Overexpression of CXCR7 induces angiogenic capacity of human hepatocellular carcinoma cells via the AKT signaling pathway

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Abstract. Angiogenesis is essential for tumor growth, especially in hepatocellular carcinoma (HCC). The hypervascularity is associated with poor prognosis and highly invasive HCC. The C-X-C chemokine receptor type 7 (CXCR7) has been implied overexpressed in many tumor types. Our study aimed to investigate the CXCR7 function in HCC. The tube formation, Transwell migration assay of human umbilical vein endothelial cells (HUVECs) and chicken chorioallantoic membrane (CAM) assay were used. We confirmed that CXCR7 induces angiogenic capacity. Moreover, overexpressing CXCR7 increased the phosphorylated (but not total) AKT expression in HCC cells. Furthermore, overexpressing CXCR7 increased the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 in HCC cells. Additionally, inhibition of AKT by LY294002 abrogated CXCR7-induced angiogenic capacity in HCC cells. Our study suggested that CXCR7 plays an important pro-angiogenic role in HCC via activation of the AKT pathway. So CXCR7 may be a potential target for anti-angiogenic therapy in HCC.

Introduction

Angiogenesis, the creation of new blood vessels, is an important effector of many physiological and pathological processes (1). Angiogenesis occurs to induce tumor growth and metastasis, and represents a key hallmark of tumor development (2-5). Tumor angiogenesis provides the nutrients and oxygen to maintain tumor growth and invasion. Thus, inhibition of tumor angiogenesis may decrease tumor cell growth and spread (6-8). Angiogenesis is considered to be an effective therapeutic target; hence, angiogenesis is a necessary area of biological research and clinical oncology (4,9,10). Tumor angiogenesis is an outcome of an imbalance between pro-angiogenic factors, including the vascular endothelial growth factor (VEGF) family, and anti-angiogenesis factors, such as endostatin and other related factors (11-13). VEGF effects the sprouting and endothelial cell proliferation, and then VEGF can stimulate tumor angiogenesis (14). Many of anti-angiogenesis drugs serve as inhibiting pro-angiogenic factors, such as the monoclonal antibody bevacizumab binds to VEGF, or other small molecules that inhibit the binding of VEGF (15,16). Nevertheless, the mechanisms of tumor angiogenesis are not yet entirely understood and specific, effective inhibitors of angiogenesis are required for cancer therapy.

Hepatocellular carcinoma (HCC) includes >90% of primary malignant liver cancers and is one of the most common reasons for cancer-related mortality (17,18). Because of the hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, the HCC incidence is increasing in Asia, especially in China (19). HCC is regarded as hypervascular, and tumor growth depends on angiogenesis (15,20). Targeting angiogenesis by pharmacologic therapy has been used in many other solid tumors. Therefore, the anti-angiogenic strategy for HCC may increase the treatment outcomes for HCC patients.

C-X-C chemokine receptor type 7 (CXCR7), a new known orphan receptor, has been shown to bind stromal cell-derived factor-1 (SDF-1) (19,21). CXCR7 also has been demonstrated important for primordial germ cell migration in zebra fish (22). Recently, high level of CXCR7 has been confirmed to be associated with aggressive tumors (23). Moreover, overexpression of CXCR7 was found to be connected to metastatic recurrence in non-small cell lung cancer. Recently, overexpression of CXCR7 was reported in tumor cell lines and tissues (24). However, the CXCR7 function in angiogenesis of HCC is not yet clear.

We demonstrated that CXCR7 is highly expressed in HCC cell lines. In addition, overexpression of CXCR7 promoted the angiogenic capacity of HCC cells via ATK signaling pathway. This study demonstrates that CXCR7 may induce angiogenesis *in vivo*; therefore, CXCR7 may have potential therapeutic effects in HCC.

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Materials and methods

Cell lines. The HCC cell lines HCCLM3 (100% lung metastatic potential), MHCC97-L (low metastatic potential) and SMMC-7721 (without lung metastatic potential) were purchased from the Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA) and 100 U penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 humidified incubator at 37˚C.

Real-time reverse transcription-PCR analyses. The CXCR7 mRNA expression was calculated as follows. Reactions were executed in 20 μl volumes, every sample including 2 μl complementary DNA (cDNA) by using the primer pairs: CXCR7 sense, 5'-GGGATGCAGCGGATAGTCAA-3' and antisense, 5'-CGGTCGTTGTCCACATCCA-3'; Taqman probe, 5'-TCGGTCTCTCCCTGCCCGTCCT-3'. Real-time reverse transcription-PCR used TaqMan PCR reagents and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) followed with an initial denaturation step at 95˚C for 10 min, then 28 cycles of denaturation at 95˚C for 60 sec, primer annealing at 58˚C for 30 sec and primer extension at 72˚C for 30 sec, with a final extension step at 72˚C for 5 min.

Western blotting. Western blotting was performed by using the Bio-Rad Transfer Cell System (Bio-Rad, Mississauga, ON, Canada). Rabbit anti-human CXCR7 antibody (1:200; R&D Systems, Inc., Minneapolis, MN, USA) followed by 1:3,000 horseradish peroxidase-conjugated goat anti-rabbit IgG F(ab')2 antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used.

Overexpression of CXCR7 in HCC cells. The HCC cell line HCCLM3 was transformed with human full-length CXCR7 cDNA by using Lipofectamine 2000 (Invitrogen). The HCC cell line HCCLM3 transformed with an empty plasmid was used as a negative control. Stable cell lines expressing CXCR7 were selected with G418 (K1, 250 μg/ml; TPC-1, 200 μg/ml; and B-CPAP, 300 μg/ml). The CXCR7 expression was evaluated by western blotting.

RNA interference (RNAi) in HCC cells. Downregulation of the expression of CXCR7 in HCCLM3 cells was performed using small interfering RNAs (siRNAs) as follows: siCXCR7-286, 5'-CGC UCU CCU UCA UUU ACA UdTdT-3' (at position 286); negative control siRNA, 5'-UUC UCC GAA CGU GUC ACG UTT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) positive control siRNA, 5'-GUA UGA CAA CAG CCU CAA GTT-3'. siRNA transfection of HCCLM3 cells was performed using the protocol.

Human umbilical vein endothelial cell (HUVEC) tube formation assay. The HUVEC tube formation assay was performed as previously described. Firstly, 200 μl Matrigel were placed into a 96-well plate for 30 min at 37˚C. HUVECs ($2x10^4$) in 200 μl conditioned medium (CM) from indicated HCC cells were added to the well and incubated for 24 h at 37˚C. Images were attained by a bright-field microscope (x100), and formation of capillary tubes was quantified by measuring their total number of each image.

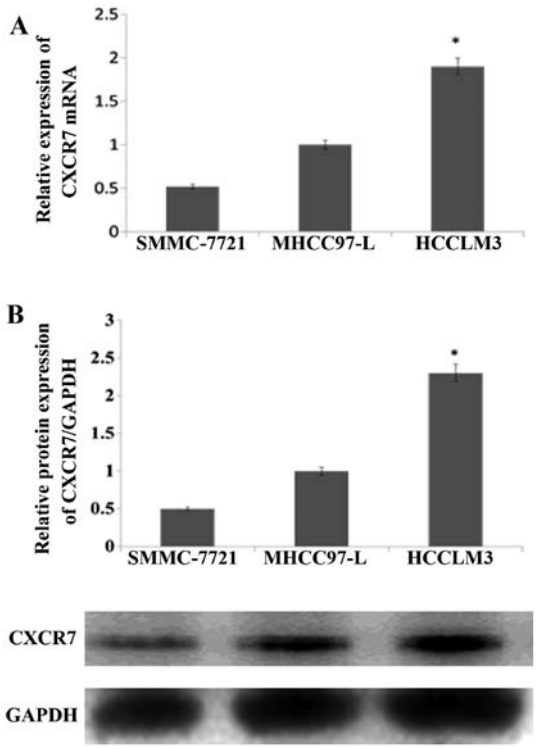

Figure 1. The CXCR7 expression in different metastatic HCC cell lines. (A) The mRNA expression of CXCR7 was examined by real-time PCR. (B) The protein expression of CXCR7 was determined by western blotting. Data are means ± SD of three independent experiments. *P<0.05. CXCR7, C-X-C chemokine receptor type 7; HCC, hepatocellular carcinoma.

Chicken chorioallantoic membrane (CAM) assay. The CAM assay was performed using 8-day-old chicken embryo. About 1-cm diameter window was shaped in the shell of chicken embryo. A diameter gelatin sponge with 100 μl CM harvested from the indicated HCC cells was placed on the CAM. The windows in the chicken embryo were closed by bandages. The chicken embryos were incubated at 37˚C for 48 h. Then the CAM was fixed with stationary solution (1:1 v/v mixture of methanol and acetone) for 15 min, the CAM was imaged by a digital camera. The number of second- and third-order vessels in the test samples was compared to control.

HUVEC Transwell migration assay. HUVECs ($1x10^4$) were cultured on the top of polycarbonate Transwell filters (pore size, 8.0 μm; Corning, Inc., Corning, NY, USA) in CM containing 5% FBS. The lower chamber was filled with 500 μl of media containing SDF-1α (100 ng/ml) and 15% FBS. The cells were incubated at 37˚C for 8 h, and the cells that migrated to the lower membrane surface were fixed in 4% paraformaldehyde, stained using hematoxylin for 15 min, and the number of cells in 10 randomly selected x200 fields of view per filter was counted and expressed relative to that of cells treated with CM from vector control cells.

Matrigel plug assay in mice. C57BL/6 mice were injected 50 μl of reconstituted CM with 50 U/ml heparin and SDF-1α

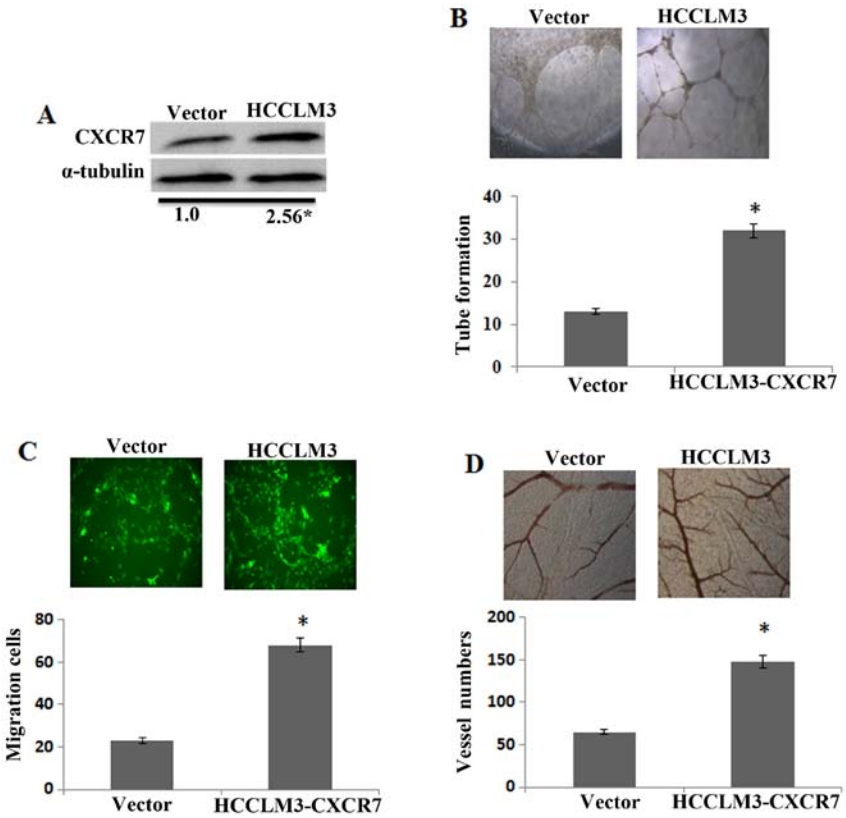

Figure 2. Overexpression of CXCR7 induces angiogenic capacity of HCC cells *in vitro*. (A) Western blotting of HCCLM3-vector, HCCLM3-CXCR7 protein expression in HCCLM3 cells; α-tubulin was used as a control. The numbers show the relative level of protein compared to the control. (B) The images (left) and quantification (right) of tube formation by HUVECs cultured in CM. (C) The migration images and quantification of HUVECs after incubation in CM. (D) The images (left) and quantification (right) of blood vessels in the CAM assay when stimulated by CM. Data are means ± SD of three independent experiments. *P<0.05. CXCR7, C-X-C chemokine receptor type 7; HCC, hepatocellular carcinoma; HUVECs, human umbilical vein endothelial cells; CM, conditioned medium; CAM, chicken chorioallantoic membrane.

(100 ng/ml) added to 0.6 ml Matrigel. Matrigel polymerizes to a solid gel at body temperature, and then the gel become vascularized after 10 days. The gel was removed, photographed, stained, and also diluted in water to measure the hemoglobin content by Drabkin's reagent kit (Sigma).

Enzyme-linked immunosorbent assay (ELISA). The tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 ELISA assay were performed by the commercial kits. All samples were added to the 96-well in triplicate, incubated at 36˚C for 90 min, washed, incubated with a specific anti-antibody (Cell Signaling Technology, Inc.) at 36˚C for 1 h, washed, incubated with secondary antibody at 36˚C for 1 h, and then substrate was added, incubated for 1 h and the absorbance values were read at OD450 by an ELISA plate reader.

Statistical analysis. All experimental data are presented as the mean ± SD of three independent biological replicates. Statistical analyses were performed using SPSS 13.0 (IBM Corp., Armonk, NY, USA). Analysis of variance (ANOVA) was used to evaluate the significance of the differences between two groups. P≤0.05 was considered statistically significant.

Results

CXCR7 is upregulated in HCC cell lines. Western blotting and qRT-PCR assays demonstrated CXCR7 protein and mRNA expression in HCCLM3 cells. The qRT-PCR showed that CXCR7 mRNA level was obviously increased in HCCLM3 compared to the SMMC-7721 (Fig. 1A). Consistent with the mRNA results, the protein level also was significantly upregulated in HCCLM3 compared to the SMMC-7721 (Fig. 1B). The data implied that CXCR7 is overexpressed in HCC cells.

Overexpression of CXCR7 promotes the angiogenic capacity in HCC cells. Firstly, overexpression of CXCR7 in the HCCLM3 was confirmed by western blotting (Fig. 2A). The effect of CXCR7 inducing angiogenesis in the HCCLM3 was investigated by tube formation assay. CM from CXCR7 overexpression in HCCLM3 notably induced the tube formation compared to the control (Fig. 2B). In addition, CXCR7 overexpressing in HCCLM3 significantly induced the HUVEC migration (Fig. 2C). Moreover, CM from CXCR7 overexpression of HCCLM3 cells increased the second- and third-order vessel number in the CAM (Fig. 2D). The results together indicated, that CXCR7 induced the angiogenesis capacity of HCCLM3 cells *in vitro*.

Silencing CXCR7 reduces the angiogenic capacity in HCC cells. To better confirm the effect of CXCR7 on angiogenesis in the HCC progression, CXCR7 was knocked down in the HCCLM3 cells and was confirmed by western blotting (Fig. 3A). Compared to the control, CM from CXCR7 knockdown cells decreased HUVEC tube formation (Fig. 3B)

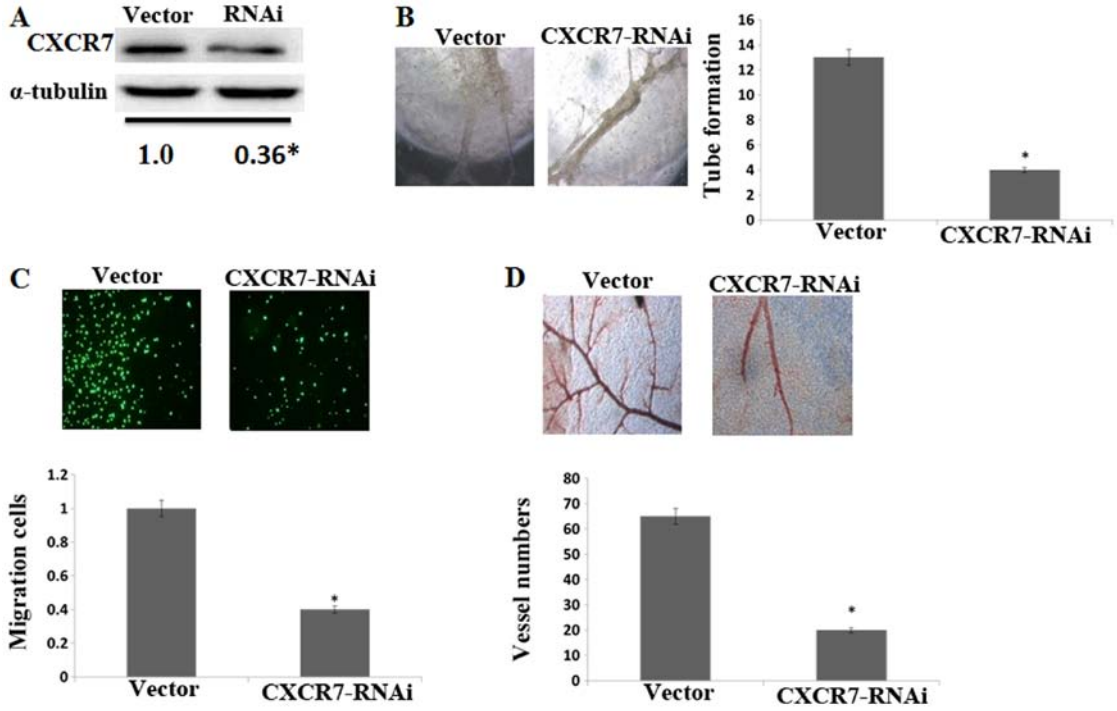

Figure 3. Knockdown of CXCR7 decreases the angiogenic capacity of HCC cells *in vitro*. (A) Western blotting of CXCR7-shRNA-transduced HCCLM3 protein expression in HCCLM3 cells; α-tubulin was used as a control. The numbers show the relative level of protein compared to the control. (B) The images (left) and quantification (right) of tube formation by HUVECs cultured in CM. (C) The migration images and quantification of HUVECs after incubation in CM. (D) The images (left) and quantification (right) of blood vessels in the CAM assay when stimulated by CM. Data are means ± SD of three independent experiments. *P<0.05. CXCR7, C-X-C chemokine receptor type 7; HCC, hepatocellular carcinoma; HUVECs, human umbilical vein endothelial cells; CM, conditioned medium; CAM, chicken chorioallantoic membrane.

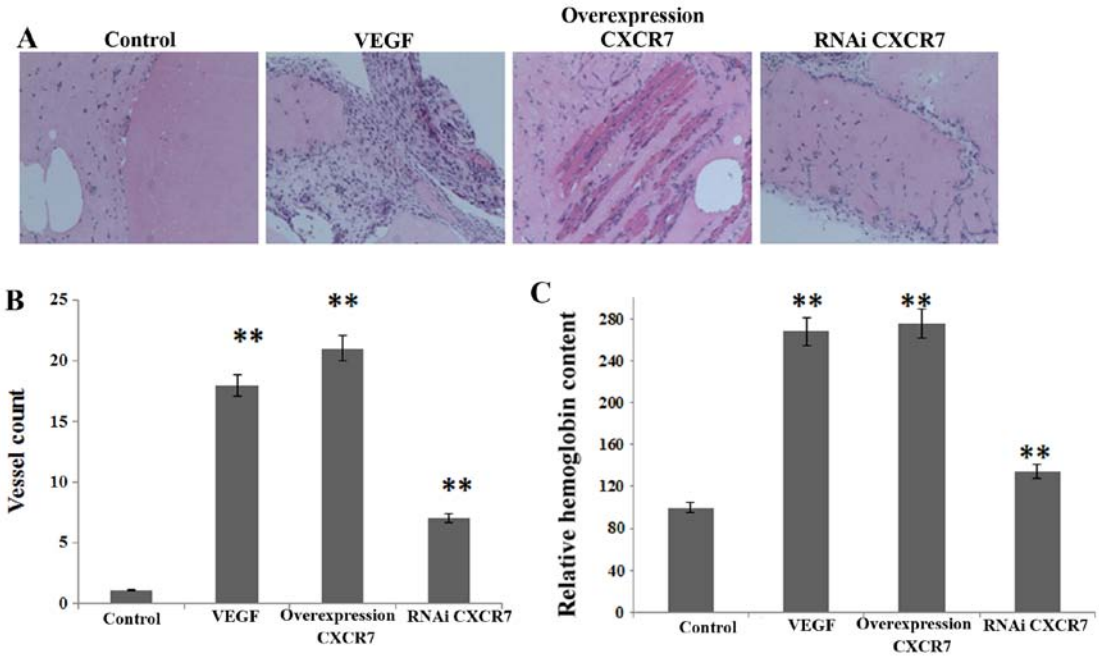

Figure 4. Matrigel plug assay of *in vivo* angiogenesis. (A) The images show vascularization of Matrigel plug under the various experimental conditions. (B) Matrigel plug assay was used quantitatively as vessel counts of the sponges under the various experimental conditions. (C) The relative Hb content of the plug under the various experimental conditions. Data are means ± SD of three independent experiments. **P<0.01. Hb, hemoglobin.

and migration (Fig. 3C). CM from CXCR7 knockdown cells also decreased the second- and third-order vessel number in the CAM assay (Fig. 3D). These data illustrated that CXCR7 is involved in angiogenesis of HCCLM3 cells.

Overexpression of CXCR7 induces the angiogenic capacity in Matrigel plug assay. Vascularization was calculated by Matrigel plug assay (Fig. 4A). The C57BL/6 mice were injected with Matrigel containing aliquots of control, overexpressing

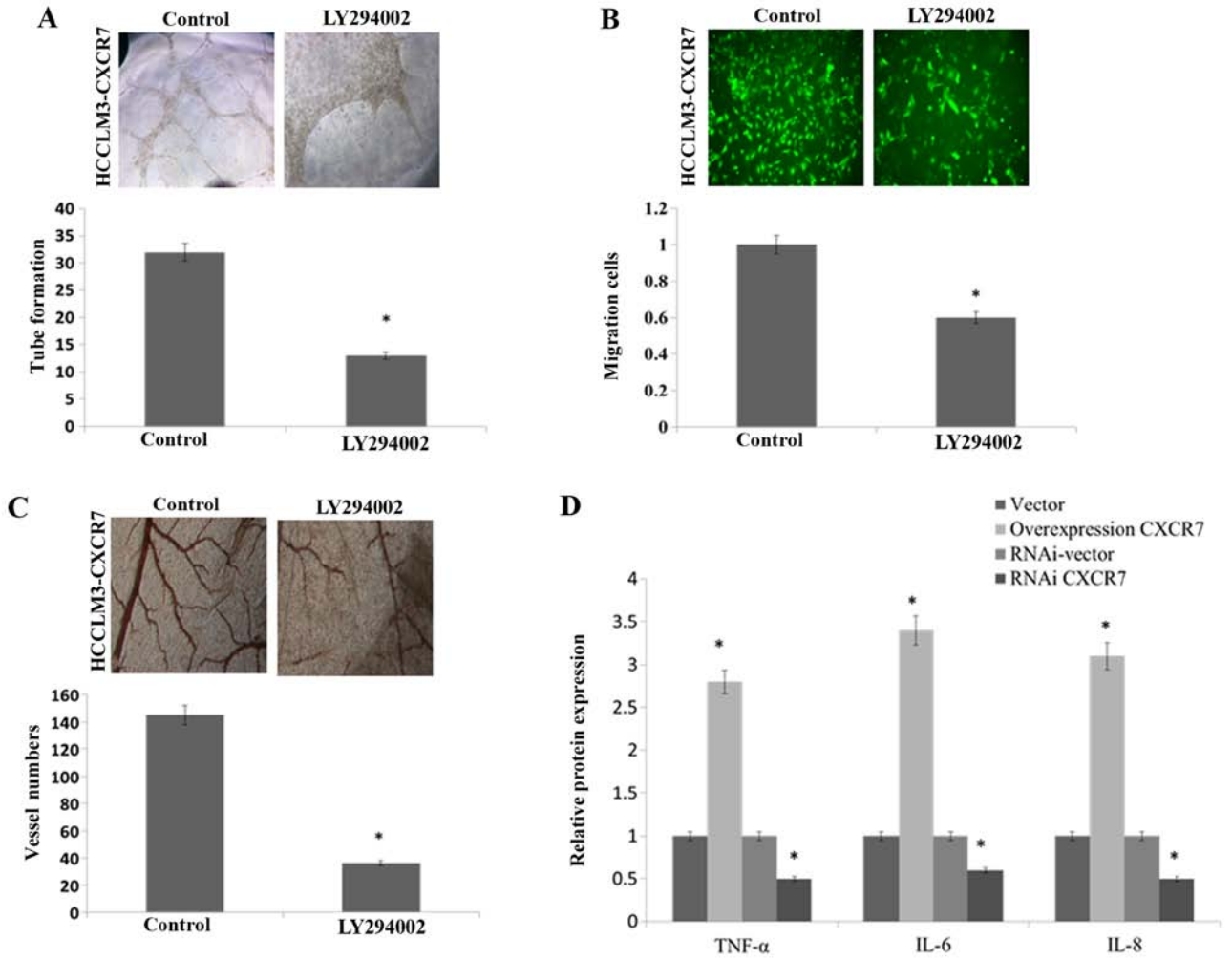

Figure 5. Overexpression of CXCR7 enhances the angiogenic capacity of HCC cells via activating the AKT pathway. CXCR7-overexpressing HCC cells were inhibited with LY294002, which uses as a specific AKT inhibitor. (A) The images and quantification of tube formation by HUVECs on CM from the indicated cells. (B) The migration images and quantification of HUVECs after incubation in CM. (C) The images and quantification of blood vessels in the CAM assay when stimulated by CM. (D) ELISA of TNF-α, IL-6 and IL-8 protein expression in the indicated cells *P<0.05. CXCR7, C-X-C chemokine receptor type 7; HCC, hepatocellular carcinoma; HUVECs, human umbilical vein endothelial cells; CM, conditioned medium; CAM, chicken chorioallantoic membrane; ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor; IL, interleukin.

or silenced CXCR7 in HCCLM3, plus heparin (50 U/ml) and SDF-1α (100 ng/ml). As shown in Fig. 4B and C, the relative hemoglobin content and vessel number showed that angiogenesis was inhibited by CM containing CXCR7-silenced HCCLM3, whereas it was partially promoted by CM containing overexpressing CXCR7.

CXCR7 promotes the angiogenic capacity of HCC cells via activating the AKT signaling pathway. The CXCR7 affected the angiogenic capacity of HCCLM3 via activating AKT signaling. We used the AKT signaling inhibitor LY294002. The stimulatory effects of CM derived from CXCR7 overexpressing HCCLM3 on HUVEC tube formation (Fig. 5A), migration (Fig. 5B) and the second- and third-order vessel number in the CAM assay (Fig. 5C) was significantly reduced by using LY294002. Taken all together, these results suggested that CXCR7 enhances the angiogenic capacity of HCCLM3 cells via activation of the AKT signaling pathway. The expression of AKT target protein, including TNF-α, IL-6 and IL-8, was induced in CXCR7 overexpressing HCCLM3 and reduced in CXCR7 knockdown HCCLM3 (Fig. 5D). Moreover,

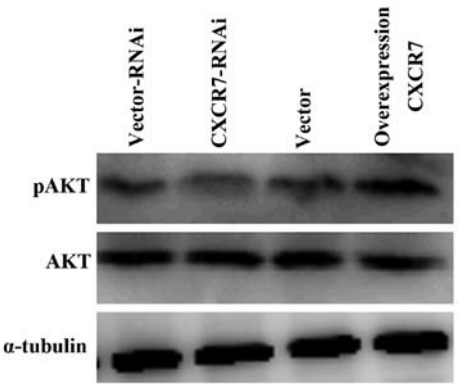

Figure 6. The protein expression analysis of the pAKT, total AKT; α-tubulin was used as a loading control. Data are means ± SD of three independent experiments. pAKT, phosphorylated AKT.

western blotting demonstrated that overexpression of CXCR7 upregulated the phosphorylated AKT expression but did not significantly impact the total AKT protein expression (Fig. 6).

These data implied that the AKT pathway may trigger angiogenesis of CXCR7 in HCCLM3 cells.

Discussion

There is abundant evidence to show that CXCR7 overexpression is involved in HCC progress, including breast, lung, prostate and pancreatic cancers (23,25). It has been demonstrated that knockdown of CXCR7 expression significantly reduces SMMC-7721 cell invasion, adhesion and angiogenesis. Moreover, decreased CXCR7 expression inhibited tumor growth in a mouse model of HCC. The highly specific CXCR7 antagonist CCX771 also was used. In the past report, CXCL12 induced Huh7, SNU449 and SNU475 cell migration, and the migration was blocked by CCX771/anti-CXCR7. Similarly, the CCX771 significantly inhibited glioma cell proliferation and invasion (26). The study provides new insights into the significance of CXCR7 in invasion and angiogenesis of tumors (27,28). Although the study shows the importance of CXCR7 in HCC invasion, angiogenesis and tumor growth, the role of CXCR7 effect the HUVEC function in tumor microenvironment is not fully established. In the present study, CXCR7 was confirmed to be involved in metastatic HCC. Both the past studies and our data showed overexpression of CXCR7 in a highly metastatic HCC. Also, increased expression of CXCR7 in HCCLM3 induced the angiogenic capacity *in vitro*. Furthermore, silence of CXCR7 decreased the angiogenic capacity of HUVECs. The data showed that CXCR7 may induce angiogenesis via activating the AKT signaling pathway. Collectively the findings suggested the potential effect of CXCR7 in angiogenic capacity of HCC cells.

The overexpression of CXCR7 suggested several potential unknown functions. Although substantial research has suggested that CXCR7 may serve as an oncogene in many tumor types, the molecular mechanism of CXCR7 has not been accurately demonstrated. In our study, we found that the overexpression of CXCR7 induced the tube formation and migration of HUVECs and significantly induced the second- and third-order vessel number of CAM. The data showed the potential effect of CXCR7 angiogenic activities in HCC cells. Furthermore, overexpression of CXCR7 notably enhanced the AKT pathway activity, suggesting that phosphorylated AKT plays a key role in the CXCR7-induced angiogenesis of HCC progression. AKT signaling pathway is known to regulate inflammatory responses, and other physiological and pathological functions including cancer progression. The findings suggested that CXCR7 induces HCC angiogenesis via activating the AKT signaling pathway.

Additionally, overexpression of CXCR7 induced TNF-α, IL-6, and IL-8 expression. TNF-α promoted angiogenesis and regulated blood vessel remodeling *in vivo*; IL-6 and IL-8 also promoted VEGF expression and tumor angiogenesis. Increasing and compelling epidemiological evidence has been suggested that the inflammatory microenvironment plays a key role in tumor cell proliferation, angiogenesis and metastasis (29-31). TNF-α is known as a key regulator of inflammation-related cancer, including HCC (32). IL-6 is necessary for HCC progression in animal models, with hepatic-associated macrophages representing a major paracrine IL-6 expression during HCC development and autocrine IL-6 inducing notably HCC initiation (33-35). IL-8 induced HCC grade, metastasis and recurrence (36,37). It would be worth exploring the TNF-α, IL-6, and IL-8 functions in HCC angiogenesis. The TNF-α, IL-6, and IL-8 expression in HCC cells remained to be clarified and should be investigated further.

In conclusion, our study indicated that CXCR7 is upregulated and induces angiogenic capacity in HCC by activation of the AKT pathway. These data may give new insight into the angiogenesis mechanism in HCC; and CXCR7 may be a new therapeutic target for HCC.

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