Legends to supplementary figures and tables

Supp Figure S1: Gating strategies for flow cytometry (A) CD45 and GlyA staining were used to gate out hematopoietic cells and erythroid progenitors respectively. Live cells (Sytox-negative) that are CD45/GlyA-negative are mostly EpCAM positive cells. (B) Quantitation for EpCAM expression level is done by determining the Median Fluorescence Intensity (MFI) of the EpCAM positive signal in the flow cytometry results. All EpCAM positive cells (gating based on isotype control) are analyzed. For each sample, MFI for EpCAM positive signal of three samples were determined using FlowJo software. (C) EpCAM+ cells initiated tumors in mice. Patient-derived cells (GC16) were isolated based on expression of EpCAM and injected subcutaneously into mice at $1X10^5$ cells per site. Tumors were formed at sites injected with either unsorted or EpCAM+ fractions but not EpCAM- fraction. Lower panel shows flow cytometry analyses of patient derived cells in the live cell fraction after gating out the CD45 and GlyA positive cells (unsorted) and cells after sorting based on EpCAM expression.

Supp Figure S2: (A) Flow cytometric analysis of specific staining of CD44v8-10+ transiently expressed in MKN28 cells using the CD44v8-10 antibody. Pan-CD44 antibody was used as a control comparison. (B) Immunoblot showing soluble CD44s (sol-CD44s) and soluble CD44v8-10 (sol-CD44v8-10) proteins in media of 293T cells that were transfected with expression plasmids of vector control, sol-CD44s and sol-CD44v8-10. Flow cytometry showing specific blocking of CD44v8-10 antibody using conditioned media containing 200ng of sol-CD44s or sol-CD44v8-10. Levels of soluble protein in the media were quantitated by ELISA prior to performing the blocking experiment. Gastric tumor xenograft (GC84) cells were stained with blocked CD44v8-10 antibody. (B) Gating strategy and isotype control for Figure 3. Flow cytometric analyses of surface expression of CD44v8-10 in EpCAM+CD45- and CD45+ cells isolated from tumor (T) and adjacent non-tumor (N) tissues of patient GC101. Proportion (%) of CD44v8-10 positive (CD44v+) and CD44v8-10 negative cells within the EpCAM+CD45- or CD45+ populations are shown in the top and lower right quadrants respectively.

Supp Figure S3: (A) Flow cytometric analyses of cell isolated from GC119 xenograft tumors showing the presence of CD44+CD44v8-10- cells. Purity of various sorted fractions for injection is shown. (B)

Tumors harvested from mice injected with 200 of the indicated cells. Scale bar represents 1cm. (C) Bright field image of spheroids derived from GC38 xenograft viewed at 200X magnification. These spheroids could be serially passaged for more than 6 months. Scale bar represents 200μm (D) Verification of spheroid identity by immunofluoresence staining. Sphere-forming cells expressed EpCAM and accumulated p53. Nuclei were stained with Hoechst. Scale bar represents 50μm. (E) Sphere-forming abilities of CD44v8-10+ or CD44v8-10- (CD44v+ or CD44v- respectively) cells isolated from spheroid culture. CD44v+ and CD44v- cells were purified from spheroids grown in serum-free culture (see supplementary method, F), plated at the indicated cell number in 2 ml of media in duplicates. 10 days after cell plating, the number of spheroids was counted at 200X magnification. The graph summarizes data (mean±SD) of 6 randomly chosen fields. Data were analyzed for statistical significance using Student's paired t-test. (F) Sphere-forming cells were dissociated from GC38 spheroids and subjected to flow cytometric analyses and cell sorting. CD44v+ and CD44v- cells in unsorted fractions and purity of sorted fractions are shown in the upper panel. Lower panel shows flow cytometry analyses of cells dissociated from CD44v+ or CD44v- spheroids.

Supp Figure S4: (A) Gating strategy for sorting of CD44 depleted TMK1 cells. Cells transfected with shRNA plasmids were tracked by GFP expression. GFP positive cells from scrambled control (sh-control) as well as CD44 depleted cells were sorted for tumor formation assay. (B) Post-sort analysis of GFP-positive cells from scrambled control (Control) and CD44 depleted (sh-CD44) cells that were sorted for tumor formation assay. Percentage purity of sorted cells is indicated. (C) Xenograft tumor formation in NSG mice by TMK1 gastric cancer cells after sh-CD44 transfection. Control indicates scrambled shRNA sequence. 200 cells were injected per site. Differences between groups were tested for statistical significance using Fisher's exact test (two-tailed). Scale bar represents 1cm. # denotes matrigel plugs. (D) Xenograft tumor formation in NSG mice by CD44-silenced TMK1 cells harboring the indicated shRNA-refractory CD44 variants. Mice were injected with indicated number of cells. Detailed data for limiting dilution analysis is presented in Supplementary Table S3B. Differences between groups were tested for statistical significance using chi-squared test (degrees of freedom=1, 95% confidence intervals). Asterisks denote statistically significant P values. Scale bar represents 1cm.

Supp Table S1: Patient clinical information. Histological classification and degree of differentiation of each tumor were determined by pathologists in National University Hospital.

Supp Table S2: Estimation of CSC frequency in unsorted, EpCAM+CD133+ and EpCAM+CD133- fractions from GC16 xenografts

Supp Table S3: (A) Effects of CD44 variant over-expression on tumor initiation potential. (B) Expression of shRNA-refractory gene *CD44v8-10* (r*CD44v*) rescued the tumor initiation potential of TMK1 cells in which the endogenous total CD44 is silenced.

Supp Table S4: p53 mutation status and proportion of CD44+ cells in gastric tumor xenografts. Mutations of p53 in the indicated xenograft lines were determined by Sanger sequencing of cDNA generated by reverse transcription polymerase chain reaction. R175C is a known mutation in colon and uterine cancer [1] that has been reported to retain wild-type functions [2]. R273C is a known mutation in multiple cancers [1]. N239 insertion is a novel mutation in the DNA binding domain of p53.

References:

1. De Vries, EMG et al. Database of mutations in the p53 and APC tumor suppressor genes designed to facilitate molecular epidemiological analyses. Human Mutation 7: 202-213 (1996)

2. Joerger and Firsht. Structure–function–rescue: the diverse nature of common p53 cancer mutants. Oncogene 26: 2226-2242 (2007)