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miR-214 promotes the proliferation and invasion of osteosarcoma cells through direct suppression of LZTS1

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ABSTRACT

Previous studies have shown that miR-214 functions either as an oncogene or a tumor suppressor in various human cancer types. The role of this microRNA in osteosarcoma (OS) is presently unclear. Here, we demonstrated that miR-214 is frequently upregulated in OS specimens, compared with noncancerous bone tissues. Bioinformatics analysis further revealed leucine zipper, putative tumor suppressor 1 (LZTS1) as a potential target of miR-214. Expression patterns of miR-214 were inversely correlated with those of LZTS1 mRNA and protein in OS tissues. Data from reporter assays showed that miR-214 directly binds to the 3′-untranslated region (3′-UTR) of LZTS1 mRNA and suppresses expression at both transcriptional and translational levels. In functional assays, miR-214 promoted OS cell proliferation, invasion and tumor growth in nude mice, which could be reversed by overexpression of LZTS1. Taken together, our data provide compelling evidence that miR-214 functions as an onco-miRNA in OS, and its oncogenic effects are mediated chiefly through downregulation of LZTS1.

1. Introduction

Osteosarcoma (OS) is the most common primary sarcoma of bone in adolescents and young adults [1]. The incidence of OS in the general population is three cases per million per year, with peak occurrence in the 15–19-year age group, in which 8–11 cases/million/year are recorded [2]. Despite extensive advancements in diagnostic methods and surgical techniques in recent years, the 5-year survival rate of osteosarcoma patients remains at 60–70% [3]. Thus, improved understanding of the mechanisms underlying osteosarcoma development and progression is urgently required to optimize therapeutic options.

MicroRNAs (miRNAs) are an emerging class of small, non-coding, single-stranded RNAs that serve as important regulators of gene expression by binding to the 3′ untranslated region (UTR) of target mRNAs, thereby leading to their translational repression and/or degradation [4,5]. miRNAs regulate a variety of biological processes, including cell proliferation, differentiation, migration, metabolism and apoptosis [6]. In cancers, homeostatic expression of miRNAs is disrupted, resulting in aberrant gene expression in tumor initiation, development and metastasis. Multiple miRNAs have been implicated in carcinogenesis and tumor progression in OS. For example, miR-143 is reported to function as a tumor suppressor by downregulating Bcl-2 and MMP-13 [7,8]. miR-199b-5p, miR-183, miR-34a, miR-340 and miR-16 exert tumor suppressor effects in osteosarcomagenesis through suppression of the Notch pathway [9], the oncogenes Ezrin [10], c-Met [11], ROCK1 [12], and Raf1-MEK1/2-ERK1/2 signaling [13] in OS cells, respectively. A recent miRNA microarray analysis by Jones et al. [1] revealed higher miR-214 expression in OS tissues, compared with normal bone tissues. However, the precise role of miR-214 in OS cells remains unclear at present.

In this study, we demonstrated that miR-214 is upregulated in OS and inversely correlated with LZTS1 levels. Our collective findings suggest that miR-214 directly targets 3′-UTR of the LZTS1 transcript and suppresses its expression, eventually promoting OS cell proliferation, invasion and tumor growth in nude mice.

2. Materials and methods

2.1. Clinical specimens

Eight OS tissues and adjacent normal bone tissues were obtained from OS patients at Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China). The study was approved by the ethics committee of Shanghai Municipality. Written informed consent was obtained from all patients.

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2.2. Cell culture

Human OS cell lines, Saos-2 and U2OS, were obtained from Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/ml of penicillin–streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.3. RNA preparation and quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). qRT-PCR for miR-214 detection was performed using 10 ng total RNA and the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. For LZTS1 mRNA detection, 1 μg of DNAse-treated RNA was retrotranscribed with the High Capacity cDNA Reverse Transcription Kit, and qRT-PCR carried out using gene-specific primers on a 7900HT Fast Real Time PCR System. Quantitative normalization was performed on the expression of RNU6 small nucleolar RNA or GAPDH genes for miR-214 and LZTS1 mRNA, respectively. Relative expression levels between samples were calculated using the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$), with a control sample as the reference point.

2.4. Plasmid construction

For ectopic expression of LZTS1, LZTS1 ORFs with and without 3′-UTR were amplified using PCR and subcloned into pEGFP-N3 (Invitrogen). To construct a luciferase reporter vector, the LZTS1 3′-UTR fragment containing putative binding sites for miR-214 was amplified using PCR and cloned downstream of the luciferase gene in the pGL3-luciferase reporter plasmid (Promega, Madison, WI), designated LZTS1-3′UTR-WT. The corresponding mutant constructs were created by substituting the seed region of the miR-214-binding site (designated LZTS1-3′UTR-MUT). Constructs were verified by sequencing.

2.5. Oligonucleotide and plasmid transfection and lentivirus infection

Cells were seeded into six-well plates and transfected with miR-214 mimics, anti-miR-214 inhibitor or controls using Lipofectamine RNAi MAX (Invitrogen) and pEGFP-LZTS1 or pEGFP-LZTS1-3′UTR plasmid using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested for assay 48 h after transfection. Stable miR-214-overexpressing cell lines were generated via lentiviral infection. The human miR-214 gene was amplified from genomic DNA and cloned into pLemiR-turbo Red Fluorescent Protein (tRFP) vector (Open Biosystems, Huntsville, AL), as described by Penna et al. [14]. Lentiviruses were produced via calcium phosphate transfection of 20 μg vector plasmid together with 15 μg packaging (pCMVdR8.74) and 6 μg envelope (pMD2.G-VSVG) plasmids into 293T cells. Supernatant fractions were harvested at 48 h post-transfection.

2.6. Cell proliferation and invasion assays

Cells were seeded at a density of 2000 cells per well in 96-well plates and cultured for 1–5 days after transfection. Next, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT;

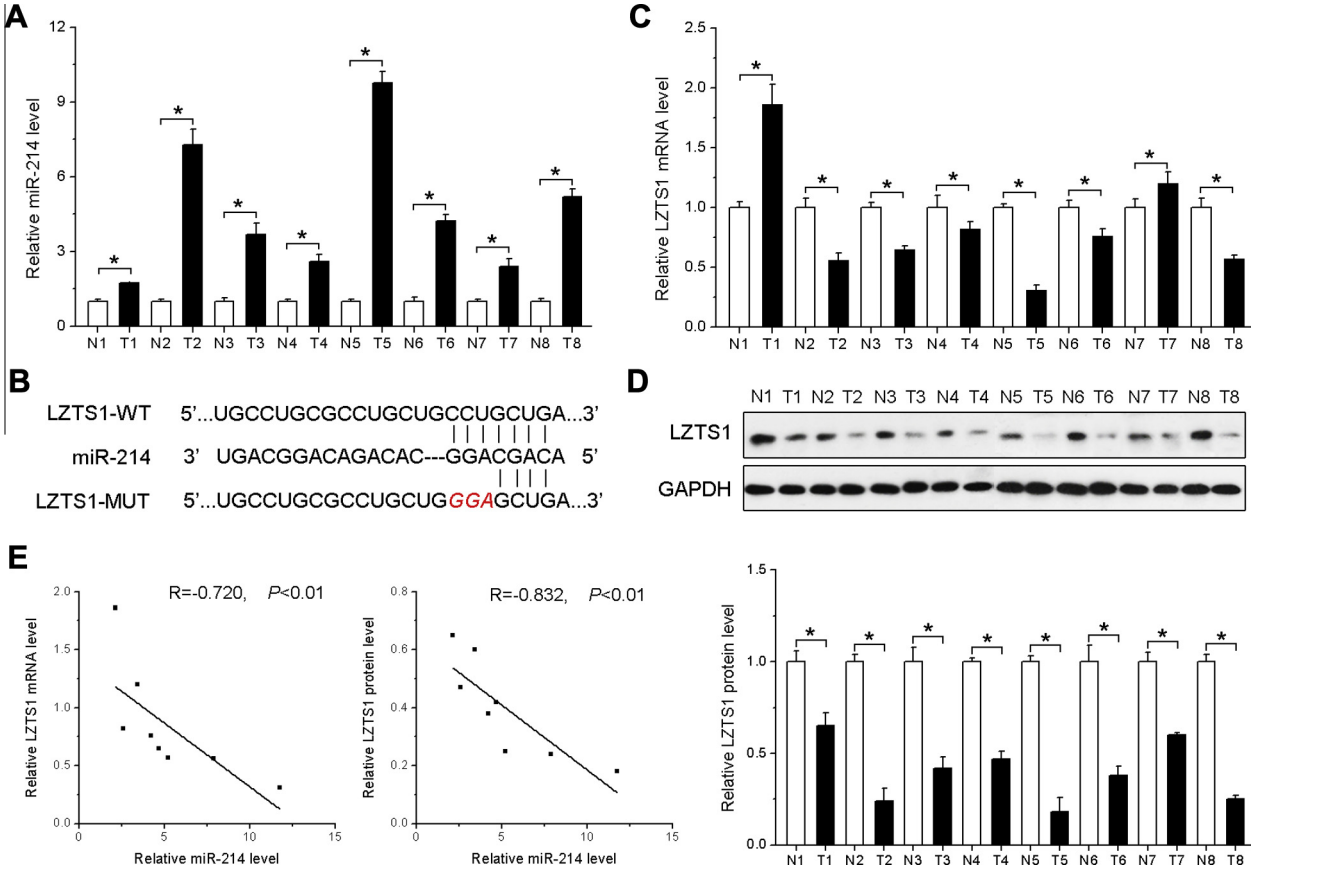

Fig. 1. miR-214 is upregulated and inversely correlated with LZTS1 levels in OS tissues. (A) Relative miR-214 expression was determined in eight human OS and adjacent normal bone tissues using qRT-PCR. (B) The putative wild-type and mutant binding sites of miR-214 in LZTS1 3′-UTR are presented. (C and D) LZTS1 mRNA and protein levels in OS and adjacent normal bone tissues were detected using qRT-PCR and Western blot, respectively. (E) Pearson's correlation analyses between relative miR-214 expression and LZTS1 mRNA and protein levels in eight human OS tissues.

Sigma, St. Louis, MO) reagent (5 mg/ml) was added, followed by incubation for 4 h. Supernatant fractions were discarded and 150 μl/well of DMSO added to terminate the reaction. Absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA). For the cell invasion assay, infected or transfected cells were harvested and resuspended in serum and calcium-free DMEM. Aliquots of cells (3×10^4) were placed in chambers coated with 150 mg Matrigel (BD Biosciences, Bedford, MD), which were fitted into 24-well plates and incubated for 24 h in DMEM with 10% fetal bovine serum. Cells remaining on the upper surface of the membranes were removed, whereas those adhering to the lower surface were fixed with ice-cold methanol and stained with 1% crystal violet. Stained cells were visualized and counted under a microscope. Results were averaged from three independent experiments.

2.7. Western blot analysis

Total proteins were extracted from tissues or cells using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5) plus 1% complete protease inhibitor. Lysates were clarified via centrifugation at 13,000 rpm for 5 min at 4 °C, and the supernatant fractionated using SDS–PAGE and subsequently transferred onto nitrocellulose membrane. Proteins were detected with anti-LZTS1 and anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were exposed to LAS 3000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.8. Luciferase reporter assay

Luciferase reporter assays were performed as described previously [15]. Saos-2 and U2OS cells were plated at a density of 4000 cells per well in a 96-well plate. At 24 h after plating, cells were transfected with 30 nM miR-214 mimics or anti-miR-214, 10 ng pGL3 and 500 ng LZTS1-3′UTR-WT or LZTS1-3′UTR-MUT vectors per well using Lipofectamine 2000, according to the manufacturer's protocol. Relative luciferase activity was calculated after 48 h by normalizing Firefly luminescence to that of Renilla using the Dual-Luciferase Reporter Assay (Promega).

2.9. In vivo models

Logarithmically growing Saos-2 cells transduced with lentiviral constructs carrying either miR-214 or vector control were harvested and resuspended in phosphate buffered solution, and aliquots of 5×10^6 cells inoculated subcutaneously into BALB/c nude mice. After 30 days, mice were sacrificed, followed by surgical excision of tumors. Tumors were weighed and their volumes calculated according to the formula: Tumor volume (mm^3) = ($A \times B^2$)/2, where A represents the longest axis (mm) and B the shortest axis (mm).

2.10. Statistical analysis

All data are expressed as means ± standard deviation from three independent experiments. Statistical analyses were performed

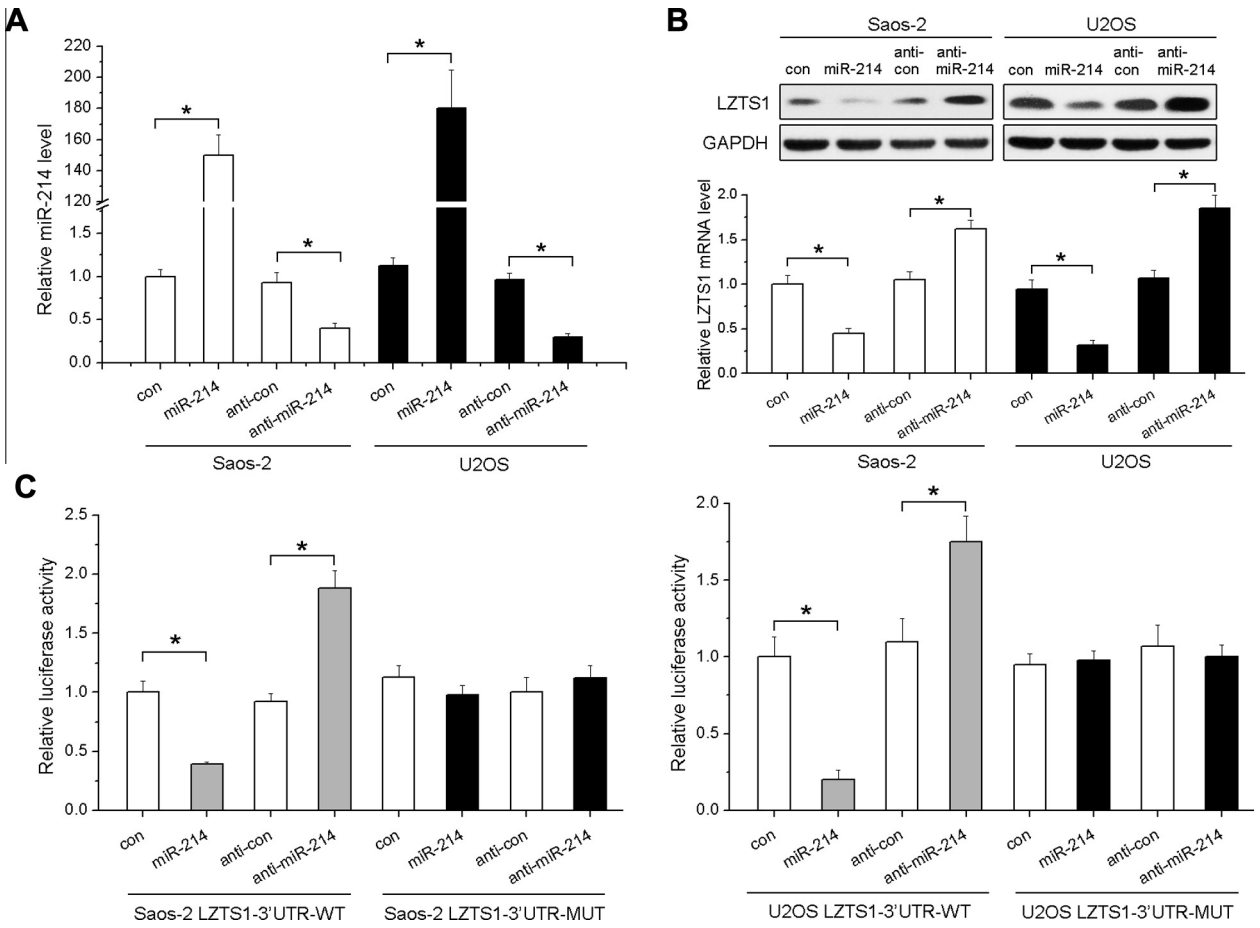

Fig. 2. miR-214 targets LZTS1 by binding to its 3′-UTR. (A) qRT-PCR analysis of miR-214 levels in OS cell lines, Saos-2 and U2OS, transfected with miR-214 mimics or anti-miR-214. (B) LZTS1 protein and mRNA levels were quantified using western blot and qRT-PCR, respectively. (C) Luciferase reporter assays in Saos-2 and U2OS cells co-transfected with miR-214 and a luciferase reporter containing full-length wild-type LZTS1 3′-UTR or a mutant with substitutions in the first three nucleotides of the miR-214 binding site.

using SPSS16.0 software (SPSS, Chicago, IL). The differences between groups were analyzed using Student's *t*-test with only two groups or one-way analysis of variance (ANOVA) when more than two groups were compared. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. miR-214 is upregulated in human OS tissues and inversely correlated with LZTS1 expression

To explore the potential role of miR-214 in OS development, its expression was evaluated in eight pairs of human OS and adjacent normal bone tissues using qRT-PCR. As shown in Fig. 1A, miR-214 expression was significantly higher in tumors than normal bone tissues. Potential targets of miR-214 were predicted using bioinformatics methods. LZTS1, a tumor suppressor gene containing a binding site for miR-214, was selected as the target for further analysis (Fig. 1B). qRT-PCR analysis of the above tissues showed that LZTS1 mRNA expression in six examined OS tissues was significantly decreased in comparison with that in paired adjacent normal bone tissues (Fig. 1C). Consistently, lower expression of LZTS1 protein was also found in tumor tissues as indicated by western blot (Fig. 1D). Furthermore, miR-214 levels were inversely correlated with those of LZTS1 mRNA and protein (Fig. 1E). In view of these findings, we propose that LZTS1 is a direct target of miR-214.

3.2. miR-214 directly targets LZTS1 by binding to its 3′-UTR

To determine whether LZTS1 is regulated by miR-214, we transfected the OS cell lines, Saos-2 and U2OS, with miR-214 mimics, anti-miR-214 or the respective controls. Relative miR-214 expression was verified using qRT-PCR (Fig. 2A), and LZTS1 protein and mRNA levels evaluated to determine the influence of specific miRNAs. Western blot and qRT-PCR findings showed that miR-214 mimics significantly inhibit LZTS1 protein and mRNA levels in Saos-2 and U2OS cells, respectively (Fig. 2B), while anti-miR-214 clearly promoted LZTS1 protein and mRNA expression. To verify

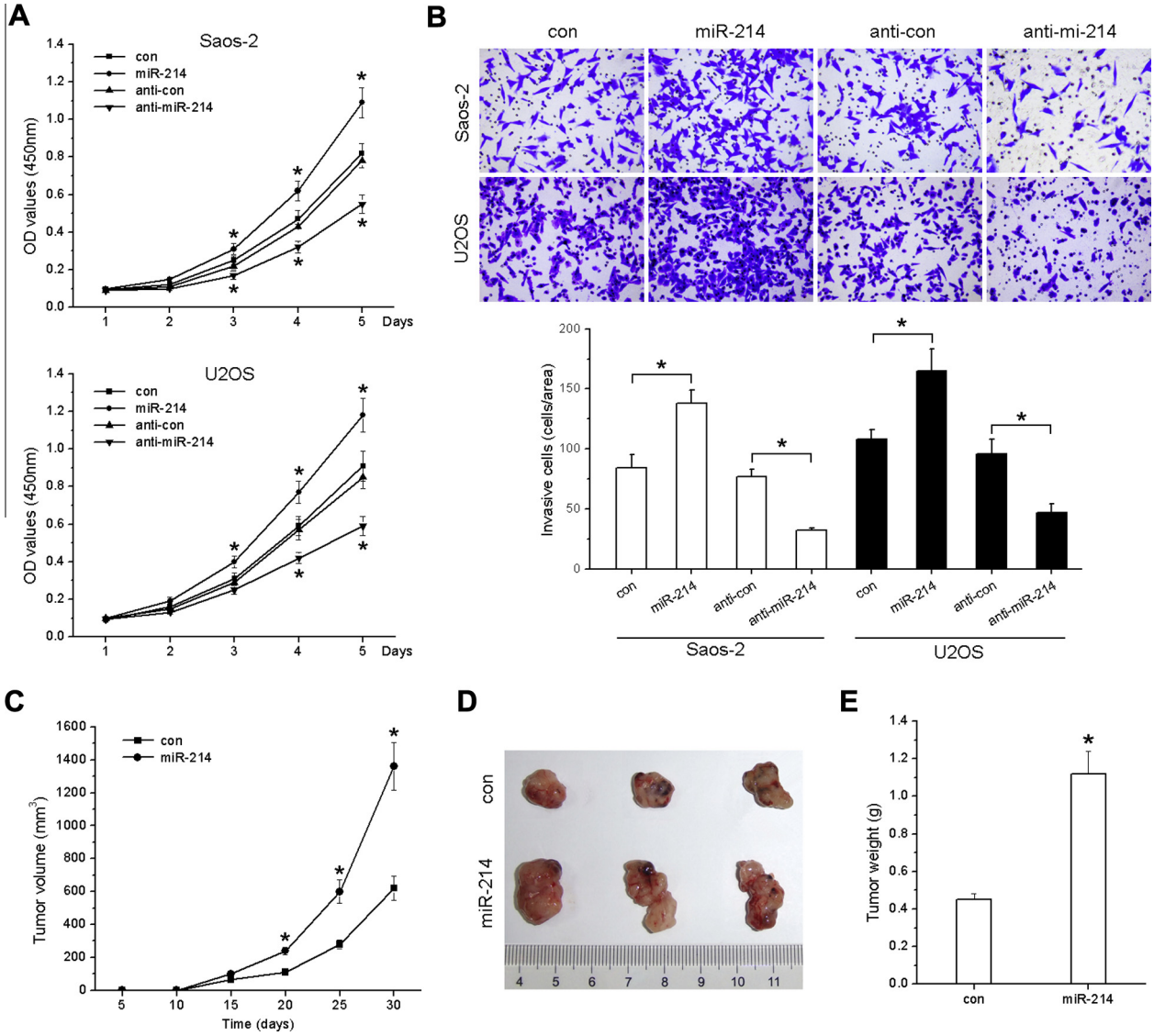

Fig. 3. miR-214 promotes OS cell proliferation, invasion and tumor growth. (A) MTT assays of Saos-2 and U2OS cells transfected with miR-214 mimic, anti-miR-214 or the corresponding controls. (B) Transwell invasion assays of Saos-2 and U2OS cells transfected with miR-214 mimic, anti-miR-214 or the corresponding controls. (C) miR-214 promotes tumor growth in nude mice. Saos-2 cells stably transduced with lentiviral constructs carrying either miR-214 or vector control were subcutaneously injected into nude mice ($n = 6$). Tumor volumes were measured on the indicated days. At the experimental endpoint, tumors were dissected, photographed and weighed (Fig. 4D and E).

whether LZTS1 is a direct target of miR-214, LZTS1 3′-UTR wild-type or mutant constructs were cotransfected with miR-214 or anti-miR-214 into Saos-2 and U2OS cells, followed by measurement of luciferase activity. As shown in Fig. 2C, overexpression of miR-214 in both OS cell lines led to significantly reduced luciferase activity for wild-type LZTS1, whereas knockdown of miR-214 increased wild-type LZTS1 luciferase activity. The effects of miR-214 mimics and anti-miR-214 on luciferase activities were completely deprived upon introduction of the 3-nucleotide mutations in LZTS1 3′-UTR, supporting its identification as a true miR-214 target site. Our data strongly suggest that miR-214 negatively regulates LZTS1 expression via direct binding to putative binding sites in the 3′-UTR region.

3.3. miR-214 promotes OS cell proliferation, invasion and tumor growth

We assessed the functional role of miR-214 in OS cells by determining the effects of miRNA overexpression and inhibition on cell proliferation and invasion using MTT and Transwell assays, respectively. Overexpression of miR-214 significantly promoted the growth of Saos-2 and U2OS cells, compared with negative control-transfected cells, whereas knockdown of miR-214 reduced the cell growth rate, as shown in Fig. 3A. Similarly, transwell assays showed that miR-214 markedly promotes invasion of OS cells while anti-miR-214 inhibits this activity (Fig. 3B). To further determine whether miR-214 promotes OS tumor growth *in vivo*, Saos-2 cells engineered to stably overexpress miR-214 were subcutaneously inoculated into nude mice and tumor growth was measured. As shown in Fig. 3C, miR-214-expressing tumors showed higher volume and larger size, compared to control tumors. The average weight of miR-214-overexpressing tumors was >2-fold higher than that of the controls (Fig. 3D). These data collectively indicate that miR-214 promotes the proliferation and invasion of OS cells.

3.4. Overexpression of LZTS1 reverses miR-214-induced proliferation and invasion of OS cells

To determine whether the oncogenic effects of miR-214 are mediated by LZTS1, we transfected Saos-2 and U2OS cells stably overexpressing miR-214 with LZTS1 constructs without (LZTS1) or with 3′-UTR (LZTS1-3′UTR), and measured LZTS1 expression using western blot (Fig. 4A). LZTS1 overexpression significantly abolished miR-214-induced proliferation and invasion of Saos-2 and U2OS cells (Fig. 4B and C). However, overexpression of LZTS1-3′UTR had no significant impact on proliferation and invasion of these cells (Fig. 4B and C). These results demonstrate that miR-214 promotes the proliferation and invasion of OS cells via suppression of LZTS1 expression.

4. Discussion

Emerging data have shown that miR-214 is downregulated and functions as a potential tumor suppressor in several human cancer types, including cervical cancer, intrahepatic cholangiocarcinoma, hepatoma and colorectal cancer [16–19]. Conversely, miR-214 is upregulated, and displays oncogenic properties in ovarian cancer, nasopharyngeal carcinoma and gastric cancer [20–22]. These dual effects of miR-214 may be attributable to organ-specific actions and different cellular contexts of tumors. No information is currently available on the specific role or molecular mechanism of miR-214 in OS. In this study, we initially examined miR-214

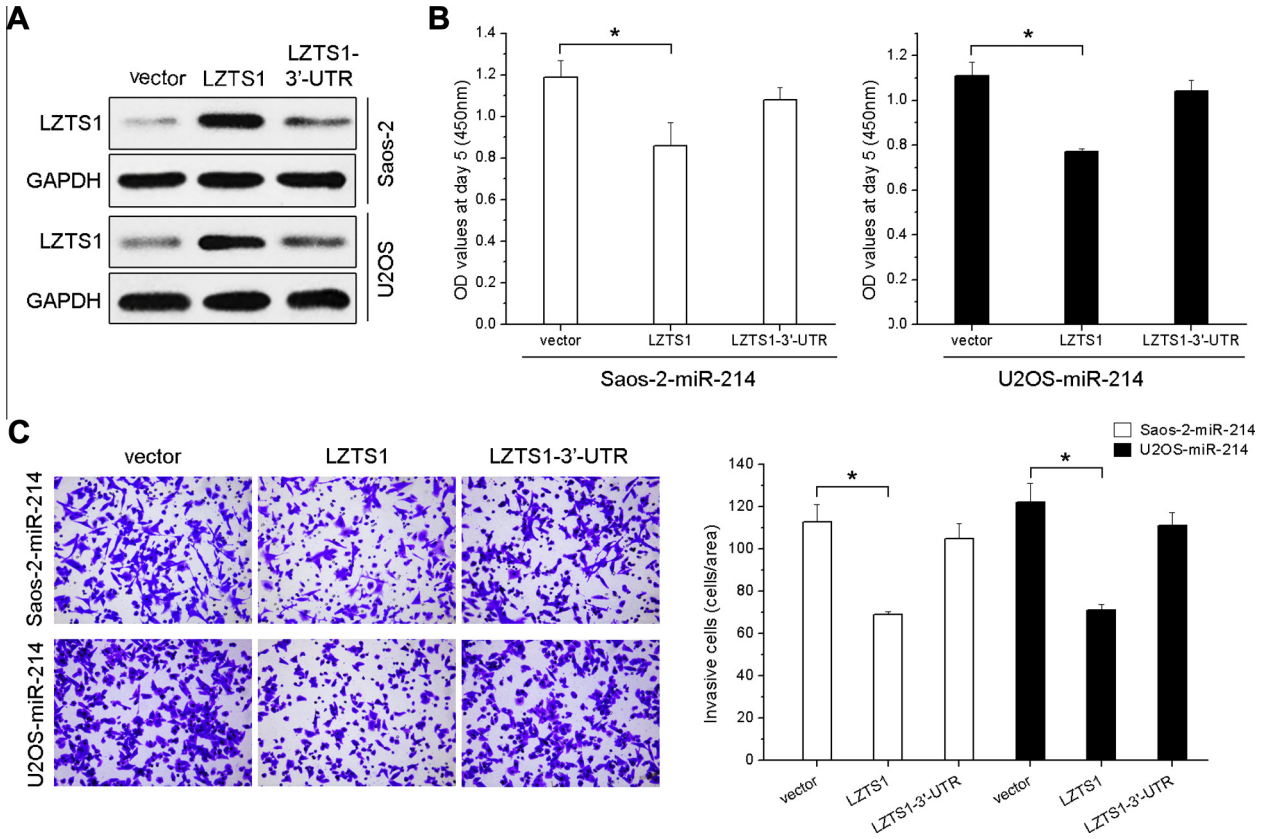

Fig. 4. Overexpression of LZTS1 suppresses miR-124-induced proliferation and invasion of OS cells. (A) Western blot analysis of LZTS1 in miR-214-overepressing Saos-2 and U2OS cells transfected with vector control, LZTS1 (without 3′-UTR) and LZTS1-3′UTR (with 3′-UTR). (B) MTT assays. (C) Transwell invasion assays.

expression in normal as well as tumor tissues from OS patients using qRT-PCR. Consistent with data from previous microarray analyses [1], miR-214 was dramatically upregulated in OS, suggesting its involvement in carcinogenesis. Our data are similar to the finding of Wang et al. [23] that miR-214 expression is linked to tumor progression and adverse prognosis in pediatric osteosarcoma.

LZTS1 was subsequently identified a candidate target of miR-214 using the Targetscan algorithm. LZTS1, previously designated FEZ1, is a tumor suppressor gene located at chromosome 8p22 [24], which is frequently downregulated or absent in human, bladder and breast cancers [25–27]. LZTS1-deficient mice have been shown to be more susceptible to spontaneous and carcinogen-induced cancers [28,29]. In the current study, we detected lower levels of LZTS1 in OS tissues, compared with normal bone tissues, which were inversely correlated with miR-214 expression patterns. Moreover, we demonstrated direct binding of miR-214 to the 3′-UTR of LZTS1 mRNA and showed that miR-214 overexpression suppresses, while its knockdown increases LZTS1 expression in OS cells.

Given that LZTS1 plays key roles in regulation of cell proliferation and invasion [30,31], we further investigated the effect of miR-214 on these phenotypes of OS cells. Notably, overexpression of miR-214 significantly enhanced *in vitro* cell proliferation and invasion and promoted *in vivo* tumor growth. Conversely, knockdown of miR-214 inhibited proliferation and invasion. Furthermore, restoration of LZTS1 expression abrogated miR-214-induced OS cell proliferation and invasion. Our results establish a functional association between miR-214 and LZTS1, and confirm that miR-214 acts as an onco-miRNA in OS cells by targeting LZTS1.

In conclusion, we demonstrated for the first time that upregulation of miR-214 is a common event underlying OS. Furthermore, miR-214 functions as an oncogene by directly targeting LZTS1. These findings collectively implicate miR-214 as a promising prognostic and therapeutic target for future OS therapy.

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