Exploration and validation of the hub genes involved in hypoxia-induced endothelial-mesenchymal transition of systemic sclerosis

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Abstract

Objective

During the development of systemic sclerosis (SSc), endothelial-mesenchymal transition (EndoMT) has been shown to be one of the mechanisms leading to pulmonary fibrosis. However, the correlation between hypoxia and EndoMT was mostly unknown.

Methods

R software was used to analyse differentially expressed genes (DEGs) in vascular endothelial cells under hypoxic conditions, and fibroblasts derived from SSc-related pulmonary fibrotic tissues, respectively. Using a web-based online Venn diagram tool, we analysed overlapping genes of DEGs between endothelial cells and fibroblasts. Finally, the protein-protein interaction network of EndoMT hub genes were constructed using the STRING database. The hub genes were knockdown by transfection of siRNAs in the hypoxia model of HULEC-5a cells constructed by liquid paraffin closure and then used to detect the effect on EndoMT-related biomarkers by western blot.

Results

In this study, we found that INHBA, DUSP1, NOX4, PLOD2, BHLHE40 were upregulated in SSc fibroblasts and hypoxic-treated endothelial cells, while VCAM1, RND3, CCL2, and TXNIP were downregulated. In the hypoxia model of HULEC-5a cells, the expression of these 9 hub genes was confirmed by western blot. In addition, through Spearman's correlation analysis and Western blot, we confirmed that these hub genes were closely related to the EndoMT-related markers. The mechanisms of these hypoxia-induced EndoMT hub genes may be related to TGF-β, Notch, Wnt, NF-κB, TNF and mTOR signalling pathways.

Conclusion

Our study provides new insights into the occurrence and development of SSc-related pulmonary fibrosis resulting from hypoxia-induced EndoMT.

Key words

systemic sclerosis, hypoxia, endothelial-mesenchymal transition, pulmonary fibrosis, bioinformatics

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Competing interests: none declared.

Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterised by progressive fibrosis of the skin and/or internal organs, immune disorders, and vascular damage (1). Skin and lung lesions are the most common symptoms of SSc, with pulmonary vascular injury and pulmonary fibrosis being the main factors affecting the progression and prognosis of SSc (2). More than 70% of SSc patients are accompanied by pulmonary lesions, mainly including interstitial lung disease (ILD) and pulmonary hypertension (PH), which account for 60% of SSc-related deaths (3). The nature of interstitial lung disease and pulmonary hypertension are both fibrotic lesions, and myofibroblasts are the effector cells that lead to fibrosis during the pathogenesis (4). It has been found that one of the important sources of myofibroblasts is the vascular endothelial cells that undergo endothelial-mesenchymal transition (EndoMT) (5).

EndoMT refers to the pathological process by which endothelial cells acquire a mesenchymal cell-like phenotype upon stimulation (6). EndoMT can occur in vascular endothelial cells of different tissue under the stimulation of various pathological factors and the combined action of various cytokines (such as TGF-β, Snail, Slug, Twist, etc.) (7). Endothelial cells undergoing EndoMT lose their own properties and exhibit mesenchymal characteristics with enhanced invasive and migratory abilities, gradually transforming into myofibroblasts, and migrating to perivascular areas to participate in the process of tissue fibrosis (8). Studies have shown that EndoMT of endothelial cells exists during the occurrence and development of SSc. Cells in the intermediate stage of EndoMT have been found in the dermal vessels of SSc patients and bleomycin-induced or urokinase-type plasminogen activator receptor (uPAR)-deficient SSc animal models (9, 10). Compared with normal skin microvascular endothelial cells, endothelial cells from SSc patients show a spindle-like shape and express the markers of both endothelial cells (CD31 and VE-cadherin) and myofibroblast (α-SMA, S100A4, collagen type I) (11). Furthermore, exposure of normal vascular endothelial cells to SSc serum or TGF-β1 triggers a transition to a myofibroblast-like morphology, contractile phenotype, downregulation of endothelial markers and upregulation of mesenchymal markers (9). Similarly, exposure of pulmonary artery endothelial cells to a cocktail of pro-inflammatory cytokines such as IL-1β, TNF-α and TGF-β also altered their "cobblestone" structure, inducing a spindle-like appearance and the upregulation of mesenchymal markers (12).

Hypoxia is one of the important manifestations of SSc patients (13, 14). Early hallmarks of SSc are endothelial cell damage, as well as intimal hyperplasia and eventual occlusion of the vascular lumen. Reduced capillary blood flow combined with insufficient angiogenesis can lead to chronic hypoxia (15). Studies have shown that hypoxia is an effective stimulus to promote EndoMT, which is one of the mechanisms of fibrosis (16, 17). However, the correlation among hypoxia, EndoMT and pulmonary fibrosis during the development of SSc remains unclear. In this study, we attempted to reveal the molecular mechanism of hub genes by which hypoxia-induced EndoMT promotes pulmonary fibrosis.

Materials and methods

Data collection and analysis

The data used in this study were obtained from GSE73674 and GSE40839, downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The GSE73674 dataset contains RNA-seq data from vascular endothelial cells from 26 SSc patients cultured under normoxic or hypoxic conditions. GSE40839 contains RNA-seq data of fibroblasts from 10 normal lung tissues and 8 patients with SSc-related interstitial lung disease. The datasets were analysed for DEGs after batch normalisation using the R packages "sva" and "limma". Adjusted *p*-values were analysed to correct for false-positive results. "Adjusted $p<0.05$ and log2(fold change) > 1 or log2(fold change) < -1" was defined as the threshold to screen for DEGs. The GO function and pathway enrichment analysis of DEGs were assessed by the ClusterProfiler package.

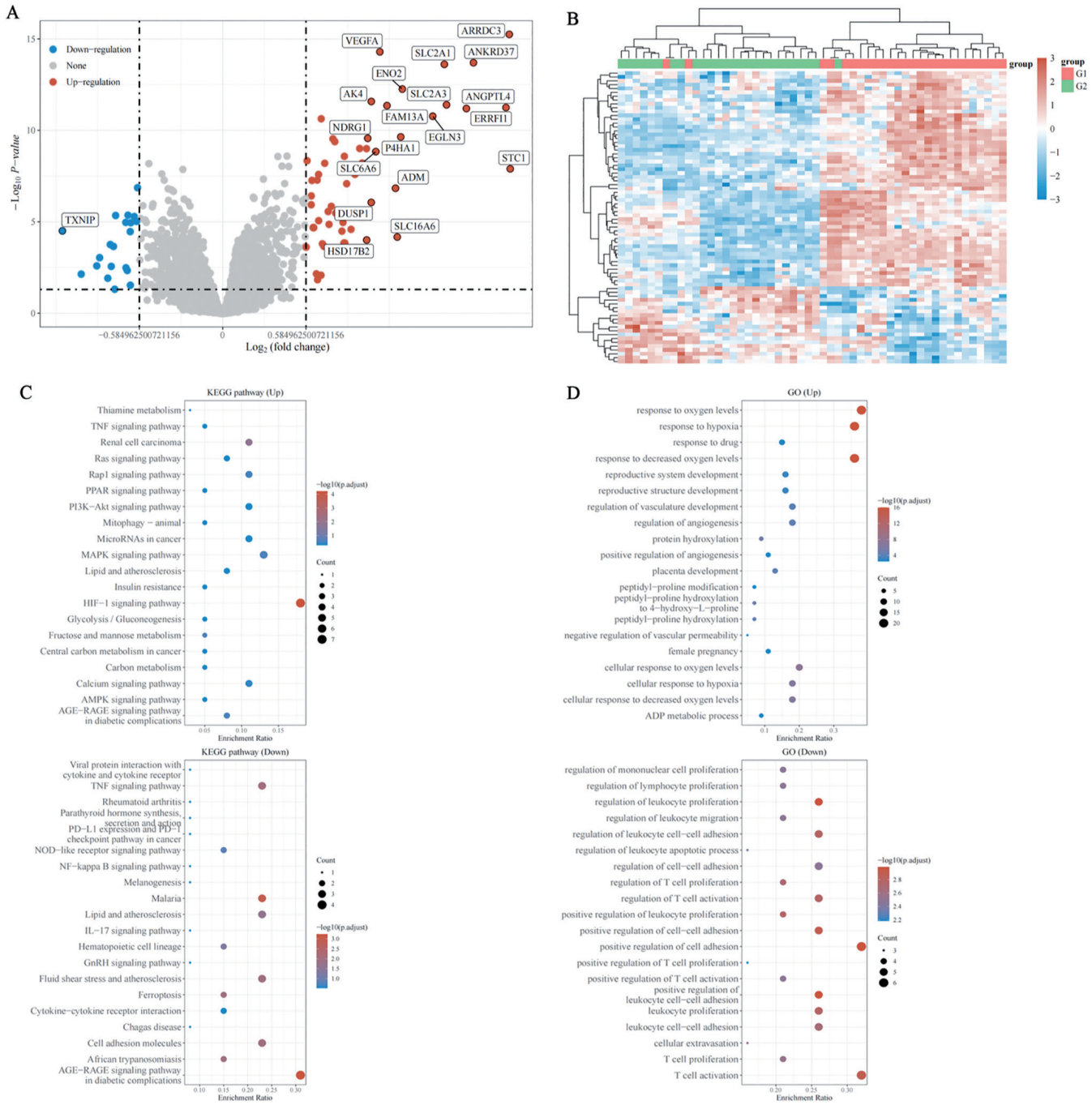

Fig. 1. Analysis of DEGs in vascular endothelial cells of SSc patients under hypoxic condition.
(**A**) Volcano plots of DEGs in vascular endothelial cells of SSc patients under normoxic and hypoxic conditions, where red dots indicate upregulated genes, blue dots indicate downregulated genes, and gray dots indicate no significant difference. (**B**) Heat map illustrating the DEGs in vascular endothelial cells of SSc patients under normoxic and hypoxic conditions, where red indicates significantly upregulated genes, blue indicates significantly downregulated genes, and white indicates genes with no significant difference. The G1 group represents hypoxic condition, and G2 group represents normoxic condition. KEGG pathway analysis (**C**) and GO analysis (**D**) of upregulated and downregulated DEGs revealed the top 20 relevant pathways and biological progresses. The dot sizes refer to the number of genes involved in the pathway, and the dot colours depict the *p*-values.

The study protocol was approved by the Ethics Committee of Nanyang Institute of Technology.

Identification of EndoMT hub genes

The differentially expressed genes in endothelial cells and fibroblasts obtained from the analysis were subjected to a web-based online Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to identify EndoMT hub genes. In the present study, intersecting DEGs between endothelial cells and fibroblasts were considered as EndoMT hub genes. In addition, the expression levels of EndoMT hub genes in different subgroups of endothelial cells and fibroblasts in the GSE73674 and GSE40839 datasets were analysed using the R packages "limma" and

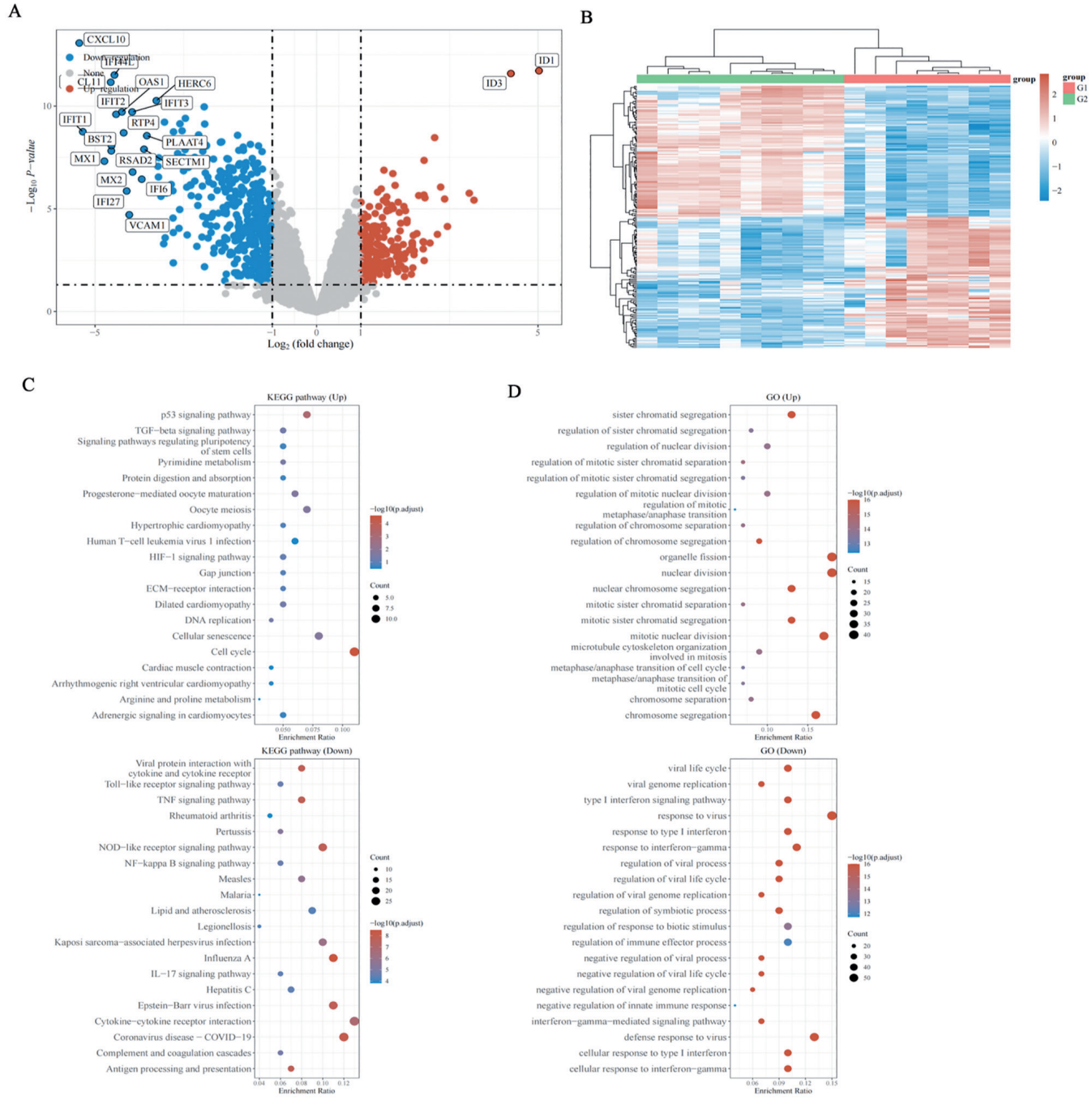

Fig. 2. Analysis of DEGs in lung fibroblasts from patients with SSc-related pulmonary fibrosis.
(**A**) Volcano plots of DEGs between fibroblasts of SSc-associated pulmonary fibrosis and normal lung tissues, where red dots indicate upregulated genes, blue dots indicate downregulated genes, and gray dots indicate no significant difference. (**B**) Heat map illustrating the DEGs between SSc-associated pulmonary fibrosis and normal lung tissues, where red indicates significantly upregulated genes, blue indicates significantly downregulated genes, and white indicates genes with no significant difference. The G1 group represents SSc-associated pulmonary fibroblasts, and G2 group represents normal pulmonary fibroblasts. KEGG pathway analysis (**C**) and GO analysis (**D**) of upregulated and downregulated DEGs revealed the top 20 relevant pathways and biological progresses. The dot sizes refer to the number of genes involved in the pathway, and the dot colours depict the *p*-values.

"boxplot". Wilcoxon Rank Sum and Signed Rank Tests were employed to compare the expression level between two groups, $p<0.05$ was considered to be statistically significant using R software and results were visualised by the ggplot2 package.

Correlation analysis of hub genes and EndoMT-related markers

In the GSE73674 and GSE40839 datasets, the correlation between hub genes and EndoMT-related markers (VIM, FN1, COL1A2, COL1A1) was analysed. The R software pheatmap package was used to perform the analysis of gene correlation. Spearman's correlation analysis was used to describe correlations between quantitative variables without a normal distribution.

A *p*-value less than 0.05 was considered statistically significant.

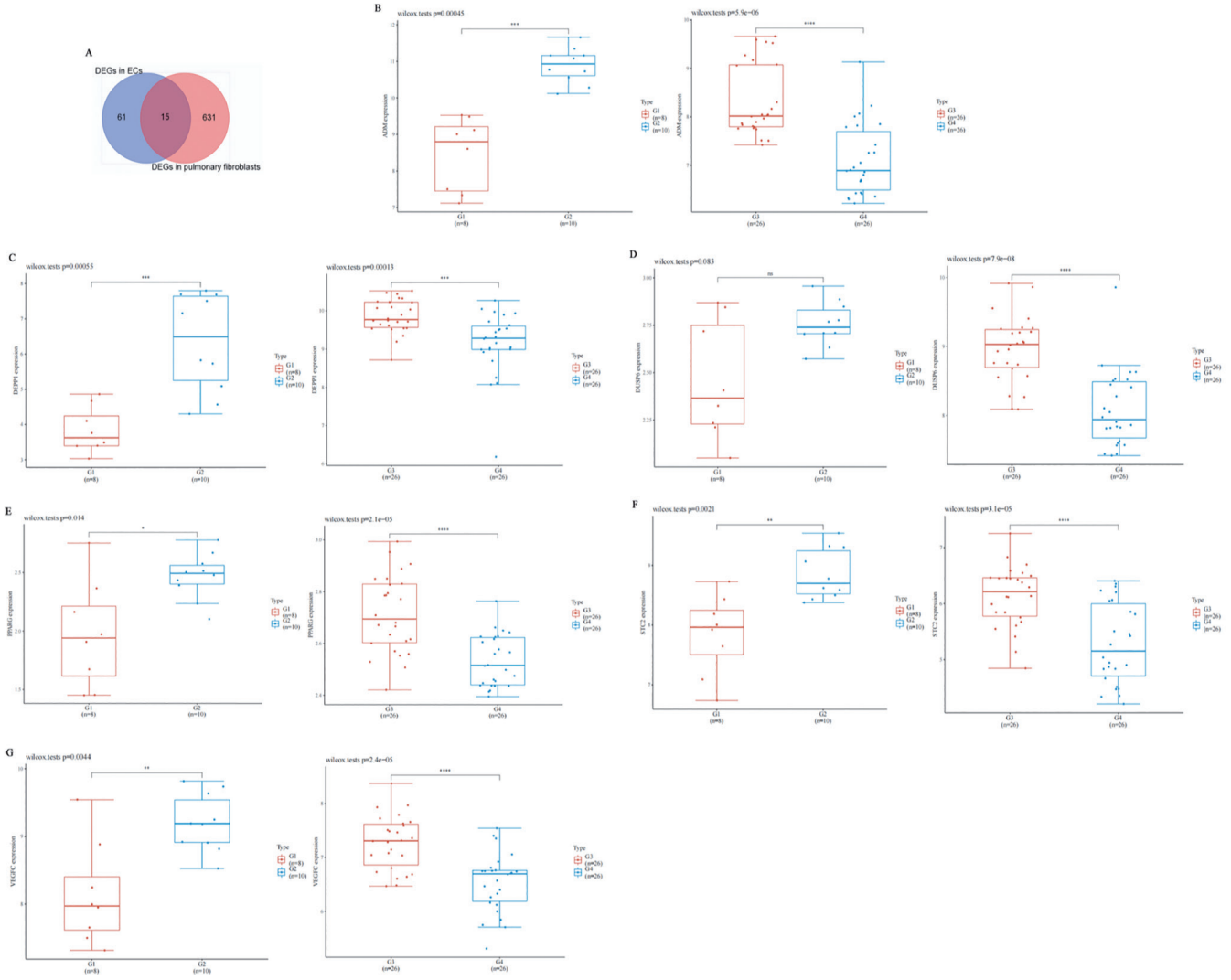

Fig. 3. Key genes of hypoxia-induced EndoMT in the development of SSc pulmonary fibrosis. (**A**) Venn diagram of the intersecting DEGs between endothelial cells and fibroblasts. Commonly intersecting genes were identified as key genes, which included DUSP6, INHBA, PPARG, BHLHE40, VCAM1, VEGFC, ADM, DUSP1, NOX4, STC2, RND3, PLOD2, DEPP1, CCL2, TXNIP. Differential expression of ADM (**B**), DEPP1 (**C**), DUSP6 (**D**), PPARG (**E**), STC2 (**F**), VEGFC (**G**) in vascular endothelial cells and fibroblasts. The G1 group represents vascular endothelial cells under hypoxic condition, and G2 group represents vascular endothelial cells under normoxic condition. The G3 group represents SSc-associated pulmonary fibroblasts, and G4 group represents normal pulmonary fibroblasts.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns represents no significance.

Construction and enrichment analysis of protein-protein interaction (PPI) network of EndoMT hub genes

PPI network and GO biological process and KEGG pathway of EndoMT hub genes were analysed using the STRING database (https://string-db.org/). The PPI data in the STRING database was imported into cytoscape software to draw the PPI network of EndoMT hub genes.

Cell transfection and construction of hypoxia models

We synthesised siRNAs for INHBA, DUSP1, NOX4, PLOD2, BHLHE40, VCAM1, RND3, CCL2, TXNIP (labelled as siR-INHBA, siR-DUSP1, siR-NOX4, siR-PLOD2, siR-BHLHE40, siR-VCAM1, siR-RND3, siR-CCL2, siR-TXNIP) and scrambled siRNA (labelled as siR-NC) (GenePharma, Shanghai, China) (Supplementary Table 1). When lung microvascular endothelial cells HULEC-5a were grown to 70%-80% confluence in six-well plates, siR-INHBA, siR-DUSP1, siR-NOX4, siR-PLOD2, siR-BHLHE40, siR-VCAM1, siR-RND3, siR-CCL2, siR-TXNIP and siR-NC were transfected into HULEC-5a using Lipofectamine RNAiMAX reagent, respectively, according to the instructions. After 36 hours of transfection, the medium was replaced with sugar- and serum-free medium, and then 2 ml/well of sterile liquid paraffin was added to cover the surface of the medium, which was used to insulate it from oxygen. HULEC-5a cells were continued to be cultured in the incubator for 8h, and then the cells were collected and proteins were extracted for Western blot assay.

Western blot

The experimental procedure for Western blot was referred to our previous study (18). Antibodies of INHBA (10651-

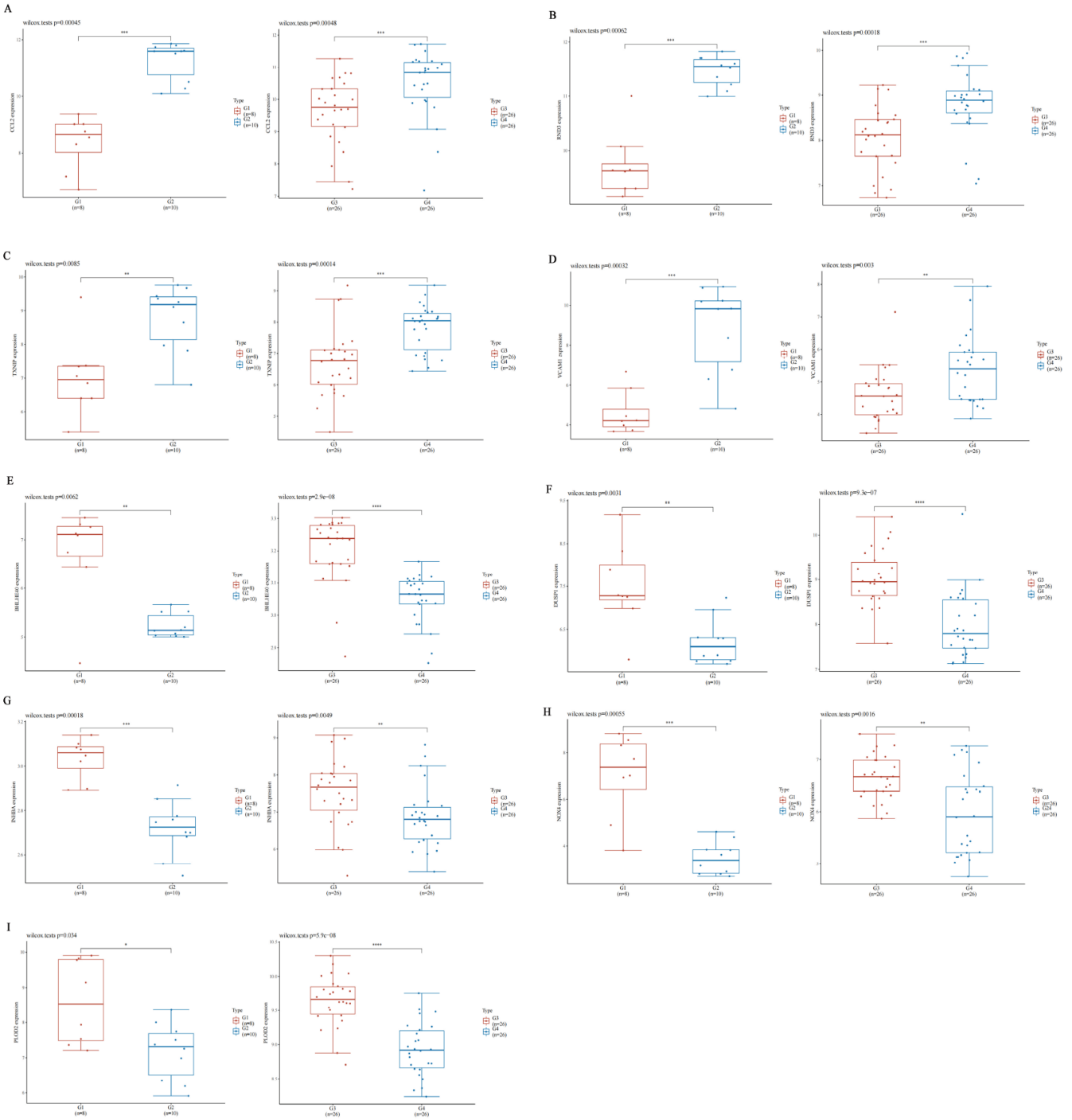

Fig. 4. Identification and analysis of hypoxia-induced EndoMT hub genes. Differential expression of CCL2 (**A**), RND3 (**B**), TXNIP (**C**), VCAM1 (**D**), BHLHE40 (**E**), DUSP1 (**F**), INHBA (**G**), NOX4 (**H**), PLOD2 (**I**) in vascular endothelial cells and fibroblasts. The G1 group represents vascular endothelial cells under hypoxic condition, and G2 group represents vascular endothelial cells under normoxic condition. The G3 group represents SSc-associated pulmonary fibroblasts, and G4 group represents normal pulmonary fibroblasts.
**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

1-AP), NOX4 (14347-1-AP), PLOD2 (66342-1-Ig), BHLHE40 (17895-1-AP), VCAM1 (11444-1-AP), RND3 (66228-1-Ig), CCL2 (66272-1-Ig), TXNIP (18243-1-AP), GAPDH (60004-1-Ig) were purchased from Proteintech. Antibodies of DUSP1 (AF5286), VE-cadherin (AF6265), CD31 (AF6191), vWF (AF3000), Tie-1 (DF4582), COL1A1 (AF7001) and vimentin (AF7013) were purchased from Affinity Biosciences company. The dilution ratio of GAPDH antibody was 1:5000 and all other antibodies was 1:500.

Statistical analysis

A *p*-value <0.05 was considered statistically significant. The analysis and visualisation of the data were made using the above-mentioned online databases and tools, R 3.6.4, Excel 2019, and Cytoscape 3.7.2.

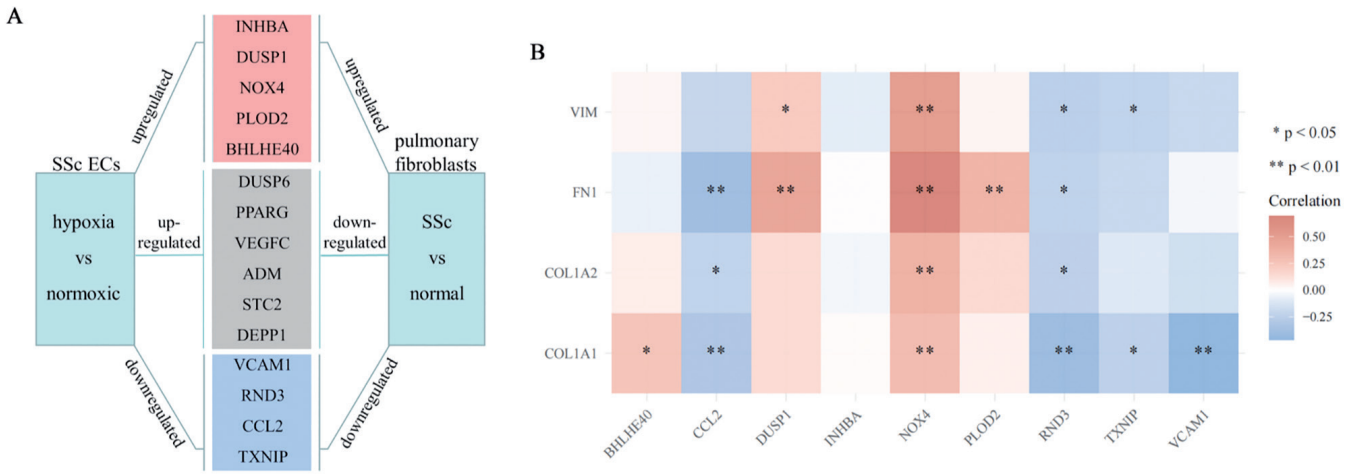

Fig. 5. Correlation analysis of the hub genes and EndoMT-related biomarkers.
(**A**) The expression levels of 15 DEGs in different groups of SSc ECs and pulmonary fibroblasts. (**B**) The correlation between the hub genes and EndoMT-related biomarkers VIM, FN1, COL1A2, COL1A2. *p<0.05, **p<0.01.

Results

Analysis of DEGs in vascular endothelial cells of SSc patients under hypoxic condition

During the development of SSc, the role of hypoxia in the development of SSc pulmonary fibrosis remains unclear. Hypoxia is an effective stimulus to promote EndoMT of vascular endothelial cells, but its molecular mechanism is still unclear. To explore the molecular mechanism of hypoxia-induced EndoMT in vascular endothelial cells of SSc patients, we downloaded and used the GSE73674 dataset to analyse the DEGs in vascular endothelial cells of SSc patients under normoxic and hypoxic conditions. The results showed that compared with normoxic condition, there were 76 DEGs in SSc vascular endothelial cells under hypoxic condition, including 56 upregulated genes and 20 downregulated genes (Fig. 1A-B, Suppl. Table S2). Since the biological effects induced by hypoxia are very extensive, we performed KEGG analysis and GO analysis on these DEGs. The results showed that the HIF-1 signalling pathway was significantly affected, which is beyond doubt. In addition, TNF signalling pathway, NF-κB signalling pathway, cell adhesion molecules, vascular permeability, etc. are also affected by hypoxia (Fig. 1C-D), both of which were closely related to the occurrence of EndoMT (19-21). The above results indicate that hypoxia may induce EndoMT in vascular endothelial cells during the development of SSc.

Analysis of DEGs in lung fibroblasts from patients with SSc-related pulmonary fibrosis

Fibroblasts are the main cells that maintain the balance of collagen metabolism and play a key role in organ fibrosis and wound healing (22). In this study, we analysed the DEGs in fibroblasts between SSc-associated pulmonary fibrosis and normal lung tissues to explore the change of gene expression patterns in fibroblasts during the development of SSc pulmonary fibrosis. The results showed that compared with normal pulmonary fibroblasts, a total of 646 DEGs were found in pulmonary fibroblasts from patients with SSc-related pulmonary fibrosis, including 223 upregulated genes and 423 downregulated genes (Fig. 2A-B, Suppl. Table S3). Then, we performed KEGG analysis and GO analysis on these upregulated and downregulated genes, respectively. The results showed that there were abnormalities in TGF-β signalling pathway, HIF-1 signalling pathway, TNF signalling pathway, NF-κB signalling pathway, gap junction in pulmonary fibroblasts from patients with SSc-related pulmonary fibrosis (Fig. 2C-D).

Identification and analysis of hypoxia-induced EndoMT hub genes in the development of SSc pulmonary fibrosis

Endothelial cells undergoing EndoMT lose their own properties and exhibit mesenchymal characteristics with enhanced invasive and migratory abilities, gradually transforming into myofibroblasts, and migrating to perivascular areas to participate in the process of tissue fibrosis (23, 24). In the present study, we found that hypoxia could induce EndoMT in SSc vascular endothelial cells. Meanwhile, dysfunction of HIF-1 signalling pathway exists in both SSc endothelial cells and lung fibroblasts. These results suggest that hypoxia may lead to pulmonary fibrosis in SSc by promoting EndoMT of vascular endothelial cells. In order to further explore the hub genes of hypoxia-induced EndoMT, we used Venn diagram to analyse the intersecting DEGs between endothelial cells and fibroblasts, and identified 15 overlapping genes, including DUSP6, INHBA, PPARG, BHLHE40, VCAM1, VEGFC, ADM, DUSP1, NOX4, STC2, RND3, PLOD2, DEPP1, CCL2, TXNIP (Fig. 3A). In addition, we further verified the expression levels of these genes in vascular endothelial cells and fibroblasts. The results showed that the expression levels of ADM, DEPP1, DUSP6, PPARG, STC2, and VEGFC were upregulated in SSc lung fibroblasts, but downregulated in SSc vascular endothelial cells (Fig. 3B-G). The expression levels of INHBA, DUSP1, NOX4, PLOD2, and BHLHE40 were all upregulated in SSc lung fibroblasts and vascular endothelial cells (Fig. 4A-E), while VCAM1, RND3, CCL2, and TXNIP were all downregulated (Fig. 4F-I). Therefore,

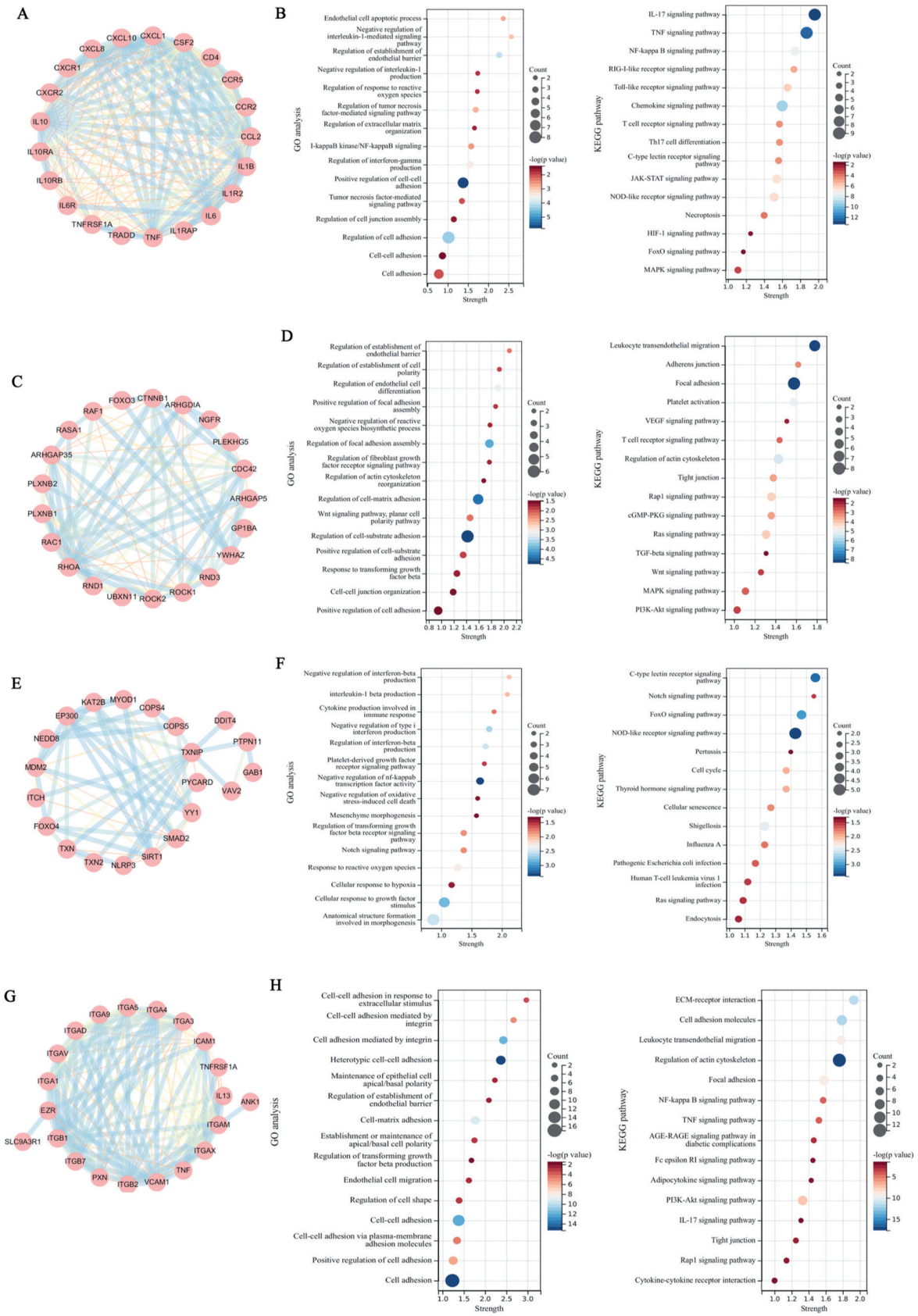

Fig. 6. Construction of protein-protein interaction (PPI) network of downregulated EndoMT hub genes. Using String database, we constructed the PPI network of CCL2 (**A, B**), RND3 (**C, D**), TXNIP (**E, F**), VCAM1 (**G, H**), and performed KEGG analysis and GO analysis. The top 15 relevant pathways and biological progresses of KEGG analysis and GO analysis were shown. In addition, the line thickness of PPI network between proteins represents the score of prediction.

we speculate that upregulated INHBA, DUSP1, NOX4, PLOD2, BHLHE40 and downregulated VCAM1, RND3, CCL2, TXNIP may be the hub genes of hypoxia-induced EndoMT.

Correlation analysis of the hub genes and EndoMT-related biomarkers

During EndoMT, endothelial cells lose their specific endothelial cell markers, such as CD31/PECAM-1, von Willebrand Factor (vWF) and VE-cadherin, and upregulate mesenchymal cell markers including fibronectin 1 (FN1), vimentin (VIM), COL1A1, COL1A2 (25-27). To further verify whether these hub genes are involved in the occurrence of EndoMT, we analysed the correlation between these hub genes and EndoMT-related biomarkers (VIM, FN1, COL1A1, COL1A2). The results showed that, except for INHBA, other hub genes were significantly correlated with EndoMT-related biomarkers. More importantly, DUSP1, NOX4, PLOD2, and BHLHE40 were significantly positively correlated with EndoMT-related biomarkers, while VCAM1, RND3, CCL2, and TXNIP were significantly negatively correlated with EndoMT-related biomarkers (Fig. 5). In addition, we also detected the correlation between CD31/PECAM1, von Willebrand Factor (VWF), VE-cadherin (CDH5), Tie-1 (TIE1) and the hub genes. The results showed that BHLHE40, CCL2, INHBA, RND3 were negatively correlated with PECAM1, VWF, CDH5 and TIE1, while DUSP1, NOX4 and PLOD2 were positively correlated with PECAM1, VWF, CDH5 and TIE1 (Suppl. Fig. S1). These results suggest that the hub genes may be closely related to the occurrence of EndoMT.

Construction of protein-protein interaction (PPI) network of EndoMT hub genes

Although we have analysed the expression levels of these hub genes in SSc lung fibroblasts and vascular endothelial cells, and speculated their correlation with EndoMT, the mechanism is still unclear. Using String database, we constructed the PPI network of CCL2, RND3, TXNIP, VCAM1, BHLHE40, DUSP1, INHBA, NOX4, PLOD2, and performed KEGG analysis and GO analysis. The results of enrichment analysis showed that CCL2 is related to endothelial cell apoptosis, establishment of endothelial barrier, reactive oxygen species response, cell-cell adhesion and other biological processes, and its molecular mechanism may be related to IL-17 signalling pathway, TNF signalling pathway, NF-κB signalling pathway, etc. (Fig. 6A-B, Suppl. Table S4). RND3 is related to the establishment of endothelial barrier, establishment of cell polarity, endothelial cell differentiation, formation of adhesion junctions, rearrangement of cytoskeleton and other biological processes, and its molecular mechanism may be related to VEGF signalling pathway, TGF-β signalling pathway, Wnt signalling pathways (Fig. 6C-D, Suppl. Table S5). TXNIP is related to the production of interleukin-1β, mesenchymal morphogenesis, reactive oxygen species response, hypoxia response and other biological processes, and its molecular mechanism may be related to TGF-β signalling pathway, Notch signalling pathway, FoxO signalling pathway and so on (Fig. 6E-F, Suppl. Table S6). VCAM1 is related to cell-cell adhesion, establishment of endothelial barrier, establishment and maintenance of cell polarity, endothelial cell migration, regulation of cell shape, regulation of cytoskeleton and other biological processes, and its molecular mechanism may be related to TGF-β signalling pathway, NF-κB signalling pathway, TNF signalling pathway and so on (Fig. 6G-H, Suppl. Table S7). BHLHE40 is associated with oxidative stress response, DNA repair and other biological processes (Fig. 7A-B, Suppl. Table S8). DUSP1 is related to biological processes such as lung morphogenesis, vascular development, microtubule polymerisation, reactive oxygen species response, and cell-cell adhesion, and its molecular mechanisms may be related to TGF-β signalling pathway, VEGF signalling pathway, TNF signalling pathway, HIF-1 signalling pathway, mTOR signalling pathway, etc (Fig. 7C-D, Suppl. Table S9). INHBA is involved in endothelial cell differentiation, extracellular matrix synthesis, epithelial-mesenchymal transition, mesenchymal morphogenesis, vascular remodelling, mesenchymal cell differentiation, hypoxia response, cell-cell adhesion, cytoskeleton rearrangement and other organisms, and its mechanism may be related to TGF-β signalling pathway, Notch signalling pathway, Wnt signalling pathway, Hippo signalling pathway, FoxO signalling pathway and so on (Fig. 7E-F, Suppl. Table S10). NOX4 is related to the generation of reactive oxygen species, cell-cell adhesion, vascular morphogenesis, migration of vascular endothelial cell, and regulation of cell morphology, and its molecular mechanism may be related to VEGF signalling pathway, NF-κB signalling pathway, HIF-1 signalling pathway, FoxO signalling pathway, JAK-STAT signalling pathway, etc (Fig. 7G-H, Suppl. Table S11). PLOD2 is related to biological processes such as collagen synthesis, extracellular matrix reorganisation, vascular development, and cell-cell adhesion, and its molecular mechanism may be related to TGF-β signalling pathway, PI3K-Akt signalling pathway, etc (Fig. 7I-J, Suppl. Table S12).

Effect of hub genes on EndoMT-related biomarkers in a cellular hypoxia model

HIF-1α is a key factor in regulating the cellular hypoxic response and plays an important role in maintaining the body's oxygen homeostasis. Therefore, we detected whether the hypoxia model was successfully constructed by detecting the protein expression level of HIF-1α. The results showed that the expression level of HIF-1α was significantly increased in the hypoxia model group induced by liquid paraffin closure (Fig. 8A), indicating that the hypoxia model was successfully constructed. Meanwhile, we also found that INHBA, DUSP1, NOX4, PLOD2, BHLHE40 were significantly upregulated while VCAM1, RND3, CCL2, TXNIP were significantly downregulated in the hypoxia model group (Fig. 8A). These results were consistent with our previous analysis. To verify the regulation of EndoMT by hub genes, we knocked down hub genes under hypoxic condition and then examined the expres-

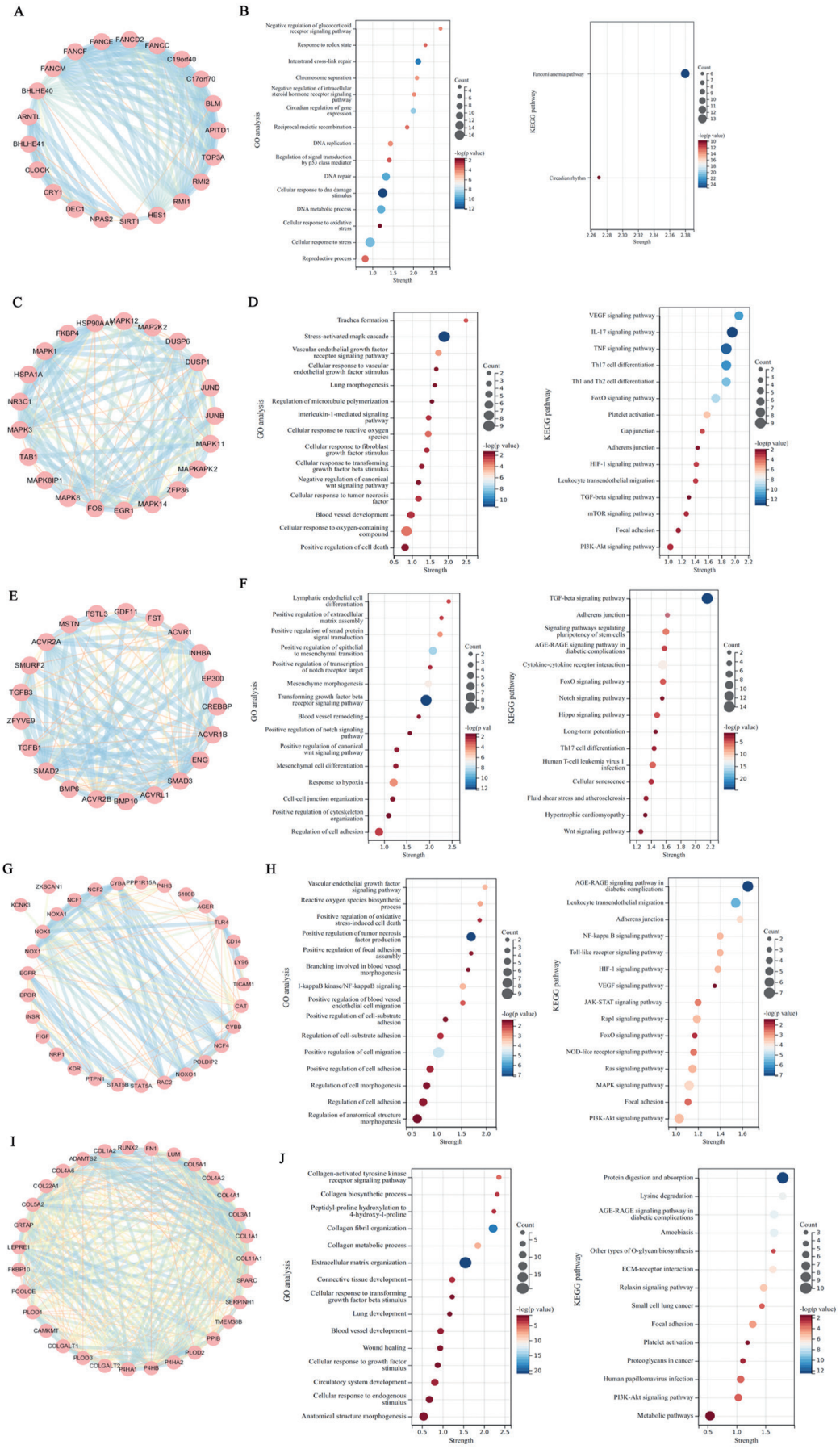

Fig. 7. Construction of protein-protein interaction (PPI) network of upregulated EndoMT hub genes. Using String database, we constructed the PPI network of BHLHE40 (**A, B**), DUSP1 (**C, D**), INHBA (**E, F**), NOX4 (**G, H**), PLOD2 (**I, J**), and performed KEGG analysis and GO analysis. The top 15 relevant pathways and biological progresses of KEGG analysis and GO analysis were shown. In addition, the line thickness of PPI network between proteins represents the score of prediction.

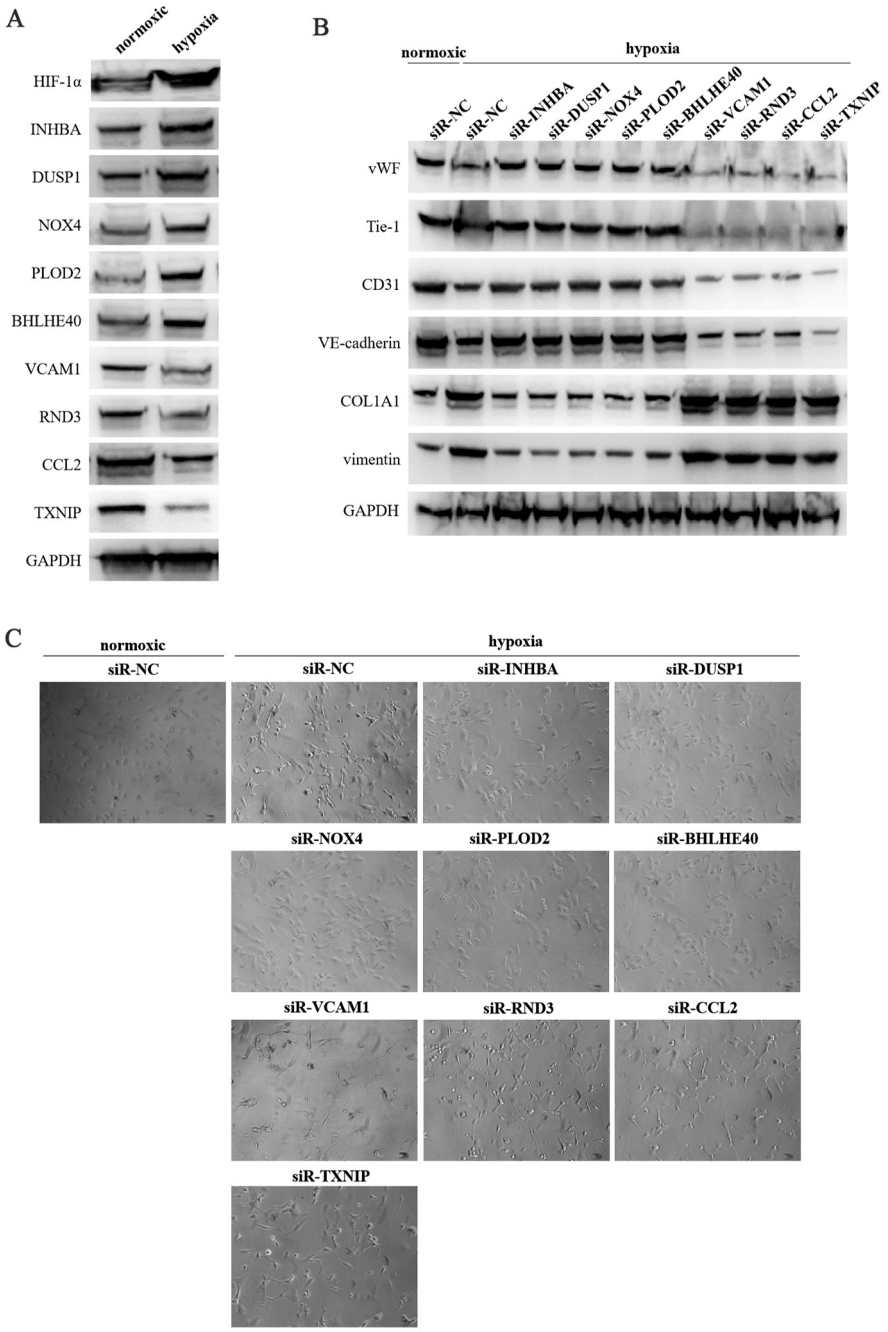

Fig. 8. Effect of hub genes on EndoMT-related biomarkers in a cellular hypoxia model. In the hypoxia model of HULEC-5a cells constructed by liquid paraffin closure. (**A**) The effect of hypoxia on HIF-1α, INHBA, DUSP1, NOX4, PLOD2, BHLHE40, VCAM1, RND3, CCL2, TXNIP was detected by Western blot. (**B**) The effect of hub genes knockdown on the expression of endothelium-associated markers VE-cadherin, CD31, vWF, Tie-1 and mesenchymal cell-associated markers COL1A1, vimentin was detected by Western blot. (**C**) The morphological changes of cells after silencing the expression of hub genes under hypoxic condition.

sion levels of EndoMT-related markers. The results showed that hypoxia was able to downregulate the expression levels of endothelial cell-related markers VE-cadherin, CD31, vWF and Tie-1 in HULEC-5a cells, while upregulate mesenchymal cell-related markers COL1A1 and vimentin (Fig. 8B). Knockdown of INHBA, DUSP1, NOX4, PLOD2, BHLHE40 significantly reversed the alterations of these EndoMT-related markers in hypoxic environment. However, knockdown of VCAM1, RND3, CCL2, TXNIP enhanced the effect of hypoxia on these EndoMT-related markers (Fig. 8B). Furthermore, we detected the morphological changes of cells after silencing the expression of hub genes. The results showed that hypoxia induced a spindle-like transition of endothelial cell morphology. In addition, knockdown of INHBA, DUSP1, NOX4, PLOD2, and BHLHE40 significantly reversed this effect, but knockdown of VCAM1, RND3, CCL2, and TXNIP did not (Fig. 8C). These results are consistent with our previous analysis and suggest that these hub genes play an important role in hypoxia-induced EndoMT. In conclusion, INHBA, DUSP1, NOX4, PLOD2, BHLHE40 can promote hypoxia-induced EndoMT, while VCAM1, RND3, CCL2, TXNIP have the opposite effect.

Discussion

The pathogenesis of SSc fibrosis is mainly due to abnormal metabolism of the extracellular matrix induced by fibroblasts and activated myofibroblasts, as well as the uncontrolled accumulation of collagen (28, 29). The persistence of activated myofibroblasts contributes to progressive fibrosis and favours the progression of interstitial and perivascular fibrosis in the lung, heart, kidney, and other organs, which is responsible for high mortality in patients with SSc (30, 31). Myofibroblasts are derived from pericytes and smooth muscle cells of the vessel wall, resident fibroblasts, and bone marrow-derived fibroblasts (32). An increasing number of studies have shown that EndoMT is a potential source of activated fibroblasts, through which endothelial cells transform into myofibroblasts (11). EndoMT is considered a unique form of EMT, because vascular endothelial cells share similar characteristics and molecular mechanisms with epithelial cells in generating fibroblasts and myofibroblasts, both of which contributes to the fibrosis of heart, lung, kidney, liver and intestinal in SSc (33).

Studies have confirmed that the mechanism of EndoMT is mainly related to hypoxia, TGF-β/smad signalling pathway, Wnt signalling pathway, Notch signalling pathway, TNF signalling pathway, PI3K/Akt/mTOR signalling pathway, interleukin-1, endothelin-1,

interferon, reactive oxygen species, etc (34-37). Among them, hypoxia is an effective promoter of EndoMT, and its mechanism is mainly related to the HIF-1 signalling pathway. Studies have shown that hypoxia can upregulate the expression of TGF-β, the main promoter of EndoMT (16). Additionally, key transcription factors that promote EndoMT, such as snail and twist-1, have also been identified as targets of hypoxia (17, 38). Recent studies have found that hypoxia can induce EndoMT by promoting autophagy (39). However, the mechanism of hypoxia in the occurrence of EndoMT is still insufficient, especially in the development of SSc. In this study, through bioinformatics analysis, we speculated that upregulated INHBA, DUSP1, NOX4, PLOD2, BHLHE40, and downregulated VCAM1, RND3, CCL2, TXNIP may play important roles in the occurrence of hypoxia-induced EndoMT. Among these genes, studies have confirmed that NOX4 and PLOD2 are upregulated in SSc, while VCAM1 and CCL2 are downregulated, and their mechanisms are mainly related to the occurrence of SSc-related skin and lung fibrosis (4, 40-43), which are consistent with our results.

No studies have investigated the association of these hub genes with EndoMT. Through correlation analysis, we found that these genes were closely related to EndoMT markers. However, some of the bioinformatics analyses were contradictory, such as the correlation between CCL2, DUSP1, NOX4, PLOD2, RND3 and endothelial cell-related markers was not as expected. Therefore, we further verified the correlation between hub genes and EndoMT in a hypoxia model of HULEC-5a cells. We demonstrated that hypoxia upregulated INHBA, DUSP1, NOX4, PLOD2, BHLHE40, and downregulated VCAM1, RND3, CCL2, TXNIP. In addition, knockdown of these hub genes was able to reverse or enhance hypoxia-induced EndoMT. Through enrichment analysis, we found that these genes were all associated with cell-cell adhesion which was closely related to the occurrence of EndoMT. During the occurrence of EndoMT, deficiency of the adhesion between vascular endothelial cells promotes the dissociation of endothelial cells from the vascular wall and destroys the endothelial barrier (43). Studies have shown that loss of VE-cadherin and β-catenin on the cell membrane disrupts cell-cell adhesion, leading to vascular lesions and EndoMT (44, 45). In SSc, iloprost can maintain the stability of cell adhesion and inhibit the occurrence of EndoMT (46). Therefore, we speculate that INHBA, DUSP1, NOX4, PLOD2, VCAM1, RND3, CCL2, TXNIP may affect the occurrence of EndoMT by regulating cell-cell adhesion. In addition, RND3, VCAM1, INHBA, and PLOD2 are related to the establishment of cell polarity, cytoskeleton rearrangement, extracellular matrix synthesis, mesenchymal morphogenesis, and collagen synthesis, which are all key events in the occurrence of EndoMT (8, 47). The production of reactive oxygen species and oxidative stress have also been shown to promote the occurrence of EndoMT (35). Therefore, CCL2, TXNIP, BHLHE40, and NOX4 may also regulate the occurrence of EndoMT through reactive oxygen species and oxidative stress.

The mechanisms of these hub genes in the occurrence of EndoMT may be similar, including TGF-β signalling pathway, Notch signalling pathway, Wnt signalling pathway, NF-κB signalling pathway, TNF signalling pathway, mTOR signalling pathway, which have been confirmed to be closely related to the occurrence of EndoMT. For example, TGF-β is currently recognised as a key inducer of EndoMT in cardiac, cancer, and SSc, as well as a major player in promoting fibrosis (48). In cardiovascular, interaction of Jagged1 and Notch induces EndoMT (49). The Wnt/β-catenin signalling pathway was shown to induce EndoMT in cardiovascular and glomerular endothelial cells, human dermal microvascular endothelial cells (50). The NF-κB signalling pathway mainly promotes the occurrence of EndoMT through its target gene snail (51). Vascular endothelial cells treated with TNF-α exhibited persistent activation of Smad2/3 signalling, suggesting that TNF-α induced EndoMT by activating the TGF-β signalling pathway (52). The occurrence of bleomycin-induced EndoMT is related to slug mediated by Akt/mTOR signalling pathway (53).

Conclusions

In this study, we speculated that upregulated INHBA, DUSP1, NOX4, PLOD2, BHLHE40, and downregulated VCAM1, RND3, CCL2, TXNIP may play key roles in the occurrence of hypoxia-induced EndoMT, and proposed possible molecular mechanism. Our study provides new insights into the pathogenesis of SSc-related pulmonary fibrosis and new strategies for its treatment. However, there are also some limitations which need to be addressed in the future study. For example, our study only preliminarily revealed the correlation between hub genes and EndoMT, and analysed the molecular mechanisms by bioinformatics. However, these mechanisms still need to be verified by experiments.

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