

ANGPTL4 Negatively Regulate the Progression of Osteosarcoma by Remodeling Branched-Chain Amino Acid Metabolism

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Abstract

Background

Angiopoietin-like-4 (ANGPTL4), a secreted glycoprotein that is mainly recognized as a regulator in lipid metabolism, now, is implied in the regulation of the growth and metastasis of various carcinomas. However, less is known about its functions in the progression of sarcomas, let alone osteosarcoma (OS), which is the most common malignant diagnosed in musculoskeletal system.

Methods

The expression of ANGPTL4 in clinical OS samples and cell lines paired with their controls were analyzed in both mRNA and protein levels. Cell functional analysis including proliferation and colony formation were carried out to detect the roles ANGPTL4 takes in the progress of OS using stable *ANGPTL4* overexpression and knockdown HOS/MNNG cell lines. The RNA-Seq and bioinformatics analysis were then employed to discover the BCAA metabolism related signaling which is involved in ANGPTL4 functioning on HOS/MNNG cell growth. Furthermore, BCAAs content measurement, and BCATs rescue experiments were performed to confirm the BCAA/mTOR signaling axis that ANGPTL4 triggered in HOS/MNNG cells. Finally, a xenograft mouse model was carried out to further verify the ANGPTL4 /BCAA/mTOR signaling axis discovered.

Results

We found that the expression of ANGPTL4 is reduced in clinical OS tissues and cell lines compared to cancellous bone tissues and BMSCs, respectively. The knockdown of *ANGPTL4* in HOS/MNNG cells results in enhanced cell growth and clone formation. Moreover, BCAA/mTOR signaling axis were discovered to be triggered by ANGPTL4 down regulation in HOS/MNNG cell using RNA-seq. It was also verified that the accumulation of BCAAs activates the mTOR signaling pathway, and in turn promotes HOS/MNNG cell growth using BCAAs content measurement, and BCAT inhibition. Finally, the IHC results of xenograft mouse model also confirmed this ANGPTL4/BCAA/mTOR signaling axis *in vivo*.

Conclusions

Taken together, our results demonstrate that the expression of ANGPTL4 were negatively related to OS progress. Moreover, it was found the down-regulation of ANGPTL4 promoted OS cell growth via BCAAs/mTOR axis.

1. Introduction

Osteosarcoma (OS) is a major health burden and cause of cancer-related death in adolescents worldwide (1). Patients diagnosed with OS face the threat of amputation and even death after tumor cells metastasize to the lungs (2). Currently, the treatment of OS includes complete tumor removal and two rounds of chemotherapy (i.e., preoperative and postoperative

chemotherapy), which are continuous for at least 6 months (2). However, such systematic therapies could only cure 60%~70% of patients. The 5-year survival rate of OS patients has not improved, although surgical techniques have improved considerably in recent decades (2, 3). To improve the prognosis of OS patients, clinical trials of some second-line or third-line drugs are in full flow (4, 5). In order to develop more drugs against OS, it is important to fully understand the mechanisms underlying the occurrence and progression of OS.

Angiopoietin-like proteins (ANGPTLs) are a protein family that includes eight members characterized as structurally similar to angiogenin (6). ANGPTL4, a member of the ANGPTL family that is mainly found in the liver, adipose tissue, and skeletal muscle, is well known as an inhibitor of lipoprotein lipase (LPL) (7, 8). Due to the organ expression bias of ANGPTL4, it was initially regarded as merely a metabolic regulator that maintains metabolic homeostasis (8, 9). Recently, ANGPTL4 has also been detected in diverse tumor cells and associated with malignant phenotypes. Chen et al. found that the expression of ANGPTL4 was higher in gastric cancer cells and that the knockdown of ANGPTL4 could suppress the development of gastric cancer (10). However, Cai et al. demonstrated that ANGPTL4 was a favorable prognostic factor in breast cancer and that upregulation of ANGPTL4 induced the suppression of adhesion and migration in cancer cells (11). Besides, ANGPTL4 is also involved in the temozolomide resistance of glioblastoma by promoting cancer stemness (12). Although researchers have started to explore the role of ANGPTL4 in OS (13), the underlying fine molecular mechanism remains barely unclear.

In the present study, a clearly lower expression of ANGPTL4 was found in both clinical OS tissue samples and OS cell lines, compared to that found in normal control groups. Knockdown of *ANGPTL4* leads to the acceleration of proliferation in OS cells. RNA-Seq analysis suggests that ANGPTL4 expression results in the remodeling of branched-chain amino acid (BCAA) metabolism. BCAAs are essential amino acids (leucine, isoleucine and valine) which play a crucial role in protein synthesis and energy, and are indispensable for cell growth (14, 15). When the accumulation of BCAAs was up-regulated in OS cell lines due to knockdown of *ANGPTL4*, the mTOR signaling pathway was activated, which results in enhanced growth of OS cells. Taken together, our results indicate the low-expression of ANGPTL4 promotes the progression of OS via mTOR signaling by remodeling BCAA metabolism.

2. Materials And Methods

2.1 Clinical samples

The clinical specimens were collected from the Department of Bone Oncology, and Department of Emergency, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, including osteosarcoma tissue and normal cancellous bone tissue. The information of patients both in osteosarcoma group and control group were shown in Supplementary table 1 and Supplementary table 2. Ethical approval was obtained from the Ethics Committee of Shanghai Sixth People's Hospital (YS-2016-064, February 24, 2016).

2.2 RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA from both clinical specimens and cell lines was extracted by TRIzol reagent (Invitrogen, USA) and then reverse transcribed to cDNA according to the instructions of a cDNA Synthesis Kit (Invitrogen, USA). The relative expression levels of genes were measured on an ABI Prism 7900HT real-time system (Applied Biosystems) and calculated by the $2^{-\Delta\Delta C_t}$ approach. All primers were shown in Supplementary Table 3.

2.3 Western blot and reagent

Total proteins were extracted by RIPA solution (EpiZyme, PC102) according to standard procedures. Then, the collected proteins were separated by electrophoresis and transferred to a PVDF membrane. The nonspecific binding sites of the PVDF membrane were blocked with 5% milk at room temperature (RT) for 60 min. Target proteins were detected by incubating in the primary antibody solution at 4 °C overnight. Finally, protein bands were detected by chemiluminescence. All Primary antibodies were shown in Supplementary Table 4.

2.4 Cell culture

Human MNNG/HOS (MNNG is used instead in the following), U2OS, and MG63 were purchased from American Type Culture Collection (ATCC). Human BMSCs were harvested from patients with open fractures who underwent debridement at Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The culture media for MNNG, MG63, and U2OS, and BMSCs were DMEM (Corning, USA), RPMI 1640 (Corning, USA), and α -MEM (Corning, USA), respectively. All culture media were supplemented with 10% fetal bovine serum (Gibco, USA). All cells were cultured at a 37 °C atmosphere containing 5% CO₂.

2.5 Stable cell line construction

For stable cell line construction, HEK293T cells were co-transfected with lentivirus packing vectors and LV shuttle plasmids containing full-length ANGPTL4 and siRNA against ANGPTL4. Forty-eight hours later, the supernatant containing lentivirus was collected by centrifugation at 400 g for 10 min, purified and titer determined. Then, the lentivirus was added to the culture medium of MNNG cells at an MOI of 10.0 to infect the cells. After 72 hours, the culture medium was changed to a new medium that contained puromycin at a concentration of 1.0 μ g/ml to select the positive cells. Finally, stable cells overexpressing (MNNG-A4) and knockdown ANGPTL4 (MNNG-siA4) were verified by RT-qPCR and WB. All the information of the vector and sequences of full-length ANGPTL4 and siRNA against ANGPTL4 has been provided in the Supplementary Table 5.

2.6 Cell proliferation assay

Real-time cellular analysis (RTCA) (ACEA Biosciences, USA) was used to evaluate the cell proliferation ability (16). First, the baseline value was measured in 100 μ l of culture medium which pre-incubated at 37 °C in a cell incubator for 1 hour. Then, the cells were seeded into wells at a density of 2.0×10^3 cells per well. The attachment and proliferation of cells were measured by the RTCA system for 6 hours and 168 hours, respectively.

2.7 Colony formation assay

The cells were seeded into a 6-well plate to ensure that there were 1000 cells per well. After incubation for 14 days, the cells were fixed with 4% paraformaldehyde (PFA), and then the cells were immersed in crystal violet for half an hour. The cells in each well were photographed and recorded, and colonies containing more than 50 cells were counted by ImageJ software.

2.8 Subcutaneous tumor model

Female nude mice ranging from 4-6 weeks were purchased from the Laboratory Animal Research Center of Shanghai Sixth People's Hospital, and all operations were approved by the Animal Research Committee of Shanghai Sixth People's Hospital. After anesthesia by pentobarbital sodium, 200 μ l of cell suspension containing 1×10^6 cells were injected into the nude mouse flank (17). Tumors were measured by researchers until the longest diameter of the largest tumor reached 200 mm. The volume of tumors was calculated as length (mm) \times width (mm)²/2.

2.9 RNA-seq and analysis

TRIZOL (Invitrogen, USA) was used to extract total RNA from MNNG-A4, MNNG-siA4, and control cell lines. Then, standard guidelines were followed to construct paired-end libraries with a TruSeq™ RNA Sample Preparation Kit (Illumina, USA). The mRNA is cleaved into small pieces and reverse transcribed into first strand cDNA. Then, DNA polymerase I and RNase H were used to generate second strand cDNA. These cDNAs then undergo the addition of a single 'A' base and then ligation of the adapters. The products were purified and enriched with PCR to create the final cDNA library. Library construction and sequencing were performed by Sinotech Genomics Co., Ltd. (Shanghai, PRC). Differentially expressed genes (DEGs) were selected based on a false discovery rate (FDR) less than 5% and changed expression higher than 1.5-or lower than 0.67-fold. All cell lines were tested three times.

2.10 Branched Chain Amino Acids (BCAAs) Assay

Cells (2×10^6) were harvested from a T75 cell culture flask. Cells were lysed, and the levels of BCAAs were measured by a BCAA assay kit according to the manufacturer's instructions (Sigma–Aldrich, USA). Briefly, cells were lysed in 100 mL of cold BCAA assay buffer to obtain the lysate, 10 µl of lysate was added to a 96-well plate, and BCAA buffer was added to bring the volume to 50 mL. Next, 50 µl reaction mixes containing 46 µl assay buffer, 2 µl BCAA enzyme mix, and 2 µl WST substrate mix were added to each well. The blank wells contained the cell lysate, BCAA assay buffer, and substrate mix but omitted the enzyme. Then, the reaction was incubated for 30 minutes at room temperature, and the absorbance was measured at 450 nm (A450). The blank absorbances were subtracted from lysate absorbances. A standard curve was generated through the above method, but the cell lysate was changed with a leucine standard. Each sample was measured in duplicate.

2.11 Immunohistochemical (IHC) analysis

The clinical OS specimens and OS tissues excised from the subcutaneous tumor model were embedded in paraffin and then cut into 4 µm sections and deparaffinized. The sections were blocked with 5% bovine serum albumin (BSA) at 37°C for 30 min after antigen retrieval. After that, specific primary antibodies were added to the samples and incubated overnight at 4 °C. Then, the cells were washed three times with PBS and incubated with HRP-linked anti-IgG at 37 °C for 30 min. Washing by PBS again and stained in DAB for 10 min. Finally, the samples were counterstained, dehydrated, covered with cover glass and photographed with DM6B (Leica, BRD).

2.12 Statistical analyses

The data were analyzed by SPSS 25.0 software and presented as the mean \pm SD. The differences between experimental and control groups were analyzed by two-tailed Student's t test while the differences between tumor tissues group and nontumor tissues group of clinical samples were analyzed by Welch's t test. ns means $P > 0.05$, * means $P < 0.05$, ** means $P < 0.01$, and *** means $P < 0.001$.

3. Results

3.1 ANGPTL4 was expressed at low levels in clinical OS samples and OS cells

To investigate the role of ANGPTL4 in the development of OS, the expression level of *ANGPTL4* in clinical OS tissue samples and control samples (normal cancellous bone) was first evaluated in mRNA level. As shown in Fig. 1A, there was a clear lower expression pattern of *ANGPTL4* found in OS tissues than those in adjacent normal bone tissues. Then, we further analyzed the expression of ANGPTL4 protein by immuno-histochemical (IHC) staining in both OS tissue sections and adjacent nontumor sections. Following the same pattern as the RT–qPCR results, OS tissues also showed

less positive area of ANGPTL4 staining compared to the control sections (Fig. 1D). Besides, the expressions of *ANGPTL4* were verified in three OS cell lines: HOS/MNNG (short for MNNG in the following text), U2OS, and MG63 and one control cell: BMSC in both mRNA and protein levels, too. As a result, the mRNA and protein expressions of *ANGPTL4* in MNNG, U2OS, and MG63 cells were 0.52 ± 0.02 , 0.69 ± 0.04 , 0.73 ± 0.02 , and 0.61 ± 0.02 , 0.62 ± 0.04 , 0.67 ± 0.06 , folds lower than that in BMSC, respectively (Fig. 1B, C). These data confirmed that OS tissues and cell lines had lower expressions of ANGPTL4 than those in cancellous bones and BMSCs in both mRNA and protein levels.

3.2 ANGPTL4 down-regulation promotes the growth of OS cells *in vitro*

Our previous results suggests that ANGPTL4 may have a negative correlation with the progress of OS. Therefore, the question how ANGPTL4 negatively affecting OS growth is raised. Among the three OS cell lines we tested, MNNG cells showed the lowest ANGPTL4 expression compared to BMSC cells. Herein, the MNNG cell was selected as an *in vitro* model for validation. Using lentivirus system, we constructed *ANGPTL4* up-regulation (MNNG-A4) and down-regulation (MNNG-siA4) stable OS cell lines, as well as their control cell lines (A4Control and siControl) in order to further detect the biological role ANGPTL4 takes in MNNG growth. As shown in Fig. 2A-D, the successful construction of MNNG-A4 (*ANGPTL4* up-regulation stable OS cell lines) and MNNG-siA4 (*ANGPTL4* down-regulated stable OS cell lines) were confirmed in both mRNA and protein levels by RT-qPCR and western blotting.

We then evaluated the cell proliferations of these two *ANGPTL4*-regulated MNNG cell lines with their control cells by RTCA assay (16). The results showed that the cell growth was significantly suppressed in the MNNG cells overexpressing *ANGPTL4* (Ratio of MNNG-A4 vs A4Control was 0.30 ± 0.01 , 0.22 ± 0.01 , 0.21 ± 0.02 , and 0.20 ± 0.01 at 48h, 72h, 96h, and 120h). Whereas, the knockdown of *ANGPTL4* in MNNG cells enhanced their growth (Ratio of MNNG-siA4 vs siControl was 1.23 ± 0.04 , 1.43 ± 0.09 , 1.75 ± 0.30 , and 1.40 ± 0.15 at 48h, 72h, 96h, and 120h) (Fig. 2 E-H). Accordingly, the colony formation assays were also consistent with the cell proliferation assays (Ratio of MNNG-A4 vs A4Control and MNNG-siA4 vs siControl was 0.77 ± 0.04 and 1.39 ± 0.04 , respectively) (Fig. 2 I-L). Taken together, these results demonstrated that the expression of ANGPTL4 showed a negative correlation with the growth of OS cells *in vitro*.

3.3 ANGPTL4 activates mTOR signal pathway via remodeling branched-chain amino acid (BCAA) metabolism

In the interest of exploring the underlying mechanism of how ANGPTL4 affects the cell growth in MNNG cells, we employed mRNA sequencing to discover the changed signaling cascades by identifying differentially expressed genes (DEGs) between *ANGPTL4*-regulated MNNG cells and their control cells. The DEGs numbers are shown in Fig. 3A and 3B based on an cut off value that changed expression

higher than 1.5-or lower than 0.67-fold. There were 6,087 DEGs (2,545 up-regulated, 3,542 down-regulated) between the control and the *ANGPTL4*-upregulated cell line, whereas in the *ANGPTL4*-downregulated group the number was 6,957 (3,356 up-regulated, 3,601 down-regulated).

Then the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was firstly performed on these DEGs to more detail the underlying mechanisms. As presented in Fig. 3C, 3D and Fig. S1, the results suggested that the DEGs in both *ANGPTL4*-regulated MNNG (overexpression and knockdown) cells were highly related to the metabolism of Branched-Chain Amino Acid (BCAA). There were 22 out of 46 genes have been found to be different expressed in this gene cluster, which were shown in Fig. 3E. To better understand the molecular action mechanism of these genes, we then introduced these genes into STRING (<https://string-db.org/>) database to constructing the protein-protein interaction network. As Fig. 3F showed, these genes are closely clustered together and form the complex network, which shows the precise inter-regulatory relationship of these genes are highly involved in BCAAs metabolism.

To verify the results of RNA-seq, we then detected the expressions of these genes in the *ANGPTL4*-regulated MNNG cells. The RT-qPCR results were in accord with the RNA-seq. As shown in Fig. 4A and 4B, the expression of *HMGCL* and *ABAT* were upregulated while *AHU*, *AOX-1*, *ALDH6A1*, *BCKDHA*, *BCKDHB*, *IL4I-1*, *SDSL*, and *ACADS* were downregulated in MNNG-A4 cells. Meanwhile, we also observed a opposite trend of these genes in MNNG-siA4 cells. Furthermore, the western blotting results also verified that the protein expression levels of some important genes (*AOX-1*, *BCKDHA*, *IL4I-1*, and *HMGCL*) were consistent with their mRNA levels (Fig. 4C). To further confirm our hypothesis, we also measured the BCAAs concentrations in both MNNG-A4 and MNNG-siA4 cells and their control cells. As shown in Fig. 4D, MNNG-A4 cells displayed a marked decrease of BCAAs compared with control cells (MNNG-A4 vs A4Control was 0.09 ± 0.01 nmol/ul vs 0.12 ± 0.01 nmol/ul), and the expression of BCAAs content was significantly increased in MNNG-siA4 cells (MNNG-siA4 vs siControl was 0.17 ± 0.01 vs 0.10 ± 0.01).

According to the previously studies, BCAAs could both be catabolized into BCKAs and entered the TCA cycle (14, 15), and activate the mTOR signaling and promote cell growth (18). Thus, we then examined the activation of mTOR signaling in *ANGPTL*-regulated MNNG cells and control cells. As shown in Fig. 4E, the phosphorylation levels of mTOR together with the downstream effectors S6 kinase and S6 were up-regulated in MNNG-siA4 cells, while a opposite expression patterns found in MNNG-A4 cells compared with control cells. Overall, these data demonstrated that low expression of *ANGPTL4* in MNNG cells increased the concentration of BCAAs, which in turn activated the mTOR pathway and promoted the progression of MNNG cells.

3.4 The inhibition of BCATs attenuated the elevated proliferation in *ANGPTL4* down-regulated MNNG cells

As mentioned above, when BCAAs imported into the cells, they are converted to branched-chain α -keto acids (BCKAs) firstly, by branched-chain amino acid transaminases (BCATs). This catalytic reaction was reversible, which also production of BCAAs via BCKAs (15). Though the RNA-seq results did not point out the expression levels of BCATs were regulated by ANGPTL4 directly, the previous data we got still held a possibility that ANGPTL4 affected BCAA metabolism via enhancing the activities of BCATs. To verify our hypothesis, we treated MNNG-siA4 and siControl cells with a BCATs inhibitor: BCATc inhibitor 2 (19). As expected, it was showed that 5 μ M of BCATc inhibitor 2 reduced the cell proliferation (Ratio of MNNG-siA4+inhibitor vs siControl+inhibitor was 0.63 ± 0.24 , 0.56 ± 0.13 , 0.58 ± 0.08 , and 0.68 ± 0.07 at 48h, 72h, 96h, and 120h, respectively) and colony formation (Ratio of MNNG-siA4+inhibitor vs siControl+inhibitor was 0.96 ± 0.02) of MNNG-siA4 cells (Fig. 5A-D). Mechanismly, the expression levels of BCAA metabolism signaling pathway related proteins (AOX-1, BCKDHA, IL4I-1, and HMGCL) were also reversed in MNNG-siA4 and siControl cells after 5 μ M of BCATs inhibitor treatment (Fig. 5E, F). Moreover, the BCAA concentrations were also measured in MNNG-siA4 and siControl cells after BCATs inhibitor treatment. The results showed that the high level of BCAA induced by knockdown of *ANGPTL4* in OS cells was reduced by BCATc inhibitor 2 (MNNG-siA4+inhibitor vs siControl+inhibitor was 0.08 ± 0.01 nmol/ul and 0.13 ± 0.01 nmol/ul, respectively) (Fig. 5G). In summary, these data confirmed that downregulation of ANGPTL4 promotes MNNG growth by regulating the metabolism of BCAAs.

3.5 ANGPTL4 attenuates OS progress via BCAA/mTOR axis *in vivo*

Based on the *in vitro* results we obtained, the downregulation of ANGPTL4 leads to the accumulation of BCAAs in cells by enhancing the activities of BCATs, which triggers mTOR signaling pathway, and ultimately promotes the proliferation of OS cells. In order to examine this signaling axis *in vivo*, we then carried out a nude mice subcutaneous implantation tumor model. Briefly, 1×10^6 cells were injected into the nude mouse flank. When the longest diameter of the largest tumor reached 200 mm, the tumors were then excised, measured and recorded. After embedded and cut into sections, the OS cell implants were detected with antibodies against ANGPTL4, BCAT1, BCKDHA, p-mTOR, and p-S6. The intensities of these IHC staining targeting above proteins was analyzed, calculated and perform statistics (Fig. 6A).

As shown in Fig. 6B, the growth of OS implantations was significantly promoted in MNNG-siA4 cells (Ratio of MNNG-siA4 vs siControl was 3.01 ± 1.40); while the MNNG cells overexpressing *ANGPTL4* showed an attenuated growth (Ratio of MNNG-A4 vs A4Control was 0.40 ± 0.30) (Fig. 6B). Then, we detected the ANGPTL4/BCAA/mTOR signaling in the OS tissues obtained from these nude mouse models by IHC staining (Fig. 6C). As shown in Fig. 6C, *ANGPTL4* down-regulated OS tissue presented high expressions of these proteins (Ratio of MNNG-siA4 vs siControl for ANGPTL4, BCAT1, BCKDHA, p-mTOR, and p-S6 was 0.87 ± 0.03 , 1.07 ± 0.02 , 1.14 ± 0.02 , 1.57 ± 0.15 , and 1.07 ± 0.01). While in the *ANGPTL4* up-regulated tissue, the expression patterns of these key proteins showed an opposite pattern except BCAT1 (Ratio of MNNG-A4 vs A4Control for ANGPTL4, BCAT1, BCKDHA, p-mTOR, and p-S6 was 1.28 ± 0.02 , 0.92 ± 0.04 , 0.83 ± 0.02 , 0.92 ± 0.03 , and 0.90 ± 0.01) (Fig. 6D).

4. Discussion

BCAAs, the essential amino acids for humans, are important participants in metabolic regulation. The BCAAs were crucial in protein synthesis and energy supply which laid a strong foundation for unrestricted division and durably growth of tumor cells (15) (20). However, it seems not all tumor cells possess the same pattern of BCAA metabolism. The increased level of circulating BCAAs as a result of the protein breakdown or BCKAs aminating could be observed in cancer patients with pancreatic adenocarcinoma and leukemia (21, 22). These do not imply that the BCAAs level was always elevated in cancer patients. Indeed, a decrease in circulating BCAAs caused by increased tumor cell uptake and breakdown has also been found in lung tumor cells (23). Mayers et al. attributed such distinct phenotypes to the difference in mutations and the origin of cancer tissues (23). Furthermore, BCAAs were recently regarded as an upstream signal input of the mTOR signaling pathway, which activates the pathway regulating the growth and proliferation of cancer cells (18) (24-26). Several recent studies have discovered that the accumulation of BCAAs in cancer cells due to the reduction of catabolism may enhance the activity of mTOR signaling pathway and promote the progression of cancers (21, 22) (27-29). However, less is known on the functions of BCAA in the progression of osteosarcomas.

In our present study, we showed the possible that ANGPTL4 triggers BCAA/mTOR signaling axis in OS cells. Knockdown of *ANGPTL4* in OS cell lines changed the metabolism of BCAAs and then enhanced the accumulation of BCAAs in these cells. This led to the activation of the mTOR signaling pathway, which result in the promotion the growth of OS cell (Fig. 7). Our findings provide further evidence for the theory that BCAA metabolism was implicated with the progress of various tumors and confirmed that BCAA metabolism can be subject to regulation by ANGPTL4.

Although the role of ANGPTL4 played in the metabolism regulation was compelling, the impacts of ANGPTL4 in the progression of the tumor were confusing. Previous studies discovered that ANGPTL4 could have a higher expression in tumor patients and evoke malignant phenotype of tumor cells, i.e., proliferation, migration, and drug resistant (10) (12) (30, 31). However, some subsequent studies demonstrated that ANGPTL4 showed an anti-angiogenesis as well as antitumor cell invasion and migration ability during the tumor progression. It should therefore be regarded as a tumor suppressor and a favorable prognostic marker of patients (11) (32-34). In a previously OS study, Zhang et al. showed that ANGPTL4 promoted tumor progression, which is inconsistent with our findings (13). However, there was no more detailed mechanism research that can be referred to in Zhang's study, which remains no definite explanation for these contradictory phenomena to date. Mechanically, ANGPTL4 could be processed and cleaved into two main functional domains, the *N*-terminal fragment (nANGPTL4) and *C*-terminal fragment (cANGPTL4) (35, 36). The difference in function of the full-length ANGPTL4 (fANGPTL4) and the cleaved form may contribute to the contrary experimental results. Except for being directly cleaved, as a secreted glycoprotein, the diverse post-translational modification of ANGPTL4 was also a significant factor that affects the functions of ANGPTL4. For example, an abnormal sialylation of ANGPTL4 was observed in nephrotic syndrome (37). Furthermore, available data suggest that the different experimental models,

different microenvironments, and even different cell lines could be the reasons why confounded results were observed in different studies.

Even if more research is needed if we want to understand the overall mechanism by which ANGPTL4 affects tumors, the present results revealed significant components of the whole mechanism. Recent research has shed new light on lipid metabolism and progression in tumors. Pascual et al. pointed out that a high-fat diet promotes the metastasis of oral squamous cell carcinoma and melanoma in an animal model through epigenetic regulation (38). In addition, the immune microenvironment of the intestinal tract was validated and could be regulated by a high-fat diet which enhanced intestinal tumorigenesis (39). Interestingly, previous studies had found a mutual effect between BCAAs and lipid metabolism (40, 41). Supplementation with BCAAs in a diet-induced obese mice model results in significant hepatic metabolic disorder that promoted gluconeogenesis and inhibited lipogenesis (24). In contrast, the suppression of the BCAA catabolism was also observed under a high-fat circumstance, and the expression of BCAA catabolism enzymes was reduced in the mice fed a high-fat diet (42). Conceivably, although the underlying mechanism is unknown to date, the interaction between BCAA metabolism and lipid metabolism will definitely affect tumor progression. According to our results, ANGPTL4 serves as not only a lipid regulator, but a bridge between BCAA metabolism and lipid metabolism as well.

Although our current results have partly clarified the organization and relationship of ANGPTL4, BCAA metabolism, and mTOR signaling pathways in the progression of OS, the precise mechanisms involved in this loop remain unclear and need to be further studied. In particular, we better understand how ANGPTL4 regulates metabolism of BCAAs, thereby regulating tumor progression of OS through the activation of mTOR signaling pathway, but we do not know exactly how various receptors are involved. The precise mechanisms involved in this loop especially the cascade between the ANGPTL4 and the BCATs were unclear and need to be further studied. Furthermore, the present results demonstrate the significant role of ANGPTL4 in the progression of OS. However, no more details were obtained, and it is still unclear whether it is the flANGPTL4, the truncated ANGPTL4 (nANGPTL4, cANGPTL4) or the coaction of all three that plays a role in tumor progression. Thus, additional studies are needed to further clarify the exact mechanism underlying the BCAA metabolism alteration after ANGPTL4 regulation during OS progression.

In summary, we discovered that low levels of expression of ANGPTL4 regulated the metabolism of BCAAs to activate the mTOR signaling pathway, leading to accelerated proliferation in OS, as presented in Fig. 7. ANGPTL4 may be a reasonable and not previously reported bridge that links BCAA metabolism and lipid metabolism to promote OS progression. Given that overexpression of ANGPTL4 reduced the progression of OS, increasing the expression of ANGPTL4 in tumor cells of patients with OS may be a promising therapeutic strategy for OS in the future.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Sixth People's Hospital

Consent for publication

All authors involved in the authorship are consent for publication in the current form.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional files).

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

LSY carried out the experiment and wrote the manuscript; MY and ZX carried out the experiment; DY and YQC prepared the clinical samples; YQJ quantified and analyzed the data; XJ, ZSM and YT designed the research, reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figures

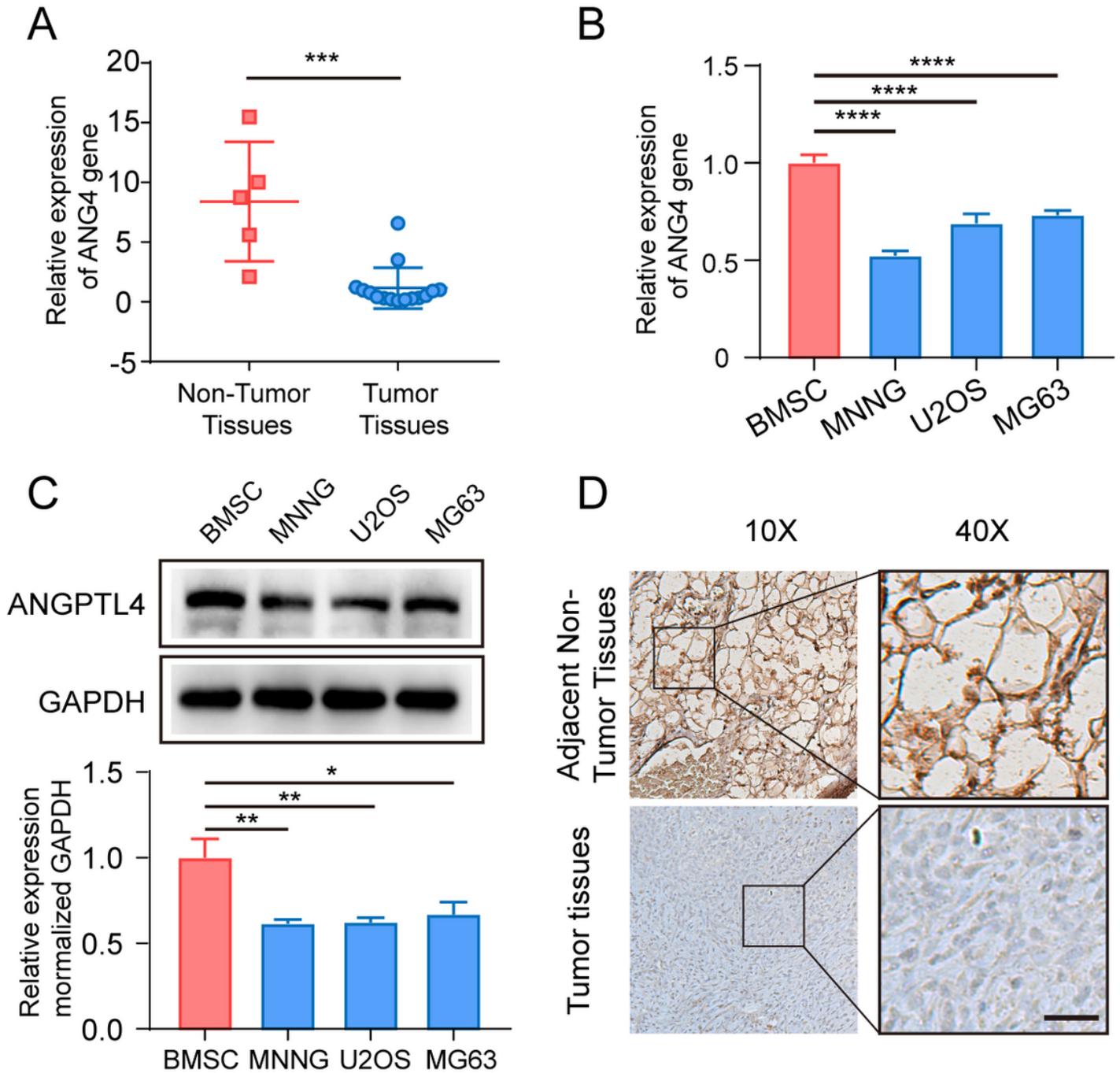


Figure 1

Expression of ANGPTL4 is lower in osteosarcoma tissue and osteosarcoma cell lines. (A) Relative expression of *ANGPTL4* mRNA in clinical osteosarcoma (OS) tissue samples ($N=15$) and adjacent nontumor cancellous bone tissue samples ($N=5$). **(B)** Relative expression of *ANGPTL4* mRNA in OS cell lines (MNNG, U2OS, MG63) and the bone marrow derived stroma cell (BMSC). Mean \pm SD ($N=3$). **(C)** *ANGPTL4* expression (upper panels) and quantitation of protein levels (lower bar graphs) in OS cell lines and BMSC cells were detected by western blotting. Mean \pm SD ($N=3$). **(D)** Representative immunohistochemical images of *ANGPTL4* in clinical OS sample and adjacent nontumor bone marrow sample. (D, scale bars, 100 μ m). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

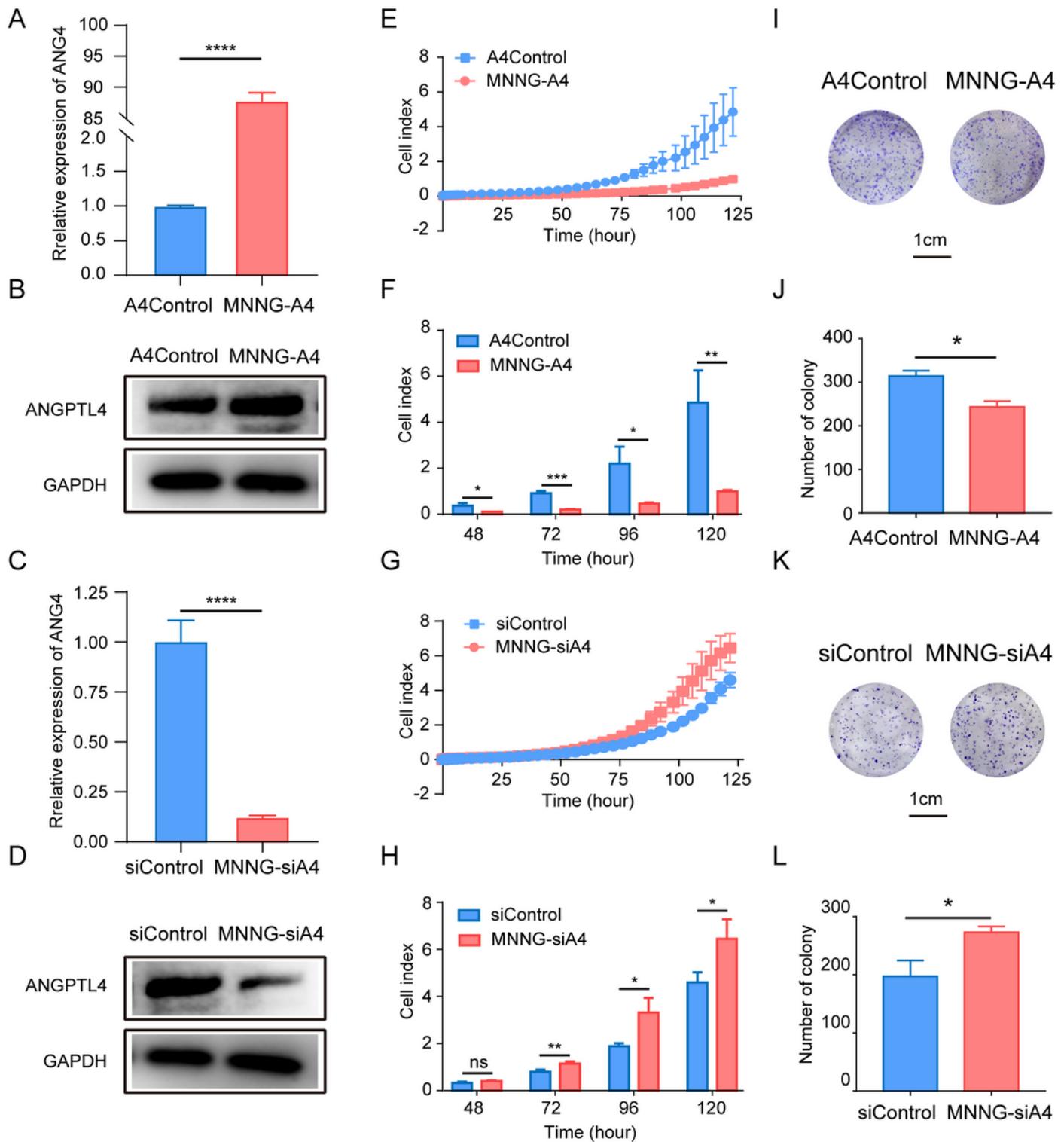


Figure 2

The Knockdown of *ANGPTL4* promotes the growth of MNNG *in vitro*. (A-D) Quantitation of *ANGPTL4* mRNA and *ANGPTL4* protein in MNNG-A4, MNNG-siA4, and control cell lines by qRT-PCR and western blot, respectively. (E, G) *In vitro* measurement of cell proliferation in two *ANGPTL4*-regulated MNNG cell lines (MNNG-A4, MNNG-siA4) and according control cell lines. Cell index results reflects that cell proliferation is enhanced in MNNG-siA4 cells, while reduced in MNNG-A4 cells compared to control

groups. Mean \pm SD ($N=4$). **(F, H)** Cell index at 48 h, 72 h, 96 h, 120 h. Mean \pm SD ($N=4$). **(I, K)** Representative images of colony formation assay in MNNG-A4, MNNG-siA4, and control cells. **(J, L)** Quantitation of colony counts after 2 weeks of *in vitro* maintenance. Mean \pm SD ($N=3$). Mean \pm SD ($N=5$). Mean \pm SD. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

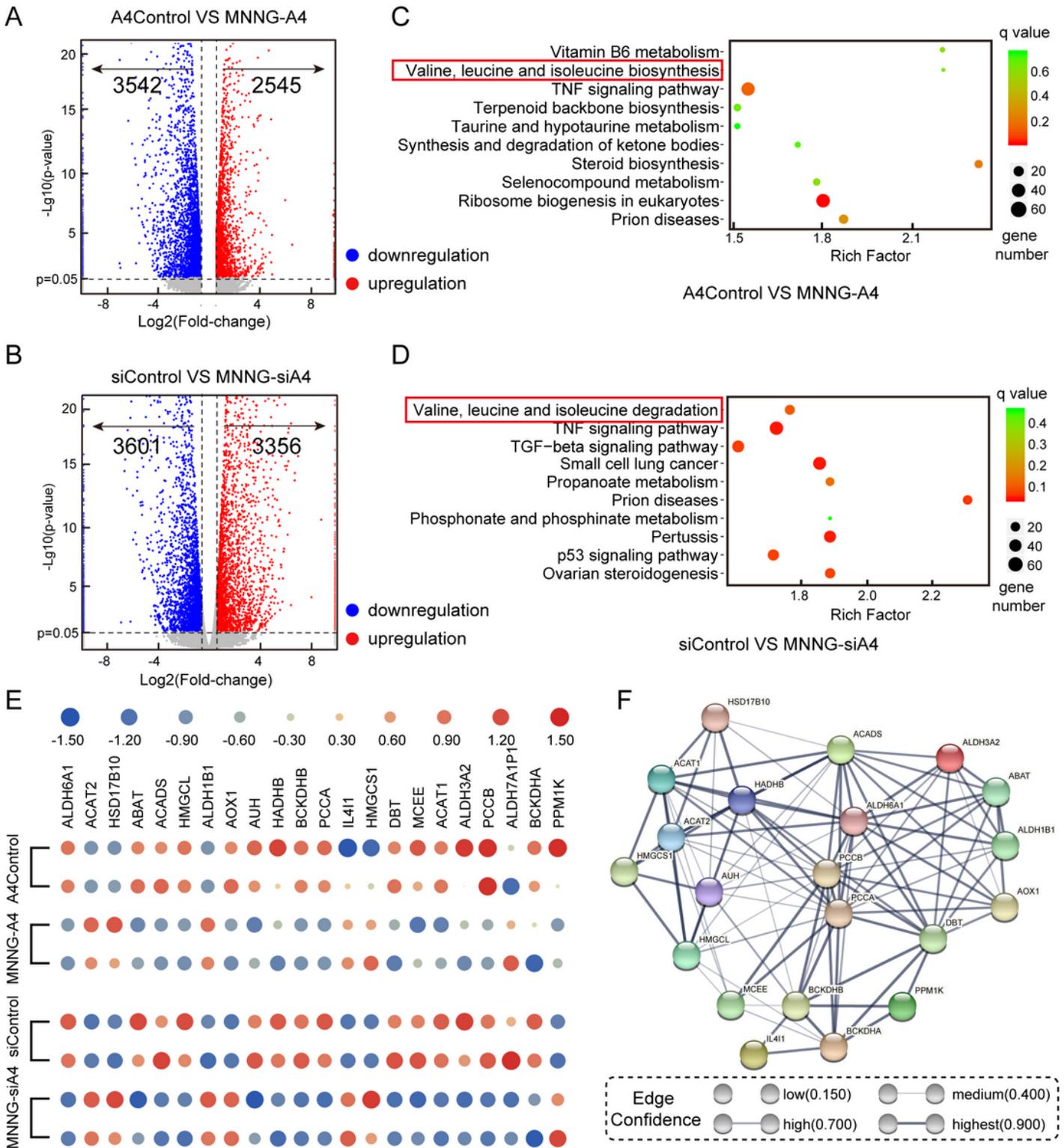


Figure 3

The signaling discovery of ANGPTL4 functioning on OS growth by RNA-seq. (A, B) Volcano plots of the differentially expressed genes (DEGs) in MNNG-A4 cells, MNNG-siA4 cells, and their control cells. Fold-change values on the X-axis were log₂-transformed while *P* values on the Y-axis were -log₁₀ transformed. The screening criteria for EDGs are positioned as *P* value < 0.05, and the expression level is higher than 1.5 folds or lower than 0.67 folds. **(C, D)** KEGG pathway enrichment analysis for DEGs in MNNG-A4 cells, MNNG-siA4 cells, and their control cells. **(E)** Heatmap of the BCAAs associated DEGs between ANGPTL4-regulated cells and control cells. **(F)** Protein-protein interaction network provided interactive information among the BCAAs associated DEGs. Mean ± SD (*N*=3) ns *P* > 0.05; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

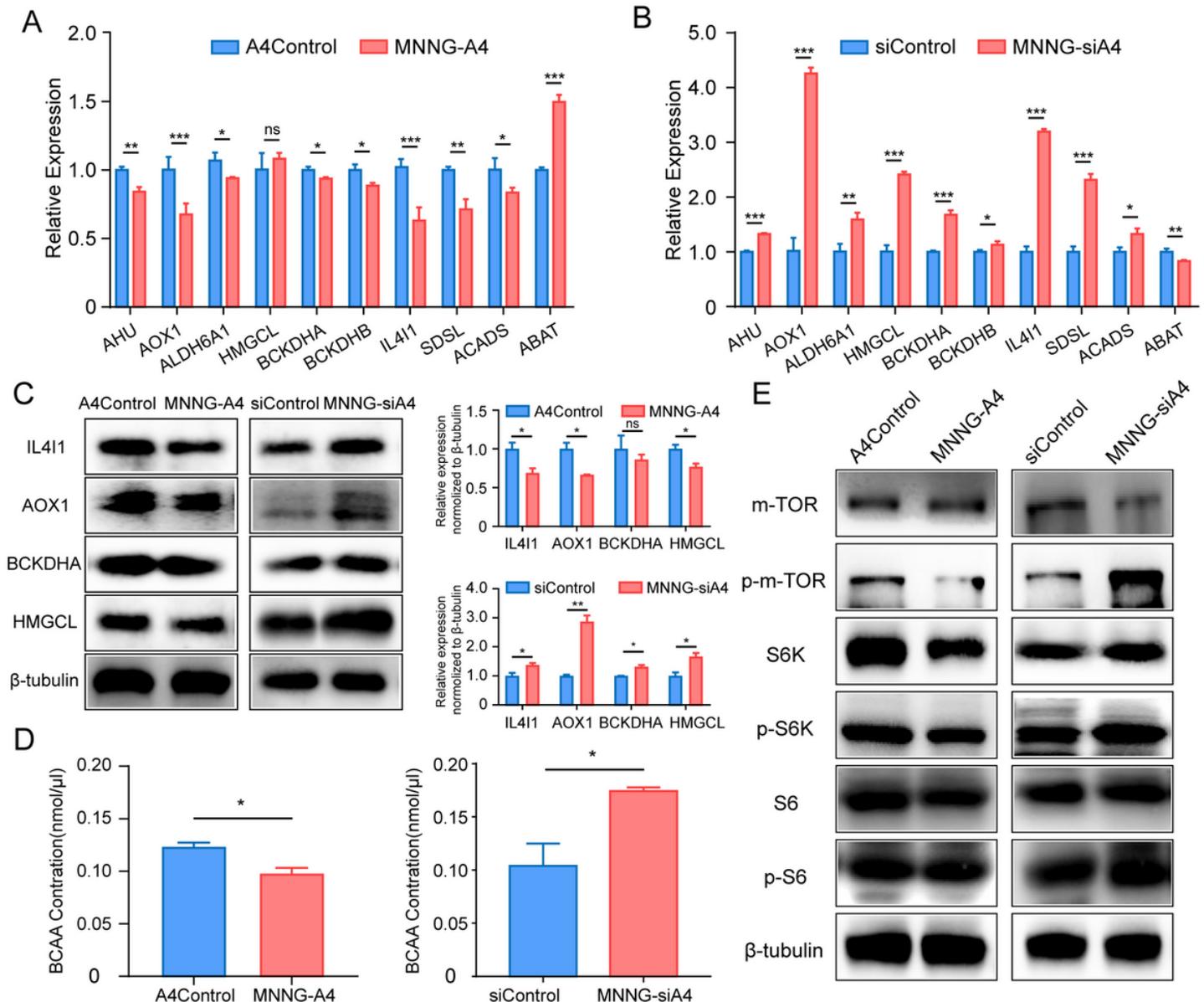


Figure 4

The low expression of ANGPTL4 promoted the accumulation of branched chain amino acid (BCAA) and activate mTOR signaling. (A, B) Among the DEGs, genes that are highly related to BCAA metabolism were verified by RT-qPCR in the ANGPTL4-regulated cells and their controls. **(C)** Proteins that are highly related

to the BCAA metabolism were verified by western blotting in the ANGPTL4-regulated cells and their controls. Quantitation of relative expression. Mean \pm SD ($N=3$). **(D)** The relative levels of BCAAs in MNNG-A4 cells, MNNG-siA4 cells, and their control cells. **(E)** western blot detected proteins in the mTOR signaling pathway show that this pathway was activated in MNNG-siA4 cells but inhibited in MNNG-A4 cells. Mean \pm SD ($N=3$). ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

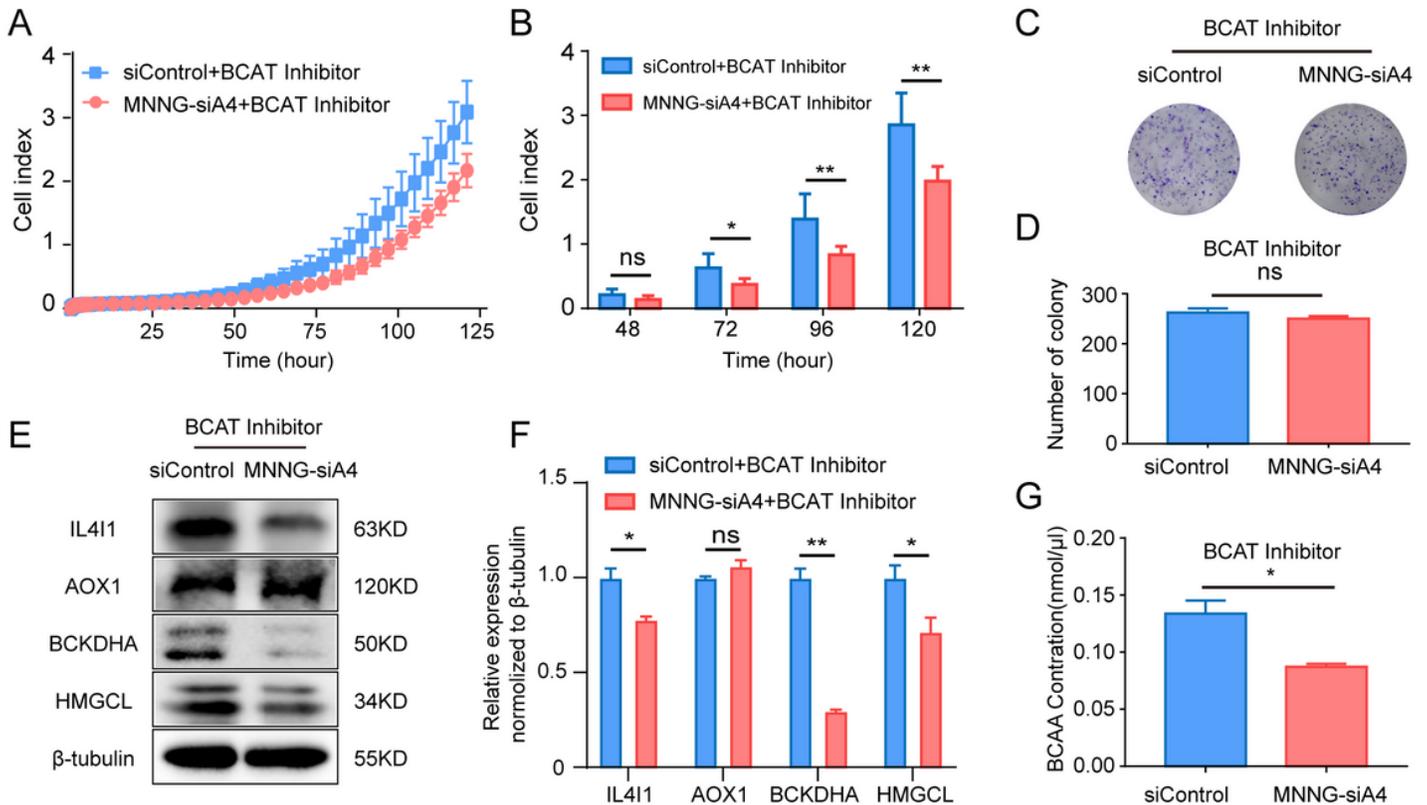


Figure 5

BCATs inhibitor attenuates the elevated growth of MNNG-siA4 cells. **(A)** *In vitro* measurement of cell attachment and cell proliferation in MNNG-siA4 and control cell lines with 5 μ M BCATs inhibitor treatment. **(B)** Cell index at 48 h, 72 h, 96 h, 120 h with 5 μ M BCATs inhibitor treatment. Mean \pm SD ($N=4$). **(C)** Representative images of colony formation assay in MNNG-siA4 and control cells after 5 μ M BCATs inhibitor treatment. **(D)** Quantitation of colony counts after 2 weeks of *in vitro* maintenance with 5 μ M BCATs inhibitor treatment. Mean \pm SD ($N=3$). **(E, F)** Proteins that are highly related to the BCAA metabolism were verified by western blotting in the MNNG-siA4 cells and control cells after 5 μ M BCATs inhibitor treatment. **(G)** The relative levels of BCAAs in MNNG-siA4 cells and control cells after 5 μ M BCATs inhibitor treatment. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$.

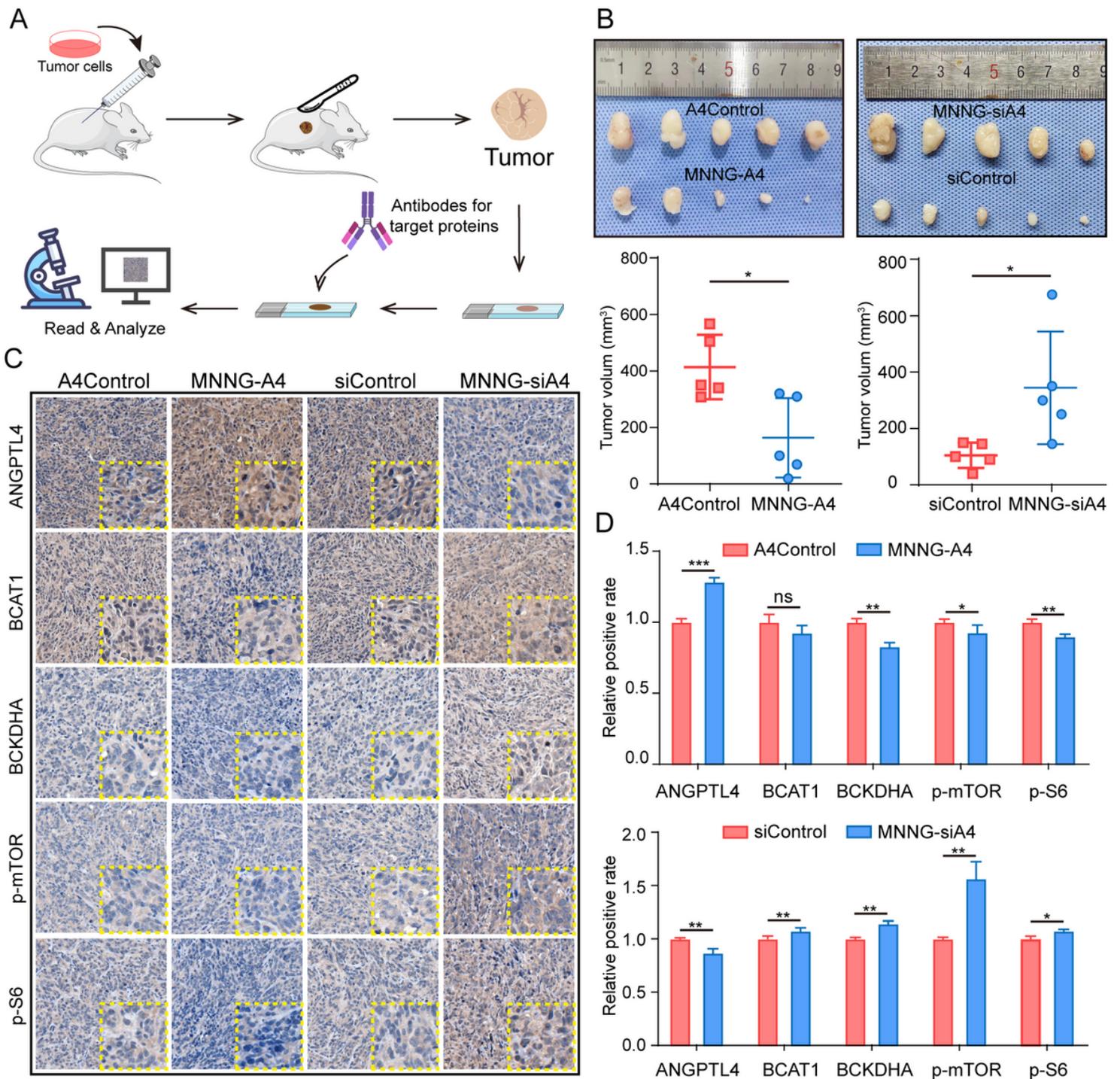


Figure 6

The expression of ANGPTL4 shows a negatively regulation with OS progress via BCAA/mTOR axis *in vivo*. **(A)** Diagram illustrating the method for constructing subcutaneous tumor model and IHC analysis. **(B)** Images of excised tumors from the nude mouse subcutaneous tumor model and quantitation of tumor volume. **(C, D)** Immunohistochemical staining of ANGPTL4, BCAT1, BCKDHA, p-mTOR, and p-S6 in xenografts of OS tissue from the subcutaneous tumor nude mouse model.

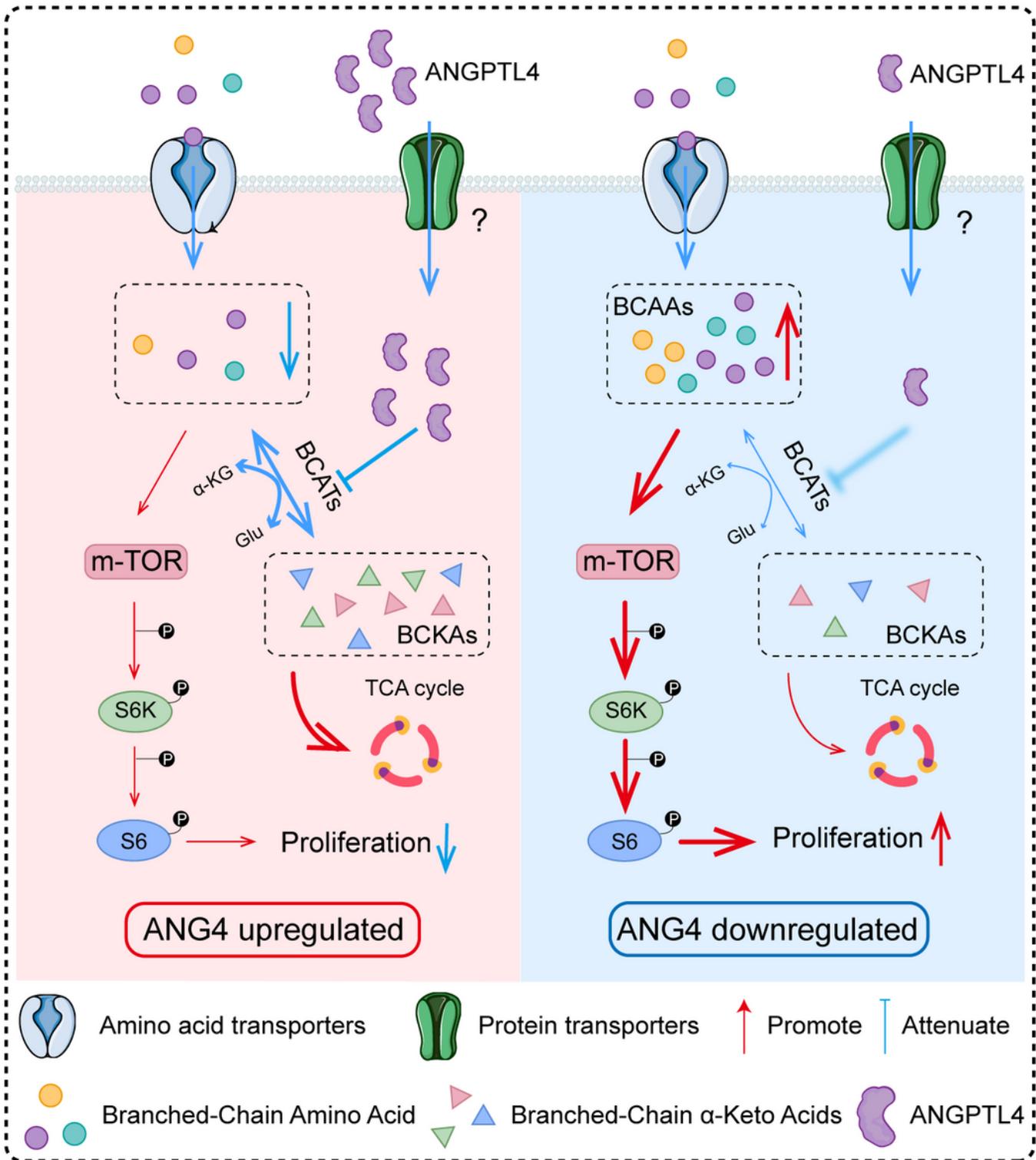


Figure 7

ANGPTL4 negatively regulates OS cell growth by remodeling BCAA metabolism. Diagram illustrating one mechanism that illustrates how ANGPTL4 influences OS cell progression through the ANGPTL4/BCAAs/mTOR axis.

Supplementary Files

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