Hypoxia induced interstitial transformation of microvascular endothelial cells through mediating HIF-1 α /VEGF signaling in systemic scleroderma

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Research article

Keywords: Hypoxia, scleroderma, endothelial interstitial transformation, Hypoxia inducible factor-1 α , vascular endothelial growth factor a

Posted Date: March 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-19468/v1>

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Abstract

Background: Endothelial mesenchymal transition (EndMT) is a key pathological event for vasculopathy which is one of the early features and hallmarks of systemic sclerosis (SSc). It is well established that hypoxia contributes to EndMT, however, little is known about the effects of EndMT induced by hypoxia on skin microvascular remodeling of SSc, as well as the underlying mechanism.

Methods: Skin biopsy was done for SSc patients and healthy controls and skin tissues were collected for iTRAQ-based proteomics and Immunohistochemical test. Human microvascular endothelial cell line (HMEC-1) cultured with hypoxic or normal conditions, treated by either tamoxifen or bevacizumab. The expression of Hypoxia inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF)-a, CD31, α -smooth muscle actin (α -SMA), VE-cadherin, fibronectin were detected at both protein and mRNA level.

Results : The iTRAQ-based proteomics indicated significantly up-regulated HIF-1 signaling in the skin tissues of SSc patients. Immunohistochemical results demonstrated a significant downregulation of the endothelial cell (EC) marker CD31 and a distinct positive staining of the interstitial cell (IC) marker α -SMA at sites lining the vessel lumens in the skin tissues of SSc. Meanwhile the positive staining of HIF-1 α , a key transcription factor in response to chronic hypoxia, and VEGF-a were found to be diffusely distributed in SSc skin tissue. Consistent with these observations, HMEC-1 cells cultured under hypoxic conditions exhibited a significant decrease in CD31 and VE-cadherin expression, alongside a marked increase in the expression of α -SMA and fibronectin, as well as a distinct up-regulation of HIF-1 α and VEGF-a, while comparing with them under normal condition. Of note, the inhibition of HIF-1 α by tamoxifen effectively down-regulated the hypoxic induction VEGF-a, α -SMA, while rescuing hypoxic suppression of CD31. In addition, a VEGF-a inhibitor bevacizumab treatment had the same effect on the hypoxic expression of α -SMA and CD31 as tamoxifen intervention, but did not reduce HIF-1 α .

Conclusion: These results suggest that the HIF-1 α /VEGF signaling pathway has a critical role in mediating the effect of hypoxia-induced EndMT on skin microvascular remodeling of SSc.

Background

Systemic sclerosis is a rare connective tissue disease with high disability and mortality rates, featured by autoimmunity, a broad microvasculopathy, and fibrosis of the skin and of visceral organs[1]. EndMT has emerged as a key player in the pathogenesis of tissue fibrosis and fibroproliferative vasculopathy in various diseases, including cardiac and intestinal fibrosis, diabetic nephropathy, portal hypertension, and pulmonary arterial hypertension (PAH) [1, 2]. As well, experimental evidence supported a role of EndMT in lung tissues of patients with interstitial lung disease associated with SSc[3, 4]. Additionally, cells in the intermediate stages of EndMT were identified in dermal microvessels of both patients with SSc and bleomycin-induced and urokinase-type plasminogen activator receptor deficient mouse models[5]. That is, EndMT causally connect two hallmarks of SSc, the persistent endothelial injury and the aberrant fibrogenesis.

In the pathogenesis of SSc, reduced capillary blood flow coupled with deficient angiogenesis leads to chronic hypoxia, enforcing a positive feed-forward loop sustaining vascular remodelling, which further promote the irreversible extensive tissue fibrosis[2]. It indicates that chronic hypoxia is a prominent trait contributing to vasculopathy and tissue fibrosis in SSc. In recent years, a large number of studies have confirmed that hypoxia could induce EndMT in vitro and in vivo[6, 7, 8]. However, the process of EndMT induced by hypoxia in the pathogenesis of SSc was not clear.

HIF-1 α is a key transcription factor in response to chronic hypoxia and is currently considered to be the "main regulatory factor" of hypoxia environment[9]. It was reported that HIF-1 α promoted fibrotic disease and its implicated function included stimulation of excessive ECM, vascular remodeling, and futile angiogenesis with further exacerbation of chronic hypoxia and deteriorate pathofibrogenesis, as well as EndMT[10, 11]. Excessive expressions of HIF-1 α has been detected in the tissues of systemic sclerosis[12, 13]. In addition, VEGF is the predominant proangiogenic factor regulated by HIF-1 α in hypoxia related diseases, and remarkable up-regulation of VEGF has been observed in SSc specimen[10, 13]. Recently, the inhibition of HIF-1 α /VEGF signaling pathway was reported to reverse the anti-angiogenesis effects of chrysophanol[14]. Nevertheless, whether hypoxia induced EndMT in the pathogenesis of SSc depended on HIF-1 α /VEGF signaling needs to be further verified.

Herein, we provided immunohistological evidence verifying the EndMT in the dermal specimens of patients with SSc, as well as the excessive increased expression of HIF-1 α and VEGF-a. We further show that HMEC-1 cells cultured under hypoxic conditions exhibited the characteristics of EndMT and the activation of both HIF-1 α and VEGF-a. Finally, the inhibition of HIF-1 α /VEGF signaling by tamoxifen and bevacizumab effectively inhibited EndMT. These observations provide strong support to the hypothesis that the HIF-1 α /VEGF signaling has a critical role in mediating the effect of hypoxia-induced EndMT on skin microvascular remodeling of SSc. This novel mechanism may represent an important and novel therapeutic target for the complications of SSc-associated fibroproliferative vasculopathy and fibrosis.

Materials And Methods

Patients and tissue samples

Skin tissue samples from 8 patients with SSc and 8 age and gender matched healthy controls were studied. Tissue samples of patients and controls were obtained by skin biopsy performed at the Affiliated Hospital of Southwest Medical University. We included only those patients who met the American College of Rheumatology (ACR)/European League Against Rheumatism(EULAR) 2013 classification criteria for SSc [15]. Eligibility criteria excluded the following key conditions: (1) Aged <18 or >75 years. (2) BMI>25. (3) Patients had severe primary diseases such as liver, kidney, hematopoietic system damage, and other skin and connective diseases. (4) Patients with recent infection. (5) Tumor patients. The control samples were obtained from the normal skin surrounding nevus, when it was removed. All the specimens were taken from the fingers, back of the hand and forearm. All the surgical biopsies were performed following

the patients' provision of informed consent and according to Institutional Review Board–approved protocols from the Affiliated Hospital of Southwest Medical University.

Histopathology, immunohistochemistry

The qualified skin tissue was fixed with 10% formalin, embedded in paraffin and sliced at 4 μm . Slides containing paraffin-embedded skin tissue sections were examined histopathologically by staining with hematoxylin and eosin, and also by immunohistochemical staining with specific EC and mesenchymal (myofibroblast) cell markers. The procedure of immunohistochemical staining was as following. After dewaxing and hydration, the specimens were washed with PBS and repaired with antigen in citric acid buffer. Catalase blockers were added, and the antibodies were added in turn. Slides were first incubated overnight with one of the following antibodies: anti-CD31 (1:50 dilution), anti- α -SMA (1:200 dilution), anti-HIF-1 α (1:100 dilution), and anti-VEGF-a (1:100 dilution). IgG binding was revealed following incubation with secondary antibodies for 1 hour. Then the slides were colored with DAB chromogenic agent. Finally, the positive signals of each index were semi-quantitatively counted by Image J, and the analysis was carried out.

Extraction of total proteins from skin tissues

The skin tissues were taken from 4 patients and matched controls, immediately soaked in liquid nitrogen before storing them at -80°C . Each tissue was ground in lysis buffer using Tissue Lyser II at a frequency of 25 Hz for a period of 30 min. The skin tissues were chopped up with scissors, then additional 30 min of sonication were added using the intelligent ultrasonic processor DH99-IIIN (lawson, China) to lyse well the tissues until a total grinding. Then, tissues were lysed in a lysis buffer (4% SDS, 0.1M DTT in 0.1M Tris-HCl, pH 7.6) at a ratio of 100 mg tissue per 1mL lysis buffer. The tissues were vortexed and incubated for 5 min at 95°C before being lysed for 20 s with 1 sec OFF 1 sec ON at 20% amplitude using ultrasonic processor probe. Then, the lysate was centrifuged at $14,000\times g$ for 20 min at 4°C to get rid of the cell debris. The supernatant was recovered in a new tube and the amount of proteins was measured using BCA Protein Assay Kit according to the manufacture's instruction. The protein assay by the BCA method is used for the quantification of total proteins in a sample.

Absolute quantitation (iTRAQ)-based proteomics

An amount of 1 mg of protein from each sample was deposited in a filtration centricon Microcon YM-30 (Millipore). Washing steps were performed with 8 M urea, 0.1M Tris-HCl, pH 8.5 buffer. The cysteine residues were blocked with 12 mM methyl methanethiosulfonate for 30 min at room temperature. Next, the proteins were digested by trypsin with enzyme to substrate ratio of 1:50 (w/w) at 37°C for 15 h. Furthermore, the digested peptides were labeled with iTRAQ Reagent Kit (Applied Biosystems, Foster city, CA, USA). The iTRAQ-labeled samples were analyzed by a NanoAquity UPLC system connected to Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). All the protein analysis were performed by Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). The amino acid sequences of identified proteins from iTRAQ were matched to the local protein

database(<https://www.ncbi.nlm.nih.gov/genbank/>) , and KEGG (<http://www.genome.jp/kegg/>) databases to search protein candidates and associated pathways.

Reagents and antibodies

For immunohistochemistry and western blot, the following primary antibodies were used: anti-CD31, anti- α -SMA, anti-HIF-1 α , anti-VEGF-a. They were all purchased from Abcam (USA). The mouse Anti-Rabbit IgG (1:5000 dilution) was purchased from Santa Cruz (USA). The anti-b-actin and Goat Anti-Mouse IgG (1:3000 dilution) were from Bioss (China). The specific HIF-1 α inhibitor tamoxifen was purchased from Solarbio (China). The specific VEGF-a inhibitor bevacizumab was purchased from MedChemExpress (USA).

Cell culture

HMEC-1 cell line was obtained from Zhongqiao Xinzhou Company (Shanghai, China) . The cells were cultured in sciencell brand ECM medium containing 5% fetal bovine serum(FBS), 1% P/S double antibody and 1%ECGS endothelial growth factor. Cells were maintained at 37°C in a humidified 5% CO₂ in air atmosphere. The anaerobic gas production bag (Hpebio, Qingdao, China) was used to promote hypoxia environment, by putting it into the anaerobic culture box. Then the HMEC-1 cells were treated with hypoxia by culturing in the anaerobic culture box for 72h. Phenol blue oxygen indicator was used to monitor oxygen concentration and hypoxia status.

Tamoxifen was dissolved in DMSO to construct a solution of 5mmol/L concentration, then filtered through a needle sterile filter and stored at -20°C in dark. The HMEC-1 cells also could be co-cultured with 5 μ mol/L tamoxifen , and with DMSO as the negative control. Bevacizumab was dissolved in PBS to construct a solution of 3mg/ml concentration, then filtered through a needle sterile filter and stored at -20 °C in dark. The HMEC-1 cells also could be co-cultured with 0.3mg/ml bevacizumab, and with PBS as the negative control.

Quantitative reverse transcription–polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA), according to the manufacturer's instructions. And β -actin was used as internal control to detect the mRNA expression of transcription factors HIF-1 α , VE-cadherin, Fibronectin, CD31, α -SMA, VEGF-a, VEGFR1 and VEGFR2. The EC transcript levels were quantified using Rever Tra Ace qPCR RT Kit (Code No.FSQ-201,TOYOBO,Japan) on the ABI PRISM 7500 system (Applied Biosystems, USA). Quantification was performed using $2^{-\Delta\Delta CT}$ method. The primers used in the PCR analyses are as followings.

HIF-1 α : forward 5'-AGCAACTTGAGGAAGTACCATT-3'

Reverse 5'-AGGTGAACTTTGTCTAGTGCTT-3'

VE-cadherin: forward 5'-AAAGAATCCATTGTGCAAGTCC-3'

Reverse 5'-CGTGTTCATCGTGATTATCCGTG-3'

Fibronectin: forward 5'-AATAGATGCAACGATCAGGACA-3'

Reverse 5'-GCAGGTTTCCTCGATTATCCTT-3'

CD31: forward 5'-TCGTGGTCAACATAACAGAACT-3'

Reverse 5'-TTGAGTCTGTGACACAATCGTA-3'

α -SMA: forward 5'-CCGGGAGAAAATGACTCAAATT-3'

Reverse 5'-CTCAGCAGTAGTAACGAAGGAA-3'

VEGF-a: forward 5'-ATCGAGTACATCTTCAAGCCAT-3'

Reverse 5'-GTGAGGTTTGATCCGCATAATC-3'

VEGFR1: forward 5'-CAAGATTTGCAGAACTTGTGGA-3'

Reverse 5'-CTGTCAGTATGGCATTGATTGG-3'

VEGFR2: forward 5'-GGAGCTTAAGAATGCATCCTTG-3'

Reverse 5'-GATGCTTTCCCAATACTTGTC-3'

Western blot

HMEC-1 cells were lysed with RIPA lytic buffer (Beyotime Biotechnology, China). The same amount of protein samples were separated by 10% SDS-PAGE and transferred to NC membrane (Millipore, USA). After 5% skim milk was blocked, the membranes were incubated overnight at 4 °C with one of the following primary antibody, β -actin (1:1000), HIF-1 α (1:1000), α -SMA (1:500), CD31 (1:500), VEGF-a (1:500). Then, the membranes were washed with PBS and incubated for 1 hour with the secondary antibodies. Finally, they were visualized by ECL reagent (Millipore, USA). Protein images were captured using Image J.

Statistical analysis

All experiments were repeated at least three times with the similar results. The values were expressed as mean $\pm$ SD of at least three independent experiments in triplicate. Statistical differences were assessed by a one-way analysis of variance (ANOVA) (Kruskal–Wallis) in SPSS17.0 software. A P value of less than 0.05 was considered statistically significant.

Results

Background of participants and iTRAQ-based proteomics

The clinical features of the SSc patients and healthy controls whose skin tissues were studied were shown in Table 1. Protein patterns from 4 patients and 4 controls were analyzed by iTRAQ-based quantitative approach. A total of 2159 proteins were identified. The amino acid sequences of identified proteins the protein dataset was matched to the local database, and 43 proteins were found to be significantly different between SSc patients and controls with P value<0.05, FC >1.2 or <0.83, shown in Additional file 1. Functional enrichment analysis was performed for the identified proteins based on KEGG databases, the top 15 up and down pathways were shown in fig. 1A. Interestingly, the HIF-1 signaling significantly up-regulated in the skin tissues of SSc patients.

Table 1. Clinical and demographic characteristics of SSc patients and controls.

Characteristics	SSc (n=8)	Controls (n=8)	P value
Age(years)	51±8.49	51±8.49	1.00
Female/male	6/2	6/2	1.00
BMI(Kg/m ²)	21.75±2.63	22.06±1.19	0.77
Duration of disease (years)	1.04±0.82	-	-
Involve organs (n/N)			
Raynaud phenomenon	7/ 8	-	-
Lung	4/ 8	-	-
Gastrointestinal tract	3/ 8	-	-
Joint	5/ 8		
Kidney	1/ 8	-	-
Heart	2/ 8	-	-
Biopsy site (n)			
Finger/Back of hand/ Forearm	4/ 2/ 2	4/ 2/ 2	1.00

EndMT and activated HIF-1α/VEGF signal were detected in the patients with systemic sclerosis

Compared with controls, all 8 skin tissue specimen from the SSc patients with displayed varying degrees of interstitial fibrosis, along with a pattern of infiltration of inflammatory mononuclear cells around vessels, marked narrowing, occlusion of the vessel lumen and vascular loss, as illustrated in Fig. 1B, C. The EC marker CD31 and IC marker α-SMA were used in immunohistologic analyses to identify EndMT present in the cutaneous tissue. As expected, CD31 positive cells significantly decreased in the SSc tissue were detected at sites lining the vessel lumens, compared with them in control tissue, as illustrated in Fig.1D, E. Meanwhile, positive α-SMA staining of α-SMA was shown perivascularly in specimens of patients and on the epidermal cells , which was negative in control specimen, as illustrated in Fig.1F,G.

The HIF-1α/VEGF signal markers were also analyzed by immunohistochemistry. All skin biopsies from SSc patients were both HIF-1α and VEGF positive, however HIF-1α was not present and VEGF expressed lowly in the healthy tissue samples (Fig. 1 H, I, J, K). The staining of the two markers were observed throughout the keratinocytes of the epidermis, as well as diffusely distributed in dermis.

Hypoxia induced interstitial transformation of microvascular endothelial cells in vitro

To test whether hypoxia is able to induce the conversion of ECs into ICs, EC cell line HMEC-1 were exposed to anaerobic gas production bag for hypoxia treatment and endothelial and fibrotic characteristics were measured. ECs exposed to anaerobic gas exhibited a decrease for the mRNA expression of the endothelial proteins VE-cadherin and CD31 (Fig. 2A and B) at every hypoxia time point that was tested. Furthermore, consistent with the establishment of a fibrotic process, ECs challenged with anaerobic gas showed an increase for the mRNA level in the fibrotic markers α -SMA and fibronectin (Fig. 2C and D) at every hypoxia time point that was tested. At protein level, the expression level of CD31 was up-regulated temporarily after 24 hours of hypoxia, and then declined gradually with the extension of hypoxia. The protein of α -SMA showed an continuous increase with the prolongation of hypoxia treatment, as illustrated in Fig. 2 E,F,G.

We also investigated the effect of hypoxia on the cellular morphology and polarity of ECs. In the absence of hypoxia, HMEC-1 cells were circular adherent cells. When the cells were anoxic for 12 hours, there was a small amount of death, but the shape was still full and the outline is clear. With the duration of hypoxia, the number of cell death increased, and the morphology changed to fusiform, losing adhesion connection and polarization gradually. The data was shown in Additional file2.

Hypoxia activated the HIF-1 α and VEGF signaling in vitro

Considering the postulation of HIF-1 α /VEGF signaling pathway involving in hypoxia-mediated EndMT, we were prompted to investigate whether hypoxia induced the activation of signal molecules and their ligands at both mRNA and protein levels. There was no change of HIF-1 α mRNA level at different time points after hypoxia (Fig. 3 A). But ECs in the presence of hypoxia exhibited increased VEGF-a mRNA level at every time point that was tested (Fig. 3 B). Meanwhile, the expression of two ligands of VEGF, VEGF-R1 and VEGFR2 were detected at different time points of hypoxia by qRT-PCR. To that end, the activation of VEGF-R1 and the inhibition of VEGF-R2 were found, as illustrated in Fig. 3 C and D. At protein level, the detection of HIF-1 α and VEGF-a were performed by western blot, which indicated the up-regulation of both molecules (Fig. 3 E,F,G).

Down-regulation of HIF-1 α inhibits hypoxia induced EndMT and VEGF Signaling

Although tamoxifen has been reported to be the specific inhibitor of HIF-1 α [16], whether HIF-1 α signaling and its probable downstream VEGF signaling induced by hypoxia could be effectively abolished needed further investigation. To test this, hypoxia-treated ECs incubated with tamoxifen for 72 hours and the signal molecules HIF-1 α and VEGF-a were detected at protein level. Results showed that hypoxia treated ECs with tamoxifen showed a sustaining decreased expression of both HIF-1 α and VEGF-a (Fig. 4 A,B,C).

Taking into account that hypoxia induces EndMT and the expression of HIF-1 α and VEGF-a, we tested whether the activation of the HIF-1 α / VEGF signaling is necessary for the EndMT induced by hypoxia. To that end, we used tamoxifen the specific inhibitor of HIF-1 α / VEGF signaling and tested the expression of CD31, α -SMA and fibronectin in hypoxia treated EC. As a result, the decreased mRNA expression of EC markers CD31 in hypoxia treated HMEC-1 cells could be inhibited by tamoxifen intervention (Fig. 5 A).

Moreover, the hypoxia treated EC with tamoxifen showed a significant decrease in the mRNA expression of fibrotic protein α -SMA and fibronectin, which could up-regulate in EC induced by hypoxia without the inhibition of HIF-1 α / VEGF signaling (Fig. 5 B,C). The protein levels of CD31 and α -SMA were also detected by Western blot. We found that the expression of EC marker CD31 could not decrease and even the IC marker α -SMA was inhibited in hypoxia treated EC, while co-culturing with tamoxifen (Fig. 5 D,E,F).

Hypoxia induced EndMT is dependent on the activation of HIF-1 α / VEGF signaling

Although the VEGF-a has been verified as the predominant proangiogenic factor regulated by HIF-1 α in hypoxia related diseases, it's unknown whether the hypoxia-induced EndMT mediated by up-regulation of HIF-1 α signaling was dependent on the activation of VEGF-a. Then the bevacizumab, the inhibitor of VEGF-a was used to treat the ECs in hypoxia conditions for 72 hours and HIF-1 α , VEGF-a, CD31 and α -SMA were detected at protein level[17]. The VEGF-a could be inhibited gradually by its inhibitor which could not intervene the activation of HIF- α induced by hypoxia(Fig.6A, B, C). The down-regulation of CD31 and up-regulation of α -SMA induced by hypoxia could be rescued perfectly by the inhibition of VEGF-a (Fig. 6 D,E,F).

Discussion

Therapeutic improvements have been reported to alleviate the relative damage of SSc complications on patients' prognosis, however previous articles did highlight a high mortality rate in SSc patients and SSc vasculopathy, in terms of pulmonary PAH, heart involvement, and scleroderma renal crisis, still represents the first cause of disease related mortality[2, 18–21]. In deed, vasculopathy is present not only early but almost invariably during the course of SSc, which is characterized by the frequent perivascular inflammation, intimal hyperplasia, medial thickening, obliteration of the lumen, vascular loss and occasionally microthrombi[22]. The mechanism of SSc vasculopathy is not fully understood, but increasing evidence indicates that endothelial injury and subsequent endothelial dysfunction is a primary event that triggers the subsequent formation of typical vascular lesions[23]. It is known that EndMT, a key player in the remodelling of injured vessels may be reversible, possibly offering novel cues for treatment[2]. Moreover, the main fibrosis orchestrators in SSc are represented by chronically activated myofibroblasts, and EC was demonstrated to be capable of trans-differentiating toward myofibroblasts, which indicated that EndMT plays a role in both vascular remodeling and tissue fibrosis[5]. However, the mechanism underlying the transformation was not completely unveiled.

EndMT refers to a trans-differentiation process during which ECs downregulate the expression of its specific EC markers, such as CD31 and VE-cadherin, acquiring a mesenchymal/myofibroblast phenotype, which is characterized by the expression of SMA, type I collagen, et al[24]. Meanwhile, ECs gain a spindle-shaped fibroblast-like morphology, disaggregate, and lose polarity during this transition[24]. In recent years, accumulative evidence supported the prominent role of EndMT in the pathogenesis of tissue fibrosis and fibroproliferative vasculopathy in various diseases, such as PAH, diabetic nephropathy, cardiac and intestinal fibrosis[25, 26]. Increasing data showed that EndMT emerged as an important

event in SSc as well[4, 25–27]. In particular, it has been proposed that the SSc-related EndMT process have differential pathogenetic roles depending on the type of affected vessels[24, 27]. When affecting capillary vessels, EndMT may lead to an increase in the number of perivascular myobroblasts, thereby contributing to tissue fibrosis and a parallel loss of endothelial cells[27]. In this study, the positive α -SMA staining of α -SMA was shown mainly around the dermis vessels in the specimen of SSc patients.

It is well known that SSc is an autoimmune disease characterized by immunological disturbances, vascular remodeling and broad fibrosis, the order of which remains to be fully determined. Hypoxia is potentially caused by a yet to be identified circuitry involving such three features that typify SSc. Once present, the hypoxia creates a vicious circle of ongoing pathology. It is clear that hypoxia is an important hallmark in SSc patients[28]. Additionally, previous studies have confirmed that hypoxia could induce EndMT in PHA and myocardial remodeling[6, 8, 29, 30], as well as in scleroderma[7]. Galectin-3, HIF-1 α /Twist1, BMP-7/Smads and TGF β 1/SMAD signaling pathways proved to be a critical mediator in PAH by regulating EndMT and a critical role in mediating the effect of hypoxia-induced EndMT in pulmonary arterial and cardiovascular remodeling[6, 8, 29, 30]. The vessels involved in these diseases are arterioles and small arteries, in which EndMT may have differential pathogenetic roles from it affecting capillary vessels. Although Liu C, et al. have demonstrated that hypoxia increased vimentin and α -SMA but decreased VE-cadherin and CD31 in HUVECs[7], the pathogenesis underlying EndMT induced by hypoxia was not well studied in SSc.

As a result of the hypoxia in skin and other affected tissue in SSc, activation of important hypoxia pathways is plausible. HIF-1 α is the master transcriptional regulator of the adaptive response to hypoxia, which is the tightly regulated form of HIF-1, and will quickly hydroxylated and degraded in normoxic conditions by prolyl hydroxylases [31–33]. But in hypoxia conditions, the expression of HIF-1 α upregulate dramatically [34, 35]. The positive staining of HIF-1 α has been detected throughout the keratinocytes of the epidermis in all skin biopsies from scleroderma patients[13], which was also observed in our study. Although HIF-1 α was postulated to be dysregulated in various pathologic conditions, including SSc[36–38] and HIF-1 α could induce hypoxia-treated EndMT in pulmonary arterial remodeling through regulating Twist1. The role of HIF-1 α in hypoxia induced EndMT of SSc has remained to be fully characterized. This study postulated HIF-1 α /VEGF signaling playing a critical role in regulating EndMT induced by hypoxia.

The VEGF has been reported to be significantly up-regulated in all stages of fibrosis and dendritic endothelial cells, which was thought to be one of the main transcriptional targets of HIF-1 α in hypoxia-related diseases [13, 39]. Traditionally, VEGF was identified as a key mediator of angiogenesis which induced differentiation, proliferation, and migration of endothelial cells, consequently contributing to the formation of vessels through both angiogenesis and vascular remodeling[39]. VEGF exerts its biological functions by binding to its receptors the tyrosine kinase receptors VEGFR1 and VEGFR2. However, unexpectedly, skin tissues of SSc patients with characterized vessel obstruction and loss demonstrated a strong upregulation of VEGF and its two receptors, with an intenser staining for VEGFR-2 than for VEGFR-1[13, 39]. Moreover, the increase of VEGF, HIF-1 α accompanied by the characteristics of EndMT were observed in the skin of SSc patients in both previous articles and in this study. Meanwhile, HMEC-1

treated by hypoxic exhibited the downregulated level of CD31, VE-cadherin expression, and a marked increase in the expression of α -SMA and fibronectin, alongside the increased expression of HIF-1 α , VEGF-a. Of note, the hypoxia induced EndMT was effectively reversed by tamoxifen and bevacizumab, the inhibitor of HIF-1 α /VEGF pathway.

Conclusion

Taken together, the results from this study shown here provide evidence that hypoxia is a crucial factor in inducing the conversion of ECs into ICs through a a HIF-1 α /VEGF dependent mechanism that consequently promotes the skin microvascular remodeling and fibrosis in SSc. This information will be beneficial for designing novel and improved therapeutic strategies against the complications of SSc associated fibroproliferative vasculopathy and fibrosis.

Abbreviations

EndMT endothelial mesenchymal transition

SSc systemic scleroderma

iTRAQ isobaric tags for relative and absolute quantification

HMEC-1 Human microvascular endothelial cell line

HIF-1 α Hypoxia inducible factor-1 α

VEGF vascular endothelial growth factor

α -SMA α -smooth muscle actin

EC endothelial cell

IC interstitial cell

PAH pulmonary arterial hypertension

ECM extracellular matrix

ACR American College of Rheumatology

EULAR European League Against Rheumatism

PBS phosphate buffer saline

DAB Diaminobenzidine

SDS	Sodium dodecyl sulfate
DTT	Dithiothreitol
BCA	bicinchoninic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
FBS	fetal bovine serum
ECGS	endothelial growth factor
DMSO	dimethyl sulfoxide
ECL	electrochemiluminescence
ANOVA	Analysis of Variance
VEGFR	Vascular Endothelial Growth Factor Receptor

Declarations

Ethics approval and consent to participate

All the surgical biopsies were performed following the patients' provision of informed consent and according to Institutional Review Board–approved protocols from the Affiliated Hospital of Southwest Medical University.

Consent for publication: Not applicable

Availability of data and materials

The iTRAQ-based proteomics data from this study will be made freely available from the link: <http://cloud.majorbio.com>; The accession number is deng123.

Competing interests

The authors declare that they have no competing interests.

Funding:

This work was supported by research grants from Southwest medical university and luzhou science and technology bureau(Grant number:090300021686).

Authors' contributions: J Mao performed the experiments, analyzed the data, and wrote manuscript. JX Liu collected the data, and revised the manuscript. JX Liu, M Zhou and GQ Wang participated in the

sample and data collection. Xia Xiong helped optimize the research and proofread the paper. YQ Deng designed and optimized the research, wrote and revised manuscript. All authors read and approved the final manuscript.

Acknowledgements: Not applicable

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Figures

Figure 1

The iTRAQ-based proteomics, histopathologic and immunohistologic assessments of skin tissue from patients with SSc. Original magnification $\times 20$. (A) The top 15 up and down pathways of functional enrichment analysis performed for the identified proteins based on KEGG databases. (B, C) Hematoxylin and eosin staining of skin tissue of a patient with SSc, indicating a large amounts of fibrous tissue in dermis and infiltration of inflammatory mononuclear cells around vessels, as well as narrowing, occlusion of the vessel lumen and vascular loss. (D,E,F,G) Immunohistochemical staining of skin tissue for the EC-specific marker CD31 and IC marker α -SMA. In the tissue from patient as in E, G, the presence of CD31-positive cells decreased and α -SMA-positive signaling increased in the dermis, compared with it in control tissue as in D, F. (H,I,J,K) Immunohistochemical staining of skin tissue for the HIF-1 α /VEGF signal. Specimens from SSc a patient were both HIF-1 α and VEGF positive as in I,K, and HIF-1 α was not present and VEGF expressed lowly in the normal tissue samples as in H,J.

Figure 2

Hypoxia-induced changes in endothelial and fibrotic markers expression. (A,B,C,D) ECs were exposed to hypoxia for 72 h and mRNA expression was analyzed. (E) Representative images from western blot experiments performed for detection of endothelial marker CD31, and fibrotic marker α-SMA as in E. (F,G) Densitometric analyses of the western blot experiments. Statistical differences were assessed by a one-

way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunn’s post hoc test. *: $P < 0.05$ against the untreated condition. Graph bars show the mean $\pm$ SD.

Figure 3

Hypoxia-induced changes of HIF-1α, VEGF-a, VEGFR1, VEGFR2 at mRNA and protein level. (A,B,C,D) ECs were exposed to hypoxia for 72 h and mRNA expressions of HIF-1α, VEGF-a, VEGFR1, VEGFR2 were analyzed. (E) Representative images from western blot experiments performed for detection of HIF-1α

and VEGF-a. (F and G) Densitometric analyses of the western blot experiments. Statistical differences were assessed by a one-way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunn’s posthoc test. *: P<0.05 against the untreated condition. Graph bars show the mean ± SD.

Figure 4

Hypoxia-treated ECs was incubated with tamoxifen,the HIF-1α inhibitor, and the expressions of HIF-1α, VGEF-a were detected. (A) Representative images from western blot experiments performed for detection of HIF-1α, VGEF-a with and without tamoxifen treatment. (B,C) Densitometric analyses of the western blot experiments. Statistical differences were assessed by a one-way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunn’s posthoc test. *: P<0.05 against the untreated condition. Graph bars show the mean ± SD.

Figure 5

Hypoxia-treated ECs was incubated with tamoxifen, the HIF-1 α inhibitor, and the expressions of CD31, fibronectin and α -SMA were detected. (A,B,C) The relative expression of CD31, fibronectin and α -SMA mRNA. (D) Representative images from western blot experiments performed for detection of HIF-1 α , VEGF-a with and without tamoxifen treatment. (E,F) Densitometric analyses of the western blot experiments. Statistical differences were assessed by a one-way analysis of variance (ANOVA) (Kruskal-Wallis) followed by Dunn's posthoc test. *: $P < 0.05$ against the untreated condition. Graph bars show the mean $\pm$ SD.

Figure 6

Hypoxia-treated ECs was incubated with bevacizumab, the VEGF-a inhibitor, and the expressions of HIF-1α, VEGF-a, CD31, and α-SMA were detected. (A,D) Representative images from western blot experiments performed for detection of HIF-1α, VEGF-a, CD31, and α-SMA with and without bevacizumab treatment. (B,C,E,F) Densitometric analyses of the western blot experiments. Statistical differences were assessed by a one-way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunn’s posthoc test. *: P<0.05 against the untreated condition. Graph bars show the mean ± SD.

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