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LncRNA FAL1 promotes the development of oral squamous cell carcinoma through regulating the microRNA-761/CRKL pathway

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Abstract. – OBJECTIVE: This study aims to elucidate the regulatory effect of long non-coding RNA (lncRNA) FAL1 on the tumorigenesis of oral squamous cell carcinoma (OSCC), and to explore its underlying mechanism.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect the expression levels of lncRNA FAL1, microRNA-761 and CRKL in 20 pairs of OSCC tissues and adjacent normal oral tissues. Meanwhile, their expressions in OSCC cell lines were also determined by qRT-PCR. The protein expression of CRKL in OSCC tissues was detected by Western blot. The cell counting kit-8 (CCK-8) assay was performed to access the proliferation of SCC25 and HN4 cells transfected with si-FAL1. The binding conditions between lncRNA FAL1 with microRNA-761, and microRNA-761 with CRKL were tested by the Dual-Luciferase reporter gene assay. Gain-of-function experiments were conducted to determine the proliferation of OSCC cells co-transfected with si-FAL1 and microRNA-761 inhibitor. Furthermore, the proliferative potential of OSCC cells was evaluated after co-transfection of si-FAL1 and CRKL overexpression plasmid.

RESULTS: LncRNA FAL1 was highly expressed in OSCC tissues and cell lines. The proliferative capacity of OSCC cells was significantly inhibited by lncRNA FAL1 knockdown. The mRNA expression of microRNA-761 was lowly expressed in OSCC tissues and cell lines. Dual-Luciferase reporter gene assay showed that lncRNA FAL1 directly bound to microRNA-761. Meanwhile, microRNA-761 expression was negatively regulated by FAL1. CRKL was verified as the target gene of microRNA-761. Both the mRNA and protein levels of CRKL were remarkably upregulated in OSCC tissues and cell lines. CRKL expression was found to be negatively regulated by microRNA-761 in OSCC cells. Lowly expressed microRNA-761 reversed the inhibitory effect of lncRNA FAL1 knockdown on the proliferative potential of OSCC cells. In addition, the overexpression of CRKL reversed the inhibitory effect of lncRNA FAL1 down-regulation on the proliferative potential of OSCC cells as well.

CONCLUSIONS: LncRNA FAL1 is highly expressed in OSCC. Moreover, it promotes the development of OSCC by regulating CRKL expression as a sponge of microRNA-761.

Key Words:
LncRNA FAL1, MicroRNA-761, CRKL, Oral squamous cell carcinoma (OSCC).

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck, which is originated from oral mucosa. The incidence of OSCC accounts for more than 95% of all oral malignant tumors, ranking sixth among all malignant tumors. Previous studies have estimated that about 400,000 people die from OSCC each year[1,2]. Risk factors for OSCC differ a lot in different regions. For example, smoking and drinking are the main causes of OSCC in Europe. However, betel nut, tobacco, low-quality edible pigment and human papillomavirus infection are risk factors for Asian OSCC patients[3]. With the characteristics of rapid progression, invasive growth, early metastasis to regional lymph nodes, high recurrence rate and poor prognosis, OSCC seriously affects life quality of the affected people[4]. Currently, surgical resection, chemotherapy, radiotherapy and biologic targeted therapy are the most applied treatments of OSCC[5]. Although huge progress has been made in the pathogenesis and therapy of OSCC, the prognosis of OSCC patients remains poor. Therefore, in-depth explorations on the molecular pathogenesis of OSCC help to develop more effective diagnostic, therapeutic and prognostic methods.

MicroRNAs are small, non-coding RNAs with about 17-25 nt in size. They negatively regulate target genes by degrading or inhibiting mRNA

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translation by binding to the 3'UTR of target mRNAs[6]. A large number of microRNAs have been identified in the pathogenesis of OSCC. For example, microRNA-211 promotes the proliferation, migration and invasion of OSCC cells by targeting bridging integrin 1 protein[7]. MicroRNA-182-5p promotes the growth of OSCC by inhibiting CAMK2N1[8]. Moreover, a competitive endogenous theory proposed that microRNA response elements can competitively bind to pseudogene transcripts, long non-coding RNA (lncRNA) and mRNA transcripts. This may eventually block the inhibitory effect of microRNA on targeted RNAs and regulate tumorigenesis[9].

LncRNAs are non-coding RNAs containing more than 200 nt in length. They exert biological functions through transcriptional, post-transcriptional and epigenetic regulation[10]. LncRNAs are closely related to tumor development by regulating the growth, differentiation and metabolism of tumor cells[11]. A growing number of studies have shown differentially expressed lncRNAs in multiple malignant tumors, such as leukemia, lung cancer, prostate and colorectal cancer[12]. For example, lncRNA MEG3 influences the development of advanced chronic myeloid leukemia by absorbing miR-147 to activate the JAK/STAT signaling pathway[13]. LncRNA IGFBP4-1 can promote lung cancer progression by regulating energy metabolism[14]. Differentially expressed lncRNAs have also been identified in OSCC[15]. LncRNA MALAT1 promotes the development of OSCC *via* the miRNA-125b/STAT3 axis[14]. LncRNA p23154 promotes the invasion and metastasis of OSCC by regulating Glut1-mediated glycolysis[16]. Further studies have indicated that lncRNA FAL1 is a crucial gene that is involved in tumorigenesis and tumor progression[17,18]. However, the specific function of FAL1 in OSCC has not been reported yet.

Materials and Methods

Research Subjects

Twenty pairs of OSCC tissues and adjacent normal tissues (1.5 cm away from the tumor edge) were collected from OSCC patients undergoing surgical resection in the Jinan Stomatological Hospital from July 2016 to September 2017. Patients were pathologically diagnosed as OSCC. None of the patients received preoperative radiotherapy or chemotherapy. Collected tissues (5 mm × 5 mm × 5 mm) were preserved in liquid nitrogen for subsequent use. The study was approved by the Ethics Committee of the Jinan Stomatological Hospital. Informed consent was obtained from each subject before surgery.

Cell Culture

The cell lines used in this experiment (HOKs, SCC6, SCC9, SCC25, HN4, and HN6) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and placed in a 37°C, 5% CO_2 incubator. The culture medium was replaced every other day.

Cell Transfection

OSCC cells were inoculated into 6-well plates. Cell transfection was performed until 60-70% of confluence according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids used in this experiment included siRNA NC, siRNA-FAL1, microRNA-761 inhibitor, and CRKL overexpression plasmid. The culture medium was replaced 6 hours after transfection. The sequences of constructed si-FAL1 were: si-FAL1-1: ACUACAGGUAUGGCCUCACAA; si-FAL1-2: CUACAGGUAUGGCCUCACA; si-FAL1-3 UACAGGUAUGGCCUCACAAGU.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells or tissues was first extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) for qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. QRT-PCR parameters were: 95°C for 10 s, 60°C for 30 s, and 70°C for 30 s, for a total of 40 cycles. Primers used in this study were as follows: CRKL, forward: 5'-CGCTCCGCCTGGTATATGG-3', CRKL, reverse: 5'-GGACACCGACAGCACATAGTC-3'; FAL1, forward: 5'-TCCGACTCTCACGGCGTATG-3', FAL1, reverse: 5'-GATACACGAGCTAGAGCACAC-3'; MicroRNA-761, forward: 5'-GCAAGAGGACACACATTGAGAC-3', MicroRNA-761, reverse: 5'-TATTGGGCTGGGTGAAGTTG-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', GAPHD, reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot

OSCC cells were lysed with radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) for extracting the protein samples. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). The protein expressions of the genes in OSCC cells were detected in strict accordance with standard protocols of Western blot.

Dual-Luciferase Reporter Gene Assay

The binding sites of microRNA-761 in FAL1 and CRKL sequences were first predicted by bioinformatics, and were then amplified by PCR. Subsequently, the amplified fragment was inserted into a vector to construct wild-type FAL1 and wild-type CRKL plasmids. Partial nucleotides were mutated by gene mutation technique, and mutant-type FAL1 and mutant-type CRKL plasmids were constructed. The cells were co-transfected with wild-type or mutant-type plasmid and microRNA-761 mimics. Finally, the Luciferase activities of each group were detected.

Cell Proliferation Assay

OSCC cells were inoculated into 96-well plates with 1×10^4 cells per well. 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) solution was added to each well, followed by incubation at 37°C for 1 h in the dark. The absorbance of each well at 450 nm was recorded by a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 18.0; Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SD ($\bar{x}\pm s$). The standard *t*-test was used to compare the differences between the two groups. The differences among multiple groups were compared using one-way ANOVA, followed by post-hoc test. $p<0.05$ was considered statistically significant.

Results

LncRNA FAL1 Was Highly Expressed in OSCC

To study the role of lncRNA FAL1 in OSCC, its expression in 20 pairs of OSCC tissues and adjacent normal tissues was detected by qRT-PCR. The results showed that lncRNA FAL1 was highly expressed in OSCC tissues (Figure 1A). Meanwhile, lncRNA FAL1 was highly expressed in OSCC cell lines when compared with normal human oral keratinocytes HOK as well (Figure 1B). Among the five selected OSCC cell lines, SCC25, HN4 and HN6 cells presented a relatively high expression of lncRNA FAL1. Then, SCC25 and HN4 cells were selected for the following experiments. Transfection efficacy of siRNA-FAL1 in OSCC cells was first verified by qRT-PCR (Figure 1C and 1D). Subsequently, CCK-8 data showed the inhibited proliferative potential of OSCC cells with lncRNA FAL1 knockdown (Figure 1E and 1F). The above results indicated that lncRNA FAL1 was highly expressed in OSCC, which might participate in the progression of OSCC by regulating the proliferative potential.

LncRNA FAL1 Sponged MicroRNA-761

Studies[19] have shown that lncRNA can competitively interact with MREs to abolish the inhibitory effect of microRNA on target RNAs. To elucidate the possible mechanism of lncRNA FAL1 in regulating the biological performances of OSCC, we first predicted the binding sites of lncRNA FAL1 by bioinformatics (Figure 2A). The results indicated that lncRNA FAL1 could bind to microRNA-761. In addition, we found that the expression level of microRNA-761 in OSCC tissues was significantly lower than that of adjacent normal oral tissues (Figure 2B). MicroRNA-761 was lowly expressed in OSCC cell lines as well (Figure 2C). To further elucidate the regulatory effect of lncRNA FAL1 on microRNA-761, we determined microRNA-761 expression in OSCC cells transfected with si-FAL1. As the data showed, the mRNA level of microRNA-761 in OSCC cells was markedly upregulated after lncRNA FAL1 knockdown (Figure 2D and 2E). Next, we constructed wild-type (lncRNA FAL1-WT) and mutant-type vectors containing lncRNA FAL1 (lncRNA FAL-MUT), and co-transfected them with microRNA-761 mimics in OSCC cells. Decreased Luciferase activity was observed in the lncRNA FAL1-WT group, whereas the lncRNA FAL-MUT group showed no significant change in Luciferase activity (Figure 2F and 2G). Dual-Luciferase reporter gene assay confirmed that lncRNA FAL1 could directly sponge microRNA-761, thereafter participating in the development of OSCC.

CRKL Was a Potential Target Gene of MicroRNA-761

MicroRNAs regulate the target gene expression by binding to the 3'UTR of target miRNAs, thus

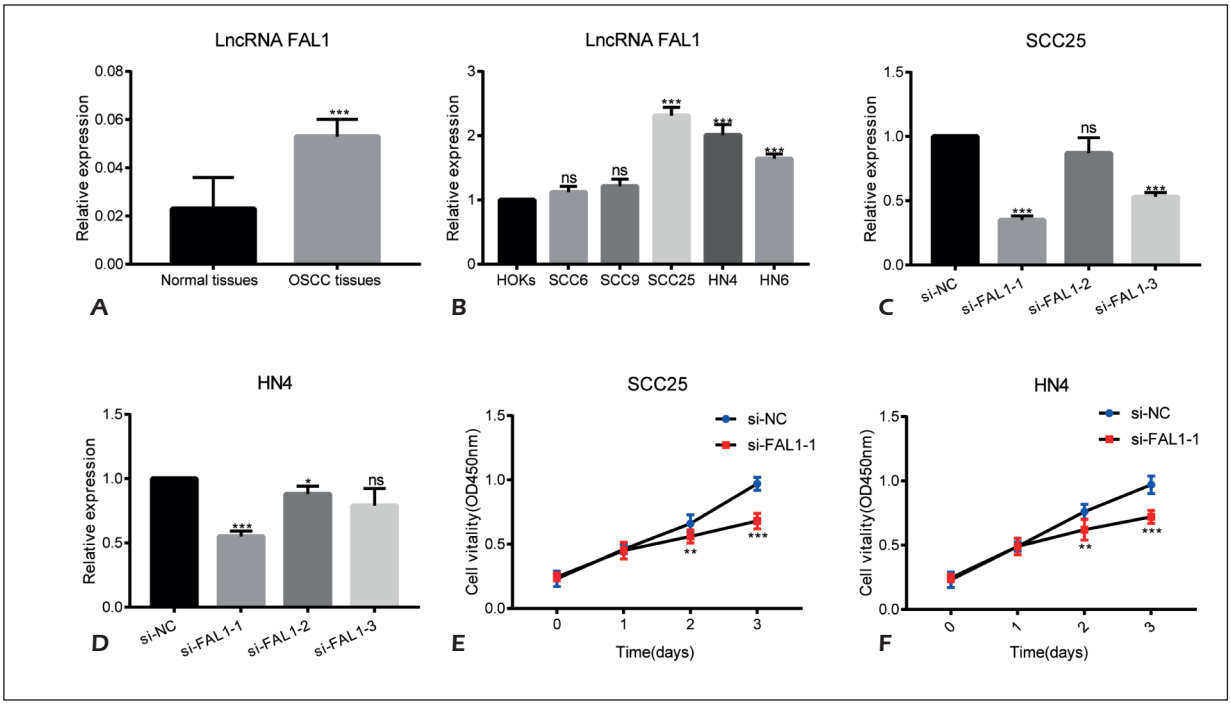

Figure 1. LncRNA FAL1 was highly expressed in OSCC. **A**, LncRNA FAL1 was highly expressed in OSCC tissues compared with adjacent normal tissues. **B**, LncRNA FAL1 was highly expressed in OSCC cell lines compared with normal human oral keratinocytes HOK. **C-D**, Transfection efficacy of siRNA-FAL1 in SCC25 and HN4 cells. **E-F**, CCK-8 data showed that the proliferative potential of SCC25 and HN4 cells after lncRNA FAL1 knockdown was significantly inhibited. *p<0.05, **p<0.01, *** p<0.001.

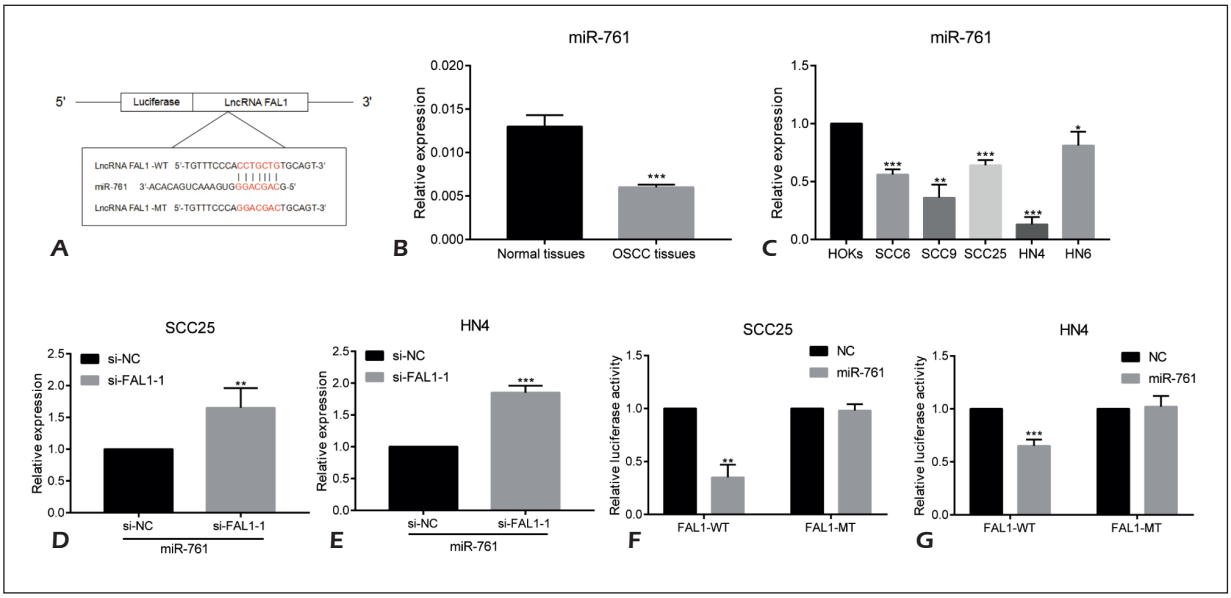

Figure 2. LncRNA FAL1 sponged microRNA-761. **A**, A potential binding site between lncRNA FAL1 and microRNA-761. **B**, MicroRNA-761 was lowly expressed in OSCC tissues compared with adjacent normal tissues. **C**, MicroRNA-761 expression was lowly expressed in OSCC cell lines. **D-E**, The mRNA level of microRNA-761 in SCC25 and HN4 cells was markedly up-regulated by lncRNA FAL1 knockdown. **F-G**, LncRNA FAL1-WT group showed significantly decreased Luciferase activity, whereas the lncRNA FAL-MUT group showed no change in Luciferase activity. *p<0.05, **p<0.01, ***p<0.001.

contributing to tumorigenesis and tumor progression[20]. To further explore the specific regulatory mechanism of microRNA-761 in OSCC, we predicted the target gene CRKL of microRNA-761 by bioinformatics (Figure 3A). CRKL expression was significantly higher in OSCC tissues at both mRNA and protein levels (Figure 3B and 3C). Consistently, CRKL was also highly expressed in OSCC cell lines (Figure 3D). To verify the regulatory effect of microRNA-761 on CRKL, we constructed a wild-type (CRKL-WT) and mutant-type sequence of CRKL (CRKL-MUT). OSCC cells were co-transfected with CRKL-WT or CRKL-MUT and microRNA-761 mimics. The results demonstrated that the Luciferase activity of the CRKL-WT group was remarkably decreased, which was not significantly altered in the CRKL-MUT group (Figure 3E and 3F). These results suggested that CRKL was a target gene of microRNA-761. Meanwhile, microRNA-761 could inhibit CRKL expression by binding to the 3'UTR of CRKL.

LncRNA FAL1 Promoted OSCC Development Through the MicroRNA-761/CRKL Pathway

To verify whether microRNA-761 could regulate CRKL in OSCC cells, we determined the protein level of CRKL after transfection with microRNA-761 inhibitor in OSCC cells. Western blot showed that the protein expression of CRKL was remarkably upregulated (Figure 4A). To further validate the role of microRNA-761/CRKL axis in the development of OSCC regulated by lncRNA FAL1, OSCC cells were co-transfected with si-FAL1 and microRNA-761 inhibitor. The CCK-8 assay demonstrated that the inhibitory effect of lncRNA FAL1 knockdown on the proliferation of OSCC cells was reversed by microRNA-761 knockdown (Figure 4B and 4C). Subsequently, CRKL was observed to be significantly downregulated after transfection of si-FAL1 (Figure 4D). Gain-of-function experiments revealed that inhibited proliferation of OSCC cells by lncRNA FAL1 knockdown was reversed by CRKL overexpression (Figure 4E and 4F). The above data illustrated that lncRNA FAL1 promoted the development of OSCC by regulating the microRNA-761/CRKL pathway.

Discussion

OSCC is a common disease in oral oncology, mostly occurring in the tongue, cheeks and gingival tissues. Tobacco and alcohol addicts with 40-70 years are the high-risk population of OSCC. High

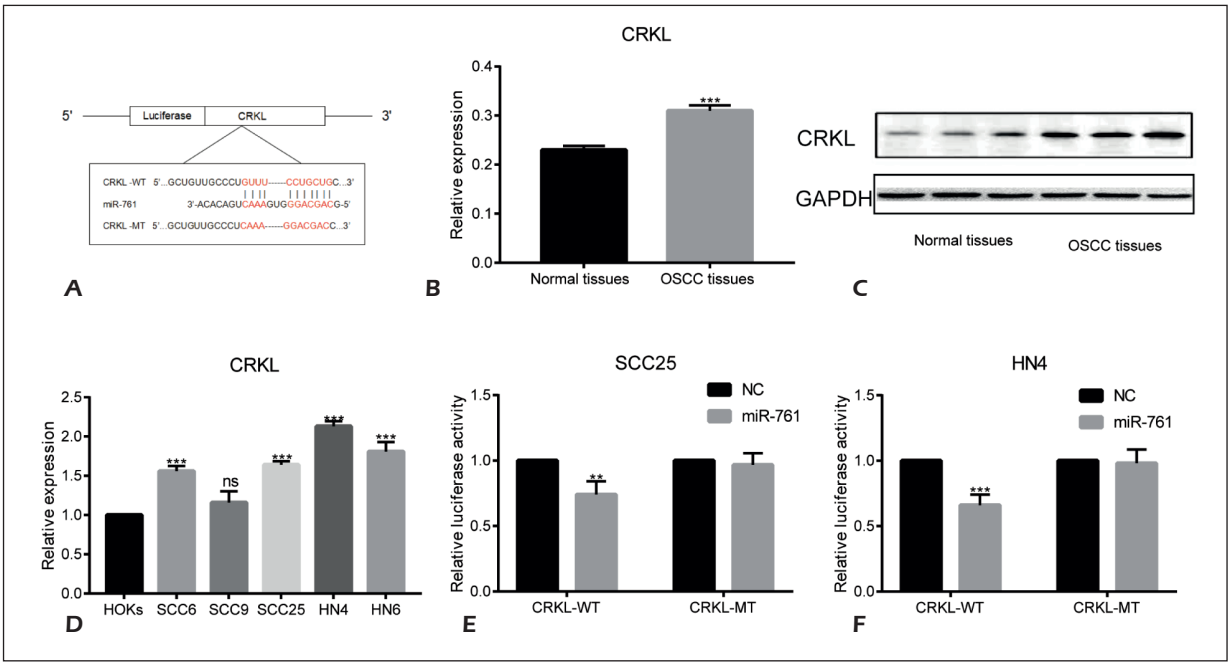

Figure 3. CRKL was a potential target gene of microRNA-761. **A**, A potential binding site between CRKL and microRNA-761. **B**-**C**, CRKL expression was highly expressed in OSCC tissues at both mRNA and protein levels. **D**, CRKL was highly expressed in OSCC cells. **E**-**F**, CRKL-WT group showed markedly decreased Luciferase activity, whereas the CRKL-MUT group showed no change in Luciferase activity. *p<0.05, **p<0.01, ***p<0.001.

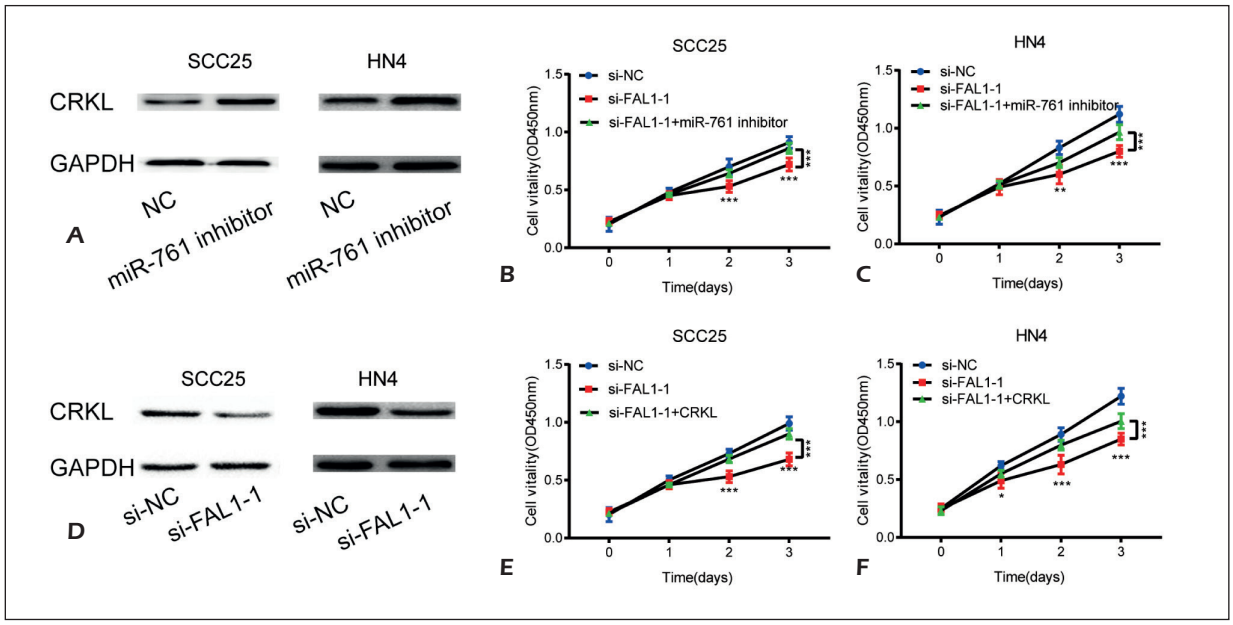

Figure 4. LncRNA FAL1 promoted OSCC development through the microRNA-761/CRKL pathway. **A**, CRKL expression was remarkably upregulated after transfection of microRNA-761 inhibitor in OSCC cells. **B**-**C**, CCK-8 assay demonstrated that the inhibitory effect of lncRNA FAL1 knockdown on the proliferative potential of SCC25 and HN4 cells was reversed by microRNA-761 knockdown. **D**, CRKL expression was remarkably downregulated after transfection of si-FAL1 in OSCC cells. **E**-**F**, Inhibited proliferation of SCC25 and HN4 cells by lncRNA FAL1 knockdown was reversed by CRKL overexpression. **p*<0.05, ***p*<0.01, ****p*<0.001.

malignancy and high lymph node metastatic rate result in poor prognosis and low survival rate of OSCC[20]. In recent years, clinical treatments for oral tumors, including chemotherapy, radiotherapy, restorative and reconstructive surgery have made great progress. However, even after combined treatment of surgery and radiotherapy, the outcome of the advanced oral tumor (stages III and IV) is unsatisfactory[21]. Therefore, specific molecular mechanism and targeted therapy of oral tumors are important issues that need to be solved.

The occurrence and development of OSCC are complex biological processes involving multiple genetic and epigenetic changes. LncRNAs are involved in stem cell differentiation and autoimmune disease regulation as splicing factors and competitive endogenous RNA (ceRNA)[22]. Recent studies have shown that lncRNAs are differentially expressed in tumors, such as gastric cancer[23], hepatocellular carcinoma[24], breast cancer[25], and other malignancies. DLEU1[26], H19[27], AFAP1-AS1[28] are confirmed to be differentially expressed in OSCC. Meanwhile, it is suggested that lncRNAs exert diagnostic and therapeutic potentials for OSCC. FAL1 is differentially expressed in many tumors as a long-term oncogenic RNA. FAL1 is associated with BMI1 and inhibits p21 expression, thereafter regulating tumorigenesis and tumor progression[18]. Moreover, it promotes cell proliferation, invasion and epithelial-mesenchymal transition by regulating the PTEN/AKT pathway in non-small cell lung cancer[17]. In this work, FAL1 was highly expressed in OSCC tissues and cells, which participated in the progression of OSCC by regulating the proliferation of OSCC cells.

The competitive endogenous RNA (ceRNA) hypothesis proposes that transcriptome members containing the same miRNA response elements (MREs), such as mRNA, tRNA, lncRNA, and circRNA, can compete with miRNAs[29]. In this work, we analyzed and predicted the presence of binding sequences in lncRNA FAL1 that could bind to microRNA-761 by bioinformatics, which was further verified by the Dual-Luciferase reporter gene assay. The regulatory role of microRNA-761 in tumors remains controversial. MicroRNA-761 promotes the progression and metastasis of non-small cell lung cancer by targeting ING4 and TIMP2[30]. Studies have also indicated that microRNA-761 inhibits ovarian cancer progression by targeting MSI1[31]. In this work, microRNA-761 expression was significantly downregulated in OSCC. Meanwhile, low expression

of microRNA-761 markedly promoted proliferation of OSCC cells. As a member of the adaptor protein CRK family, CRKL is expressed in a variety of tumors and may induce tumorigenesis[32]. Our study found that CRKL was a target gene of microRNA-761 and its expression was regulated by FAL1 in OSCC cells. The overexpression of CRKL reversed the inhibitory effect of lncRNA FAL1 knockdown on the proliferation of OSCC cells. These results suggested that lncRNA FAL1 promoted the development of OSCC by regulating the microRNA-761/CRKL pathway.

Conclusions

We indicated that lncRNA FAL1 is highly expressed in OSCC, which promotes the development of OSCC by regulating CRKL expression as a sponge of microRNA-761.

Conflict of Interests

The authors declare that they have no conflict of interest.

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