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HIF-1α Was a Key Regulator to Improve hBMSCs to Secrete Vascular Endothelial Cytokine with Astragaloside in Hypoxia

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Abstract: This study aimed to assess the combined therapeutic efficacies with gene therapy and stem cell treatment and herb on the differentiation of hBMSCs into vascular endothelial cells under hypoxia condition. The third passage of hBMSCs were randomly divided into four groups, including control group (hBMSCs transfected with empty vectors), VEGF group (VEGF gene transfected to hBMSCs), AST group (cultured with AST), and VEGF + AST group (the intervention of VEGF group plus AST group). Each group was cultured at 37°C and 5% O_2 for 2 weeks. Cell morphology was observed by inverted phase and were crowded and arranged irregularly in the control group and AST group, showing a fiber-like growth, while those in the VEGF group and VEGF plus AST group were mostly triangular or polygonal, exhibiting a colony-like growth, contrast microscope. CD31 was negative in the control group and AST group, while CD105 was positive in both groups, tested by flow cytometry assay. The positive rate of CD31 was significantly higher in the VEGF group than it in the VEGP + AST, and the positive rate of CD105 was lower in the VEGF group than it in the VEGF + AST group. The levels of VEGF and endothelial nitric oxide synthase (eNOS) by ELISA and the expression of endothelin and prostacyclin by west blot (WB) and RT-PCR were significantly higher in VEGF group and AST group and VEGF plus AST group, compared with control group. Further, the expression of endothelin and vWF and VEGFR-2 was highest in the VEGP + AST group and the expression of prostacyclin was lowest in VEGF group. AST can promote the secretion of VEGF from the differentiation of hBMSCs induced by VEGF gene transfected to hBMSCs by HIF-1α under hypoxia.

Keywords: HIF-1α, Astragaloside, Hypoxia, Bone Mesenchymal Stem Cells, VEGF Gene Transfection, Vascular Endothelial Cytokine

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death both in the developing and developed countries and constitutes a major public health problem. The rising trend of cardiovascular mortality mainly results from the rising death rate of ischemic heart disease [1]. Therapeutic angiogenesis refers to actions performed to facilitate revascularization of ischemic tissues. Bone marrow stem cells (BMSCs) have been shown to become ideal stem cells for therapeutic angiogenesis due to self-renewal, multi-trans differentiation and paracrine effects, especially secreting a variety of vascular growth factors, compared with other stem cells [2-4]. However, BMSCs are faced with a hypoxic-ischemic microenvironment after implantation into a myocardial ischemic area. A critical

question that emerges is how to utilizeparacrine cytokines to reconstruct a collateral microcirculation as part of the process to repair the myocardium.

At present, there is no clear induction agent for directional differentiation of BMSCs to VECs [30]. Members of the vascular endothelial growth factor (VEGF) family are the strongest known modulators of vascular biology. They regulate vasculogenesis, angiogenesis, and vascular maintenance during embryogenesis and in adults. Co-culture of VEGF and BMSCs can induce BMSCs to differentiate into vascular endothelial cells [5]. Our previous study has found that BMSCs transfected by a VEGF gene can differentiate into vascular endothelial cells in a hypoxic environment [6]. However, completed randomized controlled VEGF trials using VEGF delivered as are combinant protein or via gene transfer for the treatment of myocardial or peripheral ischemia have not provided convincing evidence of clinical efficacy, primarily because of their rapid elimination *in vivo* [7].

A large number of animal and clinical studies have found that Astragalusmembranaceus can significantly improve cardiac function, reduce myocardial infarction area, reduce tissue impairment and inhibit cognitive impairment [8-11].

Astragaloside IV (AST) is the major active saponin abundant in Astragalusmembranaceus and, reportedly, has a variety of pharmacological activities. AST had a significant effect on the treatment of ischemic heart disease [12-16], protected BMSCs from apoptosis and promoted proliferation *in vitro* [11]. Consequently, it is hypothesized that the combination treatment of BMSCs with VEGF gene transfection and AST will improve the differentiation from BMSCs into vascular endothelial cells and their subsequent proliferation.

2. Materials and Experiments

2.1. Main Instruments and Reagents

The main instruments and reagents listed in Table 1. Primers were synthesized by the Invitrogen Corporation. AST (purity quotient of 99.8%) were purchased from National Institutes for Food and Drug Control (batch No. 100781200613). 20mg AST was added into 200μL dimethyl sulfoxide (DMSO), then gently mixed, then added to DEM to 50mL and completely dissolved, filtered by filter membrane with a pore size of 0.22μm, resulting in a mother liquor (0.4mg/mL) which was saved at 20°C.

Table 1. Cells, reagents and instruments.

Cells, reagents and instruments	corporation
Human bone marrow mesenchymal stem cells (1st generation)	Scien Cell corporation（Cargo No. 7500）
MSCM culture medium and fetal bovine serum	500 mL, Scien Cell corporation
Adenovirus vector pAd- vegf-ires2-egfp (293A cells) The viral titer is 2 x 1010 ifu/ml	Life Technologies Corporation
TakaRa Prime ScriptTM reagent Kit	TaKaRa corporation
SDS-PAGE gel preparation kit	Solarbio Corporation
CD31 FITC、CD105 APC	BD Corporation

Cells, reagents and instruments	corporation
Human VEGF ELISA kit	Consortium creatures, batch no:218360343
Human eNOS ELISA kit	Shanghai elisa, batch no:201604
BCA protein concentration assay kit	Solarbio Corporation
inverted phase contrast microscope	OLYMPUS Corporation
flow cytometry	BD FAC Scan Corporation

2.2. Human BMSCs Culture

The human BMSCs were propagated from generation 1 to 3 in a 37°C, 5% CO_2 incubator. When the cell confluence reached 80% to 90%, they were transferred to a 37°C, 5% O_2 incubator for differentiation. All experimental tests were performed after 2 weeks incubation.

The third-generation BMSCs were randomly divided into 4 groups as follows: Normal control group (control group): DMEDM cultured for 2 weeks; VEGF induction group: DMEDM culture containing Adenovirus vector pAd-VEGF-IRES2-EGFP [5]; AST group: DMEDM culture containing AST [7]; VEGF + AST group: combination of interventions in the VEGF group and in the AST group. After 3 days induction, the VEGF group, AST group and VEGF + AST group were changed into a common DMEDM culture condition. All groups were renewed with DMEDM culture medium every 3 days and saved the culture supernatant was saved and stored at -20°C, for a period of two weeks of culture.

2.3. Characterization of Vascular Endothelial Cells (VECs) Phenotype by Flow Cytometry

Cells were collected at differentiation day 0 and at 2 weeks. After trypsin-digestion and centrifugation, the cells were incubated with anti-human CD31 (PECAM-1), a marker of white cells and endothelial cells and anti-human CD105 (endoglin), a marker of actively proliferating endothelial cells and angiogenesis (BD Inc, USA). Subsequently, the expression of CD31 and CD105 on the cell surface was analyzed by flow cytometry.

2.4. Detection of VEGF and Endothelial Nitric Oxide Synthetase (eNOS) in Supernatants

At the end of 2 weeks, the expression levels of VEGF and eNOS in supernatants were detected using VEGF and eNOS kits with ELISA according to manufacturer recommendations.

2.5. Detection of Endothelin (ET) and Prostacyclin (PGI) by Real-time qPCR

After 2 weeks of induction and culturing, the cells were collected and the total RNA was extracted and reverse transcribed to cDNA according to the instructions of the TakaRa Prime ScriptTM reagent Kit. Primers are shown in Table 2. Real-time PCR was undertaken as follows: 1μg RNA was added to 4μL 5 × PrimeScript Buffer and 1μL PrimeScript RT Enzyme Mix I and 1μL Oligo dT Random Primer and1μL 6mers. Then, RNase Free dH_2O was added to 20μL. Lastly, the samples were amplified at 37°C for 15min, 85°C for 5s,

the 4°C for 10min. Real-time PCR was used to test the cDNA obtained from reverse transcription and make the dissolution curve.

The Ct (Ct) was used to calculate the relative gene expression data using Real-Time Quantitative PCR.

Table 2. Sequence of primers detected by qPCR.

primer sequence	
ET	forward primer:5'-TGC TCC TGC TCG TCC CTG ATG GAT AAA GAG-3' downstream primers: 5'-GGT CAC ATA ACG CTC TCT GGA GGG CTT-3'
PGI	orwardprimer:5'-ACC CCC CAC TGA AAA AGA TGA-3' downstream primers:5'-CCT TCT AAG TGG TTG GAA CA-3'
GADPH	orward primer:5'-TGA AGG TCG CTG TCA ACG GA-3' downstream primers:5'-GAT GGC ATG GAC TGT GGT CAT-3'

2.6. Detection of ET and PGI2 Proteins in Cells by Western Blotting

After 2 weeks of induction and culturing, the cells were collected, pyrolysis solution was added and placed on ice for 10min. After 12000rpm centrifugation for 30min at 4°C, the solution was extracted and the protein concentration was measuredusing the BCA method. The protein was added to 7.5%, 9% gel for SDS-PAGE, and transferred using 200mA constant flow under wet conditions to PVDF membrane. Antibody I was incubated at 4°C overnight, and then incubated with the correspondingantibody IIat room temperature. Thereafter, exposure was performed using an electrochemiluminescence solution (ECL). ImageJ software analyzes were undertaken using the grayscale values of the strips.

2.7. Immunocytochemistry (ICC) for vWF, HIF-1α and VEGFR-2 After 7 Days

The expression of vWF, HIF-1α and VEGFR-2 was determined after 7 days. Cells were cultured on glass coverslips, washed with PBS 3 times. then naturally dried, cells were fixed in 4% poly formaldehyde for 30min and then immersed in PBS and washed by PBS 5min × 3 times. Then, cells were infiltrated with 0.1% Triton X-100 for 15min, and then washed by PBS 5min × 2 times. Furthermore, cells were blocked for endogenous peroxase-activity for 10min, and then washed by PBS 5min × 3times again, and were blocked for 10min and flushed by PBS 5min × 3 times. Cells were incubated with the mouse monoclonal anti-VWF (1:100), anti-VEGFR-2 (1:100), and anti HIF-1α (1:100) for 60min. After thorough washing with PBS, secondary antibodies of goat anti-mouse IgG and/or goat anti-rabbit IgG were applied for 10 min incubation, and then flushing by PBS 5min × 3 times. Next, cells were incubated with Streptavidin-HRP working liquid for 10min and washed by PBS 5min × 3 times. Finally, DAB' staining and Sumusu' re-dyeing and dehydration were performed. Fluorescent images were obtained using an Olympus DP70 microscope equipped with a digital camera (Olympus). Cells were examined randomly using 5 microscopes' FOV (field of vision) and imaged at 200x magnification. The positive rate was the ratio of positive cells to total cells.

2.8. Statistical Methods

SPSS V13.0 statistical software was used for analysis. The Kolmogorov-Smirnov test was used for tests of normality. The measurement data with normal distribution were described as mean ± standard deviation (SD). The differences among groups were compared with one-way variance analysis, and LSD was further used for pair-wise comparison between any two groups. If the data were a non-normal distribution, the data were expressed as median and quartiles and the Kruskal-Wallis H test was used for comparison between groups. α=0.05.

3. Results

3.1. Morphology of BMSCs with Inverted Phase Contrast Microscope

As shown in Figure 1, BMSCs presented spindle-shaped, oval nuclei, fibrous growth and vigorous growth in the normal control group (Figure 1A). After 2 weeks of differentiation, the number of cells in the AST group was increased, which were still spindle shaped and interwoven, without obvious morphological changes (Figure 1B). The cells in the VEGF group and VEGF + AST group were mostly triangular or polygonal, were growing in colonies and were confluent (Figure 1C, Figure 1D).

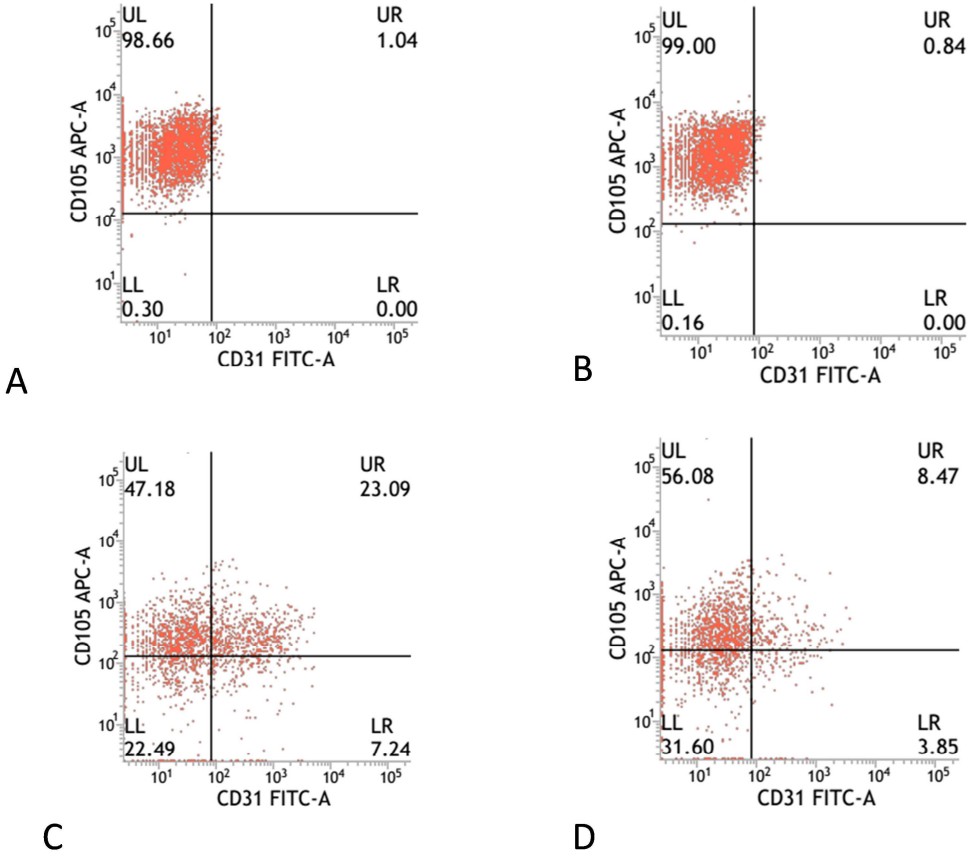A: Control group, B: AST group, C: VEGF group, D: AST + VEGF group.

Figure 1. Morphological observation of each group at 14 days after induction under inverted microscope (4×).

3.2. Detection of Cell Surface Markers by Flow Cytometer

After 2 weeks of differentiation, the cell surface marker CD31 was negative in the AST group and the control group, however, CD105 was positively expressed, with a respectively positive rate of 99.7% and 99.6%, indicating that the BMSCs phenotype was still present in the cells (Figure 2A and Figure 2B). CD31 expression was significantly higher in both the VEGF and AST + VEGF

group than it in both control group and AST group, furthermore, the positive rate in the VEGF group was higher than that in the AST + VEGF group (30.33% vs. 10.22%, $P<0.05$). CD105 expression in VEGF group was significantly lower than that in the AST + VEGF group (58.11% vs. 63.47%, $P<0.05$) (Figure 2C and Figure 2D).

A: Control group; B: AST group; C: VEGF group; D: AST + VEGF group.

Figure 2. *Expression of CD31 and CD105 in hBMSCs after 2 weeks induction.*

3.3. Expression of ET mRNA and PGI mRNA by RT-PCR

Compared with the control group, ET mRNA in the three intervention groups were significantly higher (all P<0.05), whereas PGI mRNA were lower (all $P<0.05$) (Table 3). Compared with the AST group, ET mRNA in VEGF group and VEGF + AST group both were significantly higher, however PGI mRNA were lower (all $P<0.05$). Compared with the VEGF group, ET mRNA was significantly lower in the AST + VEGF group ($P<0.05$), while PGI mRNA was increased ($P<0.05$), as shown in table 3.

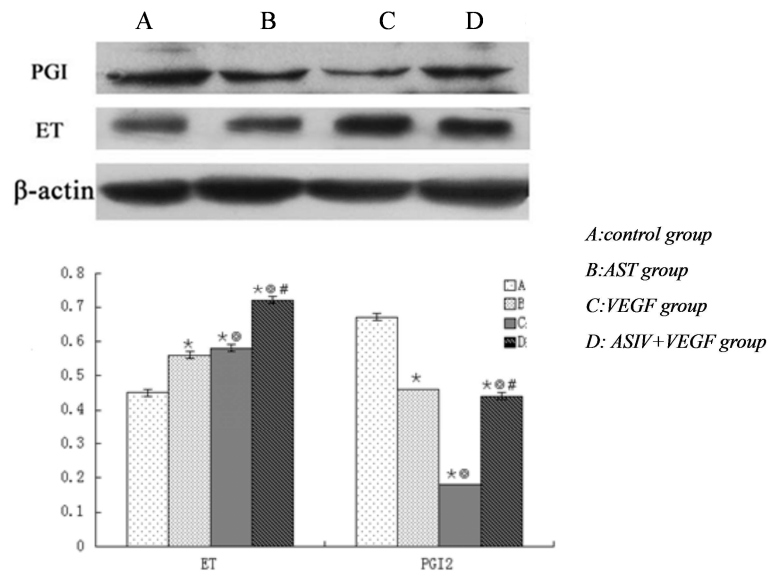***Table 3.*** *Expression of ET and PGI mRNA after 2 weeks induction (mean ± SD, n=3).*

	Control group	AST group	VEGF group	VEGF + AST group
GAPDH	16.12±0.02	15.66±0.02	15.43±0.03	16.13±0.03
ET	28.05±0.28	26.45±0.03	25.12±0.07	26.59±0.03
PGI	26.84±0.21	27.38±0.01	27.93±0.01	27.23±0.01
ET 2-ΔΔCt	1.00±0.26	2.21±0.06*#	4.71±0.24*©	3.39±0.02*#©
PGI 2-ΔΔCt	1.00±0.20	0.39±0.00*#	0.29±0.00*©	0.33±0.00*#©

*Compared with the control group, P<0.05; ©Compared with the AST group, P<0.05; #compared with VEGF group, P<0.05.

3.4. Expression of ET and PGI Proteins by Western Blotting

β-actin was expressed in each group. Compared with the control group, ET protein was expressed significantly higher in the VEGF group, and the AST group and the VEGF + AST group (all $P<0.05$), while PGI was lower (all $P<0.05$) in VEGF group and AST group and VEGF + AST group (Figure 3). Compared with the AST group, levels of ET protein were significantly higher in both VEGF group and VEGF + AST group (both $P<0.05$), while PGI was lower (both $P<0.05$). Compared with VEGF group, both levels of ET and PGI were significantly higher in the VEGF + AST group (both $P<0.05$) (Shown in Figure 3).

A: control group
B: AST group
C: VEGF group
D: ASIV+VEGF group

*Different to control group, P<0.05; ©different to AST group, P<0.05; # different to VEGF group, P<0.05.

Figure 3. Expression of ET and PGI proteins in each group after 2 weeks under hypoxia.

3.5. Expression of VEGF and eNOS by ELISA

Compared with the control group, the levels of VEGF and eNOS were significantly higher in the VEGF group and the AST group and VEGF + AST group (all *P*<0.05). Compared with the AST group, the expression levels of VEGF and eNOS were significantly higher in both the VEGF group and the VEGF + AST group (all *P*<0.05). Compared with the VEGF group, the expression levels of VEGF and eNOS were significantly higher in the VEGF + AST group (both *P*<0.05) (seen in Table 4.).

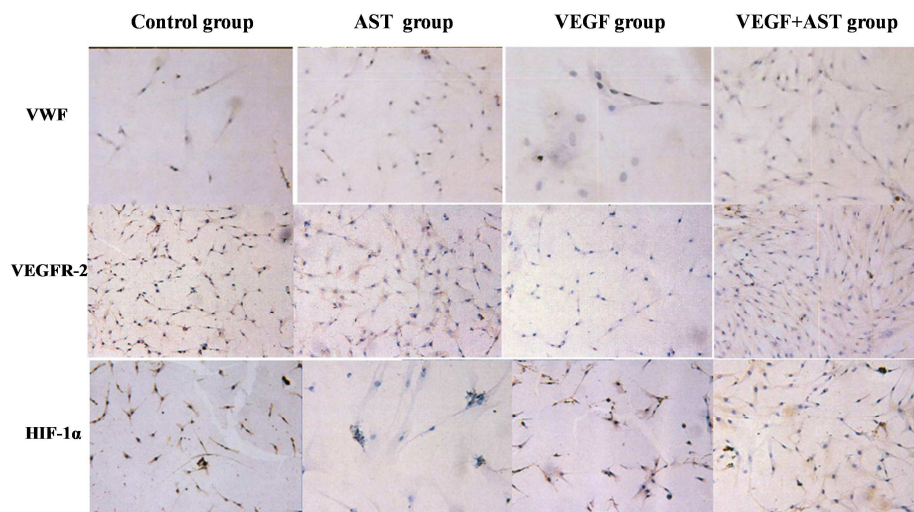**Table 4.** Expressions of VEGF and eNOS in supernatants after 2 weeks induction (mean ± SD, n =4).

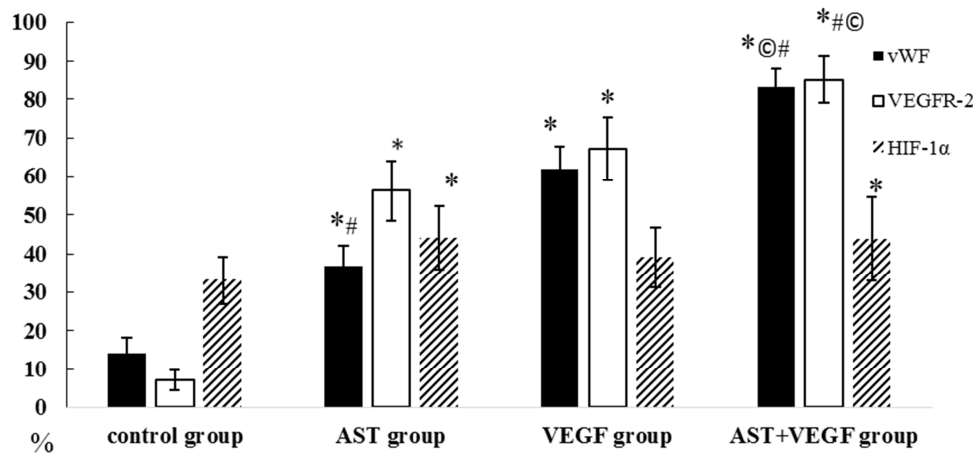|	Control group	AST group	VEGF group	VEGF + AST group
VEGF (pg/mL)	450.99±18.07	700.10±39.45*#	976.50±171.95*©	1008.35±146.34*©#
eNOS (U/mL)	21.13±0.94	24.01±0.74*#	27.40±1.14*©	30.46±2.23*©#

*Compared with control group, P<0.05; ©compared with AST group, P<0.05; #compared with VEGF group, P<0.05.

3.6. The Positive Rate of Von Willebrand Factor (vWF), VEGFR-2 and HIF-1α by ICC

Compared with the control group, the levels of vWF and VEGFR-2 were significantly higher in the VEGF group and the AST group and VEGF + AST group (all *P*<0.05); the levels of HIF-1α were significantly higher in AST group and AST + VEGF group (both *P*<0.05). Compared with AST group, VEGFR-2 was significantly higher in AST + VEGF group (*P*<0.05); the levels of vWF was significantly higher in both VEGF group and AST + VEGF group (both *P*<0.05). Compared with VEGF group, the expression levels of vWF and VEGFR-2 both were significantly higher in AST + VEGF group (both *P*<0.05) (Figure 4).

	Control group	AST group	VEGF group	VEGF+AST group
VWF				
VEGFR-2				
HIF-1α				

Different to control group, *P<0.05; different to AST group, ©P<0.05; different to VEGF group, #P<0.05.

Figure 4. The positive rate of vWF, VEGFR-2 and HIF-1α after 7 days induction.

4. Discussion

Ischemic diseases (such as cerebral embolism, myocardial infarction, osteonecrosis, etc.) result in local tissue ischemia and hypoxia. Therapeutic angiogenesis stimulates the formation and maturation of new blood vessels by introducing pro-angiogenic growth factors, thereby increasing blood perfusion, reducing tissue damage and promoting repair. BMSCs have the ability to undergo multidirectional differentiation. BMSCs can be differentiated into corresponding mature cells by specific inductions, and then are able to repair corresponding tissues at the injury sites which are mostly ischemic and hypoxic [12]. Thus, BMSCs become seed cells for cell therapy and gene therapy. After stem cell shave been transplanted, most stem cells grow within the microenvironment of tissue hypoxia. Thus, how to promote the survival and directional differentiation of BMSCs is the focus of many researchers. VEGF has been considered as the most effective vascular growth promotion factor until now [13]. Our previous study has found that VEGF gene transfection into human BMSCs induced BMSCs differentiation into VECs under hypoxic conditions [5]. AST is the main active component of Astragalus. It has been reported that AST not only increases the proliferation of human BMSCs *in vitro*, but also increases the expression of VEGF mRNA [14]. However, the role of a combination of VEGF gene transfection and AST treatment, to facilitate more effective differentiation into functional VECs and increased proliferation under hypoxic conditions, remains to be evaluated.

In this study, the cells in VEGF group and VEGF + AST group presented the phenotypic characteristics of endothelial cells, and VEGF transfection probably had a tendency to differentiate into VECs in hypoxia. This is consistent with the results of human BMSCs differentiation to endothelial cells induced by VEGF transfection under normal oxygen [15]. It also was found that the co-culture of human BMSCs [17-18], mouse BMSCs [19] or sheep BMSCs [20] with VEGF also promoted the differentiation of BMSCs into endothelial cells

under normal oxygen conditions. However, AST did not promote the differentiation of human BMSCs into VECs. The result is different from that CD31 positively expressed after BMSCs induced by Astragalus injection [21]. The differences may be due to other active components in Astragalus promote the differentiation of BMSCs into VECs. Additionally, we found that the different test time probably resulted in the different ranks of CD31, and vWF and VEGFR-2 among 4 groups. It was reported that VEGFR-2 firstly expressed, subsequently CD31 and vWF expressed in BMSCs; VEGFR-2 regulated the budding of capillary growth and played a main role in angiogenesis [29].

Mature VECs eventually play their functions through the related cytokines which are classified into endothelium-derived contraction factors (eg, ET and thrombatin, etc.) and diastolic factors (eg, NO and PGI, etc.). If or not the differentiated human BMSCs have the functions of VECs, it is necessary to verify the related functional cytokines had positive or negative expression. At the present, the relevant study is rare. Our previous study found that VEGF gene transfection increased ET and reduced PGI under hypoxia [5]. In this study, ET, ET mRNA, eNOS and VEGF all upregulated, but PGI and PGI mRNA downregulated in VEGF group and AST group and VEGF + AST group, moreover, the promotion power is different. Normal VECs can maintain homeostasis and play function [24] and promote the normal growth of VECs [25] only when the secretion of ET increases and PGI reduced. VEGF is currently known as the strongest and most specific angiogenic factor, which plays an important role in maintaining the normal structure and physiological function of VECs [22]. eNOS produces nitric oxide (NO) in vascular vessels and helps to regulate vascular function. It was found that BMSCs expressed the cytokines related with VECs such as CD31 and VEGF after the endothelium-type eNOS vector is transfected to BMSCs [23]. In addition, AST improved the VEGF expression of BMSCs although AST not had the potential directional induction ability of human BMSCs differentiation into VECs in this study. The result is consistent with the previous studies [14, 21]. We also found

that ET was highest in AST + VEGF group. AST is often used as a complementary and alternative medicine which can provide *Qi* in clinical practice. The results in this study conforms to the theory of "*Qi* produces blood" in Chinese medicine.

HIF-1α is as a key regulator of the molecular hypoxic response and a master regulator of the cellular homeostatic response to hypoxia by activating many genes, including those involved in energy metabolism, angiogenesis, apoptosis and so on [31]. In this study, HIF-1α was upregulated in AST group and AST + VEGF group. It is consistent with the results in ischemia and reperfusion injury [32]. Hypoxia increases VEGF-1 expression via up-regulation of HIF-1α [33-34]. However, VEGF was highest in VEGF group, but not AST + VEGF. The reasons need further study to explore.

There are some limitations in this study. Firstly, we not designed the comparison between hypoxia and normal oxygen. Secondly, we mainly did test at the end of experiment. The continuous changes of all test variables can't be observed and analyzed. Thirdly, although AST not induced BMSCs to differentiate into VECs, it could promote BMSCs to secrete cytokines of VECs. The mechanism of these problems needs to explore.

5. Conclusion

In hypoxic, HIF-1α probably plays a key positive regulatory role in which AST promotes the secretion of vascular endothelial cell-associated factors by hBMSCs, including the increase expression of ET, PGI, VEGF and eNOS. Additionally, via HIF-1α, AST combined with VEGF can promote the secretion of ET and vWF and VEGFR-R2 by VECs-like cells. It suggests that HIF-1α master AST promoting the differentiation of BMSCs into vascular endothelial cells by angiogenesis regulation to hypoxia.

Acknowledgements

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