MicroRNA-221-3p promotes post-burn HUVEC proliferation, migration, and angiogenesis by regulating CDKN1B

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INTRODUCTION

Burn is one of the top ten causes of death in the world, killing more than 200,000 patients each year. In addition to a significant economic burden, burn injuries have a higher risk of developing mental illness (Haagsma et al., 2016). Severe burn injuries can affect various organs and systems of the body, and their severity is related to the surface area and depth of burn throughout the body, but even relatively minor burn injury can still be life-threatening and life-altering (Kanitakis et al., 2011). The core problem of burn injury is the loss of vascular integrity, the destruction of physiological barriers, and the edema caused by increased interstitial pressure (Edgar et al., 2011). One of the key points of burn injury treatment is to maintain the blood volume, supply blood to the organs, and accelerate the exchange of skin and important nutrients, such as oxygen (Gelfand, Donelan, and Burke 1983). Vascular damage caused by burn injury can easily cause tissue edema around the wound or even systemic (van Baar et al., 2006). Therefore, post-burn angiogenesis is an important factor for a good outcome of the disease.

MicroRNA (miRNA) is a class of conservative non-coding microRNA molecules consisting of about 22 nucleotides, which can regulate gene expression (Johnson et al., 2019). With the development of next-generation sequencing technology, the research on the function and mechanism of miRNA has become increasingly in-depth, and there are also more studies on miRNA targeting mRNA in the treatment of vascular-related diseases. For example, miR-342-3p/-5p has significant anti-inflammatory and pro-angiogenic effects when targeting pannexin-2 down-regulation (Ray et al., 2020); miR-210 can inhibit the apoptosis of arteriosclerotic occlusive vascular endothelial cells through JAK-STAT (Yue et al., 2019) and miR-125b can limit the formation of the vascular lumen through translation inhibition of VE-cadherin (Muramatsu et al., 2013). This study focused on a miRNA with an important role in vascular remodeling, named miR-221-3p. miR-221-3p has also been studied to mediate vascular remodeling in perivascular adipose tissue-derived extracellular vesicles (Li et al., 2019) and regulate the dysfunction of diabetic retinal microangiopathy (Wang et al., 2020). However, it is unclear whether miR-221-3p plays a role in vascular remodeling after burn injury.

Preliminary experiments found that miR-221-3p was abnormally low expressed in the serum of burn injury patients. It was speculated that miR-221-3p may have a similar role in the process of vascular remodeling after burn injury. Therefore, this work focused on exploring the effects of miR-221-3p on the proliferation, migration, tube formation, apoptosis, and cell cycle of HU-
VECs in the burn environment, and revealed its potential downstream molecular mechanisms.

MATERIALS AND METHODS

Serum from burn injury patients

Ten burn injury patients (burns area ≥50%) in Fuzhou Second Hospital Affiliated to Xiamen University were enrolled, including 6 males and 4 females, aged 18–55 years. Venous blood (10 mL) was collected within 24 h before regular anti-shock treatment and centrifuged at 4°C to obtain the supernatant which was then stored at −80°C. Normal serum from 10 healthy volunteers was recruited as a control group. This study was approved by the Ethics Committee of Fuzhou Second Hospital Affiliated to Xiamen University (ethical number: 2015061129), and written informed consent was obtained from all participants.

Heat treatment

HUVECs (BeNA, Beijing, China) were identified by STR typing. HUVECs were seeded in Petri dishes for 48 h, harvested into 15 ml centrifuge tubes, and immersed in a circulating water bath at 52°C for 3 min. The cells in the control group were placed in a 37°C water bath for 3 min. Heat-treated cells were re-seeded in Petri dishes and further incubated at 37°C. Cells were then harvested after 6 h of heat treatment.

Cell culture and transfection

Heat-treated HUVECs were grown to 80% confluence in an endothelial cell culture medium (ScienCell, USA) with 5% fetal bovine serum, 0.05% penicillin-streptomycin (Thermo Fisher Scientific, USA). Then, HUVECs were detached with 0.25% trypsin, added with a culture medium to terminate the detachment, and prepared for a cell suspension (1×10⁶ cells/mL). Flow cytometry confirmed the positive expression of CD31 and VWF in the purchased HUVECs (Supplementary Fig. 1 at https://ojs.pbioch.edu.pl/index.php/abp/).

HUVECs with a cell density of 1×10⁶ cell/mL were inoculated into 96-well plates (100 μL/well), and 10% fetal bovine serum (Thermo Fisher) was added and incubated for 24 h. miR-221-3p-mimic, miR-221-3p-inhibitor, mimic/inhibitor-negative control (mimic/inhibitor-NC), small interfering RNA (siRNA) targeting CDKN1B (si-CDKN1B), si-inhibitor-negative control (mimic/inhibitor-NC), and NC, PCDNA-CDKN1B, and pcDNA 3.1 were purchased from Shanghai GenePharma. The reagent was transfected into HUVECs instantaneously according to the manufacturer’s instructions for Lipofectamine 2000 (Thermo Fisher). After incubation for 48 h, the transfection efficiency was detected by RT-qPCR and western blot.

MTT method

After transfection, every 4×10⁴ HUVECs in each well on the 96-well plates were combined with 20 μL of MTT solution (Sigma, USA) at 0 h, 24 h, 48 h, and 96 h. Then, the samples were further cultured for 3 h, centrifuged at 4°C for 15 min, and dissolved by adding 150 μL of dimethyl sulfoxide solution (Sigma). Finally, optical density (OD)₄₉₀ nm was measured (Zhang et al., 2022).

Colony formation method

HUVECs were cultured at 700 cells/well on the 6-well plates with the culture medium changed every 3 days. The culture was terminated when macroscopic clonal clusters appeared. Then, colonies were fixed with 4% paraformaldehyde (Leagene, Beijing, China) at 1 mL/well for 40 min, stained with crystal violet solution (Leagene) at 1 mL/well for 20 min, and counted (Zhang et al., 2021).

Transwell experiment

A total of 2×10⁴ HUVECs suspended in a serum-free DMEM (Thermo Fisher Scientific) were added to the upper chamber of the transwell plate, and 800 μL of endothelial cell culture medium and 10% fetal bovine serum were added to the lower chamber. HUVECs after 48-h culture were dyed with crystal violet solution for 20 min and counted under a microscope (Wang et al., 2022).

Flow cytometry detection

Apoptosis was assessed according to Annexin V-FITC Apoptosis Detection Kit (ThermoFisher, USA). The log-phase growing HUVECs were digested with EDTA-free trypsin, washed once with pre-cooled PBS, centrifuged at low speed for 15 min at 4°C, and centrifuged once again. After discarding the supernatant, 5 μL of Annexin V-FITC and PI were added for 10 min, and finally, 500 μL of Annexin V binding buffer was supplemented to detect cell apoptosis by flow cytometry (He et al., 2021).

Cell cycle was assessed by the Propidium Iodide Flow Cytometry Kit (Abcam, USA). HUVECs were first digested with trypsin to obtain a single cell suspension, fixed with 66% ethanol for 2 h at 4°C, and centrifuged at 500×g for 5 min. The cell pellet was resuspended in prepared 1×PI+RNase Staining solution for 20 min at 37°C in the dark and loaded into a flow cytometer.

RT-qPCR detection

Total RNA was extracted with Trizol reagent (Thermo Fisher Scientific), reverse-transcribed into cDNA with the reverse transcription kit (Thermo Fisher Scientific). All primers were synthesized by Sangon (Shanghai, China). Taking U6 and GAPDH as the internal references, miR-221-3p and CDKN1B expression was calculated by the 2⁻ΔΔCT method (Miao et al., 2020).

Western blot

Protein lysates were harvested by adding RIPA (Merck, Germany) and the concentration was examined by a BCA kit (Enzyme-Linked Biotechnology, Shanghai, China). Proteins were separated by protein electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (Thermo Fisher Scientific) and transferred to PVDF membranes for reaction with CDKN1B (1:1000, ab92742), PCNA (1:1000, sc-56) and GAPDH (1:1000, ab8245), together with goat anti-rabbit secondary antibody (1:5000, 7074). ECL solution (GlpBio, USA)-developed bands were tested to analyze the gray value (Lu & Huang, 2021).

Luciferase reporter gene assay

Prediction from the website https://starbase.sysu.edu.cn shows that miR-221-3p has a binding site for CDKN1B. Wild-type and mutant CDKN1B sequences containing the miR-221-3p binding site were cloned into the PGL4 luciferase reporter vector (Promega). The above luciferase reporter vector and miR-221-3p mimic and mimic-NC were then co-transfected into HUVECs using Lipofectamine 2000 (Invitrogen). The cells were collected 48 h after trans-
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**In vitro HUVECs tube formation model**

Matrigel (Merck, Germany) was added to the center of the μ-slide well plate and left for 15 min. Cell suspension (50 μL, 3×10⁴/mL) was centrifuged, washed twice with serum-free DMEM, and centrifuged again. Cells were resuspended in 50 μL endothelial cell culture medium (containing 10% burns serum or 10% control serum) and added to μ-slide well plates. Images were taken in 5 fields of view 6 h later and analyzed by Image-pro Plus 6.0 software (Stefanini et al., 2009).

**Statistical analysis**

All data were analyzed using SPSS 21.0. Data were presented as mean ± standard deviation (S.D.). Two groups were compared by Student’s t-test while multiple groups were compared by One-way ANOVA. All functional experiments were run in triplicate. Results were plotted using GraphPad Prism 7.0 software. *P*<0.05 indicated statistical significance.

**RESULTS**

**Abnormally low expression of miR-221-3p in burn environment**

miR-221-3p Expression was decreased in the serum of burn injury patients (Fig. 1A). Subsequently, miR-221-3p expression was found to be reduced in heat-treated HUVECs (Fig. 1B).

**Overexpression of miR-221-3p restores the angiogenic capacity of burn-injured HUVECs**

miR-221-3p-mimic was transfected into burn-injured HUVECs. miR-221-3p-mimic promoted miR-221-3p expression in burn-injured HUVECs (Fig. 2A). MTT assay and colony formation assays showed that heat treatment inhibited the proliferation and clonogenic ability of HUVECs, while overexpression of miR-221-3p alleviated this phenomenon (Fig. 2B, C). Transwell assay indicated that heat treatment inhibited the migration ability of HUVECs, whereas the migration ability of HUVECs was increased after overexpression of miR-221-3p (Fig. 2D). Western blot reported that heat treatment decreased the expression of the proliferation proteins Ki-67 and PCNA in HUVECs, whereas overexpression of miR-221-3p prevented this change (Fig. 2E, F). Flow cytometry demonstrated that heat treatment increased the apoptotic rate of HUVECs and arrested cells in G0/G1 phase, while overexpression of miR-221-3p alleviated this phenomenon (Fig. 2G, H). Tube formation experiments manifested that heat treatment reduced the angiogenic capacity of HUVECs, but overexpression of miR-221-3p increased tube formation in HUVECs (Fig. 2I).

**Targeted regulation of CDKN1B by miR-221-3p**

Subsequently, the potential downstream target genes of miR-221-3p were explored. miRNAs can often bind to the 3' UTR of mRNA to regulate their expression (Yang et al., 2021). Through the bioinformatics prediction website https://starbase.sysu.edu.cn, it was found that miR-221-3p had a binding site with CDKN1B (Fig. 3A). The luciferase reporter gene assay

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**Figure 1. miR-221-3p and CDKN1B signatures in HUVECs**

(A) RT-qPCR to detect miR-221-3p in the serum of healthy subjects and burns patients; (B) RT-qPCR to detect miR-221-3p in the normal and heat-treated HUVECs. Data are presented as mean ± S.D. *P*<0.05.

**Figure 2. Overexpression of miR-221-3p restores the angiogenic capacity of burn-injured HUVECs**

miR-221-3p-mimic was transfected into burn-injured HUVECs to upregulate miR-221-3p. (A) RT-qPCR to detect miR-221-3p in HUVECs; (B) MTT assay to detect the proliferation of HUVECs; (C) Clone formation assay to evaluate the clonogenic ability of HUVECs; (D) Transwell assay to detect the migration of HUVECs; (E–F) Western blot to measure Ki-67 and PCNA; (G–H) Flow cytometry to determine apoptosis and cell cycle; (I) Tube formation assay to assess the tube-forming ability of HUVECs; data are expressed as mean ± S.D. *P*<0.05.
results verified the binding relationship between the two. The co-transfection of WT-CDKN1B and miR-221-3p-mimic reduced luciferase activity, but that of MUT-CDKN1B and miR-221-3p-mimic had no effect on luciferase activity (Fig. 3B). In burn-injured HUVECs, an abnormal increase in CDKN1B expression (Fig. 3C) was determined. Furthermore, overexpression of miR-221-3p suppressed CDKN1B expression (Fig. 3D).

### Overexpression of CDKN1B enhances the inhibitory effect of burn on HUVECs angiogenesis

Subsequently, pcDNA-CDKN1B was transfected into burn-injured HUVECs to explore the role of CDKN1B. pcDNA-CDKN1B increased CDKN1B expression in HUVECs (Fig. 4A). Functional experiments verified that overexpression of CDKN1B further inhibited the proliferation and colony ability of burn-injured HUVECs, decreased the number of migrating cells, suppressed Ki-67 and PCNA protein expression, promoted cell apoptosis, blocked cells at G0/G1 phase, and reduced the number of tube formations (Fig. 4B–I).

**miR-221-3p Targets CDKN1B expression to improve angiogenesis in burn-injured HUVECs**

A functional rescue experiment was implemented to probe the regulatory role of the miR-221-3p/CDKN1B axis in burn-injured HUVECs. The transfection designs were as follows: Inhibitor-NC+si-NC, miR-221-3p-inhibitor+si-NC and miR-221-3p-inhibitor + si-CDKN1B. The results presented that miR-221-3p inhibitor promoted CDKN1B expression, while si-CDKN1B reversed this effect (Fig. 5A). Functional experiments manifested that after transfection of miR-221-3p-inhibitor, cell proliferation and cloning abilities were attenuated (Fig. 5B, C), the number of migrating cells was reduced (Fig. 5D), and Ki67 and PCNA protein expressions were reduced (Fig. 5E, F), the apoptotic rate was promoted, cells in G0/G1 phase were increased (Fig. 5G, H), and tube-forming ability was impaired (Fig. 5I), and these effects were reversed by knockdown of CDKN1B.

**DISCUSSION**

Burn injury damages the body caused by thermal exposure, radiation, and chemical or electrical contact, which
induce activation of peripheral nerve fibers, initiating a sustained hypersensitivity response to thermal and mechanical stimuli (Laycock et al., 2013). The consequences of burns are severe, with a high mortality rate and common in low- and middle-income countries (Atwell et al., 2020). With the development of science and technology, there have been many studies on gene technology treatment for burn injury (Zhang et al., 2021). Bioinformatics analysis has identified 43 miRNAs as potential regulators of early burn injury response among which miR-212-3p is down-regulated in dermal interstitial fluid (Foessl et al., 2021). In addition to post-burns pain management, psychological counseling, scar healing, etc., post-burns angiogenesis is the basis of many burn treatment principles (Eyuboglu et al., 2018). The physiological basis of tissue edema and body fluid extravasation after burn injury is the change of vascular permeability which is one of the main manifestations of vascular endothelial cell damage (Tian et al., 2015).

Belonging to miRNAs family which affects physiological processes (Amponsah et al., 2017), miR-221 is located in the p11.3 region of the X chromosome and is involved in the physiological regulation of hematopoiesis and angiogenesis (Liu et al., 2009). In vascular endothelial cells, miR-221 acts on CDKN1B and PI3KR1 and inhibits endothelial cell biological functions (Celic et al., 2017). In human aortic endothelial cells, miR-221-3p carries the ability to block the production of peroxisome proliferator-activated receptor λ coactivator 1α, leading to mitochondrial dysfunction and apoptosis (Xue et al., 2015). The present study found that miR-221-3p has a positive role in vascular remodeling after burn injury. Overexpression of miR-221-3p promoted the proliferation of HUVECs by reducing the ratio of HUVECs in the G0/G1 phase, thereby mediating HUVECs migration and tube formation, which will benefit the angiogenic capacity of HUVECs in the burn environment. Cell cycle changes are important for the proliferation of HUVECs. When cells are arrested in the G0/G1 phase, the proliferation of HUVECs is inhibited and their tube-forming ability is reduced (Cota Teixeira et al., 2019; Zhang et al., 2013). Several studies have demonstrated the role of miRNAs in regulating the cell cycle and proliferation of HUVECs, such as miRNAs including miR-182-5p (Su et al., 2021), miR-20b (Dong et al., 2020). This study speculated that the regulation of miR-221-3p on the cell cycle of HUVECs will affect the ability of vascular remodeling, and the effect of miR-221-3p on the cell cycle of HUVECs needs to be further explored in subsequent studies.

CDKN1B is located on chromosome 12p 13 and has a promoting regulatory role in tumors (Kim et al., 2015), such as osteosarcoma (Gao et al., 2022). As a downstream target gene of miR-221, CDKN1B can promote the proliferation of vascular smooth muscle cells in the neovascular intimia (Medina et al., 2008). MiR-221-3p/CDKN1B axis can regulate the proliferation and cell cycle of HUVECs after burn injury, and further influence cellular migration and tube formation ability. It is worth noting that this study only validated the role of the miR-221-3p/CDKN1B axis in angiogenesis in an in vitro model and could be explored in future animal burn injury models. In addition, there are few reports on the regulatory mechanism of ncRNAs in burn injury, and the regulatory mechanism of ncRNAs is crucial in vascular remodeling, skin recovery and other processes. Therefore, it is necessary to further explore the changes and mechanisms of ncRNAs in burn injury.

In conclusion, this work confirmed that miR-221-3p affects the proliferation, migration, cell cycle, apoptosis, and tube formation of burn-injured HUVECs by regulating CDKN1B. MiR-221-3p/CDKN1B axis may be of interest in future burn treatments.

**REFERENCES**


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