

LncRNA CCAL Promotes Angiogenesis Through Regulating the MiR-29b/ANGPTL4 Axis in Osteosarcoma

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Purpose: The objective of this study was to detect the expression of the long noncoding RNA (lncRNA) colorectal cancer-associated lncRNA (CCAL) in osteosarcoma tissues and to investigate its role in angiogenesis and the potential molecular mechanisms associated with this effect in osteosarcoma.

Materials and Methods: CCAL expression in 40 osteosarcoma tissues and 40 noncancerous tissues was measured by qRT-PCR (quantitative real-time polymerase chain reaction). Tube formation assays were performed to explore the role of CCAL in angiogenesis in osteosarcoma. In addition, the regulatory interaction between CCAL, miR-29b, and ANGPTL4 was investigated via luciferase reporter assay and bioinformatics predictive analysis.

Results: Compared with noncancerous tissues, the expression of CCAL was markedly upregulated in osteosarcoma tissues. Higher CCAL expression levels were closely related to shorter overall survival in patients with osteosarcoma. Additionally, functional analysis indicated that CCAL could facilitate tumour angiogenesis in vitro and in vivo in osteosarcoma. Mechanistically, CCAL upregulated ANGPTL4 expression in osteosarcoma cells, and ANGPTL4 mediated angiogenic induction by CCAL in osteosarcoma. Moreover, CCAL directly targeted miR-29b in osteosarcoma. More importantly, we demonstrated that CCAL upregulated the expression of ANGPTL4 by sponging miR-29b, which promoted angiogenesis in osteosarcoma.

Conclusion: Our results show that CCAL promotes angiogenesis by regulating the miR-29b/ANGPTL4 axis in osteosarcoma.

Keywords: lncRNA CCAL, miR-29b, ANGPTL4, osteosarcoma, angiogenesis

Introduction

Osteosarcoma is the most common primary malignant tumour derived from bone among children and adolescent individuals.^{1,2} Surgery and intensive adjuvant chemotherapy are the main treatments for patients with osteosarcoma. However, the survival rate is approximately 15–17% in osteosarcoma patients treated with surgery alone.^{3,4} For osteosarcoma patients with metastasis or relapse, survival has remained virtually unchanged in the past 30 years.^{1,5} Increasing evidence shows that angiogenesis is a pivotal process in the development and progression of osteosarcoma.^{6,7} ANGPTL4 (angiopoietin-like 4), a secretory glycoprotein consisting of N-terminal and C-terminal domains, is a member of the angiopoietin family.⁸ Previous studies have shown that ANGPTL4 plays an important role in tumour

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angiogenesis.^{9–11} However, the role of ANGPTL4 in angiogenesis in human osteosarcoma remains largely unknown.

lncRNAs (long noncoding RNAs) are a subgroup of noncoding RNAs that are more than 200 nucleotides in length.¹² Accumulating evidence indicates that lncRNAs serve as important players in cancer biology, typically resulting in aberrant expression of gene products that contribute to the progression of a variety of human tumours.^{13,14} The lncRNA CCAL (colorectal cancer-associated lncRNA) acts as an oncogenic gene in many human cancers.^{15–17} Currently, it has been reported that CCAL promotes gastric cancer metastasis by regulating the miR-149/FOXM1 axis.¹⁶ Additionally, CCAL drives the progression of colorectal cancer through activating the Wnt/ β -catenin signalling pathway by suppressing AP-2 α (activator protein 2 α).¹⁵ However, the biological role and molecular mechanisms of CCAL in the angiogenesis of osteosarcoma have not yet been clarified.

In the present study, we assessed the level of CCAL among osteosarcoma tissues via qRT-PCR. In addition, we further investigated the role and potential regulatory mechanisms of CCAL in the angiogenesis of human osteosarcoma. Ultimately, our findings suggested that CCAL promotes angiogenesis by regulating the miR-29b/ANGPTL4 axis in osteosarcoma.

Materials and Methods

Tissue Specimens

The present study followed the Helsinki declaration guidelines. It was approved by the Ethics Review Committee of Yangpu Hospital, Tongji University School of Medicine (LL-003). Informed consent was obtained from each patient. A total of 40 osteosarcoma tissues were collected from patients diagnosed with osteosarcoma who had undergone surgical resection at Yangpu Hospital, Tongji University School of Medicine between January 2015 and December 2018. All of these osteosarcoma cases were

localized primary tumours. There were no patients with metastasis or relapse. Forty normal tissues (referred to as noncancerous tissues) come from corresponding para-tumour tissues and were used as the relevant controls for osteosarcoma.

Cell Culture and Transfection

A human osteoblast cell line (hFOB1.19), osteosarcoma cell lines (HOS, SAOS2, U2OS, and MG-63), and HUVECs (human umbilical vein endothelial cells) were purchased from the Chinese Academy of Sciences (Shanghai, China). Short tandem repeat (STR) analysis and mycoplasma testing were performed in all cell lines used in this study. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) or Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan City, UT) containing 10% foetal bovine serum (Gibco, Grand Island, NY) at 37°C under a humidified atmosphere of 5% CO₂. A lentivirus-based shRNA (short hairpin RNA) targeting CCAL was obtained from GenePharma (Shanghai, China). All shRNA sequences for CCAL are shown in Table 1.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

According to the manufacturers’ instructions, total RNA was extracted from 40 osteosarcoma tissues, 40 noncancerous tissues and osteosarcoma cell lines using TRIzol reagent (Invitrogen, MA). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara, Japan). Primers for GAPDH and CCAL were acquired from Sangon Biotech (Shanghai, China). GAPDH was used as a reference control for CCAL. The primers for CCAL and GAPDH were as follows: CCAL, forward: 5'-AAGGGAGTTTTGTGCGGTGAGAA-3' and reverse: 5'-TGTGCTGGCTTGTTTGGCTTTATT-3'; ANGPTL4, forward: 5'-GGACCACAAGCACCTAGACCA-3' and reverse: 5'-GATCCCCAAACCCCGCCTT-3'; and GAPDH, forward:

Table 1 The Sequences of shRNA for CCAL

Gene Name	Sequence (5' to 3')
sh-CCAL-1	Sense: GATCCGTTTCCAGAACTGGCAGCCCTTCCTGTCTGAGAGGCTGCCAGTCTGGAACTTTTTG
sh-CCAL-2	Anti-sense: AATTCAAAAAGTTTCCAGAACTGGCAGCCTCTGACAGGAAGGGCTGCCAGT
sh-NC	Sense: CACCGCATCTTAGACTGGATCTTCATTCAAGAGATGAAGATCCAGTCTAAGATGCTTTTTTG
	Anti-sense: GATCCAAAAAGCATCTTAGACTGGATCTTCATCTCTGAATGAAGATCCAGTCTAAGATGC
	Sense: GATCCGAAGCCAGATCCAGCTTCCCTTCCTGTCTGAGGAAGCTGGATCTGGCTTCTTTTTG
	Anti-sense: AATTCAAAAAGAAGCCAGATCCAGCTTCTCTGACAGGAAGGGAAGCTGGATCTGGCTTCG

5'-TCAAGGCTGAGAACGGGAAG-3' and reverse: 5'-TCGCCCCACTTGATTTTGA-3'.

Animal Study

BALB/c nude mice (6 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). For subcutaneous tumorigenesis assays, U2OS or MG-63 cells (5×10^5) transfected with sh-CCAL or sh-NC were injected into mice. Tumour growth was monitored every 10 days. The tumour volume was calculated as follows: volume = length $\times$ width² $\times$ 0.5. Five weeks after the injections, tumours were collected for immunohistochemistry (IHC) staining. All animal assays were approved by the Ethics Review Committee of Yangpu Hospital, Tongji University School of Medicine. Animal care and procedures were performed in accordance with Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines.

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed as previously described.¹⁸ Antibodies against CD31 (ab182981) and ANGPTL4 (ab196746) were obtained from Abcam (Cambridge, UK). All images were collected via microscopy (Leica, Germany). Tissue slides stained for CD31 were examined to ascertain microvascular density (MVD) in the tissue samples. The detailed process of evaluating microvessel density in tumour tissue was performed as previously described.^{19,20}

Enzyme-Linked Immunosorbent Assay (ELISA)

The conditioned medium of U2OS or MG-63 cells transfected with sh-CCAL or sh-NC was collected. According to the manufacturer's instructions, ELISAs (Abcam, UK) were performed to measure the level of ANGPTL4 (ab99974). The concentration of ANGPTL4 was determined by measuring the absorbance at 450 nm in a microplate reader.

Tube Formation Assay

HUVECs (human umbilical vein endothelial cells) (2×10^4 /well) were seeded in 96-well plates that contained dissolved Matrigel matrix (BD Biosciences, USA). Conditioned medium from U2OS or MG-63 cells transfected with sh-CCAL or sh-NC was collected and added into the well. After incubation for 6 h at 37°C, the

structures (tube-like) in each well were photographed under a microscope (Leica, Germany).

Western Blotting

U2OS or MG-63 cells transfected with an NC mimic or a miR-29b mimic were first lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Then, extracted proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were incubated with an ANGPTL4 primary antibody (ab196746) (Cambridge, UK) for 12 h at 4°C and then were incubated with secondary antibodies (Cambridge, UK) at 37°C for 2 h. The reaction was visualized via ECL (Electrochemical Luminescence) (Millipore, USA).

Dual-Luciferase Reporter Assay

Luciferase reporter assays were performed with a dual-luciferase assay system (Promega, USA) according to the manufacturer's protocol. U2OS or MG-63 cells in 96-well plates were simultaneously transfected with a miR-29b mimic (RiboBio, China) and reporter plasmids using Lipofectamine 2000 reagent (Thermo Fisher Scientific). After 48 h, luciferase activities were measured by a dual-luciferase reporter assay kit. Then, the ratio of Renilla/firefly luciferase activities was further analysed.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. All data are expressed as the means $\pm$ SD. Pearson correlation analysis was carried out to assess the correlation among the groups. One-way analysis of variance or a Student's *t*-test was used to compare quantitative variables. $P < 0.05$ was considered statistically significant.

Results

Upregulated CCAL is Closely Associated with Poor Prognosis in Osteosarcoma

Accumulating evidence indicates that CCAL acts as an oncogene in many human cancers.^{15–17} However, there have been no studies about the relationship between CCAL and osteosarcoma prognosis. Therefore, to determine the level of CCAL among osteosarcoma, qRT-PCR was performed to detect its expression in 40 osteosarcoma

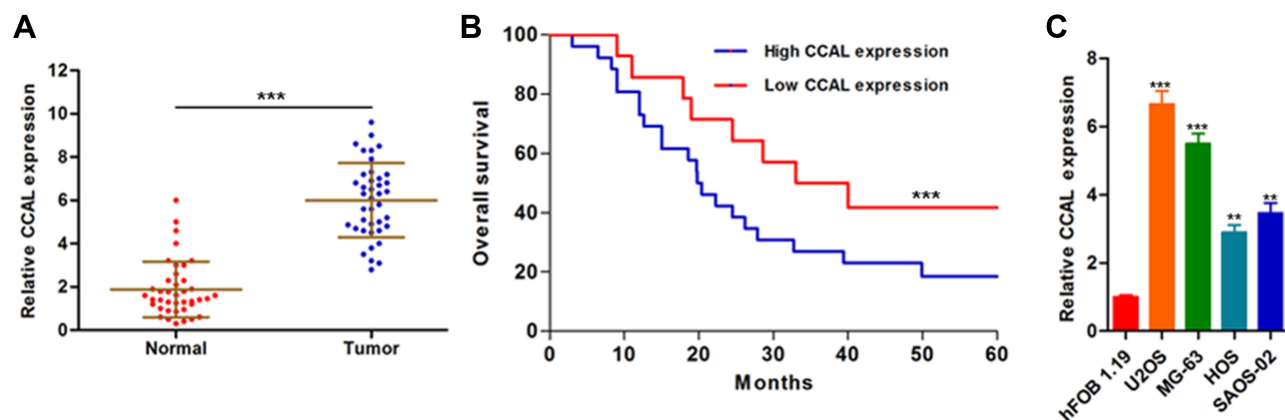


Figure 1 Upregulated CCAL is closely associated with poor prognosis in osteosarcoma. **(A)** CCAL expression was measured in 40 osteosarcoma tissues and 40 noncancerous tissues via qRT-PCR. $***P<0.001$. **(B)** Kaplan-Meier survival analysis was performed to investigate the overall survival of patients in the CCAL high ($N=20$)/low ($N=20$) groups. $***P<0.001$. **(C)** CCAL expression was determined in different osteosarcoma cell lines (U2OS, MG-63, HOS, and SAOS-02) and in a human osteoblast cell line (hFOB1.19) using qRT-PCR. $**P<0.01$, $***P<0.001$ vs hFOB1.19 group.

tissues and 40 noncancerous tissues. As shown in Figure 1A, CCAL levels were higher in osteosarcoma tissues than in noncancerous tissues. Additionally, Kaplan-Meier analysis was used to investigate the relationship between patient survival and CCAL expression. The results indicated that high expression of CCAL was associated with poor overall survival in patients with

osteosarcoma (Figure 1B). Further results also confirmed that CCAL expression was significantly higher in osteosarcoma cell lines (U2OS, MG-63, HOS, and SAOS-02) than it was in a human osteoblast cell line (hFOB1.19) (Figure 1C). These data demonstrate that CCAL expression is upregulated in osteosarcoma and is related to poor overall survival in patients with osteosarcoma.

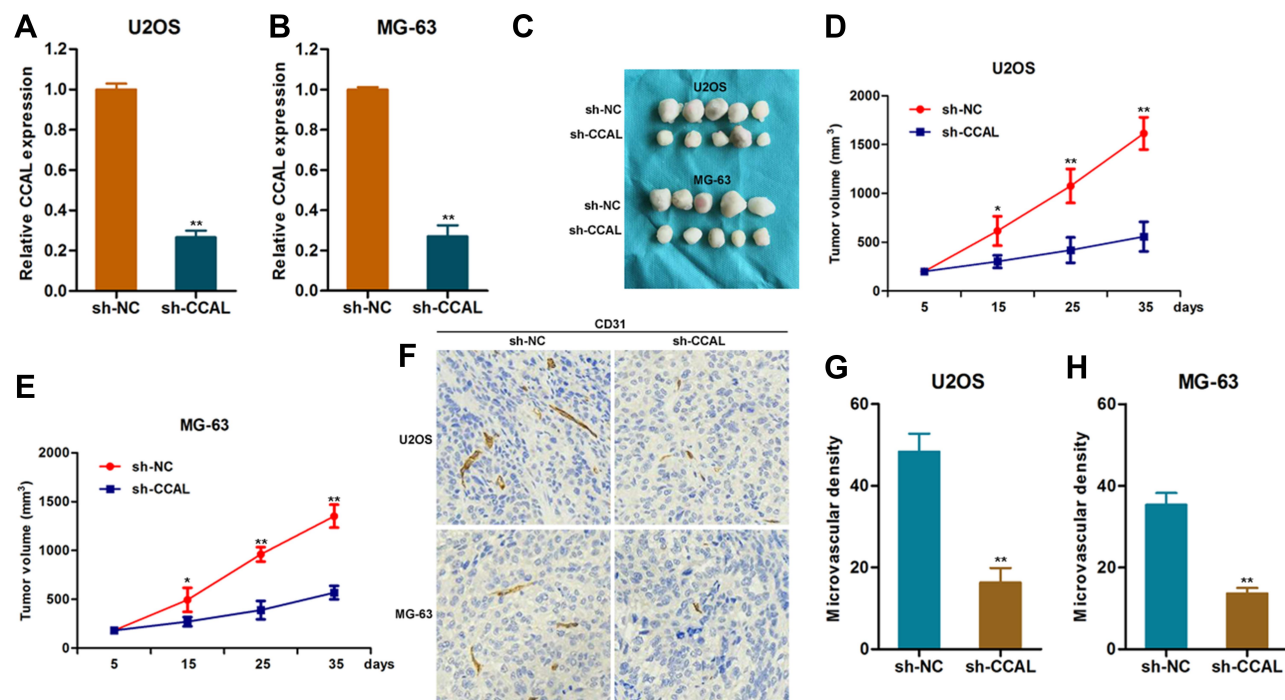


Figure 2 CCAL promotes tumour growth and angiogenesis in osteosarcoma. **(A, B)** CCAL expression was detected through qRT-PCR in MG-63 and U2OS cells treated with a lentivirus-based shRNA targeting CCAL. $**P<0.01$. **(C–E)** In vivo studies were performed with U2OS and MG-63 cells. Typical xenograft images are shown **(C)**, and the tumour volume was calculated **(D, E)**. $*P<0.05$; $**P<0.01$. **(F–H)** Immunohistochemistry was performed to measure CD31 expression in MG-63 and U2OS tumours **(F)**. The microvessel density in tumour xenografts was assessed **(G–H)**. $**P<0.01$.

CCAL Promotes Tumour Growth and Angiogenesis in Osteosarcoma

To identify the roles of CCAL in tumour growth and angiogenesis in osteosarcoma, U2OS and MG-63 cells were treated with a lentivirus-based shRNA targeting CCAL. The qRT-PCR results showed the efficiency of sh-CCAL in knocking down CCAL in U2OS and MG-63 cells (Figure 2A and B). Subsequently, *in vivo* studies were performed with U2OS and MG-63 cells. We used U2OS and MG-63 cells to form subcutaneous xenografts that in nude mice, and we found that when CCAL was silenced in those cells, there was dramatic suppression of growth in the xenografts (Figure 2C–E). Moreover, the vascular density in the tumours in the sh-CCAL group was obviously decreased compared with that in the sh-NC group (Figure 2F–H). These observations demonstrated that CCAL promotes tumour growth and angiogenesis in osteosarcoma.

CCAL Promotes Angiogenesis by Regulating ANGPTL4 in Osteosarcoma

To further explore the role of CCAL in angiogenesis in osteosarcoma, tube formation assays were performed in HUVECs treated with conditioned medium from MG-63 or U2OS cells infected with a lentivirus-based shRNA targeting CCAL. The results indicated that decreased tube formation was observed in the sh-CCAL group compared with the sh-NC group (Figure 3A and B). Next, angiogenesis-related cytokine levels were determined in the cell culture supernatants of U2OS and MG-63 cells via ELISA. As shown in Figure 3C, knockdown of CCAL significantly suppressed the secretion of ANGPTL4 in MG-63 and U2OS cells but had no obvious effects on HGF, VEGF-D, VEGF-C, and VEGF. In addition, silencing CCAL significantly inhibited ANGPTL4 mRNA expression (Figure 3D). Similar results were observed regarding the protein expression of ANGPTL4 in MG-63 or U2OS-sh-CCAL tumour tissues, as shown by IHC (Figure 3E). More importantly, treatment with an ANGPTL4 antibody abolished the tube formation induced by CCAL (Figure 3A, F and G). Collectively, CCAL promotes angiogenesis in osteosarcoma by regulating ANGPTL4.

CCAL Directly Targets MiR-29b in Osteosarcoma

Previous studies have proven that lncRNAs play important roles in the development and progression of cancer by

serving as microRNA decoys to modulate gene expression.²¹ To further investigate the molecular mechanisms involved in CCAL-induced ANGPTL4 expression, the potential interactions between lncRNAs and miRNAs were predicted by StarBase prediction software. As shown in Figure 4A, there was a binding site for miR-29b in CCAL. Dual-luciferase reporter assays indicated that luciferase activities of CCAL-WT but not CCAL-MT were dramatically decreased by treatment with miR-29b mimics (Figure 4B). Moreover, knockdown of CCAL resulted in an obvious increase in the expression of miR-29b in MG-63 and U2OS cells (Figure 4C). Additionally, Pearson correlation analysis demonstrated an inverse correlation between CCAL and miR-29b in osteosarcoma tissues (Figure 4D). Our observations indicated that CCAL directly targets miR-29b in osteosarcoma.

ANGPTL4 is a Direct Target Gene of MiR-29b

To further explore the role of miR-29b in the expression of ANGPTL4 induced by CCAL, the potential interactions between ANGPTL4 and miR-29b were predicted via bioinformatics prediction tools (Starbase, miRDB, and TargetScan). The predicted results indicated that there was a binding site for miR-29b in ANGPTL4 (Figure 5A). Next, the relative luciferase activities were determined using reporter plasmids containing miR-29b binding sequences in the wild-type (WT) ANGPTL4 3'-UTR as well as mutant (MT) counterparts. As shown in Figure 5B, the relative luciferase activity was obviously decreased in MG-63 and U2OS cells that were transfected with a miR-29b-mimic. In addition, miR-29b significantly suppressed the mRNA and protein levels of ANGPTL4 and its secretion in MG-63 and U2OS cells (Figure 5C–E). The results of Pearson correlation analysis showed that there was a negative relationship between miR-29b and ANGPTL4 in osteosarcoma tissues (Figure 5F). Furthermore, knockdown of CCAL-mediated inhibition of tube formation was blocked in MG-63 and U2OS cells that were transfected with a miR-29b inhibitor (Figure 5G). Taken together, these results suggest that ANGPTL4 is a direct target gene of miR-29b.

Discussion

Osteosarcoma is the most common primary malignant tumour derived from bone among children and adolescent individuals.^{1,2} For osteosarcoma patients with metastasis

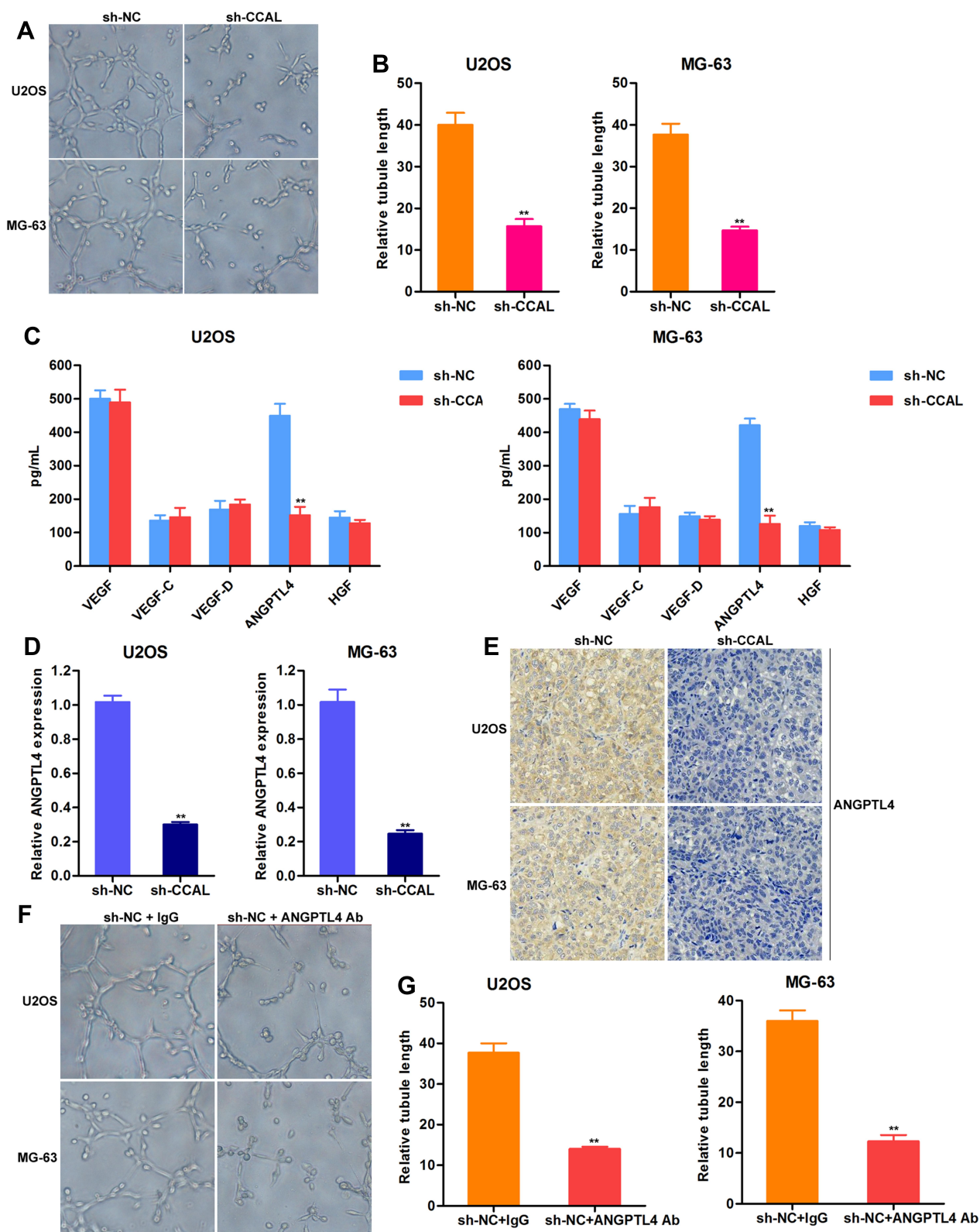


Figure 3 CCAL promotes angiogenesis by regulating ANGPTL4 in osteosarcoma. (A, B) Tube formation assays were performed in HUVECs treated with conditioned medium from MG-63 or U2OS cells infected with lentivirus-based shRNA targeting CCAL. ** $P < 0.01$. (C) Angiogenesis-related cytokines were measured in the culture supernatants of U2OS and MG-63 cells via ELISA. ** $P < 0.01$. (D) ANGPTL4 expression was measured by qRT-PCR in MG-63 and U2OS cells treated with a lentivirus-based shRNA targeting CCAL. ** $P < 0.01$. (E) Immunohistochemistry was used to detect ANGPTL4 expression in MG-63 and U2OS tumours. (F–G) HUVECs were stimulated with conditioned medium from MG-63 or U2OS cells treated with lentivirus-based shRNA targeting CCAL. An ANGPTL4 antibody (Ab) was applied to neutralize ANGPTL4 in conditioned medium. Tube formation assays were performed. ** $P < 0.01$.

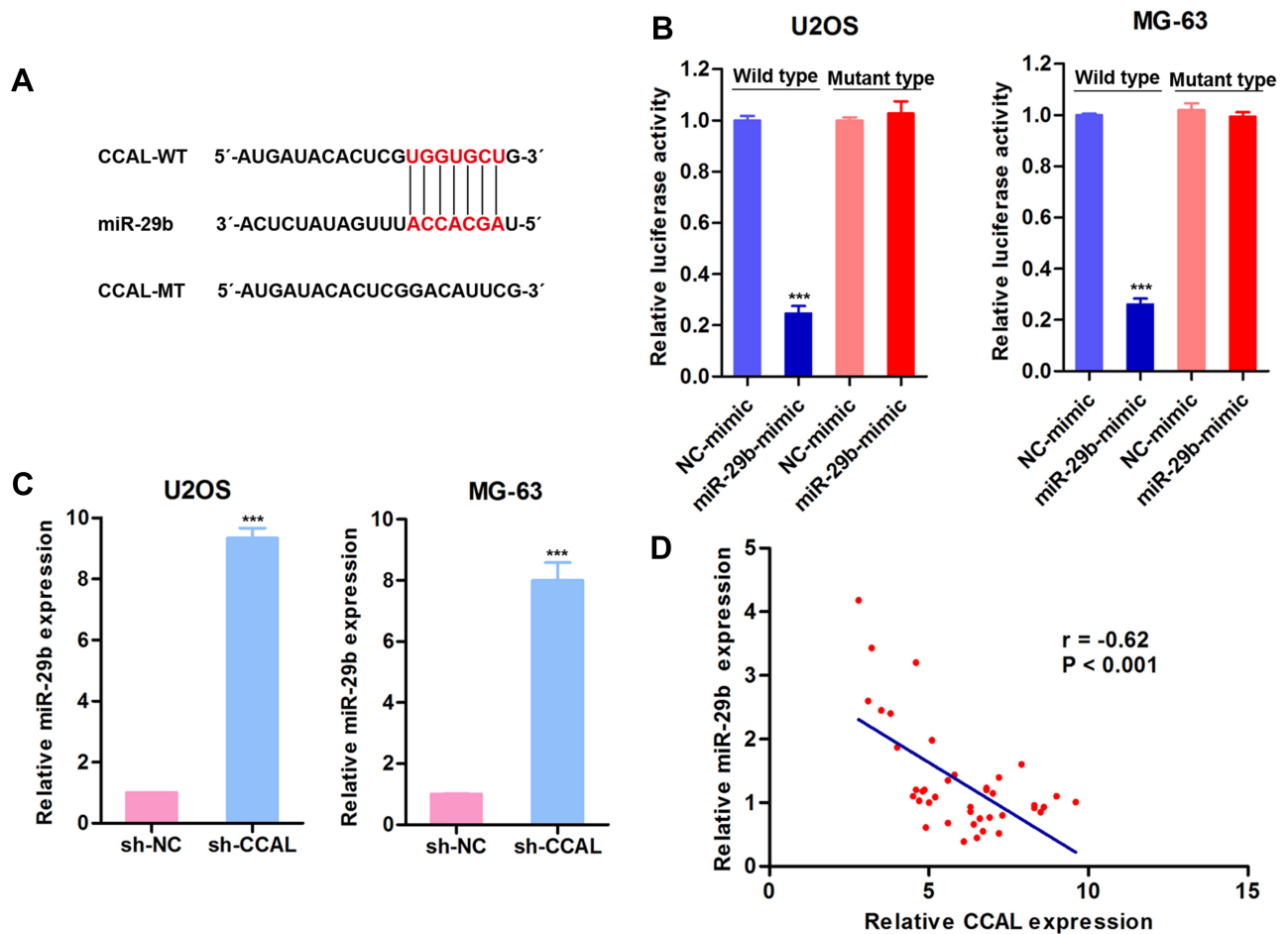


Figure 4 CCAL directly targets miR-29b in osteosarcoma. **(A)** The potential interactions between miR-29b and CCAL were predicted by StarBase prediction software. **(B)** Dual-luciferase reporter assays were performed in MG-63 and U2OS cells transfected with a miR-29b-mimic or an NC-mimic. *** $P < 0.001$. **(C)** MiR-29b expression was determined by qRT-PCR in MG-63 and U2OS cells treated with a lentivirus-based shRNA targeting CCAL. *** $P < 0.001$. **(D)** The relationship between miR-29b and CCAL in osteosarcoma tissues was explored via Pearson correlation analysis.

or relapse, survival has remained virtually unchanged in the past 30 years.^{1,5} Accumulating evidence demonstrates that angiogenesis is a pivotal process in the development and progression of osteosarcoma.^{6,7} The lncRNA CCAL (colorectal cancer-associated lncRNA) acts as an oncogene in many human cancers.^{15–17} However, little is known about the role and biological mechanisms of CCAL in the pathogenesis of osteosarcoma. In this study, we demonstrated that CCAL upregulated the expression of ANGPTL4 by sponging miR-29b, which promoted angiogenesis in osteosarcoma.

LncRNAs are involved in various types of gene regulation, including transcription and epigenetic or posttranscriptional regulation, which lead to the development of carcinomas and other diseases.²² Moreover, lncRNAs play an important role in tumour occurrence, metastasis, angiogenesis and chemoresistance by acting as ceRNAs

(competitive endogenous RNAs) toward miRNAs.^{23–25} For instance, the lncRNA DANCER promotes tumour progression and cancer stemness features in osteosarcoma by upregulating AXL via miR-33a-5p inhibition.²⁶ The lncRNA TUG1 (taurine upregulated gene 1) could promote osteosarcoma cell proliferation, angiogenesis, and metastasis in vivo and in vitro by serving as a miRNA sponge to competitively protect the HIF-1 α mRNA 3' UTR from miR143-5p.²⁷ Recently, it has been reported that CCAL could bind to miR-149, suppress the translation of FOXM1 (fork head box M1), and subsequently promote metastasis in gastric cancer.¹⁶ Additionally, CCAL drives the progression of colorectal cancer by suppressing AP-2 α to activate the Wnt/ β -catenin signalling pathway.¹⁵ CCAL promotes papillary thyroid cancer progression by activating the NOTCH1 pathway.²⁸ Furthermore, CCAL has been reported to be significantly upregulated in osteosarcoma

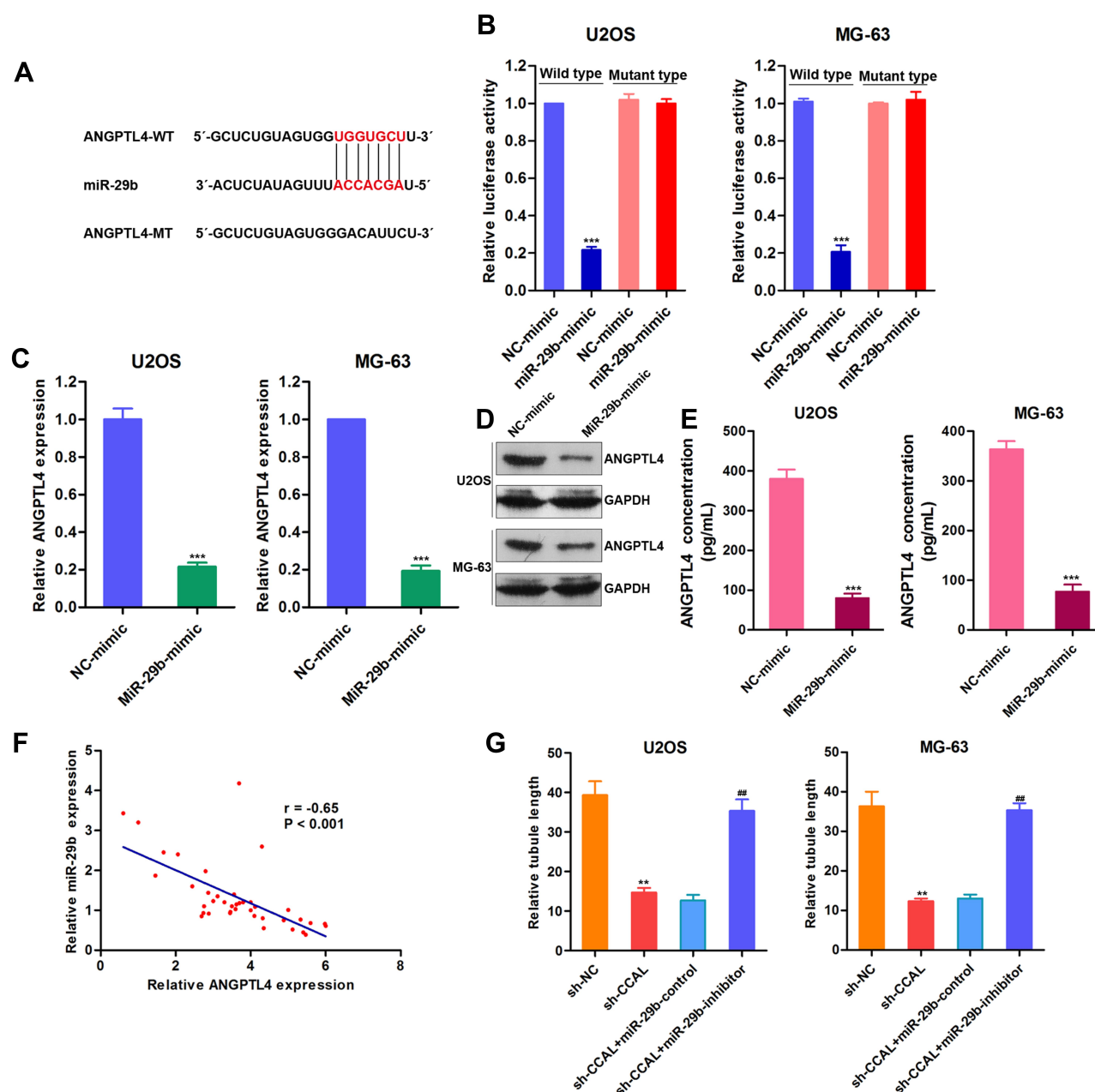


Figure 5 ANGPTL4 is a direct target gene of miR-29b. (A) The potential interactions between ANGPTL4 and miR-29b were predicted via bioinformatics prediction tools (Starbase, miRDB, and TargetScan). (B) Relative luciferase activities were determined using reporter plasmids containing either the wild-type (WT) ANGPTL4 3'-UTR with miR-29b binding sequences or mutant (MT) counterparts. *** $P < 0.001$. (C–E) The mRNA and protein expression of ANGPTL4 and its secretion by MG-63 and U2OS cells were assessed via qRT-PCR, Western blotting, and ELISA. *** $P < 0.001$. (F) The relationship between miR-29b and ANGPTL4 among osteosarcoma tissues was investigated through Pearson correlation analysis. (G) Tube formation was performed in HUVECs treated with conditioned medium from MG-63 or U2OS cells transfected with a shRNA-CCAL and a miR-29b inhibitor. The relative tubule length was calculated. ** $P < 0.01$ vs sh-NC group. *** $P < 0.01$ vs sh-CCAL + miR-29b-inhibitor group.

tissues and facilitate osteosarcoma cell proliferation, migration and invasion.²⁹ Similar to previous findings, we verified that CCAL was markedly upregulated in osteosarcoma tissues. Higher CCAL expression levels were closely related to shorter overall survival in patients with osteosarcoma. In addition, functional analysis indicated that CCAL could facilitate tumour angiogenesis in vitro

and in vivo in osteosarcoma. Mechanistically, CCAL upregulated the expression of ANGPTL4 by sponging miR-29b, thus promoting angiogenesis in osteosarcoma.

Previous studies have shown that miR-29b acts as a tumour suppressor by inhibiting tumour angiogenesis in various cancers, including cervical cancer, endometrial carcinoma, breast cancer, and hepatocellular carcinoma.^{30–33}

Insulin-like growth factor 1 (IGF1) has been reported to promote angiogenesis by functioning as a ceRNA, sponging the miR-29 family (miR-29a, miR-29b and miR-29c) in osteosarcoma.³⁴ Moreover, it has been reported that miR-29b is downregulated in osteosarcoma tissues and acts as a tumour suppressor of osteosarcoma by targeting CDK6 during the proliferation and migration processes.³⁵ ANGPTL4, a secretory glycoprotein consisting of N-terminal and C-terminal domains, is a member of the angiopoietin family.⁸ Previous studies have shown that ANGPTL4 plays an important role in tumour angiogenesis.^{9–11} In addition, ANGPTL4 is a potential molecular target for the treatment of osteosarcoma; its inhibition could conceivably target the primary tumour, inhibit angiogenesis, reduce metastatic events and prevent bone destruction.³⁶ In agreement with these studies, in the present study, bioinformatic analysis predicted a binding site for miR-29b in ANGPTL4. Luciferase assay results demonstrated that ANGPTL4 is a direct target gene of miR-29b in osteosarcoma. A negative relationship between miR-29b and ANGPTL4 in osteosarcoma tissues was also confirmed.

Conclusions

In conclusion, this study is the first to demonstrate that CCAL is an oncogenic lncRNA that promotes angiogenesis by regulating the miR-29b/ANGPTL4 axis in osteosarcoma. Therefore, this finding suggests that CCAL may be considered a novel therapeutic target for patients with osteosarcoma.

Disclosure

The authors report no conflicts of interest in this work.

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