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# Tumor-associated macrophages promote cancer stem cell-like properties via transforming growth factor-beta1-induced epithelial–mesenchymal transition in hepatocellular carcinoma

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ABSTRACT

Tumor-associated macrophages (TAMs), a crucial component of immune cells infiltrated in tumor microenvironment, have been found to be associated with progression and metastasis of hepatocellular carcinoma (HCC). In this study, we aimed to clarify the mechanism underlying the crosstalk between TAMs and cancer stem cells (CSCs) in HCC. Mouse macrophage cell line RAW264.7 cells were used to investigate the effects of TAMs on mouse hepatoma cell line Hepa1-6 cells in vivo and vitro. A total of 90 clinical samples had pathology-proven HCC were used to evaluate the distribution of TAMs and CSCs and analyze their value in predicting the prognosis. In the study, we have found that the number of TAMs has a positive correlation with the density of CSCs in the marginal of human HCC. Our result show that, cocultured with TAM-conditioned medium (CM) promoted CSC-like properties in Hepa1-6 cells, which underwent EMT and gained higher invasive capability. TAMs secreted higher transforming growth factor- beta1 (TGF-beta1) than other phenotypes of macrophage. Furthermore, depletion of TGF-beta1 blocked acquisition of a CSC-like properties by inhibition of TGF-beta1-induced EMT. High expression of CD68 in the EpCAM positive expression HCC tissues was strongly associated with both poor cancer-free survival and overall survival in patients. Our results indicate that the TAMs promote CSC-like properties via TGF-beta1-induced EMT and they may contribute to investigate prognosis in HCC.



## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. And it is the third most common cause of cancer-related death in adults [19,26]. Although hepatic resection, liver transplantation and various of minimally invasive therapies are widely used to improving outcomes of the patients of HCC. Due to recurrence and metastasis, the five-year survival of patients undergoing therapies still remains disappointingly low.

Cancer stem cells (CSCs) or cancer-initiating cells are defined as a small subpopulation of cancer cells with the ability of self-renewed and pluripotency. Currently, many studies supports that CSCs, which have many features of stem cells, are responsible for the poor prognosis of patients by promoting tumor recurrence and metastasis [32,39]. Recent data suggest that CSCs rely on a specialized tumor microenvironment (TME) or niche [3]. However, the effect of TME on cancer cells stemness remains unclearly on HCC.

Macrophages could polarize into two functionally phenotype, classically activated M1 and alternatively activated M2, in response to different microenvironment. Macrophages infiltrated in tumor microenvironment (TME) are define as tumor-associated macrophages (TAMs), expressing the similar molecular and functional characteristic of M2 phenotype [1]. TAMs is a pivotal component of tumor-infiltrating immune cells which play a critical role in

*Abbreviations:* HCC, hepatocellular carcinoma; CSC, cancer stem cell; TAM, tumor-associated macrophages; TGF-β1, transforming growth factor-β1; EMT, epithelial to mesenchymal transition; CM, conditioned medium; TME, tumor microenvironment.

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regulating tumor growth and progression. At present, some studies about TAMs have showed that an increased density of TAMs were correlate with worse prognosis in many cancers [22,31]. And many evidences has revealed that TAMs promoted cancer cells to gain CSC-like properties [7], and EMT [2,18]. However, the role of TAMs promote CSC-like properties maintenance in HCC and the detailed mechanisms underlying this process was remain elusive.

Epithelial to mesenchymal transition (EMT) is a process epithelial tumor cells lose epithelial feather and gain mesenchymal phenotypes [34]. It is considered as the key step by which tumor cells gain the higher ability of invasive and metastatic. Tumor cells take advantage of EMT as an intermediary phenotype to achieve self-renewal and adapt to microenvironments [15,28]. Transforming growth factor-β1 (TGF-β1) is one of the most important members of the transforming growth factor family. It is a potent inducer of epithelial plasticity leading to EMT in cancer cells [14,40]. Increasing studies point out the importance of TGF-β1 in cancer progression and metastasis. As reported, TGF-β1 released from the tumor microenvironment are essential to regulate CSC-like properties maintenance, differentiation and function in many epithelial cancers such as skin, gastrointestinal tract, and bladder [9,20].

In the study, we aim to analyze the role of TAMs in HCC progression with focus on TGF-β1. We found that in HCC samples, the distribution of TAMs correlated with the location of $EpCAM^+$ CSC cells at the edge of HCC. After treated with the supernatant from cultural TAMs, the hepatoma cells acquired CSC-like properties. And the expression of EMT markers, the invasion ability and tumorigenicity of these cells have been increased. As tested, TAMs produced more TGF-β1 than other macrophage phenotype. Furthermore, neutralization of TGF-b1 inhibited the process of the maintenance of CSC-like phenotype by inhibition of TGF-β1-induced EMT on hepatoma cells. We demonstrated that TAMs promote gain of CSC phenotype by TGF-β1-induced EMT in hepatoma cells.

## Materials and methods

### *Patients and specimens*

From May 1999 to February 2007, 90 patients who underwent curative liver resection and pathology-proven HCC were examined in the study. Tumor stage was determined according to the 2009 International Union Against Cancer TNM Classification system (7th edition). The available characteristics of the patients are shown in Table 1. The study was approved by the Eastern Hepatobiliary Surgery Hospital Research Ethics Committee. All specimens were obtained from the archives of formalin-fixed, paraffin tissue blocks in the Department of Pathology.

Overall survival (OS) was defined as the period between the date of surgery and death or the date of last contact of living patients, Disease-free survival (DFS) was defined as the interval from the date of surgery to the first appearance of recurrence or death, whichever occurred first, or to the date of the last follow-up.

**Table 1**
Patient characteristic of 90 HCCs.

| Variables | Value | Percent |
|---|---|---|
| Age | 41.6 ± 22.4 | |
| Gender (male/female) | 81/9 | 90.0/10.0 |
| HBsAg (positive/negative) | 74/16 | 82.2/17.8 |
| Cirrhosis (absent/present) | 21/69 | 23.3/76.7 |
| ALT (U/L) | 45.9(1.0–234.9) | |
| AST (IU/L) | 35.2(1–108.3) | |
| AFP (ng/ml) | 267.2(1.0–1000.0) | |
| CEA (ng/ml) | 4.2(0–58.2) | |
| CA19-9 (U/ml) | 15.0(0–38.0) | |
| Tumor number (solitary/multiple) | 23/67 | 25.6/74.4 |
| Tumor size (⩽5 cm/>5 cm) | 38/52 | 42.2/57.8 |
| Microvascular invasion (absent/present) | 33/57 | 36.7/53.3 |
| Majorvascular invasion (absent/present) | 85/5 | 94.45.6 |
| TNM stage (I + II + III + IV) | 79/11 | 87.6/12.4 |
| EpCAM (positive/negative) | 38/52 | 42.2/57.8 |
| CD68 (%) | 1.72 ± 1.11 | 0.12–6.7 |

### *Cell cultures*

The mouse hepatoma cell line Hepa1-6 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA), mouse macrophage-like line RAW264.7 cells were maintained in Roswell Park Memorial Institute-1640 medium (RPIM-1640, Gibco-BRL, Gaithersburg, MD, USA), Both of them were supplemented with 10% fetal bovine serum(FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a 95% humidified atmosphere containing 5% $CO_2$.

### *Conditioned medium and ELISA*

RAW264.7 cells were cultured in RPIM-1640 with 10% FBS with or without 20 ng/ml IL-4 for 24 h, then changed to serum-free medium for 24 h and collected supernatant. The TAM-CM was collected by centrifugation. TAM-CM was incubated with 1 μg/ml TGF-β1 neutralizing antibody (mouse monoclonal anti-TGF-β1; Abcam) for 24 h at 4 °C to obtain TGF-β1-depleted TAM-CM. $5 \times 10^5$ Hepa1-6 cells/well were pre-culture in 2 ml DMEM with 10% FBS in 6-well plate for 24 h. Then culture medium was replaced with 1 ml CM and 1 ml Complete Medium per well for 24 h. And the treated Hepa1-6 cells could be used in the subsequent trials.

20 ng/ml IL-4 and 100 ng/ml LPS plus 20 ng/ml IFN-γ was used to stimulate two types of macrophages for 24 h, respectively. Then the supernatants of RAW264.7 cells were centrifuged and collected. Amounts of TGF-β1 secreted by macrophage was measured using commercial ELISA kits (Shanghai Hengyuan Biotechnology Co., Ltd.), according to the manufacturer's instructions.

### *Mice and in vivo tumorigenicity experiments*

Six-week-old Male BALB/c mice were purchased from Shanghai Experimental Animal Center, Chinese academy of science and maintained in a specific pathogen-free environment. All mice were treated according to the Laboratory Animal Center care guidelines of the Second Military Medical University. Ten mice were randomized into two groups. The suspensions of cells were injected subcutaneously into the left axilla at $1 \times 10^6$ cells/injection site. Mice were sacrificed by cervical dislocation 3 weeks after injection and tumor were isolated for analysis.

### *Immunohistochemistry and immunofluorescence staining*

The protocol of immunohistochemistry (IHC) and immunofluorescence (IF) are described elsewhere [4]. The staining was performed on formalin-fixed, paraffin tissue blocks of HCC. Rabbit anti-EpCAM (1:100; Epitomics, California), Mouse anti-human CD68 (1:100; DakoCytomation, Demark), Rabbit anti-F4/80(1:100; Abcam, Cambridge, UK), Rabbit anti-TGF-β1 (1:100; BioVision, California) were used as primary antibodies. For IHC, the components of the Envision-plus detection system ((EnVision/HRP/Mo; Dako, Carpinteria, CA) were used to detection. Reaction results were shown by incubation with 3, 3′-Diaminobenzidine (DAB). Images were photographed with a microscope. CD68-positive areas in the photographs were measured by Image Pro Plus (IPP). Five fields of images per sample were taken, and the results were expressed as CD68-positive area/total area. Negative controls were treated identically but without the primary antibody. For IF, the secondary antibodies Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rat IgG (1:200; Invitrogen, Paisley, UK) were applied. The nuclear was stained by 4′, 6-diamidino-2-phenylindole (DAPI, sigma, Saint Louis, USA). Images were captured with a fluorescent microscope (Leica TCS SP2).

### *Cell immunofluorescence*

About $5 \times 10^4$ cells per well were plated on 24-well culture dish and cultured in DMEM containing 10% FBS. Different treatments were given for 24 h after these cells were adherent. Washed twice, then the cells were fixed in 4% paraformaldehyde and 0.1% Triton × 100 in PBS buffer at 4 °C for 30 min. After washed 3 times, the cells were blocked with 1% bovine serum albumin(BSA) in PBS at 37 °C for 30 min, followed by incubation with the primary antibodies Rabbit anti-E-cadherin (1:200; Cell Signaling technology, Boston, US) and anti-Vimentin (1:200; Abcam) overnight at 4 °C, washed 3 times with PBS and incubated with the Alexa Fluor 568-labeled secondary antibody (1:200; Invitrogen, Paisley, UK) for 30 min at 37 °C. Finally being stained with DAPI for 3 min. All matched samples were captured with a fluorescent microscope at identical exposure times.

### *Colony formation assay*

About $3 \times 10^2$ Hepa1-6 cells per well were seeded into a 24-well culture dish. After incubated at 37 °C for two weeks, washed twice with PBS, the cells were stained with 0.1% crystal violet solution. The number of colonies containing ⩾50 cells was counted under a microscope. The experiments were performed in triplicate.



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*Wound-healing assay*

The method for wound healing has been described [12]. About $5 \times 10^4$ Hepa1-6 cells were seeded in 24-well plates and incubated for 24 h, then the monolayer cells were disrupted by scratching with a 10 μl microsterile pipette tips. Photographs were taken at 0, 24 and 48 h in a phase-contrast microscope. The assays were performed in triplicate, and four fields of each point were measured.

*Transwell Invasion assay*

About $5 \times 10^4$ Hepa1-6 cells from different groups in 200 μl of serum-free medium were seeded in the upper chamber. And 500 ml of medium supplemented with 10% FBS were added to the lower compartment. After incubated for 24 h, the cells migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and counted under a microscope. All these samples were plated at three times.

*Quantitative real-time polymerase chain reaction*

Total RNA extraction, complementary DNA (cDNA) synthesis, and qPCR were performed as described [4,36]. Total RNA from Hepa1-6 and RAW264.7 cells was extracted by Trizol (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Expression of mRNA was determined by real-time PCR using SYBR Green Master Mix (TaKaRa, Dalian, China). The results were normalized to expression of glyceraldehyde-3-phosphatedehydrogenase (GAPDH). The primer sequences used in qPCR are shown in Supplementary Table 1.

*Western blot analysis*

Western blot was performed as described previous [20,21]. The protein of Hepa1-6 cells was extracted by RIPA Lysis Buffer (Beyotime, Haimen, China) with a protease inhibitors PMSF (Cwbiotech, Beijing, China) according to the manufacturer's instructions. Primary antibodies were rabbit against E-cadherin (1:1000; Abcam), N-cadherin (1:500; Abcam), Vimentin (1:2000; Abcam), and Snail (1:1000; Cell Signaling technology), GAPDH (1:5000; Bioworld Technology, Nan-

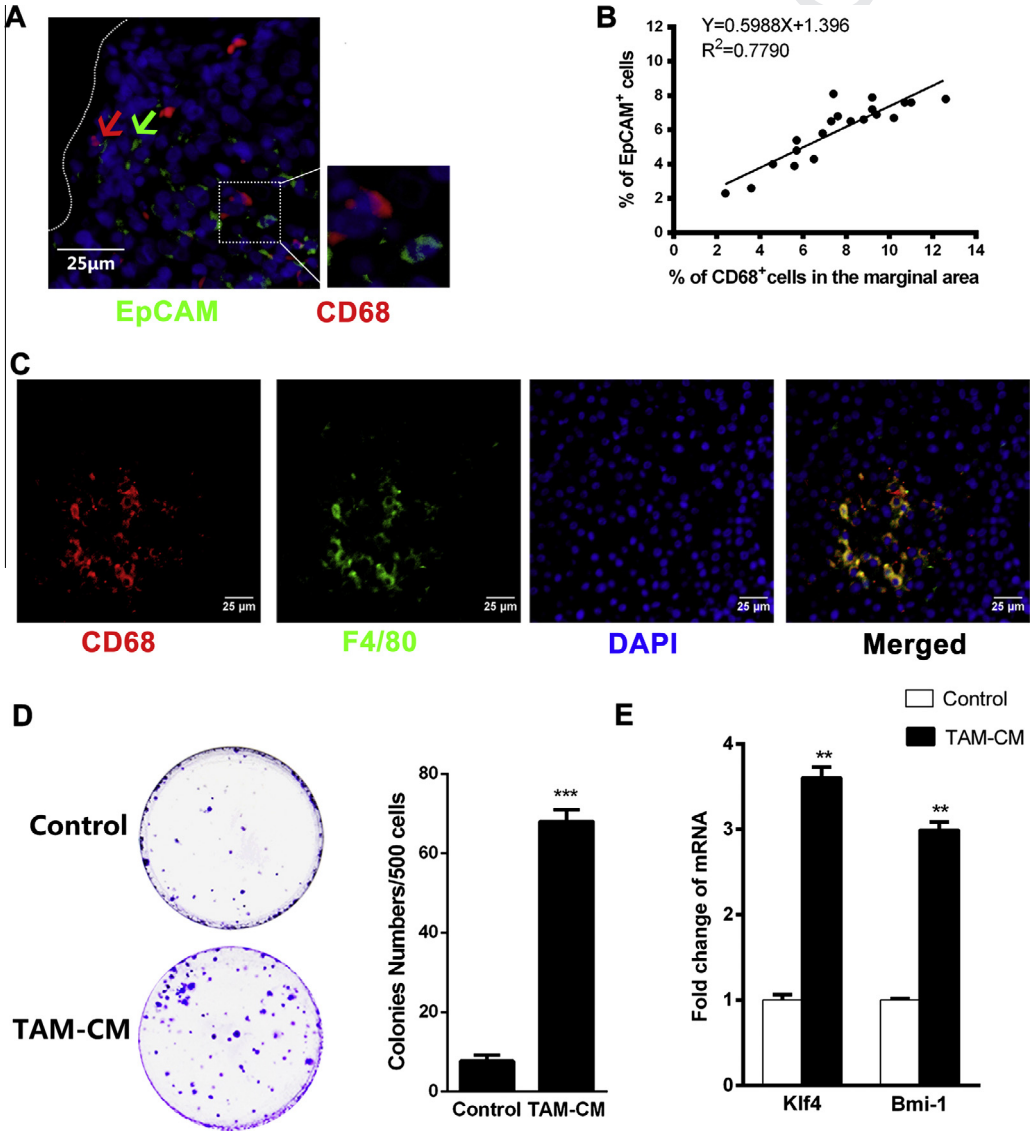


**Fig. 1.** Presence of TAM correlates with CSC-like properties s in vivo and vitro of HCC (A). Immunofluorescence staining. The distribution of $CD68^+$ macrophages (green) and $EpCAM^+$ tumor cells (red) in the invasive front area of HCC. Nuclei were counterstained with DAPI (blue). (B) Linear regression revealed a positive correlation between the distributions of $CD68^+$ macrophages and $EpCAM^+$ tumor cells. The relationship between local distributions $CD68^+$ macrophages and $EpCAM^+$ tumor cells was determined under 20 fields of fluorescence microscopy where $CD68^+$ cells and $EpCAM^+$ cells were found within a distance of 100 μm from the invasive edge of HCC. (C) Most of $CD68^+$ cells are expressing F4/80. F4/80 (green); CD68 (red); Nuclei (blue) (×400). (D) Colony formation assay was used to quantify the number of spheres of TAM-CM-treated Hepa1-6 cells and its control cells. (E) Expression of CSC transcriptional factors Bmi1 and Klf4 mRNA of Hepa1-6 cells was determined by RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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jing, China) as control. These primary antibodies were detected with Goat polyclonal Secondary Antibody to Rabbit IgG (1:10,000; Abcam). Proteins were detected by ECL detection reagent.

### *Statistical analysis*

Statistical analysis was performed with spss20.0. All date are shown as mean ± standard error of the mean (SEM). Difference between two groups was analyzed by unpaired Student's *t* test. For clinical samples, Kaplan–Meier curves analysis were performed to determine the survival. A $P < 0.05$ was considered statistically significant.

## Results

### *The relationship between the expression of macrophage marker CD68 and CSC marker EpCAM in the marginal area of HCC*

TME plays a critical role for the maintenance and function of CSCs. TAMs constitute a major cell population in TME. In order to assess the relation of TAM and HCC–CSCs, we performed immunofluorescence staining on the paraffin section of HCC specimens. We found that presence of $CD68^+$ macrophages has a correlation with location of $EpCAM^+$ CSCs in the marginal area of tumor (Fig. 1A). As shown in Fig. 1B, when the cells with a distance of 100 μm from the edge of tumor were taken into accounted, the density of $CD68^+$ macrophages showed positive correlation with $EpCAM^+$ CSCs. Whether or not these TAMs have effects on the $EpCAM^+$ CSCs remains elusive. In addition, F4/80 was also used to identify these myeloids in tumor sections, most of the $CD68^+$ cells were $F4/80^+$ as examined by immunofluorescence staining (Fig. 1C). Our data indicate that these $CD68^+$ TAMs obviously correlated with the distribution of $EpCAM^+$ CSCs in the invasion edge of HCC.

### *TAMs enhance cancer stem cell-like properties*

To investigate the role of TAMs in the regulation of CSCs activities, the mouse hepatoma cell line Hepa1-6 cells was cocultured with TAM-CM for 24 h. Then we tested the ability of Hepa1-6 cells to form colonies in vitro. The TAM-CM-treated Hepa1-6 cells were able to form more tumor spheres, compared with control cells (Fig. 1D). Transcription regulation plays a key role in maintenance of CSC-like properties. Two CSC transcriptional factors Bmi1 and Klf4 of Hepa1-6 cells treated with TAM-CM was shown a higher levels than control. The increase in Bmi1 and Klf4 levels in Hepa1-6 cells of TAM-CM treatment was detected by real-time PCR (Fig. 1E). As shown, TAMs could promote the acquisition of CSC-like properties of Hepa1-6 cells.

### *TAMs induce EMT and promote invasive capability in Hepa1-6 cells*

Cell Immunofluorescence was performed to show the expression of EMT-related regulators. While Hepa1-6 cells normally expressed a high level of E-cadherin and an undetectable level of Vimentin, TAM-CM-treated cells shown significantly reduction E-cadherin expression and increased Vimentin expression (Fig. 2A). Western blot and RT-PCR were also used to analyze EMT markers. As shown in Fig. 2B–C, the expression of epithelial marker E-cadherin was reduced, while the mesenchymal markers N-cadherin and Vimentin was up-regulation. Meanwhile, the transition marker snail was also increased. Whether TAM-CM could promote the invasive and migratory abilities of Hepa1-6 were tested by transwell assay and wound-healing assay. Compared with control, TAM-CM-treated Hepa1-6 cells had fast closure of the wound. The result was confirmed by transwell assay (Fig. 2D–E).

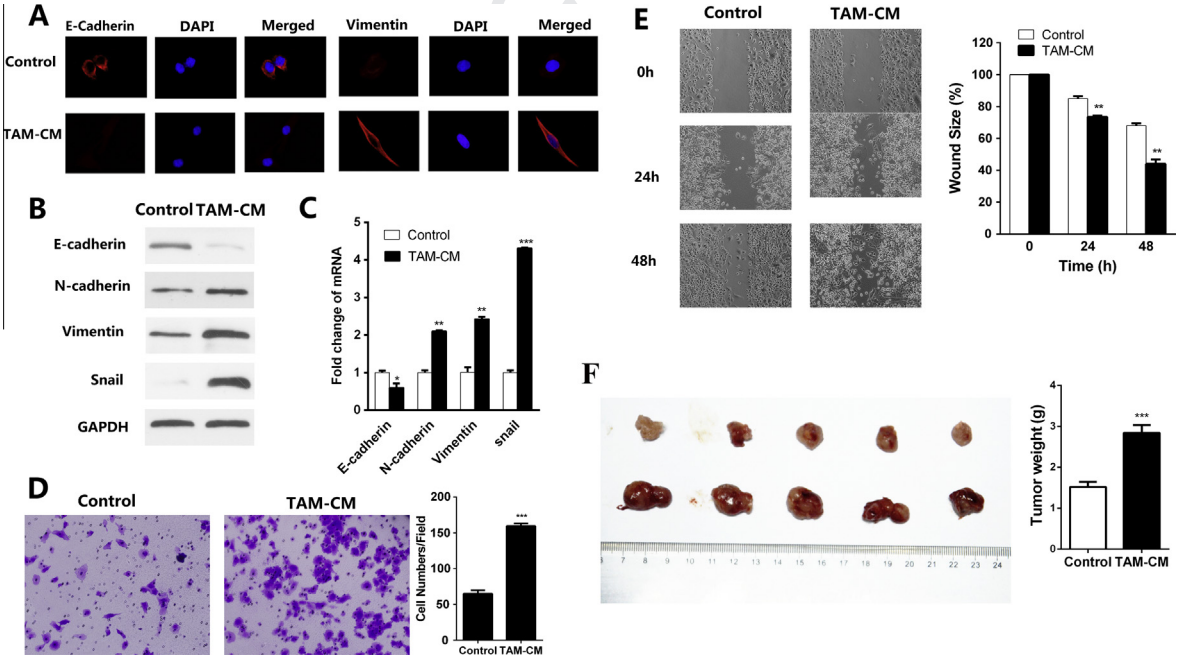


**Fig. 2.** TAM-CM promote EMT in Hepa1-6 cells. (A) Immunofluorescence staining for analysis EMT markers E-cadherin (red) and Vimentin (red) in TAM-CM treated Hepa1-6 cells and its control cells, nuclei were counterstained with DAPI. (B) Expression of EMT markers E-cadherin, N-cadherin, Vimentin and snail in TAM-CM treated Hepa1-6 cells and its control cells were analyzed by Western blotting and (C). RT-PCR. (D) Migration of TAM-CM treated Hepa1-6 cells and its control was measured by wound-healing assay (×200). (D) Invasion of TAM-CM treated Hepa1-6 cells and its control was measured by transwell (×200). (E) Migration of TAM-CM treated Hepa1-6 cells and its control was measured by wound-healing assay (×200). (F) TAM modulate tumorigenicity of Hepa1-6 cells in vivo. TAM-CM treated Hepa1-6 cells and its control were injected subcutaneously into mice to from tumors. The tumors of treatment group were larger and heavier than control $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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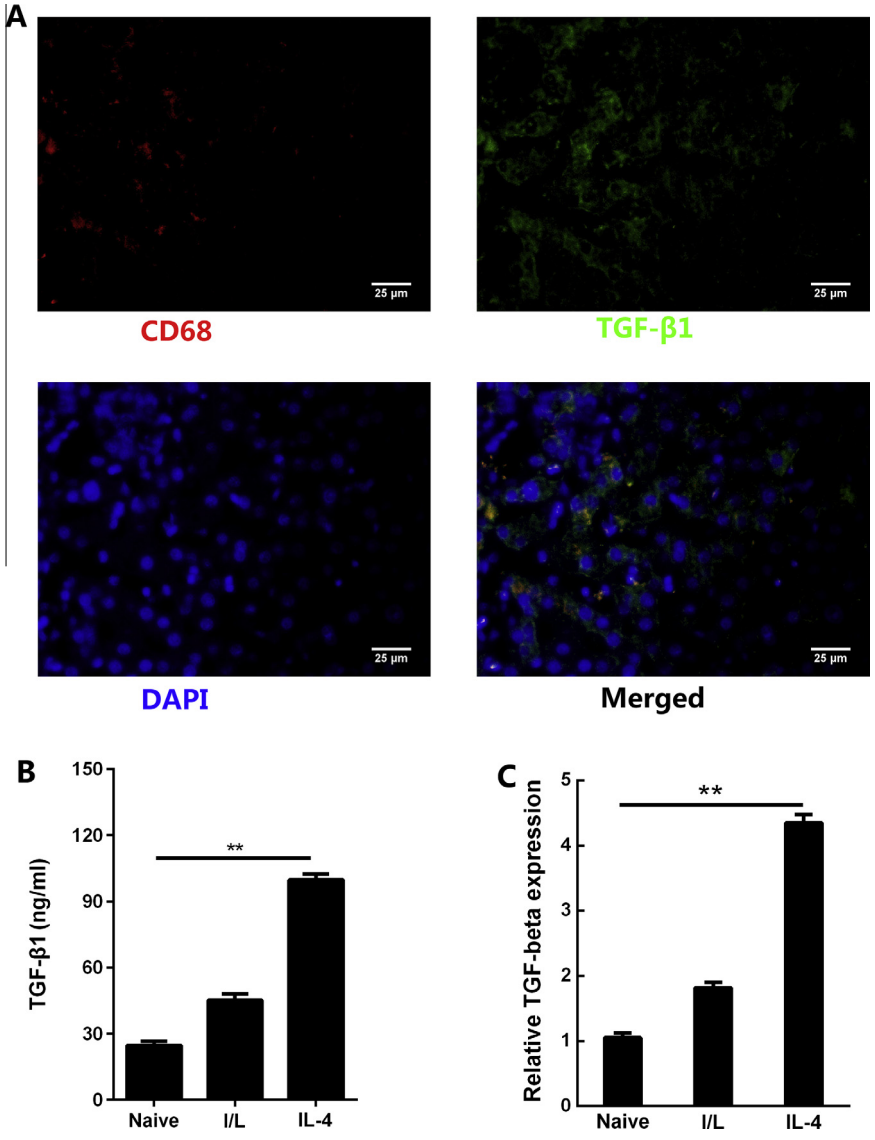


**Fig. 3.** The high expression of TGF-β1 in TAM. (A) Immunofluorescence staining, TGF-β1 (green) was detected in the extracellular matrix of CD68 + macrophages (red), nuclei were stained with DAPI (blue) (×400). (B) TGF-β1 secreted by RAW264.7 macrophages without stimulation (Naive) or stimulated with IFN-γ plus LPS (IFN-γ + LPS) or IL-4, and respectively. (C) TGF-β1 mRNA level in RAW264.7 macrophages without stimulation (Naive) or stimulated with IFN-γ plus LPS (IFN-γ + LPS) or IL-4 $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In addition, we adopted tumorigenicity experiments of BALB/c mice to evaluate whether TAM regulated hepatoma cells in vivo. Suspension of TAM-CM treated Hepa1-6 cells and unpretreated cells were injected subcutaneously into the left axilla of mice to from tumors. The effects of TAM were shown by the volume and weight of tumor. As result, the tumors of treatment group were larger and heavier than control (Fig. 2F). Accordingly, TAM has a significant promotion on the growth of tumors in mice.

In summary, TAMs mediated EMT to upregulate cancer cells stemness characteristic and promote migratory and invasive behaviors in Hepa1-6 cells in vitro and vivo.

### *TGF-β1 expression in TAMs derived from murine macrophage-like line RAW2647 cells*

TAMs exhibit an M2-like phenotype, which are characterized by high secretion of TGF-β1 and IL-10, two most important cytokines [23,25]. TGF-β1 has been previously accepted to play a major role in tumor progression and malignancy via mediating hepatocyte plasticity, EMT, secreting some proteins that act on the TME and so on [25]. A high expression of TGF-β1 was detected in the microenvironment surrounding $CD68^{+}$ TAMs in the tumor (Fig. 3A). To understand the mechanism by which TAM-CM promoted CSC-like properties of Hepa1-6 cells, TGF-β1 secreted by different phenotypes of RAW264.7 macrophages and its mRNA level was measured by ELISA and RT-PCR. 100 ng/ml LPS plus 20 ng/ml IFN-γ were used to generate classically activated M1 macrophages. In contrast, alternatively activated M2 macrophages differentiated in response to IL-4. RAW264.7 cells treated with IL-4 have a high production of TGF-β1 compared with cells treated with LPS plus IFN-γ (Fig. 3B–C). These results suggest that TAMs, derived from murine macrophage-like line RAW264.7 cells, has alternatively activated M2 phenotypes with a higher production of TGF-β1.

### *TGF-β1 neutralizing antibody inhibits the effect of TGF-β1-induced EMT on stem cell-like behavior*

Next, in order to determine whether TGF-β1 would have effects on TAM-mediated stem-like characteristic of hepatoma cells, we used TGF-β1 neutralizing antibody to deplete TGF-β1 in TAM-CM.

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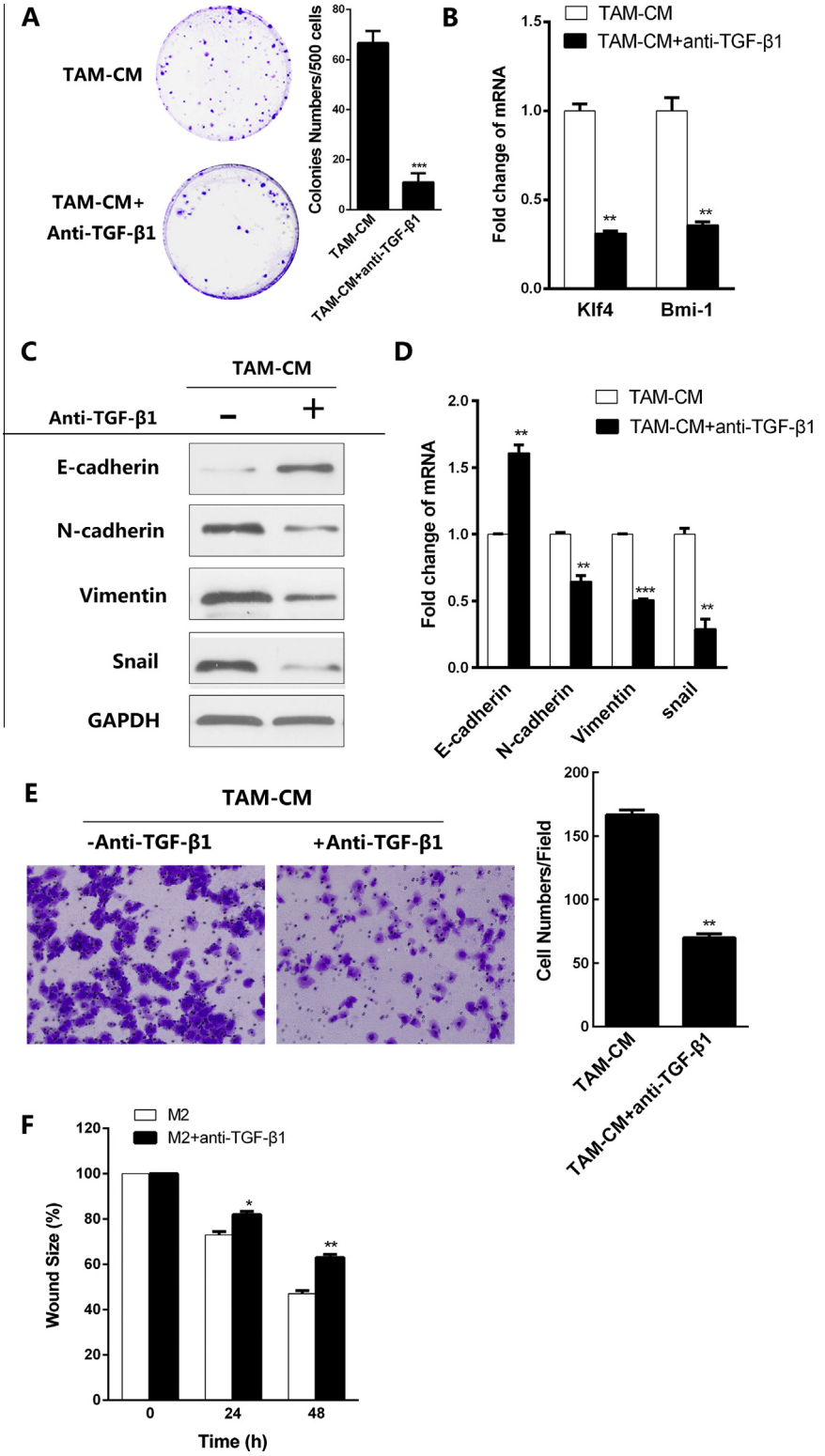


**Fig. 4.** Inhibition of TGF-β1-induced EMT block Hapa1-6 cells to acquire CSC-like properties. (A) Colony formation assay was used to quantify the number of spheres of TGF-β1 depleted TAM-CM-treated Hepa1-6 cells and TAM-CM-treated Hepa1-6 cells. (B) Real time PCR analysis of Bmi1 and Klf4 expression in Hepa1-6 cells treated by TGF-β1 depleted TAM-CM or TAM-CM. (C) Expression of EMT markers E-cadherin, N-cadherin, Vimentin and snail in TGF-β1 depleted TAM-CM-treated Hepa1-6 cells and its control cells were analyzed by Western blotting and (D) RT-PCR. (E) Invasion of TGF-β1 depleted TAM-CM treated Hepa1-6 cells and its control was measured by transwell (×200). (F) Migration of TGF-β1 depleted TAM-CM treated Hepa1-6 cells and its control was measured by wound-healing assay $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, mean ± SEM.

As shown in Fig. 4A, after applied TGF-β1 neutralizing antibody in TAM-CM, the colony formation ability of hepa1-6 cells was significantly lower than the cells treated with TAM-CM. And compared to TAM-CM treated, CSC transcriptional factors Bmi1 and Klf4 levels were also decreased in Hepa1-6 cells cocultured with TGF-β1 depleted TAM-CM (Fig. 4B). Consistently, the expression of epithelial marker E-cadherin was increased, while the mesenchymal markers N-cadherin, Vimentin and transition markers snail were decreased

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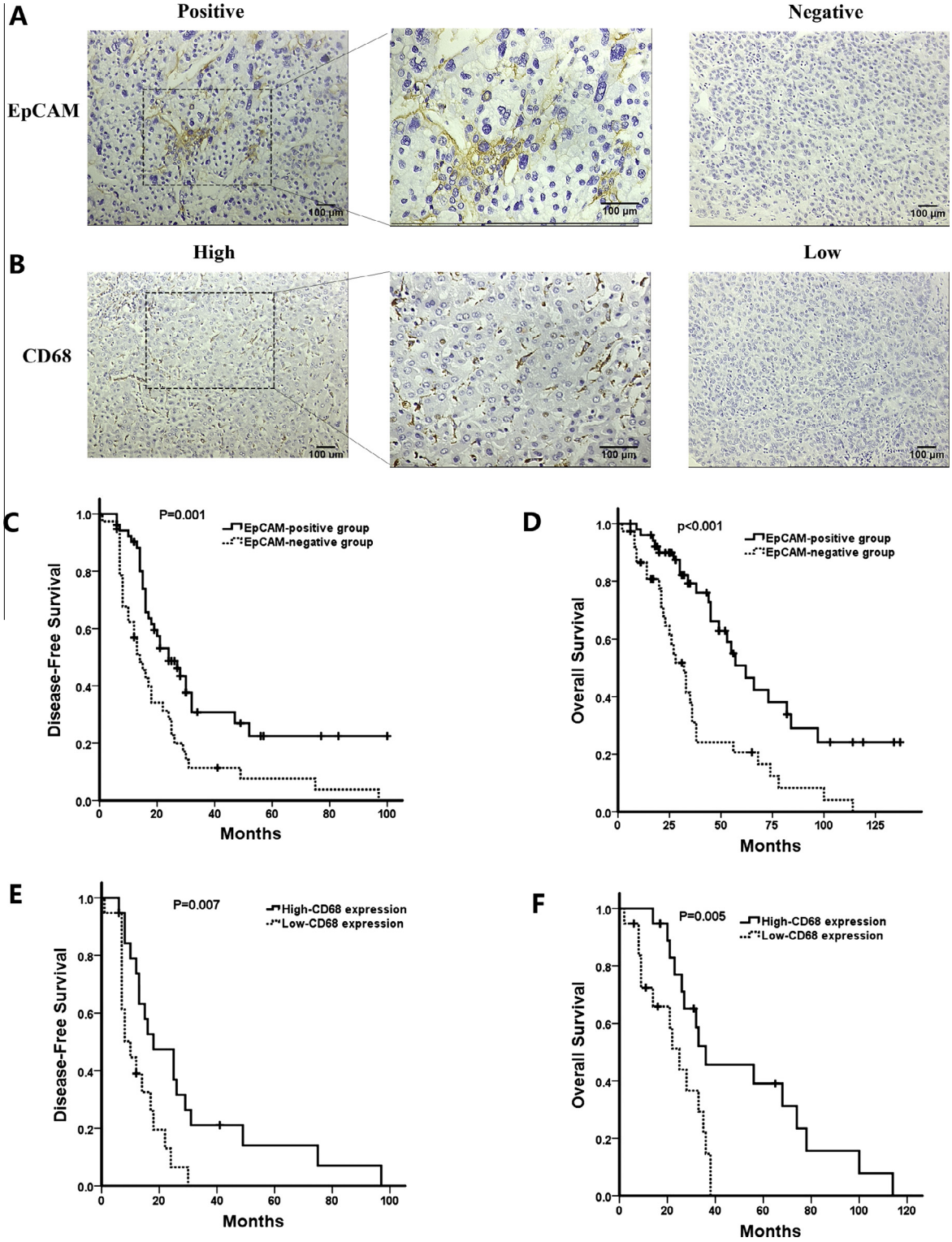


**Fig. 5.** Immunohistochemistry in HCC tissues and its prognostic implication. (A) Photographs of immunohistochemistry of epithelial cell adhesion molecule (EpCAM) in tissue microarrays, the left one showed high density of staining, the right one showed low density of staining (×200). (B) Photographs of immunohistochemistry of CD68 in tissue microarrays, the left one showed high density of staining, the right one showed low density of staining (×200). (C) Kaplan–Meier analyses of patients in the high-EpCAM expression group and in the low-EpCAM expression group. (D) In the high-EpCAM expression group, Kaplan–Meier analyses of patients in the high-CD68 expression group and in the low-CD68 expression group.

in Hepa1-6 cells cocultured with TGF-β1 depleted TAM-CM (Fig. 4C–D). The depletion of TGF-β1 in TAM-CM decreased migratory (Fig. 4F) and invasive capacities (Fig. 4E) of hepa1-6 cells in vitro.

Collectively, the results presumably suggest that depletion of TGF-β1 block the effect of TAM-induced EMT on promotion of the CSC-like properties in Hepa1-6 cells.

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*Immunohistochemistry characteristic and prognostic analyses*

Finally, in order to evaluate prognostic value of TAM in HCC patients, we collected tissue samples from 90 patients with HCC. Positive EpCAM expression was detected in 38 out of 90 HCC cases (42.2%), showing predominantly membrane and cytoplasm staining. The average levels expression of EpCAM and CD68 staining of these samples are shown in Fig. 5A and B. Our date showed that the positive expression of EpCAM were independent prognostic factors for both OS and DFS (Fig. 5C and D). These EpCAM$^+$ expression samples were equally divided in two groups according to their expression of CD68 (median of 1.31%, ranged from 0.12% to 5.4%), the median value of the density of CD68 was defined as the cutoff of subgroups. In EpCAM$^+$ samples the DFS and OS rates for patients in the low-CD68 expression group were significantly higher than the DFS and OS rates for patients in the high-CD68 expression group (Fig. 5E and F). These results demonstrated that high density of TAM would be a risk factor in EpCAM$^+$ sample of HCC. The combination of CSC and TAM density had a better power to predict patients' outcomes.

## Discussion

The critical role of the TME in modulating tumorigenesis, EMT, tumor invasion and metastasis has been widely accepted. TME is consisted of stromal cells, including carcinoma-associated fibroblasts (CAFs), mesenchymal cells, endothelial cells and various of immune cells [29]. As a main component of tumor-infiltrating leukocyte, TAMs play a decisive role in tumor progression through the expression of cytokines, chemokines, growth factors, and matrix metalloproteases [1,6]. However, a better understanding of underlying mechanism of TAMs regulate tumor initiation and development was needed.

Previous papers have reported that, tumor infiltrating macrophages in invasive margin of cancer could be useful as a prognostic marker [10,36]. In our study, by examining the HCC tissue samples, we found the density of marginal CD68$^+$ TAMs had a positive correlation with EpCAM$^+$ HCC–CSCs (Fig. 1A–C). Several evidences show that EpCAM-positive tumor cells in HCC appear to be stem-like cells and significantly shorter survival [30,33]. CD68, a highly glycosylated lysosomal membrane protein, which is expressed strongly in cytoplasmic of macrophages and monocytes, was frequently used as a marker to identify TAMs in several studies [16,41].

Several studies showed that, in the tumor microenvironment, TAMs are polarized to M2 phenotype and promote cancer cell growth, invasion, and metastasis [24]. Here we used the M2 macrophages, derived from RAW264.7, as a substitute to explore the impact of TAMs on the hepatoma cells. After incubated with TAM-CM, Hepa1-6 cells enhanced CSC-like properties. CSCs represent a cell population with biological characteristics of stemness, such as self-renewal capability, stem cell signaling pathways, generate progeny cells, resistance to chemotherapy and radiotherapy. Both Bmi1 and Klf4 are transcriptional factors which are critical for maintaining stem cell -like features and promoting cell migration and invasion [35,38]. In this study, Bmi1 and Klf4 expression in Hepa1-6 cells treated with TAM-CM were increased (Fig. 1E). And it has a higher rate of colony formation than control (Fig. 1D).

As shown in the study, Hepa1-6 cells treated by TAM-CM underwent epithelial to mesenchymal transition (EMT) (Fig. 2A–C). And its abilities of migration and invasion were increased (Fig. 2D and E). We also determined weather TAM has effect on hepatoma cells in vivo by tumorigenicity experiments of mice (Fig. 2F). EMT is a key process of the cancer cells dissemination and metastasis [5]. Moreover, as reported, cells that have undergone an EMT behave in many respects similar to cancer stem cells [21]. The loss of epithelial E-cadherin and the gain of mesenchymal N-cadherin expression is a major hallmark of EMT. N-cadherin is able to induce the mesenchymal phenotype. Vimentin is one of the type III intermediate filament protein family that is normally found in mesenchymal cells [27]. And EMT is modulated by several transcription factors, such as Snail, Twist [13,17]. These mesenchymal markers and transcription markers were upregulated in Hepa1-6 cells incubated with TAM-CM, while the expression of E-cadherin, the major component of epithelial adherens junctions [37], was downregulation. These results indicate that TAMs promote CSC-like properties of HCC by EMT.

Recent reports have stated that TAMs contribute to promote of self-renewal and maintain of CSC-like properties by several of growth and other factors [11]. TGF-β1 serves as a central regulator among inflammation and HCC [8]. Accumulating evidence has demonstrated that TGF-β1 may contribute to impaired normally differentiation of stem cells and allow for the development of cancers. It is known that TGF-β1 is considered as a potent EMT inducer in cancer. And TAMs promote tumor progression through TGF-β signaling to induce EMT in intratumoral cancer cells [2]. Immunofluorescence staining shows that CD68 was found in the area where TGF-β has a high expression in the tumor (Fig. 3A). Consistent with these finding, we detected TGF-β1 produced by different RAW264.7 macrophage phenotypes. And TGF-β1 secreted by RAW264.7 macrophages treated with IL-4 was great higher than treated with or without LPS plus IFN-γ. We can distinguish the TAMs and M1 macrophage by the level of TGF-β1 in protein and mRNA (Fig. 3B and C).

We hypothesized that TAMs has effect on the CSC-like properties and of HCC by TGF-β1 pathway. Here TGF-β1 neutralizing antibody has been used to immunodeplete TGF-β1 in TAM- CM. we showed that the upregulation of TGF-β1 on EMT was suppressed (Fig. 4C and D). Furthermore, we found that depletion of TGF-β1 block its effect on promotion of stem-like characteristics. The ability of colony formation was downregulation, and the expression of Bmi1 and Klf4 were decreased in mRNA (Fig. 4A and B). Meanwhile, the abilities of migration and invasion were inhibition (Fig. 4E and F).

In the present study, we demonstrated that in tumor tissue, the positive expression of EpCAM was associated with poor survival after resection of primary tumor (Fig. 5A, C and D), as reported previously by others. Among these EpCAM$^+$ sample, the high expression of CD68 indicated a worse outcome than those low-CD68 expression patients (Fig. 5B, E and F). In accord with our study in vitro and in vivo, TAMs would promote cancer stem cell activities maintained by TGF-β1-induced EMT. Therefore, we propose that the TME is important in understanding the mechanism of recurrence and metastasis of HCC.

Collectively, stem-like properties is characteristic of tumor cells to migration, invasion and form new tumor in distant area. Our study demonstrated that TAMs regulate the essential characteristic of tumor cells through TGF-β1 signaling pathway. M1 phenotype are tumoricidal, which play an important role anti-tumor responses of immune system [24]. To explore effective methods to inhibit tumor-infiltrating macrophages polarized into M2 phenotype, even promote it into anti-tumor M1, may new countermeasures to improve HCC outcome. Meanwhile, the therapeutic strategies against TGF-β1 pathway are also valuable to be development for HCC Therapy.

## Conflict of Interest

The authors have declared that no competing interests exist.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2014.05.008.

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