doi:10.4149/neo_2016_612

Targeting HIF-1α and VEGF by lentivirus-mediated RNA interference reduces liver tumor cells migration and invasion under hypoxic conditions

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Received May 16, 2016 / Accepted July 2, 2016

Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor to initiate the expressions of distinct pro-angiogenic growth genes, particularly the expression of vascular endothelial growth factor (VEGF).

$CoCl_2$ was used in rat liver tumor cell line McA RH-7777 to stimulate hypoxia to mimic the hypoxic conditions induced by transcatheter arterial chemoembolization (TACE). CCK8 assays were performed to examine the effect of hypoxia on cell viability. Real-time qRT-PCR, western blot and ELISA assays were used to measure the expression of HIF-1α and VEGF in McA RH-7777 cells under hypoxic conditions, respectively. Lentivirus-mediated HIF-1α and/or VEGF-specific shRNA was used to establish single or HIF-1α and VEGF double knocking-down McA RH-7777 cells. Transwell assays were performed to examine the effect of HIF-1α and VEGF knocking-down on McA RH-7777 cells migration and invasion.

The mRNA and protein expression level of HIF-1α and VEGF were remarkably up-regulated in McA RH-7777 cells under hypoxic conditions, respectively. The knockdown of HIF-1α or VEGF significantly reduced the expression of the secreted VEGF. More importantly, knockdown of both HIF-1α and VEGF resulted in the best effective inhibitory effect in VEGF expression, and in turn remarkably reduced the cell migration and invasion activity.

Our findings showed that HIF-1α play an important role in the stimulation of the secreted VEGF expression under hypoxic conditions, suggesting that targeting both HIF-1α and VEGF could represent a potential therapeutic strategy in combination with TACE in the treatment of liver tumors.

Key words: liver tumor, hepatocellular carcinoma, transcatheter arterial chemoembolization, hypoxia-inducible factor-1α, vascular endothelial growth factor

Hepatocellular carcinoma (HCC) is one of the most common and a lethal malignancy in the world. Because most HCC patients are diagnosed at advanced stages, transcatheter arterial chemoembolization (TACE) is considered the key treatment of interventional therapy for these patients [1]. TACE is a form of intra-arterial catheter-based chemotherapy, selectively delivering high doses of cytotoxic drug to the tumor bed [2]. However, recent studies have shed light on the implications of TACE-induced hypoxic microenvironment surrounding liver tumors [3, 4]. To increase oxygen supply, the residual surviving tumor cells secrete several pro-angiogenic growth factors which promote the process of angiogenesis, resulting in tumor cell growth, invasion and metastasis [5]. Therefore, hypoxia-inducible pro-angiogenic gene expression after TACE is one of the most important malignant behaviors.

Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of an oxygen-regulated HIF-1α subunit and a HIF-1β subunit, is induced under hypoxic condition which activates the transcription of genes involving in angiogenesis [6-8]. Vascular endothelial growth factor (VEGF), one of the most important angiogenic factors, is induced by HIF-1α [9-11]. The concentration of circulating VEGF was found to tightly associate with advanced HCC and tumor metastases [12]. VEGF can be secreted by tumor cells, thereby activating transduction pathways which promote migration, proliferation and prolong

cell survival of quiescent endothelial cells [13, 14]. Therefore, the better understanding of the process of HIF-1α promoting VEGF expression under hypoxic conditions is required for the improvement of the long-term efficacy of TACE. Moreover, targeting both HIF-1α and VEGF may provide a potential therapeutic strategy in combination with TACE for the treatment of liver tumors.

To test this possibility, the present study used $CoCl_2$-stimulated hypoxia to mimic the hypoxic conditions induced by TACE and explored the effects of hypoxia on the expressions of HIF-1α and VEGF in liver tumor cells. Furthermore, we sought to demonstrate the effects of knockdown of HIF-1α and/or VEGF on the behaviors of liver tumor cells.

Materials and methods

Cell culture. McA RH-7777 cells were brought form American Type Conditions Collection (no. CRL1601; ATCC, Manassas, VA, USA). McA RH-7777 cells were cultured in high glucose Dulbecco's modified Eagle's medium (HG-DMEM) (GIBCO, Scotland, UK) containing 10% fetal bovine serum (FBS) (GIBCO) at 37°C and 5% CO2 in a humidified atmosphere. For hypoxic conditions, McA RH-7777 cells were incubated in complete HG-DMEM medium with 150 μmol/L $CoCl_2$ for 24 h.

CCK8 assays. The viability of cells were assessed by Cell Counting Kit solution-8 (CCK8) assay. Cells were collected in the logarithmic growth phase and plated at a density of 1×10^3 per well in 96-well plates in triplicate. Following treatment, every well was incubated with CCK8 reagent (Dojindo Molecular Technologies Inc, Japan) in complete medium with a ratio of 1:10 for 5 h. The absorbance value of each well was measured at 450 nm using a microplate reader (Molecular Devices) with a reference wavelength of 650 nm.

RNA isolation and real-time RT-PCR. Total RNA were extracted from cells using Trizol (Invitrogen) according to the manufacturer's protocol. Quantitative real-time RT-PCR assays were performed to evaluated the expression of HIF-1α and VEGFA, and primers were designed as follows: HIF-1α forward 5'-AAGCACTAGACAAAGCTCACCTG-3', reverse 5'- TTGACCATATCGCTGTCCAC-3'; VEGF forward 5'- AGATTCTGCAAGAGCACC-3', reverse 5'- AAGGTCCTCCTGAGCTAT-3'; β-actin forward 5'- AGGGAAATCGTGCGTGACAT-3', reverse 5'- GAACCGCTCATTGCCGATAG-3'; The HIF-1α or VEGFA mRNA expression level was standardized to β-actin mRNA. All experiments were performed at least three times.

Western blot analysis. Cells were harvested and lysed by a lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 2 μg/ml leupeptin. Lysates were cleared by centrifugation (14,000 rpm) at 4°C for 30 min. The supernatant protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Normoxic lysates were boiled in electrophoresis SDS sample buffer, run on a 10% SDS-PAGE gel, and transferred to a polyvinylidine difluoride membrane (Millipore). Membranes were blocked for 1 h in Tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% skim milk. The membrane was probed with the indicated antibodies with the proper concentrations and times according to manufacturer's guidelines and washed twice with TBST for 10 min, then incubated with peroxidase-conjugated goat antibody to mouse IgG (1:5000; Amersham Pharmacia Biotech) for 1 h. After being washed three times, the membrane was developed by an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech).

ELISA assays. VEGF secreted in the cell conditions supernatants was collected and measured with the Quantikine human VEGF ELISA kits (R&D systems inc, UK) according to the manufacturer's instructions. This assay uses the quantitative sandwich enzyme immunoassay technique. HG-DMEM medium was used as control and recombinant VEGF165 was used as standard for drawing the standard curves.

Transwell invasion assay. Transwell invasion assays were performed using 8-μm pore transwell chambers in 24-well plates (Corning Costar, Cambridge, MA, USA). After being treated with serum-free HG-DMEM starvation overnight, cell suspension (1×10^5 cells/ml, 100μl) was added into the upper chamber. Transwell membranes were pre-coated with 50 μg/μl Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with 500 μl serum-free HG-DMEM medium containing 10% FBS. Subsequently, the cells were conditioned for another 24 h. After swabbing the non-migrated cells from the upper chambers, the cells that had migrated to the lower chambers were fixed with 4% paraformaldehyde in PBS and stained with Giemsa. Finally, invading cells in 5 adjacent microscope fields for each membrane were counted at x40 magnification under a microscope (Nikon, Tokyo, Japan).

Constuction of lentivirus-mediated HIF-1α and VEGF knocking-down cells. The lentiviral vector containing HIF-1α (LV-shRNA-HIF-1α) with CMV-driven mCherry RFP reporter or VEGFA (LV-shRNA-VEGFA) shRNA with CMV-driven GFP reporter was purchased from Genecopoeia Company (Guangzhou, China), respectively. A lentiviral vector containing a non-specific shRNA (LV-shRNA- scramble) was used as scramble control. Briefly, the plasmids LV-shRNA-HIF-1α, LV-shRNA-VEGFA or LV-shRNA-HIF-1α together with LV-shRNA-VEGFA were transfected into McA RH-7777 cells with packaging vectors, respectively. Cells transfected with lentiviral vector containing a non-specific shRNA (LV-shRNA- scramble) was used as scramble control. At 72 h post-transfection, YFP and GFP positive cells were by Influx™ Fluorescence-Activated Cell Sorting system according to manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with SPSS (Statistical Package for the Social Sciences) 17.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are expressed as mean±SD. Data were analyzed using the Student's

t-test between the two groups and AVOVA analysis for others. $P<0.05$ was considered as significant.

Results

Hypoxic conditions up-regulated the expressions of HIF-1α and VEGF and promoted the invasion activity of McA RH-7777 cells.

To explorer the effects of hypoxic conditions on HIF-1α and VEGF, we used $CoCl_2$ to stimulate hypoxia. At the beginning, CCK8 assays were performed to examine McA RH-7777 cell proliferation at different $CoCl_2$ concentrations. Mock cells were used as controls (100%, 0μM $CoCl_2$). Increasing the concentrations of $CoCl_2$ from 50 μM to 150 μM showed the limited effect on cell proliferation, while the concentration of 200μM resulted in a significant reduction in cell viability (64.3%) (Figure1A). Thus, the concentrations of $CoCl_2$ from 50 μM to 150 μM were used in the subsequent experiments.

Subsequently, the real-time qRT-PCR assays showed that HIF-1α mRNA expression in McA RH-7777 cells was gradually up-regulated with increasing the concentrations of $CoCl_2$ (Figure 1B). The results were further confirmed by western blot analysis using HIF-1α specific antibody (Figure 1B). Beside, no detectable protein level of HIF-1α was observed without $CoCl_2$, confirming that the stimulation of HIF-1α is required for hypoxic condition. Similarly, the real-time qRT-PCR assays also showed that increasing the concentrations of $CoCl_2$ resulted in the VEGF mRNA expression up-regulation (Figure 1C). ELISA assays confirmed that VEGF secreted in the cell conditions supernatants was increased in the presence of $CoCl_2$ (Figure 1C).

In addition, we sought to examine invasion activity of McA RH-7777 cells under hypoxic condition by using transwell assays. As shown in Figure 1D, the number of cells migrated to the lower chambers was increased according to the concentrations of $CoCl_2$ (Figure 1D), showing that hypoxic conditions could promote the activity of McA RH-7777 cells to migrate and invade. Taken together, these results indicated that hypoxic conditions increased the expressions of HIF-1α and VEGFA, and in turn promoted the activity of McA RH-7777 cells to migrate and invade.

Lentivirus-mediated high-efficiency infection of McA RH-7777 cells for HIF-1α and VEGF knocking-down. HIF-1α/VEGF HIF-1α, VEGF and HIF-1α/VEGF knocking-down McA RH-7777 cell lines were established via using lentivirus-mediated RNA interference (RNAi) technology, respectively. Briefly, the fragments targeting HIF-1α and VEGF were designed and implanted to lentiviral vectors fused mCherryRFP and GFP as reporters, respectively. A non targeting fragment was used as scramble control. For knockdown of single HIF-1α or VEGF, the lentivirus containing HIF-1α or VEGF fragments were packaged and purified to infect McA RH-7777 cells (McA RH-7777-shHIF-1α and McA RH-7777-shVEGF cells), respectively. For knockdown of both HIF-1α and VEGF, McA RH-7777 cells were co-infected with lentivirus containing HIF-1α or VEGF fragments (McA RH-7777-shHIF-1α/shVEGF cells). The infected cells were screened and sorted by flow cytometry assays (Figure 2A and 2B).

The knockdown effect was evaluated by real-time qRT-PCR. As shown in Figure 2C and 2D, knockdown of HIF-1α and VEGF resulted in approximately 70% and 50% reduction in HIF-1α and VEGF mRNA expression, respectively. Moreover, mRNA expressions of HIF-1α or VEGF in double knocking-down McA RH-7777-shHIF-1α/shVEGF cells also significantly reduced compared to that in scramble cells, respectively. Beside, western blot analysis further confirmed that HIF-1α protein expression was significantly reduced in both McA RH-7777-shHIF-1α and McA RH-7777-shHIF-1α/shVEGF cells (Figure 2E). Similarly, ELISA assays also confirmed that VEGF protein expression was significantly reduced in both McA RH-7777-shVEGF and McA RH-7777-shHIF-1α/shVEGF cells (Figure 3A). Of note, knockdown of VEGF or HIF-1α alone did not affect HIF-1α or VEGF expression, respectively, suggesting that HIF-1α is not positive correlated with VEGF under normoxic conditions.

Knockdown of both HIF-1α and VEGF reduced the secreted VEGF protein expression and liver tumor cell invasion under hypoxic condition. We sought to examine the expression of VEGF in the presence of HIF-1α and/or VEGF knocking-down under 150 μM $CoCl_2$-stimulated hypoxic conditions by using ELSIA assays. As shown in Figure 3A, VEGF protein expression in the cell conditions supernatants of McA RH-7777-shHIF-1α cells was comparable to that of the scramble cells under normoxic conditions, whereas a significantly reduction in VEGF protein expression was observed in McA RH-7777-shHIF-1α cells' conditions supernatants compared to that of the scramble cells under hypoxic conditions (2291.09±165.29 vs. 3423.76±327.83, P=0.006). These results indicated that HIF-1α plays an important role in VEGF expression under hypoxic conditions.

Beside, although VEGF protein expression still increased about 2-folds in the cell conditions supernatants of McA RH-7777-shVEGF cells under hypoxic conditions compared to that under normoxic conditions, it was significantly reduced compared to that of the scramble cells under hypoxic conditions (2324.82±104.78 vs. 3423.76±327.83, P=0.005). These results further confirmed the importance of HIF-1α in promoting VEGF expression under hypoxic conditions. Indeed, knockdown of both shHIF-1α and VEGF resulted in the most effective inhibitory effect in VEGF expression (1474.73±66.38 for KD HIF-1α/VEGF vs. 3423.75±327.83 for scramble, P=0.001; vs. 2291.09±165.29 for KD HIF-1α, P=0.001; vs. 2324.82±104.78 for KD VEGF, P<0.001).

Moreover, transwell assay was performed to examine the migration and invasion activity of McA RH-7777-shHIF-1α/shVEGF cells under hypoxic conditions (Figure 3B). Under normoxic conditions, the number of the migrated McA RH-7777-shHIF-1α/shVEGF cells was significantly reduced compared to that of the scramble cells (5.2±2.1 vs. 8.2±0.84, P=0.045). On the other hand, the number of the migrated

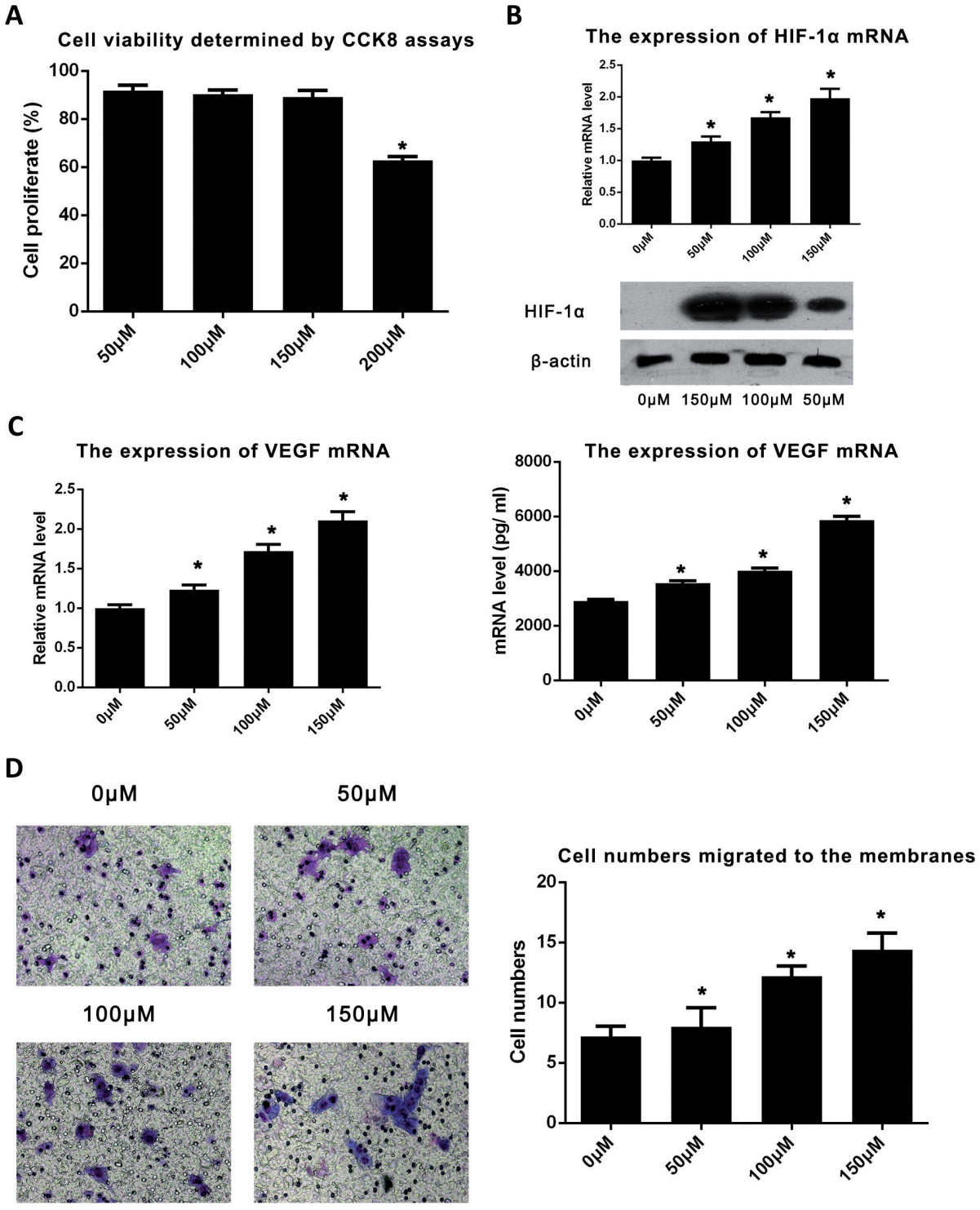

Figure 1. Hypoxic conditions up-regulated the expressions of HIF-1α and VEGF and promoted the invasion activity of McA RH-7777 cells. (A) The cell viability was determined by using CCK8 assays after 24 h incubation with $CoCl_2$ from 50 μM to 200 μM. Mock McA RH-7777 cells were used as positive control (100%) and the viability of treated groups of cells were calculated as the percentage of mock cells. (B) The expression of HIF-1α mRNA and protein in McA RH-7777 cells treated with different concentrations of $CoCl_2$ were examined by real-time qRT-PCR and western blot analysis, respectively. β-actin was used as loading control. (C) VEGF mRNA and protein expression secreted in the cell conditions supernatants in McA RH-7777 cells treated with different concentrations of $CoCl_2$ were examined by real-time qRT-PCR and ELISA assays, respectively. HG-DMEM medium was used as control and a serial dilution of recombinant VEGF165 was used as standard. (D) Cell invasion was examined by using Transwell chamber. The cells were fixed and stained (×200). The numbers of the cells migrated to the membranes were counted. Data were presented as means ± SD analyzed with AVOVA from three independent experiments.

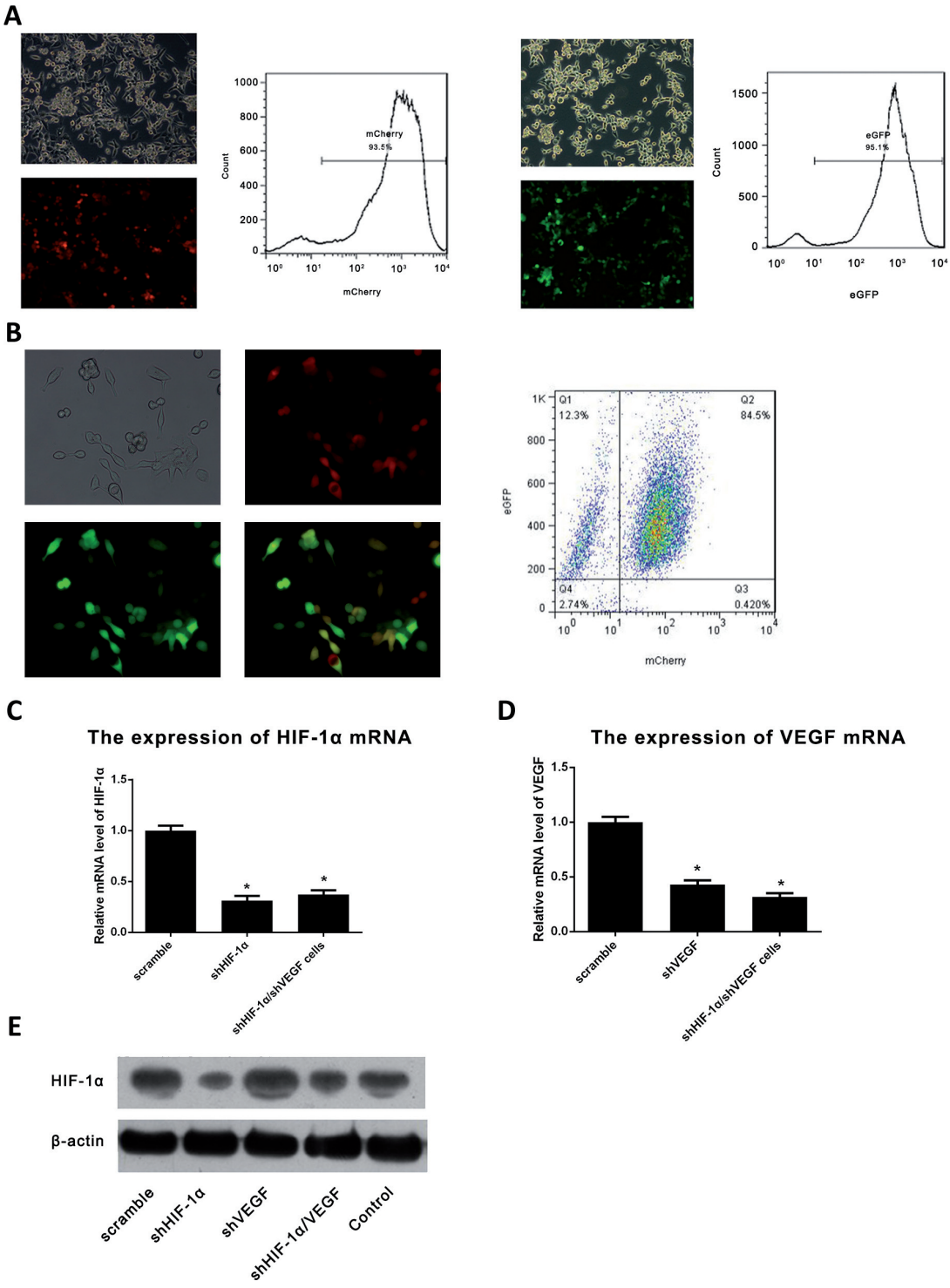

Figure 2. Knockdown of HIF-1α and/or VEGF through lentivirus-mediated shRNA in McA RH-7777 cells. (A) The recombinant lentiviruses contained the short shRNA targeting the specific sequences of HIF-1α, VEGF or scramble control were constructed as described in material and methods. The HIF-1α knocking-down McA RH-7777-shHIF-1α cells were screened by mCherry RFP reporter, the VEGF knocking-down McA RH-7777-shVEGF cells were screened with GFP reporter. (B) HIF-1α and VEGF co-silencing McA RH-7777-shHIF-1α/VEGF cells were screened by merging the RFP and GFP reporters. The real-time qRT-PCR was performed to examine the mRNA expression levels of HIF-1α (C) and VEGF (D) in McA RH-7777-shHIF-1α, McA RH-7777-shVEGF and McA RH-7777-shHIF-1α/VEGF cells, respectively. The scramble cells were used as the control. Data were presented as means ± SD analyzed with AVOVA from three independent experiments. (E) The protein expression of HIF-1α in the different treated groups of cells examined by western blot analysis. β-actin was used as loading control. Data were presented as means ± SD analyzed with AVOVA from three independent experiments.

McA RH-7777-shHIF-1α/shVEGF cells was also significantly reduced compared to that of the scramble cells (5.8±0.84 vs. 13.8±1.92, P<0.001) under hypoxic conditions. Taken together, these results indicate that knockdown of both HIF-1α and VEGF resulted in a remarkable reduction in the migration and invasion activity of McA RH-7777 cells no matter under normoxic conditions or hypoxic conditions.

Discussion

The treatment of TACE for liver tumors usually results in the stimulation of angiogenesis in the residual surviving tumor cells [3, 4, 15], since TACE is always found to lead to the hypoxic microenvironment. HIF-1α is a key transcription factor induced by hypoxia and it has been shown to initiate the expressions of distinct pro-angiogenic growth genes, particularly the activation of VEGF gene expression [5, 16-20], which is a potent growth factor stimulating proliferation of endothelial cells and promoting angiogenesis, particularly in areas of hypoxia [19]. Suppression of tumor development on the molecular level could be achieved by down-regulating pro-tumoral and pro-angiogenic factors. The present study showed that knockdown of both HIF-1α and VEGF genes resulted in a significant reduction in secreted VEGF and decreased the cell invasion activity of liver tumor cell under $CoCl_2$-stimulated hypoxic conditions. These result suggested that targeting both HIF-1α and VEGF could represent a potential therapeutic strategy in combination with TACE in the treatment of liver tumors.

The limitations of the TACE technique is that embolization of the tumor-feeding vessel is possibly incomplete, which allows the tumor to survive in a hypoxic microenvironment. This process results in the reduced blood supply as well as formation of tumor necrosis. Therefore, the residual surviving tumors respond to the hypoxia. The molecular responses to hypoxia include accumulation of active HIF-1α and β heterodimers in cell nuclei and induction of many genes involving in angiogenesis, such as VEGF. By using $CoCl_2$-stimulated hypoxia, we found that HIF-1α and VEGF protein were remarkably up-regulated under hypoxic conditions. Our results agreed with previous studies that HIF-1α protein is a critical transcription factor response to hypoxia.

The importance of HIF-1α in angiogenesis induced by hypoxia was also confirmed by our observation that HIF-1α protein was undetectable under normoxic conditions. HIF-1α has been shown to have a half-life of approximately 5 min in normoxic conditions [21, 22]. Receptor for activated C-kinase 1 (RACK1) RACK1 competes with HSP90 for binding to HIF-1α, links HIF-1α to Elongin-C, and promotes HIF-1α degradation [23]. As the cells are exposed to hypoxic conditions, HIF-1α is protected from ubiquitination and translocated to nucleus to activate the transcription of down-scream genes [22]. In addition, we found that knockdown of HIF-1α showed no effect on the expression of secreted VEGF under normoxic conditions, but resulted in a significant reduction in the secreted VEGF expression under hypoxic conditions compared to the scramble control. These results further confirmed the

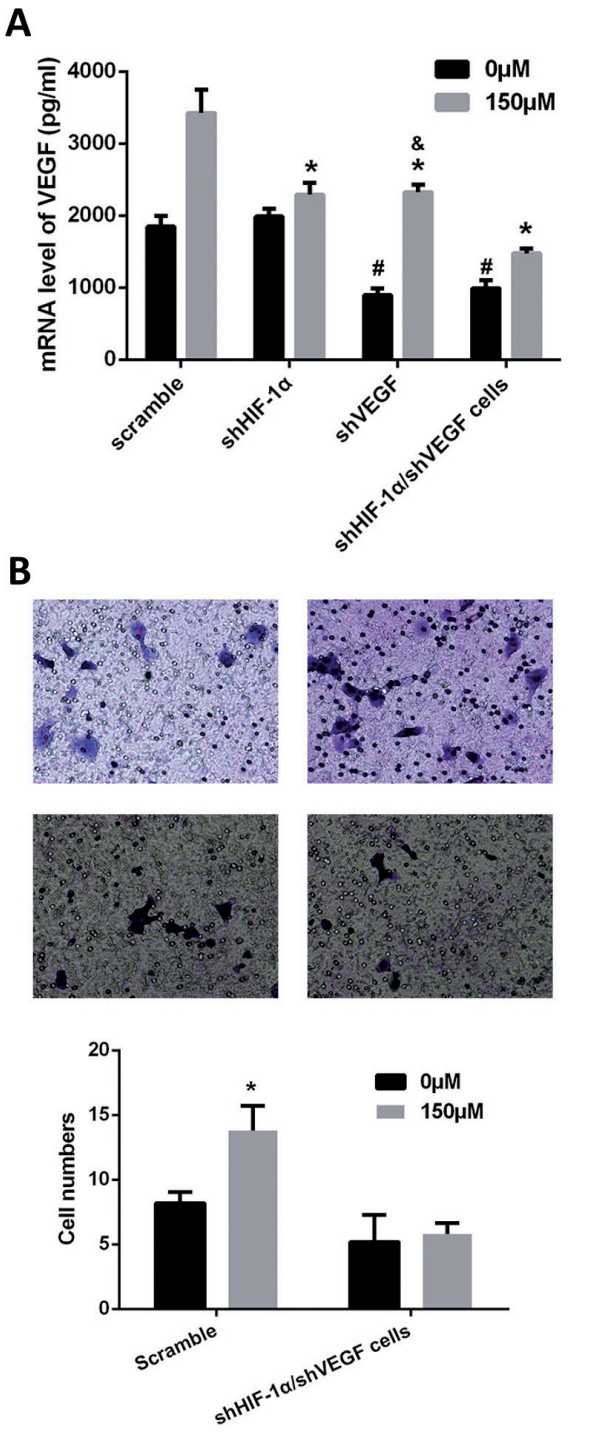

Figure 3. Knockdown of both HIF-1α and VEGF reduced the secreted VEGF protein expression and liver tumor cell invasion under hypoxic condition. (A) The secreted VEGF protein in the different treated groups of cells under normoxic conditions (blue) and under hypoxic conditions (red) were examined by ELISA assays, respectively. HG-DMEM medium was used as control and a serial dilution of recombinant VEGF165 was used as standard. (B) Cell invasion was examined by using Transwell chamber. The cells were fixed and stained (×200). The numbers of the cells migrated to the membranes were counted. Data were presented as means ± SD analyzed with Student's t-test from three independent experiments.

positive correlation of HIF-1α with angiogenesis (VEGF) in liver tumors under hypoxic conditions. Therefore, it could be postulated that tumor angiogenesis can be inhibited effectively by knocking-down both HIF-1α and VEGF. Furthermore, we found that co-silence of HIF-1α and VEGF resulted in best inhibitory effect on the expression levels of secreted VEGF and the activity of cell to invade under hypoxic conditions.

In summary, this report showed that $CoCl_2$-stimulated hypoxia significantly increased the expression of HIF-1α and VEGF in liver tumor cells. Further investigation showed that knockdown of HIF-1α gene expression significantly down-regulated VEGF expression. More importantly, knockdown of both HIF-1α and VEGF resulted in the best effective inhibitory effect in VEGF expression, and in turn remarkably reduced the cell invasion activity. Therefore, targeting both HIF-1α and VEGF may represent a potential therapeutic strategy in combination with TACE in the treatment of liver tumors.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (nos. 81172193 and 81571774) and the PhD Start-up Fund of Natural Science Foundation of Guangdong Province, China (2015A030310171)

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