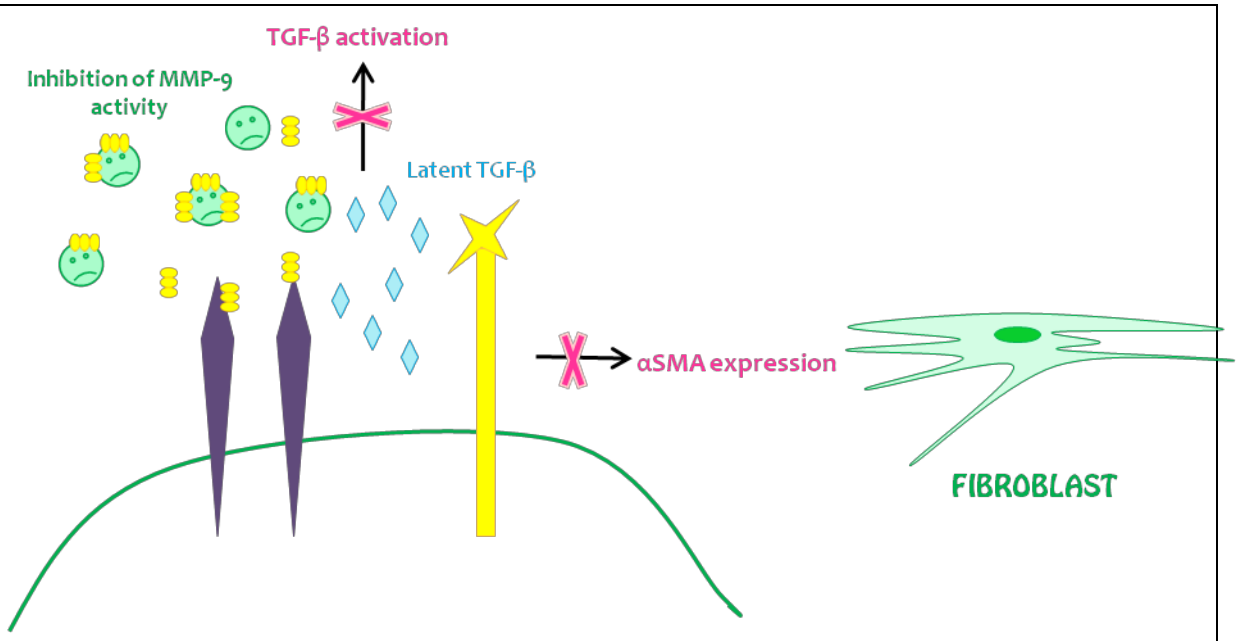
Figure 14. The presence of the FN domain of MMP-9 in the ECM tends to increase adhesion, migration and invasion of MDA-MB231 cells ; **A.** The presence of the FN domain in the ECM increases MDA-MB231 adhesion. Wt MDA-MB231 cells were labeled with 2 µM Calcein-AM and incubated on different ECMs for 10 min. Adhesion percentage was assessed as the ratio of adherent cells to total labeled cells. n = 2, *P < 0.05.. **B.** MDA-MB231 expressing the FN domain have a tendency to migrate more. MDA-MB231 expressing MMP-9, FN, ΔFN or control cells were labeled with Calcein-AM and incubated on growth factor reduced matrigel-coated inserts for 24 hours. Migration percentage was assessed as the ratio of migratory cells to total labeled cells. n = 2. **C.** The presence of the FN domain in the ECM helps MDA-MB231 to invade. Wt MDA- cells were labeled with Calcein-AM and incubated on growth factor reduced matrigel-coated inserts for 24 hours in the absence or presence of purified FN peptide. Invasion percentage was assessed as the ratio of invasive cells to total labeled cells. n = 3.

Discussion & perspectives

In this work, we have shown that pro- and active MMP-9 are recruited to the fibroblast cell surface via their fibronectin type II-like motifs, and more abundantly so than to various tumor cell lines tested. Recruitment of proMMP-9 activates latent TGF-β, which induces αSMA expression reflecting myofibroblast differentiation (*Model 1*). We provide evidence that the activity and location of MMP-9 can be modulated by its recombinant FN domain. Thus, the FN domain of MMP-9 is shown to interact directly with proMMP-9 and to sequester it in the fibroblast conditioned culture medium, which abrogates both MMP-9-induced TGF-β activation and αSMA expression in resting fibroblasts (*Model 2*).



Model 1: ProMMP-9 recruitment to the fibroblast cell surface activates latent TGF-β and induces αSMA expression reflecting myofibroblast differentiation.



Model 2: The FN domain inhibits both MMP-9-induced TGF-β activation and αSMA expression in resting fibroblasts.

Our second major observation was that MMP-9 and its FN domain display a dual and opposing role in tumor growth and invasion in a cell type-specific manner. Indeed, expression of the FN domain in HT1080 and MDA-MB231 cells increased their ability to form lung metastases in NOD/SCID mice, whereas overexpression of MMP-9 in these same cells decreased their dissemination to the lungs. Whereas MMP-9 activity in HT1080 cells does not seem to be directly affected by the FN domain, as assessed by gelatin zymography, MDA-MB231 cells overexpressing the FN domain display a decrease of an active form of MMP-9, which suggests that the FN domain may inhibit MMP-9 activity in those cells. This may prevent MMP-9-induced activation of TGF-β and other cytokines, the result of which is increased MDA-MB231 invasion that could explain the increased number of lung metastases. An alternative view is that the FN domain of MMP-9 may under defined circumstances display intrinsic pro-tumorigenic properties independent of the proteolytic activity of MMP-9, including the possibility to provide a scaffold for tumor cells to adhere to and migrate on. By contrast, the FN domain of MMP-9 decreased both tumor growth and metastasis when it was expressed in SW480 cells, whereas wt MMP-9 displayed both a pro-tumorigenic and a pro-invasive effect. In this case, the FN domain may displace MMP-9 from the cell surface where its activity may be required for growth and invasion of tumors derived from these cells. Our results demonstrate discrepant roles

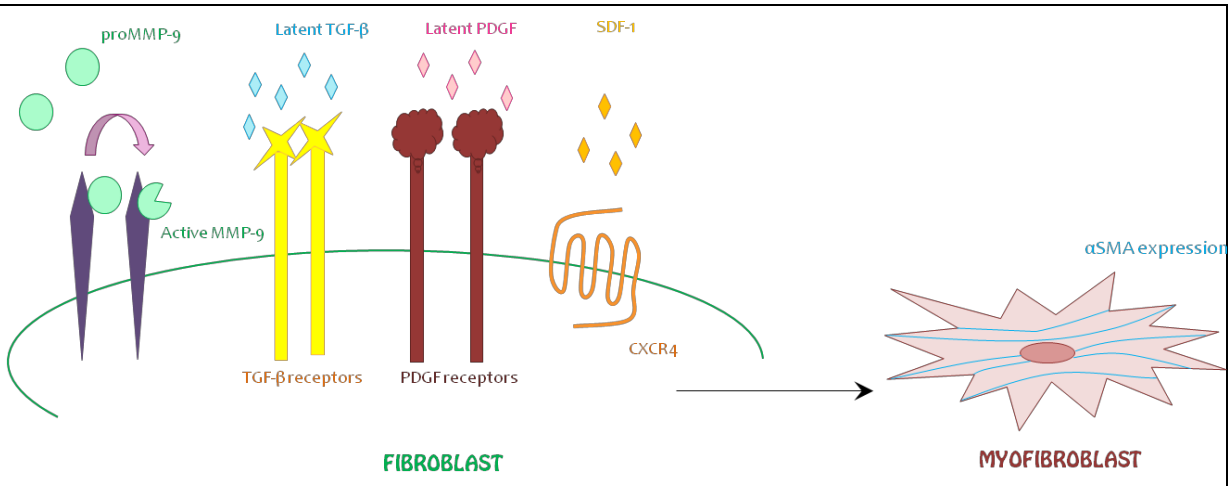
of MMP-9 and its FN domain in primary tumor growth and invasion that deserve further attention. Importantly, MMP-9 and its recombinant FN domain appear to function in opposition to each other.

Potential models of MMP-9 action and FN domain recruitment to the fibroblast cell surface

At least two MMP-9 regions provide candidate structures for recruitment of the enzyme to cell surface: the FN type II-like motifs, also known as a collagen-binding domain located in the catalytic region of the protease in the vicinity of the active site and shared only by MMP-2; and the hemopexin-like domain. It is possible, however, that catalytic activity itself may play a role in modulating MMP-9 interaction with cell surface molecules. Thus, reduced recruitment of the catalytically inactive mutant E402Q to the surface of fibroblasts (*Figure 3A*) may suggest that recruitment of proMMP-9 to the fibroblast cell surface depends in part on the presence of the FN domain, but possibly also on MMP-9 activity. Indeed, although a single amino acid mutation in the catalytic site could potentially affect recruitment to the fibroblast cell surface, an alternative explanation may be that the inability of E402Q mutant to become activated weakens interaction with fibroblast cell surface ligands. The putative requirement of MMP-9 activity for fibroblast cell surface recruitment is consistent with the observation that the active form of MMP-9 is also recruited to the MRC-5 cell surface (*Figure 5A*). Nevertheless, the observation that the ΔHEX mutant is more strongly recruited than proMMP-9 (*Figure 3B*) may possibly be explained by a favorable conformation of the enzyme, which gives the FN domain better access to fibroblast cell surface ligands. In addition, even though the HEX domain is reported to be a driver for MMP-9 recruitment to tumor cell lines [46], it is not recruited at all to the surface of fibroblasts tested in our study (*Figure 3B*), suggesting the importance of exosites or secondary binding sites in MMP binding and activity [72].

Recruitment of proMMP-9 induces αSMA expression in resting fibroblasts reflecting myofibroblast differentiation (*Figure 5C*) which appears to occur through MMP-9-induced TGF-β activation (*Figure 5B*). We noted, in some experiments, that the active form of MMP-9 induces higher αSMA expression than active TGF-β alone, which suggests that

MMP-9 activity is sufficient for myofibroblast differentiation and that pathways other than that of TGF-β alone might be involved in the process. This is in line with the notion that TGF-β is not the only substrate of MMP-9 in this context. Thus, in a colorectal cancer model, MMP-9 inhibitors have been shown to block PDGF- and TGF-β-driven cellular invasion suggesting that both TGF-β and PDGF can induce MMP-9-mediated cellular invasion [73]. PDGF may therefore provide an alternative substrate of MMP-9 in the mechanism of myofibroblast differentiation, as may other candidate substrates. In addition, Kojima and coworkers nicely demonstrated that tumor-promoting CAFs can originate from preexisting stromal fibroblasts and maintain this status by establishing TGF-β and stromal-derived factor-1 (SDF-1) autocrine signaling [74]. SDF-1 may therefore be another important factor in myofibroblast differentiation that synergizes with TGF-β in our case.



Model 3: Potential substrates and factors involved in MMP-9-induced αSMA expression in resting fibroblasts.

Dissection of potential molecular mechanisms underlying MMP-9-induced TGF-β activation and αSMA expression in resting fibroblasts

We do not have yet a complete explanation of the precise molecular mechanism whereby activation of TGF-β and myofibroblast differentiation occur. We still need to determine whether proMMP-9 bound to the fibroblast cell surface becomes activated on the cell surface and cleaves latent TGF-β in the pericellular matrix (as observed in certain tumor cells [57]) or whether it is recruited to their cell surface for proteolytic activation and then released into the ECM to free TGF-β from its latency complex. This would also address the

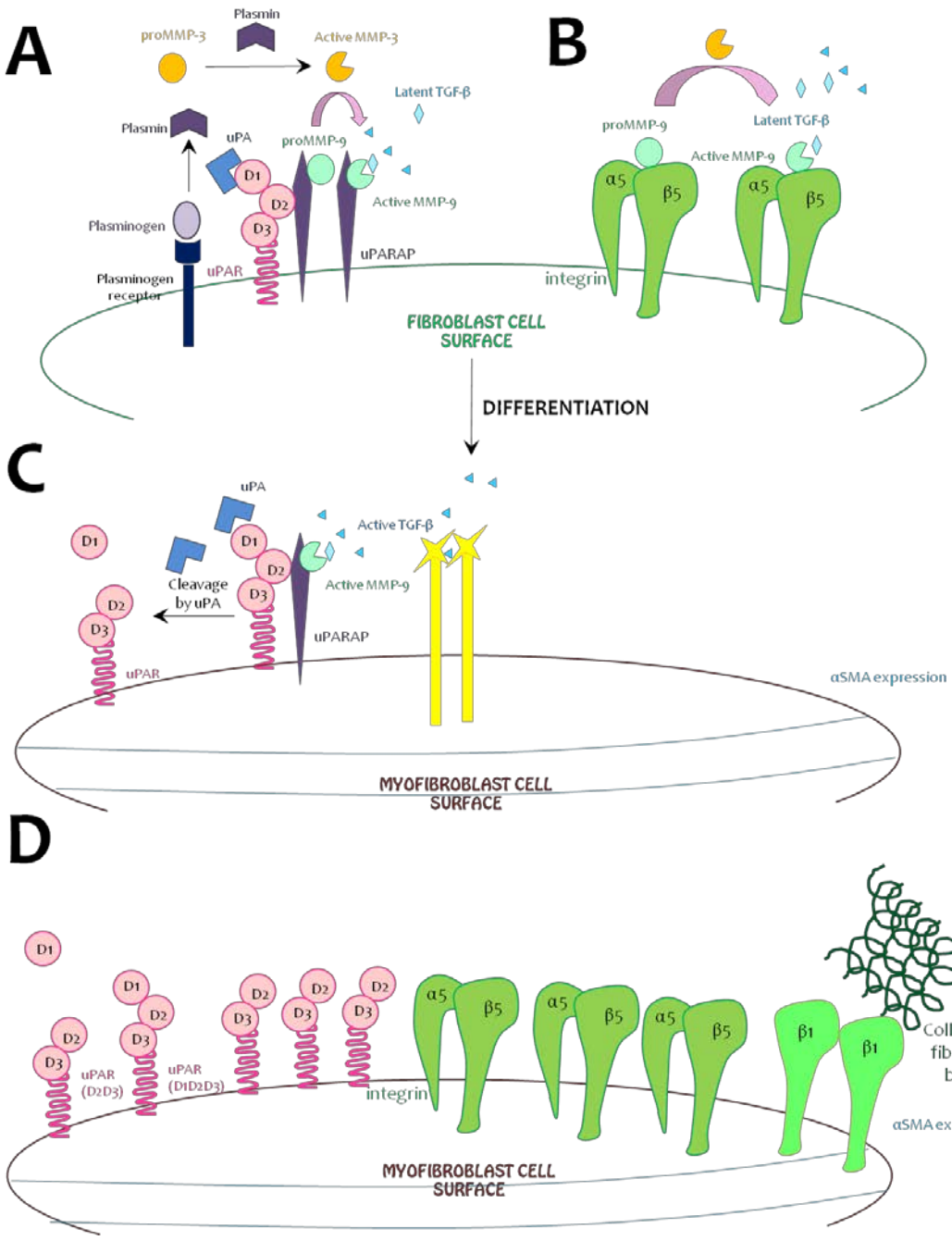
question as to whether recruitment to fibroblast membranes is a prerequisite for MMP-9 activation. Because we noted that the FN domain does not displace proMMP-9 from the fibroblast cell surface (*Figure 7A*), the FN polypeptide cannot be used to abrogate recruitment and force MMP-9 to remain in the conditioned medium. Thus, whereas it may provide a reagent of choice to modulate MMP-9 activity, FN domain does not constitute a suitable tool to investigate how physiological activation of proMMP-9 on the cell surface might occur. Indeed, we have not proven that MMP-9 is proteolytically activated by its fibroblast recruitment and thus, we cannot exclude the possibility of enzymatic activity of MMP-9 without proteolytic activation, i.e. in the presence of intact propeptide [42]. Nevertheless, the fact that the proteolytically cleaved active form of MMP-9 is recruited to the MRC-5 membrane (*Figure 5A*) suggests that proteolytic activation at the fibroblast cell surface may well occur. Consequently, we need to investigate the precise mechanism of MMP-9 activation at the cell surface in order to develop ways to block it. To do so, fluorogenic DQ-gelatin, which emits fluorescence when degraded by gelatinases and is more quantitative than gelatin zymography, has been used in MMP-2 knock down MRC-5 fibroblasts but did not give satisfying results yet. The protocol requires optimization to obtain detectable and reliable signals.

A potentially relevant pathway to investigate MMP-9 activation and downstream TGF-β-induced αSMA expression in resting fibroblasts would be uPA/uPAR system. Indeed, during tissue remodeling, urokinase-type plasminogen activator (uPA) binds urokinase-type plasminogen activator receptor (uPAR) and cleaves the zymogen plasminogen into the active protease plasmin at the cell surface, which in turn cleaves and activates pro-uPA and MMPs. uPAR is overexpressed in many human cancers and facilitates cell migration through the ECM by promoting pericellular proteolysis, in cooperation with MMPs [75]. uPAR contains 3 extracellular domains, which are called D1D2D3, where D1 is the principal uPA binding domain [76]. Moreover, uPA negatively regulates the pathway by cleaving uPAR in a D1 soluble fragment and D2D3 membrane-associated fragments, thereby inactivating uPAR proteolysis function. The contribution of uPAR proteolysis in tumors has also been shown to occur through stromal cells, including fibroblasts [77]. The maintenance of full-length uPAR (D1D2D3) on the fibroblast cell surface, as opposed to cleaved uPAR (D2D3) has been shown to prevent the transition from migratory

fibroblasts to adherent myofibroblasts, demonstrating that uPAR cleavage and subsequent downregulation are necessary for myofibroblast differentiation [76]. Recently, Wang and coworkers showed that uPA bound to uPAR regulates integrin α5β5 and that downregulation of uPA/uPAR results in increased α5β5 integrin cell-surface protein levels, which regulate β1 integrin binding to collagen/fibronectin and promote characteristics of persistent myofibroblast [78]. Moreover, uPA activity is decreased by TGF-β1. In glioblastoma, uPA has been shown to directly activate MMP-9, which in turn degrades fibronectin and promotes invasion [79]. In human breast cancer cells, colocalization of uPAR and MMP-9 in lipid rafts were found to play a critical role in promoting migration, invasion and angiogenesis [80]. Thus, it would be interesting to see whether treatment of MRC-5 fibroblasts with MMP-9 versus the FN domain might directly or indirectly modulate the uPA/uPAR balance and how, i.e. whether the presence of the FN domain and its inhibition of MMP-9 activity abrogates uPAR cleavage by uPA, therefore preventing α5β5 integrin cell surface activity and myofibroblast differentiation. In addition, uPAR-associated protein (uPARAP/Endo180), an internalization receptor containing one fibronectin type II-like motif, interacts with uPAR but also takes part in other events of matrix turnover and cooperates with other MMPs [81]. uPARAP expression is induced by TGF-β and increases tumor growth while diminishing tumor collagen content. If dimerization of the FN type II-like motifs is plausible, it may be worthwhile to investigate whether MMP-9 and its FN domain interact with uPARAP during myofibroblast differentiation and whether the uPA/uPAR pathway plays a relevant role in our model.

Hence, a hypothetic model of action that we propose here is that proMMP-9 is recruited to fibroblast cell surface through its FN domain either by uPARAP (via the FN type II-like motifs) (*Model 4A*) or integrin α5β5 (*Model 4B*). uPA present in the complex of uPA/uPAR/uPARAP activates proMMP-9, releasing it (or not) into the ECM for further latent TGF-β activation, which may occur either by proteolytic activity (*Model 4A*) or by α5β5 integrin-mediated myofibroblast contraction that releases active TGF-β [82]. TGF-β activity then triggers myofibroblast differentiation and uPA negatively regulates uPAR by cleavage in the D2D3 fragments at the cell surface (*Model 4C*). Downregulation of uPAR would maintain myofibroblasts in an adhesive phenotype by increased expression of

α5β5 integrin and β1 integrins (*Model 4D*). One of the possibilities for MMP-9 activation could be via the plasmin, which is cleaved by uPA/uPAR from plasminogen (*Model 4A*). Plasmin activates proMMP-3, and the combination of plasmin and active MMP-3 may activate proMMP-9 [41, 83].



Model 4: uPA/uPAR and MMP-9-induced-TGF-β activation and αSMA expression in resting fibroblasts.

An important initial goal of this work was the identification of the docking receptor of MMP-9 and its FN domain at the fibroblast cell surface. Since MMP-9 is known to be anchored to some tumor cell surface in a complex with CD44 and TGF-β [57, 58] and that fibroblast migration has been shown to be mediated by CD44-dependant TGF-β activation via an MMP-dependant mechanism [84], we assessed whether CD44 could be a potential target of MMP-9 anchorage to the fibroblast cell surface. However, we observed that MMP-9 was recruited to the cell surface of primary CD44 knockout fibroblasts (*unpublished data*), suggesting that MMP-9 anchorage to the fibroblast membrane occurs through CD44-independent mechanism. Given that binding of proMMP-9 to the cell surface could also be mediated by surface-associated α2(IV) collagen [59] and that fibroblasts produce large amounts of collagen, we have to take into consideration that the docking mechanism responsible for the MMP-9 recruitment to the MRC-5 cell surface may well be provided by fibroblast-secreted collagen IV, or even other collagen types. To this end, we have performed mass spectrometry analysis of anti-v5 antibody pull-down of MMP-9, FN and ΔFN cross-linked at the MRC-5 cell in an effort to identify MMP-9 fibroblast cell surface ligands. Preliminary results look promising and unveiled lysyl hydroxylase 3 (LH3) as a potential specific interactor of both MMP-9 and the FN domain. These data will be confirmed and will determine whether knocking down the docking receptor may provide a robust way to block MMP-9-induced TGF-β activation and αSMA expression in resting fibroblasts.

The fibronectin type II-like motifs of MMP-9 in TGF-β-induced αSMA expression impairment: a "specific" MMP-9 competitive substrate?

Fibronectin type-II like motifs are widespread among several classes of extracellular proteins and engage in interactions with collagens and gelatin [2, 81]. Interestingly in this work, we observed that the FN domain of MMP-9 decreases both MMP-9-induced TGF-β activation (*Figure 6A*) and αSMA expression in resting MRC-5 fibroblasts (*Figure 6B*). Blockade of MMP-9 activity by its FN domain does not appear by displacement of MMP-9 from the cell surface (*Figure 7A*), but rather via sequestration of the protease by direct interaction of MMP-9 and its FN domain in the conditioned culture medium (*Figure 7B*) and probably at the cell surface as well (*preliminary data*). Chemical compounds targeting the hemopexin domain (HEX) of MMP-9 have already been identified as potent inhibitors

of MMP-9-mediated tumor cell migration and proliferation by abrogating MMP-9 homodimerization [85, 86]. Preliminary immunoprecipitation experiments using MMP-9 and the mutant lacking the FN domain (ΔFN) suggest that the FN domain may interact with MMP-9 in homotypic manner via the FN type II-like motifs in the catalytic region of the enzyme, because ΔFN displays no interaction with the FN domain alone (*preliminary data*). This observation is of interest because the FN domain is shared only by MMP-9 and MMP-2 among all MMPs and thus, its targeting could potentially provide a selective manner of inhibiting only this subset of MMPs or even better, in our case, targeting recruitment of MMP-9 to fibroblasts themselves, and thereby fibroblast differentiation. In addition, it would be the first report of potential homodimerization of MMP-9 by the FN type II-like motifs, given that it has thus far been ascribed to the HEX domain [87]. Consequently, it appears worthwhile to explore whether the FN domain interacts with other proteins containing FN type-II motifs, including MMP-2, blood coagulation factor XII, hepatocyte growth factor activator (HGFA) and uPARAP/Endo180 [88]. If so, it may also act as a potential inhibitor of the function of various molecules and therefore, depending on the context, it may be involved in the inhibition of unexpected pathways.

In addition, we need to investigate whether the FN domain prevents potential proMMP-9 activation or whether it directly inhibits MMP-9 enzymatic activity, for instance by mimicking MMP-9 or substrate in the catalytic domain (gelatin) or by preventing homodimerization of MMP-9. We have to keep in mind the possibility that the FN domain may inhibit, by sequestration of one MMP-9 molecule, MMP-9 homodimerization via the already known HEX domain, therefore preventing any other second molecule to form a complex [86]. Saad and coworkers observed that MMP-2 was bound to collagen associated with bone marrow fibroblasts (BMFs) via the FN type-II motifs and that it could be displaced by MDA-MB231 cell-associated fibronectin for further activation by MT1-MMP/TIMP2 complexes. This mechanism is believed to facilitate invasion [89]. Preliminary experiments showed that MMP-2 seems to be recruited to the fibroblast cell surface also via its FN domain, despite the small differences between FN type-II like motifs of MMP-9 and MMP-2 [90]. MMP-2 is constitutively secreted by fibroblasts, including the MRC-5 cells that we used, whereas MMP-9 is primarily provided to the tumor by inflammatory and/or tumor cells themselves [2]. We can thus emit the

hypothesis that MMP-9 has to be recruited by stromal fibroblasts for potential cell surface activation, subsequent promotion and sustainment of tumor growth and invasion. These observations are relevant to MMP-targeting anti-cancer therapies because if the FN domain of MMP-9 could specifically inhibit MMP-9 activity and decrease latent TGF-β activation and αSMA expression induction, it would provide a selective inhibition of a defined mechanism of MMP-9 cell surface docking, via the unique gelatinase domain. Nevertheless, we still need to test the specificity of the FN domain for inhibition of other MMPs, including MMP-2, MT1-MMP or MMP-3, but this would most likely be due to other mechanisms.

Recently, enhanced selectivity of MMP inhibitors has been shown to be achieved by taking advantage of differences in secondary substrate binding sites or exosites within the MMP family [72]. Thus, a triple-helical peptide incorporating an FN type II-like motif-binding sequence was found to selectively inhibit MMP-9 type V collagen-specific activity. Paradoxically, the FN domain of MMP-2 has been found to bind different sites on collagen IV and therefore modulate MMP-9 binding to collagen IV, which in turn either enhances MMP-9 collagenolytic activity at low concentrations or acts as a competitive inhibitor at higher concentrations [91]. This study was interesting for two reasons. First, it showed that the catalytically inactive FN domain of MMP-2 regulates proteolytic processing of MMP-9 by inducing a structural change on type IV collagen and secondly it suggests that these two gelatinases may cooperate in degrading substrates because inactive forms of MMP-2 also modulate collagen IV degradation by MMP-9. Nevertheless, due to the complexity of understanding MMP-9 activation and activity and even if we showed in our situation that the FN domain inhibits MMP-9-induced TGF-β activation and αSMA expression, we have to keep in mind that it might, under other conditions and depending on the doses used, act as a competitive substrate or ligand, which simply disengages the propeptide and leads to proMMP-9 activation [42]. Consequently, we need to investigate a potential cooperation between MMP-9 and MMP-2 in TGF-β activation and αSMA expression. Moreover, we cannot exclude the possibility that their FN domains may act together in the inhibition of TGF-β and that the effect observed may also depend on the doses of the peptide that are used.

Discrepant role of CAFs in tumor growth and invasion

Our observation that MMP-9-induced TGF-β activation promotes αSMA expression in fibroblasts (*Figure 5*) does not demonstrate the direct role of these differentiated myofibroblasts in tumor growth and invasion. Indeed, the role of CAFs in tumor progression is multifaceted as they can inhibit, as well as promote, malignant growth depending on their activation state and secretion repertoire [92]. Relevant genes involved in fibroblast differentiation and that may help further characterization of our αSMA-expressing myofibroblasts include SDF-1, whose secretion is increased in CAFs, endosialin (CD248), which is a marker of CAFs and could be induced by TGF-β, Caveolin-1 and CLIC4, whose expression decreases in CAFs, focal adhesion kinase (FAK) (Y397), which is induced by TGF-β and plays a key role in adhesion and in regulation of multiple signaling pathways, Rho/ROCK which is required for leader fibroblast remodeling of the ECM to form tracks for tumor cells and finally miRNA-21, which participates in TGF-β1-induced myofibroblast differentiation in cancer stroma by targeting PDCP4. Preliminary qRT-PCR of MRC-5 fibroblasts treated with MMP-9, only the FN domain or with MMP-9 and an excess of the FN domain showed an increase of HGF expression in non-αSMA expressing fibroblasts, whereas the cells treated with active TGF-β display no HGF expression at all (*preliminary data*). Bhowmick and coworkers observed that the loss of TGF-β responsiveness in fibroblasts (TGF-β type II receptor KO) results in prostatic intraepithelial neoplasia, as well as in invasive squamous cell carcinomas of the forestomach, via increased signaling of HGF, whilst TGF-β1 suppresses HGF expression in CAFs [17, 93, 94]. CAFs have been associated with increased secretion of HGF and the autocrine loop of HGF/c-Met in CAFs promotes invasion and metastasis [95]. Moreover, in breast cancer cells, HGF secreted by CAFs enhances activation of uPA/uPAR protease [92]. Our previous observation by qRT-PCR would be in line with the blockade of TGF-β activation and signaling by the FN domain of MMP-9, which increases HGF expression and probably secretion. This raises the possibility that the FN domain blocking αSMA-expressing myofibroblast differentiation may positively affect tumor growth and invasion, while MMP-9 may be anti-tumorigenic. Indeed, CAFs are not invariably myofibroblasts and also include non-αSMA-expressing fibroblasts. Consequently, the effect of MMP-9 may depend on the cell type MMP-9 acts on. Bone marrow-derived cells

are a significant cellular source of myofibroblasts found in the tumor stroma [96], whilst here, we demonstrated the action of MMP-9 on resting fibroblasts as source of myofibroblasts. This supports the notion, yet to be proven, that residual fibroblasts in tumor stroma differentiate into myofibroblasts in response to TGF-β. But the direct effect on tumor growth and invasion still has to be investigated and an anti-tumorigenic effect of this pool of cells should not be excluded. Consequently, we now need to functionally characterize the MMP-9-induced-αSMA-expressing fibroblasts and determine their behavior *in vivo*. To do so, transcriptome analysis of MRC-5 treated with MMP-9 or the FN domain would help to identify pathways that are triggered or silenced by MMP-9 or FN. Another important *in vitro* experiment would be to coculture MRC-5 fibroblasts with carcinoma cell lines (with or without direct contact) in order to mimic an *in vivo* situation, because CAFs, as their name indicates, need crosstalk with tumoral cells to acquire their identity.

Tumor microenvironment via CAFs: combinational target for cancer therapy?

It is only in the last decades that tumor microenvironment has been studied with sufficient depth in the context of tumor development and progression and therefore its potential therapeutic targeting has given new perspectives. The observation that MMP-9 protein is more strongly recruited to the fibroblast cell surface than to the various tumor cell lines tested may have a significant impact on tumor-host crosstalk research. Here, we suggest that MMP-9 may have an effect on tumor growth and invasion not directly on tumor cells but via the stroma. Indeed, targeting the stroma in combination with tumor cells to counteract cancer progression appears to be an increasingly attractive and plausible strategy [97]. In the present study, we focused on targeting the stromal compartment not via nutrient-supplied vasculature blockade but by weakening supportive and connective tissue and more particularly, impairing myofibroblast differentiation. Targeting fibroblasts seems promising in cancer treatment, because these cells are genetically stable, which reduces the risk of drug resistance and are responsible for the structure of ECM that hampers diffusion of anticancer agents through solid tumors. Fibroblasts also favor survival, proliferation and invasive features of cancer cells [92]. Once the molecular mechanisms responsible for our observations are elucidated, we will use an osmotic pump that continuously releases the FN domain in spontaneous

mouse tumor models, where MMP-9 acts as a pro-tumoral and pro-invasive agent and has to be recruited to the fibroblast cell surface. This would be relevant because MMPs display compensatory and redundant as well as secondary effects, abrogating selectivity among MMP inhibitors [98, 99]. Hence, the FN domain could contribute to the blockade of a new potential mechanism of MMP-9 activation via the stromal compartment. Consequently, it could provide a framework to design selective MMP or gelatinase inhibitors.

Dual role of MMP-9 and its FN domain in tumor growth and invasion of HT1080, MDA-MB231 and SW480 cells

One of the most intriguing findings of this work was that MMP-9 and its FN domain display dual roles in tumor growth and invasion in a cell type-specific manner (*Figure 8*). In HT1080 fibrosarcoma and MDA-MB231 adenocarcinoma cells, expression of the FN domain increased lung metastasis in NOD/SCID mouse lungs, whereas expression of MMP-9 either caused no change or reduce the number of metastases (*Figure 8B & D*). Although we cannot yet explain the increased number of metastases in HT1080 cells expressing the FN domain of MMP-9, it does not seem to directly depend on gelatinase expression, secretion or activity as assessed by gelatin zymography (*Figure 8A & 9A*). In the case of MDA-MB231 cells, however, we noted that overexpression of the FN domain caused a decrease in an active form of MMP-9 in the conditioned medium (*Figure 9B*), that is not reflected by increased anchorage of active MMP-9 on their membranes (*Figure 11B*). The decreased MMP-9 activity observed in the conditioned medium of MDA-MB231 cells overexpressing the FN domain suggests that the FN domain may impair MMP-9 activity by either preventing its cell surface activation or by directly inhibiting an active form of MMP-9 by sequestration or degradation. In addition, MDA-MB231 cells overexpressing the FN domain displayed increased invasion that could be rescued by the addition of active TGF-β (*Figure 13*). This observation suggests potential inhibition of TGF-β by the FN domain of MMP-9 that does not seem to occur by direct interaction with either latent or active TGF-β (*preliminary data*). By contrast, SW480 colorectal adenocarcinoma cells overexpressing the FN domain of MMP-9 displayed a decrease in both subcutaneous tumor growth and lung metastasis in NOD/SCID mice (*Figure 8E & F*). Curiously, we

observed increased MMP-9 activity in the conditioned medium of SW480 cells overexpressing the FN domain (*Figure 9C*), but no MMP-9 activity at their membrane (*Figure 11C*), suggesting that the FN domain produced by SW480 cells may displace MMP-9 activity from their cell surface. This could explain the decrease in both subcutaneous tumor growth and lung metastasis. In SW480 cells, MMP-9 displayed both pro-tumorigenic and pro-invasive properties, both of which may be inhibited by the FN domain by a mechanism of cell surface displacement.

Dependence of tumor growth and invasion on MMP-9 activity

The absence of differences in NOD/SCID lung colonization by the constitutively MMP-negative BT-20 cell line overexpressing MMP-9 or the FN domain suggests that the dual effect (increase in *figure 8B & D* versus decrease in *figure 8F*) of the FN domain on lung metastasis in different cell types may be related to endogenous gelatinase activity. This notion is supported by the observations of the inhibition (*Figure 9B*) versus enhancement (*Figure 9C*) of an active form of MMP-9 in the conditioned medium of cell lines expressing the FN domain.

Nevertheless, figures 9 and 11 have to be interpreted with caution. As different forms of pro- and active MMP-9 that can be detected on gelatin zymography, one cannot automatically assume that bands of lower molecular weight than proMMP-9 are truly active forms of the enzyme [41]. Indeed, proMMP-9 undergoes glycosylation and some deglycosylated precursor forms of the enzyme that may or may not be active are also detected on gelatin zymography. Moreover, proMMP-9 activation occurs through an intermediate form that maintains zinc coordination by the cysteine residue in the active site and that is in fact inactive [40]. Detection of MMP-9 activation and activity thus remains a complex issue that warrants care. It therefore appears worthwhile to use additional functional assays where MMP-9 activity may be quantified for instance by measuring its ability to degrade a fluorescent gelatin substrate (*DQ-gelatin from Invitrogen*). The problem with such an approach, however, is that gelatinolysis due to MMP-2 activity may confound interpretation.

Elucidation of the mechanisms whereby MMP-9 can display both pro-invasive and protective functions and whereby the FN domain may act as an MMP-9 inhibitor requires

further work. A key experiment will be the use of an shRNA against MMP-9 in these different tumor cell lines, which, based on our observations thus far, should mimic overexpression of the FN that blocks MMP-9 activity. This would support the specificity of the FN domain in MMP-9 inhibition and the fact that MMP-9 can display both pro-invasive and tumor protective properties depending on the tumor cell type. Similarly, immunocompromized MMP-9 KO mice could be used for experimental metastasis assays with our tumor cell lines.

A study performed by Mira and coworkers suggests that in some cell types, membrane location may be important for MMP-9 activation but that MMP-9 that this biologically relevant for tumor development is the secreted form [64]. This study supports the notion that MMP-9 functions as a paracrine factor in tumor progression. Thus, we have to bear in mind the seemingly contradictory possibility that in all of our situations (HT1080, MDA-MB231, SW480), MMP-9 may display protective functions and that the FN domain may, depending on the context, have a dual role on MMP-9 activity. This notion is supported by our observations (*Figure 9B & C*) that the FN domain may inhibit MMP-9 activity in MDA-MB231 cell supernatants, which increases lung metastasis, but activate MMP-9 in SW480 cell supernatants, which decreases lung metastasis. MMP-9 may also act as a paracrine factor for stromal cells when secreted by MDA-MB231 cells, thus inducing fibroblast differentiation and promoting invasion, whereas it may act in autocrine fashion when it is expressed by SW480 cells by directly promoting metastatic growth.

Role of MMP-2: a compensatory effect?

Compensatory effects are important in MMPs family and always have to be taken into consideration, more particularly when this involves the same subfamily [25]. Thus, the results that we present in this work do not exclude an indirect effect of MMP-2. As mentioned above, MMP-2 bound to collagen-associated bone marrow fibroblasts (BMFs) via its FN type II-like motifs has been shown to be released by MDA-MB231 cell-associated fibronectin or fragments of fibronectin containing FN type-II motifs and this mechanism allowed MMP-2 activation and enhanced cell invasion [89]. This mechanism may provide a partial explanation for our observations that the FN domain of MMP-9 increases lung metastasis of both HT1080 and MDA-MB231 cells (*Figure 8B & D*). Active MMP-2 released

from fibroblasts by the FN domain may increase invasiveness of HT1080 and MDA-MB231 cells and therefore counteract the absence of MMP-9 activity, at least in the MDA-MB231 cell context. Although we did not observe an increase of active MMP-2 in the conditioned medium of either HT1080 or MDA-MB231 cells expressing the FN domain of MMP-9 (*Figure 9A & B*), this does not exclude such a mechanism because it may occur only when the cells grow in coculture with stromal cells. Thus, the absence of differences in MMP-2 activity between our different cell lines does not exclude a potential implication of MMP-2 *in vivo*. Nevertheless, even if the FN domain acts specifically on MMP-9 activity, its inhibitory role may affect MMP-2 in situations where the absence of MMP-9 has to be compensated for. Importantly, it should be borne in mind that the gelatinase expression, secretion and activation studied here by gelatin zymography used tumor cell lines alone in culture. Given the importance of the molecular and cellular environment in tumor growth and invasion, we cannot exclude different expression of MMP-9 and MMP-2 in cocultures or in *in vivo* situations compared to what we observed *in vitro*. In the same way, the fine balance between MMPs and TIMPs depending on their combinations, the spatial and temporal stage of tumor progression at which they are solicited are crucial for cellular homeostasis, protease regulation and activity [25, 47]. Hofmann and coworkers nicely demonstrated the importance of the cellular MMP source in relation to the mechanism of tumor dissemination (spontaneous versus experimental metastases), which may differ drastically in MMP expression [100]. Therefore, expression of gelatinases observed by zymography with single tumor cell lines may not reflect their expression and activity at the lung colonization step of metastasis. However, accurate assessment of MMP-9 expression and activity *in vivo* is challenging because of lack of adequate reagents. It is noteworthy, however, to keep in mind that proteolytic imbalance by overexpression of MMP-9 or its FN domain in certain tumor cell types or in defined experimental settings may provoke an unpredicted effect in metastasis development. Consequently, the discrepancy in tumor growth and metastasis observed in this study may possibly be explained by the differential expression of MMP-9 and MMP-2. It is conceivable therefore, that the FN domain may display anti-metastatic effects only in the cells that display activity of both gelatinases, which was the case for SW480.

MMP-9 and TGF-β effects on MDA-MB231 cells

TGF-β is a potent inhibitor of growth and proliferation of breast epithelial cells. Malignant transformation and tumorigenesis has often been associated with loss of sensitivity to TGF-β and resistance to TGF-β growth inhibitory effects has been attributed at least in part to loss of expression of TGF-β type II receptor [101]. We observed that the MDA-MB231 cells that we used are responsive to TGF-β growth inhibition and that there are no differences in TGF-β-mediated reduction of proliferation between MDA-MB231 cells overexpressing the different mutants of MMP-9 (*Figure 12*). However, abolition of the increased invasiveness of MDA-MB231 cells expressing the FN domain of MMP-9 by addition of active TGF-β1 evokes a negative regulation of TGF-β by the FN domain during MDA-MB231 invasion (*Figure 13*). The absence of differences in MDA-MB231 primary tumor growth (*Figure 8C*) when the FN domain is expressed, on the other hand, suggests that the FN domain may have no effect on TGF-β pathway when these cells are responsive to TGF-β growth inhibition. The FN domain may therefore be implicated in adhesive, migratory and invasive features of MDA-MB231 cells (*Figure 14*), providing a potential scaffold for the cells to adhere to and migrate on. Thus, we hypothesize that the increase in lung metastasis observed by the FN domain overexpression may primarily affect MDA-MB231 invasiveness rather than their proliferation and that this mechanism may occur through impairment of TGF-β activity. In this case, TGF-β may display an anti-invasive function. Our hypothesis would therefore be in contradiction with the observation of Farina and coworkers that TGF-β1 enhances MDA-MB231 invasion in an uPA/plasmin-dependent fashion [102]. This unexpected and seemingly opposite effect of TGF-β may be explained by the observation that our MDA-MB231 are still responsive to TGF-β-growth inhibition in contrast to theirs. Consequently, responsiveness or not to TGF-β-growth inhibition may guide tumor cells at discrete stages of invasion and metastasis.

The relationship between the FN domain and potential TGF-β inhibition remains unclear. Preliminary experiments revealed that the FN domain does not inhibit TGF-β directly. Thus, taking into consideration that MDA-MB231 cells expressing the FN domain display inhibition of an active form of MMP-9 in their conditioned medium (*Figure 9B*), we hint that the potential effect of the FN domain on TGF-β may occur through MMP-9 inhibition. The hypothetical model of action that we evoke here is that during invasion, MDA-MB231

cells that overexpress the FN domain can use it as a scaffold that increases their adhesion (to endothelial cells facilitating extravasation to the lungs, or to lung parenchyma to colonies). Following extravasation, the FN domain may inhibit MMP-9 activation or activity, potentially by sequestration as described above, which would prevent latent TGF-β activation and consequently increase lung adherence, invasion and metastasis. This scenario would suggest protective roles of both MMP-9 and TGF-β in organ colonization, which can be interrelated if we can show that the inhibition of MMP-9 activity by the FN domain directly prevents TGF-β activation. To this end, we are now determining whether there is a difference in TGF-β activity in MDA-MB231 cells stably expressing MMP-9, the FN domain and control cells.

It has been described that as a tumor progresses, the genome often accumulates mutations in the TGF-β receptor system that renders cancer cells unresponsive to TGF-β [22]. Moreover, Kessenbrok and coworkers evoked that given that tumor cells often acquire unresponsiveness to TGF-β, proteolytic activation of TGF-β by MMPs may have tumor promoting effects by selectively driving stroma-mediated invasion of the tumor. So despite the fact that we observed that our MDA-MB231 cells were responsive to TGF-β growth inhibition *in vitro*, we could conceive that the FN domain may specifically block TGF-β responsiveness in MDA-MB231 cells *in vivo* by a mechanism such as sequestration of the TGF-β type II receptor and that the observed increase of metastasis is due to TGF-β activity on stroma, promoting invasion. In relation with the first part of our results, TGF-β activity may induce the differentiation of fibroblasts into myofibroblasts in order to increase invasion by leading tumor cells to metastasize [14].

The effect of the FN domain in MDA-MB231 cells

MDA-MB231 cells expressing the FN domain do not display cell surface MMP-9 but lack an active form of the enzyme in their conditioned medium (*Figure 11B*). This suggests that the FN domain acts in a way that is distinct from providing cell surface anchorage of MMP-9 activity. Most studies using MDA-MB231 cell line have shown that increased MMP-9 expression correlates with increased migration and invasion. However, if we assume that the FN domain inhibits MMP-9 activation or activity and increase metastasis in NOD/SCID mouse lungs, MMP-9 should provide tumor protective and anti-invasive

properties. Nevertheless, as evoked above, it is possible that the observed decreased MMP-9 activity in the conditioned medium of MDA-MB231 cells expressing the FN domain may not reflect the *in vivo* situation. Thus, in a 3D situation, there is maybe an overexpression of MMP-9 that is not induced by MDA-MB231 cells but by stromal cells, which could explain the increased invasion and metastasis observed. However, this does not explain the lack of increased lung metastasis of MDA-MB231 overexpressing MMP-9. Consequently, transcriptome analysis and SILAC of MDA-MB231 cells expressing MMP-9 or the FN domain could be performed in order to assess their secretion repertoire and see whether other factors may be involved in the mechanism.

Recently, Maity and coworkers found that culture of MDA-MB231 cells on fibronectin induces proMMP-9 expression and activity via fibronectin-integrin α5β1 signaling pathways, which enhances MDA-MB231 migration [103]. This observation prompted us to ask whether the FN domain, which may be considered as a "fibronectin-like fragment", may have the opposite effect of that of the entire molecule of fibronectin on MMP-9 expression, secretion and activity (*Figure 9B & 8B*), while promoting adhesion and migration (*Figure 13*), possibly through a TGF-β pathway. Safina and coworkers showed that in MDA-MB231 cells, TGF-β1 activates MAP kinases, via ALK5 (TGF-β type I receptor) which upregulates MMP-9 expression via the MEK-ERK pathway and not p38 MAPK, JNK or Smad4 and thus promotes tumor angiogenesis and metastasis [104]. Ilunga and coworkers also observed that the effect of Tenascin-C (TNC) and TGF-β on MDA-MB231 cells enhances MMP-9 expression and cancer invasion [105]. By contrast, our observations rather suggest a protective role of both MMP-9 and TGF-β in metastasis formation.

Finally, it is important to bear in mind the possibility of a simple and non-enzymatic mechanism to explain pro-invasive properties of the FN domain independently from the catalytic activity of MMP-9. Because MMP-9 is shed from the cell surface and deposited in the ECM, its FN domain could help tumor cells to invade tissues by providing a scaffold to migrate on.

The role of MMP-9 in SW480 cells

The hypothetical model that we propose concerning colorectal adenocarcinoma SW480 cell metastasis is more founded and correlates with what we know about the role of MMP-9 in tumor progression. Indeed, it highlights pro-tumorigenic and pro-invasive properties of MMP-9 and the ability of the FN domain to specifically inhibit MMP-9 activity at the SW480 cell surface by displacement (*Figure 11C*). We noticed that MMP-9 activity may be required in autocrine fashion in SW480 cells without any direct implications of the stroma, because despite the increase of active MMP-9 in the conditioned medium of SW480 expressing the FN domain (*Figure 9C*), we observed loss of MMP-9 activity at the membrane compared to SW480 cells expressing MMP-9 (*Figure 11C*). This observation does not support a protective role of active MMP-9 in the supernatant (evoked above) because SW480 cells overexpressing MMP-9 display an increase in both tumor growth and lung colonization (*Figure 8E & F*).

However, and in relation with what we described above, we may take into consideration the fact that the FN domain might activate proMMP-9 and that it is this secreted active form that is biologically relevant for metastasis and not the membrane bound MMP-9 [64]. In this case, overexpression of the FN domain would release active MMP-9 from the SW480 cell surface and promote its paracrine activity. By contrast, SW480 cells overexpressing MMP-9 may retain enzyme activity on their membrane, reducing its biological efficiency. In this situation, MMP-9 may also display a protective role against tumor growth and metastasis. Consequently, the FN domain would show a dual role in MMP-9 regulation depending on the cellular context.

The metastatic potential of colon cancer cells correlates with their enhanced secretion of plasminogen activators and MMP-2 [106]. Moreover, numerous studies have demonstrated the localization of MMPs at the interface between CRC cells and the surrounding stroma, due to CRC-induced, host cell expression [107]. This supports the notion that MMPs are necessary for metastasis in SW480 cells and that the tumor-produced FN domain may inhibit both tumor- and host-derived MMP-9 activity at the SW480 cell surface.

Furthermore, colon cancers often display mutations of TGF-βRII, that correlates with lack of TGF-β responsiveness but high secretion of TGF-β for paracrine recruitment of host cells [108]. Also, various colon cancers display the need for myofibroblasts for invasion, even though E-cadherin, known to prevent invasion, is retained. The importance of tumor-associated myofibroblasts has been illustrated by their secreted factors, specifically HGF in colon cancer, which induces the Wnt signaling pathway in tumor cells and thus, probably participates in restoring the cancer-stem cell phenotype [109]. In addition, De Wever and coworkers demonstrated that myofibroblasts from colon cancer stimulate tumor cell invasion through collagen I and matrigel via scatter factor/hepatocyte growth factor (SF/HGF) and the TGF-β-upregulated extracellular matrix glycoprotein TNC [110]. EGF-like repeats of TNC through EGF-receptor signaling confer a permissive signal for the proinvasive activity of SF/HGF, which activates Rac via c-Met. This myofibroblast-stimulated invasion is characterized by a migratory morphotype with low RhoA and high Rac activity. Thus, we may hypothesize that in SW480 cell metastasis development, overexpression of MMP-9 allows its recruitment to the fibroblast cell surface, according to the model that we proposed above, which differentiates them into myofibroblasts and leads to increased SW480 cell invasion. Consequently, it may be worthwhile to determine the relationship between SW480 cells and MMP-9-induced myofibroblasts in coculture condition in the absence or presence of the FN domain by assessing their MMP-9 secretion and their invasiveness. This would address the importance of the stroma, more particularly myofibroblasts, in this type of tumor cell and the ability of the FN domain to affect tumor cell growth and invasion by inhibiting their differentiation. This model would be in relation with the observations of Gaggioli and coworkers using squamous cell carcinoma cells that retain epithelial markers and are unable to remodel the surrounding ECM. These cells migrate in the value of fibroblasts that remodel the ECM by creating tracks via a MMP and Rho dependent mechanism [14].

TGF-β-induced Epithelial-to-Mesenchymal Transition (EMT)

Epithelial-to-Mesenchymal Transition (EMT) involves loss of epithelial and acquisition of mesenchymal gene expression programs, which allows tumor cells to acquire motility and invasiveness [111]. TGF-β is an inducer of EMT and has been shown to cooperate with the mitogenic Ras pathway to induce an invasive and proliferative tumor phenotype [111, 112].

Indeed, it promotes mesenchymal features only in Ras-transformed epithelial cells, while normal cells respond to TGF-β growth inhibition. In addition, the cells that have undergone EMT start to secrete TGF-β in an autocrine fashion in order to maintain the invasive phenotype. Thus, the discrepant results that we observed in NOD/SCID lung metastases with HT1080 and MDA-MB231 versus SW480 cells (*Figure 8*) may be related to the character of these cells. HT1080 fibrosarcoma are purely mesenchymal cells and have an activated N-ras oncogene [113]. MDA-MB231 adenocarcinoma cells have already undergone EMT and have been shown to have a hyperactive Ras pathway [114]. By contrast, SW480 colorectal adenocarcinoma cells are primarily epithelial, but also display mutated Ras proteins. Thus, MMP-9 and the FN domain may display opposite roles in lung colonization depending on the status of the cells. MMP-9 may act as a protective agent when the cells are mesenchymal, such as HT1080 and MDA-MB231, but may enhance EMT via TGF-β or other pathways when the cells display predominantly epithelial features.

Discrepancy of tumor- versus stromal-derived MMP-9

A potentially important point to consider is the relative role of MMP activity derived from host or tumor cells [47]. Indeed, there are examples where the effect of an MMP is dependent upon the cell in which it is expressed (MMP-12 [115]). Studies have demonstrated that host-derived MMP-9, supplied by resident stromal and inflammatory cells, penetrates the primary tumor site, but that tumor-derived MMP-9 is the initial trigger of the angiogenic switch [27] (seen by overexpression in tumor cells = increased tumorigenicity and invasiveness, whereas downregulation in tumor cells = decreased tumorigenesis and distant metastasis). The protective role of host-derived MMP-9 also appears to be mediated via angiogenic mechanisms through generation of angiogenic inhibitors [47]. In contrast to its apparent pro-metastatic role, tumor cell-derived MMP-9 has also been shown to display protective and anti-metastatic properties. Indeed, the specific and only downregulation of tumor cell derived MMP-9 by siRNA in a highly invasive HT1080 fibrosarcoma cell variant triggers unexpected increase in intravasation and metastasis [116]. We could therefore extrapolate that the FN domain expressed by HT1080 and/or MDA-MB231 tumor cells in our experimental setting inhibits MMP-9 activity specifically in these tumor cells, and not stromal MMP-9, which blocking generation of angiogenic inhibitors and increasing metastasis. If we assume that stromal

cell MMP-9 contributes to metastatic steps [24], overexpression of MMP-9 in tumor cells could conceivably provide protection against cancer progression, possibly by generating anti-angiogenic inhibitors or even by degrading pro-tumorigenic factors. This would explain our observation of increased lung metastasis in NOD/SCID mice injected with HT1080 or MDA-MB231 overexpressing the FN domain (*Figure 8B & D*). Nevertheless, this hypothesis has to be further proved using congenic mice, which have an intact immune system. Indeed, we cannot exclude the possibility of an inflammatory effect of the FN domain, for example by immune cell recruitment.

Overview of the FN type II-like domain potential functions

The FN domain is specific for gelatinases amongst MMPs and is composed of three fibronectin type II-like motifs, necessary for the binding of gelatin [29], elastin [29] and different collagen types [28]. MMP-9 and MMP-2 CBDs are very similar but differ by exosite substrate binding specificity.

Role in fibronectin fragmentation?

The role of fibronectin (FN) in metastasis has been attributed to its increased degradation and also decreased expression of its binding receptors [117]. Interestingly, a feedback loop exists between MMP-9 and FN, as MMP-9 degrades FN and binding of FN upregulates MMP-9 expression [118]. The mechanism underlying FN fragmentation is associated with tissue remodeling and FN fragments have been detected at high levels in wound exudates from chronic inflammatory conditions. It involves synergy between FN inherent autolytic and MMP activities by interaction of FN with the HEX domain of MMP-2 [119]. FN fragments have biological activities that differ from those of intact FN and affect cell behavior and MMPs expression [30, 120]. Indeed, HT1080 cells cultured on FN fragments showed a decrease of MMP-2 and MMP-9 activation due to the competition of these fragments with gelatinases at their cell surface [120]. These observations indicate that exposure of pathologically-generated FN fragments may alter cell behavior and thus, understanding the molecular mechanisms of ligand interactions and cellular regulation in response to FN fragments is of considerable interest. FN also interacts with α5β1 integrin (via RGD sequence) to induce MMP-9 activity and modulate migration and invasion of

B16F10 murine melanoma cells [121]. However, the FN type II-like motifs of MMP-2 have been shown to support cell attachment via a β1-integrin-dependent mechanism at the fibroblast cell surface, despite the absence of an RGD sequence in these motifs [38], suggesting a role of this domain in cell adhesion and inhibition of MMP-2 activation.

By contrast to tumor cells, FN has been shown to modulate MMP-9 secretion by monocytes and thereby their migration in opposite directions according to whether it is in native or fragmented form [118]. Indeed, fragments of FN antagonize the inhibitory effects of intact FN in MMP-9 production and consequently enhance monocyte migration through ECM degradation. Thus, the differences between full-length and fragmented FN, more particularly FN type II-modules in our case, may provide a means to study MMP activation and regulation pathways.

Role as scaffold in pre-metastatic niches?

An interesting hypothesis that may explain the dramatic increase of lung metastasis due to the FN domain of MMP-9 in HT1080 and MDA-MB231-injected mice could be related to the role of the FN domain as a scaffold, allowing adhesion and pre-metastatic niche formation. Kaplan and coworkers observed that premetastatic niches are generated by circulating bone marrow-derived VEGFR1-positive hematopoietic progenitor cells that infiltrate secondary organs such as lungs to form a pre-metastatic cell cluster. These cells express α4β1 integrin and their extravasation is facilitated by high levels of FN, which is produced by stromal fibroblasts in response to tumor-specific growth factors. These clusters express MMP-9 that breaks down the basement membrane and releases VEGF and soluble kit-ligand [122]. We can therefore envisage that the FN domain secreted by HT1080 and/or MDA-MB231 cells may serve as a scaffold for pre-metastatic niches by recruiting VEGFR1-positive bone marrow cells and allowing their extravasation, which leads to increased lung adhesion of these tumor cells (*Figure 14*).

Role in platelet aggregation?

Extensive evidence shows that platelets are involved in key steps of tumor progression and that platelet aggregation correlates with metastasis [123, 124]. Tumor cells have been shown to induce platelet activation and aggregate formation in capillaries in order to

shield them from immune surveillance [123-126]. FN has been shown to inhibit platelet aggregation [127] and to thereby display anti-tumoral effects. FN fragmentation and thus potential presence of the FN domain of MMP-9 may enhance platelet aggregation, which in turn abolishes the anti-tumoral effect of intact FN and promotes HT1080 and MDA-MB231 invasion and metastasis (*Figure 8B & D*). As we performed experimental metastasis assays in NOD/SCID mice, tumor cells were directly injected in the tail vein. Therefore, components of the blood circulation, including platelets play a key role in the tumor cell journey to lungs. The FN domain of MMP-9 secreted by HT1080 or MDA-MB231 cells may directly affect platelet aggregation and function by interacting with platelet receptors (glycoprotein VI (GPVI), glycoprotein Ibα (GPIbα) or platelet integrin αIIbβ3 (GPIIb/IIIa)), necessary for adhesion and aggregation, or other molecules involved in blood coagulation. This may increase tumor cell survival, protection from the immune system, but also adhesion, migration and extravasation of tumor cells from blood vessels, thereby promoting lung metastasis. In addition, it has been proposed that the FN type II-like motifs, present only in vertebrates, originate from structural modification of the more ancestral kringle domain [128]. Kringle domains are present in proteins implicated in blood coagulation, including but not only, prothrombin, plasminogen, urokinase, tissue-plasminogen activator, hepatocyte growth factor and activator, coagulation factor XI [88] thus reinforcing the fact that the FN domain of MMP-9 may play a role in platelet aggregation by interaction with homologous domains.

Interestingly, MMP-2 has been shown be released from platelets to be activated and to induce platelet activation and aggregation [129]. By contrast, MMP-9 could display anti-aggregatory effects opposing the effects of MMP-2 [130]. Thus, the MMP-2/MMP-9 system may play a key role in the regulation of platelet-platelet and platelet-vessel wall interactions. These observations underline once again, the importance of the right balance between MMP expression, location and timing of their activation. In this case, MMP-9 may display a tumor-protective role, which may be related to our observations in HT1080 and MDA-MB231 cells and the FN domain may counteract the anti-aggregatory effect by inhibiting MMP-9 activity in blood vessels.

Do the FN type II-like motifs exist in the nature?

An interesting question in this study is the possible existence of the FN domain of MMP-9, i.e. the three FN type II modules in normal and natural conditions. Although we have no direct proofs that the FN domain exist naturally in 3 FN type II-like motifs, we may consider that they may result as fragments produced by either FN, as we described above, or degradation of ECM molecules exposing cryptic protein domains and generating specific new molecule fragments that can have pro-migratory as well as pro- and anti-angiogenic functions [6, 131].

Whereas intact FN appears to diminish metastasis, its fragments, generated proteolytically or by differential splicing, display pro-tumorigenic and pro-invasive properties. These notions could potentially provide a basis for an explanation of the observed effect of the FN domain of MMP-9 on tumor cell behavior in our study. In MDA-MB231 and HT1080 cells, the FN domain may act in a non-enzymatic pro-invasive fashion, whereas it may inhibit MMP-9 activity directly on SW480 cells.

Epilogue

In this work, we underscore a pivotal role of MMP-9 in tumor cell invasion and metastasis, showing that depending on the context, MMP-9 can be agonistic or antagonistic for tumor growth and more particularly, metastasis. We also unveil novel FN domain features and demonstrate that MMP-9 and its recombinant FN domain have opposing functions in supporting tumor progression. The overall study can be subdivided into two sets of observations:

First, we report differential recruitment of MMP-9 to the surface of fibroblasts and tumor cells, showing far more robust recruitment to the fibroblast membrane. Cell surface activity of MMP-9 is shown to be important for growth factor activation (particularly TGF-β), and probably also pericellular matrix remodeling, although we did not address this issue directly. TGF-β activation, whether on the fibroblast surface or in the immediate pericellular fibroblast microenvironment, may play a critical role in fibroblast differentiation into myofibroblasts and thereby provide a mechanism underlying the constitution of at least a subset of CAFs. In this context, the recombinant FN domain blocks MMP-9-dependent, TGF-β-mediated myofibroblast differentiation and thereby abrogates an important fuelling mechanism of tumor progression.

In our second set of observations, we determined the importance of MMP-9 activity in different tumor cell lines and showed that despite the commonly accepted notion that MMP-9 expression promotes tumor invasion and metastasis, MMP-9 can fulfill an inhibitory role in tumor dissemination. These observations underscore the all too frequently overlooked notion that despite a voluminous literature on MMP function and implication in tumor growth and metastasis, full elucidation of role of MMPs in cancer remains a field in need of investment. Studies in which MMP-9 function in tumor progression is investigated in several diverse tumor cell lines, for instance, are rare. In the present work, we highlight discrepant roles of MMP-9 in tumor growth and invasion in a cell context-dependent fashion and disclose a hitherto unrecognized property of the FN peptide, recognized as a collagen-binding domain, in the modulation of pro- and anti-tumorigenic features of MMP-9 activity.

The complexity of MMP regulation illustrates well the discrepant effects of what are often considered to be potentially key molecules in cancer development and progression. It is also a clear reminder that successful therapeutic targeting of solid tumors will require in-depth understanding in a tumor type- and probably stage-specific manner of all of the key events in tumor host interactions, including tumor cell-dependent events, signals delivered by the soil in which the tumor cells are implanted and the role of both tumor and stromal-derived bi-functional molecules that can enhance or abet hijacking of the host tissue.

References

1. Bacac, M. and I. Stamenkovic, *Metastatic cancer cell.* Annu Rev Pathol, 2008. **3**: p. 221-47.
2. Stamenkovic, I., *Matrix metalloproteinases in tumor invasion and metastasis.* Semin Cancer Biol, 2000. **10**(6): p. 415-33.
3. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
4. Tlsty, T.D. and L.M. Coussens, *Tumor stroma and regulation of cancer development.* Annu Rev Pathol, 2006. **1**: p. 119-50.
5. Pietras, K. and A. Ostman, *Hallmarks of cancer: interactions with the tumor stroma.* Exp Cell Res, 2010. **316**(8): p. 1324-31.
6. Mueller, M.M. and N.E. Fusenig, *Friends or foes - bipolar effects of the tumour stroma in cancer.* Nat Rev Cancer, 2004. **4**(11): p. 839-49.
7. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, *Stromal fibroblasts in cancer initiation and progression.* Nature, 2004. **432**(7015): p. 332-7.
8. Liotta, L.A. and E.C. Kohn, *The microenvironment of the tumour-host interface.* Nature, 2001. **411**(6835): p. 375-9.
9. Dvorak, H.F., *Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.* N Engl J Med, 1986. **315**(26): p. 1650-9.
10. Midwood, K.S., L.V. Williams, and J.E. Schwarzbauer, *Tissue repair and the dynamics of the extracellular matrix.* Int J Biochem Cell Biol, 2004. **36**(6): p. 1031-7.
11. Kalluri, R. and M. Zeisberg, *Fibroblasts in cancer.* Nat Rev Cancer, 2006. **6**(5): p. 392-401.
12. De Wever, O. and M. Mareel, *Role of myofibroblasts at the invasion front.* Biol Chem, 2002. **383**(1): p. 55-67.
13. De Wever, O. and M. Mareel, *Role of tissue stroma in cancer cell invasion.* J Pathol, 2003. **200**(4): p. 429-47.
14. Gaggioli, C., et al., *Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells.* Nat Cell Biol, 2007. **9**(12): p. 1392-400.
15. Liao, D., et al., *Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model.* PLoS One, 2009. **4**(11): p. e7965.
16. Fullar, A., et al., *Tumor cell and carcinoma-associated fibroblast interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma.* Exp Cell Res, 2012. **318**(13): p. 1517-27.
17. Bhowmick, N.A., et al., *TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia.* Science, 2004. **303**(5659): p. 848-51.
18. Akhurst, R.J., *TGF-beta antagonists: why suppress a tumor suppressor?* J Clin Invest, 2002. **109**(12): p. 1533-6.
19. Vaughan, M.B., E.W. Howard, and J.J. Tomasek, *Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast.* Exp Cell Res, 2000. **257**(1): p. 180-9.
20. Desmouliere, A., et al., *Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts.* J Cell Biol, 1993. **122**(1): p. 103-11.
21. Stamenkovic, I., *Extracellular matrix remodelling: the role of matrix metalloproteinases.* J Pathol, 2003. **200**(4): p. 448-64.
22. Kessenbrock, K., V. Plaks, and Z. Werb, *Matrix metalloproteinases: regulators of the tumor microenvironment.* Cell, 2010. **141**(1): p. 52-67.
23. Egeblad, M. and Z. Werb, *New functions for the matrix metalloproteinases in cancer progression.* Nat Rev Cancer, 2002. **2**(3): p. 161-74.

24. Martin, M.D. and L.M. Matrisian, *The other side of MMPs: protective roles in tumor progression.* Cancer Metastasis Rev, 2007. **26**(3-4): p. 717-24.
25. Deryugina, E.I. and J.P. Quigley, *Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions.* Biochim Biophys Acta, 2010. **1803**(1): p. 103-20.
26. Coussens, L.M., et al., *MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis.* Cell, 2000. **103**(3): p. 481-90.
27. Bergers, G., et al., *Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis.* Nat Cell Biol, 2000. **2**(10): p. 737-44.
28. Xu, X., et al., *Functional basis for the overlap in ligand interactions and substrate specificities of matrix metalloproteinases-9 and -2.* Biochem J, 2005. **392**(Pt 1): p. 127-34.
29. Collier, I.E., et al., *Alanine scanning mutagenesis and functional analysis of the fibronectin-like collagen-binding domain from human 92-kDa type IV collagenase.* J Biol Chem, 1992. **267**(10): p. 6776-81.
30. Steffensen, B., U.M. Wallon, and C.M. Overall, *Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen.* J Biol Chem, 1995. **270**(19): p. 11555-66.
31. Overall, C.M., *Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites.* Mol Biotechnol, 2002. **22**(1): p. 51-86.
32. Xu, X., et al., *Contributions of the MMP-2 collagen binding domain to gelatin cleavage. Substrate binding via the collagen binding domain is required for hydrolysis of gelatin but not short peptides.* Matrix Biol, 2004. **23**(3): p. 171-81.
33. Banyai, L., H. Tordai, and L. Patthty, *Structure and domain-domain interactions of the gelatin binding site of human 72-kilodalton type IV collagenase (gelatinase A, matrix metalloproteinase 2).* J Biol Chem, 1996. **271**(20): p. 12003-8.
34. Xu, X., et al., *Matrix metalloproteinase-2 contributes to cancer cell migration on collagen.* Cancer Res, 2005. **65**(1): p. 130-6.
35. O'Farrell, T.J. and T. Pourmotabbed, *The fibronectin-like domain is required for the type V and XI collagenolytic activity of gelatinase B.* Arch Biochem Biophys, 1998. **354**(1): p. 24-30.
36. Shipley, J.M., et al., *The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases. Role of the fibronectin type II-like repeats.* J Biol Chem, 1996. **271**(8): p. 4335-41.
37. Berton, A., et al., *Involvement of fibronectin type II repeats in the efficient inhibition of gelatinases A and B by long-chain unsaturated fatty acids.* J Biol Chem, 2001. **276**(23): p. 20458-65.
38. Steffensen, B., H.F. Bigg, and C.M. Overall, *The involvement of the fibronectin type II-like modules of human gelatinase A in cell surface localization and activation.* J Biol Chem, 1998. **273**(32): p. 20622-8.
39. Fridman, R., et al., *Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2).* Cancer Res, 1995. **55**(12): p. 2548-55.
40. Ogata, Y., J.J. Enghild, and H. Nagase, *Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9.* J Biol Chem, 1992. **267**(6): p. 3581-4.
41. Fridman, R., et al., *Cell surface association of matrix metalloproteinase-9 (gelatinase B).* Cancer Metastasis Rev, 2003. **22**(2-3): p. 153-66.
42. Bannikov, G.A., et al., *Substrate binding of gelatinase B induces its enzymatic activity in the presence of intact propeptide.* J Biol Chem, 2002. **277**(18): p. 16022-7.
43. Van den Steen, P.E., et al., *Matrix remodelling enzymes, the protease cascade and glycosylation.* Biochim Biophys Acta, 2001. **1528**(2-3): p. 61-73.
44. Kotra, L.P., et al., *N-Glycosylation pattern of the zymogenic form of human matrix metalloproteinase-9.* Bioorg Chem, 2002. **30**(5): p. 356-70.

45. Roomi, M.W., et al., *Patterns of MMP-2 and MMP-9 expression in human cancer cell lines.* Oncol Rep, 2009. **21**(5): p. 1323-33.
46. Malla, N., et al., *Biological and pathobiological functions of gelatinase dimers and complexes.* Connect Tissue Res, 2008. **49**(3): p. 180-4.
47. Deryugina, E.I. and J.P. Quigley, *Matrix metalloproteinases and tumor metastasis.* Cancer Metastasis Rev, 2006. **25**(1): p. 9-34.
48. Derynck, R., R.J. Akhurst, and A. Balmain, *TGF-beta signaling in tumor suppression and cancer progression.* Nat Genet, 2001. **29**(2): p. 117-29.
49. Heissig, B., et al., *Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand.* Cell, 2002. **109**(5): p. 625-37.
50. Sheu, B.C., et al., *A novel role of metalloproteinase in cancer-mediated immunosuppression.* Cancer Res, 2001. **61**(1): p. 237-42.
51. Cornelius, L.A., et al., *Matrix metalloproteinases generate angiostatin: effects on neovascularization.* J Immunol, 1998. **161**(12): p. 6845-52.
52. Ferreras, M., et al., *Generation and degradation of human endostatin proteins by various proteinases.* FEBS Lett, 2000. **486**(3): p. 247-51.
53. Kim, Y.M., et al., *Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase.* Cancer Res, 2000. **60**(19): p. 5410-3.
54. Hamano, Y., et al., *Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin.* Cancer Cell, 2003. **3**(6): p. 589-601.
55. Toth, M., et al., *Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes.* Biochem Biophys Res Commun, 2003. **308**(2): p. 386-95.
56. Fiore, E., et al., *Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity.* Oncogene, 2002. **21**(34): p. 5213-23.
57. Yu, Q. and I. Stamenkovic, *Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion.* Genes Dev, 1999. **13**(1): p. 35-48.
58. Yu, Q. and I. Stamenkovic, *Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis.* Genes Dev, 2000. **14**(2): p. 163-76.
59. Olson, M.W., et al., *High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV.* J Biol Chem, 1998. **273**(17): p. 10672-81.
60. Mantuano, E., et al., *The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein.* J Neurosci, 2008. **28**(45): p. 11571-82.
61. Takahashi, C., et al., *Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13221-6.
62. Chang, C.K., W.C. Hung, and H.C. Chang, *The Kazal motifs of RECK protein inhibit MMP-9 secretion and activity and reduce metastasis of lung cancer cells in vitro and in vivo.* J Cell Mol Med, 2008. **12**(6B): p. 2781-9.
63. Hahn-Dantona, E., et al., *The low density lipoprotein receptor-related protein modulates levels of matrix metalloproteinase 9 (MMP-9) by mediating its cellular catabolism.* J Biol Chem, 2001. **276**(18): p. 15498-503.
64. Mira, E., et al., *Secreted MMP9 promotes angiogenesis more efficiently than constitutive active MMP9 bound to the tumor cell surface.* J Cell Sci, 2004. **117**(Pt 9): p. 1847-57.
65. Bourguet, E., et al., *Pharmacomodulation of Broad Spectrum Matrix Metalloproteinase Inhibitors Towards Regulation of Gelatinases, Enzyme Inhibition and Bioapplications*, 2012.

66. Acuff, H.B., et al., *Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment.* Cancer Res, 2006. **66**(1): p. 259-66.
67. Bendrik, C., et al., *Gene transfer of matrix metalloproteinase-9 induces tumor regression of breast cancer in vivo.* Cancer Res, 2008. **68**(9): p. 3405-12.
68. Pozzi, A., et al., *Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization.* Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2202-7.
69. Chen, X., et al., *Increased plasma MMP9 in integrin alpha1-null mice enhances lung metastasis of colon carcinoma cells.* Int J Cancer, 2005. **116**(1): p. 52-61.
70. Shchors, K., et al., *Increased invasiveness of MMP-9-deficient tumors in two mouse models of neuroendocrine tumorigenesis.* Oncogene, 2013. **32**(4): p. 502-13.
71. Giambernardi, T.A., et al., *Overview of matrix metalloproteinase expression in cultured human cells.* Matrix Biol, 1998. **16**(8): p. 483-96.
72. Lauer-Fields, J.L., et al., *Selective modulation of matrix metalloproteinase 9 (MMP-9) functions via exosite inhibition.* J Biol Chem, 2008. **283**(29): p. 20087-95.
73. Matsuzaki, K., et al., *Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer.* Cancer Res, 2009. **69**(13): p. 5321-30.
74. Kojima, Y., et al., *Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts.* Proc Natl Acad Sci U S A, 2010. **107**(46): p. 20009-14.
75. Smith, H.W. and C.J. Marshall, *Regulation of cell signalling by uPAR.* Nat Rev Mol Cell Biol, 2010. **11**(1): p. 23-36.
76. Bernstein, A.M., et al., *Urokinase receptor cleavage: a crucial step in fibroblast-to-myofibroblast differentiation.* Mol Biol Cell, 2007. **18**(7): p. 2716-27.
77. Dublin, E., et al., *Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma. Fibroblastic expression has strong associations with tumor pathology.* Am J Pathol, 2000. **157**(4): p. 1219-27.
78. Wang, L., et al., *Degradation of internalized alphavbeta5 integrin is controlled by uPAR bound uPA: effect on beta1 integrin activity and alpha-SMA stress fiber assembly.* PLoS One, 2012. **7**(3): p. e33915.
79. Zhao, Y., et al., *Urokinase directly activates matrix metalloproteinases-9: a potential role in glioblastoma invasion.* Biochem Biophys Res Commun, 2008. **369**(4): p. 1215-20.
80. Raghu, H., et al., *Localization of uPAR and MMP-9 in lipid rafts is critical for migration, invasion and angiogenesis in human breast cancer cells.* BMC Cancer, 2010. **10**: p. 647.
81. Behrendt, N., *The urokinase receptor (uPAR) and the uPAR-associated protein (uPARAP/Endo180): membrane proteins engaged in matrix turnover during tissue remodeling.* Biol Chem, 2004. **385**(2): p. 103-36.
82. Wipff, P.J., et al., *Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix.* J Cell Biol, 2007. **179**(6): p. 1311-23.
83. Ramos-DeSimone, N., et al., *Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion.* J Biol Chem, 1999. **274**(19): p. 13066-76.
84. Acharya, P.S., et al., *Fibroblast migration is mediated by CD44-dependent TGF beta activation.* J Cell Sci, 2008. **121**(Pt 9): p. 1393-402.
85. Dufour, A., et al., *Small-molecule anticancer compounds selectively target the hemopexin domain of matrix metalloproteinase-9.* Cancer Res, 2011. **71**(14): p. 4977-88.
86. Dufour, A., et al., *Role of matrix metalloproteinase-9 dimers in cell migration: design of inhibitory peptides.* J Biol Chem, 2010. **285**(46): p. 35944-56.

87. Olson, M.W., et al., *Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1.* J Biol Chem, 2000. **275**(4): p. 2661-8.
88. Ozhogina, O.A., et al., *Origin of fibronectin type II (FN2) modules: structural analyses of distantly-related members of the kringle family idey the kringle domain of neurotrypsin as a potential link between FN2 domains and kringles.* Protein Sci, 2001. **10**(10): p. 2114-22.
89. Saad, S., et al., *Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts.* Cancer Res, 2002. **62**(1): p. 283-9.
90. Lambert Vidmar, S., et al., *Collagen-binding domain of human plasma fibronectin contains a latent type-IV collagenase.* Eur J Biochem, 1991. **201**(1): p. 79-84.
91. Gioia, M., et al., *The collagen binding domain of gelatinase A modulates degradation of collagen IV by gelatinase B.* J Mol Biol, 2009. **386**(2): p. 419-34.
92. Cirri, P. and P. Chiarugi, *Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression.* Cancer Metastasis Rev, 2012. **31**(1-2): p. 195-208.
93. Cheng, N., et al., *Enhanced hepatocyte growth factor signaling by type II transforming growth factor-beta receptor knockout fibroblasts promotes mammary tumorigenesis.* Cancer Res, 2007. **67**(10): p. 4869-77.
94. Cheng, N., et al., *Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion.* Mol Cancer Res, 2008. **6**(10): p. 1521-33.
95. Orimo, A., et al., *Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion.* Cell, 2005. **121**(3): p. 335-48.
96. Shimoda, M., K.T. Mellody, and A. Orimo, *Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression.* Semin Cell Dev Biol, 2010. **21**(1): p. 19-25.
97. Loeffler, M., et al., *Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake.* J Clin Invest, 2006. **116**(7): p. 1955-62.
98. Brinckerhoff, C.E. and L.M. Matrisian, *Matrix metalloproteinases: a tail of a frog that became a prince.* Nat Rev Mol Cell Biol, 2002. **3**(3): p. 207-14.
99. Coussens, L.M., B. Fingleton, and L.M. Matrisian, *Matrix metalloproteinase inhibitors and cancer: trials and tribulations.* Science, 2002. **295**(5564): p. 2387-92.
100. Hofmann, U.B., et al., *Expression of matrix metalloproteinases in the microenvironment of spontaneous and experimental melanoma metastases reflects the requirements for tumor formation.* Cancer Res, 2003. **63**(23): p. 8221-5.
101. Lynch, M.A., et al., *Responsiveness to transforming growth factor-beta (TGF-beta)-mediated growth inhibition is a function of membrane-bound TGF-beta type II receptor in human breast cancer cells.* Gene Expr, 2001. **9**(4-5): p. 157-71.
102. Farina, A.R., et al., *Transforming growth factor-beta1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity.* Int J Cancer, 1998. **75**(5): p. 721-30.
103. Maity, G., et al., *Culture of human breast cancer cell line (MDA-MB-231) on fibronectin-coated surface induces pro-matrix metalloproteinase-9 expression and activity.* Tumour Biol, 2011. **32**(1): p. 129-38.
104. Safina, A., E. Vandette, and A.V. Bakin, *ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells.* Oncogene, 2007. **26**(17): p. 2407-22.
105. Ilunga, K., et al., *Co-stimulation of human breast cancer cells with transforming growth factor-beta and tenascin-C enhances matrix metalloproteinase-9 expression and cancer cell invasion.* Int J Exp Pathol, 2004. **85**(6): p. 373-9.
106. Shah, V., S. Kumar, and K.A. Zirvi, *Metastasis of human colon tumor cells in vivo: correlation with the overexpression of plasminogen activators and 72 kDa gelatinase.* In Vivo, 1994. **8**(3): p. 321-6.

107. Mc Donnell, S., et al., *Metastatic and non-metastatic colorectal cancer (CRC) cells induce host metalloproteinase production in vivo.* Clin Exp Metastasis, 1999. **17**(4): p. 341-9.
108. Massague, J., S.W. Blain, and R.S. Lo, *TGFbeta signaling in growth control, cancer, and heritable disorders.* Cell, 2000. **103**(2): p. 295-309.
109. Vermeulen, L., et al., *Wnt activity defines colon cancer stem cells and is regulated by the microenvironment.* Nat Cell Biol, 2010. **12**(5): p. 468-76.
110. De Wever, O., et al., *Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac.* FASEB J, 2004. **18**(9): p. 1016-8.
111. Xu, J., S. Lamouille, and R. Derynck, *TGF-beta-induced epithelial to mesenchymal transition.* Cell Res, 2009. **19**(2): p. 156-72.
112. Oft, M., et al., *TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells.* Genes Dev, 1996. **10**(19): p. 2462-77.
113. Paterson, H., et al., *Activated N-ras controls the transformed phenotype of HT1080 human fibrosarcoma cells.* Cell, 1987. **51**(5): p. 803-12.
114. Chen, C.R., Y. Kang, and J. Massague, *Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program.* Proc Natl Acad Sci U S A, 2001. **98**(3): p. 992-9.
115. Kerkela, E., et al., *Metalloelastase (MMP-12) expression by tumour cells in squamous cell carcinoma of the vulva correlates with invasiveness, while that by macrophages predicts better outcome.* J Pathol, 2002. **198**(2): p. 258-69.
116. Deryugina, E.I., et al., *Unexpected effect of matrix metalloproteinase down-regulation on vascular intravasation and metastasis of human fibrosarcoma cells selected in vivo for high rates of dissemination.* Cancer Res, 2005. **65**(23): p. 10959-69.
117. Akiyama, S.K., K. Olden, and K.M. Yamada, *Fibronectin and integrins in invasion and metastasis.* Cancer Metastasis Rev, 1995. **14**(3): p. 173-89.
118. Marom, B., et al., *Native and fragmented fibronectin oppositely modulate monocyte secretion of MMP-9.* J Leukoc Biol, 2007. **81**(6): p. 1466-76.
119. Steffensen, B., et al., *Fragmentation of fibronectin by inherent autolytic and matrix metalloproteinase activities.* Matrix Biol, 2011. **30**(1): p. 34-42.
120. Steffensen, B., et al., *Human fibronectin and MMP-2 collagen binding domains compete for collagen binding sites and modify cellular activation of MMP-2.* Matrix Biol, 2002. **21**(5): p. 399-414.
121. Sil, H., T. Sen, and A. Chatterjee, *Fibronectin-integrin (alpha5beta1) modulates migration and invasion of murine melanoma cell line B16F10 by involving MMP-9.* Oncol Res, 2011. **19**(7): p. 335-48.
122. Kaplan, R.N., et al., *VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche.* Nature, 2005. **438**(7069): p. 820-7.
123. Crissman, J.D., et al., *Morphological study of the interaction of intravascular tumor cells with endothelial cells and subendothelial matrix.* Cancer Res, 1988. **48**(14): p. 4065-72.
124. Bastida, E. and A. Ordinas, *Platelet contribution to the formation of metastatic foci: the role of cancer cell-induced platelet activation.* Haemostasis, 1988. **18**(1): p. 29-36.
125. Bambace, N.M. and C.E. Holmes, *The platelet contribution to cancer progression.* J Thromb Haemost, 2011. **9**(2): p. 237-49.
126. Gay, L.J. and B. Felding-Habermann, *Contribution of platelets to tumour metastasis.* Nat Rev Cancer, 2011. **11**(2): p. 123-34.
127. Tanabe, J., et al., *Fibronectin inhibits platelet aggregation independently of RGD sequence.* J Biol Chem, 1993. **268**(36): p. 27143-7.
128. Patthy, L., et al., *Kringles: modules specialized for protein binding. Homology of the gelatin-binding region of fibronectin with the kringle structures of proteases.* FEBS Lett, 1984. **171**(1): p. 131-6.

129. Sawicki, G., et al., *Release of gelatinase A during platelet activation mediates aggregation.* Nature, 1997. **386**(6625): p. 616-9.
130. Fernandez-Patron, C., et al., *Differential regulation of platelet aggregation by matrix metalloproteinases-9 and -2.* Thromb Haemost, 1999. **82**(6): p. 1730-5.
131. Kalluri, R., *Basement membranes: structure, assembly and role in tumour angiogenesis.* Nat Rev Cancer, 2003. **3**(6): p. 422-33.