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Knockdown of Long Noncoding RNA TUG1 Inhibits the Proliferation and Cellular Invasion of Osteosarcoma Cells by Sponging miR-153

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Long noncoding RNA (lncRNA) taurine-upregulated gene 1 (TUG1) has been confirmed to be involved in the progression of various cancers; however, its mechanism of action in osteosarcoma has not been well addressed. In our study, TUG1 was overexpressed and miR-153 was downregulated in osteosarcoma tissues and cell lines. A loss-of-function assay showed that TUG1 knockdown suppressed the viability, colony formation, and invasion of osteosarcoma cells in vitro. Moreover, TUG1 was confirmed to be an miR-153 sponge. Ectopic expression of TUG1 reversed the inhibitory effect of miR-153 on the proliferation and invasion of osteosarcoma cells. Further transplantation experiments proved the carcinogenesis of TUG1 in osteosarcoma in vivo. Collectively, our study elucidated that TUG1 contributes to the development of osteosarcoma by sponging miR-153. These findings may provide a novel lncRNA-targeted therapy for patients with osteosarcoma.

Key words: Taurine-upregulated gene 1 (TUG1); miR-153; Proliferation; Invasion; Osteosarcoma

INTRODUCTION

Osteosarcoma is the most common primary bone sarcoma in humans worldwide. It is the second highest cause of cancer-related deaths in the pediatric population[1–3]. Despite the improvement of several treatments, such as surgical intervention and chemotherapy, the total survival rate of osteosarcoma patients is still low, and approximately 35% of patients will die within 5 years[4]. The poor prognosis and high recurrence rate of osteosarcoma are largely due to the frequent pulmonary metastasis[5]. Although numerous studies have been conducted to identify various molecules involved in the pathogenesis and progression of osteosarcoma, the exact molecular mechanism behind osteosarcoma metastasis and relapse remains largely unclear. Therefore, to understand the potential mechanisms of osteosarcoma development, it is valuable to explore effective target therapies for patients with osteosarcoma.

Long noncoding RNAs (lncRNAs) are a type of nonprotein coding transcript, more than 200 nucleotides in length, with the capacity to control gene expression at the level of transcription, posttranscription, or epigenetic modification[6]. Increasing documents have revealed that lncRNAs could regulate the expression of tumor-associated genes and modulate the function of tumor-associated pathways by serving as oncogenes or anti-oncogenes in cancer[7,8]. The regulatory network of lncRNAs has been demonstrated to be complicated and extensive[9]. MicroRNAs (miRNAs), small noncoding RNA molecules with a size of approximately 18 to 25 nucleotides, are able to posttranscriptionally regulate the expression of target genes by suppressing translation or destabilizing mRNA through incomplete base pairing to the 3′-untranslated region (3′-UTR) of mRNA targets[10]. Since a competitive RNA (ceRNA) hypothesis was proposed[11], a growing body of studies has reported the reciprocal repression between lncRNAs and miRNAs in tumorigenesis[12].

Taurine-upregulated gene 1 (TUG1), a 7.1-kb lncRNA that is mapped to chromosome 22q12.2, was initially identified to be upregulated in taurine-treated retinal cells of mice[13]. Accumulating evidence proved that TUG1 was aberrantly expressed and functioned as an oncogene or a tumor suppressor depending on the type of cancer[14–16]. In addition, TUG1 genes have been confirmed to affect cell proliferation, migration, invasion, apoptosis, and metastasis in various cancers[17–20]. Previous studies revealed that TUG1 expression was elevated and closely associated with a poor prognosis in osteosarcoma, and TUG1 silencing contributed to inhibiting proliferation and inducing apoptosis in osteosarcoma cells[21,22]. However, the precise molecular mechanisms by which TUG1 exerts its functional role in osteosarcoma progression have not been

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well determined. In the present study, the role and underlying molecular mechanisms of TUG1 in osteosarcoma progression were investigated.

MATERIALS AND METHODS

Tissue Specimens and Cell Culture

Ten normal osseous tissue samples and 17 osteosarcoma tissue samples were obtained from patients in the Zhoukou Central Hospital. This study was approved by the Zhoukou Central Hospital Ethics Review Committees, and all patients provided informed consent prior to enrolling in this study.

Two human osteosarcoma cell lines (MG63 and U2-OS) and a normal human osteoblastic cell line (hFOB 1.19) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen). All cultures were performed in an incubator at 37°C under humidified air with 5% CO_2.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from osteosarcoma cell lines or osteosarcoma tissues using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was conducted using M-MLV reverse transcriptase (Invitrogen). qRT-PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in the IQ5 Real-Time PCR System (Bio-Rad). The relative expression of TUG1 and miR-153 was analyzed using the $2^{-\Delta\Delta}$Ct method.

Cell Transfection

miR-153 mimic, scrambled negative control (miR-NC), siRNA duplex oligonucleotides targeting TUG1 (si-TUG1), and scrambled negative control (si-NC) were synthesized from Dharmacon (Austin, TX, USA). For TUG1 overexpression, pcDNA-TUG1 plasmid was constructed by amplifying TUG1 and cloning it into the empty pcDNA3.1 vector (Invitrogen). Cell transfections were performed using Lipofectamine 2000 (Invitrogen) as described in the manufacturer's instructions.

Cell Viability Assay

MG63 and U2-OS cells were placed into a 96-well plate at a density of 2×10^3 per well and cultured overnight. Subsequently, cells were transfected with si-TUG1, miR-153 mimic, or combined miR-153 mimic and pcDNA-TUG1. At 48 h after transfection, all cells were treated with 10 µl of CCK-8 reagent (Dojindo, Kumamato, Japan) and incubated for an additional 2.5 h. The absorbance at 450 nm was then determined by a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony Formation Assay

MG63 and U2-OS cells transfected with si-TUG1, miR-153 mimic, or miR-153 mimic + pcDNA-TUG1 were seeded into six-well plates with a density of 500 cells/well. Two weeks later, cells were fixed with 4% paraformaldehyde followed by staining with 0.1% crystal violet. Colonies of more than 50 cells were photographed and counted using a microscope (Leica Microsystems, Heerbrugg, Switzerland).

Transwell Invasion Assay

Transwell plates and cell culture inserts (BD Biosciences, San Jose, CA, USA) were applied to observe the invasive capacity of MG63 and U2-OS cells. Transfected cells (1×10^5) in serum-free DMEM were added into the upper chamber coated with Matrigel (BD Biosciences), and 500 µl of DMEM containing 10% FBS was added into the lower chamber. After 24 h, cells passing through the pores were fixed with 4% paraformaldehyde and were observed by hematoxylin and eosin staining. The cell images were photographed under a microscope (Olympus, Japan), and cell numbers were counted in five random visual fields.

Luciferase Reporter Assays

The wild or mutant TUG1 fragments harboring the binding sequences of miR-153 were amplified and inserted into pmirGLO Dual-Luciferase vector (Promega, Madison, WI, USA). MG63 and U2-OS cells were then cotransfected with 200 ng of reporter plasmids (pmirGLO, pmirGLO-TUG1-WT, or pmirGLO-TUG1-MUT) and 100 nM miR-153 or miR-NC using Lipofectamine 2000 (Invitrogen). Luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection. *Renilla* luciferase was used as a normalization control.

RNA Immunoprecipitation (RIP) Assay

MG63 and U2-OS cells were cotransfected with pLV-MS2, pLV-TUG1-WT-MS2, or pLV-TUG1-MUT-MS2 and pMS2-FLAG. After 48 h, cells were used to perform RIP assays using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) was used for RIP. The miR-153 expression level in the precipitates was detected by qRT-PCR.

Xenograft in Nude Mice

Recombinant lentivirus vectors containing shRNA-control (sh-NC) or shRNA-TUG1 (sh-TUG1) were purchased from the GeneChem Company (Shanghai, P.R. China). Suspended MG63 cells (100 µl) transfected with constructed lentivirus vectors were subcutaneously injected into the lateral tail veins of the female athymic

BALB/c null mice (4–6 weeks old) from the Shanghai Experimental Animal Center (Shanghai, P.R. China). Tumor volumes in the sh-NC group (six mice) and the sh-TUG1 group (six mice) were assessed using calipers every 3 days when the implantations were beginning to grow. Thirty days later, the mice were sacrificed for tumor weight analysis. Additionally, miR-153 expression in excised tumors was detected by qRT-PCR. All animal experiments were performed with the approval of the Research Ethics Committee of Zhoukou Central Hospital.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 statistics software (SPSS, Chicago, IL, USA). All quantified data were presented as mean ± SD. Student's *t*-test and one-way ANOVA were performed to estimate the significance of difference. A value of $p < 0.05$ was considered statistically significant.

RESULTS

TUG1 Expression Is Upregulated and miR-153 Is Downregulated in Osteosarcoma Tissues and Cell Lines

To evaluate the expression of TUG1 and miR-153 in osteosarcoma tissues, qRT-PCR analysis was performed in 17 osteosarcoma tissues and 10 normal osseous tissues. The level of TUG1 was high in osteosarcoma tissues and low in normal osseous tissues, and the expression of miR-153 was low in osteosarcoma tissues and high in normal osseous tissues (Fig. 1A and B). The expression levels of TUG1 and miR-153 in the osteosarcoma MG63 and U2-OS cell lines were then further evaluated by qRT-PCR analysis. As expected, TUG1 expression was significantly increased in MG63 and U2-OS cells compared with that in normal osteoblastic cell line hFOB 1.19 (Fig. 1C). In contrast, miR-153 expression was remarkedly lower in MG63 and U2-OS cells than in hFOB 1.19 cells (Fig. 1D). These data indicated that abnormal expression of TUG1 and miR-153 may be associated with osteosarcoma pathogenesis.

TUG1 Knockdown Suppresses Proliferation and Invasion in Osteosarcoma Cells

To investigate the functional role of TUG1 in osteosarcoma cells, we first performed gene knockdown experiments by transfecting MG63 and U2-OS cells with si-NC or si-TUG1. The CKK-8 assay revealed that TUG1 knockdown led to a significant inhibition of viability in MG63 and U2-OS cells when compared with the si-NC group (Fig. 2A). To further confirm the effect of TUG1 inhibition on the proliferation of osteosarcoma cells, colony formation assay was performed. The results showed that the colony number of MG63 and U2-OS cells was dramatically lowered following inhibition of TUG1 expression (Fig. 2B). The effect of TUG1 deletion on the cell invasive capacity was then further tested. Transwell invasion assay confirmed that silencing TUG1 expression by siRNA resulted in a significant impairment in the invasive capacity of MG63 and U2-OS cells (Fig. 2C and D). Taken together, these data demonstrate that TUG1 knockdown contributes to the inhibition of proliferation and invasion in osteosarcoma cells.

TUG1 Functions as a Sponge of miR-153

Recently, increasing evidence suggests that lncRNAs could negatively control miRNA expression by acting as sponges. Therefore, the online software starBase v2.0 was used to predict the miRNA recognition sequence. The level of TUG1 was negatively correlated with miR-153 expression in osteosarcoma tissues (Fig. 3A). TUG1 contains one conserved cognate site of miR-153 (Fig. 3B). The above results prompted us to suppose that TUG1 regulated miR-153 through binding to the predicted sites. The MS2-TUG1 plasmids with wild-type or mutant miR-153 binding sites (MS2-TUG1-WT or MS2-TUG1-MUT) were first constructed, and an anti-MS2 RIP assay was performed to pull down endogenous miR-153. As expected, the TUG1 RIP in MG63 and U2-OS cells was significantly enriched for miR-153, compared to MS2 and MS2-TUG1-MUT (Fig. 3C). To further confirm the relationship between TUG1 and miR-153, luciferase reporter assay was performed by transfecting reporter plasmid (pmirGLO, pmirGLO-TUG1-WT, or pmirGLO-TUG1-MUT) and miR-153 or miR-NC into MG63 and U2-OS cells. The results demonstrated that miR-153 overexpression dramatically reduced the wild-type reporter activity in MG63 and U2-OS cells, but no significant suppression was observed when the reporter plasmid carried a mutant TUG1 (Fig. 3D). The actual regulating role of TUG1 in miR-153 expression was examined by qRT-PCR analysis. TUG1 knockdown upregulated miR-153 expression, while TUG1 overexpression downregulated the expression of miR-153 in both MG63 and U2-OS cells (Fig. 3E). Collectively, these data demonstrated that TUG1 directly inhibited miR-153 expression by acting as an miR-153 sponge.

TUG1 Overexpression Reversed miR-153-Mediated Inhibition of Proliferation and Invasion in Osteosarcoma Cells

To investigate whether the effect of TUG1 on the proliferation and invasion in osteosarcoma cells was mediated by miR-153, MG63 cells were transfected with miR-153 mimics or cotransfected with miR-153 mimics and pcDNA-TUG1. miR-153 upregulation led to a significant reduction in viability in MG63 cells, which was attenuated by TUG1 overexpression (Fig. 4A). miR-153-induced decrease in the colony numbers of MG63

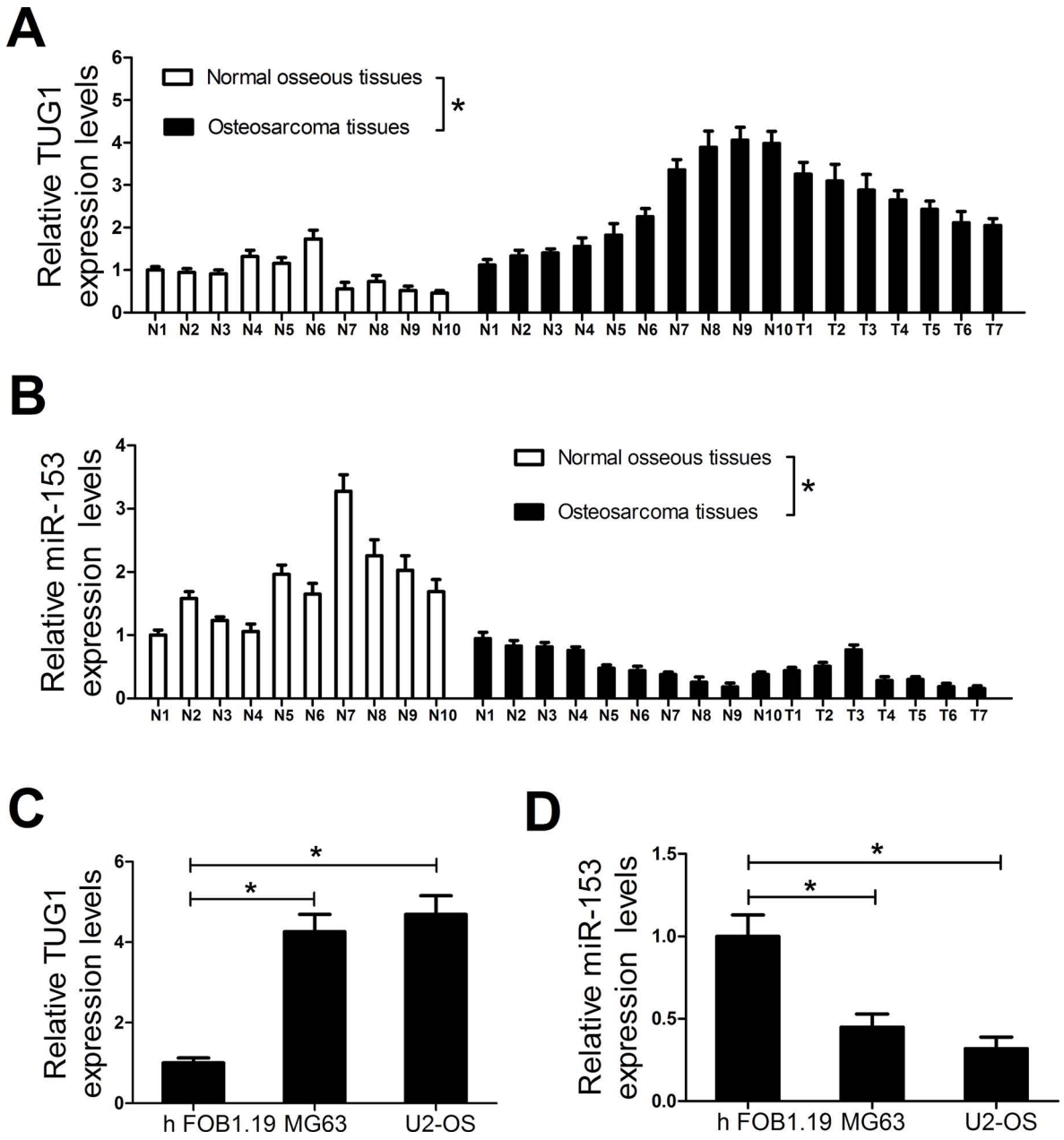


Figure 1. Taurine-upregulated gene 1 (TUG1) expression is increased and miR-153 expression is decreased in osteosarcoma. (A) The expression levels of TUG1 in 17 osteosarcoma tissues (2.55±0.95) and 10 normal osseous tissues (0.93±0.40) were determined using quantitative real-time (qRT)-PCR. (B) The expression levels of miR-153 in 17 osteosarcoma tissues (0.48±0.26) and 10 normal osseous tissues (1.77±0.67) were detected using qRT-PCR. (C, D) The TUG1 and miR-153 expression levels in osteosarcoma MG63 and U2-OS cell lines and in the normal osteoblastic cell line hFOB 1.19. Data are shown as mean±SD, $n=3$. *$p<0.05$.

cells was abolished by transfection of pcDNA-TUG1 (Fig. 4B). Similarly, the invasive ability of miR-153 mimic-treated cells was significantly suppressed, whereas cotransfection of miR-153 mimic and pcDNA-TUG1 reversed this inhibitory effect in MG63 cells (Fig. 4C). Taken together, these data illuminated that TUG1 promoted the tumorigenesis of osteosarcoma by regulating miR-153 in vitro.

TUG1 Knockdown Suppresses Osteosarcoma Growth In Vivo

To further confirm the carcinogenicity of TUG1 on osteosarcoma in vivo, a BALB/c nude mice xenograft model was established by injecting MG63 cells transfected with lentivirus vectors harboring sh-NC or sh-TUG1 into mice. Tumor growth in the sh-TUG1 group was significantly lower than that in the sh-NC control group

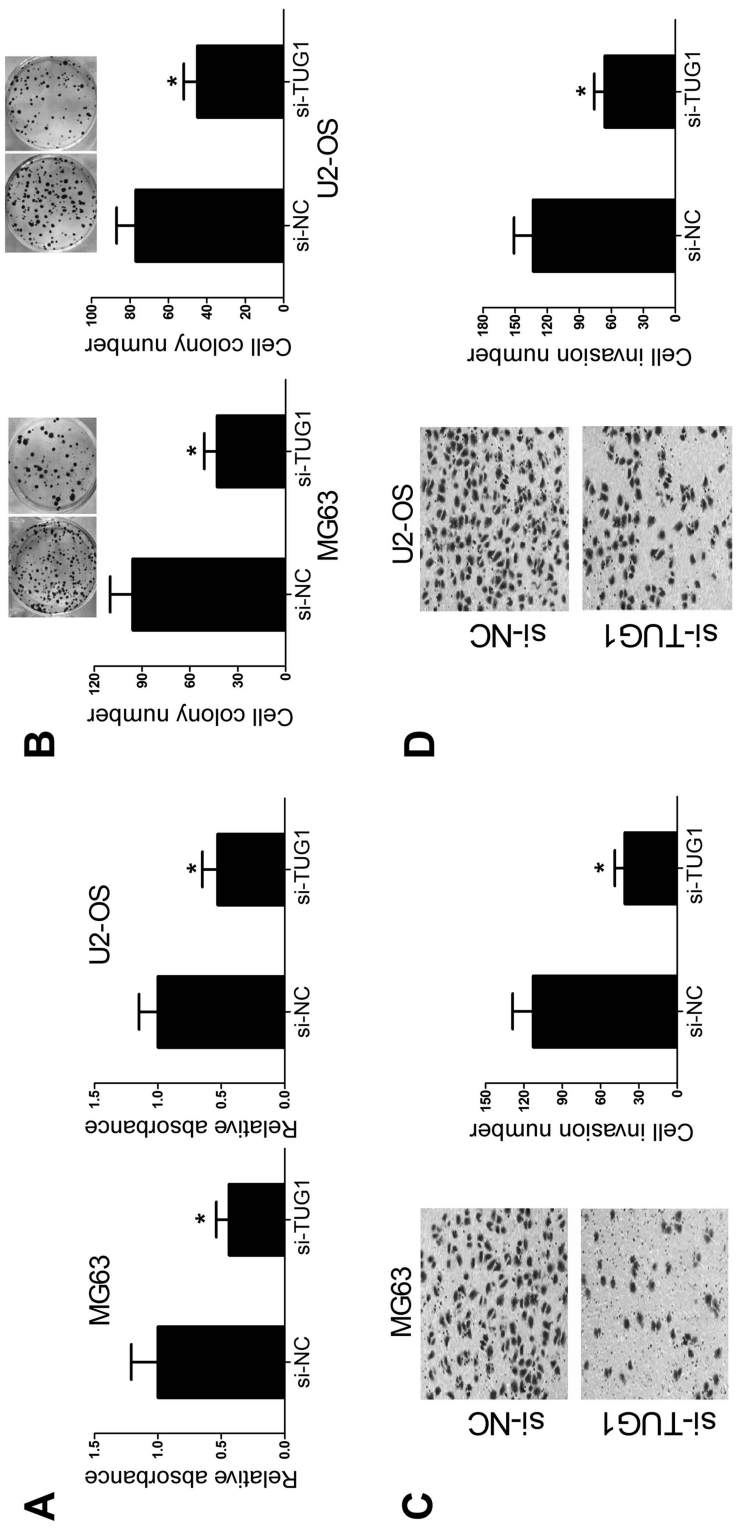
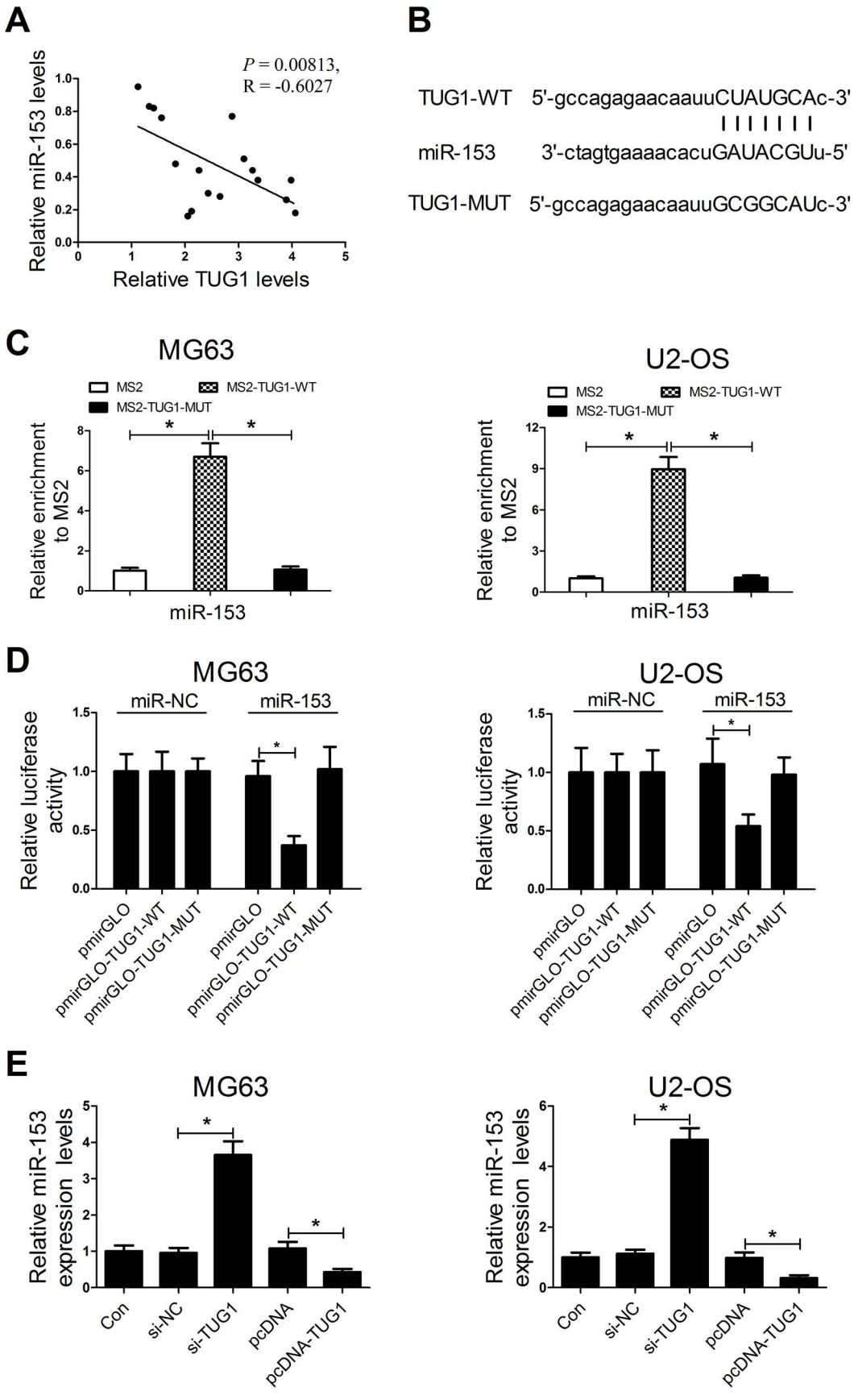


Figure 2. TUG1 knockdown inhibits proliferation and invasion in osteosarcoma cells. MG63 and U2-OS cells were transfected with si-NC or si-TUG1. (A) CCK-8 assays were performed to determine the viability of MG63 and U2-OS cells. (B) The cell colony numbers of MG63 and U2-OS cells were examined by colony formation assay. (C, D) The cell-invasive capacity of MG63 and U2-OS cells was determined by Transwell invasion assay. Data are shown as mean ± SD, $n = 3$. *$p < 0.05$ versus si-NC.


A
P = 0.00813,
R = -0.6027
Relative miR-153 levels
Relative TUG1 levels
B
TUG1-WT 5'-gccagagaacaauuCUAUGCAc-3'
miR-153 3'-ctagtgaaaacacuGAUACGUu-5'
TUG1-MUT 5'-gccagagaacaauuGCGGCAUc-3'
C
MG63
U2-OS
MS2
MS2-TUG1-WT
MS2-TUG1-MUT
Relative enrichment to MS2
miR-153
D
MG63
U2-OS
miR-NC
miR-153
Relative luciferase activity
pmirGLO
pmirGLO-TUG1-WT
pmirGLO-TUG1-MUT
E
MG63
U2-OS
Relative miR-153 expression levels
Con
si-NC
si-TUG1
pcDNA
pcDNA-TUG1

(Fig. 5A). Thirty days after inoculation, the weight of tumors developed from the sh-TUG1-transfected cells was lower than the control tumors (Fig. 5B). Moreover, miR-153 expression in isolated tumors was significantly upregulated by TUG1 deletion (Fig. 5C). These data demonstrated that TUG1 knockdown inhibited osteosarcoma growth in vivo.

DISCUSSION

Emerging evidence suggests that lncRNAs are involved in the carcinogenesis and progression of various malignant tumors, including osteosarcoma[23]. For instance, MALAT1, acting as an oncogene in osteosarcoma, was upregulated in osteosarcoma tissues and cell lines, and knockdown of MALAT1 inhibited cell proliferation, migration and invasion, and metastasis, and promoted cell cycle arrest and apoptosis in osteosarcoma[24,25]. A novel lncRNA tumor suppressor candidate 7 (TUSC7) was downregulated in osteosarcoma tissues and was closely associated with poor survival, and silencing of TUSC7 by si-TUSC7 contributed to the enhancement of cell proliferation ability and suppression of cell apoptosis in two osteosarcoma cell lines, HOS and MG63[26]. In this work, the underlying mechanism of TUG1 in the regulation of osteosarcoma progression was investigated.

TUG1 was initially identified to be upregulated in taurine-treated mouse retinal cells[13]. A handful of studies suggest that TUG1 acts as a tumor suppressor or oncogene in various cancers. For example, TUG1 was demonstrated to be upregulated in bladder tissues and cancer cell lines, and knockdown of TUG1 suppressed bladder cancer cell metastasis, radioresistance, and invasion by sponging miR-145 and derepressing ZEB2, a direct target of miR-145, through inducing the epithelial-to-mesenchymal transition[27]. In hepatocellular carcinoma (HCC), elevated TUG1 expression was positively associated with tumor size and stage, and TUG1 downregulation inhibited HCC proliferation and induced apoptosis by upregulating the tumor suppressor gene KLF2[19]. On the contrary, TUG1 was found to be downregulated and was correlated with a larger tumor size and poorer overall survival in NSCLC, and silencing TUG1 promoted NSCLC cell proliferation partly through epigenetically regulating HOXB7 expression[14]. Moreover, TUG1 has been confirmed to be upregulated in human osteosarcoma, and a high expression of TUG1 was involved in tumorigenesis of osteosarcoma[21,22]. However, the exact mechanism remains to be clarified. First, the functional role of TUG1 in osteosarcoma was further confirmed in the present study. TUG1 was found to be upregulated in osteosarcoma tissues and cell lines, which was consistent with previous studies[21,22]. In addition, a functional study revealed that knockdown of TUG1 suppressed proliferation and invasion, which was supported by recent studies[28,29]. Furthermore, our findings confirmed that TUG1 inhibition impeded tumor growth in vivo. All these studies, together with ours, demonstrate that TUG1 acts as an oncogene and promotes cancer progression in osteosarcoma.

As is well known, lncRNAs can act as molecular sponges and inhibit miRNA-mediated silencing of its target genes by binding to miRNAs[30,31]. Therefore, whether TUG1 functions as an miRNA sponge was further tested in this study. Through searching the online database starBase v2.0, a binding site for miR-153 in TUG1 was found. In addition, miR-153 expression was negatively correlated with the TUG1 level in osteosarcoma tissues. Thus, we hypothesized that TUG1 may function as an miR-153 sponge in osteosarcoma. In order to verify this hypothesis, RIP and luciferase reporter assays were performed. As expected, miR-153 could bind to TUG1. Moreover, TUG1 could suppress endogenous miR-153 expression. These results confirmed the reciprocal repression between TUG1 and miR-153. Furthermore, TUG1 overexpression could reverse the miR-153-mediated inhibition of proliferation and invasion in osteosarcoma, suggesting that TUG1 exerted its oncogenic role in osteosarcoma by sponging miR-153. Consistent with our findings, Xie et al.[28] confirmed that TUG1 acts as a sponge to directly bind to miR-9-5p and suppress miR-9-5p expression, leading to the inhibition of cell proliferation and colony formation and the induction of apoptosis in osteosarcoma. A recent study revealed that TUG1 promoted migration and invasion by functioning as an miR-335-5p sponge in osteosarcoma cells[29]. These studies demonstrate that TUG1 plays an oncogenic role in osteosarcoma by functioning as miRNA sponges, which may provide a novel therapy target for patients with osteosarcoma.

In summary, this study demonstrated that TUG1 knockdown suppresses cancer progression in vitro and inhibits its tumor growth in vivo in osteosarcoma. Mechanically, TUG1 acts as an oncogene in osteosarcoma progression by sponging miR-153. Our findings provide a further

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Figure 3. TUG1 functions as an miR-153 sponge. (A) Negative correlation between TUG1 and miR-153 exists in osteosarcoma tissues. (B) The putative binding sites of miR-153 on TUG1. (C) MS2 RIP followed by qRT-PCR to detect miR-153 endogenously associated with TUG1. (D) Luciferase activity was measured in MG63 and U2-OS cells cotransfected with miR-153 or miR-NC and luciferase reporters containing pmirGLO, pmirGLO-TUG1-WT, or pmirGLO-TUG1-MUT. (E) qRT-PCR analysis was performed to detect the expression of miR-153 in MG63 and U2-OS cells with si-TUG1 or pcDNA-TUG1. Data are shown as mean±SD, $n=3$. $^*p<0.05$ versus controls.

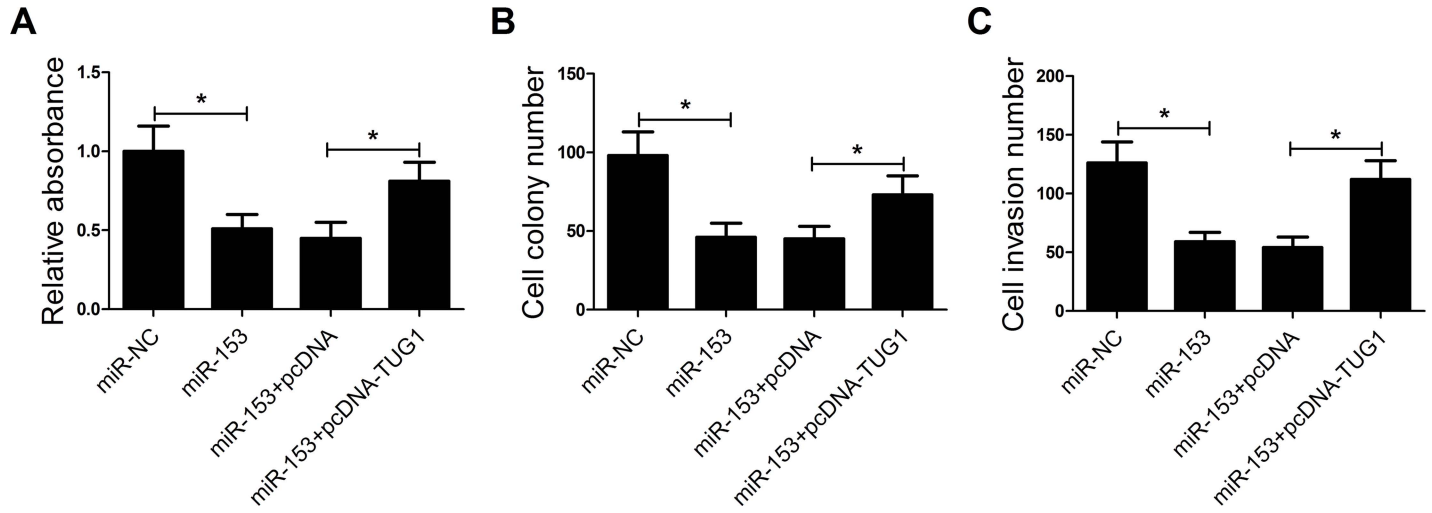

Figure 4. Exogenous expression of TUG1 reversed the inhibitory effect of miR-153 overexpression on the proliferation and invasion in osteosarcoma cells. MG63 cells were transfected with either miR-153 or in combination with pcDNA-TUG1. (A) The viability of MG63 cells was determined by CKK-8 assay. (B) Colony formation assay was performed to detect the colony numbers of MG63 cells. (C) The invasive ability of MG63 cells was determined by Transwell invasion assay. Data are shown as mean±SD, $n=3$. *$p<0.05$ versus controls.

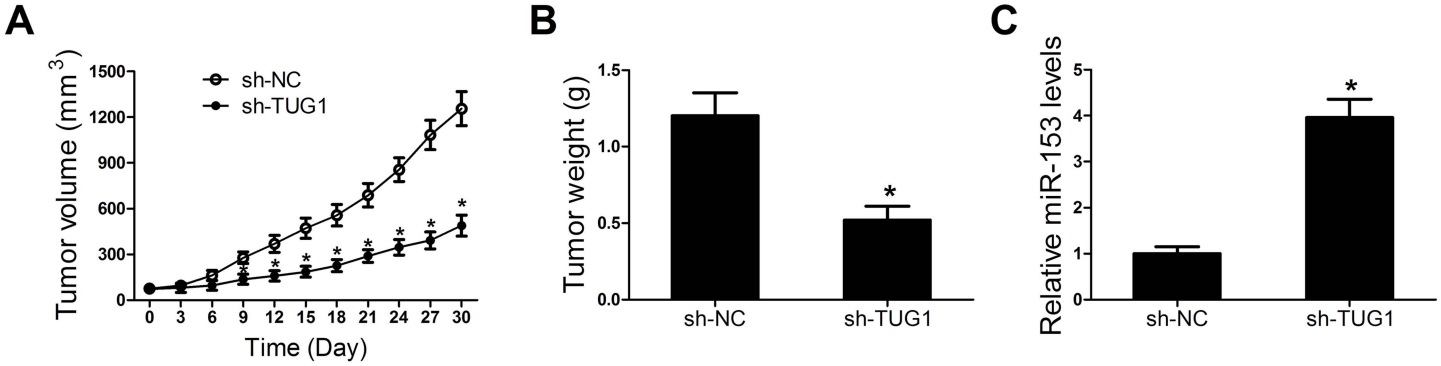

Figure 5. TUG1 knockdown suppresses tumor growth in osteosarcoma. MG63 cells transfected with lentivirus vectors carrying sh-NC or sh-TUG1 were subcutaneously injected into the mice. (A) Tumor volume was measured every 3 days from 0 to 30 days. (B) Tumor weight was measured 30 days after inoculation. (C) The miR-153 expression level in tissues of resected tumors. Data are shown as mean±SD, $n=3$. *$p<0.05$ versus sh-NC.

theoretical foundation for lncRNA as an miRNA sponge and indicates that targeting the TUG1/miR-153 interaction may be a promising therapeutic strategy for patients with osteosarcoma.

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