Syndecan-2 mediates adhesion and proliferation of colon carcinoma cells

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Key words: colorectal cancer, syndecan-2, adhesion, proliferation, tumorigenesis, extracellular matrix
ABSTRACT

Syndecan-2 is a transmembrane heparan sulfate proteoglycan whose function at the cell surface is unclear. In this study, we examined the function of syndecan-2 in colon cancer cell lines. In several colon cancer cell lines, syndecan-2 was highly expressed compared with normal cell lines. In contrast, syndecan-1 and –4 were decreased. Cell biological studies using the extracellular domain of recombinant syndecan-2 (2E) or spreading assay with syndecan-2 antibody-coated plates showed that syndecan-2 mediated adhesion and cytoskeletal organization of colon cancer cells. This interaction was critical for the proliferation of colon carcinoma cells. Blocking with 2E or antisense syndecan-2 cDNA induced G0/G1 cell cycle arrest with concomitantly increased expression of p21, p27 and p53. Furthermore, blocking of syndecan-2 through antisense syndecan-2 cDNA significantly reduced tumorigenic activity in colon carcinoma cells. Therefore, increased syndecan-2 expression appears to be a critical for colon carcinoma cell behavior and syndecan-2 regulates tumorigenic activity through regulation of adhesion and proliferation in colon carcinoma cells.
INTRODUCTION

The syndecans are a family of cell-surface heparan sulphate proteoglycans that regulate cell behavior through the binding of extracellular matrix molecules and/or soluble ligands (1-3). This interaction regulates cell-ECM adhesion, migration, cytoskeleton organization and gene expression through signal transduction pathways (2,3). This interaction may be differently regulated in cancer cells, since they are generally less adhesive and more migratory than normal counterparts. Therefore, it is probable that syndecans may influence adhesion to ECM, cell morphology, and tumorigenic activity of cancer cells. Indeed, syndecan expression has been shown to suppress transformation and migration of several tumor cells (1,4). Syndecan-1, in particular has been associated with a tumor suppressor function. Transfection of syndecan-1 cDNA dramatically reverses the transformed phenotype of the S115 mammary epithelial-derived tumor cell line, together with inhibition of soft agar colony formation (5). Expression of syndecan-1 is inhibited by malignant transformation of human keratinocytes (6). Moreover, syndecan-1 expression is decreased in a variety of cancer tissues (7-12). Similarly, syndecan-4, which is mainly involved in cytoskeletal and membrane reorganization to form stress fibers and focal adhesions at the later stage of primary fibroblast spreading (13), inhibits cell migration and tumor activity (14-16).
RH-77 lymphoma cells, which readily invade into type I collagen gels, but fail to following expression of either syndecan-1 or -4 (16,17). Consistently, mRNA expression of syndecans-1 and -4 are reduced significantly in several cancer cells including colon carcinoma cells (7,10,18,19).

On the other hand, syndecan-2 shows somewhat different characteristics. Syndecan-2 is involved in regulation of cell adhesions in several cell lines including epithelial cells (20-22), neuronal cells (23,24) and mesenchymal cells (25). Compared to syndecan-1 and -4, the role of syndecan-2 in cell migration has been less investigated. However, several reports indicate that syndecan-2 may positively regulate cell migration, since syndecan-2 is normally highly expressed in cells on migratory conditions (22,24,25). In particular, in Lewis lung carcinoma-derived P29 cells, syndecan-2 plays a major role in interaction with fibronectin and regulates actin stress fiber formation in cooperation with integrin $\alpha_5\beta_1$ (22). These reports indicate that syndecan-2 may function as a cell surface receptor in highly migratory tumor cells. Here, we present evidences that syndecan-2 plays a critical role in adhesion of colon carcinoma cells onto ECM and most importantly, this interaction is crucial for proliferation and tumorigenic activity in colon carcinoma cells.
EXPERIMENTAL PROCEDURES

Materials and antibodies—Monoclonal cyclin D1 and polyclonal p21, p27, cyclin D2, cyclin E, CDK2, CDK4 antibodies were purchased from Santa Cruz Inc (Santa Cruz, CA). Monoclonal p53 antibody was obtained from Calbiochem (San Diego, CA). Texas-Red conjugated affinity purified anti-mouse IgG1 was obtained from Rockland Inc (Gilbertsville, PA). Fluorescein (FITC)-conjugated AffiniPure F(ab')2 fragment Donkey Anti-Chicken IgY was purchased from Jackson ImmunoResearch laboratories, Inc (West Grove, PA). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco Inc (Solon, OH) and the effectene was purchased from Qiagen (Hilden, Germany). Isopropyl-β-D-thio-galactopyranoside (IPTG), glutathione-Sepharose beads and other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture, morphological and treatment—One normal colorectal cell line (CCD-18Co), three colorectal adenocarcinoma cell lines (SW403, LoVo, COLO205), three colorectal carcinoma cell lines (HCT116, KM12SM, KM1214) were purchased from Korean cell line bank. HCT116 were grown in McCoy’s 5a, KM1214 in DMEM, and KM12SM in MEM (Gibco BRL) supplemented with 10 % (v/v) fetal bovine serum.
(FBS) together with penicillin (100 units/ml) and streptomycin (10 µg/ml, Gibco BRL) at 37 °C in 5 % CO₂ in a humidified atmosphere. For treatment with EGF, KM1214 and KM12SM cells were starved for 24 hours in serum free media with or without 2E (0.5 µg/ml) and then 10 nM EGF was added for 5-30 minutes.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)—

Total RNA extracted from cultured cells was used as template for reverse transcriptase reaction. Aliquots of cDNA were amplified using the following primers: human syndecan-1 (forward) 5'-GCTCTGGGGATGACTCTGAC-3' and (backward) 5'-GTAT-TCTCCCCCGAGGTTTC-3'; human syndecan-2 (forward) 5'-ACATCTCCCCTTTG-CTAACGGC-3' and (backward) 5'-TAACTCCATCTCCTTCCCAGG-3'; human syndecan-4 (forward) 5'-GTCTGGCTCTGGAGATCTGG-3' and (backward) 5'-TGGGGGCTTTCTTGTAGATG-3', human GAPDH (forward) 5'-CCACCCATGGCAAATTCCATGGCA-3' and (backward) 5'-TCTAGACGGCAGGTCCAGGTCACC-3', integrin β1 (forward) 5'-GCGCATATCTGGAAATTTGG-3' and (backward) 5'-TCTC-CAGCAAACCC-3'. After an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s (except, GAPDH and integrin β1 at 60 °C), and extension at 72 °C for 60 s were carried out. The reaction products
were analyzed in 1.5 % agarose gels. The amplified DNA fragments (syndecan-1, 552 bp; syndecan-2, 539 bp; syndecan-4, 397 bp; GAPDH, 600 bp; Integrin β-1, 143 bp) were cloned and sequenced to confirm the PCR products.

**Immunoblotting**—After cultures were washed twice with PBS (500 µl/10 cm diameter plate), the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 % Nonidet P-40, 10 mM NaF, 2 mM Na₃VO₄) 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). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Amersham Pharmacia Biotech) and probed with appropriate antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life science). Signals were detected by enhanced chemiluminescence (ECL; Amersham Life science).

**Transient transfections of antisense syndecan-2 cDNA**—For syndecan-2 antisense cDNA, a 5’ fragment of 150 bp excised using the polylinker HindIII site and an internal
HindIII site, and relegated into pcDNA3.1 cut with HindIII. KM1214 cells (2 X 10^6) were plated on 6 cm-diameter culture dishes, incubated at 37 °C for 24 hr, and then transfected with 4 µg of mock- or antisense syndecan-2 in pcDNA3.1 using effectene reagent (Qiagen).

Recombinant syndecan-2E and –4E—The extracellular domains of syndecan-2 and syndecan-4 were cloned into pGEX-5X-1. These constructs were used to transform Escherichia coli DH5α and expressions of fusion protein, glutathione S- transferase-ectodomain of syndecan-2 (2E) and -4 (4E) were induced with 1 mM IPTG for 7 hr. The fusion proteins were purified with glutathione-Sepharose beads. Purified 2E and 4E were used after dialysis in 50 mM Tris-HCl (pH 8.0).

Plating experiment—35mm bacteria culture plates were coated with either 20 µg/ml syndecan-2 or syndecan-4 antibody in PBS overnight at 4 °C. The coated plates were washed with phosphate-buffered saline (PBS), blocked with 0.2 % heat-inactivated bovine serum albumin (BSA) for 1 hr at room temperature, and then washed again with PBS (3 x 5 min). KM1214 cells were detached with 0.05 % trypsin-0.53 mM EDTA, suspended in SFM containing 0.25 mg/ml of soybean trypsin inhibitor, and centrifuged.
Cells were resuspended in SFM, plated on the coated plates, and incubated for various periods of time at 37 °C. To study the morphological changes, cells were incubated with or without 2E for 24 hr in 5 % CO₂ in a humidified atmosphere. Cells were photographed at 20 X magnification with a digital camera (Olympus, Japan).

*Cell proliferation assay*—Cell proliferation was measured by a colorimetric assay using MTT; 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide. In brief, KM1214 and KM12SM cells were harvested with 0.05 % trypsin/EDTA and seeded into 35 mm dishes at 1 X 10⁴ cells/dish. After allowing cells to attach to the plate for 24 hr, fresh medium containing 2E or 4E (0.25 μg/ml) were added. After incubation, the medium containing 0.5 mg/ml MTT (Sigma) was added to each plate in a volume of 100 μl and cells were incubated for 1 hr. The medium was then removed and 200 μl of dimethyl sulphoxide was added to each plate for half an hour at room temperature. The mean concentration of absorbance at 570 nm in each set of all samples was measured using a 96-well microtiter plate reader (Dynatech, Chantilly, VA). Also, the growth activity of syndecan-2 antisense transfected cells was performed as described above.

*Fluorescence-activated cell sorting (FACS)*—Colon cancer cells were cultured in 10-cm
diameter dishes, then washed with PBS and released trypsin (wt/vol) /1 mM EDTA, followed by the addition of PBS. After pelleting, cells were resuspended in PBS and counted. Cells (1 x 10⁶/ml) were incubated anti-syndecan-2 or anti-syndecan-4 in 10 % FBS in PBS for 1 hr on 4 °C. Followed by PBS, contains 0.05 % Tween-20, washing three times and incubated FITC-conjugated anti-mouse or anti-chicken in 10 % FBS in PBS for 30 min. Syndecan-2 or syndecan-4 expression was analyzed by flow cytometry. For the cell cycle distribution, KM1214 cells were cultured in 10-cm diameter dishes, containing 7 ml of DMEM supplemented with or without 0.75 µg/ml 2E or 4E. After 24~36 hr, cells were collected by centrifugation (1,000 rpm, 5 min) and fixed with 70 % EtOH at 4 °C overnight. Cells were collected again by centrifugation (4,000 rpm, 10 min) and washed with PBS. Cells were treated with RNase (250 µg/ml in PBS) and then stained with propidium iodide (50 µg/ml in PBS) at 37 °C for 3 hr. The cells were analyzed for DNA content by flow cytometry and cell-cycle phase distribution was analyzed by MULTICYCLE software.

Anchorage-independent growth in Soft Agarose—Each well of a 6-well culture plate was coated with 3 ml of bottom agar mixture (DMEM/10 % fetal bovine serum/0.6 % agar). After the bottom layer had solidified, 2ml of top agar mixture (DMEM/10 %
fetal bovine serum/0.3 % agar) containing either $1 \times 10^5$ cells with 2E or antisense transfected cells was added to each well, and the cultures were incubated at 37 °C in a 5 % CO$_2$ atmosphere. Every 5 days, normal growth medium was gently layered over the cultures and either 2E or 4E were added. Colony formation was monitored daily with a light microscope. Colonies in soft agar were photographed with a digital camera after incubation for 14 days.
RESULTS

*Syndecan-2 is highly expressed in tumor cells*—We investigated mRNA expression of each syndecan family member in several colon cancer cell lines including normal (CCD-18co), weakly metastatic (COLO205, SW403, and LOVO), and highly metastatic cells (KM1214, KM12SM, and HCT116, Fig. 1A). mRNA expression of syndecan-1 was decreased in most colon cancer cell lines. On the other hand, syndecan-2 mRNA levels were increased by 2 - 5 folds in all cancer cell lines tested, compared with normal colon cell line. Syndecan-4 expression levels were decreased in highly metastatic cell lines, whereas integrin β1 expression levels were not significantly changed. Cell surface expression of syndecan-2 was correspondingly increased in colon carcinoma cell lines, while syndecan-4 was not (Fig. 1B). These data suggest that syndecan-2 may be related to tumorigenic activity in colon carcinoma cells.

*Syndecan-2 mediates adhesion of colon carcinoma cells on ECM*—Since it is known that syndecans regulate cell-ECM interactions (1,2), we investigated whether increased expression of syndecan-2 regulates adhesion of colon cancer cells to ECM. The function of syndecan-2 core proteins as a cell surface receptor was directly analyzed with purified recombinant syndecan-2 (2E) corresponding to the extracellular domain of
syndecan-2, and extracellular domain of recombinant syndecan-4 (4E) as a control (Fig. 2A). Addition of 2E completely blocked adhesion of colon cancer cells on ECM in two different experimental conditions. Firstly, cells were detached, and replated onto tissue culture plates in the presence of 0.75 µg/ml of either 2E or 4E (Fig. 2B). In the absence of 2E (control), both KM1214 and KM12SM normally attached and spread onto tissue culture plates at 24 hr after plating. In the presence of 2E, however, these cells were not attached at all even after 48 hr. In contrast, their attachment and spread was normally occurred in the presence of the same amount of 4E. Secondly, either 2E or 4E was added to exponentially growing cells, and the morphological changes were monitored (Fig. 2C). Unexpectedly, at 24 hr after addition of 2E, but not 4E, cells started rounding and floating off from the tissue culture plates. We presumed that this was due to interruption of cell interaction with ECM through syndecan-2.

In order to more directly access the involvement of syndecan-2 in adhesion on ECM, highly metastatic KM1214 and weakly metastatic LOVO cells were detached and replated onto antibody-coated plates (Fig. 3). Compared to cells on either BSA- or syndecan-4 antibody-coated plates which remained unattached, both colon carcinoma cells on syndecan-2 antibody-coated plates were normally attached (90 ± 8 %, 89 ± 6 %) and spread (46 ± 9 %, 40 ± 2 %) at 12 hr after plating. It was even more efficient
than normal culture condition on tissue culture plates. The number of either attached
or spread KM1214 cells on syndecan-2 antibody-coated plates was approximately 1.5
times and 2.2 times higher than cells on normal tissue culture plates (T/C plate),
respectively (Fig. 3A). These results strongly suggest that syndecan-2 mediates
adhesion of colon cancer cells to ECM.

*Syndecan-2 regulates proliferation of colon carcinoma cells*—Engagement of cells on
ECM is important for cell growth (35,36). Since syndecan-2 is expressed highly in
colon cancer cells, syndecan-2 may play a critical role in the tumorigenic activity in
colon cancer cells. We investigated whether syndecan-2 regulated proliferation of
cancer cells. Both KM12SM and KM1214 cells were culture in the presence of low
amounts (0.25 µg/ml) of either 2E or 4E, and cell numbers were quantified using a
colorimetric assay (Fig. 4A). In the presence of 2E, but not 4E, both cell lines showed
no net increase in cell number, implying that blocking of syndecan-2 function with 2E
caused severe growth arrest. Consistent with this data, transfection of 4 µg antisense
syndecan-2 cDNA reduced cell surface expression of syndecan-2 (Fig. 4B left panel),
and induced cell cycle arrest in KM1214 cells (Fig. 4B right panel). Both 2E-treated
and antisense syndecan-2 transfected cells showed increased expression of cdk
inhibitors, p53, p21 and p27 (28), and decreased expression of cyclin E and cyclin D2 (Fig. 5A, B). Furthermore, FACS analysis using PI staining confirmed that 2E induced cell cycle arrest at G0/G1 phase (Fig. 5C). Exposure of cells for 36 hr with 2E caused inhibition of progression from the G0/G1 to S and G2/M phase in KM1214, which resulted in increase of 1.35 times of cells in the G0/G1 phase compared with control cells. In contrast to 2E, 4E did not significantly affect cell growth. All these data strongly suggest that syndecan-2 is important for proliferation in colon carcinoma cells.

Several studies have shown that Epidermal growth factor (EGF) receptors are expressed at high levels in a variety of epithelial cancers including colon cancer, and activation of EGF receptors appears to be critical for the growth of many tumors (41-44). Thus, we investigated EGF-mediated MAP kinase activation in colon cancer cells. Compared with control cells, 2E pretreated cells showed decreased MAP kinase activation in response to 10 nM EGF (Fig. 6). Therefore, increased expression of syndecan-2 is closely correlated with increased proliferative activity in colon cancer cells.

*Increased expression of syndecan-2 is important for tumorigenic activity of colon cancer cells*—In order to investigate the effect of syndecan-2 on tumorigenic activity,
we performed anchorage-independent growth assay in soft agar. The colony forming ability of KM1214 cells was reduced approximately 70% in the presence of syndecan-2E (0.75 µg/ml) compared with normal cells (Fig. 7A). Similarly, transfection of antisense syndecan-2 cDNA into KM1214 cells significantly reduced colony formation in soft agar in a dose dependent manner (Fig. 7B). Therefore, expression of syndecan-2 was crucial for anchorage-independent growth in colon cancer cells.
DISCUSSION

Cell adhesion to the ECM is mediated by specific cell surface receptors, and progression of colon and other cancers has been associated with changes in their level of expression and/or activity. Cancer cells have changed adhesive properties and this is important for tumorigenesis and metastatic spread. In this study, we have investigated the function of a cell surface heparan sulfate proteoglycan in colon cancer cells. Among syndecans tested, syndecan-2 plays a critical role as a major adhesion receptor to mediate adhesion and regulates proliferation of cancer cells. Similar to previous reports (6-12,18,19), together with decreased expression of integrin $\beta_1$, the expression of both syndecan-1 and –4 was decreased in several colon cell lines, suggesting decreased cell adhesion and increased cell migration. In contrast to syndecan-1 and -4, the expression of syndecan-2 was increased in all colon cancer cell lines tested (Fig. 1B). Therefore, it is highly possible that syndecan-2 mediates adhesion to ECM. In fact, colon carcinoma cell lines KM1214 can spread on syndecan-2 antibody-coated plates more than normal tissue culture plates, an effect not shared by syndecan-4 antibody-coated plates (Fig. 3A, B). Blocking the interactions of syndecan-2 using recombinant syndecan-2 ectodomain (2E) resulted in detachment of colon carcinoma cells from the tissue culture plates (Fig. 2A, B). Therefore, it
seems that syndecan-2 is a major adhesion receptor in colon carcinoma cells.

As the interaction of cells with ECM regulate cell proliferation, syndecan-2 engagement is important for cell proliferation in colon carcinoma cells, since either functional blocking using 2E (Fig. 4A) or antisense cDNA expression induces cell cycle arrest (Fig. 4B). Therefore, it seems that increased expression of syndecan-2 is crucial for increased rates of cell proliferation, an important characteristic of tumor cells. In normal epithelial cell and tissues, the expression level of syndecan-2 is less than that of syndecan-4. This implies that, during transformation into cancer cell lines, there is a change of expression patterns from anti-tumorigenic syndecans (syndecan-1 and –4) to tumorigenic syndecan (syndecan-2). This is a similar mechanism in breast cancer cells (29). E-cadherins are the major cell-cell adhesion receptor in normal epithelial cells (30), and in a variety of carcinomas, their expressions are decreased (32). However, some of carcinomas, such as breast cancer, expresses similar amount of E-cadherins, but increased expression of N-cadherin, which mediates proper adhesion for cancer cells to migrate (29,31). In order for cells to migrate, they require weaker interactions. For this purpose, increased syndecan-2 expression is meaningful, since syndecan-2 is found many migratory cells, and even fibroblasts, syndecan-2 is located in cortical actin, which is involved in rapid turnover of actin filaments (26,27).
Our results clearly show the importance of increased expression of syndecan-2 for tumorigenic activity of colon cancer cells (Fig. 7). What is the role of syndecan-2 in colon cancer cell lines, related to tumorigenic activity? Firstly, as mentioned above, syndecan-2 is crucial for increased rates of cell proliferation. Cells treated with 2E or transfected with antisense syndecan-2 induce G0/G1 cell cycle arrest, suggesting that engagement of syndecan-2 and ECM transduces signals for tumor cell proliferation. Thus, it will be very interesting to identify the cytosolic protein(s) interacting with syndecan-2 cytoplasmic domain in colon cancer cells. Secondly, it may regulate the activity or localization of metrix metallo-proteases (MMPs), an important regulator of cancer cell migration/invasion (33,34). Since cell surface heparan sulfate proteoglycan is known to dock MMP into cell surface (37-39), we have tested the effect of recombinant syndecan-2 core proteins on MMP activity, but there was no significant difference (unpublished results). Thirdly, syndecan-2 may directly regulate interaction of colon cancer cells with ECM during migration. Since cancer cells have more migratory tendency with different adhesion receptors, syndecan-2 may crucial for cancer cell invasion and migration. It needs to be further investigated in detail mechanism(s) for tumorigenic activity of syndecan-2.
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ACKNOWLEDGEMENT

This work was supported by a research grant of the Ministry of Health & Welfare (HMP-00-B-20800-0080) and Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling Research at Ewha Womans University. H.Park and Y.Lim were supported by fellowship from Brain Korea 21 project.
**FIGURE LEGEND**

**FIG. 1.** **Syndecan-2 expression is increased in colon cancer cell lines.** *A,* Total RNA was extracted from human colon cancer cell lines and mRNA expression were analyzed by RT-PCR using each primer as indicated. GAPDH was used as a control. The reaction products were analyzed in 1.5 % agarose gels (*top panel*). Representative results from three independent experiments are shown. Quantified syndecan-2 mRNA levels compared with normal cells are shown (*bottom panel*). *B,* Colon cancer cells were incubated with anti-syndecan-2 or anti-syndecan-4 antibodies and each protein expression levels were analyzed by flow cytometry. IgG was used as a control.

**FIG. 2.** **Exogenous recombinant syndecan-2 extracellular domain inhibits adhesion and spreading in colon cancer cells.** *A,* Purified glutathione S-transferase-ectodomain of syndecan-2 (2E) and -4 (4E) were separated on 10 % SDS-PAGE and stained with Coomassie blue. *B,* Both KM1214 and KM12SM cells were detached with trypsin and replated onto tissue culture in the presence of 0.75 µg/ml of either recombinant 2E or 4E. *C,* Either recombinant 2E or 4E (0.75 µg/ml) was added into exponentially growing cells, and incubated at 37 °C. After 24 hrs, morphological changes were monitored, and photographs were taken under a phase-contrast
microscope attached with a digital camera. Representative results from five independent experiments are shown.

**FIG. 3. Engagement of syndecan-2 mediates adhesion and spreading in colon cancer cells.** Highly metastatic KM1214 (A) and low metastatic LOVO cells (B) were trypsinized and replated on either syndecan-2 antibody (S2Ab) or syndecan-4 antibody (S4Ab)-coated plates at a density of 1 x 10⁴ cells/dish. After incubation at 37 °C, photographs were taken under a phase-contrast microscope attached with a digital camera at the indicated time (*top panel*) and attached or spreading cells were counted (*bottom panel*). Shown are mean percentages of attached and spread cells per field ± the standard errors of the mean of three independent experiments.

**FIG. 4. Effects of syndecan-2 on cell growth in colon cancer cells.** A, Both KM1214 and KM12SM cells were incubated in the absence (*diamond*) or presence of 0.25 μg/ml of either recombinant 2E (*rectangle*) or 4E (*triangle*) for indicated time, and cell numbers were evaluated with MTT assay as described in Material and method. B, KM1214 cells were transiently transfected with 4 μg of antisense syndecan-2 cDNA and analyzed by FACS using anti-syndecan-2 antibody, followed by fluorescein
isothiocyanate-conjugated goat anti-mouse IgG (left panel). Proliferation rate of antisense syndecan-2 transfected KM1214 cells (empty triangle) were measured as described in (4). Data are shown as average value and ± S.E of three independent experiments, carried out in triplicate (right panel).

**FIG. 5. Inhibition of syndecan-2 induces cell cycle arrest.**  
*A*, After treatment with recombinant syndecan-2E for the indicated period of time, cells were lysed and analysed by immunoblotting with each antibody.  
*B*, KM1214 cells (1 X 10⁶) were transfected using effectene with the syndecan-2 antisense in pcDNA 3.1. After 3 days of selection in the presence of 0.2 µg/ml of G418, cells were lysed and analyzed by immunoblotting.  
*C*, KM1214 cells (2 X 10⁶) were incubated with recombinant syndecan-2E or syndecan-4E, and cell cycle distribution was analyzed by propidium iodide (PI) staining. Representative results from three independent experiments are shown.

**FIG. 6. EGF-stimulated MAP kinase activation is reduced in 2E-pretreated cells.**  
KM1214 and KM12SM cells were serum-starved overnight without (control) and with recombinant 2E (0.5 µg/ml) and then treated with 10 nM EGF for 5-30 min. EGF-
stimulated MAP kinase activation was analyzed by immunoblotting. Erk1/2 was used as a control for equal amounts of proteins. Representative results from three independent experiments are shown.

**FIG. 7.** **Syndecan-2 expression is important for anchorage-independent growth of colon cancer cells.** A, KM1214 cells containing either recombinant syndecan-2E or syndecan-4E were seeded in soft-agar plates as described in methods. Untreated KM1214 cells used as a control. Colonies were grown for 14 days and viable colonies were counted. B, Each of the KM1214 cells were transfected with 1, 2, 4 µg of syndecan-2 antisense cDNA. Anchorage-independent growth in soft-agar of transfected cells were tested as described above. Representative results from two independent experiments are shown.
Figure 1.

(A) Western blots showing normal and metastatic cell lines for GAPDH, Syn-1, Syn-2, Syn-4, and Integrin β-1. The relative density of Syn-2 is quantified for each cell line.

(B) Flow cytometry analysis of Ig-G, Syn-2, and Syn-4 in CCD-18Co, KM12SM, KM1214, HCT116, COLO205, SW403, and LOVO cell lines.
Figure 2.
Figure 3.

A

BSA  S2Ab  S4Ab

B

BSA  S2Ab  S4Ab

Cell number (%) attached spread

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Cell number (%) attached spread

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Figure 4.

A

KM12SM

Absorbance (570 nm)

Time (hr)

KM1214

Absorbance (570 nm)

Time (hr)

B

Log fluorescence intensity

Counts

Ig-G

Syn-2 As

mock

KM1214

Absorbance (570 nm)

Time (hr)
Figure 5.

A

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Vec  S2As

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C

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<td>36 hr</td>
<td>G0/G1 (60 %)</td>
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2E

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<td>G0/G1 (45 %)</td>
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4E

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Figure 6.

Table: Time course of EGF stimulation of ERK1/2 phosphorylation in KM1214 and KM12SM cells

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Figure 7.

A

Relative colony number (%)

Control 2E (0.75 µg/ml)

B

Relative colony number (%)

Vector S2As (1 µg) S2As (2 µg) S2As (4 µg)