Title:

Asparaginyl endopeptidase may promote liver sinusoidal endothelial cell angiogenesis via PI3K/Akt pathway

Authors:
Na Li, Chu Liu, Guifen Ma, Yujen Tseng, Duyi Pan, Jie Chen, Feng Li, Xiaoqing Zeng, Tiancheng Luo, Shiyao Chen

OR 5709

Asparaginyl endopeptidase may promote liver sinusoidal endothelial cell angiogenesis via PI3K/Akt pathway

Na Li[1], Chu Liu[1], Guifen Ma[2], Yujen Tseng[1], Duyi Pan[1], Jie Chen[1], Feng Li[1], Xiaoqing Zeng[1], Tiancheng Luo[1] and Shiyao Chen[1]

Departments of [1]Gastroenterology and [2]Radiotherapy. Zhongshan Hospital. Fudan University. Shanghai, China

Correspondence: Shiyao Chen. Department of Gastroenterology. Fudan University. Zhongshan Hospital. 180 Fenglin Road. 200032 Shanghai, China

e-mail: chen.shiyao@zs-hospital.sh.cn

ABSTRACT

Background and aims: pathological angiogenesis plays an important role in the progression of chronic liver diseases. Asparaginyl endopeptidase (AEP) participates in tumor angiogenesis and was recently shown to be associated with liver fibrosis. This study aimed to explore the effect of AEP on liver sinusoidal endothelial cell (LSECs) angiogenesis and determine the underlying mechanism.

Methods: cultured LSECs were infected with lentiviruses in order to suppress AEP expression (AEP-KD1, AEP-KD2). The effect of AEP on LSEC proliferation, apoptosis and migration were subsequently determined by a CCK8 assay, flow cytometry and wound-healing and Transwell assays, respectively, in AEP knocked-down and control LSECs. The expression of the endothelial cell surface markers CD31, CD34 and von Willebrand factor (vWF) were detected by immunofluorescence assay and western blot. The angiogenic factors, vascular endothelial growth factor receptor 2 (VEGFR2) and interleukin 8 (IL 8) were detected by real-time PCR and western blot. The effect of AEP on vessel tube formation by LSECs was examined by Matrigel™ tube-

formation assay. Phosphoinositide 3-kinase (PI3K)/Akt expression and phosphorylation were detected by western blot.

Results: AEP was effectively knocked down by lentivirus infection in LSECs. Down-regulation of AEP expression significantly decreased proliferation and migration and increased apoptosis of LSECs. Moreover, expression levels of the endothelial cell surface markers CD31, CD34 and vWF, as well as angiogenic factors VEGFR2 and IL 8, were also reduced after AEP was knocked-down. The vessel tube formation abilities of AEP-KD1 and AEP-KD2 LSECs were significantly inhibited compared with LSECs without AEP knocked-down. Down-regulation of AEP also inhibited the phosphorylation of PI3K and Akt.

Conclusion: AEP promotes LSECs angiogenesis *in vitro*, possibly via the PI3K/Akt pathway. AEP may therefore be a potential therapeutic target for preventing the progression of liver fibrosis.

Key words: Asparaginyl endopeptidase. Liver sinusoidal endothelial cells. Angiogenesis. Sinusoidal capillarization. PI3K/Akt.

INTRODUCTION

Angiogenesis occurs during the progression of chronic liver diseases, including fibrosis, cirrhosis and hepatocellular carcinoma (HCC). A close relationship between angiogenesis and liver fibrosis has been indicated in both clinical and experimental conditions (1). Liver sinusoidal endothelial cells (LSECs) form the fenestrated wall of the hepatic sinusoid and are the most important and abundant non-parenchymal cells in the liver. LSECs have also been shown to participate in pathological hepatic angiogenesis (2). The early stage of angiogenesis includes three steps: endothelial cell proliferation, migration and tube formation (3). We previously found that LSECs formed tube-like structures *in vitro* and played an important role in the pathobiology of angiogenesis (4). Differentiated LSECs have also been shown to activate hepatic stellate cells (HSCs), promote angiogenesis and therefore accelerate liver fibrosis (5). Thus, they are thought to play an essential role in pathological angiogenesis in chronic liver diseases. Furthermore, inhibition of pathological angiogenesis could be

a new treatment strategy in patients with chronic liver diseases (6).

Asparaginyl endopeptidase (AEP), also known as legumain, is a member of the C13 family of cysteine proteases (7). AEP expression is absent or low in normal tissues, including normal peritumoral tissues (8). However, AEP is overexpressed in solid tumors, the extracellular matrix and endothelial cells in the tumor microenvironment (9). The highest expression levels are in lysosomes. AEP is also involved in many physiological and pathological processes, including cell proliferation (10) and migration (8). A previous study showed that AEP promoted cell proliferation independently of endopeptidase activity (10). Shen et al. (11) found that legumain (AEP) overexpression in RAW 264.7 cells induced tube formation of endothelial cells in Matrigel™. They also showed that legumain expression and angiogenesis were increased in late-stage compared with early stage tumors and the administration of a legumain inhibitor suppressed tumor growth, angiogenesis and collagen deposition.

The phosphoinositide 3-kinase (PI3K)/Akt pathway is an important signaling pathway for endothelial cell proliferation, migration and tube formation (12). Furthermore, AEP is known to be an up-stream regulator of the Akt pathway (13). Zhu et al. (14) demonstrated that AEP may be involved in prostate cancer cell proliferation, invasion and survival, partially via the activation of the PI3K/Akt signaling pathway.

A previous study by Abdul Hameed et al. (15) indicated that legumain may be a relevant gene in liver fibrosis. However, no further studies have reported on the relationship between AEP and liver fibrosis and few studies have examined the role of AEP in liver diseases. We hypothesized that AEP may promote angiogenesis of LSECs, thus promoting the progression of liver fibrosis. The effect of AEP on the proliferation, apoptosis, migration and vessel tube formation of LSECs was investigated in the present study. AEP was found to promote proliferation and migration and inhibited apoptosis of LSECs. Furthermore, the expression of the endothelial cell markers CD31, CD34, vWF and angiogenic factors VEGFR2 and IL 8 were increased. It was also demonstrated that AEP increased vascular tube formation of LSECs. The function of AEP may be mediated through the PI3K/Akt signaling pathway. These findings may provide new evidence to aid in the treatment

of liver diseases, with the potential to reduce the incidence rates of liver cirrhosis and even liver carcinoma.

MATERIALS AND METHODS

Cells and cell culture

Primary human LSECs that were authenticated via phenotype characterization and positive for von Willebrand were purchased from Sciencell (CA, USA). Bovine plasma fibronectin (Sciencell, CA, USA) was coated on flasks (1 ug/cm^2) overnight before sub-culturing cells. LSECs were cultured with complete endothelial cell medium (ECM) supplemented with 5% heat-inactivated fetal bovine serum, 1% endothelial growth supplement and 1% penicillin/streptomycin (Sciencell, CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

Lentiviral infection

Lentiviral vectors for AEP knockdown were constructed by Hanyin Biotech (Shanghai, China). The shRNA sequences for AEP gene knockdown were: sh1: 5'-GATGGTGTTCTACATTGAA-3'; sh2: 5'-AAACTGATGAACACCAATGAT-3'. The control shRNA sequence was 5'-GTAGCGCGGTGTATTATAC-3'. The lentiviruses were packaged using psPAX2 and pMD2G (Hanyin Biotech). Lentivirus-containing supernatants were added to the LSECs cells, followed by selection with 1 μg/ml puromycin in order to obtain cells with AEP knockdown or overexpression. LSECs were subsequently infected with lentiviruses to generate AEP-knockdown (referred to as AEP-KD1, and AEP-KD2 LSECs) according to the manufacturers' instructions. Cells infected with an empty vector served as a control, referred to as NC. The infection efficacies were examined by Western blot (Fig. 6).

Cell proliferation assay

The cell counting kit-8 (CCK-8) (Obio, Shanghai, China) was used to test the effect of AEP on LSEC proliferation; 2×10^3 cells were seeded per well in a 96-well plate (Corning®, NY, USA) in 100 ul of ECM medium with 1% FBS. The cells were incubated for 1 d, 2 d, 3 d, 4 d and 5 d; 10 ul of CCK8 was added to each well and incubated at

37 °C for an additional three hours at the end of each time point. The absorbance at 450 nm was subsequently measured with FlexStation® 3 (Molecular Devices). Each sample was assessed in quintuplicate replicas and each experiment was independently replicated three times.

Flow cytometry analysis of apoptosis

Cells were trypsinized and single-cell suspensions (2×10^6 cells) were extracted and washed using phosphate-buffered saline for cell apoptosis analysis. The Annexin V-PE Apoptosis Detection Kit (BD Pharmingen™, USA) was applied to assess apoptosis according to the manufacturer's instructions. Briefly, cells were resuspended in 1x binding buffer at a concentration of 1×10^6 cells/ml and 100 µl of this suspension was combined as follows: a) only cells; b) Annexin V-PE reagent (5 µl); c) 7-AAD reagent (5 µl); and d) both Annexin V-PE reagent (5 µl) and 7-AAD reagent (5 µl). The tubes were gently vortexed and incubated for 15 minutes at room temperature and then the dark 1x binding buffer (400 µl) was added to each tube and analyzed by flow cytometry.

Wound-healing assay

An *in vitro* wound-healing assay was performed to measure the migration of LSECs. Cells were seeded in a 6-well plate (2×10^5 cells/well) and incubated in complete ECM for 24 hours. When the cells reached confluence, a straight line was scratched across the culture using a 10-200 µl micropipette tip. Subsequently, the cells were washed twice with PBS and then incubated in ECM with 1% FBS at 37 °C. Images of the cells were obtained using an inverted phase-contrast microscope (Olympus, Tokyo, Japan) at 0 hours and 24 hours. The wound width was determined using Image J 1.48 software (Bio-Rad, USA).

Transwell assay

LSECs migration was evaluated using an 8-um pore size Transwell system (Corning® Costar, MA, USA) inserted into a 24 well plate separating the upper and lower wells of the chamber. Briefly, 3×10^4 cells/well in 200 ul of 1% FBS ECM was added to the

upper chamber and the bottom wells were filled with 600 µl of complete ECM. The chamber was then incubated at 37 °C with 5% CO_2 for 24 hours. Migrated cells were stained with May-Grunwald-Giemsa and then counted in ten random fields using a phase-contrast microscope (Olympus, Tokyo, Japan). Each sample was assessed in triplicate and the experiments were repeated and performed three times.

Immunofluorescence assay

LSECs were fixed with 4% paraformaldehyde and permeabilized with a solution containing 0.5% Triton X-100 and 0.1 mg/ml RNase A in phosphate-buffered saline (PBS). They were subsequently immunostained by incubating with primary antibodies against CD31, CD34, and vWF (1:200, Abcam, USA) at 4 °C for overnight. They were subsequently incubated with Texas red-conjugated secondary antibodies (1:1000, Abcam, USA) and then DAPI at room temperature. The slides were photographed using a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted from cells using the Trizol reagent. RNA extracts were reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase using a commercial kit (SYBR® PrimeScriptTM, Takara, Japan). Real-time (RT) PCR for quantitative assessment of mRNA expression was performed using an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems®, Tokyo, Japan), as described by the manufacturer. The expression levels of VEGFR2 and IL 8 were normalized to the expression of actin mRNA in each sample using the ΔCt method. Relative differences in gene expression between subgroups were determined using the comparative Ct (ΔΔCt) method. Fold expression was calculated using the formula 2-ΔΔCt, in which ΔΔCt represents the ΔCt values normalized to the mean ΔCt of actin.

Western blot analysis

Cells were lysed and protein was extracted by RIPA lysis and then separated using 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, MA, USA). After

blocking with 10% non-fat dry milk in TBS for one hour, protein was detected using a primary monoclonal rabbit antibody specific to AEP, CD31, CD34 and vWF (1:1000, Abcam, USA), VEGFR2 and IL 8 (1:1000, Immuno Way, USA), phosphorylated (p)-PI3K, p-AKT, total-PI3K and total-AKT (1:1000, Cell Signaling Technology, USA) and actin (1:2000, Cell Signaling Technology, USA). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, Santa Cruz, USA). The protein bands were visualized using enhanced chemiluminescence (Millipore, MA, USA). Each experiment was independently replicated three times.

In vitro tubulogenesis assay with LSECs

The vessel tube formation ability of LSECs was assessed with Matrigel™-induced tube formation as previously described (16); 60 µl Growth Factor Reduced Matrigel™ Matrix (BD Bioscience, USA) was added per well to a 96-well plate and polymerized at 37 °C for 30 minutes to form a gel layer. LSECs were serum starved overnight and then 2×10^4 of LSECs in 100 µl of ECM with 1% FBS were added to each well. The cells were observed six hours later with an inverted phase-contrast microscope (Olympus, Tokyo, Japan) and photographed. The number of branching points which generated at least three tubules was counted. The experiments were performed independently three times.

Statistical analysis

Quantitative results were expressed as mean ± SD. Statistical analysis was assessed using the Student's t-test (between two groups) with the SPSS 20.0 software (SPSS, Chicago, USA). p values < 0.05 were considered as statistically significant and p values of < 0.01 were considered as highly significant.

RESULTS

AEP promotes LSECs proliferation and inhibits LSECs apoptosis

The role of the AEP gene in LSECs was assessed using the CCK8 assay. Down-regulation of AEP expression in LSECs (AEP-KD1 and AEP-KD2) significantly reduced

cell proliferation, demonstrated by a reduction in optical density (OD) compared with NC control cells at three, four and five days (p < 0.01 for all comparisons) (Fig. 1A). Cell apoptosis is another important cellular activity. The apoptotic rate of each group of cells was calculated by counting late apoptosis in the Q2 region and early apoptotic cells in the Q4 region. The apoptosis rates were significantly increased in both AEP-KD1 and AEP-KD2 LSECs compared with NC control cells, as shown by flow cytometry analysis (p < 0.001 for both comparisons) (Fig. 1B and C).

AEP promotes LSECs migration *in vitro*

Cell migration is key process in angiogenesis and therefore the migration ability of LSECs was investigated by wound-healing and Transwell assays. AEP down-regulation (AEP-KD1 and AEP-KD2) significantly impaired cell migration in a wound-healing assay compared with NC control cells (p < 0.001 for both comparisons) (Fig. 2A and B). Similarly, fewer AEP-KD1 and AEP-KD2 cells had migrated to the lower side of the filter after 24 hours in the Transwell assay (p < 0.001 for both comparisons) (Fig. 2C and D).

AEP increases the expression of endothelial cell surface markers and angiogenic factors on LSECs

Furthermore, cells were then immunostained with antibodies to CD31, CD34 and vWF, which are all important human endothelial cell surface markers indicative of hepatic sinusoid capillarization of LSECs (17). There was a lower expression of CD31, CD34 and vWF in AEP-KD1 and AEP-KD2 LSECs compared with NC control LSECs (Fig. 3A and B). This was confirmed by western blot, as shown in figure 3C. VEGFR2 and IL 8 are important angiogenic factors expressed on LSECs under pathological conditions, mRNA and protein levels of both VEGFR2 and IL 8 were reduced after AEP was down regulated in LSECs (Fig. 3D and E).

AEP promotes LSECs tube formation *in vitro*

The effect of AEP on the tubulogenesis ability of LSECs was assessed *in vitro* by the Matrigel™ tube-formation test. Down-regulation of AEP significantly inhibited the

vessel formation ability of LSECs. AEP-KD1 and AEP-KD2 cells developed incomplete and sparse networks in Matrigel™ after incubation for six hours, whereas NC control cells formed extensive and enclosed tubes (Fig. 4A and B) (both p < 0.001).

AEP regulates LSECs angiogenesis partially through activation of the PI3K/Akt signaling pathway

The importance of the PI3K/Akt signaling pathway in cell survival and migration has been well established (18). The role of AEP in the activation of this pathway was further investigated. Suppression of AEP expression in LSECs reduced the levels of phosphorylated, but not total, PI3K and Akt (Fig. 5A and B). Taken together, these results suggest that AEP might be a crucial factor that controls LSECs cell proliferation, migration and survival through the activation of the PI3K/Akt pathway.

DISCUSSION

The progression of liver disease is accompanied by pathological angiogenesis (19). Ehling et al. (20) demonstrated that angiogenesis was induced, even in the initial stages of liver fibrosis, while the fibrosis stage involved extensive hepatic neovascularization. Furthermore, anti-angiogenic multikinase inhibitors have been shown to have anti-fibrotic potential in the preclinical settings (19). However, the relationship between angiogenesis and fibrosis during the progression of chronic liver disease remains unclear (21).

An increasing number of studies have examined the role of AEP, which was first described within the endosome/lysosome system (22). Since then, it has been detected in the nucleus (10), on the cell surface and in the extracellular matrix (23). Although the expression level in most normal tissues is very low (8), it is highly expressed in many solid tumors and has been shown to be related to a more invasive and metastatic phenotype (8,24). AEP is involved in many physiological and pathological processes, including liver fibrosis (15), tumor angiogenesis and metastasis (25). However, its detailed effect and mechanism remain poorly studied. In the current study, we identified AEP as a potential pro-angiogenic factor and demonstrated that AEP down-regulation could represent an anti-angiogenic

therapeutic strategy for liver fibrosis.

Cell proliferation is the first step in angiogenesis. In this study, we demonstrated that down-regulation of AEP significantly inhibited the proliferation of LSECs. This is consistent with previous studies showing that AEP promoted the growth of cancer cells, such as breast (26) and prostate cancer cells (27). The legumain promoter is sensitive to nuclear Ca^{2+}, which has been shown to regulate legumain expression via ELK-1, affecting cell proliferation (10). Down-regulation of AEP resulted in increased apoptosis of LSECs, indicating that AEP inhibited LSEC apoptosis *in vitro.* Endothelial cell apoptosis may reduce pathological angiogenesis. For example, pigment epithelium-derived factor is a potent anti-angiogenic factor and its effect is partially mediated through the induction of endothelial cell apoptosis (28). However, further studies are needed in order to clarify if AEP promotes the proliferation of LSECs by inhibiting the apoptosis.

Collective cell migration is an important cellular event involved in many different physiological processes such as embryonic development, tissue repair, angiogenesis, and wound healing (29,30). AEP was previously shown to promote cancer cell migration *in vitro* and was identified as a poor prognostic factor in gastric cancer patients (31). The present study also showed that down-regulation of AEP sub stantially inhibited LSECs migration.

LSECs play an important role in the pathology of portal hypertension. Furthermore, sinusoidal capillarization or decreased fenestration of LSECs was shown to lead to angiogenesis and the development of portal hypertension (32). CD31, CD34 and vWF are indicators of sinusoidal capillarization in cirrhotic liver (33). CD31 and CD34 are also widely used as continuous or pan vascular endothelial markers (34). Although they are rarely expressed in normal liver tissues, both CD31 and CD34 (as markers of angiogenesis) are abundantly expressed in liver tissues from cirrhotic patients (35). CD31 expression on the surface of differentiated LSECs remains controversial and some studies have suggested that CD31 is absent on the surface of differentiated LSECs, except during *in vitro* culture or stimulation with pathological factors (36). vWF is expressed in most blood vessels but its expression levels differ among various kinds of vascular beds. vWF expression in the liver is extremely low

under normal conditions and mostly occurs in vascular endothelial cells but not LSECs (37). However, LSECs capillarization in a fibrotic liver is associated with a change in many cells from a vWF-negative to a vWF-positive immunophenotype (38). The present study showed that down-regulation of AEP inhibited the expression of CD31, CD34 and vWF in cultured LSECs *in vitro.* Meanwhile, down-regulation of AEP also inhibited tube formation of LSECs *in vitro* by reducing the number of tubes. This is consistent with previous reports showing that isolated rat LSECs could undergo capillary morphogenesis and form a vascular network in Matrigel™ (39). This suggests that AEP down-regulation was associated with LSEC differentiation, while previous *in vitro* studies notably showed that differentiated LSECs prevented HSCs activation and promoted their quiescence, thus enhancing the regression of fibrosis and preventing cirrhosis progression (40-42).

The PI3K/Akt signaling pathway is known to play an important role in cell proliferation and migration (18), which are essential processes in angiogenesis. The present study indicated that depletion of AEP inhibited the activation of phospho-PI3K and phospho-Akt. Whereas previous studies reported that activation of the PI3K/Akt signaling pathway enhanced the angiogenic capability of LSECs by promoting the production of vascular endothelial growth factor A in hepatocytes and expression of VEGFR2 in LSECs (43). Gong et al. (44) found that down-regulation of PI3K/Akt phosphorylation blocked IL 8 secretion and therefore inhibited LSECs angiogenesis. The present study also demonstrated that the expression levels of VEGFR2 and IL 8 were reduced after AEP was down regulated, which may result from the inhibition of PI3K/Akt activation.

Pathological angiogenesis occurs during the initial stages of liver fibrosis and is likely a prerequisite that favors the eventual development of HCC. Andrade et al. (10) showed that AEP expression was increased in HCC cells compared with normal hepatocytes, suggesting that AEP expression was increased in HCC, similar to the situation in other solids tumors. Up-regulation of AEP expression was shown to be positively correlated with invasive and metastatic potential of tumors (8,45,46). AEP has also been examined as a target of oral DNA vaccines that target tumor vasculature and the microenvironment and suppress tumor growth and metastasis

(47). Overall, these results suggest the need for a safe and effective pharmacological treatment to prevent the progression of liver fibrosis to HCC.

The current study had some limitations. The LSECs used were derived from healthy individuals rather than from patients with fibrosis. Furthermore, all the experiments were conducted *in vitro* and further *in vivo* studies are needed to confirm the ability of AEP to improve liver fibrosis through the maintenance of the normal LSEC phenotypes and reduction of LSECs angiogenesis.

In conclusion, we demonstrated that human LSECs can form tube-like structures *in vitro.* We also showed that AEP can promote LSEC angiogenesis by increasing their proliferation and migration. This is associated with an increased expression of angiogenesis markers and pro-angiogenic factors, while reducing their apoptosis. We anticipate that AEP will provide a potential therapeutic target in patients with liver fibrosis, which will ultimately reduce the incidence of liver cirrhosis and even HCC.

REFERENCES

1. Coulon S, Heindryckx F, Geerts A, et al. Angiogenesis in chronic liver disease and its complications. Liver Int 2011;31:146-62. DOI: 10.1111/j.1478-3231.2010.02369.x

2. Zhang Z, Zhang F, Lu Y, et al. Update on implications and mechanisms of angiogenesis in liver fibrosis. Hepatol Res 2014;45(2):162-78. DOI: 10.1111/hepr.12415

3. Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931-4.

4.   Zeng XQ, Li N, Pan DY, et al. Kruppel-like factor 2 inhibit the angiogenesis of cultured human liver sinusoidal endothelial cells through the ERK1/2 signaling pathway. Biochem Biophys Res Commun 2015;464:1241-7. DOI: 10.1016/j.bbrc.2015.07.113

5.   Sakata K, Eda S, Lee ES, et al. Neovessel formation promotes liver fibrosis via providing latent transforming growth factor-beta. Biochem Biophys Res Commun 2014;443:950-6. DOI: 10.1016/j.bbrc.2013.12.074

6.   Thabut D, Shah V. Intrahepatic angiogenesis and sinusoidal remodeling in chronic liver disease: new targets for the treatment of portal hypertension? J Hepatol 2010;53:976-80.

7.   Chen JM, Dando PM, Rawlings ND, et al. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. J Biol Chem 1997;272:8090-8. DOI: 10.1074/jbc.272.12.8090

8.   Liu C, Sun C, Huang H, et al. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. Cancer Res 2003;63:2957-64.

9.   Wu W, Luo Y, Sun C, et al. Targeting cell-impermeable prodrug activation to tumor microenvironment eradicates multiple drug-resistant neoplasms. Cancer Res 2006;66:970-80. DOI: 10.1158/0008-5472.CAN-05-2591

10.   Andrade V, Guerra M, Jardim C, et al. Nucleoplasmic calcium regulates cell proliferation through legumain. J Hepatol 2011;55:626-35. DOI: 10.1016/j.jhep.2010.12.022

11.   Shen L, Li H, Shi Y, et al. M2 tumour-associated macrophages contribute to tumour progression via legumain remodelling the extracellular matrix in diffuse large B cell lymphoma. Sci Rep 2016;6:30347. DOI: 10.1038/srep30347

12.   Jin J, Yuan F, Shen MQ, et al. Vascular endothelial growth factor regulates primate choroid-retinal endothelial cell proliferation and tube formation through PI3K/Akt and MEK/ERK dependent signaling. Mol Cell Biochem 2013;381:267-72. DOI: 10.1007/s11010-013-1710-y

13.   Liu C, Sun C, Huang H, et al. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug

therapy. Cancer Res 2003;63:2957-64.

14.   Zhu W, Shao Y, Yang M, et al. Asparaginyl endopeptidase promotes proliferation and invasiveness of prostate cancer cells via PI3K/AKT signaling pathway. Gene 2016;594:176-82. DOI: 10.1016/j.gene.2016.08.049

15.   AbdulHameed MD, Tawa GJ, Kumar K, et al. Systems level analysis and identification of pathways and networks associated with liver fibrosis. PLoS One 2014;9:e112193. DOI: 10.1371/journal.pone.0112193

16.   Zheng X, Li A, Zhao L, et al. Key role of microRNA-15a in the KLF4 suppressions of proliferation and angiogenesis in endothelial and vascular smooth muscle cells. Biochem Biophys Res Commun 2013;437:625-31. DOI: 10.1016/j.bbrc.2013.07.017

17.   Poisson J, Lemoinne S, Boulanger C, et al. Liver sinusoidal endothelial cells: physiology and role in liver diseases. J Hepatol 2017;66(1):212-27.

18.   Shen M, Zhou XZ, Ye L, et al. Xanthatin inhibits corneal neovascularization by inhibiting the VEGFR2mediated STAT3/PI3K/Akt signaling pathway. Int J Mol Med 2018;42(2):769-78. DOI: 10.3892/ijmm.2018.3646

19.   Tugues S, Fernández-Varo G, Muñoz-Luque J, et al. Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats. Hepatology 2007;46:1919-26. DOI: 10.1002/hep.21921

20.   Ehling J, Bartneck M, Wei X, et al. CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. Gut 2014;63:1960-71. DOI: 10.1136/gutjnl-2013-306294

21.   Schuppan D, Kim YO. Evolving therapies for liver fibrosis. J Clin Invest 2013;123:1887-901. DOI: 10.1172/JCI66028

22.   Chen JM, Dando PM, Stevens RA, et al. Cloning and expression of mouse legumain, a lysosomal endopeptidase. Biochem J 1998;335(Pt 1):111-7. DOI: 10.1042/bj3350111

23.   Haugen MH, Boye K, Nesland JM, et al. High expression of the cysteine proteinase legumain in colorectal cancer - Implications for therapeutic targeting. Eur J Cancer 2015;51:9-17. DOI: 10.1016/j.ejca.2014.10.020

24.   Wang L, Chen S, Zhang M, et al. Legumain: a biomarker for diagnosis and

prognosis of human ovarian cancer. J Cell Biochem 2012;113:2679-86. DOI: 10.1002/jcb.24143

25. Lewen S, Zhou H, Hu HD, et al. A legumain-based minigene vaccine targets the tumor stroma and suppresses breast cancer growth and angiogenesis. Cancer Immunol Immunother 2008;57:507-15. DOI: 10.1007/s00262-007-0389-x

26. D'Costa ZC, Higgins C, Ong CW, et al. TBX2 represses CST6 resulting in uncontrolled legumain activity to sustain breast cancer proliferation: a novel cancer-selective target pathway with therapeutic opportunities. Oncotarget 2014;5:1609-20. DOI: 10.18632/oncotarget.1707

27. Zhu W, Shao Y, Yang M, et al. Asparaginyl endopeptidase promotes proliferation and invasiveness of prostate cancer cells via PI3K/AKT signaling pathway. Gene 2016;594(2):176-82. DOI: 10.1016/j.gene.2016.08.049

28. Zhang H, Wei T, Jiang X, et al. PEDF and 34-mer inhibit angiogenesis in the heart by inducing tip cells apoptosis via up-regulating PPAR-gamma to increase surface FasL. Apoptosis 2016;21:60-8. DOI: 10.1007/s10495-015-1186-1

29. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol 2009;10:445-57. DOI: 10.1038/nrm2720

30. Rorth P. Collective cell migration. Annu Rev Cell Dev Biol 2009;25:407-29. DOI: 10.1146/annurev.cellbio.042308.113231

31. Li N, Liu Q, Su Q, et al. Effects of legumain as a potential prognostic factor on gastric cancers. Med Oncol 2013;30:621. DOI: 10.1007/s12032-013-0621-9

32. Dill MT, Rothweiler S, Djonov V, et al. Disruption of Notch1 induces vascular remodeling, intussusceptive angiogenesis, and angiosarcomas in livers of mice. Gastroenterology 2012;142:967-77. DOI: 10.1053/j.gastro.2011.12.052

33. Xu B, Broome U, Uzunel M, et al. Capillarization of hepatic sinusoid by liver endothelial cell-reactive autoantibodies in patients with cirrhosis and chronic hepatitis. Am J Pathol 2003;163:1275-89. DOI: 10.1016/S0002-9440(10)63487-6

34. Miyata Y, Mitsunari K, Asai A, et al. Pathological significance and prognostic role of microvessel density, evaluated using CD31, CD34, and CD105 in prostate cancer patients after radical prostatectomy with neoadjuvant therapy. Prostate 2015;75:84-91. DOI: 10.1002/pros.22894

35.   Kobus K, Kopycinska J, Kozlowska-Wiechowska A, et al. Angiogenesis within the duodenum of patients with cirrhosis is modulated by mechanosensitive Kruppel-like factor 2 and microRNA-126. Liver Int 2012;32:1222-32. DOI: 10.1111/j.1478-3231.2012.02791.x

36.   Zhang LJ, Sun MY, Ning BB, et al. Xiayuxue Decoction ([symbols; see text]) attenuates hepatic stellate cell activation and sinusoidal endothelium defenestration in CCl4-induced fibrotic liver of mice. Chin J Integr Med 2014;20:516-23. DOI: 10.1007/s11655-014-1862-y

37.   Arimoto J, Ikura Y, Suekane T, et al. Expression of LYVE-1 in sinusoidal endothelium is reduced in chronically inflamed human livers. J Gastroenterol 2010;45:317-25. DOI: 10.1007/s00535-009-0152-5

38.   Zhao X, Zhao Q, Luo Z, et al. Spontaneous immortalization of mouse liver sinusoidal endothelial cells. Int J Mol Med 2015;35:617-24. DOI: 10.3892/ijmm.2015.2067

39.   Yokomori H, Oda M, Yoshimura K, et al. Caveolin-1 and Rac regulate endothelial capillary-like tubular formation and fenestral contraction in sinusoidal endothelial cells. Liver Int 2009;29:266-76. DOI: 10.1111/j.1478-3231.2008.01891.x

40.   Marrone G, Maeso-Díaz R, García-Cardena G, et al. KLF2 exerts antifibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins. Gut 2015;64:1434-43. DOI: 10.1136/gutjnl-2014-308338

41.   DeLeve LD. Liver sinusoidal endothelial cells in hepatic fibrosis. Hepatology 2015;61:1740-6. DOI: 10.1002/hep.27376

42.   Xie G, Wang X, Wang L, et al. Role of differentiation of liver sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. Gastroenterology 2012;142:918-27. DOI: 10.1053/j.gastro.2011.12.017

43.   Yan Z, Qu K, Zhang J, et al. CD147 promotes liver fibrosis progression via VEGF-A/VEGFR2 signalling-mediated cross-talk between hepatocytes and sinusoidal endothelial cells. Clin Sci (Lond) 2015;129:699-710. DOI: 10.1042/CS20140823

44.   Gong WG, Lin JL, Niu QX, et al. Paeoniflorin diminishes ConA-induced IL-8 production in primary human hepatic sinusoidal endothelial cells in the involvement of ERK1/2 and Akt phosphorylation. Int J Biochem Cell Biol 2015;62:93-100. DOI:

10.1016/j.biocel.2015.02.017

45.   Briggs JJ, Haugen MH, Johansen HT, et al. Cystatin E/M suppresses legumain activity and invasion of human melanoma. BMC Cancer 2010;10:17. DOI: 10.1186/1471-2407-10-17

46.   Ohno Y, Nakashima J, Izumi M, et al. Association of legumain expression pattern with prostate cancer invasiveness and aggressiveness. World J Urol 2013;31:359-64. DOI: 10.1007/s00345-012-0977-z

47.   Xiang R, Luo Y, Niethammer AG, et al. Oral DNA vaccines target the tumor vasculature and microenvironment and suppress tumor growth and metastasis. Immunol Rev 2008;222:117-28. DOI: 10.1111/j.1600-065X.2008.00613.x
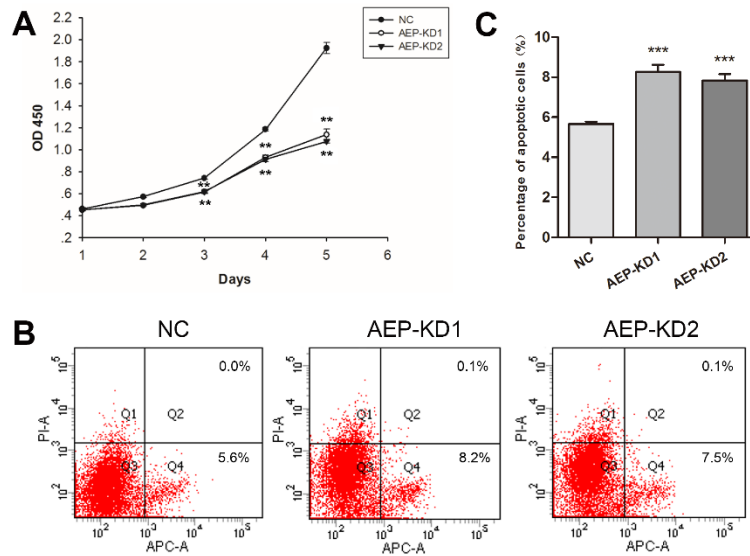
Fig. 1. AEP promotes LSEC proliferation and inhibits LSEC apoptosis *in vitro.* The effects of AEP on LSEC proliferation and apoptosis were examined using the CCK8 assay and flow cytometry analysis, respectively. A. Down-regulation of AEP in the AEP-KD1 and AEP-KD2 groups significantly decreased LSEC proliferation. Cell proliferation abilities are expressed as the mean ± standard deviation (SD; n = 5 per time point) from three independent experiments. B and C. The apoptotic rate of each group of cells was calculated by counting late apoptosis in the Q2 region and early apoptotic cells in the Q4 region. Down-regulation of AEP in the AEP-KD1 and AEP-KD2 groups significantly increased LSECs apoptosis. ***p < 0.001 compared to NC.
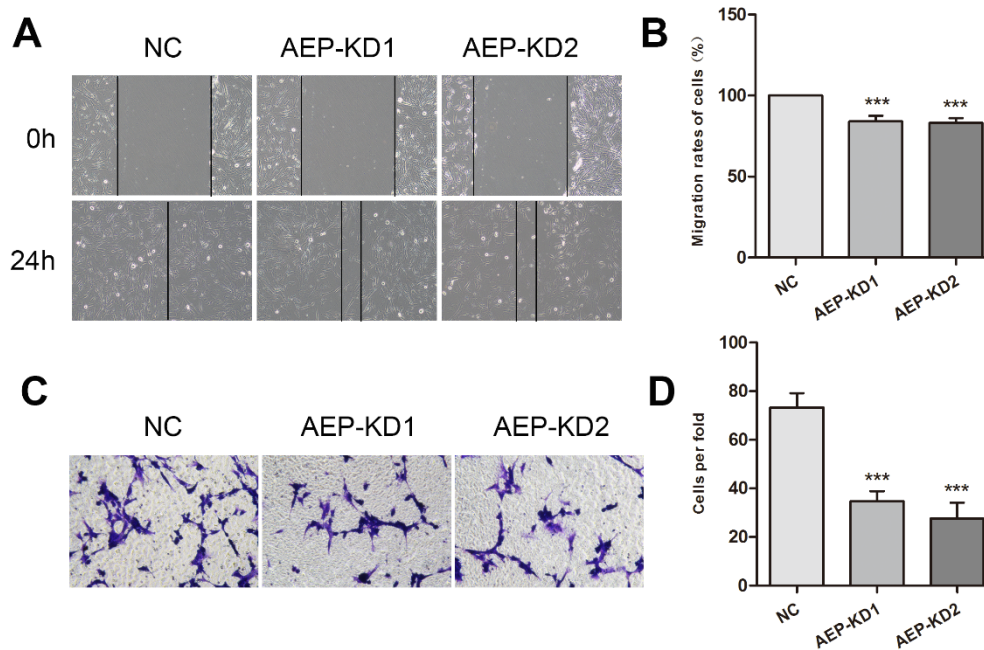
Fig. 2. AEP promotes LSECs migration ability *in vitro*. The effect of AEP on LSEC migration was examined by wound-healing and Transwell assays. A. Representative images of LSECs in a wound-healing assay (×40 magnification). B. Fewer cells migrated in the AEP-KD1 and AEP-KD2 groups compared to the NC group. ***p < 0.001. C. Representative images of LSECs in a Transwell assay (×100 magnification). Migrated cells were stained with May-Grunwald-Giemsa and counted in ten random fields using a phase-contrast microscope after 24 h incubation in a Transwell chamber. D. Fewer cells migrated after AEP was down regulated. The relative numbers of migrated cell are presented as the mean ± SD of three independent experiments. ***p < 0.001, compared to NC.
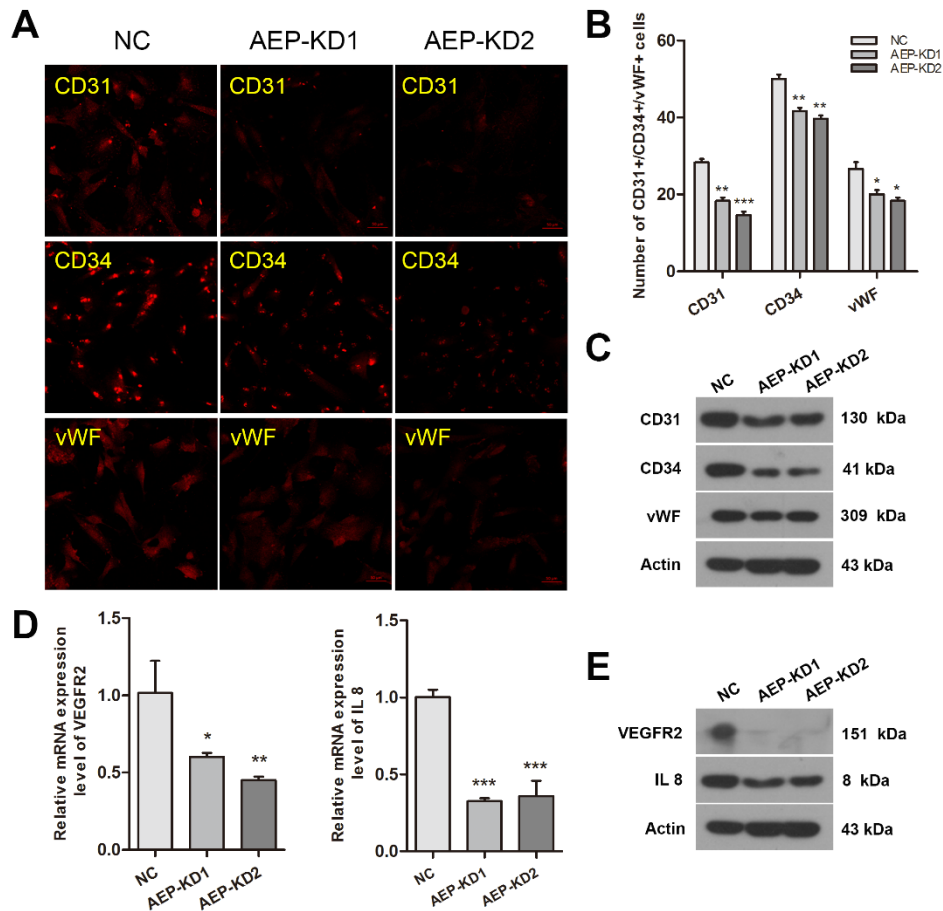
Fig. 3. Down-regulation of AEP impedes LSEC surface markers and angiogenic factors *in vitro.* A. Representative images of human endothelial cell surface markers CD31, CD34 and vWF detected by an immunofluorescence assay. B. The number of CD31+/CD34+/vWF+ cells were reduced after AEP was down regulated (*p < 0.05, **p < 0.01, ***p < 0.001). C. Representative western blots of CD31, CD34 and vWF. D and E. The mRNA and protein expression levels of VEGFR2 and IL 8 were reduced in AEP-KD1 and AEP-KD2 cells (*p < 0.05, **p < 0.01, ***p < 0.001, compared to NC).
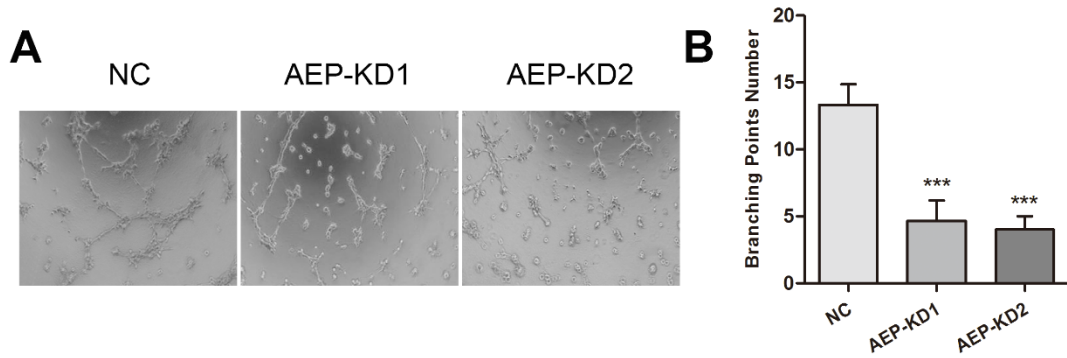
Fig. 4. Down-regulation of AEP inhibits LSECs tube formation *in vitro*. The effect of AEP on LSEC tube formation ability was assessed using Matrigel™. A. Representative photographs of tube formation in cultured LSECs (×100 magnification). B. The number of branching points, each generating at least three tubules, were counted. Down-regulation of AEP decreased the total number of branching points compared with the NC control group (***p < 0.001). Data represent the mean ± SD from three independent experiments.
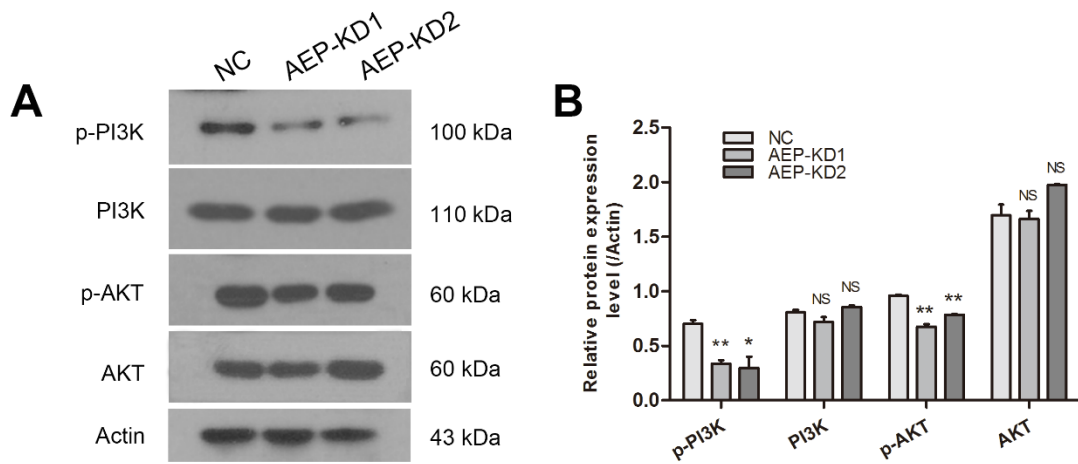
Fig. 5. AEP regulates LSECs angiogenesis partially through the activation of the PI3K/Akt signaling pathway. The effect of AEP on the PI3K/Akt signaling pathway was analyzed by western blot analysis. A. Representative western blots of the PI3K, Akt, phospho-PI3K and phospho-Akt in LSECs. B. Phospho-PI3K and phospho-Akt were inhibited after AEP was down regulated. NS: no significant difference. *p < 0.05, **p < 0.01, compared to NC.
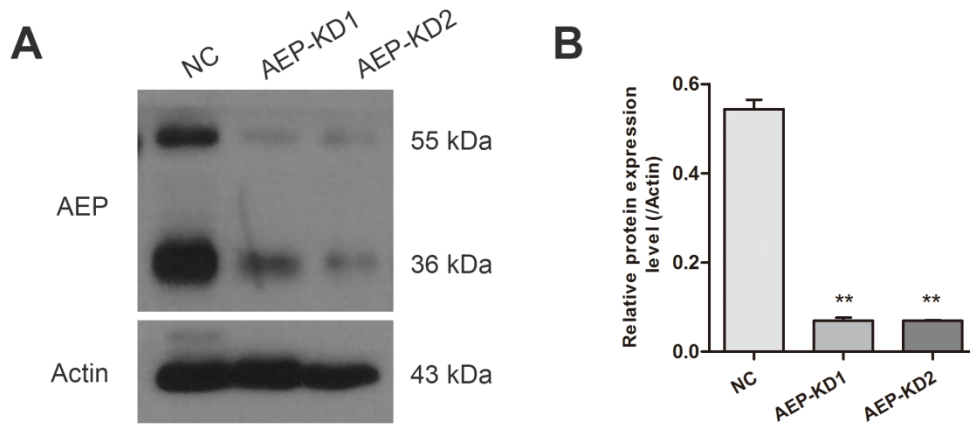
Fig. 6. Lentiviral infection efficacies in LSECs. A. AEP protein expression levels in AEP-KD1, AEP-KD2 and NC control LSECs were investigated by western blot analysis. B. AEP was effectively knocked down by lentiviruses in LSECs. **$p < 0.01$, compared to NC.