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Knockdown of long noncoding RNA DLX6-AS1 inhibits cell proliferation and invasion of cervical cancer cells by downregulating FUS

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Abstract. – OBJECTIVE: Recently, the vital role of long noncoding RNAs (lncRNAs) in human diseases have got much attention. In this research, lncRNA DLX6-AS1 is studied to verify how it affects the development of cervical cancer (CC).

PATIENTS AND METHODS: DLX6-AS1 expression was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in both CC cells and tissue samples. Besides, functional experiments including cell counting kit-8 (CCK-8) assay, colony formation assay and transwell assay were performed. Meanwhile, the underlying mechanism was explored through qRT-PCR and Western blot assay. The function of DLX6-AS1 was also identified.

RESULTS: By comparing with corresponding tissues, the DLX6-AS1 expression level was significantly higher in CC samples. Moreover, cell growth ability and invasion of CC cells were inhibited after DLX6-AS1 was knocked down. Furthermore, the expression of FUS was inhibited after knockdown of DLX6-AS1. It was found that the expression level of FUS positively correlated with the expression of DLX6-AS1 in CC tissues. In addition, knockdown of DLX6-AS1 inhibited tumor formation and metastasis of CC in nude mice.

CONCLUSIONS: These results suggest that DLX6-AS1 could enhance cell proliferation and metastasis of CC by upregulating FUS, which might be a potential therapeutic target in CC.

Long Noncoding RNA, DLX6-AS1, Cervical cancer, FUS.

Introduction

Cervical cancer (CC) is the fourth most common type of cancers in women globally, following breast, [illegible] and lung cancers, which is the fourth cause for cancer-related death in the world[1]. It is estimated that 570,000 patients were newly diagnosed of CC and 311,000 cases died due to this deadly disease in 2018[1]. The morbidity accounts for 85% of all CC patients in developing countries, and 83% of CC patients were diagnosed at advanced stages[2]. The therapeutic strategies available for CC patients such as surgery, radiotherapy, chemotherapy, and so on. The occurrence and recurrence rates of CC have been significantly increasing both in developed and developing countries[3]. Therefore, it is urgent to find out the molecular characteristics of CC and figure out a new treatment strategy for patients with CC.

Long non-coding RNAs (lncRNAs), as one subtype of non-coding RNAs, are defined as ncRNAs greater than 200 nt. Some studies have suggested that lncRNAs are important regulators in biological behaviors of various cancers. For example, LncRNA PVT1 promotes glucose metabolism, cell motility, cell proliferation and tumor progression in osteosarcoma by modulation of miR-497/HK2 axis[4]. LncRNA ENST00000547547 acts as a tumor suppressor in colorectal cancer by inhibiting cell proliferation, cell invasion, and cell metastasis[5]. LncRNA LINC00052 inhibits migration and invasion of hepatocellular carcinoma cells through upregulating EPB41L3[6]. In addition, lncRNA PlncRNA-1 functions in the progression of colorectal cancer cell through activating PI3K/Akt signaling pathway[7]. LncRNA DLX6-AS1 is a novel lncRNA and acts as an oncogene in many tumors. However, the role of lncRNA DLX6-AS1 in CC and the potential molecular mechanism haven't been studied so far.

In this investigation, DLX6-AS1 was found remarkably higher-expressed in CC tissues. Knock-

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down of DLX6-AS1 suppressed the proliferation and invasion of CC cells. The underlying mechanism of how DLX6-AS1 functioned in CC proliferation and metastasis was further explored. Furthermore, the role of DLX6-AS1 in CC progression was detected in nude mice.

Patients and Methods

Cell Lines and Clinical Samples

Human tumor tissues and adjacent non-tumor tissues were sequentially got from 60 CC patients who underwent surgery during our hospital. The Ethics Committee of our hospital approved this study protocol. Before the operation, the written informed consent was achieved from all the participants.

Cell Culture

Human CC cell lines (HeLa, SiHa, C4-1, and C-33a) and normal cervical epithelium cell line (NC104) were purchased from the Institute of Biochemistry and Cell Biology, Chinese A[illegible]emy of Science (Shanghai, China). Cultu[illegible] dium consisted of 10% fetal bovine serum ([illegible]S; Life Technologies, Gaithersburg, MD, USA), [illegible] icillin as well as Dulbecco's Modified Eagle's M[illegible] dium (DMEM; Gibco, Rockvil[illegible]SA). Th[illegible] incubator, containing 5% C[illegible]and s[illegible]t 37°C, was used for cell culture.

Cell Transfection

Lentivirus e[illegible]ing sho[illegible]pin RNA (shRNA) dire[illegible]t DLX6-A[illegible]as compounded b[illegible]eneP[illegible] (Shanghai, China). Negative [illegible]trol shRNA[illegible]) was also synthesized. [illegible]reover, pLenti-[illegible]EGFP-F2A-Puro vec[illegible]Biosett[illegible]c., San Diego, CA, USA) was use[illegible]clo[illegible]g the shRNA and negative control, which [illegible]n tran[illegible]ed in SiHa cells.

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

[illegible]was extracted from cultured CC cells [illegible]nd tissues *via* TRIzol reagent (TaKaRa Bio, Inc., [illegible]su, Shiga, Japan) and then reverse-transcribed to [illegible]lementary deoxyribose nucleic acids (cDNAs) th[illegible]ough reverse Transcription Kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan). Following are the primers used for qRT-PCR: DLX6-AS1 primers forward 5′-AGTTCCCCCCTAGATTGCCTT-3′, reverse 5′-ATTGACATGTTAGTGCCCTT-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward 5′-CCAAAACCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGCCATCCA-3′. Thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

Cell Proliferation Assay

96-well plate was used for the c[illegible]of cervical cancer cells (1×10^3 cells/wel[illegible]). The[illegible]added 10 μL of cell counting kit-8 ([illegible]K-8; Doj[illegible]Kumamoto, Japan) into thes[illegible]ls at differen[illegible] Microplate reader was[illegible]d for m[illegible]uring ab[illegible]bance at 450 nm (Bi[illegible]R[illegible]e[illegible]s, CA, USA).

Colony Formation Assay

SiHa cel[illegible]laced in a[illegible]l plate for 10 days. T[illegible]n, co[illegible]s were treated with 10% formal[illegible]de for 30[illegible]and stained for 5 min wit[illegible]crystal viol[illegible]he Image-Pro Plus 6[illegible]Silver Springs, MD, USA) was used for c[illegible]analysis.

Transwell Assay

Tra[illegible]mbers with 8 μm pores were provided by Corning (Corning, NY, USA). The membrane was precoated with 50 μL Matrigel (BD [illegible]ces, Heidelberg, Germany). Cells were then seeded into the upper chambers of a 24-well plate. 20% FBS-DMEM was added to the lower chamber of the culture inserts. After cultured for 24 h, these inserts were fixed with methanol for 30 min and stained by hematoxylin for 20 min. The number of invaded cells was counted by a light microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Cell samples were washed with precooled phosphate-buffered saline (PBS) and then lysed with cell lysis solution (RIPA; Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA; Thermo Fisher Scientific Inc., Waltham, MA, USA). The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked in Tris-Buffered Saline & Tween (TBST) (25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of FUS and GAPDH (Abcam Inc., Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3 × 10 min) with TBST, the secondary antibody was added and incubated

at room temperature for 1 h. The results were analyzed by Image J software (NIH, Bethesda, MD, USA).

Xenograft Model

After transfected, SiHa cells (6×10^5/mL) were replaced into NOD/SCID mice (6 weeks old) subcutaneously. Tumor diameters were detected every 5 days. Tumor volume was calculated as the formula (volume = length × width^2 × 1/2). Tumors were extracted after 4 weeks. Transfected SiHa cells were injected into tail vein of NOD/SCID mice (4-5 weeks old). The mice were sacrificed, and the lung was extracted after 4 weeks. Then, the number of metastatic nodules in the lung was counted. The animal experiments were approved by the Animal Ethics Committee of Changzhi Medical College.

Statistical Analysis

Statistical analysis was conducted through Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Chicago, IL, USA). Data was presented as mean ± SD. The Student *t*-test was selected when appropriate. Moreover, $p<0.05$ was considered of statistically significance.

Results

DLX6-AS1 Expression Level in C sues and Cells

We conducted qRT-PC detec DLX expression in 60 CC ents' es. DL AS1 was significa ly er ressed n CC tissue samples th at i ent tis (Figure 1A). More qRT-PC rmed to detect DL xpression cells and DLX6-AS1 as up ated in CC cells than that in NC10 ells (Figu). We chose the SiHa cell the knockdo f DLX6-AS1. Then, q PCR was utilized for detecting the transfect efficiency (re 1C).

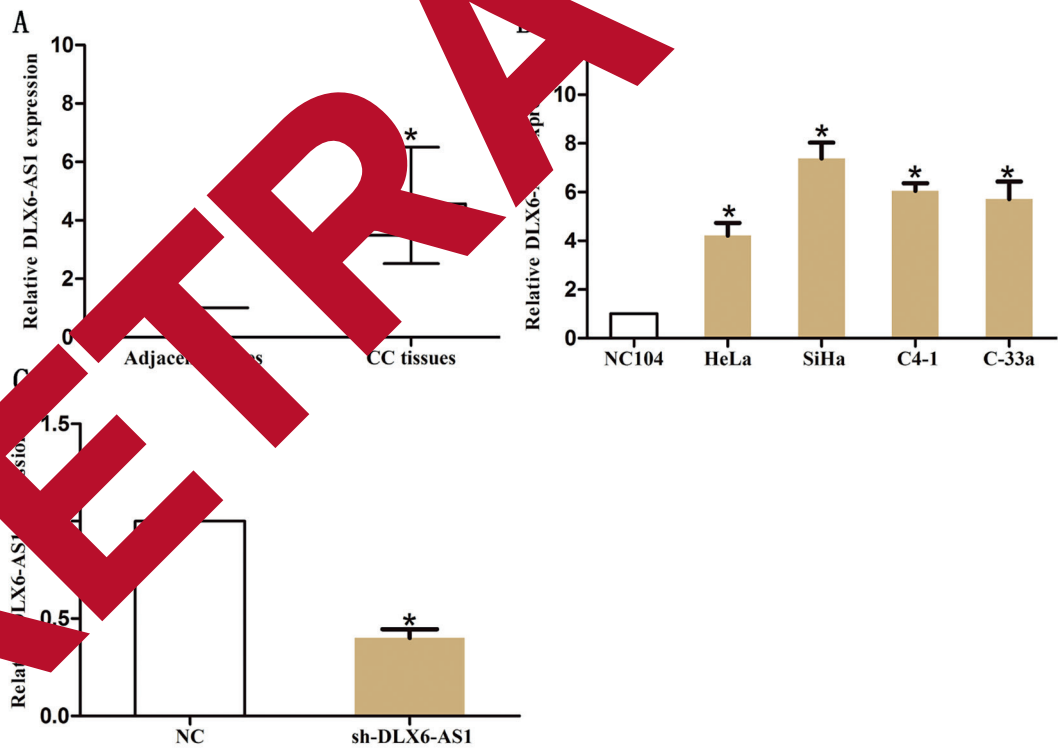

Figure 1. Expression levels of DLX6-AS1 were increased in CC tissues and cells. ***A,*** DLX6-AS1 expression was significantly increased in the CC tissues compared with adjacent tissues. ***B,*** Expression levels of DLX6-AS1 relative to GAPDH were determined in the human CC cell lines and NC104 by qRT-PCR. ***C,*** DLX6-AS1 expression in SiHa cells transduced with sh-DLX6-AS1 and NC was detected by qRT-PCR. GAPDH was used as an internal control. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

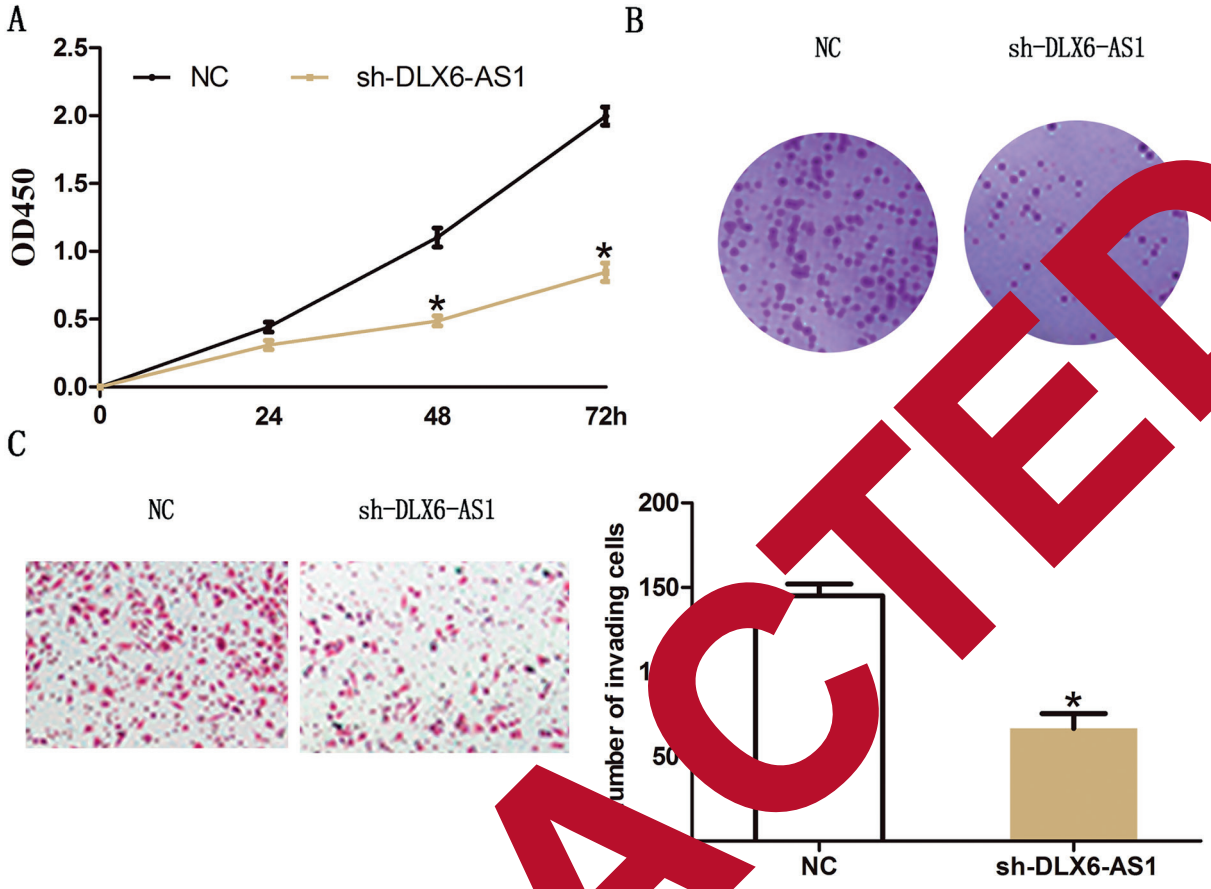

Figure 2. Knockdown of DLX6-AS1 i d CC cell ration and invasion. ***A,*** CCK8 assay showed that cell proliferation was remarkably suppressed *vi* of DLX 1 in SiHa cells. ***B,*** Colony formation assay indicated that the cell growth ability in sh-DLX6-A up wa nificantl reased compared with NC in SiHa cells (magnification: 10×). ***C,*** Transwell assay displayed th number nvaded cel was decreased *via* knockdown of DLX6-AS1 in SiHa CC cells (magnification: 40×). The resul sent three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared wit co

Knockd of DLX6- Inhibited Cell Prolif ion and Invas n CC Cells

C 8 ass howed that cell proliferation was ark suppressed *via* knockdown of DLX6- SiHa (Figure 2A). Moreover, erfo col formation assay and found th nockd DLX6-AS1 inhibited CC cell gr h ability (igure 2B). Results of transwell as- at cell invasion of CC was inhibited fter DLX6-AS1 was knocked down (Figure 2C).

Interaction Between FUS and DLX6-AS1 in CC

FUS was predicted by Starbase v2.0 as the target protein of DLX6-AS1. Moreover, qRT-PCR results demonstrated that compared with the FUS level in NC group, it was lower-expressed in sh-DLX6-AS1 group (Figure 3A). Western blot assay found out that after DLX6-AS1 was knocked down, FUS could be downregulated at the protein level (Figure 3B). In addition, FUS expression was markedly higher in CC tissues compared with that of adjacent tissues (Figure 3C). Correlation analysis demonstrated that FUS expression level positively correlated to DLX6-AS1 expression in CC tissues (Figure 3D).

Knockdown of DLX6-AS1 Inhibited Tumor Formation and Metastasis in Nude Mice

The ability of DLX6-AS1 in CC formation and metastasis was detected in nude mice. The tumor size in sh-DLX6-AS1 group was smaller compared with NC group (Figure 4A). The num-

ber of metastatic nodules in the lung from the sh-DLX6-AS1 group was less than that from NC group (Figure 4B). Moreover, the expression level of DLX6-AS1 and FUS in dissected tumor tissues was detected by qRT-PCR. DLX6-AS1 was reduced in sh-DLX6-AS1 group compared with NC group (Figure 4C). FUS was reduced in sh-DLX6-AS1 group compared with NC group (Figure 4D).

Discussion

Numerous studies have indicated that lncRNAs have emerged as important regulators in the development of CC. For instance, lncRNA HOTAIR promotes the development of cervical cancer *via* targeting Notch pathway[8]. LncRNA LINP1 promotes repair of DNA double-strand breaks and raises the sensitivity of cancer cells to radiotherapy in CC[9]. LncRNA PTENP1 depresses the proliferation and metastasis of CC cells[10].

LncRNA DLX6 antisense RNA 1 (DLX6-AS1) is abnormally expressed in several cancers and promotes tumor progression. For example, DL[illegible] AS1 induces cell invasion by regulating miR-181b in pancreatic cancer[11]. DLX6-AS1 promotes cell proliferation and metastasis through regulating miR-144 in non-small cell lung cancer[12]. In this study, DLX6-AS1 was upregulated both in CC samples and cells. CC cell proliferation [illegible] vasion were found to be inhibited thro[illegible] down of DLX6-AS1. Above results i[illegible]ated that DLX6-AS1 might act as an oncoge[illegible]CC proliferation and invasion

Fused in sarcoma (FUS) was pred[illegible] by Starbase v2.0 as the ta[illegible] protein of D[illegible] AS1. Previous resea[illegible]s[13,14] h[illegible] sugges[illegible] that the aberrant ac[illegible]va[illegible]of[illegible]S plays a fundamental role in [illegible]ariet[illegible]ologic[illegible]ehaviors of malign[illegible]umors. K[illegible]al[illegible]ows that downregula[illegible]FUS/TLS [illegible]esses cell proliferati[illegible]and i[illegible]es cell apoptosis in CC. Our w[illegible]howed tha[illegible]S expression could be inh[illegible]y knockdow[illegible]DLX6-AS1. Moreo[illegible]FUS expression was positively related to D[illegible]6-AS1 exp[illegible]sion in CC tissues. Further re[illegible]ches show[illegible]that knockdown of DLX6-AS[illegible]inhib[illegible]tumor formation and metastasis i[illegible]ce.

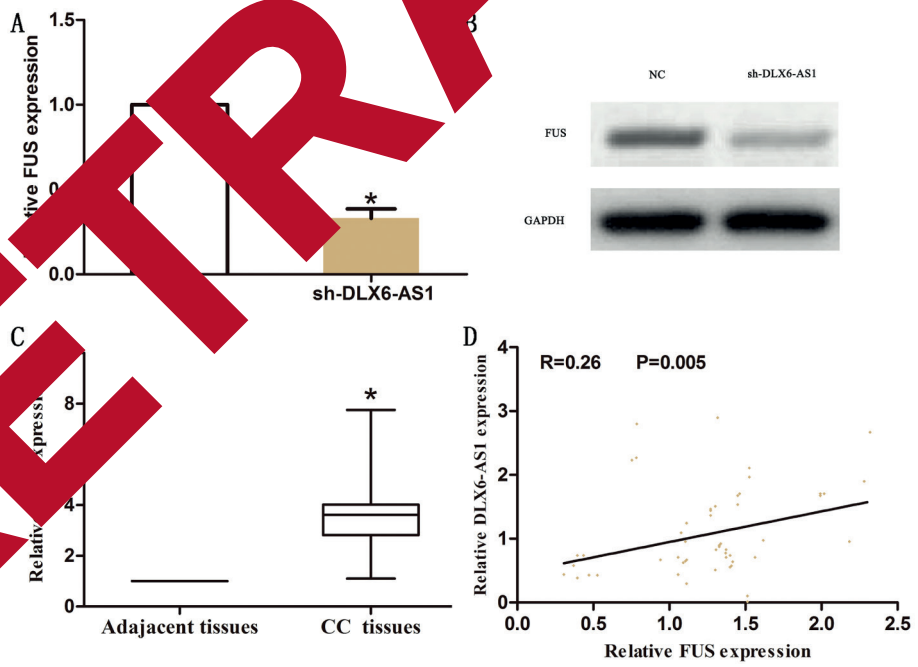

Figure 3. The association between DLX6-AS1 and FUS. ***A,*** Results of qRT-PCR showed that FUS expression was lower in sh-DLX6-AS1 group compared with the NC group. ***B,*** Western blot assay revealed that FUS protein expression was decreased in sh-DLX6-AS1 group compared with NC group. ***C,*** FUS was significantly upregulated in CC tissues compared with corresponding tissues. ***D,*** The linear correlation between the expression level of FUS and DLX6-AS1 in CC tissues. Data are presented as the mean ± standard error of the mean. **p*<0.05.

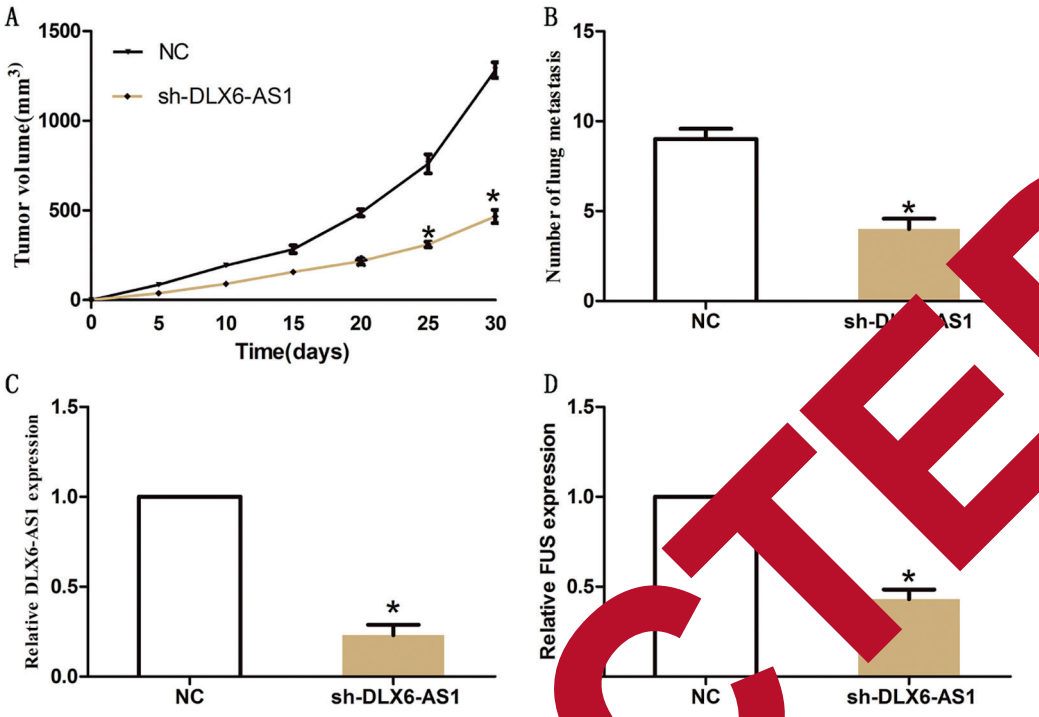

Figure 4. Knockdown of DLX6-AS1 inhibited tumor formation and [illegible]tasis of C[illegible] nude mice. ***A,*** The tumor size in sh-DLX6-AS1 group was smaller compared with NC grou[illegible]. ***B,*** The numbe[illegible]odules in the lung from the sh-DLX6-AS1 group was significantly reduced compared to N[illegible]C. DLX6-AS[illegible] dissected tumors was lower-expressed in sh-DLX6-AS1 group compared with NC group. ***D,*** F[illegible]cted tumors was lower-expressed in sh-DLX6-AS1 group compared with NC group. The results represent the a[illegible]ge of [illegible]pendent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

Conclusions

LncRNA DLX6-AS[illegible]uld enhance CC cell proliferati[illegible]and invasio[illegible]rough upregulating FUS, [illegible] implied that DL[illegible]S1 could act as a pro[illegible]ive the[illegible]utic target for CC.

Conflict of [illegible]s

Th[illegible]thors decl[illegible] that they have no conflict of interests.

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