



Effect of hematopoietic progenitor cells on the biological characteristics of colon cancer tumor stem cells

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Background: Cancer stem cells (CSCs) are a type of tumor cell that are self-sustaining and can differentiate into several different types of cells. The present study aims to investigate the effect of hematopoietic progenitor cells (HPCs) on the biological behavior of colon CSCs (CCSCs) and to determine the underlying molecular mechanisms of liver metastasis in colorectal cancer.

Methods: Subsets of CCSCs were isolated from stem-like HCT116 cells and cocultured with CD133⁺ HPCs *in vitro*. Colony formation assay, and CCK-8 were used to assess the effects of HPCs on CCSC subsets. Invasive and migration assay were done to study the effects of HPCs mediated metastatic capacity of CCSC subsets. Expression of MMP-9, VEGF, E-cadherin and β -catenin was analyzed by qPCR and Western blotting.

Results: CCK-8 and colony formation assays showed that HPCs significantly promoted proliferation and colony formation of the CCSC subsets ($P=0.031$). HPCs also significantly enhanced the migration ($P=0.011$) and invasive capacity ($P=0.001$) of the CCSC subsets. Quantitative PCR showed that MMP-9 and VEGF expression in CCSC subsets were significantly upregulated ($P=0.000$ and $P=0.005$). Western blotting showed that MMP-9, VEGF and β -catenin expression in CCSC subsets were significantly upregulated ($P=0.000$, $P=0.005$, $P=0.000$). The protein expression levels of E-cadherin in the CCSC subsets was significantly downregulated ($P=0.002$).

Conclusions: CD133⁺ HPCs enhanced migration, invasion and proliferation of CCSC subsets *in vitro*.

Keywords: Liver metastases of colorectal cancer; colon cancer stem cell (CCSC); hematopoietic progenitor cells (HPCs); serum-free medium

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the world, and liver metastasis is the most common form of CRC metastasis (1). Studies have shown that liver metastasis can occur in ~20% of newly diagnosed CRC patients within half a year (2,3). In addition, >50% of patients with advanced CRC have a median survival

of only 21.3 months (4). Currently, the “seed and soil” theory proposed by British surgeon Dr. Stephen Paget is generally accepted as the underlying developmental mechanism of colorectal cancer liver metastases (CRLM) (5). On this basis, the “cancer stem cell theory” has been expanded in recent years (6). Cancer stem cells (CSCs) are a group of tumor cells that are self-sustaining and possess the ability to differentiate into different types of cells. CSCs are the source of the

initiation and maintenance of tumor growth and serve a decisive role in tumor recurrence and metastasis (7).

CSC function may be induced by specific microenvironmental signals and cellular interactions in the tumor niche (8). CSCs were considered as “seed”, meanwhile the microenvironment of the target organ were “soil”. Based on this theory, it was shown that primary tumor cells secrete specific cytokines prior to metastasis to mobilize a group of myeloid precursor cells into the circulatory system, which are then recruited into the tissues of the target organ to remodel the premetastatic site microenvironment. This group of cells serve an important role in the formation of a microenvironment suitable for the growth of tumor cells in the premetastatic target organs (9,10). Kaplan *et al.* identified this group of hematopoietic progenitor cells (HPCs) by using mouse animal models that express molecular markers, such as VEGFR1, CD133, CD11b and scal-1 (11). It was reported that HPCs could facilitate colorectal cancer progression (12). However, few studies explore the contribution of colon cancer stem cells (CCSCs) on this phenomenon and the underlying mechanism is still undefined.

The aim of the present study was to investigate the effect of HPCs on the biological behavior of CCSCs and to examine the molecular mechanisms of metastasis in CCSCs. The results may assist in the identification of novel molecular targets for the treatment of metastatic colorectal cancer. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-2810>).

Methods

Cell lines and reagents

The human colon cancer cell line HCT116, was gifted by the Institute of Oncology, Fujian Provincial Cancer Hospital. HPCs and monocytes cryopreservation solution was obtained from iXCells Biotechnologies, Inc. The CD133⁺ HPC population was verified by flow cytometry. Human basic fibroblast growth factor (bFGF) and human epidermal growth factor (EGF) were obtained from PeproTech, Inc. B-27 supplement (50×) was obtained from Gibco; Thermo Fisher Scientific, Inc. Anti-human CD133-PE were purchased from (R&D Systems, Inc.); anti-human CD24-fluorescein isothiocyanate (FITC) was obtained from Becton, Dickinson and Company; and fluorescent secondary antibody was obtained from OriGene Technologies, Inc.

Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. Transwell chambers were obtained from EMD Millipore. HiFi-mmlv cDNA kit and Ultra SYBR mixture were purchased from KW-BIO. The bicinchoninic protein assay (BCA) kit was obtained from Beyotime Institute of Biotechnology. Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Hyclone, Inc. Goat serum sealant (ZLI-9022) were purchased from ZSGB-BIO, Inc. ACCUTASE cell digestive juice were purchased from Yeasen Biotech Co., Ltd., Shanghai.

CCSC culture and detection

The HCT116 cell line was subcultured in RPMI 1640 medium containing 10% FBS; cells were obtained during the logarithmic growth phase and inoculated in serum-free medium (SFM) supplemented with 10 ng/mL bFGF, 20 ng/mL EGF and 2% B27 in Gibco Dulbecco's Modified Eagle Medium: F-12 (DMEM/F-12) (12). In SFM, the differentiated cells in HCT116 cells were apoptotic because they could not stick to the wall for growth. However, the subpopulations of CSCs with stem cell potential could survive sustainably and grow in stem cell globulin suspension (13,14). bFGF, EGF and B27 growth factors were added to SFM at specific concentrations, in which EGF and bFGF could significantly promote the proliferation of CCSCs (15). B27 can replace serum as the basic nutrient needed to stimulate cell growth without promoting differentiation of CCSCs. Previous literature has also reported that serum-free culture and separation method is suitable for CCSCs separation (16,17). HCT116 colon cancer cells were suspended in SFM as single cells and allowed to proliferate in the form of a “bud”. After 3–7 days, they gradually formed oblate-like cell spheres with a dense structure and regular edges in microscope. After 10 days, we obtained the CCSCs spheres. After three generations of continuous passages at a density of 1×10^4 /mL, tumor cell spheres in a healthy growth state were taken for subsequent experiments at a density of 1×10^6 /mL. Mouse anti-human monoclonal antibody, CD133-phycoerythrin (PE) was added to the experimental group, and IgG was used to treat the control group. The two groups were incubated at room temperature in the dark for 60 min. After washing with 3% FBS-PBS twice, the number of CD133⁺ cells was analyzed by flow cytometry (FC500, Beckman, Inc., Fullerton, CA, USA). The number of CD24⁺ cells in both groups were also assessed using the same method with the CD24 specific antibody.

Immunofluorescence

The HCT116 cell spheres were grown for 7–10 days, and were then cultured using the hanging drop method in normal goat serum sealant and sealed at room temperature for 30 min. The primary anti-CD133 antibody was added to each well at 1:50, incubated overnight at 4 °C in the dark, and subsequently washed twice with PBS buffer. The secondary fluorescent antibody was added to each well at 1:100, incubated in dark for 60 min at room temperature. The intensity of red fluorescence after CD133-PE labeling was observed under a fluorescence microscope. The fluorescence intensity of CD24 antibody markers in tumor cells was detected using the same method except with an anti-CD24 primary antibody instead.

CCK-8 assay

A single-cell suspension of the HCT116 cell spheres were prepared, adjusted to a cell density of 5×10^3 /mL, 200 μ L per well, plated in a 96-well plate for 12–24 h and cultured. In the experimental group, CD133⁺ HPCs were added 50 cells to each well, and the same number of tumor cells were added in the control group. In the blank control group, an equivalent volume of PBS was added. Subsequently, cells were cultured for 48 h, and 20 μ L CCK-8 reagent was added to each well. After 2 h of culturing in a 37 °C incubator, the absorbance value of each well was measured at 450 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Inc.).

Colony formation assays

Single-cell suspensions of CCSCs and CD133⁺ HPCs were cocultured *in vitro* for 48 h at a ratio of 20:1, and the control CCSCs were established. Subsequently, 400 cells per well were inoculated into 6-well plates and cultured for 10–14 days. When the colonies were visible, crystal violet dye was immediately used for staining for 15 min at room temperature. Cells were air dried, and the number of colonies formed were counted by eye (were ≥ 50 cells constituted a colony).

Transwell migration and invasion assays

Single-cell suspensions of the CCSC and CD133⁺ HPC coculture as well as the control CCSCs were resuspended in 0.1% BSA, and the cell density was adjusted to

1.5×10^5 cells/mL. In the Transwell chambers (8.0 μ m aperture), 100 μ L cell suspension was added to the upper chamber, and 600 μ L RPMI 1640 supplemented with 20% FBS was added to the lower compartment. After 48 h, the cells were taken out, stained using crystal violet dye for 30 min at room temperature, and subsequently dried at room temperature. The number of cells in five randomly selected fields of view were counted using an inverted high-power microscope (200 \times) (TE200, NIKON), and the mean values were calculated. To assess invasion, a similar procedure was performed, except the Transwell chambers were first coated with Matrigel (BD Biosciences).

Reverse transcription-quantitative (RT-qPCR)

RT-qPCR was used to detect the mRNA expression levels of *MMP-9* and *VEGF* in the two groups of tumor cells as indicators of invasion and metastasis. TRIpure reagent (Roche) was used to extract total RNA. The first strand cDNA from 1 μ g of total RNA was synthesized using HiFi-mmlv cDNA kit which was carried out at 42 °C for 40 min and 85 °C for 5 min. Real-time fluorescent quantitative PCR was used to detect the quantity of RNA, and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method (18): ΔCt value is the difference between the Ct value of *GAPDH* gene and the Ct value of the target gene [$\Delta Ct = Ct(\text{target gene}) - Ct(\text{GAPDH})$]. The $\Delta\Delta Ct$ values is the difference in the Ct value between the experimental group and the control group [$\Delta\Delta Ct = \Delta Ct(\text{experimental group}) - \Delta Ct(\text{control group})$].

A two-step RT-PCR kit was used for RT-PCR, and PCR amplification. PCR amplification of *MMP-9* and *VEGF* was performed according to the protocol of the RT-PCR kit. The relative expression of genes was normalized to *GAPDH*. The primer sequences were shown in Table 1.

The thermocycling conditions for PCR were as follows: predenaturation at 95 °C for 10 min; followed by 40 cycles of 15 s at 95 °C, 20 s at 60 °C and 25 s at 72 °C; with a final extension step of 72 °C for 5 min. Temperature was then ramped from 65 to 95 °C to plot the melting curve. The differences in the mRNA expression levels of *MMP-9* and *VEGF* genes in the two groups of tumor cells were observed and evaluated.

Western blot analysis

Western blotting analysis was used to detect the protein expression levels of *MMP-9*, *VEGF*, *E-cadherin* and β -*catenin*

Table 1 The primer sequences of *MMP-9*, *VEGF*, and *GAPDH*

Gene	Primer
<i>MMP-9</i>	Forward: 5'-CTGAAGGCCATGCGAACCCCA-3'
	Reverse: 5'-GCAAAGGCGTCGTCAATCACC-3'
<i>VEGF</i>	Forward: 5'-ACTTCTGGGCTGTTCTCGCTT-3'
	Reverse: 5'-GCCGCCTCACCCGTCCATG-3'
<i>GAPDH</i>	Forward: 5'-TGGTATCGTGAAGGACT-3'
	Reverse: 5'-AGGGATGATGTTCTGGAGA-3'

in the two groups of tumor cells related to tumor invasion and metastasis. The two groups of cells were harvested by scraping the cells in ice-cold PBS and using RIPA lysis buffer to lyse the cells. The protein concentration was measured using a BCA kit. The lysed protein sample was loaded on an SDS-gel composed of a 10% resolving SDS-gel and a 5% loading SDS-gel. The proteins were resolved by electrophoresis at 80 V until the loading buffer had entered the resolving gel, and then the gel was run at 100 V until the bromophenol blue reached the bottom of the gel. Subsequently, the resolved proteins were transferred to a PVDF membrane which had been activated in methanol. After transfer, the membrane was cut according to the marker and location of the target band. The membranes were blocked for 1 h in Tris Buffer Saline Tween containing 5% milk powder. The membranes were then incubated overnight at 4 °C with the primary antibody (1:1,000). The membranes were washed 3 times with Tris Buffer Saline Tween and incubated with the secondary antibody (1:500) for 1h at room temperature. The membrane was allowed to dry, and signals were visualized using enhanced chemiluminescence.

Statistical analysis

All experiments were repeated at least three times and the data are presented as the mean \pm the standard deviation. Datasets with only two groups were analyzed using an independent sample *t*-test. SPSS version 24.0 (IBM, Corp.) was used for data analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CD133⁺ and CD24⁺ cells are significantly increased in the CCSC subsets

HCT116 colon cancer cells were suspended in SFM as single cells and allowed to proliferate in the form of a "bud". After

3–7 days, they gradually formed oblate-like cell spheres with a dense structure and regular edges (Figure 1A). After 7–10 days, cells were digested and passed by ACCUTASE cell digestive juice in preparation for the CCSC subsets. The results of the flow cytometry showed that the counts of the CD133⁺ and CD24⁺ cells were significantly higher compared with the monolayer of cells in serum supplemented medium (SSM) ($P = 0.000$; $P = 0.001$, $P < 0.05$; Figure 1B).

Immunofluorescence analysis showed that CD133-PE- and CD24-FITC-labeled fluorescence was visible on the surface of SFM cultured cells (Figure 1C).

Proliferation of CCSC subsets is promoted by HPCs

The proliferation of the two groups of cells was assessed using a CCK-8 assay at five different time points, and a growth curve was plotted. The results showed that the proliferation of CCSC subsets in the experimental group was significantly higher at the different time points (day 1 to day 5) compared with the control group ($P < 0.05$) (Figure 2A).

Colony formation in the CCSC subsets is enhanced by HPCs

In both groups, the CCSC subsets of tumor cells formed colonies in the 6-well plates, and colony formation in the CCSC subsets in the experimental group was significantly higher than that of the control group ($P = 0.031$) (Figure 2B,C).

Migration and invasion of CCSC subsets is enhanced following coculture with HPCs

In both groups, the CCSC subsets passed through the polycarbonate membrane in the Transwell chamber, and the number of CCSC subsets passing through the polycarbonate membrane in the coculture group was significantly higher than the monoculture group ($P = 0.011$) (Figure 3A,B). Both groups of tumor cells were able to penetrate the polycarbonate membrane and Matrigel. The number of CCSC subsets passing through the polycarbonate membrane and Matrigel in the coculture group was significantly higher compared with the monoculture group ($P = 0.001$) (Figure 3C,D).

MMP-9, VEGF, E-cadherin and β -catenin expression in CCSC subsets is significantly increased after coculture with HPCs

The qPCR results showed that there was a significant increase in the mRNA expression levels of *MMP-9* and *VEGF* in

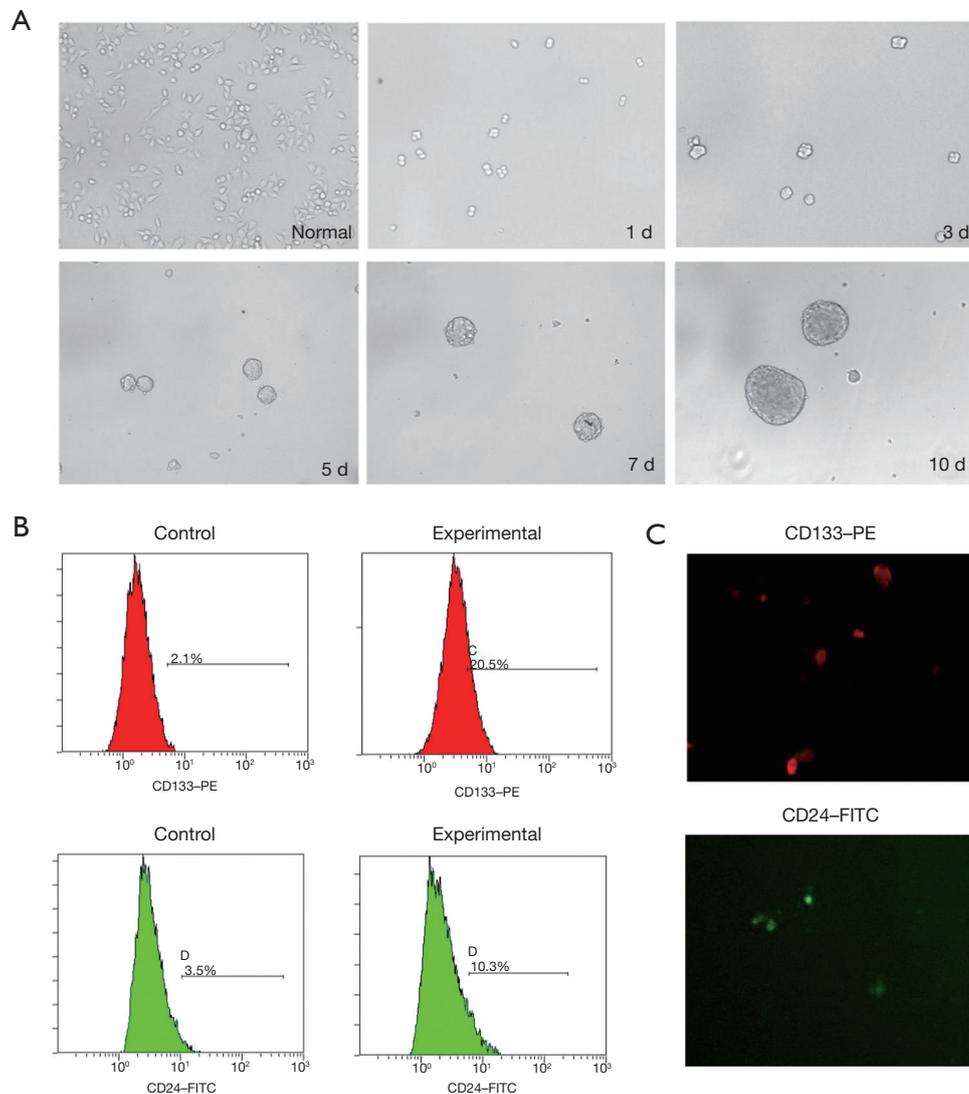


Figure 1 The formation of the CCSC subsets and expression of CD133⁺/CD24⁺ cell in it. (A) Normal: monolayer cell morphology in SSM; 1–10 days. The formation of tumor cell spheres in SFM. (B) Ratio of CD133⁺ (P=0.000) and CD24⁺ (P=0.001) cells in the two groups significantly differed. (C) Immunofluorescence analysis of tumor cell spheres in SFM for CD133 and CD24. Magnification, ×100. Experiments were independently repeated at least 3 times. CCSC, colon cancer stem cell; SSM, serum supplemented medium; SFM, serum free media.

the CCSC cocultured cells compared with the monoculture (*MMP-9*, P=0.000; *VEGF*, P=0.005) (Figure 4A). After coculture with HPCs, the protein expression levels of *MMP-9*, *VEGF*, *E-cadherin* and β -*catenin* in the CCSC subgroup cells of the experimental group were detected by Western blotting. The results showed that the protein expression levels of *MMP-9*, *VEGF* and β -*catenin* in the CCSC subgroup cells of the experimental group was significantly higher compared with the control group (*MMP-9*, P=0.000; *VEGF*, P=0.005; β -*catenin*,

P=0.000) (Figure 4B). The results showed that the protein expression levels of *E-cadherin* in the CCSC subgroup cells of the experimental group was significantly lower compared with the control group (*E-cadherin* P=0.002) (Figure 4B).

Discussion

As early as 1998, Luzzi *et al.* showed that only ~0.02% of tumor cells “escaping” from the primary site could

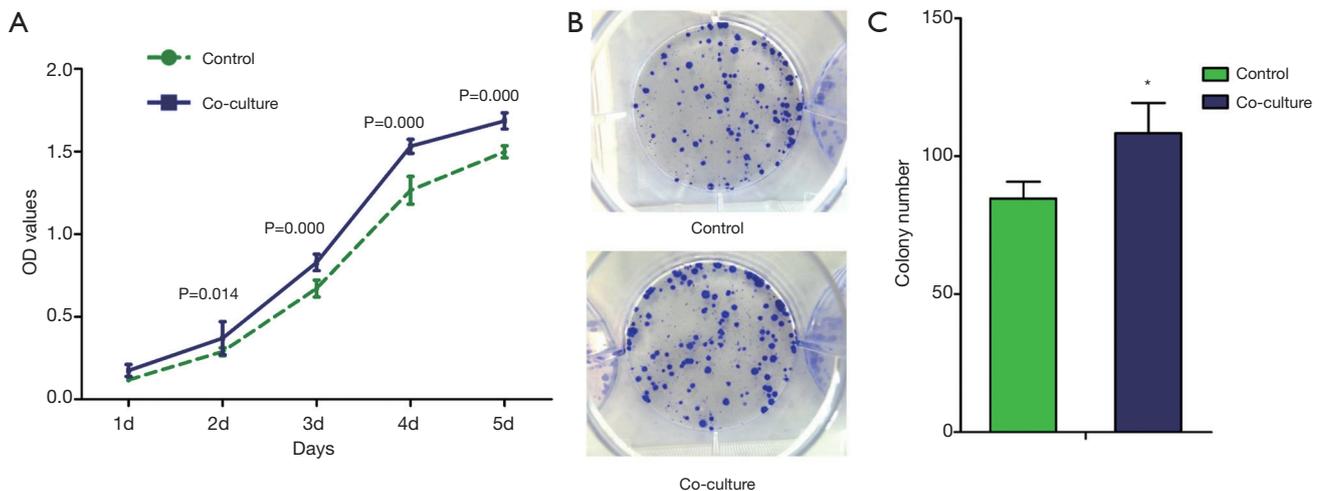


Figure 2 Proliferation and colony formation of CCSC subsets cocultured with HPCs. (A) Proliferative capacity of CCSC subsets in the two groups. (B) Number of colonies of CCSC subsets in the two groups. (C) Colony formation analysis of the CCSC subsets in the coculture group was significantly higher compared with the control group ($P=0.031$). *, $P<0.05$. Experiments were independently repeated at least three times. CCSC, colon cancer stem cell; HPCs, hematopoietic progenitor cells.

successfully reach the premetastatic target organ site (19); this shows that distant metastasis of tumor cells is extremely inefficient. However, in 2005, VEGFR1⁺ HPCs were observed forming clusters in specific target organs, and that they created the contours which eventually developed into metastatic sites by forming the premetastatic niche where the tumor cells in the circulatory system could settle, survive, divide and proliferate (11). Therefore, tumor stem cells and HPCs serve an important role in the formation of tumor metastasis. Successive studies have confirmed the existence of this phenomenon in the metastasis of multiple types of tumor, such as colorectal cancer, lung cancer and esophageal cancer, amongst others (20,21). With regards to distant metastasis of colorectal cancer, the liver is the most common site of distant metastasis, which is observed in about 15–25% of CRC patients at the first diagnosis (22).

Do HPCs promote liver metastasis of colon cancer? How do HPCs affect the biological behavior of CCSCs? To address these questions, CCK-8 and colony formation assays were used, and the results showed that HPCs significantly promoted the proliferation and colony formation capacity of CCSC subsets *in vitro* at different time periods when cocultured. When tumor metastases reach the premetastatic site to form tumor micrometastases, the tumor cells are required to invade through the basement membrane of blood vessels, which is dependent on chemotaxis. In the present study, a Transwell assay

was used to assess the migratory and invasive capacity of CCSC subsets in the coculture environment, and the results showed that both migration and invasion was significantly enhanced compared with the tumor cells cultured as a monoculture. Therefore, it is hypothesized that HPCs may enhance the ability of CCSC subsets to invade the basement membrane by secreting certain cytokines, thus promoting tumor metastasis. Zhang *et al.* also showed the presence of HPC aggregates in colorectal cancer, and demonstrated the enhancing effect of CD133⁺ HPCs on tumor metastasis (12).

The degradation of the extracellular matrix is a key process required for tumor cell metastasis (23). MMP-9 is a zinc-dependent peptide chain endonuclease with the ability to degrade the extracellular matrix, and a key factor involved in tumor invasion and metastasis (24–26). MMP-9 is secreted by tumor cells and various immune cells in the stroma. The products formed by MMP-9 activation can decompose the majority of substances present in the basement membrane and extracellular matrix (27). In addition, angiogenesis is increased by the presence of VEGF in tumors (28). Together, the combined function of MMP-9 and VEGF promote tumor invasion and metastasis (29). In the coculture, HPCs significantly upregulated the expression of MMP-9 at the mRNA and protein expression levels in the CCSC subsets. Therefore, it is hypothesized that the local microenvironment formed by the interactions between HPCs and premetastatic tissues results in recruitment of distant CCSC subsets,

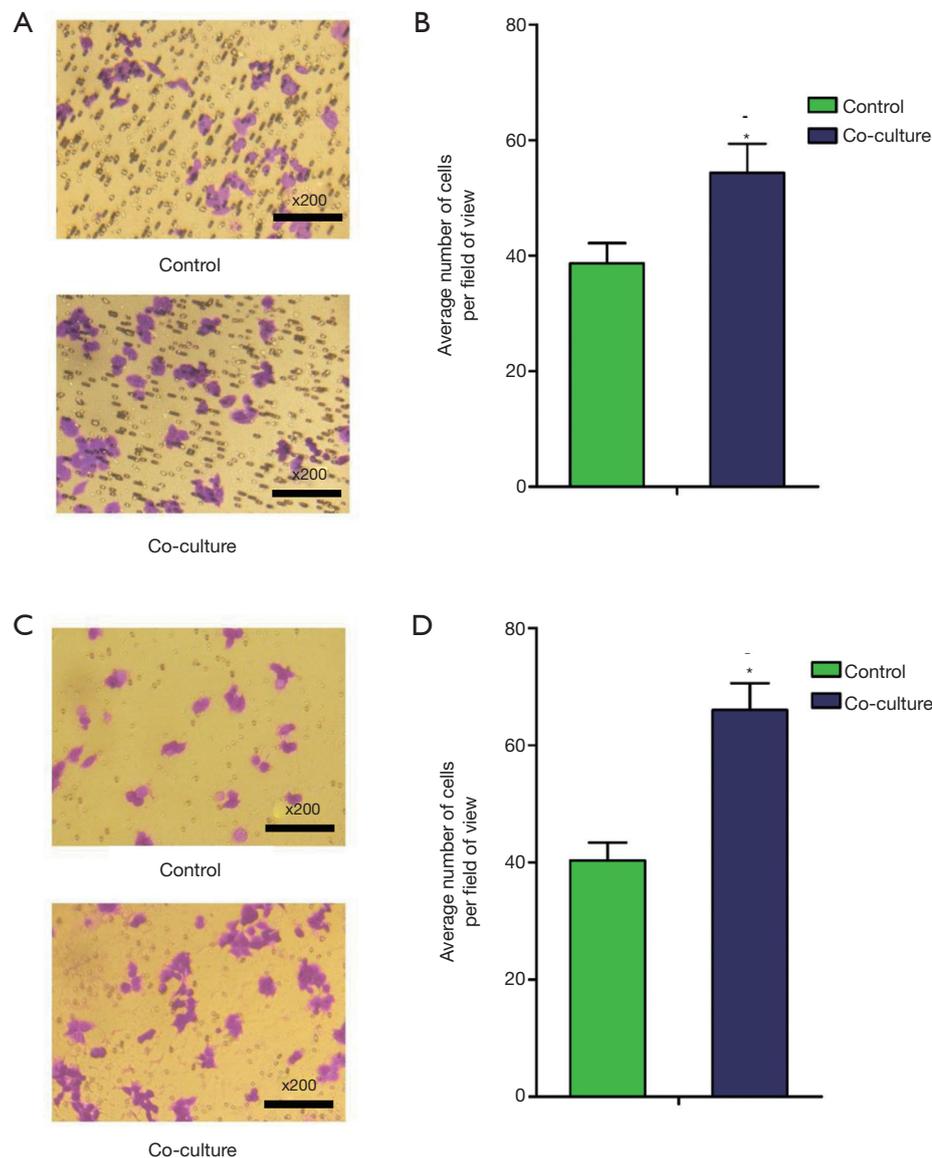


Figure 3 Migration and invasion of CCSC subsets cocultured with HPCs. (A) Cell counts in the two groups under an inverted high-power microscope. (B) CCSC subsets passing through the polycarbonate membrane in the coculture group was significantly higher compared with the control group ($P=0.011$). (C) Cell counts in the two groups counted using an inverted high-power microscope. (D) Cell counts of CCSC subsets passing through the polycarbonate membrane and Matrigel in the coculture group was significantly increased compared with the control group ($P=0.001$). Crystal violet dye, Magnification, $\times 200$. *, $P < 0.05$. Experiments were independently repeated at least three times. CCSC, colon cancer stem cell; HPCs, hematopoietic progenitor cells.

which promotes the secretion of MMP-9 by tumor cells to accelerate the process of tumor cell metastasis.

Honguero Martínez *et al.* showed that the invasion, growth and metastasis of primary tumors also requires an abundant blood supply (30). Based on the current body of knowledge, VEGF is the most important angiogenic

stimulating factor (31). VEGF primarily promotes tumor metastasis in two ways: it specifically binds and stimulates the proliferation of vascular endothelial cells to induce angiogenesis; and enhances a series of specific signaling pathways which increase the permeability of microvessels, making it easier for tumor cells to penetrate the vascular

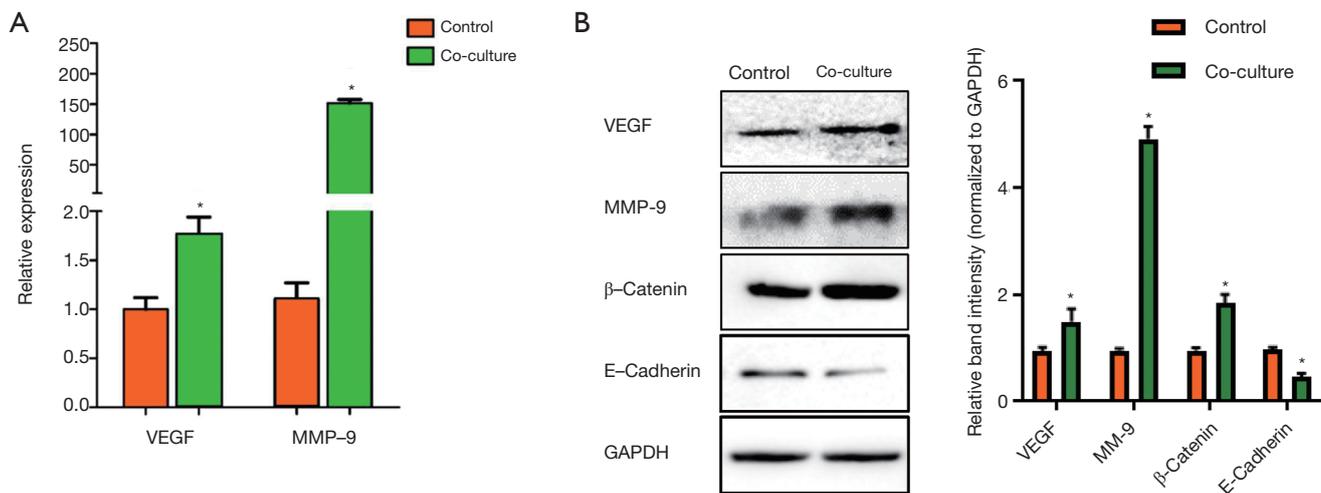


Figure 4 *MMP-9*, *VEGF*, *E-cadherin* and β -*catenin* expression in CCSC subsets by HPCs. (A) mRNA expression levels of *MMP-9* ($P=0.000$) and *VEGF* ($P=0.005$) in the cocultured CCSC subsets was significantly increased compared with the control group. (B) Protein expression levels of *MMP-9* ($P=0.000$), *VEGF* ($P=0.005$) and β -*catenin* ($P=0.000$) in the cocultured CCSC subsets was significantly increased compared with the control group. Protein expression levels of *E-cadherin* ($P=0.002$) in the cocultured CCSC subsets was significantly lower compared with the control group. *, $P<0.05$. Experiments were independently repeated at least three times. CCSC, colon cancer stem cell; HPCs, hematopoietic progenitor cells.

wall and enter the circulatory system (32). Zheng *et al.* confirmed that VEGF mRNA expression is upregulated in various types of malignant tumors, and its expression is positively correlated with tumor metastasis (33). In the present study, the mRNA and protein expression levels of VEGF in CCSC subgroup cells were significantly upregulated in the cocultured cells. Thus, it is hypothesized that when tumor cells in the circulatory system reach the target organ, the HPCs send specific stimulation signals to promote CCSC subsets to secrete VEGF, promoting the occurrence of neoplastic angiogenesis, and thus facilitating secondary metastasis.

It is well known that epithelial-mesenchymal transition (EMT) play an important role in colon cancer metastatic progression (34). Firstly, Epithelial cells lose the junctions and apical-basal polarity, then re-arrange the cytoskeletons. This procession up-regulates the motility of individual cells and promotes them to leave the tissue parenchyma, which facilitates the cancer metastasis (34). The hallmarks of EMT include the reduced expression of E-cadherin, cytokeratins and tight junction proteins, in which E-cadherin has a critical role in the whole procession (35). Furthermore, a variety of signaling pathways are associated with the activation of EMT, including Wnt/ β -catenin, tumor growth

factor- β , RTK/Ras pathways and others (36). The Wnt/ β -catenin signaling pathway has been revealed to have important roles in the promotion of EMT in colon cancer metastasis. β -catenin promotes the EMT by binding to the TCF/LEF transcription factors to trans-activate target genes. In our study, we found that HPCs significantly increase the β -catenin expression levels in CCSC subsets. Moreover, E-cadherin expression was remarkably decreased in CCSC subsets. Thus, our results showed that HPCs might promote CCSC metastasis partly by facilitating the EMT procession though increasing β -catenin expression.

Conclusions

In conclusion, through the HPC and CCSC subsets that were studied in an *in vitro* coculture, we showed that HPCs enhanced the biological properties of CCSCs, including their migratory, invasive and proliferative capacity. The underlying mechanism may be the activation of specific signaling pathways that increases the secretion of specific chemokines, cytokines and protein enzymes. In the present study, HCT116 colon cancer cells were used to study the effect of HPCs on metastasis of colon cancer cells.

Similarly, to examine the effect of HPCs on breast cancer metastasis, Meng *et al.* determined the metastatic ability of MDA-MB-435s-hm cells co-cultured with HPC (37). Although the specific pathways and mechanism of action are not fully understood, it was shown that tumor metastasis was related both to the characteristics of tumor cells, and also the premetastatic microenvironment in which HPCs are involved in generating. Therefore, further research on the mechanisms related to the establishment of a suitable premetastatic microenvironment for colorectal cancer cells may result in the development of novel methods for the treatment of liver metastasis of colorectal cancer, and the development of a series of targeted drugs, in turn, improving the outcomes of CRLM patients.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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