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CCL5/CCR5 axis induces vascular endothelial growth factor-mediated tumor angiogenesis in human osteosarcoma microenvironment

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Chemokines modulate angiogenesis and metastasis that dictate cancer development in tumor microenvironment. Osteosarcoma is the most frequent bone tumor and is characterized by a high metastatic potential. Chemokine CCL5 (previously called RANTES) has been reported to facilitate tumor progression and metastasis. However, the crosstalk between chemokine CCL5 and vascular endothelial growth factor (VEGF) as well as tumor angiogenesis in human osteosarcoma microenvironment has not been well explored. In this study, we found that CCL5 increased VEGF expression and production in human osteosarcoma cells. The conditioned medium (CM) from CCL5-treated osteosarcoma cells significantly induced tube formation and migration of human endothelial progenitor cells. Pretreatment of cells with CCR5 antibody or transfection with CCR5 specific siRNA blocked CCL5-induced VEGF expression and angiogenesis. CCL5/CCR5 axis demonstrably activated protein kinase Cδ (PKCδ), c-Src and hypoxia-inducible factor-1 alpha (HIF-1α) signaling cascades to induce VEGF-dependent angiogenesis. Furthermore, knockdown of CCL5 suppressed VEGF expression and attenuated osteosarcoma CM-induced angiogenesis *in vitro* and *in vivo*. CCL5 knockdown dramatically abolished tumor growth and angiogenesis in the osteosarcoma xenograft animal model. Importantly, we demonstrated that the expression of CCL5 and VEGF were correlated with tumor stage according the immunohistochemistry analysis of human osteosarcoma tissues. Taken together, our findings provide evidence that CCL5/CCR5 axis promotes VEGF-dependent tumor angiogenesis in human osteosarcoma microenvironment through PKCδ/c-Src/HIF-1α signaling pathway. CCL5 may represent a potential therapeutic target against human osteosarcoma.

Abbreviations: CAM, chick chorioallantoic membrane; CM, conditioned medium; EPCs, endothelial progenitor cells; HIF-1, hypoxia-inducible factor 1; HREs, hypoxia response elements; PBS, phosphate-buffered saline; PKCδ, protein kinase Cδ; shRNA, small hairpin RNA; siRNA, small interfereing RNA; VEGF, vascular endothelial growth factor.

Introduction

Tumor growth and dissemination is the result of dynamic interactions between tumor cells themselves, and also with components of the tumor microenvironment, including endothelial cells, smooth muscle cells, stromal fibroblasts and tumor-associated macrophages (1). The interaction between tumor cells and their microenvironment is dramatically facilitated by the soluble inflammatory mediators, such as cytokines and chemokines (2). Chemokines and chemokine receptors are instrumental players in inflammation, immunosurveillance and cancer progression. The chemokine–chemokine receptor axis has been indicated to regulate tumor growth, angiogenesis, invasion and tissue-specific metastasis (3,4). Regulated upon Activation Normal T cell Expressed and Secreted (RANTES, CCL5) is an inflammatory chemokine, primarily identified as potent inducers of leukocyte motility (5). CCL5 mediates its biological activities through activation of G protein-coupled receptors, CCR1, CCR3 or CCR5, and binds to glycosaminoglycans. CCL5 is associated with chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and cancer. CCL5 can be expressed and secreted either by cancer cells or by non-malignant stromal cells in the microenvironment (6). Autocrine secretion of CCL5 controls the migration and invasion of cancer cells *in vitro* (7). CCL5 expression has been implicated in the correlation with a variety of human tumors, including prostate, cervical, colon and lung cancers (8–11). The most striking findings thus far have been with breast cancer. Several investigations have indicated that CCL5 was found to be highly expressed in breast cancer patients and that expression levels correlated with advanced disease course (12,13).

Osteosarcoma is a high-grade malignant bone tumor that most frequently affects children and young adolescents (14,15). The chemotherapies regimens are not fully effective, and osteosarcoma shows a predilection for metastasis to the distant organs. Recurrence usually occurs as pulmonary metastases or, less frequently, metastases to distant bones or as a local recurrence (1,16). Angiogenesis has been demonstrated to facilitate metastatic osteosarcoma within the tumor microenvironment (17). Angiogenesis is a key event in the tumor growth and metastatic cascade of human cancers. In angiogenic processes, endothelial cells must undergo migration, proliferation and tube formation to form tumor neovessel (18). Subsequent studies indicate that tumor angiogenesis is also supported by the mobilization and functional incorporation of endothelial progenitor cells (EPCs [19]). Recently, EPCs have been proposed to mediate early tumor growth and late tumor metastasis by intervening with the angiogenic switch (20). Vascular endothelial growth factor (VEGF), one of the major angiogenic factors, that is released by most types of cancer stimulates angiogenesis to the tumour tissue. The induction of VEGF expression in tumors can be caused by numerous environmental factors, such as hypoxia, growth factors and chemokines (21). Compelling evidences indicate that chemokines exert the angiogenesis directly or as a consequence of leukocyte trafficking and/or the induction of growth factor expression such as that of VEGF in the microenvironment (3). In recent years, VEGF antagonists significantly attenuate tumor angiogenesis and successfully controls the disease progression of many cancers. Therefore, targeting VEGF may represent a potential approach for preventing osteosarcoma angiogenesis and metastasis (22).

Hypoxia-inducible factor 1 (HIF-1), a transcription factor that is critical for tumor adaptation to microenvironmental stimuli, regulates the blood supply through its effects on the expression of VEGF (23). HIF-1 exists as a heterodimeric complex consisting of HIF-1α and HIF-1β. The availability of HIF-1 is determined primarily by HIF-1α, which is regulated at the protein level in an oxygen-sensitive manner,

in contrast to HIF-1β, which is stably expressed. HIF-1α is expressed in many human tumors and renders cells able to survive and grow under hypoxic condition (24). During normoxia, HIF-1α is efficiently degraded through the von Hippel–Lindau-dependent ubiquitin-proteasome pathway (25). Under hypoxia, however, HIF-1α is not degraded and accumulates to form transcriptionally active complexes with HIF-1β. The HIF-1α and HIF-1β complex can then bind to hypoxia response elements (HREs) located in gene promoters to regulate transcription of VEGF that enhance cellular adaptation to hypoxia (26). In addition, a wide range of growth-promoting stimuli, cytokines and chemokines also induce modest HIF-1α accumulation (27). Several signaling pathways, such as phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), integrin-linked kinase (ILK) and protein kinase Cδ (PKCδ) have been shown to induce VEGF expression via HIF-1α-dependent mechanism (28–31).

Previous studies have confirmed that CCL5 is associated with the disease status and outcomes of cancers (6). VEGF is the most potent angiogenic mediator that is essential for angiogenesis and tumor growth (22). However, the regulation of CCL5 on VEGF expression in human cancer cells is mostly unknown. The mechanism underlying CCL5-mediated VEGF expression and tumor angiogenesis in human osteosarcoma microenvironment is also unclear. In this study, we investigated the relationship of CCL5 with VEGF expression and tumor angiogenesis, and further elucidated its mechanism of action in human osteosarcoma.

Materials and methods

Materials

The recombinant human CCL5 was purchased from PeproTech (Rocky Hill, NJ). ON-TARGETplus small interfering RNA (siRNA) of CCR5, PKCδ, c-Src, HIF-1α and control were purchased from Dharmacon Research (Lafayette, CO). Antimouse and antirabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for HIF-1α, HIF-1β, β-actin, CD31, control small hairpin RNA (shRNA) plasmid and CCL5 shRNA plasmid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for CCL5 and VEGF antibodies were purchased from Abcam (Cambridge, MA). Recombinant human VEGF, mouse monoclonal antibody specific for CCR5 and VEGF were purchased from R&D Systems (Minneapolis, MN). Rottlerin, PP2 and HIF-1α inhibitor were purchased from Calbiochem (San Diego, CA). The pHRE-luciferase construct was provided from Dr W.M. Fu (National Taiwan University, Taiwan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

Cell culture

The human osteosarcoma cell lines U2OS and MG63 were purchased from the American Type Culture Collection/Bioresource Collection and Research Center (BCRC) (Hsinchu, Taiwan) on August 2008 and February 2013, respectively. Short tandem repeat profiles were examined by BCRC before and after our study (July 2014) to ensure the quality and integrity of these two cell lines. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, L-glutamine, penicillin and streptomycin at 37°C with 5% CO_2.

Preparation of conditioned medium

In the series of experiments, osteosarcoma cells were treated with CCL5 alone for 24 h or pretreated with pharmacological inhibitors, including CCR5 mAb, rottlerin, PP2 and HIF-1α inhibitor followed by stimulation with CCL5 for 24 h. These inhibitors at the concentration we used did not significantly affect cell viability (Supplementary Figure S1, available at *Carcinogenesis* online). SiRNA-mediated knockdown were also applied to validate the findings from pharmacological intervention. After treatment, cells were washed and changed to serum-free medium. Conditioned medium (CM) was then collected 2 days after the change of medium and stored at −80°C until use.

Isolation and cultivation of EPCs

Ethical approval was granted by the Institutional Review Board of Mackay Medical College, New Taipei City, Taiwan (reference number: P1000002). Informed consent was obtained from healthy donors before the collection of peripheral blood (80 ml). The peripheral blood mononuclear cells were fractionated from other blood components by centrifugation on Ficoll-Paque plus (Amersham Biosciences, Uppala, Sweden) according to the manufacturer's instructions. CD34-positive progenitor cells were obtained from the isolated peripheral blood mononuclear cells using CD34 MicroBead kit and MACS Cell Separation System (Miltenyi Biotec, Bergisch Gladbach, Germany). The maintenance and characterization of CD34-positive EPCs were performed as described previously (31,32). Briefly, human CD34-positive EPCs were seeded on 1% gelatin-coated plasticware and cultured in MV2 complete medium (PromoCell, Heidelberg, Germany) with 20% defined fetal bovine serum (HyClone, Logan, UT). Experiments were performed by using cells from passages 8 to 12.

Tube formation assay

Matrigel (BD Biosciences, Bedford, MA) was dissolved at 4°C overnight, and 48-well plates were prepared with 150 µl Matrigel in each well after coating and incubating at 37°C for 30 min. After gel formation, EPCs (5×10^4 cells) were seeded per well on the layer of polymerized Matrigel in cultured media containing 50% MV2 complete medium and 50% osteosarcoma cells CM, followed by incubation for 16 h at 37°C. Then, EPCs tube formation was taken with the inverted phase contrast microscope. Tube branches and total tube length were calculated using MacBiophotonics Image J software.

Migration assay

Cell migration assay was performed using Transwell chambers with 8.0 µm pore size (Coring, Coring, NY). EPCs (1×10^4 cells/well) were seeded onto the upper chamber with MV2 complete medium, then incubated in the bottom chamber with 50% MV2 complete medium and 50% osteosarcoma cells CM. The plates were incubated for 24 h at 37°C in 5% CO2, and cells were fixed in 4% formaldehyde solution for 15 min and stained with 0.05% crystal violet in phosphate-buffered saline (PBS) for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cell migration was quantified by counting the number of stained cells in 10 random fields with the inverted phase contrast microscope and photographed.

Quantitative real-time PCR

Total RNA was extracted from osteosarcoma cells as described previously (33). Two micrograms of total RNA was reverse transcribed into cDNA using oligo (dT) primer. The quantitative real-time PCR analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). cDNA templates (2 µl) were added per 25 µl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, CA). The quantitative real-time PCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

Measurement of VEGF production

Human osteosarcoma cells were cultured in 24-well plates. After reaching confluence, cells were changed to serum-free medium. Cells were then treated with CCL5 alone for 24 h, or pretreated with pharmacological inhibitors or transfected with specific siRNA followed by stimulation with CCL5 for 24 h. After treatment, the medium was removed and stored at −80°C. Then, VEGF in the medium was determined using VEGF ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol.

Kinase activity assay

PKCδ and c-Src activities were assessed with a PKC Kinase Activity Assay Kit (Assay Designs, Ann Arbor, MI) and a c-Src Kinase Activity Assay Kit (Abnova, Taipei, Taiwan). The kinase activity kits are based on a solid phase ELISA that uses a specific synthetic peptide as substrate for PKCδ or c-Src, and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

Western blot analysis

Cells were lysed with lysis buffer as described previously (34). Cell homogenates were diluted with loading buffer and boiled for 5 min for detecting phosphorylation and protein expression. Total protein was determined and equal amounts of protein were separated by 8–12% SDS–PAGE and immunoblotted with specific primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used, and the signal detected using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously (31). DNA immunoprecipitated by anti-HIF-1α Ab was purified. The DNA was then extracted with phenol–chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by

1.5% agarose gel electrophoresis and visualized by UV light. The primers 5′-CCTTTGGGTTTTGCCAGA-3′ and 5′-CCAAGTTTGTGGAGCTGA-3′ were utilized to amplify across the VEGF promoter region.

Transfection and reporter assay

Cells were transfected with siRNA or HRE-Luc reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. After 24 h transfection, cells were pretreated with inhibitors for 30 min and then CCL5 or vehicle was added for 24 h. Cell extracts were then prepared, luciferase and β-galactosidase activities were measured.

Establishment of CCL5 shRNA stably transfected cells

CCL5 shRNA or control shRNA plasmids are transfected into cancer cells with Lipofetamine 2000 transfection reagent. Twenty-four hours after transfection, stable transfectants are selected in puromycin at a concentration of 10 μg/ml. Thereafter, the selection medium is replaced every 3 days. After 2 weeks of selection in puromycin, clones of resistant cells are isolated.

Chick chorioallantoic membrane assay

Angiogenic activity was determined using a chick chorioallantoic membrane (CAM) assay as described previously (35). Fertilized chicken eggs were incubated at 38°C in an 80% humidified atmosphere. On day 8, CM from U2OS/control-shRNA or U2OS/CCL5-shRNA cells (2×10^4 cells) deposited in the center of the chorioallantoic. CAM results were analyzed on the fourth day. Chorioallantoid membranes were collected for microscopy and photographic documentation. Angiogenesis was quantified by counting the number of blood vessel branch; at least 10 viable embryos were tested for each treatment. All animal works were done in accordance with a protocol approved by the China Medical University (Taichung, Taiwan) Institutional Animal Care and Use Committee.

In vivo Matrigel plug assay

Thirty male nude mice (4 weeks of age) were used and randomized into three groups: PBS (control), U2OS/control-shRNA or U2OS/CCL5-shRNA resuspended with Matrigel. Mice were subcutaneously injected with 0.2 ml Matrigel containing 0.1 ml osteosarcoma cells CM. On day 7, Matrigel plugs were excised, partly were fixed with 4% formalin, embedded in paraffin, and subsequently processed for CD31 staining using immunohistochemistry. They were also partly used for measuring the extent of blood vessel formation by hemoglobin assay.

In vivo tumor xenograft model

Twenty male nude mice (4 weeks of age) were used and randomized into two groups. For experimental cells growing exponentially, each implanted into 10 nude mice by subcutaneous injection; 1×10^6 cells (U2OS/control-shRNA or U2OS/CCL5-shRNA) were resuspended in 0.1 ml of serum-free RPMI-1640 and injected into the right flank. After 4 weeks, the mice were killed and tumors were excised for CD31 staining or hemoglobin assay. The mice were observed daily and the body weights were monitored for toxicity. The tumor volume and weight were also measured during this month.

Hemoglobin assay

All the sponges (Matrigel plugs or tumors) were processed for measuring blood vessel formation. Briefly, the amount concentration of hemoglobin in the vessels that have invaded the Matrigel or tumor were determined with Drabkin's reagent (Sigma–Aldrich) according to manufacturer instructions. Homogenized in 1 ml of RIPA lysis buffer and after centrifuged at 1000 r.p.m., 20 μl of supernatants were added to 100 μl of Darkin's solution. The mixture was allowed to stand 30 min at room temperature, and then readings were taken at 540 nm in a spectrophotometer. The results are expressed in milligrams per milliliter.

Immunohistochemistry

The human osteosarcoma tissue array was purchased from Biomax and Cybrdi (Rockville, MD, 15 cases for normal cartilage, 13 cases for type IIb osteosarcoma and 12 cases for type IIIb osteosarcoma). The tissues were placed on glass slides, rehydrated and incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. After trypsinization, sections were blocked by incubation in 3% bovine serum albumin in PBS. The primary antibody anti-human CCL5 or VEGF was applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. After being washed three times in PBS, the samples were treated with goat antimouse IgG biotin-labeled secondary antibodies at a dilution of 1:50. Bound antibodies were detected with an ABC kit (Vector Laboratories, Burlingame, CA). The slides were stained with chromogen diaminobenzidine, washed, counterstained with Delafield's hematoxylin, dehydrated, treated with xylene and mounted. According to the histologic scoring, the staining intensity was ranked into five groups: very strong (score 5), strong (score 4), moderate (score 3), weak (score 2), very weak (score 1) and negative (score 0) by two independent observers. The immunoreactivity score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining.

Statistics

The values given are mean ± SEM. The significance of difference between the experimental groups and controls was assessed by Student's *t* test. The difference is significant if the *P* value is <0.05.

Results

CCL5/CCR5 axis promotes VEGF expression and angiogenesis

Previous studies have shown that CCL5 directly promotes angiogenesis of endothelial cells and chemotaxis of human EPCs (36,37). However, it is still not well-recognized whether CCL5 stimulates tumor angiogenesis by VEGF production in human cancer cells, especially osteosarcoma cells. We first applied CCL5 to human osteosarcoma cell line and determined the expression of VEGF. The results showed that CCL5 concentration dependently increased VEGF mRNA expression and production in human osteosarcoma cells (Figure 1A and B). The process of angiogenesis mainly involves endothelial cells proliferation, migration and tube formation to form new blood vessels (18). We then used an *in vitro* EPCs model to examine whether CCL5-dependent VEGF expression induced angiogenesis. As shown in Figure 1C, the capillary tube like structure was facilitated by the CM from CCL5-treated osteosarcoma cells. Furthermore, CM from CCL5-treated osteosarcoma cells enhanced tube formation and migration of EPCs in a concentration-dependent manner (Figure 1D and E). To elucidate CCL5-dependent VEGF, plays an important role in angiogenesis, the VEGF antibody was used. We found that addition of VEGF-neutralizing antibody to the CCL5-treated CM significantly prevented CCL5-induced migration and tube formation of EPCs. These results indicate that CCL5-dependent VEGF expression promotes angiogenesis *in vitro*.

It has been reported that CCL5 increases angiogenesis through interaction with its specific receptor CCR5 (36). Because our previous report indicated that osteosarcoma cells expressed functional CCR5 (34), we further examined the role of CCR5. As shown in Figure 1F and G, CCL5-induced expression of VEGF could be inhibited by blocking CCR5 using either anti-CCR5 mAb or CCR5 siRNA transfection. Furthermore, CM from osteosarcoma cells demonstrated that CCR5 mAb and CCR5 siRNA both significantly reduced CCL5-mediated migration and tube formation of EPCs (Figure 1H and I). These data suggest that CCL5 and CCR5 interaction promotes angiogenesis by VEGF expression.

PKCδ/c-Src signaling pathway is involved in CCL5-mediated VEGF expression and angiogenesis

The activation of PKCδ has been reported to increase tumor angiogenesis and VEGF production (30). To examine whether PKCδ is involved in CCL5-mediated VEGF expression and angiogenesis, the PKCδ inhibitor rottlerin was used. Pretreatment of cells with rottlerin reduced CCL5-induced expression of VEGF. Additionally, transfection with PKCδ siRNA specifically reduced CCL5-induced VEGF expression (Figure 2A and B). Moreover, CCL5-mediated EPCs migration and tube formation were also diminished by treatment with PKCδ inhibitor rottlerin and PKCδ siRNA (Figure 2C and D). Incubation of U2OS cells with CCL5 increased PKCδ kinase activity in a time-dependent manner (Figure 2E). Pretreatment of cells with CCR5 mAb significantly blocked CCL5-induced PKCδ kinase activity (Figure 2F). These results demonstrate that CCL5 and CCR5 interaction promotes VEGF expression and angiogenesis via PKCδ-dependent pathway.

Several studies have demonstrated that PKCδ mediated the activation of c-Src kinase (38,39). We next examined whether PKCδ/c-Src pathway is involved in the CCL5-induced VEGF expression and angiogenesis. As shown in Figure 3A and B, CCL5-induced VEGF expression was markedly attenuated by pretreatment with c-Src inhibitor PP2 and transfection with c-Src siRNA. Furthermore, CCL5-mediated EPCs migration and tube formation were also profoundly suppressed by treatment with c-Src inhibitor PP2 and c-Src siRNA (Figure 3C and D). Importantly, CCL5-concentration dependently

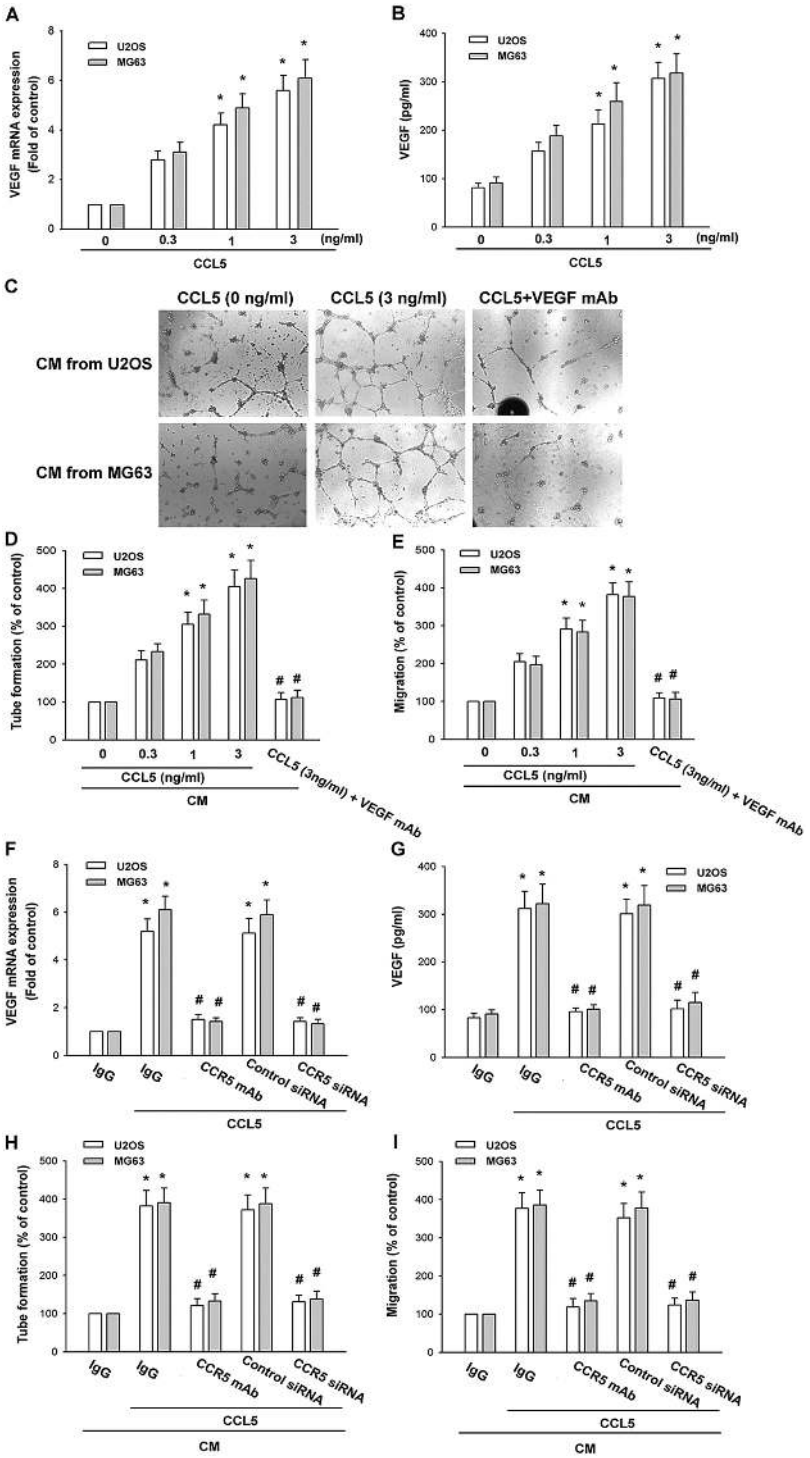

Fig. 1. CCL5 and CCR5 interaction promotes VEGF expression and angiogenesis. (**A** and **B**) Cells (U2OS and MG63 cells) were incubated with CCL5 (0–3 ng/ml) for 24 h, and VEGF expression was examined by quantitative real-time PCR and ELISA. (**C**–**E**) Cells were incubated with CCL5 (0–3 ng/ml) for 24 h. The medium was collected or treated with VEGF-neutralizing antibody (5 μg/ml) for 30 min before applied to EPCs for 24 h. The capillary-like structures formation and cell migration in EPCs were examined by tube formation and Transwell assay. (**F** and **G**) Cells were pretreated with CCR5 mAb (5 μg/ml) for 30 min or transfected with CCR5 siRNA for 24 h followed by stimulation with CCL5 (3 ng/ml) for 24 h, and VEGF expression was examined by quantitative real-time PCR and ELISA. (**H** and **I**) In addition, the medium was collected as CM and then applied to EPCs for tube formation and Transwell assay. Results are expressed as the mean ± SE. * $P < 0.05$ compared with control; # $P < 0.05$ compared with CCL5-treated group.

increased the activity of c-Src kinase, and both CCR5 mAb and PKCδ inhibitor rottlerin significantly inhibited CCL5-activeted c-Src kinase activity in U2OS cells (Figure 3E and F). Taken together, CCL5/CCR5 axis appears to act through PKCδ/c-Src signaling pathway to enhance angiogenesis and VEGF expression in human osteosarcoma cells.

CCL5/CCR5 axis induces HIF-1α activation for VEGF expression and angiogenesis

Hypoxia-inducible factor 1 (HIF-1), a pivotal transcription factor that is critical for VEGF expression in tumor microenvironment (27). We therefore sought to investigate whether HIF-1 activation was involved

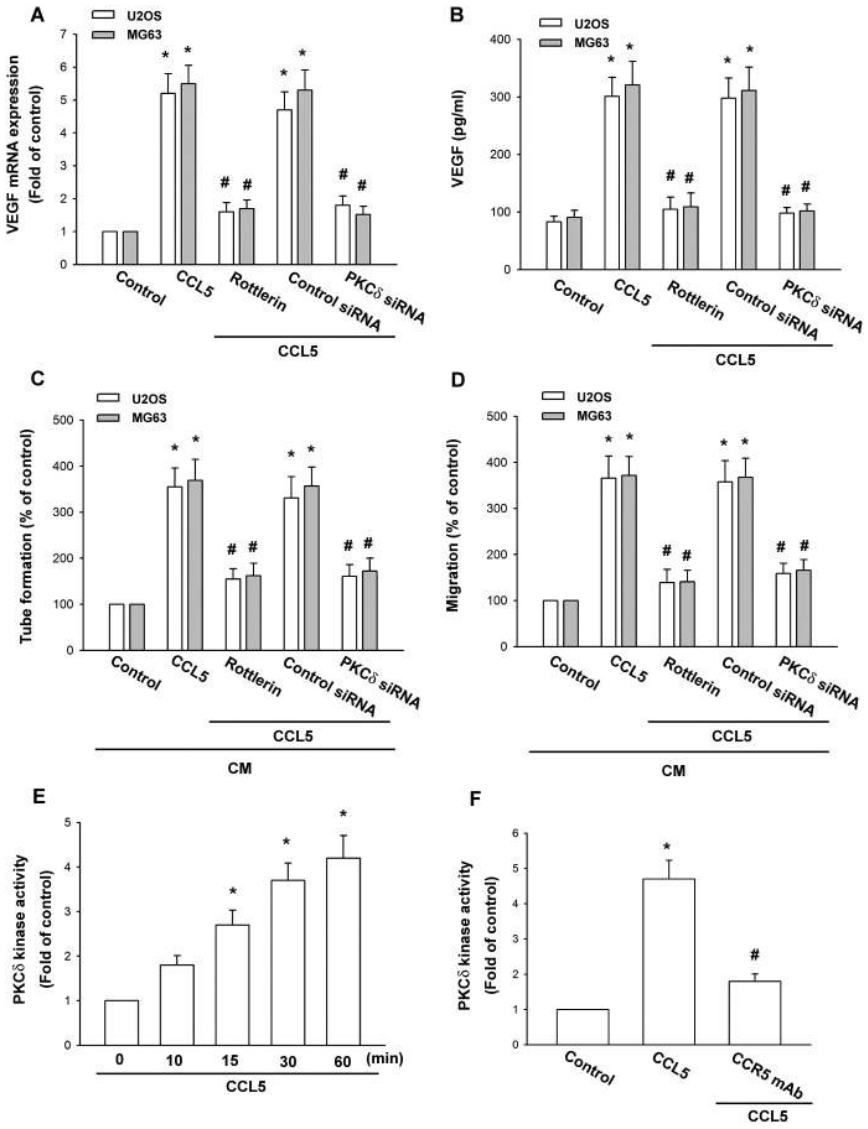

Fig. 2. PKCδ is involved in CCL5–induced VEGF expression and angiogenesis. (**A** and **B**) Cells were pretreated with the rottlerin (3 μM) for 30 min or transfected with PKCδ siRNA for 24 h followed by stimulation with CCL5 (3 ng/ml) for 24 h, and VEGF expression was examined by quantitative real-time PCR and ELISA. (**C** and **D**) In addition, the medium was collected as CM and then applied to EPCs for 24 h. The capillary-like structures formation and cell migration in EPCs were examined by tube formation and Transwell assay. (**E** and **F**) U2OS cells were incubated with CCL5 (0–3 ng/ml) for the indicated times, or pretreated with the CCR5 mAb (5 μg/ml) for 30 min followed by stimulation with CCL5 (3 ng/ml) for 60 min, and the activity of PKCδ was determined by PKCδ kinase assay. Results are expressed as the mean ± SE. $*P < 0.05$ compared with control; $\#P < 0.05$ compared with CCL5-treated group.

in CCL5-induced VEGF expression in human osteosarcoma cells. We found that pretreatment with HIF-1α inhibitor and transfection with HIF-1α siRNA both markedly antagonized CCL5-induced VEGF expression (Figure 4A and B). CCL5-mediated EPCs migration and tube formation were significantly suppressed by treatment with HIF-1α inhibitor and HIF-1α siRNA (Figure 4C and D). The results from western blot indicated that CCL5 significantly increased protein level of HIF-1α time dependently (Figure 4E). However, CCL5 did not affect the mRNA level of HIF-1α using quantitative real-time PCR analysis (Figure 4F). These results suggest that CCL5 increases the accumulation of HIF-1α, possibly by enhancing HIF-1α protein stability, which subsequently promotes VEGF expression and angiogenesis.

It has been reported that c-Src induced HIF-1α protein accumulation via a general increase in cap-dependent translation (40). We further explored whether PKCδ/c-Src signaling pathway was involved in CCL5-induced HIF-1α activation in human osteosarcoma cells. We performed chromatin immunoprecipitation assay to examine the DNA binding activity of HIF-1α in CCL5-treated cells. As shown in Figure 4G, the *in vivo* binding of HIF-1α to the HRE element of the VEGF promoter occurred after CCL5 stimulation. The binding of HIF-1α to the HRE element by CCL5 was markedly attenuated by CCR5 mAb, rottlerin and PP2. Moreover, HIF-1α activation was also evaluated using HRE-luciferase assay. We found that CCL5-induced HRE-luciferase activity was significantly reduced by pretreatment with CCR5 mAb, rottlerin and PP2 (Figure 4H). Based upon these finding, we suggest that CCR5/PKCδ/c-Src signaling pathway is involved in CCL5-induced HIF-1α activation.

Knockdown of CCL5 impairs angiogenesis in vitro and in vivo

To confirm the CCL5-mediated VEGF-dependent angiogenesis in human osteosarcoma cells, the CCL5-shRNA expression cells were established. The expression of CCL5 and VEGF was reduced by CCL5-shRNA in U2OS/CCL5-shRNA cells (Figure 5A). We found that CM from U2OS/control-shRNA cells increased tube formation and migration of EPCs. Knockdown of CCL5 significantly suppressed CM-mediated EPCs migration and tube formation (Figure 5B and C). In addition, the effect of CCL5 on angiogenesis *in vivo* was

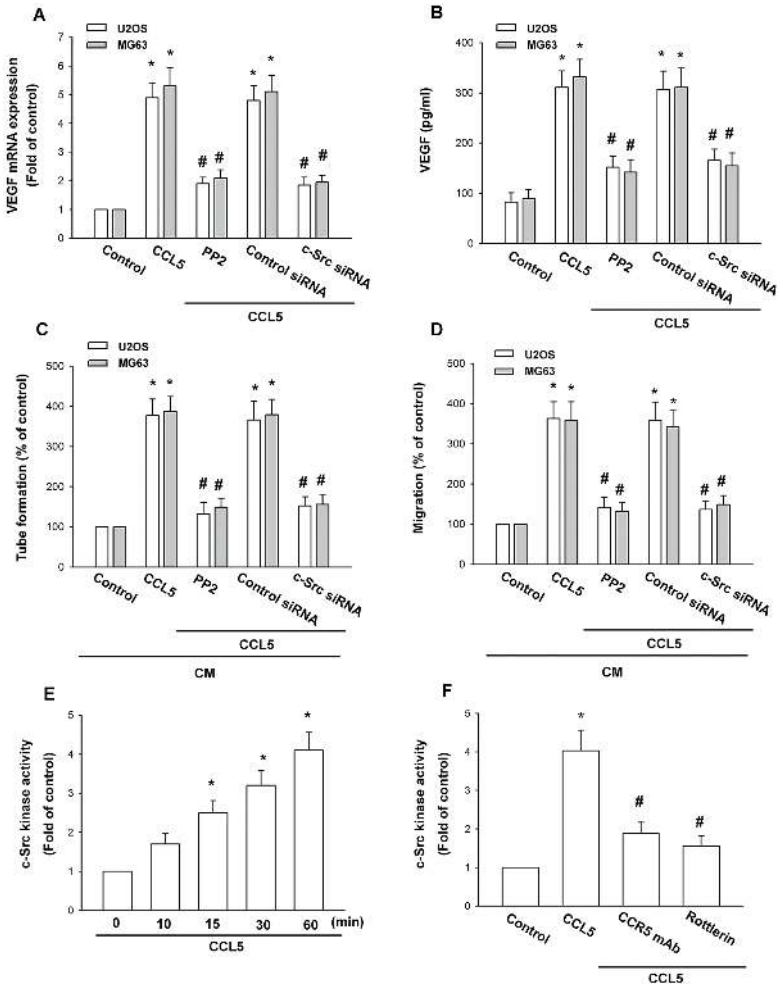

Fig. 3. c-Src is involved in CCL5–induced VEGF expression and angiogenesis. (**A** and **B**) Cells were pretreated with the PP2 (3 μM) for 30 min or transfected with c-Src siRNA for 24 h followed by stimulation with CCL5 (3 ng/ml) for 24 h, and VEGF expression was examined by quantitative real-time PCR and ELISA. (**C** and **D**) In addition, the medium was collected as CM and then applied to EPCs for 24 h. The capillary-like structures formation and cell migration in EPCs were examined by tube formation and Transwell assay. (**E** and **F**) U2OS cells were incubated with CCL5 (0–3 ng/ml) for the indicated times, or pretreated with the CCR5 mAb (5 μg/ml) and rottlerin (3 μM) for 30 min followed by stimulation with CCL5 (3 ng/ml) for 60 min, and the activity of c-Src was determined by c-Src kinase assay. Results are expressed as the mean ± SE. * $P < 0.05$ compared with control; # $P < 0.05$ compared with CCL5-treated group.

evaluated by using the *in vivo* model of chick embryo CAM assay. CM from U2OS/control-shRNA cells increased angiogenesis in CAM was clearly observed. In contrast, CCL5-shRNA markedly reduced angiogenesis in CAM (Figure 5D). We next performed the Matrigel implant assay in mice to further confirm CCL5-mediated angiogenic response *in vivo*. The results showed that Matrigel mixed with CM from U2OS/control-shRNA cells increased microvessel formation. Accordingly, CM from U2OS/CCL5-shRNA cells significantly abolished neovascularization (Figure 5E). Knockdown of CCL5 also reduced microvessel formation in the Matrigel plugs by analyzing the CD31 and hemoglobin content (Figure 5E and F). Therefore, these results indicate that CCL5 plays an important role during osteosarcoma-mediated angiogenesis.

Essential role of CCL5 for tumor angiogenesis in human osteosarcoma microenvironment

Herein, we investigated whether CCL5 promoted tumor angiogenesis and progression in osteosarcoma. To determine the effect of CCL5-shRNA on tumor angiogenesis, osteosarcoma xenograft-induced angiogenesis model was used. Human osteosarcoma cells were mixed with Matrigel and injected into the flanks of nude mice. As shown in Figure 6A and B, knockdown of CCL5 profoundly suppressed tumor growth and volume in mice. We also evaluated the level of angiogenesis in tumor specimens from animals by determining the hemoglobin content. The results demonstrated that CCL5-shRNA markedly inhibited osteosarcoma-induced angiogenesis *in vivo* (Figure 6C). We next examined human osteosarcoma tissues for the expression of CCL5 and VEGF using immunohistochemistry. The expression of CCL5 and VEGF in osteosarcoma patients was significantly higher than that in normal cartilage. In addition, the high level of CCL5 expression correlated strongly with VEGF expression and tumor stage (Figure 6D). The quantitative data also exhibited the high positive relationship between the expression of CCL5 and VEGF in tissues obtained from osteosarcoma patients (Figure 6E). Overall, these results suggest that CCL5 promotes VEGF-mediated tumor angiogenesis in human osteosarcoma microenvironment (Figure 6F).

Discussion

Increasing evidences suggest that chemokines are produced by tumor cells as well as by cells of the tumor microenvironment. In this regard, chemokines are emerging as key mediators not only in the homing of cancer cells to metastatic sites but also in the recruitment of a number of different cell types to establish tumor microenvironment, facilitating tumor-associated angiogenesis and metastasis (1,6). Osteosarcoma is the most frequent primitive malignant tumor of the skeletal system and is characterized by an aggressive clinical course and metastatic potential (14). Previously we demonstrated

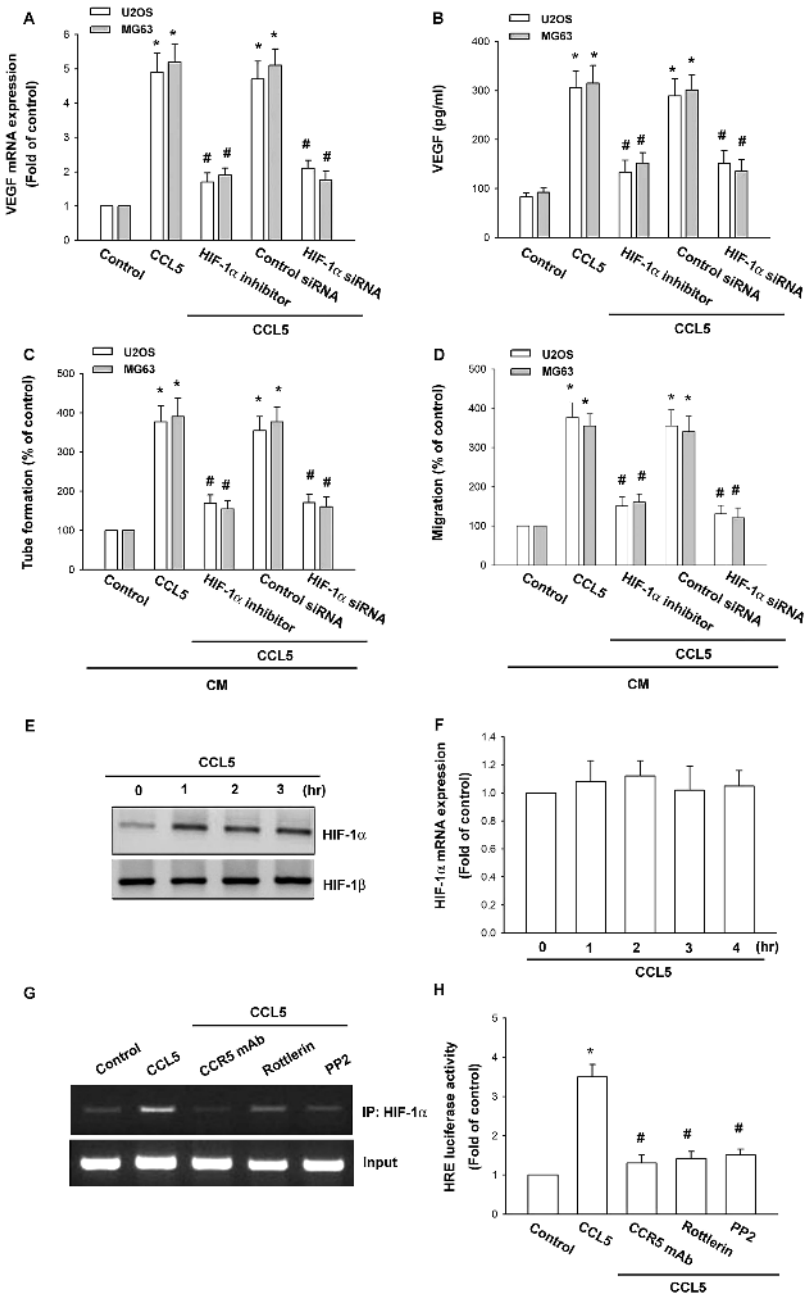

Fig. 4. HIF-1α activation is involved in CCL5-induced VEGF expression and angiogenesis. (**A** and **B**) Cells were pretreated with the HIF-1α inhibitor (10 μM) for 30 min or transfected with HIF-1α siRNA for 24 h followed by stimulation with CCL5 (3 ng/ml) for 24 h, and VEGF expression was examined by quantitative real-time PCR and ELISA. (**C** and **D**) In addition, the medium was collected as CM and then applied to EPCs for 24 h. The capillary-like structures formation and cell migration in EPCs were examined by tube formation and Transwell assay. (**E** and **F**) U2OS cells were incubated with CCL5 (3 ng/ml) for the indicated times and HIF-1α expression was determined by Western blotting and quantitative real-time PCR. (**G** and **H**) U2OS cells were pretreated for 30 min with CCR5 mAb, rottlerin or PP2 for 30 min followed by stimulation with CCL5 (3 ng/ml) for 120 min. The HIF-1α activation was examined by chromatin immunoprecipitation and HRE luciferase activity. Results are expressed as the mean ± SE. * $P < 0.05$ compared with control; # $P < 0.05$ compared with CCL5-treated group.

that CCL5/CCR5 chemokine axis promoted osteosarcoma migration through αvβ3 integrin (34). In this study, we found that CCL5 increased VEGF expression in human osteosarcoma cells, and subsequently induced tube formation and migration in human EPCs, indicating CCL5 promoted angiogenesis by the induction of VEGF. Tumor-derived chemokines have been shown to directly affect tumor cells in an autocrine manner (3). Here, we showed that knockdown of CCL5 suppressed VEGF expression and impaired angiogenesis *in vitro* and *in vivo*. We also demonstrated that CCL5-shRNA significantly abolished tumor growth and angiogenesis in human osteosarcoma. Compelling evidences indicate that CCL5 released by cells of the tumor microenvironment and acting through autocrine activities promotes proliferation, migration and invasion of tumor cells (6). Thus, we suggest that CCL5 released by osteosarcoma cells acts as an autocrine factor to stimulate VEGF expression, and contribute to tumor angiogenesis in osteosarcoma microenvironment. Furthermore, we found that the expression of CCL5 and VEGF in osteosarcoma patients were correlated with tumor stage, indicating CCL5 may be a potential predictive factor for disease progression of human osteosarcoma. Taken together, our results suggest that CCL5 promotes VEGF-dependent tumor angiogenesis in human osteosarcoma microenvironment. This is also the first study to demonstrate that CCL5 induces tumor angiogenesis by VEGF production in human cancer cells, especially osteosarcoma cells.

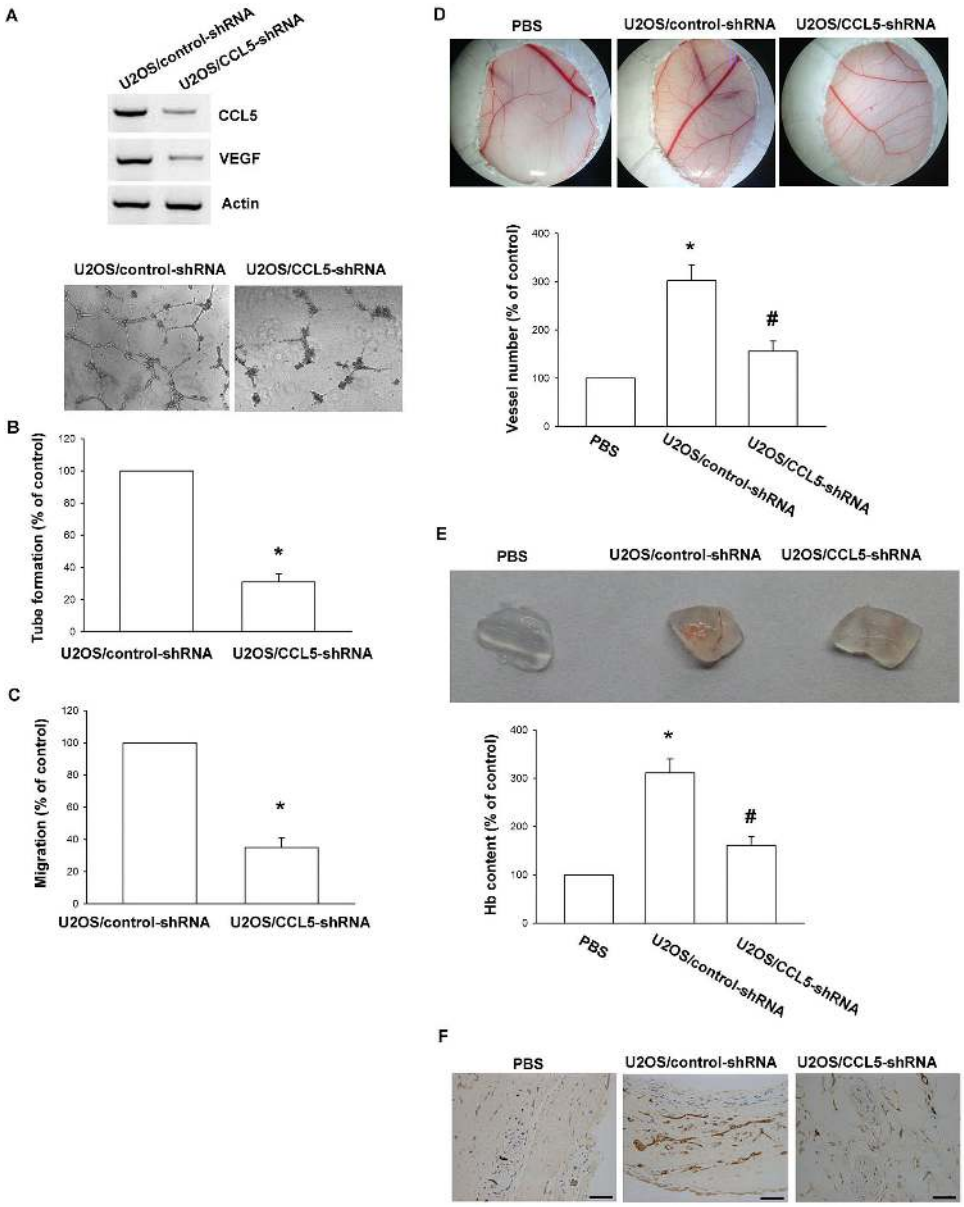

Fig. 5. Knockdown of CCL5 suppresses angiogenic effects in both *in vitro* and *in vivo* assays. (**A**) The protein expression of CCL5 and VEGF in U2OS/control-shRNA and U2OS/CCL5-shRNA cells was examined by Western blotting. (**B** and **C**) EPCs were incubated CM from U2OS/control-shRNA or U2OS/CCL5-shRNA for 24 h, and tube formation or cell migration was photographed under microscope or examined by Transwell. (**D**) Chick embryos were incubated with PBS, U2OS/control-shRNA CM or U2OS/CCL5-shRNA CM for 4 days, and then resected, fixed and photographed with a stereomicroscope. (**E** and **F**) Mice were injected subcutaneously with Matrigel mixed with PBS, U2OS/control-shRNA CM or U2OS/CCL5-shRNA CM for 7 days. The plugs were excised from mice and photographed, quantified of hemoglobin content and stained with CD31. Results are expressed as the mean ± SE. * $P < 0.05$ compared with control; #, $P < 0.05$ compared with CCL5-treated group.

Within the tumor microenvironment, chemokines and their receptors play critical roles in the regulation of angiogenesis, which enhances the progression and metastasis of many cancers (3). The first report of chemokine-dependent homing theory has demonstrated that CXC chemokine ligand 12 (CXCL12, also known as SDF-1) and its cell surface receptor CXC chemokine receptor 4 (CXCR4) coordinately modulate the metastasis in breast cancer (4). Accumulating evidences also suggest that CXCL12/CXCR4 signaling axis induce angiogenesis and progression of tumors by the activation of VEGF (41,42). Likewise, CCL5/CCR5 axis was proposed to regulate directional cancer migration, invasion and metastasis in various types of tumor, including osteosarcoma, breast and lung cancers (11,13,34). Recent studies have shown that CCL5 directly induces angiogenesis of endothelial cells and chemotaxis of human EPCs through the chemokine receptor CCR5 (36,37). Furthermore, CCL5–CCR5 interaction was highlighted and reported to promote breast cancer metastasis in tumor microenvironment (43). Current study found that CCL5-induced VEGF expression in osteosarcoma cells was attenuated by CCR5 mAb and CCR5 siRNA. Additionally, treatment of CCR5 mAb and CCR5 siRNA also significantly suppressed CCL5-indcued angiogenesis of EPCs. These results indicate that CCL5 and CCR5 interaction contributes to angiogenesis by increasing VEGF secretion from cancer cells, suggesting CCL5/CCR5 chemokine axis mediates an additional indirect effect on angiogenesis in tumor microenvironment.

The discovery of signaling pathway underlying CCL5/CCR5 axis-mediated VEGF expression helps us to understand the mechanism of tumor angiogenesis in the microenvironment and may lead us to develop effective therapy for cancer treatment. The activation of PKCδ has been implicated in the growth of several epithelial cancers

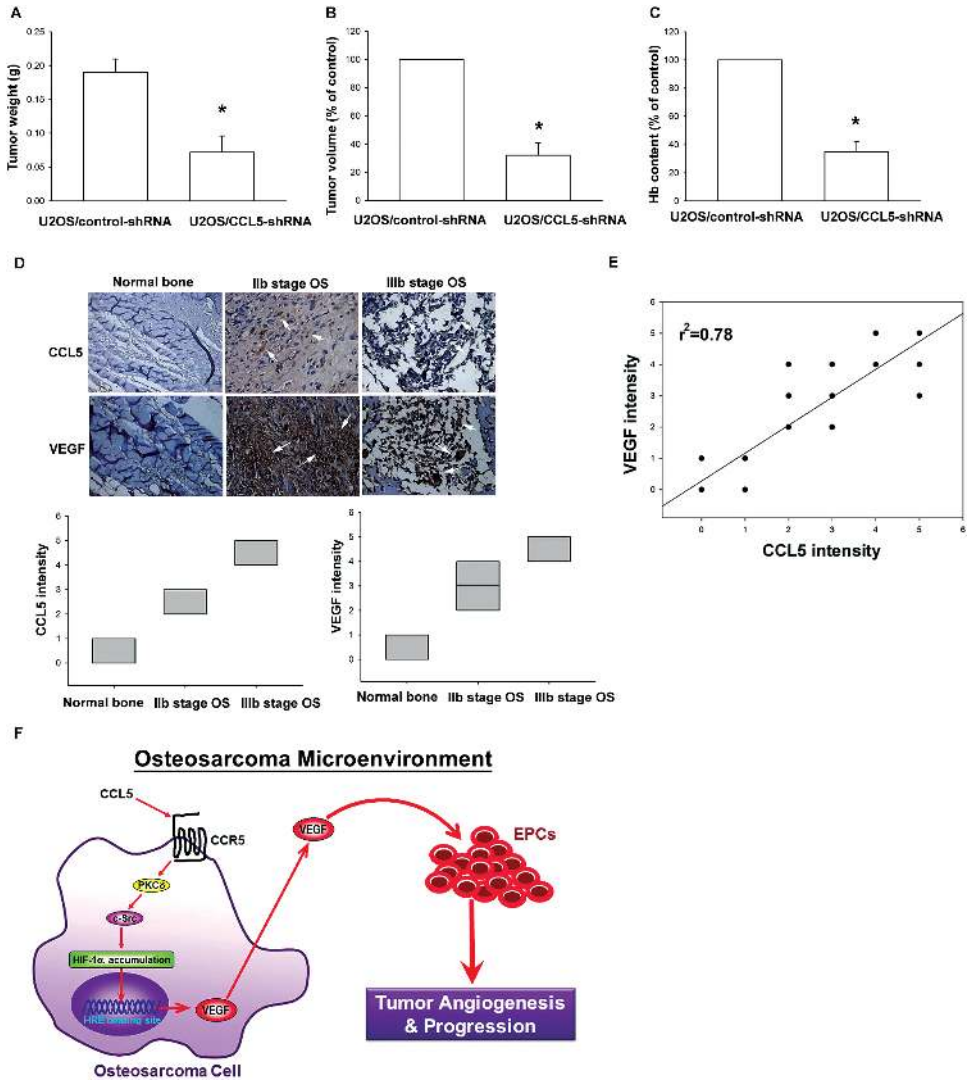

Fig. 6. Role of CCL5 on tumor angiogenesis in human osteosarcoma microenvironment. (**A**–**C**) U2OS/control-shRNA or U2OS/CCL5-shRNA cells were injected into flank sites of mice for 4 weeks, and then resected. The tumors were measured weight and volume, and quantified the hemoglobin levels. Results are expressed as the mean ± SE. * $P < 0.05$ compared with control. (**D**) The correlation between expression of CCL5 and VEGF as well as tumor stage. Three representative specimens were shown; arrows point to the positive signals of CCL5 and VEGF. (**E**) Correlation analyses between CCL5 and VEGF expression in osteosarcoma were performed by Spearman rank correlation test ($r^2 > 0.5$). (**F**) Schematic diagram summarizes the mechanism of CCL5-mediated tumor angiogenesis and progression in human osteosarcoma microenvironment. CCL5/CCR5 axis induces tumor angiogenesis by VEGF production in human osteosarcoma through the activation of PKCδ, c-Src and HIF-1α signaling cascades.

(44). Recent study further indicates that PKCδ activation promotes tumor angiogenesis by increasing the levels of HIF-1α and VEGF in human prostate cancer xenograft (30). Here, we showed that CCL5-induced VEGF expression and angiogenesis were both inhibited by the specific PKCδ inhibitor rottlerin and PKCδ siRNA. Furthermore, CCL5-activated PKCδ activity was significantly blocked by CCR5 mAb in osteosarcoma cells. These data suggest that PKCδ activation is an important signaling molecule in CCL5-induced VEGF expression and angiogenesis. Src is a non-receptor tyrosine kinase that is deregulated in many types of cancer (45). Several reports have also indicated that c-Src is a downstream effector of PKCδ, plays a critical role in tumor progression and dissemination (46,47). Thus, we examined the potential role of c-Src in the signaling pathway for CCL5-induced VEGF expression. The results of this study demonstrated that pretreatment with c-Src inhibitor PP2 antagonized CCL5-induced VEGF expression and angiogenesis. This pathway was further confirmed by transfection with PKCδ siRNA markedly attenuated the enhancement of VEGF and angiogenesis by CCL5 stimulation. Moreover, CCL5-induced upregulation of c-Src activity was profoundly suppressed by either CCR5 mAb or PKCδ inhibitor rottlerin. Therefore, we suggest that CCL5/CCR5 axis promotes VEGF-dependent angiogenesis through PKCδ/c-Src signaling pathway in human osteosarcoma cells. HIF-1 is a key transcriptional factor that regulates gene expression of VEGF (24). Our data found that CCL5 significantly increased protein level of HIF-1α time dependently but not at mRNA level in osteosarcoma cells. Pretreatment with HIF-1α inhibitor and transfection with HIF-1α siRNA both markedly attenuated CCL5-induced VEGF expression and angiogenesis. HIF-1α nuclear translocation is necessary for the transcriptional activation of HIF-1-regulated VEGF expression (26). We subsequently demonstrated that CCL5 increased the binding of HIF-1α to the HRE element on VEGF promoter by chromatin immunoprecipitation assay. Using transient transfection with HRE-luciferase as an indicator of HIF-1α activity, we found that CCL5 also dramatically increased HRE-luciferase activity in osteosarcoma cells. Collectively, we suggest that CCL5 enhances the protein stability and DNA binding activity of HIF-1α to promote VEGF expression and angiogenesis. We next explored whether the CCR5/PKCδ/c-Src pathway is an upstream signal in CCL5-mediated

HIF-1α activation. Our results showed that CCL5 induced the binding of HIF-1α to the HRE element and HRE-luciferase activity were both markedly antagonized by CCR5 mAb, rottlerin and PP2. Previous evidence implies that PKCδ regulates the stability of HIF-1α in cervical adenocarcinoma cells under hypoxia, and knockdown of PKCδ inhibits hypoxia-induced VEGF expression and angiogenesis (48). c-Src kinase also induces HIF-1α activation through the cap-dependent translation (40). Here, we present that signaling of PKCδ-dependent HIF-1α activation also exists in chemokine CCL5-treated cancer cells. We also provide promising evidences that PKCδ/c-Src/HIF-1α signaling pathway controls CCL5/CCR5 axis-induced VEGF expression and angiogenesis.

The prognosis of patients with osteosarcoma distant metastasis is generally considered as very poor. Angiogenesis facilitates metastasis formation and contributes to disease progression of osteosarcoma. Therefore, it is important to explore the novel target for preventing osteosarcoma angiogenesis and metastasis nowadays. This study showed that CCL5 and CCR5 interaction activates PKCδ, c-Src and HIF-1α pathways, leading to upregulation of VEGF expression, and contributes to tumor angiogenesis and progression in osteosarcoma microenvironment (Figure 6F). Based on the findings herein, we suggest that CCL5 may be a potential target worthy of further development to treat human osteosarcoma.

Supplementary material

Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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