Cellular Contractility and Extracellular Matrix Stiffness Regulate Matrix Metalloproteinase Activity in Pancreatic Cancer Cells

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Short Title: Mechanical Mechanisms Regulate MMP Activity

ABBREVIATIONS

Extracellular matrix (ECM)

Matrix metalloproteinase (MMP)

S-MMP (secreted MMP)

MT-MMP (membrane tethered MMP)

HEP III (heparinase III)

ABSTRACT

The pathogenesis of cancer is often driven by local invasion and metastasis. Recently, mechanical properties of the tumor microenvironment have been identified as potent regulators of invasion and metastasis, while matrix metalloproteinases (MMPs) are classically known as significant enhancers of cancer cell migration and invasion. Here we have been able to sensitively measure MMP activity changes in response to specific extracellular matrix (ECM) environments and cell contractility states. A pancreatic cancer cell line, Panc-1 cells, up-regulate MMP activities between 3- and 10- fold with increased cell contractility. Conversely, they down-regulate MMP activities when contractility is blocked to levels seen with pan-MMP activity inhibitors. Similar, albeit attenuated responses are seen in other pancreatic cancer cell lines: BxPC-3 and AsPC-1 cells. In addition, MMP activity was modulated by substrate stiffness, collagen gel concentration and the degree of collagen crosslinking, when cells were plated on collagen gels ranging from 0.5-5 mg/ml that span the physiological range of substrate stiffness (50-2000 Pa). Panc-1 cells showed enhanced MMP activity on stiffer substrates, whereas BxPC-3 and AsPC-1 cells showed diminished MMP activity. In addition, eliminating heparan sulfate proteoglycans using heparinase completely abrogated the mechanical induction of MMP activity. These results demonstrate the first functional link between MMP activity, contractility and ECM stiffness and provide an explanation as to why stiffer environments result in enhanced cell migration and invasion.

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INTRODUCTION

Pancreatic cancer is one the deadliest subtypes of cancer, with 5 year survival rates less than 5 % (1). The poor prognosis results from the combination of few early symptoms and aggressive early spreading of cancer cells to distant sites, a process called metastasis (1, 2). Metastasis relies on multiple cellular mechanisms such as directed cell migration and extracellular matrix (ECM) remodeling (3). Remodeling of the tissue microenvironment surrounding the primary tumor includes secretion of ECM and force-mediated rearrangement of the ECM structure, however much attention has been given to degradation of the ECM through the secretion of proteinases. Matrix metalloproteinases (MMPs) are the primary enzymes responsible for ECM remodeling and have long been associated with cancer cell migration and metastasis (4).

MMPs are a family of 25 zinc-dependent proteinases that have wide substrate specificities for a variety of ECM proteins. The family is split into two major groups, soluble MMPs that are secreted from cells into the extracellular space and membrane-associated MMPs that are often tethered to the cell's plasma membrane through a transmembrane domain (4, 5). Some secreted MMPs can also localize to the plasma membrane through their interaction with cell surface proteins (6, 7). The precise regulation of each MMP family member remains vague, but it has been shown that MMPs perform specific roles in the cancer microenvironment (4, 8). Increased MMP expression has been demonstrated in a variety of cancers and often increased MMP expression results in increased metastatic potential (9-12). However, cues in the tumor microenvironment can lead to an upregulation of MMP activity separate from MMP expression through enhanced secretion or post-translational modification (13, 14). This has led many researchers to investigate these stimulatory cues as upstream drivers of MMP activity and tumor invasion.

Recently, mechanical properties of the tumor microenvironment have gained much attention as potent drivers of invasion (15-20). Primary tumor tissue is often stiffer than normal tissue (17, 21). This increase in stiffness is the result of cellular contractility coupled with increased ECM crosslinking (17, 18, 20). Stiff ECM matrices often have smaller pores, but crosslinking itself does not affect pore size (19). Cells can conceivably remodel the matrix and expand pores by exerting traction force using intracellular contractility that is generated through myosin II activity, a mode of migration referred to as amoeboid (22-24). Indeed, increased traction force, has been shown to increase metastatic potential (24-26) . However, if the ECM network is stably crosslinked, MMP activity is required for cells to squeeze through pores (27, 28), a mode of migration referred to as mesechymal. Consequently, the cell must sense chemical crosslinks in the ECM and tune its MMP activity appropriately. One interesting hypothesis posits that the cell tunes MMP activity through sensing the mechanical properties of the ECM (29-31).

Mechanical properties of the ECM are sensed through ECM receptors called integrins (32, 33). Integrins link the ECM with the actin cytoskeleton and actomyosin contractility within the cell. In addition to integrins,

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heparan sulfate proteoglycans (HSPG) like syndecans are important too (34). Syndecans contain heparan sulfate groups on the outside of the cell that have been shown to be required for mechanotransduction in several cell types (35, 36). In addition, heparan sulfate groups are known to be necessary in order to activate contractility regulators, such as Rho GTPases (37, 38). On rigid substrates, Rho GTPases increase contractility by activating Rho-kinase, which in turn increases myosin II phosphorylation through a variety of mechanisms. Importantly, Rho and ROCK overexpression has been linked to malignant transformation of cancer cells, implicating a role for cellular contractility in response to ECM mechanics in metastasis (39). At first glance, enhanced contractility and force transmission by the cell might simply allow the cell to better rearrange the ECM, but perhaps there is an additional role in ECM degradation. Indeed, changes in MMP expression occur in response to changes in contractility (40-46), but expression changes do not always indicate changes in MMP activity (47). In addition, mechanical force, like that induced by cellular contractility, can act to alter the MMP cleavage rate of collagen due to a presumed change in conformation of the collagen substrate (48, 49). However, to date there is little information on whether intrinsic changes in MMP activity separate from substrate conformational changes are regulated by the cell's ability to transmit force. This ability to transmit force is driven by cellular contractility, the mechanical properties of the ECM and the cell adhesion to the ECM through receptors.

Here we demonstrate for the first time evidence that links cellular contractility, ECM mechanical properties and adhesion to MMP activity. We investigated secreted MMP (S-MMP) and membrane-tethered MMP (MT-MMP) activities in normal epithelial, breast cancer and pancreatic cancer cell lines under a variety of conditions using self-quenched cleavage peptides. In this article we show that in Panc-1 cells, increasing cellular contractility upregulates MMP activity. Conversely, decreasing cellular contractility downregulates MMP activity and to similar extents as the proteinase inhibitor, marimastat. Similar, but attenuated responses are seen in BxPC-3, AsPC-1 and MDA-MB-231 cells. However, in Panc-1 cells this regulation of MMP activity depends on the stiffness of the substrate. MMP activity on stiff substrates cannot increase after treatment with a contractility enhancer. In addition, MMP activity on soft substrates cannot be decreased by treatment with contractility inhibitors. Stiffening the ECM, by increasing the ECM concentration or crosslinking density using transglutaminase, increases MMP activity in Panc-1 cells, but BxPC-3 and AsPC-1 cells show an opposite behavior. Finally, mechanically stimulated MMP activity requires heparan sulfate groups, suggesting a role for heparan sulfate proteoglycans such as syndecans in sensing mechanics and tuning MMP activity.

MATERIALS & METHODS

Cell Culture

Human pancreatic cancer (Panc-1, BxPC-3, and AsPC-1, ATCC, Manassas, VA, USA), human keratinocyte (HaCat), and human breast cancer (MDA-MB-231, ATCC, Manassas, VA, USA) cells were used for all experiments as indicated. Panc-1, HaCat and MDA-MB-231 cultures were maintained using DMEM with phenol red + 10% FBS, 2% GlutaMAX, and 1% penicillin/streptomycin. BxPC-1 and AsPC-1 cultures were maintained using RPMI with phenol red + 10% FBS and 1% penicillin/streptomycin. All cells were harvested using trypsin (Life Technologies, Grand Island, NY, USA).

Absorbed & Gelled Collagen

Absorbed coatings of 0.1 mg/ml rat-tail collagen type I (Life Technologies, Grand Island, NY, USA) diluted in 0.5 M acetic acid were used. The 96-well high-binding plate (not tissue culture treated) 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, St. Louis, MO, USA) before plating cells. Gels were formed by adding specific concentrations of non-pepsin treated rat-tail collagen type I (BD Biosciences, San Jose, CA, USA) to phenol red free DMEM or RPMI supplemented with 2% GlutaMAX, 1% penicillin/streptomycin and 12 mM HEPES (Life Technologies, Grand Island, NY, USA). Guinea pig transglutaminase (2 U/mg (U = 1 µmole/min), Sigma Aldrich, St. Louis, MO, USA) was aliquoted, lyophilized and dissolved in phenol red free DMEM + 2% GlutaMAX, 1% penicillin/streptomycin and 12 mM HEPES and added to the gel solution at 50 µg/ml. 100 µl of neutralized collagen solution was added per well and incubated for 5 hours at 37 °C. Cells were then plated on top of gels. For glutaraldehyde crosslinked gels, 0.05% glutaraldehyde was added on top of gels after a 5 hour incubation. Glutaraldehyde was diluted in either PBS or PBS + 100 mM glycine (inactive control). Gels were washed extensively in both PBS and PBS + 100 mM glycine before seeding cells on top of the gels.

Drug Treatments

Y-27632 (Calibiochem, Billerica, MA, USA), blebbistatin (Calibiochem, Billerica, MA, USA), marimastat (R & D Systems, Minneapolis, MN, USA), ML-7 (Sigma Aldrich, St. Louis, MO, USA) and calyculin A (Santa Cruz Biotechnology, Dallas, TX, USA) were all received as lyophilized powder. Each drug was dissolved in DMSO at the following stock concentrations: 10 mM, 3 mM, 2 mM, 11 mM and 100 µM. Each drug was further diluted in media containing cells (phenol red free DMEM supplemented with 2% GlutaMAX, 1% penicillin/streptomycin and 12 mM HEPES) at the following working concentrations: 10 µM, 10 µM, 10 µM, 75 µM and 1 µM. Heparinase III (294 U/mg (U = 1 µmole/hr), Sigma Aldrich, St. Louis, MO, USA) was dissolved in buffer (20 mM Tris, 0.1 mg/ml BSA, 4 mM $CaCl_2$ in PBS) at 0.011 mg/mL. It was then aliquoted and lyophilized. HEP III was aliquoted, lyophilized and reconstituted for experiments in media containing cells (phenol red free DMEM supplemented with 2% GlutaMAX, 1% penicillin/streptomycin, and 12 mM HEPES) at a final concentration of 2.9 µg/ml.

MMP Activity Assays

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Cells were harvested with trypsin and then counted on a hemocytometer. Low density experiments for membrane-tethered MMP (MT-MMP) activity assays were all carried out at approximately 25,000 cells/well and high density experiments for secreted MMP (S-MMP) activity assays ranged between 100,000 and 200,000 cells/well. These cell densities resulted in confluences of 25% and 100% respectively. Cells were suspended in serum-free media with or without drug treatments for 1 hour, after which 100 µL (on adsorbed collagen substrates) or 50 µL (on collagen gel substrates) were transferred to a high-binding (not tissue culture treated) 96-well dish and kept in serum free media with or without drug treatment throughout the experiments. 10µM of S-MMP (Mca-PLGL-Dpa-AR-NH$_2$, R & D Systems, Minneapolis, MN, USA, ES001) or MT-MMP (Mca-PLA-C(OMeBz)-WAR(Dpa)-NH$_2$, Calbiochem, Billerica, MA, USA, 444528) quenched fluorescent cleavage peptide was added immediately following plating (spreading) or three hours after plating (spread) (50, 51). The fluorescence of these peptides is normally low due to quenching caused by close proximity of the fluorphores. Upon cleavage this quenching is released and the fluorescence dramatically increases. MMP activity during spreading was measured during the first hour after plating and MMP activity after cells have spread were measured between the third and fourth hour after plating.

Fluorescence of the sample and background was excited at 320 nm and collected at 405 nm every 40 seconds using a BioTech SynergyMx micro plate-reader (Fig. S1). Background fluorescence was measured on multiple days in wells consisting of all components less cells. Background fluorescence values were averaged at each time point under each ECM condition. The average background fluorescence was then subtracted from each sample fluorescence. This created a cleavage peptide background-subtracted fluorescence signal. The slope of the background-subtracted fluorescence over the first hour (spreading) or between three and four hours (spread) was used as a measure of MMP enzymatic activity and was divided by the cell number to generate an MMP activity per cell.

Fixed Spreading Assays

Panc-1 and HaCat cells were plated at 100 µl per well with 25,000 cells/well on absorbed collagen coatings of 0.1 mg/ml. Cells were incubated in the high-binding (not tissue culture treated) plate at 37 ºC for 30 or 180 minutes. At these time points cells were washed once with PBS and then incubated for 30 minutes at 37 ºC with 4% paraformaldehyde. After incubation cells were washed twice with phosphate buffered saline. Fixed cells were then stained with FITC-phallodin (Sigma Aldrich, St. Louis, MO, USA). Before staining cells were incubated for 30 minutes with PBS + 1% BSA. Cells were then incubated with 5µg/ml FITC-phallodin for 30 minutes at room temperature. After staining cells were washed twice with PBS and imaged.

In Vitro Proteinase Assays

1.3 µM recombinant human MT1-MMP (R & D systems, Minneapolis, MN, USA) was activated by 0.013 µM recominbinant human Furin (R & D systems, Minneapolis, MN, USA) for 90 minutes at 37°C. 10

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nM of the activated MT1-MMP was then added to 10 µM S-MMP (R & D Systems, Minneapolis, MN, USA, ES001) or MT-MMP (Calbiochem, Billerica, MA, USA, 444528) quenched fluorescent cleavage peptide in 300 µl total assay buffer with or without drug inhibitors, blebbistatin at 10µM (Calibiochem, Billerica, MA, USA,), marimastat at 10µM (R & D Systems, Minneapolis, MN, USA) or calyculin A at 1µM (Santa Cruz Biotechnology, Dallas, TX, USA). Fluorescence of the sample and background was excited at 320 nm and collected at 405 nm every 40 seconds using a BioTech SynergyMx micro plate-reader. The background, without MT1-MMP, was then subtracted from the sample at each read. The slope was then calculated from the first hour of fluorescent output.

RESULTS

Matrix metalloproteinase (MMP) activity in pancreatic cancer cells is an increasing, but saturating function of contractile state

While there is some indication that altering contractility can change the expression and/or secretion of MMPs (40-46) or localized ECM degradation (29, 30) and that tension can lead to either enhanced or diminished collagen degradation (48, 49), we were interested in examining whether the contractile state of the cell regulates MMP activity in intact live cells. Two commercially available self-quenched MMP peptide substrates were used to measure either secreted MMP activity (S-MMP) or membrane tethered MMP activity (MT-MMP). Before cleavage these self-quenched peptides exhibit a low fluorescence which increases upon cleavage (Fig. S1). Panc-1 cells were plated on collagen for one hour. Media and cells were separated and MMP activity was measured using each of the two quenched cleavage peptides. S-MMP activity in the media was ~6-fold higher than that in the cells. Conversely, MT-MMP activity in the cells was ~6-fold higher than in the media, demonstrating an ability to measure either secreted or membrane tethered MMP activity (Fig 1). These levels are statistically significant because the error bars do not overlap. Throughout the entire article error bars represent 95% confidence intervals. Consequently, non-overlapping error bars represent statistically significance to 95%. In order to optimize the MMP activity response, cells were plated at both high and low cell densities and both S-MMP and MT-MMP activity was measured. S-MMP activity on a per cell basis was higher when cells were densely plated as compared to sparsely plated (Fig. S2). Conversely, MT-MMP activity on a per cell basis was lower when cells were densely plated as compared to sparsely plated (Fig. S2). Therefore, we measured S-MMP and MT-MMP activity at high and low cell densities, respectively, for the remainder of the experiments. S-MMP and MT-MMP activity was also investigated at two time points, during the first hour after plating, and during the third hour after plating. Cells were plated on adsorbed collagen and then thoroughly rinsed to dislodge any non-attached cells at one and three hours. After rinsing, cells were fixed and stained for F-actin. Fewer cells were attached at one hour, and the cells that were attached were much less spread for both

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Panc-1 and a non-tumorigenic epithelial line (HaCat) (Fig. 2A). Consequently, the one hour time point was identified as the spreading condition and the three hour time point was identified as the spread condition. A legend of the schematics used to describe the experimental conditions throughout the paper is shown in Fig. 1B.

Spreading Panc-1 cells showed a decrease in S-MMP activity with contractility inhibitors, Y-27632 (Y), ML-7 (ML) and blebbistatin (B), but S-MMP activity could not be further increased with the contractility enhancer, calyculin A (CA) (Fig 2B). The decrease due to inhibition of contractility with either blebbistatin or Y-27632 matched closely to that seen with the pan MMP inhibitor, marimastat (M) (Fig. 2B). HaCat cells, a non-tumorigenic cell line, showed an insensitive MMP activity response to contractility inhibitors or enhancers (Fig. 2B). The change in MMP activity due to these contractility inhibitors and enhancers was not caused by direct inhibition of MT1-MMP activity as these inhibitors and enhancers did not block *in vitro* cleavage of the peptide substrate (Fig. S3). Spread Panc-1 and HaCat cells showed similar qualitative responses of S-MMP activity to contractility inhibitors and enhancers (Fig. 2C). However, marimastat, Y-27632 and blebbistatin blocked S-MMP activity to a greater extent (Fig. 2C). In addition, two other pancreatic cancer cell lines BxPC-3 and AsPC-1 cells showed a more attenuated response post spreading to contractility inhibitors and enhancers, however, it is evident that increasing contraction increased S-MMP activity (Fig. 2D). No difference in BxPC-3 or AsPC-1 cells was seen during spreading (data not shown). MT-MMP activity showed a similar dependence on the contractile state of the cell as did S-MMP activity with the exception that during spreading marimastat, Y-27632 and blebbistatin blocked MT-MMP activity equally as well as ML-7 (Fig 2E&F). In addition, MT-MMP activity under control or calyculin A treatment was increased after spreading when compared to MT-MMP activity during spreading. (Fig 2E&F). The two other pancreatic cancer cell lines again showed small, but a statistically significant increase in MT-MMP activity with increased contraction after spreading. No difference in BxPC-3 or AsPC-1 cells was seen during spreading (data not shown).

Though contractile force seems to dramatically alter MMP activity in Panc-1 cells and marginally alter MMP activity in BxPC-3 and AsPC-1 cells plated on adsorbed collagen I, this ECM condition is quite different from what is seen *in vivo*. One difference is the stiffness of the substrate. Tissue culture plastic like that used in this study is stiff and has a Young's modulus of ~ 1 GPa, whereas a 1 mg/mL collagen gel is soft and has a Young's modulus of ~ 0.2 kPa. This decrease (increase) in substrate stiffness is sensed by the cell and results in a decrease (increase) in cell contractility (32). Because Panc-1 cells increase their contractility on stiff substrates, perhaps the extremely stiff environment of the tissue culture plastic saturates the contractility under control conditions. Therefore, we conducted the same experiments on 1 mg/mL collagen gels. On these gels, S-MMP and MT-MMP activity showed no great decrease in MMP activity after either treatment with marimastat or contractility inhibitors with the exception of S-MMP activity in spreading cells (Fig. 3). However, treatment with the contractility enhancer calyculin A did show a dramatic increase in MMP activity with the exception of

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S-MMP activity in spreading cells (Fig. 3). This suggests that on stiff substrates MMP activity can be decreased by contractility inhibitors, but MMP activity is saturated and cannot be increased further by contractility enhancers. However, if cells are plated on soft substrates, MMP activity is low and cannot be decreased with contractility inhibitors, however contractility enhancers can increase MMP activity. In order to test this view, cells were plated on 5 mg/mL collagen gels, which are stiffer than 1 mg/mL collagen gels, but softer than absorbed collagen on plastic. Cells display an intermediate behavior, where both contractility inhibitors and enhancers can modulate MMP activity (Fig. S4).These consistent changes in MMP activity in response to contractility inhibitors and enhancers on absorbed and gelled collagen were not seen in non-tumorigenic HaCat cells or a breast cancer cell line (MDA-MB-231) and were attenuated, but statistically significant in BxPC-3 and AsPC-1 cells (Fig. 2, 3, S4 and S5).

MMP activity in pancreatic cancer cells increases with increased ECM stiffness

Since contractility inhibitors and enhancers significantly regulate MMP activity in Panc-1 cells and to a lesser extent in BxPC-3 and AsPC-1 cells, we then investigated whether ECM stiffness regulated MMP activities. One way in which to tune collagen gel stiffness is through the gelling collagen concentration. Low collagen concentrations generate soft gels, whereas high collagen concentrations generate stiffer gels. MMP activities in different cell lines were measured in cells plated on various collagen gel concentrations. As collagen concentration increased, Panc-1 cells show a significantly higher S-MMP activity, while HaCat and MDA-MB-231 cells display no response during spreading (Fig 4A and S6A). MT-MMP activity also increased with higher collagen gel concentration during spreading in Panc-1 cells, though less significantly. HaCat and MDA-MB-231 cells again showed no response during spreading (Fig 4B and S6B). Interestingly, the response to increasing stiffness differed qualitatively between the three pancreatic cancer cell lines after spreading (Fig 4C&D). Collagen gels of 1 mg/ml are less stiff than gels of 5 mg/ml, which are less stiff than collagen adsorbed to plastic. As stiffness increased, Panc-1 cells increased their MMP activity, but both BxPC-3 and AsPC-1 cells decreased their MMP activity. HaCat cells remain roughly the same (Fig 4C&D). However, this is not a clear demonstration of the importance of ECM stiffness on regulating MMP activity, because changes in the collagen density, a possibly important determinant of MMP activity, are used to change substrate stiffness. Consequently, we used glutaraldehyde and transglutaminase to crosslink gelled collagen and increase the stiffness of the gel without changing collagen concentration. This allowed, for the decoupling of ECM density and stiffness.

We first determined whether crosslinking using glutaraldehyde, which stiffens collagen matrices (52-54), upregulates MMP activity. There were marginal, but statistically significant increases in MMP activity on glutaraldehyde treated collagen gels (1 mg/ml) (Fig 5A). In addition, transglutaminase was added to a low concentration collagen gel (1 mg/mL) and cells were seeded on these crosslinked gels and compared to the

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uncrosslinked gels. Panc-1 cells showed no change in S-MMP activity and a decrease in MT-MMP activity on crosslinked gels during spreading (Fig. 5B). Interestingly, S-MMP and MT-MMP activities both increased dramatically in spread Panc-1 cells on crosslinked gels (Fig 5B). In order to determine if this increase in MMP activity was dependent on the contractile state of the cell we treated cells seeded on crosslinked gels with contractility inhibitors and enhancers. Increasing contractility with calyculin A could not further increase MMP activity from the control on crosslinked gels. However, both S-MMP and MT-MMP activities displayed significant inhibition with contractility inhibitors to levels seen with a proteinase inhibitor, demonstrating that the increase in MMP activity due to crosslinking is primarily a mechanical effect and is dependent on changes in cellular contractility (Fig 5C&D).

Pancreatic cells sense ECM stiffness, modulating MMP activities, via heparan sulfate groups

Considering that ECM mechanics regulate MMP activities and their response to contractile state, we then investigated how cells sense these changes in ECM mechanics in order to tune their MMP activities. Panc-1 cells were treated with Heparinase III (HEP III), a bacterial-derived lyase for heparan sulfate groups and plated them on high collagen concentration gels (5 mg/ml). During spreading and after cells spread, HEP III treatment decreased S-MMP activity, but had no effect on MT-MMP activities in Panc-1 cells (Fig 6A). We then investigated if HEP III treatment could affect MMP activity responses to crosslinked gels. Spread Panc-1 cells on transglutaminase-crosslinked collagen gels (1 mg/ml) that were treated with HEP III showed a significant decrease in both S-MMP and MT-MMP activities from what had previously been seen on crosslinked gels alone. The activity was lowered to similar levels seen on low concentration gels (1 mg/ml) without crosslinking treatment (Fig 6 B&C).

DISCUSSION

Here we demonstrate a link between ECM mechanical properties and MMP activity that is mediated by cellular myosin II-dependent contractility and HSPG-dependent adhesion in Panc-1 cells. Invading tumor cells from the primary tumor must be able to sense the density of crosslinks in the ECM, because a highly crosslinked ECM contains pores that must be opened by active MMPs in order for cell migration to proceed (55). Because the mechanical properties of the ECM are set in part by the crosslinking density, they constitute a potential signal for MMP activity regulation. A higher density of crosslinking results in a stiffer matrix, which in turn activates MMP activity. However, cells may also want to avoid over degradation of the matrix, given its requirement as a scaffold for migration. Consequently, when the crosslinked ECM network is cut and the ECM softens, MMP activity must be shut down. This gives the cell a regulated, dynamic feedback system that allows for tunable MMP activity to meet the needs of the cell under all ECM conditions. While MMP transcription or translation has been shown to be altered in response to changes in the mechanical environment, these changes

have tended to occur over long timescales (40-46). This suggests that the mechanical cue impacts a post-translational process. This post-translational process changes MMP activity and depends on both contractility and adhesion in order to function specifically and dynamically.

The necessary requirement of contractility and adhesion in order to sense mechanical properties of the surrounding environment is well-established. However, the observation that increasing contractility in soft environments leads to upregulated MMP activity is novel. Indeed, cancer cells with higher Rho GTPase signaling, a driver of enhance contractility, tend to be more invasive (56). In addition to contractility, adhesion through integrins and HSPGs is necessary for MMP upregulation in response to mechanical signals in 2D environments (35, 36). The response in 3D environments is less well understood, but a link between HSPGs and MMPs has been proposed (57). What is the mechanism by which MMP activity is upregulated or downregulated as the cell contractile state changes?

Though not investigated here, there are a variety of mechanisms that could explain how cells specifically achieve a change in MMP activity due to changing contractility. The first possibility is a change in the spatial localization of MMPs. Membrane-bound MMPs as well as secreted MMPs that bind membrane-bound receptors have been known to localize to focal adhesions, invadopodia or in bands around the pericellular region (29-31, 58-60). Focal adhesions, invadopodia and the pericellular band are likely organized by the underlying contractile actin network (29-31). In addition, HSPGs like syndecans localize to focal adhesions (34). This specific organization not only puts MMPs in the right place for degradation, but could also force their interaction with accessory proteins that enhance the intrinsic enzymatic activity. In addition to localization, the rate of exocytosis or endocytosis of MMPs could drive differences in MMP concentration and consequently activity on a per cell basis (61). The actin cytoskeleton is known to regulate both exocytosis and endocytosis, providing a possible link between myosin II contractility and MMP activity. In addition to MMPs, TIMPs may also be controlled by contractility (62). Changes in localization, endocytosis or exocytosis could impact the amount of active TIMPs as well as their interaction with MMPs. Further investigation is needed to delineate the mechanism used by pancreatic cancer cells to increase or decrease MMP activity in response to alterations in contractility and mechanical properties of the microenvironment.

Interestingly, MMP activity and its response to alterations in contractility or matrix stiffness were different among different cell lines. HaCats showed no MMP response under any condition. MDA-MB-231 cells, breast cancer cells that were taken from a pleural effusion (63) and that perhaps represent a population of cells that had already left the primary tumor site, showed an attenuated response to alterations in both contractility and matrix stiffness, when compared to Panc-1 cells. To provide clearer explanation of the cell type difference, we examined two other pancreatic cancer cell lines: BxPC-3 cells and AsPC-1 cells. BxPC-3 cells were taken from a primary pancreatic tumor, however the patient showed no signs of metastasis (64) and

consequently, this cell line perhaps constitutes an early progression state. AsPC-1 cells were taken from ascites fluid (64) and consequently represent a population of cells that had already left the primary tumor site, similar to MDA-MB-231 cells. Panc-1 cells on the other hand are adenocarcinoma cells of ductal cell origin harvested from the primary tumor, even though there were local metastases in peripancreatic lymph nodes (65). Panc-1 cells responded robustly to both alterations in contractility and matrix stiffness. As contractility or matrix stiffness increases, MMP activity increases. Both BxPC-3 and AsPC-1 cells showed attenuated but statistically significant changes in MMP activity in response to contractility enhancers and inhibitors. However, as stiffness increased, MMP activity decreased.

What explains this difference in MMP activity between pancreatic cancer cell lines? Perhaps sensitivity to the matrix stiffness at the level of MMP activity correlates with or causes pancreatic tumor invasion (Fig. 7). Certainly, matrix stiffness has been linked to invasion (17, 66). This might explain why Panc-1 cells, which were taken from the primary tumor site of an individual with metastases, sensitively respond to increases in matrix stiffness by increasing MMP activity. BxPC-3 cells have perhaps not yet acquired this sensing mechanism, cannot detect the stiff and often fibrotic ECM environment (67-69) and cannot activate MMPs required to invade and metastasize. AsPC-1 cells and MDA-MB-231 cells might not respond to mechanical cues in the same fashion that Panc-1 cells respond for a slightly different reason. Both cell lines (AsPC-1 and MDA-MB-231) were taken from fluid consisting of cells that had already metastasized and left the primary tumor. Their focus is to colonize secondary tumor sites, where the ECM environment is much different and most likely less dense than that around the tumor (67-69). Consequently, mechanical upregulation of MMP activity in response to stiff ECM is mostly likely less important. In addition, metastasizing cells can take on an amoeboid mode of migration that is independent of MMP activity (70). Indeed, AsPC-1 cells seem to be somewhat less well-spread than either Panc-1 or BxPC-3 cells, arguing that they might take on an amoeboid migratory mode. The genesis of the difference in mechanical MMP regulation between these cell types along with identifying cellular mechanisms that lead to mechanical regulation of MMP activity will be the focus of future studies.

ACKNOWLEDGEMENTS

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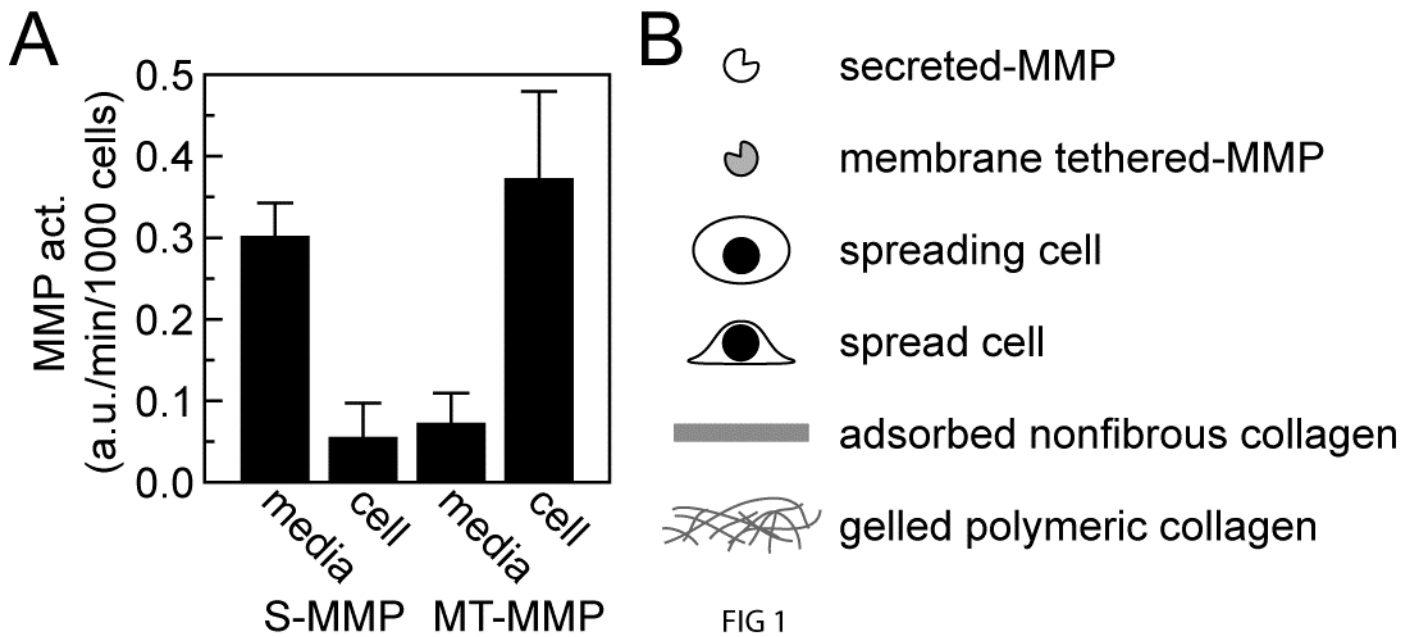
FIGURE LEGENDS

A, Panc-1 cells were incubated for 60 minutes in solution at 37 ℃ and then centrifuged 5 minutes at 5,000 RPM. The supernatant was separated and cells were suspended in new serum free media. Supernatant and cells were plated on 0.1 mg/ml absorbed collagen with a cell density of approximately 100,000 cells per well. Cleavage peptides were added at 10 μM. Error bars are 95% confidence intervals ($n = 4$). **B**, Symbols used throughout the article to illustrate the various conditions.
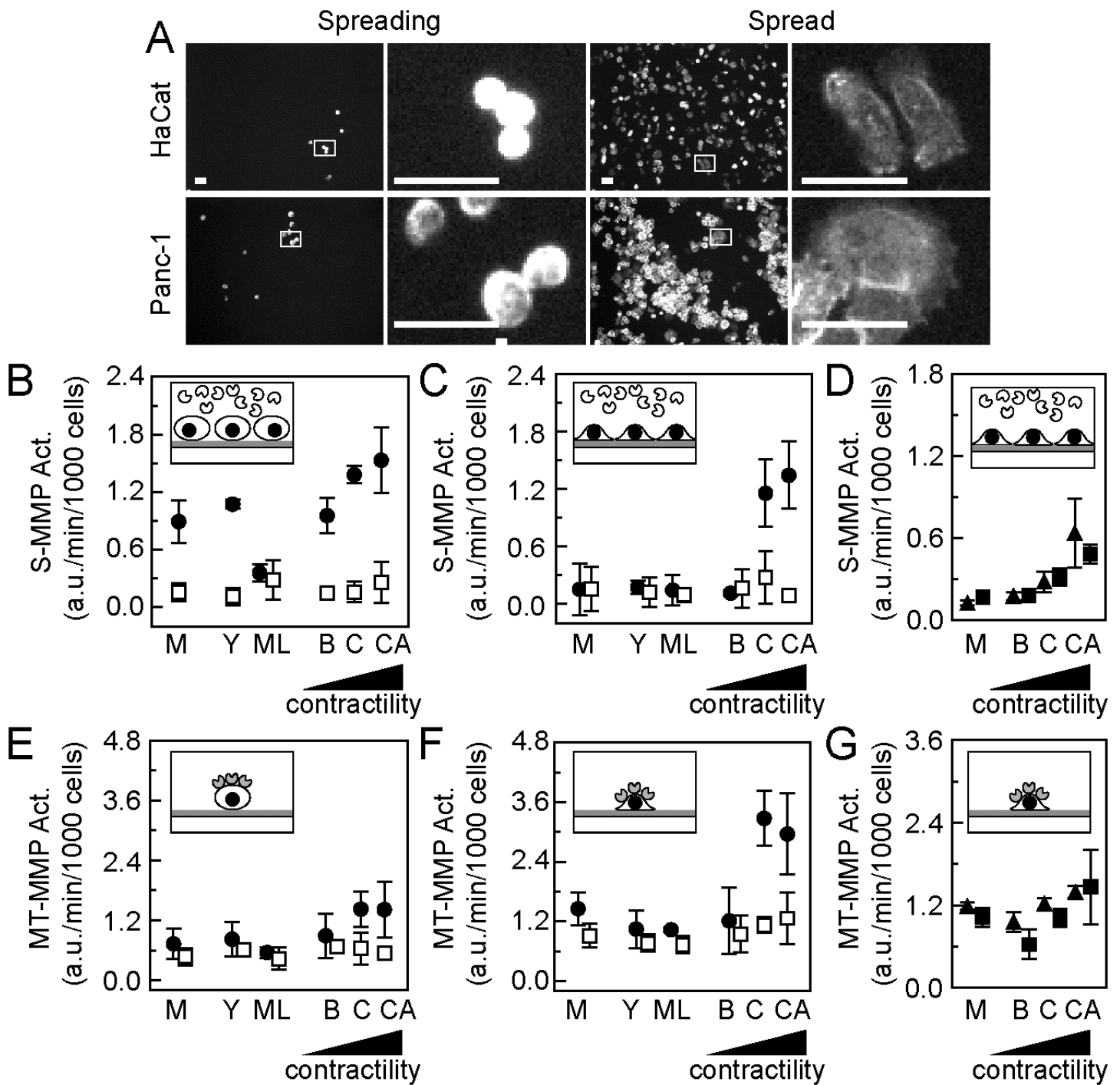
Figure 1: Self-quenched Fluorescent Cleavage Peptide Specificity

Fig. 2

Figure 2: Cellular Contractility Regulates MMP Activity on Absorbed Collagen

A, HaCat and Panc-1 cells were plated on 0.1 mg/ml absorbed collagen at 25,000 cells per well. Cells were stained for F-actin at 30 minutes (spreading) and 180 minutes (spread). Panc-1 (filled circles, B, C, E and F), BxPC-3 (filled triangles, D and G), AsPC-1 (filled squares, D and G) and HaCat (open squares, B, C, E and F) cells were plated on 0.1 mg/ml absorbed collagen under various drug treatments (C: control, M: marimastat (10 µM), Y: Y-27632 (10 µM), B: blebbistatin (10 µM) and CA: calyculin A (1 µM)). B, S-MMP activity

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measured at high cell density immediately following plating. C and D, S-MMP activity measured at high cell density 3 hours after plating. E, MT-MMP activity measured at low cell density immediately following plating. F and G, MT-MMP activity measured at low cell density 3 hours after plating. Error bars are 95% confidence intervals ($n \geq 3$).
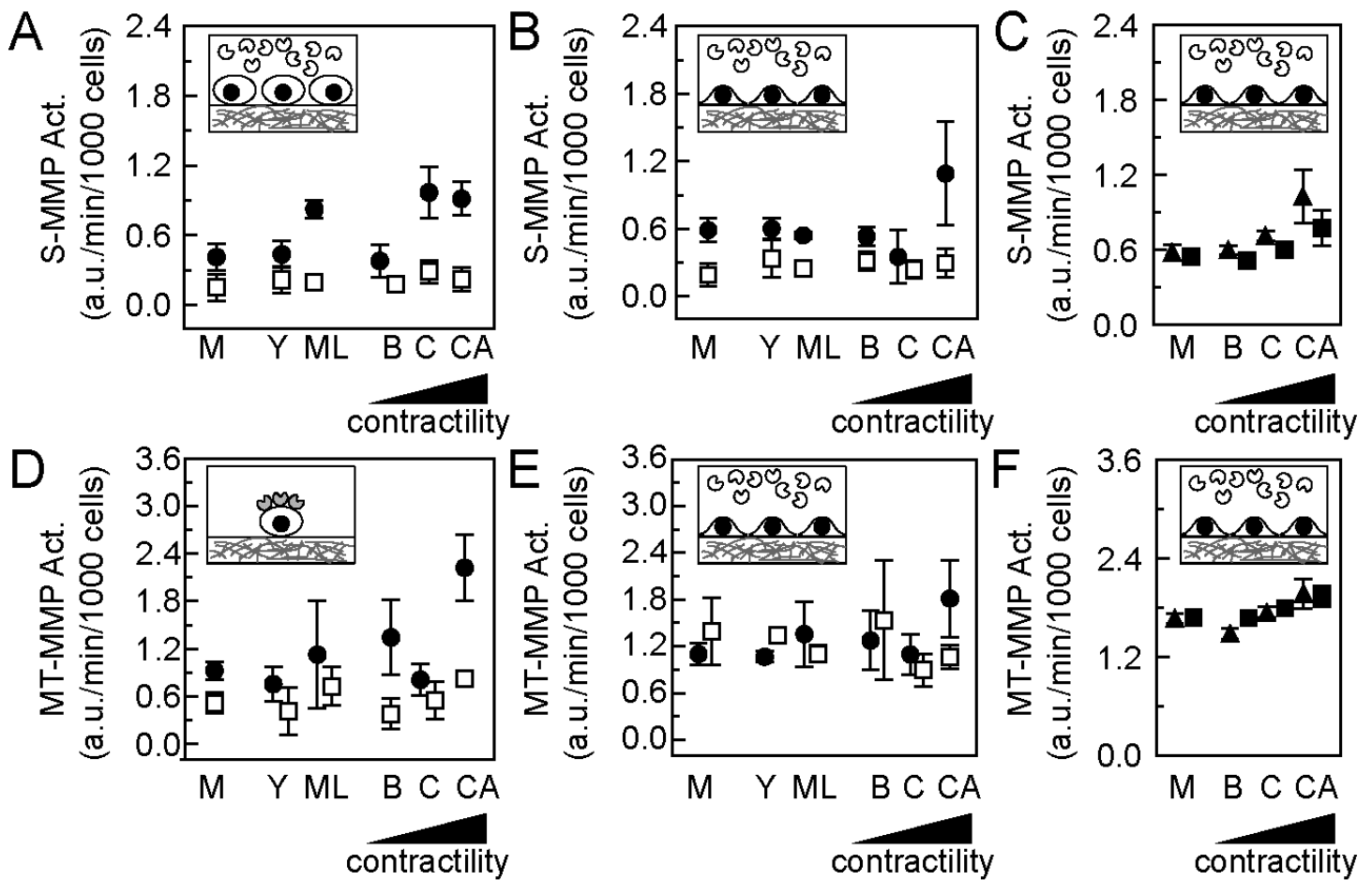

Fig. 3

Figure 3: Cellular Contractility Regulates MMP Activity on Collagen Gels

Panc-1 (filled circles, A, B, D and E), BxPC-3 (filled triangles, C and F), AsPC-1 (filled squares, C and F) and HaCat (open squares, A, B, D and E) cells were plated on 1 mg/ml collagen gel under various drug treatments (C: control, M: marimastat (10 μM), Y: Y-27632 (10 μM), B: blebbistatin (10 μM) and CA: calyculin A (1 μM)). A, S-MMP activity measured at high cell density immediately following plating. B and C, S-MMP activity measured at high cell density 3 hours after plating. D, MT-MMP activity measured at low cell density immediately following plating. E and F, MT-MMP activity measured at low cell density 3 hours after plating. Error bars are 95% confidence intervals ($n \geq 3$).

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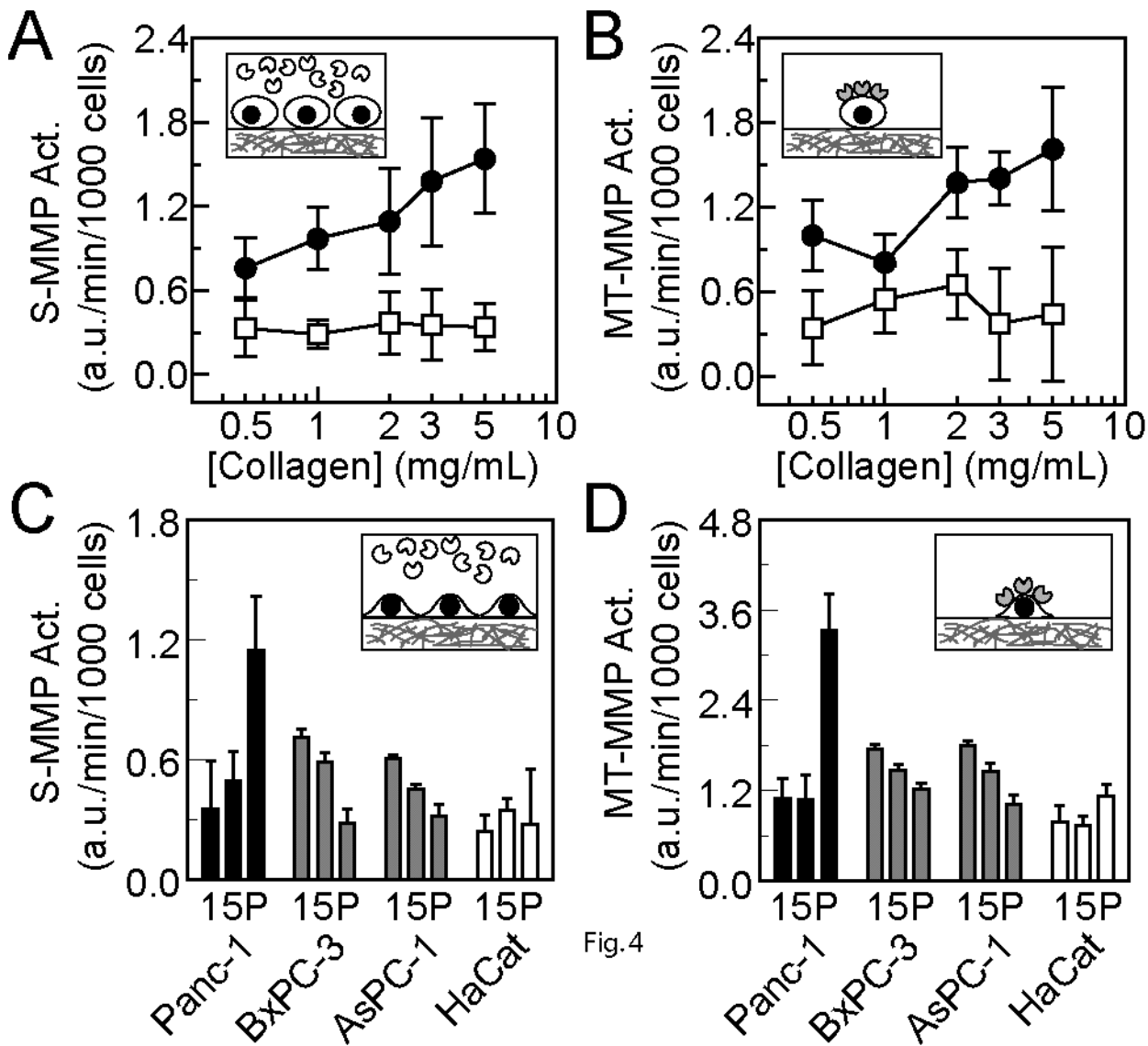
Figure 4: Collagen Concentration in Gels Effects MMP Activities

Panc-1 (filled circles, A and B) and HaCat (open squares, A and B) cells were plated on various concentrations of collagen gels and 0.1 mg/ml absorbed collagen. A, S-MMP activity measured at high cell density in cells plated on collagen gels of increasing concentration during spreading. B, MT-MMP activity measured at low cell density in cells plated on collagen gels of increasing concentration during spreading. C, S-MMP activity measured at high density after cells have spread on 1 mg/ml collagen gels (1), 5 mg/ml collagen gels (5) and 0.1 mg/ml collagen physisorbed to plastic (P). D, MT-MMP activity measured at low density after cells have spread on 1 mg/ml collagen gels (1), 5 mg/ml collagen gels (5) and 0.1 mg/ml collagen physisorbed to plastic (P). Error bars are 95% confidence intervals ($n = 6$).

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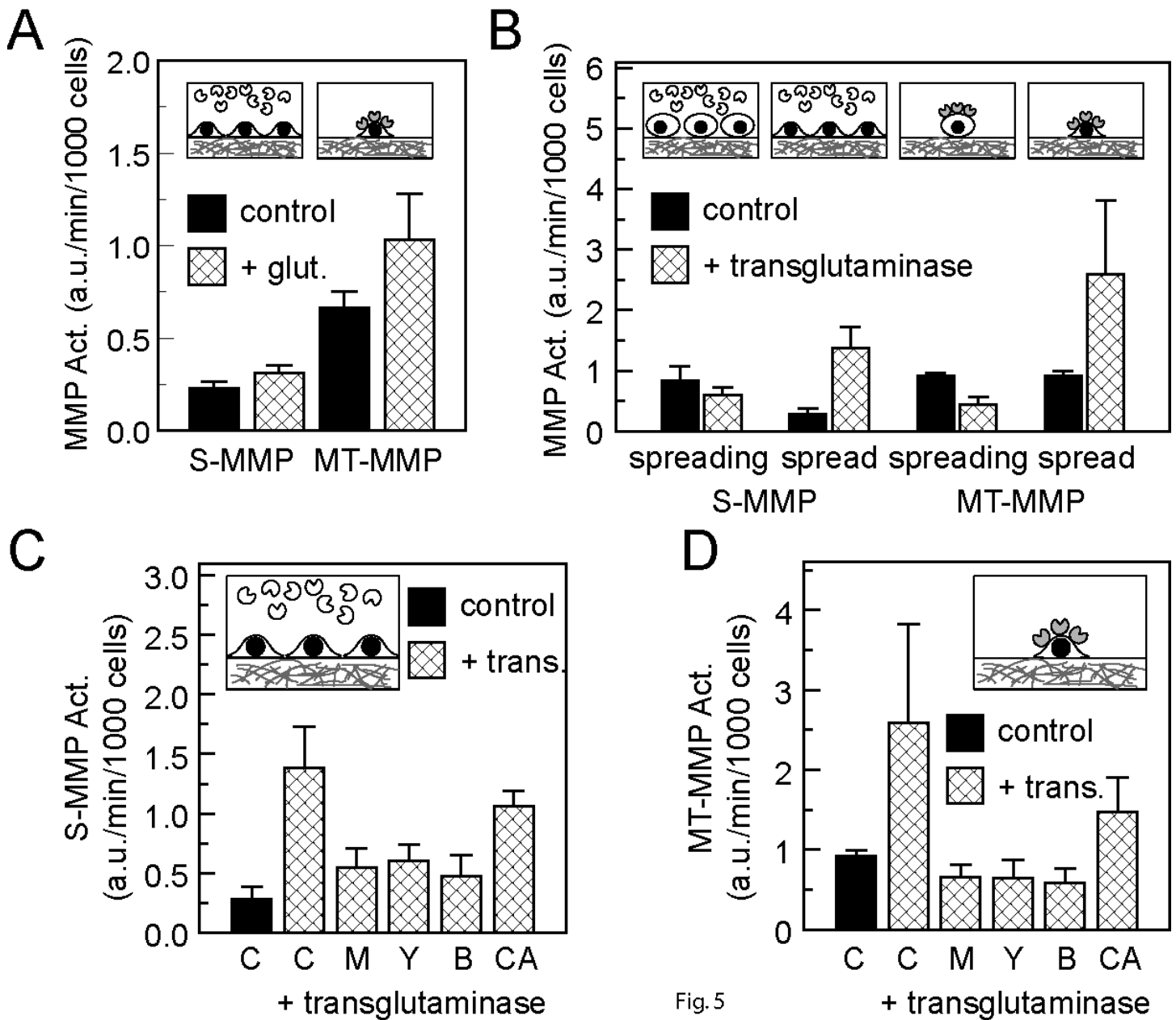
Figure 5: Crosslinked Gels Increase MMP Activities in Spread Cells

A, MMP activity measured in Panc-1 cells after spreading on glutaraldehyde treated 1 mg/ml collagen gels. B, MMP activity measured in Panc-1 cells during or after spreading with S-MMP activity measured with high cell density and MT-MMP activity measured at low cell density. Collagen gels (1 mg/mL) were gelled in the presence of 50 µg/ml transglutaminase ($n = 4$). C, S-MMP activities are measured at a high cell density in Panc-1 cells 3 hours after plating on 1 mg/ml collagen gels, 1 mg/ml collagen gels + 50µg/ml transglutaminase or on 1 mg/ml collagen gels + 50µg/ml transglutaminase under various drug treatments (M: marimastat (10 µM), Y: Y-27632 (10 µM), B: blebbistatin (10 µM) and CA: calyculin A (1 µM)) ($n = 3$). D, MT-MMP activities are measured at low density in Panc-1 cells 3 hours after plating on 1 mg/ml collagen gels, 1 mg/ml

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collagen gels + 50µg/ml transglutaminase or on 1 mg/ml collagen gels + 50µg/ml transglutaminase under various drug treatments (M: marimastat (10 µM), Y: Y-27632 (10 µM), B: blebbistatin (10 µM) and CA: calyculin A (1 µM)) ($n = 3$). Error bars are 95% confidence intervals.
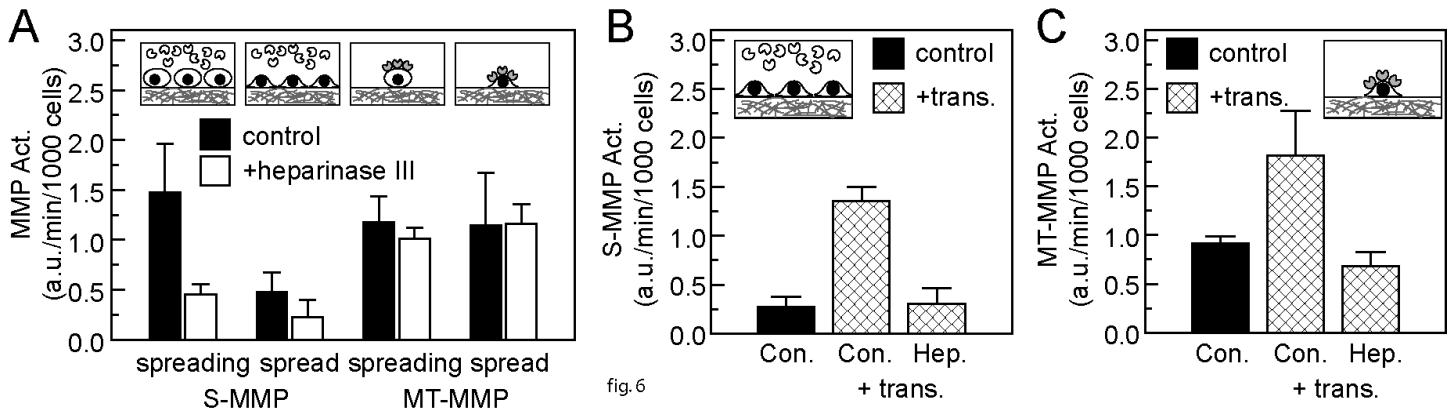

Figure 6: Cells Sense Collagen Gel Crosslinking Via Heparan Sulfate Groups.

A, MMP activity measured in Panc-1 cells during or after spreading with S-MMP activity measured at high cell density and MT-MMP activity measured at low cell density. Cells were plated on 5 mg/ml collagen gels with and without 3 µg/mL HEP III per well. B, S-MMP activities are measured in Panc-1 cells at high density 3 hours after plating on 1 mg/ml collagen gels, 1 mg/ml + 50 µg/ml transglutaminase and 1 mg/ml + 50 µg/ml transglutaminase after incubation with HEP III. C, MT-MMP activities are measured in Panc-1 cells at low density 3 hours after plating on 1 mg/ml collagen gels, 1 mg/ml + 50 µg/ml transglutaminase and 1 mg/ml + 50 µg/ml transglutaminase after incubation with HEP III. Error bars are 95% confidence intervals ($n = 3$).
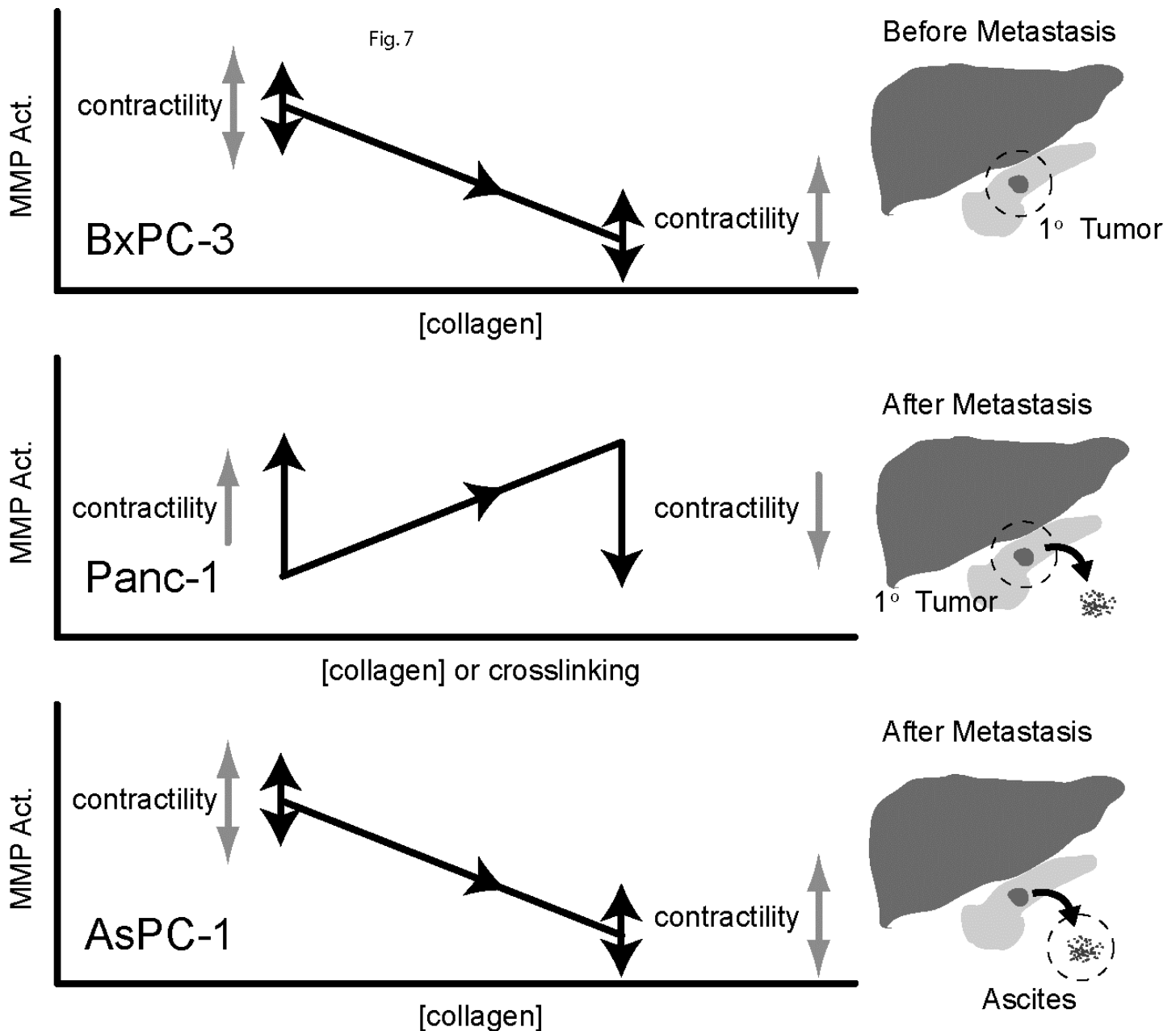
Figure 7: Contractility-mediated Changes in MMP activity Depend on the Stiffness of the Substrate which is regulated through Collagen Concentration and Crosslinking

BxPC-3 and AsPC-1 cells have marginal, but statistically significant responses to altered contractility and show decreases in MMP activity as a function of collagen concentration or matrix stiffness. Panc-1 cells are more sensitive to altered contractility; however these effects depend on the stiffness of the ECM. On stiff ECM, MMP activity is usually saturated and cannot be increased with enhanced contractility and conversely on soft ECM, MMP activity is usually low and cannot be decreased with inhibited contractility. In addition, Panc-1 cells show increases in MMP activity as a function of collagen concentration, matrix stiffness or collagen crosslinking. A schematic showing the origin of the different pancreatic cancer cell lines is shown to the left.
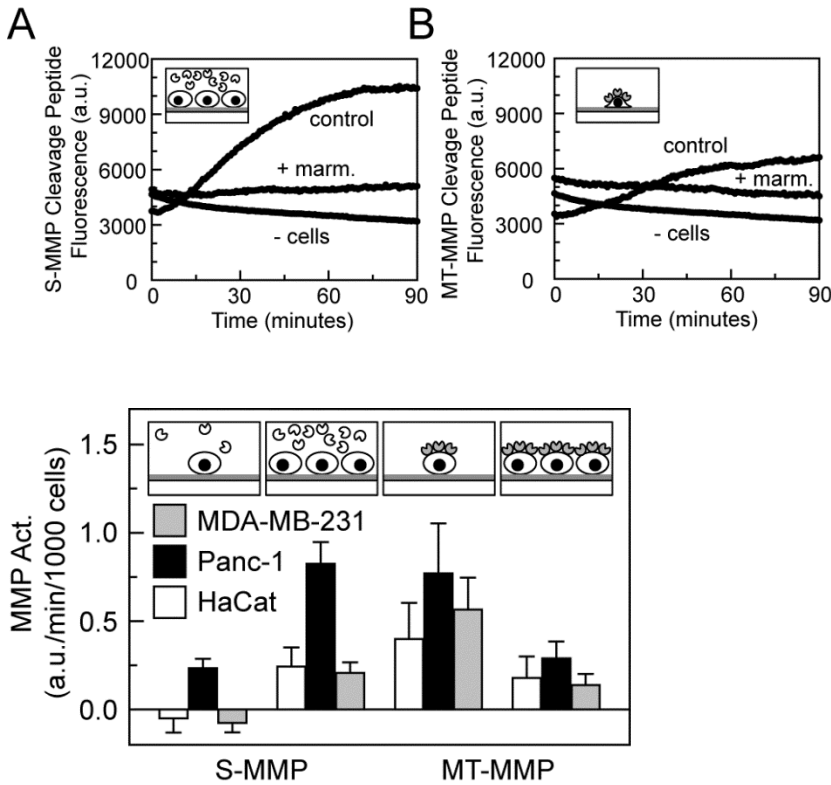
SUPPLEMENTARY FIGURES

Figure S1: Fluorescent Cleavage Peptide Output

A, S-MMP fluorescence over 90 minutes at a high cell density with or without 10μM marimastat and in the absence of cells. Measurements were taken immediately after plating cells on 0.1 mg/ml collagen. B, MT-MMP fluorescence over 90 minutes at a low cell density with or without 10 μM marimastat and in the absence of cells. Measurements were taken immediately after plating cells on 0.1 mg/ml collagen.

Figure S2: Cell Density Effects on S-MMP and MT-MMP Activities

A, S-MMP activity measured at high cell density and MT-MMP activity measured at low cell density in different cell lines. Cells were plated on 0.1 mg/ml absorbed collagen. Error bars are 95% confidence intervals (n = 4).
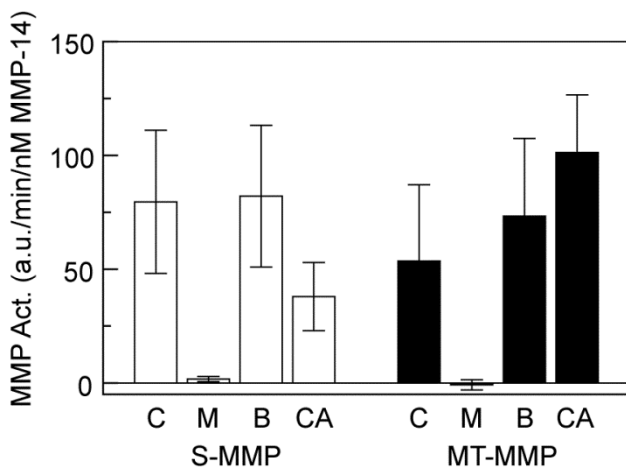

Figure S3: Blebbistatin and calyculin A do not affect MMP-14 activity

An *in vitro* assay was performed on purified MMP-14 catalytic domain after the addition of marimastat (M), blebbistatin (B) and calyculin A (CA). The activity on both peptides was measured. Error bars are 95% confidence intervals (*n* = 6).
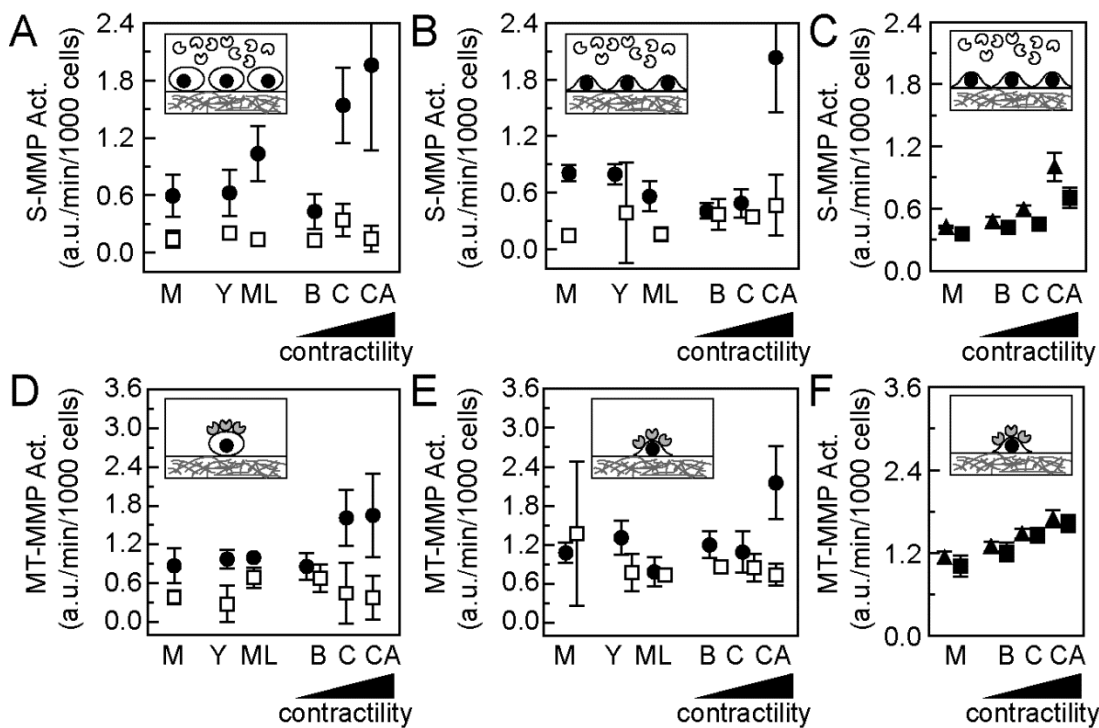
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Figure S4: Cellular Contractility Regulates MMP Activity on High Collagen Concentration Gels

Panc-1 (filled circles, A, B, D and E), BxPC-3 (filled triangles, C and F), AsPC-1 (filled squares, C and F) and HaCat (open squares, A, B, D and E) cells were plated on 5 mg/ml collagen gels under various drug treatments (C: control, M: marimastat (10 μM), Y: Y-27632 (10 μM), B: blebbistatin (10 μM) and CA: calyculin A (1 μM)). A, S-MMP activities measured at high cell density immediately after plating. B and C, S-MMP activities measured at high cell density 3 hours after plating. D, MT-MMP activities measured at low cell density immediately after plating. E and F, MT-MMP activities measured 3 hours after plating. Error bars are 95% confidence intervals (n ≥ 3).
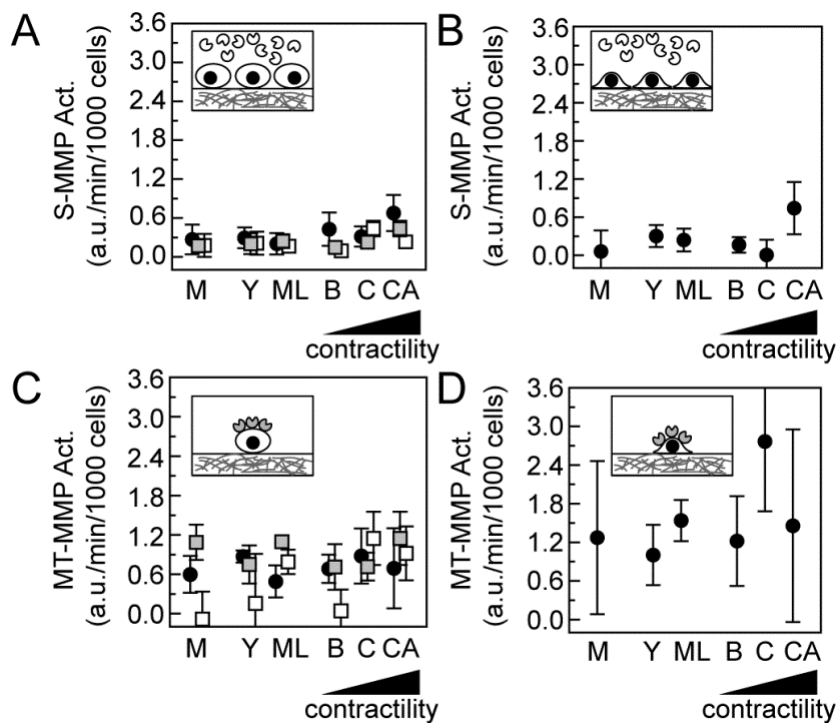
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Figure S5: Cellular Contractility Does Not Effect MMP Activities in MDA-MB-231 Cells.

A, S-MMP activities measured at high cell density of MDA-MB-231 cells immediately after plating on 0.1 mg/ml absorbed collagen (black circles) and 1 mg/ml (gray squares) or 5 mg/ml (open squares) collagen gels under various drug treatments. B, S-MMP activities measured at high cell density of MDA-MB-231 cells 3 hours after plating on 0.1 mg/ml absorbed collagen under various drug treatments. C, MT-MMP activities measured at low cell density of MDA-MB-231 cells immediately after plating on 0.1 mg/ml absorbed collagen (black cirles) and 1 mg/ml (gray squares) or 5 mg/ml (open squares) collagen gels under various drug treatments. D, MT-MMP activities measured at low cell density of MDA-MB-231 cells 3 hours after plating on 0.1 mg/ml absorbed collagen under various drug treatments. Drug treatments used (C: control, M: marimastat (10 μM), Y: Y-27632 (10 μM), B: blebbistatin (10 μM) and CA: calyculin A (1 μM)). Error bars are 95% confidence intervals (n ≥ 3).
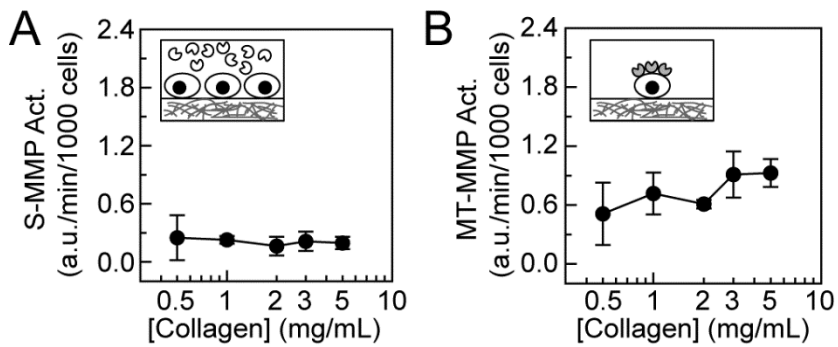
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Figure S6: Collagen Concentration in Gels Has No Effect on MMP Activities in MDA-MB-231 Cells.

A, S-MMP activity measured at high cell density in MDA-MB-231 cells immediately after cells were plated on various collagen gel concentrations. B, MT-MMP activity measured at low cell density in MDA-MB-231 cells immediately after cells were plated on various collagen gel concentrations. Error bars are 95% confidence intervals ($n \geq 3$).