Mesenchymal Stem Cells Promote Formation of Colorectal Tumors in Mice

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BACKGROUND & AIMS: Tumor-initiating cells are a subset of tumor cells with the ability to form new tumors; however, they account for less than 0.001% of the cells in colorectal or other types of tumors. Mesenchymal stem cells (MSCs) integrate into the colorectal tumor stroma; we investigated their involvement in tumor initiation. **METHODS:** Human colorectal cancer cells, MSCs, and a mixture of both cell types were injected subcutaneously into immunodeficient mice. We compared the ability of each injection to form tumors and investigated the signaling pathway involved in tumor initiation. **RESULTS:** A small number (≤10) of unsorted, $CD133^-$, $CD166^-$, epithelial cell adhesion $molecule^-(EpCAM^-)$, or $CD133^-/CD166^-/EpCAM^-$ colorectal cancer cells, when mixed with otherwise nontumorigenic MSCs, formed tumors in mice. Secretion of interleukin (IL)-6 by MSCs increased the expression of CD133 and activation of Janus kinase 2–signal transducer and activator of transcription 3 (STAT3) in the cancer cells, and promoted sphere and tumor formation. An antibody against IL-6 or lentiviral-mediated transduction of an interfering RNA against *IL-6* in MSCs or *STAT3* in cancer cells prevented the ability of MSCs to promote sphere formation and tumor initiation. **CONCLUSIONS: IL-6, secreted by MSCs, signals through STAT3 to increase the numbers of colorectal tumor-initiating cells and promote tumor formation. Reagents developed to disrupt this process might be developed to treat patients with colorectal cancer.**

Keywords: Marrow Stromal Cells; Cancer Stem Cells; JAK2; Tumor Development.

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Tumor initiating or stem cells (TICs), the initiation cells in tumors, are a minor population of tumor cells that possess the stem cell property of self-renewal and multilineage differentiation. Recently, a subpopulation of TICs were identified in colon cancer.[1,2] They are included in the high-density CD133+ population that accounts for about 2.5% of the tumor cells. Subcutaneous injection of colon cancer CD133+ but not CD133− cells readily reproduced the original tumor in immunodeficient mice. Another study also showed that the ability to engraft in immunodeficient mice was restricted to a minority subpopulation of epithelial cell adhesion molecule $(EpCAM)^{high}/CD44^+$ epithelial cells in colon cancer, and further identified CD166 as an additional differentially expressed marker, useful for TIC isolation in colon cancer.[3] These studies validate the stem cell working model in human colon cancer and provide a highly robust surface marker profile for colon TIC isolation and the small number of undifferentiated tumorigenic cells should be the target of future therapies.

Normal stem cells are controlled by a mechanism that allows them to proliferate or adapt to the microenvironment or niche of stem cells. The tumor microenvironment is composed of altered extracellular matrix and various nontransformed cells (eg, fibroblast, myofibroblast, myoepithelial, and endothelial cells). The orchestra interaction between microenvironmental components and tumor cells is bidirectional. Microenvironmental components regulate gene expression in tumor cells, thereby directing the tumor into one or several possible molecular evolution pathways, some of which may lead to tumor formation, progression,[4,5] metastasis,[6] and drug resistance[7] of neoplasms.

Among the microenvironment components of tumor, mesenchymal stem cells (MSCs) recently have attracted great interest because of their ability to migrate and engraft to areas of tumor development.[8] MSCs reside in the stroma of breast cancer and enhance tumor metastases via the Chemokine (C-X-C motif) ligand 5 (CCL5)–Chemokine (C-C motif) receptor (CCR) signaling pathways.[6] Cancer development involves a series of oncogenic transformations that may be endowed by tumor microenvironment. We have shown the integration of MSCs into tumor-associated stroma of colorectal cancer.[8] However, the involvement of MSCs (or their

Abbreviations used in this paper: αSMA, α-smooth muscle actin; CAF, cancer-associated fibroblasts; CCCs, colorectal cancer cells; CDX2, caudal-type homeobox transcription factor 2; DF, dermal fibroblast; EpCAM, epithelial cell adhesion molecule; GE, gingival epithelial; GFP, green fluorescent protein; IL, interleukin; MSC, mesenchymal stem cell; MSC-CM, MSC-derived conditioned medium; NTCs, non-transformed cells; TICs, tumor initiating or stem cells; TSM, tumor sphere medium.

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associated tumor stroma) in tumor initiation has not been addressed.

In the current study, we found that a very small number (≤10) of unsorted, CD133−, CD166−, EpCAM−, or triple-negative CD133-CD166-EpCAM- human colorectal cancer cells, when mixed with otherwise nontumorigenic bone marrow–derived human MSCs, obtain their de novo tumorigenicity when this cell mixture is introduced into a subcutaneous site and allowed to form a tumor xenograft. Moreover, the signaling pathway involved in MSC-mediated enrichment of TICs was identified.

Materials and Methods

Primary Cells and Cell Lines

The human colorectal cancer cell line HT-29 was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mmol/L glutamine, and 10% fetal bovine serum (Gibco). The MSC cell line was immortalized by retroviral transduction of HPV16 E6E7 and grown in Dulbecco's modified Eagle medium–low glucose (Gibco) supplemented with 10% fetal bovine serum.[8] Primary MSCs from different normal human volunteers were obtained from the Tulane Center for Distribution of Adult Stem Cells and were prepared and grown as described previously.[9] For preparation of fresh tumor cells, excised tumor tissue samples were digested for 4 hours with 3 mg/mL collagenase I (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline (PBS)/3% fetal calf serum at 37°C. Single-cell suspensions were obtained by repeated pipetting of cells followed by passage through a 40-μm strainer. MSC-like tumor stromal cells were isolated from colon cancer cells via their preference for migration by culturing primary tumor cells in the upper well of a Transwell (Costar, Cambridge, MA) containing 5-μm pores, where MSC-like cells passed through the base of the upper well and attached to the lower well. The details about MSCs, WI38, dermal fibroblasts (DFs), gingival epithelial (GE) cells, 293 cells, HT-29, and other tumor cells are listed in Supplementary Table 1. All cells were kept in a 37°C humidified atmosphere with 5% CO_2.

Characterization of MSCs

These immortalized or primary MSCs have been characterized to meet the definition of MSCs: plastic adherence; expression of MSC surface proteins such as CD29, CD44, CD90, CD73, CD105, and CD166; and possession of differentiation potential into osteoblast, adipocyte, and chondrocyte.[10]

Xenograft Transplantation

Study protocols involving mice were approved by the Institutional Animal Committee of Taipei Veterans General Hospital. Nonobese diabetic/severe combined immunodeficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as a colony at the National Taiwan University Animal Facility in specific pathogen-free conditions. The mice were used for experiments at 6–8 weeks of age. Tumor cells admixed without/with MSCs were injected subcutaneously without Matrigel (BD Biosciences, San Diego, CA) or other extracellular matrix. Some mice transplanted with tumor cells admixed with MSCs were injected intraperitoneally with 10 μL PBS containing 5 μg anti–IL-6 antibodies (MAB206; R&D Systems, Minneapolis, MN) every 3 days until death.

Sphere Culture Conditions and Sphere Counting Assay

Primary colorectal cancer cells or colorectal cell line were resuspended in a modified tumor sphere medium[11] (TSM) (Dulbecco's modified Eagle medium/F12 medium consisting of a chemically defined serum-free medium with N2 supplement, recombinant human [20 ng/mL; PeproTech, Rocky Hill, NJ], epidermal growth factor [20 ng/mL; PeproTech], and fibroblast growth factor [10 ng/mL; PeproTech]), and plated at a density of 10^4 cells/well of a 6-well plate. Spheres were recognized as 3-dimensional cell colonies with a blurred cell margin in Ultra-Low Attachment Microplates (Corning, Lowell, MA). For cells treated with recombinant human IL-6 (R&D) or indirect co-culture with MSCs, spheres were recognized as cell colonies with more than 50% of the area showing a 3-dimensional structure and blurred cell margin. The ratio of the sphere was calculated as the percentage of sphere number to the total colony number.

Results

Nontransformed Cells Derived From Various Tissues Promote Colorectal Tumor Initiation and Tumor Sphere Formation

To investigate the functional consequences of the heterotypic interactions between HT-29 colorectal cancer cells (CCCs) and nontransformed cells (NTCs) derived from various tissues, the growth kinetics of the nontransformed cells containing tumors (CCCs plus NTCs) was compared with those of CCCs or NTCs alone in a xenograft model of immunocompromised mice. We found that co-injection with MSCs, WI38 lung fibroblasts, primary DFs, or primary GE, but not with 293 adenoviral-transfected human embryonic kidney epithelial cells, formed tumors with a smaller amount of tumor cells, 10^4 cells compared with 10^6 cells when injected with tumor cells alone, whereas 10^6 of NTC cells did not form tumors 3 months after transplantation alone (Supplementary Figure 1*A*). To further characterize the effect of NTCs on CCCs, we first compared the tumor sphere formation ability in TSM, a property of TICs in vitro, of HT-29 cells and HT-29 cells admixed with each type of NTC cells. We observed that HT-29 cells cultured alone had a minimal ability to form 3-dimensional tumor spheres and, as expected, MSCs or other NTC cells cultured alone were not able to form tumor spheres either (Figure 1*A* and Supplementary Figure 1*B*). Although co-culture of HT-29 cells with 293 cells did not change the tumor sphere formation (determined by sphere number and sphere ratio), the sphere formation ability of HT-29 increased when directly or indirectly co-cultured with MSCs (Figure 1*A* and *B*), WI38, and DF cells, and directly co-cultured with GE (indirect co-culture with GE only slightly increased sphere formation) (Supplementary Figure 1*C* and *D*). Because MSCs have been reported to migrate and incorporate into colorectal tumor development,[8] we therefore compared the effect of MSCs on tumor initiation and sphere formation with normal or tumor colonic fibroblasts. Interestingly, the ability of MSCs to enhance tumor initiation or sphere formation was significantly higher than normal or tumor colonic myofibroblasts (Supplementary Figure 1*E* and *F*).

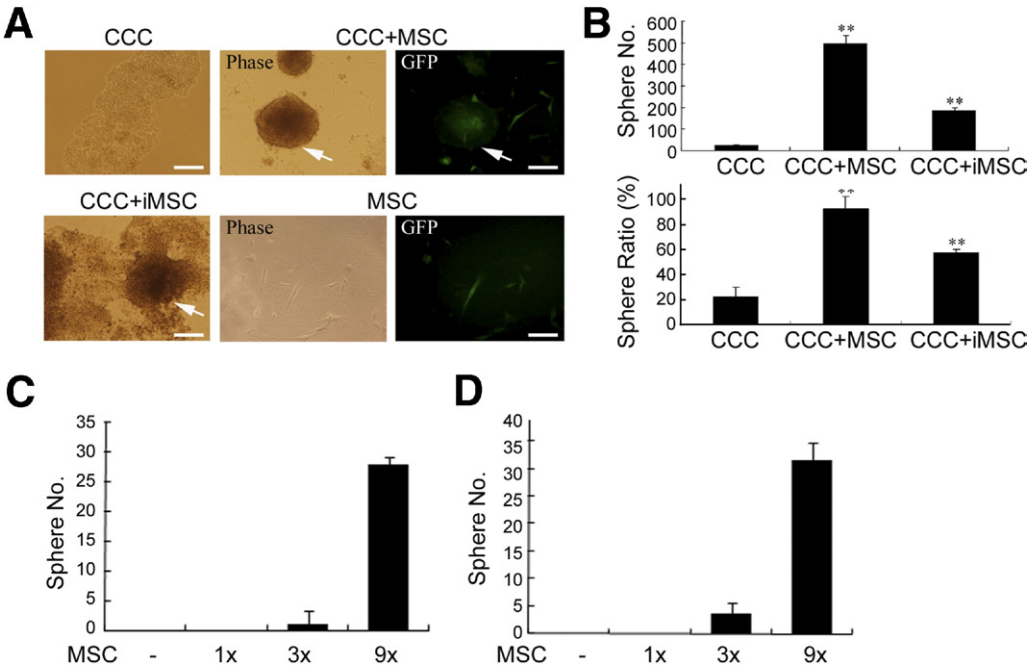


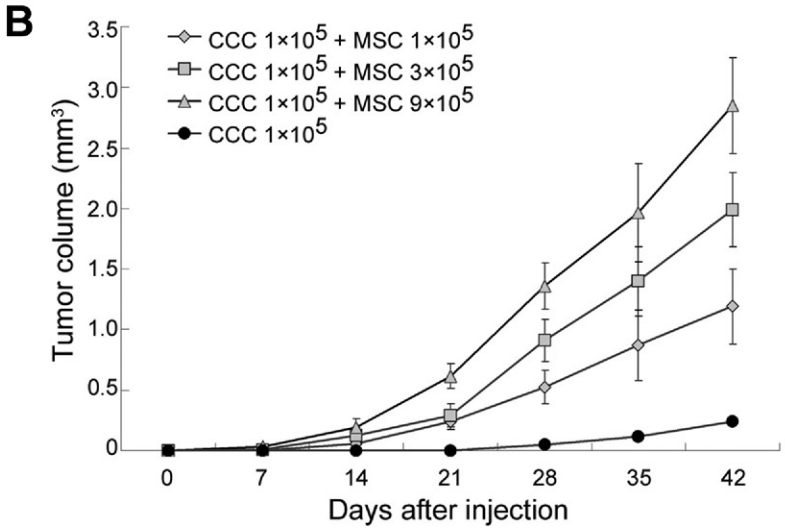
Figure 1. MSCs promote tumor sphere formation. CCC, direct co-culture of GFP-labeled MSCs (CCC + MSC), indirect co-culture of GFP-labeled MSCs (CCC + iMSC), and GFP-labeled MSCs alone were grown in TSM for 12 days. (*A*) Morphology and (*B*) sphere number and sphere ratio counted at 12 days for 10,000 HT-29 cells when grown in TSM in a 6-well plate. Experiments were performed in triplicate. The data represent 1 experiment representative of 3 separate experiments. (*Arrow* indicates sphere formation; scale bar = 50 μm.) (*C* and *D*) MSCs promote tumor sphere formation in media that do not enrich cells with TIC properties. Sphere numbers were counted on day 12 for 10,000 HT-29 cells when grown in a 6-well plate with indicated folds of MSCs. (*C*) Medium in serum-free Dulbecco's modified Eagle medium–HG was supplemented with ITS Premix (Gibco, Grand Island, NY), 10^{-8} mol/L dexamethasone, and 50 μg/mL ascorbate-2-phosphate. (*D*) Medium in serum-free Dulbecco's modified Eagle medium–HG was supplemented with N2 supplement, 10 ng/mL epidermal growth factor, and 10 ng/mL fibroblast growth factor 2.

MSCs Promote Tumor Sphere Formation and Tumor Initiation

The effects of colonic myofibroblasts, dermal, or other fibroblasts on enhancing tumor formation and the underlying mechanisms have been investigated previously,[12,13] however, little is known about the effects of MSCs on tumor sphere formation and tumor initiation. Because it also is noted that indirect co-culture of HT-29 cells with MSCs dose-dependently enhanced sphere formation in 2 different media in which HT-29 cells do not usually form tumor spheres (Figure 1*C* and *D*), it prompted us to examine the enhanced tumor initiation and sphere formation by MSCs via generating cells with TIC properties and identifying the secreted molecules and signaling pathways mediating the tumor initiation and sphere formation. Although injection of HT-29 cells alone was able to induce tumor formation with as many as 1×10^5 cells (83% within 3 weeks) or 1×10^6 cells (100% within 1 week), no tumors were formed with only 1×10^4 cells even after 6–7 months (Figure 2*A*). Interestingly, when only 1×10^4 unsorted, CD133−, CD166−, EpCAM−, or triple-negative CD133-CD166-EpCAM- HT-29 cells were mixed with 9×10^4 MSCs, tumor formation occurred within 2 (25%~75%) to 3 weeks (75%~100%), even though MSCs alone could not form tumors with up to 2×10^6 cells (Figure 2*A* and Table 1). Because MSCs induced de novo tumor formation by the number of cells that did not form tumors, these data suggest the ability of MSCs to enhance tumor initiation. In addition, we noted MSCs enhanced tumor initiation (Figure 2*A*) in a dose-dependent manner with the optimal dose at 9×10^4 cells. The ability of MSCs to enhance tumor initiation properties also was observed in primary MSCs (primary MSC1 and primary MSC2) from different individuals and other immortalized MSCs (Table 1). A similar observation was obtained with primary-derived (CCS and HCW) or fresh tumor cells collected from patients with colorectal cancer (Table 1). In addition, MSCs not only enhanced tumor initiation but also accelerated tumor growth in a dose-dependent manner (Figure 2*B*). The histology and degree of differentiation of xenografts derived from both CCCs alone (bulk) and the admixture of CCCs and MSCs was similar. They were positive for cytokeratin-20, the caudal-type homeobox transcription factor 2 (CDX2) and β-catenin (Figure 2*C*), a pattern seen almost exclusively in colonic adenocarcinoma.[14] Thus, the xenografts generated in this model matched the phenotypes of the original tumors. In addition, MSCs also induced de novo tumor formation of other gastrointestinal cancer cell lines such as gastric cancer AZ-521, liver cancer Hep-3B, and pancreatic cancer PANC-1 (Table 1). These data suggest primary and immortalized MSCs posses the ability to induce de novo tumor formation by non-TICs of a variety of gastrointestinal cancer cells.

A

	0 wk	1 wk	2 wks	3 wks	4 wks	5 wks	6 wks	12 wks*
MSC 1-2×10^6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
CCC 1×10^6	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CCC 1×10^5	0/6	0/6	0/6	5/6	6/6	6/6	6/6	6/6
CCC 1×10^4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CCC 1×10^5 + MSC 1×10^5	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CCC 1×10^5 + MSC 3×10^5	0/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6
CCC 1×10^5 + MSC 9×10^5	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CCC 1×10^4 + MSC 9×10^1	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CCC 1×10^4 + MSC 9×10^2	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CCC 1×10^4 + MSC 9×10^3	0/4	0/4	0/4	0/4	0/4	0/4	1/4	1/4
CCC 1×10^4 + MSC 9×10^4	0/4	0/4	2/4	4/4	4/4	4/4	4/4	4/4



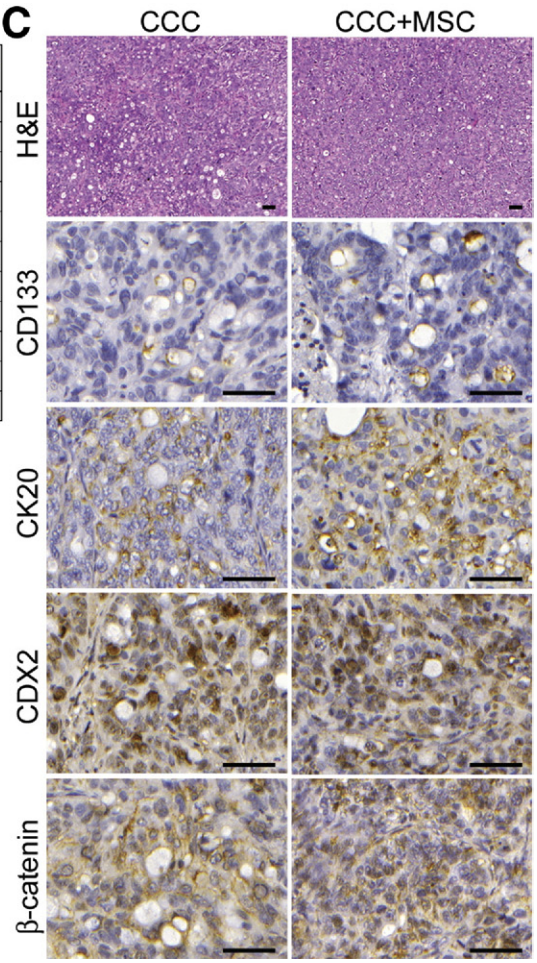


Figure 2. MSCs promote colorectal tumor initiation and growth. (*A*) Tumor formation rate at the indicated times after injection of indicated CCCs with or without indicated MSCs. *This was continued for more than half a year. (*B*) Tumor volume measurements (mean ± standard deviation) of 10^5 CCCs injected subcutaneously into nonobese diabetic/severe combined immunodeficient mice with or without 1–9 × 10^5 MSCs (n = 6 tumors per group). (*C*) Histology and immunohistochemistry for CD133, CK20, CDX2, β-catenin, and ki67 in tumors derived after a 6-week injection with CCC or CCC plus MSCs (scale bar = 50 μm).

MSC-Derived Conditioned Medium Enhances Tumor Sphere Formation, Increases Expression of TIC Markers, and Decreases Expression of Differentiated Markers

To further support whether MSCs caused ordinary tumor cells to acquire the ability to form spheres by secretory factors in vitro, the tumor sphere-forming rate of cancer cells negative for certain TIC markers was measured in TSM medium containing 1-fold of MSC-derived conditioned medium (MSC-CM) in ultra-low attachment plates. MSC-CM enhanced tumor sphere formation of unsorted, CD133−, CD166−, or EpCAM− population of HT-29, primary CCS, and fresh tumor cells (Figure 3*A* and data not shown). Moreover, enhancement of tumor sphere formation by MSC-CM also was observed in triple-negative (CD133-CD166-EpCAM-) cancer cells in serial dilution (Figure 3*B* and data not shown). Although the tumor sphere-forming rate for a single triple-negative HT-29 or CCS cell was about 0% (>100 μm) when cultured alone, the rate increased greatly to 58% (HT-29) or 67% (CCS) when co-cultured with MSCs. It has been shown that the CD133+ population includes TICs of human colon cancer.[1,2] Similarly, the current study also showed that CD133+ cells have an increased tumor initiation ability compared with bulk and CD133− cells (Supplementary Figure 2*A*). Further, tumors formed by CD133+ cells expressed the same markers of tumor formed by bulk tumor cells (Supplementary Figure 2*B*). After long-term treatment with MSC-CM in TSM, HT-29 formed 3-dimensional tumor spheres, which have increased CD133 expression (Figure 3*C*), and decreased CK20 and CDX2 expression, 2 colorectal cancer differentiated markers (Figure 3*D*).[2] Although the initial percentage of HT-29 cells was 10%, the co-culture resulted in a rapid increase of HT-29 percentage (Supplementary Figure 2*C*), suggesting a rapid loss of MSC ratio in the co-culture. Taken together, these observations suggest that MSCs caused ordinary tumor cells to acquire properties of colorectal TICs, including sphere formation in vitro and the increase of CD133 expression, and a decrease in the expression of differentiated markers through secretory factors.

Table 1. Comparison of Tumor Formation by Primary and Colorectal Cancer Cell Lines, Different Gastrointestinal Cancer Cells, Primary and Immortalized MSCs, and their Admixture

	1 wk	2 wk	3 wk	4 wk	6 wk	8 wk	12 wk
Unsorted $1*10^4$ + MSC $9*10^4$	0/4	1/4	4/4	4/4	4/4	4/4	4/4
CD133− $1*10^4$ + MSC $9*10^4$	0/4	2/4	3/4	4/4	4/4	4/4	4/4
CD166− $1*10^4$ + MSC $9*10^4$	0/4	3/4	4/4	4/4	4/4	4/4	4/4
EpCAM- $1*10^4$ + MSC $9*10^4$	0/4	2/4	4/4	4/4	4/4	4/4	4/4
CD133-CD166-EpCAM- $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CD133-CD166-EpCAM- $1*10^4$ + MSC $9*10^4$	0/4	2/4	3/4	4/4	4/4	4/4	4/4
3A6 $2*10^6$	0/6	0/6	0/6	0/6	0/6	0/6	0/6
pMSC1 $1*10^6$	0/6	0/6	0/6	0/6	0/6	0/6	0/6
pMSC2 $1*10^6$	0/6	0/6	0/6	0/6	0/6	0/6	0/6
HT-29 $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
HT-29 $1*10^4$ + 3A6 $9*10^4$	0/4	2/4	4/4	4/4	4/4	4/4	4/4
HT-29 $1*10^4$ + pMSC1 $9*10^4$	0/4	2/4	4/4	4/4	4/4	4/4	4/4
HT-29 $1*10^4$ + pMSC2 $9*10^4$	0/4	2/4	4/4	4/4	4/4	4/4	4/4
CCS $1*10^2$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CCS $1*10^3$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CCS $1*10^2$ + MSC $9*10^4$	0/4	0/4	1/4	2/4	2/4	2/4	2/4
CCS $1*10^3$ + MSC $9*10^4$	0/4	2/4	4/4	4/4	4/4	4/4	4/4
HCW $1*10^4$	0/4	0/4	0/4	0/4	1/4	1/4	1/4
HCW $1*10^4$ + MSC $9*10^4$	0/4	0/4	0/4	0/4	3/4	4/4	4/4
Fresh colon cancer cells $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Fresh colon cancer cells $1*10^4$ + MSC $9*10^4$	0/4	0/4	0/4	0/4	3/4	4/4	4/4
Gastric AZ-521 $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Gastric AZ-521 $1*10^4$ + MSC $9*10^4$	0/4	0/4	0/4	0/4	1/4	2/4	3/4
Liver Hep-3B $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Liver Hep-3B $1*10^4$ + MSC $9*10^4$	0/4	0/4	0/4	0/4	3/4	4/4	4/4
Pancreas PANC-1 $1*10^4$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Pancreas PANC-1 $1*10^4$ + MSC $9*10^4$	3/4	4/4	4/4	4/4	4/4	4/4	4/4

NOTE. Number of tumor formations/number of cell injections shown. For details about the cells please refer to Supplementary Table 1. pMSC, primary MSC.

MSCs Enhance the Tumor Initiation Rate of a Single Cancer Cell

To show in vivo the TIC ability of cancer cells rendered by MSCs, a very small number (10 or 100) or a single unsorted, triple-negative (CD133-CD166-EpCAM-) HT-29, or fresh colorectal cancer cell were co-injected with MSCs into nonobese diabetic/severe combined immunodeficient mice. All of the 10 or 100 tumor cells induced tumor formation in mice about 2 months after injection (Figure 4*A* and *B*). Moreover, these tumor cells expressed markers for normal colorectal tumors (Figure 4*C*). Interestingly, for the single unsorted triple-negative cells, we injected 100 mice and 5% developed tumor. These data together strongly suggest that MSCs caused ordinary tumor cells to acquire the ability to form xenograft tumor in vivo.

Because MSCs also increased tumor cell proliferation, as evidenced by the increase of ki67 staining in tumors admixed with MSCs (data not shown), it is uncertain whether MSC-enhanced cell proliferation contributed to in vivo tumor initiation and in vitro sphere formation. Therefore, to exclude that possibility, we administered prostaglandin E2, which enhances colorectal tumor growth (Supplementary Figure 3*A*) via transactivating the epidermal growth factor receptor,[15] in vitro and in vivo with tumor cells and found that sphere formation and tumor initiation were not enhanced (Supplementary Figure 3*B* and data not shown). These data suggest that increasing cell proliferation per se is not sufficient to enhance the tumor cells' ability to form spheres and initiate tumors, and that MSC enhancement of these properties is through another mechanism. Collectively, these data suggest that MSCs have the ability to generate cells with TIC properties.

MSCs Secrete IL-6 to Induce Tumor Sphere Formation

To examine the molecular mechanism by which MSCs enhance TIC properties in CCCs, we used a human protein cytokine array kit to explore the acting paracrine cues that MSCs supply to induce CCCs to form tumor spheres, and noticed that the levels of granulocyte-macrophage colony–stimulating factor, interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 were not detected in CCC culture but were detected in both the MSC and CCC-MSC co-culture (Figure 5*A*). Among them, IL-6 and IL-8 are associated with the development and progression of colorectal cancer and adenomas.[16,17] Secretion of IL-6 and IL-8 by MSCs but not by HT-29 CCCs, DF, GE, and 293 cells was first confirmed by enzyme-linked immunosorbent assay and by analyzing the messenger RNA (mRNA) levels (Figure 5*B* and data not shown). Interestingly, treatment with IL-6 up to 10 ng/mL enhanced tumor sphere formation by CCCs in a dose-dependent manner (Figure 5*C* and Supplementary Figure 4*A*), and also increased CD133 expression (Supplementary

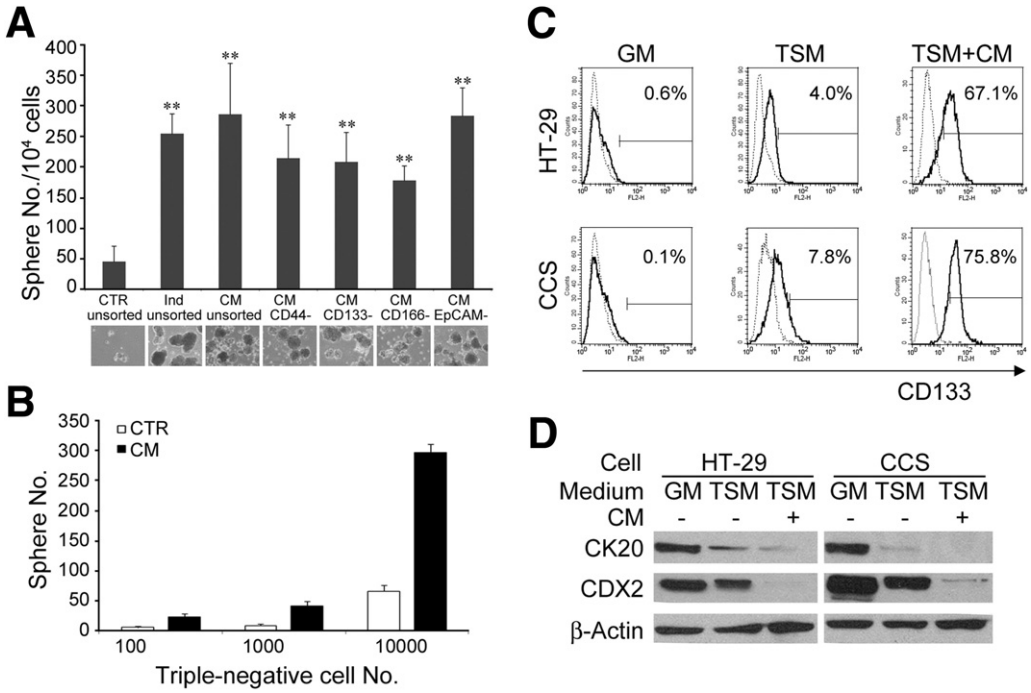


Figure 3. Treatment with MSC-conditioned medium enhances sphere formation and the expression of CD133 and reduces the expression of differentiated markers. Unsorted or each kind of negatively selected CCCs were cultured in growth medium (GM) or in TSM using Ultra-Low plates without (CTR) or with indirect co-culture with MSCs (Ind) or treatment with MSC-CM (CM) for 12 days. (*A*) Sphere number formed by 10,000 CCCs in each condition was counted. (*B*) Sphere number formed by indicated number of CD133-CD166-EpCAM- triple-negative CCCs in each condition was counted. (*C*) Flow cytometric analysis for the expression of CD133 was assayed. (*D*) Western blotting for the expression of CK20 and CDX2 was examined.

Figure 4*B*) and inhibited cytokeratin-20 and CDX-2 expression of these tumor cells (Supplementary Figure 4*C*). However, treatment with IL-8 up to 10 ng/mL did not induce any increase in tumor sphere formation (Figure 5*C*), suggesting that IL-6 but not IL-8 was involved in the ability of MSCs to generate cells with TIC properties. Compared with isotype antibodies, anti–IL-6 antibodies abrogated MSC or MSC-CM–induced enhancement of tumor sphere formation by CCCs (Figure 5*C* and Supplementary Figure 4*A*), and anti–IL-6 antibodies also down-regulated CD133 expression and increased cytokeratin-20 and CDX-2 expression (Supplementary Figure 4*B* and *C*).

Notably and consistent with the in vitro results, when we infused the mice with anti–IL-6 antibodies, HT-29 cells admixed with MSCs lost the ability to initiate tumors and for tumor growth (Figure 5*D*). In addition, knockdown of IL-6 in MSCs with short hairpin RNAs against IL-6 impaired MSCs in the ability to enhance tumor sphere formation (Supplementary Figure 5*A*–*C*) and tumor initiation in tumor cells (Supplementary Figure 5*D*). In contrast, overexpression of IL-6 in 293 fibroblasts was sufficient to enable these cells to stimulate sphere formation (Figure 5*E*) and in vivo tumor initiation of admixed HT-29 CCCs (Figure 5*F*). Collectively, these data suggest that IL-6, secreted from MSCs, is responsible for much, if not all, of the observed MSC-mediated enhancement in sphere formation and tumor initiation by the CCCs.

Involvement of JAK2-STAT3 in MSCs- and IL-6–Mediated Enhancement of Tumor Sphere Formation and Tumor Initiation

To determine what downstream signals in the tumor cells respond to IL-6 secretion by MSCs we looked at Akt, ERK, STAT3, and the Wnt pathway, which all have been reported to be activated upon stimulation with IL-6, and found that only STAT3 was activated by IL-6 (Figure 6*A* and Supplementary Figure 6*A*). The activation was as early as 30 minutes (Figure 6*A*) and in a dose-dependent manner (Figure 6*B*). When examining the downstream pathways involved in IL-6 signaling,[18] we found that JAK2 (Figure 6*A*) but not JAK1 (data not shown) was activated by IL-6. Interestingly, a JAK inhibitor 1 (inhibitor of pan JAKs) or AG490 (a specific JAK2-inhibitor) inhibited the activation of STAT3 by IL-6 to the same degree, suggesting that JAK2 is involved in IL-6–induced activation of STAT3 (Figure 6*C*). STAT3 activation also was shown upon indirect co-culture of HT-29 with MSCs, and the activation was completely inhibited by treatment with anti–IL-6 antibodies or AG490 (Figure 6*D*). Similar changes also were observed in CCS or HT-29 cells treated with MSC-conditioned medium (Supplementary Figure 6*B*). Moreover, expression of STAT3 downstream molecules such as cyclinD1, Survivin, and c-Myc also was increased upon treatment with MSC-conditioned medium, and the increase was inhibited by treatment with anti–IL-6 antibodies or AG490 (Supplementary Figure

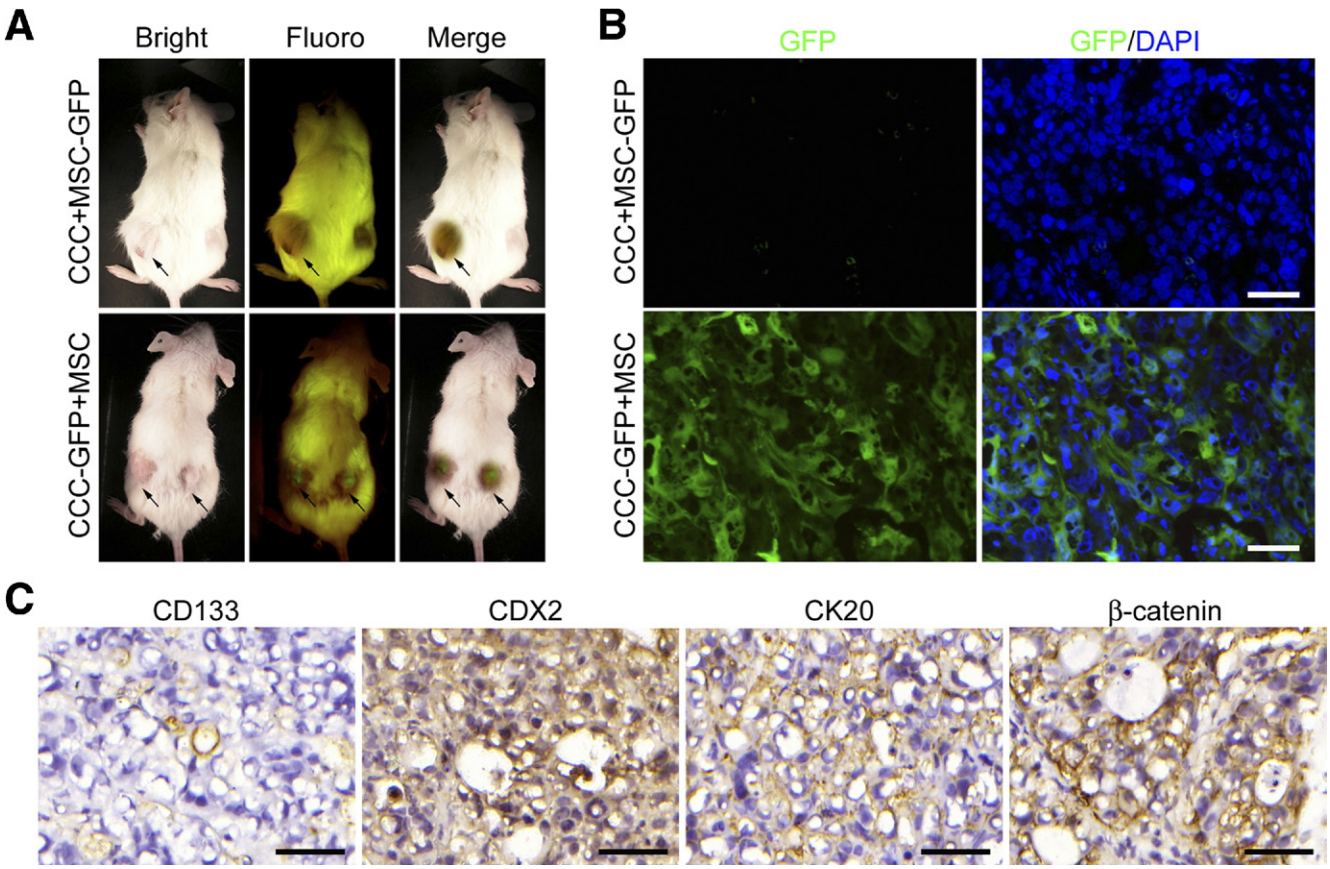


Figure 4. Single-cell CCCs admixed with MSCs form tumors with expression of colorectal cancer markers. (*A*) Gross, fluorescence, and merged pictures show tumors derived from injection with a single GFP-labeled CCC plus MSCs (CCC-GFP+MSC) but not with a single CCC plus GFP-labeled MSCs (CCC+MSC-GFP) (*arrows* indicate tumor). (*B*) Immunofluorescence for GFP (*green*), and DAPI (*blue*), and (*C*) immunohistochemistry for CD133, CDX2, CK20, and β-catenin in tumors derived by injection with single GFP-labeled CCC plus MSCs (scale bar = 50 μm). DAPI, 4′,6-diamidino-2-phenylindole.

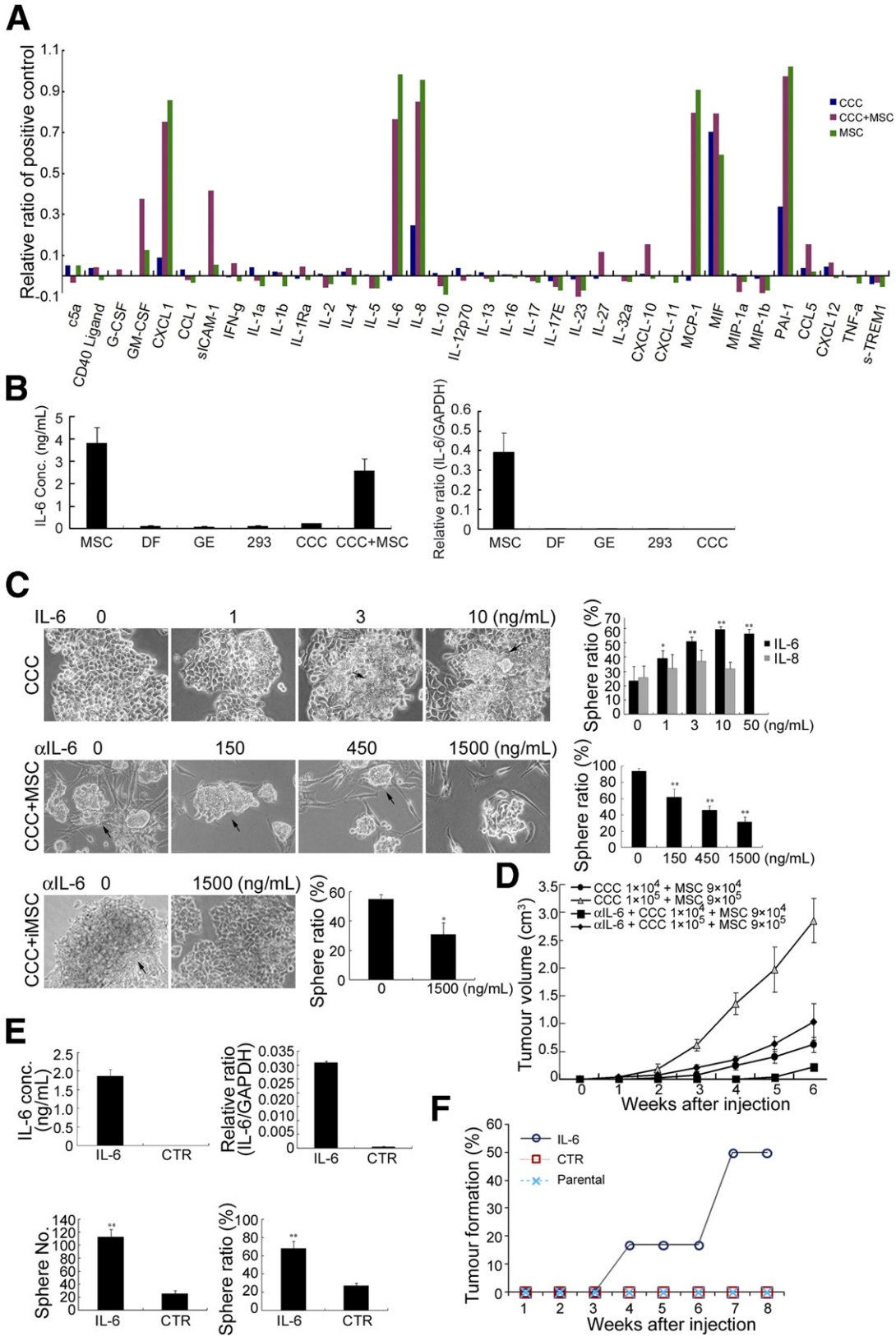
6C). Finally, MSCs and IL-6 failed to enhance tumor sphere formation by HT-29 CCCs lacking the expression of STAT3 (Figure 6*E*). To examine the involvement of this signaling pathway in MSC-enhanced CCC tumor initiation in vivo, we first showed an increase in expression of phosphorylated JAK2 and STAT3 in MSC-containing tumor sections compared with sections of the tumors without MSCs (Supplementary Figure 7). Similarly, MSCs failed to enhance tumor initiation of 10^4 CCCs lacking STAT3, although tumors still were able to be formed with 10^6 of the STAT3-knockdown cells (Figure 6*F*). Taken together, these data suggest the activation of the JAK2-STAT3 pathway is required for TICs enhanced by MSCs and IL-6.

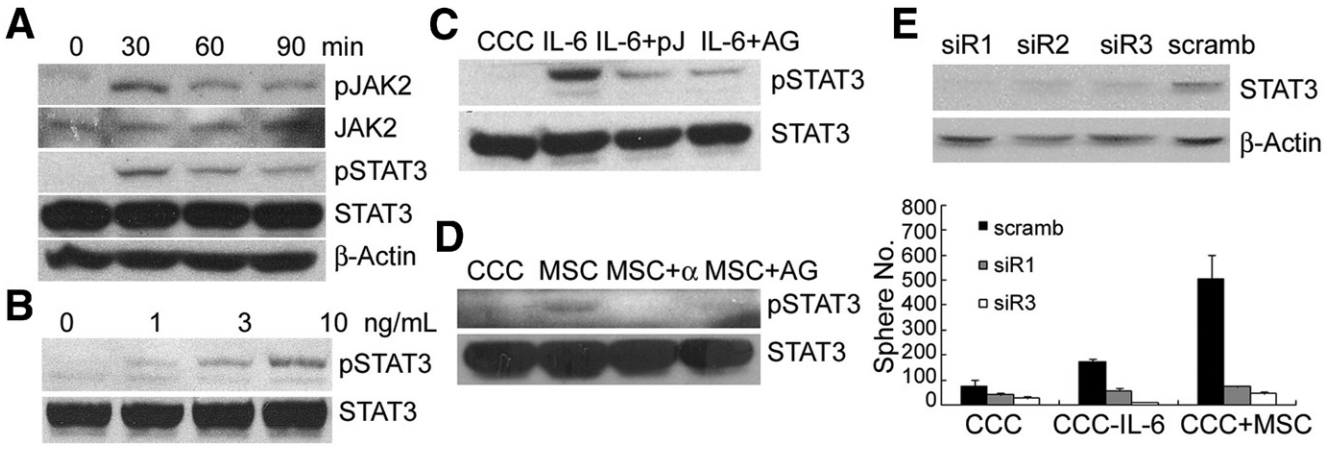
Co-injected MSCs Contribute Cancer-Associated Fibroblasts or Mesenchymal Cells

Regarding the cell fate of co-injected MSCs after tumor development, we showed that tumors formed by a mixture of cancer cells and MSCs had an increase in the

Figure 5. MSCs secrete IL-6 to induce tumor sphere formation. (*A*) Cytokine protein array for conditioned medium of CCC, CCC plus MSCs, and MSCs. (*B*) Enzyme-linked immunosorbent assay for determination of the concentration of IL-6 (10^5 in 2 mL for 48 h), quantitative reverse-transcription polymerase chain reaction for expression ratio of IL-6 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*C*) CCC or CCC with direct (CCC+MSC) or indirect (CCC+iMSC) co-culture of MSCs were grown in TSM and treated with the indicated dose of IL-6 or IL-8, or anti–IL-6 antibodies, and the sphere ratio was counted at 12 days. $^*P < .05$ and $^{**}P < .01$ compared with control as determined by the Student *t* test. (*D*) Tumor volume measurements (mean ± standard deviation) of CCC plus MSCs injected subcutaneously into nonobese diabetic/severe combined immunodeficient mice and treatment with control immunoglobulin G or anti–IL-6 antibodies ($n = 6$ tumors per group). (*E* and *F*) The 293 cells overexpressed with IL-6 promote tumor sphere formation and tumor initiation by CCCs. (*E*) Enzyme-linked immunosorbent assay (*left upper panel*) and quantitative reverse-transcription polymerase chain reaction (*right upper panel*) show 293 cells transduced with IL-6 vectors (IL-6) increase in the secretion of IL-6 and the level of IL-6 mRNA compared with 293 cells transduced with control vectors (CTR). HT-29 cells (10,000 cells) when indirectly co-cultured with IL-6 expressing 293 cells (90,000 cells) increase in tumor sphere formation both as sphere no. (*left lower panel*) and sphere ratio (*right lower panel*) compared with that co-cultured with control 293 cells. $^{**}P < .01$ compared with CTR as determined by the Student *t* test. (*F*) HT-29 cells (10,000 cells) when co-injected with IL-6 expressing 293 cells (90,000 cells) increase in tumor initiation compared with that co-injected with parental or control 293 cells. The ratio of tumor formation for each injection at the indicated time period after injection is shown.)

expression of several markers of cancer-associated fibroblasts (CAFs) or mesenchymal cells such as α-smooth muscle actin (αSMA), platelet-derived growth factor receptor-β, and NG2 chondroitin sulfate proteoglycan[19] compared with tumor formed by cancer cells alone (Supplementary Figure 8). Moreover, both immunohistochemistry and flow cytometric analysis showed that GFP-labeled MSCs expressed αSMA, platelet-derived growth factor receptor-β, and NG2 chondroitin sulfate proteoglycan (Supplementary Figure 9), suggesting that MSCs after





F

	0 wk	1 wk	2 wks	3 wks	6 wks	8 wks	16 wks	19 wks	24 wks
scramb 1×10^4 + MSC 9×10^4	0/4	0/4	2/4	4/4	4/4	4/4	4/4	4/4	4/4
siR1 1×10^4 + MSC 9×10^4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
siR3 1×10^4 + MSC 9×10^4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
scramb 1×10^6	0/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
siR1 1×10^6	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	1/4
siR3 1×10^6	0/4	0/4	0/4	0/4	0/4	1/4	3/4	3/4	3/4

Figure 6. Involvement of JAK2–STAT3 in MSCs- and IL-6–mediated enhancement of tumor sphere formation and tumor initiation. HT-29 CCCs were grown in TSM without or with IL-6 and indicated reagents. Cell lysates were used for Western blotting. (*A*) Time-course of JAK2 and STAT3 activation by IL-6 (3 ng/mL). (*B*) Dose-dependent activation of STAT3 by IL-6 at 30 minutes of treatment. (*C*) Protein level of phosphorylated STAT3 upon treatment with IL-6 without or with pan JAK inhibitor (pJ) or JAK2 inhibitor (AG). (*D*) Protein level of phosphorylated STAT3 upon indirect co-culture with MSCs (MSC) and treated with anti–IL-6 (MSC+α) antibodies, or with JAK2 inhibitor (MSC+AG). (*E*) Cells were Lentiviral transduced with STAT3 short hairpin RNA or scrambled short hairpin RNA, then co-cultured with MSC in TSM or injected subcutaneously into nonobese diabetic/severe combined immunodeficient mice with MSCs. Immunoblot for STAT3 protein levels. Cells transduced with STAT3 short hairpin RNA lose the ability to be stimulated by MSCs to form tumor sphere compared with cells transduced with scrambled short hairpin RNA. (*F*) Tumor formation rate at indicated time after injection of indicated CCCs (Scrambled, scramb; short hairpin RNA 1, siR1; short hairpin RNA 3, siR3), with or without MSCs.

homed and engrafted into an area of tumor development became part of tumor stroma and transited to CAF or mesenchymal cells. Although the recruitment of labeled MSCs to colorectal tumor xenografts has been shown in an experimental model of tumorigenesis,[8] there is currently no convenient way to quantitatively stain MSCs in human tumors, in part because no set of markers have been identified that can uniquely stain these cells without concomitantly staining other mesenchymal types in the tumor-associated stroma.[20] To address whether MSCs are mixed with tumor cells in human colon cancers, our microarray data showed that malignant colorectal tumors were increased significantly in the expression of several MSC markers such as CD29, CD44, CD90, and CD166 when compared with normal colon tissues (Supplementary Figure 10*A* and *B*). Results of principal component analysis of log-transformed gene expression values also showed that the gene expression profiles of colorectal tumors were more similar to MSCs than normal colon tissues (Supplementary Figure 10*C*). Moreover, the stromal cells isolated from primary human colorectal tumor tissue were plastic-adherent, fibroblastic in morphology (Supplementary Figure 10*D*), had the same surface protein profile as MSCs (Supplementary Figure 10*E*), and also expressed markers of CAF or mesenchymal cells. We further characterized the phenotypes of the isolated MSC-like cells and found that these cells lost MSC differentiation potential as compared with their original cells (data not shown). Supported by previous reports,[21,22] these data suggest that MSCs transit to CAF or mesenchymal cells and contribute to fibrovascular network expansion and tumor progression.

Discussion

Recently, tumors formed by an unsorted single tumor cell after transplantation into a highly immunocompromised mouse (with IL-2–receptor-γ chain deletion in nonobese diabetic/severe combined immunodeficient mice) was achieved in melanoma.[23] The current study also showed a single unsorted or CD133-CD166-EpCAM triple-negative colorectal cancer cell admixed with MSCs, up to 5%–7% of the injections formed tumors in mice. It is yet to be determined whether the

MSC-enhanced TICs in colon cancer also will be valid in melanoma.

Many studies have used putative stem cell markers or side populations to isolate unique subsets of TICs from different types of tumors. Among these markers, CD133,[1,2] CD44, CD166, and EpCAM[3] have been used successfully for isolating TICs from colorectal cancer. The number of TICs used to initiate xenograft tumor formation in these studies was more than 500–10,000 cells, which is much greater than those of unsorted or CD133-CD166-EpCAM- triple-negative cancer cells used to form tumor when mixed with MSCs. Coincidently, some of the markers such as CD44 and CD166 used to isolate TICs in a previous study[3] also are expressed in MSCs or MSC-like cells isolated from fresh tumor. Thus, it will be interesting to know whether cells isolated by these putative TIC markers contained some tumor stromal cells such as CAFs. Moreover, it will be critical to exclude the contamination of tumor stromal cells when isolating TICs for xenograft tumor formation experiments, especially with the use of fresh tumor sample–derived single-cell suspensions.

Our findings are in agreement with and extend recent reports[21,22] of transition of MSCs into αSMA+ CAFs, which showed that MSCs were activated and recruited to inflammation-induced gastric dysplasia, and contributed to tumor promotion and 20% of αSMA+ CAFs in an inflammation-related model of gastric carcinogenesis[22]; however, the study did not reveal that MSCs caused ordinary tumor cells to acquire properties of TICs. Here, we show the admixed MSCs enhanced tumor cells with the ability to form tumor spheres in vitro and form xenograft tumors when transplanted in immunodeficient mice. In contrast to these reports, Elkabets et al[24] showed Sca1+, cKit- hematopoietic bone marrow cells (BMCs) of mouse hosts bearing instigating tumors promoted the growth of responding tumors that formed with a myofibroblastic-rich, desmoplastic stroma. However, these instigating bone marrow cells did not form αSMA+ CAFs. Moreover, both MSCs and bone marrow cells expressed some of the genes associated with an "inflammatory signature" when compared with a previously reported gene signature for skin CAFs.[25] These reports indicate that bone marrow contained many subpopulations of cells, which can be recruited and incorporated into tumor during tumor development and progress.

When comparing the cytokine array data of our human MSC-CM with the gene signature of mice bone marrow–derived CAFs[22] and skin CAFs,[25] protein levels of several key inflammatory signature genes proposed by Quante et al[22] or Erez et al[25] also were increased in MSC-CM compared with colorectal cancer cell–derived CM (IL-6 and CXCL1), but only IL-6 was involved in MSC-induced enhancement of tumor sphere formation and tumor initiation. Because our cytokine array did not examine the protein levels of several key inflammatory signature genes such as stromal cell-derived factor-1α and transforming growth factor-α, we did not evaluate their involvement in MSC-induced enhancement of TIC properties. Because SDF-1α and transforming growth factor-α were reported to enhance the recruitment of MSCs and the expression αSMA in CAFs,[22] they therefore may play a role in xenograft tumor formation and their functions should be investigated in the future.

The first evidence of a microenvironment-induced increase of tumor cells with TIC properties was shown most recently in colonic myofibroblasts via Hepatocyte growth factor-c-Met signaling in colorectal tumor.[26] In the current study, the same effect also was shown in a lot of fibroblasts including tumor or normal colonic myofibroblasts or other fibroblasts. For colorectal tumor initiation, more than 100–1000 tumor cells were required when co-injected with colonic myofibroblasts,[26] whereas only 1–10 tumor cells were required when co-injected with MSCs. We further showed that MSCs had greater ability than tumor or normal colonic myofibroblasts to enhance tumor sphere formation and tumor initiation. These data imply that the tumorigenicity-enhancing effect of MSCs might be stronger than that of myofibroblasts. Moreover, MSCs increased tumor cells with TIC properties through the IL-6/STAT3 pathway, which was different from the pathway that fibroblast or myofibroblast mediated to enhance tumor initiation. IL-6/STAT3 signaling recently was reported with certain premalignancies such as inflammatory bowel diseases to induce tumor formation.[27,28] Our findings show the involvement of STAT3 activation in MSCs or IL-6–induced tumor initiation and further implies a role of MSCs in these diseases. Based on the current results, investigations of the tumor-microenvironment cross-talk involved in tumor initiation in cancer may lead to the design of novel therapeutic and preventive strategies.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.05.045.

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Conflicts of interest

The authors disclose no conflicts.

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