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MT1-MMP prevents growth inhibition by three dimensional fibronectin matrix

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Research Letters

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3 Figures

Abstract

The extracellular microenvironment plays a key role in regulation of cellular functions and growth control. We show here that membrane-type 1 matrix metalloproteinase (MT1-MMP) acts as a growth promoter in confluent culture. When MT1-MMP was silenced in HT1080 fibrosarcoma cells, cells created three dimensional (3D) fibronectin matrix in a confluent culture, and growth of cells embedded within it was retarded. Formation of 3D fibronectin matrix initiated by MT1-MMP silencing was impeded by knockdown of either FN or integrin β_1, which resulted in restoration of cell growth. When cells in 3D fibronectin matrix were treated with integrin β_1 neutralizing antibody, cells underwent S phase entry. These results suggest that MT1-MMP prevents growth suppression by 3D fibronectin matrix, which is mediated through integrin β_1.

Keywords: Tumor, ECM, Growth, Integrin, MT1-MMP

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FN, fibronectin; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP; PBS, phosphate-buffered saline; pRb, retinoblastoma protein; siRNA, small interfering RNA; 3D, three-dimensional.

Introduction

Tissue development, remodeling, and homeostasis are governed by adhesion of cells to extracellular matrix (ECM) and neighboring cells. The ECM not only provides cell anchorage but also regulates gene expression, cell proliferation, differentiation, and motility [1,2]. It is reorganized and remodeled by synthesis, contraction, and degradation. The integrin family of transmembrane proteins forms heterodimers that function as receptors for ECM proteins. Cells predominantly interact with the ECM through integrins, which induces the formation of cell-matrix adhesions including focal adhesions, fibrillar adhesions and other variants such as nascent adhesions, focal complexes and three-dimensional (3D) matrix adhesions, as well as the degradative structure podosomes and invadopodia [1-3].

Recently it has become clear that stiffness and topology of ECM surrounding cells regulates integrin signals and cell phenotype. The stiffness of ECM is regulated by its composition and organization [4]. When cells are cultured within 3D matrices including collagen gel, matrigel, and fibroblasts-derived 3D matrix, integrin signals are reduced compared with tissue culture dishes (2D), which is insufficient to support cell proliferation [5-7]. Tumor cell often have abnormal interactions with ECM due to alternations in integrins and ECM degrading enzymes such as matrix metalloproteinases (MMPs) [1,2].

MMPs are considered as key enzymes in tumor progression, as they degrade/modify ECM and cleave membrane proteins [8]. Among them, membrane-type 1 MMP (MT1-MMP) plays a significant role in tumor progression. It was originally identified as a tumor-specific activator of MMP-2 [9] and is now known to activate MMP-13 and degrade a wide range of ECM components, including collagens, laminins, and fibronectin (FN) [8]. This enzyme also processes and interacts with membrane proteins such as integrins, CD44, syndecan-1, and tissue transglutaminase [8,10-12]. It has been demonstrated that MT1-MMP promotes cell migration and invasion [13-16], and serves as a collagenolytic growth factor that provides tumor cells with the space to grow but not the ability to accelerate proliferative responses by overcoming the growth-suppressive signals from 3D collagen matrix [17-19].

FN assembly is a cell-dependent process, as binding to cell-surface integrins is necessary to initiate FN polymerization and assembly. Subsequent steps convert FN into a dense 3D meshwork of interconnected assembled FN that provides a dynamic environment for cells [20,21]. Oncogenically transformed cells are known to exhibit decreased synthesis and increased ability to degrade FN, resulting in a decrease of FN matrix [22]. Studies from our and other laboratories have indicated that MT1-MMP prevents FN matrix assembly, which causes promotion of cell motility [23,24]. In this study, we demonstrate that MT1-MMP promotes cell proliferation by impeding formation of 3D FN matrix.

1. Materials and methods

2.1. *Cell culture and reagents*

HT1080 cells were obtained from the Health Science Research Bank (Osaka, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum. An anti-MT1-MMP antibody was gifted by Fuji Fine Chemicals (Toyama, Japan). Glass coverslips were purchased from Asahi Techno Glass (Tokyo, Japan). The immunological reagents used were anti-retinoblastoma protein (pRb), anti-phosphorylated ($pSer^{807/811}$) Rb (ppRb), anti-paxillin, and anti-extracellualr signal-regulated kinase (ERK) 2 antibodies (BD Biosciences, Bedford, MD, USA); anti-FN and anti-α-tubulin antibodies (Sigma-Aldrich); anti-integrin β_1 and anti-$p21^{Cip1}$ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); an anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology, Danvers, MA, USA); an inhibitory anti-integrin β_1 antibody (4B4: Beckman Coulter, Fullerton, CA, USA); a mouse control IgG (Dako, Denmark); Rhodamine-labeled phalloidin, DAPI, and Alexa Fluor-labeled secondary antibodies (Molecular Probes, Eugene, OR, USA).

2.2. *Expression plasmids and transfection*

A fluorescent ubiqutination-based cell cycle indicator, pFucci-G_1 Orange, was purchased from MBL (Nagoya, Japan). pFucci-G_1 Orange was transfected into HT1080 cells by standard calcium phosphate methods and selected with G418 to establish stable transfectants.

2.3. *Small interfering RNA (siRNA)-mediated protein knockdown*

siRNA for negative control and human FN were purchased from Qiagen. The siRNA sequences were *mt1-mmp*, 5'-GCGAUGAAGUCUUCACUUATT; *integrin* β_1, 5'-AAUGUAACCAACCGUAGCATT. Cells were transfected with 20 nM of siRNA duplexes in Opti-MEM (Gibco) using Lipofectamine RNAi MAX (Invitrogen), according to the manufacturer's instructions, and were incubated for 48 h.

2.4. *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, Rockford, IL, USA). Samples were separated by electrophoresis on SDS-polyacrylamide gels

and transferred onto nitrocellulose membranes. The blots were analyzed using Odyssey infrared imaging system (Li-COR Bioscience, Lincoln, NE, USA).

2.5. *Immunofluorescence staining*

Cells gown on the glass coverslips were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 for 5 min. After blocking with Image-iT FX signal enhancer (Molecular Probes), cells were reacted with indicated antibodies. Samples were observed by confocal laser scanning microscopy LSM510 (Carl Zeiss, Jena, Germany).

2.6. *5-etynyl-2'-deoxyuridine (EdU) labeling and detection*

Cell proliferation and S-phase entry in cells were measured by cultivation with EdU (10 μM) for 2 h, according to the manufacturer's instructions. EdU incorporation was determined by confocal laser microscopy using Click-iT EdU Alexa Fluor 555 imaging kit (Invitrogen) and analyzed with image J software.

2.7. *Cell viability*

HT1080 cells were cultured with 96-well culture plates for indicated periods. Cell viability 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).

2. Results

3.1. *MT1-MMP induces cell growth*

HT1080 cell line, which has one activated allele of N-*ras* and expresses MT1-MMP, shows the invasive phenotype [23,25]. We previously reported that treatment of HT1080 cells with the synthetic MMP inhibitor BB94 promotes dexamethasone-induced FN assembly, and down-regulates cell motility, concluding that MT1-MMP stimulates cell migration and invasion by negatively regulating FN assembly [23].

In this study, we examined the effect of MT1-MMP silencing on cell proliferation in a dense culture condition, which results in FN assembly. First, FN assembly and growth pattern of MT-MMP-silenced HT1080 cells cultured in a dense condition were visualized three-dimensionally (Fig. 1A). FN fibrils assembled at cell-cell interface on both basal and lateral surface of MT1-MMP-silenced HT1080 cells when cells were cultured in a dense condition. Three dimensional images which are stacked up on the bottom z section demonstrated that assembled FN fibrils interconnect and then form a meshwork surrounding the cells three dimensionally. A part of FN meshwork was hidden by overlaid nuclei (red hatched circles) and some nuclei were present under FN meshwork (yellow hatched circles). Thus, MT1-MMP-silenced HT1080 cells were embedded in 3D FN matrix. Next, we examined the expression of growth-associated molecules by immunoblotting. MT1-MMP knockdown resulted in the increase of cell-associated FN level and cyclin-dependent kinase inhibitor $p21^{Cip1}$ expression and the decrease of phosphorylated ERK and pRB at confluence (Fig. 1B). The number of EdU-incorporating mitotic cells was significantly attenuated in the cells transfected with MT1-MMP siRNA (Fig. 1C); contrary, the number of cells in G1 phase was remarkably increased in MT1-MMP-silenced cells, which was accompanied with 3D FN matrix formation (Fig. 1D). Concomitantly, cell viability was reduced by MT1-MMP knockdown (Fig. 1E). These results suggest that MT1-MMP regulates cell cycle progression and thereby cell proliferation in confluent cultures.

3.2. *Disruption of FN matrix assembly by MT1-MMP stimulates cell proliferation*

Among the FN binding integrins, integrin $\alpha_5\beta_1$ is considered of major importance for the formation of FN assembly [9,10]. HT1080 cells were transfected with siRNA for MT1-MMP and/or integrin β_1, and formation of 3D FN matrix was examined. Cell-associated FN increased by MT1-MMP knockdown was reduced by double-knockdown with integrin β_1, (Fig. 2A), which caused only short FN fibrils but not 3D FN matrix formation (Fig. 2B). Formation of 3D FN matrix induced cell matrix adhesions at both basal and lateral surface of MT1-MMP-silenced cells. FN assembly is known to require cellular FN synthesis [20,21]. To

confirm it, HT1080 cells were transfected with siRNA for MT1-MMP and/or FN. Cell-associated FN increased by MT1-MMP knockdown was abrogated by double-knockdown with cellular FN, which resulted in the loss of 3D FN matrix (Fig. 2C). These results show that both cellular FN and integrin β_1 are crucial for 3D FN matrix formation induced by MT1-MMP knockdown.

In order to examine the effect of 3D FN matrix on HT1080 cell proliferation, cells were transfected with siRNA for MT1-MMP and/or integrin β_1 or FN, and cultured for 2 days at high cell density. Cell proliferation attenuated by MT1-MMP knockdown was recovered by co-transfection of siRNA for FN or integrin β_1, whereas neither integrin β_1 nor FN knockdown alone affected cell proliferation significantly (Fig. 2D). These results indicate that assembled FN matrix interferes with HT1080 cell proliferation.

3.3. *Integrin β_1 mediates growth suppression by 3D FN matrix*

To seek the molecular mechanism by which 3D FN matrix suppresses cell growth, we examined the effect of integrin β_1 inhibitory antibody 4B4 that interferes with interaction of integrin β_1 with its ligand. Cell-associated FN increased by MT1-MMP knockdown was attenuated by treatment with 4B4 for 12 h, which caused the loss of 3D FN matrix (Fig. 3A). These results further confirmed that integrin β_1 is involved in 3D-FN matrix formation. Next, we examined the role of integrin β_1 in regulation of cell proliferation in 3D FN matrix using the inhibitory antibody. After FN matrix assembly was completed by MT1-MMP silencing, cells were incubated with 4B4 antibody for 2 h, and then the number of mitotic cells was analyzed by EdU-incorporation (Fig. 3A). Although the number of EdU-incorporating mitotic cells was unaffected by 4B4 treatment in mock-transfected cells, the reduction of mitotic cells by MT1-MMP-silencing was partially rescued by treatment with 4B4 (Fig. 3B and C). These results suggest that integrin β_1 mediates growth suppression of HT1080 cells embedded within 3D FN matrix, the formation of which is inhibited by MT1-MMP.

3. Discussion

Focal adhesions are sites of integrin clustering that link the actin cytoskeleton to ECM and develop to fibrillar adhesions, which facilitates FN assembly by creating cytoskeletal tension [1,2]. This increased cytoskeletal tension is required for further formation of FN fibril and 3D FN matrix. We have previously reported that MT1-MMP degrades assembled FN on cell surface, which accelerates turnover of focal adhesions and thereby prevents maturation of focal adhesion and FN assembly, resulting in promotion of cell migration [23]. We demonstrate here that destruction of 3D FN matrix by MT1-MMP releases confluent cells from the growth suppression by 3D FN matrix.

Oncogenically transformed cells are known to exhibit decreased synthesis and increased ability to degrade FN, resulting in a decrease of FN matrix [22]. Silencing of MT1-MMP caused FN assembly in HT1080 cells to form 3D FN matrix, and cells were embedded in it (Fig. 1A). Cells on 2D matrices exhibit behaviors drastically different from cells within 3D matrices. Culturing cells on 2D substrates induces integrin clustering and robust focal adhesion formation and thereby generates signals, which promote cell proliferation and migration. In contrast, integrin signals are reduced in cells embedded in 3D matrices including collagen gel, matrigel, and fibroblasts-derived 3D matrix, and thereby cell proliferation and migration are suppressed [1-7]. Three-dimensional broad distribution of integrin on cells embedded in 3D matrices may be insufficient to generate growth signal.

MT1-MMP knockdown resulted in formation of 3D FN matrix and growth suppression in HT1080 cells (Fig. 1). Silencing of FN or integrin β_1, which interferes with FN assembly, rescued growth suppression induced by MT1-MMP knockdown (Fig. 2), indicating that 3D FN matrix formed through MT1-MMP knockdown caused growth suppression of HT1080 cells. Confluent MT1-MMP-silenced HT1080 cells cause FN assembly three-dimensionally along cell-cell adhesion and the lateral surface (Fig. 2). Paxillin was co-localized three-dimensionally with assembled FN in the dense population of cells (Fig. 2). The three-dimensionally enlarged cell-ECM adhesions of dense cell population visualized by paxillin staining suggest the reduced signal transduction for cell proliferation. Addition of neutralizing antibody against integrin β_1 to HT1080 cells interferes with FN assembly promoted by MT1-MMP knockdown. Furthermore, treatment of cells already embedded in FN matrix through MT1-MMP silencing with the neutralizing antibody recovered cell proliferation quickly (Fig. 3). It is known that the association of integrin $\alpha_2\beta_1$ with collagen fibrils generates the growth-suppressive signals in normal epithelial or cancer cells embedded in 3D collagen [26-28]. These results suggest that integrin β_1 is involved in not only FN assembly but also maintenance of cell-FN matrix adhesion, which negatively regulates integrin clustering associated with signal transduction for cell proliferation.

Recently it has become clear that stiffness and topology of ECM surrounding cells regulates integrin signals and cell phenotype [1-7]. Cell cycle progression, cell spreading and cytoskeletal organization are inhibited due to decreased integrin signals, when cells are plated on low-stiffness substrate but not deformable 2D substrates. Tumor stroma is stiffer than normal one; a randomly organized, compliant normal stroma is tumor suppressive. As tumors grow, fibroblasts reorganize a loose stroma into stiffer one [4,29]. Our findings agree with previous results demonstrating that decreased FN matrix is observed in numerous tumors and tumor-derived cell lines [25] and indicate that MT1-MMP is responsible at least in part for destruction of FN matrix. The control of not only deposition but also degradation is crucial for reorganization and remodeling of ECM. There may be a delicate balance between deposition and degradation of ECM to create stiff tumor stroma that promotes growth and invasion of tumors.

In conclusion, cells expressing MT1-MMP escape from growth suppressive condition by disrupting 3D FN matrix. As cellular functions including gene expression, cell proliferation, migration, and differentiation are controlled by ECM elasticity, further studies on regulation of ECM remodeling and stiffness by MT1-MMP might provide novel molecular targets for cancer therapy.

Conflict of interest statement

None declared.

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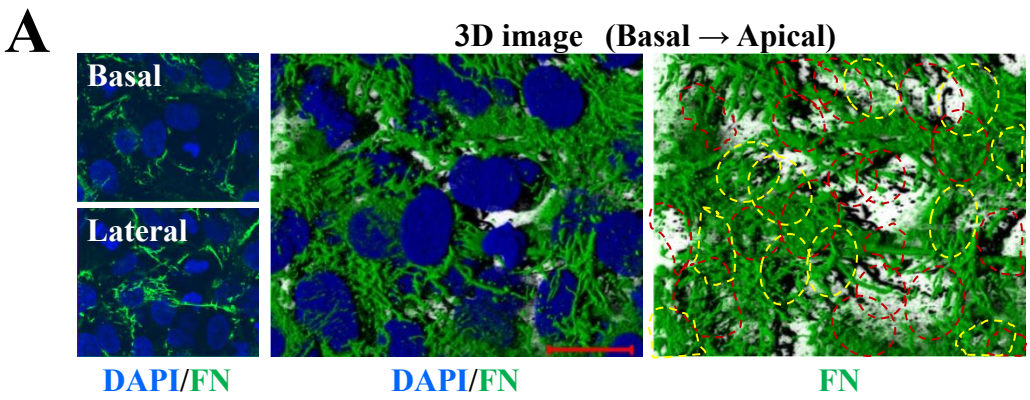
Figure legends

Fig. 1. MT1-MMP induces cell growth. (A) MT1-MMP-silenced HT1080 cells plated on the glass coverslips were stained with DAPI and anti-FN antibody. Confocal image of cells with z section were taken and analyzed. The images at basal and lateral levels were shown at left. For z section analysis, 50 images are stacked up on the bottom z section (3D images). Red hatched circles show FN meshwork hidden by overlaid nuclei. Yellow hatched circles indicate nuclei hidden by FN meshwork. (B) HT1080 cells were transfected with siRNA for control (siScr) or MT1-MMP (siMT1). The cell lysates were immunoblotted with anti-MT1-MMP (MT1), anti-FN, anti-phosphorylated ERK (pERK), anti-ERK2, anti-phosphorylated pRb (ppRb), anti-pRb, anti-$p21^{Cip1}$, or anti-tubulin antibodies. (C) HT1080 cells were transfected with siRNA for control or MT1-MMP and stained with DAPI and EdU. EdU-incorporating mitotic cells were observed by confocal laser scanning microscopy as described in "Materials and methods", and then the percentages of cells immnoreactive for EdU were counted (right panel). (D) HT1080 cells stably expressing pFucci-G_1 Orange were transfected with siRNA for control or MT1-MMP. Cells were stained with DAPI and anti-FN antibody, and then the percentages of cells in G_1 phase were counted (right panel). Scale bar, 20 μm; columns, mean; bars, SD; *, $p < 0.005$. (E) HT1080 cells were transfected with siRNA for control or MT1-MMP, and then cultured within 96-well culture plates (5×10^3 cells/well) for indicated periods. Cell viability was determined as described in "Materials and methods". Cell viabilities were normalized to the levels of the cells transfected with control siRNA at day 1. Columns, mean; bars, SD; *, $p < 0.005$ (compared to the siScr).

Fig. 2. Disruption of FN matrix assembly stimulates cell proliferation. (A) HT1080 cells were transfected with siRNA for control, MT1-MMP, or integrin β_1 (siIntβ_1). The cell lysates were immunoblotted with anti-MT1-MMP, anti-integrin β_1 (Intβ_1), anti-FN, or anti-tubulin antibodies. (B) The cells were also stained with DAPI, anti-paxillin and anti-FN antibodies. The images at basal and lateral levels were shown. Arrowheads indicate cell matrix adhesions at lateral surface. (C) HT1080 cells were transfected with siRNA for control, MT1-MMP, or FN (siFN). The cell lysates were immunoblotted with anti-FN, anti-MT1-MMP, or anti-tubulin antibodies. The cells were also stained with DAPI and anti-FN antibody. Scale bars, 20 μm. (D) HT1080 cells were transfected with siRNA for control, integrin β_1, FN and/or MT1-MMP and cultured with 96-well culture plates (1×10^4 cells/well) for 2 days. Cell viability was determined as described in "Materials and methods". Cell viabilities were normalized to the levels of the cells transfected with control siRNA. Columns, mean; bars, SD; *, $p < 0.005$ (compared to the siMT1 alone).

Fig. 3. 3D FN matrix suppresses cell growth. (A) HT1080 cells were transfected with siRNA for control or MT1-MMP. The cells were treated with 10 μg/ml of control mouse IgG or anti-integrin β_1 inhibitory antibody (4B4) for 12 h. The cell lysates were immunoblotted with anti-FN, anti-MT1-MMP, or anti-tubulin antibodies. Cells were also stained with DAPI, Rhodamine-labeled phalloidin (F-actin), and anti-FN antibody (right panel). (B) HT1080 cells transfeced with siRNA for control or MT1-MMP were reattached to coverslips for 24 h. Then, the cells were treated with control mouse IgG or 4B4 for 2h and analyzed with immunostaining with DAPI and anti-FN antibody. EdU-incorporating mitotic cells were observed by confocal laser scanning microscopy as described in "Materials and methods", and then the percentages of cells immnoreactive for EdU were counted. Scale bars, 20 μm. (C) Columns, mean; bars, SD; *, $p < 0.005$.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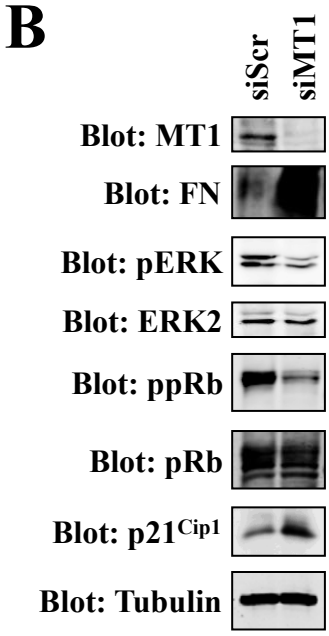
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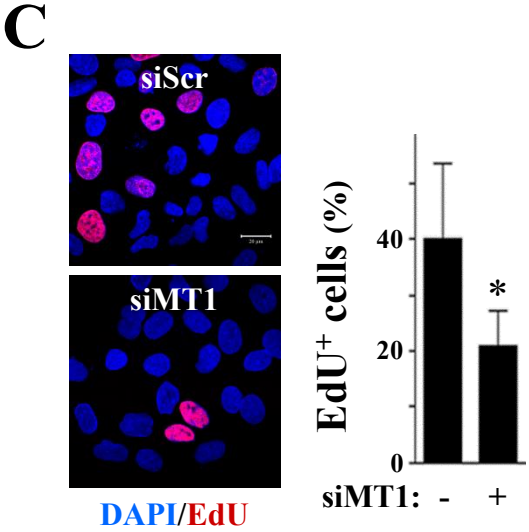
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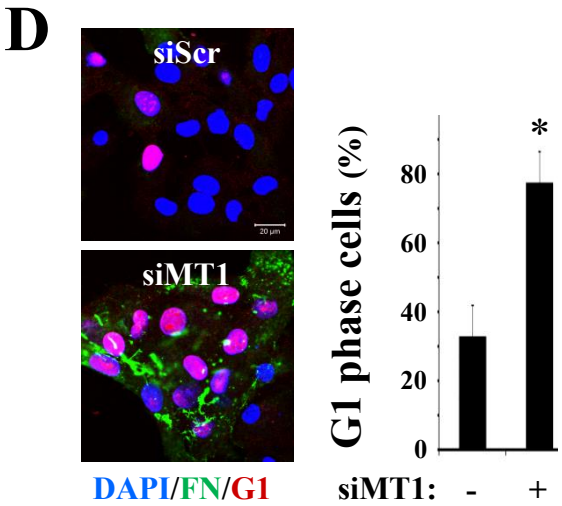
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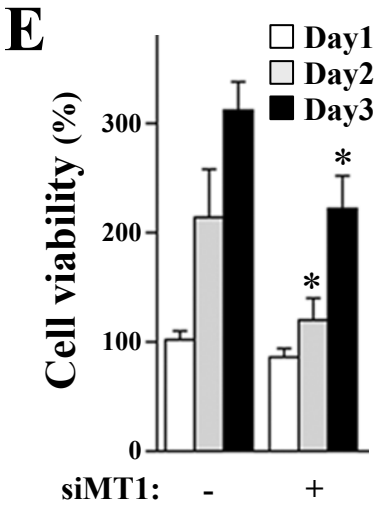
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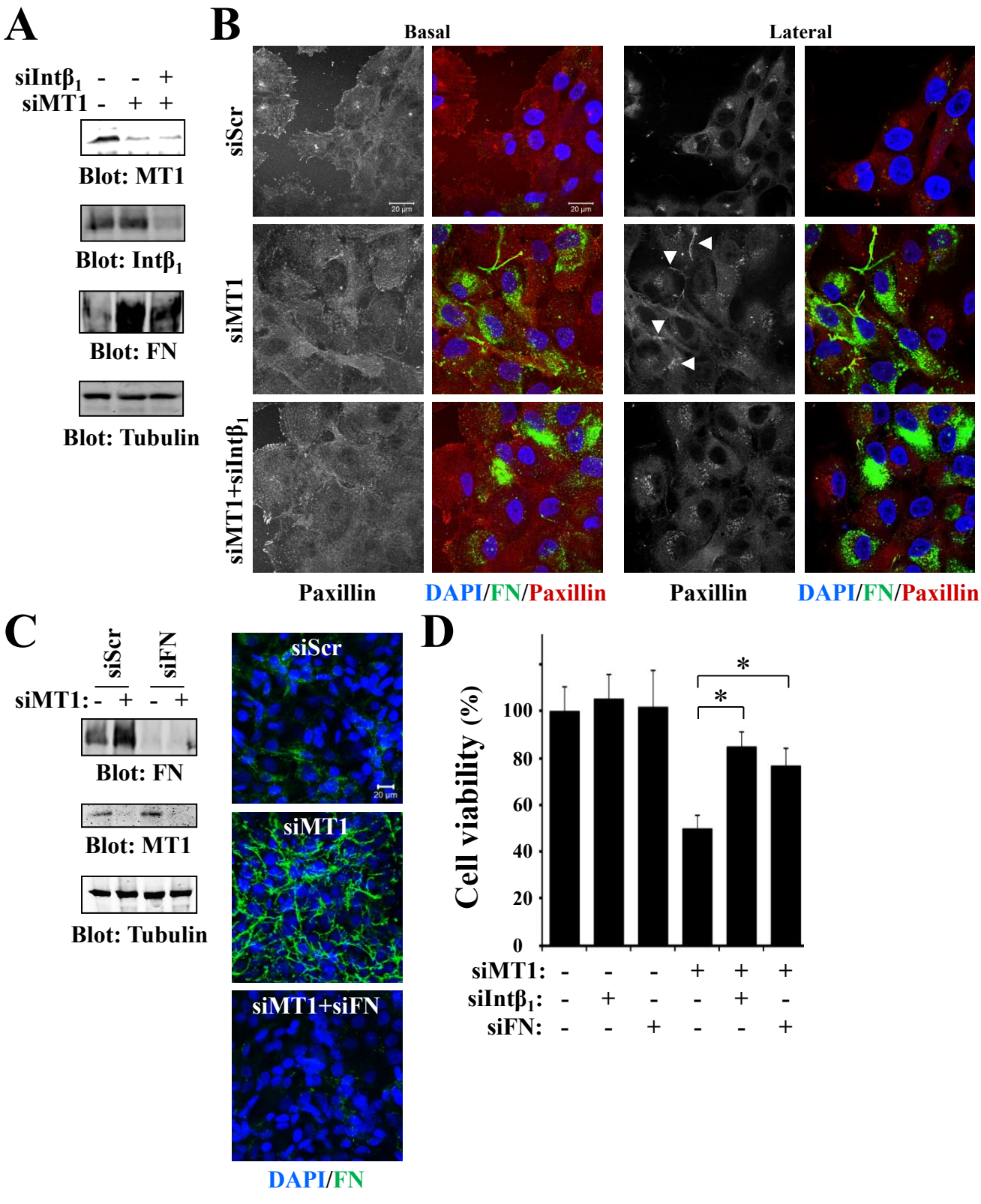
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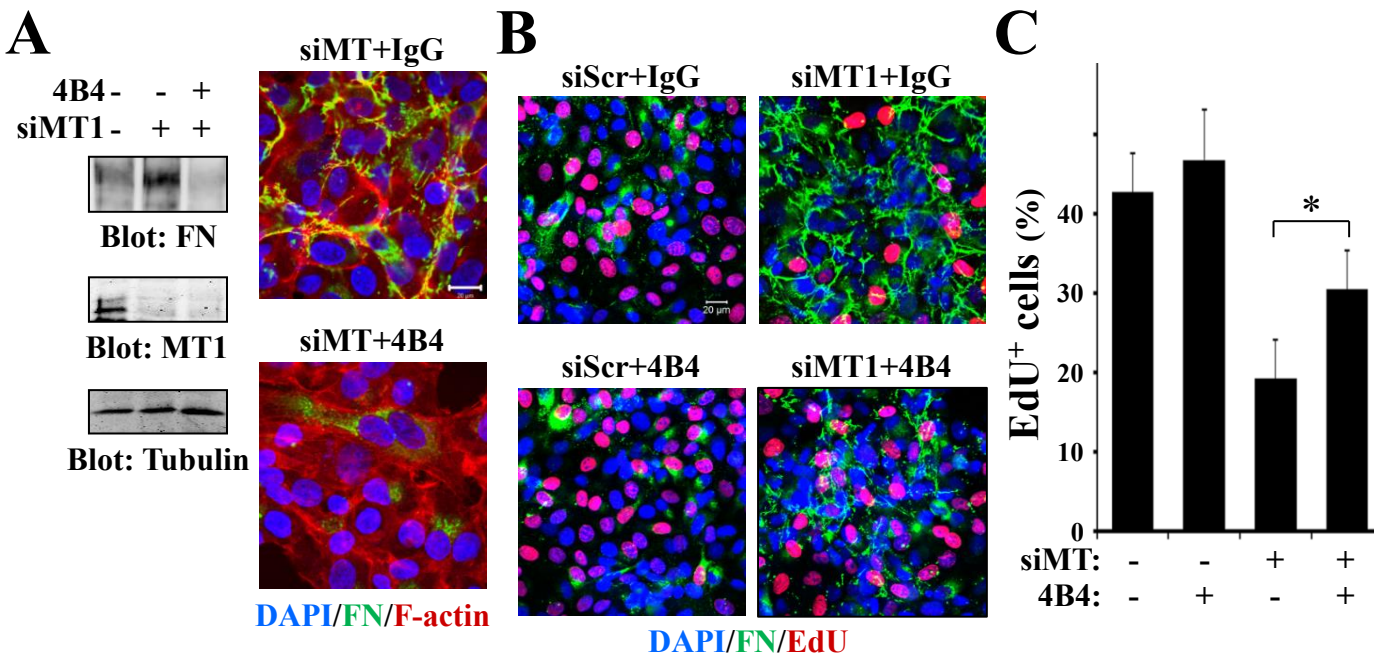
siIntβ1: - + - - + -
siFN: - - + - - +


A
4B4 - - +
siMT1 - + +
Blot: FN
Blot: MT1
Blot: Tubulin
siMT+IgG
siMT+4B4
DAPI/FN/F-actin
B
siScr+IgG
siMT1+IgG
siScr+4B4
siMT1+4B4
DAPI/FN/EdU
C
EdU+ cells (%)
0
10
20
30
40
*
siMT: - - + +
4B4: - + - +