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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; PBS, phosphate-buffered saline; PTEN, phosphatase and tensin homolog deleted on chromosomal 10; SCC, squamous cell carcinoma; siRNA, small interfering RNA

Abstract

Vinculin regulates a variety of cellular functions partly through stabilization of tumor suppressor PTEN. In order to study the role of vinculin in tumor progression other than PTEN stabilization, vinculin was knocked down in PTEN-deficient squamous cell carcinoma HSC-4 cells. Knockdown of vinculin induced phenotypical change by reducing cell-cell and cell-extracellular matrix adhesions, and enhanced MT1-MMP expression at transcription level and subsequent cell migration. Up-regulation of MT1-MMP transcription by vinculin knockdown was abrogated by ERK inhibition. These results suggest that vinculin negatively regulates malignant phenotype of tumor cells including MT1-MMP transcription through MEK/ERK pathway.

Keywords: Cancer, Cell-extracellular matrix adhesion, Migration, MT1-MMP, Vinculin

1. Introduction

A cellular adhesiveness, including cell-cell and cell-extracellular matrix (ECM) adhesions, correlates with invasive and metastatic phenotype of cancer. Viculin has been implicated as an inhibitor of cell migration, as it enhanced cellular adhesiveness [1-4]. Loss or reduced expression of vinculin is related to the metastatic potential of squamous cell carcinomas (SCC), although in situ SCC exhibits high level expression of vinculin [5]. The role of vinculin in suppression of the malignant phenotype of SCC still remains to be solved.

Vinculin is a well-known component of cell-ECM adhesions by interacting with several focal adhesion proteins such as talin, paxillin, tensin, VASP, zyxin, vinexin, filamin, α-actinin, actin and focal adhesion kinase (FAK) either directly or indirectly [6]. It possesses tumor suppressive functions. Studies using F9 embryonic mouse carcinoma cells showed that vinculin knockout cells exhibit more rounded phenotype, less spreading, smaller focal adhesions and increased motility compared with wild type [1,2]. Mouse embryonic fibroblasts from vinculin knockout mouse also showed decreased cell-matrix adhesion, increased motility and FAK phosphorylation compared with wild-type cells [7]. Vinculin competes with paxillin for binding to FAK, which modulates extracellular signal–regulated kinase (ERK) activation in the downstream of FAK/paxillin [8].

Vinculin also localizes at and modulates cell-cell adhesions [6,9]. It binds to α- and β-catenin, which bind to one another and regulate cell-cell adhesion by interacting with cadherin adhesion receptors. The interaction between vinculin and catenins facilitates the stability of E-cadherin adhesions [10,11]. This enhanced cadherin adhesion stabilize a well-known tumor suppressor phosphatase and tensin homolog deleted on chromosomal 10 (PTEN) by interfering with ubiquitin-mediated proteolytic degradation of PTEN, which results from the binding of vinculin with membrane-associated guanylate-kinase inverted 2/β–catenin complex [12]. Loss of vinculin attenuates the protein level of PTEN, which is recovered by ectopic expression of vinculin. PTEN stabilizes E-cadherin adhesions, and is often deleted in cancers [13]. Consequently, vinculin may facilitate tumor suppressive function of E-cadherin and PTEN.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) is considered to play a significant role in tumor progression, as its expression correlates most closely with the invasive phenotype of human tumors among MMPs and the inhibition of MT1-MMP suppresses tumor cell invasion both *in vitro* and *in vivo* [14]. MT1-MMP was originally identified as a tumor-specific MMP-2 activator [15]. It activates MMP-2 and -13 and degrades a wide range of ECM components, including type I, II, III collagen, laminins, and fibronectin. This enzyme also processes and interacts with membrane-tethered proteins such as integrins and CD44 [14]. The activity of MT1-MMP is up-regulated by ERK [16,17] and down-regulated by PTEN [18].

The aim of this study is to understand how vinculin regulates motility and invasiveness of

SCC cells that lacks PTEN. We analyzed the effect of vinculin knockdown on MT1-MMP activity and invasion of SCC cells deficient in PTEN.

2. Materials and methods

2.1.Cell culture and reagents

Human oral SCC HSC-3 and HSC-4 cells were obtained from the Health Science Research Bank (Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The reagents used were type I collagen (Nitta Gelatin, Osaka, Japan); MEK inhibitor (PD98059) (Merck Millipore, Temecula, CA, USA). A synthetic MMP inhibitor (BB94) was a kind gift from the Kotobuki Pharmaceutical Co., Ltd. (Nagano, Japan). An anti-MT1-MMP antibody was gifted by Dai-ichi fine chemicals (Toyama, Japan). The immunological reagents used were anti-E-cadherin, anti-β-catenin, anti-ERK2, and anti-paxillin antibodies (BD Biosciences, Bedford, MA, USA); anti-phospho-p44/42 MAPK and anti-PTEN antibodies (Cell Signaling Technology, Danvers, MA, USA); anti-α-tubulin and anti-vinculin antibodies (Sigma-Aldrich, St Louis, MO, USA); an anti-vinculin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); an anti-p53 antibody (Merck Millipore); Rhodamine-labeled phalloidin, Hoechst 33342, and Alexa Fluor-labeled secondary antibodies (Molecular Probes, Eugene, OR, USA).

2.2.Small interfering RNA (siRNA)-mediated protein knockdown

A siRNA for negative control was purchased from Qiagen (Valencia, CA, USA). The siRNA sequences used here were: *mt1-mmp*, 5'-GCGAUGAAGUCUUCACUUATT; *vinculin-1*, 5'-GGCAUAGAGGAAGCUUUAATT; *vinculin-3*, 5'-GCCAAGCAGUGCACAGAUATT. Cells were transfected with 20 nM of siRNA duplexes in Opti-MEM (Invitrogen, Carlsbad, CA, USA) using Lipofectamine RNAi MAX (Invitrogen), according to the manufacturer's instructions, and were incubated for 48 h.

2.3.Immunoblotting

Cells were washed with phosphate-buffered saline (PBS), and homogenized in SDS lysis buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 2 mM NaF, and 1% SDS. Protein concentration was determined using BCA assay (Pierce, Thermo Scientific, Rockford, IL, USA). Samples were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were reacted with the indicated antibodies. The signal was monitored using a Li-COR Odyssey IR

imaging system (Lincoln, NE, USA).

2.4.Immunofluorescence staining

Cells grown on the coverslips were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and reacted with indicated antibodies. Samples were observed by confocal laser scanning microscopy LSM510 (Carl Zeiss, Jena, Germany).

2.5.Real-time PCR quantification

cDNA were generated from total RNA using a QuantiTect reverse transcription kit (Quiagen). Real-time quantitative PCR experiments were carried out using the following PCR primers for *mt1-mmp*: forward (5'-GGAATAACCAAGTGATGGATGG) and reverse (5'-TTGTTTCCACGGAAGAAGTAGG). Hs_GAPDH_1_SG QuantiTect Primer (Qiagen) was used to monitor the amplification of GAPDH gene transcript as a control.

2.6.Cell motility

HSC-4 cells transfected with siRNA were replated on 35-mm dishes coated with 300 μg/ml of type I collagen in 10% FBS/DMEM with or without BB94 (10 μM). Two hour after replating, cell migration was monitored using an Cultured Cell Monitoring System (Astec, Fukuoka, Japan) for 6 h. Video images were collected at 20-min intervals, digitized, and analyzed using Image J software.

3. Results

3.1. Vinculin knockdown promotes MT1-MMP-mediated proMMP-2 activation

Loss of vinculin promotes ubiquitin-mediated proteolysis of PTEN, which regulates a variety of proteins as a tumor suppressor. In order to study the cellular function of vinculin other than PTEN stabilization in SCC, we employed SCC HSC-4 cells that lacked PTEN expression in this study (Fig. 1A). Consistent with previous studies [1-4], vinculin knockdown cells showed lower cell-cell and cell-ECM adhesions compared with mock-transfected HSC-4 cells (Fig. 1B). Cell-cell adhesions supported by β-catenin were reduced in vinculin knockdown cells; cell-ECM adhesion stained by anti-paxillin antibody was poor-organized and smaller in vinculin-depleted cells than mock-transfected cells. Next, the effect of vinculin knockdown on MT1-MMP-mediated proMMP-2 activation was examined. HSC-4 cells expressed endogenous MT1-MMP, and thereby induced the activation

of proMMP-2, which was suppressed by the transfection of siRNA for MT1-MMP (Fig. 1C). Enhanced proMMP-2 activation in vinculin-knockdowned cells was completely inhibited by knockdown of MT1-MMP. In parallel with proMMP-2 activation, the protein level of MT1-MMP was increased in vinculin-depleted cells. The expression level of MT1-MMP mRNA examined by real-time PCR quantification was up-regulated by 2 to 2.5-fold in vinculin knockdown cells compared with mock-transfected cells (Fig. 1D). These results indicate that knockdown of vinculin up-regulates the expression of MT1-MMP at transcription level, resulting in the promotion of MT1-MMP activity including proMMP-2 activation.

3.2. Up-regulation of MT1-MMP by vinculin knockdown requires ERK

Vinculin knockdown induces the association between FAK and paxillin, which results in the increase of ERK activation [8]. The effect of MEK/ERK inhibition on MT1-MMP activity stimulated by vinculin knockdown was tested. Treatment with MEK inhibitor PD98059 repressed both ERK activation and MT1-MMP-mediated proMMP-2 activation in vinculin-depleted HSC-4 cells (Fig. 2A). In parallel with reduced proMMP-2 activation, up-regulation of MT1-MMP mRNA was suppressed by PD98059 (Fig. 2B). These results suggest that ERK activation by FAK/paxillin is required for up-regulation of MT1-MMP in vinculin-depleted HSC-4 cells.

3.3. Vinculin suppresses cell motility on collagen

Cell migration is stimulated by facilitating focal adhesion turnover through ECM degradation by MT1-MMP [14-16]. Then, we examined whether vinculin knockdown promotes the cell motility on type I collagen (Fig. 3A). The time lapse analysis of vinculin knockdown cells showed that vinculin-depleted cells migrated a longer distance than mock-transfected cells on type I collagen, which was suppressed by treatment with MMP inhibitor BB94 (Fig. 3B). On type I collagen, treatment with BB94 attenuated both MT1-MMP-mediated proMMP-2 activation and ERK activation in vinculin-depleted HSC-4 cells (Fig. 3C). These results show that MT1-MMP activity stimulates migration and ERK activation in vinculin-depleted HSC-4 cells on type I collagen.

4. Discussion

Here we demonstrated that vinculin knockdown in SCC induced phenotypical change by reducing cell-cell and cell-ECM adhesions and stimulated MT1-MMP expression, resulting in enhanced cell migration on type I collagen. Vincuin interferes with the activation of

FAK/Paxillin pathway [8], and augments PTEN stability [12]. PTEN inhibits cell surface expression of MT1-MMP, resulting in the reduction of MT1-MMP activity [18]. As HSC-4 cells used in this study lack PTEN expression (Fig. 1), vinculin knockdown may induce MT1-MMP transcription through the activation of FAK/Paxillin/ERK pathway without involvement of PTEN reduction. As MT1-MMP activity is up-regulated by ERK [16,17], treatment of vinculin-knockdown cells with MEK inhibitor PD98059 abrogated MT1-MMP induction (Fig. 2).

Pericellular collagenolysis caused by up-regulated MT1-MMP through vinculin knockdown further activates FAK/Paxillin/ERK pathway, and induces cell migration on type I collagen (Fig. 3). The reduction of cellular adhesiveness by vinculin knockdown may also contribute to migration of HSC-4 cells. Both of MT1-MMP and paxillin are required for proliferation of HSC-4 cells during collagen gel culture [19]. Vinculin expression may also affect cell proliferation through modulation of the FAK-paxillin interaction and MT1-MMP expression.

Knockout of vinculin in F9 embryonic mouse carcinoma cells and mouse embryonic fibroblasts show increased not only cell motility but also survival by activating FAK/Paxillin/ERK pathway [8,20]. Recent studies have demonstrated that MT1-MMP protects cancer cells from apoptosis induced by collagen gel culture [21]. HSC-4 cells used here express mutant form of p53-R248Q [22] and lack the expression of PTEN (Fig. 1). MT1-MMP may facilitate further HSC-4 cell survival induced by vinculin knockdown.

The loss of vinculin links advanced poor prognosis in cancer patients [5,23,24]. Present findings revealed that vinculin is one of key molecules controlling SCC progression through FAK/Paxillin/ERK pathway and thereby MT1-MMP expression.

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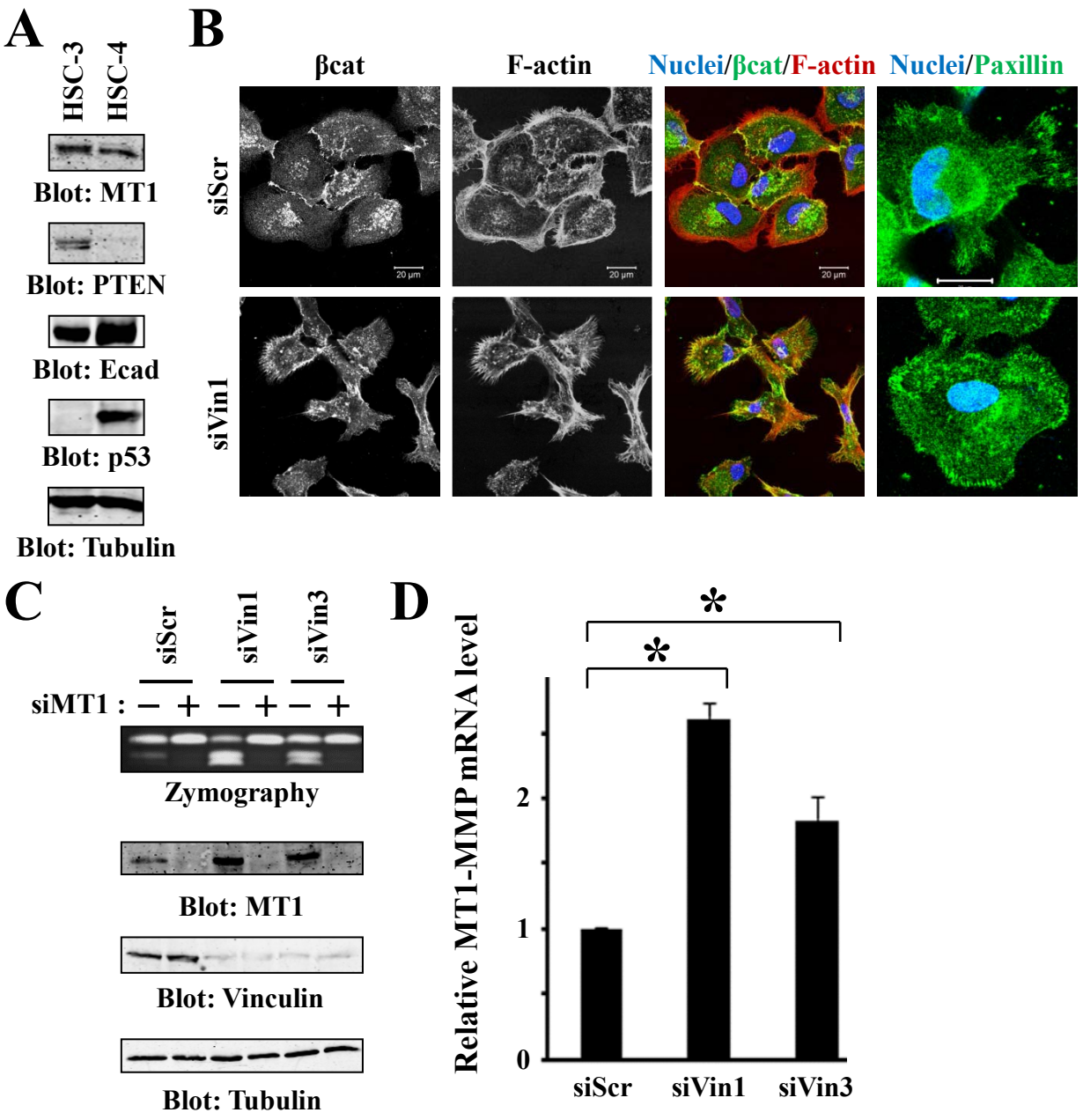
Figure legends

Fig. 1. Vinculin knockdown promotes MT1-MMP-mediated proMMP-2 activation. (A) Cell lysates from either HSC-3 or HSC-4 cells were analyzed by immunoblotting using MT1-MMP (MT1), anti-PTEN, anti-E-cadherin (Ecad), anti-p53 or anti-tubulin antibodies. (B) HSC-4 cells were cultured on glass coverslips, and transfected with siRNA for control (siScr) or vinculin (siVin1) for 48 h. The cells were analyzed by immunofluorescence staining using anti-β-catenin (βcat) or anti-paxillin antibodies, Rhodamine-phalloidin (F-actin), and Hoechst 33342 (Nuclei). Scale bars are 20 μm. (C) HSC-4 cells were transfected with siRNA for either control or vinculin (siVin1 or siVin3) plus siRNA for MT1-MMP (siMT1). The conditioned media were analyzed by gelatin zymography, and cells lysates were analyzed by immunoblotting using anti-MT1-MMP, anti-vinculin or anti-tubulin antibodies. (D) HSC-4 cells were transfected with siRNA for either control or vinculin (siVin1 or siVin3) and mRNA was prepared from these cells. Quantitative RT-PCR for mRNA expression of MT1-MMP was performed as described in "Section 2". Expressions of MT1-MMP mRNA are normalized to that of cells transfected with siRNA for control. The *error bars* are standard deviations of the mean values obtained from three independent experiments. *, $p < 0.05$.

Fig.2. Up-regulation of MT1-MMP by vinculin knockdown requires ERK activation. (A) HSC-4 cells were transfected with siRNA for either control or vinculin (siVin1 or siVin3) for 48 h. After washing, cells were cultured in the absence or presence of PD98059 (25 μM) for 24 h. The conditioned media were analyzed by gelatin zymography, and cells lysates were analyzed by immunoblotting using anti-phospho-p44/42 MAP kinase (pERK), anti-ERK2, anti-vinculin, or anti-tubulin antibodies. (B) Alternatively, mRNA was prepared from these cells, and quantitative RT-PCR for mRNA level of MT1-MMP was performed. Expressions of MT1-MMP mRNA are normalized to that of cells transfected with siRNA for control in the absence of PD98059. The *error bars* are standard deviations of the mean values obtained from three independent experiments. *, $p < 0.05$.

Fig.3. Vinculin knockdown promotes cell motility on collagen. HSC-4 cells were transfected with siRNA for either control or vinculin for 48 h. The cells were replated onto collagen-coated dishes in the absence or presence of BB94 (10 μM) and cell motility was monitored for 6 h. Tracks of migrating cells were collected, digitized, and analyzed using Image J software. (A) Tracks of migrating cells at 60-min intervals. Scale bars are 10 μm. (B) Migrating distance of individual cells were determined by individual tracking of > 20 cells pooled from three independent experiments. Data show the percentage of migrating cells for the different migrating distances. Columns, mean; bars, SE. (C) HSC-4 cells were transfected

with siRNA for either control or vinculin and were replated onto collagen-coated dishes in the absence or presence of BB94 for 24 h. The conditioned media were analyzed by gelatin zymography, and cells lysates were analyzed by immunoblotting using anti-vinculin, anti-phospho-p44/42 MAP kinase, anti-ERK2, or anti-tubulin antibodies.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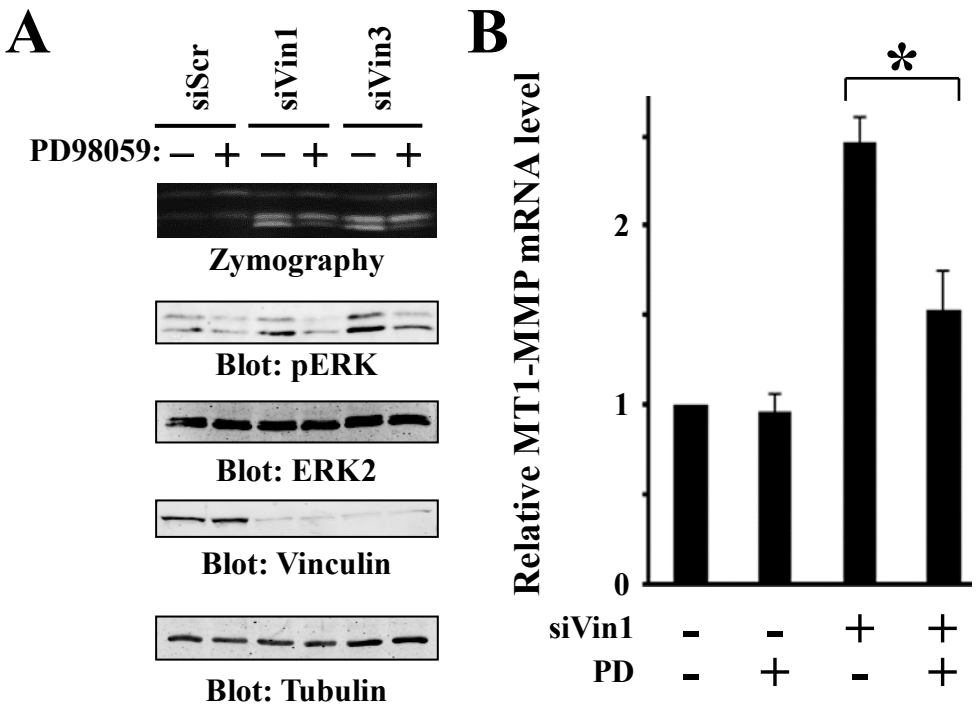
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