Long non-coding RNA NR2F1-AS1 facilitates the osteosarcoma cell malignant phenotype via the miR-485-5p/miR-218-5p/BIRC5 axis

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Abstract. Long non-coding RNA (IncRNA) NR2F1 antisense RNA 1 (NR2F1-AS1) has been reported to be an oncogene in several cancer types, including osteosarcoma (OS). However, the underlying fundamental molecular mechanism of NR2F1-AS1 in OS remains largely unknown, which the present study aimed to elucidate. The present study demonstrated that NR2F1-AS1 expression is markedly increased in OS, and NR2F1-AS1 was shown to exert oncogenic functions in OS. Further molecular mechanistic studies revealed that microRNA (miR)-485-5p and miR-218-5p were direct targets of NR2F1-AS1. More importantly, miR-485-5p and miR-218-5p exhibited low expression levels and were negatively correlated with NR2F1-AS1 expression in OS tissues. It was then identified that baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) was a direct target of miR-485-5p and miR-218-5p in OS cells. Furthermore, a series of experiments suggested that NR2F1-AS1 affects the proliferation, migration, invasion and apoptosis of OS cells by regulating BIRC5. Finally, it was identified that silencing of NR2F1-AS1 repressed the OS cell malignant phenotype by binding with miR-485-5p and miR-218-5p, and then downregulating BIRC5 expression, which suggests that the NR2F1-AS1/miR-485-5p/miR-218-5p/BIRC5 axis could be a potential target for treating OS.

Introduction

Osteosarcoma (OS), as the most common primary human malignant tumor among children and adolescents, originating from bone mesenchymal cells, has the characteristics of early distant metastasis and a high local recurrence rate (1-3). Currently, treatment of OS includes mainly neoadjuvant chemotherapy combined with surgical treatment; however, the clinical prognosis rate following current treatment strategies has not been largely improved (4). The main reason for the poor clinical prognosis is that the current treatments cannot effectively inhibit distant metastasis and the recurrence of drug resistance. In addition, the most fundamental reason is that the pathogenesis of OS has not been well elucidated. With the rapid development of molecular biology, new technologies provide opportunities for a more in-depth study of the pathogenesis of OS.

Long non-coding RNA (IncRNA) is a class of endogenous RNA sequences >200 nucleotides in length, which completely lacks or only has a small portion of protein encoding capacity (5). The abnormal sequences, abnormal spatial structure, abnormal protein binding and expression of IncRNAs in the body are all closely associated with the occurrence of human diseases. Numerous studies have shown that IncRNAs have a wide range of functions in almost all physiologic processes of an organism, and they do not only regulate cell differentiation (6,7) and metabolism (8), but also participate in a variety of pathological processes, such as cancer proliferation, invasion, metastasis (9), apoptosis (10) and drug resistance (11). NR2F1-AS1, a recently discovered IncRNA, has been found to enhance esophageal squamous cell carcinoma (ESCC) progression by regulating the Hedgehog signaling pathway (12). NR2F1-AS1 has also been reported to regulate the microRNA (miR)-338-3p/cyclin D1 axis to promote thyroid cancer progression (13). In papillary thyroid carcinoma, NR2F1-AS1 has been confirmed to exacerbate cell proliferation by regulating the miR-423-5p/SRY-box transcription factor (SOX)12 signaling pathway (14). In addition, in endometrial cancer, NR2F1-AS1 has been reported to promote Forkhead box A1 (FOXA1) expression (15). These findings demonstrate that NR2F1-AS1 acts as an oncogene in cancer. However, the function of NR2F1-AS1 in OS is not well understood and requires further investigation.

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A number of studies have demonstrated that miRNAs exhibit roles in numerous biological processes by regulating their mRNA targets, including in cell development, tumorogenesis, cell differentiation and aging (17-19). In OS, the miRNA expression profile has been extensively studied, and a variety of miRNAs has been identified to play a role in OS, such as miR-422a, miR-145 and miR-194 (20). Furthermore, the aberrant expression of miRNAs is closely associated with OS progression and prognosis. miR-485-5p and miR-218-5p have been reported to inhibit the malignant behavior of OS (21-23). Therefore, the present study investigated these miRNAs to expand our understanding of their functional effects in OS.

Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), also termed survivin, is a member of the family of apoptotic inhibitors and plays an important regulatory role in tumor progression in numerous types of cancers, including OS (24-27). In addition, accumulating evidence has confirmed that BIRC5 is negatively regulated by miRNAs in different tumor cells to play functional roles. For example, in breast cancer, BIRC5 has been demonstrated to be regulated by miR-485-5p to suppress cancer progression and chemosensitivity (24). Furthermore, in OS, miR-218 promotes apoptosis of U2OS osteosarcoma cells via targeting BIRC5 (22). However, in OS, whether BIRC5 is controlled by miR-485-5p and miR-218-5p to serve roles in tumor development and progression has not been fully elucidated, to the best of our knowledge. It is well understood that lncRNAs can combine with miRNAs to target mRNAs, which creates a network of lncRNA-miRNA-mRNA and thus exerts biological functions in cancer. Therefore, it is worth investigating whether NR2F1-AS1, miR-485-5p, miR-218-5p and BIRC5 form an axis to affect the malignant phenotype of OS.

The present study revealed that NR2F1-AS1 expression was upregulated in OS. Further studies indicated that NR2F1-AS1 knockdown inhibited cell proliferation, migration and invasion, and facilitated cell apoptosis by interacting with miR-485-5p and miR-218-5p to downregulate BIRC5 expression. In summary, the present study described the participation of NR2F1-AS1/miR-485-5p/miR-218-5p/BIRC5 axis in regulating the OS malignant phenotype.

**Materials and methods**

**Clinical samples.** A total of 32 cases of OS tissue specimens and corresponding adjacent normal tissue (age range, 8-50 years; mean age 18.8±10.1 years) specimens were collected at the Zhengzhou Orthopedic Hospital (Zhengzhou, Henan, China) between January 2016 and December 2018. All specimens were confirmed by pathological biopsy, and none of the patients underwent chemotherapy and radiation therapy before surgery. The tissue samples were quickly frozen in liquid nitrogen in vitro. The patient samples were collected with permission from the Medical Ethics Committee of Zhengzhou Orthopedic Hospital, and written informed consent was obtained from the patients before the operation.

**Cell culture and cell transfection.** Human OS cell lines (HOS, MG63 and U2OS) and a normal osteoblast cell line (hFOB1.19) were obtained from the Chinese Academy of Sciences Cell Bank. The OS cells (HOS, MG63 and U2OS) were cultured in RPMI-1640 medium (Sangon Biotech Co., Ltd.) with 10% fetal bovine serum (FBS; Hyclone; Thermo Fisher Scientific, Inc.) in a 5% CO2 atmosphere at 37°C. The hFOB1.19 cells were cultured at 33.5°C with 5% CO2 in RPMI-1640 medium with 10% FBS. The 293T cells were cultured at 37°C with 5% CO2 in Gibco (no. 12800017, containing NaHCO3 1.5 g/l) medium (Thermo Fisher Scientific, Inc.) with 10% FBS. The small interfering RNA (siRNA) specifically targeting NR2F1-AS1 (si-NR2F1-AS) and the negative control (si-NC) were synthesized and purchased from Invitrogen; Thermo Fisher Scientific, Inc. The sequences were as follows: si-NR2F1-AS#: 5' -GAU GUU CUC AAU AUU UCU AUU-3'; si-NC: 5'-CGA AAC CUA GCG UGU ACA CAA-3'. miR-485-5p mimics and negative control (NC), and miR-485-5p inhibitor (miR-485-5p in) and NC were also obtained from Invitrogen; Thermo Fisher Scientific, Inc. The sequences were as follows: miR-485-5p mimics: 5'-AGAGGCUCGCGGCUAGUAUAAU C-3'; miR-485-5p inhibitor: 5'-GAUCAUCAGCGACGCCAGCU CUC-3'; inhibitor NC: 5'-UAAUUGAUGUAAUUAUUAUUAUUA-3'. miR-218-5p mimics and negative control (NC), and miR-218-5p inhibitor (miR-218-5p in) and NC were also obtained from Invitrogen; Thermo Fisher Scientific, Inc. The sequences were as follows: miR-218-5p mimics: 5'-UGUGUCUUGUGCUACUCAA CGAUU-3'; miR-218-5p inhibitor: 5'-GAAUCAUCAGCGACGCCAGCU CUC-3'; inhibitor NC: 5'-UAAUUGAUGUAAUUAUUAUUAUUA-3'.

**Reverse primer sequences.**

| Gene       | Position | Sequence (5’ to 3’) |
|------------|----------|---------------------|
| NR2F1-AS1  | Forward  | AGCGGTGCAACCAACCACTG |
| miR-218-5p | Forward  | AAGAC ACCCT GAGGC AAGCC |
| miR-485-5p | Forward  | ACATCTCCAGCTGGGAAAG |
| miR-485-5p | Reverse  | ACGGGAGGAAAGAA |
| miR-218-5p | Reverse  | CTCACAATGGTTCGTTGGA |
| GAPDH      | Forward  | CAGTGCCAGCTCGCTTCTT |
| miR-485-5p | Reverse  | AGGGGCCATCCACAGTCTTC |
| U6         | Forward  | CTCGCTTCGCGCACACATA |
| miR-485-5p | Reverse  | AAGGTTTCAGCAATTTGGCT |
| BIRC5      | Forward  | AGGACACCCCGCATCTTCTCAT |
| miR-218-5p | Reverse  | AAGTCTGGTCTGGTCTCAGT |

**BIRC5, baculoviral inhibitor of apoptosis repeat-containing 5; NR2F1-AS1, long non-coding RNA NR2F1 antisense RNA1.**
NR2F1-AS1 NC group (transfected with 50 nM si-NR2F1-AS1 NC); ii) si-NR2F1-AS11# group (transfected with 50 nM si-NR2F1-AS11#); iii) si-NR2F1-AS12# group (transfected with 50 nM si-NR2F1-AS12#); iv) si-NR2F1-AS1 + miR-485-5p inh

Figure 1. NR2F1-AS1 expression is upregulated in OS. (A) NR2F1-AS1 expression in human OS tissues and adjacent tissues was detected by RT-qPCR. **P<0.01, tumor vs. adjacent tissues. (B) NR2F1-AS1 expression in human OS cell lines (U-2OS, 143B and MG63) and human normal osteoblast cell line (hFOB1.19) was detected by RT-qPCR. **P<0.01, compared with hFOB1.19 cells. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA NR2F1 antisense RNA 1.

Figure 2. NR2F1-AS1 exerts oncogenic function in OS. (A) Expression of NR2F1-AS1 was silenced by transfection with si-NR2F1-AS11#, si-NR2F1-AS12# and si-NR2F1-AS13# in MG63 and U-2OS cells. (B and C) The effect of NR2F1-AS1 knockdown on the proliferation abilities of MG63 and U-2OS cells was detected by performing CCK-8 assay. (D) The effect of NR2F1-AS1 knockdown on the apoptosis abilities of MG63 and U-2OS cells was identified by performing flow cytometry assay. *P<0.05 and **P<0.01, vs. the si-NC group. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA NR2F1 antisense RNA 1; NC, negative control.
miR-218-5p and miR-485-5p were calculated using the 2-ΔΔCq method (28). The NR2F1-AS1 and BIRC5 expression levels were made relative to GAPDH, and the miR-485-5p and miR-218-5p expression levels were made relative to U6. The sequences of all primers are presented in Table I.

**Table II. Association between NR2F1-AS1 expression level and clinical parameters of the patients with OS.**

| Parameters          | NR2F1-AS1 expression | P-value |
|---------------------|----------------------|---------|
|                     | Low (n=15)           | High (n=17) |       |
| Age (years)         |                      |          |         |
| <18                 | 11                   | 12       | 0.589   |
| ≥18                 | 4                    | 5        |         |
| Sex                 |                      |          |         |
| Male                | 6                    | 9        | 0.354   |
| Female              | 9                    | 8        |         |
| Tumor site          |                      |          |         |
| Femur/Tibia         | 8                    | 10       | 0.517   |
| Other               | 7                    | 7        |         |
| Enneking stage      |                      |          |         |
| I-II                | 12                   | 7        | 0.029*  |
| III                 | 3                    | 10       |         |
| Distant metastasis  |                      |          |         |
| Present             | 13                   | 6        | 0.004*  |
| Absent              | 2                    | 11       |         |

*P<0.05, significant difference. NR2F1-AS1, long non-coding RNA; NR2F1 antisense RNA 1; OS, osteosarcoma.

Flow cytometric analysis of apoptosis. Following transfection for 24 h, MG63 and U-2OS cells were digested with 0.25% trypsin and harvested. Subsequently, the cells were re-suspended with pre-cooled 1X PBS, centrifuged for 10 min (1,200 x g), and washed. The cells were stained with Annexin V-FITC and propidium iodide (PI) for 10 min and incubated at room temperature. Finally, the apoptosis rate was detected using a flow cytometer and analyzed on FACSCalibur (BD Biosciences).

Bioinformatics analysis. To investigate how NR2F1-AS1 regulates miR-485-5p and miR-218-5p, and how miR-485-5p and miR-218-5p regulate BIRC5, bioinformatics websites were used for analysis. The websites used were as follows: Diana Tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=IncaBase2/index), miRwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), Target Scan (http://www.targetscan.org/vert_71/) and mirBase (http://www.mirbase.org/).

Luciferase reporter assay. The NR2F1-AS1 fragments containing the predicted miR-485-5p and miR-218-5p binding sites were separately amplified by PCR (PCR thermocycling condition: 3 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec) and cloned into a pmirGLO dual luciferase miRNA target expression vector (Promega Corp.) to create a wild-type NR2F1-AS1 reporter vector (NR2F1-AS1-wild-type; NR2F1-AS1-Wt). To mutate the putative miR-485-5p and miR-218-5p binding sites in NR2F1-AS1, the sequences of the putative binding site were changed and replaced to form a NR2F1-AS1-mutated-type
Figure 3. NR2F1-AS1 exerts oncogenic function in OS. (A) Effect of NR2F1-AS1 knockdown on the migration abilities of MG63 and U-2OS cells was confirmed by performing Transwell assay. (B) Effect of NR2F1-AS1 knockdown on the invasion abilities of MG63 and U-2OS cells was identified by performing Transwell assay. **P<0.01 vs. the si-NC group. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA NR2F1 antisense RNA 1; NC, negative control.

Figure 4. miR-485-5p and miR-218-5p are direct targets of NR2F1-AS1 in OS cells. (A) Sequences matching between NR2F1-AS1 and miR-485-5p, and NR2F1-AS1 and miR-218-5p. (B) Luciferase assay was used to verify the binding between NR2F1-AS1 and miR-485-5p, and NR2F1-AS1 and miR-218-5p. **P<0.01 compared with miR-485-5p-NC group (verifying the binding between NR2F1-AS1 and miR-485-5p, the left panel); **P<0.01 compared with the miR-218-5p-NC group (verifying the binding between NR2F1-AS1 and miR-218-5p, the right panel). (C) Expression of miR-485-5p and the correlation between miR-485-5p and NR2F1-AS1 expression in 32 OS tissues. **P<0.01, tumor vs. adjacent tissues. (D) Expression of miR-218-5p and the correlation between miR-218-5p and NR2F1-AS1 expression in 32 OS tissues. **P<0.01, tumor vs. adjacent tissues. (E) Expression of miR-485-5p and miR-218-5p in MG63 and U-2OS cells transfected with si-NR2F1-AS1 or si-NC, **P<0.01, vs. the si-NC group. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA NR2F1 antisense RNA 1.
Subsequently, the recombinant plasmids and miRNAs (miR-485-5p mimics, miR-218-5p mimics or miR-NC) were co-transfected into 293T cells using Lipofectamine™ 2000. After 48 h, the luciferase activities were measured using the Dual Luciferase Reporter Gene Assay System (Promega Corp.). To investigate whether BIRC5 is a functional target of miR-485-5p and miR-218-5p, the aforementioned methods were followed and recombinant plasmids pmirGLO-BIRC5-wt/pmirGLO-BIRC5-mut were constructed. These recombinant plasmids were then co-transfected with miRNAs into 293T cells and luciferase activities were determined. Western blotting. MG63 and U-2OS cells were transfected for 48 h and were then harvested and lysed with RIPA lysis buffer (Thermo Fisher Scientific, Inc.). The protein concentration was measured by BCA (Thermo Fisher Scientific, Inc.) and then a 40 µg protein sample of each group was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Following blocking with 5% skim milk, the membrane was incubated with GAPDH and BIRC5 primary antibodies: GAPDH (cat. no. ab128915; 1:10,000 dilution; Abcam), BIRC5 (cat. no. ab76424; 1:5,000 dilution; Abcam) overnight at 4°C, followed by incubation with horseradish peroxidase-labeled goat anti-Rabbit secondary antibodies (cat. no. ab205718; 1:5,000 dilution; Abcam) for 2 h at room temperature. Finally, the immune reactive bands were visualized with a chemiluminescent kit. GAPDH was used as a reference gene.

Statistical analysis. SPSS 24.0 (IBM Corp.) and GraphPad Prism 5 (GraphPad Software, Inc.) were used for data analysis. All data are expressed as mean ± standard deviation. Student's t-test was used for the comparison of two groups. Univariate ANOVA followed by Fisher's LSD post hoc tests was used for comparisons between multiple groups. Correlation between NR2F1-AS1 and miRNA (miR-218-5p and miR-485-5p) expression was determined using Spearman's correlation analysis. The correlation between NR2F1-AS1 expression and clinical parameters of patients with OS was employed by a \( \chi^2 \) test. P<0.05 was considered to indicate a statistically significant difference.

Results

**NR2F1-AS1 expression is upregulated and exerts an oncogenic function in OS.** NR2F1-AS1 has been confirmed to facilitate the malignancy of multiple tumors. Therefore, the present study investigated the NR2F1-AS1 expression in OS. Consistent with previous studies, it was demonstrated
that NR2F1-AS expression was significantly increased in OS (Fig. 1A). Furthermore, among the 32 patients, a high level of NR2F1-AS1 was closely associated with more advanced Enneking stage (P=0.029) and distant metastasis (P=0.004; Table II). In addition, NR2F1-AS1 was significantly overexpressed in OS cell lines, especially in MG63 and U-2OS cells when compared with the normal osteoblast cell line hFOB1.19 (Fig. 1B). Thus, MG63 and U-2OS cells were selected for use in subsequent functional studies. Subsequently, the functions of NR2F1-AS1 in OS were evaluated and three siRNAs (si-NR2F1-AS1#1, si-NR2F1-AS1#2, si-NR2F1-AS1#3) were assessed to detect their knockdown efficiency. The results demonstrated that si-NR2F1-AS1#1 exhibited the highest knockdown efficiency, followed by si-NR2F1-AS1#2 and finally si-NR2F1-AS1#3, which also revealed that the transfections were successful (Fig. 2A). Therefore, si-NR2F1-AS1#1 and si-NR2F1-AS1#2 were selected for further experiments. Cell proliferation, invasion, migration and apoptosis were observed following NR2F1-AS1-knockdown. By performing a CCK-8 assay, it was determined that NR2F1-AS1-knockdown markedly reduced the proliferative capacity of the OS MG63 and U-2OS cells (Fig. 2B and C). Furthermore, the data of flow cytometry demonstrated that the apoptosis of OS cells was significantly increased following silencing of NR2F1-AS1 expression (Fig. 2D). Furthermore, it was identified that the migration and invasion of OS cells were significantly attenuated following NR2F1-AS1-knockdown (Fig. 3A and B). In summary, these findings indicated that NR2F1-AS1 expression is upregulated in OS and that NR2F1-AS1 exerts an oncogenic function in OS.

miR-485-5p and miR-218-5p are direct targets of NR2F1-AS1 in OS cells. A number of studies have demonstrated that lncRNAs combined with miRNAs exert biological functions in cancer (29), cardiovascular disease (30), acute megakaryoblastic leukemia (31) and diabetes mellitus (32). Therefore, the present study attempted to identify the potential binding sites of NR2F1-AS1. With biological software analysis, miR-485-5p and miR-218-5p were identified and it was determined that their seed regions could recognize NR2F1-AS1 sequences (Fig. 4A). The subsequent luciferase assays performed demonstrated the interactions of miR-485-5p with NR2F1-AS1, and miR-218-5p with NR2F1-AS1 (Fig. 4B). Subsequently, it was observed that miR-485-5p and miR-218-5p were expressed at significantly low levels in OS tissues, which was in contrast to NR2F1-AS1 expression. Furthermore, negative correlations were identified between miR-485-5p and NR2F1-AS1, and miR-218-5p and NR2F1-AS1 (Fig. 4C and D). Finally, it was validated that NR2F1-AS1 inhibition could result in a significant increase in miR-218-5p and miR-485-5p (Fig. 4E). In summary, these findings suggest that NR2F1-AS1 may interact with miR-485-5p and miR-218-5p to serve a role in the biological functions in OS.

NR2F1-AS1 affects OS cell biological behaviors by regulating miR-485-5p and miR-218-5p. First, we transfected miR-485-5p mimics and the negative control (NC), miR-485-5p inhibitor and the negative control (NC) to MG63 and U-2OS cells to determine whether the transfections were successful. From the results of qPCR, we revealed the transfections were successful (Fig. 5A). Subsequently, to investigate whether NR2F1-AS1 affects the biological behaviors of OS cells via miR-485-5p, the inhibitor of miR-485-5p (miR-485-5p inh) and the negative control (NC inh) were co-transfected with si-NR2F1-AS1# (si-NR2F1-AS1) into MG63 and U-2OS cells. A series of experiments were then performed. As presented in Figs. 5B and C and 6A and B, NR2F1-AS1-knockdown markedly reduced cell proliferation, migration and invasion,
and stimulated apoptosis. However, these effects were markedly attenuated by concurrent miR-485-5p-knockdown. In addition, we transfected miR-218-5p mimics and the negative control (NC) to MG63 and U-2OS cells to detect whether the transfections were successful. The results of qPCR showed that the transfections were successful (Fig. 7A). To validate that NR2F1-AS1 affects OS cell biological behaviors by regulating miR-218-5p, the inhibitor of miR-218-5p (miR-218-5p inh) and the negative control (NC inh) were co-transfected with si-NR2F1-AS1 into MG63 and U-2OS cells. As presented in Figs. 7B and C and 8A and B, it was demonstrated that suppression of NR2F1-AS1 could hinder the proliferation, migration and invasion, and promote the apoptosis of MG63 and U-2OS cells. Furthermore, the effects were substantially attenuated by the inhibition of miR-218-5p. These observations indicated that NR2F1-AS1 affects OS cell biological behaviors by targeting miR-485-5p and miR-218-5p.

**BIRC5 is a direct target of miR-485-5p and miR-218-5p in OS cells.** A number of studies have confirmed that miRNAs serve roles in numerous biological processes by regulating their mRNA targets. Therefore, the present study searched for potential targets of miR-485-5p and miR-218-5p. Through biological software analysis, BIRC5, a member of the apoptosis suppressor (IAP) gene family, was identified. It was revealed that the 3' untranslated region of BIRC5 mRNA directly interacts with miR-485-5p and miR-218-5p (Fig. 9A). Subsequently, luciferase assays verified the interactions of miR-485-5p with BIRC5, and miR-218-5p with BIRC5 (Fig. 9B). It was then validated that inhibition of miR-485-5p and miR-218-5p resulted in increased BIRC5 expression (Fig. 9C and D). In addition, BIRC5 expression was observed to be expressed at a high level in OS tissues as shown in Fig. 9E. Pearson's analysis demonstrated that BIRC5 expression was negatively correlated with miR-485-5p and miR-218-5p expression, but positively correlated with NR2F1-AS1 expression in OS tissues (Fig. 9F-H). As a whole, these observations demonstrated that BIRC5 is a direct target of miR-485-5p and miR-218-5p in OS cells.

NR2F1-AS1 affects OS cell biological behaviors by regulating BIRC5. As aforementioned, NR2F1-AS1 affects OS cell biological behaviors by regulating miR-485-5p and miR-218-5p, and miR-485-5p and miR-218-5p could target BIRC5. Therefore, it was next investigated with a number of experiments whether NR2F1-AS1 affects OS cell biological behaviors by regulating BIRC5 in OS cells.
We first transfected pcDNA3.1-BIRC5 and pcDNA3.1 into cells and the results of qPCR showed that the transfections were successful (Fig. 10A). And then through a series of experiments, we demonstrated that NR2F1-AS1-knockdown markedly decreased cell proliferation, migration and invasion, and promoted cell apoptosis (Figs. 10B and C and 11 A and B). However, this influence was substantially weakened following overexpression of BIRC5, which suggests that NR2F1-AS1 affects OS cell biological behaviors by regulating BIRC5. Combined with previous studies, it was concluded that NR2F1-AS1 facilitates the OS cell malignant phenotype by downregulating miR-485-5p and miR-218-5p, and upregulating BIRC5 expression.

Discussion

Previously, abnormal expression of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) has been reported to contribute to tumor progression. For example, in esophageal squamous cell carcinoma (ESCC), SNHG6 was identified to enhance cell malignancy (33). In osteosarcoma (OS), ANRIL has been reported to be closely associated with the malignant behavior of OS cells (9). In addition, APTR has been reported to inhibit miR-132-3p and upregulate YAP1 to accelerate OS progression (34). In hepatocellular carcinoma, DLGAP1-AS1 has been confirmed to facilitate cell proliferation via the miR-486-5p/H3F3B axis (35). NR2F1-AS1 has been reported to play a role in papillary thyroid carcinoma (14), ESCC (12), endometrial cancer (15), hepatocellular carcinoma (36) and OS (16). In OS, NR2F1-AS1 has been identified to sponge miR-483-3p to upregulate FOXA1 and enhance the malignant properties of OS cells (16). However, the mechanism of NR2F1-AS1 in OS remains unknown.

Initially, the present study determined that NR2F1-AS1 expression was upregulated in OS tissues. Furthermore, a high level of NR2F1-AS1 was closely correlated with a more advanced Enneking stage and distant metastasis. Subsequently, NR2F1-AS1 expression was found to be present at a high level in OS cells. To determine the roles of NR2F1-AS1, three siRNAs were first transfected into OS cells in order to identify the most effective siRNA for further functional experiments. The results demonstrated that knockdown of NR2F1-AS1 significantly repressed the proliferation, migration and invasion, and promoted the apoptosis of OS cells, which indicated that NR2F1-AS1 exerts an oncogenic function in OS, consistent with other studies.

A number of studies have reported that lncRNAs exert biological functions in cancer by interacting with miRNAs to regulate their target genes (10,13,30). Therefore, the present study attempted to identify the potential binding sites of NR2F1-AS1 using biological software analysis, and it was identified that the seed regions of miR-485-5p and miR-218-5p could recognize NR2F1-AS1 sequences. The subsequent luciferase assays confirmed the interactions of miR-485-5p with NR2F1-AS1, and miR-218-5p with NR2F1-AS1. miR-485-5p has been recognized as an oncogene in non-small cell lung cancer (37), glioma (38), breast cancer (39), colorectal cancer (40), OS (41) and esophageal cancer (42). miR-218-5p has been reported to inhibit the malignancy of cervical cancer (43), oral squamous cell carcinoma (44) and non-small cell lung cancer (45). To detect the functional roles of miR-485-5p and miR-218-5p in OS, the expression levels of miR-485-5p and miR-218-5p were first determined in OS tissues. The data
revealed that miR-485-5p and miR-218-5p expression levels were low in OS tissues, which were in contrast to NR2F1-AS1 expression, and both miR-485-5p and miR-218-5p were negatively correlated with NR2F1-AS1 expression. Finally, it was validated that NR2F1-AS1 inhibition led to an increase in both miR-218-5p and miR-485-5p. In summary, these findings demonstrated that NR2F1-AS1 may interact with miR-485-5p and miR-218-5p to exert biological roles in OS.
Figure 10. NR2F1-AS1 affects the cell proliferation and apoptosis of OS cells via BIRC5. (A) Expression of BIRC5 by qPCR was detected to evaluate whether the transfection was successful. *P<0.01, vs. the pcDNA3.1 group. (B) Effect of NR2F1-AS1 via BIRC5 on MG63 and U-2OS cell proliferation abilities was detected by performing CCK-8 assay. *P<0.01. (C) Effect of NR2F1-AS1 via BIRC5 on MG63 and U-2OS cell apoptosis abilities were identified by performing flow cytometry assay. *P<0.01. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA lncRNA NR2F1 antisense RNA 1; BIRC5, baculoviral inhibitor of apoptosis repeat-containing 5.

Figure 11. NR2F1-AS1 affects the cell migration and invasion of OS cells via BIRC5. (A) Effect of NR2F1-AS1 via BIRC5 on the migration abilities of MG63 and U-2OS cells was confirmed by performing Transwell assay. (B) Effect of NR2F1-AS1 via BIRC5 on MG63 and U-2OS cell invasion abilities was identified by performing Transwell assay. *P<0.01. OS, osteosarcoma; NR2F1-AS1, long non-coding RNA lncRNA NR2F1 antisense RNA 1; BIRC5, Baculoviral inhibitor of apoptosis repeat-containing 5.
It has been reported that in papillary thyroid cancer (PTC), miR-485-5p is sponged by LINC00460 and thereby upregulates Raf1 expression to facilitate PTC progression (46). In addition, in cholangiocarcinoma, miR-485-5p has been demonstrated to negatively regulate FLVCR1-AS1 (47). Furthermore, in non-small cell lung cancer, DGCR5 has been identified to sponge miR-218-5p to promote cancer progression (48). In prostate cancer, PCA3 sponges miR-218-5p and modulated HMGB1 (49). These findings demonstrate that lncRNAs can affect the behavior of tumor cells via miR-485-5p or miR-218-5p. The present study revealed that inhibition of miR-485-5p or miR-218-5p could attenuate the effects of NR2F1-AS1-knockdown on cell proliferation, migration and invasion, and apoptosis, which indicated that NR2F1-AS1 affects the malignancy of OS cells via miR-485-5p and miR-218-5p. Notably, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) was identified as a target gene of both miR-485-5p and miR-218-5p. BIRC5, a member of the apoptosis suppressor (IAP) gene family, is closely associated with tumor apoptosis. The present study demonstrated that BIRC5 not only has a higher expression level in OS tissues, but also that BIRC5 expression is negatively associated with miR-485-5p and miR-218-5p, and positively correlated with NR2F1-AS1. Furthermore, we observed that NR2F1-AS1 affects the proliferation, migration, invasion and apoptosis of OS cells via BIRC5. Therefore, these data demonstrated that NR2F1-AS1 facilitates the OS cell malignant phenotype via downregulating miR-485-5p and miR-218-5p, and then upregulating BIRC5 expression. Notably, NR2F1-AS1 was reported to enhance the malignant properties of OS by increasing FOXA1 expression via sponging of microRNA-483-3p (16). In our manuscript, we revealed that NR2F1-AS1 acted as an oncogene in osteosarcoma that facilitated osteosarcoma cell proliferation and migration through targeting miR-485-5p and miR-218-5p and then targeting BIRC5. The difference from that study was that we revealed NR2F1-AS1 exert roles by acting on two miRNAs simultaneously, which confirmed the diversity of target genes. And more importantly, we demonstrated that a new axis NR2F1-AS1/miR-485-5p/miR-218-5p/BIRC5 is involved in the tumorigenesis of OS, which revealed the multi-targeting and network regulation of IncRNA-miRNA-mRNA. However, there are some limitations in the present study. For example, only 32 OS tissues were utilized and the number of samples was small. In addition, in vivo results to confirm the effects of the investigated NR2F1-AS1 axis on tumor growth were not obtained at present. Therefore, the focus of future studies will be to collect more tissue specimens and simultaneity to illuminate the effect of the NR2F1-AS1/miR-485-5p/miR-218-5p/BIRC5 axis on tumor growth in vivo.

In conclusion, the present study provides further understanding of the oncogenic functions of NR2F1-AS1 in OS. To the best of our knowledge, the present study was the first to demonstrate that the NR2F1-AS1/miR-485-5p/miR-218-5p/BIRC5 axis is involved in the tumorigenesis of OS. These findings revealed a novel regulatory network in OS and may contribute to the identification of new therapeutic targets for OS.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

GJ performed the experiments and drafted the manuscript. YW performed the data analysis and figure design. YY conceptualized the study design. ZL carried out the sample selection. XW managed the project administration. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The patient samples were collected and the protocol was granted permission of the Medical Ethics Committee of Zhengzhou Orthopedic Hospital, and informed consents of all of the patients were obtained before the operation.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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