MT1-MMP promotes cell growth and ERK activation through c-Src and paxillin in three-dimensional collagen matrix

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Abstract

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is essential for tumor invasion and growth. We show here that MT1-MMP induces extracellular signal-regulated kinase (ERK) activation in cancer cells cultured in collagen gel, which is indispensable for their proliferation. Inhibition of MT1-MMP by MMP inhibitor or small interfering RNA suppressed activation of focal adhesion kinase (FAK) and ERK in MT1-MMP-expressing cancer cells, which resulted in up-regulation of $p21^{WAF1}$ and suppression of cell growth in collagen gel. Cell proliferation was also abrogated by the inhibitor against ERK pathway without affecting FAK phosphorylation. MT1-MMP and integrin $\alpha_v\beta_3$ were shown to be involved in c-Src activation, which induced FAK and ERK activation in collagen gel. These MT1-MMP-mediated signal transductions were paxillin dependent, as knockdown of paxillin reduced cell growth and ERK activation, and co-expression of MT1-MMP with paxillin induced ERK activation. The results suggest that MT1-MMP contributes to proliferation of cancer cells in the extracellular matrix by activating ERK through c-Src and paxillin.

Keywords:
Cancer, ECM, Growth, Integrin, MAPK, MT1-MMP

Abbreviations:
ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GFP, green fluorescence protein; MAPK, mitogen-activated protein kinases; MT1-MMP, membrane-type 1 matrix metalloproteinase; 3-D, three-dimensional; 2-D, two-dimensional; PCNA, proliferating cell nuclear antigen; Pyk2, proline-rich tyrosine kinase 2; siRNA, small interfering RNA

Introduction

The extracellular matrix (ECM) is a complex network of structural and functional proteins that not only provides cell anchorage but also regulates gene expression, cell proliferation, differentiation, and migration [1]. Integrins are the principle cell surface receptors for ECM. Integrin clustering induced by cell attachment to ECM promotes formation of cell-matrix adhesion and activation of focal adhesion kinase (FAK) and c-Src. When FAK is recruited to cell-matrix adhesion by binding with integrin-associated molecules such as talin and paxillin, it undergoes autophosphorylation at Tyr 397 and then associates with c-Src and phosphatidylinositol-3 kinase. c-Src phosphorylates Tyr 576 and 577 in the catalytic domain of FAK, which augments its kinase activity and induces the formation of a multimolecular complex, including Shc, JSAP1, and $p130^{Cas}$, leading to activate mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase[1-4]. In fact, elevated FAK and c-Src expression and activity has been associated with tumor progression [5].

Type I collagen, which is the most abundant protein in the human body, acts as a growth-stimulatory protein, when cells are cultured on two-dimensional (2-D) collagen. In contrast, cultivation in three-dimensional (3-D) collagen suppresses the proliferation of numerous cell types. This growth-suppressive signal may be mediated by integrins, as integrin $\alpha_2\beta_1$ mediates fibril collagen-induced inhibition of cell growth [6] and 3-D collagen induces down-regulation of cell-matrix adhesion proteins such as FAK, talin, and paxillin, which is blocked by integrin $\alpha_2\beta_1$-neutralizing antibody [7]. Recent study has demonstrated that reduction of cell-matrix adhesion in 3-D ECM causes growth inhibition [8]. A normal cell culture dish is non-flexible with a rigidity of more than 1 gigapascals, whereas 3-D collagen is a flexible substrate with the rigidity of 30-100 pascals [7]. Therefore, when cells are plated on culture dish or 2-D collagen in the presence of serum, formation of robust cell-matrix adhesions is induced, which may function as a scaffold for signaling to promote cell growth, which masks the effects of growth factors. In 3-D collagen in which cell-matrix adhesions are reduced, the signal from growth factor receptor is required for promotion of cell growth [8]. Nevertheless, Integrin-mediated signal from ECM may control cell growth.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) was originally identified as a tumor-specific MMP-2 activator [9], and has been implicated in tumor progression [10]. It is now known to activate MMP-2 and -13 and degrade a wide range of ECM components including type I collagen, fibronectin, and laminins. This enzyme also processes and interacts with membrane-tethered proteins, such as integrins and CD44, which is associated with the regulation of tumor cell invasion [10]. The inhibition of MT1-MMP suppresses tumor cell invasion both *in vitro* and *in vivo* [11-13]. Recent studies have demonstrated that MT1-MMP functions as a collagenolytic growth factor that provides cancer cells with the ability to

accelerate proliferative responses by overcoming the growth-suppressive signals from 3-D collagen [14, 15]. The modulation of the extracellular microenvironment by MT1-MMP may play an important role in tumor cell proliferation and invasion, however, the molecular mechanism underlying this still remains unknown.

We have previously reported that MT1-MMP-mediated ECM degradation activates FAK and ERK under serum-depleted condition, which in turn stimulates cell migration [11, 16]. In this study, we demonstrated that MT1-MMP modulates extracellular microenvironment, which induces ERK activation through c-Src and paxillin, and this MT1-MMP-induced ERK activation is indispensable for proliferation of cancer cells in 3-D collagen.

Materials and methods

Cell culture and reagents. HeLa cells and human oral squamous cell carcinoma HSC-4 cells were obtained from the Health Science Research Bank (Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. For transfection experiments, cells were transfected with the calcium phosphate method or with Lipofectamine 2000 (Invitorgen) according to the manufacturer's instructions. The reagents used were type I collagen (Nitta Gelatin, Osaka, Japan); the inhibitors for Src (PP1 and PP2) and MEK (PD98059) (Calbiochem). A synthetic MMP inhibitor (BB94) was a kind gift from the Kotobuki Pharmaceutical (Nagano, Japan). The immunological reagents used were anti-ERK2, anti-FAK, anti-proliferating cell nuclear antigen (PCNA), and anti-paxillin antibodies (BD Biosciences); anti-phospho-p44/42 MAPK, anti-phospho-Y576/577-FAK, anti-phospho-Y416-Src, and anti-proline-rich tyrosine kinase 2 (Pyk2) antibodies (Cell Signaling); an anti-$p21^{WAF1}$ antibody (Santa Cruz); an anti-phospho-Y397-FAK antibody (BioSource); an anti-Src antibody (Upstate); an anti-tubulin antibody (Sigma): an anti-integrin $\alpha_v\beta_3$ (LM609) antibody (Chemicon). Cells were cultured in 3-D collagen as described previously [11].

Expression plasmids. An expression plasmid for MT1-MMP tagged with FLAG was constructed as previously described.[14] Plasmids encoding the puromycin-resistance gene (pHA262pur), pRK-green fluorescence protein (GFP), and pRK-GFP-paxillin were kind gifts from Dr. Kenneth M. Yamada (National Institutes of Health, Bethesda, MD, USA).

Gelatin zymography. The conditioned media from the cells were analyzed by gelatin zymography as described previously [11].

Immunoblotting. Cells in 3-D collagen were washed with phosphate-buffered saline and homogenized in 5× NP-40 lysis buffer containing 250 mM Tris-HCl (pH 7.5), 750 mM NaCl, 5 mM EGTA, 5 mM phenylmethylsulfonyl fluoride, 10 mM Na_3VO_4, 10 mM NaF, protease inhibitor cocktail (Nacalai tesque), and 5% Nonidet P-40. Cell lysates were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes.

Cell growth. HSC-4 and HeLa cells were cultured on 2-D collagen or in 3-D collagen with 96-well culture plates (500 cells/well) for indicated periods. Cells were fed every 3 days. Cell growth was determined by assaying viable cell numbers using a Cell Counting Kit-8 (Dojin Laboratory, Kumamoto, Japan). The living cell number was assessed by measuring the absorbance of OD_{490} using a SH-1000Lab spectrophotometer (Corona Electric, Ibaraki, Japan).

Small interfering RNA (siRNA)-mediated protein knockdown. The siRNA sequences were as follows: mt1-mmp, 5'-AACCAGAAGCUGAAGGUAGTT; fak, 5'-GCGAUUAUAUGUUAGAGAUAG; paxillin, 5'-CCCUGACGAAAGAGAAGCCCU; c-Src-1, 5'-AACAAGAGCAAGCCCAAGGAU; c-Src-2,

5’-AAGCACUACAAGAUCCGCAAG. As a control, siRNA duplex with an irrelevant sequence was used (Qiagen). HSC-4 cells were transfected with 33.2 nM of siRNA duplexes in Opti-MEM using Oligofectamine (Invitrogen), according to the manufacturer’s instructions, and were incubated for 48 h. Cells were then detached and cultured in 3-D collagen.

Real-time PCR quantification. Real-time quantitative PCR experiments were performed as described previously [17].

Results

MT1-MMP is required for proliferation of cancer cells in 3-D collagen

The role of MT1-MMP in the proliferation of cancer cells was examined. Human squamous cell carcinoma HSC-4 cells expressed endogenous MT1-MMP [18], and thereby induced the activation of MMP-2, which was stimulated by 3-D collagen culture, and was blocked by MMP inhibitor (BB94) treatment (Fig. 1A). In the cells cultured on coated-collagen (2-D), ERK was phosphorylated at high level, which was slightly down-regulated by treatment with BB94. Cultivation in 3-D collagen markedly attenuated ERK activation, which was further suppressed by BB94 treatment. Phosphorylation of FAK at Tyr 576 and 577 was quite proportional to ERK activation. FAK phosphorylation at Tyr 397 was also attenuated by BB94-treatemnet in 2-D culture, but was reduced only faintly in 3-D collagen culture. Proportional to the attenuation of phosphorylation of FAK at Tyr 576 and 577 and ERK by 3-D culture and/or BB94 treatment, the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ was increased, whereas the expression of PCNA, which is expressed in replicating cells, showed reverse co-relation to $p21^{WAF1}$ expression. HSC-4 cell growth on 2-D collagen was not affected by BB94 treatment; however, 3-D collagen culture reduced cell proliferation to 37% of that on 2-D collagen, which was further suppressed to 17% by treatment with BB94 (Fig. 1B). To confirm the role of MT1-MMP, siRNA-mediated knockdown of protein expression was employed. We achieved > 90% suppression of MT1-MMP mRNA upon transfection of MT1-MMP siRNA (data not shown), and MMP-2 activation by HSC-4 cells was completely blocked by MT1-MMP knockdown (Fig. 1C). As expected, knockdown of MT1-MMP resulted in suppression of FAK and ERK phosphorylation, which induced $p21^{WAF1}$ expression. Inhibition of ERK pathway by PD98059 treatment also induced $p21^{WAF1}$ expression without affecting FAK phosphorylation. As shown in Fig. 1D, growth of HSC-4 cells in 3-D collagen was impeded by inhibition of MT1-MMP by siRNA or BB94, or ERK pathway by PD98059. These results show that MT1-MMP induces ERK activation, which plays an important role in proliferation of cancer cells in 3-D collagen.

c-Src is involved in MT1-MMP-mediated ERK activation

We found that the inhibition of MT1-MMP suppresses FAK phosphorylation at Tyr 576 and 577 in 3-D collagen, which are sites phosphorylated by c-Src, resulting in enhancement of FAK activity (Fig. 1A). It suggests that c-Src may participate in MT1-MMP-mediated FAK and ERK activation. To investigate it, the effect of MT1-MMP knockdown by siRNA on c-Src activation was examined (Fig. 2A). Knockdown of MT1-MMP reduced MMP-2 activation

and c-Src phosphorylation at Tyr 416 in 3-D collagen. Next, the effect of c-Src inhibition on FAK and ERK activation was tested. The blockade of c-Src by its inhibitors attenuated phosphorylation of FAK and ERK in HSC-4 cells cultured in 3-D collagen as BB94 did (Fig. 2B). In addition, phosphorylation of FAK and ERK was remarkably inhibited in the cells transfected with siRNA for c-Src (Fig. 2C). Activation of c-Src is induced by forming the complex with FAK or directly by engagement of integrins that recognize RGD containing protein[19]. To seek the pathway to activate c-Src, HSC-4 cells were cultured with integrin $\alpha_v\beta_3$-neutralizing antibody (LM609) in 3-D collagen. Cultivation of cells with this neutralizing antibody but not negative control antibody resulted in suppression of c-Src phosphorylation as BB94 did (Fig. 2D). Concomitantly, ERK activation was impeded by treatment with integrin $\alpha_v\beta_3$-neutralizing antibody and BB94. These results demonstrate that MT1-MMP is required for complete activation of c-Src through integrin $\alpha_v\beta_3$ in 3-D collagen, which may subsequently induce FAK and ERK activation.

Paxillin plays an important role in MT1-MMP-mediated ERK activation

To investigate the mechanism by which MT1-MMP regulates ERK activation through c-Src, HSC-4 cells were tranfected with siRNA for either FAK or paxillin, which are the substrates of c-Src, and were cultured in 3-D collagen. ERK activation was significantly down-regulated by knockdown of paxillin but not FAK. The expression level of FAK-related protein Pyk2, which can compensate some FAK functions, was not altered in these cells. Knockdown of paxillin remarkably reduced HSC-4 cell growth in 3-D collagen, although FAK knockdown did not significantly affect it (Fig. 3B). Next, to test whether co-expression of MT1-MMP and paxillin induces ERK activation in 3-D collagen, HeLa cells, which do not express MT1-MMP, were transfected with MT1-MMP plasmid and cultured in 3-D collagen (Fig. 3C). Expression of MT1-MMP or GFP-paxillin alone was not enough to induce ERK activation, and co-expression of MT1-MMP and GFP-paxillin induced ERK activation. Co-expression of GFP-paxillin did not significantly affect MMP-2 activation by MT1-MMP. These results indicate that paxillin is involved in MT1-MMP-mediated ERK activation through c-Src and proliferation of cancer cells in 3-D collagen.

Discussion

We have previously shown that ECM degradation at cell-matrix adhesions by MT1-MMP accelerates cell-matrix adhesion turnover, which stimulates FAK and ERK activation, resulting in enhancement of cell migration [11, 13, 16]. The present study confirms and extends our previous studies to demonstrate that MT1-MMP stimulates ERK activation in 3-D collagen, which is indispensable for proliferation of cancer cells. It is well documented that the interaction between collagen fibrils and integrin $\alpha_2\beta_1$ generates the growth-suppressive signals in normal epithelial or cancer cells cultured in 3-D collagen [7, 20-22], which can be abrogated by pericellular collagenolysis [14, 15]. We represent that MT1-MMP induces c-Src activation in 3-D collagen by modulating extracellular microenvironment, which subsequently activates paxillin to scaffold signaling molecules for ERK pathway and thus leads to escape cells from growth-suppressive signals.

In this study, we observed that ERK suppression by MMP inhibitor and MT1-MMP knockdown induces $p21^{WAF1}$ expression and PCNA repression in cancer cells (Fig. 1). The increase of $p21^{WAF1}$ and the decrease of PCNA expression are associated with the growth inhibition of melanoma and hepatic stellate cells on cross-linked or collagenase-resistant collagen [20, 21, 23]. Inhibition of MEK-ERK pathway down-regulates expression of PCNA and DNA methyltransferase 1, and induces promoter demethylation of the $p21^{WAF1}$ gene, which up-regulates its mRNA and protein expression in colon cancer cells [24]. Our results also showed that inhibition of ERK pathway by PD98059 induces $p21^{WAF1}$ expression without affecting FAK phosphorylation. These effects resulted in growth suppression of cancer cells. Thus, MT1-MMP-mediated ERK activation may promote cell proliferation at least in part by down-regulating $p21^{WAF1}$. To escape from a quiescent state induced by 3-D collagen and to initiate growth, cancer cells may activate MT1-MMP axis.

Although phosphorylation of FAK at Tyr 397, 576, and 577 was shown to be regulated by MT1-MMP, the level of phosphorylation at Tyr 576 and 577 of FAK, which are phosphorylated by c-Src, was remarkably reduced by 3D-collagen culture, and correlates with ERK activation. Therefore, c-Src is suggested to be involved in MT1-MMP-mediated ERK activation. We found that MT1-MMP is required for complete activation of c-Src by its autophosphorylation at Tyr 416 located in the activation loop of the catalytic center, which induces FAK and ERK activation in 3-D collagen (Fig. 2). Activation of c-Src is induced by forming the complex with FAK or directly by engagement of integrin $\alpha_{IIb}\beta_3$, $\alpha_4\beta_1$, or $\alpha_v\beta_3$, which recognize RGD containing protein such as fibronectin and vitronectin [19]. Notably, the expression of MT1-MMP in prostate cancer LNCaP cells induces fibronectin expression in 3-D collagen [25], and intact type I collagen is a poor ligand for integrin $\alpha_v\beta_3$, while degraded collagen exposes RGD epitopes [26]. In the present study, treatment of cancer cells with integrin $\alpha_v\beta_3$-neutralizing antibody impaired activation of c-Src, FAK and ERK in 3-D

collagen. Thus, MT1-MMP may induce expression of RGD-containing proteins and/or exposure of RGD epitopes in collagen fibrils, which may activate c-Src through integrins, including $\alpha_v\beta_3$.

Mouse embryonic fibroblasts derived from paxillin-deficient mouse display reduced cell migration and activation of FAK and ERK [27]. Paxillin is known to function as a scaffold for hepatocyte growth factor-stimulated ERK activation with binding to c-Raf, MEK, and ERK, which is initiated through hepatocyte growth factor-induced c-Src activation [28]. A recent study reported that paxillin is mutated and amplified in roughly 20% of lung cancers [29]. We found that knockdown of paxillin but not FAK reduces ERK activation and growth of cancer cells in 3-D collagen, and that co-transfection of MT1-MMP and paxillin induces ERK activation in 3-D collagen (Fig. 3). A previous study by other group demonstrated that ERK activation is independent from the FAK-regulated pathway in fibroblasts cultured in cell-derived 3-D matrix [30]. These results strongly suggest that paxillin may function as a down-stream effecter for MT1-MMP-mediated ERK activation through c-Src. Paxillin has been also implicated in cell survival, as the interaction of paxillin with FAK or Bcl-2 promotes cell survival [31]. Our results showing that knockdown of paxillin severely attenuates growth of cancer cells in 3-D collagen may be caused by suppression of both ERK activation and cell survival. MT1-MMP-activated c-Src through integrins including $\alpha_v\beta_3$ may utilize paxillin as the scaffold for ERK activation, which overcomes growth suppressive signal to produce $p21^{WAF1}$ in 3-D collagen (Fig. 4).

In conclusion, we demonstrated that the enhanced expression of MT1-MMP in cancer cells contributes to not only invasion, but also proliferation in 3-D matrix by activating ERK pathway through c-Src and paxillin.

Conflict of interest statement

None.

Acknowledgments

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References

[1] M. Larsen, V. V. Artym, J. A. Green, et al., The matrix reorganized: extracellular matrix remodeling and integrin signaling, Curr. Opin. Cell Biol. 18 (2006) 463-471.

[2] A. L. Berrier, K. M. Yamada, Cell-matrix adhesion, J. Cell. Physiol. 213 (2007) 565-573.

[3] T. Takino, M. Tamura, H. Miyamori, et al., Tyrosine phosphorylation of the CrkII adaptor protein modulates cell migration, J. Cell Sci. 116 (2003) 3145-3155.

[4] T. Takino, M. Nakada, H. Miyamori, et al., JSAP1/JIP3 cooperates with focal adhesion kinase to regulate c-Jun N-terminal kinase and cell migration, J. Biol. Chem. 280 (2005) 37772-37781.

[5] G. W. McLean, N. O. Carragher, E. Avizienyte, et al., The role of focal-adhesion kinase in cancer - a new therapeutic opportunity, Nat. Rev. Cancer 5 (2005) 505-515.

[6] H. Koyama, E. W. Raines, K. E. Bornfeldt, et al., Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors, Cell 87 (1996) 1069-1078.

[7] Y. K. Wang, Y. H. Wang, C. Z. Wang, et al., Rigidity of collagen fibrils controls collagen gel-induced down-regulation of focal adhesion complex proteins mediated by alpha2beta1 integrin, J. Biol. Chem. 278 (2003) 21886-21892.

[8] H. Mizushima, X. Wang, S. Miyamoto, et al., Integrin signal masks growth-promotion activity of HB-EGF in monolayer cell cultures, J. Cell Sci. 122 (2009) 4277-4286.

[9] H. Sato, T. Takino, Y. Okada, et al., A matrix metalloproteinase expressed on the surface of invasive tumour cells, Nature 370 (1994) 61-65.

[10] H. Sato, T. Takino, H. Miyamori, Roles of membrane-type matrix metalloproteinase-1 in tumor invasion and metastasis, Cancer Sci. 96 (2005) 212-217.

[11] T. Takino, H. Miyamori, Y. Watanabe, et al., Membrane type 1 matrix metalloproteinase regulates collagen-dependent mitogen-activated protein/extracellular signal-related kinase activation and cell migration, Cancer Res. 64 (2004) 1044-1049.

[12] T. Nonaka, K. Nishibashi, Y. Itoh, et al., Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 in vivo, Mol. Cancer Ther. 4 (2005) 1157-1166.

[13] T. Takino, H. Saeki, H. Miyamori, et al., Inhibition of membrane-type 1 matrix metalloproteinase at cell-matrix adhesions, Cancer Res. 67 (2007) 11621-11629.

[14] K. Hotary, E. Allen, A. Punturieri, et al., Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3, J. Cell Biol. 149 (2000) 1309-1323.

[15] K. B. Hotary, E. D. Allen, P. C. Brooks, et al., Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix, Cell 114 (2003) 33-45.

[16] T. Takino, Y. Watanabe, M. Matsui, et al., Membrane-type 1 matrix metalloproteinase

modulates focal adhesion stability and cell migration, Exp. Cell Res. 312 (2006) 1381-1389.

[17] Y. Nishida, H. Miyamori, E. W. Thompson, et al., Activation of matrix metalloproteinase-2 (MMP-2) by membrane type 1 matrix metalloproteinase through an artificial receptor for proMMP-2 generates active MMP-2, Cancer Res. 68 (2008) 9096-9104.

[18] K. Shofuda, K. Moriyama, A. Nishihashi, et al., Role of tissue inhibitor of metalloproteinases-2 (TIMP-2) in regulation of pro-gelatinase A activation catalyzed by membrane-type matrix metalloproteinase-1 (MT1-MMP) in human cancer cells, J. Biochem. 124 (1998) 462-470.

[19] S. Huveneers, E. H. Danen, Adhesion signaling - crosstalk between integrins, Src and Rho, J. Cell Sci. 122 (2009) 1059-1069.

[20] P. Henriet, Z. D. Zhong, P. C. Brooks, et al., Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27KIP1, Proc. Natl. Acad. Sci. USA 97 (2000) 10026-10031.

[21] S. J. Wall, Z. D. Zhong, Y. A. DeClerck, The cyclin-dependent kinase inhibitors p15INK4B and p21CIP1 are critical regulators of fibrillar collagen-induced tumor cell cycle arrest, J. Biol. Chem. 282 (2007) 24471-24476.

[22] M. M. Zutter, S. A. Santoro, W. D. Staatz, et al., Re-expression of the alpha 2 beta 1 integrin abrogates the malignant phenotype of breast carcinoma cells, Proc. Natl. Acad. Sci. USA 92 (1995) 7411-7415.

[23] X. Zhou, A. Jamil, A. Nash, et al., Impaired proteolysis of collagen I inhibits proliferation of hepatic stellate cells: implications for regulation of liver fibrosis, J. Biol. Chem. 281 (2006) 39757-39765.

[24] R. Lu, X. Wang, Z. F. Chen, et al., Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells, J. Biol. Chem. 282 (2007) 12249-12259.

[25] J. Cao, C. Chiarelli, O. Richman, et al., Membrane type 1 matrix metalloproteinase induces epithelial-to-mesenchymal transition in prostate cancer, J. Biol. Chem. 283 (2008) 6232-6240.

[26] E. Petitclerc, S. Stromblad, T. L. von Schalscha, et al., Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival, Cancer Res. 59 (1999) 2724-2730.

[27] M. Hagel, E. L. George, A. Kim, et al., The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling, Mol. Cell. Biol. 22 (2002) 901-915.

[28] S. Ishibe, D. Joly, Z. X. Liu, et al., Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis, Mol. Cell 16 (2004)

257-267.
[29] R. Jagadeeswaran, H. Surawska, S. Krishnaswamy, et al., Paxillin is a target for somatic mutations in lung cancer: implications for cell growth and invasion, Cancer Res. 68 (2008) 132-142.
[30] E. Cukierman, R. Pankov, D. R. Stevens, et al., Taking cell-matrix adhesions to the third-dimension, Science 294 (2001) 1708-1712.
[31] N. O. Deakin, C. E. Turner, Paxillin comes of age, J. Cell Sci. 121 (2008) 2435-2444.

Figure legends

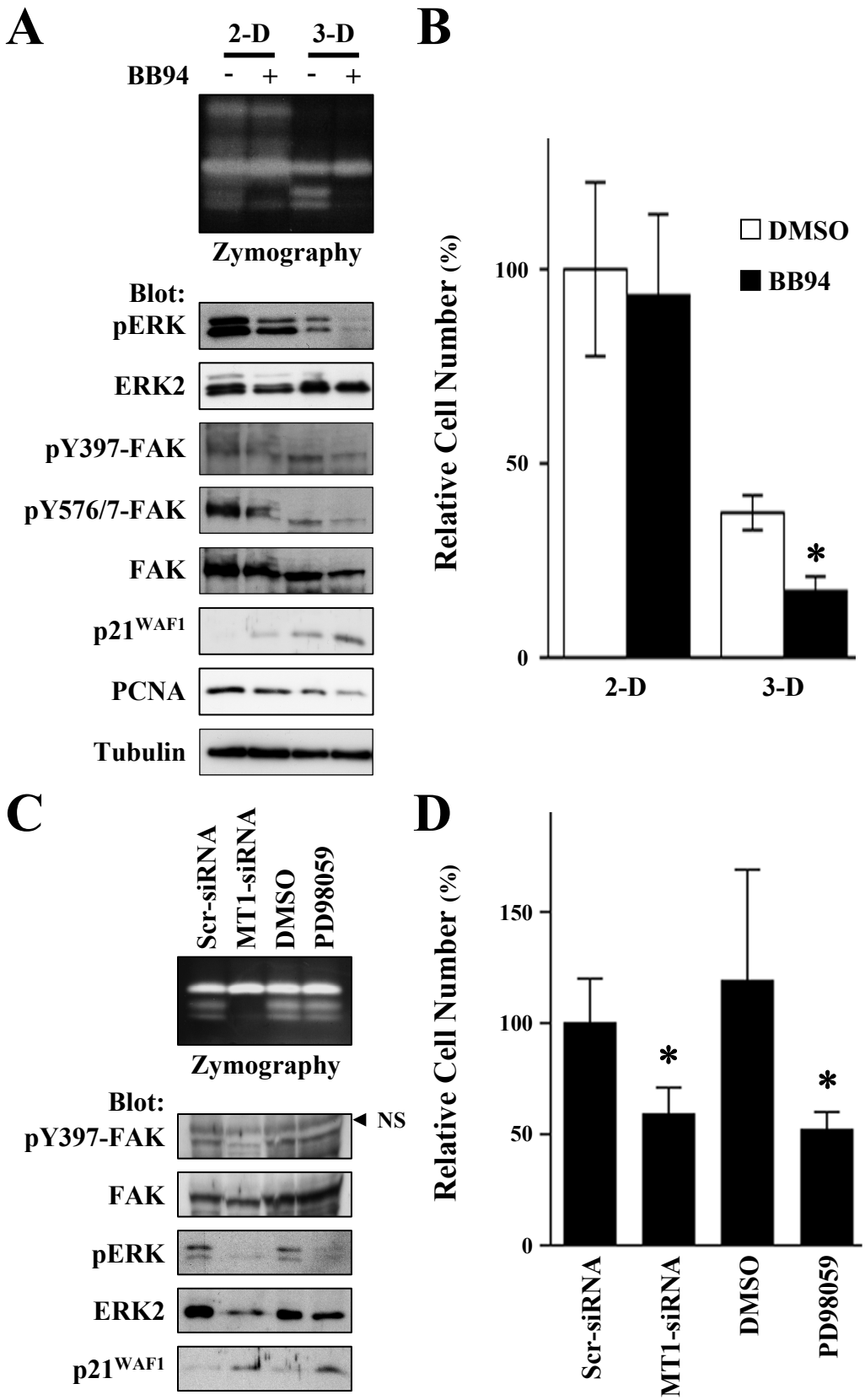
Fig. 1. MT1-MMP-dependent growth of HSC-4 cells in 3-D collagen. (A) HSC-4 cells were cultured on 2-D or in 3-D collagen with or without BB94 (10 μM) for 2 days. The conditioned media were analyzed by gelatin zymography. Cell lysates were analyzed by immunoblotting using anti-phospho-p44/42 MAP kinase (pERK), anti-ERK2, anti-phospho-Y397-FAK (pY397-FAK), anti-phospho-Y576/577-FAK (pY576/7-FAK), anti-FAK, anti-$p21^{WAF1}$, anti-PCNA, or anti-tubulin antibodies. (B) HSC-4 cells (1×10^3 cells/well) were cultured on 2-D or in 3-D collagen with or without BB94 for 7 days. Cell numbers were normalized to growth levels of 2-D-cultured cells without BB94. Columns, mean; bars, SE; n=12. *, $P < 0.0001$. (C) HSC-4 cells were cultured with scrambled siRNA (Scr) or MT1-MMP siRNA (MT1), DMSO ~~BB94,~~ or PD98059 in 3-D collagen for 24 h. The conditioned media were analyzed by gelatin zymography. Cells lysates were analyzed by immunoblotting using anti-phospho-Y397-FAK (pY397-FAK), anti-FAK, anti-phospho-p44/42 MAP kinase (pERK), anti-ERK2, or anti-$p21^{WAF1}$ antibodies. NS, non-specific immunoreactive bands. (D) HSC-4 cells (6×10^4 cells/well) were cultured as above for 3 days. Cell number was normalized to that of cells transfected with Scr-siRNA. Columns, mean; bars, SE; n=12. *, $P < 0.0001$.

Fig. 2. c-Src is involved in MT1-MMP-mediated ERK activation. (A) HSC-4 cells (1×10^5 cells) treated with scrambled siRNA (Scr) or MT1-MMP siRNA (MT1) were cultured in 3-D collagen for 24 h. Cells lysates were analyzed by immunoblotting using anti-phospho-Y416-Src (pY416-Src), anti-Src, and anti-tubulin antibodies. (B) HSC-4 cells were cultured in 3-D collagen with DMSO, BB94, PP1 or PP2 (10 μM) for 48 h. The conditioned media were analyzed by gelatin zymography. Cells lysates were analyzed by immunoblotting using anti-phospho-Y576/577-FAK (pY576/7-FAK), anti-FAK, anti-phospho-p44/42 MAP kinase (pERK), or anti-ERK2 antibodies. (C) HSC-4 cells transfected with scrambled siRNA (Scr), c-Src siRNA1 (Src-1), or c-Src siRNA2 (Src-2) were cultured in 3-D collagen for 24 h. Cells lysates were analyzed by immunoblotting using anti-Src, anti-tubulin, anti-phospho-Y576/577-FAK (pY576/7-FAK), anti-FAK, anti-phospho-p44/42 MAP kinase (pERK), or anti-ERK2 antibodies. (D) HSC-4 cells were cultured in 3-D collagen with DMSO, BB94, or anti-integrin $\alpha_v\beta_3$ blocking antibody (LM609) or negative control antibody (10 μg/mL) for 48 h. The conditioned media were analyzed by gelatin zymography. Cells lysates were analyzed by immunoblotting using anti-phospho-Y416-Src (pY416-Src), anti-Src, anti-phospho-p44/42 MAP kinase (pERK), anti-ERK2, and anti-tubulin antibodies.

Fig. 3. Paxillin plays an important role in MT1-MMP-mediated ERK activation. (A) HSC-4 cells treated with scrambled siRNA (Scr), FAK siRNA (FAK), or paxillin siRNA (Paxillin)

were cultured in 3-D collagen for 48 h. The conditioned media were analyzed by gelatin zymography. Cells lysates were analyzed by immunoblotting using anti-FAK, anti-paxillin, anti-tubulin, anti-phospho-p44/42 MAP kinase (pERK), or anti-ERK2 antibodies. (B) HSC-4 cells (6×10^4 cells/well) transected with indicated siRNA were cultured in 3-D collagen for 3 days. Cell numbers are normalized to growth levels of cells transfected with Scr-siRNA. Columns, mean; bars, SE; n=12. *, $P < 0.0001$. (C) HeLa cells were co-transfected with MT1-MMP and GFP-paxillin plus pHA262Pur as described above. After 24 h transfection, cells were cultured in 10% FBS/DMEM containing puromycin (1 μg/mL) for 48 h. Then cells were cultured in 3-D collagen for 24 h. The conditioned media were analyzed by gelatin zymography. Cells lysates were analyzed by immunoblotting using anti-paxillin, anti-phospho-p44/42 MAP kinase (pERK), or anti-ERK2 antibodies.

Fig. 4. A model for MT1-MMP-dependent cell growth in 3-D collagen culture. In 3-D collagen matrix, cell growth is suppressed by integrin $\alpha_2\beta_1$-mediated signals. Pericellular collagenolysis by MT1-MMP not only provides the space for cells to proliferate but also regulates gene expression and ERK activation through integrin $\alpha_v\beta_3$, c-Src and paxillin, and abrogates growth-suppressive signals by collagen fibrils, which consequently reduces $p21^{WAF1}$ expression and recovers cell growth.


A
2-D
3-D
BB94
- + - +
Zymography
Blot:
pERK
ERK2
pY397-FAK
pY576/7-FAK
FAK
p21WAF1
PCNA
Tubulin
B
Relative Cell Number (%)
100
50
0
DMSO
BB94
*
2-D
3-D
C
Scr-siRNA
MT1-siRNA
DMSO
PD98059
Zymography
Blot:
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NS
FAK
pERK
ERK2
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100
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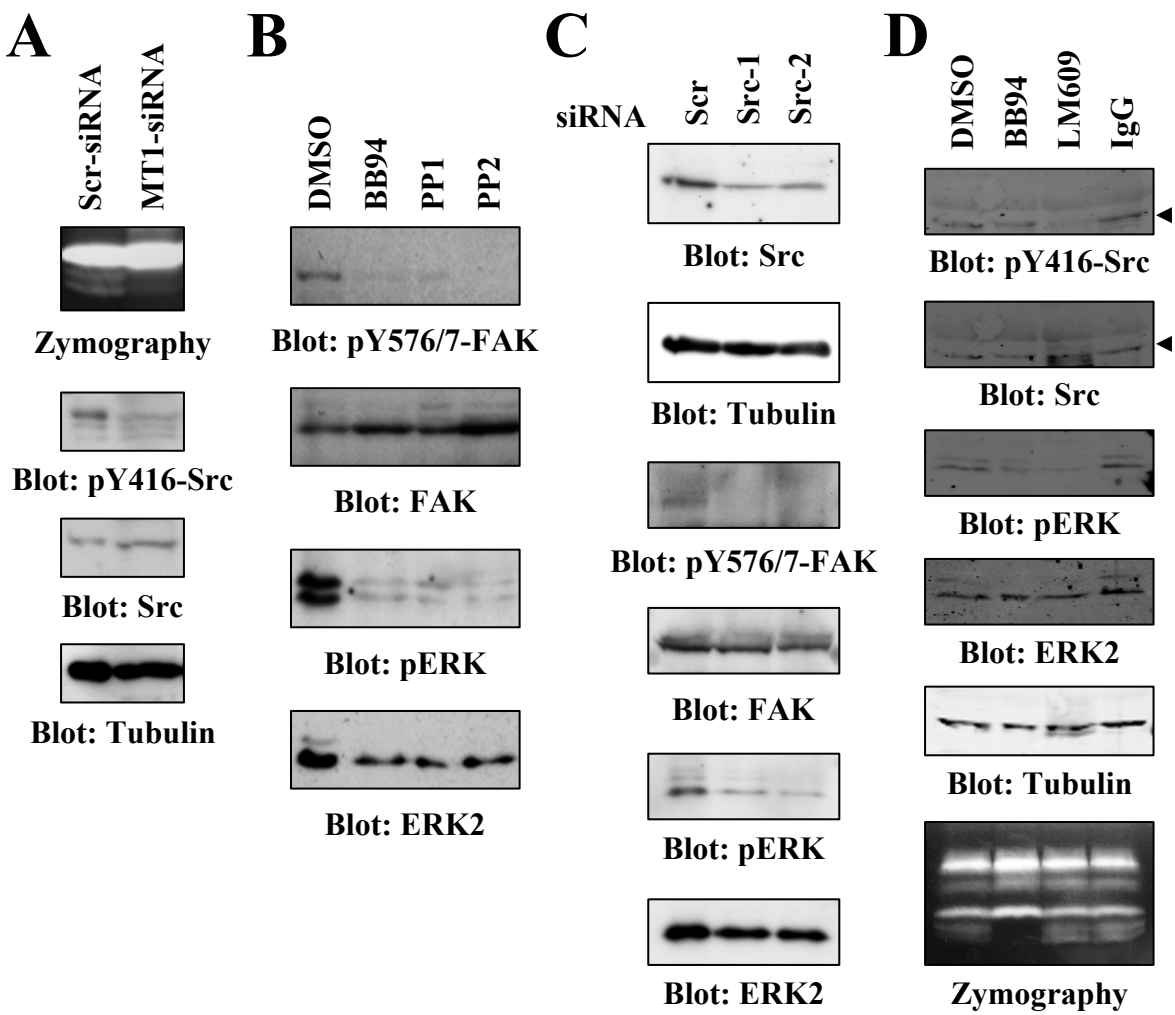
*
Scr-siRNA
MT1-siRNA
DMSO
PD98059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Scr-siRNA
MT1-siRNA
Zymography
Blot: pY416-Src
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Blot: Tubulin
B
DMSO
BB94
PP1
PP2
Blot: pY576/7-FAK
Blot: FAK
Blot: pERK
Blot: ERK2
C
siRNA
Scr
Src-1
Src-2
Blot: Src
Blot: Tubulin
Blot: pY576/7-FAK
Blot: FAK
Blot: pERK
Blot: ERK2
D
DMSO
BB94
LM609
IgG
Blot: pY416-Src
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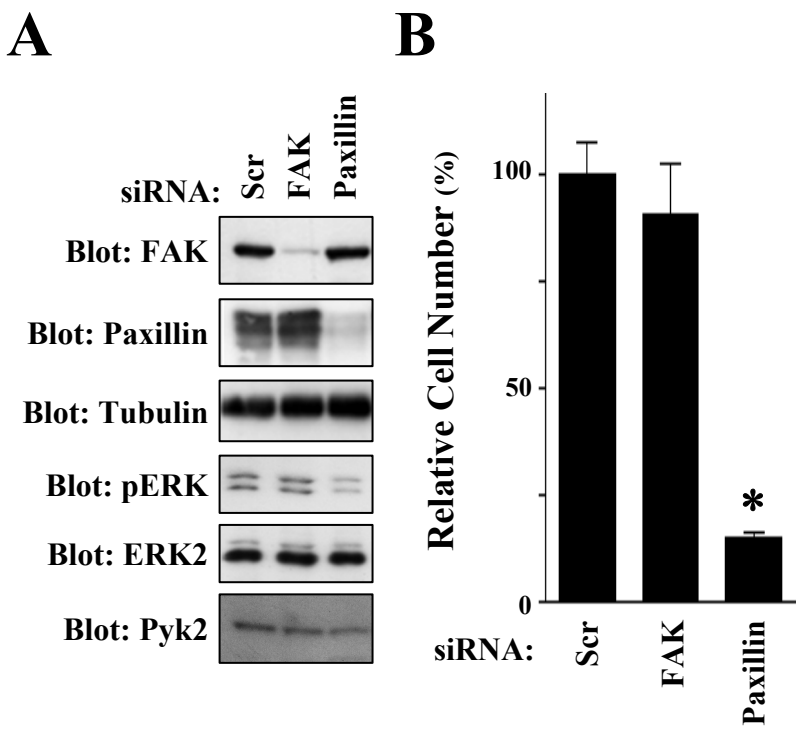
Blot: pERK
Blot: ERK2
Blot: Tubulin
Zymography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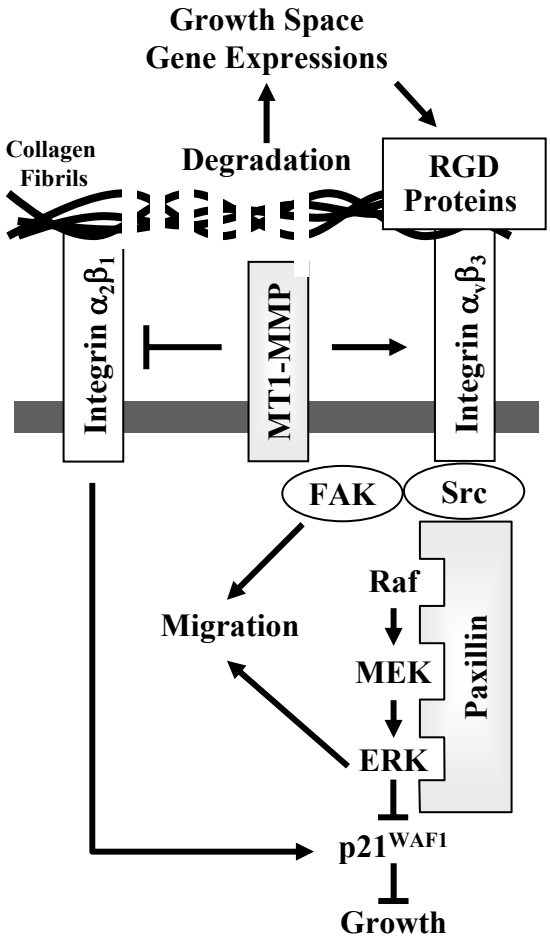
siRNA:
Scr
FAK
Paxillin
Blot: FAK
Blot: Paxillin
Blot: Tubulin
Blot: pERK
Blot: ERK2
Blot: Pyk2
B
Relative Cell Number (%)
100
50
0
*
siRNA:
Scr
FAK
Paxillin
C
GFP-Paxillin - - + +
MT1 - + - +
GFP-Paxillin
Paxillin
Blot: Paxillin
Blot: pERK
Blot: ERK2
Zymography


Growth Space
Gene Expressions
Collagen
Fibrils
Degradation
RGD
Proteins
Integrin $\alpha_2\beta_1$
MT1-MMP
Integrin $\alpha_v\beta_3$
FAK
Src
Raf
Migration
MEK
Paxillin
ERK
p21^{WAF1}
Growth