

# Cysteine-rich 61 RNA interference inhibits pathological angiogenesis via the phosphatidylinositol 3-kinase/Akt-vascular endothelial growth factor signaling pathway in endothelial cells

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Received January 30, 2015; Accepted November 18, 2015

DOI: 10.3892/mmr.2016.5772

**Abstract.** Hypoxia is a key factor in the pathogenesis of angiogenesis, and cysteine-rich 61 (CCN1), an angiogenic factor, is involved in the development of pathological angiogenesis. The aim of the present study was to investigate the mechanism of CCN1 RNA interference (RNAi)-induced inhibition of hypoxia-induced pathological angiogenesis in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were cultured under hypoxic conditions *in vitro*. The effects of inhibiting phosphoinositide 3-kinase (PI3K)/Akt signaling using LY294002 were investigated in hypoxic HUVECs. The proliferation and apoptosis of HUVECs under hypoxia were assessed using CCN1 RNAi. The CCN1-PI3K/Akt-vascular endothelial growth factor (VEGF) pathway was analyzed under hypoxic conditions using reverse transcription-quantitative polymerase chain reaction and western blotting. CCN1 RNAi inhibited the proliferation and induced the apoptosis of the HUVECs under hypoxia, with hypoxia significantly increasing the mRNA and protein expression levels of CCN1, Akt and VEGF. By contrast, CCN1 RNAi reduced the mRNA and protein expression levels of CCN1, Akt and VEGF in the HUVECs ( $P < 0.05$ ). Furthermore, LY294002 reduced the mRNA and protein expression levels of CCN1 in the hypoxic cells ( $P < 0.05$ ). These data indicated that CCN1 inhibits apoptosis and promotes proliferation in HUVECs. Therefore, CCN1 RNAi may offer a novel therapeutic strategy, which may aid in

the treatment of pathological angiogenesis via inhibition of the PI3K/Akt-VEGF pathway.

## Introduction

Hypoxia is a common pathophysiological phenomenon, which has a profound impact on endothelial cell (EC) properties in numerous pathological angiogenic diseases, including retinopathy of prematurity, proliferative diabetic retinopathy, retinal vein occlusion and age-related macular degeneration (1,2). These diseases are a major cause of blindness worldwide, however, there remains a lack of effective medical treatment options. Therefore, understanding the association between hypoxia and pathological angiogenesis may be important in characterizing the mechanisms of disease and assist in the development of novel treatment strategies.

Cysteine-rich 61 (CCN1), the first cloned member of the CCN family, mediates cell adhesion, stimulates chemotaxis, augments growth factor-induced DNA synthesis, fosters cell survival and enhances angiogenesis (3-5). Previous studies have demonstrated that hypoxic conditions are able to induce the expression of CCN1 in several types of cell (6-11). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in multiple cellular processes, including cell survival and differentiation, and it has been demonstrated to be important in angiogenesis (12). Previous studies have demonstrated that CCN1 induces monocyte chemotactic protein 1 through the activation of PI3K/Akt and nuclear factor- $\kappa$ B signaling in chorioretinal vascular ECs (13). Additionally, a previous study indicated that CCN1 can enhance the expression of vascular endothelial growth factor (VEGF) and promote tumor neovascularization via the PI3K/Akt signaling pathway (14).

However, the specific mechanisms, which are involved in CCN1-mediated pathological angiogenesis in ECs remain to be fully elucidated. The present study hypothesized that the CCN1/PI3K/AKT/VEGF signaling pathway may be associated with pathological angiogenesis and comprise possible molecular therapeutic targets. In order to confirm this hypothesis, the present study investigated the effect of reducing the expression of CCN1 in hypoxic ECs, and

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**Key words:** cysteine-rich 61, hypoxia, vascular endothelial growth factor, angiogenesis, human umbilical vein endothelial cells, RNA interference

analyzed the molecular mechanisms involved in pathological angiogenesis.

## Materials and methods

**Cell culture.** HUVECs were purchased from Cell Systems Corporation (Kirkland, WA, USA) and were cultured in Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences, Chalfont, UK) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>, with subconfluent monolayers passaged 3-10 times prior to treatment.

**Hypoxic treatment.** Hypoxic exposure was performed using a tightly sealed molecular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA), which was tightly sealed and flushed with a gas mixture containing 1% O<sub>2</sub>, 94% N<sub>2</sub> and 5% CO<sub>2</sub>, as previously described (15,16), with the cell culture dishes containing 1x10<sup>5</sup> cells/well placed in the chamber and incubated at 37°C for 24 h.

The HUVECs were divided into four groups: A normoxia group; a hypoxia group; a hypoxia-control group, which was transiently transfected with scramble small interfering (si)RNA; and a hypoxia-CCN1 siRNA group, which was transiently transfected with CCN1 siRNA. The HUVECs were transiently transfected with plasmids (500 ng/μl) using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h.

**PI3K/Akt inhibition.** The PI3K/Akt inhibitor, LY294002, was used in the present study to determine the effect of inhibiting the PI3K/Akt pathway on the normoxic and hypoxic HUVECs. The cells were cultured under normoxic or hypoxic conditions in six-well plates at a density of 1x10<sup>5</sup> cells/well as described above, in the presence of LY294002 (Sigma-Aldrich, St. Louis, MO, USA). The solution comprised 40 μmol/l dissolved in dimethyl sulfoxide (DMSO), with a final concentration of DMSO in the cell culture of 0.1%. The cells were pretreated with LY294002 for 30 min prior to being placed in the incubator for hypoxic exposure. The mRNA and protein expression levels of CCN1 were then analyzed using RT-qPCR and western blotting, respectively, following 24 h normoxia or hypoxia.

**Gene knockdown by siRNA.** Four pairs of CCN1 siRNA sequences were designed and synthesized (Shanghai GenePharma Co., Ltd., Shanghai, China), with one pair selected based on stability and effectiveness. The sequences were as follows: CCN1 (Cyr61-homo-553) forward, 5'-GGGAAA GUUCCAGCCCAACUTT-3' and reverse, 5'-AGUUGG GCUGGAAACUUCCCTT-3'; CCN1 (Cyr61-homo-789) forward, 5'-GAGGUGGAGUUGACGAGAACTT-3' and reverse, 5'-GUUUCUGUCAACUCCACCUCTT-3'; CCN1 (Cyr61-homo-1072) forward, 5'-GCAAGAAUGCAGCA AGACCATT-3' and reverse, 5'-UGGUCUUGCUGCAUUUCU UGCTT-3'; CCN1 (Cyr61-homo-1268) forward, 5'-GAUGAU CCAGUCCUGCAAUGTT-3' and reverse, 5'-CAUUGCAG GACUGGAUCAUCTT-3'. In addition, a non-silencing siRNA sequence was selected for use as a negative control (forward

5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGA CACGUUCGGAGAATT-3' reverse). The siRNAs were cloned using a pGPU6/green fluorescent protein (GFP)/Neomycin resistance screening marker (Neo) siRNA Expression Vector kit (cat. no. E-07/F-07; Shanghai GenePharma Co., Ltd.), according to the manufacturer's protocol, generating the pGPU6/GFP/Neo-CCN1 siRNA and the pGPU6/GFP/Neo-scramble siRNA plasmids, which contained *Bbs*I and *Bam*HI restriction sites. The cells were transfected, according to the manufacturer's protocol, with the mRNA and protein levels assessed 48 h following transfection. siRNA was successfully transfected into HUVECs in six-well culture plates, with each well containing 240 pmol fluorescent labelled siRNA and 8 μl Lipofectamine<sup>®</sup> 2000 for 6 h. Transfection efficiency was determined using fluorescence microscopy (FV1000; Olympus Corp., Tokyo, Japan).

**Cell proliferation assay.** A Cell Counting Kit-8 (CCK8) assay (Beyotime Institute of Biotechnology, Jiangsu, China) was used to measure cell proliferation, according to the manufacturer's protocol. Briefly, HUVECs were plated in 96-well plates at a density of 2,000 cells/well, and proliferation was measured each day for 4 days following transfection. A total of 10 μl CCK8 was added to each well and incubated for 2 h at 37°C. Following incubation, the samples were vortexed for 10 min and the absorbance of each was measured in a Sunrise<sup>™</sup> microplate reader (Tecan Group, Ltd., Männedorf, Switzerland) at 450 nm.

**Cellular apoptosis assay.** Cellular apoptosis was investigated by flow cytometry using an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (cat. no. KGA106; Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China), according to the manufacturer's protocol. The cells were washed twice in ice-cold phosphate-buffered saline at pH 7.5 (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) and resuspended in 1X binding buffer (Zhongshan Jinqiao Biotechnology Co., Ltd.) at 1x10<sup>6</sup> cells/ml. A total of 100 μl cells (1x10<sup>5</sup> cells) were gently mixed with 5 μl annexin V-FITC and 5 μl propidium iodide (PI), and incubated for 15 min in the dark at room temperature. An additional 400 μl of 1X binding buffer was added, and cellular apoptosis was detected using a flow cytometer (FACSCalibur<sup>™</sup>; BD Biosciences, San Jose, CA, USA). The apoptotic rates of the cells were calculated as the ratio of early and late apoptotic cells to the total cells (17).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from the HUVECs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse-transcribed into cDNA using a reverse transcription kit (DRR037S; PrimeScript<sup>™</sup> RT Reagent kit-Perfect Real-Time; Takara Bio Inc., Dalian, China) as previously described (18). Primers were designed using Primer Express software version 2.0 (Life Technologies; Thermo Fisher Scientific, Inc.) and are presented in Table I. qPCR was performed using SYBR Green PCR Master mix (Premix Ex Taq<sup>™</sup>-Perfect Real Time; cat. no. DRR041S; Takara Bio, Inc.). The PCR mixture contained 10 μl 2X TaqMan PCR mix, 0.4 μl PCR forward and 0.4 μl PCR reverse

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Primer sequence (5'-3')	Product length (bp)
β-actin	Forward	CGTGGACATCCGCAAAGAC	200
	Reverse	GGAAGGTGGACAGCGAGGC	
VEGF	Forward	TGCCCCTGAGGAGTCCAAC	336
	Reverse	TGGTTCCCGAAACGCTGAG	
Akt	Forward	TTGCTTTCAGGGCTGCTCA	230
	Reverse	TCTTGGTCAGGTGGTGTG ATG	
CCN1	Forward	CGAGGTGGAGTTGACGAGAA	211
	Reverse	GCACTCAGGGTTGTCATTGGT	

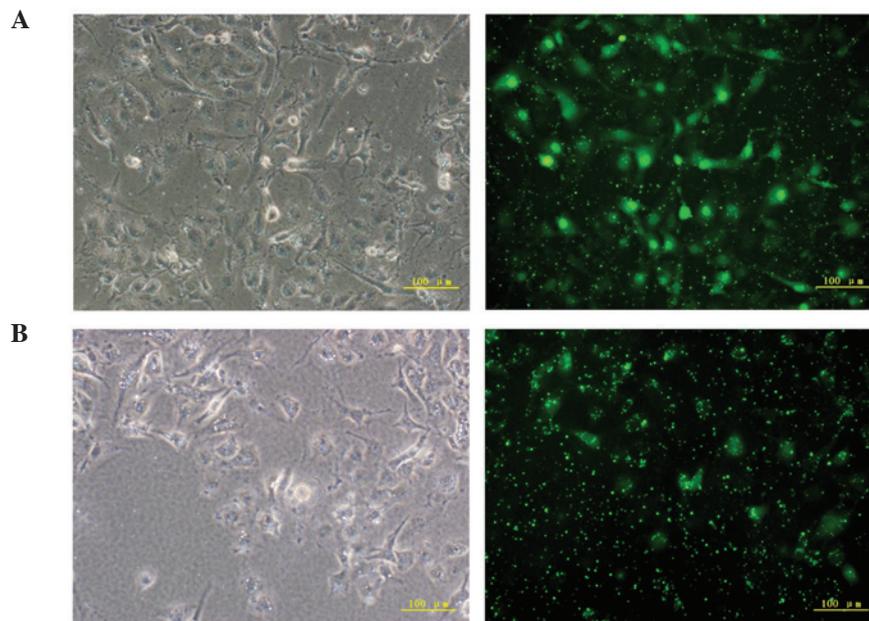


Figure 1. Transfection efficiency of (A) CCN1 siRNA and (B) scramble siRNA in HUVECs under hypoxic conditions. Transfection efficiency was detected using light and fluorescence microscopy. Scale bar=100 μm. HUVECs cultured under hypoxic conditions attached to the bottom of the well. HUVECs under the hypoxic condition lost their normal fusiform morphology and an increased number of dead cells were observed. At 6 h post-transfection, siRNA was successfully transfected into the cells with a percentage of green fluorescence protein-positive HUVECs of >80%. CCN1, cystein-rich 61; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; siRNA, small interfering RNA.

primer, 1.0 μl cDNA and 8.2 μl double-distilled H<sub>2</sub>O with a total volume of 20 μl and the reaction was performed in an Applied Biosystems 7300 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 95°C for 30 sec, 50 cycles of 95°C for 5 sec and 60°C for 31 sec. β-actin was included in each reaction as an internal control, and the relative gene expression levels were calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (19).

**Western blot analysis.** The cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and phenyl-methylsulfonyl fluoride (all from Sigma-Aldrich), and protein concentration was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Haimen, China). The samples (60 μg) were separated by 8% or 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica,

MA, USA). Following blocking with 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline-Tween-20 [20 mM Tris-HCl, 500 mM NaCl and 0.05% Tween-20 (Yesen Biotechnology Co., Ltd., Shanghai, China); TBST], membranes were washed four times for 5 min with TBST, and were then incubated with the following specific primary antibodies overnight at 4°C: Rabbit anti-CCN1 polyclonal antibody (1:2,000 dilution; cat. no. ab24448; Abcam, Cambridge, UK); rabbit anti-phosphorylated (p)AKT1/2/3 (Ser473) polyclonal antibody (1:2,000 dilution; cat. no. sc-101629); rabbit anti-VEGF polyclonal antibody (1:2,000 dilution; cat. no. sc-152) and rabbit anti-mouse β-actin polyclonal antibody (1:2,000 dilution; cat. no. sc-130656) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, membranes were incubated for 2 h at 37°C with horseradish peroxidase-conjugated anti-rabbit-immunoglobulin G secondary antibodies (1:2,000 dilution; cat. no. ZB-2010; Zhongshan Jinqiao Biotechnology Co., Ltd.). Protein bands were visualized using enhanced

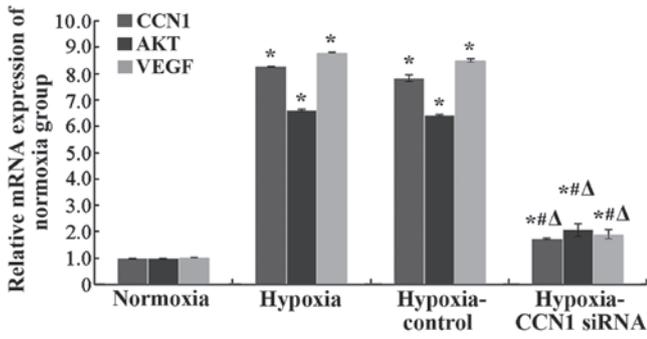


Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of the CCN1-PI3K/Akt-VEGF pathway under hypoxic conditions. The expression levels of CCN1, Akt and VEGF were assessed 2 days post-transfection under hypoxia. Data are presented as the mean ± standard deviation of three independent experiments. \*P<0.05, vs. the normoxia group, #P<0.05, vs. the hypoxia group and ΔP<0.05, vs. the hypoxia-control group. CCN1, cysteine-rich 61; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA.

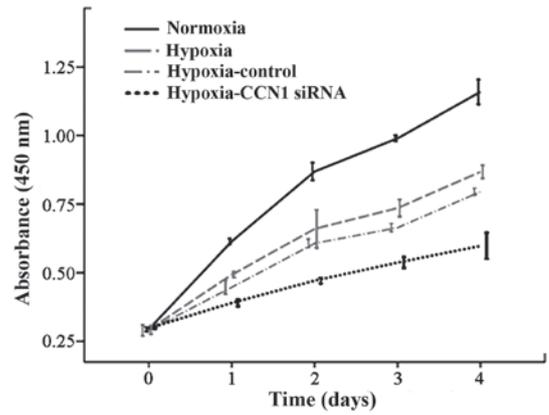


Figure 4. Hypoxia inhibits the expression of CCN1 in HUVECs under hypoxic conditions. The proliferation of the HUVECs was measured using a Cell Counting Kit-8 assay daily for 4 days post-transfection with CCN1 siRNA under hypoxic conditions. CCN1, cysteine-rich 61; HUVECs, human umbilical vein endothelial cells; siRNA, small interfering RNA.

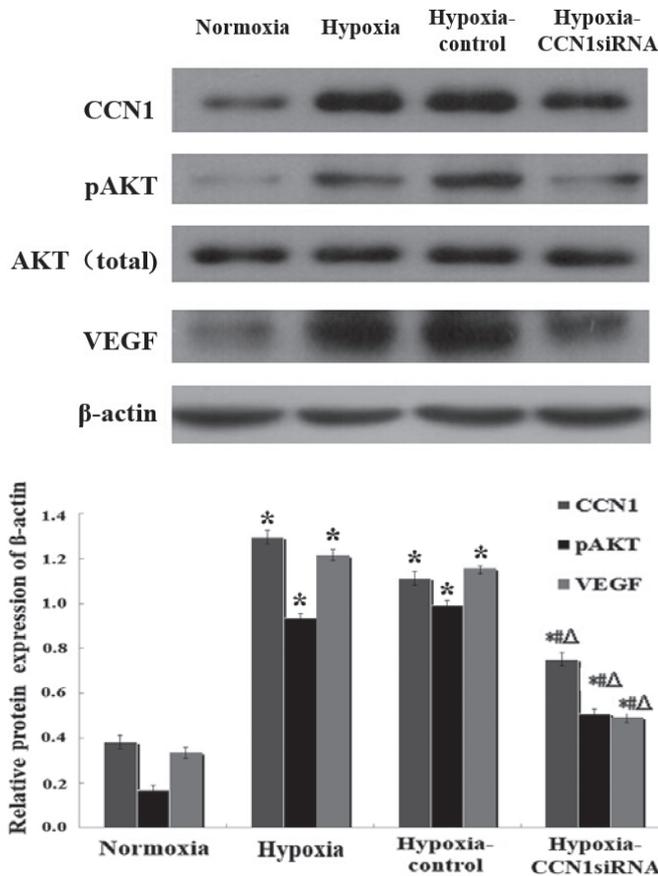


Figure 3. Western blot analysis of the CCN1-PI3K/Akt-VEGF pathway under hypoxic conditions. Data are presented as the mean ± standard deviation of three independent experiments. The protein expression levels of CCN1, pAkt and VEGF were determined 2 days post-transfection under hypoxia; \*P<0.05, vs. the normoxia group, #P<0.05, vs. the hypoxia group, ΔP<0.05, vs. the hypoxia-control group. CCN1, cysteine-rich 61; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor; pAkt, phosphorylated Akt; siRNA, small interfering RNA.

(OD) was quantified using ImageQuant LAS 4000 software (GE Healthcare Life Sciences). Protein concentrations were established by calculating the ratio between the ODs of the protein of interest and β-actin.

*Statistical analysis.* SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as the mean ± standard deviation of three independent experiments. Statistical significance was evaluated using one-way analysis of variance, with a least significant difference test for post-hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*CCN1 siRNA transfection reduces the expression of CCN1 in HUVECs.* At 6 h post-transfection with CCN1 siRNA, the percentage of GFP-positive HUVECs was >80% (Fig. 1A and B). RT-qPCR was performed to measure the mRNA expression of CCN1. Compared with the hypoxia-control, mRNA expression of CCN1 in the hypoxia-CCN1 siRNA group was downregulated by 78.21% (P<0.05; Fig. 2). Western blotting indicated that, compared with the hypoxia-control group, the protein expression of CCN1 in the hypoxia-CCN1 siRNA group was downregulated by 32.43% (P<0.05; Fig. 3).

*CCN1 siRNA inhibits the growth rate of HUVECs.* The major hallmark of angiogenesis is endothelial cell proliferation (20); therefore, HUVEC proliferation was measured using a CCK8 assay. The proliferation rate was reduced in the hypoxia-CCN1 siRNA group, compared with the proliferation rates in the hypoxia and normoxia groups (P<0.05; Fig. 4). These results indicated that CCN1 siRNA has an anti-proliferative effect on HUVECs (21), possibly due to an anti-angiogenic effect.

*CCN1 siRNA induces apoptosis in HUVECs.* To investigate whether hypoxia can induce apoptosis in HUVECs, cellular apoptotic ratios were measured using flow cytometry, in which apoptotic cells determined as annexin V-FITC positive and PI negative. The results of the flow cytometric analysis indicated

chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and an MF-ChemiBIS 3.2 (DNR Bio-Imaging Systems, Ltd., Jerusalem, Israel). Optical density

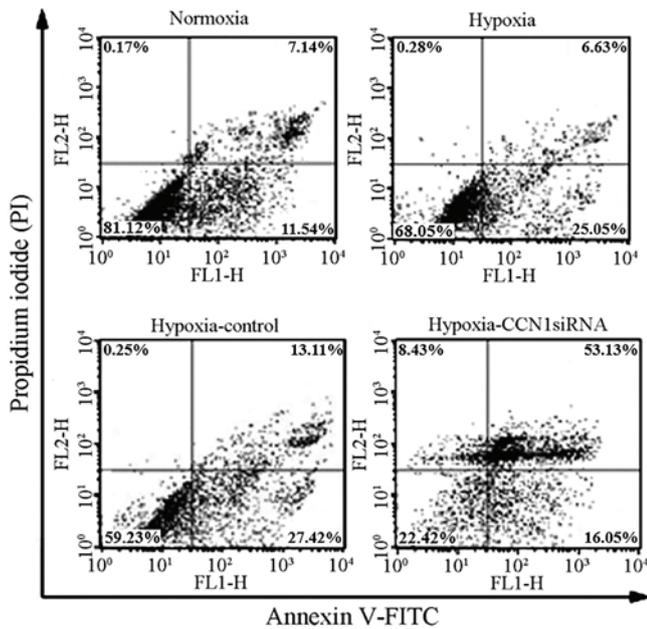


Figure 5. Inhibition of CCN1 in HUVECs promotes apoptosis under hypoxic conditions. Apoptotic cells were identified by double supravital staining with FITC-conjugated annexin-V and PI. The graphs show the percentages of late apoptotic cells (upper right quadrant), fully viable cells (lower left quadrant), early apoptotic cells (lower right quadrant) and necrotic cells (upper left quadrant). The total apoptotic rate in the CCN1 siRNA-transfected cells under hypoxic conditions was increased, compared with those in the hypoxia scramble siRNA-transfected and normoxic cells. CCN1, cysteine-rich 61; HUVECs, human umbilical vein endothelial cells; FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, small interfering RNA.

a moderate increase in apoptosis in the HUVECs transfected with CCN1 siRNA ( $69.24 \pm 0.85\%$ ;  $P < 0.05$ ), compared with the HUVECs transfected with scramble siRNA ( $40.14 \pm 0.78\%$ ), under hypoxic ( $32.28 \pm 0.23\%$ ) or normoxic ( $18.68 \pm 0.43\%$ ) conditions (Fig. 5). These results indicated that CCN1 siRNA had a pro-apoptotic effect on HUVECs (21), possibly due to an anti-angiogenic effect.

*Hypoxia induces the expression of CCN1 through the PI3K/Akt-VEGF signaling pathway.* The results of the RT-qPCR (Fig. 2) and western blot analysis (Fig. 3) indicated that the mRNA and protein levels of CCN1 and VEGF were increased in the hypoxia and hypoxia-control groups, compared with the normoxia group ( $P < 0.05$ ), however, no significant differences were observed between the hypoxia and hypoxia-control groups ( $P > 0.05$ ). The mRNA levels of Akt were increased, and western blotting indicated an increase in the expression levels of p-Akt in the hypoxia and the hypoxia-control groups, compared with the normoxia group. Additionally, the mRNA and protein expression levels were reduced in the hypoxia-CCN1 siRNA group, compared with the hypoxia and hypoxia-control groups ( $P < 0.05$ ; Figs. 2 and 3). Compared with the hypoxia-control, the hypoxia-CCN1 siRNA group demonstrated reduced mRNA expression levels of CCN1, Akt and VEGF, which were reduced by 78.21, 67.19 and 77.65%, respectively (Fig. 2). The protein levels of CCN1, Akt and VEGF were reduced by 32.43, 48.48 and 57.76%, respectively, in this group (Fig. 3). These results demonstrated that the hypoxia-induced expression of

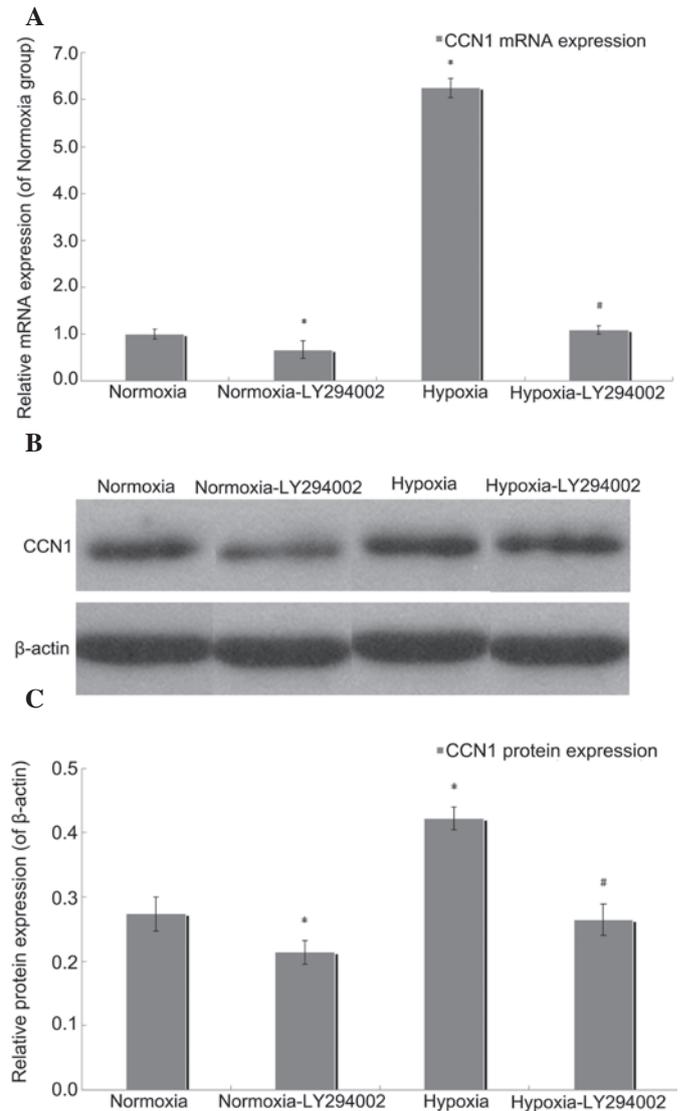


Figure 6. Effects of PI3K/Akt inhibition by LY294002 on the expression levels of CCN1 under normoxic and hypoxic conditions. (A) mRNA expression levels of CCN1 were determined using reverse transcription-quantitative polymerase chain reaction analysis.  $\beta$ -actin was used as a reference gene. (B) Protein expression levels of CCN1 were determined using western blotting. (C) Protein expression was normalized to  $\beta$ -actin. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , vs. the normoxia group; # $P < 0.05$ , vs. the hypoxia group. PI3K, phosphoinositide 3-kinase; CCN1, cysteine-rich 61.

CCN1 was mediated through the PI3K/Akt-VEGF signaling pathway.

*PI3K/Akt inhibition by LY294002 reduces the expression of CCN1.* In the present study, RT-qPCR and western blotting were performed to measure the mRNA and protein expression levels of CCN1 following exposure of the cells to LY294002 under normoxic or hypoxic conditions. Compared with the hypoxia group, the mRNA expression of CCN1 in the LY294002 hypoxia group was downregulated by 82.38% ( $P < 0.05$ ; Fig. 6A), and LY294002 treatment reduced the protein expression of CCN1 by 37.32% in the hypoxic cells ( $P < 0.05$ ; Fig. 6B and C). Compared with the normoxia group, the mRNA expression level of CCN1 in the normoxia-LY294002 group

was downregulated by 32.57% ( $P < 0.05$ ; Fig. 6A), as were the protein levels of CCN2, which were reduced by 21.87% in the normoxic group ( $P < 0.05$ ; Fig. 6B and C). These results suggested that the PI3K/Akt inhibitor, LY294002, reduced the expression levels of CCN1, and that this process involved an autocrine loop.

## Discussion

Hypoxia and ischemia trigger a multitude of responses, which are designed to compensate for the reduced oxygen availability (22). In ECs, these responses increase the expression levels of growth factors and induce angiogenesis (23). The growth of blood vessels in angiogenesis is a delicately controlled process, which involves the activation, proliferation, migration, differentiation and maturation of ECs (24,25). Physiological angiogenesis is required for normal vascular development in addition to vascular homeostasis during adulthood (26). Pathological angiogenesis, commonly induced by tissue ischemia, hypoxia or inflammation, underlies numerous vascular disorders, including retinopathy of prematurity, which is a leading cause of blindness in childhood (27).

Previous studies have directly (28,29) and indirectly (30,31) demonstrated that CCN1 is able to promote choriorretinal angiogenesis *in vitro* via the proliferation and migration of ECs, and the formation of tubular structures, indicating that CCN1 may be involved in the formation of angiogenesis in the retina. These processes all begin with EC proliferation and, mechanistically, CCN1 may promote the proliferation of ECs by upregulating the PI3K/Akt pathway (10,11,21). However, the exact role of the CCN1 pathway remains to be elucidated.

In the present study, examination of the proliferation of HUVECs following CCN1 siRNA transfection under hypoxic conditions demonstrated that treatment with CCN1 siRNA significantly inhibited cell proliferation. Furthermore, it was demonstrated that CCN1 siRNA promoted apoptosis of the cells, thus interfering with angiogenesis. However, the aim of the present study was not to determine whether apoptosis prevented angiogenesis or whether apoptosis was induced by the inhibition of angiogenesis. Despite this, these data indicated that the expression of CCN1 was involved in cell proliferation and apoptosis. These findings are supported by the findings of previous studies, which demonstrated that EC proliferation is the initial step in angiogenesis, and is an essential step prior to both cell migration and tube formation (30).

In addition, several previous studies have suggested that VEGF has central role in angiogenesis, therefore, understanding the interaction between CCN1 and VEGF is important (32,33). To further investigate the mechanisms underlying the hypoxia-induced expression of CCN1, the PI3K/Akt pathway was analyzed in the present study. PI3K/Akt is downstream effector of insulin signaling (34), in addition to being an important signaling molecule in the regulation of glycogen metabolism in myocytes, lipocytes and hepatocytes (12). Furthermore, PI3K/Akt has an important role in ECs by regulating angiogenesis, proliferation, microvascular permeability, survival, cellular transformation and embryonic differentiation (35-37). It has been reported that CCN1 induces

the expression levels of PI3K/Akt in different types of cell, including breast cancer, gastric cancer, renal cell carcinoma and glioma cells (10,38-40). The results of the present study demonstrated that hypoxia increased the mRNA and protein levels of CCN1 via the PI3K/Akt-VEGF pathway, and that CCN1 siRNA induced a significant inhibition of the PI3K/Akt-VEGF pathway. In addition, the data indicated that the mRNA and protein levels of CCN1 were reduced in the cells treated with LY294002 prior to hypoxia, compared with hypoxia-exposed cells without LY294002 treatment. These results supported the hypothesis that the hypoxia-induced expression of CCN1 acts through the PI3K/Akt-VEGF pathway.

In addition, the results of the present study demonstrated that the proliferation and of ECs, and the expression levels of CCN1, Akt, and VEGF were not completely inhibited by CCN1 siRNA. This may be associated with the actions of other growth factors, including basic fibroblast growth factor, interleukin-8, c-Jun and hypoxia-inducible factor-1 $\alpha$  (31,41). Further investigations are required to determine the precise association between these growth factors and CCN1, and their involvement in pathological angiogenesis.

Taken together, the present study demonstrated that CCN1 induced the proliferation of HUVECS, and increased the secretion of cytokines, including VEGF, which acted through PI3K/Akt activation. Therefore, CCN1 RNAi may offer a promising strategy for the treatment of pathological angiogenesis.

## Acknowledgements

The authors would like to thank Dr Juanhan Yu (Department of Pathology, First Affiliated Hospital and College of Basic Medical Sciences, China Medical University, Shenyang, China), Dr Rui Zhao (School of Forensic Medicine, China Medical University, Shenyang, China) and Dr Siyang Zhang (Center of Laboratory Technology and Experimental Medicine, China Medical University, Shenyang, China) for their technical assistance and experimental instructions. The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81371045), the Liaoning Province Technology Foundation of China (grant no. 2010225034) and the Liaoning Province Natural Science Foundation of China (grant no. 2010228).

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