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*Original Article*

# Angiopoietin-like protein 2 is an important facilitator of tumor proliferation, metastasis, angiogenesis and glycolysis in osteosarcoma

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**Abstract:** *Background:* Solid tumors are often exposed to hypoxia. Hypoxia inducible factor (HIF-1α) upregulates numerous target genes associated with the malignant behavior of hypoxic cancer cells. Angiopoietin-like protein 2 (Angptl2), a member of the angiopoietin family, is a hypoxia-inducible gene. However, the role and potential mechanism of Angptl2, and the relationship between Angptl2 and hypoxia in osteosarcoma (OS) remain unclear. *Methods:* In this study, quantitative RT-PCR was performed to detect the levels of Angptl2 and HIF-1α, and western blot assay was performed to measure the expression of Angptl2, HIF-1α, CDK2, cyclin E1, P21, MMP2, MMP9, VEGFA, Ang II and HK2 in osteosarcoma cells and tissue. Subsequently, cell viability and cycle were analyzed using CCK-8 and flow cytometer assays. Cell migration, invasion and glycolysis were analyzed with Transwell, Scratch Test and glucose/lactic acid detection kits, respectively. Experiments *in vivo* were performed to value the effects of Angptl2 on the growth of osteosarcoma xenografts in mice. Immunofluorescent and immunohistochemistry staining were conducted to detect the expression of Ki-67 and Angptl2, respectively. *Results:* The results demonstrated that Angptl2 was highly expressed in OS cells, which was induced by hypoxia (HIF-1α). Additionally, Angptl2 overexpression regulated cell proliferation, invasion, migration and G1 phase arrest in OS cells. Moreover, Angptl2 promoted OS tumor growth in vivo tumor xenografts. Angptl2 might enhance angiogenesis and glycolysis by promoting VEGFA, Ang II and HK2 both in vitro and in vivo. *Conclusion:* In conclusion, the present findings indicated that hypoxia-induced Angptl2 expression was independent of HIF-1α in hypoxic OS cells. Angptl2 might promote OS cell proliferation, metastasis, angiogenesis and glycolysis, which could be regarded as a favorable marker for predicting a long survival time in patients with OS.

**Keywords:** Osteosarcoma, angiopoietin-like protein 2, hypoxia inducible factor, angiogenesis, glycolysis

## Introduction

Osteosarcoma (OS) is the most common aggressive bone malignancy and a leading cause of cancer death in adolescents less than 20 years old due to its rapid progression. Its early stage clinical symptoms are usually not obvious [1]. However, the prognosis of OS is poor with relatively low median survival time and 5-year survival rate [2]. Although the 5-year survival rate has increased from 20% to 60% with the optimization of therapy for osteosarcoma [3], the mechanisms that orchestrate the multiple oncogenic insults required for initiation and progression of osteosarcoma are unclear.

The tumor microenvironment plays important roles in tumor progression and metastasis which contribute to tumor cell proliferation, survival, migration, and invasion [4]. Loss of oxygen or nutrients such as glucose or amino acids occurs as a primary tumor mass expands, causing increased expression of hypoxia-inducible factor (HIF), which is consist of an obligate dimer of HIF-1α and HIF-1β subunits [5, 6]. According to some studies, HIF plays an important regulatory role in tumor angiogenesis,

energy metabolism, invasion, metastasis and many other biological processes [7, 8]. Latest study revealed that long non-coding RNA TUG1 could promote osteosarcoma cell metastasis by mediating HIF-1α [9]. HIF-2α affects proliferation and apoptosis of MG-63 osteosarcoma cells through MAPK signaling [10]. However, the molecular mechanisms between hypoxia and osteosarcoma still unclear, which will be beneficial to finding novel and effective interventions.

Angiopoietin-like protein 2 (Angptl2), a member of the angiopoietin-like family, is an adipose tissue-derived secretory glycoprotein [11]. Angptl2 plays multiple important roles in metabolic syndrome [12], angiogenesis [13], inflammatory carcinogenesis and tumor metastasis [14]. Angptl2 has been reported as a chronic inflammatory mediator, which is induced by hypoxia and nutrient starvation in the tumor microenvironment [15-17]. Angptl2 promotes metastasis of osteosarcoma cells through integrin a5b1, p38-MAPK, and matrix metalloproteinases [18]. In another study, GDC-0152 attenuates the malignant progression of osteosarcoma promoted by Angptl2 via PI3K/AKT signal pathway [19]. Previous study has shown that angiopoietin-like 4 promotes osteosarcoma cell proliferation and migration and stimulates osteoclastogenesis [20]. However, the role and potential mechanism of Angptl2, and the relationship between Angptl2 and hypoxia in osteosarcoma remain elusive.

The present study evaluated the hypoxia-induced expression of Angptl2 in five osteosarcoma cells. Using U2OS cells, the study assessed whether Angptl2 expression is dependent of HIF-1α under hypoxic conditions. Meanwhile, we investigated whether Angptl2 mediated tumor proliferation, metastasis, angiogenesis and glycolysis in both vitro and vivo.

## Materials and methods

### *Cell culture and treatment*

The human OS cell lines MG63, MHM, HOS-143B, U2OS and OSA were purchased from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C with 5% $CO_2$. Then, cells were divided into hypoxic induction group and normal culture group. Hypoxic culture with parameters set at 2.5% $O_2$, 5% $CO_2$, and 92.5% $N_2$ for 24 h and 48 h were used experimentally to simulate the hypoxic microenvironment within tumors. Normal culture with parameters set at 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for 24 h and 48 h, respectively.

For induction of HIF-1α, U2OS cells were exposed to 1 mM of the prolyl-4-hydroxylase inhibitor, dimethyloxalylglycine (DMOG), which elevates HIF-1α, mimicking effects associated with hypoxia [21]. After treated with DMOG for 18 h, cells were collected.

### *Cell transfection*

The Angptl2 overexpression and HIF-1α siRNA plasmid (si-HIF-1α-1 and si-HIF-1α-2) were purchased from GenePharm (Shanghai, China). The sequence was amplified and inserted into the pcDNA3.1 (+) vector. For transfection, the U2OS cells were grown in 6-well plates to 60-70% confluence, then were transfected with the indicated molecules using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. 48 h after transfection, cells were ready for the following experiments.

### *Reverse transcription quantitative PCR (RT-qPCR)*

Total RNA from cells was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed with the PrimeScript RT Reagent Kit (Invitrogen), and reverse transcription quantitative PCR (RT-qPCR) was performed using SYBR Premix Ex Taq (TaKaRa, China), following the manufacturer's instructions. GAPDH was used as an internal control. The primer sequences were as follows: Angptl2, forward: 5'-GAACCGAGTGCATAAGCAGGA-3', and reverse: 5'-GTGACCCGCGAGTTCATGTT-3'; HIF-1α, forward: 5'-GAACGTCGAAAAGAAAAGTCTCG-3', and reverse: 5'-CCTTATCAAGATGCGAACTCACA-3'; GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3', and reverse, 5'-GGCATGGACTGTGG TCATGAG-3'. We performed qRT-PCR using the ABI PRISM 7500 PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was used in each sample as relative quantification.

*Western blot analysis*

The cells and samples were collected and lysed at 48 h after treatment using RIPA buffer. Identical amounts of proteins were resolved by 10-15% SDS-PAGE and then transferred onto PVDF membranes (Millipore Corporation, Billerica, MA, USA). The membrane was incubated with specific antibodies (Angptl2, HIF-1α, CDK2, cyclin E1, P21, MMP2, MMP9, VEGFA, Ang II and HK2; all from Cell Signaling Technology Inc.) at 4°C overnight and peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA) at room temperature for 2 h. Chemiluminescence (Milipore Corporation) and densitometry analysis (Image J software) were applied to measure the proteins expression.

*Cell proliferation assay*

The proliferation of U2OS cells was measured using the CCK-8 assay (Sigma-Aldric, USA). Cells were seeded in 96-well culture plates ($1\times10^4$ cells/well, 100 μl/well) and cultured in appropriate medium containing 10% FBS for 24, 48 and 72 h, respectively. CCK-8 reagent (10 μl/well) was added to each well and cells were incubated at 37°C for 4 h. Next, the absorbance (optical density at 450 nm) was measured on an enzyme immunoassay analyzer (Bio-Rad, USA).

*Cell invasion assay*

Cell invasion assay were performed using 24-well culture plates (Transwell; Falcon, BD Biosciences). The lower chamber was filled with 600 μl DMEM containing 10% FBS. U2OS cells ($1\times10^5$ cell/well) were seeded to the upper chamber respectively. After 72 h incubation, the number of cells in the bottom well cells were counted using counting chamber.

*Cell migration assay*

For wound healing assay, U2OS cells were seeded into the chambers of a culture dish. ($4\times10^5$ cells/well). A pipette tip was used to create a wound after 48 h transfection. The cells were then cultured in serum-free medium. Cell migration was determined by detecting the average distance of growing cells migrated into wound surface under an inverted microscopy at 0 h and 72 h under an inverted light microscope (Nikon Corporation).

*Cell cycle assay*

After Angptl2 transfection, cell cycle assay was applied to determine cell cycle distribution. Briefly, U2OS cells were collected using 0.25% trypsin, washed, and then fixed with 70% ethanol at 4°C overnight. Then, the cells were stained with RNase A and PI at 4°C for 30 min. Finally, flow cytometer (FACSort; FACSCanto II, BD Biosciences, Franklin Lakes, USA) was performed to analyze the cell cycle distribution. Tests were repeated at least for 3 times.

*Glucose consumption and lactate production detection*

The glucose consumption and lactate production were evaluated using glucose detection and lactic acid detection kits (Zhongsheng Biotech Co. Ltd., Beijing, China) according to the manufacturer's instruction [22].

*Immunofluorescence staining*

Briefly, 5 μm-thick histologic sections were incubated in 5% skim milk/TBST at room temperature for 30 min to block nonspecific immunoreactions. Next, sections were incubated overnight at 4°C with a rabbit polyclonal Ki-67 antibody (Thermo Fisher Scientific, Inc.) at 1:500 dilution at room temperature for 1 h, and then with the secondary antibodies conjugated with Alexa Fluor-488 (1:1,000) at room temperature for 1 h. Sections were then counterstained with DAPI to stain the cell nucleus. All cover slips were mounted using Prolong® Diamond Antifade Mountant (ABI, USA).

*Xenograft experiments*

The female BALB/c nude mice at the age of 5 weeks were anesthetized. Angptl2, empty vehicle and control cells were suspended in cold PBS ($1\times10^6$ cells/100 μl) and injected into the mice subcutaneously on flanks. The tumor volume and the mice weight were observed and measured after 1, 5, 10, 15 and 20 days. The tumor volume ($V/cm^3$) was calculated according to the equation: $V=(a\times b^2)/2$. Mice were sacrificed when tumors reached maximum allowed size (20 mm in diameter). Subsequently, the expression of protein factors in the tumor were detected.

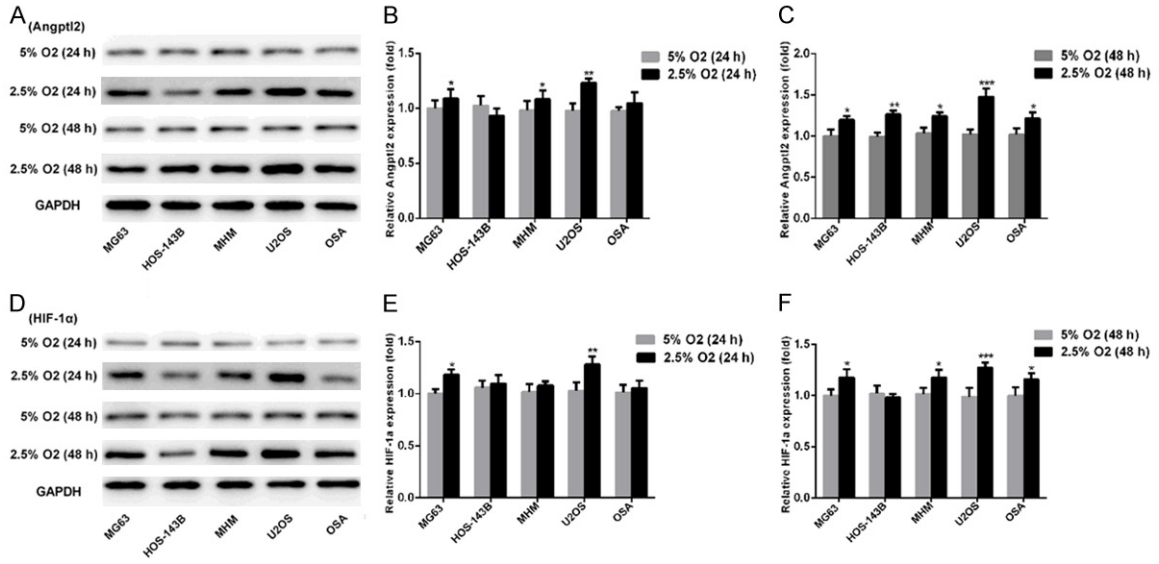



**Figure 1.** Expression of Angptl2 and HIF-1α is highly expressed in OS cells under hypoxia condition. (A) Representative picture of western blotting for Angptl2 expression in hypoxia or normal conditions cultured for 24 h and 48 h. (B and C) Histograms of the statistical results in (A). (D) Representative picture of western blot for HIF-1α expression in hypoxia or normal conditions cultured for 24 h and 48 h. (E and F) Histograms of the statistical results in (D). N=5; *P<0.05, **P<0.01 and ***P<0.001 versus 5% $O_2$.

Mice were bred in the Animal Core Facility by following procedures approved by Animal Ethics Committee in Liuzhou People's Hospital.

*Immunohistochemistry*

Immunohistochemistry (IHC) was performed for continuous sections from paraffin-embedded blocks. Antigen retrieval was performed by microwaving for 3 min in citrate-buffered solution (pH 6.0). Blocking was done by incubation with 10% goat serum at room temperature for 30 min. Sections were incubated with Angptl2 antibodies overnight at 4°C. Staining with secondary antibody (conjugated with horseradish peroxidase) was performed for 1 h at room temperature. Sections were finally stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. The images were captured with software (Leica Q-win V2.0, Germany) accompanying with Leica DMLA microscope (Leica Inc., Germany).

*Statistical analysis*

Data are expressed as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, United States) was used for all statistical analyses. Each experiment was repeated at least three times. The Student's t-test was used and P<0.05 was considered to indicate a statistically significant difference.

## Results

*Angptl2 is highly expressed in OS cells induced by HIF-1α*

In order to understand the expression of Angptl2 in hypoxia induced osteosarcoma cells, the protein level of Angptl2 in five osteosarcoma cell lines, MG63, MHM, HOS-143B, U2OS and OSA, were measured by western blot. As shown in **Figure 1**, the expression of Angptl2 and HIF-1α in five osteosarcoma cell lines cultured with 2.5% $O_2$ were significantly increased compared with normoxic condition (5% $O_2$). It was shown the highest expression of Angptl2 and HIF-1α in hypoxia for 48 h in U2OS cells. Therefore, the overexpression of Angptl2 and HIF-1α was induced under hypoxia condition in OS cells, and the U2OS cells were chosen to conduct for further experiments.

To further confirm the relationship between Angptl2 and HIF-1α, DMOG and si-HIF-1α were introduced as HIF-1α activator and inhibitor, respectively. As shown in **Figure 2A** and **2B**, the

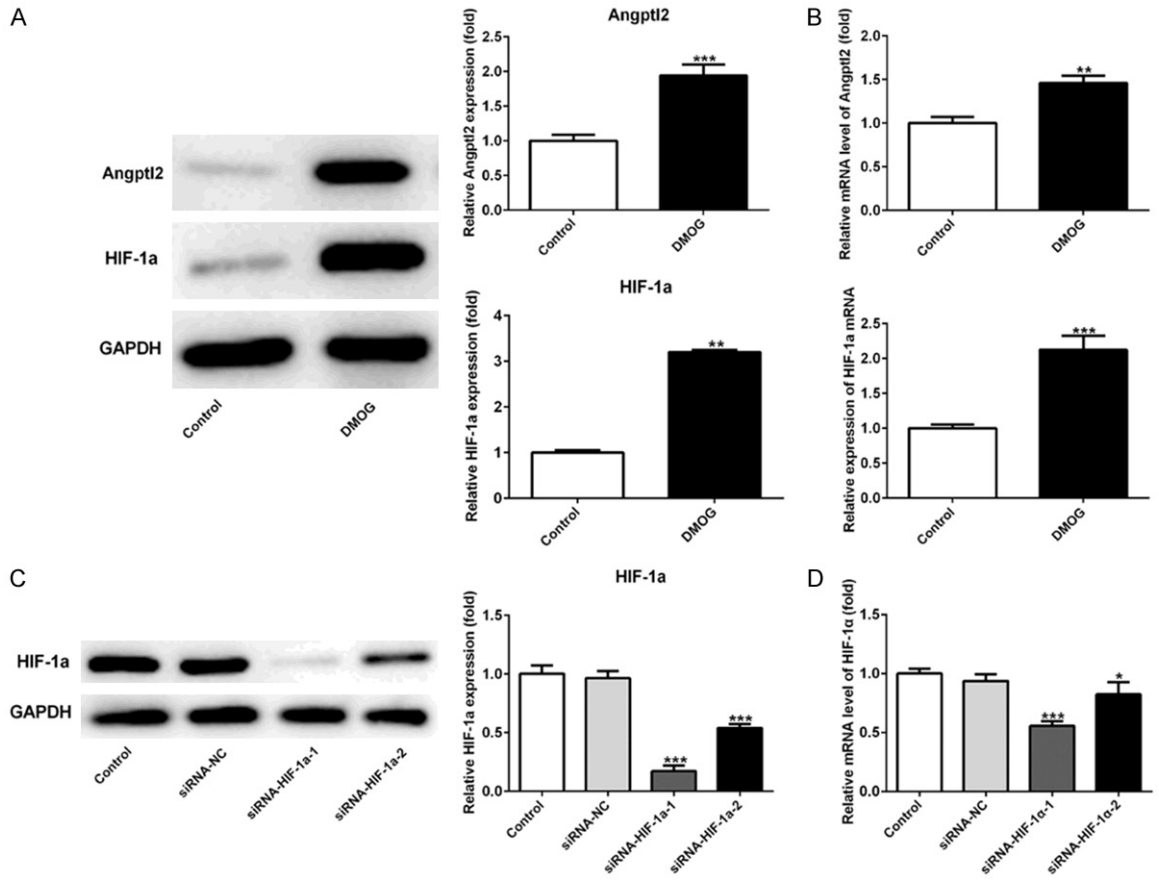



**Figure 2.** Effects of HIF-1α on Angptl2 expression in U2OS cells. (A) After treated with DMOG (1 mM) for 18 h, the protein and mRNA levels of Angptl2 and HIF-1α were detected using western blotting and (B) qRT-PCR assay. (C) After transfected with siRNA-HIF-1α-1 and siRNA-HIF-1α-2, the protein and mRNA levels of HIF-1α were assessed by western blot and (D) qRT-PCR assay. N=5; *P<0.05, **P<0.01 and ***P<0.001 versus control. DMOG, HIF-1α activator.

protein and mRNA levels of HIF-1α and Angptl2 expression were notably increased in DMOG group compared with control group (P<0.01 and P<0.001). In addition, compared with control and siRNA-NC group, HIF-1α expression was decreased remarkably after transfecting with si-HIF-1α-1 and si-HIF-1α-2, particularly in si-HIF-1α-1 group (**Figure 2C** and **2D**, P<0.05 and P<0.001). Therefore, si-HIF-1α-1 plasmid with the best interference effect was selected for subsequent experiments. Moreover, the results from western blot and qRT-PCR showed that Angptl2 was poor expressed in si-HIF-1α-1 group compared with control and siRNA-NC groups both in 2.5% $O_2$ and normoxic conditions (**Figure 3A** and **3B**, P<0.05, P<0.01 and P<0.001). Above all, the results indicated that Angptl2 was highly expressed in OS cells induced with hypoxia, and Angptl2 overexpression was induced by HIF-1α.

*Angptl2 overexpression regulates cell proliferation, invasion, migration and G1 phase arrest in osteosarcoma*

Next, the Angptl2-overexpression plasmid was transfected into U2OS cells to overexpress Angptl2. In **Figure 3C** and **3D**, the western blot and qPCR results showed that the Angptl2 expression was obviously upregulated in overexpression+Angptl2 group, compared to cells with an empty vector (overexpression NC) and control groups (P<0.01 and P<0.001). In order to determine the effects of Angptl2 on osteosarcoma cells proliferation, U2OS cells were transfected with overexpression Angptl2. Results of CCK-8 assay showed that overexpression of Angptl2 significantly increased the proliferation abilities of U2OS cells with dependence of time at 24 h, 48 h and 72 h (**Figure 3E**, P<0.05 and P<0.001). Subsequently, 72 h

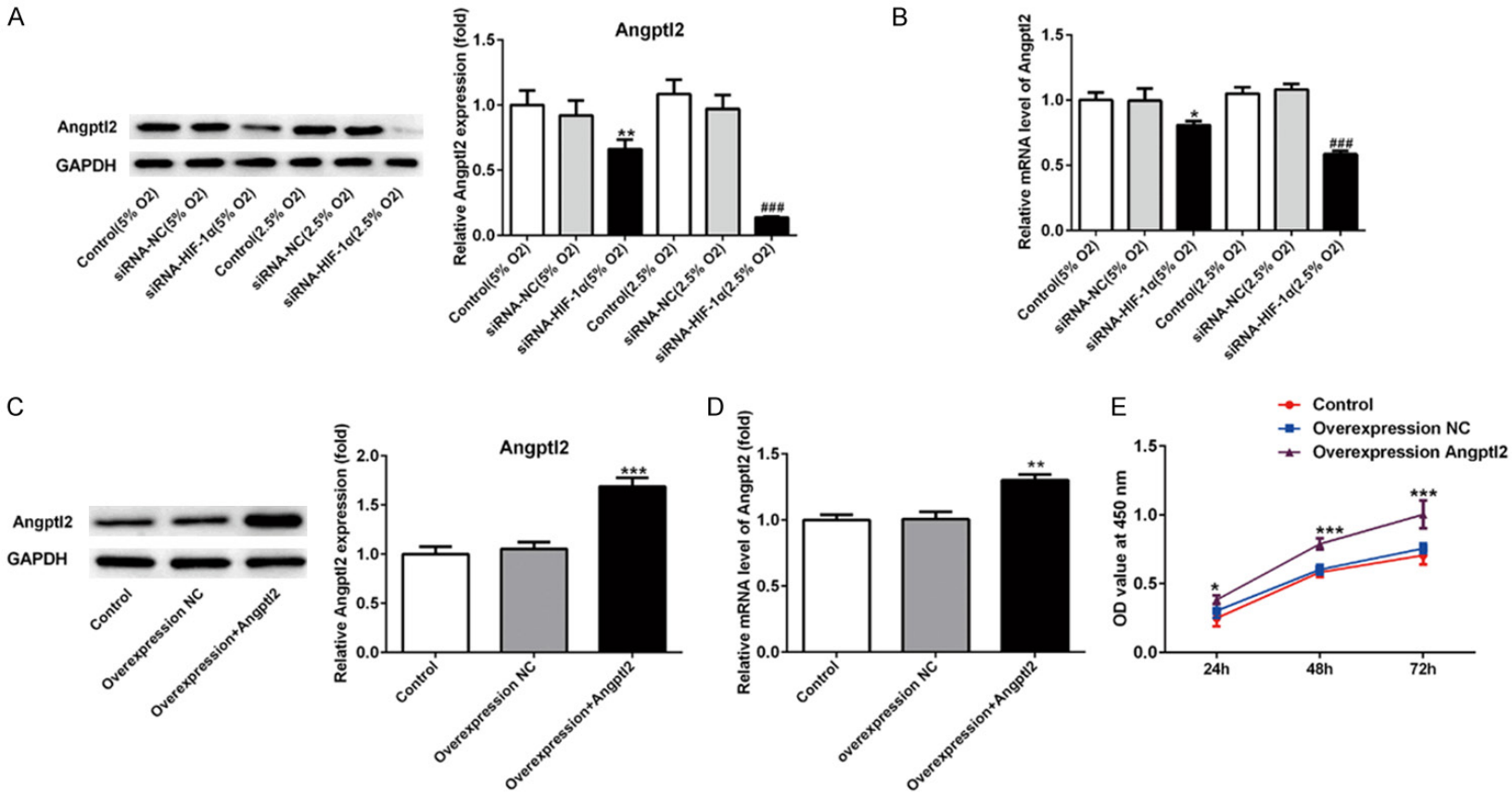



**Figure 3.** Expression of Angptl2 in U2OS cells after silencing HIF-1α or overexpressing Angptl2, and affects cell proliferation. (A) After transfected with siRNA-HIF-1α, the protein and mRNA levels of Angptl2 in U2OS cells under hypoxia or normal conditions cultured for 48 h were measured using western blot and (B) qRT-PCR assays. N=5; *P<0.05 and **P<0.01 versus control (5% $O_2$); ###P<0.001 versus control (2.5% $O_2$). (C) After transfected with overexpression Angptl2, the protein and mRNA levels of Angptl2 in U2OS cells were valued using western blot and (D) qRT-PCR assays. (E) A CCK-8 assay was conducted to assess cell proliferation of U2OS cells after transfected with overexpression Angptl2 plasmid. N=5; ***P<0.001 versus control.

transfection was chosen for cell cycle detection. As showed in **Figure 4A**, compared with the overexpression NC and control groups, Angptl2 induced G1/G0 phase arrest ($p<0.01$), and there was significant promotion of S-phase cells in overexpression Angptl2 group of U2OS cells ($P<0.01$). Additionally, cell growth-associated proteins, including CDK2, cyclin E1 and P21, were analyzed using western blot. The results indicated that overexpression of Angptl2 notably reduced p21 protein expression (**Figure 4B**, $P<0.001$), and enhanced CDK2 and cyclin E1 expressions noticeably in U2OS cells (**Figure 4B**, $P<0.001$). Thus, overexpression of Angptl2 significantly promoted cell proliferation and regulated cell cycle in OS cells.

Transwell and Scratch assays were used to detect cell invasion and migration in U2OS cells, and the results showed no significant difference between control group and overexpression NC group. Overexpression of Angptl2 dramatically promoted cell migration and invasion compared with the overexpression NC in U2OS cells (**Figure 4C-F**, $P<0.01$). Moreover, we investigated the expression of matrix metalloproteinase (MMPs), which were associated with the degradation of extracellular matrix and tumor metastasis. Overexpression of Angptl2 significantly increased MMP2 and MMP9 expression by western blot assay (**Figure 4G**, $P< 0.001$). Thus, our results suggested that Angptl2 promoted migration and invasion in osteosarcoma.

*Angptl2 overexpression enhances angiogenesis by promoting VEGFA and Ang II expression in U2OS cells*

In order to explore the effects of Angptl2 on osteosarcoma cells angiogenesis, the expression of VEGFA and Ang II were analyzed in the current study. In **Figure 5A**, western blot results indicated that the overexpression of Angptl2 significantly increased the VEGFA and Ang II expression in U2OS cells compared with control and overexpression NC groups ($P<0.001$).

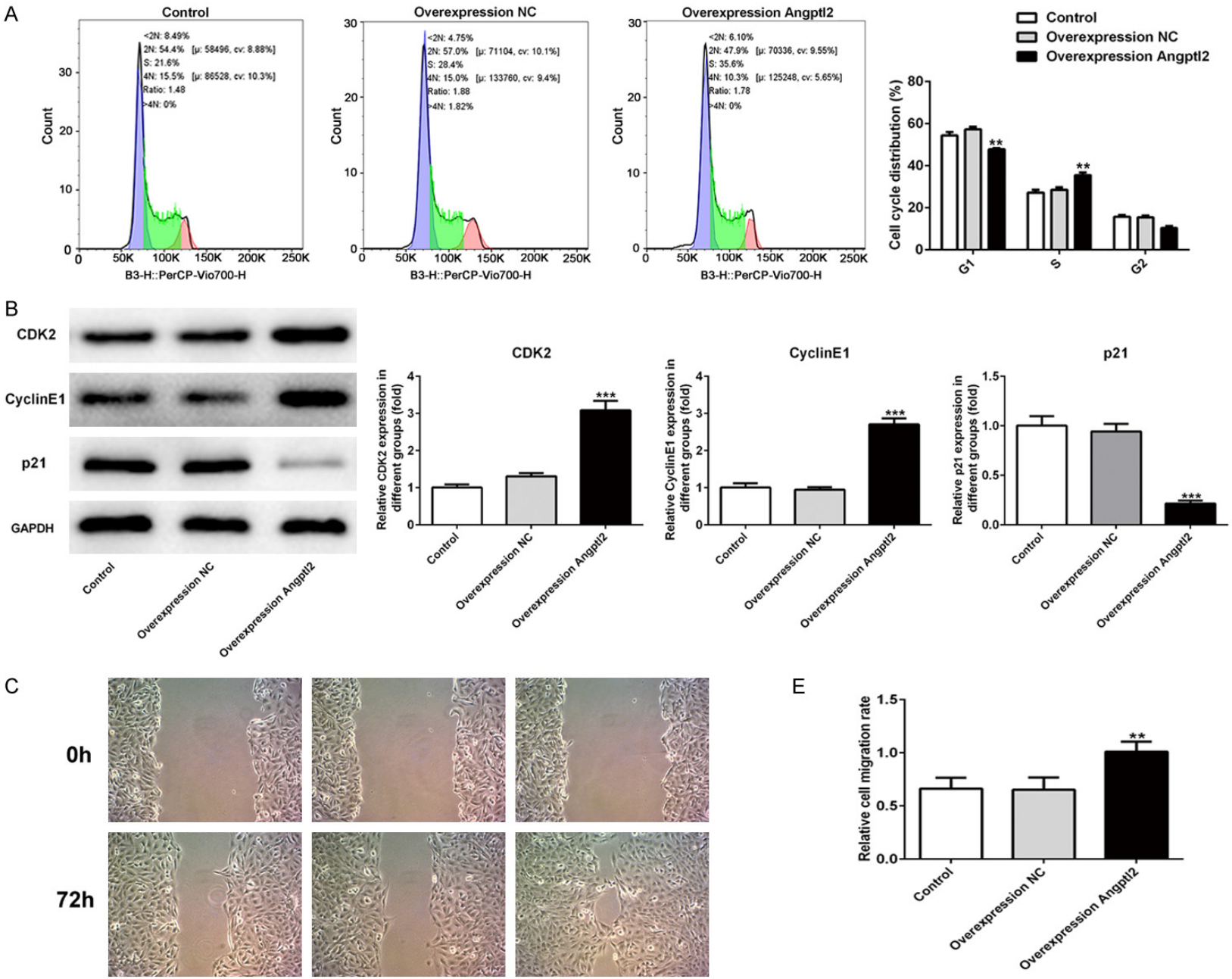
*Angptl2 overexpression enhances glycolysis by promoting HK2 expression in U2OS cells*

To analyze the effect of Angptl2 on glycolysis, the glucose consumption and lactate production were measured. As shown in **Figure 5B**, overexpression of Angptl2 facilitated the level of glucose consumption in U2OS cells ($P< 0.001$), and the level of lactate production was elevated distinctly in overexpression Angptl2 group (**Figure 5C**, $P<0.001$). Meanwhile, the inhibitor of glycolysis 2-DG could reverse the effects of Angptl2 mentioned above on glucose and lactic acid detection when compared with overexpression Angptl2 group (**Figure 5B** and **5C**, $P<0.05$ and $P<0.01$). Thus, we concluded that overexpression of Angptl2 enhanced glycolysis in osteosarcoma cells.

To explore whether glycolysis was involved in the effect of Angptl2 on the viability, the CCK-8 results have shown that cell viability was triggered by overexpression of Angptl2, while the effect was reversed by treatment with 2-DG (**Figure 5D**, $P<0.01$ and $P<0.001$). These data suggested that overexpression of Angptl2 enhanced cell viability via regulating glycolysis. Further, due to HK2 is an important enzyme during the process of glycolysis, the effect of Angptl2 on the protein level of HK2 in U2OS cells was assessed using western blot. Results denoted that HK2 expression was upregulated by overexpression of Angptl2 (**Figure 5E**, $P<0.001$), while was decreased by 2-DG treatment (**Figure 5E**, $P<0.001$). The results indicated that Angptl2 enhanced glycolysis by promoting HK2 in OS cells.

*Angptl2 promotes OS tumor growth in xenograft models*

Furthermore, the critical cancerigenic role of Angptl2 was observed *in vivo*. As indicated in **Figure 6A**, the tumor masses distinctly increased in the U2OS cells injected with overexpression Angptl2 compared with control and overexpression NC groups at 20 days after initial injection. The tumor volume of mice in three groups at 0, 5, 10, 15 or 20 days post-injection was measured and the neoplasms were stripped out at the end of the experiments. According to the statistical analyses of tumor growth curve, we could see found that control and negative control group presented a similar increasing tendency, but overexpression of Angptl2 obviously increased tumor growth and mice weight (**Figure 6A** and **6B**, $P<0.001$). Moreover, immunofluorescence staining of Ki-67, a proliferation marker, showed much higher signals in nude mice transfected with overexpression Angptl2 (**Figure 6C**). These data


A
Control
Overexpression NC
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Count
B3-H::PerCP-Vio700-H
Cell cycle distribution (%)
G1
S
G2
B
CDK2
CyclinE1
p21
GAPDH
Relative CDK2 expression in different groups (fold)
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C
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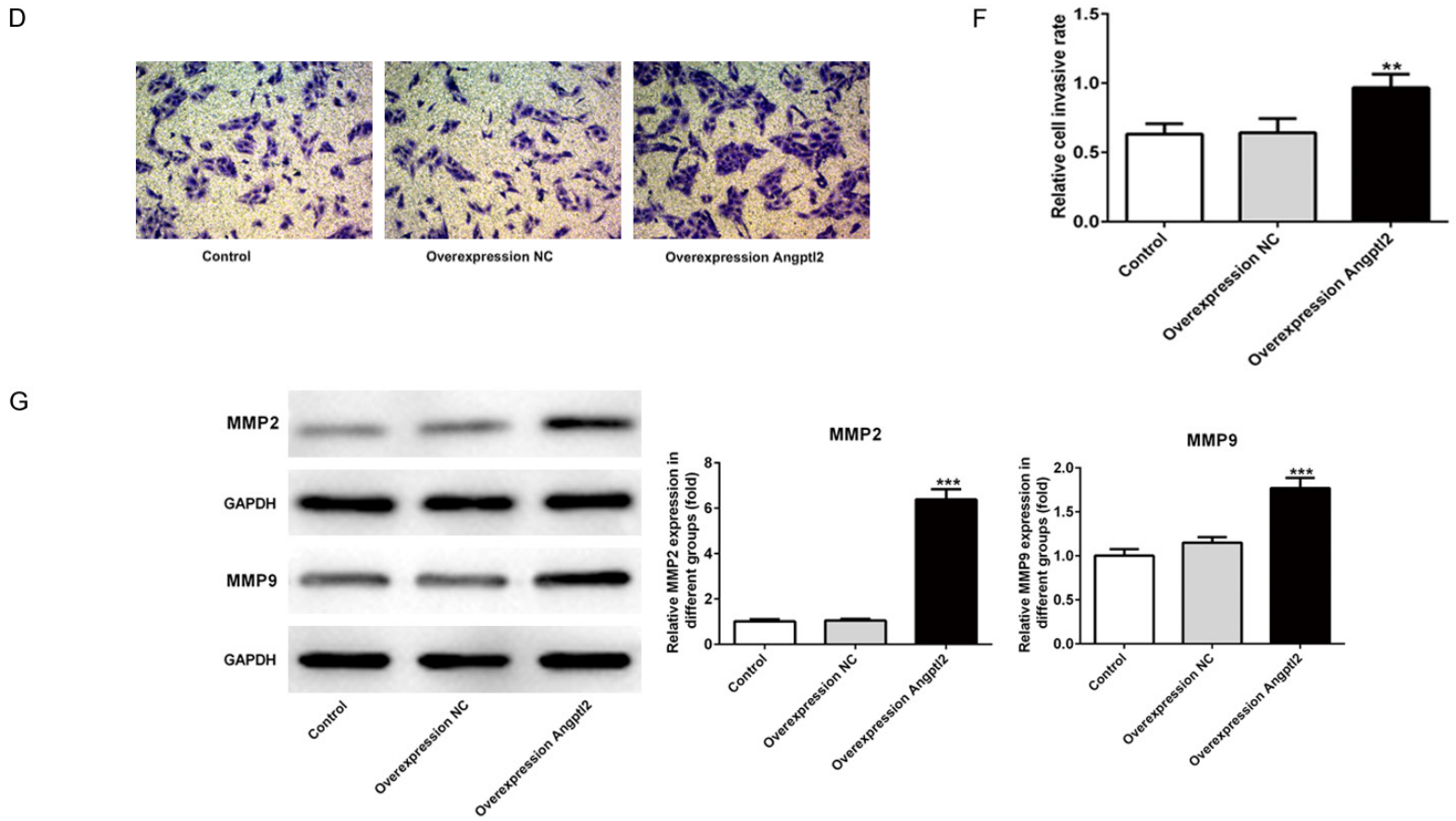


**Figure 4.** Angptl2 overexpression regulates cell invasion, migration and G1 phase arrest in U2OS cells. (A) Flow Cytometer was performed to detect cell cycle of U2OS cells after transfected with overexpression Angptl2 plasmid. (B) The proteins expression of CDK2, Cyclin E1 and p21 in U2OS cells after transfecting with overexpression Angptl2 plasmid was determinated by western blot. (C and D) Representative pictures of scratch and Transwell assays for the detection of cell invasion and migration in U2OS cells transfected with overexpression Angptl2 plasmid (magnification ×200). Results of statistical analysis results of the histograms from (E) scratch and (F) Transwell assays. (G) Western blot was used to test the expression of MMP-2 and MMP-9 when Angptl2 expressed. N=5; **P<0.01 and ***P<0.001 versus control.

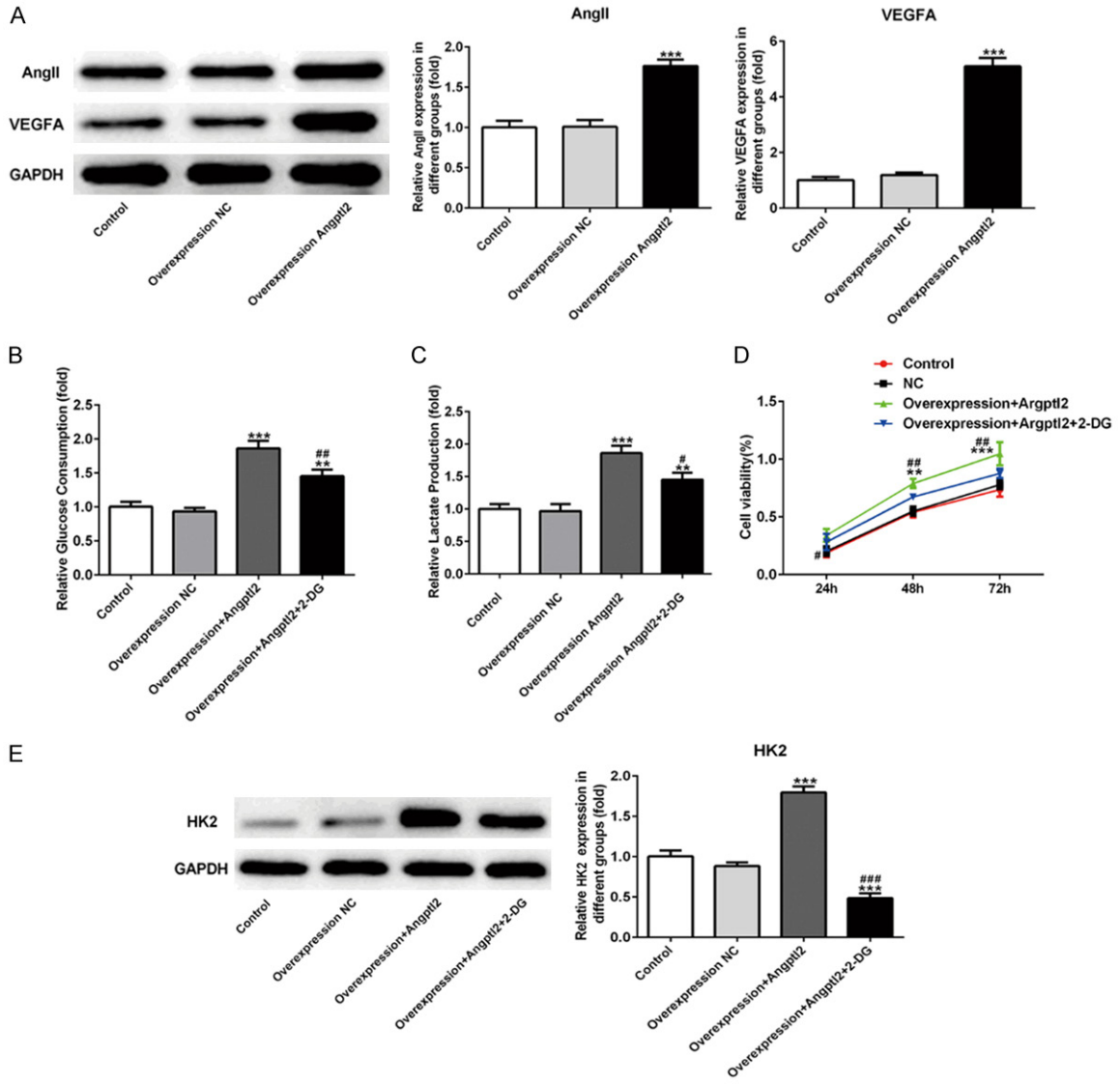


**Figure 5.** Angptl2 overexpression enhances angiogenesis and glycolysis via promoting VEGFA, Ang II and HK2 expressions in U2OS cells. (A) The proteins expression of Ang II and VEGFA in U2OS cells after transfecting with overexpression Angptl2 plasmid was determinated by western blot. The levels of (B) glucose consumption and (C) lactate production in U2OS cells after overexpression Angptl2 or following with 2-DG treatment were measured using ELISA kits. (D) Cell viability of U2OS cells after overexpression Angptl2 or following with 2-DG treatment was assessed using a CCK-8 assay. (E) Western blot was conducted to detect the glycolytic related protein HK2 expression in different groups. N=5; **P<0.01 and ***P<0.001 versus control; #P<0.05, ##P<0.01 and ###P<0.001 versus overexpression+Angptl2.

further verified the essential promotion of Angptl2 on cell proliferation *in vivo*.

*Angptl2 enhances the expression of VEGFA, Ang II and HK2 in mice*

To further investigate the effect of Angptl2 on angiogenesis and glycolysis *in vivo*, the expression of VEGFA and Ang II were measured after injecting with overexpression Angptl2 in mice. Firstly, western blot and the immunohistochemical staining of xenograft results confirmed that higher Angptl2 protein levels in tumor tissues in Angptl2 overexpressing group (**Figure 7A**, P<0.001; **Figure 7B**). Western blot showed that compared with control and overexpression NC groups, the overexpression of Angptl2 significantly increased the VEGFA and Ang II expressions (**Figure 7C**, P<0.001). In addition, HK2 expression was upregulated by

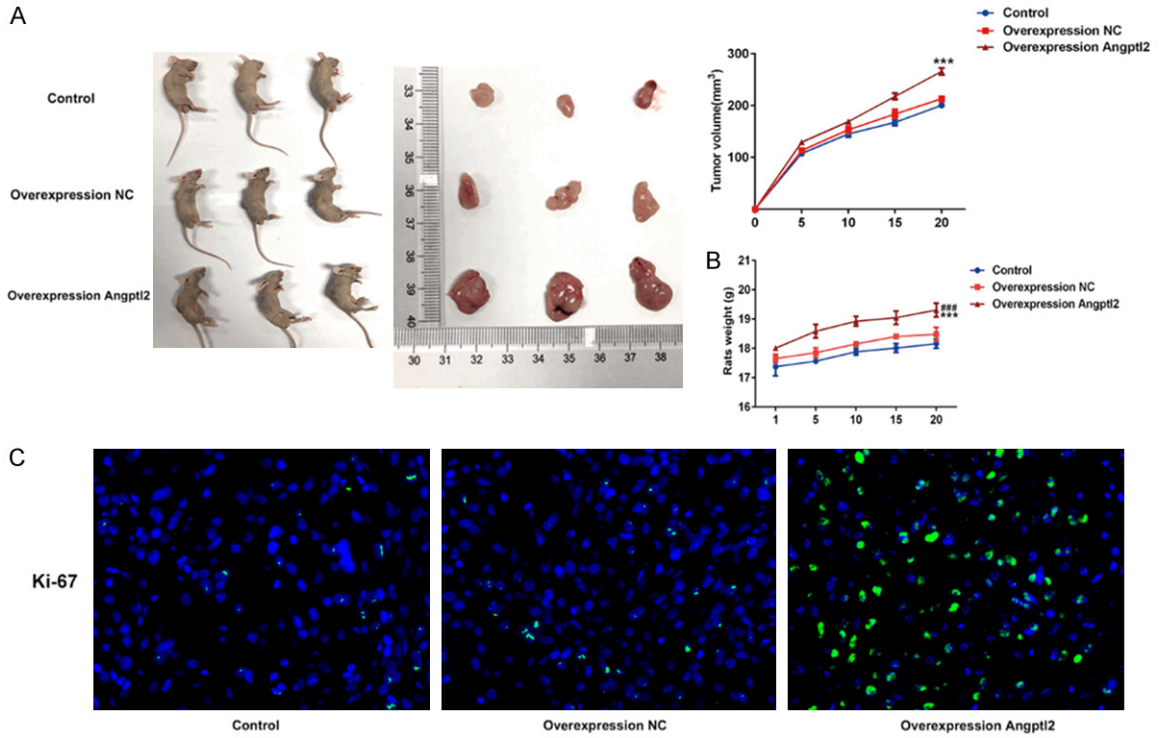


**Figure 6.** Angptl2 overexpression promotes OS tumor growth in xenograft models. A. Pictures of tumor growth and exfoliated tumor in nude mice after subcutaneous injection of cell suspension with Angptl2 overexpression. B. The nude mice wight was measured after subcutaneous injection of cell suspension with Angptl2 overexpression. C. Representative pictures of immunofluorescent staining for Ki-67 expression (magnification ×200). N=5; ***P<0.001 versus control.

overexpression of Angptl2, while was reversed by 2-DG. The results proved that Angptl2 enhanced the expression of VEGFA, Ang II and HK2 *in vivo*, which might promote the angiogenesis and glycolysis in OS cells.

## Discussion

The present study investigated the expression levels of Angptl2 in five osteosarcoma cell lines (MG63, MHM, HOS-143B, U2OS and OSA) under normoxic and hypoxic conditions. Notably, Angptl2 was shown the highest expression in U2OS, which was induced under hypoxia via HIF-1α. Further, we found that Angptl2 overexpression promoted cell proliferation, invasion, migration and G1 phase arrest, and regulate the expression of CDK2, cyclin E1 and p21 in U2OS cells. In vivo tumor xenografts, we observed that Angptl2 promotes OS tumor growth and VEGFA, Ang II and HK2 expression, which indicated the promotion of angiogenesis and glycolysis function, and these results were consistent with results *in vitro*. Therefore, our data indicated that Angptl2 might be a favorable marker for predicting a long survival time in patients with OS.

Several studies have demonstrated that Angptl2 acts as a chronic inflammatory mediator to promote tumor cell motility and invasion capacity and plays an important role in tumor metastasis. For instance, Angptl2 was overexpressed in lung cancer, gastric cancer and colorectal cancer, which was shown high invasion and motility, and was associated with the potential for lymph node and distant organ metastasis [23-25]. Demethylation of the Angptl2 promoter increased its expression and enhancing tumor cell metastasis through a mechanism involving integrin a5b1, p38 MAPK, and MMP activation in OS, which were consistent with the results of human lung and breast cancer cell lines [26]. In our study, Angptl2 was highly expressed in OS cells, and Angptl2 overexpression promoted cell proliferation, invasion, migration and G1 phase arrest via regulating MMPs and cell growth-associat-

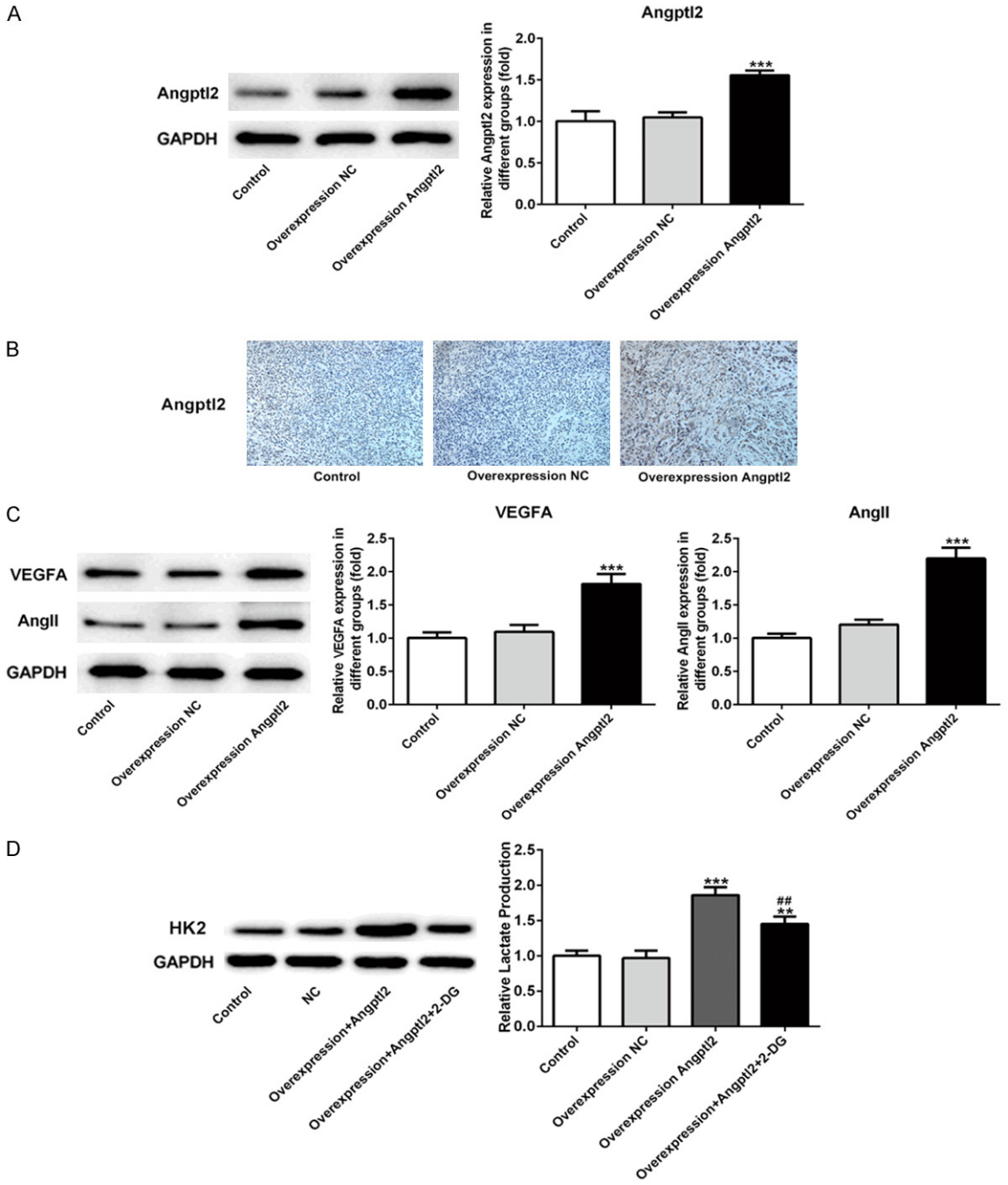


**Figure 7.** Angptl2 overexpression enhances the expression of VEGFA, Ang II and HK2 in the tumor tissue in xenograft models. A. Western blot was used to detect Angptl2 expression in tumor tissue in different groups. B. Representative pictures of immunohistochemistry for Angptl2 expression (magnification ×200). C. The expression of VEGFA and Ang II in tumor tissue was detected using western blot assay. D. Western blot was conducted to evaluate the HK2 expression in the tumor tissue of xenograft mice injected with overexpression Angptl2 or following with 2-DG treatment. N=5; **P<0.01 and ***P<0.001 versus control; ##P<0.01 versus overexpression Angptl2.

ed proteins in osteosarcoma. Furthermore, we confirmed the effects of Angptl2 *in vivo* OS xenografts. Upregulation of Angptl2 obviously increased tumor growth and mice weight. Ki-67, a proliferation marker, showed much higher signals in nude mice transfected with Angptl2 by immunofluorescence staining. Therefore, Angptl2 promotes tumor proliferation and metastasis of osteosarcoma.

Recently, the invasive potential of OS cells was increased in hypoxia condition, suggesting that

hypoxia might promote the metastasis of OS cells [27]. HIF-1α, the transcriptional master regulator that was stabilized in hypoxia, regulated the expression of multiple target genes and contributed to cell metastatic potential, angiogenesis, poor chemotherapy responses, as well as decreased overall survival and disease-free survival in OS cells and patients [28-30]. Latest studies revealed that a hypoxic environment may induce Angptl4 expression via HIF-1α-independent pathway and thus suppress tumor invasion [31]. However, the relationship between Angptl2 and HIF-1α remained unknown. In present study, Angptl2 was highly expressed in OS cells, which was induced by hypoxia/HIF-1α. Further, angiogenesis is essential for the growth of tumor and metastasis of cancer cells. The tumor would trigger the angiogenesis switch to survival under hypoxia condition [32, 33]. However, hypoxia regulated genes that involved angiogenesis in OS have not been fully explored. We found that Angptl2 enhanced the expression of VEGFA and Ang II in OS cells, which indicated the promotion of angiogenesis function. Finally, under hypoxic conditions, the uptake of glucose and glycolytic flux is increased mostly due to the HIF-1α-dependent up-regulation of gene encoding glucose transporter, glycolytic enzymes (such as HK2). Moreover, some tumor cells have been found to overexpress specific glycolytic enzymes, leading to higher rate of glycolysis [34]. HK2 is a major player in "Warburg Effect" and contributes to the immortalization of cancer cells [35]. In our study, overexpression of Angptl2 enhanced glycolysis in osteosarcoma cells, and HK2 expression was upregulated by overexpression of Angptl2, while was decreased by adding 2-DG *in vitro* and *in vivo*. The results mentioned above revealed that Angptl2 is an important facilitator of tumor angiogenesis and glycolysis induced by HIF-1α in osteosarcoma.

The present study initially elucidated the role of Angptl2 in osteosarcoma, but the occurrence and development process of osteosarcoma are complex, and the signaling pathway through which Angptl2 plays its roles needs to be further explored by more experiments in the future.

## Conclusion

In summary, we found that Angptl2 was highly expressed in OS cells, which was induced by hypoxia/HIF-1α. Angptl2 is an important facilitator of tumor proliferation, metastasis, angiogenesis and glycolysis induced by HIF-1α in osteosarcoma. Angptl2 may be a favorable marker for predicting a long survival time in patients with OS, which can be used to guide new treatment regimens for osteosarcoma.

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## Disclosure of conflict of interest

None.

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