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Effect of lncRNA ANRIL knockdown on proliferation and cisplatin chemoresistance of osteosarcoma cells *in vitro*

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ABSTRACT

Chemoresistance is a major obstacle in treating cancer, including osteosarcoma. LncRNA ANRIL (ANRIL) is involved in the growth and metastasis of osteosarcoma cells, however, its role in chemoresistance remains unclear. In this study, ANRIL shRNA was used to knock down its endogenous expression in U2-OS and Saos-2 osteosarcoma cell lines. Our data showed that ANRIL-silenced cells were more sensitive to cisplatin: apoptotic ratio was increased and cleaved caspase-3 level was upregulated. Furthermore, the expression level of miR-125a-5p, a microRNA that can bind to ANRIL, was elevated in ANRIL-silenced cells. MiR-125a-5p inhibitor attenuated ANRIL knockdown-induced chemosensitivity to cisplatin. In addition, ANRIL knockdown resulted in a reduction in STAT3, a target of miR-125a-5p, in osteosarcoma cells. Forced overexpression of STAT3 weakened the chemosensitivity of ANRIL-silenced cells to cisplatin. In conclusion, our study demonstrates that ANRIL knockdown sensitizes osteosarcoma cells to cisplatin-induced cytotoxicity, suggesting ANRIL as a therapeutic target for osteosarcoma chemotherapy.

1. Introduction

Osteosarcoma, as the common primary bone malignancy, usually arises in the long bones, such as legs and arms [1]. The children and adolescents are among the highest incidence rate [2,3]. It is reported that the survival rate of localized disease hovers around 70%, whereas that of metastatic disease could be as low as 15–30% [4]. Currently, treatments recommended for osteosarcoma include conventional chemotherapy, followed by surgical resection of tumor, and postoperative chemotherapy [5,6]. Although these treatments improve the survival of osteosarcoma patients, their prognoses still remain poor because of chemoresistance and/or residual micrometastatic deposits [7].

Long non-coding RNAs (lncRNAs), a subgroup of non-coding RNAs without protein-coding ability, are mRNA-like transcripts with more than 200 nucleotides in length [8,9]. In recent years, many studies have demonstrated that lncRNAs participated in diverse human cancer diseases [10,11]. LncRNA ANRIL (anti-sense non-coding RNA in the INK4 locus), a cancer-associated lncRNA, is transcribed from the INK4b–ARF–INK4a gene cluster [12]. It significantly up-regulates cell proliferation in gastric cancer, prostate cancer, and hepatocellular carcinoma [13–15]. Two previous studies have shown that ANRIL expression is upregulated in osteosarcoma tissues by comparison to adjacent normal tissues [16,17]. Further, ANRIL has been reported to regulate growth and metastasis of osteosarcoma cells [18]. Several lines of evidence have shown that deregulation of lncRNAs, such as LncRNA OIP5-AS1 and LINC00161, is involved in chemoresistance of osteosarcoma cells [19,20]. In other types of cancer cells, ANRIL has been noted to promote cell chemoresistance [21,22]. However, the role that ANRIL plays in chemoresistance of osteosarcoma cells is unknown. The present study was thus performed to investigate ANRIL's role in chemoresistance of osteosarcoma cells.

LncRNAs bind to shared mircoRNA (miRNA) recognition elements, act as sponge to miRNAs, and prevent miRNAs from binding to their targets [23]. ANRIL regulates the malignant phenotypes of cancer cells by interacting with miRNAs, such as miR-323 and miR-34a [24,25]. Among the miRNAs that may interact with ANRIL, miR-125a-5p attracts our attention. Earlier studies have described miR-125a-5p as a tumor suppressor. MiR-125a-5p can regulate the sensitivity of cancer cells to chemotherapy drugs [26,27]. We thus decided to explore whether ANRIL regulates chemoresistance of osteosarcoma cells by modulating miR-125a-5p.

Here we hypothesized that ANRIL serves as a clinical biomarker and potential therapeutic target for osteosarcoma. We established osteosarcoma cell models with ANRIL knockdown, and assessed alterations in proliferation, apoptosis and chemoresistance of osteosarcoma cells to cisplatin.

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2. Materials and methods

2.1. Cell line culture

MG-63, U2-OS, Saos-2 and 293 T cells were obtained from the Shanghai Zhongqiaoxinzhou Biotech (China). U2-OS, Saos-2 and 293 T cells were cultured in DMEM with 10% fetal bovine serum (FBS), whereas MG-63 cells cultured in MEM containing 10% FBS. Then they were maintained at 37 °C in a humidified atmosphere of 5% CO2.

2.2. Construction of vectors, transfection, and treatment

In order to establish the stable control and ANRIL knockdown U2-OS and Saos-2 cell models, control or shRNA for ANRIL (NR_047539.1) were inserted into PRNAH1.1 vector. The cells were first divided into three groups: parental, control and ANRIL knockdown. They were then treated with DMSO or cisplatin (1 u M/10uM; 24 h). Control and ANRIL knockdown cells with or without cisplatin treatment were then transfected via miR-125a-5p inhibitor-NC (miR-NC) or miR-125a-5p inhibitor (miR-125 inhibitor) (GenePharma, China). In addition, full-length STAT3 (NM_003150.3) was cloned into pcDNA3.1 for STAT3 overexpression. Cisplatin treated and non-treated control and ANRIL knockdown cells were transfected by STAT3 overexpression control (STAT3 ctrl) or STAT3 overexpression (STAT3-OV) as well. All transfections were performed using Lipofectamine 2000 (Invitrogen, USA).

2.3. Real-time PCR assay

Total RNA were extracted from U2-OS and Saos-2 cell lines by TRIpure (BioTeke, China). Expression levels of ANRIL, miR-125a-5p and STAT3 were detected using Real-time PCR, which was performed with Super M-MLV Reverse transcriptase (BioTeke, China) and SYBR Green (Solarbio, China). Real-time PCR primers are listed below:

ANRIL-F: 5′-CTCCAGACAGGGTCTCACTC-3′; ANRIL-R: 5′-GGAAAT TCCTAGCTCCGTAA-3′;

miR-125a-5p-F: 5′-GCCGCTCCCTGAGACCCTT-3′; miR-125a-5p-R: 5′-GTGCAGGGTCCGAGGTATTC-3′; RT-Primer: 5′-GTTGGCTCTGGTGC AGGGTCCGAGGTATTCGCACCAGAGCCAACTCACAG-3′;

STAT3-F: 5′-TGGAGAAGGACATCAGCGGT-3′; STAT3-R: 5′-TGGTC TTCAGGTATGGGGCA-3′;

β-actin was measured as the internal control for ANRIL and STAT3 expression levels, and U6 was used for normalizing miR-125a-5p expression level. All relative gene expression values were calculated via the $2^{-\Delta\Delta Cq}$ method.

2.4. Cell proliferation assay

The cell proliferation was detected via the Cell Counting Kit-8 (CCK-8) (Sigma, USA). Cells were seeded in 96-well plates with a total volume of 100 μl medium/well. After treatment with or without DMSO or cisplatin, they were incubated with 10 μl CCK-8 solution for 1 h at 37 °C. The optical density (OD) was measured at wavelength of 450 nm using a microplate reader (BIOTEK, USA).

2.5. Colony formation assay

For colony formation assay, cells were seeded onto 35 mm dishes with a concentration of 3×10^2 cells/dish and grown for 14 days. The cells were then rinsed with PBS and fixed in 4% paraformaldehyde (Sigma, USA) for 15 min at room temperature, followed by incubation with Wright-Giemsa stain (NJJCBIO, China) for 5 min. The images were captured and colony numbers were counted.

2.6. Dual luciferase reporter assay

The 293 T cells were seeded into 12-well plates and co-transfected with miRNAs (miR-NC or miR-125) and luciferase reporter vectors. The luciferase reporter vectors were subcloned by partial ANRIL sequences or STAT3 3′ UTR fragments, which contained wild-type or mutant-type miR-125 binding site. It resulted in the produce of WT-ANRIL, MUT-ANRIL, WT-STAT3-3′UTR and MUT-STAT3-3′UTR reporter plasmids. Dual luciferase reporter assay kit (Promega, USA) was used to determine the fluorescence intensity.

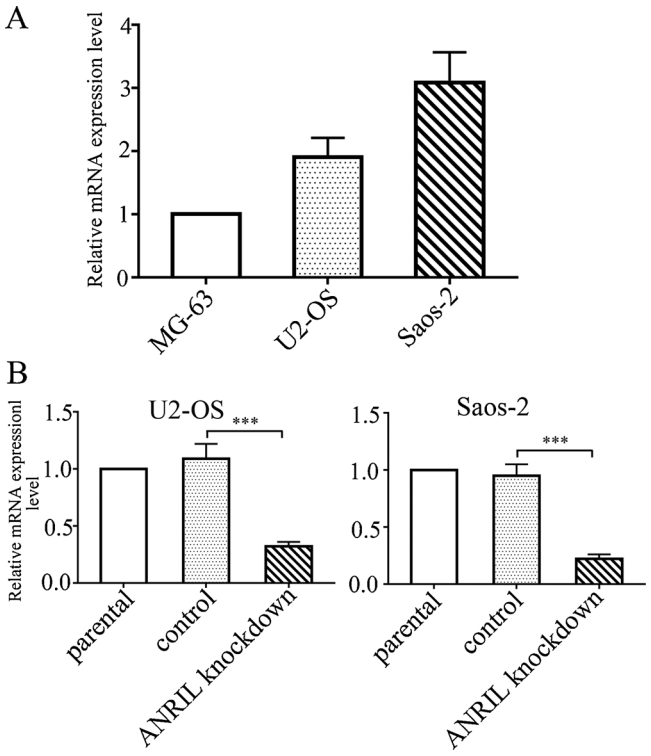

Fig. 1. ANRIL is knocked down in osteosarcoma cell lines. (A) ANRIL level in MG-63, U2-OS and Saos-2 cell lines. (B) Real-time PCR displayed that ANRIL was significantly knocked down in U2-OS and Saos-2 cell lines. Data are shown as mean ± SD. n = 3. ***p < 0.001.

2.7. Apoptosis assay

Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, China) was used to perform the cell apoptosis assay. Cells treated with or without indicated drugs were collected respectively and then washed with PBS, followed by incubation with a mixture of 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) for 15 min. Then cell apoptosis was analyzed using a flow cytometer (BD, USA).

2.8. Western blot

RIPA lysis buffer (Beyotime, China) supplemented with 1% PMSF (Beyotime, China) were prepared for cell protein lysates. Total proteins were extracted from cells and its concentrations were quantified using the BCA Protein Assay Kit (Beyotime, China). The denatured proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), and then electrophoretically transferred on a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Blocking buffer was applied to PVDF membranes for 1 h at room temperature. Subsequently, the membranes were incubated with specific primary antibodies, including cleaved-caspase-3 antibody (1:1000, CST, USA), STAT3 antibody (1:1000, CST, USA) and β-actin antibody (1:500, Bioss, China), at 4 °C overnight. After being washed with TBST buffer, the membranes were incubated with IgG-HRP (1:5000, Beyotime, China) for 45 min at room temperature. The enhanced chemiluminescence (ECL) system

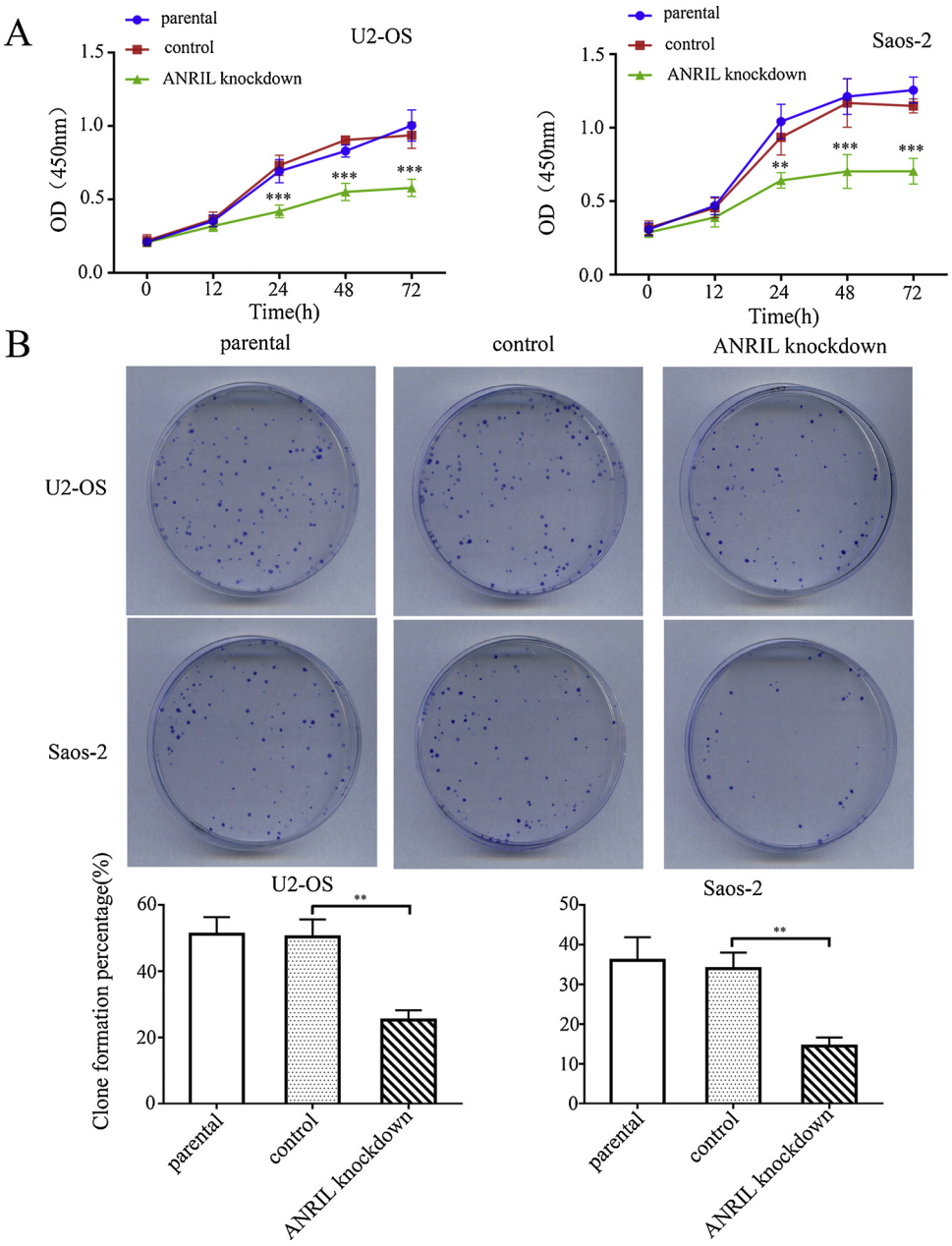

Fig. 2. Downregulation of ANRIL inhibits proliferation of osteosarcoma cells. (A) Cell proliferation was detected by CCK-8 assay in U2-OS and Saos-2 cell lines. (B) Clone formation of the osteosarcoma cells. Data are shown as mean ± SD. n = 3. **p < 0.01, ***p < 0.001.

(Beyotime, China) was used for following detection and visualization, and band densities were analyzed by Gel-Pro-Analyzer Software.

2.9. Statistical analysis

GraphPad Prism 7.0 software was used for statistical analysis. The statistical significance was performed with one-way ANOVA or two-way ANOVA followed by Turkey. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. ANRIL is knocked down in osteosarcoma cell lines

To determine whether ANRIL is knocked down successfully in U2-OS and Saos-2 cells, ANRIL level was detected in osteosarcoma cell lines (MG-63, U2-OS and Saos-2). The results showed that ANRIL levels in U2-OS and Saos-2 cells were higher than that in MG-63 cells (Fig. 1A). The efficacies of ANRIL knockdown were examined using real-time PCR, and a significant lower level of ANRIL was found in ANRIL knockdown osteosarcoma cell lines including U2-OS and Saos-2 cells than that in the control (Fig. 1B).

3.2. Downregulation of ANRIL inhibits proliferation of osteosarcoma cells

We established stable cell models to explore the effect of ANRIL knockdown on the proliferation of osteosarcoma cells. The proliferation of U2-OS and Saos-2 cells was detected via CCK-8 assay and clone formation assay. CCK-8 assay revealed that the proliferation of ANRIL knockdown cells was significantly decreased, according to the OD measured at 24, 48 and 72 h time points (Fig. 2A). A similar result was obtained from a plate clone formation assay that the clone formation percentage was markedly lower in ANRIL knockdown than that in control U2-OS and Saos-2 cells (Fig. 2B).

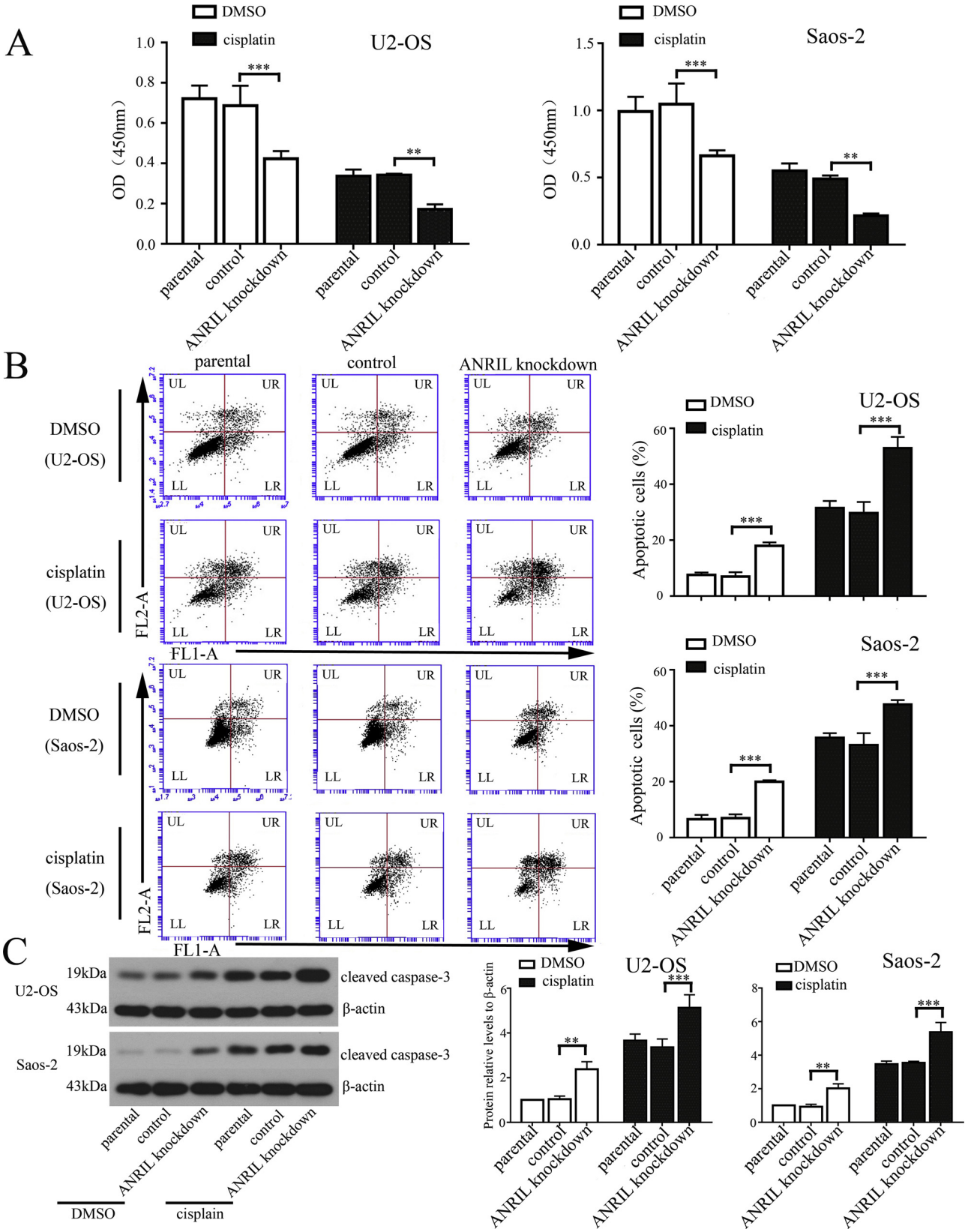

Fig. 3. The effect of ANRIL knockdown on chemoresistance in osteosarcoma cells. (A) CCK-8 assay was used to measure the cell proliferation in U2-OS and Saos-2 cell lines with DMSO or cisplatin treatment. (B) Cell apoptosis and (C) the level of cleaved caspase-3 were analyzed in U2-OS and Saos-2 cell lines with DMSO or cisplatin treatment. Data are shown as mean ± SD. n = 3. **p < 0.01, ***p < 0.001.

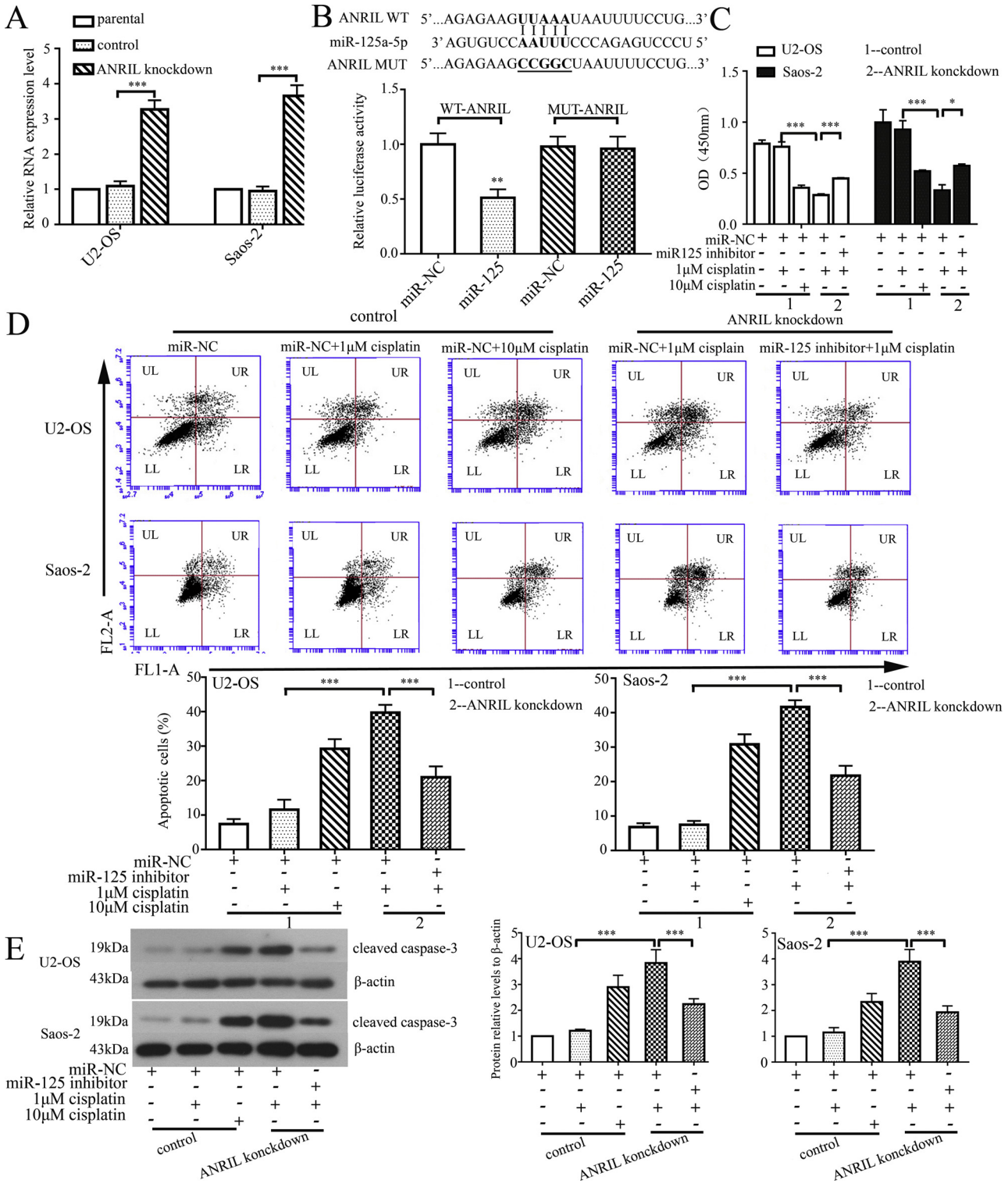

Fig. 4. MiR-125a-5p is involved in the ANRIL knockdown-mediated chemoresistance in osteosarcoma cells. (A) The level of miR-125a-5p in U2-OS and Saos-2 cell lines. (B) Effect of miR-125a-5p on the luciferase activity of WT-ANRIL and MUT-ANRIL reporter systems was detected via luciferase reporter assay. (C) CCK-8 assay was used to examine cell proliferation in U2-OS and Saos-2 cell lines. (D) Flow cytometry analysis was used to analyze cell apoptosis. (D) The expression level of cleaved caspase-3 was measured using western blot analysis. Data are shown as mean ± SD. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.

3.3. The effect of ANRIL knockdown on chemoresistance in osteosarcoma cells

To further confirm how ANRIL knockdown affects chemoresistance in osteosarcoma cells, cisplatin treatment was performed and the following experiments were performed. The result from CCK-8 assay revealed that ANRIL knockdown further decreased the cisplatin-induced cell proliferation in both U2-OS and Saos-2 cells (Fig. 3A). The flow cytometric analysis showed that ANRIL knockdown significantly enhanced cell apoptosis induced by cisplatin. In accordance with these results, knockdown of ANRIL was detected to be remarkably increased cisplatin-induced cleaved caspase-3 level via western analysis (Fig. 3B,

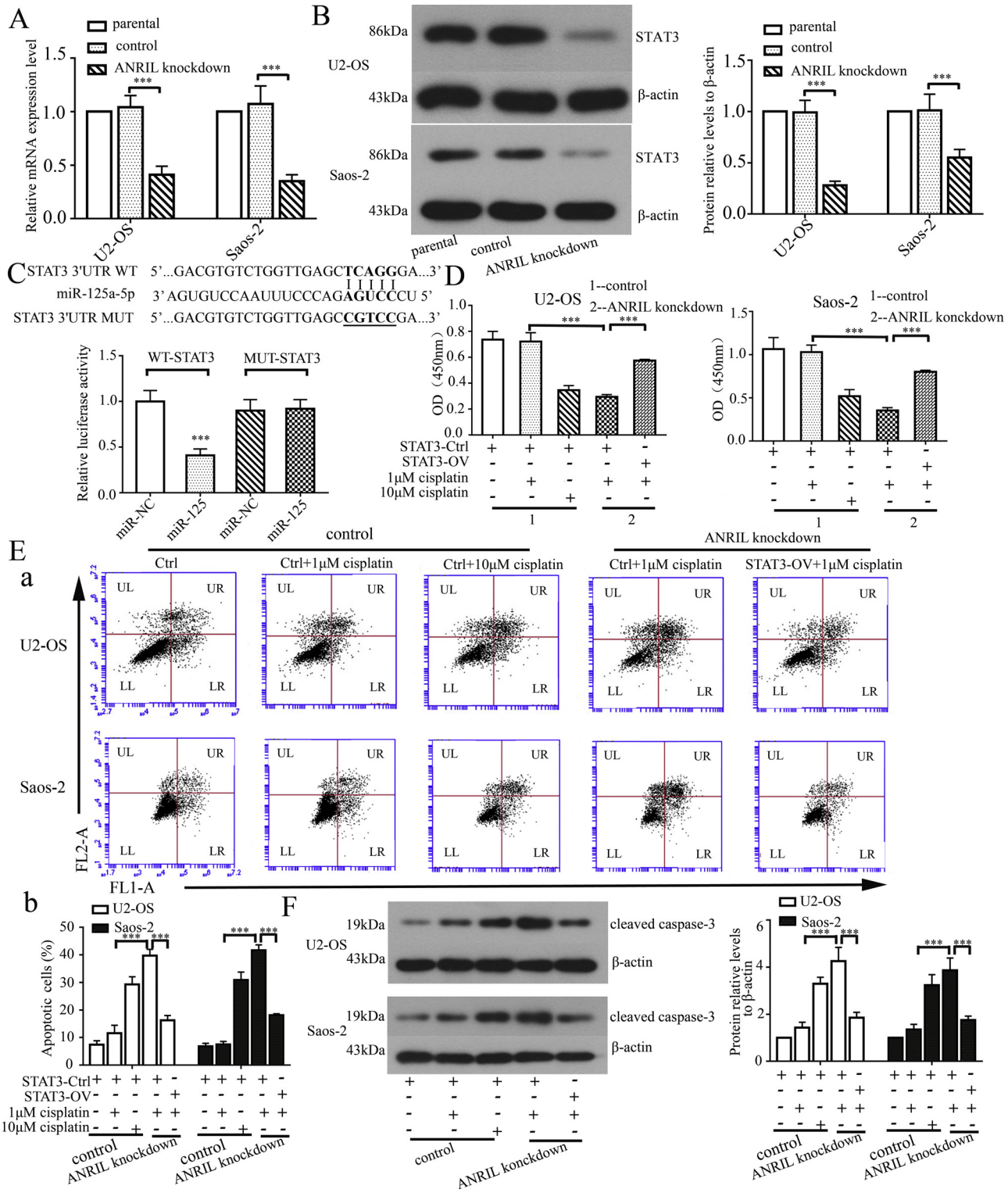

Fig. 5. ANRIL knockdown mediates chemoresistance by miR-125a-5p/STAT3 in osteosarcoma cells. (A) Real-time PCR and (B) western blot analysis were conducted to detect the level of STAT3. (C) The WT-STAT3 or MUT-STAT3 was co-transfected with miR-125a-5p control or miR-125a-5p inhibitor by luciferase reporter assay. (D) The proliferation, (E) cell apoptosis and (F) cleaved caspase-3 level were examined in U2-OS and Saos-2 cell lines by CCK-8 assay, flow cytometry and western analysis, respectively. Data are shown as mean ± SD. n = 3. ***p < 0.001.

C).

3.4. MiR-125a-5p is involved in the ANRIL knockdown-mediated chemoresistance in osteosarcoma cells

To verify whether miR-125a-5p is able to modulate ANRIL knockdown-mediated chemoresistance, we first detected miR-125a-5p level in parental, control and ANRIL knockdown osteosarcoma cells. A significant higher level of miR-125a-5p was found in ANRIL knockdown than the control using real-time PCR (Fig. 4A). In addition, ANRIL sequence was found to contain the binding target of miR-125a-5p. The result showed that miR-125a-5p inhibitor apparently suppressed

luciferase activity in the WT-ANRIL reporter systems, but had no effect on luciferase activity in MUT-ANRIL reporter systems in 293 T cells (Fig. 4B). Moreover, miR-125a-5p inhibitor was observed to elevate cell proliferation (Fig. 4C) and reduce cell apoptosis (Fig. 4D, E) in ANRIL knockdown cisplatin-induced osteosarcoma cells.

3.5. ANRIL knockdown mediates chemoresistance by miR-125a-5p/STAT3 in osteosarcoma cells

As described above, miR-125a-5p might be involved in ANRIL knockdown-mediated chemoresistance. In order to further explore the mechanism underlying ANRIL/miR-125a-5p axis mediated cisplatin chemoresistance in osteosarcoma cells, real-time PCR and western blot analysis were first conducted to detect the STAT3 level. The results indicated that the expression level of STAT3 was significantly reduced in ANRIL knockdown osteosarcoma cells (Fig. 5A, B). Additionally, STAT3 was demonstrated to be a candidate target of miR-125a-5p. Dual luciferase reporter assay displayed that miR-125a-5p inhibitor significantly suppressed luciferase activity in WT-STAT3, but no effect in MUT-STAT3, which validated the interaction between miR-125a-5p and STAT3 (Fig. 5C). In ANRIL knockdown cisplatin-induced cells, STAT3 overexpression was observed to increase cell proliferation, decrease apoptosis and reduce cleaved caspase-3 level via CCK-8 assay, flow cytometry and western analysis, respectively (Fig. 5D-F).

4. Discussion

ANRIL expression is correlated with E-cadherin. MG63 cells lose their E-cadherin in high invasive types [28], while U2-OS [29] and Saos-2 cells [30] do not. In the present study, ANRIL was knocked down in U2-OS and Saos-2 cells with relatively higher ANRIL expression. We found that ANRIL knockdown enhanced cisplatin-induced apoptosis in osteosarcoma cells, suggesting that ANRIL was involved in cisplatin resistance. In order to investigate ANRIL knockdown-mediated chemoresistance, the interaction between miR-125a-5p and ANRIL was determined and miR-125a-5p was identified to be related to ANRIL knockdown-mediated chemoresistance. Furthermore, STAT3 was validated as a target gene of miR-125a-5p in osteosarcoma cells. The effect of STAT3 overexpression on cell proliferation, cell apoptosis and chemoresistance was observed to be consistent with that of miR-125a-5p inhibitor in ANRIL knockdown osteosarcoma cells. These findings strongly suggest ANRIL plays an important role in cell growth and chemoresistance via ANRIL/miR-125a-5p/STAT3 axis in osteosarcoma.

There have been mounting evidence indicating that ANRIL act as a tumor promoter in some tumors [31,32]. For example, ANRIL was found to enhance invasion and metastasis of cells by inhibiting TGF-β/Smad signaling pathway in thyroid cancer cells [33]. Zhang et al [34] revealed that ANRIL, which was highly expressed in cervical cancer, increased E-cadherin expression and augmented migration of cervical cancer cells. It has been reported that the effect of ANRIL on proliferative activity is associated with tumor progression. Knockdown of ANRIL was showed to inhibit cell proliferation and colony-forming ability in colorectal cancer [35]. Moreover, there is evidence indicated that ANRIL, acting as a negative prognostic factor for patients with non-small cell lung cancer, promoted cell proliferation by epigenetic-silencing KLF2 and P21 transcription [31]. Then, we found that knockdown of ANRIL resulted in significant proliferation reduction in osteosarcoma cells, which is in accordance with recent observations [16,18]. Therefore, we demonstrate that ANRIL may directly or indirectly take part in cell proliferation and knockdown of ANRIL had a suppressive effect on cell proliferation in osteosarcoma.

It has been reported that ANRIL could be involved in the chemoresistance [21,22]. Up to now, accumulating evidence indicated that ANRIL was unregulated in lung adenocarcinoma promoting the chemoresistance of lung adenocarcinoma cells via modulating cleaved-PARP and Bcl-2 levels [36]. A previous literature demonstrated that ANRIL was highly expressed in nasopharyngeal carcinoma, and down-regulation of it could increase cisplatin-induced cytotoxicity [37]. Here we used cisplatin to study the effect of ANRIL knockdown on chemoresistance in osteosarcoma. Correspondingly we found that ANRIL knockdown enhanced cisplatin-induced cell apoptosis in osteosarcoma cells, suggesting that ANRIL was involved in cisplatin resistance and knockdown of ANRIL could improve cisplatin chemosensitivity.

In addition, we found that the level of miR-125a-5p was significant higher in ANRIL knockdown osteosarcoma cells. Luciferase reporter assay showed miR-125a-5p inhibitor largely weakened the luciferase activity in WT-ANRIL verifying that ANRIL interacted closely with miR-125a-5p in this study. Tong et al [38] have proved that miR-125a-5p inhibited cell proliferation and facilitated apoptosis in colon cancer. The results from our study showed that miR-125a-5p inhibitor increased cell proliferation, decreased cell apoptosis, and further attenuated ANRIL knockdown-induced chemosensitivity to cisplatin in osteosarcoma cells. The data suggests that miR-125a-5p may act as a tumor repressor and enhance cisplatin chemosensitivity of ANRIL knockdown in osteosarcoma cells.

Furthermore, our study revealed that STAT3, as a target gene of miR-125a-5p, had low expression level in ANRIL knockdown osteosarcoma cells. It has been evidenced that miR-125a-5p inhibited the proliferation and induced cell apoptosis in lung carcinoma cells by suppressing STAT3 expression [39]. In the study, we demonstrated that overexpression of STAT3 promoted cell proliferation, suppressed cell apoptosis and subsequently impaired cisplatin chemosensitivity in ANRIL knockdown osteosarcoma cells.

Although we found that ANRIL regulated the chemosensitivity of osteosarcoma cells to cisplatin by interacting with miR-125a-5p/STAT3 axis *in vitro*, we did not analyze the expression levels of ANRIL/miR-125a-5p/STAT3 in osteosarcoma samples. Osteosarcoma samples are being collected by our group. The correlation of clinic outcomes of osteosarcoma patients with ANRIL expression will be analyzed in the near future.

In conclusion, we demonstrate that ANRIL mediates proliferation, apoptosis and cisplatin chemoresistance of osteosarcoma cells by targeting miR-125a-5p/STAT3, which provides us insights into the molecular mechanism underlying the effect of lncRNA ANRIL on osteosarcoma and a therapeutic target for treatment of osteosarcoma.

Conflict of interests

No potential conflict of interest was reported by the authors.

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