



# LINC01140 regulates osteosarcoma proliferation and invasion by targeting the miR-139-5p/HOXA9 axis

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## ABSTRACT

Osteosarcoma is one of the commonest metastatic tumor in children and teenagers, and has a hopeless, prognosis. Long non-coding RNA (lncRNA) acts momentous roles as a regulator on the proliferation and migration of cancer. Here, we performed GEO database analysis and qPCR to identify differentially expressed lncRNAs in osteosarcoma cells. Knockdown of lncRNA LINC01140 was used to detect the effect of LINC01140 on the proliferation, invasion, and epithelial-mesenchymal transition (EMT) of osteosarcoma cells. Bioinformatics analysis and qPCR identified the LINC01140/miR-139-5p/Homeobox A9 (HOXA9) regulatory axis. RNA immunoprecipitation assay, Dual-luciferase assay, and rescue experiments confirmed the interaction of LINC01140/miR-139-5p/HOXA9 in osteosarcoma. LINC01140 was overexpressed in osteosarcoma and knocking down LINC01140 restrained the proliferation and invasion of osteosarcoma cells and EMT. In Saos2 and MG63 cells, LINC01140 sponged miR-139-5p, and a miR-139-5p inhibitor overturned the suppression of LINC01140 knockdown on the proliferation and migration of osteosarcoma cells. Moreover, miR-139-5p depressed the invasion, proliferation, and EMT of osteosarcoma cells via targeting HOXA9. Our results indicate that LINC01140 downregulation inhibits the invasion, proliferation, and EMT in osteosarcoma cells through targeting the miR-139-5p/HOXA9 axis. Therefore, LINC01140 is a potential therapeutic target for osteosarcoma.

## 1. Introduction

Osteosarcomas, one of the commonest primary malignant bone tumors that most commonly occur in children and teenagers are prone to invasion and distant metastasis [1]. The five-year survival rate of metastatic patients is extremely low [2]. Although in the past few decades, chemotherapy, surgery, radiotherapy, and targeted drugs have been used to remedy OS, the survival rate of metastatic patients has not ameliorated substantially [3]. Consequently, it is imperative to explore the molecular mechanism of OS growth and metastasis and identify novel therapeutic targets.

LncRNA is a subset of RNA that is not less than 200 nucleotides in length and does not encode protein [4]. LncRNAs can be used as a new type of potential tumor marker [5]. For example, MALAT1 and UCA1 can be used to detect early and metastatic lung cancer [6]. The expression of H19 and HOTAIR can be used as biomarkers for the

detection of bladder cancer [7]. In addition, the differential expression of lncRNAs can promote or inhibit tumor growth and metastasis. Downregulating the expression of HOTAIR can reduce the occurrence and metastasis of breast cancer [8]. In summary, lncRNA-targeting cancer therapy is a promising field.

There are many mechanisms of action of lncRNA. In the cytoplasm, lncRNAs are mainly used as an endogenous competitive RNAs (ceRNAs) to sponge microRNAs and regulate the expression of messenger RNAs (mRNAs) [9]. TUG1 enhances the proliferation of OS cells via miR-132-5p/SOX4 axis [10]. LINC01140 can inhibit the proliferation and invasion of bladder cancer via by regulating miR-140-5p to down-regulate FGF9 expression. Moreover, LINC01140 is involved in poor prognosis of gastric cancer [11], nonetheless the function of LINC01140 in OS has not been reported.

To this end, here we studied the function of LINC01140 in osteosarcoma cells. Studies have found that inhibiting the expression of

**Abbreviations:** GEO, Gene Expression Omnibus; EMT, epithelial to mesenchymal transition; OS, osteosarcoma; RT-qPCR, real-time quantitative polymerase chain reaction; HOXA9, Homeobox A9; FC, Foldchange; OD, optical density; WT, wild type; MUT, mutant; miRNA, miR, microRNA; ov, overexpression; NC, Negative control.

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**Table 1**

The sequence of siRNAs targeting LINC01140 and miR-139-5p mimics/inhibitor.

Name	Sequence 5'-3'
siRNA-1	CCAACUAAUUCAAUGCAAGU
siRNA-2	GUAUGUGUCUAAUAAUUAUA
siRNA-3	GAUCUAAUUUGUAAUUAUU
si-con	UUCUCCGAACGUGUCACGUTT
miR-139-5p mimics	UCUACAGUGCACGUGUCUCCAGU
mimics nc	UGGCACUACGCUUCUCUGGACAU
miR-139-5p inhibitor	ACUGGAGACACGUGCACUGUAGA
inhibitor nc	CCAGCCUAGUAGGAGAUAGCGA

LINC01140 can effectively reduce the proliferation, invasion, and EMT of OS cells. Here, we also identified that LINC01140 regulates the expression of HOXA9 by combining with miR-139-5p to modulate the proliferation, invasion, and EMT of osteosarcoma. Therefore, LINC01140 is a potential therapeutic target for osteosarcoma.

## 2. Materials and methods

**Cell culture and transfection.** OS cell lines Saos2, MG63, normal osteoblasts cell line hFOB1.19 and 293 cell line were obtained from the American type culture collection (ATCC). These cells cultured in RPMI-1640 or DMEM (Gibco, Thermo) including 10% fetal bovine serum (FBS; Hyclone) at 37 °C in 5% CO<sub>2</sub> supplemented incubator. RNA/DNA was transfected into the Saos2 and MG63 cells with Lipofectamine®2000 (Invitrogen). For LINC01140 knockdown, 20 nM of siRNA against LINC01140 (si-LINC01140, synbio-tech) or non-targeting siRNA (si-con, synbio-tech) were transfected into cells. For upregulating LINC01140 expression, 50 ng of LINC01140 (pcDNA3.1) overexpression plasmids were transfected into cells, with empty vector (synbio-tech, China). For

HOXA9 overexpression, HOXA9 was subcloned into pcDNA3.1 plasmid, and 50 ng of pcDNA3.1-HOXA9 was transfected into cells. Overexpression and inhibition of miR-139-5p were achieved by transfecting 20 nM of miR-139-5p mimic (shbio, China) or miR-139-5p inhibitor (shbio) into the Saos2 and MG63 cells, and a corresponding scrambled oligonucleotide sequence (miR-con mimic or inhibitor nc, shbio) was used as the negative control. The DNA/RNA sequences used in these experiments are listed in Table 1.

**RT-qPCR assay.** Total RNA was extracted from Saos2 and MG63 cells with TRIZOL and determined with RNA electrophoresis in terms of purity and integrity. using random primers, total RNA was reverse-transcribed into cDNA (thermo). Target genes were amplified in 20 µL reaction volume and The operation steps follow the instructions of the SYBR Green qPCR Mix (Thermo Scientific). The sequences of the primers used for RT-qPCR analysis are listed in Table 2. The expression level of mRNA/lncRNA and miRNA was assessed using the 2<sup>-ΔΔCt</sup> method [12].

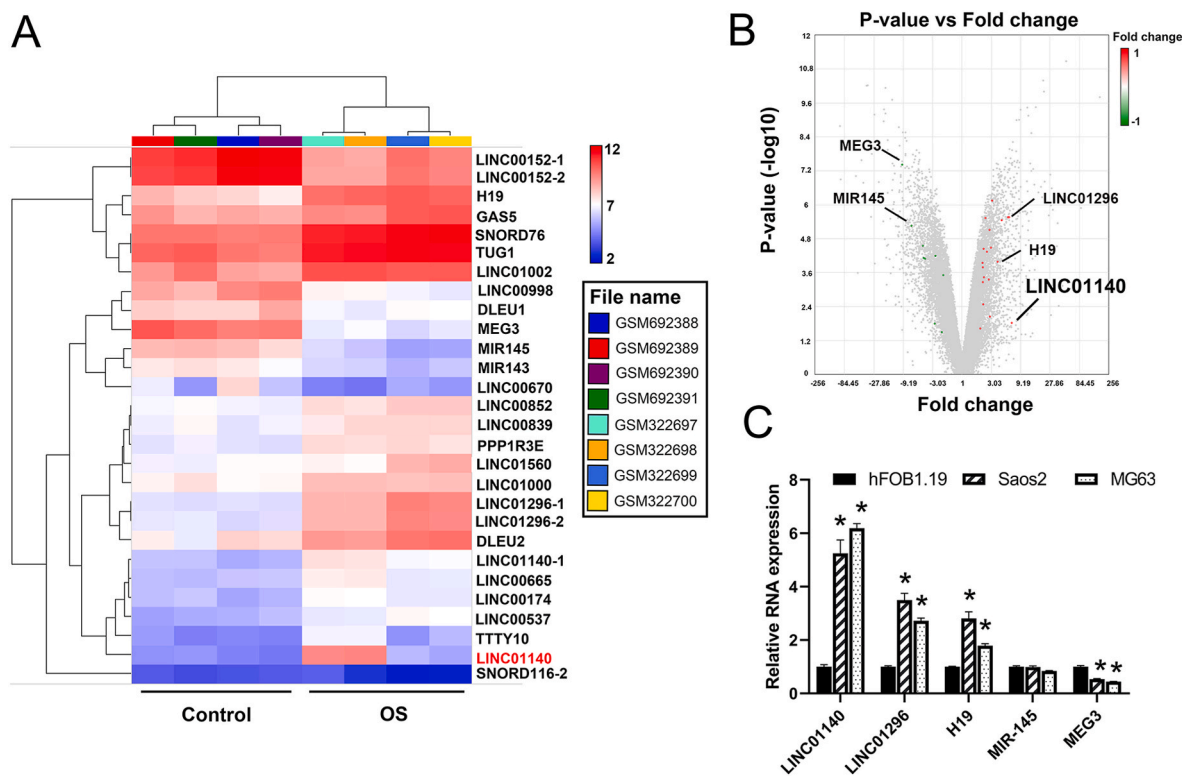
**Western blotting.** 1 × 10<sup>6</sup> cells was lysed by the lysis buffer containing protease inhibitor cocktail. Denatured protein(20µg) is subjected to gel electrophoresis to separate the protein, and then the protein is transferred to the PVDF membrane (Millipore) and incubated with primary antibody at 4 °C overnight and the secondary antibody at RT for 1 h. Finally, the binding signal was visualized using the electrochemiluminescence reagent (ECL, Thermo Scientific, Massachusetts, USA). *Anti*-GAPDH antibody was used as an internal reference (1:2000, 10494-1-AP, Proteintech). The antibody information is as follows: *anti*-HOXA9 (1:1000; ab140631, abcam), *anti*-Vimentin (1:1000; ab217673, abcam), *anti*-Fibronectin (1:1000; ab32419, abcam), *anti*-Snail (1:500; ab216347), and *anti*-Twist (1:100; ab50887, abcam), goat anti-rabbit antibody (1:5000; SA00001-2, Proteintech) or goat anti-mouse antibody (1:5000; SA00001-1, Proteintech)

**Cell Counting Kit-8 (CCK8) assay.** Briefly, 3 × 10<sup>3</sup> cells/well were plated in 96-well plates. After 72 h, 10 µL CCK8 reagent (Solarbio) was

**Table 2**

The primer sequences used to analyze the expression of various RNAs and miRNAs.

Gene symbol	Forward 5'-3'	Reverse 5'-3'
LINC01140	CCAGGCTTCTCCACTTCCGAA	CTCCTTGTTCTCCGCTCACTTTC
LINC01296	GCTCAGCTTCCACACTCAGA	TTTGGGGTCAGTGTGACCTT
H19	TCAGTACGAGTGTGCGTGAG	GAGCTTCCAGACTAGGCGAG
MIR-145	CACCTTGTCTCACGGTC	AACCATGACCTCAAGAAC
MEG3	GGAAAGGGACCTCGAATGTG	CTGTCCCGTGGGAATAGGTG
HOXA9	AGAATGAGAGCGGGGAGACAA	CTCTTTCTCCAGTTCAGGGTC
GAPDH	GTCTCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
U6	CTCGCTTCGGCAGCACAT	TTTGCGTGTCACTCTTGCC
Stem-loop universal reverse transcription primer	CTCAACTGGTGTGCTGGAGTCGGCAATTCAGTTGAGGAAAAACGC	
miR-22-3p	ACACTCCAGCTGGGAAGCTGCCAGTTGAAG	CTCAACTGGTGTGCTGGA
miR-494-3p	ACACTCCAGCTGGGTGAAACATACACGGGA	CTCAACTGGTGTGCTGGA
miR-874-3p	ACACTCCAGCTGGGCTGCCCTGGCCGAGG	CTCAACTGGTGTGCTGGA
miR-300	ACACTCCAGCTGGGTATACAAGGGCAGACT	CTCAACTGGTGTGCTGGA
miR-499a-5p	ACACTCCAGCTGGGTAAAGACTTGCAGTG	CTCAACTGGTGTGCTGGA
miR-335-5p	ACACTCCAGCTGGGTCAAGAGCAATAACGAA	CTCAACTGGTGTGCTGGA
miR-422a	ACACTCCAGCTGGGACTGGACTTAGGGTCA	CTCAACTGGTGTGCTGGA
miR-139-5p	ACACTCCAGCTGGGTCTACAGTGCAGCTGTG	CTCAACTGGTGTGCTGGA
miR-134-5p	ACACTCCAGCTGGGTGTGACTGGTTGACCA	CTCAACTGGTGTGCTGGA
PTPRD	TAGTCCCAGTGTCCAGTTCAGG	CCAAGACAGCAACCGGAAGTC
TNIK	ACAGTGGGTGTACGCGACATAC	ATACTGCCGCTGAAACTGTCCG
RASGEF1B	TTCAGGCGTTCGTGCAGAAGGA	AGCAACCAAGTAGCTGAGGCCGA
MGAT4A	TCTACCAAGGGCATACGCTGGA	GATGTTCTTGGTTGCCGCTATGG
NDRG2	CACCTGTGGAGACACCATACG	CTGAAACAGTGGCTGGAAGCAA
ATP11A	CTGACCAGAGACAACCTGTCCG	CGGCAGATTTCAGGAAGAGCT
HIPK1	GCCACTACAGATTGAGTCAGGAG	GTGTGATGGTGGCTACTTGAGG
HNRNPU	GAGATTGCTGCCCGAAAGAAGC	TTGCTGGAAGCCTGCAACAG
JAKMIP2	TGGAAGCGACTCTGGCTCAGAA	CGGTGATTTTCTGCCTCTGTTT



**Fig. 1.** LINC01140 expression in OS cell lines. A Heatmap of differentially expressed genes in GEO dataset of normal and OS patients. B Volcano Plot displaying the genes with significantly up-regulated or down-regulated expression ( $P < 0.05$  and  $FC > 2$ ) in OS patients, compared to normal controls in the GEO dataset. C RT-qPCR analysis on the expression of miR-498 in normal osteoblasts cell line (hFOB1.19) and OS cell lines. \* $P < 0.05$ . Abbreviations: GEO, Gene Expression Omnibus; OS, osteosarcoma; RT-qPCR, real-time quantitative polymerase chain reaction; FC, Fold Change.

added into the wall including cells and incubated for 60 min. The level of proliferation was estimated at 490 nm using a microplate reader (51119670DP, Thermo Scientific).

**Transwell migration and invasion assays.**  $5 \times 10^5$  cells transferred to the upper chamber of a Transwell plate containing culture medium (Serum free), and culture medium supplemented with 5% FBS was added into the lower chamber. After 48 h incubation, 4% para-formaldehyde was used to fix cells for 15 min, washed with PBS, and 0.1% crystal violet stained for 10 min. For the Transwell invasion assay, the experimental procedure was similar to the Transwell migration assay, and was performed using Transwell invasion plates.

**Dual-luciferase assay.** The HOXA9 3'UTR sequence(WT) and The HOXA9 3'UTR mutant sequence(MUT), or the LINC01140 sequence and the mutant sequence were synthesized and subcloned into the psi-CHECK2 Vector(synbio-tech). Lipofectamine 2000 (Invitrogen) was used to co-transfect the luciferase reporter plasmid with miR-139-5p mimic or mimics nc in 293 cells. After 48 h, Firefly fluorescence value and Renilla fluorescence value were estimated using the dual-luciferase reporter assay (Promega). Relative fluorescence value is Renilla fluorescence value/Firefly fluorescence value, and the experiments were performed in three independent replicates.

**RNA immunoprecipitation (RIP) assay.**  $1 \times 10^7$  cells were lysed with RIPA buffer(Beyotime, China) added protease inhibitor cocktail, take 10% Lysis mixture as Input, and incubate proteinA/G magnetic beads (millipore) coupled with anti-AGO2 antibody(ab186733, Abcam) and normal rabbit IgG(negative control) for 4 h. The magnetic beads of the coupled antibody were incubated with the lysis mixture for 6 h, and then

the RNA was eluted and RT-qPCR was performed. Experiments were performed in three independent replicates.

**Database analysis.** RNA screening: The GSE28423 dataset contained data on OS cell lines, including four OS samples (GSM322697, GSM322698, GSM322699, GSM322700) and four human normal bone samples (GSM692388, GSM692389, GSM692390, GSM692391). We used the online tool GEO2R ([ncbi.nlm.nih.gov/geo/geo2r](https://ncbi.nlm.nih.gov/geo/geo2r)) to recognize anomalously expressed lncRNA/mRNA that complied with a standard of  $p\text{-value} < 0.05$  and  $\log_2 |\text{fold change (FC)}| > 2$ . Volcano plot or Heatmap were drawn using the results of differentially expressed lncRNA/mRNAs. miRNA data were derived from dbDEMC (<https://www.biosino.org/dbDEMC/index>). The data from RNAinter (<http://www.rna-society.org/rnainter/>), TargetScan (<http://www.targetscan.org/>), and miRDB (<http://www.mirdb.org/>) were used to analyze potential genes interacting with miR-139-5p and LINC01140 or miR-139-5p and HOXA9.

**Statistical analysis.** All analysis data were analyzed by GraphPad Prism 8. The data are expressed as the mean  $\pm$  standard deviation, and the two groups of data were analyzed by Student's t-test, multiple groups were performed using one-way ANOVA followed by Bonferroni post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

### 3. Results

**lncRNA LINC01140 is overexpressed in OS cells.** The GEO database was screened to recognize the dissimilarly expressed lncRNAs in OS.

**Table 3**  
The list of differentially expressed lncRNAs.

Gene Symbol	Osteosarcoma Avg (log2)	Control Avg (log2)	Fold Change	P-val
LINC01140	7.47	4.74	6.6	0.0151
LINC01296	8.99	6.43	5.91	2.76E-06
H19	10.11	7.94	4.48	3.46E-06
DLEU2	9.47	7.53	3.84	0.0001
GAS5	11.42	9.77	3.13	7.15E-07
TTY10	6.14	4.62	2.86	0.0089
TUG1	11.46	9.95	2.84	7.75E-06
LINC01002	10.4	8.95	2.74	0.0004
LINC00537	6.82	5.46	2.58	4.62E-05
PPP1R3E	7.73	6.44	2.44	2.92E-06
LINC00839	7.86	6.65	2.31	0.0004
LINC01000	8.33	7.16	2.26	3.54E-05
GAS5	9.88	8.72	2.23	0.0034
LINC00174	6.79	5.64	2.21	0.0002
LINC00665	6.97	5.84	2.19	0.0005
LINC00852	7.89	6.77	2.17	0.0001
LINC01560	7.88	6.87	2.01	0.0237
DLEU1	6.92	7.93	-2.02	0.0003
SNORD116-22	2.64	3.74	-2.14	0.0318
MIR143	6.08	7.53	-2.73	6.32E-05
LINC00670	4.81	6.3	-2.81	0.0158
LINC00152	9.19	11.2	-4.01	8.17E-05
LINC00998	6.96	9.1	-4.39	2.80E-05
MIR145	5.66	8.42	-6.76	5.49E-06
MEG3	6.6	9.87	-9.6	3.83E-08

Among them, expression levels of 17 lncRNAs were elevated and those of 9 lncRNAs were repressed (Fig. 1A and B). The Fold change and p-values of these lists of lncRNAs are presented in Table 3.

We selected lncRNAs (LINC01140, LINC01296, H19, MEG3, MIR145) that differentially expressed Top5 for RT-qPCR detection, and the results showed that LINC01140 had the highest fold change of up-regulated expression in Saos2 and MG63 osteosarcoma cells, and MEG3 had the lowest fold change of down-regulated expression, compared with normal osteoblasts hFOB1.19 (Fig. 1C). A great deal of researches have validated that MEG3 is involved in the process of suppressing OS [13,14]. However, the molecular mechanism and potential effects of LINC01140 in OS have not yet been reported. We inferred that lncRNA LINC01140 may be involved in OS growth and metastasis.

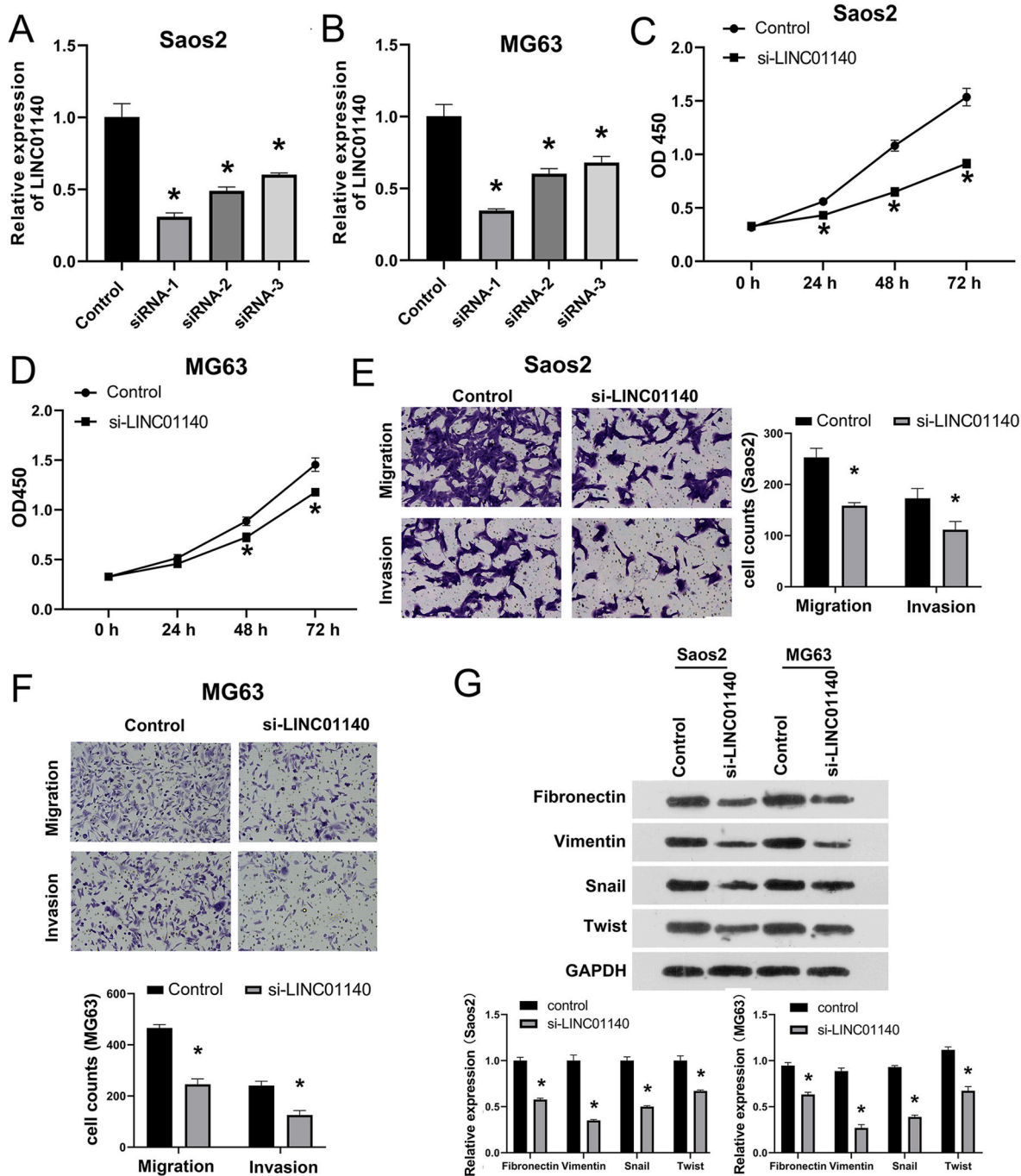
*lncRNA LINC01140 regulates the proliferation, migration, and EMT in OS.* To further confirm the effects of lncRNA LINC01140 on OS, we constructed three siRNAs targeting LINC01140 and transfected them into Saos2 and MG63 cells. RT-qPCR analysis showed that all three siRNAs significantly downregulated LINC01140 expression, confirming the effectiveness of siRNA (Fig. 2A and B). Notably, si-LINC01140-1 exhibited the best inhibitory effect, so we chose it for subsequent functional experiments. The consequences exhibited that following inhibition of LINC01140 expression, the proliferation, migration and invasion abilities of OS cells was reduced (Fig. 2C–F). In addition, Western blot analysis manifested that LINC01140 knockdown downregulated the level of EMT-related proteins Fibronectin, Vimentin, Snail, and Twist

(Fig. 2G). Therefore, we inferred that knocking down LINC01140 reduces the proliferation and migration of OS and partially prevents EMT.

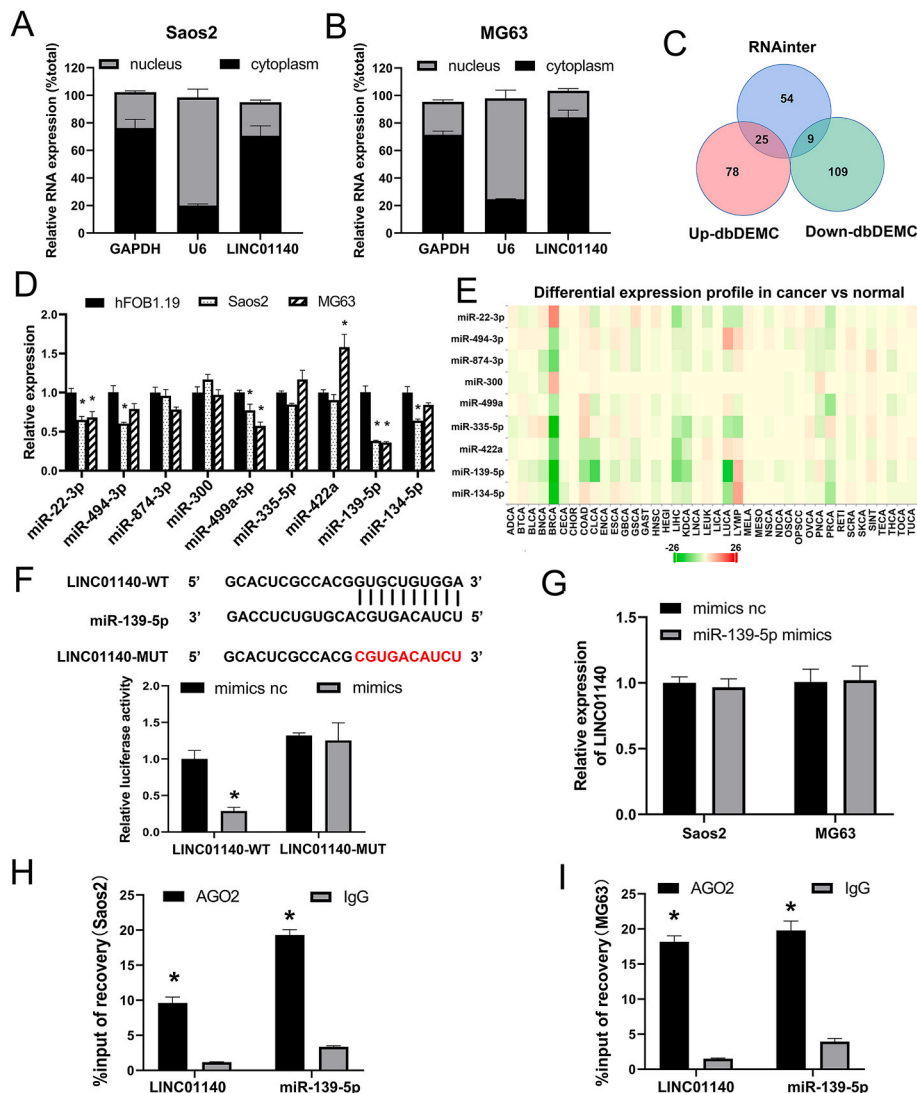
*LINC01140 sponges miR-139-5p.* To figure out the molecular mechanism underlying LINC01140-mediated regulation of OS migration and invasion, we first performed RT-qPCR analysis. After nuclear and cytoplasmic extraction the results showed that LINC01140 was overexpressed in the cytoplasm (Fig. 3A and B). The above consequences demonstrated that LINC01140 may serve as a ceRNA to sponge miRNAs and modulate the cellular functions in OS. Therefore, we subsequently used RNAinter software to predict the binding of microRNA to LINC01140, and combined the results with the results of OS microRNA expression in the dbDEMOC database to screen the miRNAs that LINC01140 may bind (Fig. 3C). RT-qPCR analysis was performed to evaluate the expression of downregulated miRNAs, and the results displayed that the expression of miR-139-5p in OS was the least, and miR-139-5p was low expression in various cancers (data derived from dbDEMOC) (Fig. 3D and E). Therefore, LINC01140 may regulate OS by adsorbing miR-139-5p. Results of dual-luciferase assay confirmed that LINC01140 binds to miR-139-5p (Fig. 3F). The RIP assay results further verified that LINC01140 bound to miR-139-5p through the AGO2 protein (Fig. 3H and I). The RT-qPCR results exhibited that the overexpression of miR-139-5p did not affect the expression of LINC01140 (Fig. 3G). Therefore, we inferred that LINC01140 can adsorb miR-139-5p.

*The effect of LINC01140 knockdown was restored by miR-139-5p inhibitor on OS cell proliferation and migration.* To further validate that LINC01140 elevates the abilities of proliferation and invasion in Saos2 and MG63 cells via miR-139-5p, under LINC01140 knocking down, we transfected miR-139-5p inhibitor, and then implemented CCK8 assay and Transwell experiments. Subsequent consequences uncovered that the downregulation of miR-139-5p expression enhanced the proliferation and migration of OS. (Fig. 4A–D). Hence, we draw the conclusion that miR-139-5p inhibitor can invert the LINC01140-knockdown-mediated inhibition on proliferation and invasion of Saos2 and MG63 cells.

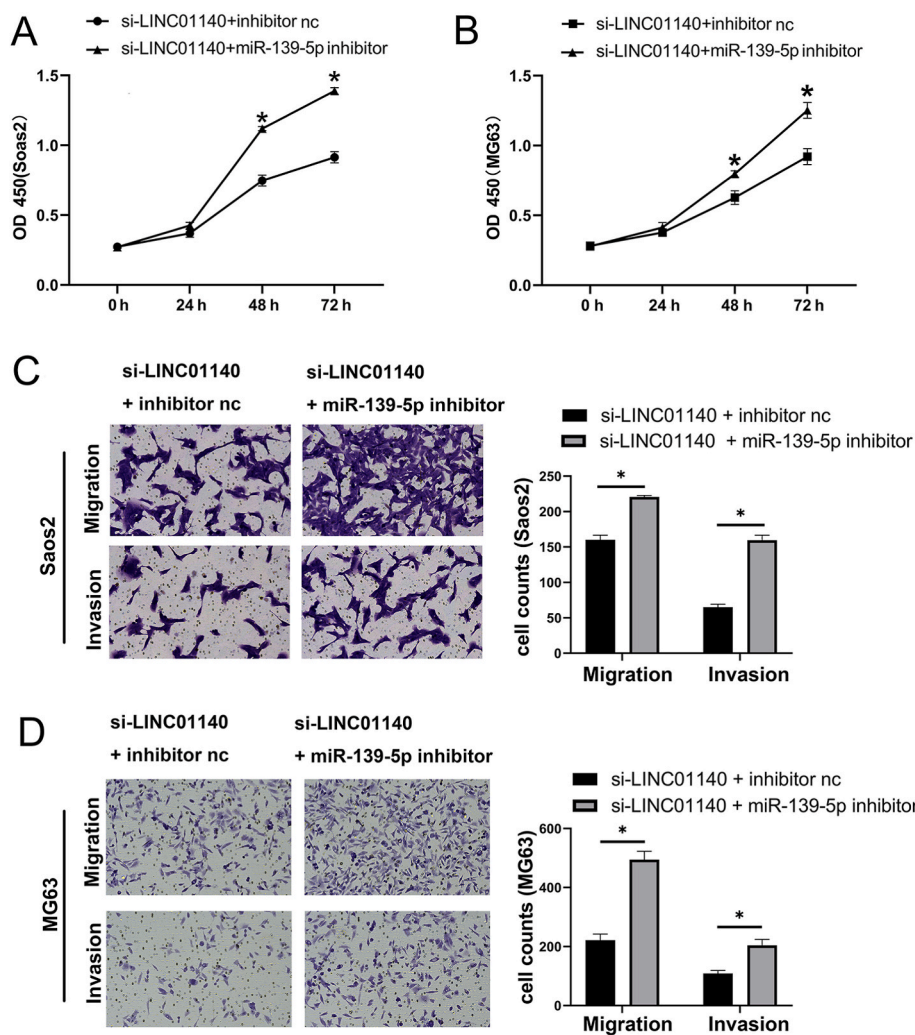
*miR-139-5p inhibits the proliferation and migration of OS cells by downregulating HOXA9 expression* Since miRNA can usually regulate the transcription and translation of downstream targets by binding to their 3' UTR region [15], we screened the mRNA interacting with miR-139-5p using the GEO database, targetscan, and miRDB database. The results showed that 65 mRNAs showed differential expression. Among them, expression levels of 34 mRNAs were upregulated, while those of 31 mRNAs were downregulated (Fig. 5A). Furthermore, we analysed the in TOP10 upregulated genes (Table 4) with RT-qPCR, compared with hFOB1.19 cells, RT-qPCR results exhibited that the expression of HOXA9 exhibited the highest degree of upregulation in Saos2 and MG63 cells (Fig. 5B). Therefore, we chose HOXA9 as a candidate target gene regulated by miR-139-5p. After miR-139-5p mimics were transfected into Saos2 and MG63 cells, and RT-qPCR and western blotting outcomes displayed that miR-139-5p constrained HOXA9 expression (Fig. 5C and D). Furthermore, the targetscan software predicted the binding region of miR-139-5p to the 3'UTR of HOXA9 (Fig. 5E), the dual-luciferase assay results corroborated the binding of miR-139-5p to HOXA9 mRNA 3'UTR region (Fig. 5F). To further validate that miR-139-5p regulates the proliferation and invasion of OS by modulating the expression of HOXA9, we co-transfected miR-139-5p mimics and ov-HOXA9 into Saos2 and MG63 cells. RT-qPCR results showed that HOXA9 was successfully overexpressed in miR-139-5p mimic transfected cells (Fig. 6A). Results of CCK8 and Transwell assays exhibited that the promotion of proliferation and migration in Saos2 and MG63 cells by miR-139-5p mimics could be inverted by HOXA9 overexpression (Fig. 6B–D). Furthermore, Western blot outcomes exhibited that miR-139-5p mimics restrained the



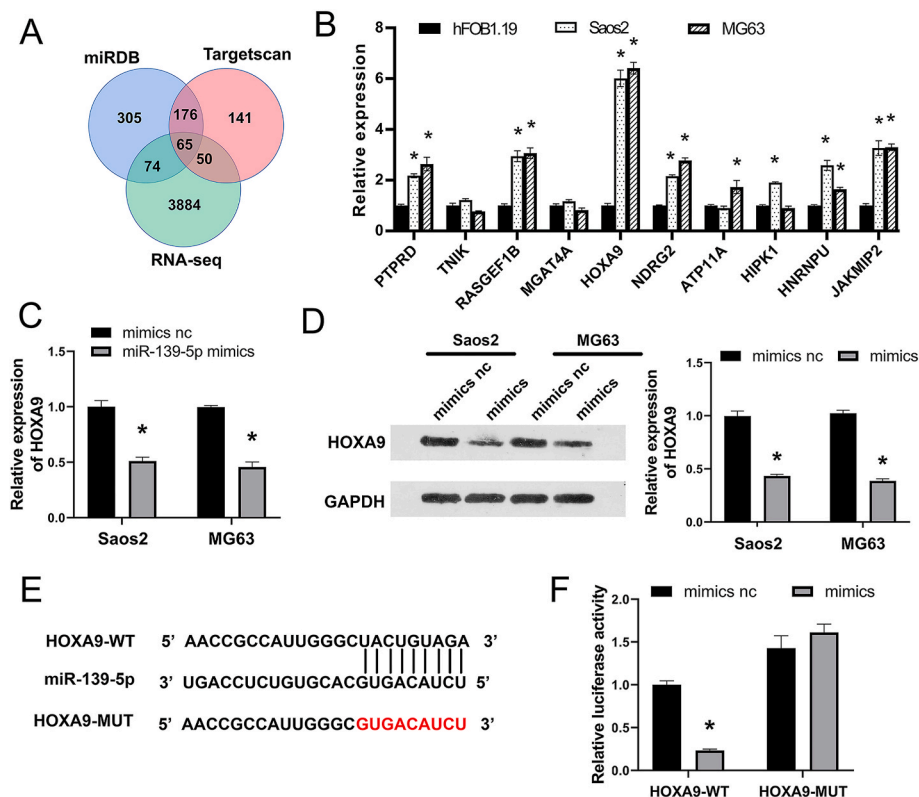
**Fig. 2.** Knocking down LINC01140 expression in OS cell lines reduced their proliferation and migration, and epithelial-mesenchymal transition (EMT). (A, B) Relative expression of LINC01140 was evaluated by RT-qPCR after transfecting Saos2 and MG63 cells with siRNA targeting LINC01140. (C, D) CCK8 assay indicated LINC01140 knockdown reduced the proliferation in Saos2 and MG63 cells. (E, F) Transwell assay results revealed that the LINC01140 knockdown reduced the ability to migrate and invade in Saos2 and MG63 cells. G Western blot results showed that the LINC01140 knockdown inhibited the expression level of EMT-related Fibronectin, Vimentin, Snail, and Twist proteins. \*P < 0.05. *Abbreviations:* CCK8, Cell Counting Kit-8; OS, osteosarcoma.



**Fig. 3.** LINC01140 acts as a sponge for miR-139-5p. (A, B) The subcellular localization of LINC01140 in Saos2 and MG63 cells was determined by RT-qPCR after extracting RNAs from cytoplasmic and nuclear extracts. C GAPDH and U6 were used as the cytoplasmic and nuclear RNA controls respectively. D Venn diagram representing the number of possible miRNAs sponged by LINC01140, identified as an overlapping area between transcripts downregulated in the dbDEM C dataset and RNAinter predicted LINC01140 target miRNAs. E Heatmap of Differential expression profile in cancer vs normal tissues as derived from the dbDEM C dataset. F psiCHECK2-LINC01140-WT and psiCHECK2-LINC01140-MUT or miR-139-5p mimics and mimics nc were transfected into 293T cells, dual luciferase reporter assay results showed that miR-139-5p could directly bind to LINC01140 and inhibit luciferase activity in 293T cells. G RT-qPCR results indicated that miR-139-5p did not impact the expression of LINC01140. (H, I) RIP assay results showed that miR-139-5p was associated with LINC01140 via the AGO2 protein in Saos2 and MG63 cells. \*P < 0.05. RIP, RNA Binding Protein Immunoprecipitation Assay.



**Fig. 4.** miR-139-5p inhibitor restored the inhibitory effect of LINC01140 knockdown on the proliferation and migration of OS cells. (A, B) CCK8 assay indicated that miR-139-5p inhibitor increased the ability to proliferate in LINC01140-knocked down Saos2 and MG63 cells. (C, D) Transwell assay results revealed that miR-139-5p inhibitor increased the ability to migrate and invade in LINC01140-knocked down Saos2 and MG63 cells. \*P < 0.05. *Abbreviations:* CCK8, Cell Counting Kit-8; OS, osteosarcoma.



**Fig. 5.** miR-139-5p targets HOXA9 3' UTR. A Venn diagram representing the number of possible miR-139-5p targets identified as an overlapping area between miRDB predicted target genes, targetscan predicted target genes, and differential expression genes derived from the GEO dataset. B RT-qPCR analysis of the differential expression of Top 10 target genes with predicted upregulated expression. qPCR results indicated that miR-139-5p inhibited the expression of HOXA9 in Saos2 and MG63 cells. (C, D) RT-qPCR and Western blot results indicated that miR-139-5p inhibited the expression level of HOXA9 protein in Saos2 and MG63 cells. The schematic diagram shows the binding site of HOXA9 and miR-139-5p and the mutant sequence of HOXA9-MUT. E Schematic diagram showing the binding site of miR-139-5p and HOXA9 predicted by targetscan software and HOXA9 mutated sequence. F psiCHECK2-HOXA9-3'UTR-WT and psiCHECK2-HOXA9-3'UTR-MUT or miR-139-5p mimics and mimics nc were transfected into 293T cells, dual luciferase reporter assays showed that miR-139-5p could directly bind to the HOXA9 3' UTR and inhibit luciferase activity in 293T cells. \* $P < 0.05$ . HOXA9, Abbreviations: Homeobox A9, WT, wild type; MUT, mutant.

**Table 4**

Top 10 target genes with predicted upregulated expression.

Gene symbol	Foldchange	P-value
PTPRD	18.01	$P < 0.05$
TNFK	6.96	$P < 0.05$
RASGEF1B	6.21	$P < 0.05$
MGAT4A	5.83	$P < 0.05$
HOXA9	5.8	$P < 0.05$
NDRG2	4.62	$P < 0.05$
ATP11A	4.38	$P < 0.05$
HIPK1	4.3	$P < 0.05$
HNRNPU	3.74	$P < 0.05$
JAKMIP2	3.63	$P < 0.05$

expression of EMT-related proteins Fibronectin, Vimentin/snail/twist, and this downregulation could be reversed via the overexpression of HOXA9 (Fig. 6E). All in all, miR-139-5p restrained the proliferation, invasion, and EMT of osteosarcoma through targeting HOXA9.

#### 4. Discussion

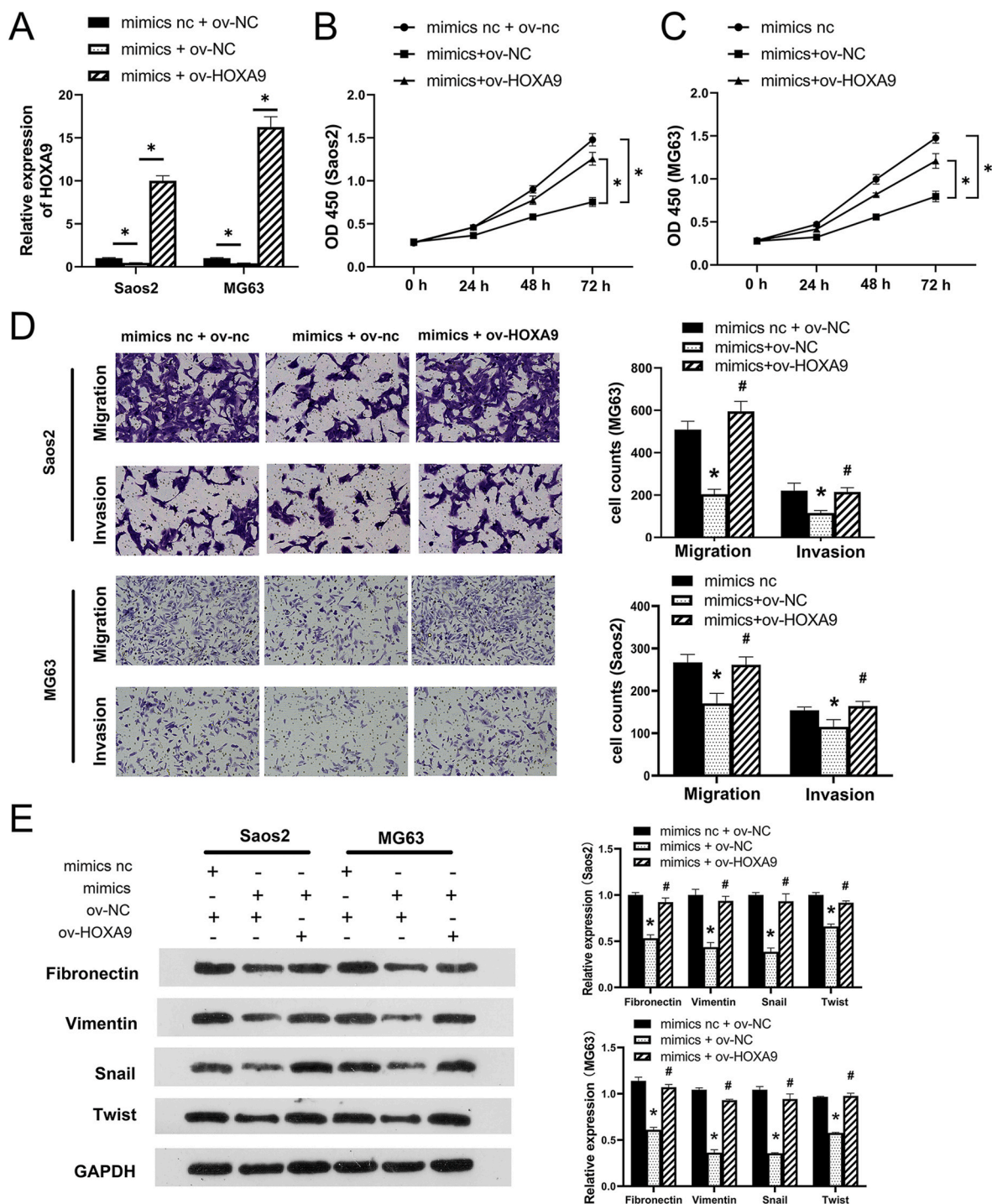
Osteosarcoma occurs in adolescents aged 15–19 years old, and the incidence is not high, but the disease develops rapidly and the mortality rate is high [1]. At present, the treatment methods for osteosarcoma include surgery, chemotherapy, targeted therapy, etc. Although these prolong the survival time of patients with osteosarcoma, their respective shortcomings, such as surgical therapy seriously reduces the quality of life of patients, chemotherapy drugs are prone to drug resistance and there are also huge individual differences in targeted drugs [3]. Guided by modern precision medicine, gene therapy brings enormous potential in the treatment of cancer, especially non-coding RNAs (previously considered non-functional RNAs) play a huge role in regulating cancer growth and metastasis [16].

In recent years, lncRNAs have been confirmed to be anomalously expressed in various diseases, especially in cancer [5,17,18]. In

addition, several investigations have demonstrated that a fraction of lncRNAs are differentially expressed in OS [19–21]. We screened the differentially expressed lncRNA, LINC01140, in osteosarcoma using the GEO database (GSE12865 and GSE27976). RT-qPCR results showed that LINC01140 is overexpressed in OS cells. This indicates that LINC01140 may contribute the occurrence and transfer of osteosarcoma. lncRNAs are participated in miscellaneous process of osteosarcoma development and distant metastasis, comprising proliferation, invasion, drug resistance, and EMT [22–24]. Here, we revealed that knocking down LINC01140 restrained OS proliferation and invasion as well as EMT. However, LINC01140 is reportedly related to poor prognosis in breast cancer [25]. We speculate that this difference in the expression and function of LINC01140 may be due to the tissue specificity of lncRNA.

lncRNAs can be used as competing endogenous RNAs (ceRNAs) to influence the occurrence and development of tumors. By targeting miRNAs to regulate target genes, lncRNA SNHG3 regulates OS proliferation and invasion via the miRNA-151a-3p/RAB22A axis [26]. In our study, miR-139-5p was selected as the candidate target miRNA for LINC01140 through biometric analysis. Analysis of dbDEMC database revealed that miR-139-5p expression is low expression in cancers of colon, lungs, and prostate, among other cancers [27–29]. Moreover, the low expression of miR-139-5p in serum is closely related to distal metastasis and poor prognosis in OS [30]. Moreover, miR-139-5p can inhibit the proliferation and invasion of OS, which is consistent with our findings. Furthermore, results of dual-luciferase and RIP assays confirmed the interaction between LINC01140 and miR-139-5p. Next, we integrated GEO sequencing data (GSE12865 and GSE27976) and combined the results of targetscan, miRDB, and other prediction software to screen for the gene targeted by miR-139-5p (HOXA9). Results of dual-luciferase assay and Western blot analysis confirmed that miR-139-5p can target the 3' UTR region of HOXA9 to downregulate its expression. HOXA9 is a transcription factor, and its overexpression could reverse the suppression of miR-647 on the proliferation and invasion of glioma cells [31]. Consistent with our results, HOXA9 has also been shown to promote EMT in breast and pancreatic cancers [32,33].





**Fig. 6.** HOXA9 reversed the repressive effect of miR-139-5p on proliferation and invasion of OS cells. A RT-qPCR analysis of HOXA9 expression after transfection of miR-139-5p mimics or HOXA9 overexpression plasmid. (B, C) CCK8 assay indicated that HOXA9 reversed the repressive effect of miR-139-5p on proliferation of Saos2 and MG63 cells. D Transwell assay indicated that HOXA9 reversed the miR-139-5p mediated repression of migration and invasion in Saos2 and MG63 cells. E Western blot results indicated that HOXA9 reversed the inhibitory effects of miR-139-5p on the expression of the EMT-related protein, Fibronectin, Vimentin, Snail, and Twist, in Saos2 and MG63 cells. \*P < 0.05. *Abbreviations:* Homeobox, WT, wild type; MUT, mutant; EMT, epithelial to mesenchymal transition.

HOXA9 expression was upregulated in Saos2 and MG63 cells, and it was validated to promote the proliferation, invasion, and EMT in OS.

Our study does have some limitations. We did not clarify the mechanism on HOXA9 regulates the proliferation, invasion, and EMT in osteosarcoma. For instance, HOXA9 could be acted as a transcription factor to bind to the promoter region or enhancer region of downstream target genes to regulate the occurrence and metastasis of OS. This should be explored in the future studies.

In conclusion, our study outcomes show that the downregulation of LINC01140 can restrain the proliferation and invasion of OS through the miR-139-5p/HOXA9 axis. Therefore, LINC01140 is a potential therapeutic target for OS.

### Ethics approval and consent to participate

Not applicable.

### Funding

Not applicable.

### Author contributions

SFZ conceived and designed the study and financed the research. SFZ and RCC performed the experiments. All authors read and approved the final manuscript.

### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Patient consent for publication

Not applicable.

### Declaration of competing interest

All authors disclosed no relevant relationship.

### Data availability

Data will be made available on request.

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