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Original Article

miR-30 inhibits the progression of osteosarcoma by targeting MTA1

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Abstract

Objectives: MicroRNAs (miRNAs) have been considered as a new class of novel diagnostic and predictive biomarker in many diseases. However, there are few studies on miRNA in osteosarcoma (OS). This study aimed to investigate the roles of miR-30 on OS occurrence and development. **Methods:** PCR was used to detect mRNA levels of miR-30 and MTA1 in cancer tissues, adjacent non-cancerous tissues from OS patients. Western blot was used to detect MTA1 protein expression in all tissues and cell lines (hFOb1.19,Saos-2, MG63, and U2OS). The correlation between miR-30 and MTA1 was predicted through bioinformatics software, and identified by a luciferase reporting experiment. *In vitro*, functional test detected the specific effects of miR-30 and MTA1 on the development of OS. **Results:** miR-30 expression was significantly reduced, while the expression of MTA1 was increased in OS tissues and cells. Luciferase reporting experiment showed that miR-30 sponged MTA1 which was negatively correlated with miR-30 expression. Furthermore, rescue tests revealed that MTA1 restrained the functions of miR-30 on cell proliferation and migration of OS. **Conclusion:** Our finding showed that miR-30 modulated the proliferation and migration by targeting MTA1 in OS.

Keywords: miR-30, MTA1, Osteosarcoma

Introduction

Osteosarcoma (OS) is the most common bone malignant tumor, which originates from mesenchymal tissue and usually occurs in adolescents[1,2]. Due to its complicated pathogenesis, there is no particularly effective treatment at present. The classic treatment method is surgery combined with neoadjuvant chemotherapy, but the highest five-year survival rate of patients can only reach about 70%[3,4]. Moreover, many patients have a recurrence and distant metastasis due to resistance to chemotherapy which seriously affects prognosis[5,6]. Hence, it is necessary to explore the mechanism of OS for target screening.

MicroRNAs (miRNAs) are small, single-stranded RNA molecules that involve in the progression of a variety of diseases[7]. MiRNA is involved in many biological processes including differentiation, metastasis, and apoptosis. It is reported that many miRNAs expression in tumors have changed either up or down, and numerous studies have identified changes in miRNA expression profiles in many malignant tumors, including osteosarcoma[8-10]. Recent study found that miR-98-5p modulates cell cycle progression by inhibiting CDC25A, thus suppressing the progression of OS[10]. In addition, miR-206 derived from bone marrow

The authors have no conflict of interest.

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Table 1. RT-qPCR primer sequence.

Genes		Primer sequences (5'-3')
miR-30	Forward	TGTAAACATCCTCGACTGGAAG
	Reverse	AGATGAGCATTGCCAGCGAG
MTA1	Forward	TGGAAGAAATATGGTGGCTTGA
	Reverse	TTCGTCGTGTGCAGATAGAAAG
U6	Forward	CTTGAACCTCCTCGTTCGACCCCGCCTC
	Reverse	AACGCTTCACGAATTTGCGT
GAPDH	Forward	GGCACAGTCAAGGCTGAGAATG
	Reverse	ATGGTGGTGAAGACGCCAGTA

mesenchymal stem cell-derived exosomes was transplanted into OS cells and suppressed cancer progression through sponging TRA2B[11]. Studies also reported that miR-30 is lowly expressed in OS[12,13]. However, the specific functions of miR-30 in OS remain unclear. Our study aims to examine the function and mechanism of miR-30 on OS proliferation and migration from the cellular and molecular biological levels, and lay the foundation for OS targeted therapy.

On the other hand, Metastasis-associated genes (MTA) are a family of genes closely related to tumor genesis and progression. Recent research has discovered that MTA family members are abnormally expressed in a variety of human tumor tissues and are involved in tumor invasion, metastasis, and angiogenesis through a variety of mechanisms. MTA may be one of the main regulatory molecular families related to the occurrence and progression of many malignant tumors[14,15]. One member of the MTA family, MTA1 is the first tumor metastasis-related gene discovered, which determines its special position in the tumor metastasis-related gene family[16]. MTA1 is often overexpressed in tumor tissues, such as colorectal cancer[17] and esophageal cancer[18], and is associated with tumor stage, grade, invasion, metastasis, prognosis, and other factors.

The present study investigated the miR-30 expression in OS, the vital role of miR-30 in the proliferation, migration, and invasion of OS *in vitro* and *in vivo*. We found that Mir-30, as an endogenous RNA, plays a tumor suppressor role in OS by targeting MTA1.

Materials and Methods

Clinical sample

Cancer tissue and adjacent non-cancerous tissues from OS patients were frozen in liquid nitrogen. All patients were informed and signed written consent. The ethics committee of Affiliated Hospital of Inner Mongolia Medical University approved and supervised the study (NO.YKD20201213).

Cell culture and treatment

Human osteoblast cell line (hFOb1.19) and OS cell lines (Saos-2, MG63, and U2OS) were purchased from EK-Bioscience (Shanghai, China), and were cultured with DMEM and DMEM/Ham's F12 mediums including 10% fetal bovine serum (FBS) plus penicillin/streptomycin at 37°C with 5% CO_2. miR-30 mimic, Negative control (NC) mimic, miR-30 inhibitor, Negative control (NC) inhibitor, si-NC, and si-MTA1 (RiboBio, Guangzhou, China) respectively treated cells by lipofectamine 3000 (Thermo-Scientific). After transfecting 2-day, cells were extracted for the following research.

PCR assay

RNA was separated from samples using Trizol reagent (Beyotime, Shanghai, China). Next, RNA was reverse transcribed to cDNA by PrimeScript RT Master Mix (Yeasen, Shanghai, China). Thereafter, RT-qPCR was implemented by using SYBR Green PCR Kit (Qiagen, Dusseldorf, Germany) through Stratagene mx3000p Real-time PCR system (Stratagene, USA). U6 and GAPDH as the internal references. The RT-PCR primer is shown in Table 1.

CCK-8 assay

Cell counting kit-8 (CCK-8; Beyotime, Shanghai, China) assay was conducted as instructed by the manufacturer for the purpose of detecting cell proliferation.Saos-2 cells (100 mL) were seeded into 96-well plates and cultured at 37°C, 5% CO_2. Then, 10 µl CCK-8 solution was added to each well and incubated in the incubator for 1-4 h. Finally, a microplate analyzer measured the optical density at 450 nm.

EdU assay

Briefly, cells (1×104) were placed in 96-well plates and cultured to the normal growth stage. Next, 100µL EdU solution was added to each well and cultured for 2 h. Next, cells were fixed with 4% paraformaldehyde for 15min following strained with 500 µL Apollo solution for 20 min. Finally, cells were observed using the fluorescence microscope (Keyence, Osaka, Japan).

Transwell analysis

A Transwell chamber was used to detect the migration activity. First, the membrane of the transwell chamber was

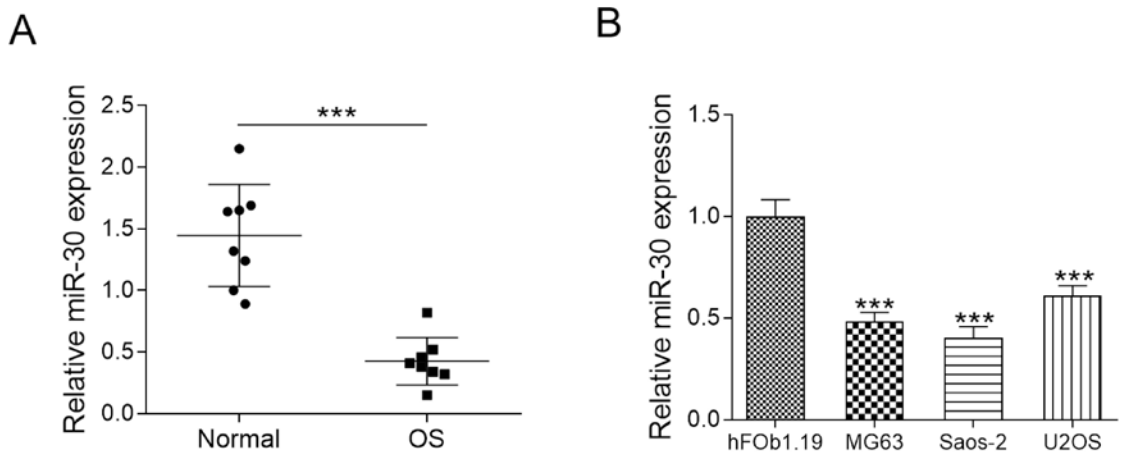

Figure 1. miR-30 expression is downregulated in OS tissues and cells. A. miR-30 expression in OS tissues and adjacent tissue. B. miR-30 expression in OS cells. ***P<0.001.

pre-coated with the Matrigel. The transfected cells were placed in serum-free medium in the upper compartment and 10% FBS medium in the lower compartment. After treatment at 37°C for 48 h, using 0.1% crystal violet stain cells at 37°C for 5 min. In the end, a fluorescence microscope was used to recorded and photographed the migrated cells.

Western blot analysis

Protein samples were lysed at low temperature with RIPA lysis buffer and using a BCA assay kit measured total protein concentration. Prepared protein samples were separated with 12.5% SDS-PAGE and transferred into PVDF membranes. Subsequently, these bands were incubated with prepared antibodies. In the end, efficient chemiluminescence kit (ECL) was used to visualize these membranes. Antibodies against Ki67, E-cad, MTA1 and GAPDH were purchased from Abcam (Cambridge, MA, USA).

Luciferase reporter assay

MTA1 containing miR-30 binding sequence was interpolated in the luciferase reporter vector (MTA1 WT). The mutation of the miR-30 binding sequence of MTA1 was inserted into the luciferase reporter vector (MTA1 MUT) to confirm its specific binding. MTA1-WT or MTA11-Mut were co-transfected with miR-30 mimic or miR-NC into cells by Lipofectamine 3000. After 48-h, luciferase activities were assessed using a luciferase assay kit (Yeasen).

Statistical Analysis

All analyses were carried out using SPSS version 21.0 software. The data were collected from at least three independent experiments and were expressed as mean ± standard deviation (SD). Analyses were made by using one-way ANOVA and student's *t*-tests. Statistical significance was set at p<0.05.

Results

miR-30 expression is decreased in OS tissues and cells

Firstly, we collected the tumor tissues and para-carcinoma tissues from OS patients to measure the levels of miR-30 and we found that miR-30 expression was decreased in OS tissues compared to their control (Figure 1A). Moreover, miR-30 expression was reduced in OS cells (MG63, Saos-2, and U2OS) than Human osteoblast cell line (hFOb1.19), especially in Saos-2 cells (Figure 1B), which were used in subsequent experiments.

miR-30 inhibits OS cell proliferation and migration

To explore the functions of miR-30 on OS progression, miR-30 inhibitor, miR-30 mimic, and their corresponding control were used to transfect into Saos-2 cells. The transfection efficiency of miR-30 was shown in Figure 2A. CCK-8 and EdU assay demonstrated that knockdown of miR-30 promoted cell viability and cell proliferation, while overexpression inhibited cell viability and cell proliferation in Saos-2 cells (Figures 2B and 2C). Additionally, the transwell assay revealed that the migration capacity was enhanced in the miR-30 mimic group, while reduced in the miR-30 inhibitor group compared to the control group (Figure 2D). And knockdown of miR-30 promoted the expression of Ki67 and E-cad, while overexpression of miR-30 restrained the expression of Ki67 and E-cad (Figure 2E).

MTA1 is downstream target gene of miR-30

Next, we further detected the specific molecular mechanism of miR-30 on OS progression. Bioinformatics

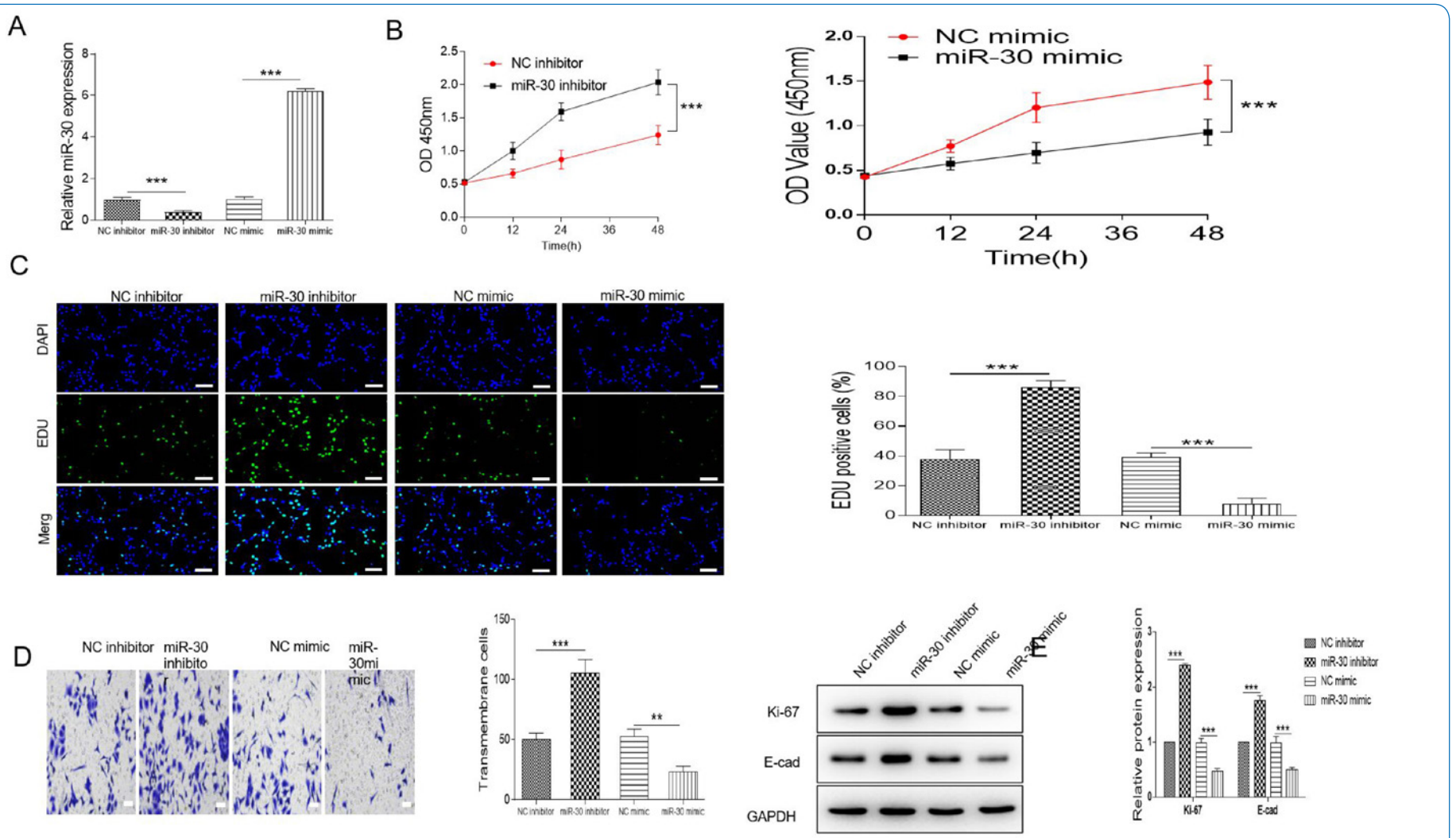

Figure 2. miR-30 suppressed cell proliferation and migration in OS. miR-30 inhibitor, miR-30 mimic and their control were used to transfect into MG63 cells. A. The transfection efficiency of miR-30 inhibitor and miR-30 mimic. B. Cell viability detected through CCK-8 assay. C. Cell proliferation measured through EdU assay. D. Transwell assay assessed cell migration. E. The expression of Ki-67 and E-cad detected by western blot assay. ***P<0.001, **P<0.01.

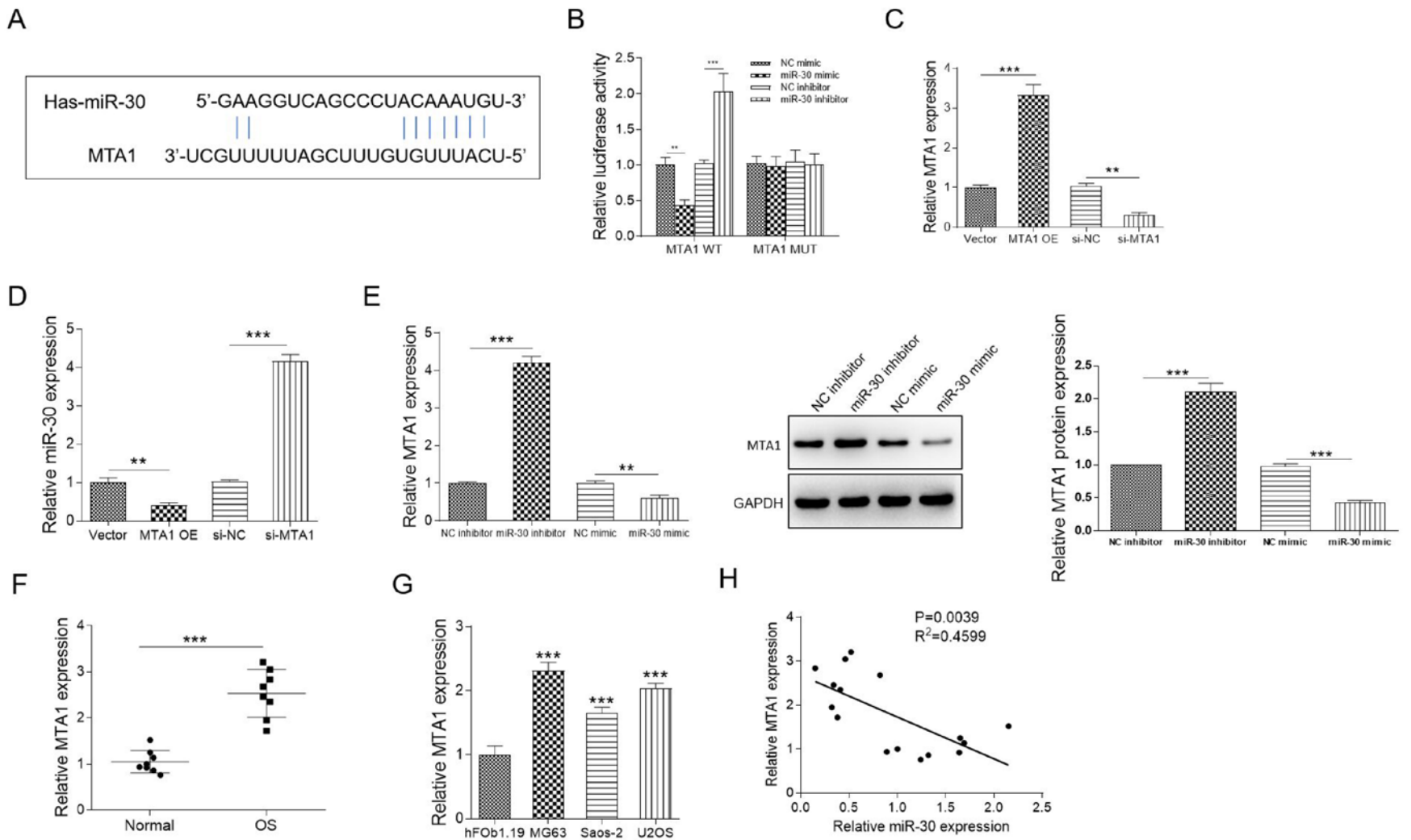

Figure 3. MTA1 is downstream target gene of miR-30. A. The binding sequence between miR-30 and MTA1. B. Luciferase reporter assay. C. The mRNA expression of MTA1. D. miR-30 expression in the MTA1 OE and sh-MTA1 group. E. MTA1 expression in the miR-30 mimic and miR-30 inhibitor group. F and G. The expression of MTA1 in OS tissues and OS cells. H. Correlation analysis of MTA1 and miR-30 in OS tumor tissues. ***P<0.001, **P<0.01.

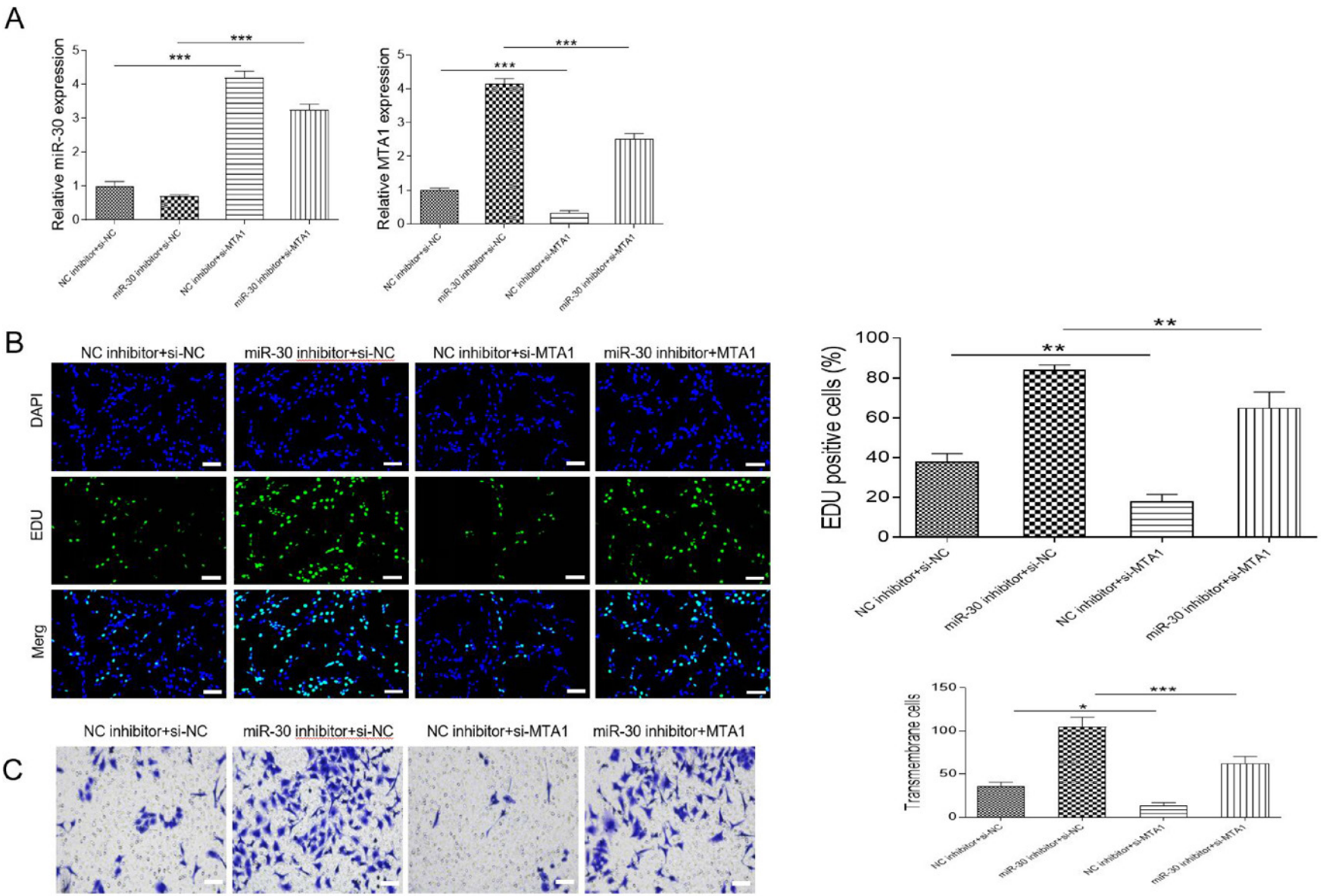

Figure 4. MiR-30 regulates the progression of OS by targeting MTA1. A. The expression of miR-30 and MTA1. B. The proliferation determined through EdU assay. C. Cell migration determined through transwell assay. ***P<0.001, **P<0.01, *P<0.05.

analysis discovered that MTA1 was the target gene of miR-30, and there were binding sites between the two (Figure 3A). Then, luciferase assay verified the relationship between MTA1 to miR-30. We found that the luciferase activity was remarkedly reduced in miR-30 mimic and MTA1 WT transfected cells compared with NC mimic and MTA1 WT transfected cells, while the luciferase activity had no change in MTA1 Mut transfected cells (Figure 3B). Subsequently, overexpression of MTA1 significantly inhibited miR-30 expression, while knockdown of MTA1 observably promoted miR-30 expression (Figure 3C, 3D), and upregulation of miR-30 significantly restrained MTA1 expression, and knockdown of miR-30 significantly promoted the expression of MTA1 (Figure 3E). Then, we discovered that MTA1 expression in OS tissues and cells group was higher than that in control group (Figure 3F, 3G). And we confirmed a negative relationship between MTA1 expression and miR-30 expression in OS tumor tissues (Figure 3H).

MiR-30 regulates the progression of OS by targeting MTA1

To further explore whether miR-30 affects the occurrence of OS by modulating MTA1, we transfected miR-30 inhibitors and si-MTA1 into MG63 separately or simultaneously. First, the transfection efficiency was detected and showed in Figure 4A, the result revealed that the transfection was successful. Then, EdU assay and transwell demonstrated that knockdown of MTA1 significantly rescued the function of miR-30 inhibitor on OS cell proliferation and migration (Figures 4B, 4C).

Discussion

MicroRNAs (miRNAs) negatively modulate gene expression through combining with specific sites in the 3' UTR of target mRNA and participate in various pathophysiological processes[19]. More and more studies have confirmed that there are significant differences in the expression of miRNAs between normal tissues and tumor tissues, and miRNAs exert a necessary regulatory role in tumor progression[20,21]. Recent studies have shown that there is a strong relationship between miR-30 expression and tumors, and it is considered a marker of metastasis in breast cancer, bladder cancer, colon cancer and lung cancer[22,23]. For example, miR-30 affects cell growth, migration, and invasion by inhibiting the HOXA1 gene in esophageal cancer[24]. In addition, miR-30 inhibits OS proliferation and metastasis through reducing MEF2D expression[12], and upregulation of miR-30 inhibits OS invasiveness[13]. In our study, miR-30 was highly expressed in OS tissues and cells. Further experiments showed that overexpression of miR-30 suppressed cell viability, proliferation, and migration of OS, while knocking down miR-30 was just the opposite.

Previous studies have shown that high MTA1 expression in OS clinical samples was related to poor prognosis[25,26]. Our study indicated the relationship between MAT1 and miR-30. In this research, we found that upregulation of miR-30 reduced MTA1 expression, while silencing of miR-30 promoted MTA1 expression in OS. Knockdown of MTA1 was shown to suppresses cell proliferation and metastasis OS cells. Our experiment confirmed the previous research results of Yi Cand Li X on the change of MAT1 expression in osteosarcoma[27]. The luciferase reporter assay discovered that MTA1 was a direct target of miR-30. In addition, knockdown of MTA1 reversed the positive functions of miR-30 inhibitor on cell proliferation and migration in OS. These data suggested that miR-30 played a tumor suppressive role in OS by targeting MTA1.

However, the roles of MAT1 and its regulation mechanism in OS are still unclear. In order to explore the relationship of miRNA and MAT1, we used luciferase reporter assay to identify to identify miR-30 specific binding to MAT1. What's more, the expression of MAT1 was upregulated in OS tissues and cell lines, and negatively regulated by miR-30. These data clearly suggested that MAT1 was a direct target gene of miR-30. Different from the normal situation, mir-30 expression was low in the cancer tissues of the object of this study, while MAT1 expression was high, indicating that there are other factors besides them that inhibit the expression of Mir-30 and enhance the invasion and proliferation ability of OS cells. This deserves further study.

Our finding showed that miR-30 acted as a tumor suppressor in OS by targeting MTA1, which may be a potential therapeutic target for OS treatment.

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Authors' contributions

AZ and YZ designed the study and drafted the manuscript. WF, ZZ and WL were responsible for the collection and analysis of the experimental data. NW, HX, LW, SC and RB revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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