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The extracellular domain of syndecan-2 regulates the interaction of HCT116 human colon carcinoma cells with fibronectin

Mi-Jung Kwon [a,1], Yeonhee Kim [a,1], Youngsil Choi [a], So Hyun Kim [b], Sungsu Park [b], Innoc Han [c], Duk-Hee Kang [d,*], Eok-Soo Oh [a,*]

[a] Department of Life Sciences, Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Seoul 120-725, Republic of Korea
[b] Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-725, Republic of Korea
[c] College of Medicine, Department of Physiology and Biophysics, Inha University, Incheon 402-751, Republic of Korea
[d] Division of Nephrology, Department of Internal Medicine, Ewha Womans University School of Medicine, Ewha Medical Research Center, Seoul, Republic of Korea

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ABSTRACT

The cell surface heparan sulfate proteoglycan, syndecan-2, is known to play an important role in the tumorigenic activity of colon cancer cells, but the function of its extracellular domain is not yet clear. Cell spreading assays showed that HCT116 human colon cancer cells attached and spread better on fibronectin compared to the other tested extracellular matrixes (ECMs). Notably, syndecan-2 overexpression enhanced the spreading of HCT116 cells on fibronectin, and the opposite effects were observed when syndecan-2 expression was reduced. In addition, an oligomerization-defective syndecan-2 mutant failed to increase cell–ECM interactions and adhesion-related syndecan-2 functions, including migration. Furthermore, analyses using a microfabricated post array detector system revealed that syndecan-2, but not the oligomerization-defective mutant, enhanced the interaction affinity of HCT116 cells on fibronectin. Taken together, these results suggest that the extracellular domain of syndecan-2 regulates the interaction of HCT116 human colon carcinoma cells with fibronectin.

1. Introduction

Syndecans are cell surface heparan sulfate proteoglycans that are known to play diverse roles in cell adhesion and mediate adhesion-dependent functions. Of the four members of the syndecan family, syndecan-2 is conspicuously pro-tumorigenic in various cancer cells including colon cancer cells [1–5]. In particular, syndecan-2 expression is increased in highly motile colon carcinoma cells, and this enhanced expression is important in the regulation of cell migration [2,6]. During cell adhesion, reorganization of the actin cytoskeleton is required to drive cell migration. Therefore, the ability of syndecan-2 to initiate intracellular signaling is dependent on interactions with cytosolic proteins that regulate downstream events and/or cytoskeletal organization. Several adaptor proteins are known to interact with the cytoplasmic domain of syndecan-2, including syntenin, a PDZ domain-containing adaptor protein that provides a framework for recruiting target molecules to membrane-bound macromolecular complexes [7–9]. Interestingly, syntenin-1 is highly expressed in several metastatic breast cancer cell lines, including MDA-MB-435, MCF7, and in the gastric cell line, Az521. Furthermore, it enhances the migratory ability of MCF7 cells [10]. Therefore, the interaction of the cytoplasmic domain of syndecan-2 and intracellular proteins is a crucial step in the regulation of cell migration.

Cancer cell adhesion to the extracellular matrix (ECM) is also a crucial prerequisite for cell migration. Although it is not clear how the ECM may regulate cancer cell migration, several reports have indicated that diverse ECM components located in the vicinity of cancer cells may induce various signals for cell motility and the regulation of cancer cell migration. For example, Lo et al. reported that the three-dimensional (3D) structure of an ECM composed of polyacrylamide sheets coated with type I collagen played an important part in controlling 3T3 fibroblast cell locomotion [11]. A 3D structure composed of collagen gel also regulated the migration abilities of LNCaP and DU-145 prostate cancer cells, and the collagen concentration around the cancer cells were found to be

Abbreviations: ECM, extracellular matrix; FGF2, fibroblast growth factor 2; FN, fibronectin; MMP, matrix metalloproteinase; mPADs, microfabricated post array detector system; MT1-MMP, membrane-type-1 matrix metalloprotease; PDMS, polydimethyl siloxane.

* Corresponding authors. Addresses: Division of Nephrology, Ewha University School of Medicine, 911 Mok-dong Yangchun-ku, Seoul 158-710, Republic of Korea (D.-H. Kang). Address: Department of Life Sciences, Ewha Womans University, 52, Ewhayeodae-gil, Seodaemoon-Gu, Seoul 120-750, Republic of Korea (W.-A. Oh). Fax: +82 2 2655 2076 (D.-H. Kang), fax: +82 2 3277 3760 (E.-S. Oh).

E-mail addresses: dhkang@ewha.ac.kr (D.-H. Kang), OhES@ewha.ac.kr (E.-S. Oh).

[1] These authors contributed equally to this study.

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modulated by matrix protease [12]. Notably, proteolytic collagen remodeling by membrane-type-1 matrix metalloprotease (MT1-MMP) was shown to regulate the invasive migration of HT1080 fibrosarcoma and MDA-MB-231 breast cancer cells [13]. In addition, the tissue stiffness induced by tumorigenesis seems to be important for the metastatic ability of cancer cell [14]. Collectively, these previous studies seem to indicate that the ECM surrounding cancer cells may regulate various aspects of their cell migration.

Syndecan-2 reportedly acts as a docking receptor for pro-matrix metalloproteinase (MMP)-7 [15], strongly suggesting that the extracellular domain of syndecan-2 may regulate both interactions with and the turnover of the ECM in colon cancer cells. In general, the binding of an extracellular ligand induces conformational changes in its receptors, inducing them to cluster together in a process known as receptor dimerization or oligomerization [16,17]. This, in turn, triggers changes in various cellular processes. As seen for many other cell surface receptors, transmembrane domain-induced oligomerization is crucial for the activation of syndecan-2 signaling and cell migration [18]. The interaction with syntenin is decreased in oligomerization-defective mutant syndecan-2 and the interactions of syndecan-2 with neurofibromin and calcium/calmodulin-dependent serine protein kinase (CASK), two membrane-associated signaling and scaffolding proteins, are also dependent on oligomerization [18,19]. These studies collectively indicate the importance of the transmembrane domain in the function of syndecan. It is highly possible that transmembrane domain-induced oligomerization regulates the valency of syndecans, thereby enhancing their interaction with the ECM ligands. However, the relationship between dimerization and the function of the extracellular domain of syndecan-2 is not yet clear. In this study, we demonstrate that the extracellular domain of syndecan-2 plays a crucial role in regulating cancer cell–ECM interactions and subsequent cell migration, and that transmembrane domain-induced oligomerization is crucial for the interaction of syndecan-2 with fibronectin.

2. Materials and methods

2.1. Cell culture, transfection, antibodies, and reagents

HCT116 cells were maintained in McCoy's 5A (Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (1000 units/ml), and streptomycin (1 mg/ml). Fibroblast growth factor 2 (FGF2) and fibronectin were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Transient transfections were carried out using Effectene (Qiagen, Hilden, Germany) according to the provided instructions. Monoclonal antibody (mAB) against syndecan-2 was produced by AdipoGen Inc. (Korea) [4]. All antibodies against integrin subunits were purchased from Cell Signaling (Beverly, MA). FITC-conjugated anti-phalloidin was purchased from Sigma–Aldrich (USA).

2.2. Molecular constructs

Wild type (SDC2) and oligomerization defective transmembrane domain mutant of syndecan-2 (2GL) constructs were subcloned into a pcDNA3 vector [18]. Syndecan-2 antisense cDNA was constructed as previously described [9].

2.3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extracted from transfected cells was used as template for reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers: rat syndecan-2 (forward) 5′-ATGCGGGTACGAGCCACGTC-3′ and (backward) 5′-CGGGAGCAGCACTAGTGAGG-3′; β-actin (forward) 5′-TGGAATCCTGTGGCATCCATGAAA-3′ and (backward) 5′- TAAAACGCAGCTCAGTAAC AGTCCG-3′. After an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 60 s were carried out. The reaction products were analyzed in 1.5% agarose gels.

2.4. Immunoblotting

Cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4) containing a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS–PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, NJ, USA), incubated with the appropriate primary and secondary antibodies. The signals were visualized by enhanced chemiluminescence (ECL; iNtRON Biotechnology, Korea).

2.5. Fluorescence-activated cell sorting (FACS) analysis

Transfected HCT116 cells were washed with PBS and released by 1 mM EDTA, followed by the addition of PBS. After pelleting, cells were resuspended in PBS and counted. Cells (1×10^6/ml) were incubated anti-syndecan-2 in 10% FBS in PBS overnight on 4 °C. After washing three times with PBS containing 0.05% Tween-20, cells were incubated FITC-conjugated anti-mouse antibody in 10% FBS in PBS for 1 h. Syndecan-2 expression was analyzed by FACSCalibure (BD Bioscience).

2.6. Cell spreading assay

ECM molecules (e.g. gelatin, laminin, collagen type I, and fibronectin) were diluted in serum-free medium (1 μg/cm^2), added to the above described ECM-bearing plates, and incubated at 25 °C for at least 1 h. After washing with PBS, the plates were blocked with 0.2% heat-inactivated BSA for 1 h. After washing with PBS, cells were detached with 0.05% trypsin and 0.53 mM EDTA, suspended in SFM containing 0.25 μg/ml of soybean trypsin inhibitor, and plated to the ECM-coated plates, and incubated for various time periods at 37 °C. Cells were either photographed with a phase-contrast microscope attached to digital camera (Zeiss, Germany) or monitored using the xCELLigence system (Roche Diagnostics GmbH, Switzerland).

2.7. Centrifugal detachment assay

Cell–substratum adhesiveness was quantified using an inverted centrifugal detachment assay. Fibronectin was diluted in serum-free medium (10 μg/ml), added to the plates, and incubated at 25 °C for 1 h. The plates were then washed with PBS and blocked with 0.2% heat-inactivated BSA for 1 h. After washing with PBS, cells were incubated for additional 2 h at 37 °C in 5% CO_2. After removing unattached cells, plates were then filled with SFM, sealed with parafilm and centrifuged inverted for 5 min at 300*g* at room temperature using a large capacity Table-top centrifuge (Hanil science industrial, Korea). After the centrifugation, retained cells were counted using a hemocytometer.

2.8. Proliferation assay

Cell proliferation was measured by a colorimetric assay using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium

bromide) assays. In brief, HCT116 cells were seeded at 1×10^4 cells/well in 96 well plate and allowed to attach for 24 h. After exchanging the medium, 0.5 mg/ml MTT was added to each plate, and cells were incubated for 1 h. The medium was then removed, and 200 μl dimethyl sulfoxide was added to each plate for 30 min at room temperature. The mean of absorbance at 570 nm in each set of samples was measured using the 96-well microplate reader (SpectraMax190, Molecular devices, USA).

2.9. Transwell migration assay

Fibronectin (10 μg/ml) was added to each well of transwell plate (Costar; 8-μm pore size), and then the membranes were allowed to dry at 25 °C for 1 h. The transwell plates were assembled in a 24-well plate, and the lower chambers were filled with the culture medium containing FGF2 (10 μg/ml). Cells were added to each upper chamber, and the plates were incubated at 37 °C in 5% CO_2 for 24 h. The cells that had migrated to the lower surface of the filters were stained with 0.6% hematoxylin and 0.5% eosin, and counted.

2.10. Wound healing assay

HCT116 cells were transfected with indicated cDNAs. After 24 h post transfection, confluent monolayers were scratched once with a sterile pipette tip, and the cells were washed twice with PBS and cultured in fresh media. During the assay, cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Pictures of the wound distance were taken at various time points.

2.11. Synthesis of small interfering RNA constructs

Oligonucleotides were designed targeting human syndecan-2, containing a 9-bp hairpin loop. Oligonucleotides were annealed and cloned into a pSUPER vector. Sequences of the primers are as follows: human syndecan-2 sense primer, 5′-GATCCCCTGACGATGACTACGCTT *CTTTCAAGAGA***ACTGTACTGATGCGAAGA**TTTTTGGAAA-3′; human syndecan-2 antisense primer, 5′-AGCTTTTCCAAAAATGACGATGACTACGCTTCTTCTCTT*GAA* **ACTGCTACTGATGCGAAGA**GGG-3′. Boldface characters indicate syndecan-2 sequences, and italics indicate the hairpin loop.

2.12. Measuring traction force

The polydimethyl siloxane (PDMS) (Dow Corning, USA) substrate was fabricated by replica molding from the master mold. The base and curing agent (ratio = 30:1 w/w) were mixed by hand and then degassed under a vacuum until all air bubbles were removed (~30 min). The prepolymer was poured onto the master mold in a flat polystyrene Petri dish, cured overnight at 80 °C, and then peeled off the template in 7:3 (v/v) ethanol:water solution. The PDMS substrates were washed twice with PBS buffer, coated for 2 h in 10 μg/ml fibronectin at 37 °C, and transferred to 12-well plates containing 10% FBS in McCoy's 5A modified media. Transfected cells (6×10^4 cells/well) were seeding to the PDMS substrate and incubated for 18 h at 37 °C. After incubation, the mPADs apparatus was washed twice with PBS buffer, and the substrate-bound cells were fixed in 3.5% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked with 0.5% BSA in PBS for 1 h, incubated with an FITC-conjugated anti-phalloidin antibody and DAPI for 1 h, and then washed three times with PBS. Finally, the PDMS substrates were mounted with VECTASHIELD mounting medium (VECTOR Laboratories, Burlingame, CA) and then analyzed via confocal laser scanning microscopy (LSM 510 meta; Zeiss, Germany).

3. Results and discussion

3.1. Syndecan-2 mediates the adhesion of colon cancer cells to fibronectin matrix

To investigate the role of the extracellular domain of syndecan-2, we investigated the ECM interactions of HCT116 human colon carcinoma cells. Cells were detached and replated on various ECM-coated plates. On fibronectin (FN)-coated plates, the HCT116 cells attached and began to spread in 10 min, and 53% cells were completely spread within 5 h (Fig. 1A). In contrast, HCT116 cells replated on either BSA, laminin (LN), collagen (Col), or gelatin (Gel)-coated plates failed to show efficient spreading, suggesting that fibronectin plays a major role in the cell adhesion of HCT116 cells. We next investigated the involvement of syndecan-2 in the adhesion of these cells to fibronectin. Transfection of vectors encoding syndecan-2 cDNA into HCT116 cells enhanced the expression levels of both the encoded mRNA and the syndecan-2 protein at the cell surface (Fig. 1B). Compared with vector-transfected cells (Vec), cell spreading was markedly increased in HCT116 cells overexpressing syndecan-2 (Fig. 1C). Real-time monitoring of the adhesion rates of HCT116 cells, performed using the xCELLigence system, revealed that HCT116 cells expressing syndecan-2 attached and spread most effectively on fibronectin (Fig. 1D). Therefore, it is likely that syndecan-2 regulates the adhesion of colon cancer cells on fibronectin.

3.2. Syndecan-2 expression enhances the ECM interaction of HCT116 cells

Since cell adhesion is regulated by interactions between cell surface receptors and substrates in the ECMs, we next investigated whether syndecan-2 was involved in regulating the cell–ECM interaction of HCT116 cells. We transfected with the cells vectors encoding the syndecan-2 cDNA, plated them on fibronectin-coated plates for 2 h, and then measured their ECM interactions with a centrifugal detachment assay. The strength of cell–substratum adhesion was quantified by enumerating the remaining adherent cells after the application of a given detaching force for 5 min. Overexpression of syndecan-2 increased the population of adherent cells on the plates (Fig. 2A), without affecting the expression of integrin α5β1 and αvβ3, are known receptors for fibronectin (Fig. 2B) [20]. In contrast, siRNA-mediated downregulation of syndecan-2 reduced the population of cells that remained after centrifugation (Fig. 2C), suggesting that the syndecan-2 extracellular domain is involved in cell–substratum adhesion. Together, these findings suggest that syndecan-2 is involved in regulating the attachment of HCT116 cells to fibronectin.

3.3. Transmembrane domain-induced syndecan-2 dimerization plays a role in adhesion-related functions

Transmembrane domain-induced oligomerization is reportedly crucial for the functions of syndecans [18], particularly the interaction of the cytoplasmic domain with intracellular signaling proteins. Thus, we further investigated whether the transmembrane domain regulates the interaction of syndecan-2 with the ECM. HCT116 cells were transfected with empty vectors (Vec) or vectors encoding syndecan-2 (SDC2) or an oligomerization-defective mutant (2GL) of syndecan-2 in which the conserved glycine residues in the transmembrane domain were replaced with leucine residue [18]. Both RT-PCR and FACS analysis showed equal expression of wild type and mutant of syndecan-2 (Fig. 3A). When cell–ECM interactions were analyzed by centrifugal detachment assays, enhanced cell adhesion on fibronectin was seen among cells

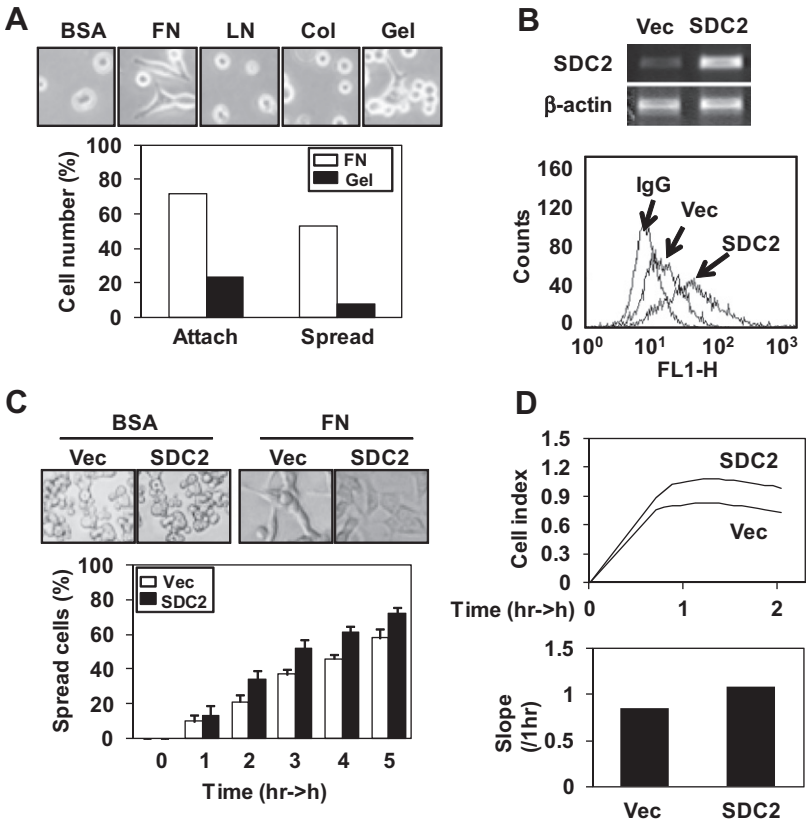

Fig. 1. Syndecan-2 mediates the adhesion of colon cancer cells on fibronectin matrix. (A) HCT116 cells were plated on plates pre-coated with ECM molecules, as described in Section 2. After 5 h, morphological changes were monitored. Photographs were taken under a phase-contrast microscope attached to a digital camera, and adherent and spread cells were counted. The results are shown as the mean percentages from three independent experiments. (B) HCT116 cells were transfected with either empty vector (Vec) or a vector encoding the syndecan-2 cDNA (SDC2). Total RNA was extracted from transfected cells, and mRNA expression levels were analyzed by RT-PCR (*top panel*). Transfected cells were incubated with anti-syndecan-2 antibodies, and protein expression levels were analyzed by flow cytometry. IgG was used as a control (*bottom panel*). (C) Transfected cells were plated on fibronectin (FN) pre-coated dishes for the indicated time periods, and spread cells were counted. Representative results from three independent experiments are shown. BSA was used as a control. (D) Transfected cells were plated on FN pre-coated E-plates. After the indicated time periods, cell spreading curves were monitored using the xCELLigence system (*top panel*). The rates of cell spreading over 1 h were analyzed using the RTCA software (*bottom panel*).

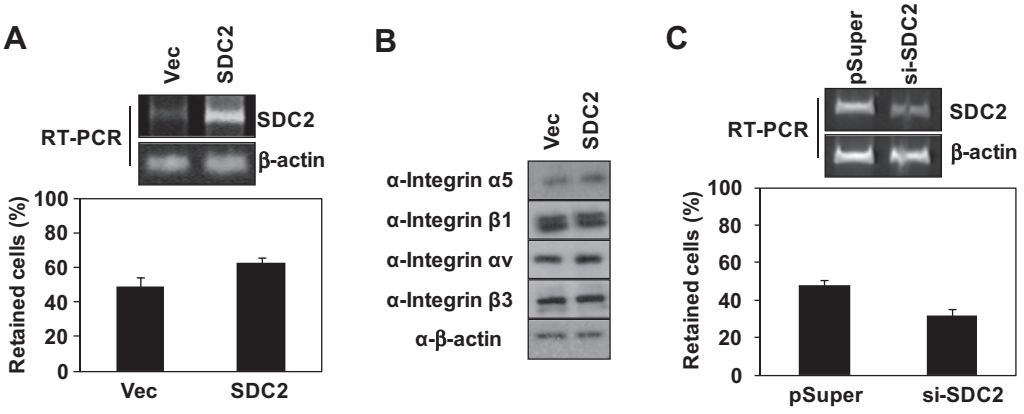

Fig. 2. Syndecan-2 expression enhances the interaction of HCT116 cells with the ECM. (A) Cell–substratum adhesiveness was quantified using an inverted centrifugal detachment assay. HCT116 cells transfected with indicated cDNAs were plated on fibronectin. After 2 h, each dish was filled with SFM, sealed with Parafilm and centrifuged at 300*g* for 5 min. Retained cells were collected and counted with a hemocytometer (*bottom panel*). Total RNA was extracted and the expression of syndecan-2 was analyzed by RT-PCR (*top panel*). The results are shown as the mean percentages of retained cells per field ± the SEM from three independent experiments. (B) HCT116 cells were lysed at 24 h after transfection, and integrin expression was analyzed by immunoblotting with the specific antibody against integrin subunit. Total β-actin was detected as a control. (C) HCT116 cells transfected with indicated cDNAs were plated on fibronectin. Cell–substratum adhesiveness was quantified using an inverted centrifugal detachment assay as described in (A). The results are shown as the mean percentages of retained cells per field ± the SEM from three independent experiments.

overexpressing syndecan-2 but not the oligomerization-defective transmembrane domain mutant (Fig. 3B), suggesting that transmembrane domain-induced oligomerization of syndecan-2 plays a role in regulating the interaction of its extracellular domain with the ECM. Since cell–matrix interactions are generally accepted as common features of cell adhesion and migration, we investigated

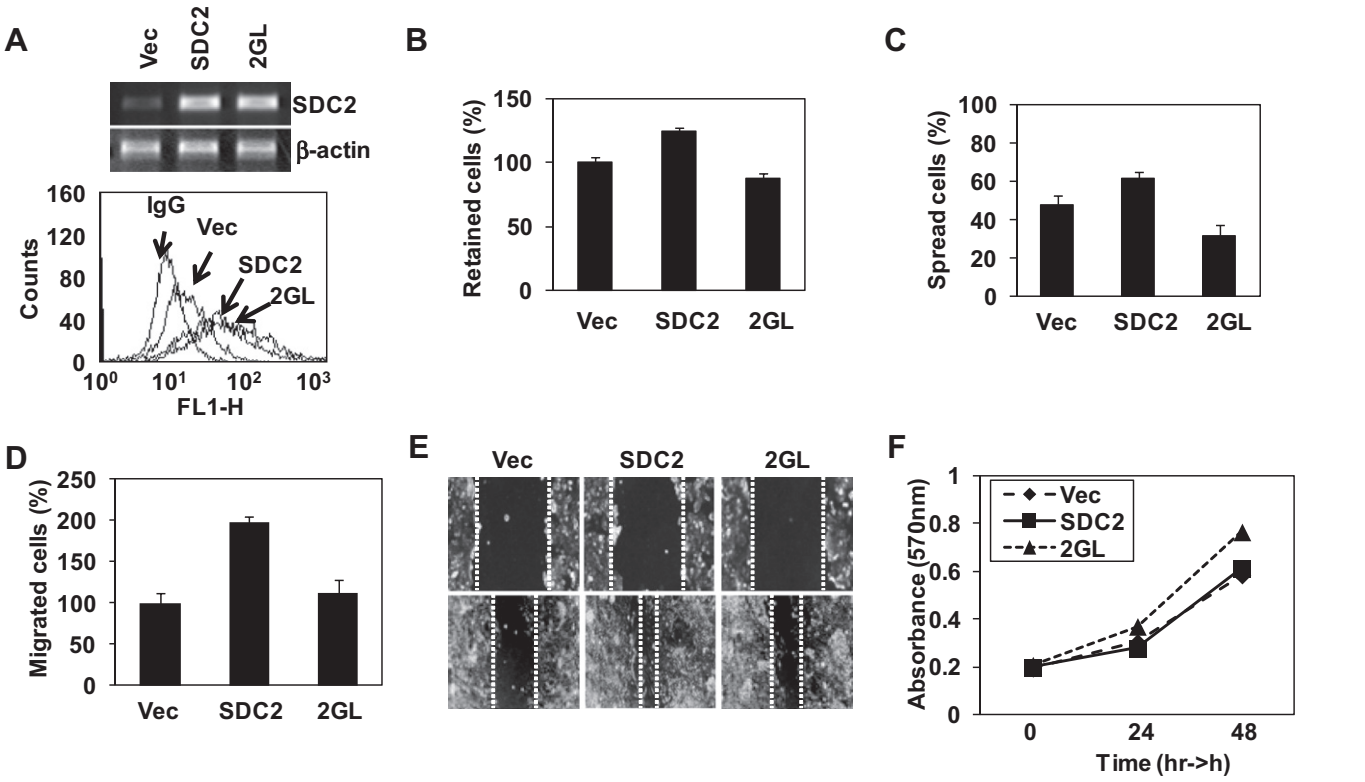

Fig. 3. Transmembrane domain-induced syndecan-2 dimerization plays a role in adhesion-related functions. (A) HCT116 cells were transfected with vector encoding the cDNAs of either wild-type syndecan-2 (SDC2) or the oligomerization-defective syndecan-2 mutant (2GL). Total RNA was extracted and mRNA expression levels were analyzed by RT-PCR (*top panel*). Cell surface expression levels of syndecan-2 were analyzed by flow cytometry. IgG was used as a control (*bottom panel*). (B) HCT116 cells transfected with indicated vectors were plated on FN pre-coated plates, and centrifugal detachment assays were performed as described in Fig. 2. (C) Transfected cells were plated on FN pre-coated plates, and a spreading assay was performed as described in the Materials and Methods section. The results are shown as the mean percentages of spread cells per field ± the SEM from three independent experiments. (D) Migration assays across fibronectin (10 μg/ml)-coated Transwells were performed with HCT116 cells transfected with SDC2 or 2GL. Shown is the relative proportion of cell migration. (E) HCT116 cells were transfected with the indicated vectors and grown to confluence. Confluent monolayers were scraped with a pipet tip to create two intersecting wounds and washed with PBS to remove damaged and detached cells. The wound area was photographed and monitored for 24 h. (F) Cell proliferation was measured by a colorimetric assay using MTT, as described in Materials and Methods.

whether reduced cell–ECM interactions affected the cell adhesion and migration of HCT116 cells. As expected, expression of the oligomerization-defective transmembrane domain mutant reduced syndecan-2-mediated cell spreading (Fig. 3C). In addition, both Transwell cell migration (Fig. 3D) and wound healing (Fig. 3E) assays showed enhanced migration (as compared to vector control cells) among HCT116 cells overexpressing syndecan-2, but not those expressing the oligomerization-defective mutant. On the other hand, expression of the oligomerization-defective transmembrane domain mutant did not affect cell proliferation (Fig. 3F). These data indicate that transmembrane domain-induced oligomerization plays a major role in regulating the functions of syndecan-2 during cell spreading and migration.

3.4. Syndecan-2 dimerization regulates the interaction affinity of HCT116 cells to fibronectin

Finally, we directly investigated the interaction affinity of HCT116 cells using a microfabricated post array detector system (mPADs). When cells adhere to the ECM molecule-coated top surfaces of the mPADs, the posts beneath the cells bend in proportion to the cell-generated force that arises during adhesion of the cells to the ECM [21]. Thus, we were able to calculate the force of cells on the fibronectin-coated mPADs by acquiring confocal image slices at appropriate intervals (Fig. 4A) and analyzing the distance between the centroid positions at the top and bottom of each post. Overexpression of syndecan-2 induced a higher average traction force on posts bearing HCT116 colon cancer cells compared with the oligomerization defective mutant and the vector control (Fig. 4B). Furthermore, according to our analysis of the distribution of traction forces at the cell periphery region, wild-type syndecan-2-expressing cells were associated with a higher proportion of posts that experienced forces over 500 pN (Fig. 4C). These data suggest that transmembrane domain-induced oligomerization increases the interaction between the extracellular domain of syndecan-2 and fibronectin.

The present work suggests that fully understanding the role of the syndecan-2 extracellular domain may be essential for a thorough molecular dissection of syndecan-2-mediated colon cancer cell adhesion. Although we previously showed that syndecan-2 interacts with intracellular signaling regulators, such as syntenin-1, to regulate Rac activation and the subsequent adhesion-related functions (e.g., cell migration) of colon cancer cells [9], it remained unclear whether the extracellular domain of syndecan-2 stimulates cell migration. In this study, we show that syndecan-2 regulates the adhesion of HCT116 cells on fibronectin through its extracellular domain, and that this adhesion is modulated by transmembrane domain-induced oligomerization. As ligand binding of the transmembrane domain induces the cytoplasmic domain to form a multimeric structure required for the specific functions of the syndecan protein, transmembrane domain also strengthens the affinity of the extracellular domain for interactions with substratum components, such as fibronectin, and is thus critical to the specific functions of syndecan-2. However, we do not yet know how oligomerization regulates ecto/cytoplasmic signals. Further work will be required to clarify the precise interactions between

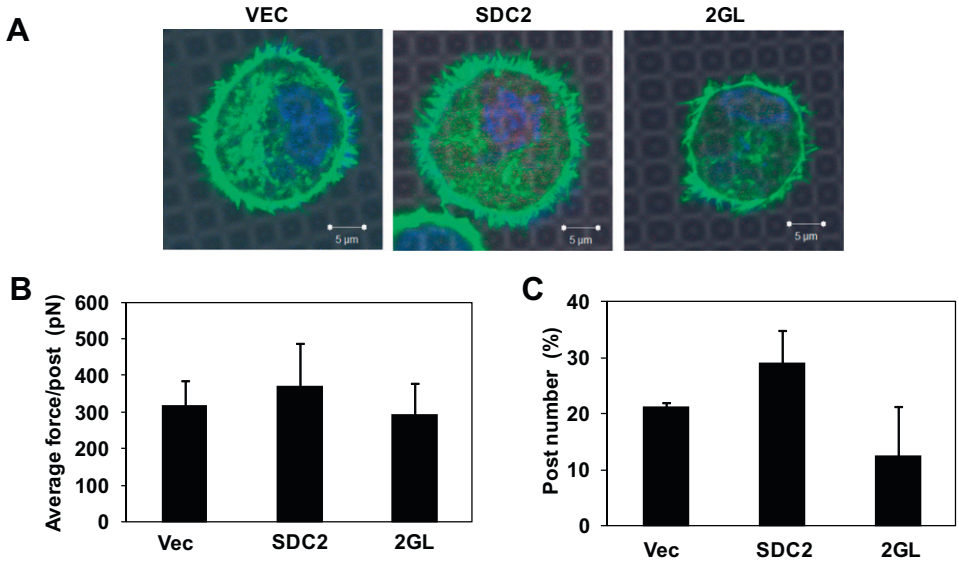

Fig. 4. Syndecan-2 dimerization regulates the interaction affinity of HCT116 cells to fibronectin. Comparison of the cell-generated force of HCT116 colon cancer cells expressing wild-type syndecan-2 and the oligomerization-defective mutant (2GL) on fibronectin. (A) A HCT116 colon cancer cells transfected with Vec, SDC2 and 2GL were plated on fibronectin (10 μg/ml)-coated mPADs, and confocal microscopy was used to image the tops of the posts. (B) The average force (pN) was calculated as the sum of forces divided by the total number of posts under the cells. (C) The relative proportion of post number generating traction forces over 500 pN is shown.

the extracellular domain of syndecan-2 and the ECM, and how it results in enhanced colon cancer cell migration via transmembrane domain-induced oligomerization.

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