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Research Article

HMGB1 regulates pathological angiogenesis in moyamoya disease via affecting TNF-α and IL-1β release

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Abstract As known moyamoya disease (MMD) is a significant contributor to stroke. And in stroke, HMGB1 increases inflammation and injury in the acute phase, but enhances angiogenesis in the delayed phase. Little is known about the role of HMGB1 in endothelial activity or inflammation-driven angiogenesis during moyamoya disease. In this study single-cell RNA-sequencing (scRNA-Seq) was performed to identified the cell cluster difference between the donor and receptor blood vessels from one moyamoya disease patient. And qRT-PCR assay revealed that the mRNA expression of HMGB1, VWF, ALDH1A2, RANBP2, AOAH, and NBEA could be upregulated in the receptor blood vessels. We further revealed that HMGB1 modulated angiogenesis by regulating endothelial cell proliferation, migration, and tube formation. Overexpression of HMGB1 increased the TNF-α and IL-1β in HMEC-1 endothelial cells, while HMGB1 knockdown impaired angiogenesis in Matrigel plug assay. Taken together, these results indicated that HMGB1 could be a key regulator of angiogenesis, whose increasement could be an early pathological mechanism leading to Moyamoya disease.

Keywords: Moyamoya disease; HMGB1; scRNA-Seq; Angiogenesis; TNF-α;

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1 Introduction

As a rare cerebrovascular disorder, moyamoya disease (MMD) is marked by the gradual narrowing and/or blockage of the circle of Willis, accompanied by an abnormal proliferation of blood vessels at the base of the skull, known as moyamoya vessels. MMD is a significant contributor to stroke, presenting a relatively high likelihood of recurrent cerebrovascular incidents [1-3].

The pathological characteristics of MMD have been documented through comprehensive scientific autopsies of the intracranial large arteries, while the natural progression of moyamoya vessels has been thoroughly outlined using the Suzuki staging system [4]. Nevertheless, the underlying causes and mechanisms of MMD remain incompletely understood. Effective treatments to halt disease progression and improve outcomes are still not available. Consequently, understanding the molecular mechanisms of this disease may help in identifying better treatment options. The characteristic histopathologic features of occluded arteries in MMD suggest thickening of fibrocytes in the intima, which contain hyperplastic smooth muscle cells. A few genes including RNF213 (ring finger protein 213), DIAPH1 (diaphanous related formin 1), and ACTA2 (actin alpha 2) have been identified be associated with susceptibility to MMD [5-9].

Moyamoya disease and angiogenesis are closely related because the condition prompts the formation of new blood vessels as a compensatory response to the narrowing or

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occlusion of major cerebral arteries. In Moyamoya disease, the major arteries at the base of the brain, particularly the internal carotid arteries and their main branches, become progressively stenotic or occluded. This significantly reduces the blood flow to the brain. As a compensatory mechanism, the body attempts to form new blood vessels, known as angiogenesis, to bypass the occluded arteries and restore blood flow to the brain [10].

As a nuclear factor and a secreted protein, high-mobility group box 1(HMGB1) serves as an alarmin to drive the pathogenesis of inflammatory and autoimmune disease [11-12]. It was identified as a delayed mediator of inflammation released from macrophages [13]. In nucleus HMGB1 functions as an architectural chromatin-binding factor, where it binds to DNA and facilitates the assembly of proteins on specific DNA targets; while at cellular outside it also exhibits a high affinity for binding to the receptor for advanced glycation endproducts (RAGE) [14]. As a classic signaling molecule, HMGB1 is involved in a variety of biological roles in both pathological and pathophysiological processes, including inflammation, immune responses, cell migration, differentiation, proliferation, tissue repair, aging and cell death. HMGB1 also recruits cells across endothelial barriers and promotes the local production of tumour-necrosis factor (TNF), interleukin-6 (IL-6) and interferon-γ [15]. And TNF-α is a cytokine released from macrophage, mast cells and T-lymphocytes. It acts as a macrophage activating factor and activates these cells to secrete angiogenic factors [16].

In this study, we utilized single-cell sequencing to compare the differences in cell types and gene expression within each type between donor and recipient vessels in patients with moyamoya disease. Furthermore, we revealed that HMGB1 may be involved in the progression of moyamoya disease by regulating pathological angiogenesis.

2 Material and methods

2.1 Human participants

All patients were consented and enrolled in our institutional review board approved research studies. Consenting was performed in accordance with the ethical standards of the medical ethics committee of Punan hospital, Shanghai (NO.2023011) and in keeping with international standards. We received and archived written patient consent for all patient data included in this manuscript. All experiments were conducted by following the “Guiding opinions on treating experimental animals” issued by the Ministry of Science and Technology and approved by the Animal Ethics Committee of Punan hospital, Shanghai.

2.2 Cell Culture and Transfection

HMEC-1 (ATCC, CRL-3243) were maintained in MCDB-131 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor (EGF) (Sigma), 1 μg/ml hydrocortisone, 5 mg/ml l-glutamine, and antibiotics (penicillin/streptomycin) at 37 °C with 5% CO2 [17]. For transfection vector, obtained from GenePharma (Shanghai, China), carrying interference sequence against HMGB1 (5’-GGACAAGGCCCGTTATGAA-3’) or the control sequence (5’-TTCTCCGAACGTGTACGT-3′) were mixed with Lipofectamine 3000 reagent (Invitrogen) according to the instruction manual.

2.3 Lentivirus-based Overexpression

For virus-based overexpression of HMGB1, the open reading frame (ORF) of HMGB1 was subcloned into the

pLVX-IRES-ZsGreen1 vector. Virus was collected from the supernatant of 293T cells 48 hours post-transfection and subsequently used to infect target cells (3×10^5) at a concentration of 6×10^6 TCID50. The cells were not utilized for proliferation assays, angiogenesis experiments, or Western blot analyses until they had been cultured without the virus for 24 hours.High-titer virus was prepared by ultracentrifugation at 1.5×10^5 g at 4°C and then resuspended in PBS.

2.4 Single-cell RNA sequencing (scRNA-Seq)

Single-cell RNA sequencing analysis (contract ID: 80-1482487154) was supported by Azenta, Inc. in Suzhou, China. Cell suspensions were prepared by dissociating vessels, and RNA was isolated using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). For single-cell analysis, the sample was prepared using the Genewiz 10x Genomics Chromium single-cell RNA-seq protocol. Following confluency, HRTPT cells cultured in three separate T-25 flasks were trypsinized, centrifuged, and resuspended in growth media in separate tubes. To obtain a single-cell suspension, the cells were gently pipetted up and down for several minutes. Viable cells were counted using a haemocytometer by mixing with 4x trypan blue, diluted to at least $1x10^6$ cells/ml, centrifuged, and resuspended in 500 µl of ice-cold DMEM containing 20% serum and 10% DMSO. The cells were then transferred to cryotubes, placed between two styrofoam tube holders, and stored in a −80°C freezer for at least 4 hours. The samples were packed in dry ice and shipped to Genewiz for single-cell RNA sequencing.

2.5 RNA isolation and qRT-PCR

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen). The RNA quality was assessed through electrophoresis, the OD 260/OD 280 ratio, and the RNA Integrity Number (RIN) determined by an Agilent Bioanalyzer 4150 system (Agilent Technologies, CA, USA). For cDNA synthesis, 500 ng of RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio, Kusatsu, Japan). The resulting cDNA served as a template and was amplified in triplicate using qRT-PCR with the LightCycler® 480 real-time PCR system (Roche, CA, USA) and SYBR Premix Ex Taq (Takara Bio), following the manufacturer's instructions. All PCR primer pairs used are:

β-actin-F: 5’-GGCTGTATTCCCCTCCATCG-3’, β-actin-R-: 5’-CCAGTTGGTAACAAT GCCATGT-3’. HMGB1-F: 5’-CGCTGGCTGGAGAGTAATGTT-3’, HMGB1-R: 5’-AACGAGCCTTGTCAGCCTTT-3’; RANBP2-F:5’-AAAACATGGCCTTCAACCTG-3’, RANBP2-R:5’-TCAACAATTTCTGATGCCTGA-3’; ALDH1A2-F:5’-GACTTGCTGGCA GAATCCTT-3’, ALDH1A2-R:5’-GACGTCCCCTTTCTGAAGCA-3’; VWF-F:5’-CGGCT TGCACCATTCAGCTA-3’, VWF-R:5’-TGCAGAAGTGAGTATCACAGCCATC-3’; AOAH-F:5’-TGACAAGCCAGACACTGGAC-3’, AOAH-R:5’-GTTTACCCAAGCCTCT GCCT-3’ ; NBEA-F:5‘- GGCACACAAACTAAAGGTTCTT-3’, NBEA-R:5‘-TTGTGGCA GACCATAGCTGG-3’; The PCR cycling conditions were set as follows: an initial denaturation at 95°C for 35 seconds, followed by 40 cycles of denaturation at 95°C for 30 seconds, and annealing at 60°C for 35 seconds. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with β-actin serving as the internal control. Fluorescent signals were measured after each annealing step at 60°C.

2.6 Western blot analysis

The cells were lysed for 30 minutes at 4°C using a commercial cell lysis buffer for Western blotting and immunoprecipitation (cat: P0013, Beyotime Biotechnology, Shanghai, China), which contained 2 mM AEBSF, 0.3 μM aprotinin, 130 μM bestatin, 14 μM E64, and 10 μM leupeptin. The lysate was then centrifuged at 13,500 × g for 15 minutes at 4°C to obtain the supernatant. The protein concentration of the supernatant was determined using the Lowry protein assay. Following this, 20–80 μg of protein was separated by PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hours at 25°C and then incubated overnight with primary antibodies at 4°C. Horseradish peroxidase-conjugated anti-mouse (1:5000; ORIGENE, China) or anti-rabbit IgG (1:5000; ORIGENE) was used as the secondary antibody, with a 2-hour incubation at 25°C. Immunoreactive bands were detected using enhanced chemiluminescence (Pierce™ ECL Western; Thermo Scientific). The ECL signals were digitized for quantification using Image J software (v1.50i). Antibodies used in this study include: Anti-TNF-α antibody (ab183218, Abcam), Anti-IL-1 beta antibody (ab2105, Abcam), Anti-HMGB1 antibody (ab79823, Abcam), β-actin (A5441, Sigma).

2.7 Matrigel Plug Assay Matrigel plug assay

Lentivirus expressing 1×10^9 pfu of pll3.7 or HMGB1 shRNA was mixed in the Matrigel solution at 4 °C containing 500 ng/ml VEGF-A (Sigma) and 100 μg/ml heparin (Sigma) [18]. A total of 500 μl of Matrigel containing lentivirus was injected subcutaneously into the abdomen of male C57BL/6 mice. Six mice were employed for each group. The mice were killed 7 days after the injection. The Matrigel plugs with adjacent subcutaneous tissues were recovered by en bloc resection, and images were then taken using a stereomicroscope (Leica). Thereafter, each sample was embedded in paraffin before being sectioned at a thickness of 5 μm.

2.8 Statistical analysis

Values are presented as means ± standard deviations (SDs). Statistical significance was determined using two-tailed non-parametric tests followed by the Mann–Whitney U-test for comparisons between two groups, or one-way ANOVA followed by the Kruskal–Wallis test for comparisons among three or more groups. A P-value of less than 0.05 was considered to indicate statistically significant differences. All calculations were performed using GraphPad Prism software (v9.0) or R Project for Statistical Computing software (v4.1.2).

3 Results

3.1 The donor and receptor blood vessels had different cell clusters in moyamoya disease patient

We collected donor and recipient blood vessels from a patient with Moyamoya disease, and through single-cell sequencing, we found detectable changes in the cell populations of the donor and recipient blood vessels (Figure 1A. We found that Single-cell sequencing results indicate significant differences in cell types between donor and recipient vessels. As shown in Figure 1b, clusters 12-15, as well as clusters 0-2, show significant changes (Figure 1A). There was notable difference in total endothelial cells (cluster 1 and 2), smooth muscle cells (cluster 0), osteoblasts and tissue stem cells from the top 9

cell populations between receptor vessels and the donor ones.

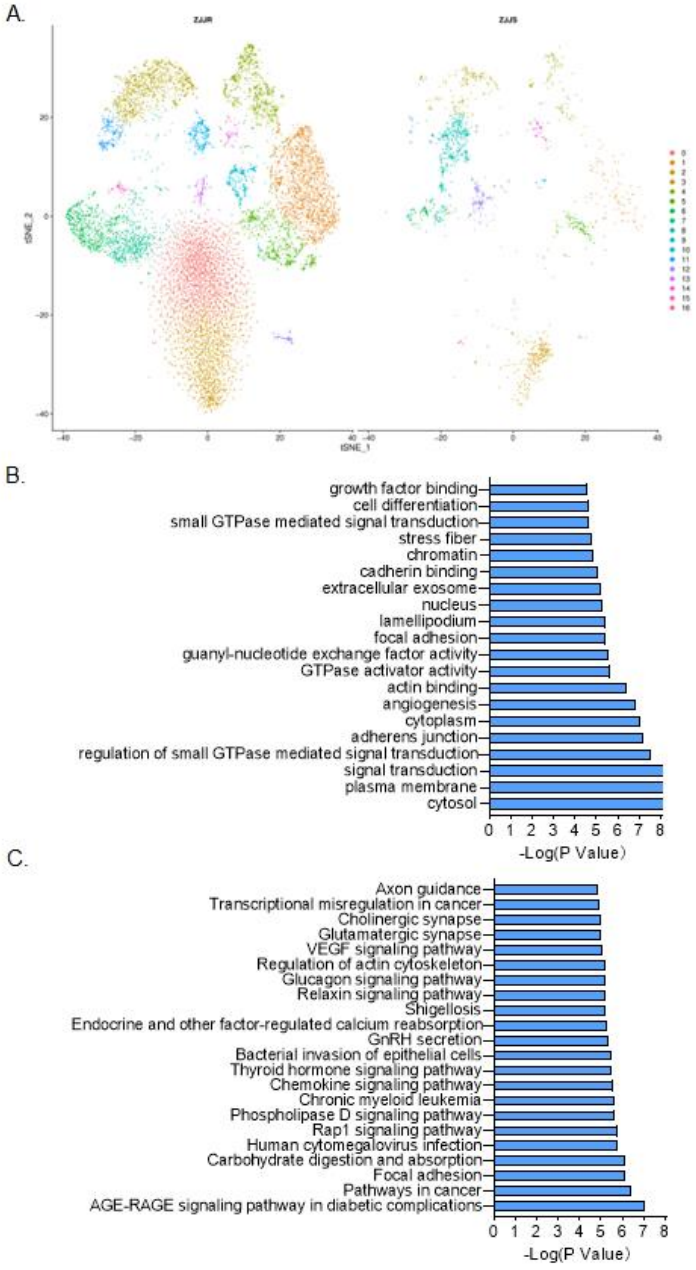

Figure 1 The donor and receptor blood vessels had different cell clusters in moyamoya disease patient

(A) The changes in cell populations of the donor and recipient blood vessels, with clusters 12-15 as well as clusters 0-2 had significant changes were showed. There was notable difference in endothelial cells(cluster 1 and 2), smooth muscle cells (Cluster 0), osteoblasts and tissue stem cells from the top 9 cell populations between receptor vessels and the donor ones.

(B) Differential Gene GO enrichment in cluster1 expressing Flt1 and TSHZ2. From the GO analysis, the functional differences in endothelial cells between recipient and donor vessels involve angiogenesis.

(C) KEGG enrichment of differentially expressed genes in the two vessels showed that receptor vessels had obvious change in the chemokine signaling pathway, axon guidance, pathways in cancer, shigellosis, rap1 signaling pathway, phospholipase D signaling pathway, regulation of actin cytoskeleton and focal adhesion.

Differential Gene GO enrichment in cluster1 expressing Flt1 and TSHZ2 (endothelial cells), intuitively reflecting the distribution of differential genes on GO terms enriched in biological process, cellular components, and molecular functions, showed that receptor vessels had obvious enrichment in plasma membrane (GO:0005886), regulation of small GTPase mediated signal transduction (GO:0051056), adherens junction (GO:0005912), angiogenesis (GO:0001525), GTPase activator activity (GO:0005096), focal adhesion (GO:0005925), and extracellular exosome (GO:0070062) (Figure 1B).

KEGG enrichment of differentially expressed genes in the two vessels showed that receptor vessels had obvious change in the chemokine signaling pathway, axon guidance, pathways in cancer, shigellosis, rap1 signaling pathway, phospholipase D signaling pathway, regulation of actin cytoskeleton and focal adhesion (Figure 1C).

From the GO analysis, the functional differences in endothelial cells between recipient and donor vessels involve angiogenesis. Therefore, we selected HMGB1, a gene that shows significant changes in endothelial cells, for further investigation to determine whether this gene is involved in regulating pathological angiogenesis in Moyamoya disease. Figure 1 shows The donor and receptor blood vessels had different cell clusters in moyamoya disease patient.

3.2 HMGB1 expression was upregulated in receptor blood vessels in moyamoya disease patient

The single-cell sequencing also showed that the HMGB1, VWF, ALDH1A2, RANBP2, AOAH, and NBEA were the mainly changed in the recipient blood vessels (Figure 2A). Using qRT-PCR we also identified that HMGB1, ALDH1A2, RANBP2, AOAH, and NBEA were significantly changed in the recipient blood vessels (Figure 2B). Western blot also supported that the HMGB1, ALDH1A2 and AOAH were significantly increased in the recipient blood vessels of the Moyamoya disease patient (Figure 2C).

These data indicated that HMGB1, ALDH1A2 and AOAH may participated in the process of pathological development of Moyamoya disease. Figure 2 shows HMGB1 expression was upregulated in receptor blood vessels in moyamoya disease patient.

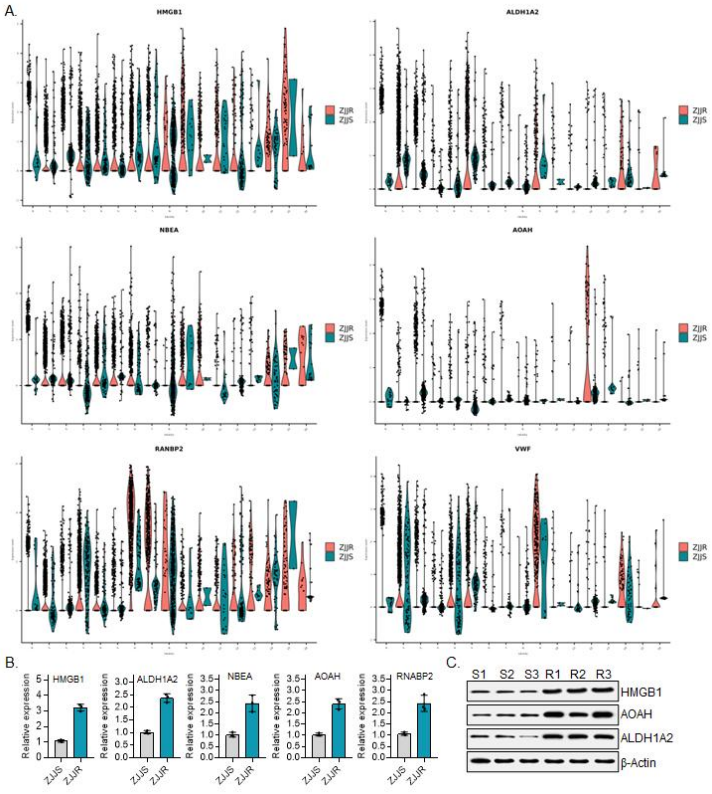

Figure 2 HMGB1 expression was upregulated in receptor blood vessels in moyamoya disease patient

(A) The single-cell sequencing showed that the indicated genes were changed in the recipient blood vessels.

(B) The indicated genes were significantly changed in the recipient blood vessels in qRT-PCR.

(C) Western blot showed that the HMGB1, ALDH1A2 and AOAH were significantly increased in the recipient blood vessels of 3 Moyamoya disease patients.

3.3 HMGB1 regulated endothelial cell proliferation, migration, and tube formation

Moyamoya disease is often characterized by abnormal vascular network development. And we hypnosis that the increased expression of HMGB1 in the recipient blood vessels may enhance the angiogenesis. Firstly, we investigate whether HMGB1 affect the cell viability of HMEC-1 endothelial cells. The result showed that knockdown of HMGB1 was actively involved in HMEC-1 proliferation (Figure 3A).

Next, we evaluated the migration properties of endothelial cells with down-regulation of HMGB1. Considering the role of HMGB1 in cell proliferation, we pretreated the cells with mitomycin C to inhibit cell proliferation before Boyden chamber cell migration assays. The number of migrated cells in HMGB1 knockdown cells decreased significantly compared with the control cells in Boyden chamber cell migration assay (Figure 3B, 3C).

We further evaluated the effects of HMGB1 on endothelial tube formation. Strikingly, HMGB1 knockdown in HMEC-1 cells inhibited the number of cell-cell contacts and the overall complexity of the tubular network (Figure 3D). Our data indicate that HMGB1 regulates endothelial cell viability and migration and tube formation. Figure 3 shows HMGB1 regulate angiogenesis in Matrigel plug assay.

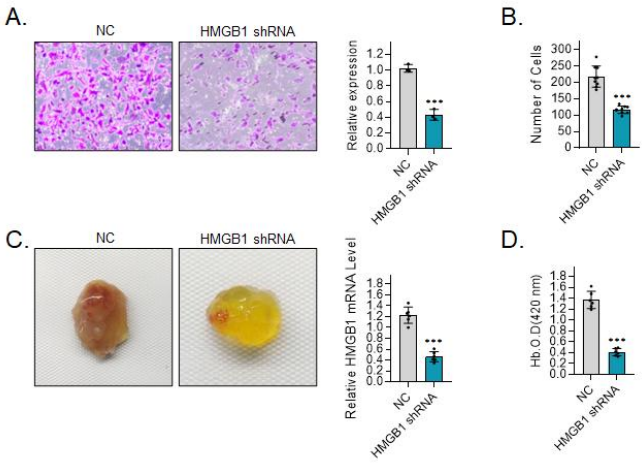

Figure 3 HMGB1 regulate angiogenesis in Matrigel plug assay

(A, B) Knockdown of HMGB1 impaired the migration of HMEC-1 cells.

(C) Matrigel mixed with VEGF, heparin, and shRNA lentivirus targeting mouse HMGB1 mRNA or control shRNA lentivirus was injected subcutaneously into the C57BL/6 strain mice; The RNA interference efficiency of lentiviral shRNA against HMGB1 showed in the right column was determined by quantitative PCR assays using RNA extracted from Matrigel plugs treated with the indicated lentivirus.

(D) The color of the plugs containing lentiviral shRNA against HMGB1 was lighter than those plugs containing control lentivirus, verified by the determination of homogenate in Matrigel plugs.

3.4 HMGB1 regulate angiogenesis in Matrigel plug assay

Matrigel consists mainly of laminin, collagen IV, nidogen-1/entactin, heparan sulfate proteoglycans and other extracellular matrix (ECM) proteins, as well as growth factors bound to the ECM. It mimics the physiological cell matrix and is the most commonly used to study in vivo angiogenesis [19].

To further confirm the roles of HMGB1 in mice models, Matrigel mixed with VEGF, heparin, and shRNA lentivirus targeting mouse HMGB1 mRNA or control shRNA lentivirus was injected subcutaneously into the C57BL/6 strain mice following the well-established protocols [20]. The RNA interference efficiency of lentiviral shRNA against HMGB1 was determined by quantitative PCR assays using RNA extracted from Matrigel plugs treated with the indicated lentivirus (Figure 4A). In Matrigel plug assay, the color of the plugs containing lentiviral shRNA against HMGB1 was lighter than those plugs containing control lentivirus (Figure 4B), suggesting that fewer red blood cells entered the plugs containing lentiviral shRNA against HMGB1. This in vivo experiment demonstrated that HMGB1 knockdown dramatically inhibited vascular formation. After being homogenized, the homogenate OD values of the plugs in HMGB1 shRNA virus group were significantly decreased (Figure 4C) which suggested that HMGB1 knockdown suppressed endothelial angiogenesis in vivo. Figure 4 shows HMGB1 regulated the TNF- α and IL-1 β release in HMEC-1.

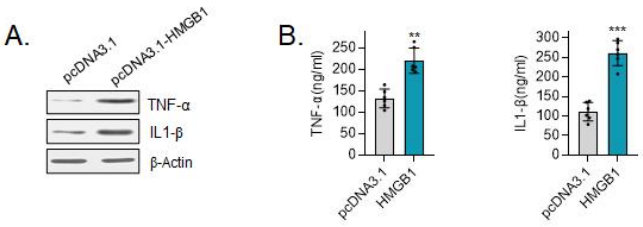

Figure 4 HMGB1 regulated the TNF-α and IL-1β release in HMEC-1

(A) Immune blot showed that overexpression of HMGB1 increased intracellular TNF-α and IL-1β protein expressions.

(B) ELISA showed that overexpression of HMGB1 increased TNF-α and IL-1β level in cell culture medium.

3.5 HMGB1 regulated the TNF-α and IL-1β release in HMEC-1

HMGB1, may as an inflammation driver, can be induced by stimulation TNF-α, and it may also regulate the release of multiple cytokines, including TNF-α, IL-1β, IL-6, and IL-8 through TLR4-MD2 [21-22]. Therefore, we

investigated whether HMGB1 regulates the release of TNF-α and IL-1β in endothelial cells.

The results indicate that the overexpression of HMGB1 leads to an increase in intracellular TNF-α and IL-1β protein expressions, accompanied by an increase in the amount of TNF-α and IL-1β released into the cell culture medium. These results suggest that HMGB1 could generally promote the expression TNF-α and IL-1β in vascular cells, thereby potentially enhancing the inflammatory response within endothelial cells.

4 Discussion

In this study, we used single-cell sequencing to compare the cell types and gene expression differences between donor and recipient vessels in Moyamoya disease. Subsequently, we identified a significant increase in HMGB1 expression in the endothelial cells of recipient vessels. Experimental results demonstrated that HMGB1 promotes angiogenesis and the release of pro-inflammatory factors TNF-α and IL-1 β , potentially contributing to the progression of Moyamoya disease.

Utilizing the single-cell sequencing, we found there were significant differences in cell numbers of smooth muscle cells (Cluster 0), endothelial cells (Cluster1 and Cluster2), cells expressing CYFIP2 and ADGRE5 (Cluster 12), cells enriched with TBX18 and RCAN2 (Cluster 14), cell population expressing COL1A2 and SLPI (Cluster 15). However, these differences may be due to changes in the composition of certain cells altering the functional characteristics of the vessels, or they may be due to inflammatory responses during the progression of Moyamoya disease changing the cell composition. Further work is needed to address this question. Differential Gene GO enrichment in cluster1 showed that receptor vessels had obvious enrichment in regulation of small GTPase mediated signal transduction (GO:0051056) and angiogenesis (GO:0001525), GTPase activator activity (GO:0005096) and focal adhesion (GO:0005925). These signals have commonly cross talk with angiogenesis pathways, suggesting that pathological angiogenesis is a major event in the progression of Moyamoya disease [23-27].

We identified that HMGB1 was significantly increased in smooth muscle cells (Cluster 0) and endothelial cells (Cluster1 and Cluster2) (Supplement material) in single-cell sequencing; And we utilized in vitro cultured cell models and a Matrigel plug model to demonstrate that HMGB1 promotes angiogenesis. Additionally, high expression of HMGB1 was found to increase the levels of TNF and IL-1β both intracellularly and extracellularly. This indicates that HMGB1 promotes vascular inflammation in Moyamoya disease by regulating angiogenesis and potentially exacerbating the expression of TNF-α and IL-1β released by cells [28-30]. These findings help to deepen our understanding of the role and mechanisms of pathological angiogenesis in Moyamoya disease. They provide data support for the development of drugs targeting HMGB1 to inhibit vascular inflammation in Moyamoya disease.

We also revealed that the expressions of ALDH1A2, RANBP2, AOAH, and NBEA were changed in the recipient blood vessels. Apart from the extensive research on HMGB1, the roles of these other genes in vascular endothelial cells are still relatively unexplored. We will continue to investigate whether these genes regulate Moyamoya disease by modulating pathological angiogenesis [31-33].

5 Conclusion

Overall, our small study compared the differences in cell populations between donor and recipient vessels from the same Moyamoya disease patient. We identified that the increased expression of HMGB1 within the vessels may contribute to the development of Moyamoya disease by regulating angiogenesis and increasing the intracellular expression of TNF-α and IL-1β.

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Data Availability

Not applicable

Ethical approval

Not required.

Declaration of Competing Interest

The authors declare no competing interests.

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