# Interdependency of cell adhesion, force generation and extracellular proteolysis in matrix remodeling

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## Summary

It is becoming increasingly evident that the micromechanics of cells and their environment determine cell fate and function as much as soluble molecular factors do. We hypothesized that extracellular matrix proteolysis by membrane type 1 matrix metalloproteinase (MT1-MMP) depends on adhesion, force generation and rigidity sensing of the cell. Melanoma cells (MV3 clone) stably transfected with MT1-MMP, or the empty vector as a control, served as the model system. $\alpha 2\beta 1$ integrins (cell adhesion), actin and myosin II (force generation and rigidity sensing) were blocked by their corresponding inhibitors ($\alpha 2\beta 1$ integrin antibodies, Cytochalasin D, blebbistatin). A novel, anisotropic matrix array of parallel, fluorescently labeled collagen-I fibrils was used. Cleavage and bundling of the collagen-I fibrils, and spreading and durotaxis of the cells on this matrix array could be readily discerned and quantified by a combined set-up for fluorescence and atomic force microscopy. In short, expression of the protease resulted in the generation of structural matrix defects, clearly indicated by gaps in the collagen lattice and loose fiber bundles. This key feature of matrix remodeling depended essentially on the functionality of $\alpha 2\beta 1$ integrin, the actin filament network and myosin II motor activity. Interference with any of these negatively impacted matrix cleavage and three-dimensional matrix entanglement of cells.

**Key words:** Atomic force microscopy, Cancer metastasis, Matrix remodelling, Membrane type 1 matrix metalloproteinase, Myosin II, Rigidity sensing

## Introduction

In 1962 Jerome Gross and Charles M. Lapiere reported that cut off tadpole tails placed on collagen-coated culture dishes degraded fibrillar collagen (Gross and Lapiere, 1962). This led to the discovery of amphibian collagenase-I, which founded the protease family now commonly known as matrix metalloproteinases (MMPs) (Brinckerhoff and Matrisian, 2002; Nagai et al., 1966). Since the discovery that proteases are linked to tissue remodeling, the interest in this connection has been constantly growing. Today, extracellular proteases are being credited as potent regulators of cellular microecology and as a driving force in fundamental tissue remodeling processes, ranging from embryogenesis to cancer metastasis (Catania et al., 2006; Ludwig et al., 2005; Page-McCaw et al., 2007; Sternlicht et al., 2006; Uzui et al., 2002).

A potent and abundant driver of tissue remodeling is the membrane type 1 MMP (MT1-MMP, MMP-14). It is considered the prototype of a membrane-tethered protease, with a broad spectrum of proteolytic capabilities and a prominent involvement in cancer spreading (Golubkov et al., 2006; Rozanov et al., 2008; Zhai et al., 2005). Solid cancer cells differ in many aspects when comparing one type of cancer to another. However, the function of proteases constitutes a common pathway during metastasis and tissue invasion (e.g. Egeblad and Werb, 2002; Sternlicht and Werb, 2001). The dependency of the metastatic potential of cancer cells on their ability to degrade the extracellular matrix (ECM) of basement membranes was recognized early (Liotta et al., 1980). Notwithstanding, the function of proteolytic activity has to go beyond a simple 'path-clearing process' (Stamenkovic, 2003). This means that despite being a physical obstacle, the matrix still has to provide sufficient resistance to act as a structural scaffold, to allow migrating cancer cells to develop traction for their locomotion.

Theoretically, proteases would pose efficient means for cancer cells to modulate (1) attachment to and detachment from the ECM, (2) the local biomechanical properties of the ECM, and (3) the overall structure of their microenvironment. In other words, it would be beneficial for cancer cells to utilize mechanisms to sense and regulate matrix proteolysis in relation to their microenvironment. Also, the ability to develop traction on the remodeled ECM is essential to promote tissue invasion. Basic principles of these processes and the integration of feedback signals for their spatiotemporal regulation remain to be elucidated.

Here, we used a novel in vitro approach to probe the putative interdependency of cellular matrix adhesion, force exertion on the matrix and proteolysis for ECM remodeling. A key feature of this approach is based on the anisotropy of the matrix. Anisotropy as such is a physical property that has a different value when measured in different directions. A simple example for anisotropy is wood, which is mechanically stronger along the grain than across it. Here, the anisotropy was accomplished by generating a homogeneous monolayer of parallel and evenly spaced fibers of collagen-I (collagen type-I). The parallel alignment of the collagen fibers results in high tensile strength and rigidity along the fibers and high pliability perpendicular to it. In addition, we succeeded in generating a fluorescently labeled matrix. This highly ordered collagen-I matrix simplifies the visualization of cellular responses to the substrate (Friedrichs et al., 2007; Jiang et al., 2004; Poole et al., 2005). For this, a combination of fluorescence and atomic force microscopy (AFM) was used (Fig. 1).

A human melanoma cell line (MV3) served as a model. MV3 cells have previously been shown to express $\alpha 2\beta 1$ integrin for adhesion to collagen-I, and have been used for cell migration studies (Friedl et al., 1997; Maaser et al., 1999). MV3 cells stably
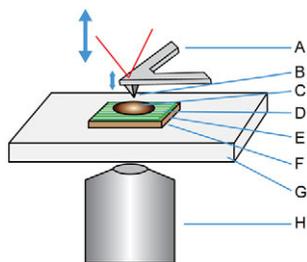
**Fig. 1. Experimental set-up of parallel collagen assay.** Melanoma cells (MV3 clone) (C) were seeded on fluorescently labeled, fibrillar collagen-I matrices (D). The matrices were generated on ultrapure mica discs (E) that were glued with an optical adhesive (F) to coverslips (G) mounted in a temperature controlled microperfusion chamber on an inverted optical microscope (H). In parallel to optical microscopy, samples were simultaneously imaged with an atomic force microscope (AFM). AFM generates 3D topographies with a nanoscale resolution through line-wise scanning of surfaces with a thin silicone or silicon nitride tip (B). The tip emanates from the flexible cantilever (A) with a reflective coating on its back. Based on the principle of an optical lever (arrows), movements of the tip can be detected with a laser reflected by the cantilever onto a photo diode.

expressing MT1-MMP were seeded on the collagen-I matrices. Their response to various conditions influencing their ability to adhere to, or generate force (e.g. pull) on, the collagen fibers was investigated.

## Results

### Anisotopic parallel collagen-I matrices simplify evaluation of cellular responses

Mica discs were coated with a parallel collagen-I matrix (anisotropic collagen) and glass coverslips were coated with randomly oriented collagen-I (isotropic collagen) (Fig. 2). Use of FITC-labeled collagen-I made the matrix accessible for fluorescent microscopy and AFM. After seeding MV3 cells stably expressing MT1-MMP on the isotropic matrix on the glass coverslips for 1 hour, no polarized effects on the collagen were observed during, or after, the spreading of the cells (Fig. 2A). Furthermore, no substantial alterations of the collagen could be detected by AFM imaging and fluorescence microscopy. As a consequence, it was extremely difficult to evaluate discrete cell–matrix interactions in this setting.

Conversely, seeding of MV3 cells expressing the empty vector (MOCK) on the anisotropic collagen yielded a visibly distinct response (Fig. 2B). Cells aligned along the collagen fibers, adopted a spindle-like shape, and actively intertwined with the matrix to generate a structured, three-dimensional environment. Upon the generation of traction, the cells pulled the collagen fibers into bundles close around themselves. Most of the fibers remained intact (Fig. 2B, middle). The collagen bundles run undisturbed along the cells, as confirmed by the fluorescent image. AFM supported this observation by revealing intact collagen fibers that were bundled by the lamellipodia, but continued parallel above the cell (Fig. 2B, right)

In contrast, the interaction of MV3 cells expressing MT1-MMP with the anisotropic collagen was easily distinguishable from that of wild-type MV3 cells. The cells adopted a more rounded shape because they pulled collagen fibers into bundles and cleaved them in the process (Fig. 2C). The cleavage of the collagen fibers was readily detectable in both the fluorescence and AFM images. The

fluorescent image (Fig. 2C, middle) showed that cleaved collagen fibers assembled into curly knots around and on top of the cells and that the fibers were not continuing further (Fig. 2C*). AFM confirmed these findings. Around the lamellipodia loose and open-ended collagen fibers were found, in addition to fibers that were curled and pulled over the original collagen layer (Fig. 2C, arrows). In conclusion, an anisotropic matrix enables a direct observation and easy quantification of cell–matrix interactions with a near-binary, 'yes' or 'no' readout, which was used to analyze the following experiments.

In addition to proteolytic cleavage of the collagen fibers, bundling of collagen fibers, cell spreading and durotaxis were analyzed. Durotaxis is a phenomenon where cells 'squeeze' themselves between the mica and the collagen matrix (Lo et al., 2000), preferring the stiffer substrate. Notably, the basic structure of the collagen coating stayed intact during durotaxis. Therefore cells were seeded on the anisotropic collagen-I matrix and analyzed by AFM, exploiting the higher resolution to assess collagen integrity, and by fluorescence microscopy, to collect data for a quantitative analysis.

### Expression of MT1-MMP promotes collagen-I cleavage

Proteolysis of the ECM is a major defining feature of tissue invasion and ECM remodeling. The influence of MT1-MMP on a cell's ability to cleave the collagen fibers was quantified under control conditions (Fig. 3A). Without any inhibitors or antibodies present, DMSO was used as solvent control. On the parallel collagen matrix, the effects of proteolysis were easily recognized. MV3 cells stably expressing MT1-MMP cleaved and displaced fibers compared with wild-type control cells expressing an empty vector (MOCK) (Fig. 3A). Upon MT1-MMP expression, 'holes' in the collagen matrix were visible in the fluorescent images and loose fibers were detected by AFM.

In addition to the presented images (Fig. 3), we took images at 20× magnification to capture a larger number of cells for analysis. Cells that exhibited only bundled continuous collagen-I fibers (compare Fig. 2B and Fig. 3A, MOCK) were counted as 'no cleavage', whereas cells that induced gaps in the parallel collagen matrix and had curled collagen present were counted as 'cleaved collagen' (compare Fig. 2C, Fig. 3A, MT1-MMP and Fig. 4A). Likewise, cells that showed thick bundles of collagen, had a spread out shape on the collagen matrix or squeezed themselves between the collagen matrix and the mica, and were classified as positive for 'bundling', 'spreading' or 'durotaxis'. On average, 450 cells for each MT1-MMP and MOCK condition (see the Materials and Methods for statistical procedures) were analyzed. The errors are given as s.e.m.

Cleavage of fibrillar collagen could be detected within the first hour of cell–matrix interaction in about 80% of MT1-MMP-transfected MV3 cells (Fig. 3A, Fig. 5A, Table 1). Conversely, matrix cleavage of the MOCK-transfected cells was significantly lower, with only around 13% of visible cleavage (Fig. 3A, Fig. 5A and Table 1). Interestingly, there was no significant difference in the bundling of collagen fibers, cellular spreading on the matrix or durotaxis under control conditions when comparing cells expressing MT1-MMP and MOCK transfected cells (Fig. 3A, Fig. 5A, Table 1).

### Changes in cellular adhesion impact collagen-I cleavage

MV3 cells rely on the expression of $\alpha2\beta1$ integrin to adhere to collagen-I (Maaser et al., 1999). Thus, the effect of integrin-
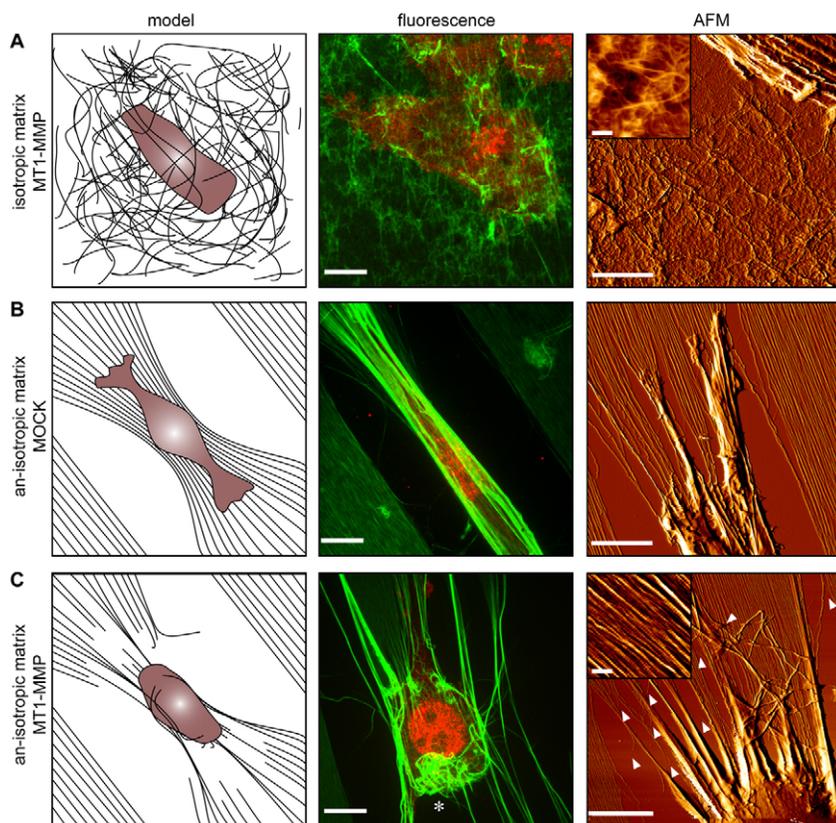
**Fig. 2. Cellular response to isotopic collagen versus parallel anisotropic collagen.** Sketches (left), spinning disc confocal (middle) and atomic force microscopy (AFM; right) images of representative melanoma cells (MV3 clone). The images were acquired 1 hour after seeding the cells onto the isotropic (**A**) and, anisotropic (**B,C**) matrices of fluorescently labeled collagen-I (green). The parallel collagen fibrils (B,C) lead to a polarization of matrix properties characterized by high tensile strength along the fibril direction that enables strong cellular traction and a high pliability in the perpendicular orientation with the opposite effect. Cells, whether stationary or migrating, exert forces against their substrates as they anchor to, and pull on, their surroundings (B,C). The MV3 cells were transfected with either an empty vector (MOCK, B) or MT1-MMP vector (A,C). Proteolysis by MT1-MMP of collagen-I and curled up fibers can be easily detected in the anisotropic matrix array (C, asterisk and arrowheads). On an isotropic matrix (A), however, such an effect cannot be readily discerned. Inset in C shows the characteristic D-banding for collagen-I of ~67 nm. Scale bars: 100 µm (confocal image); 10 µm (AFM image); 500 nm (AFM image insert).

blocking antibodies on the ability of a cell to cleave collagen-I was tested. The cellular responses were evaluated in the same way as described above. For this condition, on average, 210 cells each for MT1-MMP and MOCK transfectants were analyzed. The most prominent feature after blocking this particular integrin with antibodies was the abolished collagen cleavage in cells transfected with both MT1-MMP (0.6±0.5%) and MOCK vector (0.6±0.5%) (Fig. 3B, Fig. 5B and Table 1). Interestingly, the cells expressing MT1-MMP showed decreased collagen-I bundling, whereas no difference was detected for the MOCK cells. Conversely, spreading was only affected in MOCK cells, which showed an increase when compared with the control conditions. Durotaxis was promoted for both MT1-MMP and MOCK cells by the administration of antibodies against α2β1 integrin.

The fact that collagen fibers were physically displaced under control conditions by the cells pulling them close led us to the hypothesis that either (1) the cell's ability to exert force on the fibers is vital for proteolysis, or (2) the α2β1 integrin antibodies interfere sterically with MT1-MMP function. Consequently, we next focused on the relevance of the actin cytoskeleton and non-muscular myosin II for collagen cleavage.

### Cytochalasin D and blebbistatin impede extracellular proteolysis

A cell's ability to migrate and pull on the collagen-I fibers depends on a functional actin cytoskeleton and motor proteins. We targeted both independently by pharmacological intervention. For this, cells were seeded onto the collagen matrix in the presence of Cytochalasin D or blebbistatin (Fig. 3C,D). Cytochalasin D disrupts the formation of actin filaments, whereas blebbistatin specifically inhibits non-muscular myosin II (Dou et al., 2007; Fenteany and

Zhu, 2003; Schliwa, 1982). We counted and evaluated on average 90 cells for the Cytochalsin D and 130 cells for the blebbistatin treatment.

We found that both Cytochalasin D and blebbistatin completely inhibited the cleavage of collagen fibers by MT1-MMP and MOCK transfectants (0±0% for both cell lines under both conditions) (Fig. 3C,D, Fig. 5C,D, Table 1). In addition, Cytochalasin D significantly reduced or even inhibited fiber bundling, cell spreading and durotaxis (Fig. 5C). In general, cells adopted a round shape and resided on the collagen matrix. However, cell adhesion was severely affected, because we found fewer adherent cells. In comparison to the control conditions, blebbistatin inhibited not only the proteolysis of collagen-I fibers, but also negatively affected bundling and durotaxis. However, cell spreading was not significantly altered.

Besides the analysis of how the cells interact with the collagen matrix, we were interested in dose-dependent effects of blebbistatin. Therefore, increasing concentrations of blebbistatin [0 (solvent control), 1, 20 and 50 µM] were used. MT1-MMP transfectants treated only with DMSO (solvent control) showed collagen fiber cleavage and bundling (Fig. 4). Upon blebbistatin treatment, however, the cell's ability to pull on the collagen-I fibers was clearly reduced. Strikingly, a blebbistatin concentration that was 50 times lower than that commonly used, abolished collagen cleavage (Fig. 4B, 1 µM blebbistatin). Cells were still able to bundle a few fibers around them, as shown in the fluorescent image (Fig. 4B), yet with AFM, no cleaved fibers were visible. In the presence of 20 µM blebbistatin (Fig. 4C), the cell's ability to pull the collagen into bundles around itself decreased even further (Fig. 4C, fluorescence). AFM confirmed that no collagen cleavage occurred. Finally, 50 µM blebbistatin (Fig. 4D) nearly abolished the cell's ability to pull on the fibers. Only slight distortions in the collagen matrix were
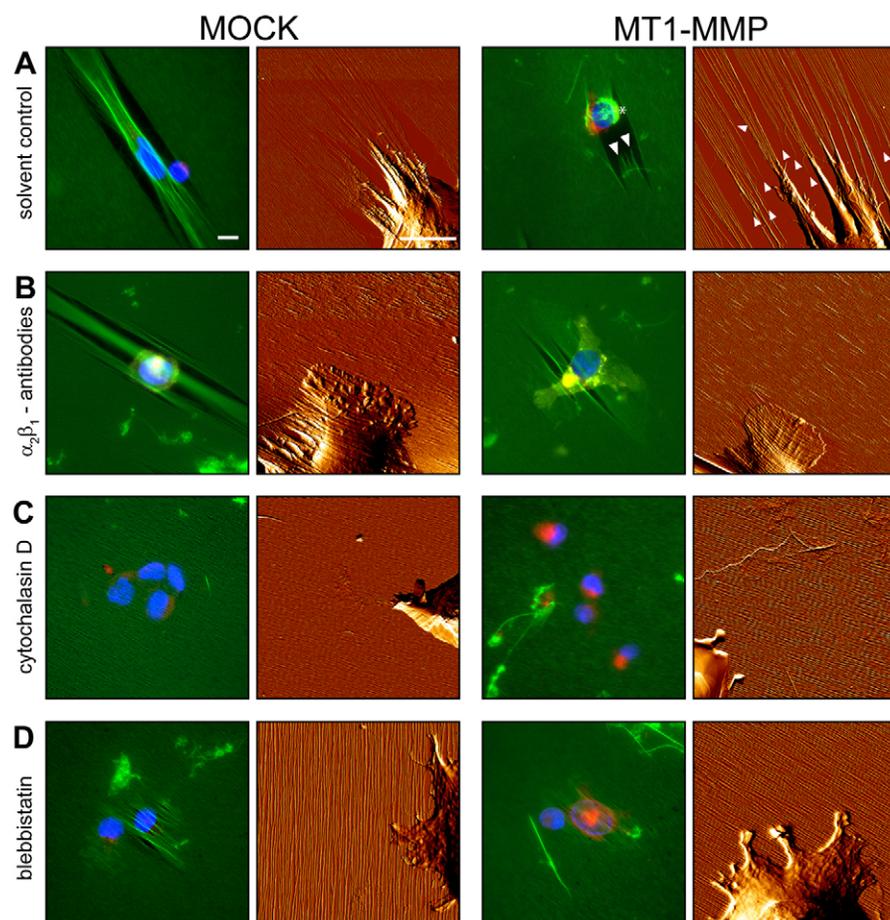
MOCK          MT1-MMP



**Fig. 3. Modulation of fibrillar collagen-I proteolysis.** Fluorescent (left columns) and AFM images (right columns) of MV3 cells that were transfected with either an empty vector (MOCK, left) or MT1-MMP (right; red fluorescence). (**B**) Yellow fluorescence indicates the integrin antibodies. Images were acquired 1 hour after seeding of the cells on parallel collagen-I fibrils (green fluorescence) either in the presence of solvent control (DMSO; **A**), 2 µg/ml anti-α2β1-integrin antibodies (B), 5 µg/ ml Cytochalasin D (**C**), or 50 µM blebbistatin (**D**). Cytochalasin D inhibits polymerization of the actin cytoskeleton, whereas blebbistatin is an inhibitor of myosin II motor protein. Arrowheads indicate cleaved open ended collagen fibers. Asterisk indicates curled up collagen the cells pulls around itself. Blue fluorescence indicates nuclear DAPI counterstain. Scale bars: 20 µm.

detected in the fluorescent image (Fig. 4D, fluorescence). AFM confirmed that the matrix, with the cell sitting mostly on top of the fibers, is essentially undisturbed. From these experiments (applying Cytochalasin D and blebbistatin), we assumed that either (1) the trafficking of MT1-MMP is influenced or (2) cells need both the ability to exert force (e.g. pull on the collagen fibers) and proteolysis to actively cleave the collagen-I fibers.

## Cytochalasin D and blebbistatin have no impact on cell-surface expression of MT1-MMP

Next, we examined whether blebbistatin and Cytochalasin D influence the trafficking of MT1-MMP to the cell surface, leading to reduced collagen proteolysis. A previously established model describes the sequential processing of this protease (Ludwig et al., 2008b). MT1-MMP is expressed and activated in the cell and the
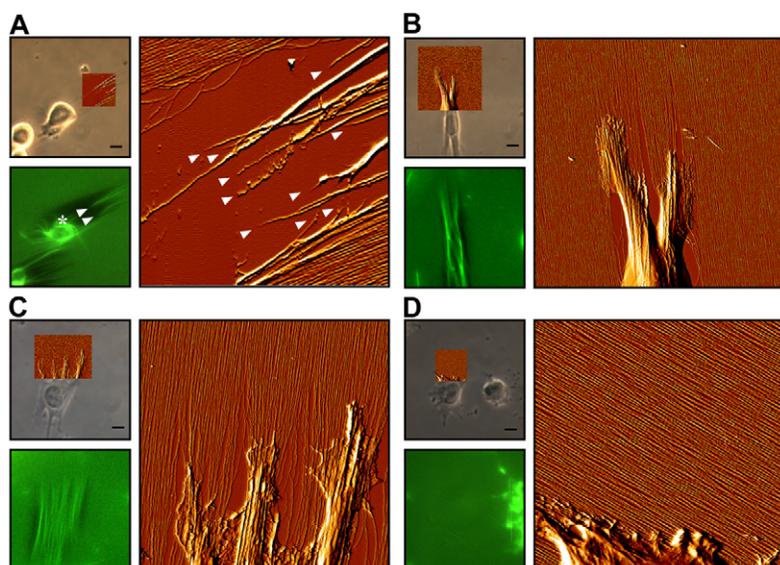


**Fig. 4. Effects of myosin II inhibition on cell-matrix interactions.** MV3 cells stably expressing MT1-MMP were seeded on mica coated with parallel collagen-I fibrils and incubated for 1 hour in the presence of solvent control (DMSO) (**A**), and 1 µM (**B**), 2 µM (**C**) and 50 µM (**D**) concentrations of blebbistatin. Representative fluorescence microscopy, atomic force microscopy images and DirectOverlay phase-contrast images that indicate the area scanned by AFM are shown. Arrowheads indicate cleaved open-ended collagen fibers. Asterisk indicates curled up collagen that the cell pulls around itself. Scale bars: 10 µm.

proteolytically active form is transported to the cell surface. We performed western blot analysis of cell-surface biotinylated MT1-MMP after cells were grown on collagen-I in the presence and absence of integrin-blocking antibodies, Cytochalasin D and blebbistatin. By utilizing antibodies against the HA-tagged MT1-MMP it could be distinguished whether the proteolytic form of MT1-MMP is present at the cell surface or not. In addition, surface immunofluorescence was used to visually assess the cell-surface expression of MT1-MMP.

In summary, the addition of integrin antibodies, Cytochalasin D or blebbistatin had no effect on MT1-MMP trafficking to the cell surface (Fig. 6A). Only in cells treated with the integrin-blocking antibodies did a minor increase in the cell-surface expression of the active MT1-MMP form become apparent. This is in accordance with previous studies, where antibodies against $\alpha2\beta1$ integrin led to an increase in MT1-MMP expression levels (i.e. Zigrino et al., 2001). Interestingly, in MV3 cells expressing MT1-MMP, $\alpha2\beta1$-integrin-blocking antibodies and Cytochalasin D caused an accumulation of the protease's 44 kDa metabolite. This has been demonstrated to be an inactive remnant, which is generated by auto-proteolytic shedding of the catalytic domain (Ludwig et al., 2008b). After Cytochalasin D treatment, this accumulation of total cell-surface MT1-MMP (including both the active and inactive metabolites) is also visible in the cell-surface immunofluorescence staining (Fig. 6B). Remarkably, under either condition, we detected no cleaved collagen fibers (Fig. 5B). Blebbistatin treatment caused no changes in the cell-surface expression pattern of MT1-MMP compared with controls. Moreover, no accumulation of the 44 kDa metabolite could be detected. Overall, the results for blebbistatin resembled the control conditions. This suggested that the trafficking of MT1-MMP in blebbistatin-treated cells is not compromised.

In summary, blocking $\alpha2\beta1$ integrins, breaking down the actin network and inhibiting myosin II directly impaired the ability of a cell to dislocate collagen-I fibers. Collagen fiber bundling observed during $\alpha2\beta1$ integrin block is most likely based on passive dislocation by the cells squeezing themselves between the matrix and the solid support. To exert a pulling force on the matrix, cells need (1) functional motorproteins (myosin II), (2) an active cytoskeleton, and (3) the means to attach to the collagen ($\alpha2\beta1$ integrins). The trafficking of MT1-MMP to the cell surface was not disturbed by the pharmacological interventions. In conclusion, collagen fiber cleavage by cells requires both the capacity to proteolyse collagen-I and the ability to attach to and pull on the matrix.

## Discussion

The functionally relevant proteolytic net activity in the cell microenvironment results from the fragile and complex local balance of distinct proteases, their substrates and inhibitors (Ludwig, 2005). However, it is becoming increasingly evident that the micromechanics of cells and their environment determine cell fate and function as much as soluble molecular factors do (Chen et al., 1997; Discher et al., 2005; Ludwig et al., 2008a; Vogel and Sheetz, 2006). Here, we show that modulating different cornerstones of that local balance – actin integrity, myosin II motor function or cell adhesion – rendered MT1-MMP ineffective and stalled extracellular proteolysis completely (Fig. 7).

In this context, we have demonstrated that the parallel fluorescent collagen-I matrix had substantial benefits over isotropic collagen coatings. Features of cell–matrix interactions, such as collagen-I
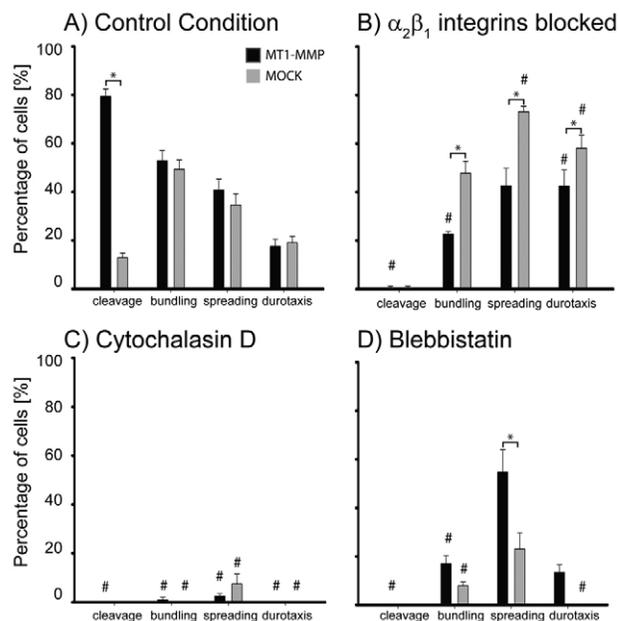


Fig. 5. **Effects of inhibition of β-integrin, actin and myosin II on features of cell–matrix interactions.** MV3 cells that were stably transfected with either an empty vector (MOCK) or MT1-MMP were each seeded on an anisotropic matrix of fluorescently labeled fibrillar collagen-I in the presence of either DMSO (**A**) as solvent control, (**B**) $\alpha2\beta1$-integrin blocking antibodies, (**C**) Cytochalasin D or (**D**) blebbistatin. Cells were allowed to interact with the matrix for 1 hour before fixation. Afterwards, oversight images with a low magnification (20×) were taken and collagen-I cleavage, bundling, cellular spreading and durotaxis were quantified. The total number of counted cells (n between 90 and 400) for each condition was set to 100%. Results are shown as the ratio of cells that display the investigated feature (cleavage, bundling, spreading, and durotaxis) to the total number of cells. Error bars display ± s.e.m. *$P<0.05$, MT1-MMP vs MOCK, same treatment. #$P<0.05$, compared with control condition.

fiber cleavage and bundling, cellular spreading and durotaxis, can be easily discerned. These parameters could be readily evaluated with a binary 'yes' or 'no' read-out after 1 hour of cell–matrix interaction. In this study, we took advantage of AFM and fluorescence microscopy imaging techniques. AFM offered 3D topography images at a higher resolution and a higher speed compared with the acquisition and processing of confocal image stacks. AFM imaging revealed the location of the cell and the structure of the collagen lattice. As such, cells squeezed between the solid support and the matrix could be easily distinguished from cells located on top of the collagen by AFM. These images proved, in addition, the structural integrity of the matrix after the addition of Cytochalasin D, blebbistatin or $\alpha2\beta1$ integrin antibodies to MT1-MMP expressing cells.

Fluorescence microscopy offered notwithstanding high-speed multicolor bulk analysis of cellular responses. It was instrumental in efficiently capturing multiple bigger image areas to analyze a large number of cells. In conclusion, the parallel fluorescent collagen coating allows for easy image analysis and the possibility to use both AFM and fluorescence microscopy in an efficient and complementary way.

Of all interventions, administration of Cytochalasin D proved most efficient in the inhibition of the bundling of collagen fibers, cell spreading and cellular durotaxis. This is not surprising because

**Table 1. Cell behavior on the parallel collagen-I matrix**

| | Control condition | | α2β1-integrin block | | Cytochalasin D | | Blebbistatin | |
|---|---|---|---|---|---|---|---|---|
| | MT1-MMP | MOCK | MT1-MMP | MOCK | MT1-MMP | MOCK | MT1-MMP | MOCK |
| Cleavage | 79.4±3 | 12.9±1.9 | 0.6±0.5 | 0.6±0.5 | 0±0 | 0±0 | 0±0 | 0±0 |
| Bundling | 52.9±4.1 | 49.3±3.7 | 22.6±1.2 | 47.6±5 | 1±1 | 0±0 | 17.1±3.2 | 7.9±1.5 |
| Spreading | 40.8±4.5 | 34.6±4.7 | 42.6±7.3 | 73.1±2.3 | 2.5±0.9 | 7.4±4.1 | 54.7±9.3 | 22.97±6.7 |
| Durotaxis | 17.6±2.9 | 19.1±2.5 | 42.3±6.6 | 57.9 ±5.5 | 0±0 | 0±0 | 13.5±3.1 | 0±0 |

All values give the percentage of cells that exhibited cleavage, bundling, spreading or durotaxis under the given condition. Values are mean±s.e.m.

Cytochalasin D breaks down the actin filament network and therefore interferes broadly with cell migration. Hence, cells had a round shape and were unable to migrate or extend lamellipodia. Conversely, the absence of collagen-I cleavage is surprising because the proteolysis of the collagen fibers is biochemical in nature. Yet, not even AFM could detect cleaved collagen fibers, at least after the given time of 1 hour for the assay. Despite the major effect of Cytochalasin D on collagen cleavage, the diverse functions of actin make a specific deduction of the underlying molecular mechanisms difficult.

Interestingly, it has been shown previously that the secretion of diverse MMPs is linked to the integrity of the actin cytoskeleton. Several findings pointed towards higher expression levels of MMPs upon Cytochalasin D administration and built the rationale of its application in this study (Chintala et al., 1998; Ogier et al., 2006; Upadhya and Strasberg, 1999). In line with the previous literature, we have found an increase in cell-surface levels of MT1-MMP by Cytochalasin D. In particular, the 44 kDa metabolite of MT1-MMP was elevated. However, despite increased MT1-MMP levels, no collagen cleavage was detectable in the parallel collagen assay after 1 hour.

Conversely, effects of actin stabilizing compounds such as phalloidin and jasplakinolides on MMP expression levels have also been reported (e.g. Fenteany and Zhu, 2003; Upadhya and Strasberg, 1999). One study demonstrated a decreased secretion after phalloidin treatment (Upadhya and Strasberg, 1999). This is particularly interesting because the intracellular trafficking of MMPs, including that of MT1-MMP, has previously been linked to microtubules (Remacle et al., 2005; Schnaeker et al., 2004). However, there seems to be a direct link between MMP trafficking along microtubules and the integrity of the actin filament network.

This makes actin filaments an interesting target for interfering with MMP secretion.

F-actin stabilizers, such as jasplakinolides, have previously been demonstrated to inhibit lamellipodia formation (Cramer, 1999). Their effects are, however, complex and might still enable the cell to develop traction and exert a pulling force on its surroundings. Because we showed in this study that collagen-I cleavage is linked to biochemical, as well as mechanical processes (e.g. the cells ability to pull on the collagen fibers), this suggests that using F-actin stabilizers probably will not have the drastic effect that destabilizers have. Regardless, the detailed effects of actin-stabilizing drugs, such as jasplakinolide, differ in vitro and in vivo (Fenteany and Zhu, 2003). As a result, their impact on MT1-MMP function in combination with the cell's ability to pull in its surroundings remains to be elucidated in the future.

In our study, effects similar to that of Cytochalasin D were obtained by the inhibition of myosin II motor function with blebbistatin. Unlike Cytochalasin D, blebbistatin leaves the actin network unaffected, but collagen-I cleavage was completely silenced and cells showed less bundling of collagen fibers. In MOCK transfectants, durotaxis was decreased, but cell spreading was not affected. MT1-MMP transfectants showed no increased cellular spreading or durotaxis. This might indicate that MT1-MMP acts not only as a protease, but also as an extracellular matrix receptor. Previously, its hemopexin domain has been shown to bind to native collagen-I (Tam et al., 2004). Although the cells were still able to extend lamellipodia and visibly dislocate some collagen fibers, collagen-I cleavage was inhibited by a concentration of blebbistatin that is 50-times lower than generally used. This stresses the importance of physical force generation in efficient matrix remodeling.
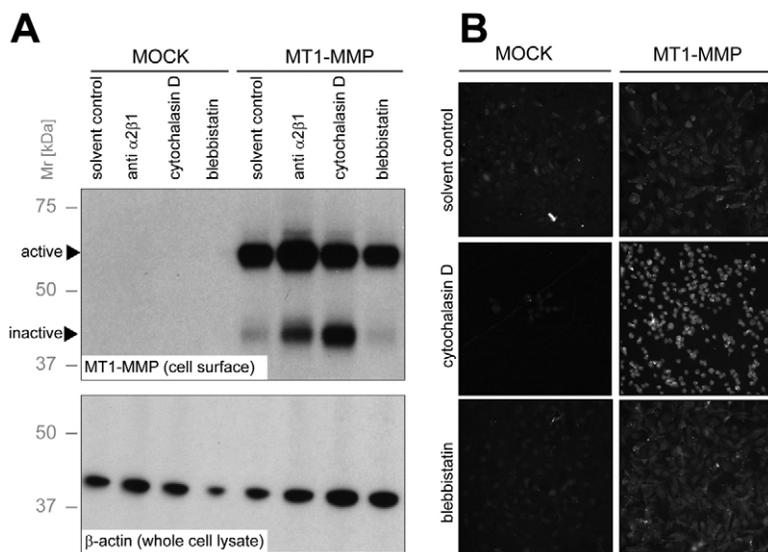


**Fig. 6. Cell-surface expression of MT1-MMP.** (**A**) Western blot analysis of cell-surface biotinlyated MV3 cells which were either stably transfected with the empty vector (MOCK) or MT1-MMP. The cells were seeded on collagen coated cell culture dishes and treated with antibodies against α2β1 integrin, Cytochalasin D and blebbistatin. The arrows indicated the catalytically active (57 kDa) and inactive (44 kDa) MT1-MMP metabolite; (A bottom) β-actin loading control of the whole cell lysate. (**B**) Cell surface immunofluorescence of MOCK and MT1-MMP expressing MV3 cells 1 hour after seeding them onto collagen coatings. Alexa-Fluor-568-labeled goat anti mouse antibody, bound to the α2β1 integrin antibodies. Thus, it was not possible to acquire data for this setting.
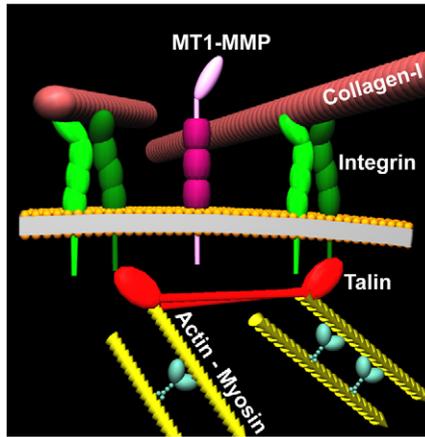
**Fig. 7. Schematic model of focal adhesion mechanics and their interaction with MT1-MMP.** Effective proteolysis by MT1-MMP depends essentially on cell adhesion and force generation. Cells establish focal adhesions to the extracellular matrix via the α2β1 integrin. The cell cytoskeleton generates pulling forces via the actin–myosin network, which is transmitted through these focal adhesions to the extracellular matrix. The extracellular headpieces of the **integrin family** transmembrane receptors bind extracellular matrix proteins, in this case **fibrillar collagen-I**. Their cytoplasmic tails bind adaptor proteins such as the **talin dimer**, which in turn binds **actin**. **Myosin II** generates force that is transmitted through integrins to the extracellular matrix. **MT1-MMP** has been described to be linked directly to β1 integrins, and indirectly to the actin network via CD44 (Galvez et al., 2002; Ludwig et al., 2008a; Mori et al., 2002).

In principle, the inhibition of myosin II could affect the regulation of MT1-MMP by preventing its localization to sites of focal adhesion. However, no effects on cell-surface trafficking of MT1-MMP compared with the control conditions were apparent after cell-surface biotinylation of the protease.

The effect of inhibition of α2β1 integrin on collagen-I cleavage is highly interesting. It literally links intracellular myosin II via the action cytoskeleton to extracellular protease function. Integrins are an integral part of focal adhesions that serve a dual function. First, they provide structural continuity between the actin cytoskeleton and the extracellular matrix and thus constitute the structural support required to maintain adhesion to the substrate (Sastry and Burridge, 2000). Second, they are strong signaling centers, which activate many important signaling cascades, including those regulated by the small GTPases of the Rho family (Burridge and Chrzanowska-Wodnicka, 1996). As such, focal adhesions appear to be the main decision-making centers for cells in regard to the physical properties of their environment. Although stretch-activated ion channels are the main molecular mechanism underlying the mechanotransductory senses of, for example touch and hearing, they are thought to be of minor significance in rigidity sensing of cells on soft substrates (Gillespie and Walker, 2001; Schwarz and Bischofs, 2005). Most evidence suggests that mechanotransduction based on rigidity sensing is localized to integrin-based focal adhesion sites (Bershadsky et al., 2003; Geiger et al., 2001).

However, an interesting link between myosin II and focal adhesions has been described previously. In naive mesenchymal stem cells, elasticity was found to direct stem cell lineage commitment (Engler et al., 2006). In brief, the stiffness of biologically inert polyacrylamide gels was tuned to mimic tissue-

like conditions in vitro. For instance, bone-like rigid matrices gave rise to an osteoblast-like phenotype, whereas soft matrices, which mimic brain-like conditions, induced neurogenesis. In this setting, matrix-stiffness-driven stem cell programming tended to be even more selective than soluble induction factors. This sensing of matrix stiffness by the stem cells depended on myosin II contractility transmitted to the outside through focal adhesions. With this indication of a functional connection between focal adhesion and myosin II function in mind, our results for collagen fiber cleavage show a coherent pattern for the pharmacological inhibition of α2β1 integrin, the actin cytoskeleton and myosin II in MT1-MMP- and MOCK-transfected cells.

Notwithstanding, unexpected differences in the behavior between MT1-MMP- and MOCK-transfected cells could be found. In the presence of α2β1-integrin-blocking antibodies, cellular spreading was facilitated in MOCK-transfected cells, in which the antibodies have otherwise no effect on fiber bundling. By contrast, in MT1-MMP transfectants, blocking α2β1 integrin has no effect on cell spreading, but significantly inhibited the bundling of collagen-I fibers. It can thus be established that, on the anisotropic matrix, spreading of cells and bundling of collagen fibers are independent processes. In principle, cells do not have to exert force for spreading, but it is required to dislocate collagen fibers. Cell spreading and collagen-I fiber bundling are unaffected per se by the expression of MT1-MMP in this model system, unless the cells are being otherwise challenged, i.e. by the addition of integrin-blocking antibodies, Cytochalasin D or blebbistatin.

Durotaxis, which is characterized in this model system by the spreading of cells underneath the sheet of fibrillar collagen, was promoted significantly by inhibition of α2β1 integrin in both MT1-MMP- and MOCK-transfected cells. This implies that integrin functionality is, in this case, not strictly linked to rigidity sensing. Interestingly, MT1-MMP has previously been demonstrated to be directed to focal adhesions by focal adhesion kinase (FAK), but also regulates focal adhesion stability and turnover by itself, via FAK cleavage (Shofuda et al., 2004; Takino et al., 2007; Takino et al., 2006). In endothelial cells, the ECM binding by β1 integrins has been proved to regulate the targeted trafficking, expression and activation of MT1-MMP (Galvez et al., 2002). Later, a mechanistic link was established between β1-integrin-mediated adhesion to collagen, and MT1-MMP delivery to invasive structures by Rab8 GTPase (Bravo-Cordero et al., 2007). This points towards a fragile balance of adhesion and de-adhesion events in focal adhesion stability (Fig. 7). Nonetheless, application of blebbistatin inhibited durotaxis only in MOCK-transfected cells, not in MT1-MMP-transfected cells.

It must be considered that proteases such as MT1-MMP have numerous targets in addition to extracellular matrix proteins, which includes growth hormones and their receptors, chemokines and their receptors, adhesion molecules, chromatin-stabilizing proteins, clotting factors, protease inhibitors, proteases and itself (Deryugina et al., 2002; Golubkov et al., 2005; Koshikawa et al., 2010; Mitsiades et al., 2001; Osenkowski et al., 2004; Overall and Blobel, 2007; Overall and Dean, 2006; Powell et al., 1999; Ratnikov et al., 2002; Schlondorff and Blobel, 1999; Sternlicht and Werb, 2001; Yu et al., 2002). Moreover, the expression of MT1-MMP has been shown to have an impact on the expression of diverse genes, including regulators of apoptosis, transcription, mRNA splicing, protein trafficking and energy metabolism (Rozanov et al., 2008). It is thus not surprising that MT1-MMP-transfected cells display complex alterations in their behavior.

In summary, the presented data stress the coherency of biochemical and biophysical processes in matrix remodeling, although the biological basis for the interactions of cells with their microenvironments is just beginning to emerge. The mechanisms by which proteases such as MT1-MMP drive fundamental tissue-remodeling events, ranging from embryogenesis to cancer cell invasion, are far from understood. A more thorough knowledge of the basic mechanisms and parameters that define the functions of MT1-MMP is vital to the development of novel approaches for specific therapeutic interventions. We focused on collagen-I cleavage, fiber bundling, cell spreading and durotaxis using a novel assay that utilizes fluorescent, parallel collagen-I fibers. These parameters represent biochemical and biophysical hallmarks of a cell's interaction with its microenvironment. We showed experimentally that key features of matrix remodeling depend fundamentally on the synergy of cellular traction, adhesion and proteolysis. It can thus be concluded that on the anisotropic matrix, spreading of cells and bundling of collagen fibers are independent processes.

## Materials and Methods

### General reagents and antibodies

All buffers and solutions were sterile filtered with Milliex-GS 0.22 μm syringe-driven filters (Millipore Schwalbach, Germany). Blebbistatin was purchased from Sigma. Cytochalasin D was ordered from Enzo Life Sciences (Enzo Life Sciences, Loerrach, Germany). Integrin antibodies were purchased from Santa Cruz (Santa Cruz Biotechnologie, Heidelberg, Germany). Primary antibodies: anti-HA mAb (MMS-101R, Covance, Berkeley, CA); anti β-actin mAb (ab6276; Abcam, Cambridge, MA, USA. Secondary antibody: Alexa-Fluor-568-labeled goat anti mouse antibody (A11031, Invitrogen).

### DNA constructs

The cDNA of FLAG-tagged, full-length wild type (WT), human MT1-MMP was kindly provided by the laboratory of Motoharu Seiki (Institute of Medical Science, University of Tokyo, Japan). The insertion of the hemagglutinin (HA) tag between residues Gly501 and Gly502 in the stalk (linker-2) region of MT1-MMP has been described previously (Ludwig et al., 2008b). The HA-tagged MT1-MMP construct was cloned into the pcDNA3.1/Hygro vector (Invitrogen, Carlsbad, CA, USA). The DNA construct was first gel purified as a PCR product and then cloned into the pCR2.1 Topo vector (Invitrogen) according to the manufacturer's instructions. The construct was cloned into the pcDNA3.1 vector after digestion with *Hin*dIII and *Not*I, followed by agarose gel purification. The primers used in this strategy were 5′-TAGTAGATGTCTCCCGCCCCAAGACCCCCCCG-3′ and 5′-GATGATT-CAGACCTTGTCCAGCAGGGAACGC-3′. The accuracy of all DNA constructs was confirmed by automated fluorescence sequencing.

### Cells and cell culture

Unless otherwise specified, cell culture media and reagents were purchased from Invitrogen. Human melanoma cells (MV3 clone) (Friedl et al., 1997) were grown in flasks under standard conditions (37°C, 5% CO2, 100% humidity) in phenol-free RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and L-glutamine (Gibco GlutaMAX™-I Supplement, Invitrogen). Cells were stably transfected with MT1-MMP or the empty pcDNA 3.1 vector (MOCK) as a control.

### Stable transfections

Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were trypsinized 48 hours after transfection, diluted in fresh medium and transferred to 10 cm dishes (Greiner Bio-One, Frickenhausen, Germany) coated with 0.2% gelatin. Stable transfectants were selected with Hygromycin B (Invitrogen; 0.2 mg/ml final concentration) for 14 days. Clonal colonies were isolated and further propagated in medium containing 0.1 mg/ml Hygromycin B. Functional expression of MT1-MMP constructs was quantified by western blot analysis.

### Coatings of isotropic and anisotropic collagen-I

Coatings of fibrillar, isotropic collagen-I were generated on APTMS (3-aminopropyltrimethoxysilane)-treated glass coverslips. After cleaning with acetone and extensive rinsing in MilliQ water, 22 mm coverslips were pretreated by immersing them for 10 minutes in a 5% APTMS solution (v/v in MilliQ water) at room temperature followed by extensive washing in MilliQ water and drying them for 1 hour at 160°C. In vitro fibrillogenesis of FITC-labeled collagen-I (C4361, Sigma; 1 mg/ml collagen-I from bovine skin in 10 mM acetic acid) was initiated by diluting

the collagen-solution tenfold in fibrillogenesis buffer (50 mM glycine, 200 mM KCl, pH 9.0). After incubation for 10 minutes at room temperature, 60 μl of the solution were added to the APTMS-treated coverslips. After overnight incubation in a humidified chamber at room temperature, coverslips were extensively rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 10 mM Na2HPO4, pH 7.4) directly before use.

The basic protocols for the generation of a single layer of parallel fibers of unlabelled collagen-I have been described elsewhere (Cisneros et al., 2007; Franz et al., 2007). These fibers are generated on a mica crystal matrix. To enable optical microscopy of parallel collagen fibers, 9.9 mm disks of thin and optically ultrapure mica (Plano GmbH, Wetzlar, Germany) were glued to 24 mm glass coverslips, using an optical adhesive (DYMAX OP-29, DYMAX Europe, Frankfurt, Germany). 30 μl of fibrillogenesis buffer were added onto the freshly cleaved mica surface. Then, 3 μl of the FITC labeled collagen-I solution were injected into the droplet and fibrillogenesis was allowed to proceed overnight at room temperature in a humidified chamber. Before use, the collagen-coated mica discs were extensively rinsed with PBS.

### Parallel collagen assay

Stably transfected MV3 cells were released from the cell culture vessel by EDTA treatment and seeded on fibrillar isotropic or anisotropic FITC-labeled collagen-I on glass coverslips or mica discs, respectively. Phenol-free RPMI 1640 served as culture medium containing either dimethyl sulfoxide (DMSO, solvent control), α2β1-antibodies (5 μg/ml), Cytochalasin D (2 μg/ml) (Schliwa, 1982) or 50 μM blebbistatin for standard experiments (Figs 2–6) and in addition, 1 and 20 μM dilutions of blebbistatin (Fig. 4). Cells were allowed to interact with the collagen coating for 1 hour under standard conditions (37°C, 5% CO2, 100% humidity). If indicated, cells were fixed by rinsing in HANKS buffer followed by addition of 4% paraformaldehyde (PFA) in PBS and incubation for 20 minutes at 4°C. Subsequently, PFA was quenched with 50 mM ammonium chloride in PBS for 20 minutes. Fixed cells were extensively washed and stored in PBS afterwards.

### Immunofluorescence

Cells were fixed with 4% PFA in PBS for 20 minutes at 4°C. PFA was subsequently quenched with 50 mM ammonium chloride in PBS and cells were permeabilized with 0.1% Triton X-100 in PBS. PBS supplemented with 0.1% Triton X-100 and 1% bovine serum albumin (BSA) was used for blocking cells and dilution of antibodies. Sequential incubation steps with anti-HA mAb (1:200) and secondary antibody combined with DAPI (5 mg/ml in PBS diluted 1:10.000; Invitrogen) were performed in a humidified chamber at room temperature for 1 hour each. Between the incubations and afterwards, mica discs were extensively rinsed in blocking buffer. The mica discs were finally rinsed in MilliQ-water, attached with superglue to glass slides and covered with glass coverslips using ProLong Gold antifade reagent (Invitrogen).

### Cell-surface immunofluorescence staining

For cell-surface immunofluorescence staining, cells were seeded on poly-L-lysine-coated coverslips and treated with DMSO (solvent control), α2β1 antibodies, Cytochalasin D or blebbistatin according to the aforementioned procedures (see 'Parallel collagen assay'). Unless otherwise noted, cells were placed on ice and all manipulations were done at 4°C with ice-cold buffers. First, cells were washed twice with Hanks and were then blocked with PBS supplemented with 1.5% BSA for 30 minutes before incubation with monoclonal anti-HA antibody diluted 1:100 in blocking buffer. Then, cells were washed with Hanks after 30 minutes and fixed with 4% PFA in PBS for 20 minutes. After fixation, samples were treated as described above, but omitting the incubation step with the primary antibody.

### Cell-surface biotinylation

MV3 cells stably transfected with MT1-MMP or the empty vector without insert (MOCK) were grown to confluence on collagen coated six-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) and treated with DMSO (solvent control), α2β1 antibodies, Cytochalasin D or blebbistatin. Protocols and procedures for cell-surface protein biotinylation have been described previously (Ludwig et al., 2008b). In brief, manipulations were performed on a rocking platform at 4°C with ice-cold reagents and buffers. Cell-surface biotinylation was carried out at 95% confluency 24 hours after seeding of cells.

### SDS-PAGE and western blot analysis

Cells were grown to confluency in six- well plates (Greiner Bio-One, Frickenhausen, Germany), washed twice with ice-cold PBS before the addition of 700 μl lysis buffer (50 mM Tris-HCl, 150 mM NaCl, supplemented with 1% Triton X-100, pH 7.5) per well. In addition, the lysis buffer contained a 2× final concentration of complete protease inhibitor cocktail tablets with ethylenediaminetetraacetic acid (EDTA, Roche Diagnostics, Penzberg, Germany). Cells were scraped from the wells lysed on ice for 45 minutes and centrifuged (17,000 *g*, 30 minutes, 4°C) afterwards. 10 μl aliquots of supernatant were mixed with reducing, 6× SDS-PAGE sample buffer [350 mM Tris-HCl, 10% (w/v) SDS, 30% (v/v) glycerol, 600 mM dithiothreitol (DTT), 0.01% (w/v) Bromphenol Blue, pH 6.8] and heated (10 minutes, 56°C). Samples were loaded on 10% NuPage Novex Bis-Tris gels (Invitrogen) and separated electrophoretically under constant current in a MOPS-buffered running buffer

(Invitrogen). Proteins were transferred to nitrocellulose membranes using the iBlot dry blotting system (Invitrogen). Western blots were blocked with 5% dry milk in TBS-T and incubated overnight with anti-β-actin antibodies (1:4000) or anti-HA (1:1000).

### Atomic force microscopy

Imaging was performed with a NanoWizard II AFM (JPK-Instruments, Berlin, Germany) mounted on an inverted microscope (Axiovert 200, Zeiss, Jena, Germany) (Fig. 1). Samples were imaged by AFM in a temperature-controlled perfusion chamber (BioCell™, JPK-Instruments, Berlin, Germany) at 37°C in PBS. Scanning of samples was performed in contact mode with silicon nitride tips (D=0.06 N/m, DNP; Veeco, Santa Barbara, CA) at a scan rate of 0.3–0.5 Hz. Optical images were calibrated with the software routines built into the AFM control software prior to image acquisition (DirectOverlayTM, JPK-Instruments, Berlin, Germany). This allowed the direct correlation of optical and AFM images. Image analysis was performed with the software supplied with the instrument (IP, JKP-Instruments, Berlin, Germany) and ImageJ (http://rsbweb.nih.gov/ij/).

### Light microscopy

A Nikon Eclipse Ti inverted microscope (Nikon Instruments, Europe) equipped with an ERS-6FE spinning disc module (Perkin Elmer, Waltham MA) at 100× (1.4 NA; Nikon Instruments) and an ECLIPSE 90i (Nikon) at 40× and 60× were used for confocal and epifluorescence microscopy. Image stacks were processed with the software supplied with the instrument (UltraView; Perkin Elmer) and ImageJ.

### Statistical analysis

For statistical analysis and in addition to the data shown in Fig. 3, low magnification (40–60×), epi-fluorescence overview images were acquired. For each image, the total number of cells was counted and each cell was dichotomically assessed as to whether one of the features, collagen cleavage, durotaxis or fiber bundling, were present. The relative number of cells, which displayed one of the features, was calculated and expressed in percentage of the total number of cells in the individual epi-fluorescence image. On average, 450 cells were analyzed for solvent control, 210 for α2β1-antibodies, 130 for blebbistatin and 90 cells for Cytochalasin D were assessed. Values were expressed as the mean percentage of all analyzed images ± s.e.m. Statistical analysis was performed with a one-way ANOVA test. The Holm–Sidak method was used for pair-wise comparisons ($P≤0.01$).

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