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Membrane-type 1 matrix metalloproteinase regulates fibronectin assembly and N-cahderin adhesion

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; GFP, green fluorescence protein; MT1-MMP, membrane-type 1 matrix metalloproteinase; PBS, phosphate-buffered saline; siRNA, small interfering RNA

Abstract

Fibronectin matrix formation requires the increased cytoskeletal tension generated by cadherin adhesions, and is suppressed by membrane-type 1 matrix metalloproteinase (MT1-MMP). In a co-culture of Rat1 fibroblasts and MT1-MMP-silenced HT1080 cells, fibronectin fibrils extended from Rat1 to cell-matrix adhesions in HT1080 cells, and N-cadherin adhesions were formed between Rat1 and HT1080 cells. In control HT1080 cells contacting with Rat1 fibroblasts, cell-matrix adhesions were formed in the side away from Rat1 fibroblasts, and fibronectin assembly and N-cadherin adhesions were not formed. The role of N-cadherin adhesions in fibronectin matrix formation was studied using MT1-MMP-silenced HT1080 cells. MT1-MMP knockdown promoted fibronectin matrix assembly and N-cadherin adhesions in HT1080 cells, which was abrogated by double knockdown with either integrin β_1 or fibronectin**.** Conversely, inhibition of N-cadherin adhesions by its knockdown or treatment with its neutralizing antibody suppressed fibronectin matrix formation in MT1-MMP-silenced cells. These results demonstrate that fibronectin assembly initiated by MT1-MMP knockdown results in increase of N-cadherin adhesions, which are prerequisite for further fibronectin matrix formation.

Keywords: cell-matrix adhesion, Fibronectin, MT1-MMP, N-cadherin, Tumor

1. **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. ECM remodeling involves alterations in its synthesis, deposition, and degradation, and has an impact on cell behavior, affecting cell motility, survival and proliferation [1-3]. It is well documented that fibronectin (FN) assembly is a cell-dependent process, as binding to cell-surface integrins is necessary to induce FN polymerization and assembly. FN assembly promotes the deposition of a number of ECM proteins, including type I and III collagen. Continuous FN assembly causes the formation of FN matrix. This process requires interaction between integrin cytoplasmic domain and actin-associated proteins, which is promoted by the increased cytoskeletal tension generated by cadherin adhesions [4,5].

Cadherins are transmembrane receptors that mediate calcium-dependent cell-cell adhesion by homophilic association of their ectodomains [6]. The cadherin receptor intercellularly associates with several structural and signaling molecules, most notably the catenines. β-catenin binds directly to the cadherin cytoplasmic tail and to α-catenin to connect the actin cytoskeleton. N-cadherin is broadly expressed by cells in nervous, fibrous, muscular, adipose, tumor tissue can interact with α-, β-, and p120-catenins. Likewise

E-cadherin, N-cadherin can form adherens junction-like structures, which play important roles in the regulation of cell attachment, motility, growth, and survival [6].

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 [7-12]. This enzyme was originally identified as a tumor-specific processing enzyme for MMP-2 [12], and is now known to activate MMP-13 and degrade a wide range of ECM components, including type I, II, III collagen, laminins, and FN [7,8]. MT1-MMP also processes/interacts with membrane proteins such as integrins, CD44, syndecan-1, and tissue transglutaminase [7-10].

Coordination and interdependence of integrin-based cell-ECM adhesions and cadherin-dependent cell-cell adhesions regulates multiple cellular functions and are crucial for tissue homeostasis [13]. For instance, integrin-based cell-matrix adhesions are known to promote stability of cell-cell adhesion [14]. Studies from our and other laboratories have shown that MT1-MMP regulate FN assembly by cleaving it [15,16]. In this study, we demonstrate that MT1-MMP inhibits nascent FN assembly by not only cleaving it but also controlling cell-matrix adhesions in close proximity of cell-cell contacts, resulting in the reduction of N-cadherin adhesions and FN matrix assembly.

2. Materials and methods

2.1.Cell culture and reagents

Human fibrosarcoma HT1080 cells and Rat1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). An anti-MT1-MMP antibody was gifted by Dai-ichi fine chemicals (Toyama, Japan). The immunological reagents used were anti-FN, anti-α-tubulin, and anti-N-cadherin neutralizing (GC-4) antibodies (Sigma-Aldrich); anti-β-catenin and anti-N-cadherin antibodies (BD Biosciences); anti-N-cadherin and anti-integrin β_1 antibodies (Santa Cruz Biotechnology); a mouse control IgG (Dako); Hoechst33342 (Molecular Probes).

2.2.Expression plasmids and transfection

Plasmids encoding the puromycin-resistance gene (pHA262pur), green fluorescence protein (GFP), and GFP-tagged Paxillin were kind gifts from Dr. Kenneth M. Yamada (National Institute of Health, Bethesda, MD, USA). Expression plasmids were transfected into cells by a modified calcium phosphate method.

2.3.Stable transfection

HT1080 cells were co-transfected with pHA262Pur and either GFP or GFP-Paxillin plasmids. After transfection, the cells were cultivated in 10% FBS/DMEM containing puromycin. Single colonies were isolated after 3 weeks of selection.

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

siRNA for negative control and FN were purchased from Qiagen. The siRNA sequences were *mt1-mmp*, 5'-GCGAUGAAGUCUUCACUUATT; *n-cadherin*, 5'-GACUGGAUUUCCUGAAGAUTT; *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.

2.5.Direct co-culture

GFP- or GFP-Paxillin-HT1080 cells were transfected with siRNA for either control or MT1-MMP for 48h. Then, 1:1 mixture of Rat1 and labeled HT1080 cells were seeded and

cultured on the coverslips and cultured for 24 h

2.6.Immunoblotting and Immunoprecipitation

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).

For immunoprecipitation analysis, cells were washed twice with ice-cold PBS and homogenized in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 2 mM NaF, protease inhibitor cocktail (Nacalai tesque), 10% glycerol, 0.1% SDS, and 1% NP-40. Cell lysates were precipitated with indicated antibodies and protein G sepharose at 4°C. Samples were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes.

2.7.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).

3. Results

3.1. MT1-MMP disrupts initiation of FN assembly and N-cadherin adhesions

To analyze the role of endogenous MT1-MMP in the initial stage of FN assembly and N-cadherin adhesions, we used Rat1 fibroblasts and HT1080 cells. The level of N-cadherin and cell-associated FN were higher in Rat1 fibroblasts than in HT1080 cells, and only HT1080 cells expressed MT1-MMP that induced MMP-2 activation (Fig. 1A). To distinguish the two cell types, HT1080 cells were labeled with GFP. In a co-culture of Rat1 and control HT1080 cells at a low density (1.25×10^4 cells/mL each), FN assembled under the cell body of Rat1 but not HT1080 cells; however, in a co-culture using MT1-MMP-silenced HT1080 cells, short FN fibrils extended from Rat1 to adjacent HT1080 cells across the cell-cell borders (Fig. 1B). FN assembly was observed to be processed along cell-matrix adhesions [1,2]. MT1-MMP accelerates turnover of cell-matrix adhesions [17-19]. To visualize cell-matrix adhesions, GFP-Paxillin-expressing HT1080 cells were established. In MT1-MMP-silenced HT1080 cells, the number and size of cell-matrix adhesions visualized by GFP-Paxillin were larger than in control cells, and FN assembled to form short fibrils, which extended from Rat1 to cell-matrix adhesions of adjacent HT1080 cells in close proximity of cell-cell contact (Fig. 1C). In control HT1080 cells, cell-matrix adhesions were oriented toward migrating side

of cells, and decreased at the cell-cell contact side, and the extension of assembled FN from Rat1 to HT1080 cells was not observed. These observations suggest that FN assembly is initiated at stable cell-matrix adhesions in close proximity of cell-cell contacts, and FN assembles to link cell-matrix adhesions of adjacent cells.

In parallel with FN assembly linking Rat1 and MT1-MMP-silenced HT1080 cells, not only homotypic but also heterotypic N-cadherin adhesions were observed in Rat1 fibroblasts, and were co-localized with β–catenin (Fig. 1D). Monitoring of interaction between Rat1 and GFP-HT1080 cells with time-lapse video microscopy revealed that Rat1 and control HT1080 cells contact transiently and migrate away quickly; however, MT1-MMP-silenced HT1080 cells form stable cell-cell adhesions and aggregate with Rat1 fibroblasts (Fig. 1E). When Rat1 fibroblasts were co-cultured with MT1-MMP-silenced HT1080 cells at a high density (5×10^4 cells/mL each), the level of cell-associated FN and N-cadherin was higher than that of co-culture with control HT1080 cells. FN matrix was constructed surrounding Rat1 and MT1-MMP-silenced but not control HT1080 cells (Fig. 1F). These results suggest that rapid turnover of cell-matrix adhesions induced by MT1-MMP reduces FN assembly and N-cadherin adhesions, which may function for FN matrix formation.

3.2. FN assembly augments N-cadherin adhesions

To examine the relationship between FN matrix assembly and N-cadherin adhesions, HT1080 cells were transfected with siRNA for MT1-MMP and/or FN, and FN matrix assembly and N-cadherin adhesions were analyzed. MT1-MMP knockdown induced accumulation of cell-associated FN and N-cadherin, which was reduced by knockdown of FN (Fig. 2A). This accumulation of FN and N-cadherin by MT1-MMP knockdown resulted in formation of FN matrix and N-cadherin adhesions, which was abrogated by knockdown of FN (Fig. 2B). Integrin β_1 is responsible for FN assembly [5,20]. To test whether integrin β_1 is involved in N-cadherin accumulation induced by MT1-MMP knockdown, integrin β_1 was doubly knock-downed with MT1-MMP in HT1080 cells. The increase of N-cadherin and cell-associated FN by MT1-MMP knockdown was abrogated by double-knockdown with integrin β_1 (Fig. 2C). As N-cadherin is known to associate with integrin β_1 and β_3 [11], we examined whether N-cadherin accumulation induced by MT1-MMP knockdown affects the interaction between N-cadherin and integrin β_1. Immunoprecipitation analysis demonstrated complex formation of integrin β_1, FN and N-cadherin, which was augmented in MT1-MMP-silenced cells (Fig. 2D). These results indicate that FN assembly induced by MT1-MMP silencing facilitates the interaction between N-cadherin and integrin β_1, resulting in the accumulation and thereby promotion of N-cadherin adhesions.

3.3. FN matrix assembly requires increased N-cadherin

FN matrix assembly requires the increased cytoskeletal tension generated by cadherin adhesions [1-5]. We hypothesized that N-cadherin adhesions augmented by MT1-MMP knockdown facilitates further FN matrix assembly. To assess it, HT1080 cells were transfected with siRNA for MT1-MMP and/or N-cadherin, and FN matrix assembly was examined. The increase of cell-associated FN induced by MT1-MMP knockdown was reduced by double-knockdown with N-cadherin (Fig. 3A), which caused the loss of FN matrix formation (Fig. 3B). In addition, treatment of MT1-MMP-silenced cells with a neutralizing antibody against N-cadherin (GC-4) eliminates FN matrix assembly (Fig. 3C). These data demonstrate that FN assembly initiated by MT1-MMP knockdown increases N-cadherin adhesions, which are essential for further FN matrix formation.

4. Discussion

We have previously reported that degradation of ECM by MT1-MMP modulates cell motility by accelerating turnover of cell-matrix adhesions [17-19]. FN assembly requires interaction between FN-bound integrins and actin-associated proteins. The increased cytoskeletal tension generated by cadherin adhesion promotes further FN and FN fibril assembly, resulting in formation of FN matrix [1-5]. The present study demonstrates that FN assembly initiated by MT1-MMP knockdown causes the enhancement of N-cadherin adhesions, which are prerequisite for further FN matrix formation.

To understand the role of MT1-MMP in nascent FN assembly, we utilized a co-culture system of Rat1 fibroblasts and HT1080 cells. Short FN fibrils extended from Rat1 fibroblasts to cell-matrix adhesions on adjacent MT1-MMP-silenced but not control HT1080 cells in close proximity of cell-cell contacts (Fig.1). In parallel with FN assembly bridging cell-matrix adhesions of adjacent cells, not only homotypic but also heterotypic N-cadherin adhesions were formed among Rat1 fibroblasts and MT1-MMP-silenced but not control HT1080 cells. FN assembly and N-cadherin adhesions subsequently resulted in FN matrix formation. MT1-MMP functions at cell-matrix adhesions by interacting with focal adhesion kinase binding protein $p130^{Cas}$, resulting in the promotion of cell-matrix adhesion turnover [21,22]. Integrin-based cell-matrix adhesions are known to promote stability of cell-cell

adhesion [13,14]. Rapid turnover of cell-matrix adhesions by MT1-MMP may reduce nascent FN assembly and N-cadherin adhesions, both of which are prerequisite for FN matrix formation.

MT1-MMP knockdown promoted FN matrix assembly and N-cadherin adhesions in HT1080 cells, which requires FN synthesis (Fig. 2). Accumulation of N-cadherin adhesions was not due to enhanced mRNA expression, or suppression of N-cadherin cleavage (data not shown). Additionally, the increase of N-cadherin and cell-associated FN was reduced by knockdown of integrin β_1 in MT1-MMP-silenced HT1080 cells, as integrin β_1 is responsible for FN assembly [5,20]. The autocrine FN promotes the establishment and maintenance of cadherin based adhesions by inducing FN assembly in endothelial cells [23]. MT1-MMP on tumor cell can destroy pre-existed FN matrix [15]. FN matrix assembly triggered by MT1-MMP knockdown facilitated formation of N-cadherin adhesions (Fig. 2). N-cadherin interacts with integrin β_1 and β_3 on the cell surface [13,14]. MT1-MMP knockdown augmented the interaction among N-cadherin, FN and integrin β_1 (Fig.2). The resent study has shown that cleavage of FN by MT1-MMP promotes endocytosis of FN receptor integrin $\alpha_5\beta_1$ [16]. Formation of FN matrix by MT1-MMP silencing may interfere with endocytosis of not only integrin $\alpha_5\beta_1$ but also N-cadherin, which results in accumulation of N-cadherin.

FN matrix assembly requires cadherin adhesions that increase cytoskeletal tension to accumulate FN fibrils [1-5]. Accumulation of N-cadherin may be essential for further FN matrix formation.

An increasing body of evidence indicates that FN matrix assembly is controlled by MT1-MMP and N-cadherin. MT1-MMP knockdown increases total FN levels and FN matrix assembly in gastrula embryos of Zebrafish [24,25]. In frog and Zebrafish embryos, ectopic expression of N-cadherin induces FN matrix assembly while loss of N-cadherin function decreases it [4,25]. The biological function of N-cadherin is known to regulate correct migration of the neural crest cells during embryonic development; the down-regulation of N-cadherin expression is a pre-requisite for migration of neural crest cells and its re-expression correlates with a migration stop [26]. The balance between MT1-MMP and N-cadherin may determine cell motility and FN matrix assembly.

In conclusion, MT1-MMP regulates nascent FN assembly at cell-matrix adhesions, resulting in the attenuation of N-cadherin adhesion and FN matrix formation, which may contribute to not only tissue organization but also tumor invasion and metastasis.

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References

[1] J.E. Schwarzbauer, J.L. Sechler, Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly, Curr. Opin. Cell Biol. 11 (1999) 622-627.

[2] Y. Mao, J.E. Schwarzbauer, Fibronectin fibrillogenesis, a cell-mediated matrix assembly process, Matrix Biol. 24 (2005) 389-399.

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

[4] B.J. Dzamba, K.R. Jakab, M. Marsden, M.A. Schwartz, D.W. DeSimone, Cadherin adhesion, tissue tension, and noncanonical Wnt signaling regulate fibronectin matrix organization, Dev. Cell 16 (2009) 421-432.

[5] I. Wierzbicka-Patynowski, J.E. Schwarzbauer, The ins and outs of fibronectin matrix assembly, J. Cell Sci. 116 (2003) 3269-3276.

[6] T.Y. El Sayegh, A. Kapus, C.A. McCulloch, Beyond the epithelium: Cadherin function in fibrous connective tissues, FEBS Lett. 581 (2007) 167-174.

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

[8] H. Sato, T. Takino, Coordinate action of membrane-type matrix metalloproteinase-1 (MT1-MMP) and MMP-2 enhances pericellular proteolysis and invasion, Cancer Sci.

101 (2010) 843-847.

[9] K. Endo, T. Takino, H. Miyamori, H. Kinsen, T. Yoshizaki, M. Furukawa, H. Sato, Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration, J. Biol. Chem. 278 (2003) 40764-40770.

[10] T. Takino, N. Koshikawa, H. Miyamori, M. Tanaka, T. Sasaki, Y. Okada, M. Seiki, H. Sato, Cleavage of metastasis suppressor gene product KiSS-1 protein/Metastin by matrix metalloproteinases, Oncogene 22 (2003) 4617-4626.

[11] T. Takino, H. Sato, A. Shinagawa, M. Seiki M, Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family, J. Biol. Chem. 270 (1995) 23013-23020.

[12] H. Sato, T. Takino, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, M. Seiki, A matrix metalloproteinase expressed on the surface of invasive tumour cells, Nature 370 (1994) 61-65.

[13] G.F. Weber, M.A. Bjerke, D.W. DeSimone, Integrins and cadherins join forces to form adhesive networks, J. Cell Sci. 124 (2011) 1183-1193.

[14] N. Chattopadhyay, Z. Wang, L.K. Ashman, S.M. Brady-Kalnay, J.A. Kreidberg, Alpha3beta1 integrin-CD151, a component of the cadherin-catenin complex, regulates PTPmu expression and cell-adhesion, J. Cell Biol. 163 (2003) 1351-1362.

[15] T. Takino, R. Nagao, R.I. Manabe, T. Domoto, K. Sekiguchi, H. Sato, Membrane-type 1 matrix metalloproteinase regulates fibronectin assembly to promote cell motility, FEBS Lett. 585 (2011) 3378-3384.

[16] F. Shi, J. Sottile, MT1-MMP regulates the turnover and endocytosis of ectracellualr matrix fibronectin, J. Cell Sci. 124 (2011) 4039-4050.

[17] T. Takino, H. Miyamori, Y. Watanabe, K. Yoshioka, M. Seiki, H. Sato, 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.

[18] T. Takino, Y. Watanabe, M. Matsui, H. Miyamori, T. Kudo, M. Seiki, H. Sato, Membrane-type 1 matrix metalloproteinase modulates focal adhesion stability and cell migration, Exp. Cell Res. 312 (2006) 1381-1389.

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

[20] T. Takino, L. Guo, T. Domoto, H. Sato, MT1-MMP prevents growth inhibition by three dimensional fibronectin matrix, Biochem. Biophys. Res. Commun. 436 (2013) 503-508.

[21] Y. Wang, M.A. McNiven, Invasive matrix degradation at focal adhesions occurs via

recruitment by a FAK-p130Cas complex, J. Cell Biol. 196 (2012) 375-385.

[22] M.A. McNiven, Breaking away: matrix remodeling from the leading edge, Trends Cell Biol. 23 (2013) 16-21.

[23] B. Cseh, S. Fernandez-Sauze, D. Grall, S. Schaub, E. Doma, E.V. Odderghen-Schillimg, Autocrine fibronectin directs matrix assembly and crosstalk between cell-matrix and cell-cell adhesion in vascular endothelial cells, J. Cell Sci. 123 (2010) 3989-3999.

[24] R.C. Coyle, A. Latimer, J.R. Jessen, Membrane-type 1 matrix metalloproteinase regulates cell migration during zebrafish gastrulation: Evidence for an interaction with non-canonical Wnt signaling, Exp. Cell Res. 314 (2008) 2150-2162.

[25] M.R. Dohn, N.A. Mundell, L.M. Sawyer, J.A. Dunlap, J.R. Jessen, Planar cell polarity proteins differentially regulate extracellular matrix organization and seembly during zebrafish gastrulation, Dev. Biol. 383 (2013) 39-51.

[26] M.A. Nieto, The early steps in neural crest development. Mech. Dev. 105 (2001) 27-35.

Figure legends

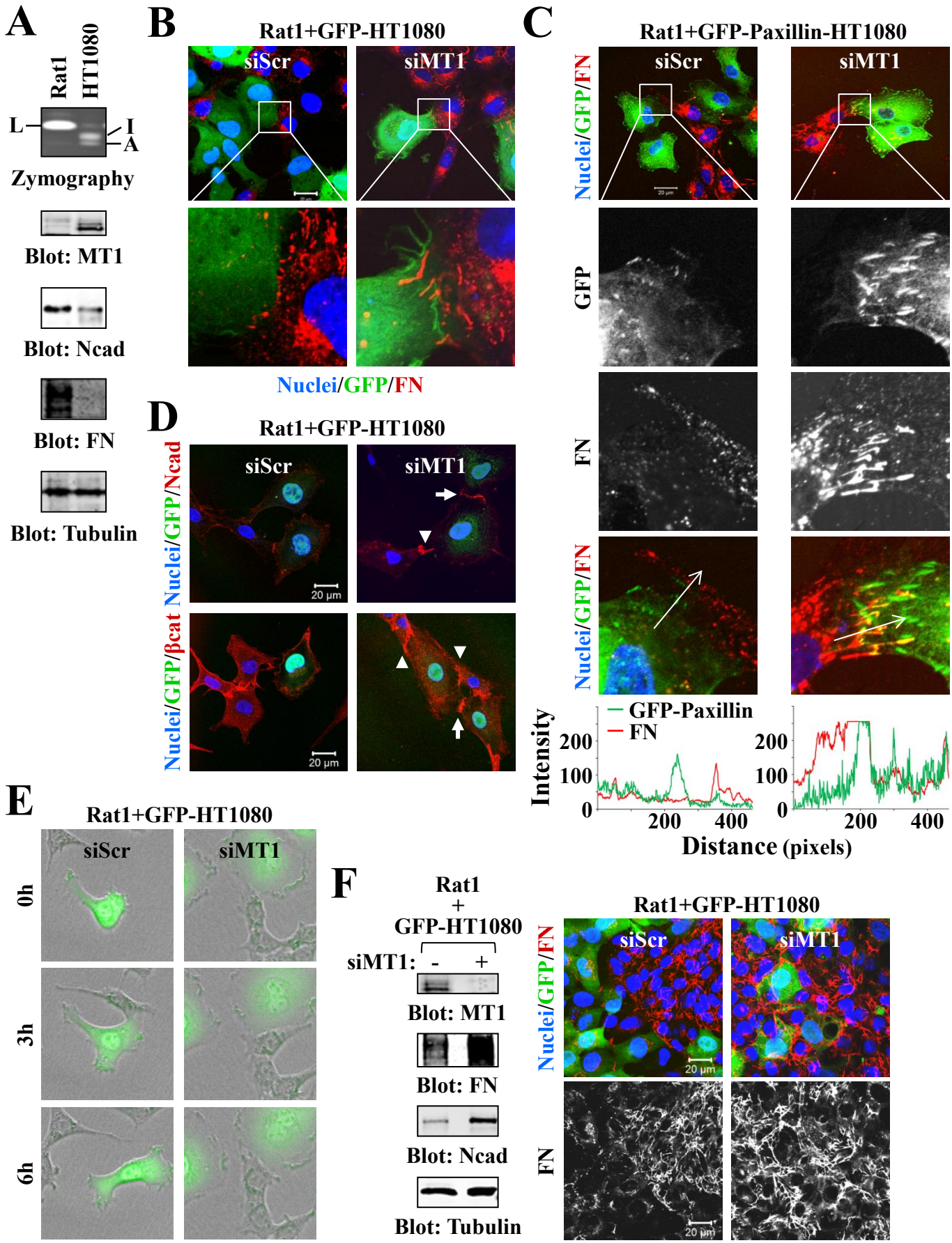
Fig. 1. MT1-MMP disrupts initiation of FN assembly by regulating cell-matrix adhesions. (A) Rat1 and HT1080 cells (1×10^5 cells/mL) were cultured for 48 h. Then, the conditioned media were analyzed by gelatin zymography. The cell lysates were immunoblotted with anti-MT1-MMP (MT1), anti-N-cadherin (Ncad), anti-FN, and anti-tubulin antibodies. L, latent form; I, intermediate active form; A, fully active form of MMP-2. (B) GFP-HT1080 cells were transfected with siRNA for either control (siScr) or MT1-MMP (siMT1). Then, 1:1 mixture of Rat1 and them (1.25×10^4 cells/mL each) were simultaneously seeded and co-cultured for 24 h. The cells were analyzed by immunofluorescence staining using Hoechst 33342 (Nuclei) and anti-FN antibody. (C) GFP-Paxillin-HT1080 cells were transfected with siRNA for either control or MT1-MMP. Then, 1:1 mixture of Rat1 and them were cultured for 24 h. The cells were analyzed by immunofluorescence staining using Hoechst 33342 and anti-FN antibody. The fluorescence intensity linescan profile was generated across indicated cell-cell contacts (Arrows). (D) GFP-HT1080 cells were transfected with siRNA for either control or MT1-MMP. Then, 1:1 mixture of Rat1 and labeled HT1080 cells were co-cultured for 24 h. The cells were analyzed by immunofluorescence staining using Hoechst 33342 and anti-β-catenin (βcat) or anti-N-cadherin antibodies. Arrows indicate homotypic N-cadherin adhesions. Arrowheads indicate heterotypic N-cadherin adhesions. (E) Cell motility was

monitored using Cultured Cell Monitoring System (Astec, Fukuoka, Japan) for 6 h. (F) GFP-HT1080 cells were transfected with siRNA for either control or MT1-MMP. Then, 1:1 mixture of Rat1 and GFP-HT1080 cells (5×10^4 cells/mL each) were co-cultured for 24 h. The cell lysates were immunoblotted with anti-MT1-MMP, anti-FN, anti-N-cadherin, and anti-tubulin antibodies. The cells were also analyzed by immunofluorescence staining using Hoechst 33342 and anti-FN antibody. Scale bars are 20 μm.

Fig. 2. FN assembly augments N-cadherin adhesions. (A) HT1080 cells were transfected with siRNA for control, MT1-MMP, and/or FN (siFN) and analyzed by immunoblotting using anti-FN, anti-MT1-MMP, anti-N-cadherin, or anti-tubulin antibodies. (B) The cells were also stained with Hoechst 33342, anti-N-cadherin or anti-FN antibody. Scale bars are 20 μm. (C) Cells were transfected with siRNA for control, MT1-MMP, and/or integrin β_1 (siIntβ_1) and analyzed by immunoblotting using anti-MT1-MMP, anti-integrin β_1 (Intβ_1), anti-N-cadherin, or anti-tubulin antibodies. (D) Cells were transfected with siRNA for control or MT1-MMP and subjected to immunoprecipitation using anti-N-cadherin (IP: Ncad) or anti-integrin β_1 (IP: Intβ_1) antibodies. The immunoprecipitates were immunoblotted with anti-FN, anti-integrin β_1 or anti-N-cadherin antibodies.

Fig. 3. FN matrix assembly requires increased N-cadherin. (A) HT1080 ells were transfected

with siRNA for control, MT1-MMP, and/or N-cadherin (siNcad) and analyzed by immunoblotting using anti-N-cadherin, anti-FN, anti-MT1-MMP or anti-tubulin antibodies. (B) The cells were also analyzed by immunofluorescence staining using Hoechst 33342, anti-FN and anti-N-cadherin antibodies. (C) MT1-MMP-silenced cells were replated and cultured onto culture dishes with 10 μg/ml of control IgG (IgG) or N-cadherin neutralizing antibody (GC-4) for 24 h. Then, immunofluorescence staining using anti-FN antibody was carried out. Scale bars are 20 μm.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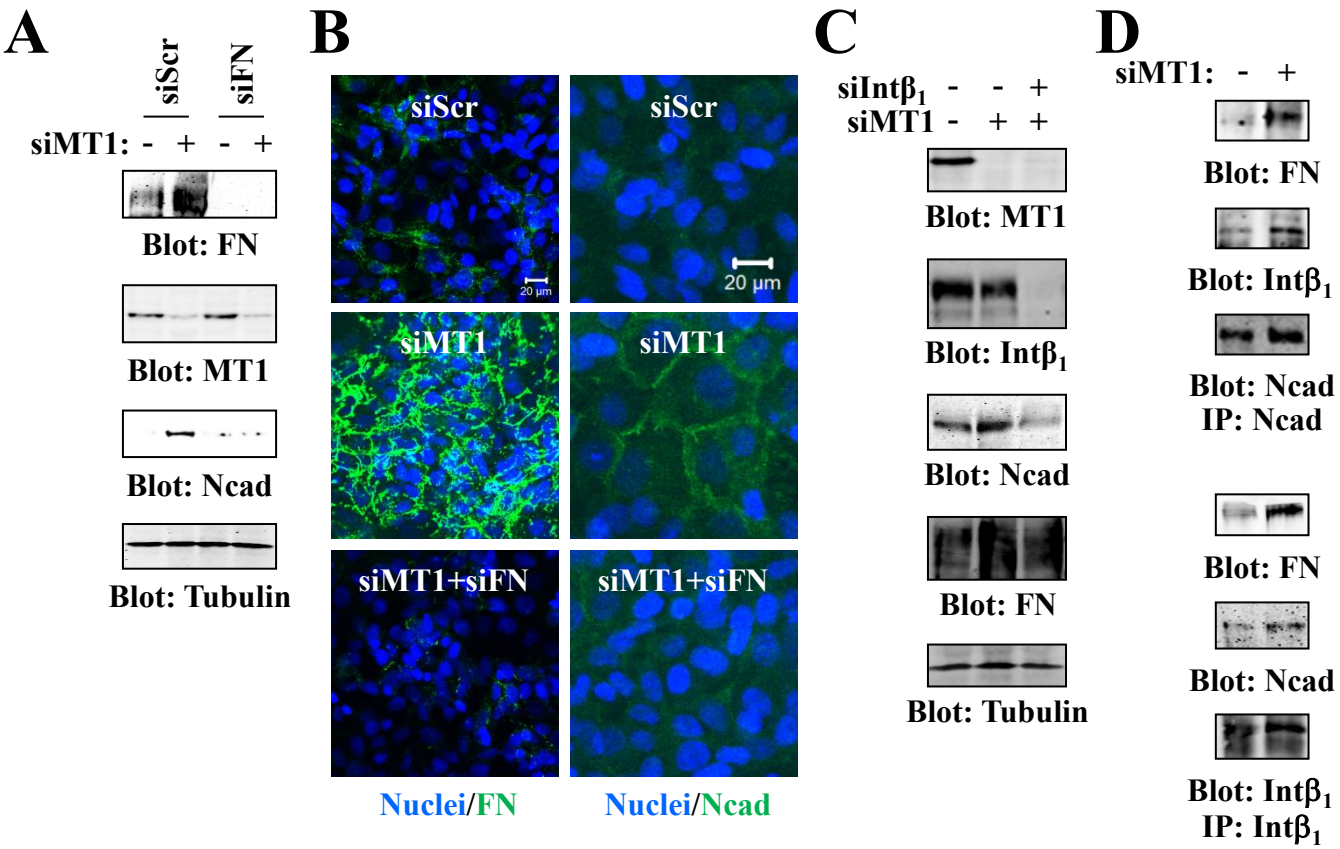
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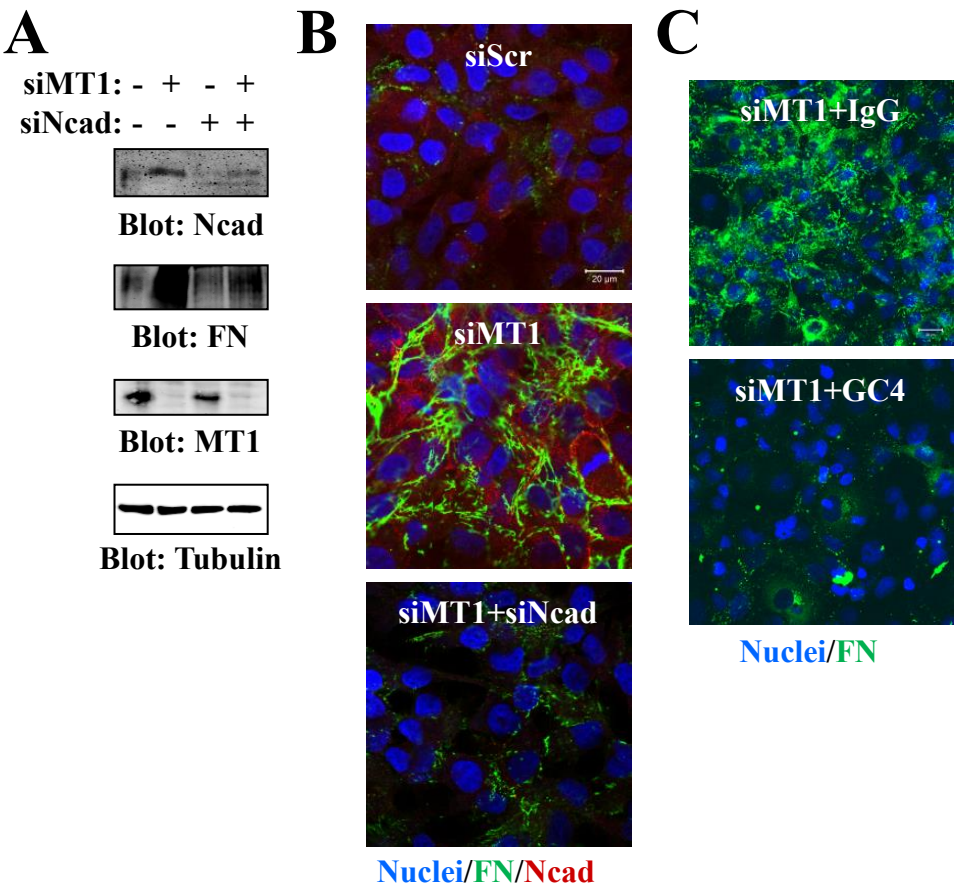
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