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Kruppel-like factor 2 inhibit the angiogenesis of cultured human liver sinusoidal endothelial cells through the ERK1/2 signaling pathway

Xiao-Qing Zeng [a, 1], Na Li [a, 1], Du-Yi Pan [a], Qing Miao [a], Gui-Fen Ma [a], Yi-Mei Liu [a], Yu-Jen Tseng [a], Feng Li [a], Li-Li Xu [a], Shi-Yao Chen [a, b, *]

[a] Department of Gastroenterology of Zhongshan Hospital, Fudan University, Shanghai, China
[b] Institute of Endoscopic Research of Zhongshan Hospital, Fudan University, Shanghai, China

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ABSTRACT

Kruppel-like factor 2 (KLF2) is a crucial anti-angiogenic factor. However, its precise role in hepatic angiogenesis induced by liver sinusoidal endothelial cells (LSECs) remain unclear. This study was aimed to evaluate the effect of KLF2 on angiogenesis of LSECs and to explore the corresponding mechanism. Cultured human LSECs were infected with different lentiviruses to overexpress or suppress KLF2 expression. The CCK-8 assay, transwell migration assay and tube formation test, were used to investigate the roles of KLF2 in the proliferation, migration and vessel tube formation of LSECs, respectively. The expression and phosphorylation of ERK1/2 were detected by western blot. We discovered that the up-regulation of KLF2 expression dramatically inhibited proliferation, migration and tube formation in treated LSECs. Correspondingly, down-regulation of KLF2 expression significantly promoted proliferation, migration and tube formation in treated LSECs. Additionally, KLF2 inhibited the phosphorylation of ERK1/2 pathway, followed by the function of KLF2 in the angiogenesis of LSECs disrupted. In conclusion, KLF2 suppressed the angiogenesis of LSECs through inhibition of cell proliferation, migration, and vessel tube formation. These functions of KLF2 may be mediated through the ERK1/2 signaling pathway.

1. Introduction

Angiogenesis is a recurring factor in many chronic liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), and it also plays an important role in the progression of these diseases [1]. The early stages of angiogenesis can be divided into three steps: endothelial cell proliferation, migration, and tube formation [2]. Inflammation, hypoxia, or ischemia may equally contribute to angiogenesis [3,4].

Liver sinusoidal endothelial cells (LSECs) are the most abundant non-parenchymal cells in the liver, which are located at the interface between the hepatic sinusoid and liver parenchyma. LSECs form a protective barrier that identifies pathogens and regulates inflammation, leukocyte recruitment and immune responses [5,6]. *In vitro* experiments have shown that immortalized LSECs form tube-like structures and play an important role in the pathobiology of the hepatic sinusoidal endothelium [3]. In addition, LSECs have been found to participate in liver angiogenesis during pathological states such as liver fibrosis, cirrhosis and tumor development [7]. Recently, Sakata K et al. [8] demonstrated that LSECs secrete latent transforming growth factor-β (TGF-β) that activates HSCs, increases angiogenesis, and thus accelerates liver fibrosis. LSECs have also been shown to secrete cyclooxygenase-2 (COX-2), which is associated with angiogenic molecules that promote angiogenesis in HCC [9]. Therefore, LSECs that are associated with pathological angiogenesis are considered to play an essential role in the progression of chronic liver diseases, and thus, their inhibition could potentially serve as a new treatment strategy [10].

Kruppel-like factor 2 (KLF2) is a member of the zinc finger transcription factor family, which regulates cellular growth and tissue development [11]. KLF2 is highly expressed in the vascular endothelium and is required for normal vessel development

* Corresponding author. Department of Gastroenterology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Shanghai 200032, China.
E-mail addresses: zeng.xiaoqing@zs-hospital.sh.cn (X.-Q. Zeng), Linala.2009@163.com (N. Li), lasikesmi@hotmail.com (D.-Y. Pan), sadsadvenus@163.com (Q. Miao), ma.guifen@zs-hospital.sh.cn (G.-F. Ma), liuyimei1988@163.com (Y.-M. Liu), dianatseng14@gmail.com (Y.-J. Tseng), li.feng2@zs-hospital.sh.cn (F. Li), xu.lili3@zs-hospital.sh.cn (L.-L. Xu), chen.shiyao@zs-hospital.sh.cn (S.-Y. Chen).
[1] Xiao-qing Zeng and Na Li contributed equally and share first authorship.

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[12,13]. It is also a prominent anti-angiogenic factor and modulates the expression of multiple endothelial vasoprotective genes [14]. Kawanami et al. [15] showed that in human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs), overexpression of KLF2 inhibits the hypoxia-mediated secretion of hypoxia-inducible factor-1α (HIF-1α) and its target angiogenic genes, angiopoietin-2 (Ang-2), interleukin-8 (IL-8) and vascular endothelial growth factor-A (VEGF-A). Conversely, knockdown of KLF2 increases the expression of HIF-1α and its target genes. These results indicate that KLF2 plays an important inhibitory role in the process of angiogenesis. However, very few studies have reported the direct effect of KLF2 on LSECs.

ERK1/2 is mainly activated by growth factor and is associated with cell proliferation, differentiation and development [16]. ERK1/2 is also demonstrated to inhibit angiogenesis in many endothelial cells, like retinal endothelial cell and HUVEC [17,18]. Therefore, ERK1/2 may be involved in the inhibitory effect of KLF2 on LSEC angiogenesis.

In the present study, we investigated the effect of KLF2 on the proliferation, migration and vessel tube formation of LSECs, as well as its underlying mechanism. Our results showed that KLF2 suppressed the angiogenesis induced by LSECs by inhibiting its proliferation, migration, and vessel tube formation, which may be mediated by the ERK1/2 signaling pathway. Our findings may provide new evidence in treating angiogenesis related diseases.

2. Materials and methods

2.1. Cells and cell culture

Primary human LSECs authenticated with phenotype characterization, positive for von Willebrand (Factor Ⅷ) were purchased from Sciencell (CA, USA) and cultured in complete endothelial cell medium (ECM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% endothelial growth supplement (ECGS) and 1% penicillin/streptomycin (Sciencell, CA, USA). LSECs were grown on 1 ug/cm^2 bovine plasma fibronectin (Sciencell, CA, USA)-coated flasks overnight before being subcultured and incubated in 5% CO_2-humidified atmosphere at 37 °C.

2.2. Lentiviral infection

The lentiviruses overexpression or suppression of KLF2 expression and their vehicles were synthesized by Genepharma (Shanghai, China). As previously described [19], the LSECs were infected with different lentiviruses (multiplicity of infection = 15) to generate KLF2-overexpression (referred to as LV5-KLF2) and KLF2-suppression (referred to as LV3-shKLF2) LSECs. Cells infected with an empty vector served as control cells (referred to as LV5-NC and LV3-NC, respectively). The efficacies of the infection were examined by real-time PCR and Western blot analysis.

2.3. Cell proliferation assay

Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8) assay (Obio, Shanghai, China) according to the manufacturer's instructions. Briefly, the cells were seeded in a 96-well cell culture cluster (Corning, NY, USA) at a concentration of 2×10^3 cells/well in 100 ul of ECM medium with 1% FBS and incubated at 37 °C for 24 h, 48 h, and 72 h. At the end of each time point, 10 ul of CCK8 was added to each well and incubated at 37 °C for an additional 3 h. The absorbance at 450 nm was subsequently measured using an automated plate reader (Flexstation3, Molecular Devices). Each experiment was performed independently three times, and each sample was assessed in quintuplicate.

2.4. Transwell assay

Migration ability of LSECs was further evaluated with a transwell system (Corning Costar, MA, USA) consisting of 8-μm polyvinyl-free polycarbonate membrane inserted in a 24-well plate separating the upper and lower wells of the chamber. The bottom wells were filled with 600 ul of complete ECM, while a concentration of 3×10^4 cells/well in 200 ul of FBS-free ECM was added to the upper chamber. The chamber was then incubated at 37 °C with 5% CO_2 for 12 h. The migrated cells were stained with May–-Grunwald–Giemsa and counted in ten random fields using a phase-contrast microscope (Olympus, Tokyo, Japan). The experiments were performed three times, and each sample was assessed in triplicate.

2.5. Matrigel-induced tube formation test

The vessel tube formation ability of LSECs was assessed using Matrigel-induced tube formation as previously described [20]. Matrigel™ Basement Membrane Matrix (BD Bioscience, USA) was added to a 96-well plate to a total volume of 60 ul per well and polymerized at 37 °C for 30 min to form a gel layer. After gel formation, LSECs (2×10^4 cells/well) in 100 ul of ECM with 1% FBS were added to each well. After incubation for 6–8 h, the cells were observed with an inverted phase-contrast microscope (Olympus, Tokyo, Japan) and photographed. The number of branching points, generating at least three tubules, was counted. Three independent experiments were performed.

2.6. Western blot analysis

Cells were lysed, and total protein was extracted by RIPA lysis, separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, MA, USA). After blocking with 5% non-fat dry milk in TBS for 2 h, the membrane was probed with primary monoclonal rabbit antibody specific to KLF2 (1:200, Abcam, USA), phosphorylated (p)-ERK1/2 and total-ERK1/2 (both 1:1000, Cell Signaling Technology, USA) or GAPDH (1:2000, Beyotime, China). The membrane was subsequently probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4000, Santa Cruz, USA) and the protein bands were visualized using enhanced chemiluminescence (Millipore, MA, USA). Each experiment was conducted in triplicate.

To detect p-ERK1/2, 1% PMSF (Beyotime, China) and 1% phosphatase inhibitor p-5726 (Sigma, USA) were added to the RIPA lysis for protein extraction. To explore LSEC function after ERK signaling pathway was blotted, cells were pretreated with the ERK1/2 inhibitor PD98059 (Sigma–Aldrich, St Louis, MO, USA) for 2 h at a concentration of 20 μM in ECM containing 1% FBS. The cells were then washed twice with PBS and resuspended in fresh complete ECM. The RNA and proteins were extracted as previously described for further experiments.

2.7. Statistical analysis

All of the data were analyzed using SPSS 16.0 software (SPSS, Chicago, USA). Quantitative results are expressed as mean ± standard deviation (SD) and were compared using the Student's *t* test (between two groups) or the Newman–Keuls test (among three or more groups). $P < 0.05$ was considered statistically significant.

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