Title: Alternative splicing variant of NRP/B promotes tumorigenesis of gastric cancer

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Supplementary materials: Supplemental Methods, Table and legend.

MATERIALS AND METHODS

Cell culture

The AGS cells were purchased from ATCC (Manassas, VA, USA), and the SNU638 and SNU668 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humid atmosphere containing 5% CO₂. RPMI1640, FBS, and antibiotics were purchased from welegene (Gyeongsan, Korea).

To generate knockdown cells using shRNA against NRP/B-2, we construct the PCR-generated sequence-specific shRNA cassettes and a scrambled RNA (Origene, Rockville, MD) were into the pLVTSH lentiviral vector using the Gateway cloning system (Invitrogen, Waltham, MS) as described (23). AGS cells were transduced with the respective lentiviruses: pLV-shRNA plasmid (sh control); pLV-NRP/B-2 (shNRP/B-2). followed by puromycin. Post-incubation for puromycin selection, we picked each colony up and cultured in the medium containing puromycin, then chose two clones: pLV-NRP/B-2#1 (shNRP/B#1); pLV-NRP/B-2#2 (shNRP/B#2), and used them for experiments. For overexpression of NRP/B-2, SNU638 cells were transfected with the empty vector as a control or the pcDNA3.1-NRP/B-2 construct using Lipofectamine 2000 (Invitrogen, Waltham, MS) according to the manufacturer’s instructions. NRP/B-2-overexpressing SNU638 cells were selected by 7-day post-transfection with neomycin.

Isolation of crypts from intestinal organoids

We obtained 5–7 weeks old C57/BL6 mice from ORIENT BIO (Seongnam, Korea). All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Bundang CHA hospital (Protocol No.
Small intestine was washed in ice-cold Dulbecco’s Phosphate-Buffered Saline (DPBS) and cut longitudinally to isolate the crypts from it. The small intestine was then cut into approximately 5 mm long and washed with iced DPBS for several times until its supernatant became clear enough. The small intestine then rinsed with chelating buffer (2mM EDTA in DPBS) for 30 minutes and the crypts were isolated by filtering with DPBS containing 0.1% BSA after using dissociation buffer (D-sorbitol and sucrose in DPBS) to obtain the intestinal crypts. Isolated crypts were mixed with Matrigel (BD Biosciences, Franklin Lakes, NJ) ratio of 1:1 and plated in 48-well plates. After polymerization of Matrigel by incubating at 37°C for 10 minutes, 400ul of our manufactured organoid growth medium containing R-Spondin-1 conditioned medium (from HA-R-Spondin 1-Fc 293T cells, Cultrex®, Sigmaaldrich, St. Louis, MA), mEGF (Peprotech, Rocky Hill, NJ), mNoggin (Peprotech, Rocky Hill, NJ), 1 N2 (Gibco®, Waltham, MS), 1 B-27 (Gibco®, Waltham, MS) in Adavanced DMEM/F12. The culture medium was replaced every 2 days. For passage, the OGM was discarded and the Matrigel was disaggregated with ice-cold DPBS. The pellet suspended in DPBS passed through 1mL Insulin Syringe (BD Bioscience, East Rutherford, NJ) to be dissociated, mixed in Matrigel ratio of 1:1 for culture.

**Immunocytochemistry staining**

Culture medium was removed and organoids in Matrigel were washed with Dulbecco’s Phosphate-Buffered Saline (DPBS). Fixation was done by using 4% Paraformaldehyde until the Matrigel (BD Biosciences, East Rutherford, NJ) was disintegrated. Permeabilization step was performed by using 0.1% Tween-20 with 0.2% Triton-X 100 in PBS buffer then 5% BSA in PBS was used for blocking step. Samples were covered up with primary antibodies at 4°C, overnight. Next day, samples were embedded with secondary antibodies at room temperature for 2hrs. Primary antibodies used for staining are: Lgr5 Antibody (Abgent, San Diego, CA), Purified Mouse Anti- ENC-1 (BD Bioscience, East Rutherford, NJ); Secondary antibodies used for staining are: Alexa Flour 488 conjugated IgG and Alxa Flour 594 conjugated IgG.
conjugated IgG (Life Technologies, Carlsbad, CA).

**Semi-quantitative RT-PCR**

Total RNA from the cultured gastric cancer cells was purified using Trizol reagent (Invitrogen, Carlsbad, USA); subsequently 1.5 μg was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Next, 1 μL of the synthesized cDNA was added into AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) with forward and reverse primers for the PCR reaction. The PCR primers and sequences for NRP/B were forward1 5’-ATGTTCAGTGGTGGCCTGAA-3’, forward2 5’-ATGTCAGTCTAGTGTGCATGA-3’, and reverse 5’-GCCAGTTGGTGGAATGCAGG-3’. The forward2 primer was made for the detection of the alternatively spliced exonic region in exon II. The GAPDH primer sets were forward 5’-ACCACAGTCCATGCCCATC-3’ and reverse 5’-TCCACCCCTCTGCTGTA-3’. Finally, the PCR products were loaded into an 1.8% agarose gel and the PCR bands were imaged using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Seoul, Korea).

**Proliferation assay**

The proliferation of human gastric cancer cells was measured using the EZ-Cytox Cell viability assay kit (Daeillab Service, Seoul, Korea). The cells were seeded in 96-well plates at a concentration of 5 × 10^3 cells/well. After 0, 24, 48, and 96 h, 10 μL of EZ-Cytox solution was added into each well and incubated at 37 °C for 2 h. The absorbance at 450 nm was measured spectrophotometrically. All proliferation assays were performed in triplicate.

**Clonogenic assay (Colony formation assay)**

Cells were seeded into 6-well plates at a density of 2 × 10^2 cells/well. Colony expansion of single cells was allowed to progress for 14 days at 37 °C in a 5 % CO₂
atmosphere; then, the cells were washed and fixed with 4% paraformaldehyde for 10 min. Colonies grown on 6-well plates were stained with 1% crystal violet and counted using a microscope.

Wound-healing migration assay
The wound-healing migration assay was performed using a SPLScar™ Block (SPL Life Sciences, Pocheon, Korea). SNU638, SNU668, SNU719, AGS, and AGS shNRP/B cells (5 × 10⁴ cells) in 100 μL of RPMI 1640 medium (Welgene, Gyeongsan, Korea) were seeded into each chamber. After 24 h, the cultured cells were treated with 50 μg/mL of mitomycin C at 37°C for 40 min. The cell culture insert was gently removed, and 1 mL of fresh medium was added to the culture chamber. Cell migration toward the wound area was observed under an inverted light microscope (Olympus IX51, Olympus America Inc., Melville, USA), and the images of each chamber were taken after 0, 12, and 24 h. The cell migration percentage was calculated from the formula (W - w)/W × 100%, where W is the width of gap at T = 0-h and w is the width of the gap at T = 12-h or 24-h.

Matrigel invasion assay
A PET (polyethylene terephthalate) membrane (8.0-μm diameter pore size) was coated with 100 μL Matrigel (1-mg/mL) and incubated at RT for 1 h. The cells (5 × 10⁵ cells) in 500 μL of serum-free medium were added to the upper chamber, and 700 μL of fresh medium was added to the lower chamber. The chambers were incubated for 24 h at 37°C. The upper chamber was removed from the 24-well plate and gently washed with PBS. The cells on the membrane of upper chamber were fixed in 3.8% paraformaldehyde and stained with crystal violet. The stained cells that invaded through the pores to the lower surface of the membrane were counted under a microscope.
**Transient transfection**

293T cells were seeded in 10-cm plates at a density of $5 \times 10^5$/well for transfection. When the cells reached 80% confluence, they were transiently transfected with not only HA-NRP/B-pCS4 and GAL4-HDAC5-pCMV plasmids, but also with c-Myc-NRP/B-pCMV and GAL4-HDAC5-pCMV plasmids using Lipofectamine 2000 (Invitrogen, Waltham, MS) according to the manufacturer’s instructions.

**Immunoprecipitation and immunoblotting**

Transfected 293T cells were harvested and lysed at 48 h post-transfection. The total cell lysates were clarified by centrifugation at 12,000 $g$ for 15 min. Then, 500 $\mu$g of protein from each sample was incubated overnight at 4 °C with anti-mouse IgG (Genetex, Irvine, CA), mouse anti-HA-probe antibody (Santa Cruz, TX, USA), and rabbit-anti-c-Myc antibody [9E10] (Genetex, Irvine, CA), followed by incubation for 1 h at 4 °C with 30 $\mu$L of IgG magnetic beads (Novex, Waltham, MS). After incubation, the bound proteins were solubilized in 2× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). The samples were separated by 10 % SDS-PAGE and then transferred to PVDF membranes. After non-specific binding to the membranes was blocked using 3 % BSA, the membranes were incubated with HRP-conjugated GAL4 antibody (Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. Subsequently, immunoreactive bands were visualized using ECL reagent (Bio-Rad Laboratories, Hercules, CA). For detection of NRP/B, primary antibodies, anti-VD2 and anti-SY5, obtained from Dr. Shalom Avraham (Harvard Medical School) and anti-ENC-1 purchased from BD Bioscience were used.
Supplemental Table S1. Patient characteristics

|                          | Gastric cancer patients (n = 10) |
|--------------------------|----------------------------------|
| **Age, mean ± SD**       | 59.9 ± 15.7                      |
| **Sex, n (%)**           |                                  |
| Male                     | 8 (80.0%)                        |
| Female                   | 2 (20.0%)                        |