Supplementary materials for methods

Antibodies used

Miltenyi Biotec - EpCAM-PE (130-091-733), EpCAM-APC (130-091-254), CD44-APC (130-095-177), CD117-PE (130-091-735), CD117-APC (130-091-733), CD133/1-PE (130-080-801), CD133/1-APC (130-090-826); BD Biosciences - CD44-PE (555479), H2Kd-FITC (553565); eBioscience - GlyA-FITC (11 -9987), CD45-PE (12-0459); Biolegend - CD45-FITC (304006), H2Kd-Pacific Blue (116616) EpCAM (9C4); Beckman Coulter – CD24-PE (IM1428U); R&D Biosystems - CD166-PE (FAB6561P); Santa Cruz - p53 (FL-393).

Cloning and transfection of CD44 constructs

pBabe-CD44s was purchased from Addgene. Sequence analysis revealed three amino acid differences between this construct and CD44s sequence (NM_001001391). We performed three rounds of mutagenesis (T255C, A326C, A716G) to generate the correct CD44s sequence, which was cloned into pcDNA3 (Invitrogen). pcDNA3-CD44v8-10 was amplified from GC21 xenograft cells by PCR and cloned into pcDNA3. pcDNA3-soluble (sol-) CD44s construct was generated by introducing a stop codon just before the transmembrane region (CD44s aa268). Soluble (sol-) CD44v8-10 was generated from sol-CD44s by addition of variant exons 8-10, which were amplified by PCR from GC21 xenograft cells. Transfections were performed using Lipofectamine 2000 (Invitrogen). Briefly, DNA plasmids diluted in Opti-MEM (Invitrogen) were incubated with Lipofectamine 2000/Opti-MEM mixture and incubated for 20 minutes at room temperature before adding to cells in complete media (10% FBS/DMEM). Amount of DNA and Lipofectamine 2000 was used in accordance to manufacturer's protocol (Invitrogen).

Immunoblotting

Total protein was extracted from primary gastric cancer cells GC38-adh or MKN28 gastric cancer cells stably expressing CD44s and CD44v8-10. Cells were sorted for the respective populations using CD44s or CD44v8-10 specific antibodies and passaged twice before immunoblot analysis. Cells were lysed for 15 minutes on ice in buffer containing 150mM NaCl, 10mM Tris-HCl pH 7.4, 5mM EDTA, 1% Triton X-100 and 1x complete protease inhibitor cocktail (Roche) before collection of lysate supernatant by centrifugation at 14,000g, 4°C. Equal amounts of protein (80-100µg) as quantitated by Bradford assay were resolved by 8% SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes (Whatman), blocked in 5% milk-TBST and probed with CD44s antibody (BBA10, R&D

Biosystems) or actin (A2066, Sigma). Membranes were incubated with horseradish peroxidase-conjugated antibodies against mouse or rabbit IgG (Amersham) and binding detected by chemiluminescence (Immobilon-P, Millipore) signal on x-ray film (Thermo Scientific).

PCR and real-time quantitative PCR (qPCR) analysis

RNA was purified from finely minced xenograft tumors and sorted cells ($7x10^4$ to $2x10^5$) using RNeasy Micro kit (Qiagen), and reverse transcription and PCR amplification was performed using the OneStep RT-PCR kit (Qiagen). Conventional PCR was performed using GoTaq (Promega) and qPCR analysis was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the following parameters: 1 cycle (95°C for 3 min) and 40 cycles (95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec). Amplification of 18S rRNA was used as internal control. Relative expression among samples was calculated by comparison with internal controls using the comparative quantitation method (Rotorgene). Gene specific primers are listed in Supplementary Table M1.

In vitro sphere-forming assay

Primary spheroids were derived from GC38 xenograft tumor by plating the tumor explant on ultralow attachment plates (Corning Costar) in serum-free DMEM/F12 medium (Invitrogen) containing 20 ng/ml human recombinant epidermal growth factor (EGF; PeproTech), 10 ng/ml human recombinant basic fibroblast growth factor (bFGF; PeproTech), B27 (Invitrogen), N2 (Invitrogen), 1 ng/ml hydrocortisone (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), and 0.4% BSA Fraction V (Sigma-Aldrich). Spheroids formed were dissociated using TrypLE™ Select (Invitrogen) that can be inactivated by dilution. For in vitro sphere-forming assay, cells were seeded in six-well ultralow attachment plates (Corning Costar) at densities of 50, 500 and 5000 cells per well and cultured in 2ml of the above mentioned serum-free media. 10 days after plating of the cells, the number of spheres was counted under phase-contrast microscopy (IX71, Olympus).

Spheroid immunofluorescence studies

Spheroids were deposited by cytospin onto glass slides, fixed in ice-cold 4% paraformaldehyde (4°C, 10 min), and blocked (1 h with normal serum). An indirect immunofluorescent labeling technique was used to identify EpCAM-expressing and p53-expressing cells using anti-EpCAM (9C4) and anti-p53 (FL-393) antibodies in PBS with 1% Triton X-100 and 2% normal serum (1 h at room temperature). Slides were washed (PBS, 5 min) and incubated in the dark at room temperature for 30 min with

Alexa Fluor® 488-conjugated goat anti-mouse (against anti-EpCAM) and Alexa Fluor® 546-conjugated goat anti-rabbit (against anti-p53).

Table M1. Primer sequences

Gene	Accession Number (NCBI)	Sequences (5'- 3')	Product size
CD44	NM_001001391	Fw: 5' AAGACATCTACCCCAGCAAC 3' Rv: 5' CCAAGATGATCAGCCATTCTGG 3'	324 bp 720 bp
β-Actin	NM_001101	Fw: 5' CGTGGGGCGCCCCAGGCACCA 3' Rv: 5' GATCTGGGTCATCTTCTCGCG 3'	265 bp
18S rRNA	NR_003286	Fw: 5' CCTGCGGCTTAATTTGACTC 3' Rv: 5' CGC TGA GCC AGT CAG TGT AG 3'	319 bp
CD44s	NM_001001391	Fw: 5' CATCTACCCCAGCAACCCTA 3' Rv: 5' CTTGGTCTCTGGTAGCAGGGA 3'	176 bp
CD44v8-10	NM_001001390	Fw: 5' AGAATCCCTGCTACCAATATGGACTC 3' Rv: 5' AGGTCACTGGGATGAAGGTC 3'	334 bp
CD44v6	(v1) NM_000610 (v2) NM_001001389	Fw: 5' AGGAACAGTGGTTTGGCAAC 3' Rv: 5' CGAATGGGAGTCTTCTCTGG 3'	68 bp
PTPRC (CD45)	(v1) NM_002838 (v2) NM_080921	Fw: 5' AGCACCTACCCTGCTCAGAA 3' Rv: 5' TTCAGCCTGTTCCTTTGCTT 3'	159 bp
shRNA sequences (Origene)			
Gi356314	NM_000610	GCTGACCTCTGCAAGGCTTTCAATAGCAC	
Gi356315	NM_000610	GACAGAAAGCCAAGTGGACTCAACGGAGA	
Scrambled control	NM_000610	GCACTACCAGAGCTAACTCAGATAGTACT	
Mutagenesis primers for CD44s and CD44R shRNA-resistant constructs			
CD44_mut314	NM_001001390 NM_001001391	Fw: 5' CTGACCTCTGCAAAGCCTTCAATAGCACCTTGCC 3' Rv: 5' GCAAGGTGCTATTGAAGGCTTTGCAGAGGTCAGC 3'	
CD44_mut315	NM_001001390 NM_001001391	Fw: 5' GGACAGAAAGCCAAGCGGTCTCAACGGAGAGGCCA 3' Rv: 5' TGGCCTCTCCGTTGAGACCGCTTGGCTTTCTGTCC 3'	