Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells

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Recent evidence has unveiled the critical role of tumor cells with stem cell activities in tumorigenicity and drug resistance, but how tumor microenvironments regulate cancer stem/initiating cells (CSCs) remains unknown. We clarified the role of tumor-associated macrophages (TAMs) and their downstream factor milk-fat globule–epidermal growth factor-VIII (MFG-E8) in the regulation of CSC activities. Bone marrow chimeric systems and adoptive cell transfers elucidated the importance of MFG-E8 from TAMs in conferring to CSCs with the ability to promote tumorigenicity and anticancer drug resistance. MFG-E8 mainly activates signal transducer and activator of transcription-3 (Stat3) and Sonic Hedgehog pathways in CSCs and further amplifies their anticancer drug resistance in cooperation with IL-6. Thus, the pharmacological targeting of key factors derived from tumor-associated inflammation provides a unique strategy to eradicate therapy-resistant tumors by manipulating CSC activities.

Chemoresistance | Tumor progression

Because tumor cells acquire multiple layers of anticancer drug resistance through the alteration of genetic and epigenetic profiles (1, 2) and activation of multidrug resistance transporters (3), it is extremely difficult to treat tumors in which multiple drug resistance is achieved systematically. Recent evidence has clarified the critical role of rare tumor cell populations, termed cancer stem/initiating cells (CSCs), in restraining the drug sensitivities of tumor cells (4). However, the molecular mechanisms whereby CSCs acquire tumorigenicity and drug-resistant machineries remain largely unknown.

Besides intrinsic genetic and epigenetic signatures in tumor cells, the tumorigenicity is regulated by extrinsic signals delivered from microenvironments or niches, which are composed of endothelial cells, stromal fibroblasts, and inflammatory cells (5–7). In addition, accumulating evidence has validated the critical role of tumor-associated myeloid cells in tumor progression and metastasis (5, 6). Thus, the molecular events linking intrinsic oncogenic signals with tumor-associated microenvironments may play an important role in rendering CSCs with the ability to modulate tumorigenicity and drug responses.

Milk-fat globule EGF-8 (MFG-E8) has been identified as a growth factor involving phagocytosis, angiogenesis, and immune tolerance (8–10). MFG-E8 was also highly produced from tumor-associated macrophages (TAMs) (11), but it remains largely unknown whether TAM-derived MFG-E8 regulates CSC activities. Here we found that TAMs produced large amounts of MFG-E8 in stimulation with CSCs. The MFG-E8 increased tumorigenicity and anticancer drug resistance in CSCs derived from murine and human tumor cells and primary tumor samples. MFG-E8 triggered anticancer drug resistance through the coordinated activation of Stat3 and Sonic Hedgehog signals in CSC populations. Furthermore, MFG-E8 and IL-6 from TAMs synergistically mediate tumorigenicity and drug resistance in subsets of CSCs including primary human tumors. These findings provide evidence that TAM serves as a source of key components in inflammatory microenvironments, such as MFG-E8 and IL-6, which trigger tumorigenicity and resistance to anticancer therapeutics by regulating CSC activities.

Results

Tumor-Infiltrating Macrophages Produce Large Amounts of MFG-E8. MFG-E8 is produced in large amounts by myeloid cells such as follicular dendritic cells and tangible macrophages and tumor-infiltrating myeloid cells (11, 12). However, it remains largely unknown which tumor cell subset is responsible for inducing MFG-E8 in tumor microenvironments. Because accumulating evidence demonstrates that CSCs are responsible for rendering tumor cells with the ability to promote tumorigenicity and drug resistance (4, 13–16), we examined the role of CSCs for the MFG-E8 expression in tumor microenvironments.

We inoculated CD44⁺/ALDH1⁺ colon tumor cells (MC38-CSCs) and CD133⁺/ALDH1⁺ lung cancer cells (3LL-CSCs), which have been validated as CSCs by the in vivo serial transplantation procedures (Fig. S1) and their nonstem cell counterpart (non-CSCs) into C57BL/6 mice. Various cell types were isolated from established tumors and splenocytes at the time when each tumor reached 100 mm³ and the MFG-E8 expression was quantified by RT-PCR. MFG-E8 expression was largely confined into CD11b⁺ and F4/80⁺ populations in MC38-CSC-challenged sites but not spleen, and other populations did not express MFG-E8 (Fig. S2). Furthermore, MFG-E8⁺ populations were enriched in F4/80⁺/CD11b⁺ macrophages derived from MC38- or 3LL-CSCs–derived tumors but not those from their non-CSC counterparts, tumor-draining lymph node (TLN), or splenocytes (Fig. 1 A and B and Fig. S3). MFG-E8 proteins were detected at much higher levels in TAM than splenic macrophages isolated from MC38-CSCs bearing wild-type mice, as quantified by ELISA (Fig. S4 A). Furthermore, F4/80⁺ splenic macrophages expressed MFG-E8 when directly cocultured with MC38-CSCs in vitro, whereas those cultured with MC38 alone did not trigger MFG-E8 induction (Fig. 1C). The supernatants from CSCs, but not other tumor cells, were sufficient for MFG-E8 expression in splenic macrophages, suggesting that soluble factors specifically released from CSCs are responsible for MFG-E8 induction (Fig. S4B). Furthermore, the inhibitors for IL-4, IL-10, TGF-β, CCL-2, and...
arginase-I, which are critical for M2 macrophage differentiation and activities (17), and GM-CSF, which is responsible for MFG-E8 production by peritoneal macrophages (9), had little effect on CSC-mediated MFG-E8 induction by TAM (Fig. S4B). Together, these results validate the CSC-specific role in MFG-E8 induction of macrophages.

To evaluate MFG-E8 expression in primary human tumor samples, we used pleural effusion cells isolated from stage IV nonsmall cell lung cancer (NSCLC) patients. Notably, most of the EpCAM + epithelial cells in pleural effusions were isolated from pleural effusion at much higher levels than EpCAM + tumor cells or CD68 + macrophages from peripheral blood mononuclear leukocytes (PBMC) of the same donors (Fig. 1D and Fig. S5).

TAMs expressed genes characteristic of tumor-promoting functions, such as hypoxia-inducible factor-1α, arginine-II, and Ets-2 (17, 19, 20). However, CSC-derived TAMs expressed these effectors at levels similar to those from tumors depleted of CSCs (Fig. S6). In addition, the macrophage mannose receptor (MMR) and TIE-2, which served as a marker for alternative (M2) and angiogenic subsets of TAMs (21, 22), was expressed on TAMs from wild-type and MFG-E8–deficient mice at similar levels. However, MFG-E8 was highly detected in TAMs expressing MMR or TIE-2 (Fig. S7), indicating that tumorigenic macrophages characterized by M2 and angiogenic profiles may regulate CSC activities in an MFG-E8–dependent manner. Collectively, these results demonstrate that CSCs are responsible for triggering MFG-E8 induction from macrophages.

**TAM-Specific MFG-E8 Renders CSCs with the Ability to Promote Chemoresistance.** Although MFG-E8 has been reported to accelerate tumorigenicity of certain spontaneously arising tumors (23), it remains unknown whether MFG-E8 modulates CSC functions. Thus, MC38-CSCs or 3LL-CSCs were inoculated into MFG-E8–deficient mice or their wild-type counterparts, and the CSC frequencies in established tumors were evaluated by measuring CSC-specific marker expression 1 mo after in vivo inoculation. The CSC markers in established MC38-CSCs and 3LL-CSCs were largely lost but still detectable 1 mo after in vivo inoculation, consistent with previous finding that CSCs differentiate into heterogeneous cell populations (4). In contrast, the frequencies of original CSC populations were largely undetectable in tumors grown into MFG-E8–deficient mice (Fig. 2B, C). The CSC growth in MFG-E8–deficient mice was compatible to that in wild-type mice, excluding the possibility that the different growth kinetics in MFG-E8–deficient and wild-type mice have an impact on the CSC frequencies (Fig. S8).

To further define whether TAM influences CSC activities in an MFG-E8–dependent manner, we evaluated the role of TAM-derived MFG-E8 in modulating anticancer drug sensitivities. To do so, CD11b + F4/80 + macrophages were isolated from established tumors from wild-type or MFG-E8–deficient CD45.1 + mice and were injected along with MC38-CSCs or their non-CSC counterparts into CD45.2 + MFG-E8–deficient mice. We first confirmed that there were little differences between wild-type and MFG-E8–deficient TAMs in the recruitment in tumor tissues (Fig. S9). Transfer of wild-type TAMs resulted in the impaired antitumor effect of chemotherapeutic agent cisplatin (CDDP) against MC38-CSCs, but the same regimens regressed tumor growth when MFG-E8–deficient TAM was transferred with MC38-CSCs (Fig. 2B). In contrast, neither wild-type nor MFG-E8–deficient TAMs had any effect on the drug sensitivities of non-CSC–derived tumors (Fig. 2B).

We next evaluated the role of TAM-derived MFG-E8 in the in vitro drug-induced apoptosis. The supernatant of wild-type TAMs expressed genes characteristic of tumor-promoting functions, such as hypoxia-inducible factor-1α, arginine-II, and Ets-2 (17, 19, 20). However, CSC-derived TAMs expressed these effectors at levels similar to those from tumors depleted of CSCs (Fig. S6). In addition, the macrophage mannose receptor (MMR) and TIE-2, which served as a marker for alternative (M2) and angiogenic subsets of TAMs (21, 22), was expressed on TAMs from wild-type and MFG-E8–deficient mice at similar levels. However, MFG-E8 was highly detected in TAMs expressing MMR or TIE-2 (Fig. S7), indicating that tumorigenic macrophages characterized by M2 and angiogenic profiles may regulate CSC activities in an MFG-E8–dependent manner. Collectively, these results demonstrate that CSCs are responsible for triggering MFG-E8 induction from macrophages.
TAM-Specific MFG-E8 Renders CSCs with the Ability to Promote Tumorigenicity. Because long-term sphere-forming capacity is a common characteristic of CSCs, we evaluated the role of MFG-E8 in tumor sphere-forming activities. The supernatant of wild-type, but not MFG-E8-deficient TAM increased sphere numbers and diameters in bulk MC38 cells. In addition, the recombinant murine MFG-E8 protein promoted sphere formation in a concentration-dependent manner (Fig. 3A). The addition of human MFG-E8 protein also accelerated sphere formation of human colon cancer cells (HCT116) (Fig. S11). MFG-E8 also regulates the PKH-26 dye-retaining ability of HCT116 cells, suggesting that MFG-E8 maintains CSCs as quiescent-state populations linking with their chemoresistant phenotype (24–26) (Fig. S12).

To further define the contribution of TAM-derived MFG-E8 in self-renewal in vivo, TAM isolated from wild-type or MFG-E8-deficient mice were injected along with MC38-CSCs into MFG-E8-deficient mice, and the isolated tumor cells were serially transplanted with wild-type or MFG-E8-deficient TAM into tumor-free MFG-E8-deficient mice. TAMs from wild-type mice accelerated tumor formation with high potency even when small amounts of tumor cells (1 × 10^2 per mouse) were inoculated, whereas TAMs from MFG-E8-deficient mice could not stimulate tumorigenicity even at more than 1 × 10^3 cells inoculated (Fig. 3B).

The tumorigenic activities induced by TAM-derived MFG-E8 were also observed in primary human tumors because CD68+ TAMs isolated from NSCLC patients stimulated sphere-forming activities of autologous CD133+EpCAM+ CSCs in an MFG-E8-dependent manner (Fig. S13). To further evaluate whether TAM-derived MFG-E8 plays a critical role in accelerating tumorigenic activities of CSCs in clinically relevant settings, EpCAM+ CD133+ primary NSCLC-CSCs were injected s.c. into NOD-SCID mice at small doses (1 × 10^2 per mouse) in conjunction with autologous CD68+ macrophages isolated from pleural effusions (TAMs) or peripheral blood (PBM), and the tumor formations were evaluated in vivo. In this experiment, NOD-SCID mice were pretreated with clodronate liposome to remove endogenous macrophages. TAMs elicited large tumor formation in NOD-SCID mice, whereas EpCAM+CD133+ CSCs alone or that inoculated with peripheral blood-derived macrophages (PBM) formed small tumors at final evaluation periods (28 d). Importantly, TAM-activated CSC tumorigenesis was suppressed by the human MFG-E8 blocking Ab (Fig. 3C). Overall, these results demonstrate that TAM-derived MFG-E8 may be responsible for regulating self-renewal and tumorigenic activities of CSCs of mice and human origins.

MFG-E8 Mediates Tumor Drug Resistance by Activating Stat3 and Hedgehog Signals. We next evaluated how MFG-E8 modulates oncogenic signals and triggers chemoresistance in CSCs. We found that MFG-E8 induced Stat3 phosphorylation and smoothened (SMO) expression, the downstream regulator of Sonic Hedgehog (shh) pathways, to a greater extent in CSCs than non-CSCs in stimulation with supernatant of wild-type but not MFG-E8-deficient TAMs (Fig. 4A). The up-regulation of Stat3 and shh activities was also confirmed in CSCs stimulated with supernatant of wild-type TAM by flow cytometry, but MFG-E8-deficient TAM or splenic macrophage (SPM) had little effect on Stat3 and shh activation in CSCs (Fig. 4B). Moreover, the target gene expression for Stat3 (SOCS3, myeloid cell leukemia protein, and vascular endothelial growth factor-A) and shh (GLI1, PTCH1, and GAS1) were up-regulated in CSCs stimulated with wild-type, but not MFG-E8-deficient, TAM (Fig. S14). These results demonstrate that TAM-derived MFG-E8 plays an important role in Stat3 and shh activation of CSCs.

We next examined whether Stat3 and shh signals are responsible for regulating CSC activities in TAM-derived MFG-E8-dependent manner. The importance of Stat3-mediated signals in MFG-E8-mediated tumorigenic activities have been validated by the observation that a constitutively active form of Stat3 (Stat3C) abrogated the tumor suppressive effects of anti-MFG-E8 Ab in MC38-CSCs stimulated with wild-type TAMs (Fig S15). The transcriptional activation of the Hedgehog effector Gli-1 was also increased in MC38-CSCs treated with wild-
We found that the combined inhibition of MFG-E8 and IL-6 increased CDDP-induced apoptosis (Fig. 5B) and suppressed sphere-forming activities (Fig. 5C and Fig. S18) at greater degrees than anti–MFG-E8 Ab or anti–IL-6 Ab alone in primary NSCLC-CSCs stimulated with TAM supernatant but not PBM. In contrast, MFG-E8 serves as a main factor for tumorigenic activities of murine MC38-CSCs (Figs. 2D and 4D). These results indicate that coregulation of MFG-E8 and IL-6 may be required for the tumorigenicity and drug resistance in subsets of CSCs including primary NSCLCs.

We finally examined the interplay between MFG-E8 and IL-6 in regulating CSC tumorigenic activities in vivo. The combined blockade of MFG-E8 and IL-6 markedly suppressed primary NSCLC-CSC–derived tumor growth in co-injection with autologous TAM, whereas the anti–MFG-E8 Ab or anti–IL-6 Ab alone had partial antitumor effects (Fig. 5D). These results demonstrate that IL-6 amplifies MFG-E8–mediated activities in increasing tumorigenic activities in subsets of CSCs including primary human tumors.

Discussion
Recent evidence has revealed that tumorigenic cells are infrequent and heterogeneous populations as measured by CSC marker expression (30). However, the identification of tumorigenic cells, including CSCs, has been largely based on the tumor formation of purified patient-derived cell suspensions in immuno- nodeficient animals, and it is difficult to clarify the role of environmental differences between tumors in modulating tumorigenicity and anticancer drug sensitivities. Therefore, it is urgent to elucidate the possibility that extrinsic signals delivered by distinct microenvironments may regulate the plasticity of CSC phenotypes and functions.

Because inflammatory cells in tumor microenvironments play an important role in affecting tumor progression via inflammatory and angiogenic signals (5, 6, 24), they may have a role in modulating tumorigenicity and stem cell activities.

MFG-E8 has been identified as a growth factor that signals through integrin-αvβ3 and αvβ5. Although MFG-E8 exerts various physiological processes, such as apoptotic cell phagocytosis and angiogenesis (8, 9, 31), it also plays a critical role for tumor progression through coordinated interplay of oncogenic and...
immune-dependent mechanisms (10, 11, 23). In this study, we have identified that MFG-E8, mainly derived from CSC-associated macrophages, is a major contributor in triggering the tumorigenicity and resistance to anticancer drugs of CSCs. Interestingly, \( \alpha \)-integrins, which serve as MFG-E8 receptors, were expressed on CSCs at higher levels than non-CSCs (Fig. S19), implying that MFG-E8 interaction with \( \alpha \)-integrins may be critical for triggering tumorigenic activities and drug resistance in a CSC-specific manner. Thus, it is of great interest to clarify whether \( \alpha \)-integrins serve as functional markers of particular CSC subsets that specifically communicate with tumor-associated myeloid cells.

We also demonstrated that IL-6 coordinates with MFG-E8 in further amplifying anticancer drug resistance by boosting specific oncogenic signals, the Stat3 and Hedgehog pathways, in subsets of CSCs including primary NSCLCs. Together, these findings clarified the indispensable role of inflammatory signals in tumor microenvironments to determine the clinical efficacy of various anticancer modalities against therapy-resistant tumor cells (Fig. S20).

There is a growing appreciation that myeloid cells, including macrophages and dendritic cells, are composed of several distinct populations that may influence the quality of tumor microenvironments. Recent genetic profiling and phenotypic analyses have also revealed that tumor cells manipulate tumor-infiltrating myeloid cells.
macrophages to induce distinct factors, such as versican and IL-13, which differentiate myeloid cells into specialized subsets with tumor-promoting capacities (32, 33). Because MFG-E8 and IL-6 were up-regulated in macrophages infiltrating the microenvironment formed by CSCs, the genetic and phenotypic profiles in CSCs distinct from bulk tumor cells may manipulate macrophages to induce tumorigenesis and drug resistance in a paracrine fashion. Thus, it is of great interest to clarify the molecular pathways and identify the distinct factors induced by CSCs.

Tumorigenic cells possessing anticancer therapy resistance, in particular CSC, have unique characteristics with which to manipulate complex signal cascades leading to oncogenic addiction, stem cell maintenance, and angiogenesis (4). Several oncogenic pathways, such as Wnt/β-catenin, mTOR, TGF-β, FOXP3, etc., regulate anticancer drug responsiveness and tumorigenicity in chemotherapy-resistant tumor cell populations (34, 35). Stat3 also plays a critical role in positively regulating self-renewal of embryonic stem cells stimulated with leukemia inhibitory factor (36). Hedgehog signals have been identified as sentinel in linking oncogenic aberration with the developmental programs of normal and cancer stem cells (37). Consistent with the importance of Stat3 and Hedgehog signals in stem cell activities, our findings clarify the coordinated interplay between Stat3 and Hedgehog signals in rendering CSCs tumorigenic and resistant to anticancer drugs. Although the molecular interplay that connects two different signaling pathways in CSCs remains largely unresolved and must necessarily be further clarified, recent studies have unveiled the critical role of epigenetic alterations in generating tumorigenic cells in NF-kB-dependent inflammatory signals (27). Thus, it is possible that tumor-associated inflammation may modify chromatin structure and epigenetic signals, leading to the transcriptional activation of subsets of target genes in CSCs.

In summary, we have unveiled the critical role of MFG-E8 and IL-6 in combination with inflammatory tumor environments in determining the clinical efficacies of anticancer therapeutics against therapy-resistant CSC populations. Recent breakthroughs in comprehensive approaches along with the progress of stem cell biology have led to the identification of CSC-specific markers and multiple genetic pathways suitable for specifically targeting cancer stem cells (38). In addition to the novel therapeutic candidates identified from CSCs, our findings demonstrate that the targeting of components derived from tumor microenvironments may provide new therapeutic strategies, in combination with inhibitors of CSC-specific pathways, to eradicate treatment-resistant tumors across the different genetic and epigenetic alterations.

Materials and Methods
MFG-E8 KO mice, C57BL/6, and NOD-SCID animals were used as hosts of tumor inoculations. Pleural effusion cells and PBMCs were obtained from advanced NSCLC patients according to the protocols approved by the institutional review board (no: 2010-0114). Tumor-associated macrophages isolated from tumors inoculated into wild-type or MFG-E8 KO mice, or from pleural effusion of advanced NSCLC patients were used for assessing MFG-E8 expression and role of CSC activities. Detailed information is provided in SI Materials and Methods.

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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/6 and NOD-SCID animals were purchased from SCL and Charles River, respectively. All experiments were conducted under a protocol approved by the animal care committee 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, LVTLDTRGDIFTEY and the sera was collected 8 wk after initial immunization. The antisera were further purified by protein-A columns (Fierce). 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+-initiating cell (CSC) populations. Bulk tumor cells or those isolated for CSC marker positivity (CD44+ for MC38; CD133+ for Colon-26) were injected s.c. into MFG-E8–deficient mice at 1 × 10⁶ 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, tumordraining 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′AACACACCAAGCCCTCCGGTGTTCT3′ and reverse, 5′ACAGACAGGCAGGGGAAATCTGTGAA3′ for murine MFG-E8; forward, 5′TCCCAAGAAGTGCAGGAGAAGTGT3′ and reverse, 5′ATGCCGCAACCAAGAAGGTCCAC3′ 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 FACS Caliber.

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-1+ granulocytes, CD11c+CD11b+ myeloid dendritic cells, and CD11c+ 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+ALDEFLUOR+MC38-CSCs or CD44+ALDEFLUOR–non-CSCs (1 × 10⁶ 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 × 10⁶ 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 epidermal 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 anti-cancer 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.

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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 \( (1 \times 10^5) \) 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′-CCTTCAGATGGATTTGCA-ATCTT-3′ and 5′-GAAGGCTCATGACAGTGCTTCGT-3′, respectively) and murine shh siRNA (5′-CGGCCATTCTACG-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.


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 C57BL/6 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 (αIL-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. 56. 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, arginase-2 (ARG2), or HIF-1α in macrophages was quantified by real-time PCR. Data are representative of three independent experiments.

Fig. 57. 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. 58. 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⁻/⁻ mice (MFG-E8KO). MC38-CSCs were injected into tumor-free MFG-E8⁻/⁻ 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⁺ALDEFLOUR⁺ (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.
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.

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.

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