CCL5 secreted from bone marrow stromal cells stimulates the migration and invasion of Huh7 hepatocellular carcinoma cells via the PI3K-Akt pathway

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Abstract. Bone metastases from hepatocellular carcinoma (HCC) seem to be increasing. Previous studies showed that soluble factors secreted by host cells and direct cell-to-cell interactions contributed to the preferential metastasis and growth of cancer cells in bone, while the underlying mechanism(s) of the metastasis of HCC in the bone are poorly understood. Here, we determined the effect of HS-5 cells on Huh7 cell proliferation, and investigated the role of CCL5 from HS-5 cells on the development of Huh7 cells. In addition, the underlying mechanisms on the influence in Huh7 cells were investigated. Our results showed that HS-5 cells could promote the proliferation, migration and invasion of Huh7 cells, and inhibited apoptosis. CCL5 downregulation was able to inhibit the effects of HS-5 cells on Huh7 cell migration and invasion via the PI3K-Akt signaling pathway and reduce MMP-2 expression. Therefore, these findings suggest that CCL5 secreted from MSCs can promote the migration and invasion of Huh7 cells and could be an important factor in HCC related to occurrence of bone metastases.

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy, the third cause of cancer-related mortality worldwide (1). Frequent intrahepatic and extrahepatic metastases are the main factors contributing to the high mortality of HCC patients (2). Previously, bone metastases were considered less common in patients with HCC. However, due to the improved duration of intrahepatic primary tumors, bone metastases from HCC seem to be increasing and frequently recorded (3,4). Thus, early diagnosis of bone metastasis plays a pivotal role in the therapeutic regimen and the assessing prognosis (5). It is generally considered that cancer is a dynamic exchange between tumor cells and surrounding host cells, as first proposed in 1889 by Stephen Paget who indicated that the seeding of metastatic cancer cells depended on the host organ microenvironment (the 'seed and soil' concept) (6). To our knowledge, soluble factors secreted by host cells and direct cell-to-cell interactions are deemed to contribute to the preferential metastasis and growth of cancer cells in bone (7,8), however, the underlying mechanism of metastasis of HCC in the bone is poorly understood.

Marrow/mesenchymal stromal cells (MSCs) play a major role of tumor stroma in bone microenvironment and have an effect on growth and metastasis of human malignancies. However, the exact effect of MSCs on tumor growth and progress is still under debate (9). The contradiction exists in some cancer cells, including melanoma (10,11), breast cancer (12), colon cancer (13), Kaposi's sarcoma (14), and prostate cancer (15). In HCC, some studies demonstrated that MSCs contributed to tumor progression (16-18), several other studies demonstrated that MSCs could inhibit metastasis (19-21) and tumorigenesis (22). MSCs in HCC metastasis remains controversial. MSCs secrete various cytokines that have both paracrine and autocrine functions, besides MSCs are able to generate a direct effect through intercellular signaling via physical contact with tumor cells (23).

CCL5 (also known as regulated upon activation, normally T cell expressed and secreted, or RANTES) belongs to the CC family of inflammatory chemokines and is expressed by many liver and infiltrating cells (24), and interacts with the G-protein coupled receptors CCR1, CCR3 and CCR5. CCL5 is also secreted from tumor or stromal cells, and may act in an autocrine or a paracrine manner on cancer cells to enhance their migration and invasion (12,25,26). It was shown that CCL5 exhibited *in vitro* migratory and invasive stimulus on HCC

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cells (27,28), but some other studies reported that increased CCL5 expression might be connected with favorable outcomes in some cancer diseases (29,30). However, the effects of CCL5 mediated by HS-5 cells and detailed mechanisms of Huh7 cell progress are largely unknown, thus, there is an urgent need for increased understanding.

Based on the studies above, we aimed to investigate the effects of CCL5 from HS-5 cells on Huh7 cells, as well as the underlying mechanisms. Our investigation found that HS-5-CM could promote the proliferation, migration and invasion of Huh7 cells, and inhibited apoptosis. CCL5 down-regulation inhibited the effects of HS-5 cells on Huh7 cells migration and invasion via PI3K-Akt signaling pathway and reduced MMP-2 expression. These results suggest that MSCs mediated CCL5 promoting migration and invasion of Huh7 cells and it may offer a novel strategy to efficiently inhibit metastasis.

Materials and methods

Cell culture and reagents. The human hepatocellular carcinoma cell lines Huh7, SMMC-7721, HepG2 and normal human liver cell lines LO2 were kindly provided by Dr Tongchuan He (University of Chicago Medical Center), and bone marrow stromal cell lines HS-5 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/ml streptomycin/penicillin at 37˚C in 5% CO_2. The primary antibodies used in this study were: rabbit anti-Akt monoclonal antibody, rabbit anti-phosphor-Akt monoclonal antibody, rabbit anti-ERK1/2 monoclonal antibody and rabbit anti-phosphor-ERK1/2 monoclonal antibody and were obtained from Cell Signaling Technology. Rabbit anti-MMP-2 polyclonal antibody was purchased from Immunoway (Immunoway, USA). Mouse anti-β-actin monoclonal antibody was purchased from Santa Cruz Biothechnology. Secondary antibodies included HRP-conjugated goat anti-mouse IgG antibody and anti-rabbit IgG antibody were purchased from Zhongshan Goldenbridge Biotechnology. Specific inhibitors of PI3K (LY294002) and ERK1/2 (PD98059) were obtained from Cell Signaling Technology. CCL5 neutralization antibody was purchased from Peprotech.

Collection of HS-5 cell conditioned medium (HS-5-CM). HS-5 cells were plated in a 100 mm^2 culture dish at a density of 80%, for 6 h, for cells adherence, culture medium was changed to serum-free DMEM (SF DMEM). The culture supernatant was harvested every day for 3 days and then all medium were pooled, and sterile filtered. The HS-5-CM was stored at -20˚C.

Co-culture experiment. The co-culture experiment was set up in duplicates using 6-well Transwell inserts (Millipore, USA) with 0.4 μm pore size. In Transwell chamber, HS-5 cells were plated at the density of 10^5 cells/well in 1 ml, whereas Huh7 or SMMC-7721 or LO2 cells were plated in the 6-well plates at the density of $2x10^5$ cells/well in 2 ml. Cells were left to adhere for 6 h before being put together in the SF DMEM for 3 days, and cells alone were used as control.

Cell viability assay. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was used to detect cell viability. A total of $2x10^3$ cells/well were cultured in 96-well plates and treated with fresh DMEM, 50% HS-5-CM (CM:DMEM=1:1) or 100% HS-5-CM containing 1% FBS for 24, 48 and 72 h. After the indicated hours of culture, the MTT reagent (Sigma, St. Louis, MO, USA) was added (20 μl/well), and incubated for 4 h at 37˚C. Dimethyl sulfoxide was added to dissolve the formazan product for 10 min at room temperature. Finally, the absorbance was measured daily for the next three days at 492 nm using a microplate reader. Each group was done in sextuplicate, and the overall experiment was repeated three times.

Flow cytometry. Cell cycle and cell apoptosis analysis were assessed by flow cytometry. Cells were divided into 3 groups: i) control group, Huh7 cells; ii) co-culture group, Huh7 cells were co-cultured with HS-5 cells; and iii) HS-5-CM group, Huh7 cells were treated with HS-5-CM. In brief, cells were seeded into 6-well plates at a density of $2x10^5$ cells/well. Every experiment was repeated twice in each group. Cells were harvested, re-suspended in 1 ml cold PBS. Samples were fixed with pre-cold 75% enthanol for 1 h at 4˚C by cell cycle assay, and other samples were added using propidium iodide (PI) and FITC-Annexin V for cell apoptosis analysis according to the manufacturer's protocol (Invitrogen, USA). Respectively, data were analyzed using FACsorter (Becton Dickinson, CA, USA).

Wound-healing assay, Transwell chamber migration and invasion assay. For wound-healing assay, cells were grown to 95% confluence and the monolayer was scratched with a pipette tip, and then culture medium was replaced with fresh DMEM or 50% HS-5-CM or 100% HS-5-CM containing 1% FBS. Cells that migrated into the scratched area were compared using bright field microscopy at 48 h. Cell migration and invasion assay were performed by 24-well Transwell chambers (8 μm pore size, Millipore) without or with ECM gel (Sigma). Briefly, cells ($5x10^4$/400 μl) in SF DMEM were seeded onto the upper chambers, and fresh DMEM or 50% HS-5-CM or 100% HS-5-CM (600 μl/well) containing 20% FBS added to the lower chambers. After 24 h, cells that migrated to the underside of the filter were fixed with methanol and stained with hematoxylin and eosin (H&E), and counted by brightfield microscopy. All the experiments were repeated three times.

Animal studies. The *in vivo* experiments were performed in accordance with the guidelines established by the Animal Care and Use Committee, of Chongqing University Laboratory Animal Research. The 4-6-week old male NOD/SCID mice were randomly divided into 2 groups (n=4/group), respectively, and were injected subcutaneously with a mixture of equal number of HS-5 cells ($3x10^6$) and Huh7 cells ($3x10^6$) or with Huh7 cells ($6x10^6$) alone. Tumor volume was measured every 10 days with vernier calipers, and calculated in mm^3 as $ab^2/2$, where a and b represent the largest and smallest tumor diameters, respectively. The mice were sacrificed after 70 days, and tumor tissues, livers and lungs were collected and stained using H&E in order to examine the histopathology.

Table I. The sequence of PCR primers for each gene.

Genes	Forward primers (5'→3')	Reverse primers (5'→3')
CXCL12	TGAGCTACAGATGCCCATGC	TTCTCCAGGTACTCCTGAATCC
CXCR4	ACGCCACCAACAGTCAGA	ACAACCACCCACAAGTCA
CXCR7	TTATCGCTGTCTTCTACTTCC	CTTGGTGAGCCCTGTTTT
CX3CL1	AAGGAGCAATGGGTCAAGG	ACGGGAGGCACTCGGAAA
CX3CR1	CACCGACATTTACCTCCT	TTGGCTTTCTTGTGGTTCTT
CXCL8	ACTCCAAACCTTTCCACC	CTTCTCCACAACCCTCTG
CXCR1	AAAATGGCGGATGGTGTT	GACGAAGAAGTGTAGGAGGT
CCL5	TTGCTACTGCCCTCTGCG	CACTTGGCGGTTCTTTCG
CCR1	CCCTTGGAACCAGAGAGAAG	CAAACTCTGTGGTCGTGTCA
CCR3	TCGTTCTCCCTCTGCTCG	AGATGCTTGCTCCGCTCA
CCR5	CTGCTACTCGGGAATCCTAAA	CATAGCTTGGTCCAACCTGTTA
MMP-2	CTACGATGATGACCGCAAGT	CAGTCCGCCAAATGAACC
MMP-7	GGAGGAGATGCTCACTTCGAT	AGGAATGTCCCATACCCAAAGA
MMP-9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC
GAPDH	CAGCGACACCCACTCCTC	TGAGGTCCACCACCCTGT

Total RNA isolation, RT-PCR, and real-time quantitative PCR (qRT-PCR) analysis. Huh7 cells were co-cultured with HS-5 cells or alone in SF DMEM for 3 days, total RNA was isolated using TRIzol Reagents (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was used to generate cDNA templates for reverse transcriptase-PCR. Touchdown PCR analysis determined the gene expression levels and was performed by using the following program: 95˚C x 2 min for one cycle, 10 cycles at 92˚C x 20 sec, 65˚C x 30 sec, and 70˚C x 40 sec with a decrease of one degree per cycle, and 25 cycles at 92˚C x 20 sec, 55˚C x 30 sec, and 70˚C x 40 sec. 70˚C x 5 min for one cycle. Real-time PCR was run in the Rotor-Gene 6000 Real-Time PCR machine (Corbett Research, Australia) using SYBR Premix Ex Taq (Takara) with the following protocol: initial activation of HotStar Taq DNA polymerase at 95˚C for 10 min, then 45 cycles of 95˚C for 5 sec and 60˚C for 20 sec. GAPDH was used as an internal control. The primer efficiency was confirmed to be high (>90%) and comparable (Table I). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The expression levels of mRNA were normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA). To determine quantification of the levels of CCL5 secreted in the supernatants from co-culture or non co-culture, the culture medium was collected and detected by using ELISA Kits (RayBiotech, ELH-RANTES-001), according to the manufacturer's protocol. Briefly, samples were added to the coated wells with CCL5 mAb of 96-well plates and incubated for 2.5 h at room temperature, washed four times and then incubated with an HRP-linked streptavidin solution for 30 min at room temperature in the dark. The absorbance was then measured at 450 nm on the microplate reader (Sunrise Remote, Switzerland) immediately. All the experiments were repeated for three times.

Gelatin zymography of MMP-2 and MMP-9 activity. Equal amount of protein from Huh7 cells co-cultured with HS-5 cells or alone were mixed with SDS buffer, and were added onto a 5% (w/v) stacking polyacrylamide gel and separated on a 10% (w/v) polyacrylamide gel containing 1.0 mg/ml porcine gelatin (Sigma) for the detection of MMP-2 and MMP-9 activity. After electrophoresis, gels were washed twice for 40 min in 2.5% Triton X-100 (Sigma) to remove SDS, and incubated for 42 h at 37˚C in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% Brij-35, and gels were stained for 3 h in 45% methanol/10% acetic acid containing 0.5% Coomassie brilliant Blue R-250 (w/v). Finally, destained with a solution containing 5% acetic acid until clear bands of gelatinolysis appeared on a dark background, proteolytic activity was detected as clear bands on a blue backgroup of the Coomassie Blue staining gel.

Western blot analysis. Western blot was done using standard protocols. In brief, Huh7 cells co-cultured or non co-cultured were washed three times with cold PBS and lysed in RIPA lysis buffer, and cell lysate was denatured after boiling. The protein concentration was measured according to NanoDrop100 UV-Vis Spectrophotometer (Thermo Scientific, USA). Equal amount of proteins were loaded onto 10% SDS-PAGE gels, and transferred subsequently onto PVDF membranes. After blocking with 5% BSA (Bovine Serum Albumin, Solarbio) in TBST, the membranes were probed with the primary antibody, and followed by incubation with a secondary antibody conjugating with horseradish peroxidase. Protein levels were quantified with SuperSignal West Pico Chemiluminescent Substrate kit.

Statistical analysis. Results are presented as mean ± standard deviation (SD). Comparisons were made using independent sample Student's t-test in GraphPad prism 5. Statistical significance is indicated as $p<0.05$.

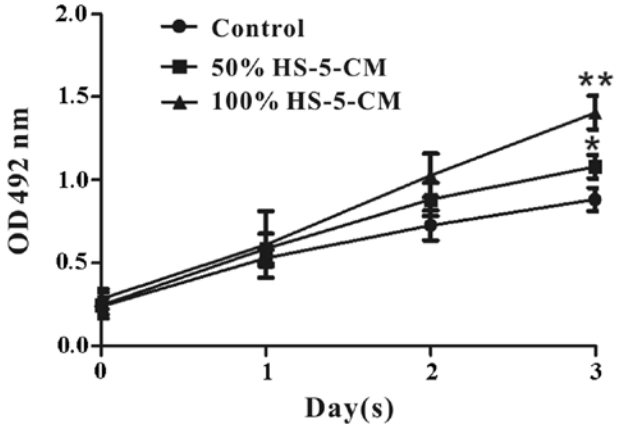

Figure 1. HS-5 cells increase Huh7 cells viability *in vitro*. Cell viability as measured by MTT assay. Huh7 cells were treated with fresh DMEM or 50% HS-5-CM or 100% HS-5-CM for 3 days, fresh DMEM was used as control group. The results represent the mean absorbance ± SD of three independent experiments. $^{*}p<0.05$ or $^{**}p<0.01$ vs. control.

Results

HS-5 cells stimulate proliferation and suppress apoptosis of Huh7 cells in vitro. The effects of HS-5 cells on Huh7 cell viability *in vitro* were evaluated by MTT assay. It revealed that Huh7 cell viability treated with HS-5-CM increased obviously and time-dependently compared with control (Fig. 1). To further investigate the mechanisms of HS-5 cell-induced proliferation of Huh7 cells, flow cytometry was performed to measure the cell cycle. In order to study how HS-5 cells affect Huh7 cell growth in tumor microenvironment, we adopted two ways to culture Huh7 cells: co-cultured with HS-5 cells, and treatment with HS-5-CM, subsequent experiments were done using 100% HS-5-CM. Respectively, Huh7 cells in co-culture group (37.85±1.52%) and in HS-5-CM group (29.56±2.03%) were at S phase of cell cycle at day 3, which was significantly higher than the control (8.96±2.34%) (Fig. 2A). These two ways had different degrees of increase in S phase, but the data demonstrated that HS-5 cells could promote Huh7 cell proliferation.

The proportion of Huh7 cells undergoing apoptosis was determined by flow cytometry analysis. After 3 days, the data showed the apoptosis ratio (early + late) of Huh7 cells in co-culture group was approximately 6.50%, and it was significantly decreased compared to the control group, although HS-5-CM was not distinct in decreasing the apoptotic percentage of Huh7 cells, the small change implied that HS-5 cells could suppress Huh7 cell apoptosis (Fig. 2B).

HS-5 cells significantly promote migration and invasion of Huh7 cells in vitro. As cell migration and invasion abilities are important determinants in the progression of HCC metastasis, the potential to migrate was tested in the commonly used wound-healing assay. After cultured with HS-5-CM for 48 h, the motility of Huh7 cells was significantly accelerated compared with control, especially in 100% HS-5-CM (Fig. 3A). Transwell assay was carried out to verify the migration potency. In line with the wound-healing experiment, it demonstrated that 100% HS-5-CM prominently increased the migration potency of Huh7 cells compared to the control (Fig. 3B). Applying Transwell assay to test the invasion property of Huh7 cells for 24 h, respectively, the number of transmembrane cells in 50% HS-5-CM group and 100% HS-5-CM group was remarkably increased compared to the control group (Fig. 3C).

HS-5 cells promote tumor growth of Huh7 cells in vivo. A critical experiment for determining whether HS-5 cells affect tumor growth of HCC is the *in vivo* tumorigenicity assay. Therefore, we sought to validate our *in vitro* findings by using an *in vivo* model. It found that HS-5 cells accelerated the growth of Huh7 tumors, up to 20 days, there was no significant difference in the two groups, while the tumor volumes became palpable after 20 days, the Huh7 group grew from 150 to 2,522.4 mm^3 and the co-culture group grew from 200 to 3,321.8 mm^3 (Fig. 4A and B). These results were consistent with the *in vitro* observation that HS-5 cells markedly promoted Huh7 cells growth. H&E staining showed no variance in heterogeneity between the two groups (Fig. 4C). Tumor cells were not found in the liver and lung tissues between the two groups (data not shown).

Co-culture with Huh7 cells increases CCL5 expression in HS-5 cells. The molecular mechanisms of these pro-metastatic impacts in tumor microenvironment are still unclear. To investigate whether it is mediated by soluble factors secreted from HS-5 cells in the capacity of migration and invasion, we identified several chemokines by RT-PCR using RNA prepared from HS-5 cells co-cultured with Huh7 cells or alone at day 3. It was found that CXCL12, CX3CL1, CXCL8, CCL5 mRNA levels were upregulated in HS-5 cells after co-culture, among which CCL5 showed the highest level of increase (Fig. 5A). We also examined these chemokine special receptor mRNAs in Huh7 cells. RT-PCR assay showed that CXCR4 and CCR5 mRNA expression was increased, and CX3CR1 mRNA was decreased in Huh7 cells co-cultured with HS-5 cells compared with Huh7 cells alone at day 3 (Fig. 5B).

To confirm the change on CCL5 expression by qRT-PCR and ELISA assay. qRT-PCR analysis demonstrated that CCL5 mRNA expression had an approximate 5-fold increase in HS-5 cells co-cultured with Huh7 cells compared with HS-5 cells alone at day 3 (Fig. 5C). In accordance with CCL5 mRNA level, ELISA assay revealed that co-culture specifically increased CCL5 protein level more than 3-fold compared with the secretion from Huh7 cells or HS-5 cells (Fig. 5D).

ELISA assay also verified that the change of CCL5 upregulation was not an accidental event with SMMC-7721 or HepG2 or LO2 cells. It found that CCL5 protein levels were increased in HS-5 cells co-cultured with SMMC-7721 or HepG2 cells in different degrees, but not with normal liver cells LO2 (Fig. 5E). The aforementioned data illustrated that interaction with HCC and HS-5 cells could increase CCL5 secretion in HS-5 cells.

CCL5 secreted from HS-5 cells promotes Huh7 cell migration and invasion in vitro. Whether HS-5 cells can modulate Huh7 cells progression via CCL5 secretion remains unreported. We took a loss-of-function approach to confirm its impact on Huh7 cells, utilizing anti-CCL5 neutralizing antibody to

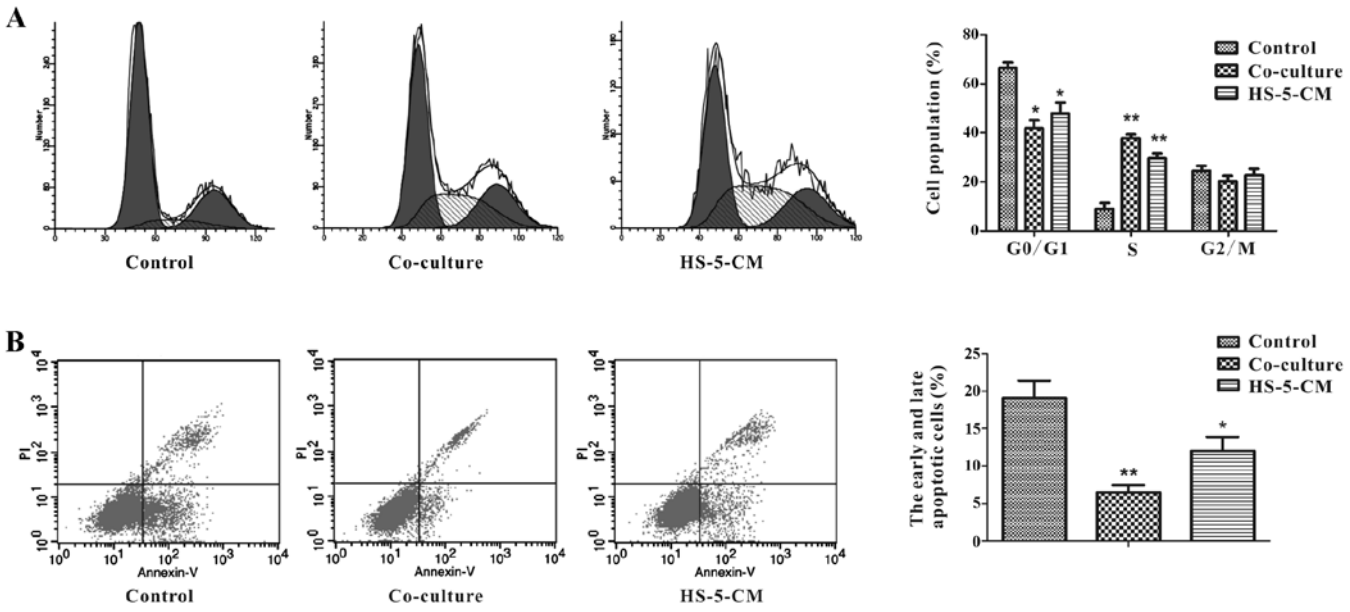

Figure 2. HS-5 cells induce S cell cycle arrest and inhibit apoptosis of Huh7 cells *in vitro*. (A) Cell cycle and (B) apoptosis as measured by flow cytometry. Huh7 cells were co-cultured with HS-5 cells or cultured with HS-5-CM for 3 days. Data are presented as mean values ± SD of three independent measurements. $^{*}p<0.05$ or $^{**}p<0.01$ vs. control.

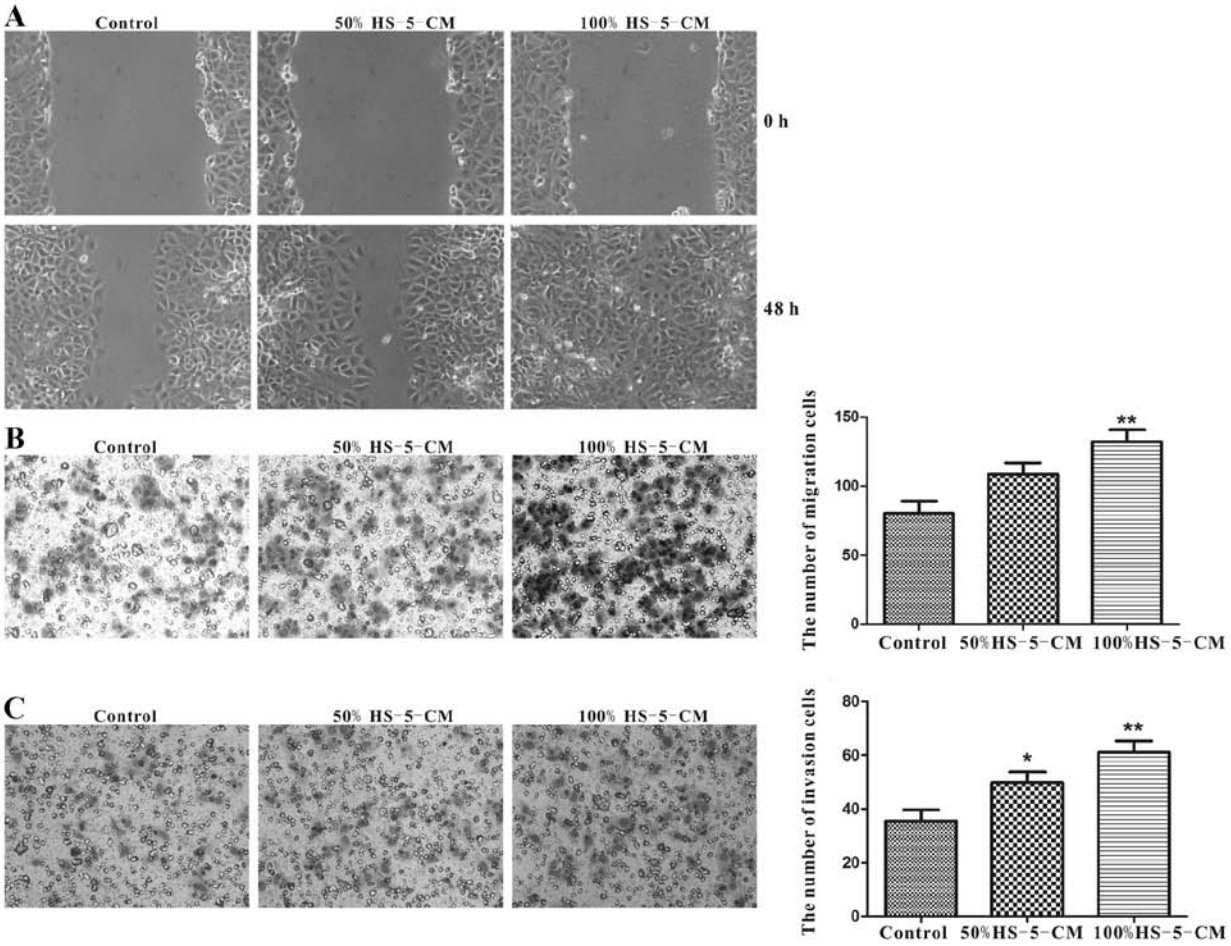

Figure 3. Increase of the migration and invasion of Huh7 cells by HS-5 cells *in vitro*. (A) Cell migration as measured by wound healing assay. Huh7 cells were treated with fresh DMEM or 50% HS-5-CM or 100% HS-5-CM for 48 h. Data are reported as mean values ± SD of three individual measurements. Magnification, x100. (B) Cell migration and (C) invasion as measured by Transwell assay. Huh7 cells were treated with fresh DMEM or 50% HS-5-CM or 100% HS-5-CM for 24 h. Data are reported as mean values ± SD of three individual measurements. The quantification data are on the right. Magnification, x150. $^{*}p<0.05$ or $^{**}p<0.01$ vs. control.

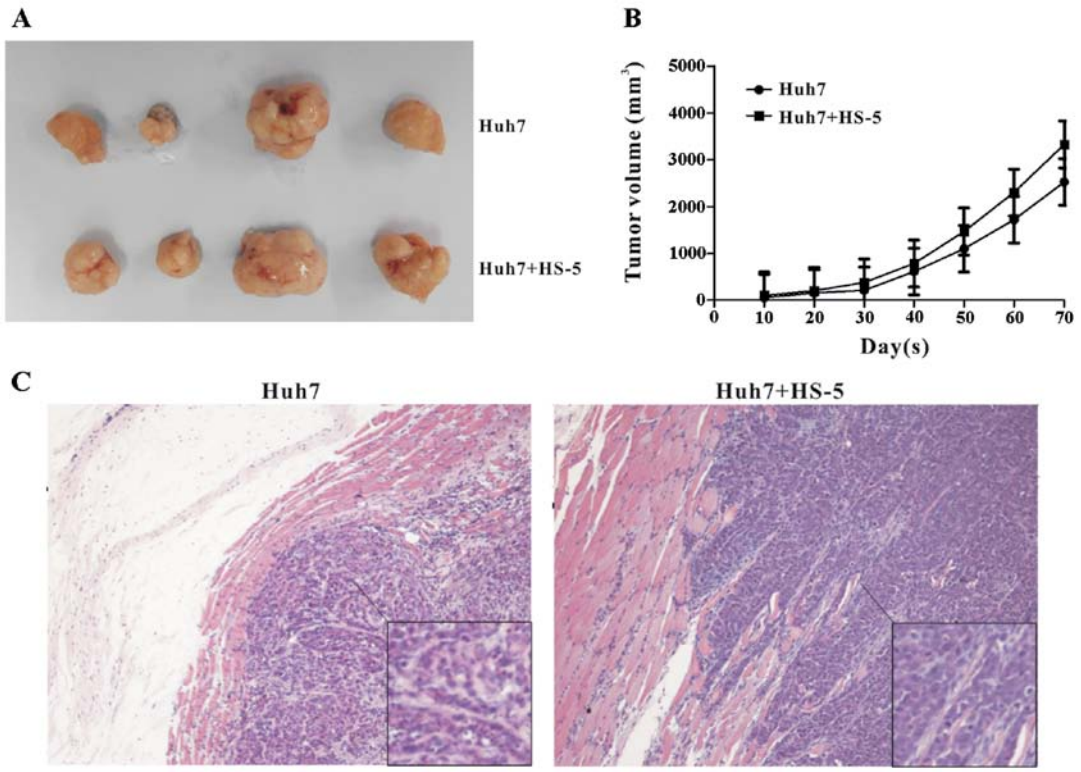

Figure 4. HS-5 cells promote Huh7 cells tumor formation *in vivo*. (A) Excised tumor tissues from the two groups. (B) Tumor growth curves of the two groups. HS-5 cells promoted Huh7 cell tumor growth *in vivo*. n=4 mice/group, data are reported as mean ± SD. (C) H&E staining of tumor tissues. All tumors showed a large nucleus, a high nucleus/cytoplasm ratio, an irregular nuclear shape and variable nuclear size, while no variation in heterogeneity was found between the two groups. The images were taken at x100 magnification.

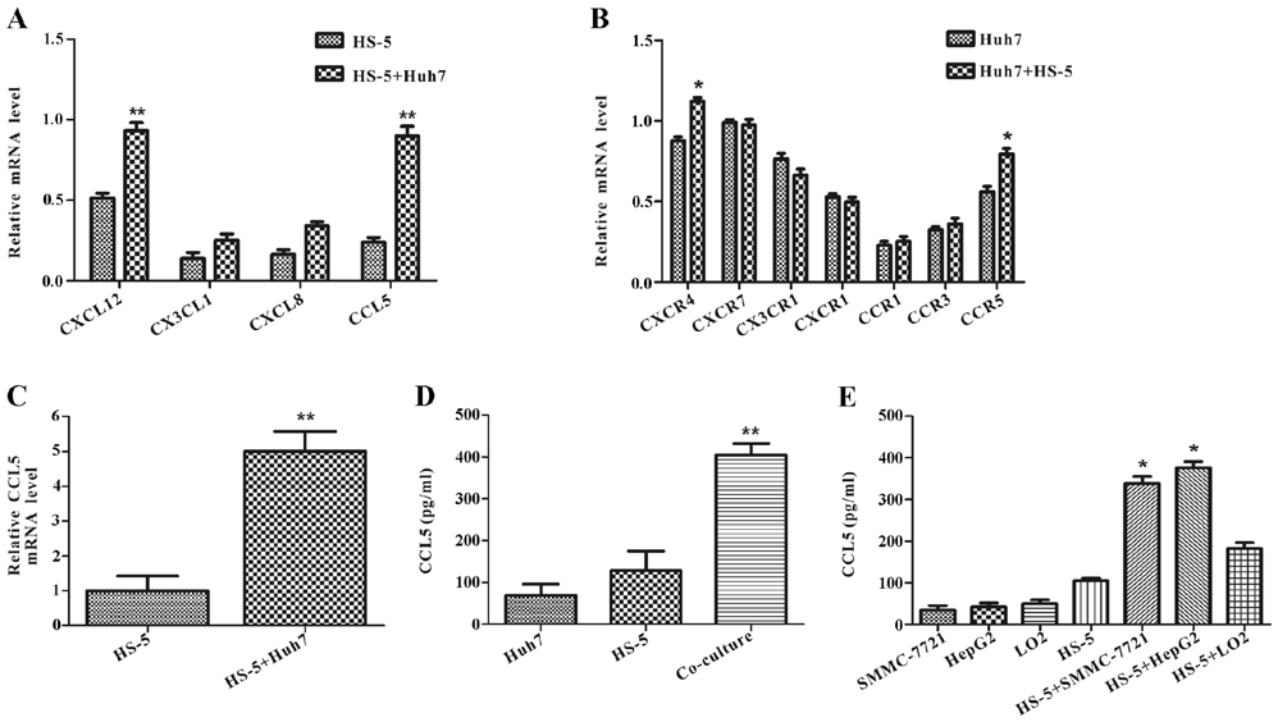

Figure 5. Effects of chemokines and receptors expression after co-culture. RT-PCR assay examined (A) chemokine mRNA levels in HS-5 cells and (B) these special receptor mRNA levels in Huh7 cells after more than 3-day co-culture. Data are reported as mean ± SD of three individual measurements. *p<0.05 vs. Huh7 cells; **p<0.01 vs. HS-5 cells. (C) qRT-PCR assay verified CCL5 mRNA expression in HS-5 cells co-cultured with Huh7 cells or alone at day 3. Data are reported as mean values ± SD of three individual measurements. **p<0.01 vs. HS-5 cells. (D) CCL5 protein expression as detected by ELISA assay. Co-culture or cells alone culture supernatant at day 3 was collected. Data are reported as mean ± SD of three individual measurements. **p<0.01 vs. Huh7 or HS-5 cells alone. (E) CCL5 protein expression in SMMC-7721, HepG2 and LO2 cells, SF culture supernatant from non co-cultured or co-cultured with HS-5 cells at day 3 as detected by ELISA assay. Data are presented as mean ± SD of three individual measurements *p<0.05 vs. HS-5 or SMMC-7721 or HepG2 cells alone.

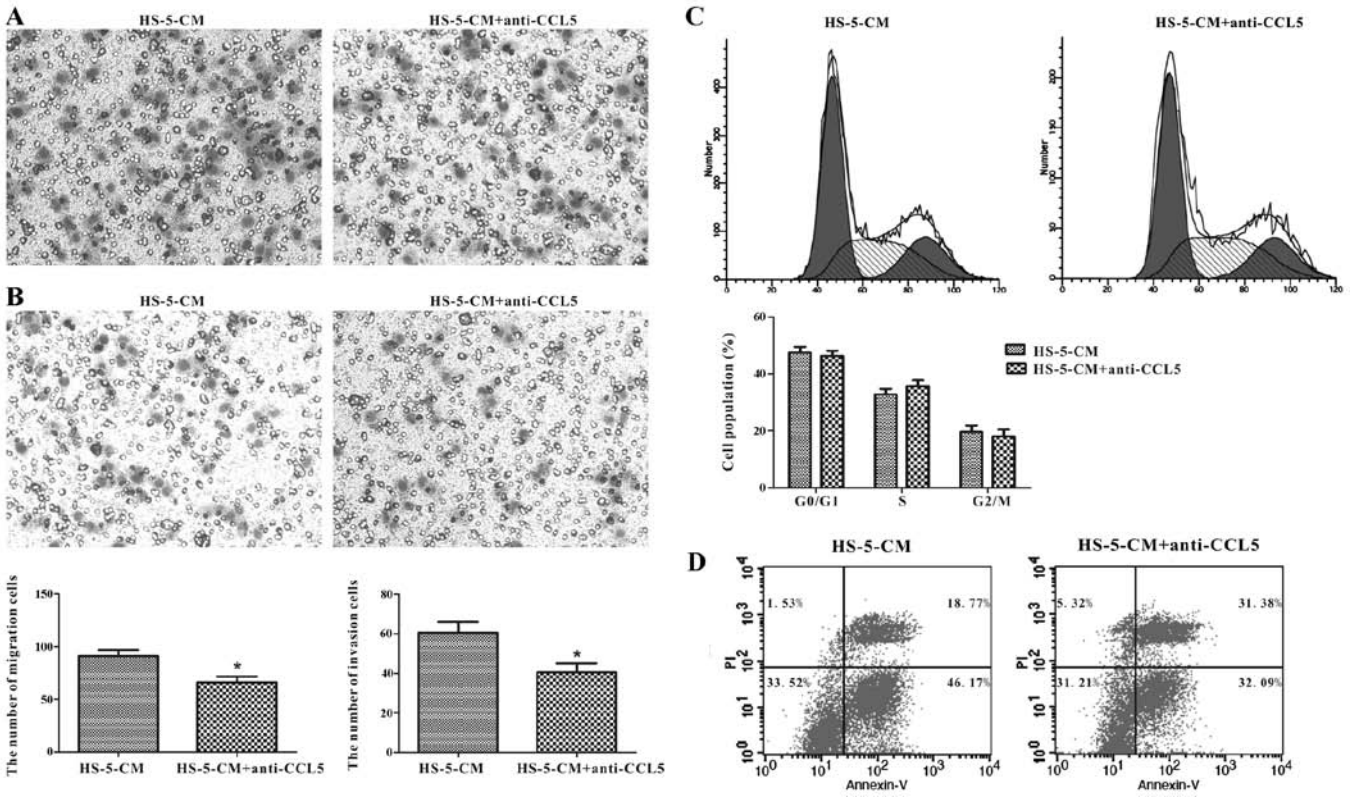

Figure 6. Effect of anti-CCL5 neutralizing antibody in HS-5-CM induced Huh7 cell progression. (A) Cell migration and (B) invasion as measured by Transwell assay. (C) Cell cycle and (D) apoptosis as measured by flow cytometry. Huh7 cells were treated with HS-5-CM containing control (irrelevant) IgG or anti-CCL5 neutralizing antibody (1 μg/ml) for 24 h. Data are shown as mean ± SD of three individual measurements (A, left below; B, right below). *p<0.05 vs. control IgG. Magnification, x150.

HS-5-CM which was cultured in Huh7 cells for 24 h. We tested the migration and invasion abilities by Transwell assay, and it illustrated that anti-CCL5 neutralizing antibody caused a decrease of 27.47% in HS-5-CM-induced migration activity of Huh7 cells compared with the control (Fig. 6A). Consistently, Huh7 cell invasion ability was inhibited by 33.06% compared to the control (Fig. 6B). However, not as imagined, CCL5 downregulation in HS-5-CM did not overtly change proliferation and apoptosis of Huh7 cells (Fig. 6C and D). It was thus possible that CCL5 from HS-5-CM did not play a leading role in Huh7 cell proliferation and apoptosis impact, but in migration and invasion potency.

Effects of CCL5-secreted from HS-5 cells on Huh7 cells via PI3K-Akt signaling pathway. The underlying mechanisms of the interaction with MSCs and HCC within HCC microenvironment remain an enigma, so we concentrated on the mechanisms of HS-5 cells inducing migration and invasion of Huh7 cells. It has been reported that stimulation of MSCs with certain chemokines causes the activation of Akt kinase and ERK1/2 MAP kinase (31). Therefore, we examined the potential changes of Huh7 cells in the presence of HS-5 cells further. The transwell co-culture with HS-5 cells for 3 days, demonstrated by western blot analysis, revealed that Huh7 cells had enhanced expression of p-Akt (Ser473) (Fig. 7A), but not p-ERK1/2 (Fig. 7B). The aforementioned data showed that CCL5 expression was upregulated in HS-5 cells after co-culture (Fig. 5C and D). Previous research showed that CCL5 activated Gαi-PI3K-Akt and Gαi-MEK-ERK signaling pathways (32). So it was postulated that CCL5 is associated with the activation of p-Akt (Ser473) and p-ERK1/2 in co-culture system. We applied anti-CCL5 antibody for 24 h to the co-culture system to evaluate the hypothesis, the data showed the activation of p-Akt (Ser473) was reduced by adding anti-CCL5 neutralizing antibody to Huh7 cells co-cultured with HS-5 cells (Fig. 7A), however, there was no significant change in p-ERK1/2 (Fig. 7B). We next focused on CCL5 expression in response to p-Akt (Ser473) and p-ERK1/2 signaling pathways. Before we investigated the roles of p-Akt (Ser473) and p-ERK1/2 signaling pathways, western blot analysis demonstrated PI3K and ERK1/2 specific inhibitors which, respectively, were LY294002 (20 μM) and PD98059 (10 μM) could decrease the expression of p-Akt (Ser473) and p-ERK1/2 (Fig. 7A and B). Transwell assay illustrated that the migration and invasion of Huh7 cells induced by HS-5-CM were partly reversed by treatment with LY294002 but not by PD98059 (Fig. 7C and D). In addition, ELISA assay demonstrated that LY294002 reduced the secretion of CCL5 in co-culture system (Fig. 7E). Thus, our results illustrated that CCL5 derived from HS-5 cells had a pivotal role in migration and invasion of Huh7 cells via PI3K-Akt signaling pathway.

CCL5 downregulation from HS-5 cells decreases MMP-2 expression in Huh7 cells. Cell movement is involved in the proteolytic activity of MMPs, which regulate the dynamic

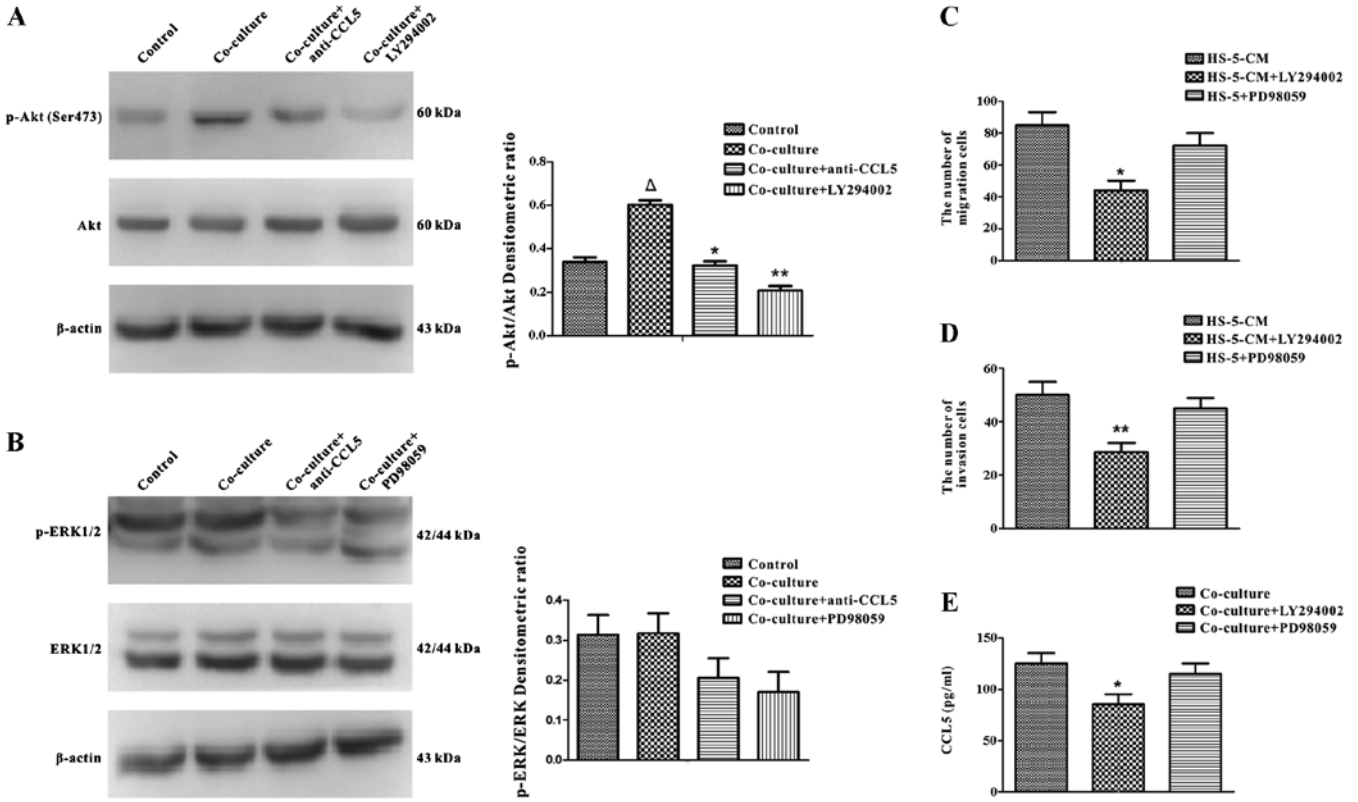

Figure 7. Interaction with the activation of p-Akt (Ser473) and ERK1/2 and the secretion of CCL5. (A) p-Akt (Ser473) protein in Huh7 cells as determined by western blot analysis. Huh7 cells were co-cultured with HS-5 cells for 3 days, and then anti-CCL5 neutralizing antibody (1 μg/ml) or LY294002 (20 μM) was added for 24 h, control IgG antibody and DMSO were used as control. Densitometric quantification data are on the right. Data are shown as mean ± SD of three individual measurements. *p<0.05, **p<0.01 vs. co-culture group; $^{\Delta}$p<0.05 vs. control group. (B) p-ERK1/2 protein in Huh7 cells as determined by western blot analysis. Huh7 cells were co-cultured with HS-5 cells for 3 days, and then anti-CCL5 neutralizing antibody (1 μg/ml) or PD98059 (10 μM) was added for 24 h, control IgG antibody and DMSO were used as control. Densitometric quantification data are on the right. Data are shown as mean ± SD of three individual measurements. (C) Cell migration and (D) invasion as measured by Transwell assay. Huh7 cells were treated with HS-5-CM containing DMSO or LY 294002 (20 μM) or PD98059 (10 μM) for 24 h. Data are shown as mean ± SD of three individual measurements. *p<0.05 or **p<0.01 vs. HS-5-CM. Magnification, x150. (E) CCL5 protein level in co-culture supernatant as detected by ELISA assay. Huh7 cells were co-cultured with HS-5 cells for 3 days, and then DMSO or LY294002 (20 μM) or PD98059 (10 μM) was added for 24 h. Data are shown as mean ± SD of three individual measurements. *p<0.05 vs. co-culture.

ECM (extracellular matrix)-cell and cell-cell interaction during migration (33). High expression of MMPs is related to HCC progression (34,35). We investigated whether CCL5 regulated the levels of MMP-2, MMP-7 and MMP-9 in Huh7 cells co-cultured with HS-5 cells. RT-PCR analysis showed that MMP-2 expression was up-regulated in Huh7 cells co-cultured with HS-5 cells at day 3, extraordinary, MMP-2 mRNA was downregulated through the addition of anti-CCL5 neutralizing antibody. Nonetheless, the expression of MMP-7 and MMP-9 mRNAs remained significantly unchanged (Fig. 8A). To further characterize the effect of CCL5 in MMP expression of Huh7 cells, we applied gelatin zymography analysis to determine expression and enzymatic activities of MMP-2 and MMP-9. The results revealed that treatment of anti-CCL5 neutralizing antibody pointed to a decrease in MMP-2 upregulation of Huh7 cells co-cultured with HS-5 cells, whereas MMP-9 was not detected (Fig. 8B). Consistently, western blot assay confirmed that anti-CCL5 neutralizing antibody could reduce MMP-2 protein upregulation mediated by HS-5 cells in Huh7 cells (Fig. 8C). The data indicated that CCL5 downregulation from HS-5 cells decreased MMP-2 expression in Huh7 cells.

Discussion

HCC invasion and metastasis are very strongly related to cancer patient survival, but the detailed mechanisms are still unknown (36). Advanced HCC patients presenting with bone metastases are different from breast and prostate cancer patients, particularly in liver function impairment, prognosis and available effective treatments (37). Cirrhosis is an independent prognostic factor for osteoporosis (38). Bone marrow is the most common source of MSCs, which are found to take part in tumor microenvironment that mediates tumor cell progress. However, crosstalk of the tumor-MSCs is complicated, the controversy on the effects of MSCs on the tumor cell progression remains to be debated, especially in HCC. Herein, we adopted HS-5-CM to eliminate the interaction with HCC cells, and it demonstrated that HS-5 cells could promote proliferation, migration and invasion of Huh7 cells *in vitro*, and the increase of proliferation was verified by the *in vivo* results. Our results are consistent with a large proportion of research (16,17,20). However, the tumor metastasis in liver and lung regions were not found, demonstrating that Huh7 cell invasion ability is limited *in vivo*. Despite the above, some reports

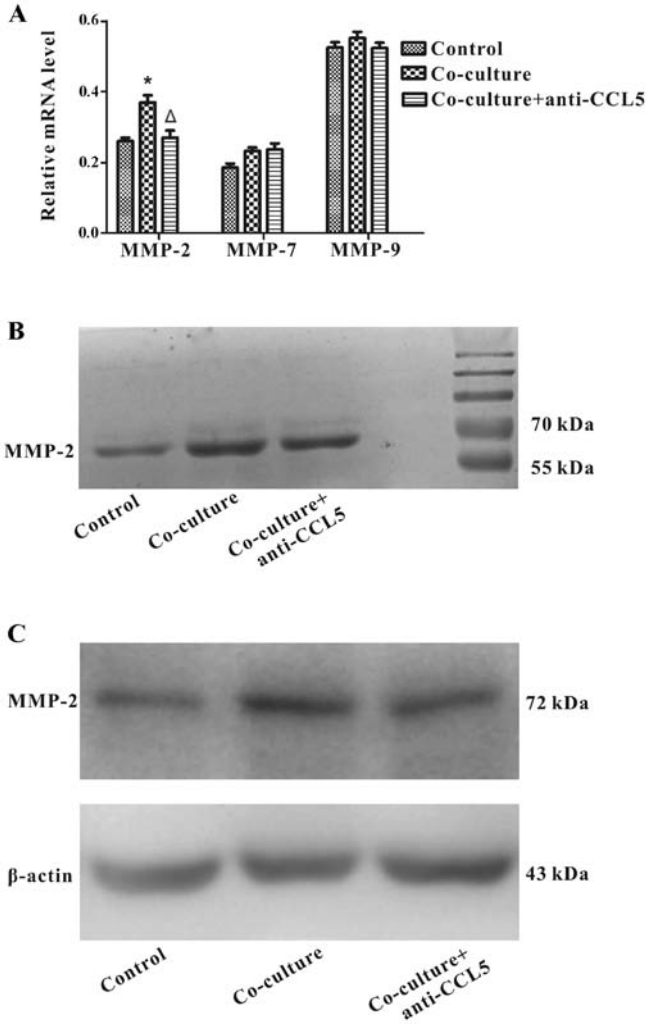

Figure 8. Expression of MMPs in Huh7 cells. (A) MMP mRNA levels in Huh7 cells as showed by RT-PCR analysis. (B) MMP-2 (72 kDa) protein as showed by gelatin zymography analysis. (C) MMP-2 protein level as confirmed by western blot assay. Huh7 cells were co-cultured with HS-5 cells for 3 days, and then control IgG or anti-CCL5 antibody (1 μg/ml) was added for 24 h. Data are shown as mean ± SD of three individual measurements. *p<0.05 vs. control; ^p<0.05 vs. co-culture.

stated that MSCs could inhibit HCC cell growth (22,39). It is proposed that the time of MSCs introduction into tumors might be a critical factor for the contradicting results (9). In this study, we consider that MSCs are helpful for HCC progression.

MSC secretions include a complex mixture of cytokines, growth factors and chemokines, which have an influence on cancer cell migration and invasion. In this study, the data determined that in co-culture with Huh7 cells, HS-5 cells expressed an increased level of CCL5. To circumvent variability in cell-cell interaction subsequent experiments were done using conditioned medium from the 'co-culture' cell lines, and it was shown to consistently alter the CCL5 level. To our knowledge, chemokines and receptors play a number of non-redundant roles in tumor progression. Besides, some reports indicate that CCL5 is an effective inducer of tumor cell migration and invasion, such as in breast, colorectal, osteosarcoma and prostate tumor cells, acting through paracrine and autocrine manners (40-44). It was demonstrated that stroma-derived CCL5 was particularly important in inducing pro-malignancy impact in CCR5-expressing breast cancer cells (12). However, in tumor microenvironment, whether HS-5 cells mediated CCL5 plays a pivotal role in HCC migratory and invasion, has not been extensively studied. We found that Huh7 cells revealed a higher level of CCR5 mRNA than CCR1 and CCR3, and an obvious increase in CCR5 mRNA after co-culture. Furthermore, a recent report determined that CCR5 was involved to HCC inflammation (45). CCR5 is more important than CCR1 and CCR3 in HCC progress in our study. Anti-CCL5 neutralization antibody decreased the migration and invasion of Huh7 cells mediated by HS-5-CM. The results indicated that the CCL5/CCR5 axis was associated with migration and invasion of Huh7 cells in tumor microenvironment. It cannot be denied that other cytokines play a pivotal role of malignant progression like CXCL12 promoting tumor cell proliferation and survival via paracrine and autocrine mechanisms (46,47). It was proposed that CXCL12 could promote HCC cells migration (48). Nevertheless, in mimicking HCC microenvironment, it cannot be ignored that CCL5-secreted from MSCs is a critical factor in Huh7 cell migration and invasion.

Activation of PI3K contributes to invasion and metastasis of HCC (49). ERK1/2 pathway is connected with the migratory or invasive behavior of a variety of malignancies (50). Thus, whether the progression of HCC induced by MSCs is involved in the PI3K/Akt and ERK1/2 signaling pathways has not been yet established. In particular, we demonstrated that the PI3K inhibitor LY294002 antagonized enhancement in migration and invasion of Huh7 cells, and also reduced CCL5 secretion. However, co-culture did not activate the p-ERK1/2 signaling pathway and ERK1/2 specific inhibitor PD98059 did not reduce the mobility and invasiveness of Huh7 cells. It is possible that ERK1/2 signaling pathway regulates another biological function in HCC microenvironment. It is conceivable that other signaling pathways may synergistically or antagonistically regulate the HCC progress. Our data indicated that PI3K/Akt signaling pathway could play an important part in CCL5-mediated migration and invasion of Huh7 cells.

MMPs play a key role in cancer cell invasion. Some data showed that the expression of MMP-2 increased in hepatic fibrosis (51), others reported that certain cytokines could increase MMP-2 and MMP-9 secretion in human sarcoma cells (52). Xiang *et al* showed that MMP-2 was an independent prognostic factor for lymph node metastasis in HCC (53). Previous studies showed that CCL5 promoted the expression of MMP-9 in tumor cells (44,54,55). In this study, we demonstrated that HS-5 cells did not elevate MMP-9 expression but MMP-2 expression in Huh7 cells. The results suggest that anti-CCL5 neutralization antibody could depress the secretion of MMP-2 in Huh7 cells. Therefore, MMP-2 may be a CCL5-responsive mediator, and it causes the degradation of ECM, which may lead to subsequent HCC migration and metastasis.

In conclusion, the current observations indicate that HS-5 cells can promote the proliferation, migration and invasion, and inhibit apoptosis of Huh7 cells. Exocrine CCL5 secreted from MSCs promotes migration and invasion of Huh7 cells via PI3K/Akt signaling pathway, and accompanies the MMP-2 upregulation. Hence, CCL5 may be an important factor in HCC with bone metastases.

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