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LncRNA FOXC2-AS1 stimulates proliferation of melanoma *via* silencing p15 by recruiting EZH2

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the role of FOXC2-AS1 in promoting the proliferative ability and inhibiting apoptosis of melanoma by silencing p15, thereafter regulating the progression of melanoma.

PATIENTS AND METHODS: FOXC2-AS1 levels in melanoma patients with or without metastasis and those with the tumor in different stages were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Regulatory effects of FOXC2-AS1 on viability and apoptosis in melanoma cells were assessed, and subcellular distribution of FOXC2-AS1 was analyzed. Subsequently, the interactions of FOXC2-AS1 with EZH2 and SUZ12 were explored by RNA-Binding Protein Immunoprecipitation (RNA-RIP) assay. Through chromatin immunoprecipitation (ChIP) assay, the role of FOXC2-AS1 to regulate p15 transcription by recruiting EZH2 was verified. At last, regulatory effects of FOXC2-AS1/p15 axis on viability and apoptosis in melanoma cells were investigated.

RESULTS: It was found that FOXC2-AS1 was upregulated in melanoma tissues, especially those with metastasis or stage II-IV. Melanoma patients expressing high level of FOXC2-AS1 showed worse survival than those with low level. Knockdown of FOXC2-AS1 inhibited viability, and stimulated apoptosis in A375 and sk-mel-110 cells. Besides, P15 level was upregulated in melanoma cells transfected with si-FOXC2-AS1, and FOXC2-AS1 was mainly distributed in cytoplasm. RNA-RIP assay confirmed that FOXC2-AS1 was mainly enriched in anti-EZH2 and aniti-SUZ12. Knockdown of EZH2 could markedly upregulate protein level of p15 in melanoma cells. Furthermore, it was verified that FOXC2-AS1 inhibited p15 transcription *via* recruiting EZH2, and the knockdown of p15 could partially reverse the regulatory effects of FOXC2-AS1 on viability and apoptosis in melanoma.

CONCLUSIONS: FOXC2-AS1 stimulates proliferative ability in melanoma *via* silencing p15.

Key Words:
Melanoma, FOXC2-AS1, P15.

Introduction

Skin malignant melanoma, basal cell carcinoma, and squamous cell carcinoma are three forms of skin cancer. Melanoma originates from melanocytes, which are responsible for producing pigments. It mainly affects skin, and sometimes, it also occurs in the eyes, nasal membrane, vagina, and oral, pharyngeal, and anal mucosa[1]. Generally speaking, melanoma arises from the existing tendons or black spots on the skin. In the early phase, it develops slowly and eventually metastasizes into lymph nodes and distant organs[2,3]. About 80% of skin cancer-related deaths are attributed to melanoma[3]. Therefore, it is of significance to uncover the potential mechanisms of melanoma.

The majority of human genomes are transcribed into non-coding RNAs. Among them, those with over 200 nt long are known as long non-coding RNAs (lncRNAs)[4]. Although lncRNAs lack the ability to encode proteins, they contain abundant ncRNAs and transcriptional regulatory elements. Functionally, lncRNAs serve as oncogenes or tumor-suppressor genes and they keep in a balance under the normal circumstance[5]. LncRNA FOXC2-AS1 is believed to be a carcinogenic gene. In osteosarcoma, FOXC2-AS1 triggers adriamycin resistance by targeting ABCB1[6].

P15 is a tumor-suppressor gene located on chromosome 9p21. Competitive binding of p15 with cyclin D1 to CDK4 and CDK6 inhibits phosphorylation of Rb products, thus preventing cell cycle progression from G1 phase to S phase[7]. P15 is mutated, deleted, and methylated during the development of many tumors, thereby losing its activity[8-10]. It is reported that p15 is lowly expressed in melanoma[11]. In non-small cell lung cancer (NSCLC), FOXC2 stimulates disease progression by downregulating p15[12]. In this paper,

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the potential functions of FOXC2-AS1 in regulating the progression of melanoma were mainly explored.

Patients and Methods

Baseline Characteristics

Melanoma tissues were surgically resected from melanoma patients treated in Zhejiang Provincial People's Hospital, while normal skin tissues were collected from patients undergoing skin transplantation. Tissues were stored at –80°C. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of Zhejiang Provincial People's Hospital and conducted in accordance with the Declaration of Helsinki.

Cell Culture and Transfection

Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL penicillin and 100 μg/mL streptomycin. After 4-5 times of cell passage, those in good condition were inoculated and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) until 80% confluence. The original medium was replaced with fresh medium 24 h later. si-FOXC2-AS1-1#: 5'-GCCTAATATAACCGGACTTT-3', si-FOXC2-AS1-2#: 5'-GCTTATAGTACATACCCCAT-3', si-FOXC2-AS1-3#: 5'-CGTGATATAACTCCTAACCT-3', and p15 siRNA: 5'-UGUUCGGCUUAGCAGUGGUUG-3'.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Through reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. The primer sequences are listed as follows: FOXC2-AS1: Forward: 5'-CACAGTGTAGGAGAGTAATCAG-3', Reverse: 5'-AAGCTCCATTTCGCAACCTTAC-3', p15: Forward: 5'-TTGATTGAAGGGCAGAGGA-3', Reverse: 5'-TTGGTCAGCACAGATCATCG-3', GAPDH: Forward: 5'- TGACTTCAACAGCGACACCCA -3', Reverse: 5'- GGAGTGTTGGAGAAGTCATATTAC -3'.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 1×10^3 cells/well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Flow Cytometry

Cells were inoculated in 6-well plate with 5×10^5 cells per well. After 800 rpm centrifugation for 3 min, cells were re-suspended in 200 μL of binding buffer, and subsequently incubated with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) in the dark for 20 min. At last, cell apoptosis was determined by flow cytometry (FACSCalibur, BD Biosciences, Detroit, MI, USA).

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

RNA-RIP (RNA-Binding Protein Immunoprecipitation) Assay

Cells were collected and processed according to the procedures of Millipore Magna RIP (RNA-Binding Protein Immunoprecipitation) Kit (Millipore, Billerica, MA, USA). Thereafter, they were incubated with corresponding antibodies or anti-IgG at 4°C overnight. A protein-RNA complex was obtained after capturing intracellular specific proteins by the antibody. Subsequently, proteins were digested by proteinase K and the RNAs were extracted. During the experiment, the magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. The immunoprecipitant RNA was finally subjected to qRT-PCR for determining the relative level.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using the Magna ChIP A/G Kit (Millipore, Billerica, MA, USA). Chromatin immunoprecipitated DNA was eluted, reversely X-linked, purified, and subjected to qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing differences between two groups. Survival analysis was conducted by Kaplan-Meier method, followed by Log-rank test. $p<0.05$ indicated the significant difference.

Results

FOXC2-AS1 Was Upregulated in Melanoma

Compared with normal skin tissues, FOXC2-AS1 was upregulated in melanoma tissues (Figure 1A). FOXC2-AS1 level remained higher in metastatic melanoma patients than those without metastasis (Figure 1B). In particular, melanoma patients in stage 0-I expressed a lower level of FOXC2-AS1 than those in stage II-IV (Figure 1C). Besides, survival analysis revealed that melanoma patients expressing high level of FOXC2-AS1 suffered worse prognosis (Figure 1D).

Silence of FOXC2-AS1 Inhibited Proliferative Ability and Stimulated Apoptosis in Melanoma

In vitro, FOXC2-AS1 was highly expressed in melanoma cell lines (Figure 2A). Among the four tested melanoma cell lines, A375 and sk-mel-110 cells expressed the highest abundance of FOXC2-AS1, so they were selected for the following experiments. Subsequently, transfection efficacy of three FOXC2-AS1 siRNAs was tested in A375 and sk-mel-110 cells, and si-FOXC2-AS1-2# was selected due to its best performance (Figure 2B). Moreover, CCK-8 results showed that knockdown of FOXC2-AS1 decreased viability in melanoma (Figure 2C). In addition, apoptotic rate increased after silence of FOXC2-AS1 in A375 and sk-mel-110 cells (Figure 2D).

FOXC2-AS1 Inhibited p15 Transcription Through Recruiting EZH2

Subcellular distribution analysis depicted that FOXC2-AS1 was mainly distributed in the cytoplasm (Figure 3A). LncRNAs are able to inhibit downstream gene expressions by recruiting PRC2, a polycomb-group protein containing methyltransferase EZH2[13,14]. Here, RNA-RIP as-

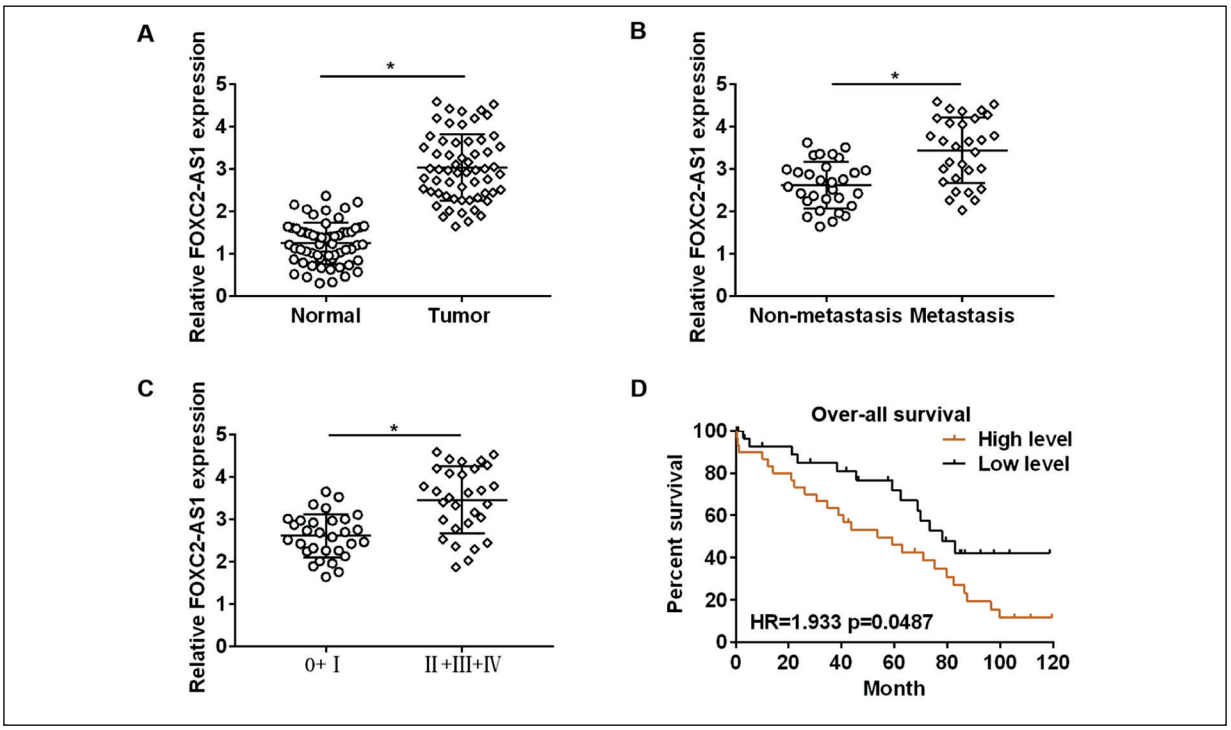

Figure 1. FOXC2-AS1 is upregulated in melanoma. **A,** FOXC2-AS1 levels in melanoma tissues and normal skin tissues. **B,** FOXC2-AS1 levels in melanoma patients with or without metastasis. **C,** FOXC2-AS1 levels in melanoma patients in stage 0-I or stage II-IV. **D,** Overall survival in melanoma patients with high level or low level of FOXC2-AS1.

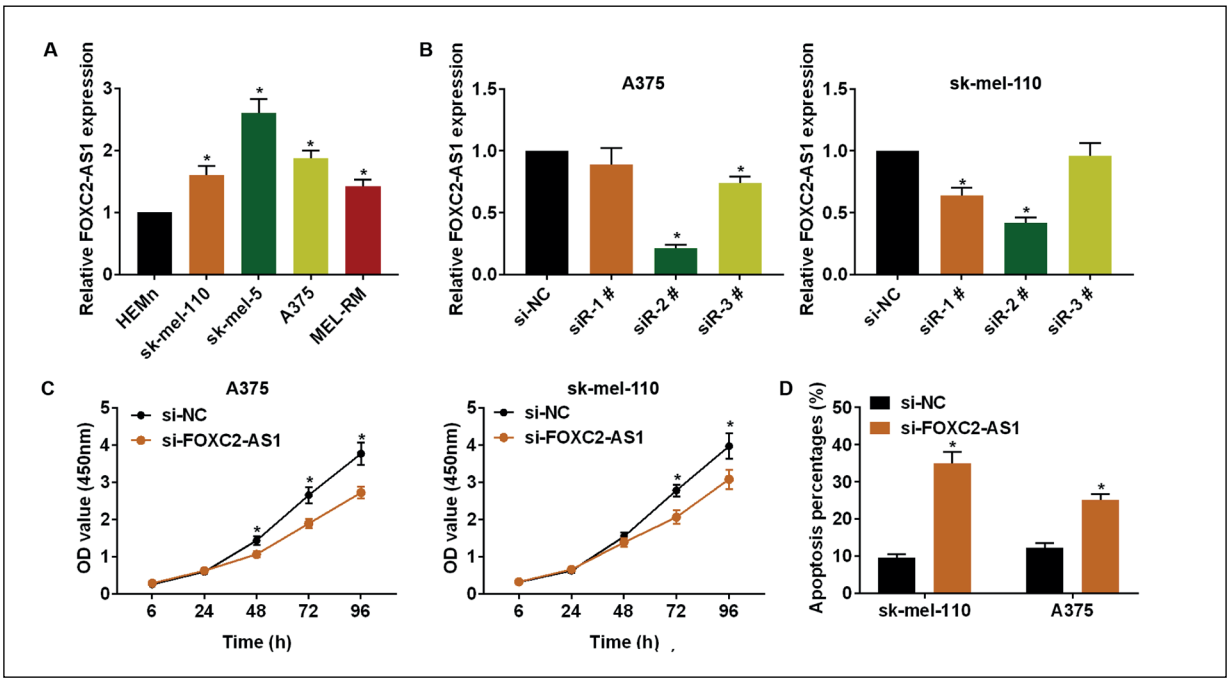

Figure 2. Silence of FOXC2-AS1 inhibits proliferative ability and stimulates apoptosis in melanoma. **A,** FOXC2-AS1 level in melanoma cell lines. **B,** Transfection efficacy of si-FOXC2-AS1-1#, si-FOXC2-AS1-2# or si-FOXC2-AS1-3#. **C,** Viability in A375 and sk-mel-110 transfected with si-NC or si-FOXC2-AS1. **D,** Apoptosis in A375 and sk-mel-110 transfected with si-NC or si-FOXC2-AS1.

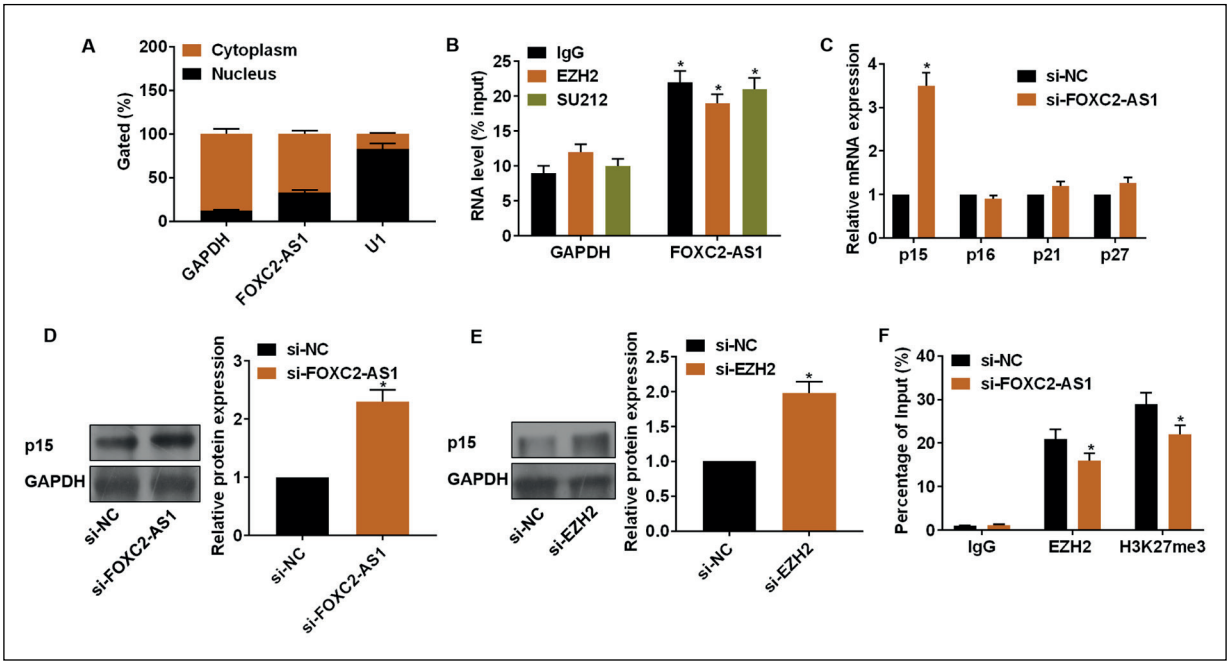

Figure 3. FOXC2-AS1 inhibits p15 transcription by recruiting EZH2. **A,** Subcellular distribution of FOXC2-AS1. GAPDH and U1 are the internal references for cytoplasm and nucleus, respectively. **B,** Enrichment of GAPDH or FOXC2-AS1 in anti-IgG, anti-EZH2, and anti-SUZ12. **C,** Relative levels of p15, p16, p21, and p27 in A375 cells transfected with si-NC or si-FOXC2-AS1. **D,** Protein level of p15 in A375 cells transfected with si-NC or si-FOXC2-AS1. **E,** Protein level of p15 in A375 cells transfected with si-NC or si-EZH2. **F,** Immunoprecipitant of FOXC2-AS1 in anti-IgG, anti-EZH2 and anti-H3K27me3 in A375 cells transfected with si-NC or si-FOXC2-AS1.

say demonstrated that FOXC2-AS1 mainly interacted with EZH2 and SUZ12 (Figure 3B). After knockdown of FOXC2-AS1, mRNA and protein levels of p15 were upregulated (Figure 3C, 3D). Moreover, p15 was upregulated by knockdown of EZH2 (Figure 3E). Subsequently, ChIP assay confirmed that knockdown of FOXC2-AS1 decreased the binding ability between FOXC2-AS1 and EZH2 (Figure 3F). It is suggested that FOXC2-AS1 inhibits p15 transcription by recruiting EZH2.

Knockdown of p15 Partially Reversed the Role of Downregulated FOXC2-AS1 on Proliferative Ability in Melanoma

In A375 cells transfected with si-p15, protein level of p15 was markedly downregulated, which was partially reversed by co-transfection of si-FOXC2-AS1 (Figure 4A). The decreased viability in A375 cells transfected with si-FOXC2-AS1 was reversed by knockdown of p15 (Figure 4B). In addition, the increased apoptosis in melanoma cells with FOXC2-AS1 knockdown was reversed by co-transfection of si-p15 (Figure 4C). It is indicated that p15 is involved in FOXC2-AS1-regulated progression of melanoma.

Discussion

Melanoma is a newly developed malignancy evolving from cytochrome nevus, dysplasia nevus, and neurospinal melanocytes. The pathogenesis of melanoma is complicated. Sun exposure, traumatic stimulation, immunosuppression, and genetic variation are all risk factors for melanoma[15]. As a highly fatal skin malignancy, melanoma is characterized as high level of malignancy, rapid progression, high rate of metastasis, and insensitivities to chemotherapy or radiotherapy. The prognosis of advanced melanoma is extremely poor, with shorter than 1 year of median survival and lower than 15% of 5-year survival[16].

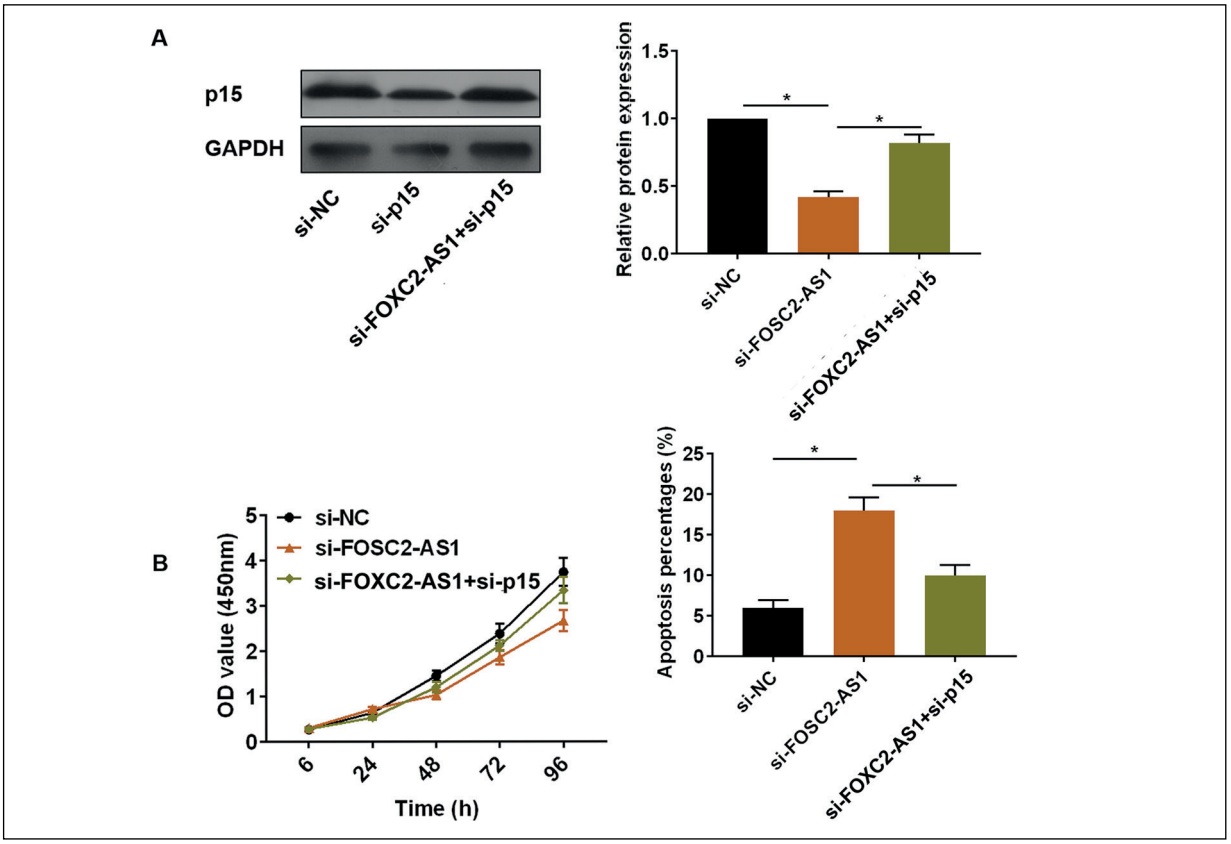

Figure 4. Knockdown of p15 partially reverses the role of downregulated FOXC2-AS1 on proliferative ability in melanoma. **A,** Protein level of p15 in A375 cells transfected with si-NC, si-p15 or si-p15+si-FOXC2-AS1. **B,** Viability in A375 cells transfected with si-NC, si-FOXC2-AS1 or si-FOXC2-AS1+si-p15. **C,** Apoptosis in A375 cells transfected with si-NC, si-FOXC2-AS1 or si-FOXC2-AS1+si-p15.

Increasing differentially expressed lncRNAs have been identified in human cancer tissues due to the rapid progression made on the new generation of sequencing technology[17-19]. Growing evidence[20] supports that lncRNAs can function as competing endogenous RNAs (ceRNAs) by competitively binding to miRNAs through their miRNA response elements (MRE) and subsequently regulate target RNA expression. FOXC2-AS1 is discovered to be closely linked to tumor proliferation[21,22]. In prostate cancer, FOXC2-AS1 is upregulated, which stimulates tumor cell growth by sponging miR-1253 to further upregulate EZH2[23]. FOXC2-AS1 is associated with the poor prognosis of breast cancer, which affects proliferative ability, cell cycle progression, and apoptosis[24]. In this paper, FOXC2-AS1 was upregulated in melanoma tissues, and its level remained higher in those with metastasis or in the advanced stage. High level of FOXC2-AS1 predicted a poor prognosis in melanoma patients.

P15 is a well-known tumor-suppressor gene that is capable of arresting cell cycle progression in G1 phase. Mutation of p53 leads to carcinogenesis[25]. Mutant p15 is observed in many types of human cancers[12,26]. Sun et al[12] showed that FOXC2-AS1 stimulates the malignant progression of NSCLC *via* downregulating p15. Our findings uncovered that FOXC2-AS1 inhibited p15 transcription by recruiting EZH2. Overexpression of FOXC2-AS1 was found to raise the expressions of EZH2 and SU212, but after FOXC2-AS1 was knocked down, the recruitment of EZH2 decreased. Notably, knockdown of p15 partially reversed the regulatory effects of downregulated FOXC2-AS1 on viability and apoptosis in melanoma cells. Collectively, FOXC2-AS1 promoted proliferative ability and inhibited apoptosis by targeting p15, thus aggravating the progression of melanoma.

Conclusions

The results of this study suggested the carcinogenic role of FOXC2-AS1 in the development of melanoma. FOXC2-AS1 can promote the proliferation of tumor cells by recruiting EZH2 to silence p15, which provides a new strategy for understanding and treating melanoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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