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Mesenchymal Stem Cells Promote Epithelial to Mesenchymal Transition and Metastasis in Gastric Cancer Though Paracrine Cues and Close Physical Contact

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ABSTRACT

Mesenchymal stem cells (MSCs) have been shown to integrate into the tumor stroma; however, the precise mechanisms of this process are still elusive. In this study, the EMT phenotype and the enhanced metastatic ability of tumor cells were observed using transwell and trans-endothelial migration assays, respectively, as well as by using electron and laser confocal microscopy. Critical genes were screened and validated using gene arrays and clinical samples, and the changes at the protein level were examined both in vitro and in vivo. Cancer cells acquired an "activated" carcinoma-associated fibroblasts (CAFs) phenotype after being in close contact with MSCs and enhancing tumor metastasis and growth in vivo. Paracrine signals also induced EMT and promoted transwell and trans-endothelial migration, the changes were dependent on β-catenin, MMP-16, snail and twist. Notably, the higher expression levels of β-catenin and MMP-16 were correlated with tumor invasion and distant organ and lymph node metastases in intestinal type gastric cancer. MSCs within the tumor niche significantly facilitated tumor growth and metastasis by paracrine cues and close physical connection. This occurred partly through snail, twist and its downstream targets, specifically β-catenin/MMP-16. J. Cell. Biochem. 116: 618–627, 2015.

KEY WORDS: EPITHELIAL-MESENCHYMAL TRANSITION; MESENCHYMAL STEM CELLS; MMP-16; β-CATENIN

Invasion and metastasis are the hallmarks of malignant tumor cells and are the main causes of cancer death [Mehlen and Puisieux, 2006]. Tumor metastasis is a multi-step process that includes escaping from the primary tumor, surviving in the circulatory system, and re-growing at distant sites. Each step of tumor metastasis is influenced by stromal cells and paracrine signals in the tumor microenvironment, especially from bone marrow-derived cells [Joyce and Pollard, 2009]. One of the candidate cell types involved in promoting metastasis is the MSC, which can be recruited to the tumor stroma and can modulate the behavior of

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neighboring cancer cells [Karnoub et al., 2007; Joyce and Pollard, 2009]. Human MSCs significantly increase the dissemination of weakly metastatic breast cancer cells in part through the elevation of a paracrine loop involving CCL5 and TGF-β [Karnoub et al., 2007; Schepers et al., 2012].

Recently, a close relationship has been identified between gastric cancer and MSCs. The female recipients of male allogeneic hematopoietic stem cell transplantation diagnosed with solid organ tumors following transplantation, who has been found gastric cancer-associated myofibroblasts developing from bone marrow-derived cells, however, this has not been identified in other cancers [Worthley et al., 2009]. In addition, MSCs may be the most likely cell of origin in gastric cancer [Houghton et al., 2004; Quante et al., 2011]. EMT is one characteristic of tumor aggressiveness. Initiation of the EMT transdifferentiation program has been proposed to be the key mechanism in the early acquisition of malignant ability by epithelial cancer cells [Ansieau et al., 2008]. In epithelial cancers, such as breast, colon and prostate cancers, cells exhibiting an EMT phenotype lose their epithelial characteristics and acquire mesenchymal markers that facilitate their invasiveness and intravasation [Acloque et al., 2009; Thiery et al., 2009]. The molecular mechanisms by which MSCs enhance tumor EMT and the involved paracrine signals are still unclear. To understand the close relationship between MSCs and gastric cancer, we sought to determine the roles of MSCs in tumor metastasis.

In the present study, we developed a multi-migration model by mixing MSCs with cancer cells in a co-culture system to gain insight into the roles of MSCs within the gastric cancer stroma. Using this model, a significant cancer cell phenotype transition that was influenced by MSCs and their paracrine signals was observed using electron microscopy and demonstrated in vivo.

MATERIALS AND METHODS

MSCS ISOLATION AND IDENTIFICATION

Upon informed consent, human MSCs were collected from two healthy male volunteers (28 and 40 years old) at the bone marrow transplantation center of Xijing Hospital of the Fourth Military Medical University (Approved by the ethical committee of Xijing Hospital No: 2010–8-H101). Heparinized bone marrow cells were recovered by centrifugation at 1000 r/min for 3 minutes and separated by centrifugation over a Ficoll (1.077) gradient. The mononuclear cells were resuspended using low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C with 5% CO_2. Cells from passages 1 through 4 were frozen in liquid nitrogen for future use, and cells at passage 5 were used for identificationthe differentiation assay and other studies. (Modified from our previous report [Cui et al., 2014]).

CANCER CELL LINES

The human gastric adenocarcinoma cell lines SGC7901, SGC7901-GFP, MKN28, and GFP-MKN28 were obtained from the Academy of Military Medical Science (Beijing, China). The other cancer cell lines, MKN28, MKN45, SW620, SW480, MCF-7, HepG 2, and AGS, were purchased from the ATCC (Rockville, MD). Primary human umbilical vein endothelial cells (HUVEC, passages 3–5) were isolated and cultured in our laboratory using endothelial growth medium consisting of M200 basal culture media (Cascade Biologics, Portland, OR, www.invitrogen.com) [Liang et al., 2006]. Tumor cells were maintained in RPMI-1640 containing 10% heat-inactivated FBS and incubated 37 °C in 5% CO_2.

CO-CULTURE SYSTEMS

MSCs or fibroblasts (NIH-3T3 cells) were mixed with cancer cells (i.e., SW620, SW480, HepG2, MCF-7, SGC7901, MKN28, MKN45, or AGS). Different ratios (MSCs:cancer cells, 10:1, 2:1, 1:1, 1:2, or 1:10) were tested in this experiment. MSCs were able to more quickly adhere to the plate, and the same result was observed whether the MSCs were seeded first or second and then co-cultured with the cancer cells. All co-cultured cancer cells were maintained in DMEM containing 10% heat-inactivated FBS.

IN VIVO EXPERIMENTS

Nude mice were divided into three groups and injected subcutaneously with MKN28 cells using PKH26-stained (PKH26 MINI kit, Sigma–Aldrich) MSCs and MKN28 (ratio: 5×10^5:4×10^4; n = 5); PKH26-stained MKN28 and MSCs (ratio: 5×10^5:5×10^4; n = 5); or MKN28 alone (5×10^5; n = 10). Eight weeks after injection, the mice were sacrificed and the lungs and livers were examined for tumor metastases. Metastatic tissues from various organs were collected and examined histologically. Frozen sections were stored at −70°C.

MULTI-MIGRATION ASSAY MODEL

MSCs (1×10^4) and cancer cells (1×10^3 with MKN28 or SGC7901) were layered respectively or mixed together at one point of an equilateral triangle on 6-cm plate (Fig. 1A) under the same culture conditions to allow for paracrine signals to be freely exchanged. Cells were fixed and stained using 1% crystal violet at days 3, 7, and 14. The culture medium used was DMEM+ 10% FBS. Each experiment was repeated three times.

STATISTICS

Differences among the variables were assessed using a χ^2 analysis, a 2-tailed Fisher's exact test, Paired student's *t*-test. Correlation was assessed using Bivariate and Partial correlations. A $P < 0.05$ (2-tailed) was considered to be statistically significant. Statistical analyses were performed using SPSS 13.0 (Chicago, IL).

ADDITIONAL METHODS

Additional methods are available in the Supplementary Materials/Methods.

RESULTS

IDENTIFICATION OF MSCS WITH SURFACE ANTIGENS AND DIFFERENTIATION

MSCs were isolated from bone marrow using density gradient centrifugation and purified by their plastic-adherence characteristic using tissue culture flasks. Passage 5 was collected for identification.

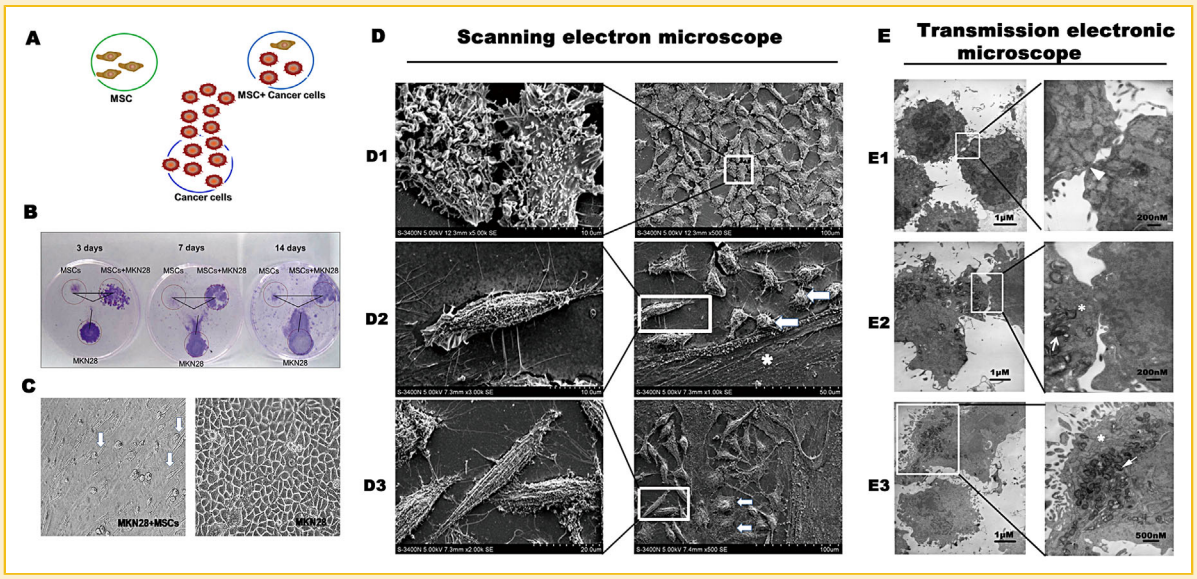

Fig. 1. A multi-migration assay model demonstrating that MSCs promote gastric cancer EMT. (A) Schematic diagram of a multi-migration assay model. (B) MKN28, MSCs or a mixture of both cell types were grown on the three vertices of an equilateral triangle for 3, 7, or 14 days. The dark line exhibited the migration trend. (C) MKN28 directly co-cultured with MSCs exhibited a greater fibroblast-like phenotype (arrow) than MKN28 alone. (D) MKN28 gastric cancer cells in close physical contact and microvilli (D1). After co-culturing with MSCs (asterisk) for 2 days (D2) or 3 days (D3), an increasing number of gastric cancer cells displayed a mesenchymal phenotype with fewer villi, had a ruffled membrane and a ridge-like protrusion along the long axis, as determined using scanning electron microscopy. (E) The phenotype transition examined using transmission electron microscopy. A tight junction was observed between MKN28 cells (E1). A similar close adherence pattern was observed between MSCs and MKN28 (E2). Activated MKN28 exhibited an abundance of lysosomes (*) and rough endoplasmic reticulum (arrow) (E2, E3).

The cells typically expressed the surface antigens CD90, CD44, CD29, and CD105 (>95%) and were negative for CD14, CD133, and CD45 (<2%) (Supplementary Figure 1 A). MSCs can differentiate into adipogenic, osteogenic and myogenic lineages in vitro under the appropriate differentiating conditions (Supplementary Figures 1B-D).

MESENCHYMAL PHENOTYPE INDUCED BY CLOSE PHYSICAL CONTACT WITH MSCS

To gain further insight into the effects of MSCs on human cancer cells, we performed a multi-migration assay model in which cancer cells, MSCs and the mixture of MSCs and cancer cells were allowed to migrate and freely and exchange paracrine signals (Fig. 1A). Active human gastric cancer cells (MKN28) migrated separately to MSCs or the mixture of MSCs and cancer cells (MSCs + MKN28) (Fig. 1B). MKN28 cancer cells migrated much more readily to the MSCs + MKN28 group than to the MSCs group (3 days vs. 7 days vs. 14 days).

To further study the aforementioned observations, we disassembled the multi-migration model and examined the phenotype by mixing MSCs and MKN28 cells (co-culture system). After 2 days, MKN28 cells displayed a fibroblast-like shape and scattered on the surface of MSCs (Fig. 1C). There were no significant changes when gastric cancer cells were co-cultured with NIH-3T3 fibroblast cells (Supplementary Figure 2 A). The ratio of fibroblast-like cancer cell was about 70% in co-culture system. To determine whether the fibroblast-like appearance was a general phenomenon, gastric cancer cell lines SGC7901, MKN45 and AGS, colon cancer cell lines SW480 and SW620, hepatic cancer lines HepG2, mammary cancer cell line MCF-7 and were co-cultured directly with MSCs (the ratios of MSCs and cancer cells 1:10). The significant morphologic change ratio observed was gastric cancer cell lines > breast cancer cell lines > colon cancer cell lines > hepatic cancer cell lines (Supplementary Figures 2B-F).

Finally, the mesenchymal phenotype changes were confirmed using both scanning and transmission electron microscopy (Fig. 1D). Close physical contact and microvilli were common in MKN28 cells (Fig. 1D1). In contrast, MKN28 + MSCs exhibited a significant phenotypic change than MKN28 alone. Indeed, these cells also displayed a fibroblast-like phenotype and had fewer microvilli on their cell surface (Fig. 1D2). Furthermore, loss of close conjunctions and the possession of longitude ridging, ruffled membranes and finger-like extensions were significantly enhanced after a longer time period (Fig. 1D2, 2 days vs. D3, 3 days). The MKN28 cells (Fig. 1E2, E3) exhibited an abundance of both rough endoplasmic reticulum and lysosomes, and the significant polarity edge contained microvilli than control (Fig. 1E1) by transmission electron microscopy. Therefore, the significant internal structure changes influenced cancer cells phenotype transition.

THE MUTUAL INTERACTION BETWEEN CANCER CELLS AND MSCS

Next, we used immunofluorescence to evaluate the protein levels of the epithelial marker E-cadherin and the mesenchymal markers α-SMA and vimentin. The fibroblast-like MKN28 cells down-regulated E-cadherin and up-regulated both α-SMA and vimentin in the MSCs + MKN28 co-culture system (Fig. 2A). Furthermore, similar patterns of the mesenchymal phenotype changes were observed when MKN28 cells and MCF-7 cancer cells were

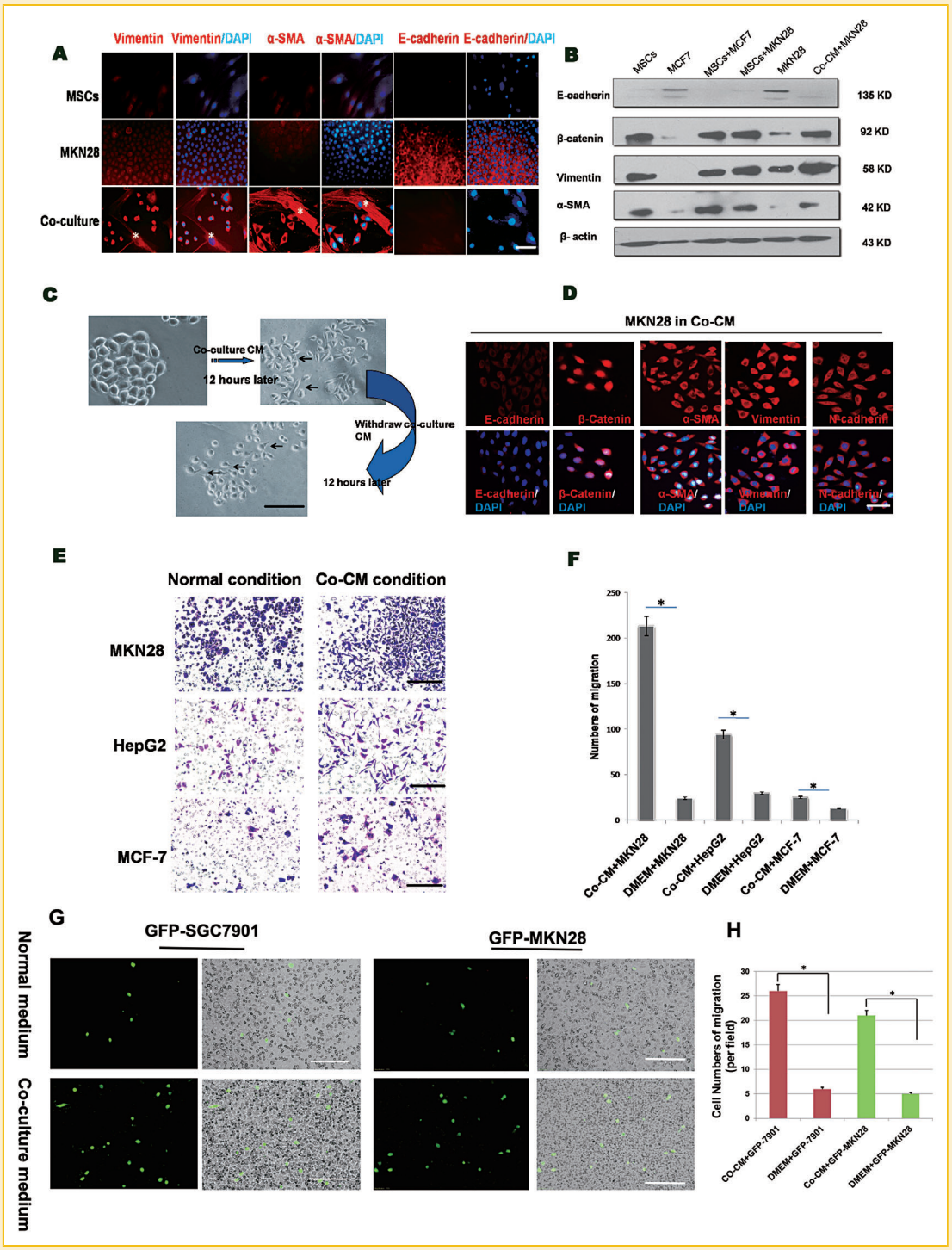

Fig. 2. The mesenchymal phenotype is induced by close cellular location and co-cultured conditioned medium (Co-CM). (A) The mesenchymal markers α-SMA and vimentin were up-regulated, and the epithelial marker E-cadherin was down-regulated, which indicated EMT in co-culture systems. MSCs also acquired a myofibroblast phenotype with the upregulaton of α-SMA and vimentin (asterisk) relative to the control. The up-regulation of mesenchymal makers α-SMA, vimentin and N-cadherin, and the down-regulation of E-cadherin was determined by Western blot (B) (MSCs: cancer cells was 2:1). (C) The mesenchymal phenotypic changes are accompanied by cancer cell dispersion or re-aggregation by the addition or removal of Co-CM after 12 h, which demonstrated that paracrine signaling could initiate EMT in Co-CM, which was determined by Western blot (B) and immunofluorescence (D). β-catenin translocated to the nucleus following stimulation with Co-CM. β-catenin translocated to the nucleus following stimulation with Co-CM (D). The transwell migration assay determined that MKN28, HepG2 and MCF-7 cells had increased migration abilities in Co-CM compared with these cells in MKN-28 conditioned medium. Additionally, the active cells that passed through the transwell kept a mesenchymal phenotype (E). Trans-endothelial migration (HUVEC, bright field) of GFP-7901 cells (green) in the presence of Co-CM or normal medium (G, H). The number of trans-endothelial GFP-7901 and GFP-MKN28 cells were significantly higher in Co-CM than in normal medium ($P < 0.05$, paired student t-test; repeated 3 times). Scale, 200 μm. (C), Original magnification, 200×.

co-cultured with MSCs (MSCs + MKN28 and MSCs +MCF-7) (Fig. 2B; Supplementary Figure 3). In addition, β-catenin also translocated to the nucleus in both MSCs +MKN28 and MSCs +MCF-7 groups (Supplementary Figures 3 and 4).

To evaluate the contributions that close physical contact had on the observed phenotypic changes, a 0.4-μm membrane was used to separate MSCs and MKN28 using a transwell assay. We determined that there were no detectable changes without close physical contact between MSCs and MKN28(1:1 or 10:1 ratio). Therefore, close contact is necessary to initiate the phenotype changes of gastric cancer cells. Even suitable cell medium from gastric cancer cells or MSCs alone failed to induce the phenotypic changes (data not shown).

PARACRINE SIGNALING ENHANCED CANCER CELL MOBILITY VIA EMT

We decided to explore the paracrine signaling effects exerted on cancer cells. To do so, we collected conditioned medium (Co-CM, co-culture for 48 h) from the MSCs + MKN28 group after MKN28 cells exhibited a significant mesenchymal phenotype. When exposed to Co-CM, MKN28 cancer cells also dispersed and acquired a similar fibroblast-like phenotype within 12 hours (Fig. 2C). Interestingly, we replaced this medium with non-conditioned medium and found fibroblast-like cells retrieved their "pseudopodia" and close to each other after 12 h (Fig. 2C). Consistently, an immunofluorescence assay and Western blot analysis determined that the spindle-shaped MKN28 cells had less expression of E-cadherin and increased expression of α-SMA, vimentin and N-cadherin (Figs. 2B and D). β-catenin translocated to the nucleus when replaced with Co-CM (Fig. 2D). Also, MKN45 cells exposed to Co-CM exhibited phenotypic changes including the down-regulation of E-cadherin and up-regulation of the nuclear expression β-catenin, which provides further proof that the paracrine signaling within Co-CM promoted EMT through the Wnt signaling pathway (Supplementary Figure 5).

Different cell lines (MKN28, MCF7 and HepG2) were layered in the upper wells, thereby allowing cells to migrate toward the Co-CM through the 8-μm membrane. Cancer cells gained a fibroblast-like phenotype and acquired an increased migratory ability relative to the cells cultured in normal medium (1.7- to approximately 8-fold, $P < 0.05$, Figs. 2E and F).

Finally, we explored a trans-endothelial migration assay in which the Co-CM medium was placed in the bottom chamber. We demonstrated that the Co-CM was approximately 4-fold more active at inducing the trans-endothelial migration of GFP-SGC7901 and GFP-MKN28 cells than control cells ($P < 0.05$, Figs. 2G and H). Together, our data indicated that paracrine signals initiated cancer cell EMT alone and maintained the phenotype, which facilitated tumor migration and invasion.

ACTIVATED β-CATENIN/MMP16 PROMOTES EMT

To better understand the crosstalk, cDNA profiles were screened between MKN28 cells treated with Co-CM and MKN28 cells treated with normal CM. The mRNA levels of 113 genes were up-regulated, and the expression levels of 115 genes were down-regulated (fold >1.5). The most EMT-related pathways included the Wnt signaling pathway, the TGF-beta signaling pathway, adherence junctions, the regulation of the actin cytoskeleton and the focal adhesion pathway ($P < 0.01$). Interesting genes were validated in triplicate experiments using qRT-PCR. The mesenchymal markers vimentin and S100A4 were up-regulated by approximately 1.1- and 1.5-fold

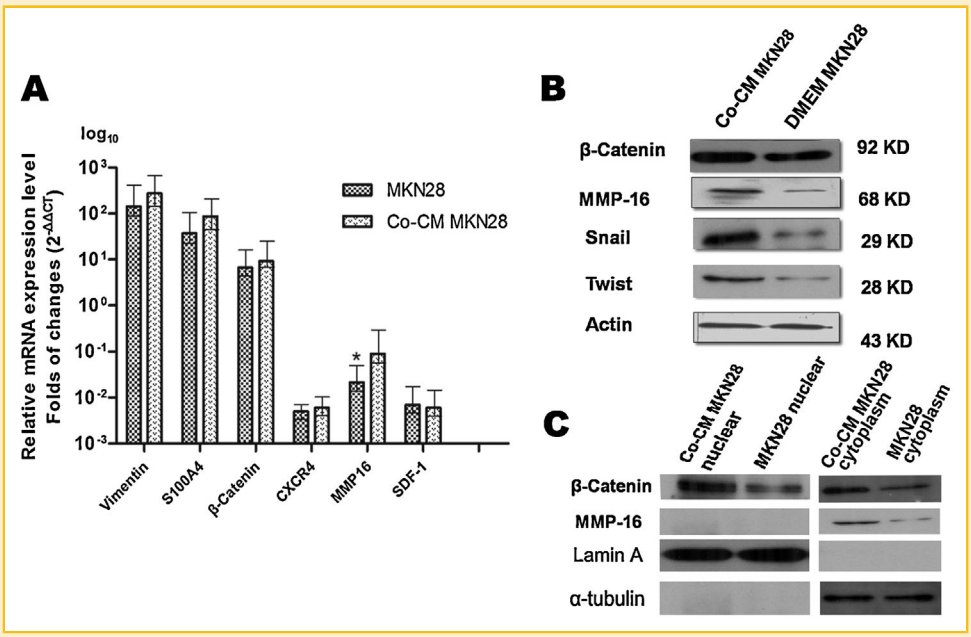

Fig. 3. β-catenin and MMP-16 were activated by Co-CM. (A) Interesting targets from the global gene expression screen were verified using real-time PCR. Vimentin, S100A4 and MMP-16 were up-regulated. β-catenin, CXCR4 and SDF-1 were down-regulated. (B) Co-CM induced MKN28 cell EMT by upregulating snail and twist and the downstream β-catenin/MMP-16 proteins. (C) Co-CM stimulated β-catenin nuclear translocation upon MMP-16 up-regulation in cytoplasm. *$P < 0.05$, Paired student's *t*-test.

in Co-CM-treated MKN28 cells. According to the aforementioned observations, we also examined β-catenin mRNA levels, which were up-regulated by approximately 1.5-fold; however, its related gene, MMP-16 (MT3-MMP), up-regulated approximately threefold (Fig. 3A).

Moreover, the nuclear translocation of the β-catenin protein was detected upon the up-regulation of MMP-16 in Co-CM (Figs. 3B and C). Upstream factors of β-catenin, including snail and twist, were also significantly up-regulated (Fig. 3B). These proteins have been demonstrated to interact with β-catenin via a positive feedback loop.

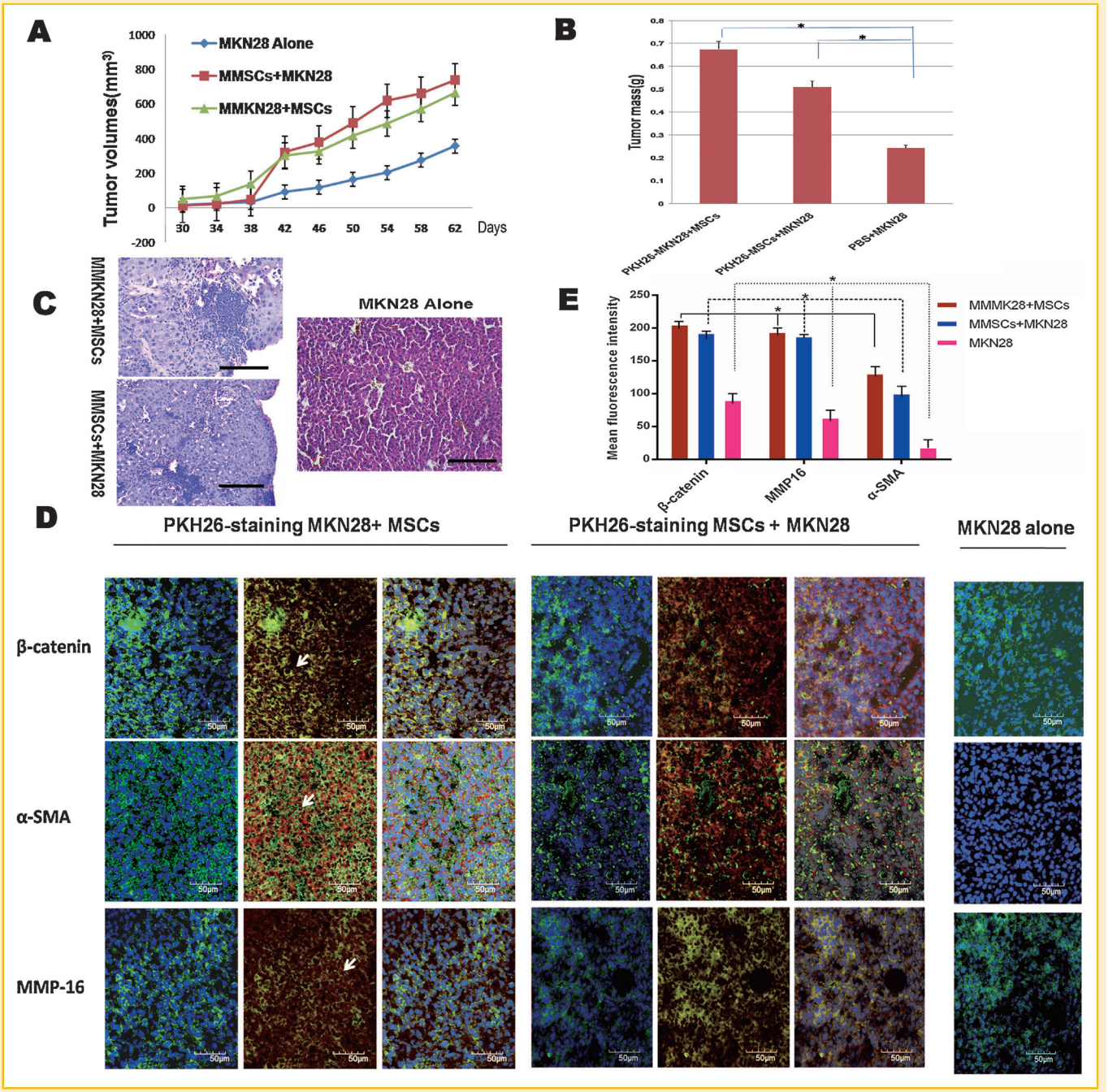

Fig. 4. MSCs promote EMT and metastasis of MKN28 in vivo. (A)PKH-26-stained MSCs(MMSC) were mixed with MKN28, or PKH-26-stained MKN28 (MMKN28) cells were mixed with MSCs or MKN28 cells alone were inoculated subcutaneously (10^5 tumor cells, n = 5). Tumor growth was measured every 4 days. (B) MSCs promote tumor growth, as determined by measuring tumor mass (asterisk, $P < 0.05$). (C) Liver micro-metastases were observed and verified by HE staining. Scale, 200 μm. (D) Both of PKH-26-staining MKN28+ MSCs and PKH-26-staining MSCs+ MKN28 groups show EMT maker up-regulatiion than MKN28 group alone: α-SMA (green) displayed increased expression when co-cultured with MSCs and MKN28 with no effect from PKH-26 (red). MSCs enhance tumor metastasis of MKN28 cells via the up-regulation of β-catenin (green) and MMP-16 (green) compared to MKN28 cells grown alone. Merged PKH-26-stained MSCs or MKN28 expressing α-SMA, β-catenin and MMP-16 (arrow). (E) The mean intensity of florescence was measured. (*$P < 0.05$).

MSCS DIRECTLY PROMOTE TUMOR GROWTH AND METASTASIS IN VIVO

To investigate the contribution of MSCs to tumor growth and metastasis in vivo, we subcutaneously injected nude mice with PKH26-MSCs + MKN28 (1:10), PKH26-MKN28 + MSCs (1:10) (n = 5 per group) or MKN28(n = 10; control). MSCs + MKN28 generated tumors with greater volumes (Fig. 4A) and weights (Fig. 4B). The mean growth rate of the tumor mass with MSCs increased by 2.1- or 2.8-fold ($P = 0.022$ or $P = 0.003$) compared to the control. There are no significant difference without labeling or not PKH26. More importantly, mice injected with co-cultured MSC tumors displayed a significant increase in the number of liver metastases, which were checked by histopathology (Fig. 4C, n = 4/10 vs n = 0/10).

We next examined the contribution of MSCs to tumor metastasis and growth by tracking PKH26 epifluorescence using confocal microscopy (Fig. 4D). Cancer cells in the PKH26-MSCs + MKN28 or PKH26-MKN28 + MSCs groups had significantly increased expression levels of β-catenin and MMP-16 relative to the MKN28 group alone (Fig. 4D). In addition, the myofibroblast marker α-SMA was also significantly up-regulated in PKH26-labeled MSCs and cancer cells, which was consistent with our in vitro observation (Figs. 2A, 2B and 4D).

β-CATENIN/MMP16 CORRELATED TO THE METASTATIC TISSUE

Immunohistochemistry was performed on serial paraffin sections from 194 patients to explore β-catenin and MMP-16 expression levels in diffuse-type and intestinal-type gastric cancers. β-catenin expression levels in the nucleus, the membrane and the cytoplasm was 22.1%, 34%, and 44.8%, respectively (Figs. 5A–C). The total expression level of MMP-16 at the cell membrane and cytoplasm was 72.2% (Fig. 5). Positive β-catenin staining in the cell membrane was 70.2%, 39.3% and 28.6% at tumor stages N0, N1 and N2,

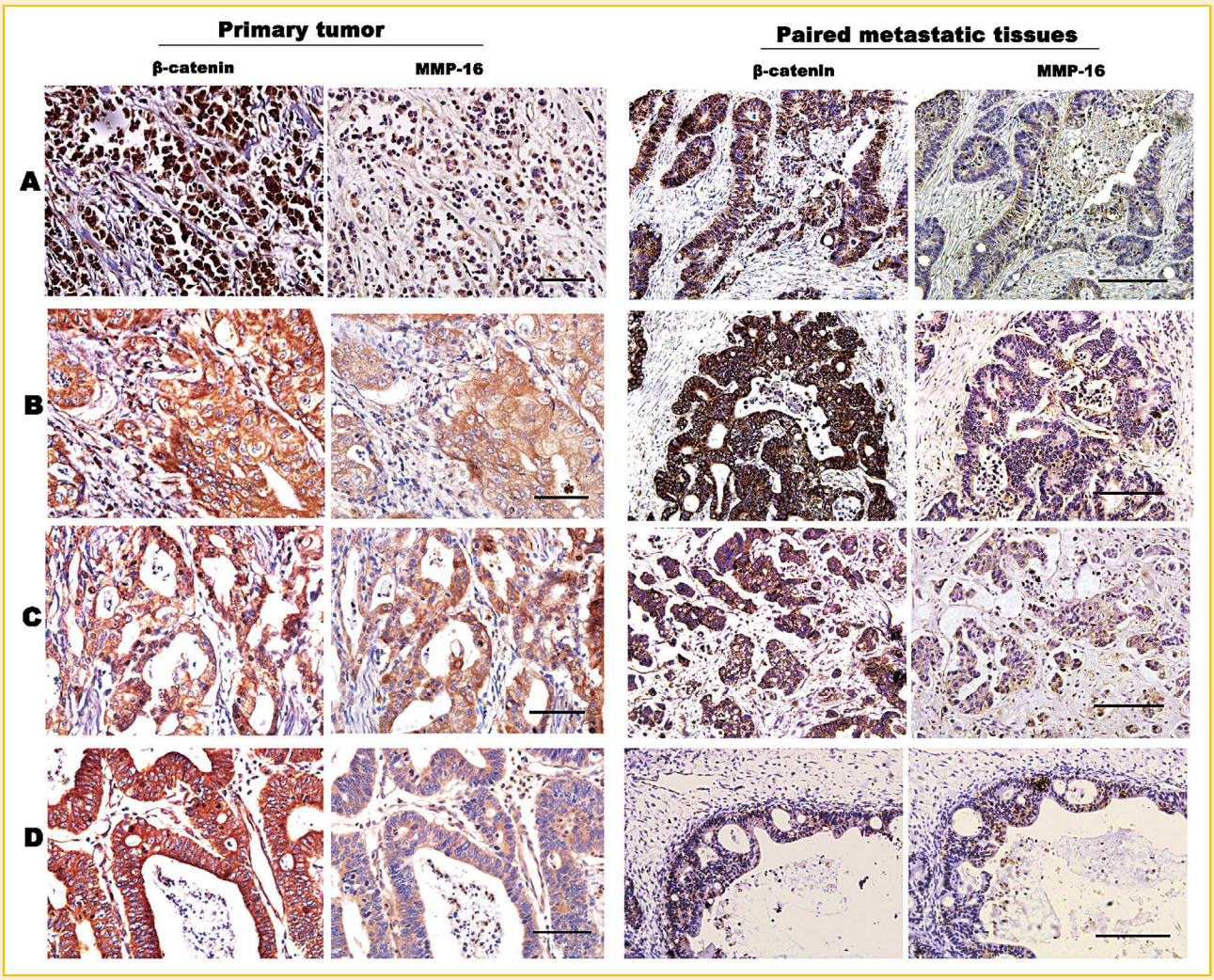

Fig. 5. β-catenin and MMP-16 are expressed in both primary gastric tumors and their paired metastatic tissues. β-catenin was explored in the nucleus (A), membrane (B), and cytoplasm (C, D). MMP-16 was expressed in the membrane and cytoplasm (A, B, C, D) in serial sections. β-catenin and MMP-16 were expressed in the paired metastatic tissues, such as the lymph node (A right), liver (B right), ovary (C right) and peritoneum (D right). Scale, 200 μm.

TABLE I. Association of Abnormal Expression of β-Catenin and MMP-16 With Histopathologic Features of Gastric Cancer

		MMP-16 n (% of total)			β-catenin n (% of total)			
	Cases n (total)	−	+	*P*	Nuclear	Cytoplasm	Membrane	*P*
N stage	194							
N0	47	16 (34)	31 (66)	0.506	4 (8.5)	10 (21.3)	33 (70.2)	<0.001
N1	112	30 (26.8)	82 (73.2)		31 (27.7)	37 (33)	44 (39.3)	
N2	35	8 (22.9)	27 (77.1)		6 (17.1)	19 (54.3)	10 (28.6)	
T stage	194							
T1	1	1 (100)	0	0.373	0	0	1 (100)	0.032
T2	46	12 (26.1)	34 (73.9)		9 (19.6)	9 (19.6)	28 (60.9)	
T3	147	41 (27.9)	106 (72.1)		32 (21.8)	57 (38.8)	58 (39.5)	
Lauren	194							
Diffuse	31	15 (48.4)	16 (51.6)	0.008	16 (51.6)	10 (32.3)	5 (16.1)	<0.001
Intestinal	163	39 (23.9)	124 (76.1)		25 (15.3)	56 (34.4)	82 (50.3)	
Intestinal type	163							
N Stage	163							
N0	41	14 (34.1)	27 (65.9)	0.036	2 (4.9)	9 (22)	30 (72.3)	0.005
N1	95	23 (24.2)	72 (75.8)		19 (20)	33 (34.7)	43 (45.3)	
N2	27	2 (7.4)	25 (92.6)		4 (14.8)	14 (51.9)	9 (33.3)	
T stage	163							
T1	1	1 (100)	0 (0)	0.342	0 (0)	0 (0)	1 (100)	0.025
T2	39	9 (23.1)	30 (76.9)		5 (12.8)	7 (17.9)	27 (69.2)	
T3	123	29 (23.6)	94 (76.4)		20 (16.3)	49 (39.8)	54 (43.9)	
Diffuse type								
N Stage								
N0	6	2 (33.3)	4 (66.7)	0.276	2 (33.3)	1 (16.7)	3 (50)	0.034
N1	17	7 (41.2)	10 (58.8)		12 (70.6)	4 (23.5)	1 (5.9)	
N2	8	6 (75)	2 (25)		2 (25)	5 (62.5)	1 (12.5)	
T stage								
T1								
T2	7	3 (42.9)	4 (57.1)	1.0	4 (57.1)	2 (28.6)	1 (14.3)	1.0
T3	24	12 (50)	12 (50)		12 (50)	8 (33.3)	4 (17.6)	

Statistic calculated by χ^2 analysis, 2-tailed Fisher's exact.

respectively, which significantly correlated with lymph node metastasis ($P<0.001$; Table I). In addition, the loss of β-catenin expression in the membrane significantly correlated with T stage (T1 = 100%, T2 = 60.9% and T3 = 39.5%; $P=0.032$, Supplementary Table 1). Moreover, β-catenin immunoreactivity was significantly enhanced in intestinal-type gastric cancer with lymph node metastasis and tumor invasion ($P=0.005$ and $P=0.025$; Table I). MMP-16 expression was also significantly up-regulated in intestinal type gastric cancer ($P=0.008$) and associated with lymph node metastasis ($P=0.036$; Table I). MMP-16 was up-regulated in 78% of the cancer samples when β-catenin was translocated to the nucleus (Table I).

To explore the roles of β-catenin and MMP-16 in cancer metastasis, we compared MMP-16 in 79 primary tumors, which were paired 40 metastatic tissues and 39 metastatic lymph nodes. MMP-16 was positively expressed in the paired metastatic tissues: lymph nodes, livers, ovaries and peritoneum (Figs. 5A–D). Moreover, MMP-16 was positivity correlated with distant organ metastasis (r = 0.409, $P=0.009$, Spearman's rho test; Table II), and the hazard ratio was 6.67 (95% confidence interval (CI) 1.46 to 30.43; Table II). MMP-16 was also positively correlated with lymph node metastasis (r = 0.516, $P=0.009$, Spearman's rho test; HR = 12.8, 95% CI 2.29–71; Table II). Therefore, MMP-16 is a new predictor for distant organ metastasis.

TABLE II. Relationship Between MMP-16 and Paired Metastatic Tissues and Lymph Node

			MMP-16					
	Cases (n, total)		Paired metastatic Tissues n(% total)			Cases (n,total)	Paired lymph node n(% total)	
	40		−	+	*P*	39	−	+	*P*
Primary	19	−	10 (52.6)	9 (47.4)	0.017	25	17 (68)	8 (32)	0.002
tumor	21	+	3 (14.3)	18 (85.7)		14	2 (14.3)	12 (85.7)	

DISCUSSION

In this study, we found that MSCs played several roles in regulating the behavior of tumors including: (1) both MSCs and gastric cancer cells exhibited myofibroblast or mesenchymal phenotype transition when they were co-cultured and along with the microstructure transition; (2) MSCs induced gastric cancer cell EMT and promoted transwell and trans-endothelial migration; (3) MSCs remarkably increased tumor mass and liver metastasis; and (4) EMT was promoted by the translocation of β-catenin/MMP-16 and their upstream factors snail and twist; β-catenin and MMP-16 were correlated with metastasis in intestinal type gastric cancer.

An ideal laboratory model is vital to study metastatic mechanisms. Here, we established a new model using a multi-migration assay (Fig. 1A) and the crosstalk of various secreted cytokines and growth factors was allowed to occur freely. The deprivation of these signals can, in part, reverse the tumor′s metastatic ability [Karnoub et al., 2007]. We have now demonstrated that the ability of MSCs to promote cancer metastasis is not only affected by a mesenchymal phenotype, but it is also enhanced by mutual paracrine signals (Fig. 1). A close physical connection between MSCs and tumor cells appeared to be necessary to induce EMT in vitro. Without close contact, no significant phenotypic change was observed. The "transdifferentiated" gastric cancer cells accumulated an abundance of rough endoplasmic reticulum and lysosomes in the cytoplasm, which is similar to activated fibroblasts. Moreover, the observation of transdifferentiation was confirmed by the up-regulation of both α-SMA and vimentin. MSCs also exhibited a myofibroblast phenotype both in vivo and in vitro when directly mixed with gastric cancer cells. The recruitment of α-SMA-positive MSCs to tumor xenografts has also been observed in recent study [Witz, 2008].

Using a multi-migration, transwell and trans-endothelial migration assay, paracrine signals of Co-CM attracted tumor cells more readily than MSCs alone. These model are similar to the in vitro tumor "self-seeding" model (Fig. 6). Tumor self-seeding refers to circulating tumor cells from the blood or bone re-infiltrating the primary tumor and promoted tumor progression [Kim et al., 2009]. In our models, MSCs in the tumor stroma (MSCs co-cultured with cancer cells) first promoted gastric cancer cell EMT by close contact and secreted cytokines, and then attracted gastric cancer infiltrating (cancer cell migrated to co-culture cells, Fig. 6). Circulating tumor cells have also been identified in gastric cancer, often residing in the bone marrow [Ebert et al., 2003]. The main cytokines, IL-6 and IL-8, attract circulating tumor cells and infiltrate primary tumors though MMP1 and recruit stroma-associated cells through CXCL1 [Norton and Massague, 2006; Kim et al., 2009].

We further confirmed that β-catenin′s upstream factors, twist and snail, accounted for the upregulation of β-catenin/MMP-16 and gastric cancer EMT. Paracrine signal promote GSK3-βphosphorylation and β-catenin nuclear translocation, which can initiate EMT. Paracrine signal promote GSK3-βphosphorylation and β-catenin nuclear translocation, which can initiate EMT. β-catenin translocation disrupted E-cadherin′s cell-cell adhesion and intracellular signaling functions. E-cadherin loss contributes to metastatic dissemination and EMT [Thiery et al., 2009; Cordonnier et al., 2014]. Mutated β-catenin has been reported to up-regulate MMP-16 expression to promote gastric cancer invasion in vitro [Lowy et al., 2006] (21). Recent studies have found HB-EGF to activate gastric cancer cell EMT along with the up-regulation of snail. Snail directly interacts with beta-catenin at its N-terminus and increased expression of Wnt target gene [Stemmer et al., 2008]. Twist acting upstream from Snail suppressed the level of E-cadherin and promote EMT [Smit et al., 2009]. Up-regulation of MMP-16 is critical to regulate the tumor invasive phenotype in vitro [Kang et al., 2000]. To examine the mesenchymal phenotype in vivo, PKH-26 staining of MSCs and gastric cancer cells was performed. Using PKH26 staining, we determined that both labeled MSCs and cancer cells can produce α-SMA protein.

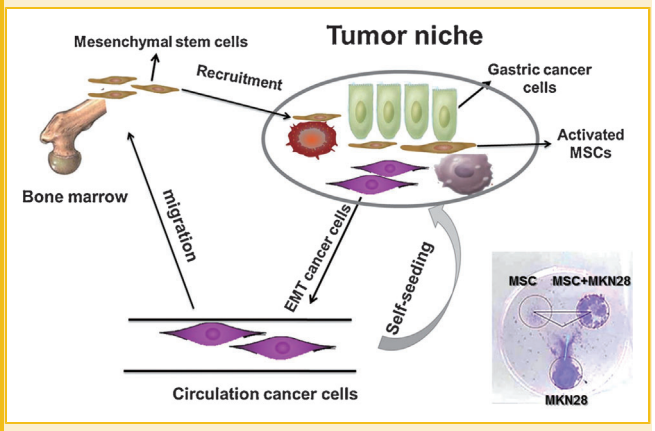

Fig. 6. Metastasis provoked by tumor stroma and tumor self-seeding. Schematic diagram of the "tumor self-seeding" model in vitro. MSCs secrete paracrine signals, similar to bone marrow. MSCs + MKN28 cells represent the primary tumor and its niche. MKN28 cells moved in a manner similar to circulating tumor cells. The tumor stroma, which includes MSCs, promotes tumor metastasis by infiltrating vessels and hijacking EMT. The circulating tumor cells were attracted to the bone marrow and also to the primary tumor by paracrine or endocrine signals. These cells were self-seeded into the primary tumor and caused mesenchymal phenotype changes and enhanced tumor aggressiveness.

In a clinical study, we found β-catenin expression to be significantly correlated with lymph node metastasis ($P < 0.001$; Table I). The nuclear expression of β-catenin was 29% in 311 gastric cancer of previous report is similar with our report 22.1% in 194 cases [Clements et al., 2002]. Aberrant β-catenin expression was significantly increased in intestinal-type gastric cancer and was correlated with metastasis [Miyazawa et al., 2000; Ebert et al., 2003; Ogasawara et al., 2006]. Mutations of the β-catenin gene were found in 45% intestinal-type gastric cancer regions with nuclear β-catenin localization in 39 gastric adenocarcinomas [Ogasawara et al., 2006]. The total expression of MMP-16 was 72.2%, similar with others report 71% [Lowy et al., 2006]. In addition, MMP-16 expression was significantly up-regulated in intestinal type gastric cancer and was associated with lymph node metastasis ($P = 0.008$ and $P = 0.036$; Table I). MMP-16 positive staining in primary tumors was an independent predictor of metastasis (HR= 6.67 with lymph node metastasis and HR = 12.8 with distant organ metastasis; Table II).

Collectively, we have observed that MSCs promote gastric cancer metastasis via close physical cellular contact and paracrine signals

released from the tumor niche both in vivo and in vitro. Using several migration assay model, we found that stroma-associated MSCs promoted gastric cancer cell EMT and then attracted circulating tumor cells to self-seed the primary tumor, again though EMT. Finally, MSCs activated snail and twist and upregulated nuclear β-catenin levels. Additionally, MMP-16 expression correlated with tumor EMT and metastasis. These data demonstrated that MSCs within the tumor niche significantly facilitated metastasis and primary tumor self-seeding by eliciting cancer cell EMT.

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