ONLINE DATA SUPPLEMENT

Lung Adenocarcinoma Syndecan-2 Potentiates Cell Invasiveness

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Running title: Syndecan-2 is elevated in lung adenocarcinoma

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Supplemental Materials and Methods:

Gelatin Zymography

MMP9 activity in A549 culture medium was determined using gelatin zymography as described previously (1). Cells were plated in equal numbers onto 6-well plates. After treatment, the conditioned medium was collected and concentrated 20-fold using Centricon filters (Millipore, Bedford, MA). Equal amounts of concentrated medium were mixed with 2x buffer and resolved using 8% SDS-PAGE gels containing 1 mg/mL gelatin. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min at room temperature and incubated with developing buffer (50 mM Tris–Cl, pH 7.6, 5 mM CaCl2, 0.02% Brij-35) overnight at 37 °C. Gels were subsequently stained with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol and 10% glacial acetic acid, and then destained in the same solution without the dye. MMP9 activity was visualized as clear bands within the stained gel.

Lentiviral Transfection

PLKO.1 plasmids carrying either the human shSDC2 target sequence CCGGCCAGCCGAAGAGGATAC (consortium number TRCN0000286560), shMMP9 (a) target sequence CCACAACATCACCTATTGGAT (consortium number TRCN0000051438), shMMP9 (b) target sequence CATTCAGGGAGACGCCCATTT (consortium number TRCN0000373008) or a scrambled (Scr) sequence were purchased from Sigma-Aldrich (St. Louis, MO). Lentiviral particles were generated by use of a commercially available packaging mix (Cat. #: SHP001; Sigma-Aldrich, St Louis, MO) in human embryonic kidney 293 T cells according to the manufacturer's instructions. A549 cells were infected with the lentiviral particles, and stably selected by use of puromycin (10 μg/mL). To overexpress human syntenin-1 and syndecan-2, pLenti-C-Myc-DDK plasmids carrying the syntenin-1 open reading frame and pLenti-C-mGFP plasmids carrying the syndecan-2 open reading frame were purchased from Origene (Rockville,

MD). Lentiviral particles were generated by use of a commercially available packaging mix (Cat. #: TR30037; Origene, Rockville, MD) in human embryonic kidney 293 T cells, according to the manufacturer's instructions.

Transient Transfection

SiScr and sip65 were provided by Cell Signaling Technology (Beverly, MA); pCMV6 and pCMV6-MMP9 were purchased from Origene, Rockville, MD. Transient transfection was performed using Superfect from Qiagen (Valencia, CA) according to the manufacturer's protocol. Briefly, 1 x 10^5 cells were plated onto 60-mm dishes (or 1 x 10^7 cells onto 100 mm dishes) the day before transfection and grown to approximately 70% confluence. The transfections were allowed to proceed for 8 h. Transfected cells were washed with 4 mL of PBS and used for further experiments.

Real Time-PCR

Total RNA was isolated using the RNeasy Mini Kit from Qiagen (Valencia, CA) and reverse transcribed. SDC2, MMP9 and GAPDH expression were analyzed by quantitative real-time PCR using SYBR Green qPCR Master Mix (GenDEPOT, Barker, TX), and specific primers for human syndecan-2 (sense: 5'CAACATCTCGACCACTTCCA3'; anti-sense: 5'TGGGTCCATTTTCCTTTCTG3'), MMP-9 (sense: ACGACGTCTTCCAGTACCGA; anti-sense: TTGGTCCACCTGGTTCAACT), GAPDH (sense: CGCTGAGTACGTCGTGG AGTC; anti-sense: GCTGATGATCTTGAGGCTGTTGTC). Signal generation was normalized to GAPDH. Fold changes for transcripts were normalized to control samples using the ΔΔCT formula.

Western Immunoblotting

Western blot experiments were performed as previously described (2-4). Cells were lysed using radio-immunoprecipitation assay (RIPA) buffer containing sodium ortho-vanadate (Sigma, St

Louis, MO) and a cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN). In select experiments, fractionation of nuclear and cytoplasmic proteins was performed using the Qproteome cell compartment kit (Qiagen, Valencia, CA). Cells were harvested and lysed according to the manufacturer's protocol. The fractionated proteins were concentrated and desalted by acetone precipitation for Western blot analysis. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to a Polyscreen PVDF membrane (Perkin Elmer Life Sciences, Waltham, MA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline + 0.05% Tween®20 (TBS-T) and incubated overnight at 4 °C with primary antibody dilutions in TBS-T. After washing in TBS-T, the membranes were incubated in cognate horseradish peroxidase (HRP)-conjugated secondary antibody for an hour at room temperature, washed again with TBS-T, and proteins were detected by chemiluminescence (Amersham Biosciences, Pittsburgh, PA). The β-actin antibody was used as a standard for protein loading.

Co-immunoprecipitation

For co-immunoprecipitation (Co-IP) of syntenin-1 with syndecan-2, Scramble and shSDC2-transfected A549 cells were plated onto 100 mm dishes. Cells were lysed using RIPA buffer, lysates were clarified by centrifuging at 15,000 g for 15 min, and pre-cleared by incubating with protein A-Sepharose (Santa Cruz, CA) for 2 hours at 4 °C. Supernatants were transferred to separate 1.5 mL microcentrifuge tubes containing syndecan-2 primary antibody or appropriate controls (beads alone, normal IgG) prebound to protein-A Sepharose. After incubation by rotating overnight at 4 °C, immunoprecipitates were washed 5 times with RIPA buffer and subjected to immunoblot analysis with the anti-syntenin-1 antibody.

Matrigel Invasion Assay

Invasion assays with A549 or NCI-H23 cells were performed as previously described (1). Briefly, cells were cultured for 3 days. The upper chamber of 24-well cell culture inserts (8 μm pore size, Falcon, Franklin Lakes, NJ) were washed with a serum-free medium, coated with 100 μL of Matrigel (1 mg/mL) and dried for 30 min at 37 °C. 2 x 10^5 cells per insert were seeded to the upper chambers and 500 μL of DMEM containing 10% FBS were added to the lower chambers. The invasion chambers were incubated for 24 h in a 37 °C cell culture incubator. Non-invasive cells that remained on the upper surface of the insert membranes were removed by scrubbing. Cells on the lower insert membrane were stained with 0.6% hematoxylin and 0.5% eosin and were counted under light microscopy. Each sample was assayed in triplicate and each experiment was repeated 3 times.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed by use of an enzymatic Chromatin IP kit from Cell Signaling Technology (Cat. #: 9002, Danvers, CA) according to the manufacturer's instructions. In brief, A549 cells were fixed in 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding glycine. DNA was digested by use of micrococcal nuclease to the length of ~150-900 bp. Before incubation with antibodies, 10 μL of input control solution was taken from each sample. The remaining chromatin solution was incubated with 10 μg anti-p65 antibody at 4 °C overnight (Cell Signaling Technology). Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65 °C for 2 h, and 10 μL of each sample was used as a template for qRT-PCR. MMP-9 oligonucleotide sequences for PCR primers were forward 5′- ATTCAGCCTGCGGAAGACCAG-3′ and reverse 5′-ACTCCAGGCTCTGTCCTCTT-3′. This primer set encompasses the MMP9 promoter segment from -634 to -484. The relative quantity of target MMP9 promoter was calculated by use of the CT method (Sequence Detection System software, version 1.7; Applied Biosystems), or $2^{-\Delta\Delta CT}$,

as described (5). The $2^{-\Delta\Delta CT}$ from immunoprecipitation samples by use of p65 antibodies was normalized with the $2^{-\Delta\Delta CT}$ from the input control samples and graphed as a percentage of the control samples.

Xenograft tumor model

Severe combined immunodeficient (SCID) female mice (n=12, 8-10 weeks old) were purchased from Jackson Laboratory. 3 x 10^6 A549 cells were suspended in Matrigel and injected subcutaneously. Tumor growth was measured 2-3 times a week. Tumor volume was estimated as the product of the longest diameter and the square of its perpendicular (i.e, width) divided by two. Thirty days after cell injection, tumors and lungs were harvested. To verify the presence of human cells in mouse lungs, RT-PCR was used to detect the alpha-satellite DNA sequence of the centromere region of human chromosome 17, as previously described by Becker et al. (6).

Supplemental Figure Legends:

Figure E1: **Syndecan-2 is increased in different types of lung carcinoma.** Anti-human syndecan-2 (sdc2) antibody was applied to a Lung Cancer Tissue MicroArray (LTMA) with normal lung tissue (n=8), squamous cell carcinoma (n=24), adenocarcinoma (n=24), adenoaquamous carcinoma (n=6), small cell carcinoma (n=6) and papillary adenocarcinoma (n=8). Staining intensity was scored from 1 to 4 (lowest to highest, respectively). Sdc2 staining intensity was increased in carcinoma tissue compared to control (*P<0.05).

Figure E2: **Syndecan-2, NF-κB (p65) and mmp9 staining are increased in lung adenocarcinoma and A549 cells.** Normal lung (n=3), lung adenocarcinoma (n=5) tissue slides and A549 cells (n=3) were incubated with sdc2, p65 and mmp9 antibodies and then

counterstained with hematoxylin. Representative images are shown. The scale bar represents 100 μm.

Figure E3: **Syndecan-2 regulates mmp9 expression and cell invasion in NCI-H23 cells.** NCI-H23 cells were transfected with Scr, shSDC2 or shMMP9 lentiviral particles. Transfected cells were transferred onto Matrigel-coated inserts, which were incubated for 24 h. Invaded cells were then stained and counted. Cells transfected with shSDC2 and shMMP9 had significantly decreased invasion compared to Scr-transfected controls. The data are presented as mean ± SEM, n=3/group, with testing by Student unpaired t-test ($P<0.05$; significant comparisons: * vs Scr).

References:

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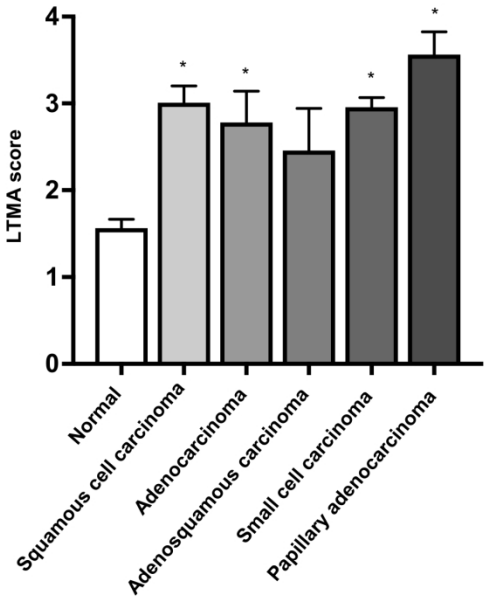

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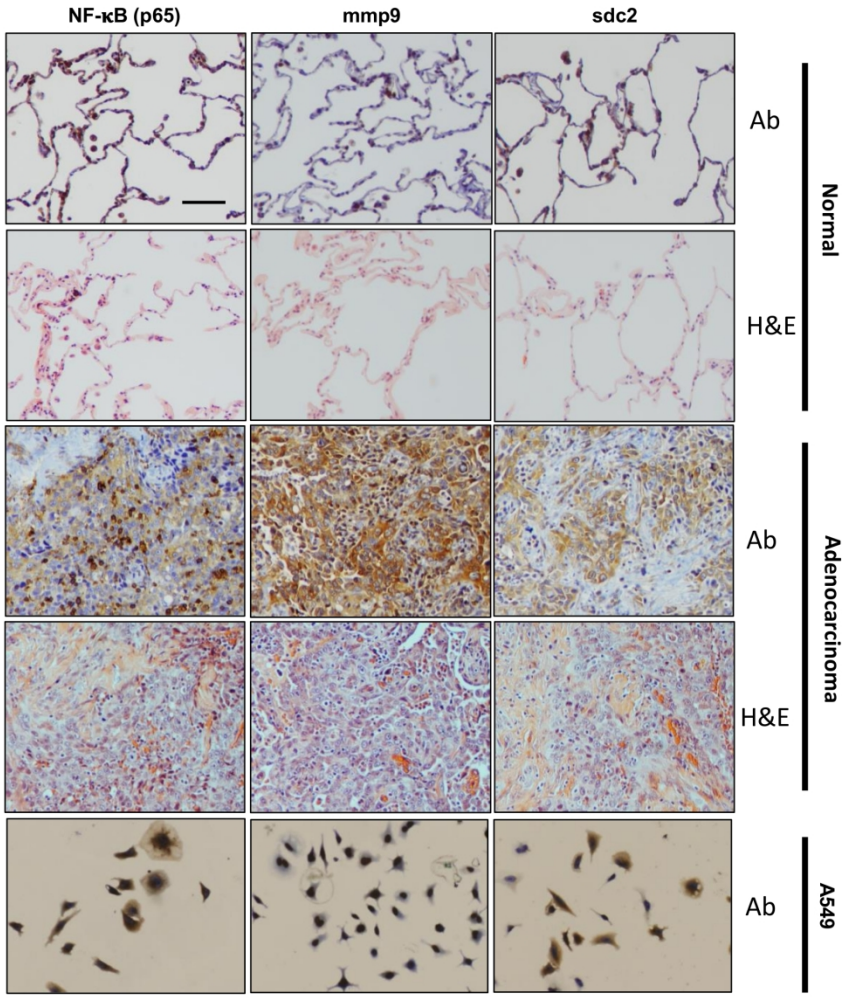

Figure E2

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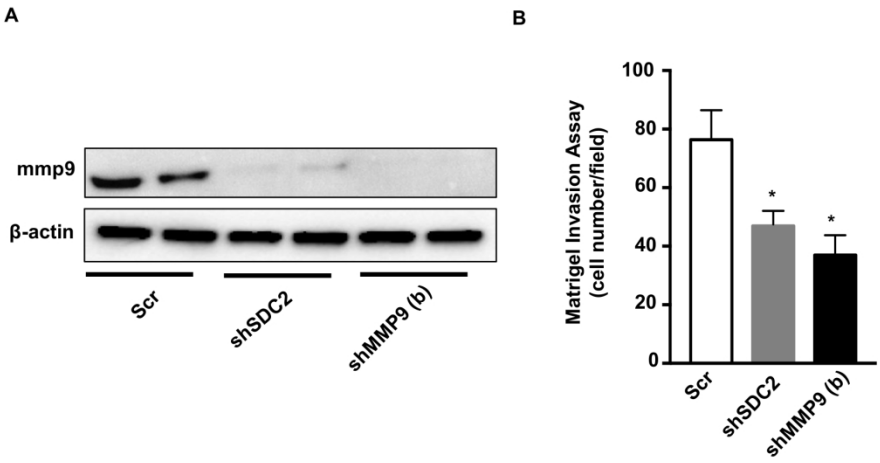

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