

## RESEARCH ARTICLE

# Targeting EGFL7 Expression through RNA Interference Suppresses Renal Cell Carcinoma Growth by Inhibiting Angiogenesis

Han-Feng Xu<sup>1,2</sup>, Lei Chen<sup>3</sup>, Xian-Dong Liu<sup>1</sup>, Yun-Hong Zhan<sup>1</sup>, Hui-Hui Zhang<sup>2</sup>, Qing Li<sup>2</sup>, Bin Wu<sup>1\*</sup>

## Abstract

Renal cell carcinoma (RCC) is the most lethal of all urological cancers and tumor angiogenesis is closely related with its growth, invasion, and metastasis. Recent studies have suggested that epidermal growth factor-like domain multiple 7 (EGFL7) is overexpressed by many tumors, such as colorectal cancer and hepatocellular carcinoma; it is also correlated with progression, metastasis, and a poor prognosis. However, the role of EGFL7 in RCC is not clear. In this study, we examined how EGFL7 contributes to the growth of RCC using a co-culture system *in vitro* and a xenograft model *in vivo*. Downregulated EGFL7 expression in RCC cells affected the migration and tubule formation of HMEC-1 cells, but not their growth and apoptosis *in vitro*. The level of focal adhesion kinase (FAK) phosphorylation in HMEC-1 cells decreased significantly when co-cultured with 786-0/iEGFL7 cells compared with 786-0 cells. After adding rhEGFL7, the level of FAK phosphorylation in HMEC-1 cells was significantly elevated compared with phosphate-buffered saline (PBS) control. However, FAK phosphorylation was abrogated by EGFR inhibition. The average size of RCC local tumors in the 786-0/iEGFL7 group was noticeably smaller than those in the 786-0 cell group and their vascular density was also significantly decreased. These data suggest that EGFL7 has an important function in the growth of RCC by facilitating angiogenesis.

**Keywords:** Renal cell carcinoma - EGFL7 - migration - tubule formation - focal adhesion kinase - vascular density

*Asian Pac J Cancer Prev*, 15 (7), 3045-3050

## Introduction

Renal cell carcinoma (RCC) is the seventh most common cancer in men and the ninth most common in women. It also accounts for 2% to 3% of all malignant diseases in adults (Rini et al., 2009). The incidence of this cancer has increased, probably contributing to the aging of population (Rini et al., 2009; Cairn, 2010). Radical or partial nephrectomy is the major surgical treatment for local RCC according to the size of carcinoma. For patients having recurrent metastatic RCC, systemic treatment is preferred. However, the overall survival of patients with RCC remains unsatisfactory because of the high incidence of recurrence and metastasis after treatment, especially for those having metastatic RCC (Cohen et al., 2005; Rini et al., 2009; Grunwald et al., 2014). Thus, inhibition of the progress, recurrence, and metastasis of the tumor is of great importance in the treatment of RCC.

Angiogenesis is indispensable in the promotion of invasive tumor growth and metastasis; it is also an important factor that controls cancer progression. For

tumors to develop in size and obtain metastatic potential, they must make an “angiogenic switch” by breaking the local balance between proangiogenic and antiangiogenic factors (Chung et al., 2010; Carmeliet et al., 2011; Shojaei, 2012). Tumor angiogenesis develop by the activation of signal pathways playing an important role in the development of RCC. Cytokines like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) and associated signal pathways are therapy targets in treatment of RCC (Tanriverdi, 2013). However, whether or not other angiogenic factors those are required for the development of RCC is not well known.

Epidermal growth factor-like domain multiple 7 (EGFL7) is a secreted protein that contains two EGF-like domains and is conserved across species (Parker et al., 2004). EGFL7 is believed to be expressed exclusively in endothelial progenitors and in endothelial cells (ECs) during embryonic and neonatal development. In adults, the expression is downregulated and still detectable in blood vessels of the lungs, heart, and kidney (Fitch et al., 2004; Parker et al., 2004; Campagnolo et al., 2005).

<sup>1</sup>Department of Urology, Shengjing Hospital of China Medical University, Shenyang, <sup>2</sup>Department of Urology, First Affiliated Hospital of Nanhua University, <sup>3</sup>Department of Surgery, Hunan Polytechnic of Environment and Biology, Hengyang, China \*For correspondence: wubin\_1959@163.com

However, EGFL7 is aberrantly expressed by tumor cells in human cancers. In colorectal cancer, high levels of EGFL7 correspond to tumors with higher pathologic stages and to the presence of lymph node metastases (Diaz et al., 2008). EGFL7 is also overexpressed by tumor cells in human hepatocellular carcinoma, and overexpression is significantly higher in tumors with multiple nodules, without capsules, and with vein invasion. Levels of EGFL7 are thus correlated with markers of metastasis and with poor prognosis (Wu et al., 2009). In glioma, EGFL7 expression levels correlate with tumor grade. EGFL7 expression, cell proliferation, and microvessel density also correlate with each other (Huang et al., 2010). However, the role of EGFL7 in RCC is not clear. In the present study, we carried out co-culture experiments targeting the expression of EGFL7 by RNA interference to determine the function of EGFL7 in the progression of RCC by characterizing its role in angiogenesis using both *in vitro* and *in vivo* models.

## Materials and Methods

### Cell lines and culture conditions

HMEC-1, a human microvascular endothelial cell line, and 786-0, a human RCC cell line, were purchased from the Cell Center of Chinese Academy of Sciences. The cell lines were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp.) and cultured in a 37°C humidified atmosphere containing 5% CO<sub>2</sub>. Cells were trypsinized upon confluence and propagated to passage 3 to 10 for further experiments.

### RNA Interference

Synthetic small interfering RNA (siRNA) was used to downregulate EGFL7 expression. The following sequences used were purchased from Sangon Biotech (Shanghai) Co., Ltd.: EGFL7: SiRNA1 sense primer, 5'-GUACAUCAUUAUAAG CUGTT-'3'; anti-sense primer, 5'-CAGCUUAUAAUGGAUGUACTT-'3'; SiRNA2 sense primer, 5'-GCCGGCGACGACUUCUCC CTT-'3'; anti-sense primer, 5'-GGGA GAAGUCGUCGC CGGCTT-'3'; non-specific sense primer, 5'-AGCGUUCA CUCCAACCUGTT-'3'; anti-sense primer, 5'-CAGGUU GGGAGUGAACGCUTT-'3'. The oligonucleotide pairs were annealed and ligated into the BglII-HindIII sites of pSUPER.retro.GFP vector following the protocol of the manufacturer. The plasmid constructs (pSUPER.retro.iEGFL7-GFP vector) were packaged into infectious retrovirus in HEK293T cells expressing gag, pol, and env proteins. For control experiments, empty pSUPER.retro.GFP vector was packaged into the retrovirus. For transfection, 786-0 cells were plated in a 4×10<sup>5</sup> cells/60 mm dish and incubated for 24h in complete media. The viral supernatants and fresh culture medium mixed at a ratio of 1:1 were added to the cells in the presence of 4 µg/mL polybrene. The cells were incubated for 48 h at 37°C in 5% CO<sub>2</sub>.

### Flow cytometric analysis

Flow cytometric analysis was performed to determine the effects of 786-0/iEGFL7 on the cell cycle and cell

apoptosis of HMEC-1 cells. Briefly, 786-0 cells or 786-0/iEGFL7 cells were plated in the lower compartment of six-well Transwell Permeable Supports (Corning Costar, Cambridge, MA) in complete media at a density of 1×10<sup>5</sup> cells per well. HMEC-1 cells were seeded into 0.4 µm pore size transwell membrane inserts at the same density in complete media. After being co-cultured for 48h the HMEC-1 cells were harvested by trypsinization. For cell cycle analysis, the cells were fixed with 70% ethanol and analyzed by flow cytometry (FACSCalibur, BD Biosciences). DNA histograms were measured using FCS Express software. The percentages of G0/G1, S, and G2/M cells were calculated. For cell apoptosis analysis, cell viability was determined through flow cytometry after double staining with annexin-V and propidium iodide (BD Pharmingen) according to the manufacturer's instructions.

### Cell proliferation assay

A total of 104 786-0 cells or 786-0/iEGFL7 cells per well were plated in 96-well plates and cultured for 48h under normal conditions. Cell proliferation was assessed using Cell Counting Kit 8 (CCK-8, Dojindo, Tokyo, Japan) according to the manufacturer's protocol. Up to 10 µl of CCK-8 solution was added to each well plate, after which it was incubated for 2h. The absorbance was measured at 450 nm using a microplate reader.

### Cell migration assay

Migration assays were performed in 24-well transwell dishes (Corning Costar, Cambridge, MA) to determine the effects of 786-0/iEGFL7 on the cell migration of HMEC-1 cells. HMEC-1 cells (5×10<sup>4</sup> cells) in 200 µl complete medium were added to the upper chamber, and 786-0 cells or 786-0/iEGFL7 cells with 600 µl complete medium were added to the bottom chamber. After the cells were incubated in the transwells for 16h at 37°C in 5% CO<sub>2</sub>, the transwell filters were carefully washed with cold phosphate-buffered saline (PBS), stained with crystal violet for 1h and mounted on glass slides. The migrated cells were counted using light microscopy at 400× magnification. Assays were performed three times using a triplicate well.

### Capillary-like tube formation assay

A Matrigel-based capillary-tube formation assay was performed to evaluate the effects of 786-0/iEGFL7 on the ability of HMEC-1 cells to form an organized tubular network. A day before the experiment, confluent HMEC-1 cells were starved overnight at 37°C with RPMI 1640 medium containing 1% FBS. Matrigel (Becton Dickinson) was thawed overnight at 4°C, plated in the 0.4 µm pore size transwell membrane of six-well plates, and left for 1h at 37°C for gel formation. Then, 786-0 cells or 786-0/iEGFL7 cells were plated in the lower compartment of six-well Transwell Permeable Supports in complete media at a density of 1×10<sup>5</sup> cells per well. The 1×10<sup>5</sup> HMEC-1 cells were seeded into a 0.4 µm pore size transwell membrane coated with Matrigel in complete media. Cells were incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere for 48h. Tube formation was examined under

an inverted light microscope (Nikon, Japan) at 100× magnification. Three images were randomly taken from different areas of the well. Each treatment was performed in triplicate.

#### Western blot

After co-culturing of 786-0 cells or 786-0/iEGFL7 cells and HMEC-1 cells for 48h or treatment with 50 ng/ml recombinant human EGFL7 (Peprotech, 100-61) and 10 μM EGFR inhibitor Gefitinib (LC Laboratories, G-4408) for 48h. HMEC-1 cells were centrifuged and lysed with RIPA lysis buffer. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked in TBST containing 5% nonfat milk for 1h at room temperature. The blots were then incubated with primary antibody overnight at 4°C. Antibodies used for western blot analysis included focal adhesion kinase (FAK) (Cell signaling, 3285), p-FAK (Cell signaling, 3283), and EGFL7 antibody (Abcam, ab102796). After washing with TBST, the membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell signaling). The signals were visualized using ECL kit (Bio-Rad) and then exposed to X-ray films.

#### Xenograft model

Six-week-old female nude mice were obtained from Vital River Laboratories (VRL), China. All procedures were performed according to internationally accepted ethical guidelines. To establish RCC tumors in mice, cultured 786-0 cells or 786-0/iEGFL7 cells were detached with trypsin and resuspended in RPMI-1640 medium containing 10% FBS. The 786-0 cells or 786-0/iEGFL7 cells ( $5 \times 10^6$  cells in 0.1 ml RPMI-1640 medium) were injected subcutaneously into the right flank of each mouse. The tumor volume was measured weekly using a slide caliper. Five weeks after implantation, all the mice were euthanized and the tumors were excised and weighed. Tumor volume was determined by the following formula:  $(\text{length} \times \text{width}^2)/2$ .

#### Histologic staining

Extracted tumor specimens were fixed in 10% neutral buffered formalin and tissue processed for paraffin embedding. Four micrometer-thick sections were obtained from the paraffin-embedded tissue and histochemically stained with hematoxylin and eosin, or immunohistochemically stained with CD31 (Abcam, ab28364). MaxVision™ HRP-polymer anti-mouse/rabbit was added at room temperature for 15 min. The slides were developed using DAB for 1 min to 5 min. The nucleus was counterstained briefly with hematoxylin and then washed thoroughly.

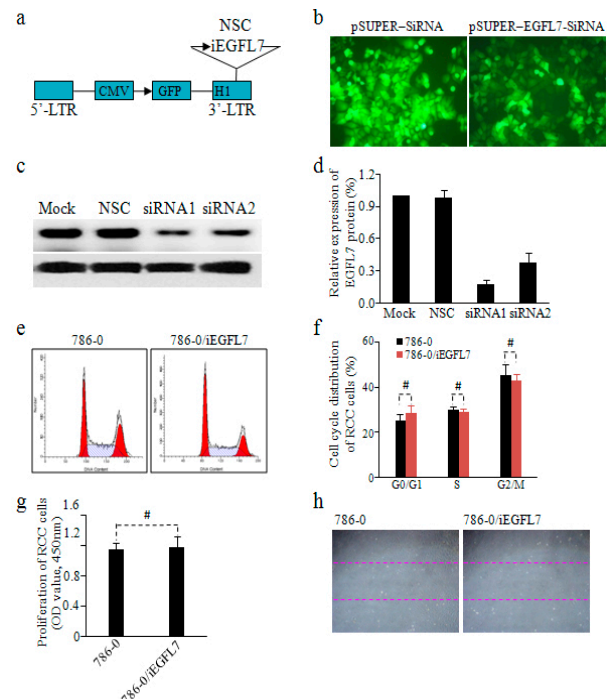
#### Statistical analysis

Data were represented as mean ± standard error (SE). The comparison between different tissues was assessed by Student's t-test. Statistical significance of differences was determined by analysis of variance (ANOVA), followed by Dunnett's test for individual group comparison.  $p < 0.05$  was considered statistically significant.

## Results

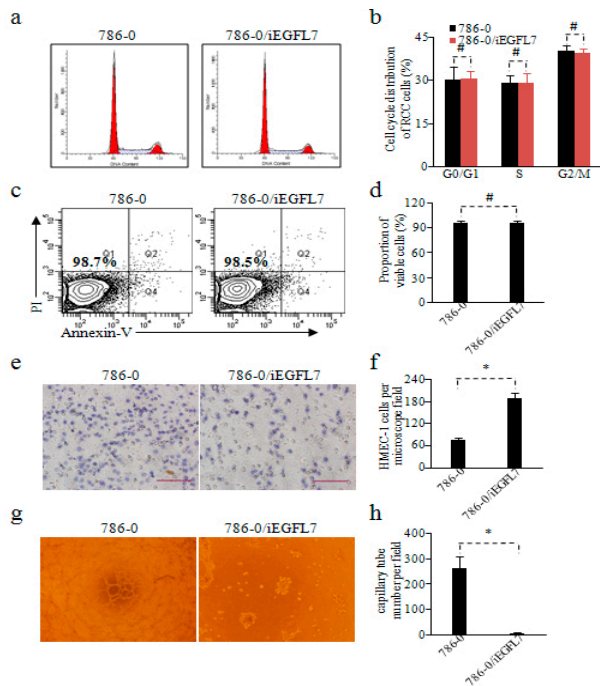
### Suppression of EGFL7 expression by siRNA in RCC cells

Our study has found that RCC cells upregulated the expression of EGFL7 protein. To determine how EGFL7 contributes to the growth of RCC, we employed siRNA to inhibit the expression of EGFL7 in RCC cells, and then assessed the cells for their tumorigenicity. Three oligonucleotide sequences were designed to interfere with the expression of EGFL7. The oligonucleotide pairs were ligated into the pSUPER.retro.GFP vector (Figure 1a). A high titer of retrovirus was obtained from a packaging vector in HEK293T cells (Figure 1b). Western blot was performed to detect the ability of two candidate sequences and a non-specific control sequence (NSC) to downregulate EGFL7 in 786-0 cells. The results showed that SiRNA1 inhibited the expression of EGFL7 protein by more than 80%. The other sequence, SiRNA2, only resulted in 50% to 70% inhibition, whereas the NSC sequence hardly caused detectable changes in EGFL7 expression (Figures 1c and 1d). Therefore, SiRNA1 was used to perform the following experiments. The 786-0 cells infected with empty virus and siRNA1 virus were termed 786-0 and 786-0/iEGFL7, respectively.



**Figure 1. Knockdown of EGFL7 Expression in RCC Cell Line 786-0.**

**a)** The short-hairpin oligos of iEGFL7 and NSC were cloned in a retroviral vector driven by H1 promoter in the 3'-long terminal repeat. **b)** The transfection efficiency of iEGFL7 in 786-0 cells. **c)** Western blot analysis of EGFL7 expression in 786-0 cells after retroviral infection. **d)** Statistical diagram of the relative expression of EGFL7 in 786-0 cells as shown in panel (c). **e)** Flow cytometric analysis of 786-0 cells after knockdown of EGFL7. **f)** Cell cycle distribution of (e) was counted. Data from three separate experiments are presented in the statistical diagram ( $\#p > 0.05$ ). **g)** Cell proliferation of 786-0 cells after knockdown of EGFL7 was detected by Cell Counting Kit-8 (CCK-8) assay ( $\#p > 0.05$ ). **h)** Cell migration of 786-0 cells after knockdown of EGFL7 was measured by wound healing assay

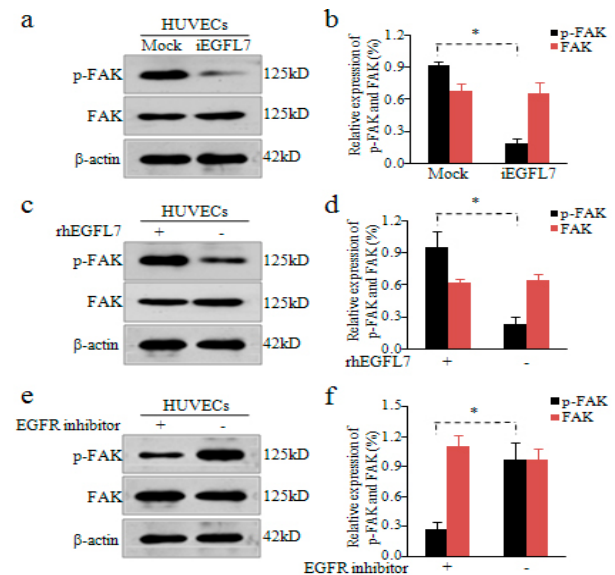


**Figure 2. Effects of iEGFL7 in 786-0 Cells on Biological Characteristics of Human Umbilical Vein Endothelial Cells (HUVECs).** **a**) Cell cycle analysis of HUVECs after co-culturing with 786-0 cells or 786-0/iEGFL7. **b**) Cell cycle distribution of (a) was counted. Data from three separate experiments are presented in the statistical diagram ( $^{\#}p < 0.05$ ). **c**) Apoptosis of HUVECs after co-culturing with 786-0 cells or 786-0/iEGFL7 was analyzed by flow cytometry. **d**) Cell apoptosis of (c) was counted ( $^{\#}p > 0.05$ ). **e**) Migration of HUVECs after co-culturing with 786-0 cells or 786-0/iEGFL7. **f**) Cell migration of (e) was counted ( $^*p < 0.01$ ). **g**) Vascular tube formation of HUVECs after co-culturing with 786-0 cells or 786-0/iEGFL7. **h**) Vascular tube numbers of (g) were counted ( $^*p < 0.01$ )

To observe if EGFL7 knockdown affects the growth of RCC cells, we compared the cell cycle and proliferation of 786-0 and 786-0/iEGFL7 cells. The results of cell cycle analysis showed that the cell cycle distribution was not affected by iEGFL7 in 786-0 cells (Figures 1e and 1f). The results of CCK-8 assay also showed that the proliferation rate exhibited no significant difference between 786-0 cells and 786-0/iEGFL7 cells (Figure 1g). To verify if EGFL7 knockdown affects the migration of RCC cells, we utilized the wound healing assay to compare the abilities of cell migration of 786-0 and 786-0/iEGFL7 cells. As shown in Figure 1h, the closure of 786-0/iEGFL7 cells was similar to that of 786-0 cells after culturing for 24h suggesting that EGFL7 has no role in RCC cell migration.

*Downregulated EGFL7 expression in RCC cells affects migration and tubule formation of HMEC-1 cells in vitro*

Numerous studies have shown that EGFL7 expression by tumor cells promotes the angiogenesis *in vivo* model (Wu et al., 2004; Huang et al., 2010). Therefore, we determined if EGFL7 depletion could affect the biological characteristics of HMEC-1 cells. Co-culture system was performed to observe the effects of EGFL7 knockdown in 786-0 cells on cell cycle, apoptosis, migration, and tubule formation of HMEC-1 cells. The results showed that the cell cycle of HMEC-1 cells was not affected by iEGFL7



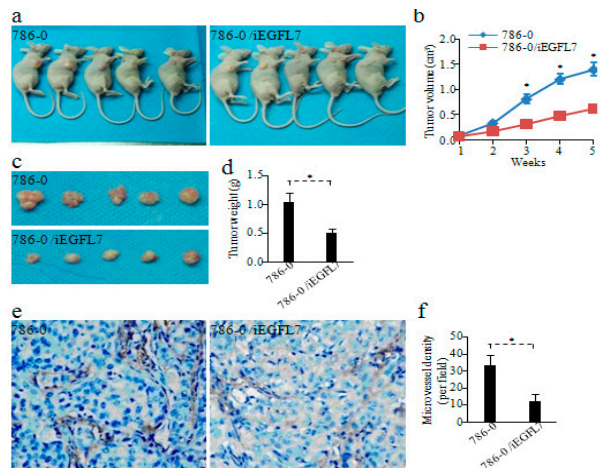
**Figure 3. RCC-Induced Migration and Vascular Tube Formation of HUVECs Were Regulated by Activating EGFR-FAK Signal Pathway.** **a**) The phosphorylated FAK and total FAK level of HUVECs after co-culturing with 786-0 cells or 786-0/iEGFL7 was detected by Western blot. **b**) Relative expression of (a) was counted. Data from three separate experiments are presented in the statistical diagram ( $^*p < 0.01$ ). **c**) The phosphorylated FAK and total FAK level of HUVECs after incubating with rhEGFL7 were detected by Western blot. **d**) Relative expression of (c) was counted ( $^*p < 0.01$ ). **e**) The phosphorylated FAK and total FAK level of HUVECs after co-culturing with 786-0 cells and incubating with EGFR inhibitor were detected by Western blot. **f**) Relative expression of (e) was counted ( $^*p < 0.01$ )

in 786-0 cells. Simultaneously, no significant difference in the cell cycle distribution was observed between 786-0 cells and 786-0/iEGFL7 cells (Figures 2a and 2b). We next determined if the cell survival of HMEC-1 cells was altered by downregulating EGFL7 expression in 786-0 cells. The results of apoptosis analysis showed that the survival rate of HMEC-1 cells exhibited no significant difference between 786-0 cells and 786-0/iEGFL7 cells (Figure 2c and 2d).

ECs migration and tubule formation are important processes in angiogenesis induced by tumor cells. Therefore, transwell assay was conducted to confirm if the cell migration of HMEC-1 cells demonstrates a distinction between 786-0 cells and 786-0/iEGFL7 cells. The data indicated that the numbers of HMEC-1 cells that passed through the transwell membrane were significantly less in 786-0/iEGFL7 cells than in 786-0 cells ( $p < 0.01$ ; Figures 2e and 2f). The vascular tubule formation of HMEC-1 cells was also disturbed by downregulating EGFL7 expression in 786-0 cells ( $p < 0.01$ ; Figures 2g and 2h). Together, our results suggest that EGFL7 is involved in HMEC-1 cell migration and tubule formation, but has little effect on HMEC-1 cell proliferation and survival.

*Secreted EGFL7 by RCC cells facilitates migration and tubule formation of HMEC-1 cells by activating EGFR-FAK signal pathway*

The EGFR-FAK signal pathway has an important function in angiogenesis induced by EGFL7. EGFL7



**Figure 4. Knockdown of EGFL7 inhibits growth of RCC tumor in nude mice.** **a)** In vivo tumor xenograft model. (Left) Tumor from 786-0 cells, (Right) tumor from 786-0/iEGFL7 cells. **b)** The volume of tumors at different time points. The tumor size was measured using a caliper. Tumor volume was calculated using the formula  $(\text{length} \times \text{width}^2)/2$  ( $*p < 0.01$ ). **c)** The weight of tumors at five weeks. Tumor from 786-0 cells (upside) and tumor from 786-0/iEGFL7 cells (below). **d)** Tumor weight of five mice was counted ( $*p < 0.01$ ). **e)** CD31 expression by immunohistochemistry staining among RCC xenograft tumors, 400 $\times$ . Brown chromogen represents positive staining. (Left) Tumor from 786-0 cells, (Right) tumor from 786-0/iEGFL7 cells. **f)** Microvessel density of tumor from five mice was counted ( $*p < 0.01$ ).

promotes endothelial cell motility and vascular tubule formation by facilitating FAK phosphorylation (Schmidt et al., 2007; Wu et al., 2009; Nikolic et al., 2013). We therefore compared the phosphorylation status of FAK in HMEC-1 cells co-cultured with 786-0 cells or 786-0/iEGFL7 cells. The results demonstrated that the level of FAK phosphorylation in HMEC-1 cells decreased significantly when co-cultured with 786-0/iEGFL7 cells compared with 786-0 cells, whereas the total of FAK levels were similar in these cells (Figures 3a and 3b). To verify if the FAK phosphorylation was mediated by EGFL7, we added 50 ng/mL recombinant human EGFL7 protein or PBS to the medium of 786-0/iEGFL7 cells and detected the phosphorylation status of FAK in HMEC-1 cells. We found that the level of FAK phosphorylation in HMEC-1 cells was significantly induced by recombinant human EGFL7 protein compared with PBS control (Figures 3c and 3d).

EGF-like protein, just like EGF, is a secreted protein that activates the downstream signal pathway by combining with the EGF receptor (EGFR). We therefore determined whether or not EGFL7 phosphorylates FAK through the EGF receptor (EGFR). To prove this hypothesis, we added 10  $\mu\text{M}$  EGFR inhibitor, Gefitinib, or PBS to the medium of 786-0 cells and detected the phosphorylation status of FAK in HMEC-1 cells. The data showed that the level of FAK phosphorylation in HMEC-1 cells was significantly decreased by the EGFR inhibitor compared with the PBS control (Figures 3e and 3f). Taken together, our data indicate that EGFL7 is specifically required for vascular tubulogenesis by activating the EGFR-FAK signal pathway in RCC.

### Targeting EGFL7 expression suppresses the growth of 786-0 cell xenografts in nude mice by inhibiting angiogenesis

In view of the observations obtained from *in vitro* studies, we investigated the role of EGFL7 in tumorigenesis of RCC in a xenograft mouse model. We found that the average size of RCC local tumors in the 786-0/iEGFL7 group was noticeably smaller than those in the 786-0 cells group (Figure 4a). The weekly results also showed that the growth rate of RCC tumors was significantly inhibited in the 786-0/iEGFL7 group than in the 786-0 cell group ( $*p < 0.01$ ; Figure 4b). Tumors were dissected in the mice and weighed; the average weights of tumor in the 786-0/iEGFL7 group were markedly lighter than those in the 786-0 cells group (Figures 4c and 4d). We next examined the vascular density of tumors by staining the endothelial cell markers CD31. Immunohistochemical staining showed significant decrease in the mean percentage of CD31-positive ECs in the 786-0/iEGFL7 group compared with the 786-0 cell group ( $p < 0.05$ ; Figures 4e and 4f). Overall, these data suggest that EGFL7 has an important function in the growth of RCC by facilitating angiogenesis.

## Discussion

Previous studies have reported that EGFL7 expression was upregulated during tumorigenesis. However, how EGFL7 contributes to the progression of human cancer remains unclear (Diaz et al., 2008; Wu et al., 2009; Huang et al., 2010; Sun et al., 2010). We found that both EGFL7 mRNA and protein increased significantly in RCC cells compared with normal renal cells (data not shown). These results indicated that EGFL7 may be involved in the development of RCC. However, the function of EGFL7 in the progression of RCC is still unknown.

To clarify the role of EGFL7 in RCC tumorigenicity, we employed siRNA to knockdown the EGFL7 expression in RCC cell line 786-0. First, we determined if knockdown EGFL7 expression affects the proliferation and migration of 786-0 cells. Our results showed that depletion of EGFL7 expression did not affect the proliferation and migration of RCC cells, indicating that EGFL7 affects the growth of RCC by other ways.

Angiogenesis is the key step for the growth of tumor (Carmeliet et al., 2000; Folkman, 2002; Weis et al., 2011). Therefore, we determined if knockdown EGFL7 expression in 786-0 cells influences the biological characteristics of human umbilical vein endothelial cells (HUVECs). We compared the cell cycle, apoptosis, migration, and tube formation of HUVECs by co-culturing with 786-0 cells or 786-0/iEGFL7 cells. The data showed that knockdown EGFL7 expression in 786-0 cells did not affect the proliferation and apoptosis of ECs. These results indicated that EGFL7 promotes the growth of RCC, but not by stimulating the proliferation or inhibiting the apoptosis of ECs. However, the results of transwell and tube-formation assay illustrated that downregulated EGFL7 expression in 786-0 cells significantly reduced the migration and tube formation of HUVECs. Our data reveal the critical role of EGFL7 secreted by RCC cells

in regulating cell migration of ECs.

Schmidt et al. reported that the level of FAK phosphorylation was significantly reduced in EGFL7-deficient mice, resulting in the significant inhibition of migration, adhesion, and growth of ECs (Schmidt et al., 2007). The results indicated that EGFL7 may promote cell motility by facilitating FAK phosphorylation. Another study by Wu et al. also showed that the phosphorylated FAK level in HCC cells was decreased significantly by knockdown EGFL7 (Wu et al., 2009). Indeed, our data showed that knockdown of EGFL7 expression in 786-0 cells significantly decreased the phosphorylated FAK level but not the total FAK level. We also found that recombinant human EGFL7 protein could restore the level of EGFL7 phosphorylation. EGFL7, as a secreted protein, may induce the phosphorylation of FAK by combining with cell surface receptor as other growth factors such as EGF and PDGF (Sieg et al., 2000). As an EGF like protein, two EGF-like domains are included in the structure of EGFL7, indicating that EGFL7 may activate FAK phosphorylation through EGFR, similar to EGF (Fitch et al., 2004; Parker et al., 2004). Treatment of HUVECs with EGFR inhibitor significantly blocked the effects of EGFL7 protein on FAK phosphorylation by co-culturing 786-0 cells, supporting the idea that FAK activation by EGFL7 was mediated by the EGFR-FAK pathway.

To confirm whether EGFL7 secreted by RCC cells has a role in the growth of RCC *in vivo*. We subcutaneously implanted both 786-0 cells and 786-0/iEGFL7 cells into nude mice. The data showed that the suppression of EGFL7 inhibited the growth of the xenotransplants of 786-0 cells in mouse models. In view of the important role of EGFL7 in angiogenesis, we detected the vascular density of *in situ* tumors by immunostaining the marker of ECs, namely, CD31. We found that the suppression of EGFL7 significantly reduced the number of microvessels, which indicated that EGFL7 slows the growth of RCC by inhibiting the angiogenesis of the tumors.

Taken together, our study is the first to show that EGFL7 is expressed in RCC cells, and its overexpression significantly correlates with the progress of RCC. We also demonstrated that EGFL7 promotes the growth of RCC by facilitating the migration and tube formation of ECs. These effects were produced by EGFL7-mediated FAK phosphorylation through combination with EGFR. Collectively, our data suggest that EGFL7 is a potential therapeutic target for the treatment of RCC.

## References

- Cairns P (2010). Renal cell carcinoma. *Cancer Biomark*, **9**, 461-73.
- Campagnolo L, Leahy A, Chitnis S, et al (2005). EGFL7 is a chemoattractant for endothelial cells and is up-regulated in angiogenesis and arterial injury. *Am J Pathol*, **167**, 275-84.
- Carmeliet P, Jain RK (2000). Angiogenesis in cancer and other diseases. *Nature*, **407**, 249-57.
- Carmeliet P, Jain RK (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature*, **473**, 298-307.
- Chung AS, Lee J, Ferrara N (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer*, **10**, 505-14.

- Cohen HT, McGovern FJ (2005). Renal-cell carcinoma. *N Engl J Med*, **353**, 2477-90.
- Diaz R, Silva J, Garcia JM, et al (2008). Deregulated expression of miR-106a predicts survival in human colon cancer patients. *Genes Chromosomes Cancer*, **47**, 794-802.
- Fitch MJ, Campagnolo L, Kuhnert F, Stuhlmannh (2004). Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. *Dev Dyn*, **230**, 316-24.
- Folkman J (2002). Role of angiogenesis in tumor growth and metastasis. *Semin Oncol*, **29**, 15-8.
- Grunwald V, Ravaud A (2014). Systemic therapy of renal cell carcinoma. *World J Urol*, **32**, 1.
- Huang CH, Li XJ, Zhou YZ, et al (2010). Expression and clinical significance of EGFL7 in malignant glioma. *J Cancer Res Clin Oncol*, **136**, 1737-43.
- Nikolic I, Stankovic ND, Bicker F, et al (2013). EGFL7 ligates alphavbeta3 integrin to enhance vessel formation. *Blood*, **121**, 3041-50.
- Parker LH, Schmidt M, Jin SW, et al (2004). The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. *Nature*, **428**, 754-8.
- Rini BI, Campbell SC, Escudier B (2009). Renal cell carcinoma. *Lancet*, **373**, 1119-32.
- Schmidt M, Paes K, De Maziere A, et al (2007). EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. *Development*, **134**, 2913-23.
- Shojaei F (2012). Anti-angiogenesis therapy in cancer: current challenges and future perspectives. *Cancer Lett*, **320**, 130-7.
- Sieg DJ, Hauck CR, Ilic D, et al (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol*, **2**, 249-56.
- Sun Y, Bai Y, Zhang F, et al (2010). miR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7. *Biochem Biophys Res Commun*, **391**, 1483-9.
- Tanriverdi O (2013). Review on targeted treatment of patients with advanced-stage renal cell carcinoma: a medical oncologist's perspective. *Asian Pac J Cancer Prev*, **14**, 609-17.
- Weis SM, Cheresh DA (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*, **17**, 1359-70.
- Wu F, Yang LY, Li YF, et al (2009). Novel role for epidermal growth factor-like domain 7 in metastasis of human hepatocellular carcinoma. *Hepatology*, **50**, 1839-50.