Supporting Information

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SI Materials and Methods

Mice and Tumor Cells. MFG-E8 KO mice were backcrossed at least nine generations onto the C57BL/6 strain and housed under specific pathogen-free conditions. Genotypes were confirmed by PCR, and the experiments were conducted as described previously (1). C57BL/5 and NOD-SCID animals were purchased from SCL and Charles River, respectively. All experiments were conducted under a protocol approved by the animal care committees of the University of Tokyo and Hokkaido University.

The tumor cells (MC38, HCT116, and Colon-26 for colorectal carcinoma cells; MCF-7 for breast carcinoma cells; A375 for melanoma cells; 3LL and A549 for NSCLC cells) were obtained from the American Tissue Culture Collection.

Human Samples. The clinical protocols for this study project were approved by the institutional review board of Hokkaido University Hospital (approval no. 10–0114). Pleural effusion cells and peripheral blood mononuclear leukocytes (PBMCs) were obtained from patients with stage IV nonsmall cell lung carcinomas after written informed consent had been obtained. The cells were isolated by Ficoll Hypaque density centrifugation, and further purified as $EpCAM^+$ epithelial tumor cells from pleural effusion and $CD68^+$ macrophages from pleural effusion and PBMCs.

Generation of Anti–MFG-E8 Blocking Ab. Rabbits were immunized three times with the following KLH-coupled peptides: human, EISQEVRGDVFPSY and mouse, LVTLDTQRGDIFTEY and the sera was collected 8 wk after initial immunization. The antisera were further purified by protein-A columns (Pierce). We assessed specificity and blocking activities of the sera on ELISA, using recombinant MFG-E8 (R&D Systems). Neither of the antibodies was cross-reacted with MFG-E8 from the other species.

In Vivo Self-Renewal Assay. To assess the self-renewal capabilities of tumor cells, serial transplantation assays were performed to evaluate the frequency and tumorigenicity of cancer stem/initiating cell (CSC) populations. Bulk tumor cells or those isolated for CSC marker positivity ($CD44^+ALDH1^+$ for MC38; $CD133^+ALDH1^+$ for 3LL) were injected s.c. into MFG-E8–deficient mice at 1×10^5 per mouse. The growing tumors were isolated, single cell suspensions prepared, and tumors and macrophages further transplanted into tumor-free wild-type or MFG-E8–deficient mice using reduced numbers of cells, as indicated. The tumor growth was measured on the indicated days. In some instances, in vivo serial dilutions were performed using tumor cells and tumor-infiltrating $F4/80^+CD11b^+$ macrophages.

MFG-E8 mRNA Analysis. C57BL/6 mice were challenged with CSCs or their CSC counterparts, and $F4/80^+CD11b^+$ macrophages were isolated from established tumors using a FACSAria. The mRNA was isolated from the lymphocytes from the tumors, tumor-draining lymph nodes (DLN), and spleens from the tumor-bearing mice. For primary human samples, $CD68^+$ macrophages were isolated from tumors or peripheral blood. MFG-E8 mRNA in macrophages was quantified by real-time PCR using SYBR Green Gene Expression Assays (Applied Biosystems) with the following primers: forward, 5′AACAACCTAGCCTCCCGTTGTTCT3′ and reverse, 5′ACAGACGAGGCGGAAATCTGTGAA3′ for murine MFG-E8; forward, 5′TTCCCAAGAAGTGCGAGGAGATGT3′ and reverse, 5′ATGCTGCAAACCCAAGAAGGTCAC3′ for human MFG-E8. The abundance of mRNA was normalized to that of GAPDH mRNA.

Flow Cytometry. The intracellular expression levels of MFG-E8 were evaluated with antimurine MFG-E8 Ab (MBL International) for mouse samples or antihuman MFG-E8 Ab (BD Bioscience) for primary human samples according to the manufacturer's instructions (BD Bioscience). In phospho-stat3 or shh expression, MC38-CSCs were treated with supernatant of TAM or splenic macrophages for 6 h and stained with Ab for phospho-stat3 (BD Bioscience) or shh (Abcam) 3 h after brefeldin-A treatment. The cells were subjected to intracellular FACS analysis with a FACSCaliber.

MFG-E8 ELISA. The protein levels of murine MFG-E8 were quantified by ELISA using supernatant obtained from cultured TAM, splenic macrophages, or those treated with supernatant of CSCs or non-CSCs, according to the manufacturer's instructions (RayBiotech).

In Vivo Adoptive Transfer of Macrophages. $CD11b^+$ $F4/80^+$ macrophages, $CD11b^+Gr\text{-}1^+$ granulocytes, $CD11c^{high}$ $CD11b^+$ myeloid dendritic cells, and $CD11c^{int}$ $B220^+$ plasmacytoid dendritic cells were isolated from growing murine tumors inoculated s.c. into wild-type or MFG-E8–deficient mice, and purified by FACS-based sorting methods with FACSAria. The purity of each population was more than 90%. $CD44^+ALDEFLOUR^+$MC38-CSCs or $CD44^-ALDEFLOUR^-$ non-CSCs (1×10^6 per mouse) were then injected into MFG-E8–deficient mice either alone or with each type of cell isolated from tumor-bearing mice at 2×10^6 per mouse.

For the primary tumor experiments, $EpCAM^+$ tumor cells and $CD68^+CD115^+$ macrophages were isolated from pleural effusions of NSCLC patients by FACSAria and inoculated s.c. into NOD-SCID mice. Seven days before tumor challenge, NOD-SCID mice were treated with clodronate i.v. to remove endogenous macrophages. The established tumors were treated with CDDP on days 10, 12, 14, and 16 after tumor inoculation, and tumor growth was measured on the indicated days.

CSC Characterization. For sphere-forming assays, the bulk tumor cells of murine tumor cell lines or primary NSCLC tumors were cultured in ultra-low attachment culture dishes (Corning) in serum-free medium. DMEM/F-12 serum-free medium was supplemented with 20 ng/mL epithelial growth factor and 10 ng/mL basic-fibroblast growth factor-2 (PeproTech). Digestion and cell passage were performed every 3 d.

For PKH-26 dye retention assays, the bulk tumor cells were labeled with 20 μM PKH-26 (Sigma-Aldrich) according to the manufacturer's instructions. The cells were cultured with TAM supernatant (1:10 dilution) in the presence of anti–MFG-E8 Ab (30 μg/mL) or recombinant murine MFG-E8 protein (R&D Systems) at a concentration of 0.1, 1, or 10 μg/mL in Celltight 96-well plates (Sumitomo Bakelite) to facilitate sphere formation. The spheres were isolated 7 d later, and the PKH-26–retaining populations in bulk tumor cells were analyzed by flow cytometry.

Detection of Apoptosis. The tumor cells were treated with anticancer drugs CDDP (10 μg/mL), 5-FU (100 μg/mL), etoposide (10 nM), and epidermal growth factor tyrosine kinase inhibitor gefitinib (at concentrations of 0.1–1 μM) for 16 h. Active caspase-3 was quantified with intracellular flow cytometry and colorimetric ELISA according to the manufacturer's instructions (BD Bioscience). For colorimetric assay, active caspase-3 was measured by a commercially available kit that uses a biotinylated caspase inhibitor (biotin-ZVKD-fluoromethylketone) that covalently modifies only the large subunit but not the inactive caspase-3 zymogen.

With a caspase-3 mAb coated on the microtiter plate to capture total caspase-3 and the biotinylated caspase inhibitor that binds to the large subunit added sequentially followed by HRP-streptavidin, only activated caspase-3 is detected.

In some experiments, blocking antibodies against MFG-E8 were used for further evaluation.

Evaluation of Chemotherapy-Mediated Antitumor Activities. For in vivo tumor experiments, mice were challenged s.c. in the flank with tumor cells (1×10^5) on day 1. For the therapy model, mice were injected on days 7, 10, and 13 with anti–MFG-E8 Ab (250 μg per mouse), anti–IL-6 mAb (250 μg per mouse; Biomol), various anticancer drugs (CPT-11, 5 mg/mL; 5-FU, 10 mg/mL; and CDDP, 10 mg/mL), Stat3 inhibitor AG490 (10 mg/mL), Hedgehog inhibitor cyclopamine (25 mg/mL), or neutralizing antibodies specifically acting on Hedgehog pathways (MAB4641; 30 μg/mL). Tumor growth was monitored and the product of tumor diameters recorded. In some instances, the $CD44^+ALDEFLOUR^+$ cells were purified by cell sorting with a FACSAria (BD Bioscience) and then injected s.c. into NOD-SCID mice at varied numbers of cells.

Immunoblotting. HCT116-CSCs or bulk cells stimulated with CDDP (10 μg/mL for 4 h) were subjected to Western blotting. Antibodies used for immunoblotting included anti–phospho-Stat3 (Y705), anti–β-actin Ab (BD Bioscience), and anti-SMO (Cell Signaling).

siRNA Transduction. The expression plasmids containing human and murine stat3 siRNA (5′-CCTTCAGGATGGATTTGCCA-ATCTT-3′ and 5′-GAAGGCTCATGACAGTGCTTCCTGT-3′, respectively) and murine shh siRNA (5′-CGGCCATCATTCAG-AGGAGTCTCTA-3′) were designed and obtained from Invitrogen and transfected into tumor cells according to the manufacturer's instructions. The gene knockdown efficacy was assessed by protein immunoblot analysis and proved to be more than 90% in cells.

Statistics. The differences between two groups were determined with Student's *t* test or the two-sample *t* test with Welch correction. The differences among three or more groups were determined with a one-way ANOVA.

1. Hanayama R, et al. (2004) Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304:1147–1150.

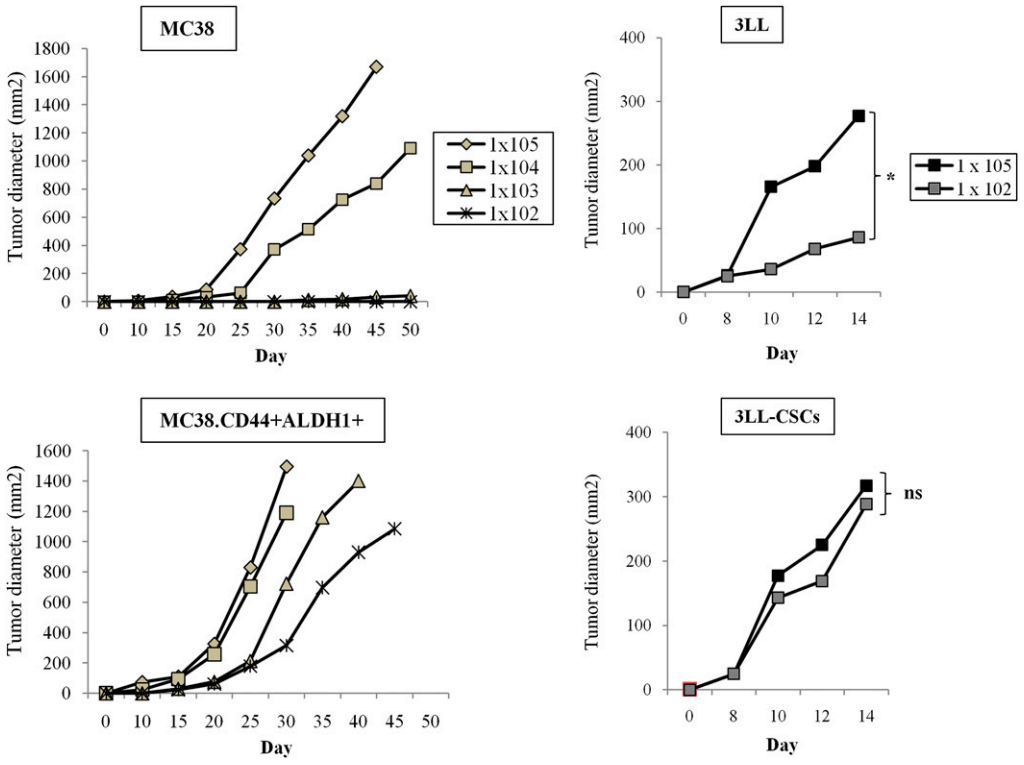

Fig. S1. Serial transplantation assays were performed to evaluate the CSC frequencies. Bulk mouse MC38 cells or their $CD44^+ALDH1^+$ CSC populations and 3LL cells or their $ALDH^+$ $CD133^+$ CSC populations were injected s.c. into C57BL/6 mice at 1×10^5 per mouse ($n = 4$ per group). Single cell suspensions prepared from the tumors were further transplanted into tumor-free C57/BL6 mice using reduced numbers of cells, as indicated. Tumor growth was measured on the indicated days. Data are representative of three independent experiments.

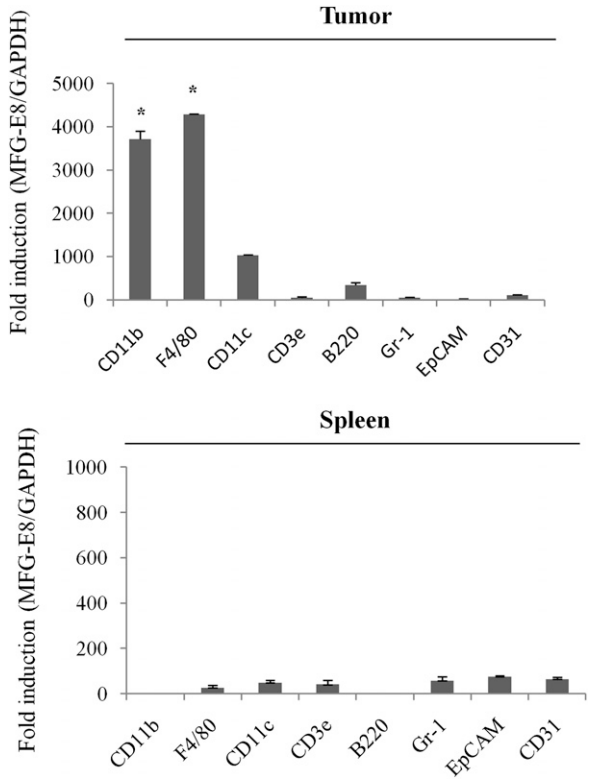

Fig. S2. Tumor-infiltrating cells were isolated from established MC38-CSC tumors according to the expression of various markers for leukocytes (CD11b, F4/80, CD11c, CD3ε, B220, and Gr-1), endothelial (CD31), and epithelial (EpCAM) cells and analyzed for MFG-E8 expression by quantitative PCR. The results are described as fold induction of MFG-E8 relative to a reference gene (GAPDH). Similar results were observed in three experiments.

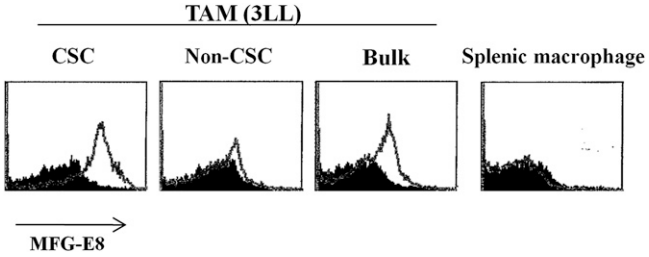

Fig. S3. F4/80^{+}CD11b^{+} macrophages were isolated from 3LL tumors of bulk cells, CD133^{+}ALDH1^{+}3LL-CSCs, CD133^{-}ALDH1^{-}3LL^{-} non-CSCs, or spleen, and the MFG-E8 expression was evaluated by flow cytometry. Data are representative of three independent experiments.

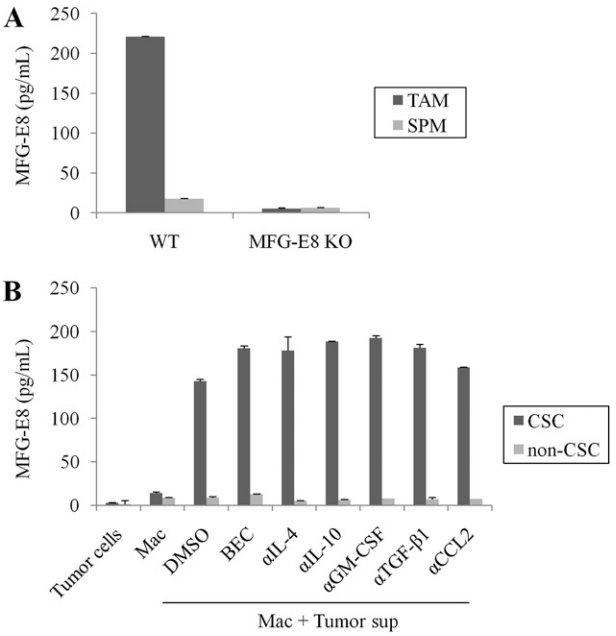

Fig. S4. (*A*) TAM was isolated from established tumors CD44$^+$ALDH1$^+$MC38-CSCs and CD11b/F4/80$^+$ splenic macrophages (SPM) from the same tumor-bearing mice, respectively. TAMs and SPMs were cultured overnight. MFG-E8 in culture supernatant was quantified by ELISA. (*B*) SPM were stimulated with supernatant of MC38-CSCs or their non-CSC counterparts (Mac + Tumor sup) in the presence of arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC), anti–IL-4 neutralizing Ab (αL-4), anti–IL-10 neutralizing Ab (αIL-10), anti–GM-CSF neutralizing Ab (αGM-CSF), anti–TGF-β1 neutralizing Ab (αTGF-β1), or anti–CCL-2 neutralizing Ab (αCCL2) for 24 h. Tumor cells (CSCs and non-CSCs) or splenic macrophages without tumor supernatant (Mac) served as controls. MFG-E8 in the culture supernatant was quantified by ELISA.

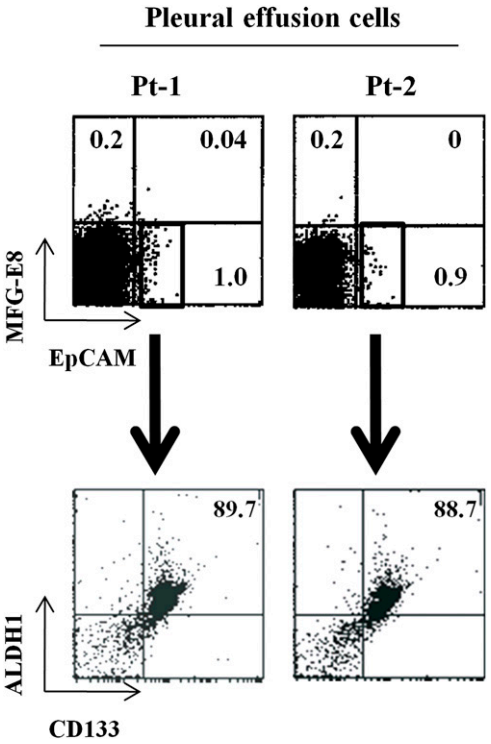

Fig. S5. MFG-E8$^+$EpCAM$^+$ populations in two NSCLC pleural effusion cells were analyzed by flow cytometry (*Upper*).The expression of CD133 and ALDH1 was further evaluated in pleural effusion cells gated by EpCAM$^+$ epithelial cells (*Lower*). Data are representative of two independent experiments.

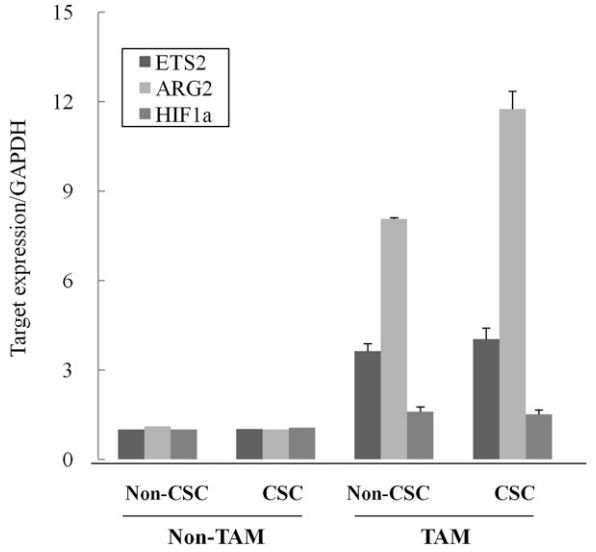

Fig. S6. TAM or CD11b/F4/80^{-} tumor-infiltrating lymphocytes (non-TAM) were isolated from established tumors of CD44^{+}ALDH1^{+}MC38-CSCs or those depleted of CSC population (non-CSC). The Ets-2, ariginase-2 (ARG2), or HIF-1α in macrophages was quantified by real-time PCR. Data are representative of three independent experiments.

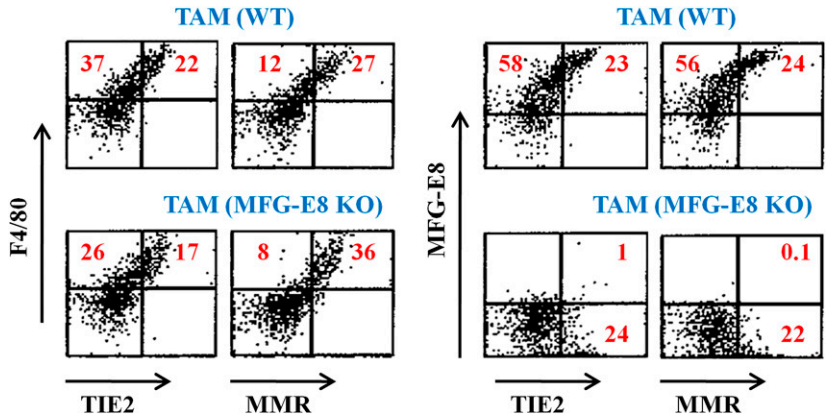

Fig. S7. CD11b/F4/80^{+}TAMs were isolated from established tumors arising from CD133^{+}ALDH1^{+}3LL-CSCs inoculated into wild-type (WT) or MFG-E8–deficient (MFG-E8 KO) mice. The expression of macrophage mannose receptor (MMR), TIE-2, and MFG-E8 in F4/80^{+} cells was examined by flow cytometry. Data are representative of two independent experiments.

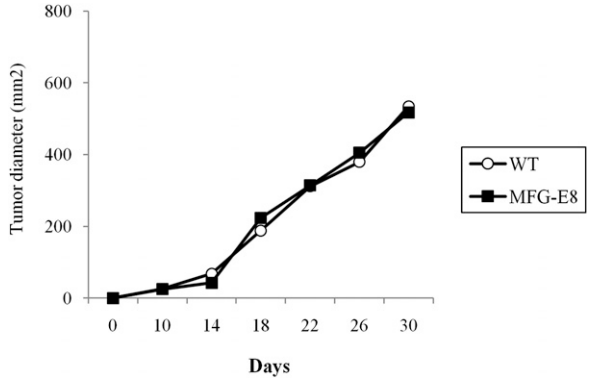

Fig. S8. MC-38-CSCs were s.c. inoculated into MFG-E8–deficient mice and their wild-type counterpart (n = 5 per group), and the tumor growth was evaluated on the indicated days. Data are representative of three independent experiments.

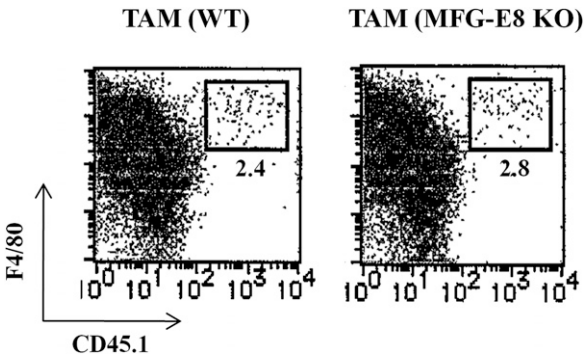

Fig. S9. CD11b^{+}F4/80^{+}macrophages were isolated from MC38-CSC–derived tumors of wild-type (WT) or MFG-E8–deficient CD45.1^{+} mice (MFG-E8KO). MC38-CSCs were injected into tumor-free MFG-E8–deficient CD45.2^{+}mice along with each type of TAM. One month after the transfer, the percentage of donor-derived TAMs (CD45.1^{+}F4/80^{+} cells) in established tumors was quantified by flow cytometry. Data are representative of three independent experiments. *$P < 0.05$.

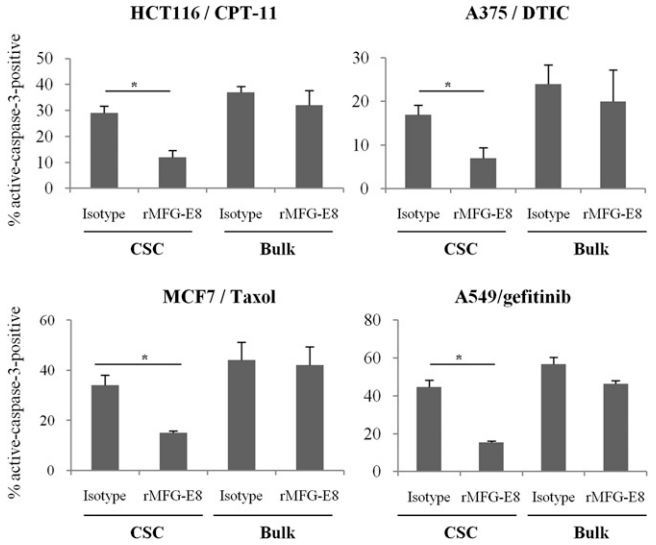

Fig. S10. CD44^{+}ALDEFLOUR^{+}(CSC), or bulk (Bulk) populations of HCT116 cells, CD271^{+}(CSC), or bulk A375 melanoma cells, CD44^{+}ADEFLOUR^{+} (CSC), or bulk MCF7 breast cancer cells, CD133^{+}ALDEFLOUR^{+}(CSC), or bulk A549 nonsmall cell lung cancer cells were purified from bulk tumor populations and incubated with CPT-11, DTIC, taxol, or gefitinib, respectively, with or without recombinant human MFG-E8 protein (100 mg/mL) for 24 h. The cell viability was quantified using cleaved caspase-3 by intracellular flow cytometry. Data are representative of three independent experiments. *$P < 0.05$.

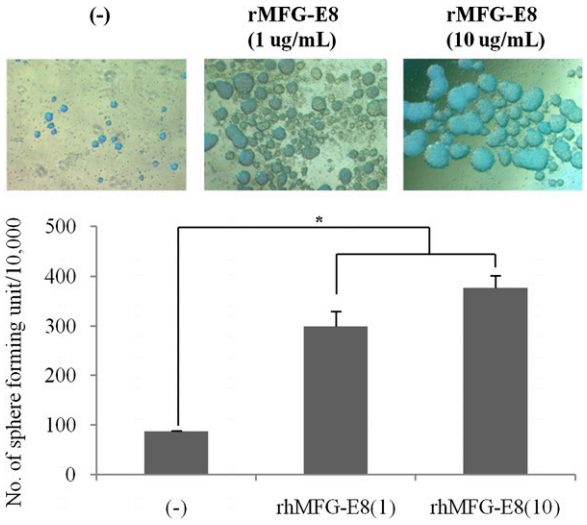

Fig. S11. HCT116 cells were treated with recombinant human MFG-E8 protein at 1 or 10 mg/mL in ultra-low attachment plates. The cells were then cultured for three passages, and the numbers of formed spheres generated per 10,000 cells were determined. Data are representative of three independent experiments. *$P < 0.05$.

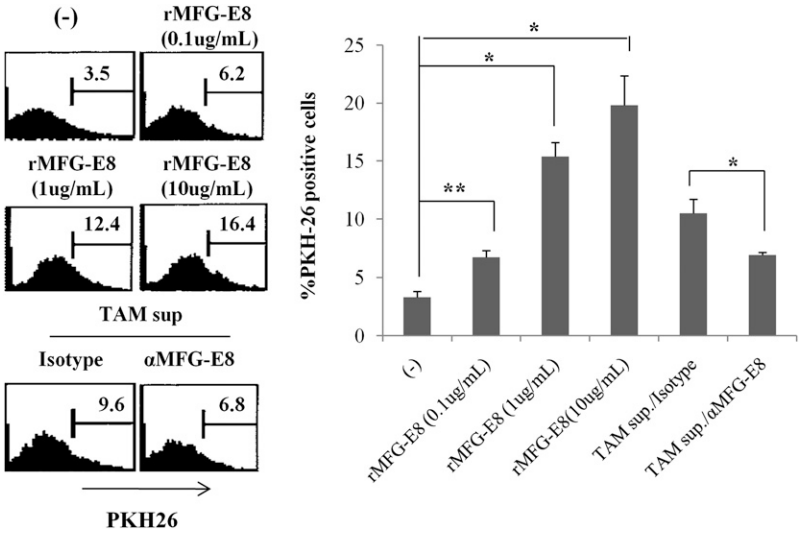

Fig. S12. Bulk tumor cells (HCT116) were labeled with PKH26 dye and cultured with TAM supernatant (1:10 dilution) in the presence of anti–MFG-E8 Ab (30 μg/mL) or an isotype control IgG or cultured with recombinant murine MFG-E8 protein at 1 or 10 μg/mL in Celltight 96-well plates to facilitate sphere formation. After 7 d, the spheres were isolated and the PKH-26–retaining populations analyzed by flow cytometry. Data are representative of four independent experiments.

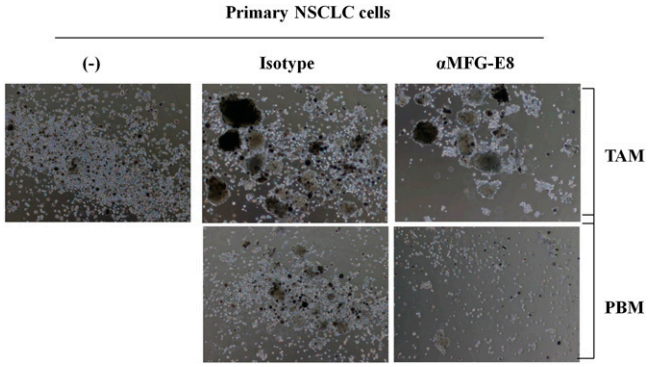

Fig. S13. EpCAM$^+$CD133$^+$primary NSCLC-CSCs isolated from pleural effusion of NSCLC patients were untreated (–) or treated with supernatant of autologous CD68$^+$ macrophages from tumors (TAM) or peripheral blood (PBM) in the presence of anti–MFG-E8 (αMFG-E8) Ab or isotype-matched control IgG (isotype). The cells were passaged three times, and the sphere formation was evaluated. Data are representative of three independent experiments.

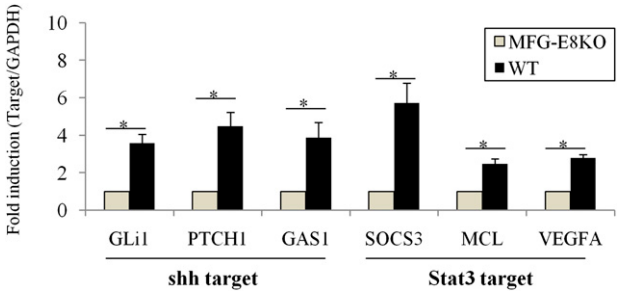

Fig. S14. Target gene expression for Stat3 (SOCS3, MCL, VEGFA) and shh (GLi1, PRCH1, GAS1) in CSCs stimulated with supernatant of TAM from wild type (WT) or MFG-E8-deficient mice (MFG-E8 KO) was quantified by RT-PCR. *$P < 0.05$.

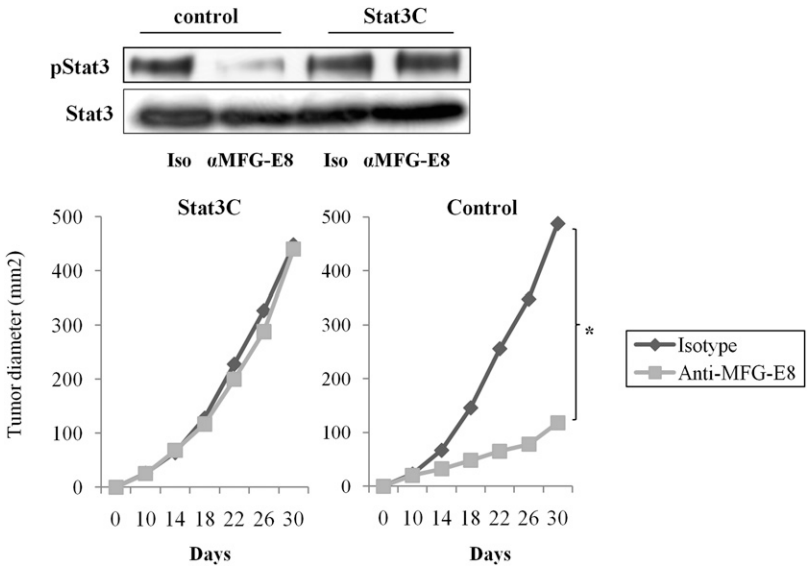

Fig. S15. The plasmids of control or a constitutive active form of Stat3 (Stat3C) were introduced into HCT116-CSCs, and the cells were inoculated with $CD68^+$ macrophages isolated from NSCLC pleural effusion (TAM) into NOD-SCID mice ($n = 5$ per group). The mice were then treated with anti–MFG-E8 blocking Ab or an isotype control Ab. Tumor growth was evaluated at the indicated times. Data are representative of two independent experiments.

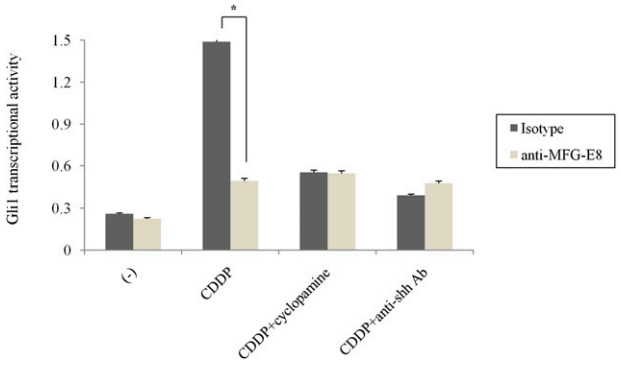

Fig. S16. HCT116-CSCs were stimulated with $CD68^+$ macrophages isolated from NSCLC pleural effusion (TAM), transfected with firefly Gli-1 reporter plasmids, and treated with CDDP with or without anti-shh neutralizing antibodies or cyclopamine. Luciferase assays were performed and results are presented as fold relative to control reporter activities. Data are representative of three independent experiments.

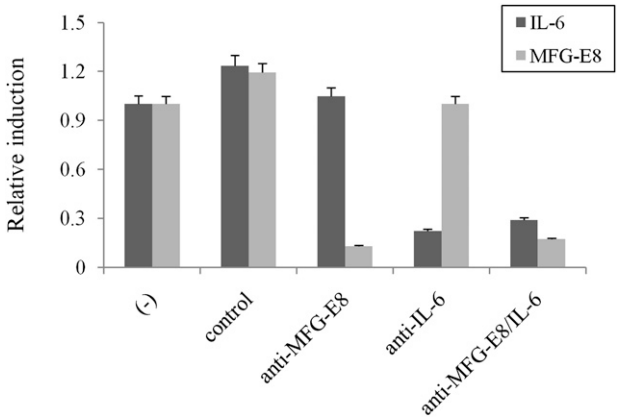

Fig. S17. CD68$^+$ macrophages isolated from NSCLC pleural effusions (TAM) were treated with isotype-matched Ig (control), anti–MFG-E8, and/or anti–IL-6, and the expression levels of MFG-E8 and IL-6 were quantified by RT-PCR. Data are representative of three independent experiments.

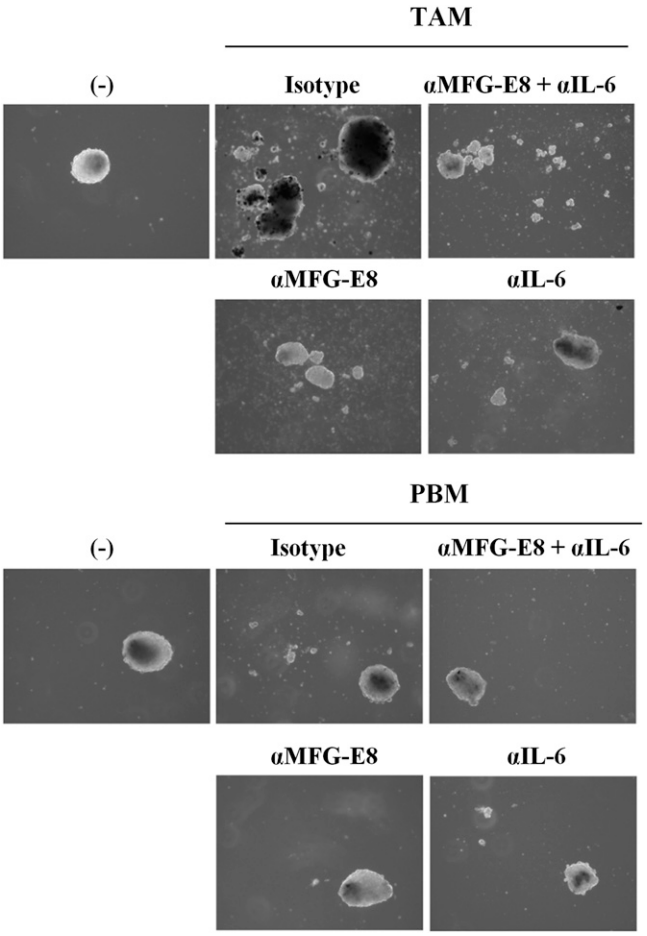

Fig. S18. EpCAM$^+$CD133$^+$primary NSCLC-CSCs were treated with supernatant of CD68$^+$ macrophages from pleural effusion (TAM) or peripheral blood leukocytes (PBM) in the absence (–) or presence of anti–MFG-E8 Ab, anti–IL-6 mAb, or both, in an ultra-low attachment plate. The cells were propagated with three passages, and the spheres generated per 1,000 cells were shown. Data are representative of three independent experiments.

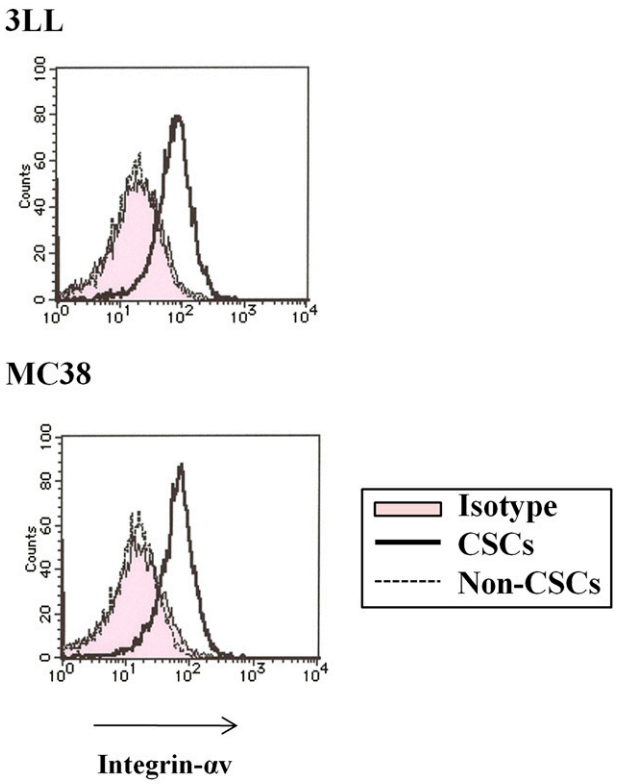

Fig. S19. Integrin-αv expression on 3LL (CD133^{+}ALDH1^{+}3LL-CSCs or CD133^{-}ALDH1^{-}3LL^{-} non-CSCs) or MC38 (CD44^{+}ALDH1^{+}MC38-CSCs or CD44^{-}ALDH1^{-}MC38-non-CSCs) was evaluated by flow cytometry. Data are representative of two independent experiments.

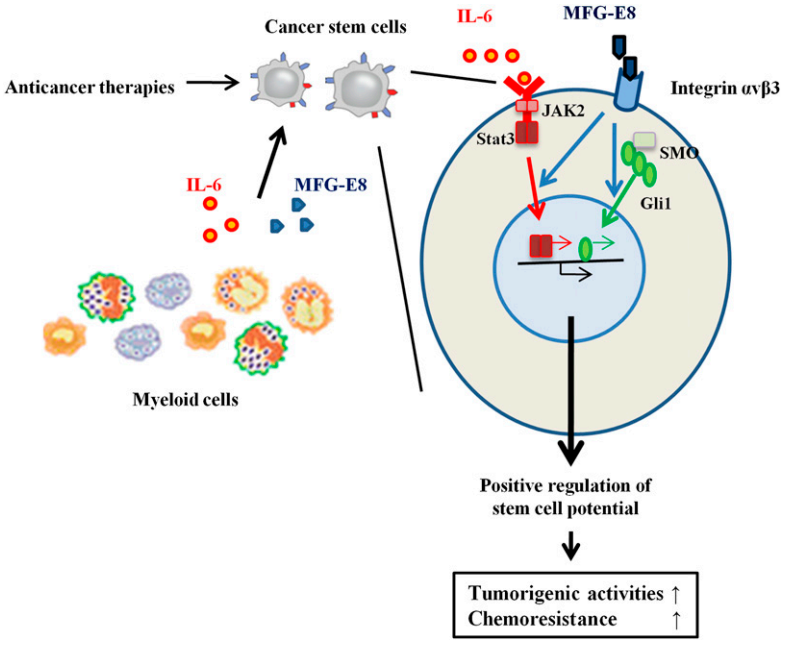

Fig. S20. The molecular mechanism by which tumor-associated macrophages activate CSCs. MFG-E8 secreted from tumor-associated macrophages triggers tumorigenesis and anticancer drug resistance in CSCs through the coordinated activation of the Stat3 and Hedgehog pathways. Inflammatory cytokines, such as IL-6, also coordinate with MFG-E8 to further amplify CSC activities in subsets of tumor cells such as primary NSCLC cells..