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Interleukin-17F Suppresses Hepatocarcinoma Cell Growth via Inhibition of Tumor Angiogenesis

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ABSTRACT

Previous studies have shown that interleukin-17F (IL-17F) can markedly inhibit the angiogenesis of endothelial cells, implying that it may play a role in antiangiogenic therapy for tumors. To explore its effect on antiangiogenic therapy for hepatocellular carcinoma (HCC), we constructed a recombinant retrovirus vector RV-IL-17F expressing IL-17F, transfected SMMC-7721 human hepatocarcinoma cells with RV-IL-17F, and investigated the effect of transgene IL-17F expression on human hepatocarcinoma cells *in vitro* and *in vivo* in animal model. We demonstrated that IL-17F expression exerted no direct effect on *in vitro* proliferation and cell cycle of SMMC-7721 hepatocarcinoma cells, while it downregulated IL-6, IL-8, and VEGF expression in SMMC-7721 cells at both protein and mRNA levels and IL-17F-expressing supernatant from SMMC-7721/RV-IL-17F directly inhibited ECV304 vascular endothelial cell growth. Moreover, SMMC-7721/RV-IL-17F exhibited a significant decrease in tumor size and microvessel density as compared to the SMMC-7721/RV control when transplanted in nude mice. This retarded tumor growth *in vivo* elicited by IL-17F was associated with direct suppression of vascular endothelial cells and reduced expression of proangiogenic factors IL-6, IL-8, and VEGF leading to the inhibition of tumor angiogenesis. Thus, our results indicate that IL-17F, a novel antiangiogenic factor, may be useful in antiangiogenic therapy for HCC.

INTRODUCTION

Interleukin-17 (IL-17), termed CTLA8, was originally described and isolated from an activated T-cell hybridoma (1). The recent large-scale sequencing of expressed sequence tags (EST) and genomes of several vertebrate species has led to the identification of additional genes that bear clear homology to IL-17 and thus define an emerging cytokine family. The prototype member of the family has been designated IL-17A. There are at least six members of the family in the human genome, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25, and IL-17F (2). IL-17F was first identified and isolated from activated $CD4^+$ T cells and monocytes (3). Recently, ML-1, IL-17F isoform 2 (shorter form), has also been identified in subjects with asthma (4). IL-17F (longer form) was expressed only in activated $CD4^+$ T cells and monocytes (3), whereas ML-1 (short form) expression was upregulated in activated peripheral blood mononuclear cells (PBMCs), $CD4^+$ T cells, allergen-specific Th0, Th1, and Th2 clones, activated basophils, and mast cells (4), suggesting that the expression pattern of IL-17F is more complex than those seen for other members. IL-17F exists as a homodimer, adopting a cysteine knot motif, which is found in the TGF-β, bone morphogenetic protein, and nerve growth factor superfamilies (5).

Previous studies have shown that IL-17F can induce several cell types to express cytokines, chemokines, and adhesion molecules, including granulocyte macrophage-colony forming factor (GM-CSF), growth-related oncogene a (GROa), epithelial cell-derived neutrophil-activating protein-78 (ENA-78), and intercellular adhesion molecule-1 (ICAM-1) (4, 6, 7). These molecules play a crucial role in leukocyte recruitment and activation, and remodeling of asthmatic airways. IL-17F also can

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stimulate production of some cytokines, and regulates cartilage matrix turnover by increasing matrix release and inhibiting new matrix synthesis (5). Recently, a new subset of Th cells named Th17 was identified (8, 9). The hallmark of Th17 subset is the production of IL-17A and IL-17F. IL-17A and IL-17F genes are localized in the same chromosome region and undergo chromatin remodeling associated with Th17 differentiation (10). IL-17A and IL-17F, as the markers of the novel Th17 cells, may play important and unique roles in the exertion of Th17 functions. Moreover, IL-17F/IL-17A heterodimer has been shown to be expressed by Th17 cells (11, 12). Interestingly, IL-17F can markedly inhibit the angiogenesis of endothelial cells and induced endothelial cells to produce IL-2, TGF-β, and monocyte chemoattractant protein-1 (MCP-1) (3), indicating that it may play a role in cancer gene therapy by inhibiting the generation of tumor vascular supply.

To investigate the effect of IL-17F on antiangiogenic therapy for hepatocellular carcinoma (HCC), in this study, we cloned human IL-17F cDNA and constructed a IL-17F-expressing recombinant retroviral vector, RV-IL-17F; we assessed the effect of retrovirus-mediated IL-17F expression on a heptocarcinoma cell line SMMC-7721 *in vitro* and *in vivo* in animal model and also elucidated its potential mechanism.

MATERIALS AND METHODS

Vectors, cell lines, reagents, and mice

The pUCm-T vector and Trizol total RNA extraction kit was purchased from Sangon (Shanghai, China). The pSIV-1, pHIT456, and pHIT60 vectors and human embryonic kidney cell line 293T were kindly provided by Dr. Yongjing Chen, Soochow University (Suzhou, China). The fresh human anticoagulated peripheral blood was kindly provided by Suzhou Central Blood Station. The SMMC-7721 human hepatocarcinoma cell line and ECV304 human umbilical vein endothelial cell line were purchased from the American Type Culture Collection (ATCC, Rockville, MD). PBMCs were cultured in RPMI-1640 (GIBCO, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 μg/mL phytohemagglutinin (PHA) (Sigma, Shanghai, China). SMMC-7721 and ECV304 cells were cultured in RPMI-1640 (GIBCO, Shanghai, China) supplemented with 10% FBS (Hyclone, Logan, UT). Methyl-thiazolyl-tetrazolium (MTT), G418, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) were purchased from Sigma (Shanghai, China). Rapid plasmid DNA daily mini-prep kit, DNA gel extraction kit, PCR product purification kit, and cellular genomic DNA extraction kit were purchased from V-gene (Hangzhou, China). Lipofectamin^{TM} was purchased from Invitrogen (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-8, and vascular endothelial growth factor (VEGF) were purchased from Jingmei (Shanghai, China). The goat anti-human IL-17F (C-16), the rabbit anti-goat IgG-AP, the mouse anti-human IL-6, the mouse anti-human IL-8, the rabbit anti-human VEGF, and the mouse anti-mouse CD34 Abs were purchased from Santa Cruz (Shanghai, China). The UltraSensitive^{TM} SP kit was purchased from Maixin (Fuzhou, China). The female athymic nude mice were obtained from Shanghai Experimental Animal Center (Shanghai, China) and maintained in the animal facility at Soochow University according to the animal research committee's guidelines of Soochow University.

Construction of recombinant retroviral vectors

The sense primer 1 (P1) (5′-cagcgcaacatgacagtgaagac-3′) and the antisense primer 2 (P2) (5′-cacctcttactgcacatggtggat-3′) were used for cloning the gene coding for human IL-17F from RNA of PHA-activated PBMCs by reverse transcriptase polymerase chain reaction (RT-PCR). The human IL-17F 492 bp cDNA (GenBank accession no. AF384857) was subcloned into a pUCm-T vector to form the pUCm-T-IL-17F clone vector. The 462 cDNA coding for human IL-17F with a 20-residual signal sequence (3) and synonymous mutation (gaattc→gaactc, both for Asn) of internal EcoRI restriction site was further amplified by SOE-PCR using pUCm-T-IL-17F as templates and four primers, namely, P3 (5′-gcagaattcatggtcaagtacttgctg-3′), P4 (5′-ctcggatccttactgcacatggtggatg-3′), P5 (5′-ctccatgaactccgttccatccagcaag-3′), and P6 (5′-ggaacggagttcatggagatgtcttcct-3′), and then subcloned into a retroviral vector pSIV-1 at the EcoRI and BamHI sites to form the expression vector pSIV-1-IL-17F. The mixture of retroviral vectors pSIV-1-IL-17F, and two helper vectors pHIT456 and pHIT60 in proportion as 2:1:1 was cotransfected into 293T packaging cell line by Lipofectamin to form the mature and infectious IL-17F-expressing recombinant retroviral vectors (RV-IL-17F). The retroviral vector (RV) without the IL-17F cDNA used as a control was similarly prepared.

Transfection of SMMC-7721 cells with IL-17F-expressing recombinant retroviral vectors

Retroviral vectors containing or lacking the IL-17F cDNA (RV-IL-17F or RV) were stably transfected by infection into SMMC-7721 human hepatocarcinoma cells and G418-resistant selection. Infection with RV-IL-17F or RV obtained from 293T packaging cells containing 8 ng/mL polybrene was performed every other day for a total of three times. At 72 hr after infection, transfectants were selected for 3 weeks by culture in RPMI-1640 medium supplemented with 10% FBS and G418 (500 μg/mL). G418-resistant clones were expanded in selection medium and tested for genomic integration, transcription, and secretion expression of IL-17F gene by PCR, RT-PCR, and Western blot analysis. Total cellular genomic DNA and RNA were purified with cellular genomic DNA extraction kit and Trizol total RNA extraction kit, respectively. The first cDNA strand was reverse-transcribed with RNA as template and Oligo d(T)18 as primer. The PCR was carried out with genomic DNA and first cDNA strand as templates using primers P3 and P4 specific for IL-17F under conditions, respectively [1 cycle at 94°C (2 min) and then 72°C (10 min) followed by 35 cycles at 94°C (50 s), 58°C (50 s), and 72°C (45 s)]. All PCR products were analyzed on 1%

agarose gel electrophoresis with ethidium bromide staining. The cell supernatants were resolved by SDS-PAGE (12%) and transferred onto a nitrocellulose membrane (NC membrane). The membrane was blocked by incubation with 5% (w/v) nonfat dry milk in Tris-buttered saline containing 0.05% Tween 20 (TBST) for 1 hr at 37°C. For Western blot analysis, the membrane was incubated with primary antibody polyclonal goat anti-IL-17F (1:1000) in blocking solution for 1 hr at 37°C. The membrane was then washed with TBST and incubated with alkaline phosphatase (AP)-conjugated secondary antibody rabbit anti-goat IgG (1:1000) (Sigma, Shanghai, China) in blocking solution for 1 hr at 37°C. The blots were washed and then detected by NBT/BCIP.

MTT assay

The effect of IL-17F on SMMC-7721 human hepatocarcinoma cell's growth was determined by MTT assay. Briefly, RV-IL-17F-transfected SMMC-7721 (SMMC-7721/RV-IL-17F), RV-transfected SMMC-7721 (SMMC-7721/RV), and SMMC-7721 tumor cells were dispensed in 96-well culture plate at a density of 1×10^4 cells per well and incubated at 37°C, respectively. After different time periods of culture (1–3 days), the cells were incubated with 10 μL MTT (5 mg/mL) for 4 hr at 37°C. The formazan crystals in the cells were solubilized with stop solution (100 μL/well). The plate was then read at 570 nm using a Microplate Reader Model 550 (BIO-RAD, Shanghai, China). The effect of IL-17F on the proliferation of ECV304 human umbilical vein endothelial cells was also determined by MTT assay. Briefly, the ECV304 endothelial cells were dispensed in 96-well culture plate at a density of 1×10^4 cells per well and incubated at 37°C. After 24-hr incubation, they were treated with medium containing 50% (V/V) IL-17F-expressing supernatant from SMMC-7721/RV-IL-17F for the indicated time periods (0–4 days). The medium containing 50% (V/V) supernatant from SMMC-7721/RV or SMMC-7721 was used as a blank retrovirus control and a cell control, respectively. After treatments for different time periods, the ECV304 endothelial cells' growth was examined as described above.

Cell cycle analysis

Cell cycle conditions were determined using propidium iodide (PI) staining by fluorescence activated cell sorting (FACS) analysis. SMMC-7721/RV-IL-17F, SMMC-7721/RV, and SMMC-7721 tumor cells (1×10^6) were cultured at 37°C, respectively. Two days later, the cells were harvested and washed in cold PBS (pH 7.4). The cell pellets were fixed in 70% cold alcohol for more than 24 hr at 4°C, washed in cold PBS, stained with PI solution at 4°C in the dark for 30 min, and washed in cold PBS. The cells were then analyzed by flow cytometry.

IL-6, IL-8, and VEGF expression determined by ELISA

SMMC-7721/RV-IL-17F, SMMC-7721/RV, and SMMC-7721 tumor cells (1×10^5) were cultured at 37°C, respectively. The supernatants were collected after 48-hr incubation, and the amounts of IL-6, IL-8, and VEGF in the supernatants were assessed by ELISA.

Real-time RT-PCR analysis

To further determine the expression levels of IL-6, IL-8, and VEGF in RV-IL-17F-transfected SMMC-7721 tumor cells, the total RNA was prepared for two-step real-time RT-PCR analysis based on SYBR Green I detection. Real-time RT-PCR assay was performed using MJ Research Opticon^{TM}2 system (MJ Research). Briefly, SMMC-7721/RV-IL-17F, SMMC-7721/RV, and SMMC-7721 tumor cells (5×10^6) were cultured at 37°C, respectively. The cells were collected after 48-hr incubation, and the total cellular RNA was isolated using Trizol total RNA extraction kit. The first cDNA synthesis was performed as described above in larger volumes such that each sample could be tested in different subsequent PCRs. Primers used in the experiment were as follows: (a) IL-6-F (5′-cagacagccactcacctcttcag-3′) and IL-6-R (5′-ctcatctgcacagctctggcttg-3′); (b) IL-8-F (5′-atgacttccaagctggccgtgg-3′) and IL-8-R (5′-ttatgaattctcagccctcttcaaaa-3′); (c) VEGF-F (5′-tggtagagttcatggatgtctatca-3′) and VEGF-R (5′-gcatggtgatgttggactcctca-3′); and (d) β-actin-F (5′-tgcgtgacattaaggagaag-3′) and β-actin-R (5′-ctgcatcctgtcggcaatg-3′). The subsequent real-time PCRs contained 5 μL of the RT reaction product in a total volume of 25 μL, consisting of 1 × PCR buffer, 2.5 mM MgCl2, 250 μM total dNTPs, 300 nM forward primer, 300 nM reverse primer, 1 × SYBR Green I, and 0.5 U Taq DNA polymerase. The PCRwas performed using the following program: 94°C for 2 min, then 72°C for 10 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The cDNA quantities were normalized to the internal control gene β-actin measured in the same samples. Relative gene expression was calculated using the pooled cDNA from all samples by the $2^{-\Delta\Delta CT}$ method as previously described (13). The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. Each sample was analyzed in duplicate in independent reactions and the experiment was repeated three times.

Animal studies

Female athymic nude mice (five each group) were subcutaneously (s.c.) inoculated on their armpits of right anterior limbs with 2×10^6 SMMC-7721/RV-IL-17F and SMMC-7721/RV (control) tumor cells, respectively. Tumor size was measured every other day using an external caliper. Tumor volumes (V) were determined by the formula $V = ab^2/2$, where a is the larger and b is the smaller of the two dimensions. The standard for tumor formation was the diameter of tumors >0.5 cm. Tumor-bearing mice were sacrificed 5 weeks after tumor inoculation, and the tumors were removed, weighed, and fixed by 10% neutral formalin and embedded in paraffin for Haematoxylin & Eosin (HE) staining and immunohistochemistry.

Immunohistochemistry

The IL-17F, CD34, IL-6, IL-8, and VEGF expression were tested by immunohistochemistry analysis using UltraSensitive

SP kit. All sections were counterstained with HE. Sections were evaluated for the presence of brown diaminobenzidine precipitates indicative of positive reactivity by microscopy. The brown staining was read as positive reactivity for IL-17F, CD34, IL-6, IL-8, and VEGF. Each value represents the number of positive cells counted at a high power view (×400). Any endothelial cell cluster immunoreactive for CD34 clearly separated from adjacent microvessels was considered as a single countable vessel (14). The mean value represents for the average number derived from five high power fields of each case.

Statistical analysis

All data are presented as mean ± SD. The significance of the difference between groups was evaluated by one-way repeated measures ANOVA and multiple comparisons and Student's *t* test with SPSS 10.0 software. A value of $p < .05$ was considered statistically significant.

RESULTS

Transgene IL-17F expression in vitro and in vivo

To explore its biological activity, we cloned the human IL-17F 492 bp cDNA encoding a protein of 163 amino acids containing a 30-residue signal sequence (5) from PHA-activated human PBMCs by RT-PCR (Figure 1(A)) and constructed a retroviral expression vector RV-IL-17F encoding IL-17F protein of 153 amino acids, including a 20-residue signal sequence, according to Kozak consensus for translation initiation (3). To address its effect on the SMMC-7721 human hepatocarcinoma cells, SMMC-7721 cells were transfected with RV-IL-17F retroviral vectors, and positive clones (SMMC-7721/RV-IL-17F) stably expressing IL-17F mediated by retroviral vectors were selected by G418 (500 μg/mL). To assess stable expression of IL-17F in G418-resistant SMMC-7721 cells, the cellular genomic DNA, RNA, and supernatants extracted or harvested from transfected SMMC-7721 cells were subjected to PCR, RT-PCR, and Western blot analysis. As shown in Figure 1(B), IL-17F gene mediated by retroviral vector was integrated into genomic DNA of SMMC-7721 cells (a) and a significant amount of IL-17F expression was also found in RV-IL-17F-transfected SMMC-7721 tumor cells, but not in RV-transfected and control SMMC-7721 cells (b and c), indicating that IL-17F is stably expressed in RV-IL-17F-transfected SMMC-7721 cells at both transcriptional and translational levels. To determine the *in vivo* persistence of the IL-17F transgene in SMMC-7721 tumor cells transfected with RV-IL-17F and transplanted in nude mice, the transgene IL-17F expression was further tested by immunohistochemistry analysis. As shown in Figure 1(C), IL-17F expression was detected in sections derived from mice transplanted with SMMC-7721/RV-IL-17F cells but not in mice previously inoculated with the control SMMC-7721/RV transfectant, indicating that the IL-17F transgene was not lost during transplanted tumor development *in vivo*.

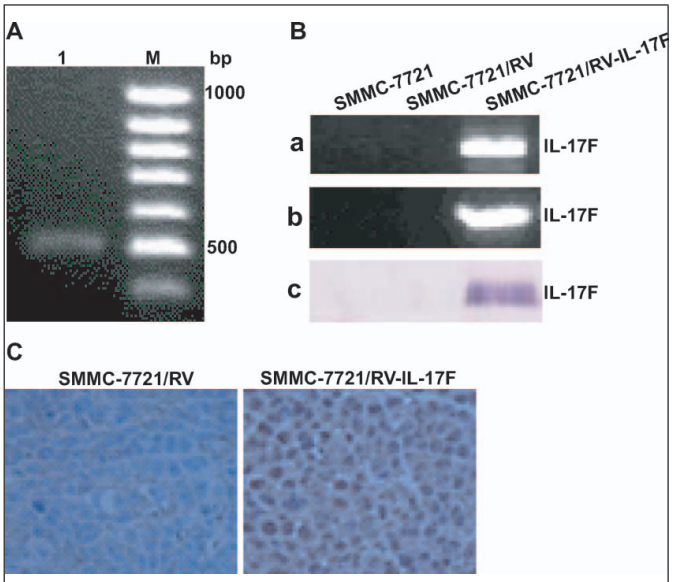

Figure 1. The expression of IL-17F in SMMC-7721 cells transfected with IL-17F retroviral vectors. (A) Human IL-17F gene cloning by RT-PCR. Total RNA was obtained from PHA-activated PBMCs. The first-strand cDNA was synthesized from RNA using reverse transcriptase; PCR was conducted using primers P1 and P2 specific for IL-17F 492 bp cDNA. 1: human IL-17F 492 bp RT-PCR products encoding a protein of 163 amino acids containing a 30-residue signal sequence; M: 100 bp DNA ladder. (B) Transgene IL-17F expression analysis. (a) PCR analysis genomic integration of IL-17F gene in SMMC-7721 cells. Total genomic DNA was obtained from SMMC-7721 cells transfected with RV-IL-17F, RV, and SMMC-7721 control cells. PCR was conducted using primers P3 and P4 specific for IL-17F 462 bp cDNA. (b) RT-PCR analysis transcriptional expression of IL-17F in SMMC-7721 cells. Total RNA was obtained from SMMC-7721 cells transfected with RV-IL-17F, RV, and SMMC-7721 control cells. RT-PCR was performed as described above using primers P3 and P4 specific for IL-17F 462 bp cDNA. (c) Western blot analysis secretion expression of IL-17F in SMMC-7721 cells. The supernatants were analyzed by Western blot with IL-17F antibody (Santa Cruz). (C) The expression of IL-17F in tumor tissues was assessed by immunohistochemistry. Representative pictures for different groups are shown. IL-17F expression was detected in sections derived from mice transplanted with SMMC-7721/RV-IL-17F tumor cells but not in the mice previously inoculated with the control SMMC-7721/RV transfectant. Data shown are representative of three independent experiments.

Effect of IL-17F on the growth and cell cycle of SMMC-7721 hepatocarcinoma cells in vitro

To investigate its effect on the growth of SMMC-7721 tumor cells *in vitro*, the RV-IL-17F-transfected, RV-transfected, and control SMMC-7721 cells were cultured and their growth was examined daily for 3 days using MTT assay. As shown in Figure 2(A), RV-IL-17F-transfected and RV-transfected or control SMMC-7721 tumor cells showed identical *in vitro* growth ($p > .05$), indicating that IL-17F did not influence the proliferation of SMMC-7721 tumor cells *in vitro*. To further explore its effect on cell cycle of SMMC-7721 tumor cells, RV-IL-17F-transfected, RV-transfected, and control SMMC-7721 cells were cultured for 48 hr and harvested for FACS analysis. As shown in Figure 2(B),

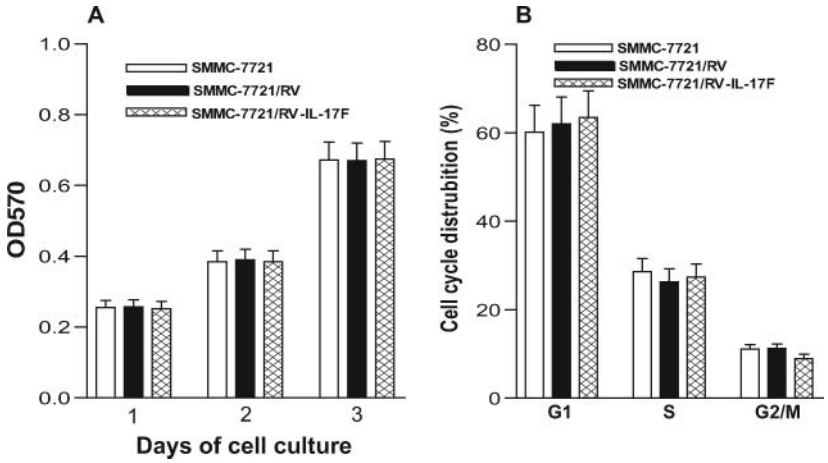

Figure 2. The effect of IL-17F on the growth and cell cycle of SMMC-7721 hepatocarcinoma cells *in vitro*. (A) The effect of IL-17F on SMMC-7721 tumor cells' growth *in vitro*. SMMC-7721/RV-IL-17F, SMMC-7721/RV, and SMMC-7721 tumor cells were cultured for 3 days. The survived cells were evaluated 1, 2, 3 days using MTT assay. RV-IL-17F-transfected and RV-transfected or control SMMC-7721 tumor cells showed identical *in vitro* growth ($p > .05$, compared with SMMC-7721/RV or SMMC-7721 control, respectively, one-way repeated measures ANOVA; $n = 4$ replicates per condition). Data shown are representative of three independent experiments. (B) The effect of IL-17F on cell cycle of SMMC-7721 tumor cells. SMMC-7721/RV-IL-17F, SMMC-7721/RV, and SMMC-7721 tumor cells were cultured for 2 days. The cell cycle conditions were determined using PI staining by flow cytometry analysis. RV-IL-17F-transfected and RV-transfected or control SMMC-7721 tumor cells also showed identical cell cycle distribution *in vitro* ($p > .05$, compared with SMMC-7721/RV or SMMC-7721 control, respectively, one-way repeated measures ANOVA; $n = 3$ replicates per condition). Data shown is representative of three independent experiments.

RV-IL-17F-transfected and RV-transfected or control SMMC-7721 tumor cells showed identical cell cycle distribution *in vitro* ($p > .05$), indicating that IL-17F did not also influence the cell cycle of SMMC-7721 tumor cells *in vitro*.

Tumor growth of IL-17F-transfected SMMC-7721 hepatocarcinoma cells in nude mice

To determine the effect of IL-17F on hepatocarcinoma cell's growth *in vivo*, RV-IL-17F-transfected and RV-transfected (control) SMMC-7721 tumor cells were transplanted s.c. into athymic nude mice, respectively. The tumor growth was monitored daily using a vernier caliper. As shown in Figure 3(A), SMMC-7721/RV-IL-17F hepatocarcinoma cells delayed tumor formation on day 19.2 ± 0.8 compared with day 11.6 ± 1.1 in SMMC-7721/RV tumor cells ($p < .05$). The tumor volume and weight at the time of sacrifice in SMMC-7721/RV-IL-17F (0.43 ± 0.05 cm^3, 0.44 ± 0.10 g) were significantly lower than those in SMMC-7721/RV control group (0.66 ± 0.06 cm^3, 0.77 ± 0.10 g) ($p < .05$) (Figures 3(B)–(D). Thus, RV-IL-17F-transfected SMMC-7721 hepatocarcinoma xenografted tumor growth was significantly retarded, indicating that transgene IL-17F expression suppresses *in vivo* in athymic nude mouse model SMMC-7721 hepatocarcinoma growth.

IL-17F inhibits tumor vessel CD34 expression and microvessel formation

The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in vascular endothelial cells. In all tumors collected, the CD34 expression of vascular endothelial cell in the SMMC-7721/RV-IL-17F group was weaker or less, compared with the SMMC-7721/RV control group (Figure 4(A)), indicating that transgene IL-17F expression downregulates CD34 expression of tumor vessels. In addition, the density of microvessel counted (Figure 4(B)) in the SMMC-7721/RV-IL-17F group was significantly less than that in the SMMC-7721/RV control group ($p < .05$), indicating that IL-17F also inhibits microvessel formation in SMMC-7721 human hepatocarcinoma transplanted tumor.

IL-17F suppresses vascular endothelial cell growth and downregulates IL-6, IL-8, and VEGF expression

To address the direct antiangiogenic effect of IL-17F on the vascular endothelial cells *in vitro*, the ECV304 human umbilical vein endothelial cells were cultured in presence or absence of IL-17F from supernatant of SMMC-7721/RV-IL-17F. The ECV304 endothelial cells' growth was examined daily for 4 days using MTT assay. As shown in Figure 5(A), IL-17F treatment significantly inhibited ECV304 endothelial cell growth in a time-dependent manner, compared to the RV or cell control group ($p < .05$). To examine the effect of IL-17F on proangiogenic factors' expression *in vitro*, we analyzed IL-6, IL-8, and VEGF expression in RV-IL-17F-transfected SMMC-7721 tumor cells by ELISA. As shown in Figure 5(B), the amounts of IL-6, IL-8, and VEGF in RV-IL-17F-transfected SMMC-7721 cells were less than that in RV-transfected or control SMMC-7721 cells ($p < .05$), indicating that transgene IL-17F expression significantly downregulates the expression of proangiogenic factors,

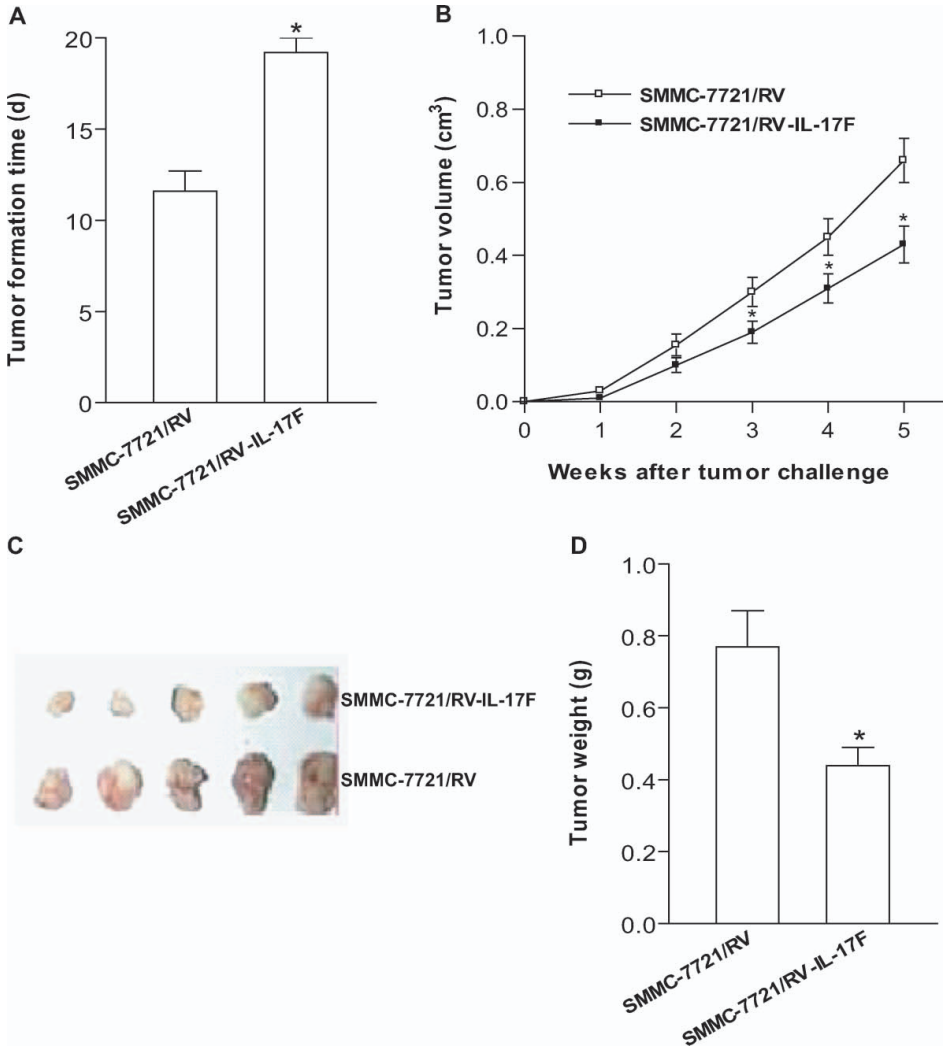

Figure 3. IL-17F suppresses the SMMC-7721 tumor growth *in vivo* in animal model. SMMC-7721/RV-IL-17F and SMMC-7721/RV (control) tumor cells were transplanted s.c. into athymic nude mice, respectively. The tumor formation time (A) and tumor volume (B) were measured ($^*p < .05$, compared with SMMC-7721/RV control, Student's *t* test; $n = 5$ mice per condition). The tumors were removed 5 weeks after tumor inoculation (C) and tumor weight (D) were measured ($^*p < .05$, compared with SMMC-7721/RV control, Student's *t* test; $n = 5$ mice per condition). IL-17F significantly suppresses the SMMC-7721 tumor growth *in vivo* in animal model. Data shown are representative of three independent experiments.

such as IL-6, IL-8, and VEGF, in SMMC-7721 tumor cells. The reduced expression of IL-6, IL-8, and VEGF was confirmed by real-time PCR analysis (Figure 5(C)) ($p < .05$). To determine the potential mechanism associating with antiangiogenic effect of IL-17F *in vivo* in nude mouse model, the expression of IL-6, IL-8, and VEGF in xenografted tumor tissues was further assessed by immunohistochemistry analysis. As shown in Figures 5(D) and (E), the expression of IL-6, IL-8, and VEGF was significantly downregulated in the SMMC-7721/RV-IL-17F group compared with the SMMC-7721/RV control group ($p < .05$). The data indicate that transgene IL-17F expression suppresses *in vivo* in athymic nude mouse model SMMC-7721 tumor growth very possibly via directly inhibiting vascular endothelial cell growth and indirectly downregulating the expression of angiogenesis-related factors, such as IL-6, IL-8, and VEGF, leading to the inhibition of tumor angiogenesis.

DISCUSSION

IL-17 family members belong to a distinct category of cytokine, and play a role in coordinating local tissue inflammation by inducing release of proinflammatory and meutrophil-mobilizing cytokines and the progression of disease, such as inflammatory diseases, autoimmune diseases, and cancer. IL-17F was first identified and isolated from activated $CD4^+$ T cells and activated monocytes (3). Among the IL-17 family members, IL-17F is most closely related to IL-17, showing a striking 50% homology with IL-17A (4, 5). Genes encoding

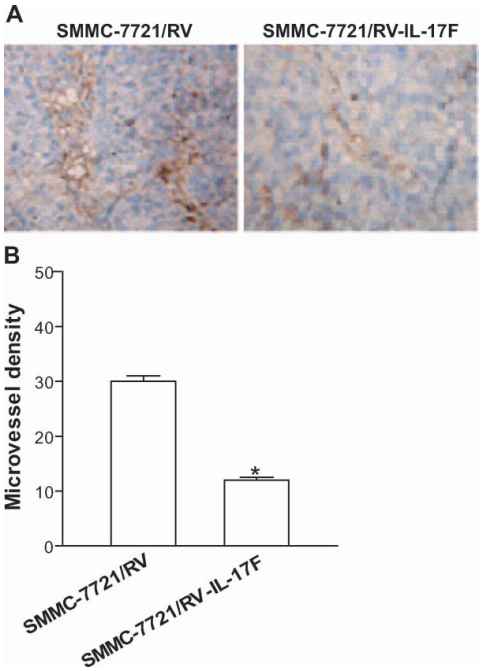

Figure 4. Inhibition of tumor angiogenesis *in vivo*. (A) Immunohistochemical detection of vascular endothelial cells for CD34 expression. Representative pictures for different groups are shown. The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in vascular endothelial cells. (B) The tumor microvessel density in different groups. The microvessel density in SMMC-7721/RV-IL-17F group was significantly lower than that in SMMC-7721/RV control group ($^*p < .05$, compared with SMMC-7721/RV control, Student's *t* test; $n = 5$ mice per condition, $n = 5$ observations per representative section). Data shown are representative of three independent experiments.

IL-17A and IL-17F lie next to each other on chromosome 6 (5) and likely arise from a gene duplication event. Recent studies showed that IL-17RC can associate with IL-17RA, composing a heteromeric receptor complex that mediates IL-17A and IL-17F signaling pathway (15). Thus, the biological activities mediated by IL-17F are similar to those of IL-17A. Of note, IL-17F significantly induces the expression of ICAM-1 in primary bronchial epithelial cells (4), while IL-17A fails to induce its expression. Furthermore, in addition to Th1 and Th2 cells, activated human mast cells and basophils express IL-17F (4), and the expression of IL-17F shows a wider tissue distribution, suggesting a wider role of IL-17F in inflammatory responses. More recently, the examination of IL-17A- and IL-17F-deficient animals in multiple models has revealed the distinct requirements of these two genes in different inflammatory responses (16), suggesting that IL-17F plays a critical role in the regulation of inflammatory reactions. Interestingly, IL-17F can markedly inhibit the angiogenesis of endothelial cells and induce endothelial cells to produce IL-2, TGF-β, and MCP-1 (3), indicating that it may play a role in cancer gene therapy by inhibiting the generation of tumor vascular supply.

HCC is one of the five most common cancers worldwide, with a particularly high prevalence in Asian countries due to endemic hepatitis B virus infection (17). The incidence of HCC is also rising in Western countries as a result of increasing hepatitis C virus infection (18). More than 80% of patients with HCC have associated cirrhosis and impaired liver function, making treatment of HCC more difficult than many other cancers. Hence, the search for a novel systemic therapy for HCC is of paramount importance. HCC is a hypervascular tumor characterized by neovascularization, which plays an important role in the growth and progression of HCC (19). Angiogenesis provides a target for novel prognostic and therapeutic approaches to HCC. The emerging success of antiangiogenic therapy for other cancers such as colorectal cancer (20, 21) and lung cancer (22) has brought hope of the potential use of antiangiogenic therapy for HCC, which is a particularly attractive approach because of the vascularity of HCC and the current lack of effective systemic therapies for HCC.

Recently, increasing interests have focused on IL-17F for their roles in the pathogenesis of inflammatory diseases, such as allergic asthma (4). However, the role of IL-17F, a novel antiangiogenic factor, in HCC and other tumors is unclear. Moreover, the effect of IL-17F on hepatocarcinoma cells and retrovirus-mediated IL-17F expression in application of antiangiogenic therapy for HCC has not been reported. In this study, we demonstrated that IL-17F expression exerted no direct effect on *in vitro* proliferation and cell cycle of SMMC-7721 hepatocarcinoma cells, while it downregulated IL-6, IL-8, and VEGF expression in SMMC-7721 cells at both protein and mRNA levels and IL-17F-expressing supernatant from SMMC-7721/RV-IL-17F directly inhibited ECV304 vascular endothelial cell growth. Moreover, SMMC-7721/RV-IL-17F exhibited a significant decrease in tumor size and microvessel density as compared to the SMMC-7721/RV control when transplanted in nude mice. The progressive growth and metastasis of solid tumors is dependent upon the process of angiogenesis, which is regulated by equilibrium between proangiogenic and antiangiogenic molecules. Previous studies showed that IL-6, IL-8, and VEGF are important proangiogenic cytokines, which are involved in tumor angiogenesis (23–26). IL-8 (also termed CXCL8) is a chemokine that exerts a potent angiogenic activity, binding the receptors CXCR1 and CXCR2 present on endothelial cells (27, 28). Tumor cell expression of IL-8 has been linked to the metastatic potential solid tumors (27, 29). VEGF is important to the growth of many solid tumors conferring survival advantage by inducing vascular formation (30). Overexpression of VEGF is the characteristic of most malignant tumors including HCC (31–35), which is highly related to microvessel density of cancer, grade of malignance, and metastasis (36–38). Thus, this retarded tumor growth *in vivo* elicited by IL-17F was associated with direct suppression of vascular endothelial cells and reduced expression of proangiogenic factors IL-6, IL-8, and VEGF leading to the inhibition of tumor angiogenesis.

It has been reported that overexpression of IL-17 (IL-17A) ectopically in tumor cells either suppresses tumor progression through enhanced anti-tumor immunity by means of a

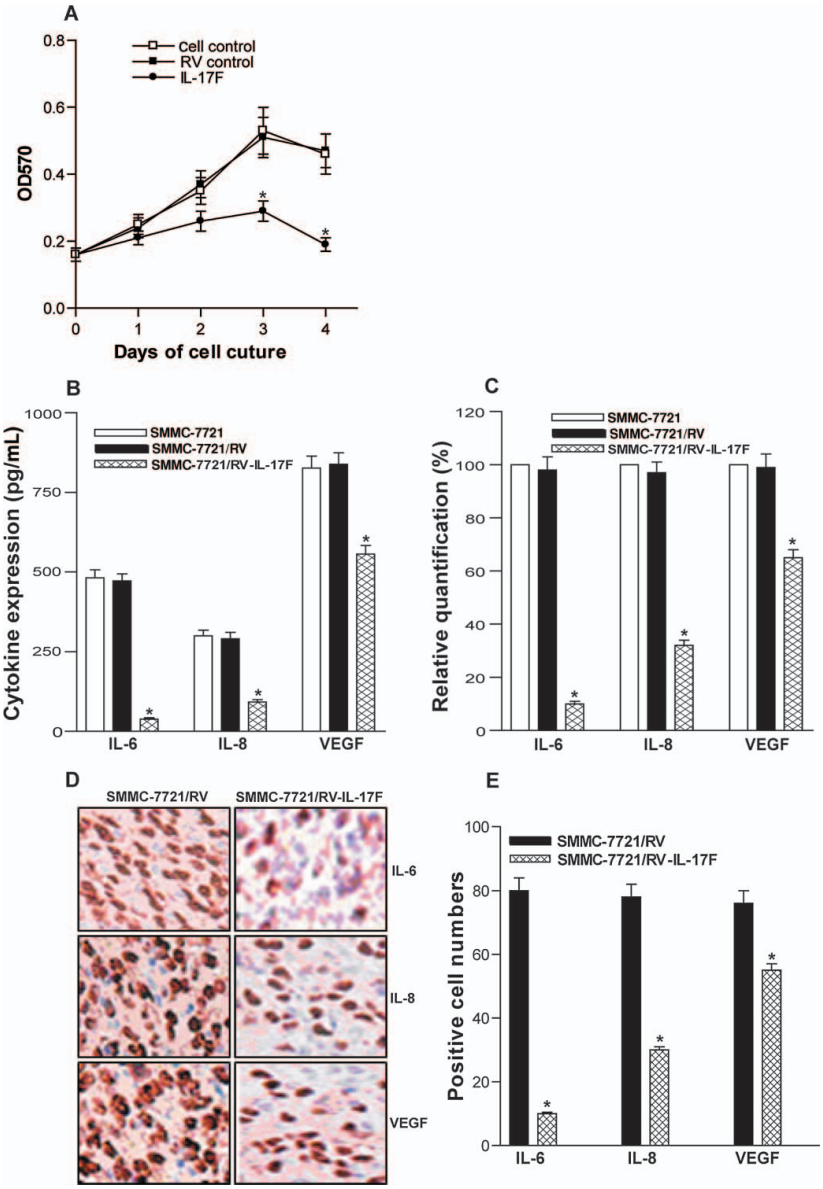

Figure 5. IL-17F suppresses vascular endothelial cell growth and down-regulates proangiogenic factors IL-6, IL-8, and VEGF expression. (A) The effect of IL-17F on the proliferation of ECV304 human umbilical vein endothelial cells. The ECV304 endothelial cells were treated with IL-17F-expressing supernatant from SMMC-7721/RV-IL-17F and with supernatant from SMMC-7721/RV or SMMC-7721 served as a control for 4 days. The survived cells were evaluated 0, 1, 2, 3, 4 days using MTT assay. IL-17F treatment significantly inhibited ECV304 endothelial cell growth ($^*p < .05$, compared with RV or cell control, respectively, one-way repeated measures ANOVA; $n = 4$ replicates per condition). Data shown are representative of three independent experiments. (B) The expression of IL-6, IL-8, and VEGF determined by ELISA. IL-17F significantly downregulates the IL-6, IL-8, and VEGF expression in SMMC-7721 cells ($^*p < .05$, compared with SMMC-7721/RV or SMMC-7721 control, respectively, whereas $p > .05$ when SMMC-7721/RV compared with SMMC-7721, one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition, $n = 3$ replicates per sample). Data shown are representative of three independent experiments. (C) Relative quantification of IL-6, IL-8, and VEGF expression by real-time RT-PCR analysis. In each case, the data were normalized to the expression level of β-actin and expressed as relative mRNA level. SMMC-7721/RV-IL-17F transgenic tumor cells express significant lower level in IL-6, IL-8, and VEGF than SMMC-7721/RV or SMMC-7721 control ($^*p < .05$, compared with SMMC-7721/RV or SMMC-7721 control, respectively, whereas $p > .05$ when SMMC-7721/RV compared with SMMC-7721, one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition, $n = 2$ replicates per sample). Data shown are representative of three independent experiments. (D) Immunohistochemical detection for IL-6, IL-8, and VEGF expression in tumor tissues. Representative pictures for different groups are shown. The expression of IL-6, IL-8, and VEGF was significantly downregulated in the SMMC-7721/RV-IL-17F group compared with the SMMC-7721/RV control group. (E) The numbers of IL-6-, IL-8-, and VEGF-expressing positive cells in different groups. The numbers of IL-6-, IL-8-, and VEGF-expressing positive cells in SMMC-7721/RV-IL-17F group were significantly lower than those in SMMC-7721/RV control group ($^*p < .05$, compared with SMMC-7721/RV control, Student's t test; $n = 5$ mice per condition, $n = 5$ observations per representative section). Data shown are representative of three independent experiments.

T-cell-dependent mechanism in immune-competent mice (39, 40) or promotes tumor progression through an increase in angiogenesis associating with stimulating vascular endothelial cell migration and regulating the production of a variety of proangiogenic factors in immune-deficient mice (41–43). These results suggest that IL-17F and IL-17A exhibit distinct roles in angiogenic regulation and tumorigenesis. More recently, Kryczek *et al*. (44) reported that endogenous IL-17 (IL-17A) contributes to reduced tumor growth and metastasis through enhanced tumor immunity. Therefore, our future work will focus on the roles of exogenous or endogenous IL-17F in tumor immunity using immune-competent mice and IL-17F-deficient mice animal models.

CONCLUSIONS

Taken together, IL-17F, a novel antiangiogenic factor, directly inhibited ECV304 vascular endothelial cell growth and downregulated the expression of proangiogenic factors IL-6, IL-8, and VEGF in hepatocarcinoma cells. IL-17F expression exerted no direct effect on *in vitro* proliferation and cell cycle of SMMC-7721 hepatocarcinoma cells. However, SMMC-7721/RV-IL-17F exhibited a significant decrease in tumor size and microvessel density as compared to the SMMC-7721/RV control when transplanted in nude mice. This retarded tumor growth *in vivo* elicited by IL-17F was associated with direct suppression of vascular endothelial cells and reduced expression of proangiogenic factors IL-6, IL-8, and VEGF leading to the inhibition of tumor angiogenesis.

Declaration of Interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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