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MicroRNA-152 Suppresses Human Osteosarcoma Cell Proliferation and Invasion by Targeting E2F Transcription Factor 3

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MicroRNA-152 (miR-152) expression has been reported to be downregulated in osteosarcoma (OS). However, the role of miR-152 in OS is not well documented. In the present study, we aimed to explore the function and underlying mechanism of miR-152 in OS. We found that miR-152 was underexpressed in OS tissues and cell lines. Decreased miR-152 was inversely correlated with lymph node metastasis and advanced clinical stage. Overexpression of miR-152 significantly inhibited cell proliferation, colony formation, migration, and invasion of OS cells. Bioinformatics analyses showed that miR-152 directly targeted E2F transcription factor 3 (E2F3), as further confirmed by a dual-luciferase reporter assay. E2F3 expression was upregulated and inversely correlated with miR-152 expression level in human OS tissues. Moreover, the inhibitory effects of miR-152 on OS growth and invasion were attenuated by E2F3 overexpression. Taken together, our findings indicated that miR-152 reduced OS growth and invasion by targeting E2F3 and provided new evidence of miR-152 as a potential therapeutic target for OS.

Key words: Osteosarcoma (OS); miR-152; E2F transcription factor 3 (E2F3); Proliferation; Invasion

INTRODUCTION

Osteosarcoma (OS) is one of the most common bone malignancies and mostly occurs in children and young adults[1]. Despite considerable improvements in therapeutic strategies, including chemotherapy, radiotherapy, and tumor excision, the survival rate of patients with OS still remains poor, and the molecular mechanisms of this disease are elusive[2,3]. Therefore, it is very urgent to elucidate the molecular mechanisms underlying carcinogenesis and progression in OS, which may help to treat this devastating cancer.

MicroRNAs (miRNAs) are endogenous 18–25 nt noncoding RNAs that can affect the expression of genes at the posttranscriptional level by directly binding to the 3′-untranslated region (3′-UTR) of messenger RNAs (mRNAs), promoting their degradation or inhibiting their translation[4]. miRNAs have an important role in a number of biological processes, such as cell proliferation, cell migration, apoptosis, differentiation, development, immunity, and metabolism[5,6]. Increasing evidence has shown that dysregulated expression of miRNAs plays critical roles in initiation and development in various cancers[7,8]. To date, numerous miRNAs have been identified to be involved in OS progression as oncogenes or tumor suppressor genes[9,10].

MicroRNA-152 (miR-152), a member of the miR-148/152 family, has been reported to be implicated in a series of cellular activities such as cell proliferation, invasion, and angiogenesis[11,12]. Accumulating evidence has suggested that miR-152 is frequently underexpressed and functions as a tumor suppressor in multiple malignancies[12–18]. On the contrary, miR-152 functions as an oncogene miRNA in neuroblastoma and nasopharyngeal carcinoma[19,20]. A previous study showed that miR-152 expression was downregulated in OS tissues, and its expression was associated with the diagnosis and prognosis of patients with OS[21]. Yet the biological role and underlying mechanism of miR-152 in human OS remain unknown. In the current study, we investigated the role of miR-152 in OS by a series of experiments and found that miR-152 significantly suppressed OS cell proliferation, colony formation, migration, and invasion through targeting *E2F3*. These results suggest that miR-152 might emerge as a potential therapeutic target in the treatment of OS.

MATERIALS AND METHODS

Sample Collection

This study protocol was recognized by the Research Ethics Committee in The First Hospital of Jilin University (Changchun, P.R. China). Written informed consent was

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obtained from each participant. OS samples and corresponding adjacent normal tissues were collected from 45 patients diagnosed with OS in The First of Hospital of Jilin University. None of the patients had received chemotherapy or radiotherapy before surgery. All samples were stored in liquid nitrogen until RNA extraction.

Cell Culture and Treatment

Human OS cell lines MG63, 143B, U2OS, and SaOS and the human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco). The cells were plated in tissue culture dishes at 37°C in a humidified 5% CO_2 incubator and cultured for 1–3 days for further study.

qRT-PCR

Total RNA was extracted from culture cells or tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For detection of the expression levels of miR-152 in cells, the TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used under ABI 7900 Sequence Detection System (Applied Biosystems). PrimeScript RT Reagent Kit (Promega, Madison, WI, USA) and the One Step SYBR PrimeScript (Promega) were used for testing the mRNA expression levels of *E2F3* in cells. The primers for *E2F3* and *GAPDH* used in this study were as described previously[22]. The internal control genes were *GAPDH* for *E2F3* mRNA and *U6* for miR-152. Relative expressions of miR-152 and *E2F3* were calculated by the relative quantification ($2^{-\Delta\Delta}$Ct) method.

Cell Transfection

The cells were cultured in a 6-well or 24-well plate for 24 h and then were transfected with plasmids (100 ng) or miRNAs (100 nM) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. miR-152 mimic and miRNA control mimic (miR-NC) were synthesized by RiboBio (Guangzhou, P.R. China). Overexpression E2F3 plasmid (pCDNA3.1-E2F3) was given by Dr. Han (Jilin University, Changchun, P.R. China).

Cell Proliferation and Colony Formation Assays

Cell proliferation was analyzed by MTT assay. Briefly, cells (2×10^3/well) were seeded in a 96-well plate, and cell proliferation was determined every 24 h for 72 h. At the indicated time point (24, 48, and 72 h), 20 µl of MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well; 150 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to each well to stop the reaction, and optical density was measured at 490 nm using a microplate reader (Bio-Tek Company, Winooski, VT, USA).

For colony formation, transfected cells were cultured in six-well plates at a density of 1×10^3 cells/well for 14 days. The resulting colonies were fixed in 4% paraformaldehyde for 5 min and stained with 1.0% crystal violet for 1 min. The colony formation rate was calculated with an X71 inverted microscope (Olympus, Tokyo, Japan) as number of colonies/number of seeded cells$\times$100%.

Cell Migration and Invasion Assays

Cell migratory distance was determined by wound healing assay. Each well of a six-well culture plate was seeded with cells to a final density of 100,000 cells/well, and these cells were grown to a 100% confluent monolayer. Wounds were created in the cell monolayer by scratching with a plastic pipette tip. After washing with serum-free culture medium three times, the cells were cultured for another 24 h. Wound closure was monitored by collecting digitized images at 0 and 24 h after the scratch was performed. Cell migration distance was observed and photographed under a light microscope and was analyzed using ImageJ software 3.1.

The invasion ability of cells was evaluated using Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, transfected cells (1×10^5 cells per assay) were seeded into the upper chamber coated with Matrigel (BD Biosciences) in serum-free medium, whereas DMEM containing 20% FBS was added to the lower chamber as chemoattractant. Twenty-four hours later, cells that had migrated to the lower surface were fixed with 70% ethanol and stained with 0.1% crystal violet, photographed under a light microscope (magnification: 200×), and counted in five randomly selected fields.

Luciferase Reporter Assay

Prediction of potential miR-152 target genes was performed using the four public bioinformatics algorithms: miRanda, TargetScan, miRBase, and PicTar. One of the identified possible targets, namely, *E2F3*, was chosen for experimental verification of its ability to bind miR-152 via luciferase assays. The human E2F3-3′-UTR, containing the putative miR-152 binding site, was amplified and subcloned into the pGL3 vector (Ambion, Austin, TX, USA) and named as WT-E2F3-3′-UTR. A mutated E2F3-3′-UTR with an altered binding sequence was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Dallas, TX, USA), referred to as MT-E2F3-3′-UTR. For the luciferase reporter assays, U2OS cells

were cotransfected with an oligo (miR-152 or miR-NC) and one of the two reporter plasmids (WT-E2F3-3′-UTR or MT-E2F3-3′-UTR), using Lipofectamine 2000 according to the manufacturer's protocol. Forty-eight hours after cotransfection, cells were lysed, and the luciferase activity was determined using the Dual-Luciferase Reporter Assay Kit (Promega) per the manufacturer's protocol. *Renilla* luciferase activity was normalized to that of firefly luciferase.

Western Blotting Analysis

Total cell or tissue extracts were extracted using cell lysis buffer followed by immunoblotting with anti-E2F3 (1:1,000; Cell Signaling Technology, Danvers, MA, USA) or anti-GAPDH (1:4,000; Cell Signaling Technology) as previously described[22].

Statistical Analysis

All statistical analyses were performed using the SPSS 19 statistical software (Chicago, IL, USA). The values are presented as mean ± standard deviation (SD) and are considered significant with a value of $p < 0.05$. Statistical analysis between two samples was performed using Student's *t*-test, and more than two groups were performed using one-way ANOVA. Kaplan–Meier analysis was used to analyze patients' survival. The correlations between miR-152 and E2F3 were analyzed in OS tissues using Pearson's correlation analysis.

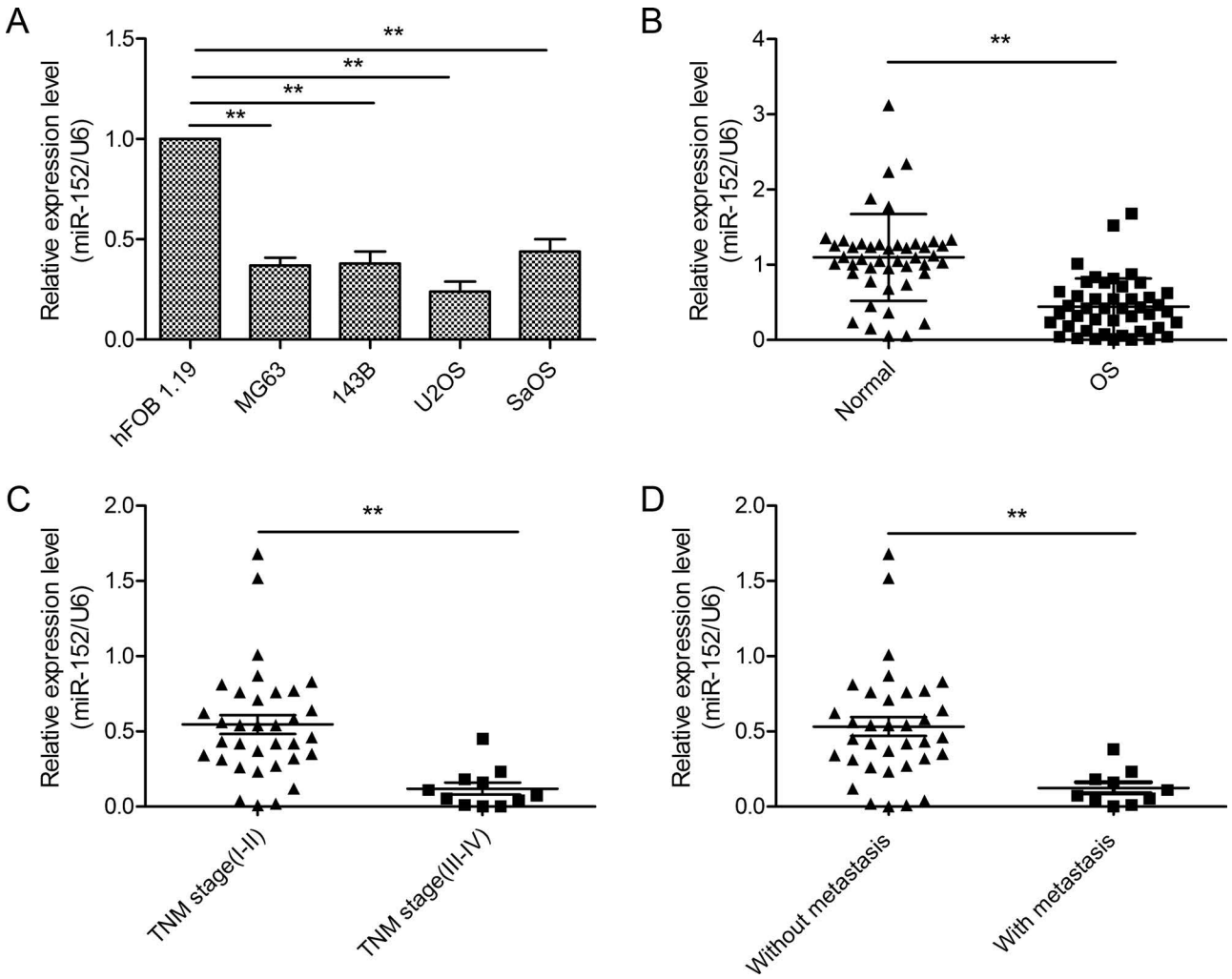

Figure 1. MicroRNA-152 (miR-152) is downregulated in osteosarcoma (OS) tissues and cell lines. (A) The expression of miR-152 in four human OS cell lines (MG63, 143B, U2OS, and SaOS) and a human osteoblast cell line (hFOB 1.19) was measured by qRT-PCR analysis. (B) Expression of miR-152 in OS tissues and corresponding normal tissues from 45 OS patients. (C) Expression of miR-152 in different clinical stages. (D) Expression of miR-152 in OS tissues with or without lymph node metastasis. $^{**}p < 0.01$.

RESULTS

miR-152 Is Downregulated in OS Tissues and Cell Lines

In order to determine the expression level of miR-152 in human OS cell lines (MG63, 143B, U2OS, and SaOS) and in the human osteoblast cell line (hFOB 1.19), qRT-PCR was performed. Expression analysis results showed that relative expression of miR-152 was significantly downregulated in four OS cell lines compared with the human osteoblast cell line (Fig. 1A). Moreover, we found that OS tissues had significantly lower expression levels of miR-152 compared to adjacent normal tissues ($p<0.01$) (Fig. 1B). We also found that miR-152 expression was lower in OS tissues with lymph node metastasis ($p<0.01$) (Fig. 1C) and those at an advanced clinical stage ($p<0.01$) (Fig. 1D). These results suggested that miR-152 expression was downregulated in OS tissues and cell lines.

miR-152 Inhibits the Proliferation and Colony Formation of OS Cells

To explore the biological functions of miR-152 in OS, we transfected miR-152 and miR-NC mimics into U2OS cells and found that miR-152 expression was upregulated in U2OS cells transfected with miR-152 mimics compared with cells transfected with miR-NC mimics (Fig. 2A). MTT assay showed that cell proliferation was significantly reduced in miR-152-treated cells in comparison with the miR-NC-treated cells (Fig. 2B). Consistent with this result, colony formation assay showed that miR-152 significantly decreased the colony formation of U2OS cells (Fig. 2C).

miR-152 Inhibits the Migration and Invasion of OS Cells

Given the miR-152 expression to be lower in tissues with lymph node metastasis, we hypothesized that miR-152 may affect metastatic activity in OS. To test this hypothesis, wound healing and Transwell invasion assays were performed to assess the effect of miR-152 on migration and invasion. We found that the ectopic expression of miR-152 significantly inhibited the migration and invasion activities of U2OS cells (Fig. 3A and B), indicating that miR-152 indeed reduces the metastatic potential of OS cells.

E2F3 Is a Target of miR-152 in OS

In order to investigate the molecular mechanism underlying the role of miR-152 in OS, we searched for potential miR-152 targets using the four public bioinformatics algorithms (miRanda, TargetScan, miRBase, and PicTar). *E2F3* mRNA was predicated as a potential binding target for miR-152 for further study (Fig. 4A). Luciferase reporter assays further confirmed that miR-152

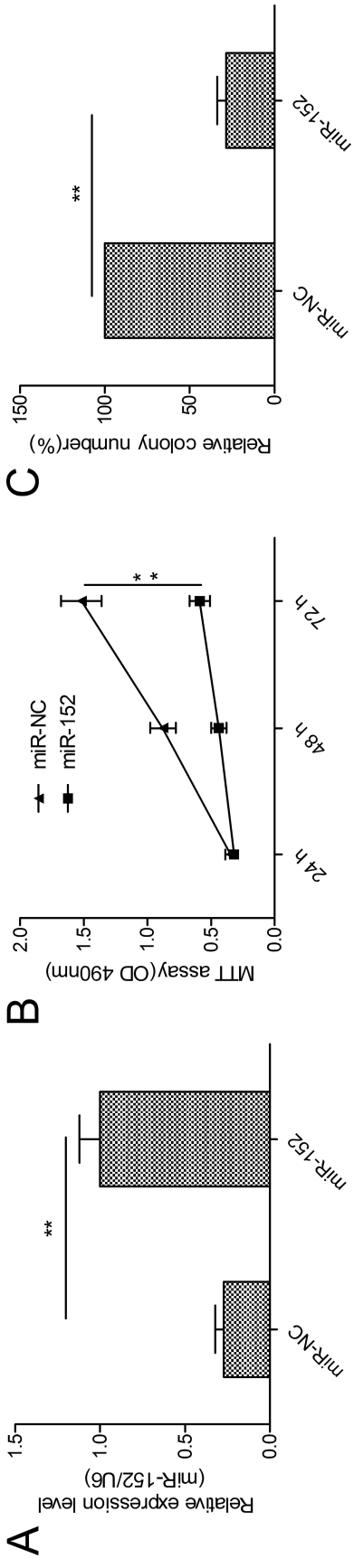

Figure 2. miR-152 inhibits OS cell proliferation and colony formation in vitro. (A) The expression level of miR-152 was measured in U2OS cells transfected with miR-152 mimic or its negative control (miR-NC) by qRT-PCR. (B, C) Cell proliferation and colony formation were determined in U2OS cells transfected with miR-152 mimic or miR-NC. $^{**}p<0.01$.

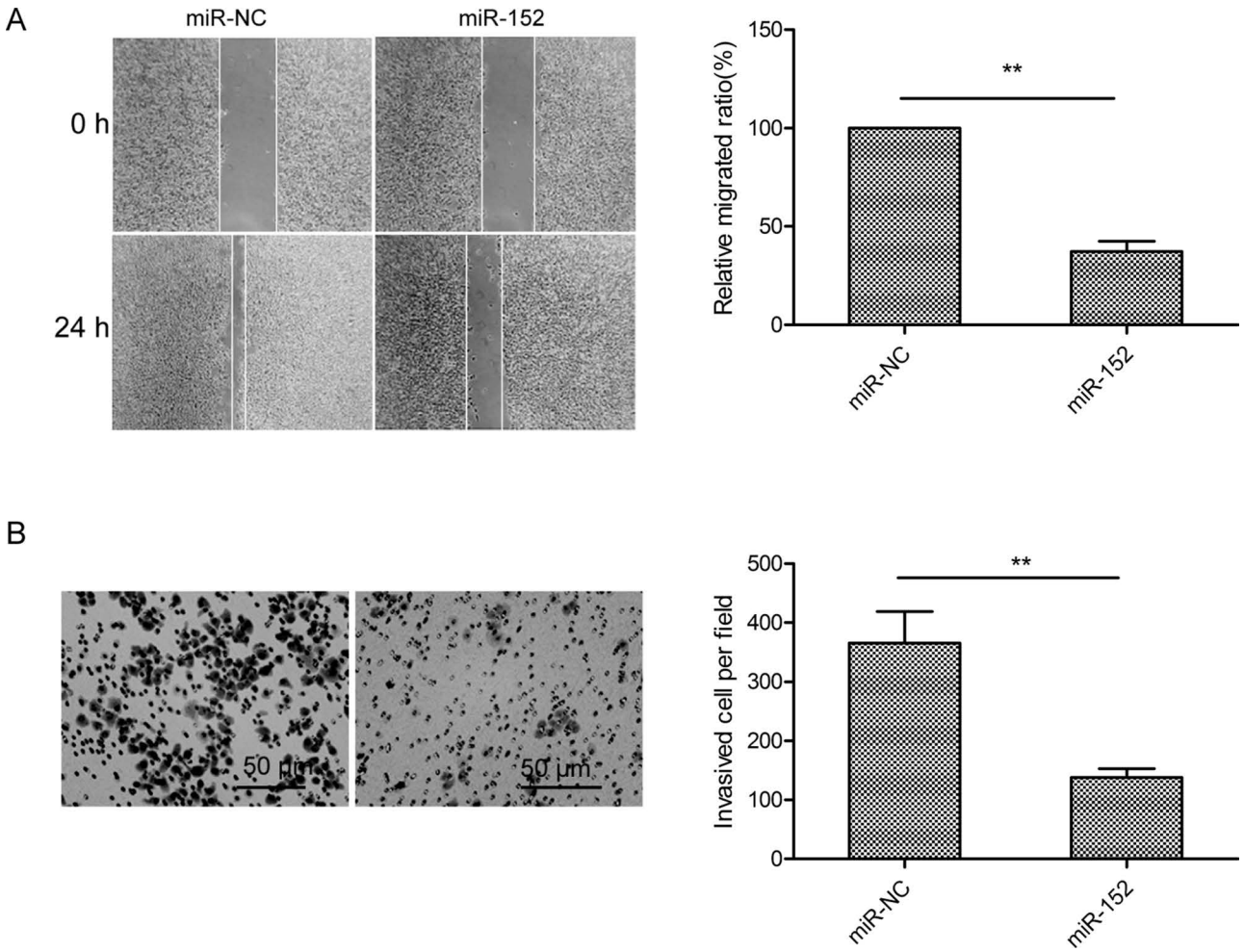

Figure 3. miR-152 inhibits OS cell migration and invasion in vitro. (A) Cell migration was detected in U2OS cells transfected with miR-152 mimic or miR-NC by wound healing. (B) Cell invasion was detected in U2OS cells transfected with miR-152 mimic or miR-NC by Transwell invasion assay. $^{**}p<0.01$.

overexpression decreased the luciferase activity of the WT-E2F3-3′-UTR reporter construct ($p<0.05$) (Fig. 4B) but did not affect the activity of the MT-E2F3-3′-UTR construct (Fig. 4B). Moreover, we found that the miR-152 overexpression dramatically decreased the expression of U2OS both at the mRNA (Fig. 4C) and the protein levels (Fig. 4D) in U2OS cells. These results suggested that E2F3 was a target of miR-152 in OS.

E2F3 Was Upregulated and Negatively Correlated With miR-152 in OS Tissues

To further explore the relationship between miR-152 and E2F3 in OS, we examined the *E2F3* mRNA expression level in OS tissues and adjacent normal tissues by qRT-PCR. As shown in Figure 5A, *E2F3* mRNA expression was higher in OS tissues than in adjacent normal tissues. Pearson's correlation analysis demonstrated that *E2F3* mRNA expression was inversely correlated with miR-152 levels in OS tissues ($r=-0.377$, $p=0.01$) (Fig. 5B).

Overexpression of E2F3 Ablates the Inhibitory Effects of miR-152 in OS Cells

As E2F3 was a target of miR-152 in OS cells, we speculated that E2F3 might be involved in miR-152-mediated inhibition of OS cell growth and metastasis. To test this hypothesis, miR-152 mimic and an E2F3 overexpressing vector (pCDNA3.1-E2F3) were cotransfected into U2OS cells. qRT-PCR and Western blot assays showed that both the mRNA and protein levels of E2F3 were increased by transfection with pCDNA3.1-E2F3 and were reduced by miR-152 mimic ($p<0.05$), and the levels were restored after cotransfection with PCDNA3.1-E2F3 and miR-152 mimic ($p<0.001$) (Fig. 6A and B), indicating the high efficiency of transfection. Moreover, transfection of PCDNA3.1-E2F3 reversed the inhibitory effects of

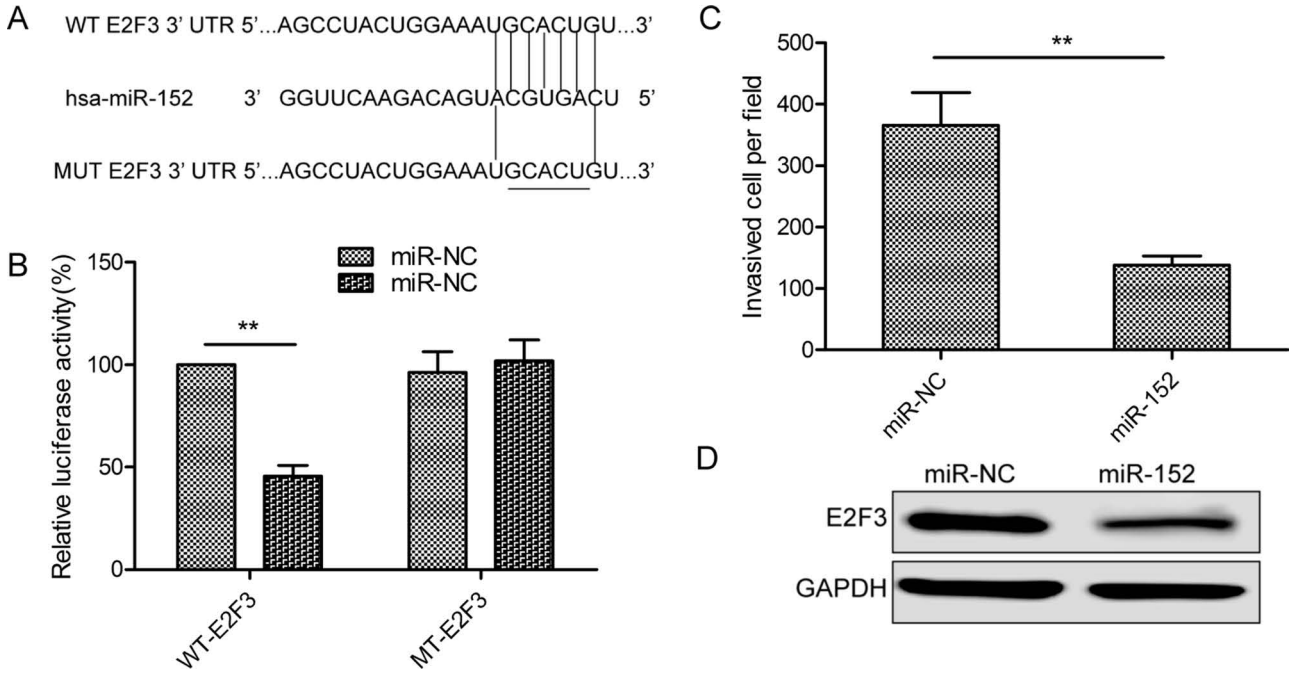

Figure 4. E2F3 is a direct target of miR-152 in OS cells. (A) The putative miR-152-binding sites and mutant 3′-UTR-E2F3 sites are shown. The replaced site is underlined. WT, wild type; MT, mutant type. (B) Relative luciferase activity was detected in U2OS cells cotransfected with WT/MT-E2F3-3′-UTR reporter plasmid and miR-152 mimic or miR-NC. (C, D) The E2F3 expression on mRNA and protein levels was determined in U2OS cells transfected with miR-152 mimic and miR-NC. GAPDH was used as an internal control. $^{**}p<0.01$.

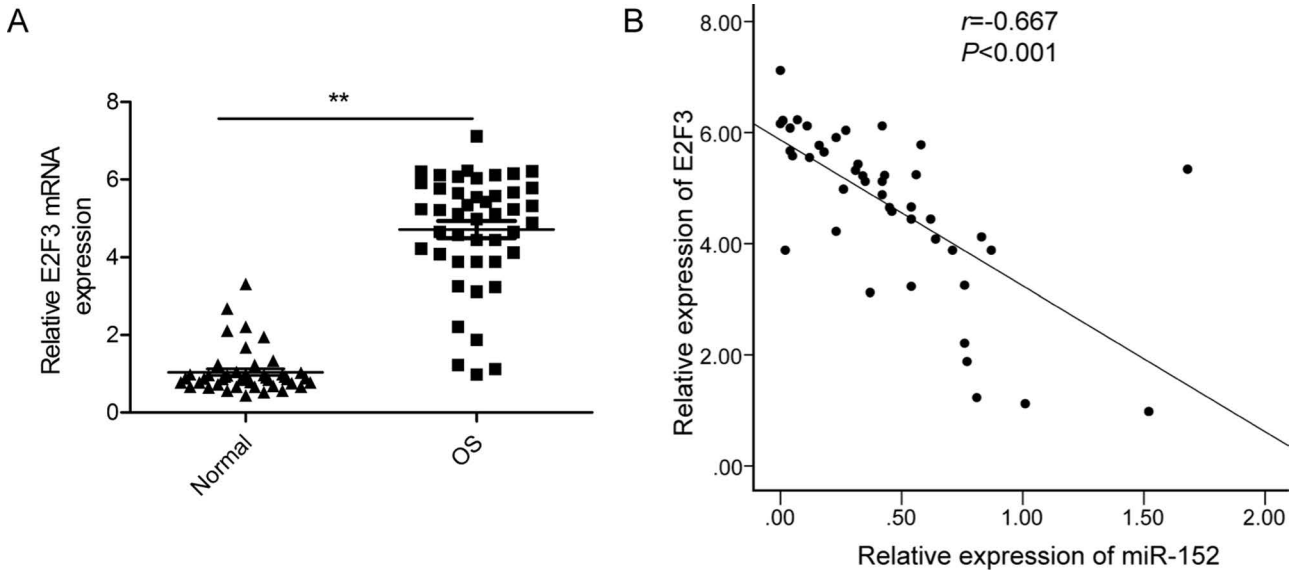

Figure 5. E2F3 expression was upregulated and was negatively correlated with miR-152 in ovarian cancer tissues. (A) The *E2F3* mRNA expression level was measured in 45 pairs of OS tissues and adjacent normal tissues by qRT-PCR. GAPDH was used as an internal control. (B) The correlation of the expression levels of E2F3 and miR-152 was analyzed by Pearson's correlation assay in OS tissues ($n=45$). $^{**}p<0.01$.

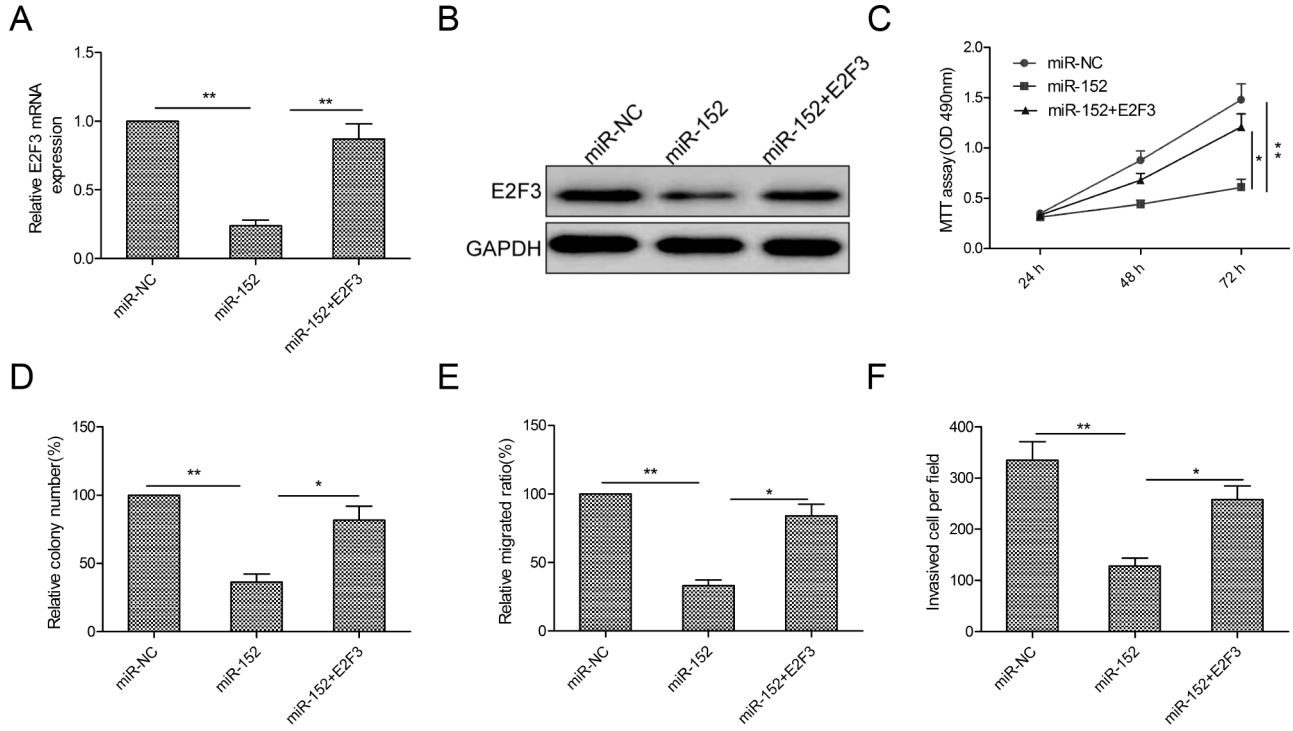

Figure 6. Overexpression of E2F3 ablates the inhibitory effects of miR-152 in OS cells. (A, B) E2F3 mRNA and protein levels were determined in U2OS cells after transfection with miR-152 mimic or miR-NC, with or without E2F3 cDNA vector (pCDNA3.1-E2F3). GAPDH was used as an internal control. (C–F) Cell proliferation, colony formation, migration, and invasion were determined in U2OS cells after transfection with miR-152 mimic or miR-NC, with or without pCDNA3.1-E2F3. *$p<0.05$, **$p<0.01$.

miR-152 on U2OS cell proliferation, colony formation, migration, and invasion (Fig. 6C–F). These results suggested that miR-152 exerts a suppressive role in OS by repressing E2F3.

DISCUSSION

Growing evidence has suggested that miRNAs play important roles in regulating initiation and development of OS as oncogenes or tumor suppressor genes[9,10]. For example, Dong et al. showed that overexpression of miR-874 in OS cells could remarkably inhibit proliferation, migration, and invasion and induce cell apoptosis by targeting E2F3[22]. Qu et al. found that restoration of miR-150 expression in OS cells could inhibit cell proliferation, migration, and invasion and induced apoptosis in vitro as well as suppressed tumor growth of OS in vivo by repressing IGF2 mRNA-binding protein 1 (IGF2BP1)[23]. Lin et al. demonstrated that exogenous miR-203 overexpression inhibited OS cell proliferation and invasion and promoted apoptosis by targeting Runt-related transcription factor 2 (RUNX2)[24]. In the present study, we found that miR-152 was underexpressed in OS tissues and cell lines. Decreased miR-152 was inversely correlated with lymph node metastasis and advanced clinical stage, which was consistent with a previous study[21]. Moreover, we found that overexpression of miR-152 in OS cells significantly inhibited proliferation, colony formation, migration, and invasion. These findings suggested that miR-152 may be a novel therapeutic target for OS.

Having a tumor-suppressive role in tumorigenesis, miR-152 was reported to regulate cell proliferation, migration, cell cycle, apoptosis, and invasion by directly or indirectly repressing oncogene expression, such as such as B7-H1[13], ALCAM[14], PIK3R3[15], WNT1[16], ERBB3[16], neuropilin-1[17], PIK3CA[25], and RTKN[26]. Here E2F3 is identified to be another important target of miR-152 by luciferase reporter assay, qRT-PCR, and Western blot. E2F3, a member of the E2F family, is a major regulator of cell cycle, apoptosis, and differentiation[27]. E2F3 expression was upregulated and functioned as an oncogene in many types of cancer, such as lung cancer[28], bladder cancer[29], colorectal cancer[30], and gastric cancer[31]. In OS, it has been shown that E2F3 expression was upregulated in human OS tissue samples compared with paired normal tissues[32] and that knockout E2F3 significantly inhibited OS cell proliferation, colony formation, migration, and invasion[22], suggesting E2F3 was an oncogene in OS. In the current study, our results demonstrated that E2F3 expression was upregulated in OS tissues and was inversely correlated with miR-152 expression. Of note, our result

showed that restoring E2F3 expression attenuated miR-152-induced inhibitory effects on cell proliferation, colony formation, migration, and invasion in OS cells. In all, these findings indicated that miR-152 exerts a suppressive role in OS, at least in part, by repressing E2F3.

This study has several limitations. First, a larger number of OS samples were needed to increase the strength of the study. Second, the roles of miR-152 in OS should be tested in two or more OS cell lines. Third, in vivo application of gene therapy in animal models of OS may provide in vivo data to better comprehend the suppressive role of miR-152.

To summarize, here we provide evidence that miR-152 was downregulated in both OS cell lines and primary OS human tumors. Overexpression of miR-152 inhibited proliferation, colony formation, migration, and invasion of OS cells. We also demonstrated that E2F3 was the downstream molecular target of miR-152 in OS cells. These results suggested that miR-152 could serve as a novel therapeutic target for inhibiting the growth of OS.

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