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# LncRNA FOXD2-AS1 accelerates the progression of cervical cancer *via* downregulating CDX1

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**Abstract. – OBJECTIVE: To uncover the role of FOXD2-AS1 in aggravating the progression of cervical cancer (CC) by negatively regulating caudal-related homeobox 1 (CDX1).**

**MATERIALS AND METHODS: FOXD2-AS1 levels in CC tissues with different tumor sizes and tumor staging were detected. Meanwhile, FOXD2-AS1 levels in CC patients either with vascular invasion, lymphatic metastasis or not were detected. Survival analysis on CC patients expressing high level or low level of FOXD2-AS1 was conducted by introducing the Kaplan-Meier method. After the silence of FOXD2-AS1, proliferative changes in SiHa and HeLa cells were assessed through cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay. Subcellular distribution of FOXD2-AS1 in CC cells was analyzed. Next, CDX1 level in CC tissues and para-tumor tissues was determined. The potential correlation between CDX1 level and FOXD2-AS1 level was evaluated by the linear regression analysis. At last, the regulatory effects of FOXD2-AS1/CDX1 on the proliferative ability of CC were examined.**

**RESULTS: FOXD2-AS1 was upregulated in CC tissues relative to those of para-tumor tissues, especially in those with larger tumor size and advanced tumor staging. Its level was not correlated to vascular invasion and lymphatic metastasis of CC. CC patients expressing a high level of FOXD2-AS1 suffered worse prognosis than those with low level. The silence of FOXD2-AS1 attenuated SiHa and HeLa cells to proliferate. FOXD2-AS1 was found to be mainly enriched in the nucleus. CDX1 was downregulated in CC tissues and its level was negatively regulated by FOXD2-AS1. The silence of CDX1 could reverse the regulatory effect of FOXD2-AS1 on the proliferative ability of CC cells.**

**CONCLUSIONS: FOXD2-AS1 is upregulated in CC and its high level predicts a poor prognosis of CC patients. It accelerates the malignant progression of CC *via* negatively regulating CDX1 level.**

*Key Words:*
Cervical cancer, FOXD2-AS1, CDX1.

## Introduction

Cervical cancer (CC) is the second most prevalent malignant tumor that threatens females' health. Globally, there are about 490,000 new cases of CC occurring each year. In recent years, the incidence of CC in China has markedly increased, presenting a younger onset[1]. The pathogenesis of CC is complex, involving multiple factors, genes, and pathways. Surgical resection is preferred for early-stage CC, with a good prognosis. However, the prognosis of advanced CC is relatively poor, since it is mainly treated by chemotherapy or radiotherapy[2]. Sensitive and specific hallmarks that monitor the progression, metastasis, and prognosis of CC are lacking. It is necessary to develop effective tumor biomarkers for improving the clinical outcomes of CC.

Long non-coding RNAs (lncRNAs) are an important class of epigenetic expression-related molecules. They are extensively involved in various physiological processes by interfering with the binding of transcriptional factors and promoters, chromosome modification, etc[3]. Increasing evidence[4,5] has proved the diagnostic and prognostic potentials of lncRNAs. Several lncRNAs are differentially expressed in CC as oncogenes or tumor-suppressor genes that influence tumor cell behaviors[6]. FOXD2-AS1 is a 2509-nucleotides lncRNA, which is able to promote the proliferative and invasive capacities of many types of tumor cells[7]. The regulatory effect of FOXD2-AS1 on CC, however, is rarely reported.

Caudal-related homeobox 1 (CDX1) is a member of the CDX family[8]. It is reported that CDX1 exerts a crucial role in intestinal metaplasia. It maintains the differentiation, growth and normal functions of intestinal epithelial cells[9,10]. Researches[11,12] have shown that CDX1 is downregulated in gastric cancer tissues compared with adjacent



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intestinal metaplasia tissues. Overexpression of CDX1 suppresses gastric cancer cells to proliferate. In colorectal cancer tissues, CDX1 expression is deficient. In this paper, we mainly clarified the potential function of FOXD2-AS1 in influencing the progression of CC through the regulation of CDX1.

## Materials and Methods

### Cell Culture

H8, SiHa, and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% $CO_2$ at 37°C.

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

Total RNA in cells or tissues was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). FOXD2-AS1, F: 5′-TGTTCGTGGGAAGAGGGTTG-3′, R: 5'-TACCACTCCGGGAACTCTGT-3′; CDX1, F: 5'-CGGTGGCAGCGGTAAGAC-3', R: 5'-GATTGTGATGTAACGGCTGTAATG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5′-ACTGCCACCCAGAAGACT-3′, GAPDH, R: 5'-GCTCAGTGTAGCCCAGGAT-3′.

### Cell Transfection

Cells were seeded in a 6-well plate with 2 ml of suspension ($1\times10^5$ cells/mL) per well. After overnight culture, cells were subjected to transfection using Lipofectamine™ 2000. Transfection efficacy was tested at 48 h by qRT-PCR. si-FOXD2-AS1 1*: forward: 5′-GCGCGGUUGUUGAGA-CCAAGG-3′ and reverse: 5′-UUGGUCUCAACAACCGCGCAG-3′; si-FOXD2-AS1 2*: forward: 5′-GCGAAGAGUACGUUGCUAUTT-3′ and reverse: 5′-AUAGCAACGUACUCUUCGCTT-3′; si-FOXD2-AS1 3*: forward: 5′-GGCUUUUCCACUAGUUACUGA-3′ and reverse: 5′-AGUAACUAGUGGAAAAGCCCA-3′.

### Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates with $5.0\times10^3$ cells per well. At the established time points, 10 μL of CCK-8 solution (cell counting kit-8, Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader.

### 5-Ethynyl-2′- Deoxyuridine (EdU) Assay

Cells were inoculated into 96-well plates with $5\times10^3$ cells per well and labeled with 50 μM EdU reagent for 2 h. After washing with phosphate buffered saline (PBS), cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. After washing with PBS once, cells were stained with 100 μL of Hoechst 33342 in dark for 30 min. EdU-positive cells were observed under a fluorescent microscope.

### Determination of Subcellular Distribution

RNAs in cytoplasmic and nuclear fractions were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA). Cytoplasmic and nuclear RNAs were subjected to qRT-PCR for determining relative levels. U6 was the internal reference of the nucleus and GAPDH was that of cytoplasm.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Intergroup data were compared using the *t*-test. The Kaplan-Meier method was used for survival analysis. The correlation between FOXD2-AS1 and CDX1 was analyzed by linear correlation analysis. $p<0.05$ considered the difference was statistically significant.

## Results

### FOXD2-AS1 was Upregulated in CC

Compared with para-tumor tissues, FOXD2-AS1 was upregulated in CC tissues (Figure 1A). Moreover, FOXD2-AS1 level was higher in CC tissues larger than 4 cm in tumor size than those smaller than 4 cm (Figure 1B). According to the tumor staging, our data showed a higher abundance of FOXD2-AS1 in CC patients with stage III+IV than those with stage I+II (Figure 1C). No

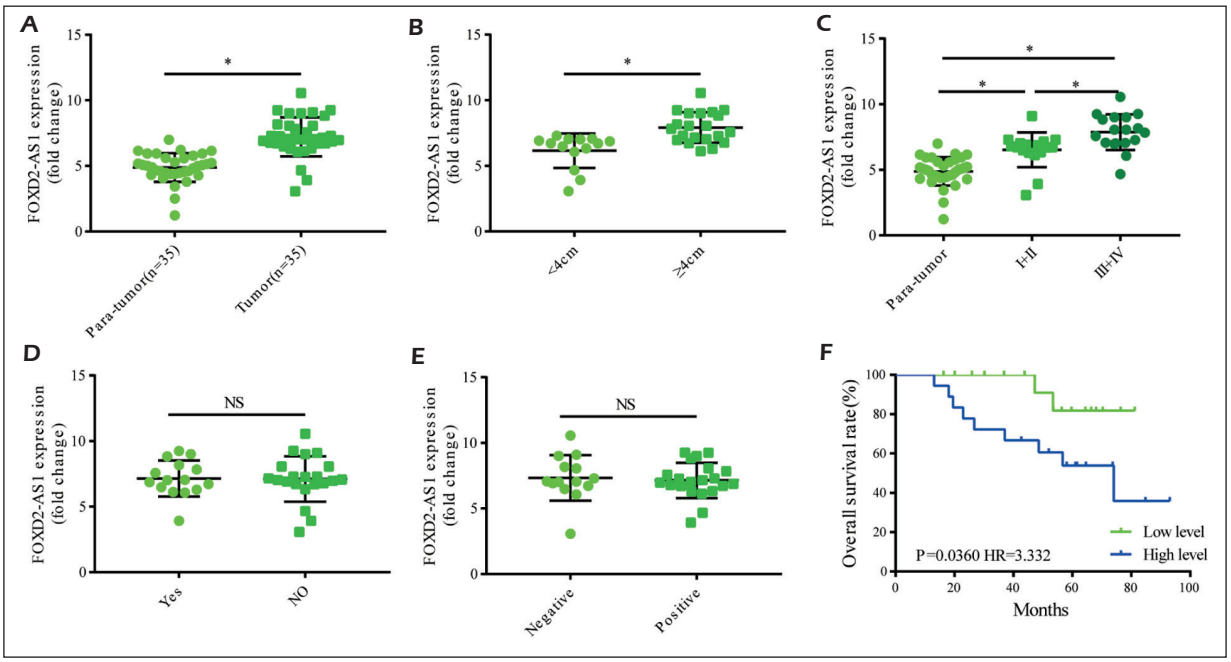


**Figure 1.** FOXD2-AS1 was upregulated in CC. **A,** FOXD2-AS1 level in para-tumor tissues (n=35) and CC tissues (n=35). **B,** FOXD2-AS1 level in CC tissues with <4 cm and ≥4 cm in tumor size. **C,** FOXD2-AS1 level in para-tumor tissues, CC tissues in stage I+II and stage III+IV. **D,** FOXD2-AS1 level in CC patients either with vascular invasion or not. **E,** FOXD2-AS1 level in CC patients either with lymphatic metastasis or not. **F,** Overall survival in CC patients with high level or low level of FOXD2-AS1 (HR=3.332, $p$=0.0360).

significant difference in FOXD2-AS1 level was found in CC patients either with vascular invasion, lymphatic metastasis or not (Figures 1D, 1E). Kaplan-Meier curves revealed worse prognosis in CC patients expressing a high level of FOXD2-AS1 compared to those expressing low level (Figure 1F). The above data suggested that the upregulation of FOXD2-AS1 in CC predicted a poor prognosis.

### *FOXD2-AS1 Accelerated CC to Proliferate*

FOXD2-AS1 was identically upregulated in CC cells than that of immortalized cells of human cervical epithelium (Figure 2A). We constructed three FOXD2-AS1 siRNAs, namely si-FOXD2-AS1 1*, si-FOXD2-AS1 2*, and si-FOXD2-AS1 3*. Transfection of any of the three could sufficiently downregulate FOXD2-AS1 level in SiHa and HeLa cells (Figures 2B, 2C). In the following experiments, si-FOXD2-AS1 1* was utilized. Transfection of si-FOXD2-AS1 1* markedly reduced viability in SiHa and HeLa cells at day 2 and 3 (Figures 2D, 2E). Similarly, EdU-positive ratio decreased after the silence of FOXD2-AS1 in CC cells (Figures 2F, 2G). Hence, it is believed that knockdown of FOXD2-AS1 attenuated proliferative ability of CC.

### *FOXD2-AS1 Negatively Regulated CDX1 Level*

Considering that the lncRNA function is linked with its subcellular distribution, we thereafter analyzed FOXD2-AS1 enrichment in CC cells. It is shown that FOXD2-AS1 was mainly enriched in the nucleus of SiHa and HeLa cells (Figures 3A, 3B). In addition, CDX1 was found to be downregulated in CC tissues than those of para-tumor ones (Figure 3C). A negative correlation was identified between expressions of FOXD2-AS1 and CDX1 in CC tissues (Figure 3D). In addition, transfection of si-FOXD2-AS1 1* could remarkably upregulate CDX1 level in CC cells, further confirming the negative regulation of FOXD2-AS1 on CDX1 (Figures 3E, 3F).

### *FOXD2-AS1 Influenced the Progression of CC Via Regulating CDX1*

Transfection of si-CDX1 remarkably downregulated CDX1 level in CC cells (Figures 4A, 4B). Interestingly, the attenuated viability in CC cells with FOXD2-AS1 knockdown was reversed by the silence of CDX1 (Figures 4C, 4D). EdU assay consistently demonstrated that co-transfection of si-CDX1 could reverse the decreased EdU-positi-

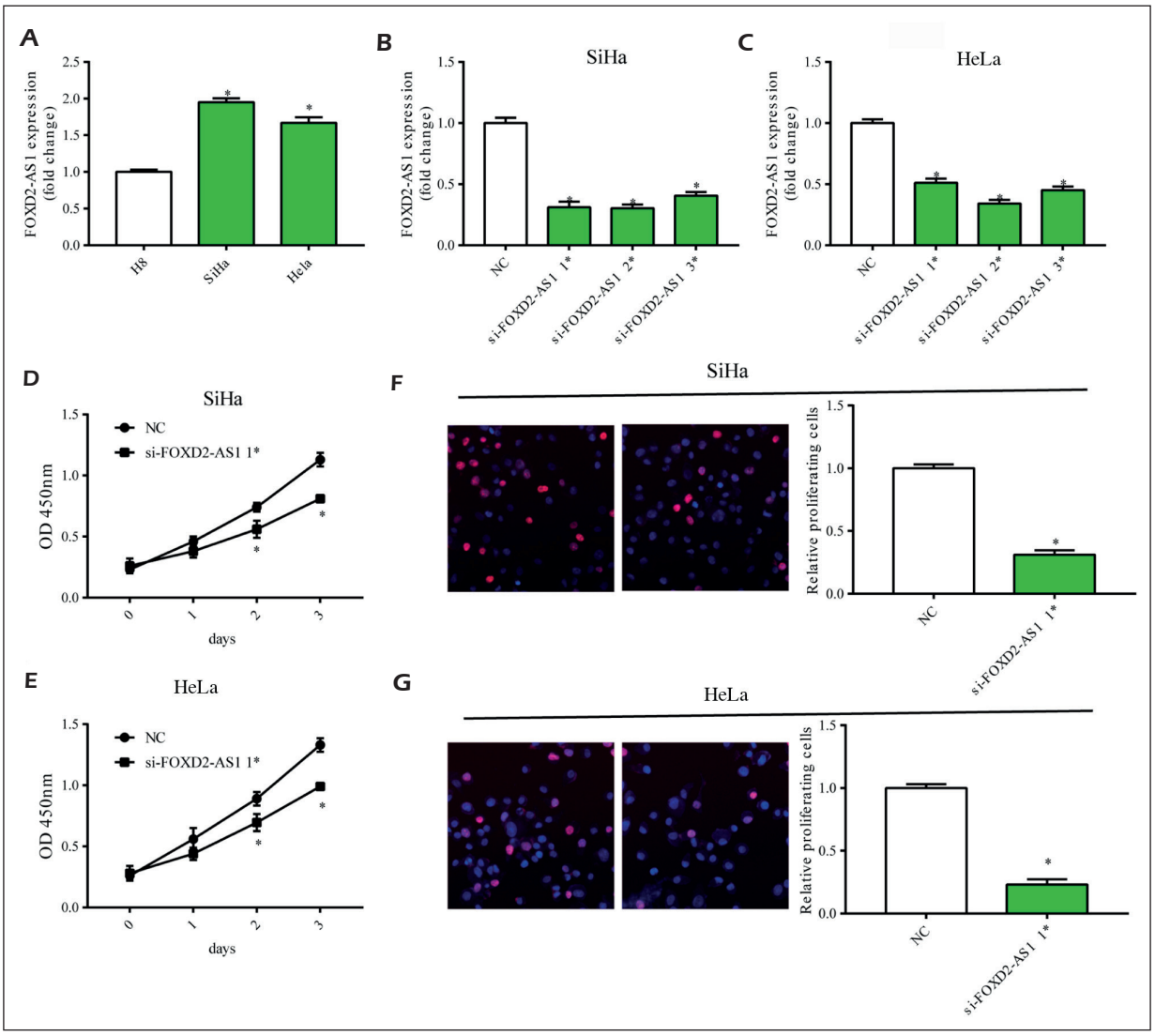


***Figure 2.*** FOXD2-AS1 accelerated CC to proliferate. **A,** FOXD2-AS1 level in CC cells (SiHa and HeLa) immortalized cells of human cervical epithelium (H8). **B,** Transfection efficacy of si-FOXD2-AS1 1*, si-FOXD2-AS1 2* and si-FOXD2-AS1 3* in SiHa cells. **C,** Transfection efficacy of si-FOXD2-AS1 1*, si-FOXD2-AS1 2* and si-FOXD2-AS1 3* in HeLa cells. **D,** Viability in SiHa cells transfected with NC or si-FOXD2-AS1 1* at day 0, 1, 2 and 3. **E,** Viability in HeLa cells transfected with NC or si-FOXD2-AS1 1* at day 0, 1, 2 and 3. **F,** EdU-positive ratio in SiHa cells transfected with NC or si-FOXD2-AS1 1* (magnification 200 ×). **G,** EdU-positive ratio in HeLa cells transfected with NC or si-FOXD2-AS1 1* (magnification: 200 ×).

ve ratio in CC cells with FOXD2-AS1 knockdown (Figures 4E, 4F). As a result, it is verified that FOXD2-AS1 accelerated the proliferative ability of CC through negatively regulating CDX1 level.

## Discussion

CC is a highly popular malignancy in females. HPV infection, certain genetic and environmental factors are risky for the tumorigenesis of CC[13]. Accurate diagnosis and early-stage intervention of CC based on molecular diagnostics and biochemical methods are urgently required[14]. Potential molecular markers for predicting the clinical efficacy of CC are of significance[15].

Non-coding RNAs that are related to gene expression regulation account for a major part of the whole genome, and they are well-known in tumor biology[16]. LncRNAs are 200-nucleotides non-coding RNAs without protein-encoding function. They are extensively involved in chromatin remodeling, regulation of nuclear protein substructures and transcriptional genes[17].

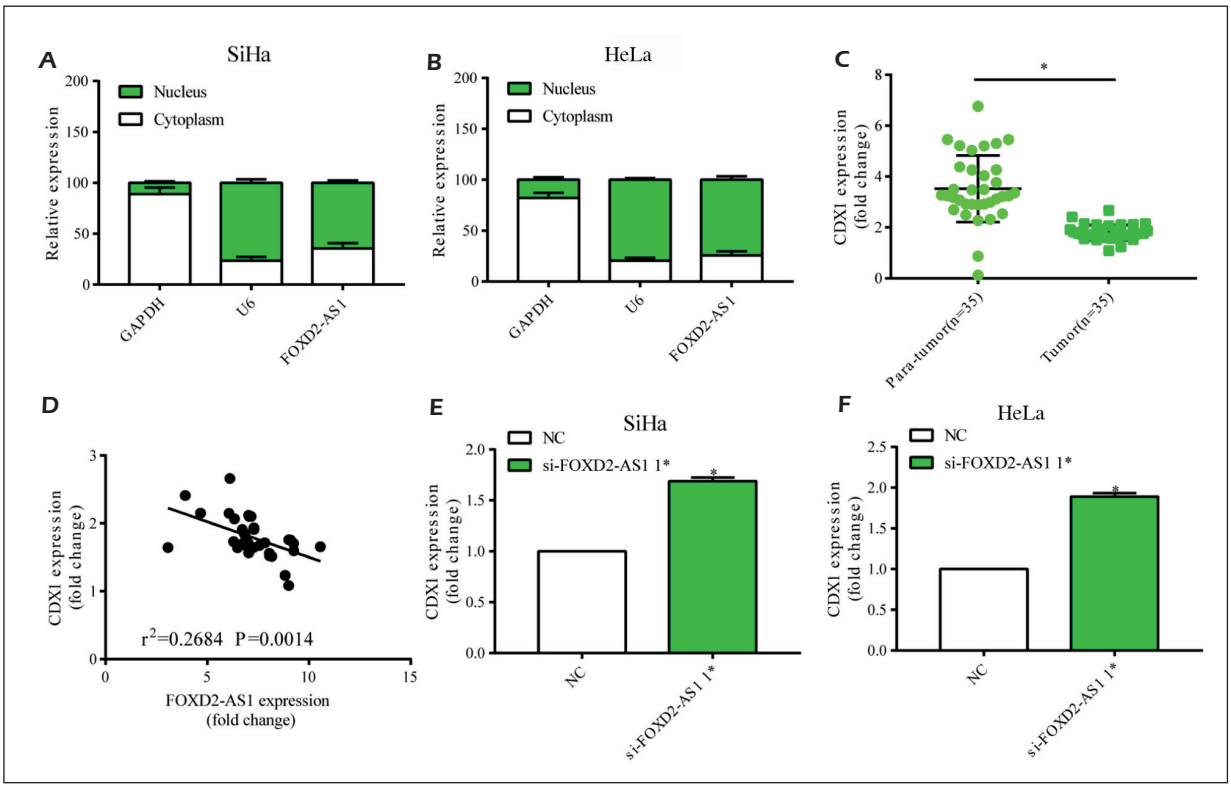


**Figure 3.** FOXD2-AS1 negatively regulated CDX1 level. **A,** Subcellular distribution of FOXD2-AS1 in SiHa cells. GAPDH and U6 were cytoplasmic and nuclear internal references, respectively. **B,** Subcellular distribution of FOXD2-AS1 in HeLa cells. GAPDH and U6 were cytoplasmic and nuclear internal references, respectively. **C,** CDX1 level in para-tumor tissues (n=35) and CC tissues (n=35). **D,** A negative correlation between expression levels of FOXD2-AS1 and CDX1 in CC tissues. **E,** CDX1 level in SiHa cells transfected with NC or si-FOXD2-AS1 1*. **F,** CDX1 level in HeLa cells transfected with NC or si-FOXD2-AS1 1*.

Jin et al[18] found that TCONS_00026907 is abnormally expressed in CC through analyzing the lncRNA microarray. TCONS_00026907 is upregulated in CC tissues, which promotes cell cycle, proliferation, and metastasis, and inhibits apoptosis of tumor cells. Fan et al[19] demonstrated that IL7R level is positively correlated to tumor size, FIGO staging and lymphatic metastasis of CC. CC patients expressing a high abundance of IL7R predicts a poor prognosis. Consistently, our experiment showed the upregulation of FOXD2-AS1 in CC tissues and cell lines. FOXD2-AS1 level increased with the enlargement of tumor size and worsen of tumor staging of CC. Survival analysis also illustrated a poor prognosis of CC patients expressing a high level of FOXD2-AS1.

In embryonic tissues, CDX1 expression varies along the intestine, with high expression in intestinal crypts and diminishing expression along intestinal *villi*[20]. CDX1 is of significance in inducing and maintaining intestinal metaplasia of the stomach and esophageal mucosa[21]. CDX1 deficiency is considered to be closely related to colorectal cancer[22]. Overexpression of CDX1 greatly attenuates colorectal cancer cells to proliferate[23]. Our findings pointed out that CDX1 was downregulated in CC tissues, indicating a potential anti-tumor role in CC. CDX1 level was negatively regulated by FOXD2-AS1. Notably, the silence of CDX1 could reverse the regulatory effect of FOXD2-AS1 on the proliferative ability of CC cells. Collectively, FOXD2-AS1 was confirmed to negatively regulate CDX1 level, thus accelerating the proliferative ability of CC. Our results provide a novel idea for the clinical treatment of CC.

## Conclusions

We showed that FOXD2-AS1was upregulated in CC and predicted a poor prognosis. It accelerated the malignant progression of CC *via* negatively regulating the CDX1 level.

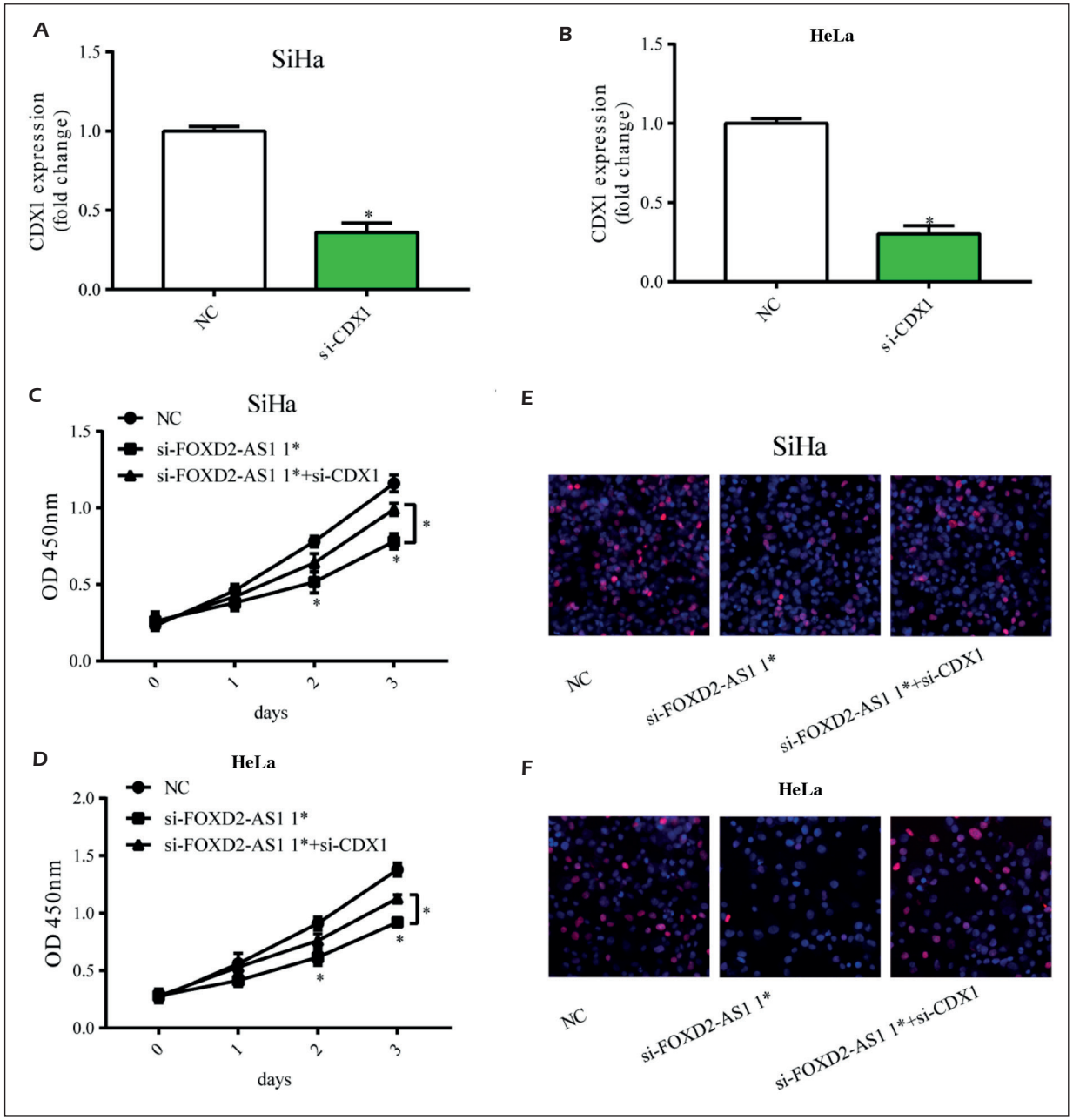


**Figure 4.** FOXD2-AS1 influenced the progression of CC *via* regulating CDX1. **A,** Transfection efficacy of si-CDX1 in SiHa cells. **B,** Transfection efficacy of si-CDX1 in HeLa cells. **C,** Viability in SiHa cells transfected with NC, si-FOXD2-AS1 1* or si-FOXD2-AS1 1* + si-CDX1. **D,** Viability in HeLa cells transfected with NC, si-FOXD2-AS1 1* or si-FOXD2-AS1 1* + si-CDX1. **E,** EdU-positive ratio in SiHa cells transfected with NC, si-FOXD2-AS1 1* or si-FOXD2-AS1 1* + si-CDX1 (magnification: 200 ×). **F,** EdU-positive ratio in HeLa cells transfected with NC, si-FOXD2-AS1 1* or si-FOXD2-AS1 1* + si-CDX1 (magnification: 200 ×).

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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