Pathology - Research and Practice 215 (2019) 152454

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

ANGPTL6-mediated angiogenesis promotes alpha fetoprotein-producing gastric cancer progression

Erbao Chen[a,1], Cheng Tang[a,1], Ke Peng[a], Xi Cheng[a], Yichou Wei[a], Tianshu Liu[a,b,*]

[a] Department of Medical Oncology, Zhongshan Hospital, Fudan University, Shanghai, China
[b] Center of Evidence-based Medicine, Fudan University, Shanghai, China

ARTICLE INFO

Keywords:
ANGPTL6
Angiogenesis
Proliferation
Alpha-fetoprotein producing-gastric cancer
Prognosis

ABSTRACT

Alpha-fetoprotein (AFP)-producing gastric cancer (AFPGC) is regarded as a rare but highly malignant gastric adenocarcinoma subtype and its clinic pathological presentation mimics hepatocellular carcinoma. However, the underlying mechanism of this disease remains elusive. The level of ANGPTL6 in AFPGC cell lines is much higher than that of common types of gastric cancer cells. A high level of ANGPTL6 confers a poor prognosis and is correlated with the expression of CD34 (an endothelial cell marker). ANGPTL6 promotes endothelial cell migration and tube formation, Moreover, ANGPTL6 knockdown inhibits cancer cell apoptosis and invasiveness. Mechanistically, ANGPTL6 activates the ERK1/2 and AKT pathways. Treatment of ERK1/2 or AKT inhibitor can attenuated cell migration and tube formation. ANGPTL6 loss results in tumor growth in vivo. Our study revealed that ANGPTL6 is an important driver gene of angiogenesis in AFPGC development. These findings provide not only an effective biomarker for diagnosis but also an attractive therapeutic target for use in AFPGC patients.

1. Introduction

Alpha-fetoprotein (AFP) is known as an embryonal serum glycoprotein that is normally synthesized from fetal hepatocytes and yolk sac cells during the gestational period [1]. In adulthood, AFP is commonly regarded as a characteristic tumor biomarker for screening and supervising hepatocellular carcinoma or yolk sac tumors. Nevertheless, several studies have uncovered that some other types of malignant solid tumors, such as tumors of the stomach, ovary, gallbladder, lung, and pancreas can also secrete AFP, with gastric cancer being the most common [2]. Gastric cancer with a high serum AFP level is defined as alpha-fetoprotein-producing gastric cancer (AFPGC) [3]. The incidence of AFPGC is merely 1.3–15.0% worldwide [4]. In most studies, the patients with AFPGC have been found to have aggressive characteristics and a high incidence of synchronous and metachronous liver and lymph node metastasis, even undergoing radical surgery; accordingly, the survival rates of AFPGC indicate an extremely poor prognosis compared that of with common gastric cancer [5]. Although surgical treatment or systemic chemotherapy is conducted to treat this malignancy in the clinic, the benefit to patients with AFPGC is still controversial [6], therefore, the molecular mechanism and standardized treatment process of AFPGC remain unclear. Therefore, it is essential to identify effective predictive biomarkers to understand the mechanism and improve the current treatment for this more malignant subtype of gastric cancer.

Angiogenesis is a sophisticated biologic process involving endotheliocyte multiplication, migration, invasion, and neovascularization. Furthermore, angiogenesis is necessary for various physiologic processes, such as body growth and tissue reproduction. To date, Angiogenesis has been identified as one of the hallmarks of tumor development [7]. The new vascularization infiltrates the tumor and supplies it with oxygen and nutrients that assist in tumor growth beyond a certain size. New tumoral vessels that exhibit abnormally high proliferation and the migration of these new tumoral vessels usually result in a poorly functioning vasculature. The loose connections between endothelial cells and cells surrounding tumoral blood vessels are conducive to tumor cells entering the circulation through the blood vessel wall, thereby promoting metastasis and spreading to secondary sites. Thus, antiangiogenic therapy has become one of the most important anticancer therapies. Antiangiogenic therapy has obvious advantages over systemic chemotherapy, which usually leads to rapid mutation and treatment resistance.

Neovascularization of a tumor relies on the production of specific angiogenic molecules; both host and tumor cells transfer from an angiogenic balance to a proangiogenic phenotype [8]. To date, many angiogenic growth factors that have been identified as critical

* Corresponding author at: No. 180, Fenglin Rd, Xuhui District, Shanghai, 200032, China.
E-mail address: liu_tianshu99@163.com (T. Liu).
[1] These authors contribute to this work equally.

https://doi.org/10.1016/j.prp.2019.152454
Received 6 April 2019; Accepted 17 May 2019
0344-0338/

dominators of the proliferation, apoptosis, and mobility of endothelial cells, including vascular endothelial growth factor (VEGF) and angiopoietin. So far, a new family of genes whose molecular structure is similar to that of angiopoietins, has been reported and subsequently denominated “angiopoietin-like gene family”.

Angiopoietin-like proteins (ANGPTLs) contain a set of eight secreted glycoproteins, from ANGPTL1 to ANGPTL8, and are similar to angiopoietin proteins; they have a specific structure: a C-terminal fibrinogen-like domain (FLD) and an N-terminal coiled-coil domain (CCD). Nevertheless, in contrast with angiopoietins, angiopoietin-like proteins do not bind to Tie 1 and Tie 2 receptors [9]. Interestingly, ANGPTLs are widely expressed in numerous organs such as the heart, vascular system, and have small intestine and hematopoietic system and have multibiological properties involved in angiogenesis [10], inflammation and lipid metabolism [11]. Emerging evidence has shown that some ANGPTLs (including ANGPTL2 [12,13], ANGPTL3, ANGPTL4 [14], and ANGPTL7(15)) are critical factors in tumor growth, migration, development, and drug resistance. Initially known as angiopoietin-related growth factor, ANGPTL6 has been reported to be a proangiogenic molecule [16], although it also plays an important role in regulating energy metabolism [17]. More recently, overexpression of ANGPTL6 through the downregulation of miRNA-128 was related to tumor proliferation of glioblastoma undifferentiated cells [18]. The intrahepatic ANGPTL6 and the interaction between tumoral α6 integrin and E-cadherin drive homing and colonization by colorectal cancer cells [19]. However, the role of ANGPTL6 in gastric cancer, especially AFPGC, remains elusive.

In the present study, we examined the expression of eight ANGPTLs in six human gastric cancer cell lines (two AFPGCs and four common types of gastric cancer cell lines). We explored how the proliferation, migration, invasion and tube formation ability changed in HUVECs treated with recombinant protein ANGPTL6 or AFPGC cellular supernatant. AFPGC cell lines were transfected with specific short hairpin RNAs. We also investigated how these changes in ANGPLT6 expression influenced AFPGC cell apoptosis, migration and invasion. Finally, we investigated whether the ANGPTL6 knockdown influenced the tumor growth in vivo.

2. Materials and methods

2.1. Cell lines and culture conditions

Human gastric cancer cells were maintained in culture using RPMI 1640 medium (GIBCO, Carlsbad, USA) (AGS, MKN1, SGC-7901, MGC-803) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). GCIY was maintained in MEM (GIBCO, Carlsbad, USA) supplemented with 10% FBS, and FU97 was maintained in DMEM (GIBCO, Carlsbad, USA) supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were conducted in endothelial cell growth medium (Cedarlane, Burlington, NC). All cell lines were cultured in a humidified chamber supplemented with 5% CO2 at 37 ˚C. Experiments were conducted using cells at a logarithmic growth phase. Recombinant human angiopoietin-like protein 6 (Catalog Number: 8466-AN, R&D systems) was purchased.

2.2. Western blotting analysis

WB analysis was conducted as previously described [20]. The anti-ANGPTL6 antibody ab155189 was purchased from Abcam, and the anti-phospho-AKT antibody (4060), anti-phospho-ERK1/2 (4370), anti-rabbit lgG, and HRP-lined antibody (7074) were purchased from Cell Signaling Technology. The anti-Ki 67 (BA2888) and anti-PNCA(BM0104) were purchased from Boster Biological Technology Co.

2.3. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as previously described [20]. The information regarding primers were ANGPTL1(forward prrimer:5′-GATT TAATGCCACCACCTGATC-3′ and reverse primer:5′-CTGTTT

TCACACCATAACTGCA -3′), ANGPTL2 (forward prrimer:5′-AAGTG CACCTACACCTTCAT-3′ and reverse primer:5′- CTCATTGTTGAGCAGC TCTAGC -3′), ANGPTL3 (forward prrimer:5′-GATCACAAAGCAAAAGG ACACT-3′ and reverse primer:5′- GGTTGTTTTCTCCACACTCATC -3′), ANGPTL4(forward prrimer:5′- TCCTGGACCACAAGCACCTAG AC-3′ and reverse primer:5′- CGGTTGAAGTCCACTGAGCCATC -3′), ANGPTL5 (forward prrimer:5′- AATGCACTTAGGACG

GTATTCA-3′ and reverse primer:5′- ATTTGCTAGACCACACTCG TTA-3′), ANGPTL6(forward prrimer:5′- ACAGAGTGGAGTGTA TGAA CTG-3′ and reverse primer:5′- AATAGGAGTCTCGGTC

CCTATC -3′), ANGPTL7(forward prrimer:5′- CACTTTGTTTTGGGC AATGAAC-3′ and reverse primer:5′- TTTGAGGGAGTAGGTAGATCCA -3′), ANGPTL8(forward prrimer:5′- ATGGAGGAGG

ATATTCTGCAG-3′ and reverse primer:5′- AAGACCTCAAATTCTCG GTAGG -3′), and universal probe the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward prrimer 5′-AGCCACATCGC

TCAGACAC-3′ and reverse primer 5′-GCCCAATACGACCAAA TCC-3′)

2.4. Transfection with shRNA plasmid

The following shRNA and vector controls were purchased from Shanghai GeneChem Co. Transfection was performed using Lipofectamine 200 according to the manufacturer’s instructions. ANGPLT6 shRNA (shANGPTL6) and the control shRNA (Ctrl) vectors were transfected into GCIY cells. After transfection, the cells were cultured in culture medium containing 3 mg/ml puromycin (Invitrogen, USA).

2.5. In vitro HUVEC tube formation assay

When the cells grew to 80% confluence, they were starved for 24 h (serum-free culture). After thawing at 4 ˚C overnight, 10 u l of Matrigel (growth factor reduced, BD Biosciences, USA) was precoated onto a u-Slide Angiogenesis (ibidi, USA) and then incubated at 37 ˚C for at least 2 h to conform Matrigel solidification. HUVECs at a density of 2×10^5 cells/ml in different culture conditions were seeded onto each u-slide. After incubation at 37 ˚C for 5 h, networks images were taken under an inverted microscope.

2.6. HUVEC proliferation assay

HUVECs during the logarithmic growth period were used for a proliferation assay. Then, 100 ul (2×10^3/well) cells were added to a 96-well plate and replenished in the conditioned medium after 24 h. Cell growth was calculated using a Cell Counting Kit-8 (CCK8) (Dojiindo, Kumamoto, Japan), according to the protocol provided by the manufacturer. The HUVECs were stained with 10 µl CCK8 for 2 h at 37 ˚C in a CO2 incubator for 24 h, 48 h, and 72 h, respectively. The spectrophotometric absorbance values at 450 nm (OD value) were measured by a microplate reader. All assays were performed in triplicate.

2.7. Migration and invasion assays

Migration and invasion assays were assessed using chambers with a size diameter of with 8 microns (Corning, USA). The upper chambers were separately inculated with 1×10^4 each type of cell. Then, the different conditioned media were added to the lower chamber. For the invasion experiment, the inner surface of the membrane was precoated with 100 µl of 1 mg/ml Matrigel (BD, USA) to form an artificial

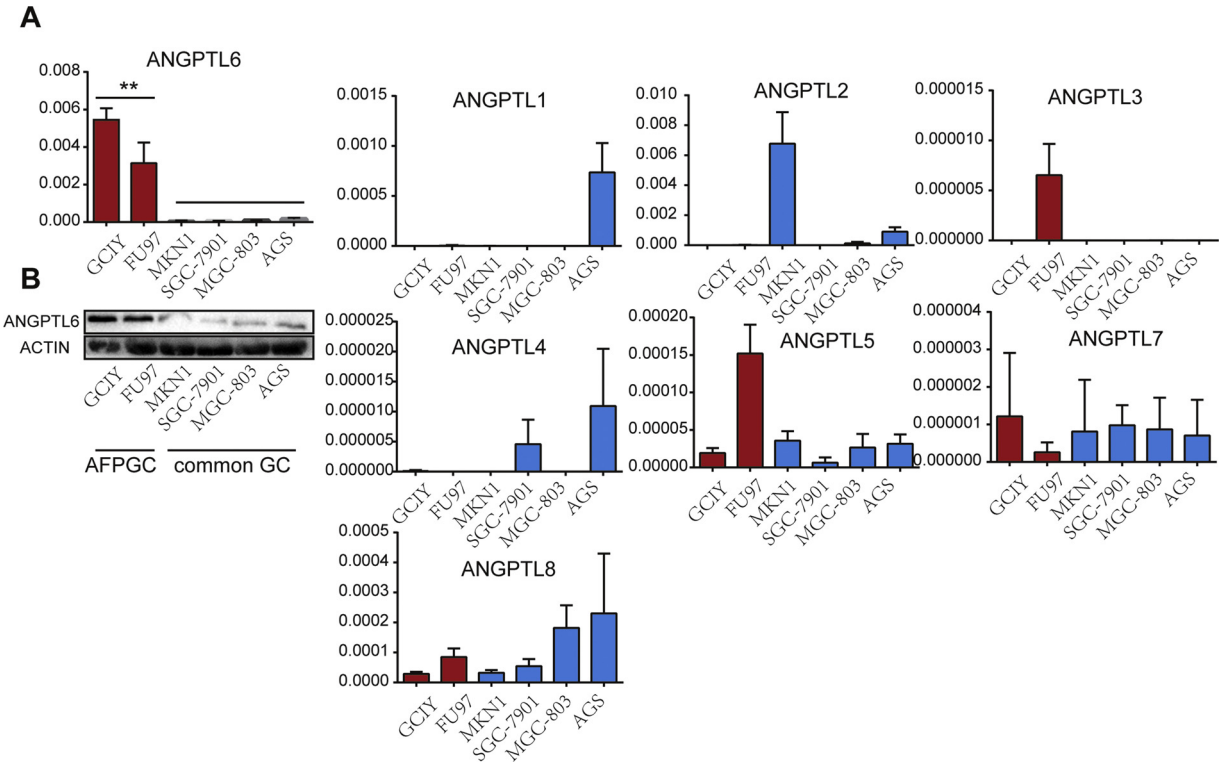

Fig. 1. ANGPTL6 is overexpressed in AFP-producing gastric cancer cell lines. (**A**) The RNA expression of ANGPTL1-8 in six gastric cancer cell lines (GCIY, FU97, MKN1, SGC-7901, MGC-803, AGS). (**B**) The protein level of ANGPTL6 in AFPGC is much higher than that in common gastric cancer. The data are expressed as the mean ± standard deviation (SD) and are representative of three independent experiments. *P < 0.05; **P < 0.01 compared with control.

basement. After 24 h incubation and fixation with formaldehyde, the cells that did not migrate through the holes and removed by cotton swabs. Subsequently, the cells inside the filter membrane were subjected to 0.1% crystal violet for 20 min. The cells that penetrated the lower membrane were enumerated. Cells in five random fields represent as the average number of cells/field of each membrane. In each case, triplicate independent experiments were conducted for each group.

2.8. Cell apoptosis assay

An Annexin V-FITC/PI Apoptosis Kit (BD Biosciences, San Jose, CA, USA) was used to investigate cell apoptosis. Briefly, a total of 1×10^5 cells were harvested for 24 h, washed using PBS and resuspended in 100 µl binding buffer at room temperature. Annexin V-FITC (5 µl) was then added and the cell sample was incubated in the dark for 5 min before incubation for another 15 min in the dark with 5 µl PI. The fluorescence intensity analysis was calculated by flow cytometry (FACS Calibur, BD Biosciences). Details are subject to the manufacturer's instructions.

2.9. The Kaplan- Meier plotter

ANGPTL6 was entered into an online database, the Kaplan-Meier Plotter (www.kmplot.com). To obtain a Kaplan-Meier plot to assess the prognostic value of ANGPTL6 RNA levels. A total of 882 clinical gastric cancer patients were included to analyze overall survival (OS). Patients were separated into two groups by best cutoff (high vs. low expression) and evaluated via a Kapan-Meier survival curve. Hazard rations (HRs) with 95% confidence intervals (95% CIs) and log rank P value were used to assess statistical significance.

2.10. Immunohistochemistry analysis

All specimens were fixed in 4% formalin and embedded in paraffin before IHC analysis. All procedures were performed according to our previous reports [20]. The staining of tumor sections was performed by three independent researchers under blinded methods. Five microscope fields were selected randomly for investigation to determine the positive staining area and staining intensity of tumor cells.

2.11. In vivo tumor growth inhibition experiments

Ten five-week-old male nude mice (4–6 weeks old, Chinese Academy of Science) were maintained under specific pathogen-free conditions and given treatment in accordance with institutional guidelines. Each mouse was independently injected subcutaneously (5 × 10^6 GCIY cells or GCIY-shANGPLT6 per site) into the right flanks. Tumor volume was calculated by the equation: tumor volume = 1/2(length × width^2). Xenograft samples were collected, fixed in formalin and embedded in paraffin. The Committee approved all animal work.

2.12. Statistical analysis

Statistical analysis was performed as described previously [20]. In comparisons of ANGPTL1-8 expression levels, statistical significance was evaluated using rank sum test. All data are presented as the mean ± standard error of the mean (SEM) of three independent experiments. Student's *t*-test was used to compare the mean value of any two groups. SPSS (version 23.0) was used for the statistical analysis. P < 0.05 was considered statistically significant.

3. Results

3.1. Overexpression of ANGPTL6 in AFPGC cell lines compared to that of common gastric cell lines

The clinical features of AFPGC are similar to those of hepatocellular carcinoma (HCC). It is well known that HCC is a typical hypervascular solid tumor. The invasive tumor cells exhibit vasculogenic mimicry, simulating the angiogenesis network, which plays a vital role in the malignancy of HCC. Therefore, we hypothesized that AFPGC secretes certain molecules that promote angiogenesis mimicking HCC. To investigate whether angiopoietin-like proteins (ANPGTLs) respond to gastric cancer progression, we first screened all eight members of the ANGPTL family and examined their endogenous mRNA expression levels of in six human gastric cancer cell lines by qRT-PCR. Strikingly, the results demonstrated that the mRNA expression of ANGPTL6 was abundant in all ANGPTL family members, while the expression of the other seven members was nearly undetectable in all gastric cancer cell lines (Fig. 1A). Furthermore, the mRNA expression of ANGPTL6 in two AFPGC cell lines (GCIY and FU97) was much higher than that in four common gastric cancer cell lines (MKN1, SGC-7901, MGC-803, AGS) (Fig. 1A). Western blot analysis confirmed that the endogenous protein level of ANGPTL6 in AFPGC cell lines was much higher than that in CGC cell lines (Fig. 1B).

3.2. ANGPTL6 expression was associated with the microvessel density of AFPGC patients

To investigate the importance of ANGPTL6 in gastric cancer, compared to patients with a low expression of ANGPTL6 (Fig. 2A), patients with high levels of ANGPTL6 had a poorer prognosis. In addition, we found that the expression of ANGPTL6 was positively correlated with the expression of CD34 (a specific biomarker of vascular endothelial cells) (Fig. 2B). These findings revealed that the upregulation of ANGPTL6 might have a significant role in AFPGC progression and is associated with angiogenesis.

3.3. ANGPTL6 regulates HUVEC migration and tube formation in vitro

Previous studies have reported that ANGPTL family members (ANGPTL2 [21], and ANGPTL4 [22]) can exert angiogenic or growth-promoting effects on endothelial cells. Therefore, we performed proliferation, chemotactic migration, and tubule formation of endothelial cells to investigate whether ANGPTL6 has a similar biological effect on endothelial cells in vitro.

We used the CCK8 assay to evaluate the pro-proliferation effect of endothelial cells administered ANGPTL6. The proliferation of endothelial cells treated with ANGPTL6 was increased with no significant difference compared with that in the control group, even with a high dose of recombinant ANGPTL6 (Fig. 3A), whereas ANGPTL6 induced a concentration-dependent chemotactic migration of endothelial cells in vitro (Fig. 3B). Finally, we performed tube formation assays to detect the effect of ANGPLT6. Consistent with the results above, ANGPLT6 facilitated the formation of a loop (Fig. 3C). To gain insight into the molecular mechanisms underlying this ANGPTL6-mediated chemotaxis, we conducted western blotting analysis to examine several important signaling pathways involved in angiogenesis. We found that transient stimulation of HUVECs with recombinant ANGPTL6 caused a significant increase in the phosphorylation of pro-survival kinase (AKT) and extracellular signal-related kinase 1 and (ERK1/2) (Fig. 3D). Furthermore, pretreating HUVECs with a specific AKT inhibitor or ERK1/2 inhibitor for two hours significantly abolished ANGPTL6-induced migration and tube formation, suggesting that the activation of AKT and ERK1/2 signaling is involved in ANGPTL6-mediated endothelial cell chemotaxis (Fig. 3E, F). Above all, ANGPTL6 can promote the chemotaxis, migration, and tube-forming ability of endothelial cells.

3.4. ANGPTL6 decreased the apoptosis but not the mitotic activity, migration and invasion of AFPGC

To interrogate the loss-of-function regulation of ANGPTL6, we performed shRNA-mediated knockdown of ANGPTL6 through control siRNA (shCtrl) or two independent shRNAs against ANGPTL6(shANGPTL6-1, shANGPTL6-2) in GCIY cells (an AFPGC cell line). qRT-PCR and immunoblotting were performed to assess the effective knockdown (Fig. 4A, B). Compared to the condition medium of GCIY-shControl, the conditioned medium of GCIY-shANGPTL6-1 or shANGPTL6-2 significantly impaired the tube formation ability of HUVECs (Fig. 4C). Previous studies have reported that some ANGPTL family members can exert anti-apoptotic effects on cells. Therefore, investigated whether the biological behavior changed in AFPGC cells. As expected, our results showed that silencing of ANGPTL6 significantly increased the levels of cell apoptosis compared to that in the control (Fig. 4D). In addition, we investigated how ANGPTL6 influences AFPGC cell viability in vitro. As shown in Fig. 4 E and F. ANGPTL6 loss led to reduced cell viability measured by cell migration and invasion assay. Above results suggest that ANGPTL6 may prevent cancer cells from undergoing apoptosis and promote cell viability.

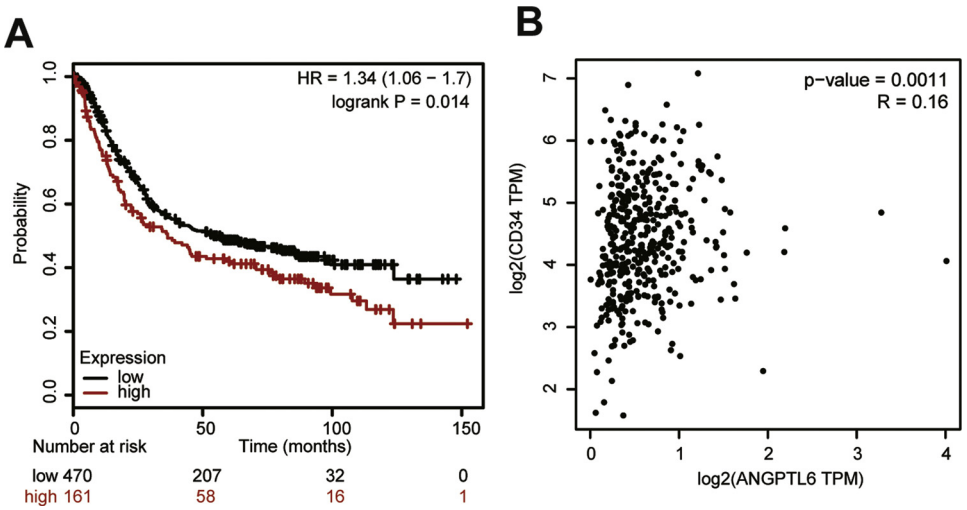

Fig. 2. ANGPTL6 has clinical significance. (**A**) A high expression of ANGPTL6 confers a poor prognosis. (**B**) The level of ANGPTL6 is positively correlated with CD34 (an endothelial cell marker).

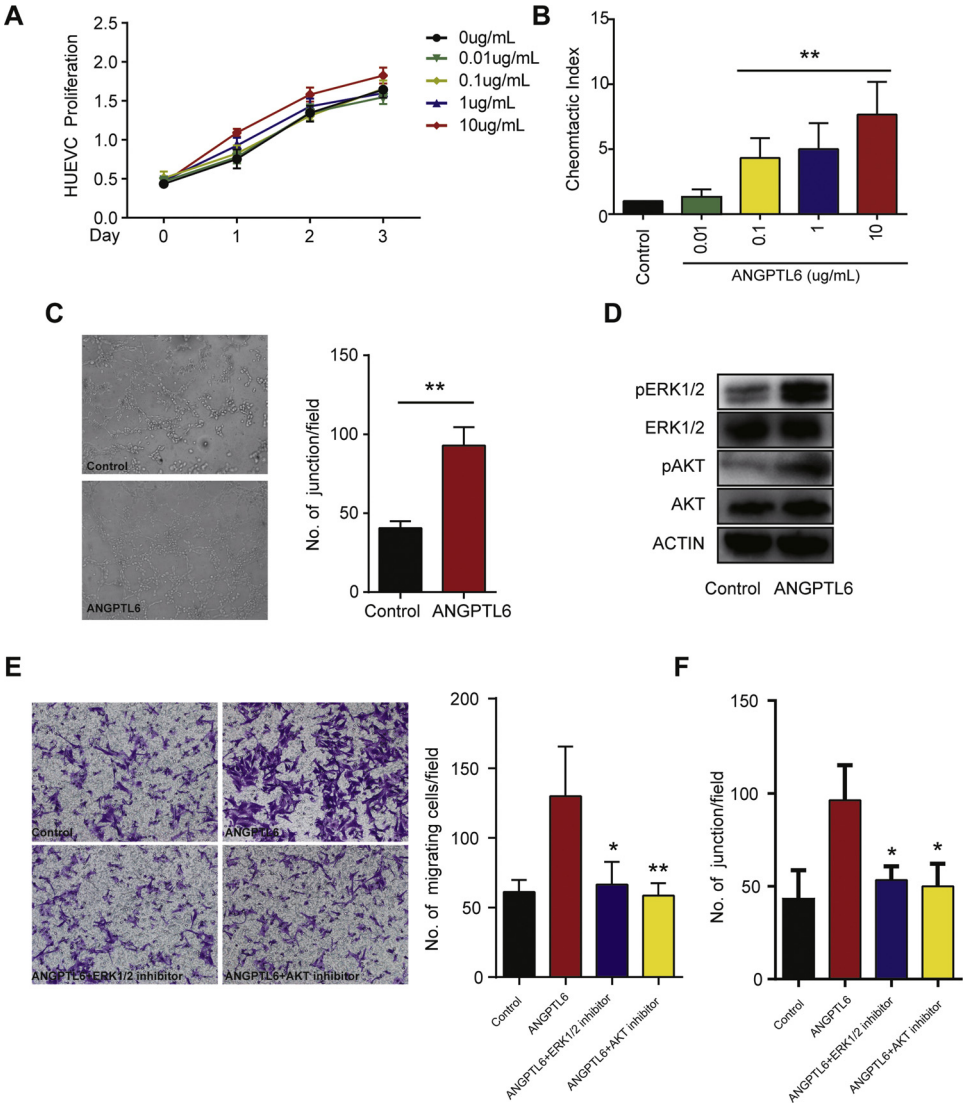

Fig. 3. ANGPTL6 promotes endothelial cell migration and tube formation via ERK1/2 and AKT. (**A**) The proliferation of endothelial cells was increased without statistical significance after exposure to ANGPTL6. (**B**) ANGPTL6 simulated the migration of endothelial cells. (**C**) Tube formation ability was increased by pretreatment with ANGPTL6. (**D**) Western blotting revealed that phospho-AKT and ERK1/2 were upregulated on exposure to ANGPTL6. (E) AKT or ERK1/2 inhibitor abrogated the pro-migration effect of ANGPTL6. (**f**) AKT or ERK1/2 inhibitor attenuated the pro-formation ability of ANGPTL6. *P < 0.05; **P < 0.01 compared with the control. The data are expressed as the mean ± SD and are representative of three independent experiments.

3.5. Targeting ANGPTL6 impairs AFPGC tumor growth in vivo

To further assess the contribution of ANGPTL6 to AFPGC tumorigenesis, we performed in vivo studies using a lentivirus shRNA approach. As expected, the mean tumoral volume or weight of the shANGPTL6 group was significantly smaller than that of the control group (Fig. 5A, B and C). Moreover, the expression of Ki-67 (an cell proliferation markers) and the expression of CD34 was remarkably decreased in the shANGPTL6 group compared to that in the controls (Fig. 5 D). These results indicated that ANGPTL6 may be a possible therapeutic target in AFPGC.

4. Discussion

Angiogenesis is considered a fundamental step in tumor development at all stages. Cancer cells produce and secrete numerous growth factors to induce tumor neovascularization and improve the blood supply and nutrient deficiency of the vascular system in response to environmental stimuli. Upregulated expression of proangiogenic factor can activate dormant microvascular endothelial cells, facilitating them to lose the their cell–cell connections, migrate into the surrounding space of blood vessels, decrease endothelial cell apoptosis, proliferate extensively, and form vascular structures.

To the best of our knowledge, the present study provides important data concerning the role of ANGPLT6 in AFPGC development. First, ANGPTL6 was identified as a major driver gene for angiogenesis among eight members of the ANGPTL family in AFPGC. Second, recombinant ANGPTL6 or cell supernatants from AFPGC promoted the endothelial cell migration, invasion, and tubule formation, and decreased apoptosis, but not cell proliferation. Furthermore, ANGPTL6 prevented cancer cells from undergoing apoptosis and enhanced cancer cell migration and invasion. Importantly, anti-angiogenesis drugs, such as bevacizumab may be a useful drug for AFPGC.

ANGPTL6 is a versatile member of the ANGPTL family. Most ANGPTLs are acknowledged to have angiogenic effects [23] and to modulate glucose, lipid, and energy metabolism [24–28]. Although some of them are not fully characterized and, ANGPTL6 in particular is a less prominent member of the family. The protein is secreted as 52 kDa monomers. Originally, ANGPTL6 was discovered in human liver [29]. As a hepatocyte-derived factor, it appears to be involved in the regulation of metabolic homeostasis and epidermal regeneration [29]. Several studies have reported that some ANGPTLs exert angiogenesis in several types of human tumors, including breast cancer, nonsmall lung cancer, colorectal cancer, prostate cancer, pancreatic cancer, ovarian cancer and glioblastoma. ANGPTL3 induces endothelial cell adhesion, and migration and promoted blood vessel formation [30]. ANGPTL7 was revealed as a pro-angiogenic factor and led to the formation of blood vessels in a Matrigel sponge experiment [15]. In contrast, it was

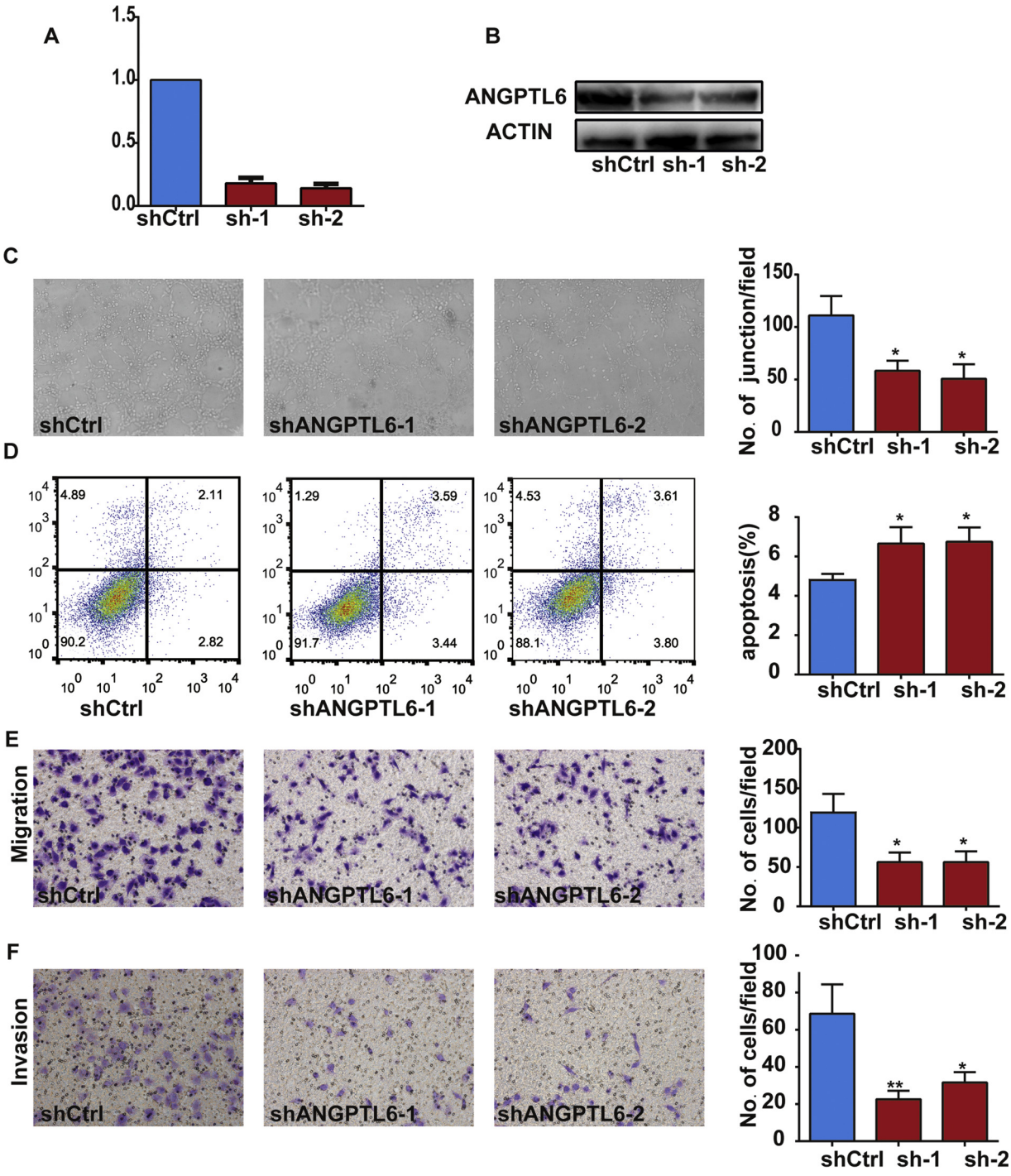

Fig. 4. ANGPTL6 loss promoted the apoptosis, migration, and invasion of AFPGC. (**A** and **B**) RT-PCR and western blotting revealed downregulation of ANGPTL6 in GCIY cells. (**C**) Tube formation assays and quantification of the numbers are shown. Magnification, 200 × . Migration assays (**D**) and invasive experiments (E) were performed after the knockdown of ANGPTL6 in GCIY cells. Magnification, 200 × . The data are expressed as the mean ± SD of three independent experiments, each performed in triplicate. *P < 0.05; **P < 0.01 compared with the control.

hypothesized that certain ANGPTLs may play an inhibitory role in tumor development. ANGPTL1 inhibits epithelial-mesenchymal transition and suppresses lung cancer by abrogating SLUG [31]. ANGPTL1 also decreases angiogenesis by inhibiting the phosphorylation of ERK1/2 and AKT and interacts with integrin α1β1 to suppress the JAK2/STAT3 signaling pathway [32]. Until now, the role of ANGPTL6 in gastric cancer has not been clearly defined. The current study is the first to show that ANGPTL6 is preferentially expressed in AFPGC compared to common gastric cell lines, while the other seven members of ANGPTLs were nearly undetectable in eight gastric cancer cell lines. Moreover, our results revealed that the expression of ANGPTL6 is positively correlated with the expression of micro-vessel density in AFPGC patients. The above results indicated that the overexpression of ANGPTL6 is involved in AFPGC progression.

ANGPTL6, signals through ERK1/2. However, the role of this secretory protein in the tumor microenvironment remains largely unknown. Indeed, we consistently found that ANGPTL6 enhances the migration and capillary network formation ability of HUVECs. Notably, we also consistently observed that ANGPTL6 is able to support the survival and motility of endothelial cells, as indicated by activation of

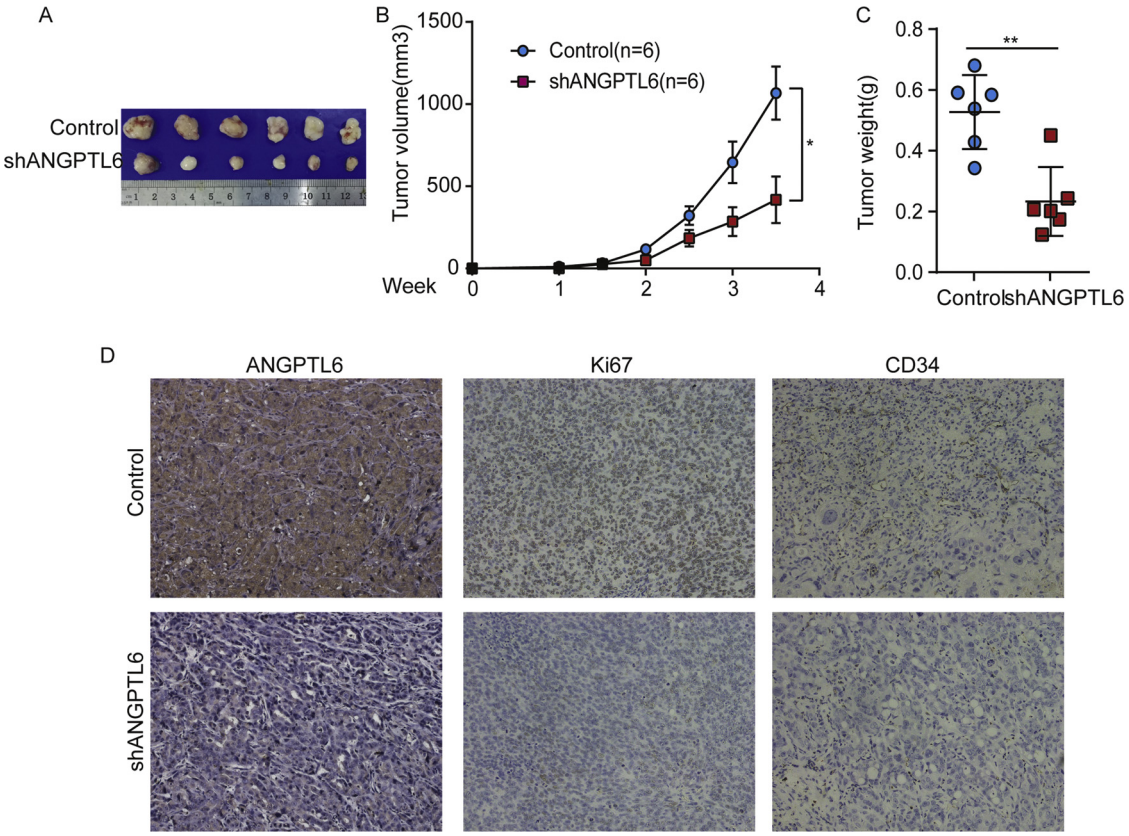

Fig. 5. ANGPTL6 knockdown inhibits tumor growth in vivo. (**A**) Tumors are shown, along tumor volumes (**B**) and tumor weights (**C**). All tumors derived from the indicated cells were shown and tumor volumes and weights were measured. The results are shown as the mean ± SEM of tumor volumes (n = 6) (**P < 0.01, Mann–Whitney test). (**D**) Tumor samples were formalin-fixed, paraffin-embedded, and sliced for IHC assay. Representative images of the indicated ANGPTL6, Ki-67, and CD34 staining are shown. Magnification, 200 × . *P < 0.05; **P < 0.01 compared with the control.

the pro-survival kinase AKT and by ERK activation, as well as by migration and invasion assays. In sum, ANGPTL6 is likely to exert a wide and complete biofunction in endothelial cells, thereby favoring in cancer angiogenesis. This finding represents an attractive therapeutic target for anti-tumor therapy. In vitro studies revealed that the biofunction of ANGPTL6 in gastric cancer progression not only increased endothelial cell migration and formation tube structures, but also decreased cancer cell apoptosis. In previous studies, ANGPTL6 was confirmed to have a powerful effect on the migration of endothelial cells, and we demonstrated that ANGPTL6 leads to endothelial cell proliferation and tube formation. Together, these observations underlie the notion that neovascularization of tumors is mediated by ANGPTL6 in AFPGC. Further investigation confirmed that the activation of FAK-, ERK1/2 and signaling pathways in endothelial cells may exert a key role in these ANGPLT6-mediated effects.

In addition to the effect on endothelial cells, we also examined the effect of ANGPTL6 on tumor cells. Different types of cytokines, chemokines, and other secretory proteins in the surrounding microenvironment were secreted into the tumor bed, and these proteins not only affect the activity of stromal cells, such as endotheliocytes, fibroblasts, immune cells and inflammatory cells, but also modulate the biological behavior of the tumor cells themselves. ANGPTL2 loss suppresses gastric cell growth, invasion, and migration. ANGPTL2 is a proinflammatory factor whose overexpression leads to EMT and promotes the aggressiveness in pancreatic cancer [33]. ANGPTL2 accelerated the invasiveness and metastasis of colorectal cells, contributing to tumor development via the promotion of vessel formation and epithelial-mesenchymal transition. ANGPTL3 knockdown decreased oral cancer proliferation with inactivated extracellular regulated kinase, increased cyclin-dependent kinase inhibitors and arrested the cell-cycle. Nevertheless, ANGPTL4 plays a completely different role depending on the tumor type. ANGPTL4 can restore the melanoma metastasis process by decreasing vascular activity and tumor cell activity and mobility, through dual effects on blood vessels and tumor compartments. Moreover, ANGPTL4 accelerates venous invasion and tumor metastasis in colorectal cancer [34] and gastric cancer [35]. Substantial experimental evidence indicated that downregulation of ANGPTL6 in cancer cells leads to cell apoptosis and migration. We investigated whether knockdown of ANGPTL6 induced cell apoptosis. Regarding cell viability and invasiveness, the numbers of migratory and invasive of cells were remarkably decreased by shRNA-ANGPTL6 compared with corresponding controls. The tumor volume and size were significantly inhibited after knockdown of ANGPLT6 in vivo. The above findings suggest that ANGPTL6 promotes not only tumor endotheliocyte angiogenesis but also the malignancy of the tumor cells themselves.

In conclusion, our study describes the role of ANGPTL6 in AFPGC angiogenesis and progression. The features of ANGTL6-endothelial cell-angiogenesis are not only an effective biomarker for AFPGC diagnosis but also an attractive therapeutic intervention for future studies.

Disclosure statement

The authors disclose no competing interests in this research.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (81772511) and the Shanghai Science and Technology Commission (17411951400,15411961900, 15411961900).

References

[1] D. Gitlin, A. Perricelli, G.M. Gitlin, Synthesis of -fetoprotein by liver, yolk sac, and gastrointestinal tract of the human conceptus, Cancer Res. 32 (1972) 979–982.

[2] H. Ishikura, Y. Fukasawa, K. Ogasawara, T. Natori, Y. Tsukada, M. Aizawa, An AFP-producing gastric carcinoma with features of hepatic differentiation. A case report, Cancer 56 (1985) 840–848.

[3] X.D. Li, C.P. Wu, M. Ji, J. Wu, B. Lu, H.B. Shi, J.T. Jiang, Characteristic analysis of alpha-fetoprotein-producing gastric carcinoma in China, World J. Surg. Oncol. 11 (2013) 246.

[4] X. Liu, Y. Cheng, W. Sheng, H. Lu, Y. Xu, Z. Long, H. Zhu, Y. Wang, Clinicopathologic features and prognostic factors in alpha-fetoprotein-producing gastric cancers: analysis of 104 cases, J. Surg. Oncol. 102 (2010) 249–255.

[5] D. Wang, C. Li, Y. Xu, Y. Xing, L. Qu, Y. Guo, Y. Zhang, X. Sun, J. Suo, Clinicopathological characteristics and prognosis of alpha-fetoprotein positive gastric cancer in Chinese patients, Int. J. Clin. Exp. Pathol. 8 (2015) 6345–6355.

[6] Z. Li, X. Hou, J. Chen, H. Sun, Y. Mi, Y. Sui, Y. Li, J. Xie, Y. Qiao, X. Lei, X. Che, J. Liu, Efficacy and safety of SOX chemotherapy with or without surgery in AFP-producing advanced gastric cancer, Oncol. Lett. 14 (2017) 579–586.

[7] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.

[8] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat. Med. 1 (1995) 27–31.

[9] C. Carbone, G. Piro, V. Merz, F. Simionato, R. Santoro, C. Zecchetto, G. Tortora, D. Melisi, Angiopoietin-like proteins in angiogenesis, inflammation and Cancer, Int. J. Mol. Sci. (2018) 19.

[10] G. Santulli, Angiopoietin-like proteins: a comprehensive look, Front. Endocrinol. 5 (2014) 4.

[11] S. Kersten, Regulation of lipid metabolism via angiopoietin-like proteins, Biochem. Soc. Trans. 33 (2005) 1059–1062.

[12] R. Sato, M. Yamasaki, K. Hirai, T. Matsubara, T. Nomura, F. Sato, H. Mimata, Angiopoietin-like protein 2 induces androgen-independent and malignant behavior in human prostate cancer cells, Oncol. Rep. 33 (2015) 58–66.

[13] H. Horiguchi, M. Endo, Y. Miyamoto, Y. Sakamoto, H. Odagiri, T. Masuda, T. Kadomatsu, H. Tanoue, I. Motokawa, K. Terada, M.S. Morioka, I. Manabe, H. Baba, Y. Oike, Angiopoietin-like protein 2 renders colorectal cancer cells resistant to chemotherapy by activating spleen tyrosine kinase-phosphoinositide 3-kinase-dependent anti-apoptotic signaling, Cancer Sci. 105 (2014) 1550–1559.

[14] A.S. El-Shal, H.E. Zidan, N.M. Rashad, F.M. Wadea, Angiopoietin-like protein 3 and 4 expression 4 and their serum levels in hepatocellular carcinoma, Cytokine 96 (2017) 75–86.

[15] M. Parri, L. Pietrovito, A. Grandi, S. Campagnoli, E. De Camilli, F. Bianchini, S. Marchio, F. Bussolino, B. Jin, P. Sarmientos, G. Grandi, G. Viale, P. Pileri, P. Chiarugi, R. Grifantini, Angiopoietin-like 7, a novel pro-angiogenetic factor over-expressed in cancer, Angiogenesis 17 (2014) 881–896.

[16] Y. Oike, K. Yasunaga, T. Suda, Angiopoietin-Related/Angiopoietin-Like proteins regulate angiogenesis, Int. J. Hematol. 80 (2004) 21–28.

[17] Y. Oike, M. Akao, K. Yasunaga, T. Yamauchi, T. Morisada, Y. Ito, T. Urano, Y. Kimura, Y. Kubota, H. Maekawa, T. Miyamoto, K. Miyata, S. Matsumoto, J. Sakai, N. Nakagata, M. Takeya, H. Koseki, Y. Ogawa, T. Kadowaki, T. Suda, Angiopoietin-related growth factor antagonizes obesity and insulin resistance, Nat. Med. 11 (2005) 400–408.

[18] J.G. Cui, Y. Zhao, P. Sethi, Y.Y. Li, A. Mahta, F. Culicchia, W.J. Lukiw, Micro-RNA-128 (miRNA-128) down-regulation in glioblastoma targets ARP5 (ANGPTL6), Bmi-1 and E2F-3a, key regulators of brain cell proliferation, J. Neurooncol. 98 (2010) 297–304.

[19] S. Marchio, M. Soster, S. Cardaci, A. Muratore, A. Bartolini, V. Barone, D. Ribero, M. Monti, P. Bovino, J. Sun, R. Giavazzi, S. Asioli, P. Cassoni, L. Capussotti, P. Pucci, A. Bugatti, M. Rusnati, R. Pasqualini, W. Arap, F. Bussolino, A complex of alpha6 integrin and E-cadherin drives liver metastasis of colorectal cancer cells through hepatic angiopoietin-like 6, EMBO Mol. Med. 4 (2012) 1156–1175.

[20] E.B. Chen, S.L. Zhou, X.G. Pang, D. Yin, P.Z. Miao, Y. Yang, Q. Chen, K. Zhu, D.M. Gao, T.S. Liu, X.Y. Wang, Y.H. Shi, W.Z. Wu, J. Zhou, Z.J. Zhou, Z. Dai, Prostate-derived ETS factor improves prognosis and represses proliferation and invasion in hepatocellular carcinoma, Oncotarget 8 (2017) 52488–52500.

[21] M.R. Richardson, E.P. Robbins, S. Vemula, P.J. Critser, C. Whittington, S.L. Voytik-Harbin, M.C. Yoder, Angiopoietin-like protein 2 regulates endothelial colony forming cell vasculogenesis, Angiogenesis 17 (2014) 675–683.

[22] D. Padua, X.H. Zhang, Q. Wang, C. Nadal, W.L. Gerald, R.R. Gomis, J. Massague, TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4, Cell 133 (2008) 66–77.

[23] M. Dhanabal, W.J. LaRochelle, M. Jeffers, J. Herrmann, L. Rastelli, W.F. McDonald, R.A. Chillakuru, M. Yang, F.L. Boldog, M. Padigaru, K.D. McQueeney, F. Wu, S.A. Minskoff, R.A. Shimkets, H.S. Lichenstein, Angioarrestin: an antiangiogenic protein with tumor-inhibiting properties, Cancer Res. 62 (2002) 3834–3841.

[24] M. Endo, Y. Yamamoto, M. Nakano, T. Masuda, H. Odagiri, H. Horiguchi, K. Miyata, T. Kadomatsu, I. Motokawa, S. Okada, H. Iwase, Y. Oike, Serum ANGPTL2 levels reflect clinical features of breast cancer patients: implications for the pathogenesis of breast cancer metastasis, Int. J. Biol. Markers 29 (2014) e239–245.

[25] T. Masuda, M. Endo, Y. Yamamoto, H. Odagiri, T. Kadomatsu, T. Nakamura, H. Tanoue, H. Ito, M. Yugami, K. Miyata, J. Morinaga, H. Horiguchi, I. Motokawa, K. Terada, M.S. Morioka, I. Manabe, H. Iwase, H. Mizuta, Y. Oike, ANGPTL2 increases bone metastasis of breast cancer cells through enhancing CXCR4 signaling, Sci. Rep. 5 (2015) 9170.

[26] L. Yang, T. Shu, Y. Liang, W. Gu, C. Wang, X. Song, C. Fan, W. Wang, GDC-0152 attenuates the malignant progression of osteosarcoma promoted by ANGPTL2 via PI3K/AKT but not p38MAPK signaling pathway, Int. J. Oncol. 46 (2015) 1651–1658.

[27] M. Endo, M. Nakano, T. Kadomatsu, S. Fukuhara, H. Kuroda, S. Mikami, T. Hato, J. Aoi, H. Horiguchi, K. Miyata, H. Odagiri, T. Masuda, M. Harada, H. Horio, T. Hishima, H. Nomori, T. Ito, Y. Yamamoto, T. Minami, S. Okada, T. Takahashi, N. Mochizuki, H. Iwase, Y. Oike, Tumor cell-derived angiopoietin-like protein ANGPTL2 is a critical driver of metastasis, Cancer Res. 72 (2012) 1784–1794.

[28] C. Carbone, G. Piro, M. Fassan, A. Tamburrino, M.M. Mina, M. Zanotto, P.J. Chiao, C. Bassi, A. Scarpa, G. Tortora, D. Melisi, An angiopoietin-like protein 2 autocrine signaling promotes EMT during pancreatic ductal carcinogenesis, Oncotarget 6 (2015) 13822–13834.

[29] Y. Oike, K. Yasunaga, Y. Ito, S. Matsumoto, H. Maekawa, T. Morisada, F. Arai, N. Nakagata, M. Takeya, Y. Masuho, T. Suda, Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 9494–9499.

[30] G. Camenisch, M.T. Pisabarro, D. Sherman, J. Kowalski, M. Nagel, P. Hass, M.H. Xie, A. Gurney, S. Bodary, X.H. Liang, K. Clark, M. Beresini, N. Ferrara, H.P. Gerber, ANGPTL3 stimulates endothelial cell adhesion and migration via integrin alpha vbeta 3 and induces blood vessel formation in vivo, J. Biol. Chem. 277 (2002) 17281–17290.

[31] T.C. Kuo, C.T. Tan, Y.W. Chang, C.C. Hong, W.J. Lee, M.W. Chen, Y.M. Jeng, J. Chiou, P. Yu, P.S. Chen, M.Y. Wang, M. Hsiao, J.L. Su, M.L. Kuo, Angiopoietin-like protein 1 suppresses SLUG to inhibit cancer cell motility, J. Clin. Invest. 123 (2013) 1082–1095.

[32] Q. Yan, L. Jiang, M. Liu, D. Yu, Y. Zhang, Y. Li, S. Fang, Y. Li, Y.H. Zhu, Y.F. Yuan, X.Y. Guan, ANGPTL1 interacts with integrin alpha1beta1 to suppress HCC angiogenesis and metastasis by inhibiting JAK2/STAT3 signaling, Cancer Res. 77 (2017) 5831–5845.

[33] C. Carbone, T. Moccia, C. Zhu, G. Paradiso, A. Budillon, P.J. Chiao, J.L. Abbruzzese, D. Melisi, Anti-VEGF treatment-resistant pancreatic cancers secrete proinflammatory factors that contribute to malignant progression by inducing an EMT cell phenotype, Clin. Cancer Res. 17 (2011) 5822–5832.

[34] B. Mikhak, S. Weinsheimer, L. Pawlikowska, A. Poon, P.Y. Kwok, M.T. Lawton, Y. Chen, J.G. Zaroff, S. Sidney, C.E. McCulloch, W.L. Young, H. Kim, Angiopoietin-like 4 (ANGPTL4) gene polymorphisms and risk of brain arteriovenous malformations, Cerebrovasc. Dis. 31 (2011) 338–345.

[35] T. Nakayama, H. Hirakawa, K. Shibata, K. Abe, T. Nagayasu, T. Taguchi, Expression of angiopoietin-like 4 in human gastric cancer: ANGPTL4 promotes venous invasion, Oncol. Rep. 24 (2010) 599–606.