MicroRNA-124 suppresses growth and aggressiveness of osteosarcoma and inhibits TGF-β-mediated AKT/GSK-3β/SNAIL-1 signaling

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Abstract. Osteosarcoma is one of the most common malignant tumors in adolescent populations and the prognosis remains incompletely understand. Previous reports have demonstrated that microRNA-124 (miR-124) has inhibitory effects on various human malignancies and is associated with tumor progression. However, the clinical significance and potential mechanisms of miR-124 in the progression of osteosarcoma is not clearly understood. In this study, the potential molecular mechanism of miR-124 in osteosarcoma tumorigenesis, growth and aggressiveness was investigated. The growth, proliferation, apoptosis, migration and invasion of osteosarcoma cells were investigated following miR-124 transfection were determined by colony formation assay, western blotting, immunofluorescence, migration/invasion assays and reverse transcription-quantitative polymerase chain reaction. *In vivo* anti-cancer effects of miR-124 were analyzed by a tumor growth assay, immunohistochemistry and survival rate observations. The results demonstrated that miR-124 transfection significantly decreased integrin expression in osteosarcoma cells, and further inhibited growth, proliferation, migration and invasion of osteosarcoma cells. Flow cytometry assays indicated that miR-124 transfection attenuated apoptosis resistance of osteosarcoma to tunicamycin, potentially via the down-regulation of P53 and Bcl-2 apoptosis regulator expression. Mechanistic assays demonstrated that miR-124 transfection suppressed TGF-β expression in osteosarcoma. An animal study revealed that tumor growth was reduced in tumor cells transfected with miR-124 compared with control cells, and the survival rate was prolonged in mice with miR-124 transfected xenografts compared with control tumors. In conclusion, these results indicate that miR-124 transection inhibits the growth and aggressive of osteosarcoma, potentially via suppression of TGF-β-mediated AKT/GSK-3β/snail family transcriptional repressor 1 (SNAIL-1) signaling, suggesting miR-124 may be a potential anti-cancer agent/target for osteosarcoma therapy.

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Introduction

Osteosarcoma is a malignant tumor that cells occur in bone, which has been reported to exhibit aberrant growth and migration in osseous tissues (1,2). In recent years, new strategies have been proposed, but the overall survival of patients with osteosarcoma remained limited improvement for a stubborn resistance of osteosarcoma cells (3,4). However, malignant osteosarcoma cancer is a typical systemic malignant disease, which causes common symptoms including bone and joint pain, and fatigue in patients (5,6). Although multiple reports demonstrated that treatment advances and neoadjuvant chemotherapy lead to improved 5-year survival in patients with osteosarcoma, the overall survival has had minimal improvement since the introduction of neoadjuvant chemotherapy, radiotherapy and surgery (7,8).

Currently, tumor apoptosis and necrosis is an important predictor of patient prognosis during the treatment of osteosarcoma (9). Resistance to drug-induced apoptosis for osteosarcoma cells contributes to growth and invasion of tumor cells in patients (10-12). Although the emergence of adjuvant and neoadjuvant chemotherapy has been greatly improved the survival rate of patients with osteosarcoma, the morbidity and mortality rate of osteosarcoma is still steadily increasing (13). p53 has a key role in mediating cellular response to various stress signals, mainly by inducing or repressing a number of proteins that are involved in cancer cell cycle progression and apoptosis (14). Clinically, apoptotic resistance has become one of the greatest challenges in cancer therapy due to fierce resistance of tumor cells through various molecular mechanisms (15,16). Thus, underlying molecular mechanism of apoptotic resistance and exploring more efficacy target therapies are urgently required to improve the overall survival rate for patients with osteosarcoma.

MicroRNAs (miRNA) are a subgroup of small non-coding RNAs of 18-25 nucleotides, which have been identified as regulators of disease initiation (17). Reports have suggested that miRNAs have a crucial role in tumorigenesis and progression by

regulating the expression of oncogenes (18,19). In addition, it has been reported that miRNA may be associated with anti-cancer treatments, which have been identified as promoters for tumor apoptosis through upregulated expression of pro-apoptosis genes (20,21). Furthermore, a report has demonstrated that cancer-associated miRNA expression was highly associated with apoptosis in gastric tumors (22). Furthermore, miRNAs are reported to be crucial in the tumorigenesis, development, apoptosis, treatment and prognosis of osteosarcoma cancer (23). Therefore, understanding of the pathogenesis of osteosarcoma may identify candidate miRNAs that are promising biomarkers for osteosarcoma diagnosis and treatment (24).

In this study, the role of miRNA-124 (miR-124) in progression and metastasis of osteosarcoma was investigated *in vitro* and *in vivo*. The role of miRNA in the progression of osteosarcoma and chondrosarcoma has been investigated (25); however, the molecular mechanism of transforming growth factor-β (TGF-β)-mediated AKT serine threonine kinase (AKT)/glycogen synthase kinase-3β (GSK-3β)/snail family transcriptional repressor 1 (SNAIL-1) signaling pathway regulated by miR-124-mediated integrin down-regulation has not been well established in osteosarcoma cells. The efficacy of miR-124 transfection in inhibiting tumor growth, proliferation, aggressiveness and promoting apoptosis was determined in osteosarcoma-bearing mice.

Materials and methods

Ethics statement. This study was performed out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ethics Committee (26). The protocol was approved by the Ethics Committee of Renji Hospital Shanghai Jiao Tong University School of Medicine. All surgery and euthanasia were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Cell culture. Mg63 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Normal bone cell line MC3T3-E1 was cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS. All cells were cultured at 37˚C and 5% CO_2. No mycoplasma contamination was detected.

TGF-β overexpression. Mg63 cells ($1x10^6$) were cultured in six-well plate until 85% confluence and the media was then removed from culture plate followed three PBS washes. The TGF-β complementary DNA (cDNA) sequence was amplified from total RNA with the following primers: Forward, 5'-CAT TGGAGAGAAAGGAAAGTGTG-3' and reverse, 5'-GCTTGC ATGTACGAAGAGGAT-3'. The TGF-β lentiviral vector was co-transfected with 1,000 ng/μl packaging plasmid (Invitrogen; Thermo Fisher Scientific, Inc.) and 2.0 μg envelope plasmid into Mg63 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols. A total of 10 μl TGF-β fresh vector supernatants in 3 ml RPMI 1640 medium were mixed with Mg63 cells in the presence of 8 μg/ml polybrene (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were cultured for 24 h at 37˚C in DMEM + 20% FBS medium. TGF-β-overexpressing-Mg63 cells were transfected with miR-124. Cells were analyzed for further analysis after 48 h transfection.

Transfection of miRNA. All miRNAs were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) including miR-124 (3'-CCGUAAGUGGCGCACGGAAU-5') and siR-vector (3'-UUAAUUCCCCUGUUG-5'). Mg63 cells ($1x10^6$) were transfected with 100 pmol miR-12 or siR-vector mimics (Control; Ambion; Thermo Fisher Scientific, Inc.) using a Cell Line Nucleofector kit L (Lonza Group, Ltd., Basel, Switzerland) and a Nucleofector I electroporation device (Lonza Group, Ltd.). All procedures were performed according to the manufacturer's instructions. Subsequent experiments were performed after 48 h transfection.

MTT cytotoxicity assays. Mg63 cells were transfected with miR-124 with miR-vector as control and cultured in 96-well plates for 72 h at 37˚C. A total of 20 μl MTT (5 mg/ml) in PBS solution was added to each well, the plate was further incubated for 4 h. The majority of the medium was removed and 100 μl dimethyl sulfoxide was added into the wells to solubilize the crystals. The optical density was measured by a Bio-Rad (ELISA) reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from Mg63 cells using RNAeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). Total RNA (1 μg) was transcribed to cDNA by using an RT kit (Qiagen, Inc.) and quality was confirmed by electrophoresis. The cDNA (10 ng) was subjected to qPCR (Bio-Rad Laboratories, Inc.) using SYBR-Green Master Mix system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All the forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.; miR-124 forward, 5'-ACG GGATCCTCTTATTCCATCTTCTACCC-3' and reverse, 5'-CGGAATTCCTGGCTCGGTCGGTCGCTC-3'; β-catenin forward, 5'-GCAGTTCGCCTTCACTATGGA-3' and reverse, 5'-GCAGTTCGCCTTCACTATGGA-3'; E-cadherin forward, 5'-TCATGAGTGTCCCCCGGTAT-3' and reverse, 5'-TCA AACACGAGCAGAGAATCATAAG-3'; α-SMA forward, 5'-GAGTTACGAGTTGCCTGATG-3' and reverse, 5'-GGT CCTTCCTGATGTCAATATC-3'; Vimentin forward, 5'-GGC TCAGATTCAGGAACAGC-3' and reverse, 5'-AGCCTC AGAGAGGTCAGCAA-3'; and GAPDH forward, 5'-GAA GGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGT GATGGGATTTC-3'. The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 45 cycles of 95˚C for 5 sec and 60˚C for 40 sec. Relative mRNA expression changes were calculated by $2^{-\Delta\Delta Cq}$ (27). The results are expressed as the n-fold way compared with the control.

Flow cytometry. Mg63 cells were cultured until 90% confluence was reached. Apoptosis was assessed by incubation these cells with tunicamycin (2 mg/ml) for 24 h. Cells were trypsinized, collected and then washed in cold PBS, adjusted to

$1x10^6$ cells/ml with PBS, labeled with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Annexin V-FITC kit; BD Biosciences, San Jose, CA, USA), and analyzed with a FACScan flow cytometer (BD Biosciences) using CellQuest Pro software (v5.1, BD Biosciences).

Colony formation assay. Mg63 or miR-124-transfected cells were seeded into 6-well plates ($1x10^3$/cells). After culturing for 12 days, proliferating colonies were stained with 1% crystal violet for 15 min at room temperature (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and colonies containing ≥50 cells were counted. All experiments were performed in triplicate.

Cells invasion and migration assays. Mg63 cells were transfected with miR-124 and used to analyze aggressiveness. Mg63 cells were cultured in a 24-well culture plate with chamber inserts (BD Biosciences). For migration assays, $1x10^4$ cells/well were placed into the upper chamber with the non-coated membrane. For invasion assays, cells ($1x10^4$ cells/well) were placed into the upper chamber with a Matrigel-coated membrane. In both assays, the cells were transfected with miR-124 or miR-vector and subjected to the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) for 24 h at 37˚C according to the manufacturer's instructions. The lower chamber of the Transwell plates were filled with 500 μl RPMI-1640 medium containing 10% FBS (Gibco; Thermo Fischer Scientific, Inc.) Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) to quantify cell migration and invasion for 15 min at 37˚C. The tumor cells invasion and migration were counted in at least three randomly stained microscope fields for every membrane.

Western blot analysis. Mg63 cells were transfected with miR-124 and harvested by scraping and lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) followed by homogenization at 4˚C for 10 min. Protein concentration was measured with a Bicinchoninic Acid protein assay kit (Thermo Scientific, Pittsburgh PA, USA). Protein (20 μg) were separated by 12% SDS-PAGE followed transfer to polyvinylidene fluoride membranes (Merck KGaA) and incubated with primary rabbit anti-human antibodies: Integrin (ab150361), β-catenin (ab32572), Vimentin (ab92547), α-SMA (ab108424), E-cadherin (ab11512), TGF-β (92486), Apaf-1 (ab2001), Bad (ab220116), Bcl-2 (ab59348), P53 (ab32049), AKT (ab8805), pAKT (ab38449), GSK-3β (ab93926), pGSK-3β (ab131097), SNAIL-1 (ab82846) and β-actin (ab8227; all 1:500 dilution; Abcam, Shanghai, China) for 12 h at 4˚C post-blocking (5% skimmed milk) for 1 h at 37˚C. Subsequently, proteins were incubated with the corresponding rabbit horseradish peroxidase-labeled Immunoglobulin G (ab6728; 1:2,000; Abcam) for 12 h at 4˚C. The proteins expression levels were detected using a chemi-luminescence detection system with Quantity One software (v3.0; Sigma-Aldrich; Merck KGaA).

Animal study. Female specific pathogen-free nude mice (6-8 weeks old; n=80, body weight, 32-38 g) were purchased from Shanghai Slack Experimental Animals Co., Ltd. (Shanghai, China). All mice were housed at 22-24˚C under a 12-h light-dark cycle and fed *ad libitum* at 40-70% humidity. Mice were subcutaneously implanted with miR-124-transfected or miR-vector-transfected Mg63 tumor cells ($1x10^7$/ml) in 2 ml. Mice were divided into two groups (20 per group). The tumor volumes were calculated according to a previous study (28): Length x width^2 x 0.52. On day 24, mice (n=20 in each group) were sacrificed under 1.5% pentobarbital sodium (100 mg/kg) for further analysis. Remaining mice (n=20 in each group) continued to be housed in order to analyze the long-term survival rate (considered to be 100 days). In the survival rate experiments, the largest tumor size was ~2,000 mm^3. Mice were sacrificed with 1.5% pentobarbital sodium (100 mg/kg) by intravenous injection when tumor diameter reached 12 mm.

Immunohistochemistry. Tumors from Mg63-bearing xenograft mice were fixed using 10% formaldehyde for 12 h at 25˚C, embedded in paraffin wax, and cut into serial sections of 4-μm thickness. Tumor samples were prepared to tumor sections and antigen retrieval (Tris-EDTA buffer solution, pH 9.0; cat. no. SRE0063; Sigma-Aldrich; Merck KGaA) was also performed in tumor sections for 60 min at 65˚C. Tumor sections were incubated with primary antibodies at 4˚C overnight: Integrin (ab150361), AKT (ab8805), pAKT (ab38449), TGF-β (92486; all 1:1,000; Abcam) then with horseradish peroxidase (HRP)-labelled streptavidin (Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min and 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA) and re-stained with hematoxylin (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C, and samples were visualized using a microscope (YS100; Nikon Corporation, Tokyo, Japan). A Ventana Benchmark automated staining system was used for observation of integrin.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) analysis. Tumor sections were permeabilized by immersing cells slides in 0.2% Triton X-100 solution in PBS for 30 min at 4˚C. Subsequently, sections were incubated with equilibration buffer for 30 min at 4˚C. The sections were then incubated with 50 μl of the reaction mixture at 37˚C for 60 min and washed three times with PBS. The TUNEL Apoptosis assay kit (cat. no. 8088; ScienCell, Carlsbad, CA, USA) were used to detect TUNEL-positive cells. Finally, images were captured with a ZEISS LSM 510 confocal microscope at 488 nm.

Statistical analysis. All data are presented as the mean ± standard error in triplicate. Unpaired data was analyzed using Student's t-test and comparisons of data between multiple groups were analyzed by one-way analysis of variance followed by Tukey honest significant difference test. Kaplan-Meier analysis was used to estimate the risk of relapse and re-treatment during the 100-day treatment. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of integrin expression in miR-124-transfected osteosarcoma cells. miR-124 expression was determined in Mg63 and MC3T3-E1 cells. As shown in Fig. 1A, miR-124 expression levels were downregulated in Mg63 osteosarcoma

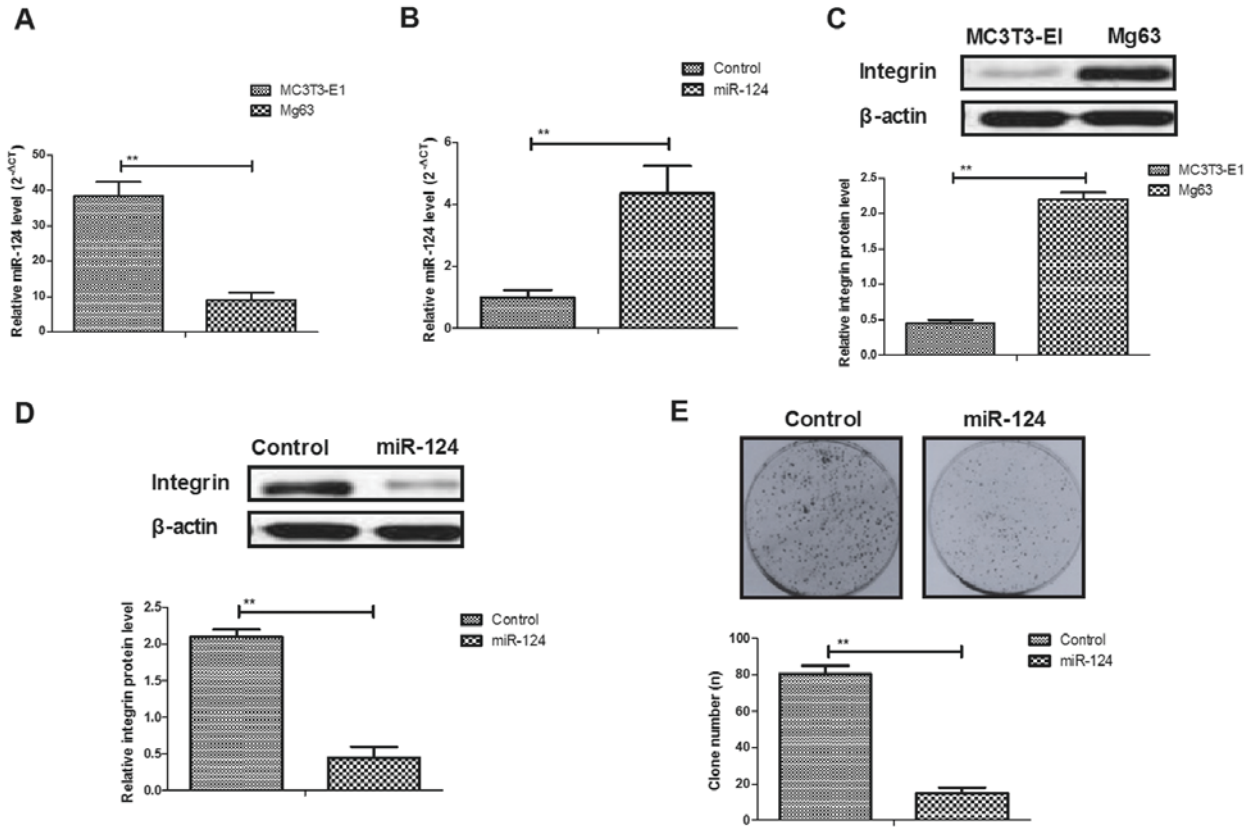

Figure 1. Effects of miR-124 transfection on integrin expression and proliferation in osteosarcoma cells. (A) Expression levels of miR-124 in osteosarcoma cells and normal bone cell lines. (B) Transfection of miR-124 increases miR-124 gene expression in Mg63 cells. (C) Expression levels of integrin-α5 in osteosarcoma cells and normal bone cell lines. (D) Effects of miR-124 transfection on integrin expression in osteosarcoma cells. (E) Effects of miR-124 transfection on the proliferation of Mg63 cells compared with the control. miR-124, microRNA-124. Data represent the mean ± standard error in triplicate (n=3). **P<0.01.

cells compared with the MC3T3-E1 bone normal cell line. Transfection of miR-124-increased miR-124 gene expression in Mg63 cells (Fig. 1B). The results also showed that integrin expression was upregulated in Mg63 cells compared with MC3T3-E1 cells (Fig. 1C). miR-124 transfection significantly decreased integrin (integrin-α5) expression in Mg63 cells (Fig. 1D). Results also demonstrated that miR-124 transfection inhibited proliferation of Mg63 cells (Fig. 1E).

Evaluation of cytotoxicity, migration and invasion in osteosarcoma cells after transfected with miR-124. As shown in Fig. 2A, miR-124 transfection significantly inhibited Mg63 cell growth compared with the control group. Transfection of miR-124 also suppressed migration and invasion of Mg63 cells after 24 h of incubation (Fig. 2B and C). Western blot demonstrated that expression levels of vimentin and α-smooth muscle actin were downregulated by miR-124 transfection (Fig. 2D). RT-qPCR and immunofluorescence revealed that miR-124 transfection significantly inhibited E-cadherin and β-catenin expression in Mg63 cells (Fig. 2E and F).

Effects of miR-124 transfection on TGF-β expression and apoptosis of osteosarcoma cells. As shown in Fig. 3A, miR-124 transfection inhibited TGF-β expression in Mg63 cells. Flow cytometry analysis demonstrated that tunicamycin-induced apoptosis was promoted by miR-124 in Mg63 cells (Fig. 3B). Western blot analysis demonstrated that P53 and Bcl-2 apoptosis regulator (Bcl-2) expression levels were downregulated, and apoptotic peptidase activating factor 1 (Apaf-1) and Bcl-2 associated agonist of cell death (Bad) expression levels were upregulated in miR-124-transfected Mg63 cells compared with control cells (Fig. 3C and D). Transfection of pTGF-β significantly increased TGF-β expression in Mg63 cells (Fig. 3E). Results demonstrated that TGF-β overexpression blocked miR-124-mediated apoptotic sensitivity to tunicamycin in Mg63 cells (Fig. 3F). Upregulation of P53 and Bcl-2 expression levels induced by miR-124 transfection was also reversed by TGF-β overexpression in Mg63 cells (Fig. 3G).

Evaluation of AKT/GSK-3β/SNAIL-1 signaling pathway in osteosarcoma cells following miR-124 transfection. In order to analyze the potential mechanisms of miR-124-meidated inhibition of osteosarcoma cells, AKT/GSK-3β/SNAIL-1 signaling was investigated in Mg63 cells. As shown in Fig. 4A, miR-124 transfection inhibited AKT, GSK-3β and SNAIL-1 expression in Mg63 cells. However, miR-124 transfection did not change phosphorylation levels of AKT and GSK-3β in Mg63 cells (Fig. 4B). The results demonstrated that TGF-β overexpression promoted AKT, GSK-3β and SNAIL-1 expression in Mg63 cells (Fig. 4C). Migration and invasion assays demonstrated that TGF-β overexpression canceled miR-124-inhibited aggressiveness of Mg63 cells (Fig. 4D and E). TGF-β overexpression improved viability of Mg63 cells transfected by miR-124 (Fig. 4F).

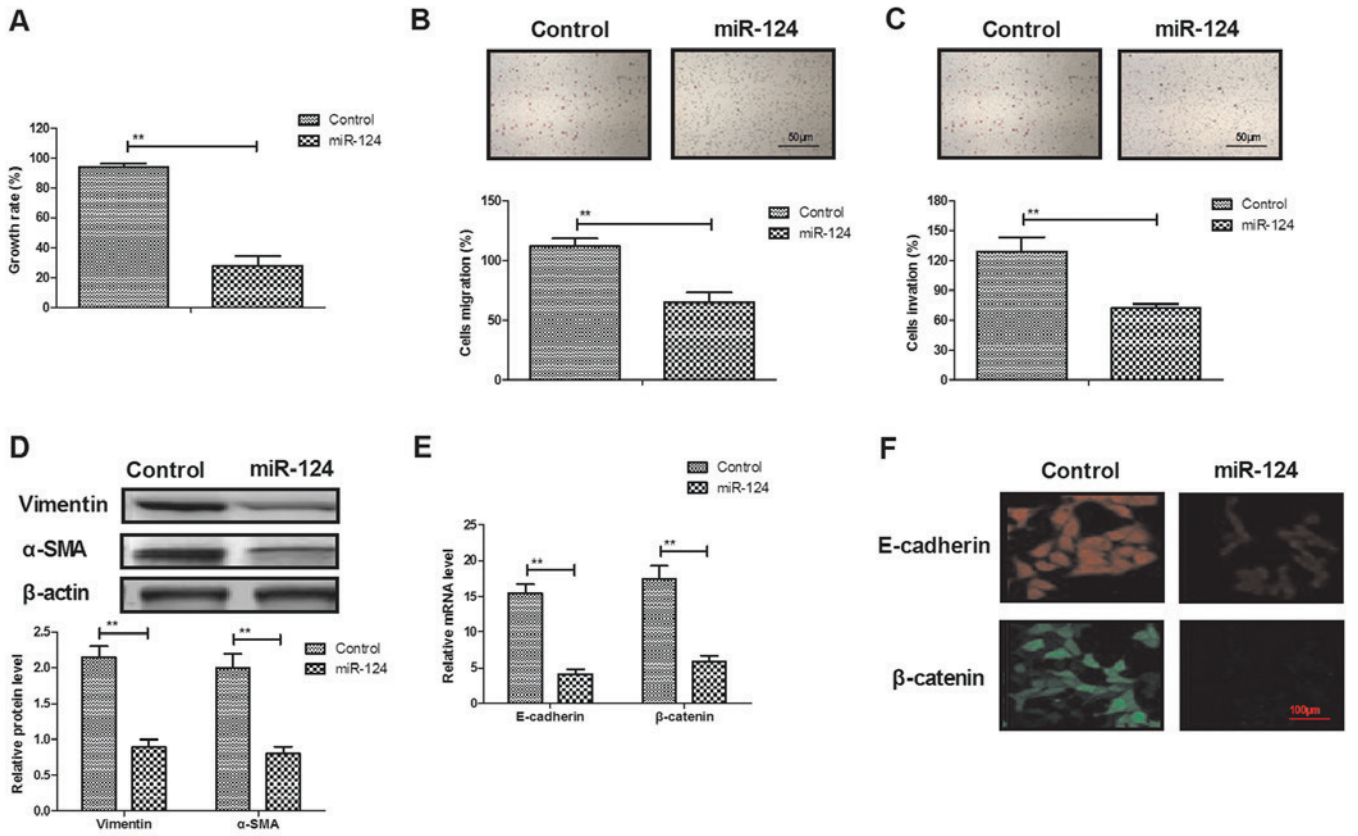

Figure 2. Effects of miR-124 transfection on cytotoxicity, migration and invasion in osteosarcoma cells. (A) miR-124 transfection inhibits Mg63 cells growth. (B and C) miR-124 transfection inhibits Mg63 cell (B) migration and (C) invasion. (D) Expression levels of vimentin and α-SMA in Mg63 cells after miR-124 transfection. (E) Reverse transcription-quantitative polymerase chain reaction and (F) immunofluorescence analyzed E-cadherin and β-catenin expression in miR-124-transfected Mg63 cells. Magnification, x400. miR-124, microRNA-124; α-SMA, α-smooth muscle actin. Data represent the mean ± standard error in triplicate of triplicate samples (n=3). **P<0.01.

Effects of miR-124 transfection on tumor growth and survival rate in osteosarcoma-bearing mice. As shown in Fig. 5A, miR-124 transfection markedly inhibited tumor growth in Mg63-bearing mice compared with the miR-vector group. Immunohistochemistry indicated that integrin and TGF-β expression levels were decreased in tumors generated from miR-124-transfected Mg63 cells compared with untransfected cells (Fig. 5B). The number of apoptotic tumor cells was increased, and AKT expression and phosphorylation levels were decreased in miR-124-transfected tumors compared with the control tumors (Fig. 5C). The survival of tumor-bearing mice was prolonged in mice with tumors overexpressing miR-124 compared with control tumor mice (Fig. 5D). These results suggest that miR-124 transfection can inhibit tumor growth and prolong survival of tumor-bearing mice, potentially by increasing the apoptosis of tumor cells.

Discussion

miRNAs are small noncoding RNAs, which have a complex role in post-transcriptional gene expression regulation, and can theoretically be used as a diagnostic, therapeutic or prognostic tool in tumor biology (29-31). Reports have indicated that the expression levels of certain miRNAs are associated with growth, proliferation, aggressive phenotypes and apoptosis in human osteosarcoma (32,33). The aim of the present study was to examine the inhibitory effects of miR-124 in osteosarcoma tumorigenesis and development. The results indicated that miR-124 transfection inhibits growth, migration and invasion, and also promotes apoptosis of osteosarcoma cells. Mechanism analysis demonstrated that miR-124 transfection may inhibit tumor growth and aggressiveness through effects on AKT/GSK-3β/SNAIL-1 signaling in osteosarcoma cells. *In vivo* experiments suggested that miR-124 transfection inhibited tumor growth and prolonged the survival of tumor-bearing mice, potentially by increasing the apoptosis of tumor cells. These findings indicate that miR-124 may serve as potential molecular therapy for osteosarcoma.

Currently, increasing the apoptosis of osteosarcoma tumor cells contributes to an efficient clinical regiment for patients with cancer (34,35). Acquired resistance protects osteosarcoma tumor cells against apoptosis induced by radiotherapy, chemotherapy and combined therapy in the treatment of tumors (36,37). Reducing the apoptotic resistance of cancer cells and tumors tissues will contribute to the clinical treatment for of with osteosarcoma who have undergone oncotherapy and other comprehensive treatments (38,39). In this study, the data indicated that miR-124 transfection promoted apoptosis of osteosarcoma cells, potentially via regulation of P53, Bcl-2, Apaf-1 and Bad expression. However, TGF-β overexpression significantly blocked miR-124 transfection-mediated tumor apoptosis in osteosarcoma cells.

A previous study suggested that miR-124 may be associated with tumor growth and aggressiveness (40). Han *et al* (38)

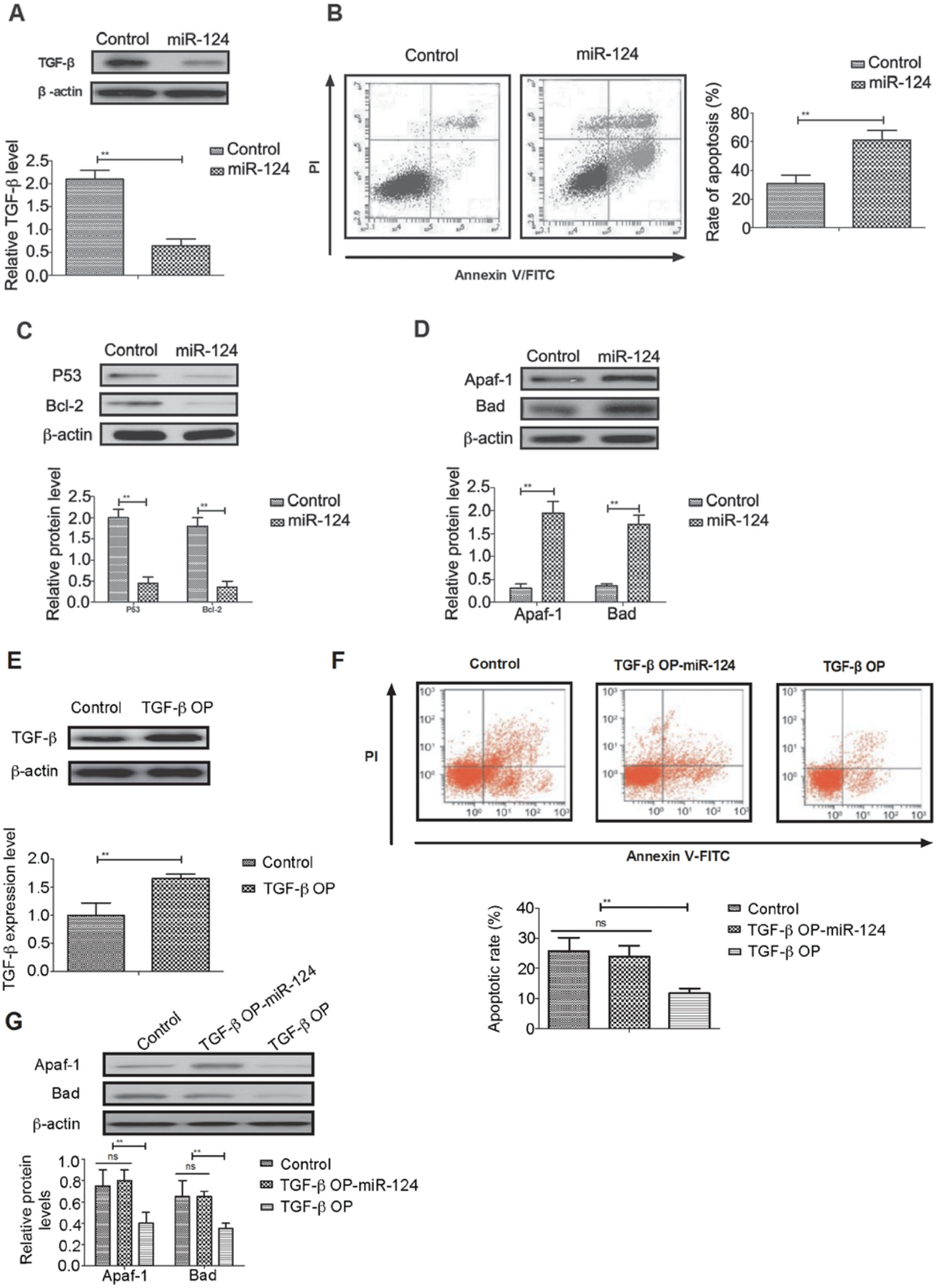

Figure 3. Effects of miR-124 transfection on TGF-β expression and apoptosis of osteosarcoma cells. (A) miR-124 transfection inhibits TGF-β expression levels in Mg63 cells. (B) miR-124 transfection promotes apoptosis of Mg63 cells induced by tunicamycin. (C) Expression levels of P53 and Bcl-2 in Mg63 cells following miR-124 transfection. (D) Expression levels of Apaf-1 and Bad in Mg63 cells following miR-124 transfection. (E) Transfection of pTGF-β (TGF-βOP) increases TGF-β expression in Mg63 cells. (F) Effects of TGF-βOP on apoptosis of Mg63 cells. (G) Effects of TGF-βOP on Apaf-1 and Bad expression levels in Mg63 cells. Control, pvector; miR-124, microRNA-124; TGF-β, transforming growth factor-β; Bcl-2, Bcl-2 apoptosis regulator; Apaf-1, apoptotic peptidase activating factor 1; Bad, Bcl-2 associated agonist of cell death; TGF-βOP, TGF-β overexpression. Data represent the mean ± standard error in triplicate of triplicate samples (n=3). **P<0.01.

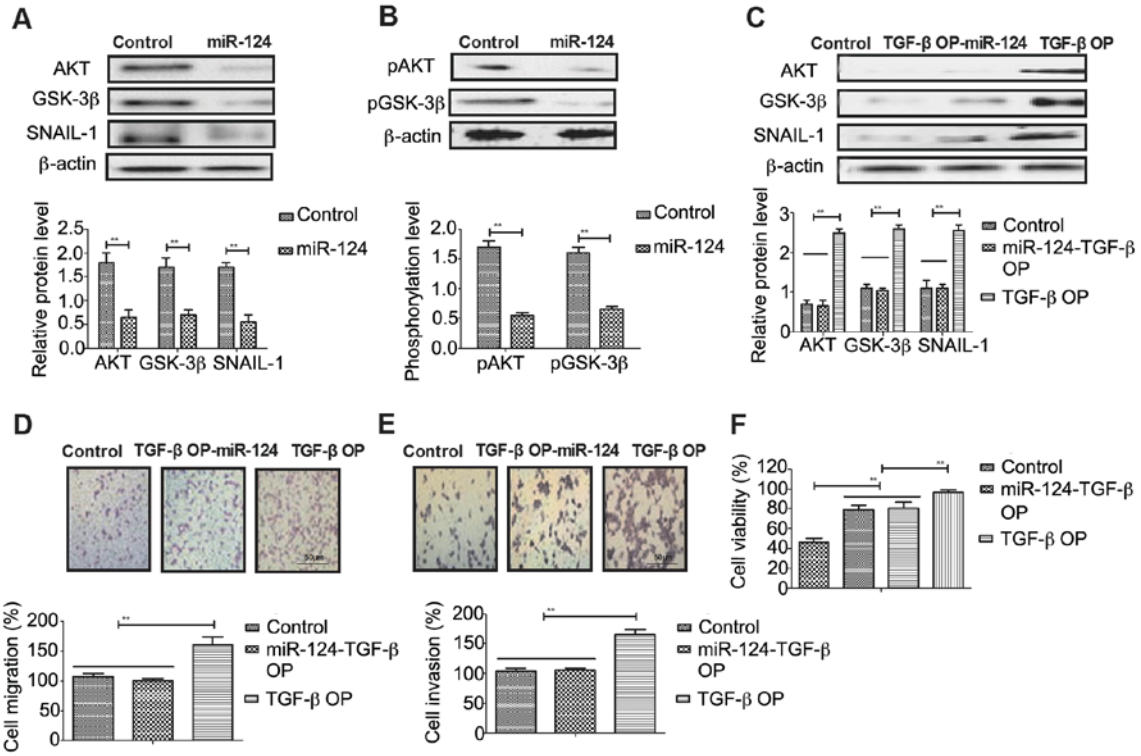

Figure 4. miR-124 transfection regulates Mg63 cell migration/invasion and downregulates AKT/GSK-3β/SNAIL-1 signaling. (A) miR-124 transfection inhibits AKT, GSK-3β and SNAIL-1 expression levels in Mg63 cells. (B) Effects of miR-124 transfection on pAKT and pGSK-3β in Mg63 cells. (C) Effects of TGF-βOP on AKT, GSK-3β and SNAIL-1 expression levels in Mg63 cells. Effects of TGF-βOP on (D) migration and (E) invasion in Mg63 cells (magnification, x400). (F) Effects of TGF-βOP on viability of Mg63 cells. Control, pvector; miR-124, microRNA-124; AKT, AKT serine/threonine kinase; GSK-3β, glycogen synthase kinase-3β; SNAIL-1, snail family transcriptional repressor 1; p, phosphorylated; TGF-βOP, transforming growth factor-β overexpression. Data represent the mean ± standard error in triplicate of triplicate samples (n=3). **P<0.01.

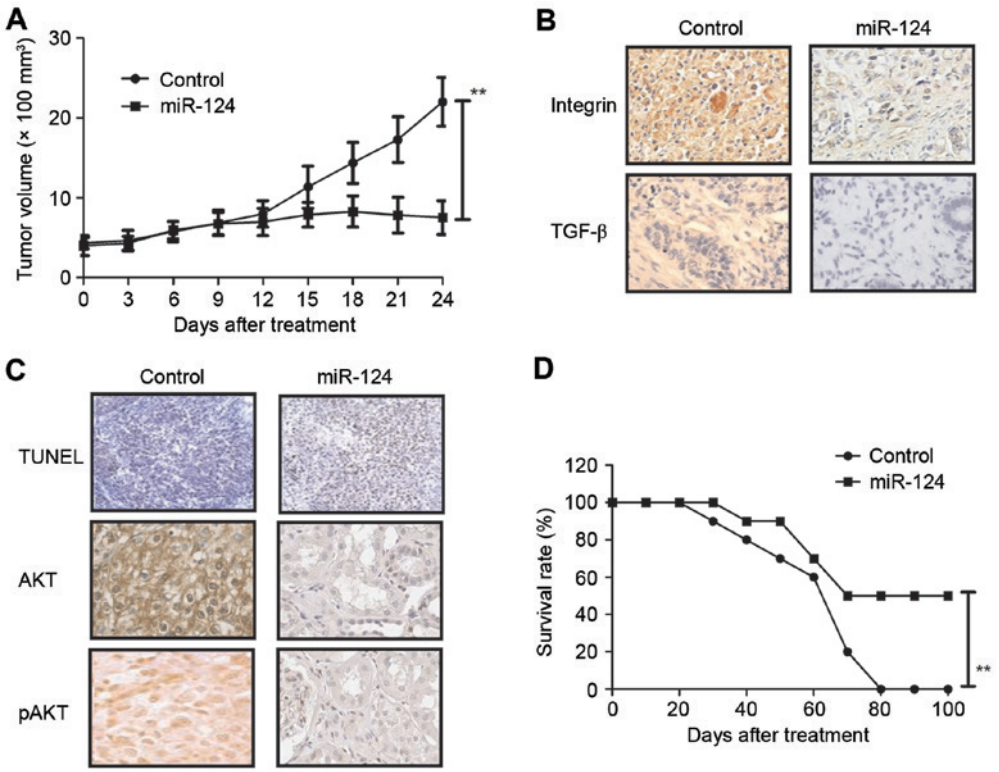

Figure 5. Anti-cancer efficacy of miR-124 on tumor growth and survival rate in osteosarcoma-bearing mice. (A) miR-124 transfection inhibits tumor growth in Mg63-bearing mice. (B) Immunohistochemistry analysis of integrin-α5 and TGF-β expression levels in tumor tissues. (C) TUNEL and immunohistochemistry analysis of AKT expression and phosphorylation levels in tumor tissues. Magnification, x20. (D) Survival rate of Mg63-bearing mice. miR-124; microRNA-124; TGF-β, transforming growth factor-β; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; AKT, AKT serine/threonine kinase; p, phosphorylated. Magnification, x40. Data represent the mean ± standard error in triplicate of triplicate samples (n=3). **P<0.01.

reported that miR-124 exerts tumor suppressive functions on growth, proliferation, motility and angiogenesis. In this study, the anti-tumor efficacy and potential mechanism of miR-124 in osteosarcoma cells was analyzed. Research has demonstrated that TGF-β-mediated AKT/GSK-3β/SNAIL-1 signaling directly induces epithelial-mesenchymal transition in human bronchial epithelial cells, which suggested these proteins as potential novel targets in the development of therapeutic and preventive approaches for human cancer (41). The findings of the present study suggested that miR-124 inhibits growth and aggressiveness by downregulation of integrin expression via TGF-β-mediated AKT/GSK-3β/SNAIL-1 signaling in osteosarcoma cells. These results indicate that miR-124 may have potential as an anti-osteosarcoma agent.

In conclusion, the levels of miR-124 and its potential molecular mechanisms in osteosarcoma cells were investigated in the current study. Although previous studies have reported the tumor suppressor role of miR-124, miR-124-regulated integrin expression through TGF-β-mediated AKT/GSK-3β/SNAIL-1 signaling has not been reported in osteosarcoma cells (42,43). The findings in the current study indicate miR-124 promotes apoptotic sensitivity of osteosarcoma to tunicamycin, suppresses growth and aggressiveness of osteosarcoma, and downregulates of TGF-β-mediated AKT/GSK-3β/SNAIL-1 signaling, which provides a potential mechanism tumor suppressor mechanism of miR-124 in osteosarcoma cells and tumor tissues.

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