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Omental milky spots in screening gastric cancer stem cells

L. CAO[1], X. HU[1], Y. ZHANG[2], X. T. SUN[1]

[1]Department of General Surgery, The First Affiliated Hospital, Dalian Medical University ,Dalian 116011,China, e-mail: caoliang913@yahoo.com.cn; [2].Plastic and Cosmetic Medical College, Dalian Medical University, Dalian 116044,China

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The existence of cancer stem and progenitor cells in solid tumors has been widely postulated. However, neither the cancer stem cells nor the cancer progenitor cells have been definitively identified and functionally characterized. Here we propose a new strategy to identify and isolate gastric cancer stem cells –using omental milky spots to screen gastric cancer stem cells in peritoneal metastasis mouse models of gastric cancer. In this study, we used the property that the macrophages in omental milky spots are cytotoxic against tumor cells and so able to screen and collect cancer stem cells. Our findings suggest that macrophages in omental milky spots have not only cytotoxic properties against tumor cells but also provide a microenvironment within milky spots in which cancer stem cells are capable to survive and grow into micrometastasis. Omental milky spot become a cancer stem cell niche in this situation. Further we studied the omental milky spots for screening gastric cancer cells (OMSS-GCCs) and found that omental milky spot enriched the volume of gastric cancer stem cells. Tumors were consistently generated after an injection of 1×10^3 OMSS-GCCs. OMSS-GCCs high express CD133 and low express CD324. Omental milky spots are a highly efficient "natural filter" for screening gastric cancer stem cells.

Key words: *cancer stem cell, gastric cancer, omentum, omental milky spot*

In the light of cancer stem cell theory, tumor growth and metastasis are driven by a small population of cancer stem cells(CSCs), and there is a hierarchical organization of cell populations in cancer cells which hierarchy includes CSCs that produce committed progenitor cells that, in turn, produce rapidly proliferating cells, finally resulting in the generation of fully differentiated cells [1,2] .Current chemotherapeutic agents and radiation therapy largely target proliferating and differentiated cells that form the bulk of the tumor but not the CSCs and progenitor cells[3]. Thus, this quiescence and resistance may account for many treatment failures. If this is the case, then the only effective way to treat cancer is to target the CSCs and progenitor cells. Until now, there are three strategies that have been employed to identify CSCs as follows: (1) expression of cell surface markers,(2) ability to exclude fluorescent dye Hoescht33342 in a cell population termed"side population"(SP) [4], (3) formation of tumor spheres in suspension culture. But these methods lead us to focus cancer stem cells on a minor subset of tumor cells, instead of initial CSCs and progenitor cells. How to target and isolate initial CSCs and progenitor cells is the key for further study.

Milky spots are primitive lymphoid tissues in the peritoneal cavity of humans and animals, and exist mainly in the great omentum, the mesenterium, and the pelvic floor. In particular, omental milky spots are composed of numerous macrophage and lymphocyte aggregation and are involved in the clearance of particles, bacteria and tumor cells from the peritoneal cavity [5-11]. Omental milky spot is a well-known site of metastases of carcinomas of the ovaries, stomach and colon. Many studies have indicated that gastric cancer cells selectively infiltrate the milky spots in the early stages of peritoneal cancer dissemination, and then form micrometastasis[5-8]. Macrophages in omental milky spots have not only cytotoxic properties against tumor cells but produce also growth stimulatory factors [9-11].The tumor cells are attached to the milky spots in the great omentum and find a microenvironment within milky spots in which they are capable to survive and grow into solid metastases. Combining the theory of cancer stem cell, we propose that a gastric cancer stem cell infiltrate into the milky spot, then proliferate and differentiate into some gastric cancer progenitor cells, which is the basis of forming metastasis.

Omental milky spot provide a chance for us to target a single gastric cancer stem cell. Oosterling and colleagues have identified that tumor cells mainly adhered on omental milky spots 4 h after tumor cell injection [7]. This preferred

attachment can be explained by the fact that mesothelial cells lining milky spots showed higher levels of cellular adhesion molecules such as intercellular adhesion molecule-1 than other omental regions, which may contribute to enhanced adhesion as well[8-11] . However, a sharp decline in numbers of tumor cells was seen in specific milky spot areas, then few proliferating tumor cell clusters were found in milky spots and the infiltrated cancer cell forming micrometastasis[7]. According to the cancer stem cell hypothesis that part of CSCs and mature cancer cells in some milky spots have been eliminated by the killing function of macrophages, but only survival CSCs in some milky spot proliferate and differentiate into gastric cancer progenitor cells and form micrometastasis. We prepared to collect the omental milky spot screening gastric cancer cells (OMSS-GCCs), and then transplanted into NOD/SCID mice in order to demonstrate its tumorigenic ability.

In most solid tumors, cancer stem cells are rare. Many cancers contain a small but significant proportion (<1%) of CSCs[12]. Catherine and colleagues calculated by limiting dilution analysis that there was only one CSC in 5.7×10^5 unfractionated human colon cancer cells [13]. So it is very difficult to find the unique marker to isolate and purify CSCs and progenitor cells which were covered by thousands of mature cancer cells. In many cases, a combination of multiple markers is used to identify a particular cancer stem cell type. So now, researchers often identify stem cells in shorthand by a combination of marker names reflecting the presence (+) or absence (–) of them [14,15]. But omental milky spots help us to resolve this problem. Cancer stem cell and progenitor cells consist of total cancer cells at the early stage of milky spot micrometastasis, the proportion of the cancer stem cell and progenitor cells markers may increase accordingly. Through comparing the change of the expression of cell surface molecules of cancer cells, we may primarily screen the cancer stem cell markers. So if express of a marker is higher on the surface of cancer cells which were isolated from milky spot micrometastasis, but lower express on surface of the bulk tumor cells or even negative. This marker may be the unique maker used for isolating and purifying CSCs and progenitor cells.

In the current study, we therefore used a milky spot model to investigate the biological significance of the cells in micrometastasis and its tumorigenic ability, and whether the omental milky spots can be a highly efficient "natural filter" for screening gastric cancer stem cells or not.

Materials and methods

Tumor cell line and animals. A murine gastric cancer cell line MFC was derived from the strain 615 murine carcinoma of proximal stomach, and obtained from the central laboratory of the First Affiliated Hospital of Dalian Medical University. The cells were revitalized and cultured in RPMI 1640 (GIBCO), supplemented with 10% fetal bovine serum (FBS, Sigma) in an incubator of 5% CO_2 with a temperature of 37°C. And near confluence, the cells were trypsinized and rinsed in media D-Hanks. A viable cell count was performed using trypan blue exclusion.

Six week-old strain 615 mice and NOD/SCID mice were obtained from Dalian Medical University of China. The mice were kept under standard laboratory conditions and had free access to standard laboratory food and water. All study protocols were approved by the Committee for Animal Research of the Dalian Medical University of China, according to national guidelines.

Transmission electron microscopy. We used 6 omental fat bands, obtained from three 615 mice of each sex. They were immersed in a fixative solution containing 2% glutaraldehyde for 2 h, then postfixed in a solution containing 2% osmium tetroxide and 1.5% sucrose in 0.05 M phosphate buffer for 2 h at 4°C. The specimens were dehydrated in a graded series of ethanol. After substituting acetone for ethanol, they were embedded in epoxy resin blocks. Ultrathin sections were stained with uranyl acetate and lead citrate. They were observed with a transmission electron microscope. (JEM-2000EX).

Tumor cell attachment on the omentum . The MFCs were incubated in complete RPMI 1640 in a concentration of 2mg/l DiI (Sigma) for 60 min at 37°C. Cells were washed three times with Hanks' balanced salt solution. Cell labeling rate was 100% and 1×10^4 cells were injected intraperitoneal. Animals were killed after 4h, 12h, 36h, 48h, 72h, 120h, 7d, 10d and 14d after intraperitoneal injection. Omenta were excised, stretched on microscope glasses for further processing.

Immunohistochemistry. For immunohistochemistry, omenta were fixed in formalin for 60 min, washed three times with PBS . Omenta were incubated for 60 min at 37°C with FITC-conjugated anti-mouse F4/80(murine macrophage marker)(1μg/ml, Biolegend) , washed in PBS (3 times) ,and then air-dried in darkroom for 12 h .Immunohistochemical staining was directly scored and images were captured with a Fluorescence Microscope (BX-51 TR32000, Olympus).

Collected OMSS-GCCs implantation into NOD/SCID mice. After 72h of intraperitoneal injection, animals were killed and omenta were excised. The surface of omental milky spots was rinsed with RPMI 1640 and collected the OMSS-GCCs. The OMSS-GCCs were suspended in a 1:1 mixture of media and injected subcutaneously into the right and left flank of NOD/SCID mice with anesthesia. After 14 weeks all mice were sacrificed by cervical dislocation, and tumors were removed , fixed in formalin and analyzed by histology (H&E, CK-20 and CEA). All of the OMSS-GCCs were originally implanted into NOD/SCID mouse subcutaneously and never cultivated or expanded in vitro. To study the tumorigenecity of OMSS-GCCs, a different number of OMSS-GCCs was injected subcutaneously into NOD/SCID mice. Tumorigenecity was evaluated at 14 weeks after NOD/SCID transplantation. To determine whether this xenotransplant system was quantitative and able to detect the doses unable to initiate tumor growth, we performed limiting dilution experiments. Groups of NOD/SCID mice were transplanted with doses of OMSS-GCCs over a range from doses unable to initiate tumor growth to doses

that always initiated tumor formation and normal MFCs as control group. (Table1).

Collected OMSS-GCCs and Immunocytochemical staining of CD133 and CD324. Cell suspensions from OMSS-GCCs were plated in chamber slides, which were coated with poly-L-Lysine, allowed to attach for 48 hours and then used for immunocytochemistry. The cells were fixed with 0.3% H_2O_2 in methanol for 60 minutes at room temperature, washing 4 times in PBS and protein block with 3% BSA and permeabilized with PBS containing 0.1% Triton X-100 for 60 minutes at room temperature. Antibodies used were FITC-conjugated anti-mouse CD324(1μg/ml,eBioscience) and PE-conjugated anti-human CD133 (1μg/ml, Santa Cruz Biotechnology) incubated for 60 min at 37°C, washed 4 times, inspected under a Fluorescence Microscope (BX-51 TR32000, Olympus). Normal MFC was used as a positive control for the immunocytochemistry.

Statistical analysis. Ten random fields were scored for percentage staining. The score was expressed as the mean of the ten regions. Statistical analysis was performed with SPSS13.0 software for Windows. Data are expressed as mean±SD. Statistical comparisons were based on One-Way ANOVA and statistical significance was defined at $p<0.05$.

Results

Observations of omental milky spots. The great omentum of the 615 mouse was bordered by a narrow fat tissue stripe (Fig. 1A), along which were some cellular aggregates known as milky spots (Fig. 1B). Electron microscopy revealed more detailed cell composition of the milky spots which were largely composed of abundant macrophages (Fig. 1C), and also with some lymphocytes, eosinophils, neutrophils, and various stromal cells (Fig. 1D).

Visualization of tumor cells on the omentum at early time points. In order to investigate the tumor cells on the omentum at early time points and find the time of forming micrometastasis we injected 1×10^4 DiI-labelled MFCs (DiI-MFCs) and removed omenta at several early time points. In vitro, no differences in adhesion abilities and proliferation rates were shown between DiI-MFCs and non-labelled MFCs. DiI-MFCs displayed high signal intensity after 10 days of culture (data not shown). At 12 h post injection, MFCs were particularly concentrated in milky spots (Fig. 2A), and no tumor cells clusters were found in non-milky spot areas of omenta. At 48 h post injection, numbers of DiI-MFCs declined in milky spot areas (Fig. 2B). After 72 h, numbers of DiI-MFCs further decreased in milky spot areas and proliferating tumor cells were observed in milky spots and forming micrometastasis (Fig. 2C). After 72h, sporadic tumor cells were found in non-milky spot areas, and no cell clusters were detected at the same time. After 10 days, some tumor cell clusters were increased in size and cell number and forming metastasis. (Fig. 2D)

Tumorigenicity in NOD/SCID mouse. To determine whether omental milky spots enrich the gastric cancer stem cells, we collected the OMSS-GCCs and injected into NOD/SCID mice. Of 40 mice (dose range: 1×10^1 to 1×10^3) injected with OMSS-GCCs, one mouse transplanted with 5×10^2 cells generated a tumor. Two mice transplanted with 7.5×10^2 cells generated a tumor and tumors were consistently generated after injection of 1×10^3 OMSS-GCCs. In contrast, one tumor was generated after injection of 5×10^4 unscreened gastric cancer cells, one mouse transplanted with 1×10^5 cells generated a tumor and tumors were consistently generated after injection of 5×10^6 unscreened gastric cancer cells. (Table 1). The histology of the tumors, as expressed by H&E (haematoxylin and eosin) staining, shows same poor differentiated adenocarcinomas. The immunohistochemical markers CK-20 and CEA reveal similar staining patterns in both the bulk and OMSS-GCC xenografts. (Fig. 3)

Expression of CD133 and CD324 in OMSS-GCCs. In order to find a marker expression higher on the surface of cancer cells which were isolated from milky spot micrometastasis, but lower expression on the surface of the bulk tumor cells or even negative, we detected the change of expression of CD133 and CD324 respectively.

Ten random fields were respectively taken for counting the CD133 positive cells and total cells, CD133 positive cells quantity in OMSS-GCCs (7.66±0.32%) was more than it was in unscreened gastric cancer cells(2.93±0.38%) ($P<0.01$). Counting the CD324 negative cells and total cells in ten random fields, CD324 negative cells quantity in OMSS-GCCs (14.56±1.68%) was more than it was in unscreened gastric cancer cells(4.93±0.91%) ($P<0.01$). (Fig.4)

Table 1. Limiting dilution analysis of the omental milky spots screening gastric cancer cells

Cancer cell source	Cell dose	Number of mice with tumors/total number injected (%)
Bulk	5×10^4	1/10 (10)
	1×10^5	1/8(12.5)
	5×10^5	3/8(37.5)
	1×10^6	4/7(57.1)
	5×10^6	7/7(100)
OMSS-GCCs	5×10^1	0/9(0)
	2.5×10^1	0/10(10)
	5×10^2	1/10(10)
	7.5×10^2	2/6(33)
	1×10^3	5/5(100)

Discussion

The existence of cancer stem and progenitor cells had been hypothesized for many decades, however, it was not until 1997 that cancer stem cells (CSCs) were isolated and identified from patients with acute myeloid leukemia [14]. And subsequently cancer stem cells have been verified in tumors of the breast

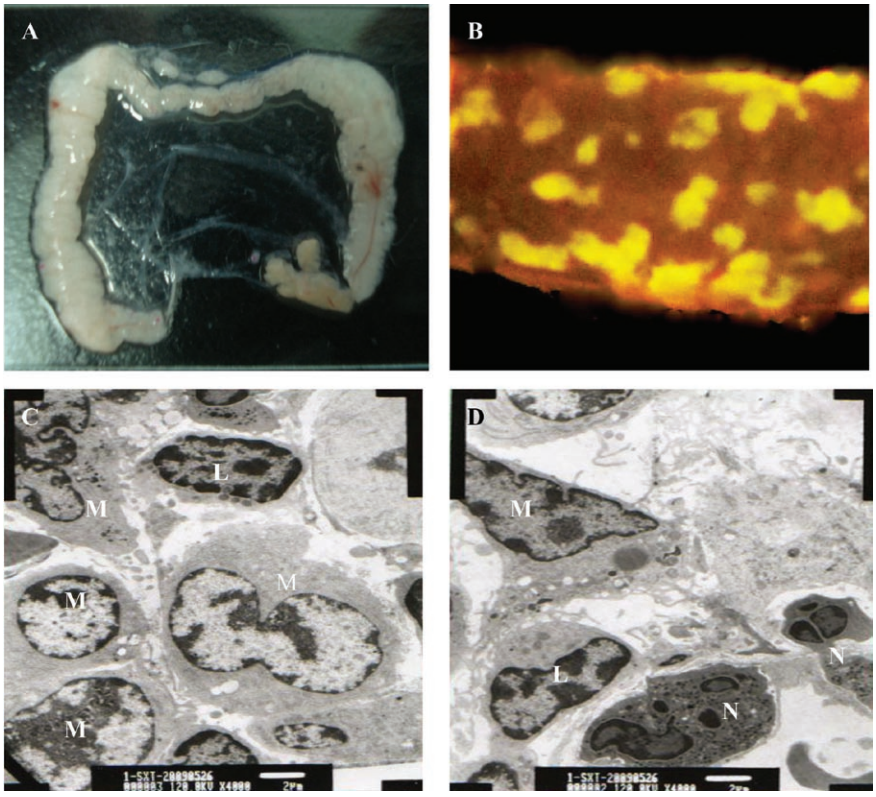

Figure. 1(A): The greater omentum of the 615 mice was bordered by a narrow fat tissue stripe; (B): Along which were some cellular aggregates known as milky spots(×100); (C)Electron microscopy revealed the cell composition of the milky spots which largely were composed of abundant macrophages (M) (×4000); (D)and also with some lymphocytes (L), neutrophils (N) (×4000).

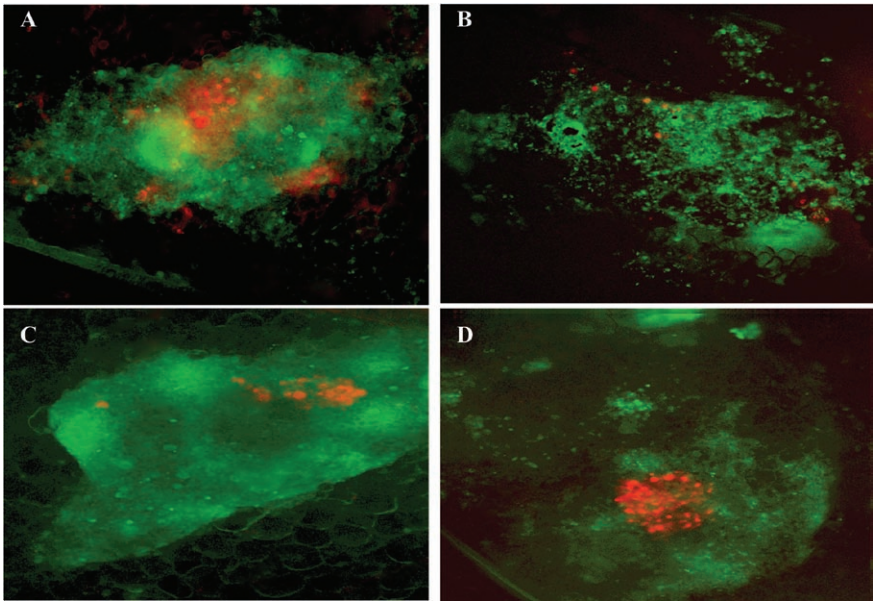

Figure 2. Gastric cancer cells adhere on the milky spots at different time points. (A): Image of milky spot macrophages (green) and large numbers of DiI-MFCs (red) concentrated in milky spot areas 12h after intraperitoneal injection. (B): After 48 h numbers of DiI-MFCs (red) cells in milky spots decreased . (C): After 72 h, numbers of DiI-MFCs further decreased in milky spot areas and proliferating tumor cells were observed in milky spots and forming micrometastasis. (D): After 10 days, some tumor cell clusters that were increased in size and cell number and forming metastasis. (Magnification: ×200).

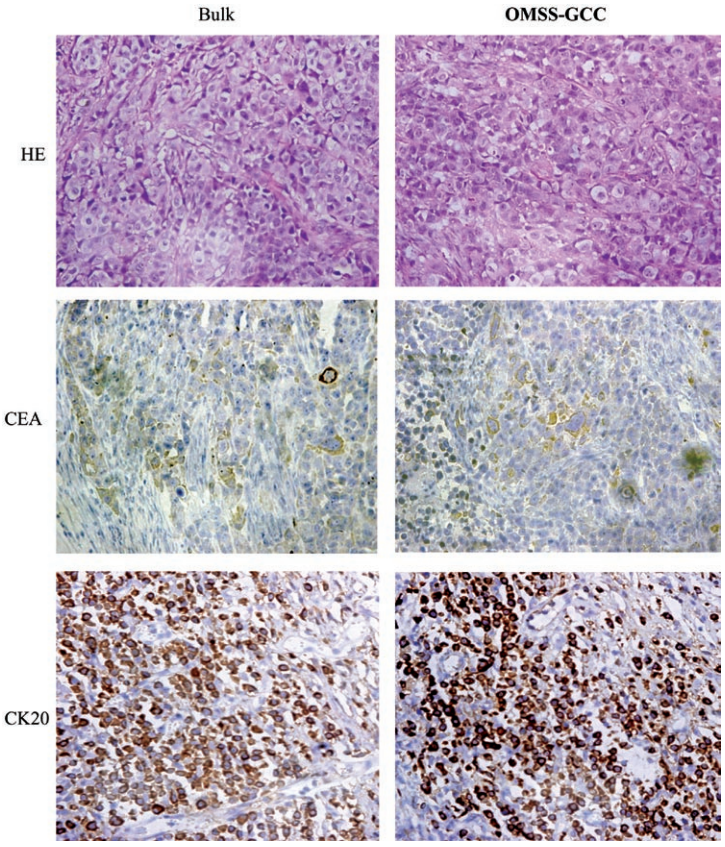

Figure 3. Xenografts generated from the OMSS-GCC and bulk. The two xenografts generated from the injection of 5×10^4 bulk human gastric cancer cells and 5×10^2 OMSS-GCCs respectively. The histology of the two tumors, as expressed by H&E (haematoxylin and eosin) staining, shows poor differentiated adenocarcinomas. The immunohistochemical markers CK-20 and CEA reveal comparable staining patterns in both the bulk and OMSS-GCC xenografts. Images for each stain are taken at the same magnification (×400).

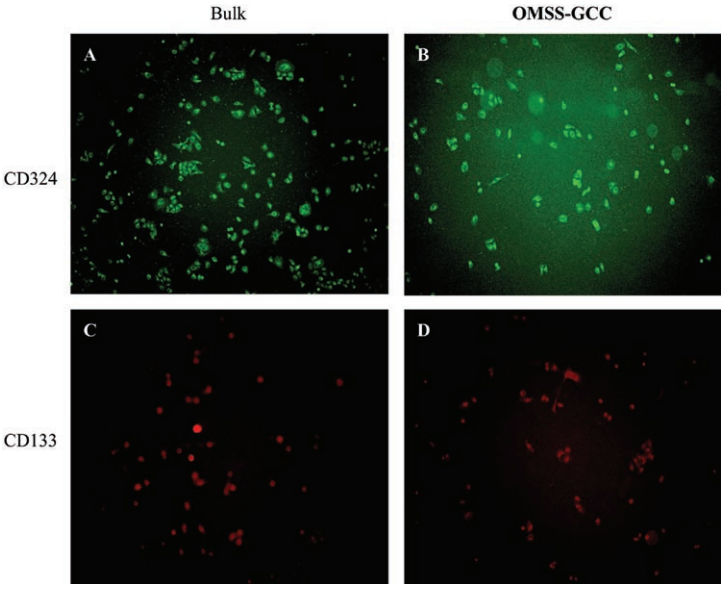

Figure 4. Expression of CD133 and CD324. (A-B) CD324 negative cells quantity in bulk gastric cancer cells(4.93±0.91%) was less than it in OMSS-GCCs (14.56±1.68%) (P<0.01). (C-D) CD133 positive cells quantity in OMSS-GCCs (7.66±0.32%) was more than it in bulk gastric cancer cells (2.93±0.38%) (P<0.01). (Magnification: ×200)

[15], brain [16], head and neck [17], prostate [18], colon [13], liver [19], and pancreas [20]. And there are many strategies that have been employed to identify CSCs, but these methods lead us to focus cancer stem cells on a minor subset of tumor cells, instead of initial CSCs and progenitor cells.

How to target and isolate initial CSCs and progenitor cells? In this regard, omental milky spots have a special character: Milky spots largely consist of an accumulation of macrophages, which have been shown cytotoxic against tumor cells. This function is just a screening process. In the present study, we found that tumor cells began to adhere on milky spots 4 h after injection, at 12 h post injection, DiI-MFCs were particularly concentrated in milky spots, and no tumor cells clusters were found in non-milky spot areas of omenta. At 48 h post injection, numbers of DiI-MFCs declined in milky spot areas as result of the killing function of macrophages. After 72 h, numbers of DiI-MFCs further decreased in milky spot areas and proliferating tumor cells were observed in milky spots and forming micrometastasis. In this process, the killing function by macrophages plays a very important role in screening tumor cells. Most of CSCs and mature cancer cells in some milky spots have been eliminated by the killing function of macrophages, the other surviving tumor cells, only CSCs in some milky spot can proliferate and differentiate into gastric cancer progenitor cells and forming micrometastasis. In this stage, the micrometastasis is full of CSCs and progenitor cells. Here we have identified and characterized OMSS-GCCs on the basis of their ability to initiate human gastric cancer after transplantation into NOD/SCID mice. OMSS-GCCs possessed the key criteria that define stem cells: after transplantation at limited dilution, OMSS-GCCs proliferated extensively and differentiated to produce tumors. Of 40 mice (dose range: 1×10^1 to 1×10^3) injected with OMSS-GCCs, one mouse transplanted with 5×10^2 cells generated a tumor. Two mice transplanted with 7.5×10^2 cells generated a tumor and tumors were consistently generated after injection of 1×10^3 OMSS-GCCs. In contrast, one tumor was generated after injection of 5×10^4 unscreened gastric cancer cells, and tumors were consistently generated after injection of 5×10^6 gastric cancer cells. Thus, while significantly enriched, not every OMSS-GCC represents a cancer stem or progenitor cell. The immunohistochemical markers (including CK-20, CEA) reveal comparable staining patterns in both the OMSS-GCC xenografts, as compared to the xenografts generated from the bulk gastric cancer cells.

Micrometastasis has now been classified into two types: single cells and small cell clusters under 0.2mm in size [21]. According to the theory of cancer stem cell, the disseminated cancer cells in some of the patients arose from the spread of nontumorigenic cells and only when cancer stem cells disseminate and subsequently self renew will micrometastases form. Many studies have proved the hierarchy includes CSCs that produce committed progenitor cells that, in turn, produce rapidly proliferating cells, finally resulting in the generation of fully differentiated cells and forming metastases [1,2,12]. So at the early stage of forming metastasis, the micrometastasis is composed of CSCs and progenitor cells. The biological significance of CSCs and progenitor cells in micrometastasis remains essentially unknown because of the lack of appropriate animal models. In the present study, we developed a milky spot micrometastasis model, which allows visualization of even isolated CSCs and progenitor cells in the development of metastasis without histological procedure. At 72h post intraperitoneal injection, numbers of gastric cancer cells decreased in milky spot areas for the reason of the killing function by macrophages and proliferating tumor cells were observed in milky spots and forming micrometastasis. Tumor cells attach to milky spots very slightly at this stage, and in the method applied in this study the omentum were rinsed twice and OMSS-GCCs in milky spot micrometastasis could be collected easily. This has significant importance, indicating that CSCs and progenitor cells in micrometastasis can be collected, which is the key for further study.

Many studies have been made in the identification of biomarkers for stem cells [1,12,17]. It is now possible to isolate cancer stem cells as well as their malignant counterparts from total cell mass in cancer patients' malignant tissue specimens and well-established cancer cell lines .Several stem cell-like surface markers, such as CD34, CD24, CD20, CD90, CD133 and CD44 have been used to isolate these cells[21]. CD133 (also known as Prominin-1 or AC133), a transmembrane pentaspan protein, was initially described as a marker for murine neuroepithelial cells and several other embryonic epithelia [23]. The specific functions and ligands of the prominins are still relatively unclear, but CD133 has been shown to be a marker for cancer stem cells in some tumors, including neural [16] and colon stem cells [13]. CD324 (also known as E-cadherin) is a transmembrane calcium-dependent cell adhesion molecule, which is capable of maintaining the polarity of epithelial cells and cell junction. Reduced expression of CD324 has been regarded as one of the main molecular events involved in dysfunction of the intercellular adhesion and maintenance, triggering cancer invasion and metastasis [24]. In the present study, marker expression was higher on the surface of cancer cells which were isolated from milky spot micrometastasis, but lower expression on the surface of the bulk tumor cells or even negative. We detect the change of expression of CD24, CD44, CD133 and CD324 respectively. Quantity of CD133 positive cells in OMSS-GCCs (7.66±0.32%) was higher than it was in unscreened gastric cancer cells (2.93±0.38%) ($P<0.01$); CD324 negative cells quantity in OMSS-GCCs (14.56±1.68%) was higher than it was in unscreened gastric cancer cells(4.93±0.91%) ($P<0.01$). So $CD133^+CD324^-$ express higher significance on the surface of cancer cells which were isolated from milky spot micrometastasis, but lower expression on the surface of the bulk tumor cells. This marker may be a marker used for isolating and purifying CSCs and progenitor cells.

In conclusion, the model has a significant importance in screening and sustaining the development of cancer stem cells. And this model provides the means of further purify-

ing and functionally characterizing the biological properties of the cancer stem cells fraction, with the goal of developing new therapeutic strategies directed specifically against cancer stem cells.

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