

Original Research Article

Suppression of KLF7 gene expression inhibits proliferation and induces apoptosis of hemangioma cells via NF- κ B signaling pathway

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Abstract

Purpose: To assess the role of Kruppel-like factor 7 (KLF7) in hemangioma (HA) progression, KLF7 expression and regulation of downstream targets in HA tissues and hemangioma-derived endothelial cells (HemECs) have been evaluated.

Methods: Quantitative polymerase chain reaction (qPCR) and immunoblotting were performed to assess KLF7 expression in HA tissues and normal tissues. Endothelial cells were isolated from HA tissues, and Cell Counting Kit-8 assay and flow cytometry were carried out to analyze proliferation and apoptosis of the isolated HemECs. Immunoblotting was used to determine the effect of KLF7 on expression of members of nuclear factor kappa B (NF- κ B) pathway in HemECs.

Results: High KLF7 expression occurred in infantile HAs during the proliferative stage. Knockdown of KLF7 expression in HemECs inhibited proliferation and induced apoptosis via suppression of NF- κ B pathway ($p < 0.001$).

Conclusion: KLF7 is a promising therapeutic target for the treatment of HA.

Keywords: Hemangioma (HA), Kruppel-like factor 7 (KLF7), Endothelial cell, Apoptosis, NF- κ B pathway

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INTRODUCTION

Hemangiomas (HAs) are congenital benign tumors or vascular malformations caused by proliferation of angiogenic cells [1]. HAs usually occur at or shortly after birth and are characterized by a rapid proliferation phase followed by a degenerative phase [2]. Although the majority of HAs shrink spontaneously, ~10 –

15 % of lesions in high-risk areas, such as the eyes and throat, can lead to serious complications and lifelong sequelae [3]. The main cell type in HAs are endothelial cells, and the accumulation of immature endothelial cells is the central feature of proliferative HA [4].

Kruppel-like factors (KLFs) are a class of zinc finger transcription factors [5]. The KLF family

consists of 17 members and regulates a wide range of biological events, including cell proliferation and embryonic development. Kruppel-like factor 7 (KLF7) is located in the nucleus and activates transcription. Elevated KLF7 expression in lung tissue is an adverse, independent prognostic factor for lung adenocarcinoma [6]. Inhibition of KLF7 expression inhibits cancer cell proliferation and invasion [7]. It has also been shown that KLF7 is overexpressed in tongue squamous cell carcinoma (TSCC) and that patients with high KLF7 expression have poor overall survival [8]. Depletion of KLF7 reduces migration and adhesion of TSCC. However, there are limited studies on the mechanism of KLF7 in HA. This study explores the effects and mechanism of KLF7 on proliferation and apoptosis of endothelial cells in HA.

KLF7 is expressed in the digestive, respiratory, and immune systems; plays an important role in transcriptional activation; and affects development of cardiovascular disease, cancer, and inflammation [9]. Therefore, we aimed to determine the effects of KLF7 on infantile HA endothelial cells during the proliferative stage. We found that KLF7 is highly expressed in proliferative infant HA tissues and that downregulation of KLF7 inhibits proliferation and induces apoptosis of HA endothelial cells by inhibiting the nuclear factor kappa B (NF- κ B) signaling pathway *in vitro*.

EXPERIMENTAL

Samples

A total of 50 human tumor tissues and corresponding normal skin tissues were obtained from Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University. The experiments were in accordance with the requirements of the Declaration of Helsinki [10]. All patients were treated with surgery only, and no chemoradiotherapy was used. All patients provided informed consent, and all studies were approved by the Ethics Committee of Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University (approval no. 2021-PFK-003).

Antibodies, primers, and siRNAs

Antibodies to β -Actin (1:10000, A1978 Sigma), KLF7 (1:1000, ab197690 Abcam), PCNA (1:1000, ab29 Abcam), Bax (1:1000, ab32503 Abcam), Bcl-2 (1:1000, ab182858 Abcam), p65 (1:500, ab32536 Abcam), p-p65 (Ser536, 1:1000, ab76302 Abcam), I κ B α (1:1000, #9242 CST), p-

I κ B α (Ser32, 1:500, #2859 CST), and CD31 (1:100, ab28364 Abcam) were used. The qPCR primer sequences are listed in Table 1. Control and KLF7 siRNAs were purchased from Riobio.

Table 1: PCR primer sequences

Gene	Forward primer	Reverse primer
KLF7	TTTCCTGGCAGT CATCTGCAC	GGTCTGTTTGTGTTG TCAGTCTGTC
GAP	AACGGATTGGTC	TCGCTCCTGGAAGAT
DH	GTATTGGG	GGTGAT

Cell isolation, identification, and culture

The proliferative HA specimens were digested with collagenase, and cells were sorted and purified with CD31 immunomagnetic beads. The cells were inoculated in a culture dish with a fibrin layer. The CD31-positive cells began to adhere after 4 h and were fusiform. After 7–10 days of growth and fusion, the HA-derived endothelial cells (HemECs) were harvested and used in subsequent experiments. CD31 expression was measured by immunofluorescence, and the number of CD31-positive cells was counted by flow cytometry (FCM). HemECs were cultured in MEM supplemented with 10 % fetal bovine serum (FBS) at 37°C in a 5 % CO₂ incubator.

Cell transfection

siRNAs were transfected into HemECs with lipofectamine 2000 (11668019, Invitrogen, USA). The siRNA targeting KLF7 (si-KLF7) or the negative control siRNA (si-NC) was transfected into HemECs as follows: Opti-MEM was added to 10 μ L of siRNA and the mixture was incubated for 5 min, then lipofectamine was added and the mixture was incubated for 5 min. The mixture was then added to serum-free HemECs and incubated for 20 min. After 24 h, subsequent assays were performed.

Immunoblot assay

Protein concentration was quantified by the BCA method; 15 μ g of total protein from HemECs were separated on 10 % SDS-PAGE gels, and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes for 2 h. The membranes were blocked in 5 % bovine serum albumin in TBST buffer at room temperature for 2 h before incubation with primary antibodies at room temperature for 2 h. Subsequently, membranes were incubated with horseradish peroxidase-conjugated antibodies for 1 h.

Signals were visualized using an enhanced chemiluminescence kit.

Quantitative PCR (qPCR)

Trizol (15596026, Invitrogen, USA) was used to extract total RNA from tissues or cells, and then the RNA was reverse-transcribed using M-MLV reverse transcriptase (M1701, Promega, USA). Total mRNA was reverse-transcribed to generate cDNA. qPCR was performed with the SYBR Ex Taq kit (638319, Takara, Japan), and relative KLF7 mRNA expression was normalized to GAPDH mRNA expression.

Cell Counting Kit-8 (CCK-8) assay

HemECs were inoculated into 96-well plates at a density of 1000 cells per well. After 24 h, cells were treated with CCK-8 reagent for 3 h, and absorbance at 450 nm was measured.

FCM

HemECs were incubated with annexin V-FITC and propidium iodide at room temperature for 10 min and then analyzed using a FACS Calibur flow cytometer. The number of apoptotic cells were quantified.

Statistical analysis

GraphPad 6.0 software was used to perform statistical analysis. Data are presented as mean \pm standard error of the mean (SEM). The student's t-test was used for statistical comparisons with $p < 0.05$ considered significant. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively.

RESULTS

High KLF7 expression in infantile HA tissues during the proliferative stage

To determine the effects of KLF7 on progression of infantile HA, we examined KLF7 expression levels in 50 infantile HA tissues and 50 normal skin tissues. qPCR showed high KLF7 expression in HA tissues when compared with normal skin tissues (Figure 1 A). In addition, three representative HA tissues and corresponding normal tissues were used to evaluate KLF7 protein expression by immunoblot. KLF7 protein expression in HA tissues was significantly higher than in normal skin tissues, which is consistent with the qPCR results (Figure 1 B). Therefore, KLF7 is highly

expressed in infantile HA tissues during the proliferative stage.

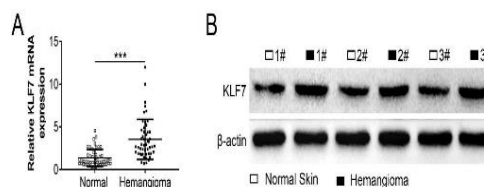


Figure 1: KLF7 expression is high in infantile HA tissues during the proliferative stage. (A) qPCR was performed to measure relative KLF7 mRNA levels in 50 normal skin tissues and in 50 HA tissues. (B) Immunoblotting was conducted to evaluate KLF7 protein levels in three representative normal skin tissues and three HA tissues. Results are presented as mean \pm SEM; *** $p < 0.001$

Isolation and identification of endothelial cells from infantile HAs during the proliferative stage

Endothelial cells were isolated from infantile HA tissues. CD31 is a known marker of endothelial cells; therefore, we used immunofluorescent staining of CD31 to analyze the isolated endothelial cells. The endothelial cells displayed yellow-green fluorescence, but there was no fluorescence in the negative control group (NC, PBS treatment), indicating isolation of highly pure endothelial cells (Figure 2 A). FCM confirmed that 90 % of the cells were CD31-positive (Figure 2 B). Therefore, endothelial cells were successfully isolated from infantile HAs during the proliferative stage.

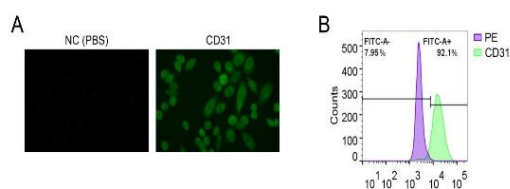


Figure 2: Isolation and identification of endothelial cells from infantile HA during the proliferative stage. (A) Immunofluorescence was performed to detect CD31 in HemECs. (B) FCM was performed to measure the percentage of CD31-positive cells and to confirm purity

Depletion of KLF7 inhibits proliferation and induces apoptosis of HemECs

To knockdown KLF7 expression, si-KLF7 was transfected into HemECs. Immunoblots showed that KLF7 protein expression decreased in HemECs transfected with si-KLF7 when compared to the control and si-NC groups (Figure 3 A). The CCK-8 assay showed that

depletion of KLF7 suppressed proliferation of HemECs when compared to the control and si-NC groups (Figure 3 B). FCM was performed to determine the effect of KLF7 on apoptosis of HemECs. KLF7 ablation stimulated apoptosis of HemECs when compared to the control and si-NC groups (Figure 3 C). Expression of proliferating cell nuclear antigen (PCNA) and Bcl-2 decreased and expression of Bax increased in HemECs upon KLF7 depletion, indicating inhibition of proliferation and stimulation of apoptosis (Figure 3 D). Therefore, depletion of KLF7 inhibits proliferation and induces apoptosis of HemECs.

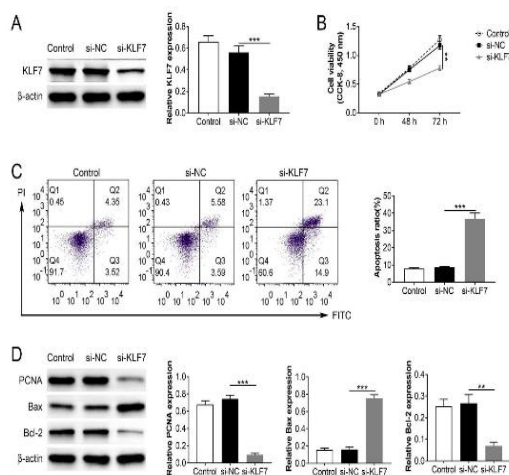


Figure 3: Depletion of KLF7 inhibits proliferation and induces apoptosis of HemECs. (A) KLF7 protein levels in the control, si-NC, and si-KLF7 groups were evaluated by immunoblot. (B) The CCK-8 assay was performed to evaluate proliferation in the control, si-NC, and si-KLF7 groups. (C) FCM was performed to evaluate apoptosis in the control, si-NC, and si-KLF7 groups. (D) Immunoblotting was performed to evaluate PCNA, Bax, and Bcl-2 expression in the control, si-NC, and si-KLF7 groups. Results are presented as mean \pm SEM; ** $p < 0.01$, *** $p < 0.001$

Downregulation of KLF7 inhibits the NF- κ B signaling pathway in HemECs

A previous study showed that the NF- κ B signaling pathway plays a role in HA progression. Therefore, we hypothesized that KLF7 promotes HA progression in HemECs through the NF- κ B signaling pathway. Immunoblots showed that depletion of KLF7 reduced phosphorylation of p65 and I κ B α , two downstream proteins in the NF- κ B pathway (Figure 4). Therefore, downregulation of KLF7 inhibits the NF- κ B signaling pathway in HemECs. Depletion of KLF7 also inhibited proliferation and induced apoptosis of HemECs, perhaps through the NF- κ B pathway.

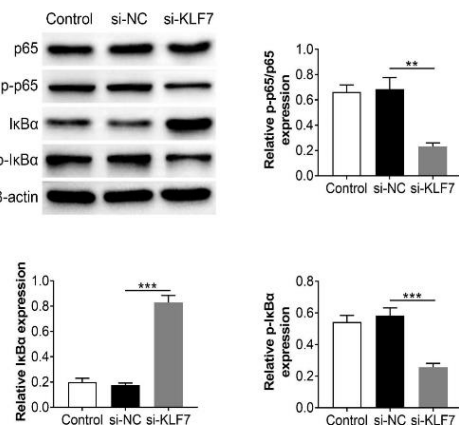


Figure 4: Downregulation of KLF7 inhibits the NF- κ B signaling pathway in HemECs. Levels of p65, p-p65, I κ B α , and p-I κ B α in the control, si-NC, and si-KLF7 groups were evaluated by immunoblotting. Results are presented as mean \pm SEM, ** $p < 0.01$, *** $p < 0.001$

DISCUSSION

HAs are tumors in the skin and soft tissue that usually occur shortly after birth [11]. Targeted therapy has promise as a method to combat HAs, but new biomarkers and therapeutic targets still need to be identified [12]. This study showed that KLF7 is highly expressed in infantile HA and affects proliferation and apoptosis of HemECs. Thus, KLF7 can function as a biomarker for and a therapeutic target of HAs.

The roles of KLF7 in cancer progression and development have been studied extensively [13]. KLF7 regulates cell motility and adhesion in tongue cancer, lung adenocarcinoma, and squamous carcinoma [14]. KLF7 has also been suggested as a biomarker and therapeutic target for serous ovarian cancer [15]. KLF7 may promote growth and metastasis of pancreatic cancer by activating interferon-stimulated gene expression and by stimulating polyamine biosynthesis and glioma development via activation of argininosuccinate lyase expression.

In this study, we found that KLF7 is highly expressed in infantile HAs by qPCR and immunoblot of infantile HA tissues and normal skin tissues. Knockdown of KLF7 with an siRNA inhibits proliferation and induces apoptosis of HemECs, as shown by the CCK-8 assay and FCM. KLF7 has been shown to exist in the nucleus and transcriptionally activate a wide range of biological events, including cell proliferation, differentiation, and embryonic development [16]. Therefore, we hypothesized

that KLF7 activates HA progression by activating expression of several downstream proteins.

NF- κ B is a protein complex that regulates transcription in eukaryotic cells, is highly expressed in tumors, and promotes angiogenesis [17]. The NF- κ B family of transcription factors regulates several genes involved in cell proliferation and migration and plays an important role in HA function [18]. Constitutive activation of NF- κ B is essential for endothelial cell survival, and it has been shown that expression of NF- κ B signaling pathway-related genes and proteins is increased in endothelial cells of infant proliferative HA specimens and in BALB/c nude mouse HA models [17]. In addition, propranolol promotes thrombocytopenia-1 mediated anti-angiogenesis and blocks NF- κ B mediated angiogenesis [19].

It has also been shown that expression of inflammatory factors and KLF7 is significantly increased in adipose tissue of patients with coronary heart disease [20]. Knockdown of KLF7 inhibited the release of inflammatory factors and significantly inhibited LPS-induced c-Jun N-terminal kinase/mitogen-activated protein kinase phosphorylation and activation of p-p65 and p-I κ B α . Therefore, KLF7 may be a potential therapeutic target for cardiovascular disease. Similarly, knockdown of KLF7 inhibited endothelial cell proliferation and induced endothelial cell apoptosis via suppression of the NF- κ B pathway, indicating that the NF- κ B pathway may be a promising target for HA treatment.

CONCLUSION

KLF7 is highly expressed in infantile HA. Endothelial cells have been successfully isolated from neonatal infantile HA. Depletion of KLF7 suppresses endothelial cell proliferation and induces endothelial cell apoptosis via suppression of NF- κ B pathway. Therefore, KLF7 is a promising target for the treatment of HAs.

DECLARATIONS

Conflict of interest

There is no conflict of interest to disclose regarding this work.

Availability of data and materials

All data generated and analyzed in this study are included in the article.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yang Wu and Fang Jin designed the study and supervised the data collection. He Huang analyzed and interpreted the data. Shi Wang prepared and reviewed the manuscript. All authors read and approved the manuscript.

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