Matrix Metalloproteinase-14 is a Mechanically Regulated Activator of Secreted MMPs and Invasion

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Short Title: Mechanical Regulation Through an MMP Hierarchy

Abbreviations:

MMP – matrix metalloproteinase

ECM – extracellular matrix

CAT – catalytic domain

MT-MMP – membrane-tethered MMP

S-MMP – soluble MMP

Abstract:

Matrix metalloproteinases (MMPs) are extracellular matrix (ECM) degrading enzymes and have complex and specific regulation networks. This includes activation interactions, where one MMP family member activates another. ECM degradation and MMP activation can be initiated by several different stimuli including changes in ECM mechanical properties or intracellular contractility. These mechanical stimuli are known enhancers of metastatic potential. MMP-14 facilitates local ECM degradation and is well known as a major mediator of cell migration, angiogenesis and invasion. Recently, function blocking antibodies have been developed to specifically block MMP-14, providing a useful tool for research as well as therapeutic applications. Here we utilize a selective MMP-14 function blocking antibody to delineate the role of MMP-14 as an activator of other MMPs in response to changes in cellular contractility and ECM stiffness. Inhibition using function blocking antibodies reveals that MMP-14 activates soluble MMPs like MMP-2 and -9 under various mechanical stimuli in the pancreatic cancer cell line, Panc-1. In addition, inhibition of MMP-14 abates Panc-1 cell extension into 3D gels to levels seen with non-specific pan-MMP inhibitors at higher concentrations. This strengthens the case for MMP function blocking antibodies as more potent and specific MMP inhibition therapeutics.

Keywords: MT1-MMP, contractility, force, pancreatic cancer, function-blocking antibody, cleavage peptide

Introduction:

In order for cells to penetrate dense extracellular matrix (ECM), they must degrade ECM, allowing them to squeeze through small, fixed pores. Matrix metalloproteinases (MMPs) comprise a large family of enzymes that degrade ECM. MMP activity has been tightly associated with cancer progression, most notably during metastasis [1]. MMP activation is tightly controlled and upregulated in response to inputs including hypoxia, growth factors and ECM composition. However, metastasis is also controlled through extracellular mechanical inputs such as ECM crosslinking or ECM density [2,3] as well as intracellular mechanical responses such as contractility and traction force [4,5,6,7]. This has led to work examining if mechanical inputs regulate MMP

activity. Indeed, MMPs cleave collagen at different rates when collagen is under different amounts of tension [8,9]. In addition, bulk MMP activity and ECM degradation at the sites of invadopodia depends on cellular contractility, traction force and ECM stiffness [10,11,12,13]. However, it is not known which MMP family member is mediating the responses to mechanical inputs. Because cancer cells appear to sense mechanical inputs differently, it is particularly important to know which MMP is transducing the mechanosensitive response if therapeutic approaches to block mechanosensitive MMP activity are to be designed.

MMP-14 is a membrane-tethered MMP that cleaves several cell adhesion proteins and is involved in growth factor processing [14,15], but its primary role is in localized ECM degradation. Recently, it was discovered that MMP-14 can be secreted in exosomes [16], however MMP-14 often works while at the cell membrane and in close proximity to ECM attachment. For instance, MMP-14 localizes to perinuclear regions that are in close contact with ECM that form small pores, hindering the advance of the nucleus [17,18]. MMP-14 also localizes to other focal degradation structures including invadopodia and focal adhesions [19,20]. At these sites MMP-14 activity acts as a collagenase; however, once collagen is cleaved gelatinases like MMP-2 and -9 can fully degrade these partially degraded collagen fibers [1]. Different MMPs might cooperate in order to achieve a required ECM degradation. This idea of cooperation is strengthened by evidence showing that MMP-14 can activate other MMPs by cleaving their pro-domains and can bind soluble MMPs and localize them to the surface of the cell [1]. The localized degradation by MMP-14 as well as its ability to activate soluble MMPs suggest that MMP-14 is a powerful point of control over ECM degradation that is needed to facilitate cell migration though dense ECM [21].

Due to the roles outlined above, MMPs remain one of the most appealing drug targets for prevention of cancer metastasis. However, the failure of small molecule pan-MMP inhibitors has dampened the outlook, presumably due to the fact that these inhibitors block several of the MMP family members with advantageous or homeostatic activities [1,22]. More specific and potent inhibition of MMPs like MMP-14 is desired [23,24,25]. Function blocking antibodies have provided a hopeful outlook in blocking individual MMPs [24]. Characterizing how these very specific MMP-14 inhibitors affect cell migration in dense ECM as well as using them to understand how the MMP-14 inhibition regulates the activity of other MMPs is critical. This is

particularly relevant in pancreatic cancer, where cancer progression is marked by the fibrotic nature of the ECM surrounding and throughout the tumor [26]. This fibrotic ECM is dense and stiff and requires high pericellular MMP activity in order for cells to invade through it suggesting that this disease might be sensitive to specific MMP-14 inhibition as an approach to block invasion and metastasis.

Materials & Methods:

DX-2400 Fab Production and Inhibition Assay:

The V_H and V_L domains of DX-2400 were PCR amplified using pMopac-DX-2400 scFv as the template [27], then cloned into NsiI/HindIII and BglII/BsmBI sites on pHP153 phagemid expression vector [28]. A 6xHis tag followed by a stop codon was inserted after heavy chain constant CH1 domain for purification. After overnight cultivation at 30 ^{o}C in 1L 2 x YT media, soluble DX-2400 Fab was recovered from the periplasmic fraction by osmotic shocks [29] and purified by affinity chromatography using Ni-NTA resin (Qiagen). The homogeneity of purified DX-2400 Fab was verified by SDS-PAGE, and its concentration was measured by NanoDrop (Thermo Scientific). Purified DX-2400 Fab was dialyzed in 50 mM Tris-HCl (pH 7.5) overnight to remove excessive imidazole for in vitro inhibition assays and cell-based bioassays.

MMP-14 catalytic domain (MMP-14 CAT) was constructed, expressed and refolded as previously described [27]. Typically, 250 mL culture yielded 5 mg purified chMMP-14 with 35% refolding efficiency. Inhibition assay of DX 2400 Fab was performed by measuring catalytic activity of purified MMP-14 CAT in the presence of 0-10 μM of DX-2400 Fab using quenched fluorescent cleavage peptides S-MMP (Mca-PLGL-Dpa-AR-NH_2, R & D Systems, ES001) or MT-MMP (Mca-PLA-C(OMeBz)-WAR(Dpa)-NH_2, Calbiochem, 444528) as the substrate.

Cell Culture, ECM Conditions and Pharmacological Inhibitors:

Human pancreatic cancer cells (Panc-1, ATCC) were used for all experiments as indicated. Cultures were maintained using DMEM with phenol red + 10% FBS, 2% GlutaMAX, and 1% penicillin/streptomycin. Absorbed coatings of 0.1 mg/ml rat-tail collagen type I (Life Technologies) diluted in 0.5 M acetic acid were used. The 96-well high-binding plate was incubated in the dark at 37 °C for 90 minutes. Each well was washed

twice with phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} (Sigma Aldrich) before plating cells. Gels were formed by adding specific concentrations of non-pepsin treated rat-tail collagen type I (BD Biosciences) to phenol red free DMEM supplemented with 2% GlutaMAX, 1% penicillin/streptomycin and 12 mM HEPES (Life Technologies). Guinea pig transglutaminase (2 U/mg (U = 1 μmole/min), Sigma Aldrich) was added to the collagen gel solution at 50 μg/ml. Cells were then plated on top of gels. Blebbistatin (Calbiochem) and calyculin A (Santa Cruz Biotechnology) were used at the working concentrations of 10 μM and 1 μM, respectively.

MMP Activity Assays in Cells:

MMP activity was measure as elsewhere [13]. Cells were harvested and suspended in serum-free media with or without drug treatments for 1 hour and transferred to a high-binding 96-well dish. 10μM of S-MMP (Mca-PLGL-Dpa-AR-NH2, R & D Systems, ES001) or MT-MMP (Mca-PLA-C(OMeBz)-WAR(Dpa)-NH2, Calbiochem, 444528) quenched fluorescent cleavage peptide was added immediately following plating or three hours post plating, as indicated [30,31]. Fluorescence of the sample and background was excited at 320 nm and collected at 405 nm over one hour using a BioTech SynergyMx micro plate-reader. The slope of the background-subtracted fluorescence over this hour was used as a measure of MMP enzymatic activity and was normalized by the cell number to generate an MMP activity per cell.

Hanging Drop Protrusion:

Panc-1 cells were harvested and suspended to a concentration of approximately 500,000 cells/15μl. 15μl of cell solution was placed directly on a small tissue-culture dish lid. Dishes were incubated with inverted lids for 24 hours at 37 °C with 5% CO_2. Cell drops were then transferred to coverslips by touch. Coverslips were placed on 4-wall chamber slide containing gel solution and sealed. Gelled drops were incubated at 37 °C for 18-28 hours and then imaged using a 10x objective (NA = 0.3, Nikon). Cell edge length was determined using ImageJ. Extensions were included in quantification if ≥ 5 μm in length.

Results & Discussion:

Monoclonal antibody DX-2400 can block MMP-14 catalytic activity *in vitro* with high specificity [24] [27]. Here we produced DX-2400 in its Fab format and compared its inhibitory functions with a pan-MMP small molecule inhibitor (marimastat) for block cleavage of two cleavage peptides. The first cleavage peptide (Mca-PLA-C(OMeBz)-WAR(Dpa)-NH_2) is fairly specific for MMP-14, so we call it the membrane-tethered (MT)-MMP cleavage peptide [31]. The second cleavage peptide (Mca-PLGL-Dpa-AR-NH_2) can measure MMP-14 as well as MMP-2, -9, and -13. However, the rate of MMP-14 cleavage is roughly 50% that of MMP-2, -9 and -13 [30] and accumulation of MMP-2, -9 and -13 in the supernatant likely limits the MMP-14 detection (Fig. 2E&F). Consequently, we call it the soluble (S)-MMP cleavage peptide [30]. Both of these peptides fluoresce when cleaved. DX-2400 was able to block *in vitro* MMP-14 catalytic activity at 10-fold lower concentrations than marimastat (Fig. 1A). This 10-fold more potent response was also seen when measuring MMP activity on live pancreatic cancer cells (Panc-1). DX-2400 inhibitory effects were significant down to 10 nM while the effect of marimastat was only robustly seen above 100 nM (Fig. 1). Given that DX-2400 is more potent (Fig. 1) and more specific [24] than marimastat, we were interested if we could use it to examine mechanical stimulation of MMP activity.

Mechanical properties of the ECM are sensed by and control the contractile state of the cell. Others have shown a role for mechanical regulation over MMP activity and invadopodia, which locally degrade ECM [10,11,12,32,33]. In addition, MMP-14 is known to cleave and activate MMP-2 [1]. Here we aimed to investigate whether the activity of MMP-14 regulates S-MMP activation in response to mechanical perturbation. Consequently, we measured both MT-MMP activity and S-MMP activity in the absence or presence of DX-2400 when Panc-1 cells were treated with either a contractility inhibitor (blebbistatin) or an enhancer (calyculin A), different concentrations of collagen, which modulate the ECM stiffness or gels with and without transglutaminase, which crosslinks collagen and modulates the ECM stiffness.

As to be expected, MT-MMP activity is significantly inhibited by DX-2400 under each ECM condition (Fig 2A-C). On soft collagen gels (1 mg/ml), S-MMP activity is regulated by contractility, decreasing with blebbistatin and increasing with calyculin A treatment (Fig. 2A). Blocking MMP-14 activity abates this response. DX-2400 treatment resulted in significantly decreased S-MMP activity with or without an additional

contractility drug when compared to control (Fig 2A). S-MMP activity is always positively correlated with contractile state and this positive correlation gradually increases when stiffness increases (Fig 2A-C), becoming saturated at high stiffness under calyculin A treatment (Fig 2C). However, S-MMP activity is consistently decreased to low levels upon treatment with DX-2400. This suggests that MMP-14 responds to contractility and activates secreted MMPs like MMP-2 in Panc-1 cells, resulting in changes in the S-MMP activity.

However, because MMP-14 can be secreted in exosomes into the surroundings and MMP-2 can bind to the cell surface through MMP-14, we decided to separate the supernatant and cell fractions to see if we could determine the location of the enhanced MMP activity and build a better case for the mechanical activation of soluble MMPs by MMP-14 (Fig 2D). We measured MT-MMP activity in the supernatant and saw no change in response to DX-2400, suggesting that MMP-14 is not secreted in exosomes in our system (Fig 2E). Conversely, we saw large changes in S-MMP activity in the supernatant in response to DX-2400. Given that we observe no change in response of the MT-MMP in the supernatant after DX-2400 treatment, it is likely that changes in S-MMP activity in the supernatant are due to changes in soluble MMP activity (Fig 2E). When examining the cell fraction, MT-MMP decreased in response to DX-2400 as would be expected (Fig 2F). We examined the S-MMP activity on cells and saw roughly no change in response to DX-2400, suggesting that the vast majority of soluble MMPs that are activated result in enhance soluble MMP activity not enhanced cell surface MMP activity due to binding of soluble MMPs to the cell surface (Fig 2F). Since MMP-14 does cleave S-MMP, why do we not see a decrease in S-MMP activity on the surface of cells? Perhaps decreasing cell surface MT-MMP activity is compensated by increased surface-bound soluble MMP activity. This explanation is possible given that MMP-14 binds MMP-2 through TIMP-2, which is known to block MMP-14 activity [34]. Consequently, if MMP-14 was merely used as a receptor, blocking its activity would not necessarily decrease S-MMP activity on the cell surface, because MMP-2 activity could replace lost MMP-14 activity. What is certain is that soluble MMP activity goes up in response to contractile state and blocking MMP-14 blocks this increase supporting the notion that MMP-14 activates soluble MMPs, like MMP-2, in response to changes in contractile state.

Since MMP-14 appears to be the major contractility-regulated MMP, modulating S-MMP activity as well, we wanted to test if MMP-14 was also responsible for the increase in S-MMP activity when ECM was

crosslinked using transglutaminase. ECM crosslinking enhances the stiffness by immobilizing collagen fibers with respect to each other. The resulting ECM contains pores that cannot be opened by contractile force, requiring proteinase activity for cell migration through the ECM. DX-2400 treatment significantly lowered MT-MMP activity on both uncrosslinked and crosslinked gels when compared to control (Fig 3). In addition, DX-2400 treatment also inhibited S-MMP activity on both uncrosslinked and crosslinked gels (Fig 3). This evidence suggests that MMP-14 drives a secreted MMP activity response to highly cross-linked ECM environments.

The experiments above have only explored how MMP-14 regulates S-MMP activity responses to changes in contractility, ECM concentration and degree of crosslinking in various 2D environments. We were interested in the efficacy of DX-2400 to block cell migration. Previous work has shown that DX-2400 does block growth of MDA-MB-231 orthotopic tumors and that siRNA knockdown of MMP-14 inhibits cell invasion into 3D matrices [24,35]. We were interested in determining if DX-2400 could block Panc-1 cell invasion under different contractility conditions. DX-2400 or pan-inhibition of MMPs do not significantly affect Panc-1 cell spreading on absorbed collagen 2D environments (data not shown). However, Panc-1 spheroids generated by the hanging drop technique and embedded in 1 mg/ml collagen gels did respond to DX-2400 treatment. Panc-1 spheroids were imaged after 24 hours in the collagen gels (Fig 4) and the number of protrusions per spheroid edge length was quantified (Fig 4B). Panc-1 cells under normal conditions had a moderate amount of single cells invading the surrounding ECM with various lengths of extensions (Fig 4A). Extensions were almost completely absent with a pan-MMP inhibitor, marimastat (Fig 4B). In addition, many fewer of these invasive cells were found after treatment with DX-2400, even at 100-fold lower concentrations than marimastat. We examined these extensions under decreased contractility conditions. With the addition of blebbistatin, many more Panc-1 cells extended protrusions, usually in connected groups with many extensions (Fig 4D). DX-2400 treatment again decreased the extension density and groups of extensions no longer formed, however there were a few single cells extending into the matrix (Fig 4A). Marimastat showed similar inhibition results at higher concentrations. Conversely, calyculin A treatment caused Panc-1 cells to round up with no protrusions (Fig 4A). This response largely did not change with DX-2400 or marimastat treatment (Fig 4B). This evidence indicates that both specifically blocking MMP-14 and generally blocking MMPs both decrease

basal level invasiveness of Panc-1 cells, but DX-2400 can inhibit cell invasion at 100-fold lower concentrations than marimastat. The higher potency and specificity of this function blocking antibody make it an attractive therapeutic target. With the recent development of a high-throughput proteinase screening approach [27], protein engineering techniques aimed at affinity maturing scFv antibodies for MMP-14 and other MMPs provide a reasonable approach for both developing tools to examine cancer cell biology as well as therapeutics that block invasion. Antibody inhibitor cocktails could be used in combination therapies with cytotoxic chemotherapeutic approaches for a wide range of cancer types including pancreatic cancer.

Here we employed a previously described MMP-14 function blocking antibody and quenched cleavage peptides to characterize MMP-14's role in the regulation of S-MMPs through mechanical stimuli. The MMP-14 function blocking antibody, DX-2400, inhibited MMP-14 activity *in vitro* and on cells at levels roughly 10-fold lower than marimastat, a pan-MMP inhibitor. We then used this antibody to show that increases in secreted MMP activity with increased contractility are dependent on MMP-14. By examining both supernatant and cell fractions, we were able to determine that the vast majority of S-MMP activity regulation by mechanical inputs occurs in solution rather than at the cell surface. In addition, MMP-14 is involved in enhancing secreted MMP activity in response to enhanced crosslinked collagen gels. Finally, we demonstrated that DX-2400 was as efficient as marimastat at inhibiting cell extension from pancreatic tumor spheroids into a surrounding 3D collagen gel network, even at 100-fold lower concentrations. The enhanced specificity and potency of MMP-14 function blocking antibodies makes them attractive therapeutic approaches for blocking invasion in a variety of cancers.

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Figures:

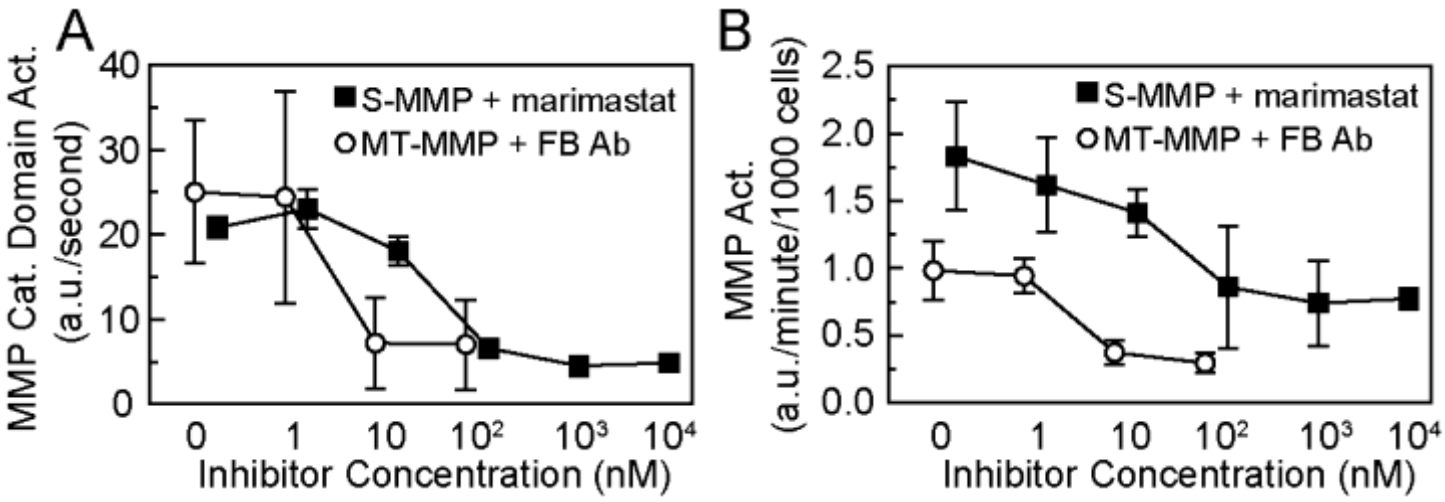

Figure 1: MMP-14 function blocking Ab blocks MT-MMP activity at low concentrations

(A) Activity of purified MMP-14 catalytic domain is measured with both S-MMP and MT-MMP quenched cleavage peptides at various concentrations of DX-2400 and marimastat. (B) S-MMP and MT-MMP activities are determined in intact Panc-1 cells during cell spreading onto 0.1 mg/ml absorbed collagen. S-MMP is measured at high cell density, while MT-MMP is measure at low cell density. Results are presented with 95% confidence intervals with $n \geq 3$.

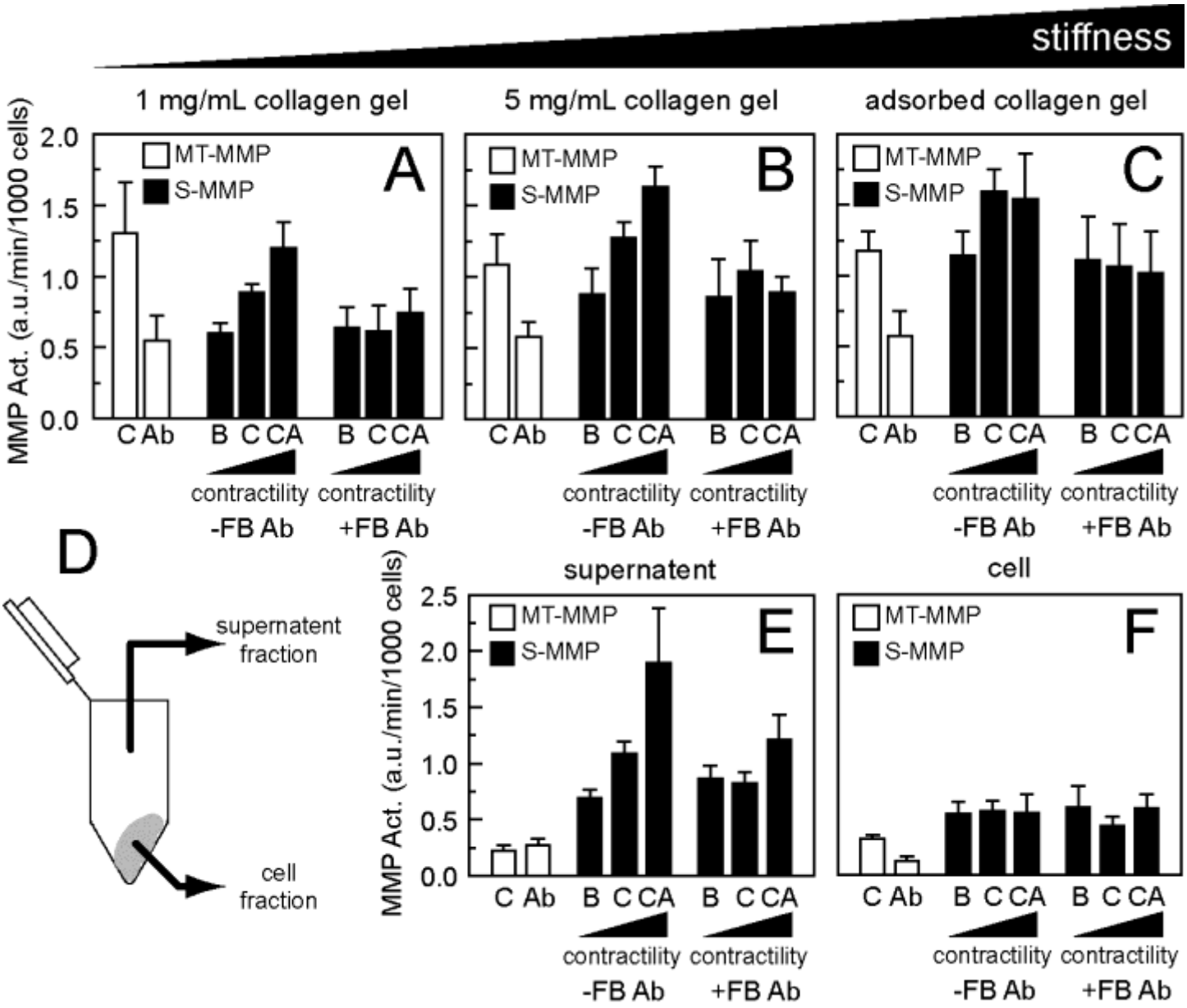

Figure 2: MMP-14 mediates S-MMP activity response to cellular contractility.

(A-C) S-MMP and MT-MMP activities are measuring in Panc-1 cells during active cell spreading on 1 mg/ml collagen gels (A), 5 mg/ml collagen gels (B), and 0.1 mg/ml absorbed collagen (C). S-MMP is measured at high cell densities, while MT-MMP is measured at low cell conditions. C is control condition, B is addition of 10 μM blebbistatin and CA is addition of 100 nM calyculin A. Beneath each graph indicates addition (+) or absence (-) of 100 nM DX-2400. (D) Diagram indicating the cell fraction (cell pellet) separated from the supernatant fraction. (E-F) After cells were incubated in solution for 60 minutes with each drug treatment, they were centrifuged and the supernatant was collected and placed in wells coated with 0.1 mg/ml absorbed collagen. The cells were resuspended and plated into wells coated with 0.1 mg/ml absorbed collagen. Both S-MMP and MT-

MMP is measured under high cell densities for the cell fraction. Results are presented with 95% confidence intervals with $n \geq 3$.

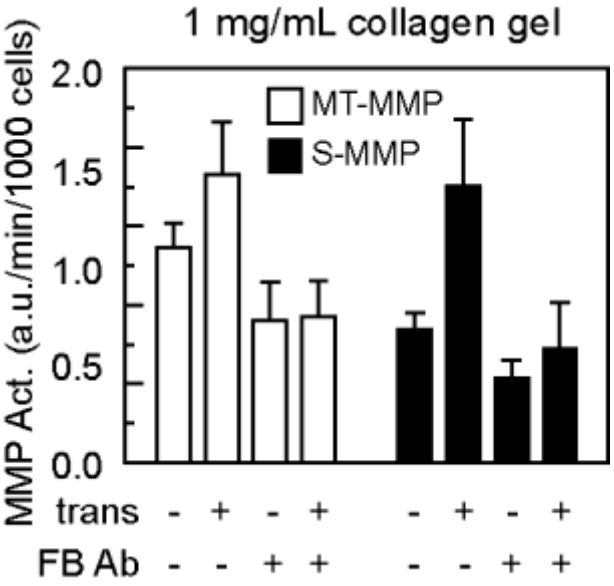

Figure 3: MMP-14 mediates S-MMP response to ECM crosslinking

MT-MMP is measured under low cell density conditions and S-MMP is measured under high cell density conditions. Transglutaminase was added to gels at 50 μg/ml and DX-2400 was added to cells at 100 nM. Results are presented with 95% confidence intervals with $n \geq 3$.

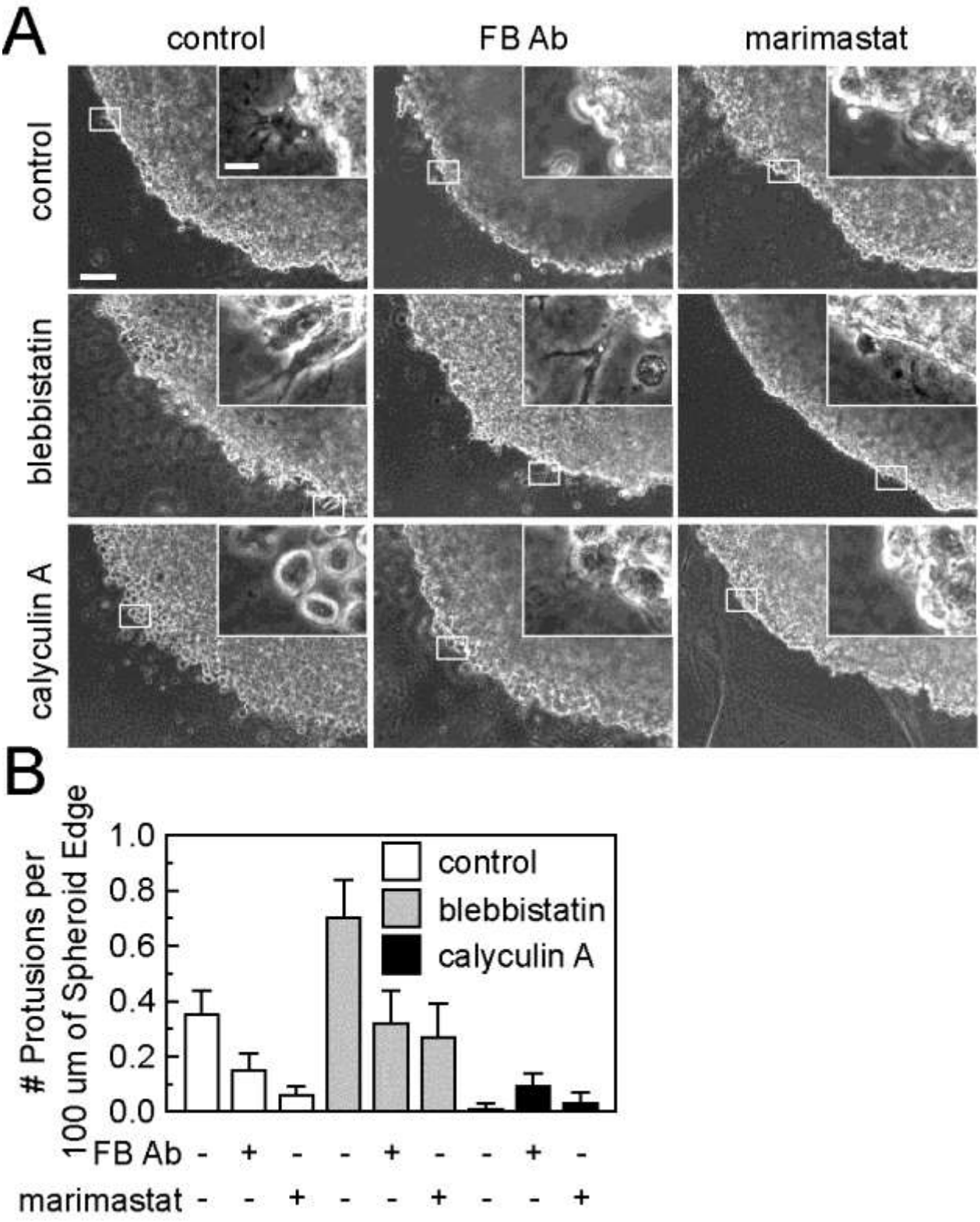

Figure 4: FB Abs effectively inhibit extension of Panc-1 cells into 3D collagen matrices

(A) Example images are shown of Panc-1 cells in 1 mg/ml hanging drop gels. Drug treatments were as follows: DX-2400 100 nM, marimastat 10 μM, blebbistatin 10 μM, calyculin A 100 pM. Inset locations are indicated with white boarder. (B) The number of cell protrusions was determined per cell edge length for all conditions

presented above. Four images of each drop were taken and quantified for at least three gels for each condition. Results are presented with 95% confidence intervals.