1. Introduction

Osteosarcoma, a high-grade primary osteosarcoma, has tumor stem cells that may originate from mesenchymal stem cells, mostly in young people and children. Although the combination of surgery, radiotherapy and chemotherapy is already used in the treatment, the prognosis of advanced osteosarcoma still remains very poor [1, 2]. A majority of patients suffered from its recurrence on account of potential/present distant metastasis. Although there have been therapeutic breakthroughs for patients with recurrence [3], severe adverse effects urgently call for the need to explore the intricate mechanism of osteosarcoma pathogenesis, progression, and metastasis.

Circ-RNA, as the subclass of endogenous non-coding RNAs, is located in eukaryotic cells and has been confirmed to sponge miRNAs, interact with proteins, and serve as the transcription factor [4]. Meanwhile, circRNAs are associated with specific types of cancer and distinctly regulate carcinogenesis and progression [5]. For instance, various circRNAs, including circ_0096041, have been shown to be upregulated in osteosarcoma [6]. However, the functional characterization and mechanistic of circRNAs in osteosarcoma are largely unknown [5]. On the other hand, miRNAs are vital regulators of oncogenicity; miR-556-5p is involved in regulating different cancer prognoses, such as breast cancer, prostate cancer, and cholangiocarcinoma [7-9].

Highly conserved RNA binding proteins Lin28A and Lin28B shared analogous structure and function. First discovered in Caenorhabditis elegans, Lin28A regulates the development duration [10, 11], and Lin28B was first found to be located in hepatocellular carcinoma (HCC) with high expression [12]. Previous experiments elucidated that Lin28A and Lin28B have relatively high expression in numerous cancers [13, 14]. There was also researches showing that Lin28A and Lin28B would serve as oncogenes in specific cancers via inhibiting the microRNA let-7s biogenesis or reserving the oncogenic transcription [15]. It was proposed that highly-expressed Lin28A or Lin28B is associated with malignant tumor and negative prognosis [15]. Recently, LIN28A was reported as a vital regulator of osteogenic differentiation [16]. Although LIN28B was found vital in osteosarcoma tumorigenesis [17], no reports have yet investigated the role of LIN28A in bone cancer. Therefore, we investigated the potential role of LIN28A in the progression of osteosarcoma.

Although significant progress has been made in understanding cancer prognosis and therapeutics, limited tumor
response rates, absence of novel tumor targets, and other unforeseen hurdles retard the development of efficacious therapeutics that preeminently target osteosarcoma. In this research, we tended to demonstrate the association of circ_0096041 with osteosarcoma progression and explicated the effect of circ_0096041/miR-556-5p/LIN28A axis in osteosarcoma pathogenesis.

2. Materials and methods

2.1. Patients and samples

Osteosarcoma patients (n = 35, age 11-24, mean age 17.35) were recruited at The Affiliated Changzhou No. 2 People’s Hospital of Nanjing Medical University. The study was approved by The Affiliated Changzhou No. 2 People’s Hospital of Nanjing Medical University. All patients were informed about the purpose and methods of this work with written consent. Tumors were acquired by complete resection without chemotherapies and were classified according to the Enneking staging system, including 6 cases in stage 1, 21 cases in stage 2, and 8 cases in stage 3. Tumors and adjacent tissues were collected surgically and placed in liquid nitrogen and stored at -80 °C.

2.2. Cell culture

Osteosarcoma cell lines MNNG/HOS, U2OS, and MG63, human osteoblast line hFOB 1.19 were purchased from Procell (Wuhan, China). DMEM culture medium (Thermo Fisher Scientific, USA) + 10% fetal bovine serum (FBS) (#1201005P, Gibco, USA) was used for cultivation. All cell lines were examined for Mycoplasma presence.

2.3. In silico prediction of circRNA and miRNA targets

Circular RNA Interactome was utilized to predict the interactions between miRNA and circRNA [18]. Besides, miRDB and miRWalk [19-21] were taken to analyze the binding of miRNA and target genes.

2.4. MTT assay

Previously description was taken as references [22]. Briefly, 1 × 10^4/well cells in 0.1 ml DMEM were placed in 96-well plates and treated accordingly for 12, 24, 36, and 48 h. Then, the mixture was treated with MTT solution (10 μl, 5 mg/mL) and incubated at 37 °C for 4 h. Dimethyl sulfoxide solution (150 μl) was added for a 10 min incubation. Optical density (OD, 490 nm) was recorded using a microplate reader (Molecular Devices, USA).

2.5. Migration assay

The migration of U2OS and MG63 cells was determined using modified Boyden chambers (MERCK, Darmstadt, Germany). The upper chamber was filled with 1 × 10^5 cells suspended in 0.2 mL DMEM while the lower was coated with 600 μL DMEM supplemented with FBS (10%). Each cell group was treated accordingly and incubated for 24 h at 37 °C. The migrated cells were stained and examined using a high-power microscope.

2.6. Western blotting

Total proteins from cells or tissues were extracted using lysis buffer containing protease inhibitor PMSF. Proteins were electrophoresed on SDS-PAGE gel and transferred onto nitrocellulose membranes (PVDF). Non-fat milk (5%) was used for 1 hour blocking. Then the mixture was co-incubated with primary antibodies against LIN28A (#MA1-016, Invitrogen), c-Myc (#MA1-980, Invitrogen), and Ras (#33-7200, Invitrogen) overnight at 4 °C. Secondary antibody against IgG (#MA5-42729, Invitrogen) was added for another one hour at room temperature. ECL kit (#E411-04, Vazyme, Nanjing, China) was employed to visualize protein bands.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (#15596026, Invitrogen) was used for RNA extraction. RT reaction kit (#K1691, Thermo Scientific) and a real-time PCR system (Agilent, Beijing, China) were used for the cDNA synthesis of 50 μg mRNA. The PCR system included 40 cycles, with each containing 15 s at 93 °C, 10 s at 55 °C, and 20 s at 72 °C. 2^ΔΔCq method was used for calculation, with data normalized to GAPDH [23]. The utilized primer sequences in this study are as follows: circ_0096041, F: 5′-GGGCCCTGCCTGGACATACTC-3′; R, 5′-CTGATGCTGACGGCTGAGG-3′; GAPDH: F: 5′-TCCCCACACACTGAATCT-3′; R, 5′-AACAGGAGGAGAGAGGCG-3′; miR-556-5p: F: 5′-GATATGAAAGAAGATGAG-3′; R, 5′-TGGTGAAGGGTAGTAAATAAAA-3′; U6: F: 5′-CGAGCA-CAGAATGCTGTTCA-3′; R, 5′-CTCGCTCGCCGAC-3′; LIN28A, F: 5′-GGTGAGGAGCGGCCAAGAAA-3′; R, 5′-TGATGATCGAGACCTCAGGCTG-3′; cMYC, F: 5′-TGAGGAGACCGCCACAC-3′; Ras, F: 5′-GTGCTGCTGATATGTCGACT-3′; R, 5′-ACTCATGAAAATGTCTGAGACACCTTAT-3′; β-actin, F: 5′-GGGCCCTGCTGGACATACTC-3′; α-TUB, F: 5′-GATATGAAAGAAGATGAG-3′; R, 5′-TGGTGAAGGGTAGTAAATAAAA-3′; U6: F: 5′-CGAGCA-CAGAATGCTGTTCA-3′; R, 5′-CTCGCTCGCCGAC-3′.

2.8. Luciferase reporter assay

The previous description was used as a reference [24]. Briefly, 1 × 10^4 cells were cultivated into 96-well plates. Twenty-four hours later, miR-556-5p/NC, along with 100 ng LIN28A/LIN28A-DEL, were transfected into cells. pmIR-REPORT^TM vector (#AM5795, Invitrogen) contained cloned 3′-UTR of LIN28A/LIN28A-DEL. Twenty-four hours later, Luciferase Reporter Assay System (Promega) was used to measure relative luciferase activity.

2.9. In vivo analysis

Eighteen female nude BALB/c mice (5–6 weeks) were purchased from TROPHIC Animal Feed High-tech Co Ltd (Nantong, China) and used to investigate tumor growth and pancreatic metastasis. MG63 cells (2 × 10^6) were subcutaneously injected into mice to form the tumor xenografts or injected via spleen in each mouse to form pancreatic metastasis. The animals were allocated into 3 groups (control, circ_0096041, and circ_0096041 + miR-556-5p) at random. Tumor tissues were harvested on day 28 for immunohistochemical analysis. All the procedures were in accordance with relevant guidance [25].

2.10. Hematoxylin and eosin (H&E) staining

Procedures were in accordance with the previous description [26].

2.11. Immunohistochemistry

The paraffin-embedded tumor xenografts were deparaffinized and rehydrated. Heat-induced epitope re-
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miR-556-5p could sponge circ_0096041 (Figure 1D). Previously, miR-556-5p was shown to be engaged in different types of cancer [8, 9]. Compared with adjacent tissues, the expression of miR-556-5p was decreased in osteosarcoma samples (Figure 1E). These results were in accordance with in vitro assays, which showed the expression of miR-556-5p in U2OS and MG63 was apparently decreased compared to hFOB 1.19 (Figure 1F). As shown in correlation analysis (Figure 1G), circ_0096041 was correlated negatively with miR-556-5p in osteosarcoma, with the

2.12. Immunofluorescence analysis

MG63 cells transfected with corresponding vectors were cultured in a six-well plate on a coverslip until reached a confluency of 60–80%. Cells were prepared as previously described [27]. Primary antibody anti-LIN28A was added for an incubation at 4°C overnight. After being washed 3 times with PBS, the cells were stained with DAPI and goat anti-mouse anti-lgG conjugated with FITC for 45 min at room temperature. The LIN28A was visualized and imaged using a confocal microscope (Olympus, Tokyo, Japan). The images were obtained from five random fields of view.

2.13. Statistical analysis

All experimental data were expressed as means ± SD. The correlation between circ_0096041 and miR-556-5p was calculated using Spearman’s correlation coefficient. One-way analysis of variance and the LSD test were taken for evaluation. P<0.01 was considered statistically significant. GraphPad version 7.0 (GraphPad, San Diego, CA, USA) was used in the statistical analysis.

3. Results

3.1. Circ_0096041 expression denotes shorter overall survival of osteosarcoma patients and negatively correlates with the expression of miR-556-5p

Previously, 10 circRNAs with differential expression were found in osteosarcoma samples; among them, circ_0096041 expression was significantly expressed [6]. The expression of circ_0096041 is highly expressed in osteosarcoma than in adjacent tissues (Figure 1A). U2OS, MG63 and HOS cells showed higher circ_0096041 expression than HFOB 1.19 (Figure 1B). Highly expressed circ_0096041 was confirmed to be a key factor in patient’s poor prognosis (Figure 1C) and advanced stages (Table 1). Patient’s gender, age, family history, etc. showed no relevance with circ_0096041. As the in silico prediction from the Circular RNA Interactome database suggested, miR-556-5p could sponge circ_0096041 (Figure 1D).

Table 1. The relationship between circ_0096041 expression and stages of osteosarcomas.

<table>
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<tr>
<th>Variables</th>
<th>Description</th>
<th>N</th>
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<th>P-value</th>
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<tr>
<td>Gender</td>
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<td>15</td>
<td>Low 7 High 8</td>
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<td>0.4333</td>
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<tr>
<td></td>
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<td>Age (years)</td>
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<td>19</td>
<td>Low 9 High 19</td>
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<tr>
<td></td>
<td>&gt;15</td>
<td>16</td>
<td>Low 7 High 9</td>
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<td>0.9003</td>
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<tr>
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<td>2</td>
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<tr>
<td></td>
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<td>33</td>
<td>Low 15 High 18</td>
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<td>5</td>
<td>Low 4 High 1</td>
<td></td>
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<tr>
<td></td>
<td>II</td>
<td>18</td>
<td>Low 12 High 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>12</td>
<td>Low 3 High 9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T primary tumor, N regional lymph nodes, M metastasis. *P<0.05.
circ_0096041 promotes osteosarcoma cell proliferation

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MicroRNA-556-5p expression inhibited by circ_0096041 confirming this finding (Figures 1H-J). These results revealed that circ_0096041 promoted osteosarcoma malignancy and inhibited the expression of miR-556-5p.

3.2. MiR-556-5p targets LIN28A in osteosarcoma

LIN28A was one of the predicted targets for miR-556-5p by STarMir Database (Figure 2A). Spearman’s rank correlation analysis showed that miR-556-5p was negatively correlated with LIN28A (Figure 2B). Luciferase reporter assay manifested that miR-556-5p inhibited the activity of wild LIN28A promoter, compared to those of mutant LIN28A promoter, LIN28A DEL 3′-UTR (the sequences within the predicted binding sites were deleted) (Figures 2C, D). Overexpressed miR-556-5p downregulated LIN28A expression, whereas inhibited miR-556-5p enhanced LIN28A expression in MG63 and U2OS cells (Figures 2E, F). These results suggested that miR-556-5p targeted LIN28A.

3.3. MiR-556-5p inhibits the proliferation and migration of osteosarcoma cells by targeting LIN28A

Previously, it has been proposed that several miRNAs blocked LIN28A expression, suppressing the proliferation and migration of osteosarcoma cells [28]. MiR-556-5p mimic significantly hampered cell proliferation (Figure 3A) and migration (Figure 3B). On the contrary, the miR-556-5p inhibitor significantly activated cell proliferation (Figure 3A) and migration (Figure 3B). These results suggested that miR-578 blocked LIN28A-mediated singling pathways. For instance, it has also been proved that LIN28A contributed to the regulation of bone deformities [16] and regulated c-MYC and Ras proteins [29].

3.4. Circ_0096041 promotes osteosarcoma cell proliferation and migration

As shown in Figures 4A, the expression of circ_0096041 boosted the proliferation significantly compared to the negative control. Circ_0096041 group had an increased number of migrated cells compared to control cells (Figure 4B). Western blot and qRT-PCR results indicated that circ_0096041 increased LIN28A, cMYC, and Ras expression significantly in MG63 and U2OS cells (Figures 4C, D). These results demonstrated that circ_0096041 boosted the proliferation and migration by promoting LIN28A, cMYC, and Ras.

3.5. Circ_0096041 promotes the proliferation and migration of osteosarcoma cells via abolishing the inhibition of LIN28A by miR-556-5p

It has been proved that circRNAs/miRNAs/mRNAs axis had a regulatory effect on the initiation and progression of osteosarcoma [30]. Significantly boosted proliferation was observed in circ_0096041 group (Figure 5A) while the proliferation of circ_0096041 + miR-556-5p group was merely affected. Also, the expression of circ_0096041 notably increased the migration of MG63 and U2OS cells while circ_0096041 + miR-556-5p group barely changed (Figure 5B). Western blot and qRT-PCR
results suggested that circ_0096041 + miR-556-5p significantly restored the enhanced LIN28A, cMYC, and Ras expression (Figures 5C, D). The immunofluorescence assay showed that LIN28A expression was increased by circ_0096041 in MG63 cells (Figure 5E) and decreased by additional miR-556-5p. These results suggested that circ_0096041 boosted LIN28A-mediated migration and proliferation by regulating miR-556-5p.

3.6. Circ_0096041 promotes osteosarcoma cell metastasis in vivo

Further in vivo analysis was conducted to determine the regulation of circ_0096041 on osteosarcoma metastasis. Three weeks later, the animals’ pancreatic tissues were harvested. As shown in Figure 6A, in the circ_0096041 group, tumor cells are arranged in a disorder with obvious cell necrosis. Co-expression of circ_0096041 and miR-556-5p significantly reversed this effect. Meanwhile, circ_0096041 enhanced pancreatic metastases while in the combination group, pancreatic metastasis was decreased (Figure 6B). In addition, immunohistochemical analysis confirmed the expression of LIN28A in tumor xenografts (Figure 6C). Overexpressed circ_0096041 elevated LIN28A expression, which was canceled out by additional miR-556-5p. Circ_0096041 evidently promoted the expression of LIN28A, cMYC, and Ras, while additional miR-556-5p significantly obstructed this effect (Figures 6D, E).

4. Discussion

In recent studies, several results have elucidated the regulatory role of circRNA in various biological processes, including the initiation, progression, and metastasis of carcinomas. For instance, it was revealed that circTADA2A directly sponge miR-203a and upregulates the expression level of CREB, resulting in metastasis [31]. CircFAT1 was confirmed to restore Yes-associated protein 1 expression, which was suppressed by miR-375 and progressed tumorigenesis [32]. CircNASP was reported to sponge miR-1253 and regulate forkhead box F1, promoting osteosarcoma from proliferation and invasion [33]. However, there is still considerable room for excavation in the function of the circRNA. In this study, we have explored the regulatory role and mode of circ_0096041 in osteosarcoma.

Upregulated expression of circ_0096041 was detected in osteosarcoma cells and tissues. The association of circ_0096041 with osteosarcoma stages was determined. Meanwhile, the high expression of circ_0096041 also indicated a poor prognosis in patients with osteosarcoma. Although the evidence associated with the circ_0096041 distributions was lacking, our work indicated that circ_0096041 greatly activated the deterioration of osteosarcoma. The circRNAs/miRNAs/mRNAs axis is known to be an important regulatory network, where miRNAs act as a circRNA sponge, targeting mRNA. In the present study, lowly expressed miR-556-5p was observed in osteosarcoma tissues and cells. Furthermore, a negative correlation exists between miR-556-5p and circ_0096041, indicating that circ_0096041 sponges miR-556-5p. The miR-556-5p was demonstrated to induce apoptosis in cancer cells.

![Fig. 4. Circ_0096041 promotes the migration and proliferation in vitro.](image)

**Fig. 4.** Circ_0096041 promotes the migration and proliferation in vitro. (A) Proliferation of cells transfected with control/circ_0096041, detected by MTT assay. (B) Migration of MG63 and U2OS cells transfected with control/circ_0096041, detected by transwell assay. (C,D) Relative expression in cells expressing control or circ_0096041, detected by western blot and real-time PCR. **P<0.01, ***P<0.001.

![Fig. 5. Circ_0096041 promotes cell migration and proliferation via promoting LIN28A inhibited by miR-556-5p.](image)

**Fig. 5.** Circ_0096041 promotes cell migration and proliferation via promoting LIN28A inhibited by miR-556-5p. (A) Proliferation in cells transfected with control/circ_0096041 + miR-556-5p. (B) Migration of MG63 and U2OS cells transfected with control/circ_0096041 + miR-556-5p. (C,D) Gene expression in MG63 and U2OS cells, detected by western blot and real-time PCR. (E) Immunofluorescent staining of LIN28A in MG63 cells. Green, LIN28A; Blue, DAPI. **P<0.01, ***P<0.001, ##P<0.01, ###P<0.001.
and osteosarcoma in stage-specific ones, as well as in os-

terinal expression of circ_0096041 in different subtypes

erated for the lack of research on LIN28A-

osteoarcoma deterioration via the LIN28A/cMYC/Ras

knew. This study confirmed that circ_0096041 advances

oncogene in the progression

of osteosarcoma. It regulates the proliferation and

migratory activities involved in LIN28A regulation by

sponing miR-556-5p. These results help to advance the

inquiry into the function of circ_0096041 and miR-556-5p

in cancer, providing a new explanation for the regulation

of the LIN28A signaling cascade.

Informed Consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
ZF conducted the experiments and wrote the paper; WH, YZ and ZW analyzed and organized the data; CW conceived, designed the study and revised the manuscript.

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tal of Nanjing Medical University approval our study. We declared that we embedded all data in the manuscript.

Availability of data and material
We declared that we embedded all data in the manuscript.

References

cancer cells [8, 9]. MiR-556-5p was also proven to modulate
cMyc and Ras expression in prostate cancer [29]. It was
 speculated that the tissue-specific expression of miR-556-
5p caused its versatility, regulating different mRNA and
protein networks. Alternatively, circ_0096041 acts by mo-
dulating the signaling cascades in cancer, both temporally
and in space.

It has shown that let-7, as a key RNA-binding protein,
underlies tumor growth, invasion and metastasis and regu-
lates carcinogenesis, progression and recurrence of osteo-
sarcoma [13, 15, 16, 34]. The increasing number of studies
revealing the role of the LIN28 pathway has also produced
corresponding targeted therapies against LIN28A / B for
the treatment of recurrent/advanced prostate cancer [29].
However, how LIN28A regulates cancer development
and the associated signaling pathways remains largely un-
known, hindering the targeted therapeutic advancement of
LIN28A. LIN28 is involved in regulating osteogenic dif-
ferentiation, compensating for the lack of research on LIN28A-
related pathways. Further experiments explored the dif-
ferential expression of circ_0096041 in different subtypes
and osteosarcoma in stage-specific ones, as well as in os-

teoarcoma tissue and serum. Furthermore, the correlation
between circulating LIN28A and circ_0096041 confirmed that
 circulating LIN28A is strongly associated with the de-
velopment of metastatic prostate cancer [29].

In conclusion, this study determined the role and me-
chanism of circ_0096041 as an oncogene in the progres-
sion of osteosarcoma. It regulates the proliferation and
migratory activities involved in LIN28A regulation by
sponging miR-556-5p. These results help to advance the
inquiry into the function of circ_0096041 and miR-556-5p
in cancer, providing a new explanation for the regulation
of the LIN28A signaling cascade.

Fig. 6. Circ_0096041 promotes osteosarcoma metastasis in vivo.
(A and B) The xenograft and pancreatic metastatic foci were valida-
ted by hematoxylin and eosin staining. (C) Immunohistochemistry of
LIN28A expression in tumor xenografts. (D) Real-time PCR of LI-
N28A gene expression and its subsequent signaling genes cMyc and
Ras. (E) Western blotting analysis of LIN28A protein expression and
its subsequent signaling proteins cMyc and Ras. Results are repre-
sented as the mean ± SD of three independent experiments. **P<0.01,
***P<0.001 vs control; ###P<0.05, ##P<0.01, vs circ_0096041.


