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# MicroRNA-1294 targets HOXA9 and has a tumor suppressive role in osteosarcoma

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**Abstract. – OBJECTIVE: MicroRNA-1294 (miR-1294) was reported to act as a tumor suppressor in several cancers. However, the biological function of miR-1294 in osteosarcoma (OS) has not been investigated. We, therefore, investigated the clinical significance and underlying mechanisms of miR-1294 in OS.**

**PATIENTS AND METHODS: Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was conducted to detect the levels of miR-1294. Targets of miR-1294 were validated by luciferase reporter assay and Western blot. *In vitro* functional assays were performed to investigate the effects of miR-217 on cell proliferation and invasion.**

**RESULTS: We found miR-1294 was downregulated in OS tissues and cell lines. Downregulation of miR-1294 has a significant negative impact on the overall survival of OS patients. Overexpression of miR-1294 suppresses OS cell proliferation and invasion *in vitro*. Then, luciferase reporter assay validated Homeobox A9 (HOXA9) was a downstream target of miR-1294. Expression patterns of miR-1294 were inversely correlated with HOXA9 in OS tissues, strengthening the findings from the luciferase reporter assay. Further functional assays revealed that overexpression of HOXA9 could reverse the inhibition effects of miR-1294 on cell proliferation and invasion.**

**CONCLUSIONS: These results suggested miR-1294 functions as a tumor suppressor in OS progression by targeting HOXA9.**

*Key Words:*
MiR-1294, Osteosarcoma, HOXA9, Tumor suppressor.

## Introduction

Osteosarcoma (OS) often occurred in children and adolescents and is one of the most lethal cancer type worldwide[1,2]. OS has a high potential for metastasis that results in the unsatisfactory OS prognosis despite the great achievements in the treatment measures in recent decades[3-5]. Hence, there is an urgent requirement to deeply investigate the abnormally expressed molecules related to the metastasis of OS to provide novel suggestions to develop anti-cancer therapeutic strategies. Extensive studies have revealed the initiation and progression of OS is associated with the abnormal expression of tumor suppressor genes or oncogenes[5,6]. MicroRNAs (miRs) are a family of non-coding RNAs and have dual functions in tumors: namely tumor suppressive role and oncogenic role[7,8]. MiRs are reported to exert its biological roles in tumors through complementary binding to the 3'-untranslated region (3'-UTR) of targeted genes[9]. Involvement of miRs in the progression and metastasis of OS has also received considerable attentions[10]. For example, miR-34a-5p expression was found elevated in OS tissues and negatively regulate angiotensin II type 1 receptor (AGTR1) expression to affect the chemoresistance of OS[11]. Li et al[12] revealed that miR-202-5p downregulation induced OS cell migration and invasion inhibition, showing a potential to develop miR-202-5p as a treatment target for OS. However, the mechanism of action of miRNAs in OS is not understood clearly. HOXA cluster belongs to the Homeobox (HOX) gene family and is reported to play a crucial role in regulating cell differentiation[13]. Hence, it is reasonable to deduce that the dysregulation of these genes has the potential to regulate cancer progression[14]. HOXA9 protein has been reported to function as a tumor suppressor or oncogene in a context-dependent


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manner[15-17]. HOXA9 can inhibit HIF-1α-mediated glycolysis to suppress the progression of cutaneous squamous cell carcinoma[15]. Recently, HOXA9 was found overexpressed in gastric cancer, implicating poor prognosis of these patients[16]. Notably, Zhang et al[17] showed HOXA9 was directly regulated by miR-182 to regulate OS cell behaviors. However, more researches are still needed to reveal the exact mechanism of HOXA9 in OS. In this study, we demonstrated the aberrantly expressed status of miR-1294 in OS specimens and cell lines. Next, the dual-luciferase reporter assay was conducted to validate HOXA9 as a direct target of miR-1294. Functional studies were utilized to investigate the roles of miR-1294 in OS.

## Materials and Methods

### Cell Culture and Collection of Tissues

Normal human osteoblastic cell line hFOB 1.19 and OS cell lines (MG-63 and HOS) used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were maintained with Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in a 37°C incubator containing 5% $CO_2$. Sixty-four OS tissues and matched normal tissues were obtained from Yantai Yuhuangding Hospital and stored at -80°C until further usage. The study protocol was approved by the Ethics Committee of Yantai Yuhuangding Hospital. These patients received treatment between October 2011 and December 2012 and written informed consent was obtained from all the participated patients.

### Cell Transfection

MiR-1294 mimic, inhibitor, HOXA9 construct were synthesized by GenePharm (Shanghai, China). The corresponding negative controls (NC) were also purchased from GenePharm. Cell transfection was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After transfection for 48 h, cells were collected for following experiments.

### Western Blot Analysis

Protein samples were extracted by radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA) protein determination kit (Beyotime) and separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (30 μg). Subsequently, protein samples were transferred to polyvinylidene difluoride (PVDF) membrane (Beyotime) and blocked with fat-free milk. After washed with TBST, membranes were cultured with mouse monoclonal antibodies (anti-HOXA9: ab51236; anti-GAPDH: ab8245; Abcam, Cambridge, MA, USA) at 4°C for overnight and horseradish peroxidase (HRP) conjugated goat-anti-mouse secondary antibody (ab6789; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Protein signals were detected using enhanced chemiluminescent reagent (Beyotime, Shanghai, China) and analyzed with ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA).

### Total RNA Isolation From Tissues and Cell Lines

RNA samples were extracted by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in line with the supplier's recommendations. RNA concentration was measured by Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

### Quantitative Reverse Transcription PCR (qRT-PCR) Analysis

Extracted RNA samples were reverse transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). qRT-PCR was conducted using SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) at Applied Biosystems StepOne Plus Real Time-PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences for miR-1294 were 5'-ACACTCCAGCTGGGTGTGAGGTTGGCATTG-3' (forward) and 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGACAACA-3' (reverse). Primer sequences for U6 snRNA were 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse). Relative expression levels were calculated using the comparative threshold cycle (Ct) method. All experiments were conducted in triplicate.

### Luciferase Reporter Assay

The wild-type (wt) or mutant (mut) sequence of HOXA9 3'-UTR were cloned into pGL3-M vector (Promega, Madison, WI, USA) and designated as HOXA9-3'-UTR-wt or HOXA9-3'-UTR-mut.

Cells were co-transfected with HOXA9-3'-UTR-wt or HOXA9-3'-UTR-mut and miR-1294 mimic or NC using Lipofectamine 2000. Luciferase activity was detected by dual-luciferase reporter assay system (Promega, Madison, WI, USA) after 48 h transfection.

### Cell Proliferation Assay

Cell counting kit-8 (CCK-8) assay was conducted to measure cell proliferation. Cells were plated in 96-well plate at the density of $5 \times 10^3$ cells/well and cultured for 0 h, 24 h, 48 h, and 72 h. Subsequently, 10 μl CCK-8 reagent (Beyotime, Shanghai, China) was added to each well and cultured for an additional 2 h. After that, the optical density of each well was detected using microplate reader (BioTek, Winooski, VT, USA) at 450 nm. All experiments were conducted in triplicate.

### Cell Invasion Assay

Transwell invasion assay was performed to measure cell invasion. Cells were seeded in the upper Matrigel-coated transwell chamber (Corning Costar Co., Corning, NY, USA) at the concentration of $1 \times 10^3$ cells/well. Dulbecco's Modified Eagle Medium (DMEM) contains fetal bovine serum (FBS) was added to the lower chamber. After incubation for 24 h, cotton swabs were used to remove the non-invaded cells. Then, these cells were fixed with methanol and stained with crystal violet. The stained cells were observed and counted using a microscope. Invasion numbers were calculated from five random fields.

### Statistical Analysis

Data were analyzed using GraphPad Prism 6 (La Jolla, CA, USA) and presented as mean ± standard deviation. Differences in two groups were measured with the Student's *t*-test, whereas one-way ANOVA and post hoc Tukey test was performed for difference analysis among more than two groups. Correlations between miR-1294 and HOXA9 were detected using Pearson's correlation. Kaplan-Meier curve along with log-rank test was used to evaluate the effect of miR-1294 expression on overall survival of OS patients. $p < 0.05$ was regarded as statistically significant.

## Results

### MiR-1294 Directly Target HOXA9 in OS

We searched the potential modulator of HOXA9 using TargetScan. The results demonstrated miR-1294 might be a regulator of HOXA9 as they can perfectly bind with each other (Figure 1A). Luciferase activity assay showed that miR-1294 mimic significantly reduced the luciferase activity of OS cells transfected with HOXA9-3'-UTR-wt but not HOXA9-3'-UTR-mut (Figure 1B). Western blot analysis results showed that miR-1294 mimic markedly decreased HOXA9 protein expression in OS cells (Figure 1C). Correlation analysis showed that miR-1294 and HOXA9 expression in OS tumor tissues was inversely correlated (Figure 1D).

### MiR-1294 Was Downregulated in OS Tissues and Cell Lines

We further investigated the expression status of miR-1294 in both OS tissues and cell lines using qRT-PCR. The results revealed that miR-1294 was frequently downregulated in OS tissues compared with their matched normal tissues (Figure 2A). Then, miR-1294 expression in OS cell lines was also analyzed by qRT-PCR and the results showed that its expression was also reduced in OS cell lines investigated than in normal human osteoblastic cell line hFOB 1.19 (Figure 2B). The median value of miR-1294 expression was used to classify the 64 OS patients into high miR-1294 (n=30) or low miR-1294 (n=34) group. Survival analysis revealed that patients with low miR-1294 expression predict worse 5-year overall survival of OS patients ($p = 0.031$, Figure 2C).

### MiR-1294 Inhibits OS Cell Proliferation and Invasion In vitro

To measure the biological role of miR-1294 in OS progression, we transfected the synthetic miRNAs into the OS cell lines investigated. It was showed that miR-1294 mimic significantly increased the expression level of miR-1294, while miR-1294 inhibitor decreased the amounts of miR-1294 (Figure 3A). The CCK-8 assay demonstrated that miR-1294 mimic reduced cell proliferation rate, while miR-1294 inhibitor has the opposite effect (Figure 3B). The transwell invasion assay revealed that cell invasion was enhanced by miR-1294 inhibitor but reduced by miR-1294 mimic (Figure 3C). These findings revealed that miR-1294 functions as tumor suppressive RNA in OS.

### MiR-1294 Regulates OS Cell Behaviors by Targeting HOXA9

Next, the HOXA9 construct was transfected into OS cell lines to regulate the expression of HOXA9.

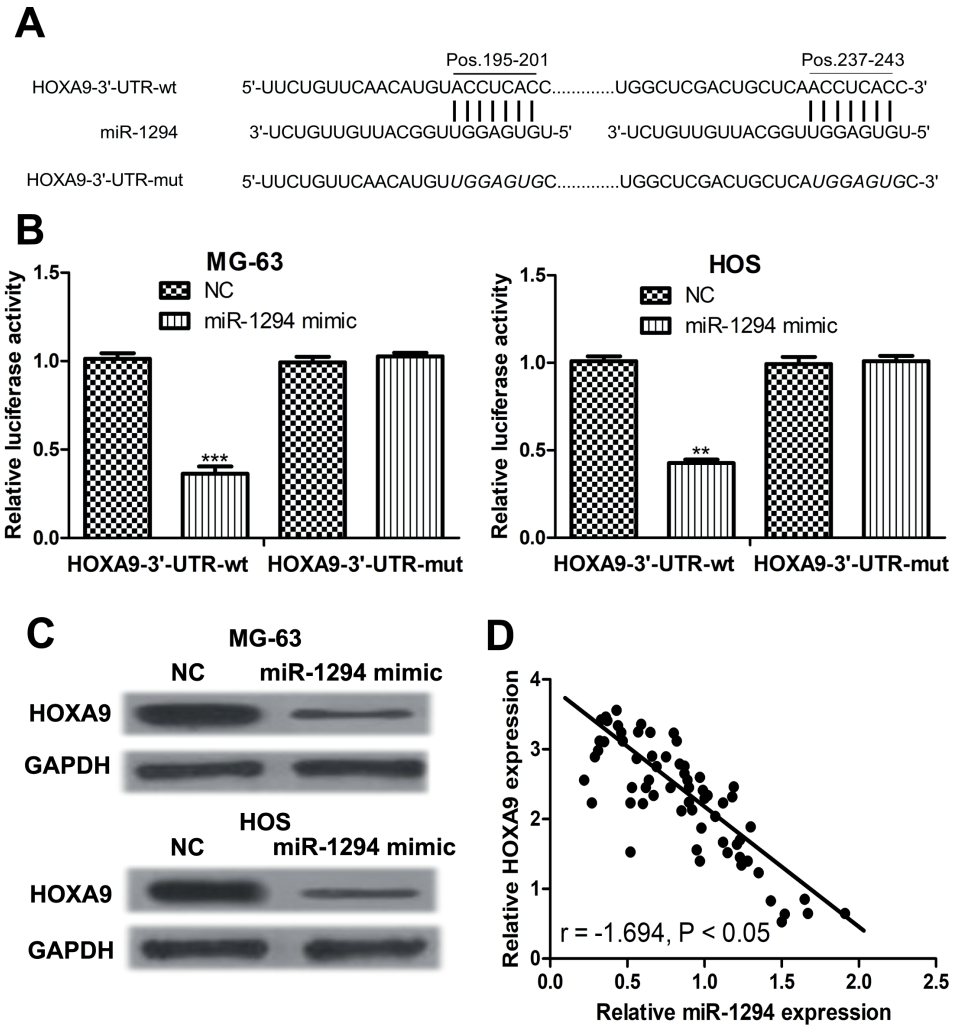


**Figure 1.** HOXA9 was a direct target of miR-1294 in OS. ***(A)*** Binding sites in the 3'-UTR of HOXA9 for miR-1294 were predicted by TargetScan. ***(B)*** Luciferase activity in MG-63 and HOS cell lines with miR-1294 mimic or NC and HOXA9-3'-UTR-wt or HOXA9-3'-UTR-mut co-transfection. ***(C)*** HOXA9 expression in MG-63 and HOS cell lines with miR-1294 mimic or NC transfection. ***(D)*** Inversely correlation between miR-1294 and HOXA9 expression in OS tissues. (**$p < 0.01$, ***$p < 0.001$) miR-1294: microRNA-1294; HOXA9: Homeobox A9; OS: osteosarcoma; UTR: untranslated region; NC: negative control; wt: wild-type; mut: mutant.

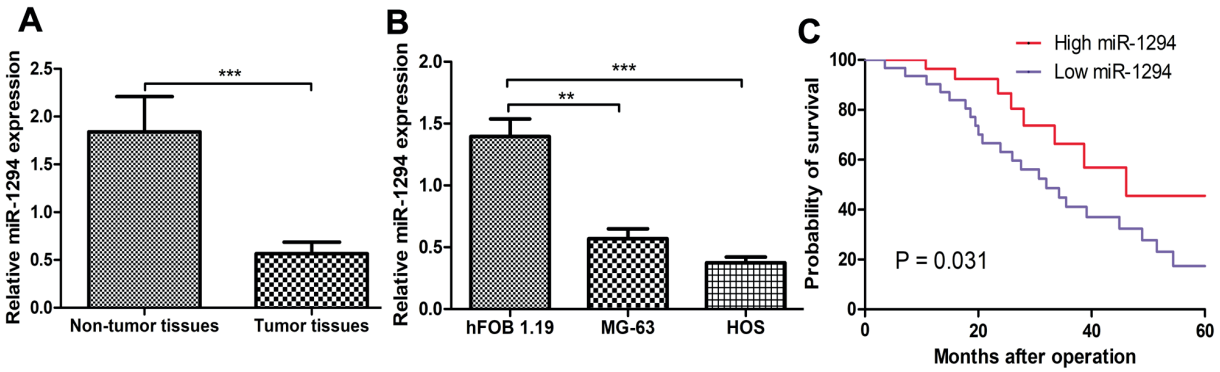


**Figure 2.** Downregulation of miR-1294 in OS tissues and cell lines. ***(A)*** Reduced expression of miR-1294 in OS tumor tissues compared with non-tumor tissues. ***(B)*** Reduced expression of miR-1294 in MG-63 and HOS cell lines compared with hFOB 1.19 cell line. ***(C)*** Low miR-1294 predicts poor 5-year overall survival of OS patients. (**$p < 0.01$, ***$p < 0.001$). miR-1294: microRNA-1294; OS: osteosarcoma.

HOXA9 construct significantly increased HOXA9 expression in OS cell lines and could abolish the effect of miR-1294 mimic on HOXA9 expression (Figure 4A). As expected, the proliferation and invasion abilities were increased by HOXA9 construct (Figure 4B and 4C). Meanwhile, HOXA9 construct impaired the inhibitory effects of miR-1294 mimic on OS cell proliferation and invasion (Figure 4B and 4C). These results revealed that miR-1294 regulated OS cell proliferation and invasion through targeting HOXA9.

## Discussion

To date, over 1,000 miRNAs were found associated with the development and progression of cancers including OS[8,10]. OS cell behaviors were also found influenced by miRNA expression[11,12,17-20]. Even though no miRNAs have been established as therapeutic targets for OS treatment[10], it is still imperative to investigate the mechanisms related to cancer metastases since the prognosis of patients with metastases remains poor. MiR-1294 is a newly identified tumor suppressive miRNA that could inhibit the growth and regulate the chemosensitivity of glioma through regulating TPX2[21]. Also, miR-1294 was found linked to oral squamous cell carcinoma growth and migration regulation through targeting c-Myc[22]. However, the expression and role of miR-1294 in OS progression remain unclear. Therefore, in this study, we established the downregulation status of miR-1294 in OS tissues. Notably, we demonstrated that low miR-1294 ex-

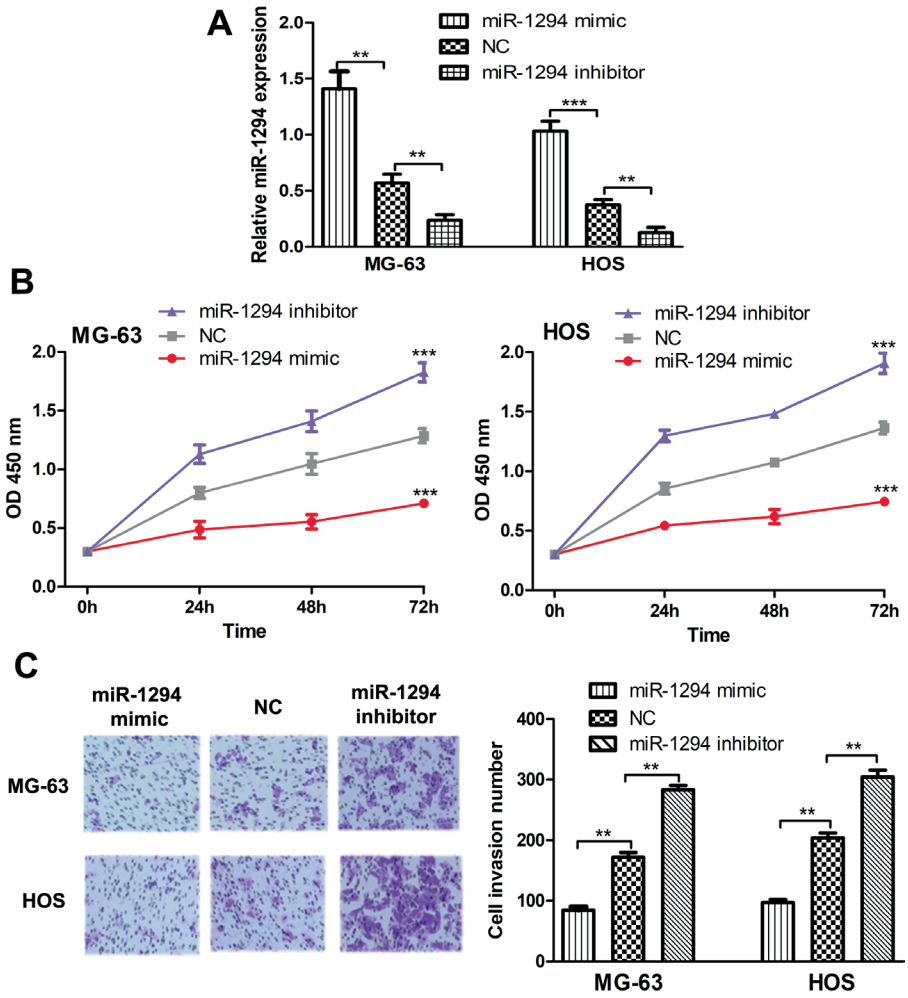


**Figure 3.** Overexpression of miR-1294 inhibits OS cell proliferation and invasion. ***(A)*** MiR-1294 expression, ***(B)*** cell proliferation, (C) cell invasion in MG-63 and HOS cell lines with synthetic miRNAs transfection. (**$p < 0.01$, ***$p < 0.001$). miR-1294: microRNA-1294; OS: osteosarcoma; NC: negative control.

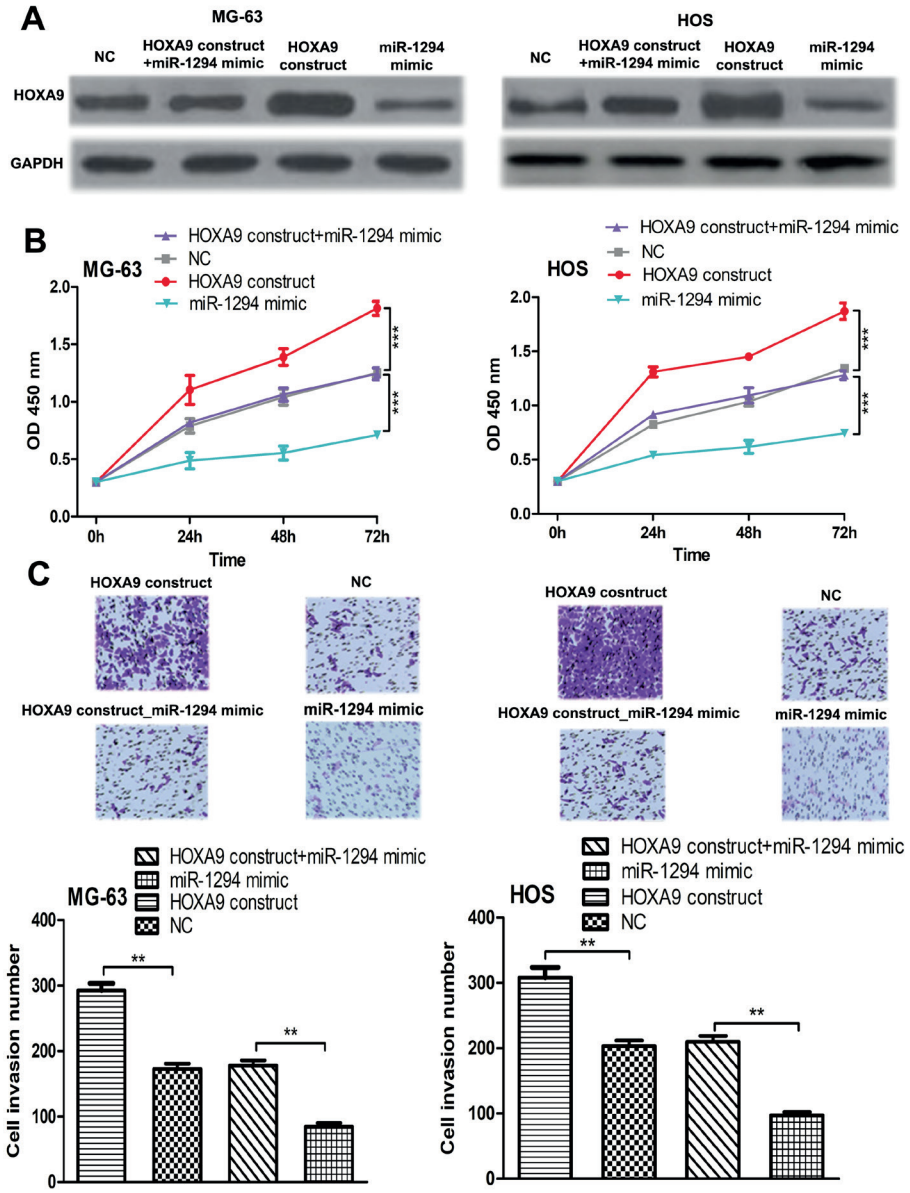


**Figure 4.** HOXA9 overexpression reverses the inhibitory effects of miR-1294 on OS cell proliferation and invasion. ***(A)*** HOXA9 expression, ***(B)*** cell proliferation, ***(C)*** cell invasion in MG-63 and HOS cell lines after miRNAs or HOXA9 construct transfection. (**$p < 0.01$, ***$p < 0.001$). miR-1294: microRNA-1294; HOXA9: Homeobox A9; OS: osteosarcoma; NC: negative control.

pression was correlated with poor 5-year overall survival of OS patients. Since miR-1294 is reported could regulate cancer cell behaviors[21,22], therefore *in vitro* functional assays were also conducted to explore how miR-1294 participated in OS progression. We manipulated miR-1294 expression in OS cell lines using the synthetic miRNAs. The cell proliferation and cell invasion assays revealed that miR-1294 overexpression inhibits both proliferation and invasion of OS cells. Collectively, we established the tumor suppressive role of miR-1294 in OS. Downstream target is crucial for the biological roles of miRNAs in a specific cell[9,11,12]. We found HOXA9 contains a putative binding site for miR-1294 using the online prediction algorithm. Therefore, HOXA9 was selected for the following investigations as it has been validated as an oncogene in multiple cancers including OS[14-17]. The combination of luciferase reporter assay and western blot assay revealed that HOXA9 was a direct target of miR-1294 in OS. Functional assay revealed that HOXA9 overexpression could promote OS cell proliferation and invasion, which was the similar stimulation effect of miR-1294 downregu-

lation. Importantly, we divided the cells into NC, miR-1294 mimic, HOXA9 construct, and miR-1294 mimic + HOXA9 construct groups. The data revealed that HOXA9 construct transfection could reverse the inhibition effects of miR-1294 mimic on cell proliferation and invasion, which suggested that miR-1294 exert its role through regulating HOXA9.

## Conclusions

We revealed miR-1294 was downregulated in OS tissues and correlated with the poor 5-year overall survival, which highlighted that miR-1294 function as a tumor suppressor in OS. Moreover, we found the inhibition effects of miR-1294 on OS cell proliferation and invasion were exerted through regulating HOXA9. The miR-1294/HOXA9 axis may be used as therapeutic targets for the treatment of OS.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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