MT1-MMP regulates the turnover and endocytosis of extracellular matrix fibronectin

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Summary

The extracellular matrix (ECM) is dynamically remodeled by cells during development, normal tissue homeostasis and in a variety of disease processes. We previously showed that fibronectin is an important regulator of ECM remodeling. The deposition and/or polymerization of fibronectin into the ECM controls the deposition and stability of other ECM molecules. In addition, agents that inhibit fibronectin polymerization promote the turnover of fibronectin fibrils and enhance ECM fibronectin endocytosis and intracellular degradation. Endocytosis of ECM fibronectin is regulated by β1 integrins, including α5β1 integrin. We have examined the role of extracellular proteases in regulating ECM fibronectin turnover. Our data show that membrane type matrix metalloproteinase 1 (MT1-MMP; also known as MMP14) is a crucial regulator of fibronectin turnover. Cells lacking MT1-MMP show reduced turnover and endocytosis of ECM fibronectin. MT1-MMP regulates ECM fibronectin remodeling by promoting extracellular cleavage of fibronectin and by regulating α5β1-integrin endocytosis. Our data also show that fibronectin polymerization stabilizes fibronectin fibrils and inhibits ECM fibronectin endocytosis by inhibiting α5β1-integrin endocytosis. These data are the first to show that an ECM protein and its modifying enzyme can regulate integrin endocytosis. These data also show that integrin trafficking plays a major role in modulating ECM fibronectin remodeling. The dual dependence of ECM fibronectin turnover on extracellular proteolysis and endocytosis highlights the complex regulatory mechanisms that control ECM remodeling to ensure maintenance of proper tissue function.

Key words: MT1-MMP, MMP14, Endocytosis, Extracellular matrix, Fibronectin, Integrin, Trafficking

Introduction

The extracellular matrix (ECM) undergoes dynamic changes in its organization and composition as part of normal homeostasis and tissue repair (Clark, 1996; Liotta and Kohn, 2001; Streuli, 1999). ECM remodeling involves alterations in the synthesis, deposition and degradation of ECM. Perturbations in ECM remodeling are known to contribute to the development of many pathologies, including fibrosis, atherosclerosis and heart failure (Berk et al., 2007; Graham et al., 2008; Heeneman et al., 2003; Intengan and Schiffrin, 2001; Spinale, 2007). ECM remodeling can have a profound impact on cell behavior, influencing cell migration, survival and proliferation (Frisch and Ruoslahti, 1997; Hocking and Chang, 2003; Mercurius and Morla, 1998; Sechler and Schwarzbauer, 1998; Sottile et al., 1998; Sottile et al., 2007). The ECM is also a major contributor to the mechanical properties of tissues (Gildner et al., 2004; Kjaer, 2004; Paszek et al., 2005; Peyton and Putnam, 2005), and remodeling of the ECM can dramatically alter the mechanical cues that cells sense from the environment. ECM remodeling can also affect cell signaling events that are downstream of integrins and other ECM receptors (Bourdoulous et al., 1998; Klein et al., 2003). Hence, a better understanding of the molecular mechanisms that regulate ECM remodeling could provide insight into strategies to maintain tissue homeostasis, promote tissue repair and/or prevent excessive ECM accumulation during fibrosis.

ECM remodeling is a complex and highly regulated process. Many ECM proteins, including fibronectin and type I collagen, form supramolecular structures (Magnusson and Mosher, 1998; van der Rest and Garrone, 1991). How cells degrade and remove large ECM fibrils is not completely understood. We previously demonstrated that the process of depositing fibronectin into the ECM plays an important role in governing ECM architecture (Sottile and Chandler, 2005; Sottile and Hocking, 2002). Fibronectin polymerization promotes the deposition of a number of ECM proteins, including type I and type III collagen (Sottile and Hocking, 2002; Velling et al., 2002). Furthermore, inhibition of fibronectin polymerization leads to turnover of ECM fibronectin and the concomitant loss of type I and type III collagen from the ECM (Sottile and Hocking, 2002; Sottile et al., 2007). We previously showed that the major fate of fibronectin that is lost from the matrix is endocytosis followed by lysosomal degradation (Sottile and Chandler, 2005). Endocytosis of ECM fibronectin is regulated by β1 integrins and caveolin-1 (Shi and Sottile, 2008; Sottile and Chandler, 2005). Given the large size of ECM fibronectin fibrils, it is unlikely that cells can effectively endocytose uncleaved ECM fibrils. Hence, we hypothesized that extracellular proteolysis was a necessary step for endocytosis of ECM fibronectin.

Fibronectin is a substrate for many proteases, including matrix metalloproteinases (MMPs) (Gronski et al., 1997; Kenny et al., 2008; Marchina and Barlati, 1996; Ohuchi et al., 1997; Watanabe et al., 2000). MMPs are also known to be crucial regulators of ECM remodeling (Filippov et al., 2005; Holmbeck et al., 1999; Shapiro, 1998). Perturbations in the levels or activities of MMPs

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contribute to the development and progression of cancer, fibrosis, atherosclerosis and heart failure (Berk et al., 2007; Chun et al., 2004; Filippov et al., 2005; Hemmann et al., 2007; Holmbeck et al., 1999; Hotary et al., 2003; Li et al., 2000). Extracellular proteases can also regulate the production of ECM fragments, which can have potent biological effects (Sasaki et al., 2000; Schenk and Quaranta, 2003; Sottile, 2004; Xu et al., 2001). Hence, we investigated whether extracellular proteolysis plays a key role in regulating the turnover and endocytosis of ECM fibronectin. Our data show that membrane type matrix metalloproteinase 1 (MT1-MMP; also known as MMP14) promotes the turnover of ECM fibronectin by regulating both the cleavage of large extracellular fibronectin fibrils and the endocytosis of the fibronectin endocytic receptor α5β1 integrin. This suggests that ECM fibronectin is cleaved by MT1-MMP before it is endocytosed by α5β1 integrin. Our data further show that MMP-regulated ECM remodeling plays an important role in regulating fibronectin-dependent cell migration. In addition we provide evidence that an ECM protein can regulate the trafficking of a receptor that regulates its removal from the ECM, and thus provide a new paradigm for understanding the reciprocal regulation of fibronectin and integrins that could be crucial for controlling many fundamental cell functions, such as cell migration and growth.

Results

MMPs are involved in ECM fibronectin turnover, endocytosis and intracellular degradation

ECM fibronectin turnover occurs through endocytosis and intracellular degradation (Shi and Sottile, 2008; Sottile and Chandler, 2005). ECM fibronectin exists in a polymerized multimeric form (Magnusson and Mosher, 1998; Singh et al., 2010). Little is known about how endocytosis of supramolecular fibronectin is initiated. To test whether extracellular proteolysis is involved in this process, various protease inhibitors were screened to test their effects on ECM fibronectin turnover. A long-term fibronectin matrix turnover pulse–chase assay was performed, in which fibronectin-null myofibroblasts (FN-null MFs) were cultured with 10 μg/ml fluorescein isothiocyanate (FITC)- or Texas Red (TR)-conjugated fibronectin overnight. A robust fibronectin matrix was elaborated by the cells at the end of the pulse (Fig. 1A; supplementary material Fig. S1A). Cells were washed and then chased in the presence (+FN) or absence (−FN) of unconjugated fibronectin for approximately 24 hours. As we previously showed (Sottile and Hocking, 2002), continuous fibronectin polymerization (+FN) inhibited ECM fibronectin turnover (supplementary material Fig. S1C), whereas disruption of ongoing fibronectin polymerization (−FN) promoted fibronectin fibril turnover (Fig. 1C; supplementary material Fig. S1B). Various protease inhibitors were added to the cells during the chase. GM6001 (Fig. 1B), a broad spectrum MMP inhibitor, prevented the loss of fibronectin fibrils in comparison with control cells (Fig. 1C). However, compounds that inhibit serine, cysteine or aspartate proteases were not able to prevent the loss of fibronectin fibrils (supplementary material Fig. S1). To investigate the effect of GM6001 on endocytosis of ECM fibronectin, we tested the ability of cells to endocytose fibronectin from cell-derived pre-assembled matrix. FN-null MFs were seeded onto a pre-assembled matrix containing TR-conjugated fibronectin and cultured for 36 hours. Endocytosed TR–fibronectin was readily detected (Fig. 1H). When GM6001 was added to the culture medium, the level of internalized fibronectin was drastically reduced (Fig. 1G). Similar results were obtained with smooth muscle cells (SMCs; data not shown). We previously showed that endocytosed ECM fibronectin is degraded in lysosomes (Sottile and Chandler, 2005). Hence ^{125}I-fibronectin was used to determine whether GM6001 inhibits the intracellular degradation of ECM fibronectin. GM6001 inhibited intracellular degradation of ECM fibronectin by 47% in FN-null MFs (Fig. 1I) and 42% in SMCs (Fig. 1J) compared with the control compound. Consistent with our previously published data (Sottile and Chandler, 2005), continuous fibronectin polymerization (Fig. 1I, +FN) inhibited ECM fibronectin degradation by 61% compared with that in cells where fibronectin polymerization was disrupted (−FN). Taken together, these data strongly suggest that MMPs play a role in ECM fibronectin turnover, endocytosis and intracellular degradation.

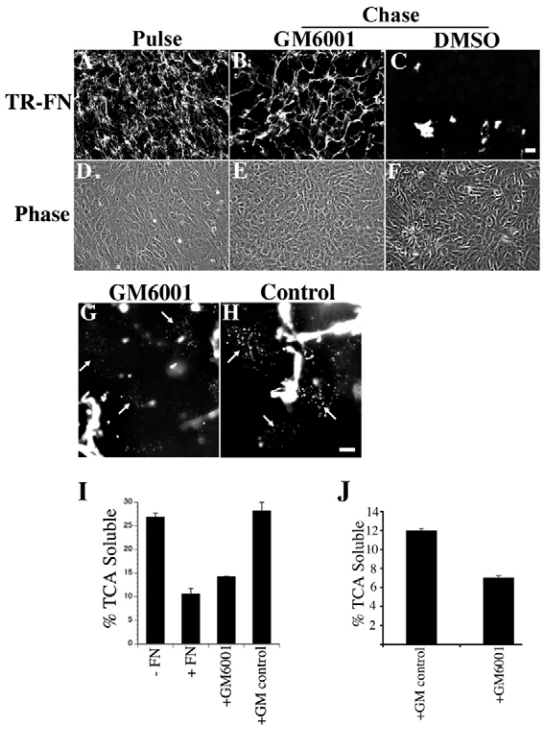

Fig. 1. GM6001, an MMP inhibitor, prevents ECM fibronectin turnover, endocytosis and degradation. (**A–F**) GM6001 prevents ECM fibronectin turnover. FN-null MFs were incubated with 10 μg/ml TR–fibronectin overnight (A, Pulse). Cells were washed and then incubated for 27 hours in medium lacking fibronectin, but containing 20 μM GM6001 (B) or vehicle control (C). Scale bar: 50 μm. Corresponding phase-contrast images are shown in D–F. (**G**,**H**) GM6001 inhibits ECM fibronectin endocytosis. FN-null MFs were seeded onto pre-assembled matrix containing TR–fibronectin and cultured for 1 hour. The medium was then supplemented with 20 μM GM6001 (G) or control compound (H), and the cells cultured for an additional 36 hours. Arrows point to intracellular TR–fibronectin. Scale bar: 10 μm. (**I**,**J**) GM6001 inhibits intracellular degradation of ECM fibronectin. FN-null MFs (I) or rat SMCs (J) were incubated overnight with ^{125}I-fibronectin. Cells were washed and then incubated with culture medium lacking or containing 10 μg/ml unlabeled fibronectin (I), 20 μM GM6001 or control compound (I,J) for 24 hours. The counts in the TCA-soluble fraction of the medium were used to determine intracellular degradation of ECM fibronectin, as described in the Materials and Methods (means ± s.d., n=3).

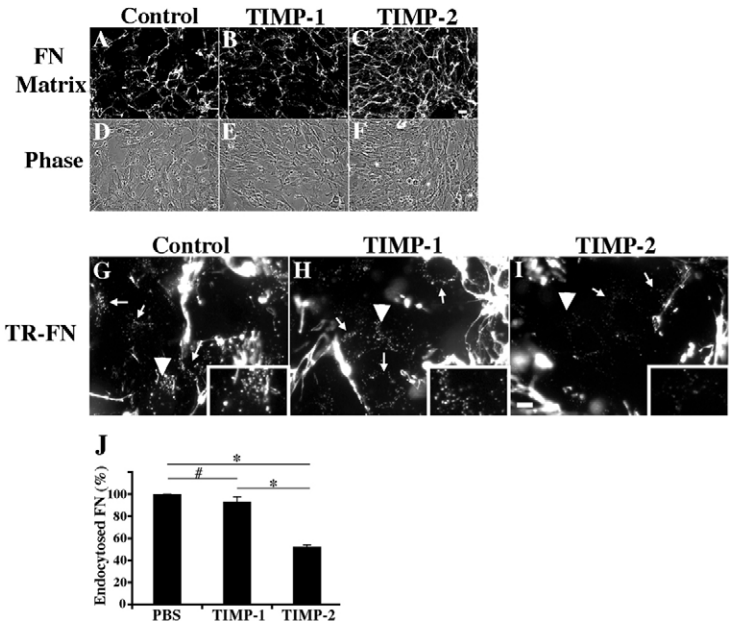

Fig. 2. Membrane-type MMPs are involved in endocytosis and turnover of ECM fibronectin. FN-null MFs were seeded onto pre-assembled matrix containing TR–fibronectin (TR-FN; **A–I**) or Alexa-Fluor-488–FN (**J**) and cultured for 1–2 hours. The medium was then supplemented with 50 nM TIMP-1 (B,E,H), 50 nM TIMP-2 (C,F,I), or PBS (A,D,G), and the cells cultured for 24 hours. Cells were then fixed for imaging assay (A–I) or processed for flow cytometry analysis (J). Images in A–C were taken at low magnification and at a focal plane that would best depict fibronectin fibrils. High magnification views are shown in G–I to allow intracellular vesicles to be distinguished from bright fibrils. Arrows point to intracellular TR–fibronectin, and arrowheads point to areas that are enlarged in the insets. Scale bars: 50 μm (A–F) and 10 μm (G–I). (J) Flow cytometry was used to quantify endocytosed fibronectin as described in the Materials and Methods. The data are expressed as the percentage change of mean fluorescence intensity (MFI) of endocytosed Alexa-Fluor-488–fibronectin (means ± s.e.m., $n=3$; $^{*}P<0.001$, $^{\#}P>0.05$). MFI in PBS-treated cells was set as 100%.

MT1-MMP is involved in ECM fibronectin turnover and endocytosis

MMPs belong to a multigene family with over 20 different members, belonging to two major groups: secreted and membrane-type MMPs (Nagase et al., 2006; Visse and Nagase, 2003). To determine the specific MMPs that are involved in ECM fibronectin turnover, we used more specific inhibitors, tissue inhibitor of metalloproteinase-1 and -2 (TIMP-1 and TIMP-2). TIMP-1 preferentially inhibits secreted and glycosyl phosphatidylinositol (GPI-anchored) MMPs. By contrast, TIMP-2 potently inhibits secreted MMPs, as well as membrane-type MMPs (MT1-, 2-, 3- and 5-MMP) (Nagase et al., 2006; Visse and Nagase, 2003). FN-null MFs were seeded onto pre-assembled matrix containing fluorescently labeled fibronectin, and cultured in the presence TIMP-1 or TIMP-2. After 24 hours, there was a substantial loss of fibronectin fibrils in control (Fig. 2A) and TIMP-1-treated cells (Fig. 2B) compared with TIMP-2-treated cells (Fig. 2C). Fibronectin endocytosis was detected in control and TIMP-1-treated cells (Fig. 2G,H), whereas there was a dramatic reduction in the level of internalized fibronectin in cells treated with TIMP-2 (Fig. 2I). Flow cytometry analysis showed that fibronectin endocytosis was reduced by 48% in TIMP-2-treated cells, but was unaffected by TIMP-1 treatment (Fig. 2J). These data suggest that MT-MMPs are likely to be involved in ECM fibronectin turnover and endocytosis.

To test directly whether MT1-MMP plays a role in ECM fibronectin turnover, MT1-MMP-null or wild-type (WT) cells were seeded onto pre-assembled matrix containing fluorescently labeled fibronectin. Fewer fibronectin fibrils were retained in wells containing WT cells (Fig. 3F) than in wells containing MT1-MMP-null cells (Fig. 3H). There was a corresponding increase in levels of endocytosed fibronectin in WT cells (Fig. 3A) than in MT1-MMP-null cells (Fig. 3C). Flow cytometry analysis showed that there was a 75% reduction in fibronectin endocytosis in MT1-MMP-null cells (Fig. 3E). Conversely, MT1-MMP-null and WT cells endocytosed similar amounts of soluble, non-matrix associated fibronectin (Fig. 3J–L). MT1-MMP similarly promoted the turnover and endocytosis of endogenously produced ECM FN (supplementary material Fig. S2).

To confirm the role of MT1-MMP in ECM fibronectin turnover, we re-expressed MT1-MMP in MT1-MMP-null cells by adenoviral transduction. MT1-MMP expression levels were slightly lower than in WT cells (supplementary material Fig. S3). As shown in Fig. 4C, expression of MT1-MMP–EGFP resulted in an increase in the level of endocytosed fibronectin. By contrast, very little intracellular fibronectin was detected in MT1-MMP-null cells expressing EGFP alone, but extensive fibronectin fibrils were retained (Fig. 4A). Flow cytometry analysis showed that expression of MT1–EGFP, but not EGFP, in MT1-MMP-null cells increased fibronectin endocytosis to a level similar to that in WT cells (Fig. 4E). These data indicate that MT1-MMP pays a crucial role in ECM fibronectin turnover and endocytosis.

MT1-MMP is involved in turnover and endocytosis of matrix collagen I, but not matrix fibrinogen

Our previous data showed that loss of ECM fibronectin results in the loss of several other proteins from the ECM, including collagen I (Shi et al., 2010; Sottile and Hocking, 2002). Therefore, we investigated whether MT1-MMP is involved in turnover of other ECM proteins. MT1-MMP-null or WT cells were seeded onto cell-derived pre-assembled matrix containing TR–collagen I. As shown in Fig. 5A, intracellular TR–collagen I was readily detected in WT cells. However, MT1-MMP-null cells endocytosed very little collagen I. By contrast, more collagen I fibrils were retained in MT1-MMP-null cell culture wells (Fig. 5C). To determine whether the requirement for MT1-MMP is specific for matrix collagen I, we used a short-term collagen I endocytosis pulse–chase assay to monitor the endocytosis of soluble collagen I. MT1-MMP-null (Fig. 5G) and WT cells (Fig. 5E) endocytosed similar amounts of soluble TR–collagen I. We also tested whether turnover of other ECM proteins is regulated by MT1-MMP. As shown in Fig. 5I–L, similar levels of internalized fibrinogen were detected in MT1-MMP-null and WT cells when these cells were cultured on top of pre-assembled matrix containing fluorescently labeled

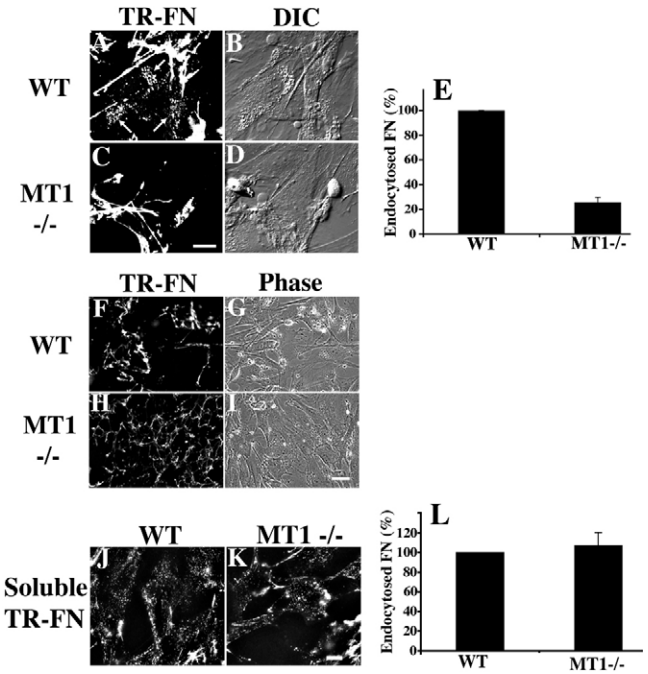

Fig. 3. MT1-MMP is involved in endocytosis of ECM, but not soluble fibronectin. (**A–I**) MT1-MMP is involved in endocytosis and turnover of ECM fibronectin. MT1-MMP-null or WT cells were seeded onto pre-assembled matrix containing TR–fibronectin (A–D and F–I) or Alexa-Fluor-488–fibronectin (E) and cultured for 25–41 hours. Cells were then fixed for imaging (A–D and F–I) or processed for flow cytometry analysis (E). A and C are confocal images that were taken in a plane of focus to best show intracellular vesicles containing TR–fibronectin (arrows). Low magnification images in F and H were taken in a plane of focus to best show fibronectin fibrils. Scale bars: 20 μm (A–D) and 100 μm (F–I). (E) Flow cytometry was used to quantify endocytosed fibronectin. The data are expressed as the percentage change of MFI of endocytosed Alexa-Fluor-488–fibronectin (means ± s.e.m., $n=3$, $P<0.0001$). MFI in WT cells was set as 100%. (**J–L**) MT1-MMP is not required for soluble fibronectin endocytosis. MT1-MMP-null or WT cells were incubated with 10 μg/ml TR–fibronectin at 4℃ for 45 minutes (pulse). Unbound fibronectin was removed and cells were then chased at 37℃ for 1 hour. Endocytosed TR–fibronectin is shown in J and K (scale bar: 20 μm). Flow cytometry analysis is shown in L. The data are expressed as the percentage change of MFI of endocytosed soluble Alexa-Fluor-488–fibronectin (means ± s.e.m., $n=3$; $P>0.05$). MFI in WT cells was set as 100%.

fibrinogen. Taken together, these data indicate that MT1-MMP is involved in endocytosis and turnover of fibronectin and collagen I, but not fibrinogen, from the ECM.

MT1-MMP enzymatic digestion rescues ECM fibronectin turnover and endocytosis in MT1-MMP-null cells

In addition to its proteolytic activity, recent studies indicate that MT1-MMP has non-enzymatic functions (Alfranca et al., 2008; Genis et al., 2007; Gonzalo et al., 2010a; Gonzalo et al., 2010b). To determine whether the ability of MT1-MMP to regulate ECM fibronectin endocytosis requires its proteolytic activity, pre-assembled matrix containing TR–fibronectin was prepared and then treated with recombinant MT1-MMP catalytic domain. Interestingly, the global structure of ECM fibronectin fibrils was similar in enzyme-treated (Fig. 6C) and untreated matrices (Fig. 6A,B). However, western blot analysis showed that there was a loss of full-length fibronectin in MT1-MMP-enzyme-treated matrices. The loss of full-length fibronectin was

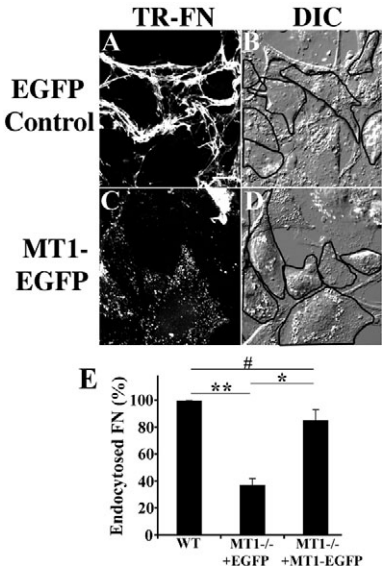

Fig. 4. Re-expression of MT1-MMP rescues endocytosis and turnover of ECM fibronectin. MT1-MMP-null cells were seeded onto pre-assembled matrix containing TR–fibronectin (**A–D**) or Alexa-Fluor-633–fibronectin (**E**). Cells were cultured for 2 hours before being transduced with MT1-MMP–EGFP (C,D) or EGFP (A,B)-expressing adenoviruses. Cells were cultured for a total of 48 hours before fixation for confocal imaging. Scale bar: 20 μm. Cells expressing EGFP or MT1–EGFP were detected by fluorescence imaging and are outlined in B and D. Some cells were processed for flow cytometry analysis (E). The graph in E shows the percentage change of the MFI of endocytosed Alexa-Fluor-633–fibronectin. MFI in WT cells was set as 100% (means ± s.e.m., $n=4$; $^{*}P<0.001$, $^{**}P<0.00001$ and $^{\#}P>0.05$).

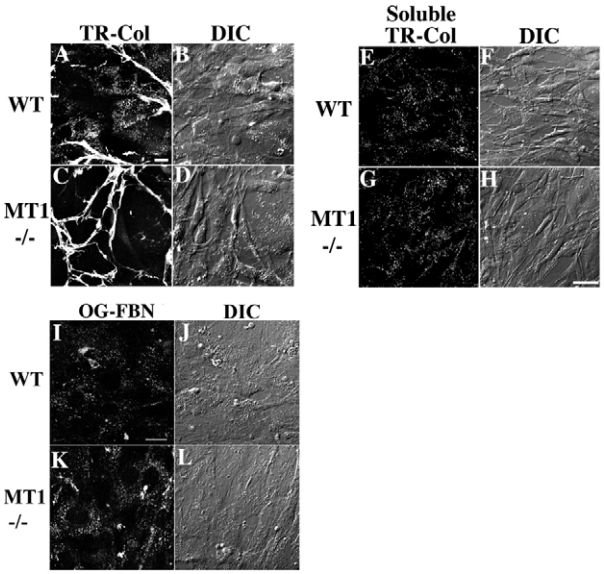

Fig. 5. MT1-MMP is involved in endocytosis of ECM collagen I, but not ECM fibrinogen. (**A–D**,**I–L**) Pre-assembled matrix containing TR–collagen I (A–D) or Oregon-Green–fibrinogen (OG-FBN, I–L) was prepared as described in Materials and Methods. MT1-MMP-null or WT cells were cultured on pre-assembled matrices for 40–48 hours. Cells were then fixed for confocal imaging. Scale bar: 20 μm. (**E–H**) MT1-MMP is not required for soluble collagen I endocytosis. MT1-MMP-null or WT cells were incubated with 10 μg/ml TR–collagen I at 4℃ for 50 minutes (pulse). Cells were washed and then chased at 37℃ for 1 hour. Intracellular TR–collagen I was visualized by confocal imaging. Scale bar: 10 μm.

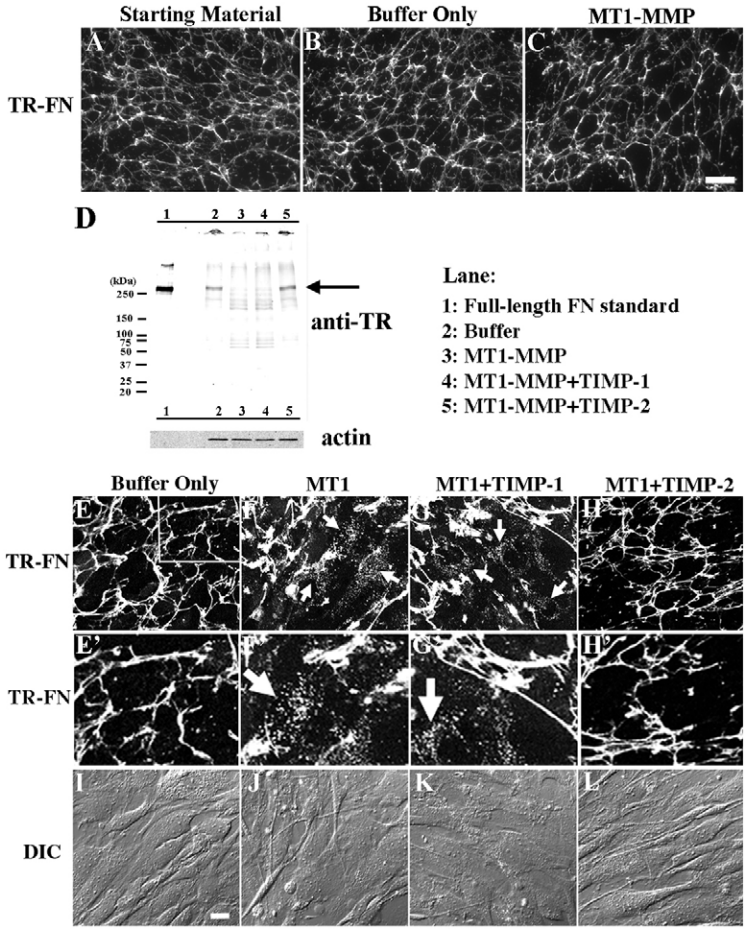

Fig. 6. MT1-MMP enzymatic digestion rescues ECM fibronectin endocytosis and turnover. (**A–C**) MT1-MMP digestion does not disrupt global ECM structure. Pre-assembled matrix containing TR–fibronectin was prepared and then treated with MT1-MMP catalytic domain in the presence or absence of 50 nM TIMP-1 or TIMP-2. Matrices treated with buffer only were used as a negative control. Matrices were fixed before (A) or after (B,C) digestion. Scale bar: 40 μm. (**D**) Cleavage of TR-conjugated ECM fibronectin by MT1-MMP. Some matrices were collected into SDS sample buffer after digestion, and then subjected to western blot analysis using anti-Texas Red antibodies. The arrow points to full-length fibronectin. Actin was used as loading control. (**E–L**) MT1-MMP enzymatic digestion increases endocytosis and turnover of ECM fibronectin. MT1-MMP-null cells were cultured on MT1-MMP-enzyme-treated or control matrices for 48 hours. Cells were then fixed for confocal imaging. (E–H) TR–fibronectin (arrows point to intracellular TR–fibronectin); (E′–H′) enlarged views of the upper right sections of E–H (shown as boxed area in E). Scale bar: 20 μm. (I–L) Corresponding DIC images of E–H.

accompanied by the appearance of many fibronectin fragments (Fig. 6D, lane 3), indicating that fibronectin had been proteolytically digested. By contrast, pre-assembled matrix fibronectin treated with only digestion buffer was largely intact (Fig. 6D, lane 2). The cleavage of fibronectin by MT1-MMP enzyme was inhibited by adding TIMP-2 (Fig. 6D, lane 5), but not TIMP-1 (Fig. 6D, lane 4), to the digestion mixture. These data indicate that MT1-MMP can degrade fibronectin from cell-derived pre-assembled matrix.

To determine whether ECM fibronectin endocytosis in MT1-MMP-null cells could be rescued by pre-treating cell-derived matrix with the MT1-MMP catalytic domain, we subjected pre-assembled matrices containing TR–fibronectin to overnight digestion by the enzyme, and then seeded MT1-MMP-null cells on top of digested or control matrices. Endocytosis and turnover of ECM fibronectin were both dramatically increased in cells seeded on matrices that were digested with exogenous MT1-MMP (Fig. 6F). The presence of TIMP-2 (Fig. 6H), but not TIMP-1 (Fig. 6G), during the digestion process prevented the rescue of ECM fibronectin endocytosis and turnover. These data strongly suggest that proteolytic cleavage of ECM fibronectin by MT1-MMP facilitates its endocytosis from the ECM.

MT1-MMP-null cells are defective in endocytosing α5β1 integrin

We previously showed that ECM fibronectin endocytosis is mediated by β1 integrins, mainly α5β1 integrin, and that endocytosis of ECM fibronectin is substantially reduced in β1-integrin-null cells (Shi and Sottile, 2008). Thus, we examined whether reduced ECM fibronectin endocytosis in MT1-MMP-null cells is correlated with altered cell surface levels of β1 integrin or α5 integrin or altered α5β1 integrin endocytosis. Flow cytometry analysis showed that the cell surface levels of β1 integrin and α5 integrin are similar in MT1-MMP-null and WT cells (supplementary material Fig. S4). We also tested whether MT1-MMP expression affected α5β1 integrin endocytosis. As shown in Fig. 7A,B, the levels of internalized α5β1 integrin were 69% lower in MT1-MMP-null cells (Fig. 7A, lane 6 and 8; quantification in Fig. 7B) compared with WT cells (Fig. 7A, lane 5 and 7). Re-expression of MT1-MMP by adenoviral transduction rescued α5β1 integrin endocytosis in MT1-MMP-null cells (Fig. 7C, lane 9) to a level comparable with that in WT cells (Fig. 7C, lane 7; quantification in Fig. 7D). These data indicate that MT1-MMP is involved in regulating α5β1 integrin endocytosis.

Fibronectin polymerization regulates endocytosis of α5β1 integrin

We previously showed that continuous fibronectin polymerization inhibits ECM fibronectin endocytosis and stabilizes fibronectin fibrils (Sottile and Chandler, 2005; Sottile and Hocking, 2002). Similarly, cells lacking MT1-MMP also endocytose less fibronectin and retain more fibronectin fibrils (Fig. 3A–I). Thus, we investigated whether ongoing fibronectin

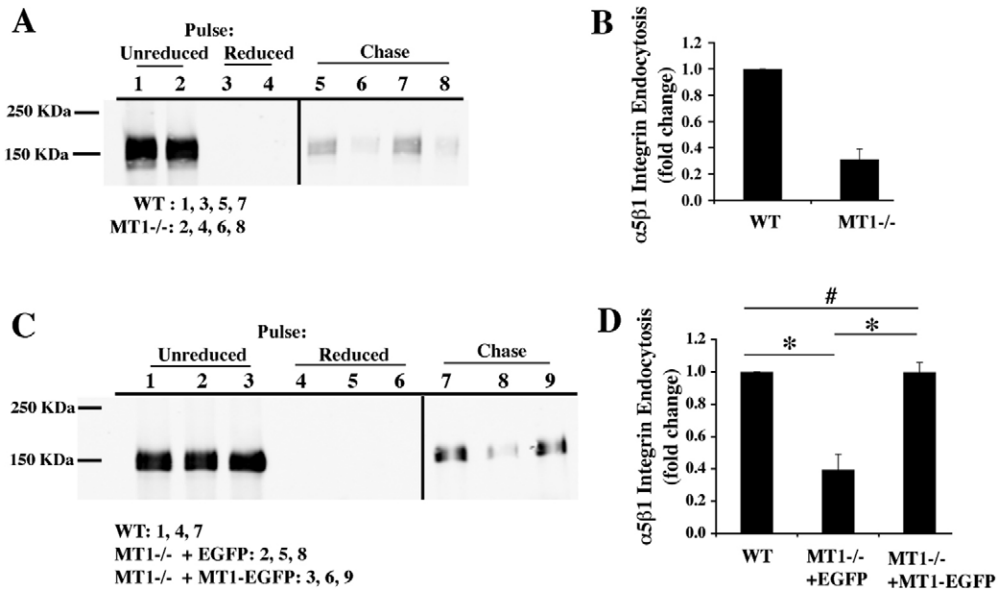

Fig. 7. MT1-MMP regulates α5β1 integrin endocytosis. (**A**,**B**) Impaired α5β1 integrin endocytosis in MT1-MMP-null cells. Integrin endocytosis was measured using a biotinylation assay as described in the Materials and Methods. Western blot analysis using anti-α5 integrin antibodies was used to detect endocytosed α5 integrin in MT1-MMP WT (lanes 1,3,5,7) or-null (lanes 2,4,6,8) cells. Total levels of biotinlyated cell surface α5 integrin (lane 1 and 2); internalized α5 integrin after 0 minutes (lane 3 and 4) and 30 minutes (lane 5–8) of chase. In panel A, duplicate samples are shown in lanes 5 and 7; 6 and 8. (B) The relative fold change of α5 integrin endocytosis in MT1-MMP-null cells compared with that in WT cells, which was set as 1 (means ± s.e.m., $n=3$; $P<0.0005$). (**C**,**D**) Re-expression of MT1-MMP rescues α5β1 integrin endocytosis. MT1-MMP-null cells were transduced with adenoviral MT1-MMP–EGFP (lanes 3,6,9) or EGFP control (lanes 2,5,8), cultured for 48 hours, and then subjected to biotinylation assay to measure α5 integrin endocytosis. The blot was probed with anti-α5 integrin antibody. Total levels of biotinlyated cell surface α5 integrin (lane 1–3); internalized α5 integrin after 0 minutes (lane 4–6) and 30 minutes (lane 7–9) of chase. (D) The relative fold change of α5 integrin endocytosis in MT1-MMP-null cells compared with that in WT cells, which was set as 1 (means ± s.e.m., $n=3$, $^{*}P<0.001$, $^{\#}P>0.05$).

polymerization regulates α5β1 integrin endocytosis. FN-null MFs were cultured with 10 μg/ml fibronectin overnight in the presence or absence of pUR4, a fibronectin polymerization inhibitor. Previous studies have shown that cells elaborate a robust fibronectin matrix in the presence of the control peptide, but that pUR4 drastically reduces the deposition of ECM fibronectin (Chiang et al., 2009; Tomasini-Johansson et al., 2001). As shown in Fig. 8A,B, pUR4 treatment resulted in a 2.2-fold increase in the level of endocytosed α5β1 integrin compared with that in control-peptide-treated cells.

ECM fibronectin retention contributes to reduced α5β1 integrin endocytosis in MT1-MMP-null cells

MT1-MMP-null cells are defective in turning over ECM fibronectin (Fig. 3H) and endocytosing α5β1 integrin (Fig. 7). We speculated that the retention of ECM fibronectin fibrils in these cells contributes to the reduction in integrin endocytosis. Thus, we tested whether culturing MT1-MMP-null cells in the presence of pUR4 could rescue α5β1 integrin endocytosis. Cells were cultured in the absence or presence of pUR4 for 24 hours to allow or inhibit the establishment of a robust matrix. As shown in Fig. 8, α5β1 integrin endocytosis was increased 2.9-fold in pUR4-treated MT1-MMP-null cells (Fig. 8D, lane 5 and 7) compared with that in control cells (Fig. 8D, lane 6 and 8; quantification showed in Fig. 8E). Interestingly, α5β1 integrin endocytosis was also increased 1.9-fold in pUR4-treated WT cells (Fig. 8D, lane 1 and 3) compared with that in control peptide-treated WT cells (Fig. 8D, lane 2 and 4). This is consistent with the observation made in FN-null MFs (Fig. 8A,B). Taken together, these data suggest that ongoing fibronectin polymerization, and the presence of robust ECM fibronectin fibrils partially accounts for reduced α5β1 integrin endocytosis in MT1-MMP-null cells.

Disruption of ongoing fibronectin polymerization induces ECM fibronectin turnover and accelerates cell migration

ECM remodeling plays an important role in regulating cell migration (Galis et al., 2002; Giannelli et al., 1997; Hocking and Chang, 2003; Sottile et al., 2007). Fibronectin has been shown to regulate cell migration in a biphasic fashion (Hocking and Chang, 2003). In FN-null MFs, migration peaks when cells are incubated with 5–10 μg/ml soluble fibronectin. In epithelial cells, fibronectin polymerization inhibitors can promote cell migration under conditions where excess soluble fibronectin is present (Hocking and Chang, 2003). To determine whether inhibition of fibronectin polymerization increases cell migration in FN-null MFs that contain a robust fibronectin matrix, FN-null MFs were cultured in the presence of soluble fibronectin overnight to allow the establishment of extensive fibronectin fibrils. The cell monolayer was then wounded and the culture medium was supplemented with soluble fibronectin (Fig. 9A,C) or buffer as a control (Fig. 9B,D). We previously showed that removal of soluble fibronectin from the culture medium disrupts fibronectin polymerization and induces the turnover of pre-existing ECM fibronectin (Sottile and Chandler, 2005; Sottile and Hocking, 2002). As shown in Fig. 9, migration of FN-null MFs was

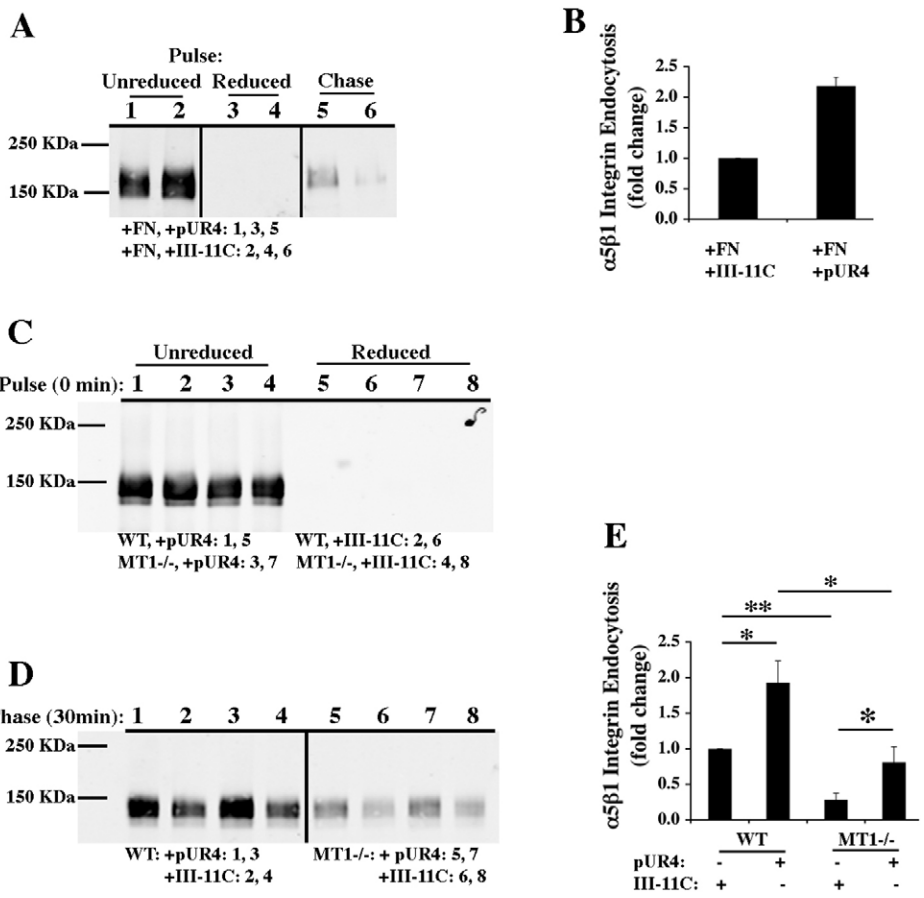

Fig. 8. Fibronectin polymerization regulates α5β1 integrin endocytosis. (**A**,**B**) Reduced α5β1 integrin endocytosis in the presence of ECM fibronectin. FN-null MFs were incubated with 10 μg/ml fibronectin in the presence of 250 nM pUR4 (FN polymerization inhibitor; lanes 1,3,5) or control peptide III-11C (lanes 2,4,6) overnight. Endocytosis of α5β1 integrin was analyzed as described in the Materials and Methods. Total levels of biotinlyated cell surface α5 integrin (lane 1 and 2); internalized α5 integrin after 0 minutes (lane 3 and 4) and 30 minutes (lane 5 and 6) of chase. (B) The relative fold change of α5 integrin endocytosis in pUR4-treated cells (+FN, +pUR4) compared with that in control cells (+FN, +III-11C), which were set as 1 (means ± s.e.m., $n=3$, $P<0.00005$). (**C**–**E**) Inhibition of fibronectin polymerization partially rescues α5β1 integrin endocytosis in MT1-MMP-null cells. MT1-MMP-null and WT cells were incubated with 250 nM pUR4 or control peptide (III-11C), and cultured for 24 hours. Endocytosis of α5β1 integrin was analyzed. (C) Western blot analysis showing total levels of biotinlyated cell surface α5 integrin (lane 1–4), and internalized α5 integrin after 0 minutes of chase (lane 5–8). (D) Levels of internalized α5 integrin after 30 minutes of chase. Duplicate samples are shown. (E) The relative fold change of α5 integrin endocytosis in cells under different treatments compared with that in WT cells treated with control peptide, which was set as 1 (means ± s.e.m., $n=3$; *$P<0.05$, **$P<0.001$).

accelerated 1.75-fold and 1.5-fold at 4 hours and 8 hours post wounding under condition where soluble fibronectin was removed (Fig. 9D, −FN; quantification in Fig. 9E,F).

To verify that inhibition of fibronectin polymerization promotes the migration of cells with a pre-existing fibronectin matrix, we performed experiments in the presence of soluble fibronectin, and in the presence or absence of pUR4, post wounding. As shown in Fig. 9E,F, addition of pUR4, but not the control peptide, promoted cell migration. These results suggest that prevention of ongoing fibronectin polymerization accelerates cell migration by inducing the turnover of pre-existing ECM fibronectin.

To determine whether the acceleration in cell migration induced by inhibiting fibronectin polymerization requires MMP activity, we performed wound healing experiments as described above in the presence of MMP inhibitors. FN-null MFs with a pre-existing fibronectin matrix were wounded, then cultured in the absence of soluble fibronectin, and in the presence or absence of TIMP-2 or TIMP-1. As shown in Fig. 10A,B, TIMP-2, but not TIMP-1, significantly reduced the accelerated cell migration induced by removing the soluble fibronectin from the culture medium. By contrast, when exogenous MT1-MMP catalytic domain was supplied to the medium, cell migration was increased by an additional ~20% (Fig. 10C). Taken together, these data suggest that MT1-MMP proteolytic activity contributes to increased cell migration that is induced by disrupting ongoing fibronectin polymerization.

Discussion

At least two mechanisms exist for the degradation and removal of proteins from the ECM: extracellular proteolysis (Filippov et al., 2005; Marchina and Barlati, 1996; Ohuchi et al., 1997) and endocytosis followed by intracellular degradation (East et al., 2003; Odrljin et al., 2001; Panetti et al., 1995; Sottile and Chandler, 2005). Our published data show that endocytosis is a major mechanism that regulates turnover of ECM fibronectin (Shi and Sottile, 2008; Sottile and Chandler, 2005). The data in this manuscript show that MT1-MMP promotes the turnover of ECM fibronectin fibrils and accelerates the endocytosis of ECM fibronectin. Taken together, these data indicate that extracellular proteolysis and endocytosis coordinately regulate the turnover of ECM fibronectin. In this manuscript we also show that both MT1-MMP and fibronectin polymerization regulate the endocytosis of α5β1 integrin. This suggests that one mechanism by which ECM proteins and ECM-modifying enzymes regulate ECM remodeling is by controlling integrin-mediated endocytosis of ECM.

Integrin trafficking has been well studied. β1 integrins are known to be constitutively endocytosed (Bretscher, 1989; Caswell et al., 2009; LaFlamme et al., 2008). However, integrin endocytosis can also be regulated by various intracellular signaling and adaptor proteins, including certain growth factors, PKCs and Rab-GTPases (Caswell et al., 2008; Caswell et al., 2007; Ivaska et al., 2002; Ng et al., 2001; Pellinen et al., 2006; Roberts et al., 2001). Integrins can regulate the

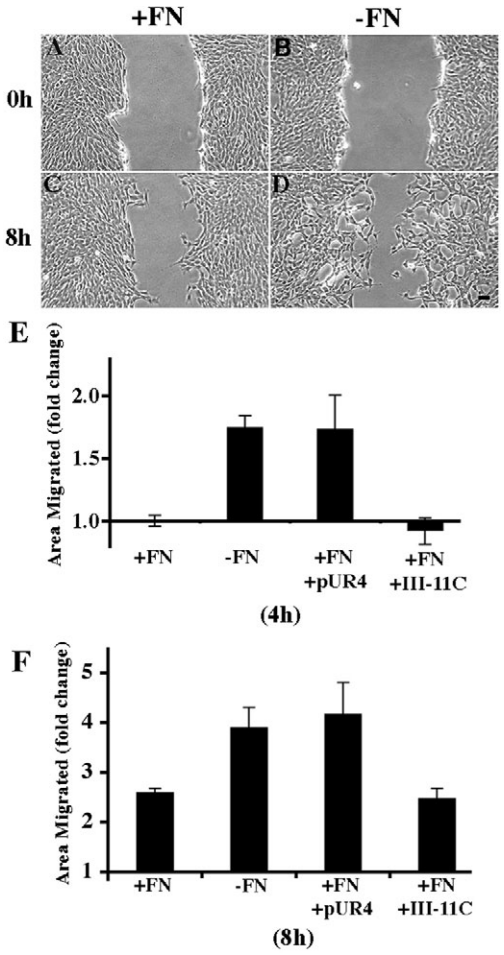

Fig. 9. Turnover of pre-existing fibronectin matrix accelerates cell migration. (**A–D**) FN-null MFs were cultured until 90% confluence and then supplied with 10 μg/ml fibronectin overnight. Cell monolayers were wounded, washed and then incubated with culture medium containing (+FN) or lacking (−FN) 10 μg/ml fibronectin. Representative phase images of wound areas are shown (scale bar: 20 μm). (**E**,**F**) Inhibition of fibronectin polymerization accelerates cell migration. Wound healing assay was performed as described above. Following wounding, cells were cultured in the presence or absence of 10 μg/ml fibronectin, and in the presence of 250 nM pUR4 or control peptide III-11C. The area migrated was analyzed as described in the Materials and Methods. Graphs show the relative fold change in the area migrated 4 hours (E) and 8 hours (F) post wounding by cells under various treatments to that in the presence of fibronectin, which is set to 1 (means ± s.e.m., $n=3$).

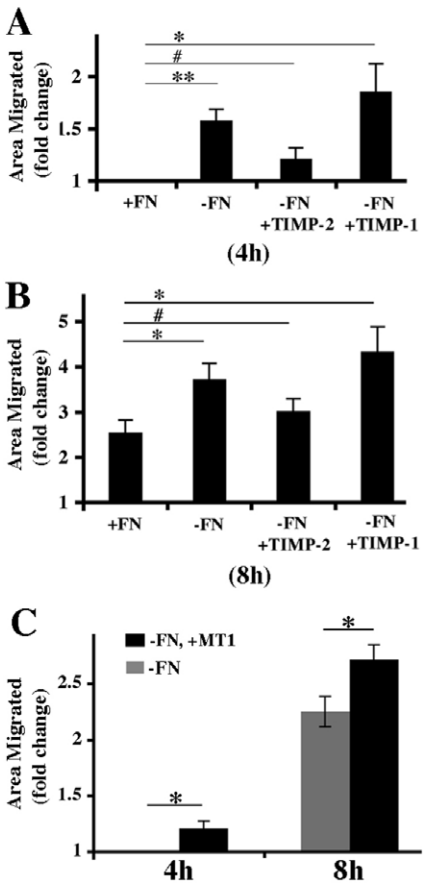

Fig. 10. TIMP-2 blunts, whereas MT1-MMP catalytic domain further accelerates, ECM fibronectin turnover-induced cell migration. FN-null MFs were cultured until 90% confluence and then incubated with 10 μg/ml fibronectin overnight to establish a robust fibronectin matrix. Would healing assay was performed as described in the legend to Fig. 9. (**A**,**B**) Following wounding, some cells were cultured in the presence (+FN) or absence (−FN) of 10 μg/ml fibronectin, and in the presence of 50 nM TIMP-1 or TIMP-2. The graphs show the relative fold change of the area migrated by cells under various treatments to that in the presence of fibronectin, which was set as 1 (means ± s.e.m., $n=3$; $^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P>0.05$). (A) 4 hours post wounding; (B) 8 hours post wounding. (**C**) After wounding, some cells were incubated with MT1-MMP catalytic domain (−FN, +MT1) or buffer control (−FN). The graph shows the relative fold change of area migrated by cells cultured in the presence of MT1-MMP to that in the absence of MT1-MMP, which was set as 1 (means ± s.e.m., $n=3$; $^{*}P<0.05$).

endocytosis of several ECM proteins, including fibronectin, collagen I and vitronectin (Odrljin et al., 2001; Panetti et al., 1995; Shi and Sottile, 2008). However, heretofore there has been little evidence that ECM proteins can regulate integrin endocytosis. In one study, plasminogen activator inhibitor was shown to regulate the endocytosis of uPA–uPAR–αv-integrin complexes by binding to the complex and regulating integrin activation (Czekay et al., 2003). Our data show that ECM fibronectin remodeling and α5β1 integrin endocytosis are closely linked. It will be interesting to determine whether fibronectin-induced changes in integrin activation play an important role in regulating integrin endocytosis.

Two previous reports have shown that fibronectin can influence integrin recycling or degradation. Soluble fibronectin was shown to stimulate Rab25-mediated recycling of α5 integrin (Caswell et al., 2007). In addition, soluble fibronectin was shown to induce the ubiquitylation and lysosomal degradation of a subset of α5β1 integrins (Lobert et al., 2010). Integrin degradation occurs relatively slowly (~50% degradation over 24 hours) in comparison to integrin recycling (~50% within 1 hour) (Lobert et al., 2010). Our data show that fibronectin polymerization is a potent inhibitor of integrin endocytosis (Fig. 8A,B). However, we have not investigated the fate of the small pool of integrins that are endocytosed under conditions where there is abundant ECM fibronectin. It is possible that fibronectin polymerization both decreases α5β1 integrin

endocytosis and promotes the degradation of the pool of integrins that are endocytosed.

Our data show that extracellular cleavage of fibronectin by MT1-MMP is necessary for efficient endocytosis of ECM fibronectin. Cells lacking MT1-MMP show a 75% reduction in FN endocytosis (Fig. 3E). This suggests that MT1-MMP is the major protease in myofibroblasts that contributes to ECM fibronectin turnover. MT1-MMP-null cells express MT3-MMP, but not MT2-MMP (Hotary et al., 2002). MT1-, MT2- and MT3-MMP have overlapping substrate specificities, and can all promote cell migration in three-dimensional environments (Chun et al., 2004; Hotary et al., 2002). Thus, it is possible that other MT-MMPs also contribute to the turnover of ECM fibronectin. It has been shown that endocytosis of soluble collagen I can be regulated by MMP-13 (Madsen et al., 2007). However, clearance of collagen I from in vitro collagen polymerized gels requires MT1-MMP and/or MMP-2, but not MMP-13 (Madsen et al., 2007). Our data show that MT1-MMP promotes the endocytosis of collagen I from cell-derived ECM (Fig. 5A–D), but is not required for endocytosis of soluble collagen I (Fig. 5E–H). These data suggest that co-regulation by extracellular proteolysis and endocytosis is a common mechanism for controlling matrix protein degradation and removal from the ECM. This could be especially important for proteins that form supramolecular structures.

MT1-MMP cleavage of ECM fibronectin results in the production of multiple fibronectin fragments (Fig. 6D). Interestingly, cleavage of ECM fibronectin fibrils with MT1-MMP resulted in complete loss of intact fibronectin, but did not alter the global structure of ECM fibronectin fibrils (Fig. 6A–C). Certain fibronectin fragments can promote inflammation (Barilla and Carsons, 2000; Norris et al., 1982), whereas other fibronectin fragments are known to regulate cell migration, growth and survival (Clark et al., 1988; Dai et al., 2005; Homandberg et al., 1985). Therefore, tight regulation of ECM fibronectin degradation and endocytosis provides a mechanism for limiting the accumulation of bioactive matrix fragments.

Our work is the first to demonstrate that an MMP can regulate integrin endocytosis (Fig. 7), and suggests that MT1-MMP regulates α5β1 integrin endocytosis by promoting ECM fibronectin proteolysis and by enhancing the endocytosis of fibronectin fibrils. The ability of the MT1-MMP catalytic domain to promote fibronectin endocytosis (Fig. 6) coupled with the ability of a fibronectin polymerization inhibitor to partially rescue integrin endocytosis (Fig. 8C,D) in MT1-MMP-null cells suggests that the accumulation of uncleaved ECM fibronectin in MT1-MMP-null cells contributes to the defect in α5β1 integrin endocytosis. This conclusion is supported by data showing that ongoing fibronectin polymerization and abundant ECM fibronectin inhibit α5β1 integrin endocytosis (Fig. 8A,B). We previously showed that ECM fibronectin endocytosis is regulated by both β1 integrin and caveolin-1 (Shi and Sottile, 2008; Sottile and Chandler, 2005). Hence, ECM fibronectin could prevent or delay integrin endocytosis by preventing integrin association with caveolae. However, previous work has shown that fibronectin fibrils, which colocalize with α5β1 integrin in fibrillar adhesions, are found in caveolin-rich regions of the cells (Hocking and Kowalski, 2002). Alternatively, ECM fibronectin could inhibit α5β1 integrin endoctyosis by sequestering integrins and/or by altering signaling pathways that are important for integrin endocytosis.

MT1-MMP could also influence α5β1 integrin endocytosis by directly associating with α5β1 integrin. We speculate that the association of MT1-MMP with α5β1 integrin could serve to localize MT1-MMP to areas of the cell surface that are enriched in fibronectin fibrils, thereby promoting the localized degradation of ECM fibronectin and the subsequent co-endocytosis of fibronectin and α5β1 integrin. However, to our knowledge, there is no published evidence that α5β1 integrin associates with MT1-MMP. Published data show that MT1-MMP can associate with β1 integrins in endothelial cells (Galvez et al., 2002). However, the α-integrin subunit involved in this interaction was not identified. Moreover, we have not been able to demonstrate an association between α5β1 integrin and MT1-MMP in fibroblasts by co-immunoprecipitation (data not shown), suggesting that the interaction between these two proteins, if it exists, could be weak and/or transient.

ECM remodeling plays an important role in regulating cell migration (Mercurius and Morla, 1998; Sabeh et al., 2004; Sottile et al., 2007). Previous data indicate that excess deposition of ECM fibronectin in epithelial cells inhibits cell migration (Hocking and Chang, 2003). In this manuscript we show that inhibiting fibronectin polymerization, which induces the turnover of pre-existing matrix fibronectin, accelerates myofibroblast migration (Fig. 9). This process requires MT1-MMP activity (Fig. 10), suggesting that MT1-MMP plays a role in cell migration that is modulated by ECM remodeling. MT1-MMP is known to promote cell migration (Filippov et al., 2005; Galvez et al., 2001; Hotary et al., 2000; Matias-Roman et al., 2005). ECM proteolysis can promote migration by generating ECM fragments that are pro-migratory, by reducing suboptimal adhesive interactions with cells and/or by creating a path for cell migration. Our data suggest that ECM proteolysis can also influence integrin trafficking, which has also been implicated in regulating cell migration (Caswell et al., 2009). Hence, the interaction between fibronectin, α5β1 integrin and MT1-MMP, which is highlighted in this work, might provide a finely tuned regulatory system that controls ECM remodeling, the bioavailability of ECM fragments, and integrin trafficking. Our manuscript provides new insights into mechanisms that regulate ECM remodeling, and could lead to the development of novel strategies for limiting excess ECM accumulation during fibrosis.

Materials and Methods

Immunological reagents, chemicals and proteins

Antibody to α5 integrin (AB1928) was from Millipore (Billerica, MA) and antibody to β1 integrin (HMβ1) was from Pharmingen, BD Bioscience (Franklin Lakes, NJ). Monoclonal antibody 04.11.041 to fibronectin was a gift from Albert Millis (Millis et al., 1985). 04.11.041 recognizes human, but not mouse or bovine fibronectin. EZ-link sulfo-NHS-SS-biotin was from Pierce, Thermo Scientific (Rockford, IL). GM6001 and control compound, and MT1-MMP catalytic domain, were from Calbiochem, EMD Biosciences (San Diego, CA), TIMP-1 and TIMP-2 were from R&D System (Minneapolis, MN). Type I collagen was from BD Biosciences or UBI (Lake Placid, NY). Texas Red, Alexa-Fluor-488- and Alexa-Fluor-633-conjugated fibronectins were made according to the manufacture's protocol (Molecular Probes/Invitrogen). Texas Red-conjugated collagen I was made as described previously (Shi et al., 2010). pUR4, a fibronectin polymerization inhibitor, was prepared as described previously (Chiang et al., 2009). Oregon-Green-conjugated fibrinogen was a gift from Patricia J. Simpson-Haidaris (Pereira, 2002).

Cell culture

Fibronectin-null myofibroblasts (FN-null MFs), rat aortic SMCs and TJ6F normal human foreskin fibroblasts were cultured as previously described (Sottile and Hocking, 2002; Sottile et al., 1998). MT1-MMP-null and WT control cells were generous gifts from Kenn Holmbeck. MT1-MMP-null and WT cells were

spontaneously immortalized using procedures similar to those used to produce 3T3 cells (Todaro and Green, 1963).

Long-term fibronectin matrix turnover pulse–chase assays

FN-null MFs were incubated (pulsed) overnight with 10 μg/ml fluorescently conjugated fibronectin. Cells were washed and then incubated (chased) with culture medium containing or lacking 10 μg/ml unconjugated fibronectin at 37℃ for various lengths of time. Some cells were also supplied with various protease inhibitors during the chase.

Short-term soluble fibronectin or collagen I endocytosis pulse–chase assays

Cells were incubated with 10 μg/ml fluorescently labeled fibronectin or collagen I for 45–50 minutes at 4℃ (pulsed). Cells were washed and then incubated with culture medium lacking fibronectin or collagen I at 37℃ for 1 hour (chased).

Preparation of cell-derived pre-assembled matrix

The procedure for preparing pre-assembled matrix containing fluorescently labeled fibronectin was as previously described (Shi and Sottile, 2008). In brief, FN-null MFs were incubated overnight with 10 μg/ml TR– or Alexa-Fluor-488–fibronectin to allow the assembly of a robust ECM fibronectin. Cellular components were then extracted with lysis buffer (20 mM Na_2HPO_4, pH 9.6, 1% NP-40). Fibronectin fibrils were largely preserved after extraction (Shi and Sottile, 2008) (Fig. 6A). The matrix was washed and stored in PBS at 4℃ until use. Some pre-assembled matrices were further treated with 0.9 ng/μl MT1-MMP catalytic domain in collagenase digestion buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) at 37℃ for ~20 hours. For some matrices, 50 nM TIMP-1 or TIMP-2 was added during the digestion period.

To prepare pre-assembled matrix containing collagen I or fibrinogen, FN-null MFs were incubated with 7 μg/ml TR–collagen I or 20 μg/ml Oregon-Green-conjugated fibrinogen. Because fibronectin deposition is required for the assembly of ECM collagen I and ECM fibrinogen, 10 μg/ml unlabeled fibronectin was also supplied to the cells. After overnight incubation, cellular components were extracted as described above.

Determination of intracellular degradation of ECM fibronectin

ECM fibronectin degradation was measured as described previously (Sottile and Chandler, 2005). In brief, after an overnight incubation with ^{125}I-labeled fibronectin, FN-null MFs were washed and then incubated in culture medium containing or lacking unlabeled fibronectin, and in the presence or absence of various inhibitors, for 24 hours. The cell culture supernatant was then collected to determine the amount of released fibronectin. The cell layers were washed and then solubilized in 0.2 N NaOH to determine the amount of cell- and matrix-associated radioactivity. The culture supernatant was precipitated with trichloroacetic acid (TCA) as described previously (Sottile and Chandler, 2005). The counts in the TCA-soluble fraction represent the fibronectin that is degraded intracellularly, which is expressed as a percentage of the total counts in each well.

Wound healing assays

FN-null MFs were grown to 90% confluence, and then cultured with 10 μg/ml fibronectin overnight. Cell monolayers were wounded with a pipette tip to generate an area devoid of cells. After washing, cells were incubated with culture medium containing or lacking 10 μg/ml fibronectin, in the presence or absence of various chemicals or proteins, as indicated in the figure legends. Photographs of the wound area were taken at various times (0–8 hours) post wounding. Wound areas were measured using a MATLab-based program as described previously (Sottile et al., 2007). The area migrated was determined by subtracting the area at 0 hours from the area at other time points.

Integrin endocytosis assays

Integrin endocytosis assays were performed as previously described (Roberts et al., 2001). In brief, cells were incubated with 0.1–0.3 mg/ml NHS-SS-biotin in Hank's balanced salt solution on ice for 30 minutes. Cells were washed and then chased at 37℃ for 30 minutes to allow endocytosis to occur. At the end of the chase, biotinlyated proteins remaining on the cell surface were reduced by incubating cells with 90 mM sodium 2-mercaptoethanesulfonate (MesNa) (50 mM Tris, 100 mM NaCl, pH 8.6) on ice for two 15-minute periods. MesNa was quenched by incubating cells with 15 mg/ml iodoacetamide on ice for 10 minutes. Cell lysates were then collected and subjected to precipitation with streptavidin agarose. Biotinylated proteins (representing endocytosed proteins) isolated by precipitation were then subjected to western blotting analysis. The blot was probed with anti-α5 integrin antibodies, and then quantified using an Odyssey infrared imaging system (Li-COR Bioscience, Lincoln, NE).

Imaging assays

Immunostaining was performed as previously described (Sottile and Hocking, 2002). Cells were examined using an Olympus microscope equipped with epifluorescence optics, or with an Olympus scanning confocal microscope.

Flow cytometry to quantify endocytosed fibronectin

Quantification of endocytosed fibronectin by flow cytometry was performed as described previously (Shi and Sottile, 2008). In brief, after being cultured on top of Alexa-Fluor-488-labeled ECM fibronectin, cells were treated with a mixture of 0.02% EDTA (ethylenediamine tetraacetic acid), 0.1% trypsin and 200 μg/ml proteinase K for 10 minutes at 37℃ to remove cell surface fibronectin. Cells were detached and washed three times with ice-cold PBS and 0.01% azide, and then fixed with 3.5% paraformaldehyde. The intracellular Alexa-Fluor-488–fibronectin signal was measured immediately using a FACSCalibur flow cytometer (Becton Dickinson).

Adenoviruses

MT1-MMP–EGFP construct (a kind gift from Alicia Arroyo) was subcloned into the vector pENTR1A (Invitrogen). MT1-MMP–EGFP–pENTR1A clone was recombined with the adenoviral vector pAdCMVDEST (Invitrogen) using a Gateway LR Clonase kit according to the manufacturer's protocol. The resulting MT1-MMP–EGFP–pAD plasmid was transfected into HEK293 cells. Preparation and transduction of adenoviruses were performed as described previously (Shi and Sottile, 2008; Sottile and Chandler, 2005). Recombinant protein expression was detected at 8 hours post transfection, and persisted for more than 48 hours (data not shown).

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