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Angiotensin II Induces Angiogenic Factors Production Partly Via AT1/JAK2/STAT3/SOCS3 Signaling Pathway in MHCC97H Cells

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Key Words

Angiotensin II • Angiogenesis • Hepatocellular carcinoma • Janus kinase 2 • Suppressor of cytokine signaling 3

Abstract

Angiotensin II (Ang II) has been shown to function as a key role in neovascularization of hepatocellular carcinoma (HCC), but little is known its underlying mechanisms. The aim of this study was to explore the role of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway in Ang II-induced HCC angiogenic factors production. Herein, we found that Ang II upregulated angiogenic factors production such as vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2) and Tie-2 in MHCC97H cells in a time- and concentration-dependent manner. And VEGF and Ang-2 caused a significant increase in angiogenic tube formation. Especially, Ang II-induced angiogenic tube formation was blunted by VEGF small interfering RNA (siRNA) and Ang-2 siRNA, respectively. The JAK2 inhibitor AG490 partly attenuated the effects of Ang II. Moreover, Ang II- induced JAK2 and STAT3 phosphorylation was significantly suppressed by losartan but not PD123319. Meanwhile, STAT3 phosphorylation and suppressor of cytokine signaling 3 (SOCS3) expression induced by Ang II were evidently impaired by AG490. More importantly, SOCS3 siRNA remarkably reinforced Ang II-induced VEGF, Ang-2 and Tie-2 generation in MHCC97H cells. Taken together, the present study demonstrates that Ang II induces angiogenic factors production partly via AT1/JAK2/STAT3/SOCS3 signaling pathway in MHCC97H cells. These findings may provide important insights into the potential mechanism with respect to the AT1/JAK2/ STAT3/SOCS3 signaling pathway associated with Ang II-induced angiogenesis in the pathogenesis of HCC.

Introduction

Hepatocellular carcinoma (HCC), a typical hypervascular tumor, is the sixth most common neoplasm and the third leading cause of cancer death worldwide [1, 2]. Tumor angiogenesis plays a critical role in the invasion, metastasis and progression of HCC [3]. Controlling tumor angiogenesis remains a crucial goal for the successful treatment of HCC. However, the lack of effective therapies for HCC is related to poor

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understanding of the molecular mechanism underlying tumor angiogenesis [4]. Accordingly, an analysis of the precise mechanism which underlies tumor angiogenesis is essential, and prevention of angiogenesis could be a useful strategy for tumor growth of HCC.

Angiogenesis in HCC is associated with upregulation of vascular endothelial growth factor (VEGF). It has also been reported that overexpression of VEGF exerted a marked increase in HCC development accompanied by augmentation of neovascularization. Furthermore, VEGF knockdown can be related to reduced endothelial cell proliferation and tube formation *in vitro* and decreased tumor growth and microvessel density *in vivo* [5].Tie-2, as identified an angiopoietin-2 (Ang-2) receptor, is absolutely required for normal vascular development, apparently by regulating vascular remodeling and maturation in HCC [6]. Recent data demonstrated that tumorigenicity with neovascularization was suppressed by *in vivo* gene transfer and a soluble Tie-2 expression in a murine HCC model, suggesting a possible role for Tie-2 expression in the induction of HCC neovascularization and disease progression [7]. In addition, the expression of Ang-2 in HCC with hypervascularity is significantly higher than HCC without hypervascularity [8]. Meanwhile, Ang-2 switching regulates angiogenesis and progression of HCC [9]. Therefore, Ang-2/Tie-2 signal transduction cascade plays an important role in HCC angiogenesis [7].

Angiotensin II (Ang II), a pivotal bioactive peptide of the rennin-angiotensin system (RAS), plays a fundamental role as a vasoconstrictor in regulating cardiovascular function and renal homeostasis [10]. Importantly, there is now increasing evidence of implication of Ang II in neovascularization and development of tumor [11]. And the proangiogenic effects of Ang II are mediated by the Ang II type 1 (AT1) receptor, which is overexpressed in several human cancers [12]. Blockage of AT1 may be a promising anti-tumor strategy by interfering with angiogenesis [13]. Furthermore, a large scale clinical trial for hypertension demonstrates that inhibitors of angiotensin-converting enzyme (ACEI) reduce mortality rates not only in cardiovascular diseases but also in malignant tumors [14]. Especially, it has also been reported that ACEI significantly attenuates the experimental HCC growth and hepatocarcinogenesis along with suppression of neovascularization [15, 16]. As a result, Ang II may play an essential role in promoting angiogenesis of HCC.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is one of the most important components of cytokine signaling cascades, which is involved in multiple cell functions such as differentiation, survival, proliferation and apoptosis [17, 18]. Accumulating evidence indicates that constitutive activation of JAK/STAT pathway occurs frequently in diverse types of tumor cells and contributes to malignant progression [19]. Moreover, recent study reveals the importance of JAK/STAT pathway in HCC development and suggests the use of JAK/STAT inhibitors for the treatment of HCC [20]. Of note, the JAK/STAT pathway is modulated by negative regulators such as suppressors of cytokine signaling (SOCS) proteins. SOCS can bind both the cytokine receptor and JAK and is recruited to the tyrosine phosphorylated receptor, facilitating inhibition of JAK [21, 22]. SOCS3, one of SOCS family members, may also be involved in the suppression of tumour growth and metastasis of HCC [23, 24]. Although Ang II has been shown to function as a key role in tumor neovascularization [25], the underlying mechanism regarding JAK/STAT/SOCS pathway related to Ang II-induced angiogenesis in HCC remains to be fully elucidated. Consequently, the objective of this study was to explore the role of JAK/STAT/SOCS signaling pathway in Ang II-induced angiogenic factors production of MHCC97H cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Carlsbad, CA, USA). Ang II, PD123319 and VEGF were provided by Sigma (St. Louis, MO, USA). Recombinant human Ang-2 was supplied by R&D Systems. Losartan was produced by Merk (Darmstadt, Germany). AG490 was from Cayman (Ann Arbor, MI, USA). Polyclonal anti-human vascular endothelial growth factor (VEGF), Ang-2, Tie-2, SOCS3, AT1, AT2 and anti- β-smooth muscle actin antibodies and phospho-Tie-2 antibody were produced by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against JAK2, STAT3, phospho-JAK2 and phospho-STAT3 were supplied by Epitomics (Burlingame, CA, USA). Human VEGF and Ang-2 ELISA kits were obtained from Invitrogen (Carlsbad, CA, USA). siRNA specific for SOCS3 (siGENOME SMARTpool, M-004299- 02-0005), VEGF (siGENOME SMARTpool, M-003550-02-0005) and Ang-2 (siGENOME SMARTpool, M-006315-01-0005) and negative control siRNA (siGENOME Non-Targeting siRNA Pool, D-001206-13-05) and DharmaFECT 4 transfection reagent (T-2002-04) were purchased from Dharmacon (Lafayette, CO, USA).

Cell culture

Human MHCC97H cell, a typical HCC cell line with high metastatic potential [26], was obtained from Liver Cancer

Gene	Primer sequence	Accession number	Expected size (bp)
VEGF	5′-GCAGAAGGAGGAGGGCAGAATC-3′, 5′-GGGCACACAGGATGGCTTGAAG-3′	NM_001025366.2	150
Ang-2	5′-CAAAGGATGGAGACAACGACAAATG-3′, 5′-CCTTGAGCGAATAGCCTGAGC-3′	NM_001147.2	183
Tie-2	5′-GCTTGGACCCTTAGTGACATTCTTC-3′, 5′-CATTCTTGCCTTGAACCTTGTAACG-3′	AB086825.1	151
SOCS3	5′- CAGGAATGTAGCAGCGATGGAA -3′, 5′- CCTGTCCAGCCCAATACCTGA -3′	NM_003955.3	125
β-actin	5′- ATCGTGCGTGACATTAAGGAGAAG-3′, 5′- AGGAAGGAAGGCTGGAAGAGTG -3′	NM_001101	179

Table 1. Primers used for real-time PCR analysis

Institute of Fudan University (Shanghai, China). MHCC97H cells were cultured with DMEM supplemented with 10% FBS in a humidified incubator at 5% CO_2 and 37°C. In an another experiment, primary human umbilical vein endothelial cells (HUVECs) (ATCC) were cultured at 37 °C and 5% CO_2 in DMEM, supplemented with 10% FBS and endothelial cell growth supplement (ECGS) (BD Biosciences, Bedford, MA). HUVECs between passages 3 and 8 were used for all experiments.

Luciferase assay

MHCC97H cells were plated on cover slips and grown to confluence, and treated with Ang II (100 nM) for 48 h. After the treatment, the cells were fixed with 4% formaldehyde- PBS for 15 min. The cell membranes were fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum. The cells were incubated with rabbit anti-human antibody including VEGF (1:100) or Ang-2 (1:100) or Tie-2 (1:100) and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC) or rhodamine. The immunolabeled cells were observed under fluorescence microscope (Nikon, Japan) and fluorescent intensity of VEGF, Ang-2 and Tie-2 were detected. Data were expressed as relative to control (%).

Small-interfering RNA transfection

MHCC97H cells ($1x10^5$) were seeded into 6-well plates and were grown until 60%–80% confluent. The cells were transiently transfected with 25 nM of SOCS3 small-interfering RNA (siRNA) or negative control siRNA (NC siRNA) using DharmaFECT 4 transfection reagents according to the manufacturer's instructions. After 48 h, protein expression and mRNA levels of SOCS3 were detected by western blot, quantitative real time-PCR and RT-PCR. Transfection rates of 60%–70% of the cells were accepted for all the experiments.

Tube formation assay

In vitro tube formation assay was performed as described previously [27]. HUVECs were incubated with VEGF (50 ng/ml), Ang-2 (300 ng/ml) and Ang II (100 nM) for 24 h before seeding and plating onto matrigel. Matrigel (BD Biosciences, San Jose) was placed in the well of a pre-chilled 24-well cell culture plate and incubated at 37°C for 1 h to allow polymerization. HUVECs ($4x10^4$) were plated into the Matrigel coated wells, and incubated at 37°C in 5% CO_2 in the conditioned media. After 16 h incubation, the plates were photographed. Tube formation was quantified by counting the number of connected cells in five randomly selected fields. All experiments were performed in triplicates. Then, HUVECs tubes were treated with VEGF siRNA (25 nM) or Ang-2 siRNA (50 nM) for additional 24 h before observation with an inverted microscope, cell images were captured and analyzed.

Enzyme-linked immunosorbent assay (ELISA)

MHCC97H cells were seeded into 6-well plates at a density of $5x10^5$ cells/well, and then incubated with Ang II at the indicated concentrations for the indicated time. In another experiment, the cells were pretreated with the JAK2 inhibitor AG490 (50 and 100 μM) for 1 h and stimulated with Ang II (100 nM) for 48 h. Furthermore, after application of negative control siRNA (NC siRNA) or SOCS3 siRNA for 48 h, cells were stimulated with Ang II (100 nM) for 48 h. VEGF and Ang-2 levels in the supernatant were measured with ELISA kits according to the manufacturer's instructions.

Western blotting

As described previously [28], protein samples (25 μg) were separated on SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and incubated with specific antibodies against VEGF (1:200), Ang-2 (1:200), Tie-2 (1:100), AT1(1:200), AT2(1:200), SOCS3(1:200), JAK2 (1:1000), phospho-JAK2 (1:5000), STAT3 (1:500), phospho-STAT3 (1:1000), phospho-Tie-2 (1:200) and β-actin (1:400). The expression of β-actin was used as a loading control. Reagents (Pierce Corp., Rockford, IL, USA) for the enhanced chemiluminescence were applied to the blots, and the light signals were detected by X-

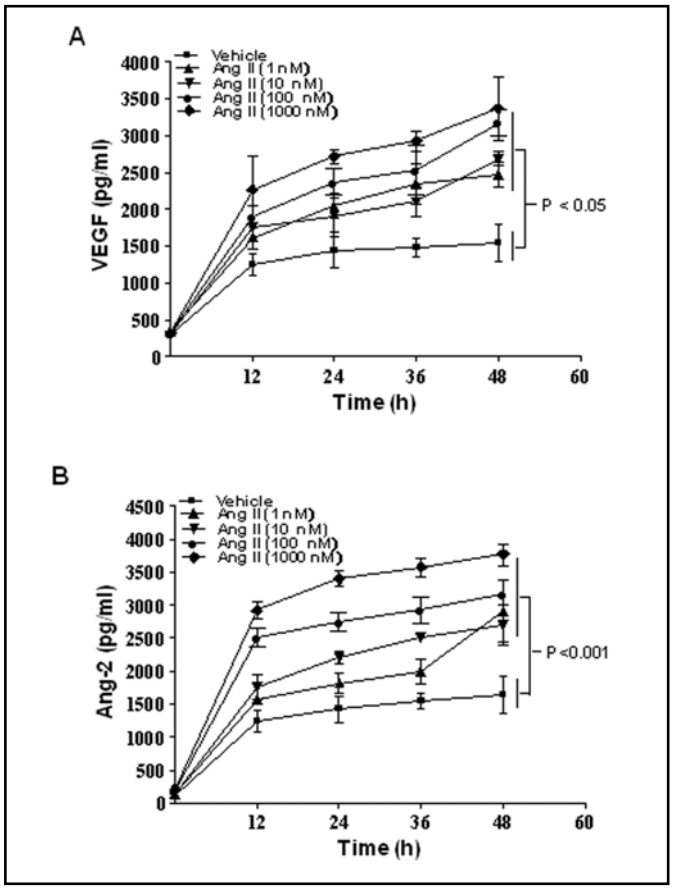

Fig. 1. Ang II-induced VEGF and Ang-2 secretion in MHCC97H cells. Cells were incubated with Ang II (1, 10, 100 and 1000 nM) for 12, 24, 36 and 48 h. Concentration of VEGF (A) and Ang-2 (B) in the supernatant was measured by ELISA. Data are presented as the mean±SEM from three independent experiments.

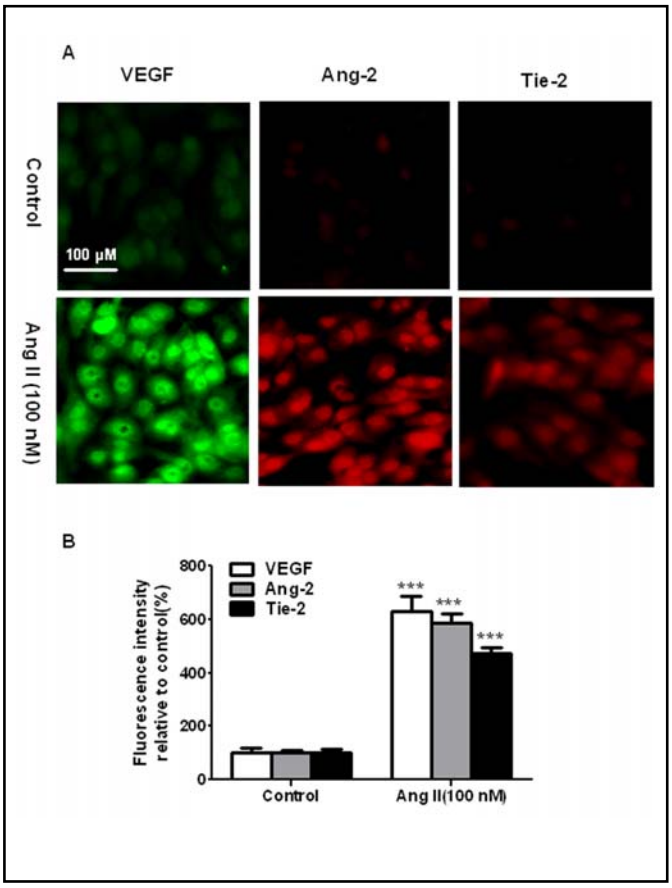

Fig. 2. Immunofluorescent analysis of VEGF, Ang-2 and Tie-2 expression induced by Ang II in MHCC97H cells. Cells were incubated with Ang II (100 nM) for 48 h. (A) VEGF, Ang-2 and Tie-2 expression were identified with immunocytofluorescence. (B) Fluorescent intensity of VEGF, Ang-2 and Tie-2 were also detected. Data are presented as mean±SEM from three independent experiments. ***P<0.001 vs. control.

ray film. Optical densities of the bands were scanned and quantified with the Syngene Gene Tools (Syngene Corp., Cambridge, UK). Three independent experiments were carried out to study protein expressions.

Quantitative real-time PCR and RT-PCR

mRNA levels were determined by our previous method [28]. Total RNA was isolated using a TRIzol Kit (Invitrogen Corp., Carlsbad, CA, USA). cDNA was synthesized from 1 μg samples of total RNA using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with the SYBR Premix Ex Taq™ II Perfect Real Time kit (Takara, Japan) on an ABI QPCR System (Applied Biosystems, CA, USA) following the manufacturer's instructions. The samples were run in triplicate. Primers for human VEGF, Ang-2, Tie-2, SOCS3 and β-actin were designed with Beacon designer v 4.0 (Premier Biosoft, USA) (see Table 1 for the sequences). Traditional PCR was performed according to the manufacturer's instructions. The RT-PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. Quantization of relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta CT}$) as described by the manufacturer. Data were normalized to rat β-actin mRNA levels. Three independent experiments were carried out to study mRNA levels.

Statistical analysis

All data are reported as mean±SEM. Student's t-test or one- or two-way ANOVA test was used to determine the significance among groups. A value of $p < 0.05$ was considered to be statistically significant.

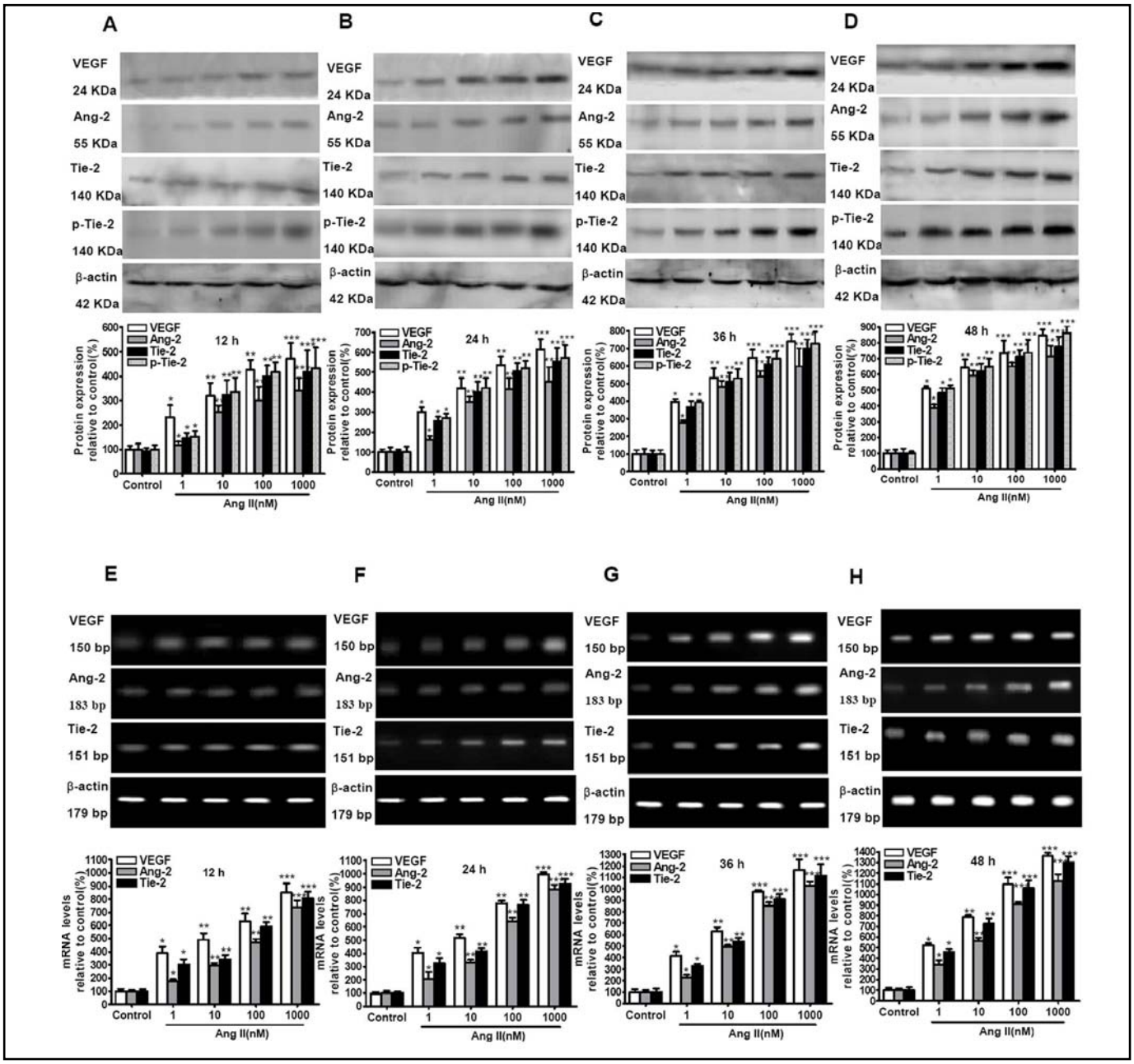

Fig. 3. Ang II-induced VEGF, Ang-2 and Tie-2 protein and mRNA expression in MHCC97H cells. Cells were incubated with different concentrations of Ang II (1, 10, 100 and 1000 nM) for 12, 24, 36 and 48 h. VEGF, Ang-2 and Tie-2 protein expression and Tie-2 phosphorylation (A-D) were measured by western blotting, and VEGF, Ang-2 and Tie-2 mRNA levels (E-H) were analyzed by RT-PCR and quantitative real-time PCR after normalization to β-actin mRNA. Data are presented as mean±SEM from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

Results

Ang II increases angiogenic factors VEGF and Ang-2 secretion in MHCC97H cells

To explore effects of Ang II on angiogenic factors VEGF and Ang-2 secretion, MHCC97H cells were incubated with Ang II (1, 10, 100 and 1000 nM) for 12, 24, 36 and 48 h. The results illustrated that Ang II increased VEGF and Ang-2 secretion in MHCC97H cells in a time- and concentration-dependent fashion (Fig. 1A, B).

Ang II induces VEGF, Ang-2 and Tie-2 fluorescent protein expression in MHCC97H cells

To examine whether Ang II induces VEGF, Ang-2 and Tie-2 protein expression in MHCC97H cells, the cells were incubated with Ang II (100 nM) for 48 h, and

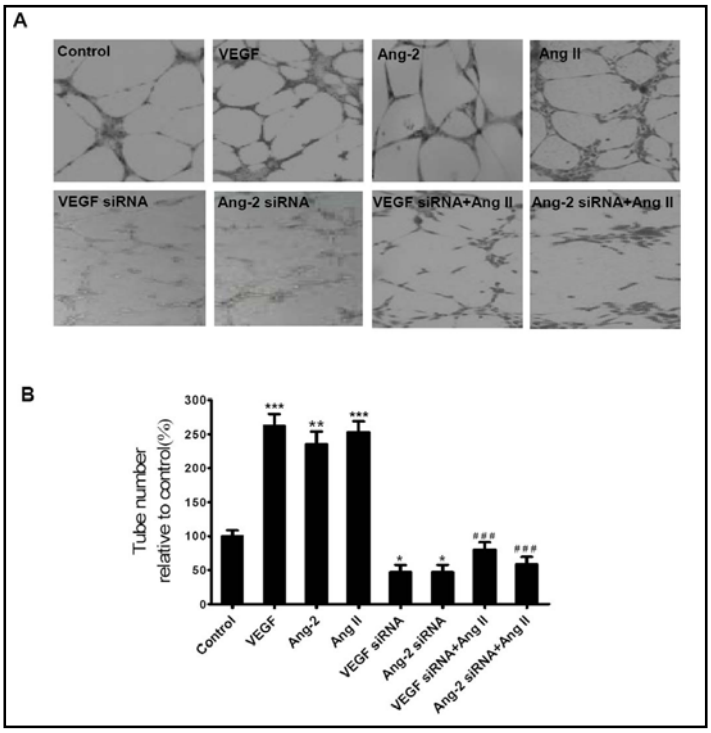

Fig. 4. Requirement of VEGF and Ang-2 *in vitro* tube formation. HUVECs were seeded onto Matrigel alone (control), with VEGF (50 ng/ml), Ang-2 (300 ng/ml) and Ang II (100 nM). Then, HUVECs tubes were treated with VEGF siRNA (25 nM) or Ang-2 siRNA (50 nM) for additional 24 h before observation with an inverted microscope, the cells were photographed to visualize and quantify tube formation. (A) The representative photograph of each treatment was shown (original magnification, x200). (B) The quantification of tube number was detected. Data are presented as mean±SEM from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control; $^{\#\#\#}P$ <0.001 vs. Ang II.

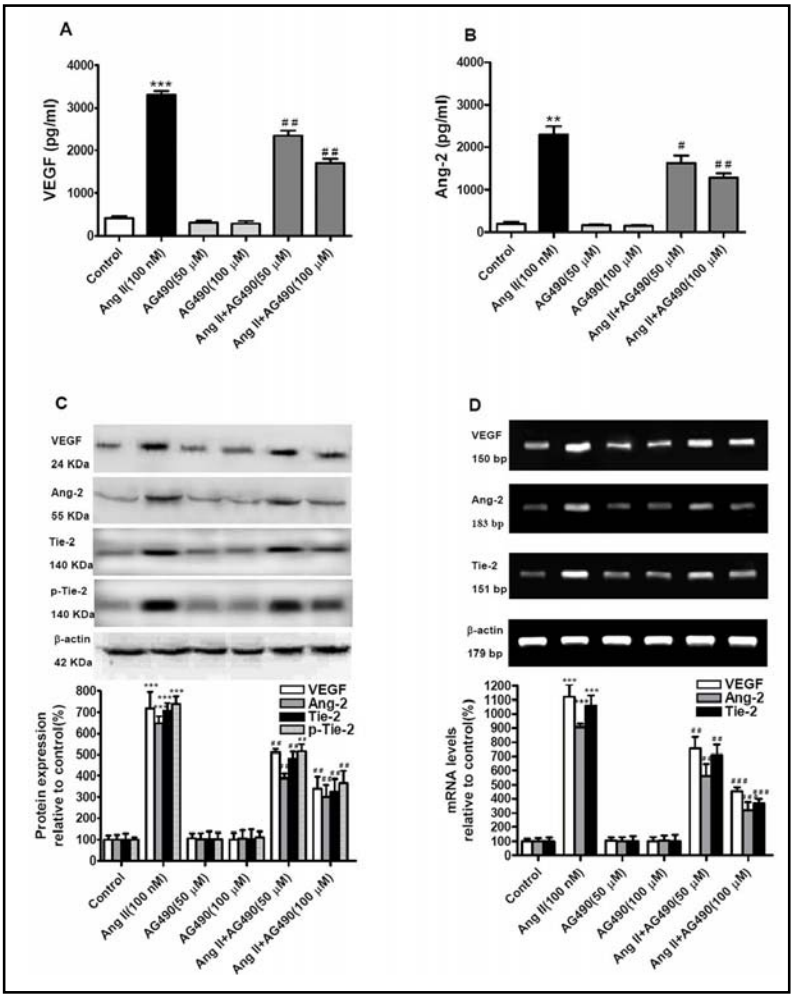

Fig. 5. Ang II-induced VEGF, Ang-2 and Tie-2 generation partly via JAK2 in MHCC97H cells. Cells were pretreated with AG490 (50 and 100 μM) for 1 h and stimulated with Ang II (100 nM) for 48 h. The conditioned media were collected and VEGF (A) and Ang-2 (B) were determined with ELISA. VEGF, Ang-2 and Tie-2 protein expression and Tie-2 phosphorylation (C) were assayed by western blotting. VEGF, Ang-2 and Tie-2 mRNA levels (D) were analyzed by RT-PCR and quantitative real-time PCR after normalization to β-actin mRNA. Data are presented as mean±SEM from three independent experiments. $**P < 0.01$, $***P < 0.001$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P$ <0.001 vs. Ang II.

VEGF, Ang-2 and Tie-2 expression were identified with immunocytofluorescence. The results showed that Ang II elicited VEGF, Ang-2 and Tie-2 fluorescent protein expression in MHCC97H cells (Fig. 2).

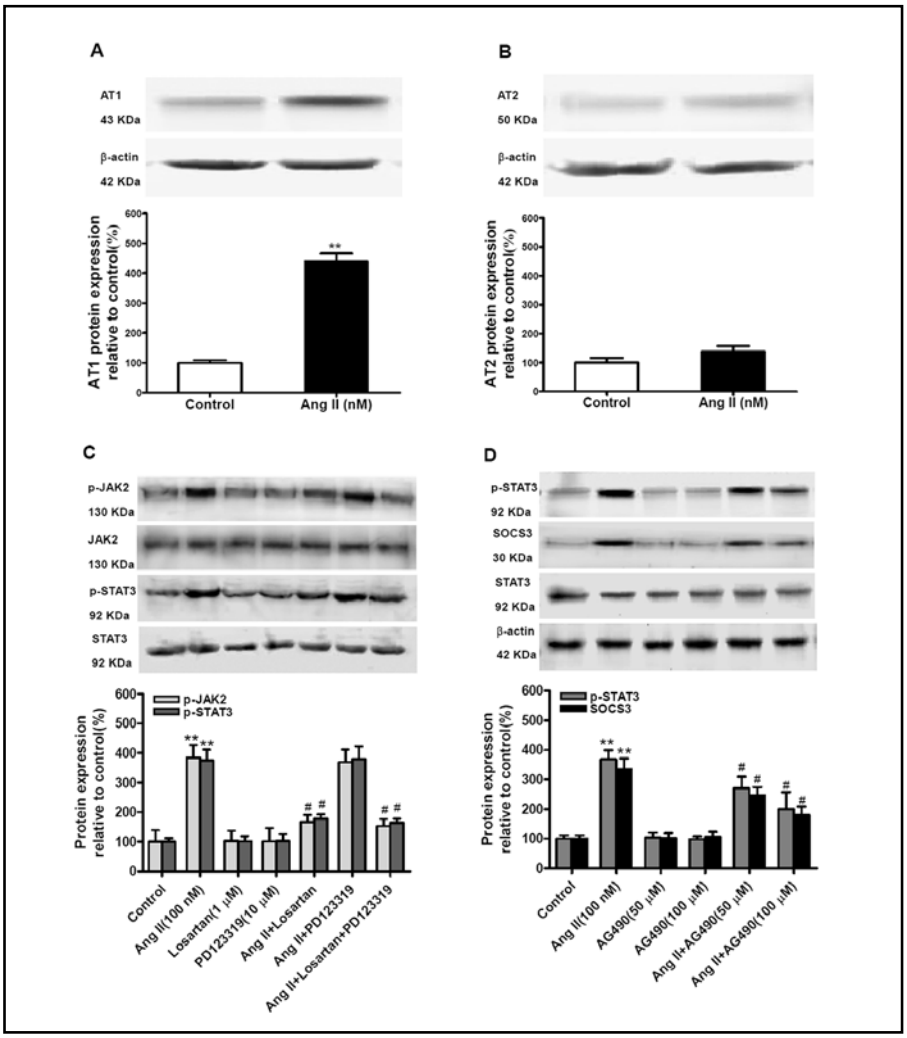

Fig. 6. Ang II-elicited activation of AT1/JAK2/STAT3/ SOCS3 signaling pathway in MHCC97H cells. AT1 (A) and AT2 (B) protein expression were assayed by western blotting. (C) Ang II-induced JAK2 and STAT3 phosphorylation through AT1. (D) Involvement of JAK2 in Ang II-induced STAT3 phosphorylation and SOCS3 protein expression. Data are presented as mean±SEM from three independent experiments. $^{**}P < 0.01$ vs. control; $^{\#}P<0.05$ vs. Ang II.

Ang II enhances VEGF, Ang-2 and Tie-2 protein and mRNA expression and Tie-2 phosphorylation in MHCC97H cells

Under suggestion of the above-mentioned results with the immunofluorescent method, we further observed effects of Ang II on VEGF, Ang-2 and Tie-2 protein and mRNA expression and Tie-2 phosphorylation in MHCC97H cells. As shown in Fig. 3, Ang II upregulated VEGF, Ang-2 and Tie-2 protein and mRNA expression in MHCC97H cells in a time- and concentration-dependent manner. In addition, Ang II dramatically elevated Tie-2 phosphorylation of MHCC97H cells in a time- and concentration-dependent way. And there was a strong relationship between increase in Ang-2 protein expression and elevation in Tie-2 phosphorylation.

Requirement of VEGF and Ang-2 in vitro tube formation

In order to elucidate functions of VEGF and Ang-2 in angiogenesis, we performed tubulogenesis *in vitro* assay. As shown in Fig.4, VEGF and Ang-2 caused a significant increase in tube formation by HUVECs. When functions of VEGF and Ang-2 were disrupted by VEGF siRNA or Ang-2 siRNA, tube formation by HUVECs was compromised indicating the role of VEGF and Ang-2 *in vitro* tube formation. Strikingly, the formation of Ang II- induced numerous tubules was remarkably reversed by the addition of VEGF siRNA or Ang-2 siRNA, suggesting that the angiogenic effect of Ang II was dependent on VEGF and Ang-2.

Ang II induces VEGF, Ang-2 and Tie-2 generation in MHCC97H cells partly via JAK2

The above-mentioned results show that Ang II may induce VEGF, Ang-2 and Tie-2 generation in MHCC97H cells. To assess the role of JAK2 in the process, we further evaluated effects of the JAK2 inhibitor AG490 pretreatment on VEGF, Ang-2 and Tie-2 generation in Ang II-stimulated MHCC97H cells. As shown in Fig. 5, 50 and 100 μM of AG490 remarkably but not completely attenuated Ang II-induced VEGF and

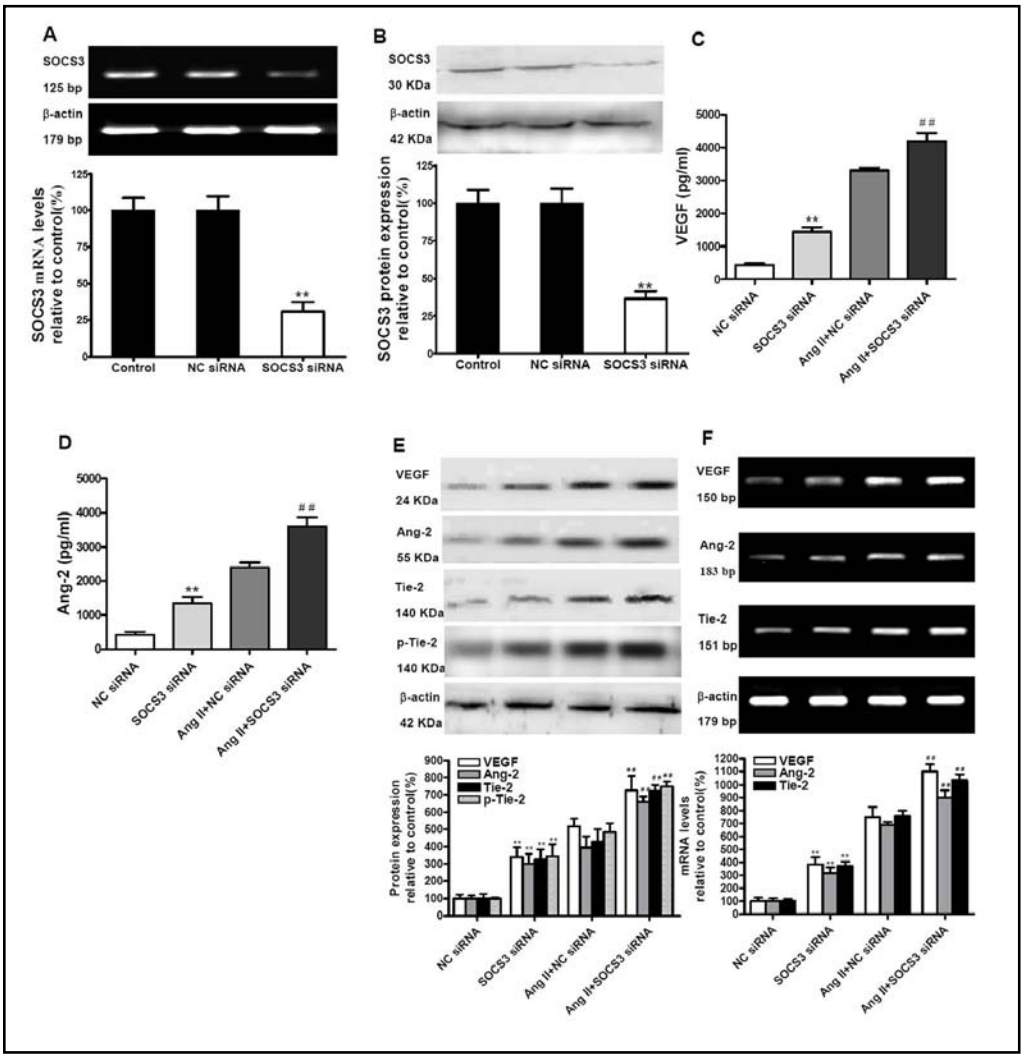

Fig. 7. Effect of SOCS3 siRNA on Ang II-induced angiogenic factors in MHCC97H cells. After application of negative control siRNA (NC siRNA) or SOCS3 siRNA for 48 h, cells were stimulated with Ang II (100 nM) for 48 h. (A and B) SOCS3 mRNA and protein expression in MHCC97H cells were detected by RT-PCR, quantitative real-time PCR and western blotting at 48 h post-transfection. (C and D) VEGF and Ang-2 were determined by ELISA. (E and F) VEGF, Ang-2 and Tie-2 protein and mRNA expression were analyzed by western blotting, RT-PCR and quantitative real-time PCR after normalization to β-actin mRNA. Tie-2 phosphorylation was also depicted. Data are presented as mean±SEM from three independent experiments. $^{**}P<0.01$ vs. NC siRNA; $^{\#\#}P<0.01$ vs. Ang II +NC siRNA.

Ang-2 secretion. Moreover, VEGF, Ang-2 and Tie-2 protein and mRNA expression and Tie-2 phosphorylation in response to Ang II were significantly but not completely downregulated in the cells pretreated with AG490. Consequently, these results demonstrate that Ang II induces VEGF, Ang-2 and Tie-2 generation partly through JAK2.

Ang II activates JAK2/STAT3 signaling pathway via AT1 in MHCC97H cells

To determine whether Ang II induces AT1 and AT2 expression in MHCC97H cells, the cells were incubated with Ang II (100 nM) for 48 h, and AT1 and AT2 protein expression were identified with western blotting. The results showed that Ang II induced AT1 expression in MHCC97H cells, but had no obvious effect on AT2 expression (Fig. 6A, B). In addition, to further certify which AT receptor subtype is required for in Ang II-induced JAK2/STAT3 activation of MHCC97H cells, the cells were stimulated with Ang II (100 nM) for 15 min after pretreatment for 30 min with AT1 antagonist losartan (1 μM) and AT2 antagonist PD123319 (10 μM) alone or in different combinations. As described in Fig. 6C, Ang II- induced JAK2 and STAT3 phosphorylation were markedly suppressed by pretreatment of the cells with losartan but not PD123319, and the inhibitory effect had no obvious change when the cells were pretreated with the combination of losartan and PD123319. Meanwhile, STAT3 phosphorylation induced by Ang II treatment for 15 min was evidently impaired by the JAK2 inhibitor AG490 (Fig. 6D). These confirm that Ang II activates JAK2/STAT3 signaling pathway via AT1 in MHCC97H cells.

Involvement of JAK2/STAT3 in Ang II-induced SOCS3 expression of MHCC97H cells

To probe role of JAK2/STAT3 in Ang II-induced SOCS3 expression, cells were preincubated with 50 and

100 μM of AG490 for 1 h prior to Ang II (100 nM) stimulation for 48 h. As shown in Fig. 6D, AG490 partially but significantly blunted Ang II-induced SOCS3 protein expression, further illustrating that JAK2/STAT3 signaling pathway mediates downstream molecule SOCS3 expression in MHCC97H cells.

Effect of SOCS3 siRNA on Ang II-induced angiogenic factors in MHCC97H cells

To further ascertain the role of SOCS3 in Ang II-induced angiogenic factors of MHCC97H cells, cells were transiently transfected with SOCS3 siRNA for 48 h, and then SOCS3 mRNA and protein expression were measured (Fig. 7A, B). Knock down efficiency of SOCS3 was 69.2% or 63.5% as determined by quantitative real-time PCR or western blot. The transfected cells were stimulated with Ang II (100 nM) for 48 h. As described in Fig. 7C and D, compared with the negative control, stimulating the cells with Ang II led to elevation of VEGF and Ang-2, whereas lack of SOCS3 remarkably aggravated Ang II-induced VEGF and Ang-2 secretion in MHCC97H cells, suggesting that Ang II elicited VEGF and Ang-2 production via SOCS3. The similar results to VEGF and Ang-2 secretion were achieved for VEGF, Ang-2 and Tie-2 protein and mRNA expression and Tie-2 phosphorylation (Fig. 7E, F). Furthermore, SOCS3 siRNA also increased VEGF, Ang-2 and Tie-2 generation in MHCC97H cells compared to negative control. Taken together, these results implied that Ang II induced angiogenic factors generation in MHCC97H cells partly via AT1/JAK2/STAT3/SOCS3 signaling pathway.

Discussion

Tumor angiogenesis begins with the activation of endothelial cells by a few specific angiogenic factors, among which VEGF is an elemental player [29]. VEGF enhances microvascular permeability, potentially facilitating tumor cell penetration into the circulation [30]. Several studies have shown that VEGF may be a potent factor for predicting tumor progression in HCC [31-33]. Moreover, VEGF is one of the most important angiogenic factors interrelated to HCC invasiveness and metastasis [34]. In the present study, effects of Ang II on VEGF secretion and expression in MHCC97H cells were assessed by ELISA, immunocytofluorescent, western blotting, RT-PCR and quantitative real-time PCR analyses. It was clear from our data that Ang II obviously enhanced VEGF production in MHCC97H cells. And VEGF caused a significant increase of tube formation *in vitro* cultured HUVECs. Especially, addition of Ang II could promote angiogenic tube formation, whereas this Ang II- induced angiogenic tube formation could be inhibited by VEGF knockdown. The results suggest that Ang II makes contribution to angiogenesis in MHCC97H cells via increasing angiogenic factor VEGF.

Recently, a novel family of angiogenic factors, Angs, has been identified as a group of ligands for the tyrosine kinase Tie-2 receptor [35, 36]. With respect to HCC, Ang-2 expression distinctly increases in the tumor compared with adjacent noncancerous tissues. Moreover, nude mice injected with a HCC cell line overexpressing Ang-2 displays faster tumor growth with greater neovascularisation in the tumor [37, 38]. Additionally, a high preoperative Ang-2 level in the hepatic vein is a good predictor for portal invasion and also poor prognosis in human HCC [39]. Tie-2 receptor depends on the balance of its ligands Ang-2, which may control vascular plasticity, angiogenesis, and vascular permeability [40]. The Ang-2/ Tie-2 system may also play a crucial role in promoting tumor angiogenesis and its progression in human HCC. The current results showed that Ang-2 led to an evident enhancement of tube formation *in vitro* cultured HUVECs. Interestingly, addition of Ang II was able to promote angiogenic tube formation, whereas this Ang II-induced angiogenic tube formation was impaired by Ang-2 knockdown. In the present study, the activated state of Tie-2 induced by Ang II was determined by phosphotyrosine immunoblotting. The present results implied that Tie-2 phosphorylated level was highly correlated with the increase of Ang-2 expression in Ang II-induced MHCC97H cells, which seems to be consistent with the recent findings that Ang-2 could induce Tie-2 phosphorylation [41, 42]. Therefore, our novel finding is the capability of Ang II to elicit angiogenesis in MHCC97H cells by enhancing angiogenic factor Ang-2 and the direct activation of Tie-2.

RAS, in addition to its central role in arterial hypertension, also modulates blood vessel formation during cancer [25]. In several organs including the brain, heart and liver, there exist a local RAS system which may affect tumorigenesis through Ang II [43]. Our study directly evaluates the role of Ang II on tumor angiogenesis of HCC, which conduces to explore the relationship between RAS and HCC. So far, even though Ang II could induce angiogenesis via upregulation of angiogenic factors VEGF, Ang-2 and Tie-2 in MHCC97H cells, the precise mechanism regarding the contribution of Ang II in angiogenesis of HCC is still elusive.

JAKs are non-receptor tyrosine kinases involved in upstream intracellular signaling pathways that become activated after extracellular ligand binding to a variety of cytokine and growth-factor receptors [44]. JAK2, an important member of the JAKs family, is linked to the activation of STAT3 [45]. STAT3 is a critical transcription activator biomarker in tumor therapy and involved in fundamental events of tumor development including angiogenesis [46, 47]. Additionally, several data reveal the oncogenic potential of JAK2 leading to the constitutive activation of the JAK2/STAT3 signaling pathway [48]. Recent knowledge also implicates that the JAK2/STAT3 signaling pathway modulates various fundamental biological processes and cancer pathogenesis. Especially, deregulation of the JAK2/STAT3 signaling pathway is frequently observed in primary tumors and results in increased survival of tumors and enhanced angiogenesis [49]. In the present study, we presumed that JAK2 could mediate the proangiogenic effect of Ang II, and examined the effect of the JAK2 inhibitor AG490 pretreatment on angiogenic factors VEGF, Ang-2 and Tie-2 production in Ang II-stimulated MHCC97H cells. The results illustrated that Ang II induced VEGF, Ang-2 and Tie-2 generation partly via JAK2 signaling pathway. These may provide a strong evidence for the direct involvement of JAK2 signaling pathway in Ang II-induced angiogenic factors production of MHCC97H cells.

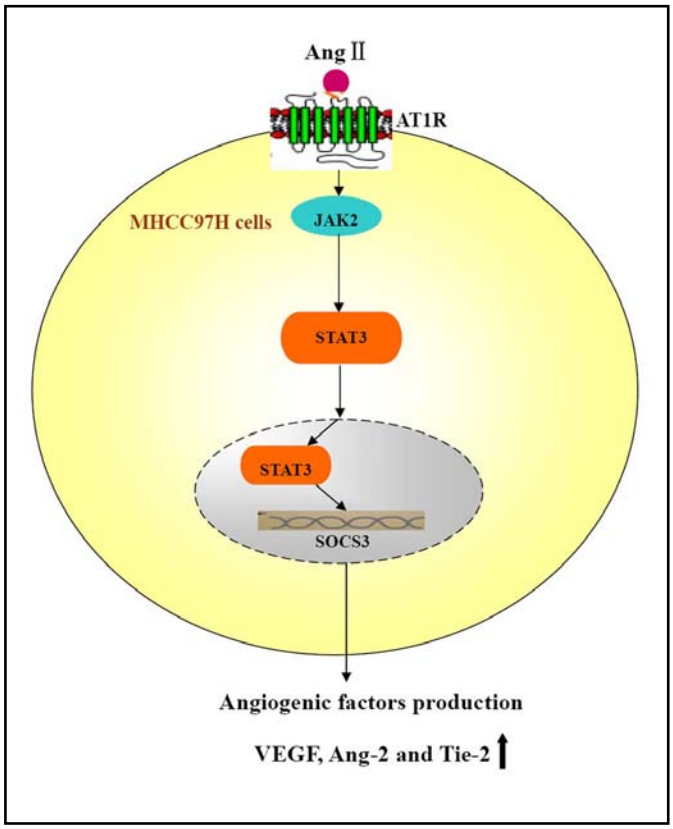

Fig. 8. A scheme for signaling pathway relevant to Ang II-induced angiogenic factors VEGF, Ang-2 and Tie-2 production in MHCC97H cells.

Ang II, the main effector of the RAS, acting through G-protein-coupled receptors, Type 1 (AT1) and Type 2 (AT2). Most of the physiological effects of Ang II have been attributed to AT1 [50]. Interference with AT1 by declined levels of the ligand Ang II or by inhibition of the receptor may interrupt angiogenesis and impair tumor growth [51]. The present results showed that Ang II activated the JAK2/STAT3 signaling pathway through AT1 in MHCC97H cells, because AT1 antagonist losartan significantly suppressed Ang II- induced JAK2 and STAT3 phosphorylation, but AT2 antagonist PD123319 did not have the same effect. Meanwhile, our results also indicated that Ang II induced AT1 expression in MHCC97H cells, but had no obvious effect on AT2 expression. Additionally, STAT3 phosphorylation elicited by Ang II was dramatically depressed by the JAK2 inhibitor. Based on the above finding, it is most likely that Ang II mediates activation of the JAK2/STAT3 signaling pathway via AT1 in MHCC97H cells, which seems to corroborate the previous study [52].

SOCS3 acts as a key negative regulator of JAK/STAT pathway and represents one of the candidate tumor suppressor genes [21]. SOCS3 markedly reduces tumor growth and potently enhances the efficacy of chemotherapy in HCC [53]. Alternatively, SOCS3 h-KO mice develops HCC at an accelerated rate in the model of chemical-induced carcinogenesis [23]. The present experiments verify the involvement of the JAK2/STAT3 signaling pathway in Ang II-mediated SOCS3 expression in MHCC97H cells, further supporting that SOCS3 lies downstream from JAK2/STAT3 in the signal cascades (Ang II/AT1/JAK2/STAT3/SOCS3). More importantly, our results manifested that angiogenesis in MHCC97H cells elicited by Ang II required SOCS3 at least in part, as angiogenic factors VEGF, Ang-2 and Tie-2 production were strengthened by SOCS3 siRNA. Since silencing of SOCS3 is one of the most important mechanisms of constitutive activation of JAK/STAT pathway in cancer pathogenesis, it seems plausible to infer that SOCS3 deficiency may augment Ang II-induced angiogenic factors generation of MHCC97H cells in association with elevated activation of the JAK2/STAT3 signaling pathway.

In conclusion, the present study demonstrates that Ang II induces angiogenic factors production partly via

AT1/JAK2/ STAT3/SOCS3 signaling pathway in MHCC97H cells. Ang II activates JAK2 and subsequent STAT3 through AT1 in MHCC97H cells, which elicits downstream molecule SOCS3 expression, finally mediating angiogenic factors such as VEGF, Ang-2 and Tie-2 generation (Fig.8). These findings provide important insights into the underlying mechanism with respect to the AT1/JAK2/ STAT3/ SOCS3 signaling pathway related to Ang II-induced angiogenesis in the metastasis and progress of HCC.

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