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Long noncoding RNA PROX1-AS1 promoted ovarian cancer cell proliferation and metastasis by suppressing KLF6

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Abstract. – OBJECTIVE: Recently, the role of long noncoding RNAs (lncRNAs) in tumor progression has attracted much attention worldwide. Numerous studies have identified lncRNA PROX1-AS1 as an oncogene in cancers. Therefore, the aim of this research was to explore the function of PROX1-AS1 in the development of ovarian cancer.

PATIENTS AND METHODS: PROX1-AS1 expression in both ovarian cancer patients and normal subjects was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Subsequently, PROX1-AS1 shRNA was constructed and transfected *in vitro*. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, colony formation assay, transwell assay, and Matrigel assay were utilized to detect the function of PROX1-AS1 in ovarian cancer. In addition, the potential mechanism was explored using qRT-PCR and Western blot assay.

RESULTS: PROX1-AS1 was highly expressed in ovarian carcinoma samples and cell lines ($p<0.05$). The proliferation, migration and invasion of ovarian cells were significantly inhibited after PROX1-AS1 was downregulated *in vitro* ($p<0.05$). Besides, the mRNA expressions of KLF6 were significantly promoted after PROX1-AS1 knockdown in ovarian cancer cells ($p<0.05$). Further functional assays showed that KLF6 expression was negatively correlated with PROX1-AS1 expression in ovarian cancer samples.

CONCLUSIONS: PROX1-AS1 enhances the metastasis and proliferation of ovarian cancer cells by suppressing KLF6. Our findings suggest that PROX1-AS1 may be applied as a novel target for therapy of ovarian cancer.

Key words:
Long noncoding RNAs, PROX1-AS1, Ovarian cancer, KLF6.

Introduction

Ovarian cancer remains the most fatal and common tumor in women globally, accounting for 5-6% cancer-related deaths[1,2]. It has been reported that 22,000 patients are newly diagnosed of ovarian cancer in America in 2017, with 14,100 deaths[3,4] due to the vagueness of symptoms and lack of early detection tests, 70% of patients have already been in advanced stages when first diagnosed[5]. Whereas, almost 50% of the patients may develop resistance to chemotherapy or recurrence after surgery[6,7]. Therefore, the severe situation underscores the urgency of early detection and the establishment of new therapeutic interventions for cancer patients.

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs that do not encode proteins. Currently, lncRNAs have emerged as an important role in tumorigenesis. Multiple studies have found that lncRNAs have the potential to regulate gene expression. By regulating vasculogenic angiogenesis, lncRNA MALAT1 promotes tumorigenicity and metastasis in gastric cancer[8]. LncRNA AC132217.4 promotes the metastasis of oral squamous cell carcinoma by regulating IGF2 expression modulated by KLF8[9]. By sponging miR-124-3p, lncRNA OGFRP1 has been reported to take part in the proliferation of non-small cell lung cancer cells[10]. Upregulation of lncRNA LINC01510 has been confirmed negatively associated with the prognosis of colorectal cancer (CRC) patients. Meanwhile, lncRNA LINC01510 may serve as a potential independent prognostic biomarker for CRC[11]. LncRNA HCCL5 activated by ZEB1 accelerates cell viability, cell migra-

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tion, epithelial-mesenchymal transition, and the malignancy of hepatocellular carcinoma[12]. However, few studies have uncovered the exact role of PROX1-AS1 in ovarian cancer, as well as the underlying mechanism.

In our study, we found that PROX1-AS1 was remarkably upregulated in ovarian cancer tissues. PROX1-AS1 enhanced the proliferation and metastasis of ovarian cancer *in vitro*. Moreover, KLF6, a tumor suppressor gene, was associated with the function of PROX1-AS1 in ovarian cancer.

Patients and Methods

Tissue Specimens

Paired ovarian carcinoma tissues and adjacent normal tissues were sequentially collected from 50 ovarian carcinoma patients undergoing surgery from December 2017 to December 2019 in The First Affiliated Hospital of XinJiang Medical University. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC) ... study was approved by The Ethics Comm... of The First Affiliated Hospital of XinJiang ... ical University. The protocol of the study ... performed as Declaration of Helsinki Principl... required.

Cell Culture

Human ovarian cancer cel... s (A... V112D, HO-8910, and SKO... al ovarian cell line (ISOE80) we... cult... Dulbecco's Modified Eagle's M... m (DMI... mo Fisher Scientific, Inc., ... ham, MA, US... on-sisting of 10% fetal b... m (FBS; Thermo Fisher Scientific, I... Wal... A, USA) and penicillin, and m...intained in a ... ed atmosphere with 5% ... at 37°C.

Lentivirus ...pressi... Short-Hairpin RNA and Cell ...fe...n

Lentivirus ...ing ...rt-hairpin RNA (shRNA) directed ... X1-AS1 were provided ... ePharm... nghai, China). The com... DNA encoding PROX1-AS1 was an... ed a... into pcDNA3.1 (Gene-P... S... ina). Subsequently, they wer... ted into ovarian cancer cells, according ... tructions of Lipofectamine 3000 (Invitrogen, ... bad, CA, USA). PROX1-AS1 expression in transfected cells was conducted using quantitative Real Time ... Chain Reaction (RT-qPCR).

RNA Extraction and ...PCR

Total RNA in tissues a... was e... cted by using TRIzol re... ent (In... sbad, CA, USA). Subse... tly, extract... RNA was reverse-tran... ed t... omplem... tary deoxyribose nucle... ids (c... As) th... ugh Reverse Transcription ... Bio... nology Co., Ltd., Dalian, Chin... ao-... ng conditions were as fo... s: 30s a... or 40 cycles at 95°C, an... at 60°C. $2^{-\Delta\Delta}$... thod was utilized for cal... lative expression of genes. The prime... que... d in this study were as follows: PROX1-AS... d 5′-CTAGTTAGCAG-G... AGCAC-3′, P... X1-AS1 reverse 5′-AA-C... AGAGGCGTGGAAGAA-3′; β-actin, forward 5′-GATGG... AATCGTCAGAGGCT-3′ and reverse 5′-TGGC... TTAGTTGGAAATGC-3′.

...Analysis

P... ins were collected from cells *via* radioimmunoprecipitation assay (RIPA) buffer and ...ntified by using a protein assay (bicinchoninic ...od; Beyotime, Shanghai, China). Target proteins were separated by sodium dodecyl ...lphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene ...ifluoride (PVDF) membranes. Then, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with rabbit anti-β-actin (Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-KLF6 (Cell Signaling Technology, Danvers, MA, USA). The Pierce enhanced chemiluminescence (ECL) was utilized for visualizing Western Blotting Substrate Immunoreactive bands (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide] Assay

MTT (Roche, Basel, Switzerland) assay was used to detect cell proliferation. 200 μL of transfected cells were first seeded into 96-well plates at a density of 2×10^3 cells per well. Following the manufacturer's protocol, cell proliferation was assessed every 24 h.

Colony Formation Assay

To detect the long-term effect of PROX1-AS1 on cell proliferation, colony formation assay was conducted. 5×10^2 cells/well cells were seeded into 6-well plate, and culture medium was replaced

every day. 7 day later, formed colonies were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet. Finally, colonies were photographed and counted.

Wound Healing Assay

After transfection, the cells were seeded into 6-well plates and incubated in DMEM medium overnight. On the next day, the cells were scratched with a plastic tip and cultured in serum-free DMEM. Each assay was repeated in triplicate independently. Relate distance was viewed under a light microscope (Olympus Corp., Tokyo, Japan) at 48 h.

Transwell Assay and Matrigel Assay

After transfection, 1 $\times 10^5$ cells in 200 µL serum-free DMEM were replanted in top chamber (Corning, Inc., Corning, NY, USA) with or without 50 µg Matrigel (BD, Bedford, MA, USA). Meanwhile, DMEM and FBS were added to the lower chamber. These chambers were then cultured overnight in an incubator supplemented with 5% CO_2 at 37°C. The top surface of chambers was fixed with methanol for 30 mi... wiped by cotton swab, followed by staini... crystal violet for 20 min. Five fields wer... domly selected for each sample. The numbe... migrating and invasive cells was finally count... under a Leica DMI4000B microsc... eica Microsystems, Heidelberg, German...

Statistical Analysis

Statistical Product and ... olutions (SPSS) 18.0 (SPSS, Chica... IL, ... as used for all statistical analysis... e differe... en two groups were compared ... t-test. $p<0.05$ was considered stati... ly sig... t.

Resu...

PROX1-AS1 Was ...ghly Expr... in Ovarian Cance... Tissue... nd Cel...

PROX1-AS1 ...essio... 50 p...rs of ovarian cancer tissues ... norma... sues was detected *via* qRT-PC... 1-A... was highly expressed in ...ian can... when compared with adja... normal tissues ... 0.05, Figure 1A). Moreov... 1-AS1 level in ovarian cancer cells ... sig... higher than that of normal ovar...n cell line ... <0.05, Figure 1B).

... Proliferation Was Repressed Via ...ence of PROX1-AS1 in Ovarian Cancer

...n our study, ...-8910 cells were chosen for ...knockdow... PROX1-AS1 *in vitro*. qRT-... d for detecting PROX1-AS1 expres... ...re 2A). MTT assay indicated that the growth ability of HO-8910 cells was signifi... ...tly repressed after PROX1-AS1 was knocked ...0.05, Figure 2B). Colony formation ...ay d...monstrated that the number of colonies ...ecreased significantly after PROX1-AS1 was knocked down ($p<0.05$, Figure 2C).

...ell Migration and Invasion Was Repressed Via Silence of PROX1-AS1 in Ovarian Cancer

Wound healing assay, transwell assay, and Matrigel assay were performed to explore how PROX1-AS1 affected ovarian cancer metasta-

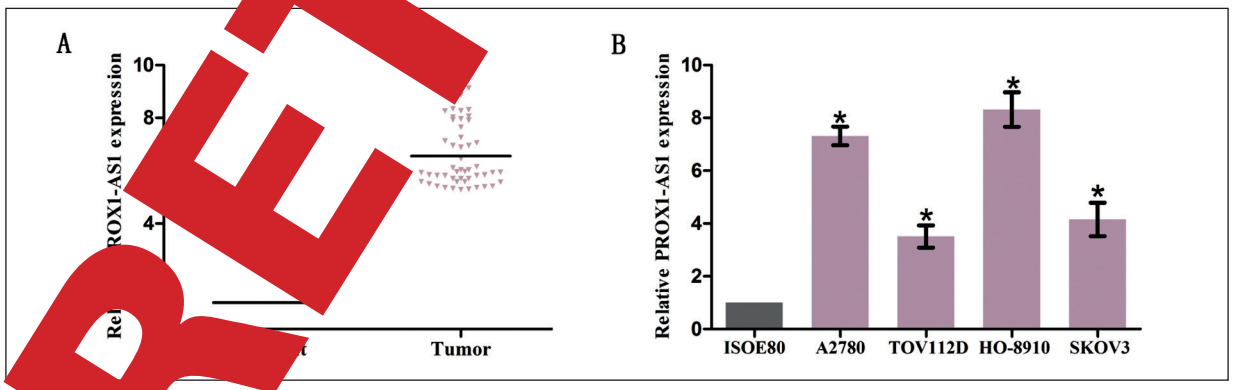

Figure ... ...sion levels of PROX1-AS1 increased significantly in ovarian cancer tissues and cell lines. **A,** QRT-PCR results sho... ROX1-AS1 expression was significantly up-regulated in ovarian cancer tissues compared with adjacent normal tissues. ... ...ression levels of PROX1-AS1 relative to β-actin were determined in human ovarian cancer cell lines and normal ovarian cell ISOE80 by qRT-PCR. Data were presented as the mean ± standard error of the mean. *$p<0.05$.

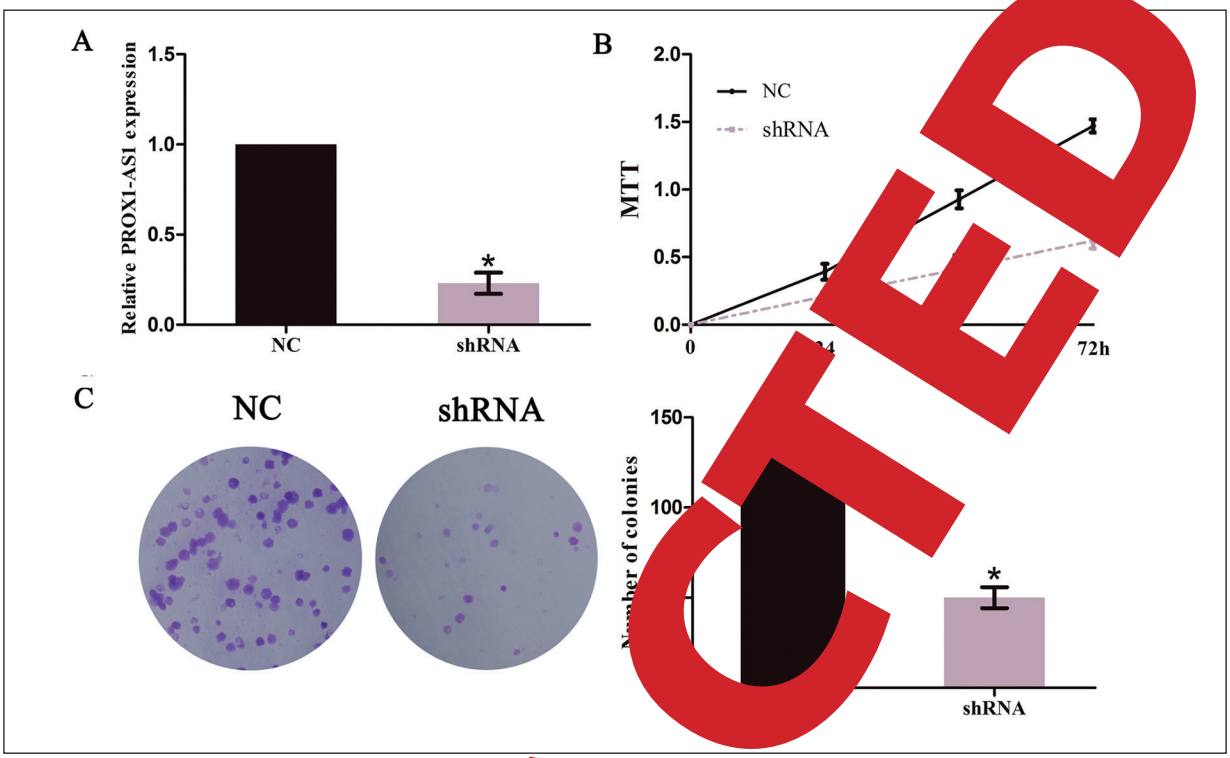

Figure 2. Knockdown of PROX1-AS1 inhibited liferation. **A,** PROX1-AS1 expression in ovarian cancer cells transduced with PROX1-AS1 shRNA (A) an ol (NC) was detected by qRT-PCR. *β-actin* was used as an internal control. **B,** MTT assay showed tha kdown -AS1 significantly inhibited the growth of ovarian cancer cells. **C,** Colony formation assay showed that ki w ROX1-AS1 significantly reduced the number of colonies in ovarian cancer cells (magnification: 40×). The results ed the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the l cells.

sis *in vitro*. Wound healing y rev the migrated length of ovari lls was significantly repressed a r PR S1 was knocked down ($p<0.05$ ure 3A). lts of transwell assay sho that the migra of ovarian cancer cells rkably repressed after PROX1-AS1 nreg (Figure 3B). Furthermore, Matrigel assay i that the number of inva cells decrease emarkably after PROX1- was knocked down *in vitro* ($p<0.05$, Fig 3C).

The Interaction between KLF6 and PROX1-AS1 in Ovarian Cancer

The xp on level of KLF6 in ovarian cancer cel was hi X1-AS1 shRNA (shRNA) s he ith negative control (control) <0.05, Figure 4A). Western blot assay found ou protein expression level of KLF6 was signific upregulated after PROX1-AS1 was knocked down ($p<0.05$, Figure 4B). Moreover, KLF6 expression in ovarian cancer tissues was significantly lower than that of adjacent normal tissues ($p<0.05$, Figure 4C). These findings all suggested that there was a negative association between KLF6 expression and PROX1-AS1 expression in ovarian cancer (Figure 4D).

Discussion

Many studies[13-16] revealed the abnormal expression and function of lncRNAs in the development of malignant tumors. They have also been found to directly participate in the proliferation, apoptosis, metastasis, immune regulation, and drug resistance in tumors. Meanwhile, lncRNA in involved in the regulation of various cellular functions, whose disorders are usually associated with human diseases, including malignant tumor.

Reports have indicated that lncRNA is an important regulator in the progression of ovari-

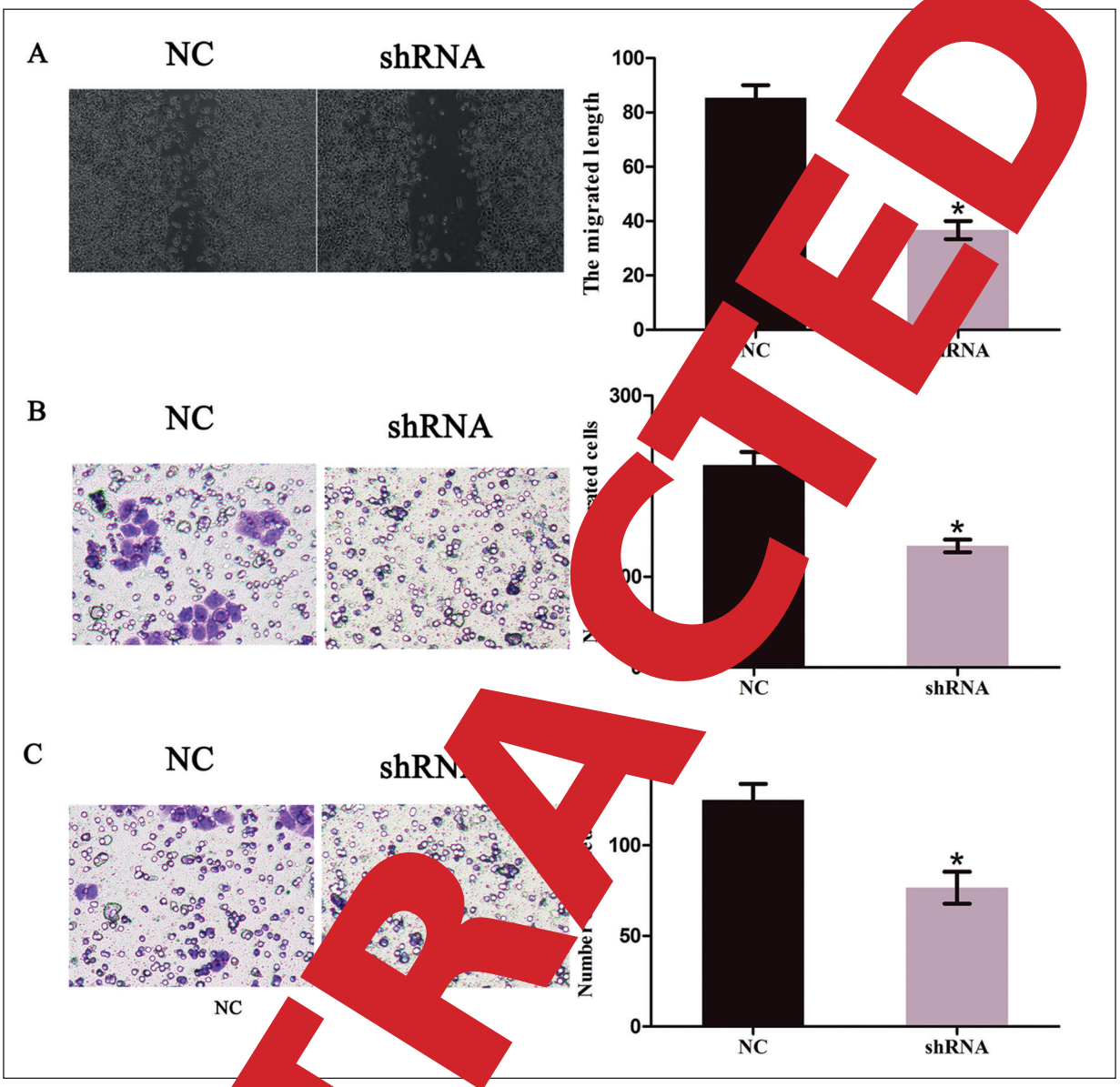

Figure 3. Knockdown [illegible] hibited ovarian cancer cell migration and invasion. **A,** Wound healing assay showed that knockdown of PRO[illegible]AS1 sig[illegible]duced migrated length in ovarian cancer cells (magnification: 40×). **B,** Transwell assay showed that k[illegible]ockdown of PRO[illegible]ignificantly inhibited the migration of ovarian cancer cells (magnification: 40×). **C,** Matrigel [illegible] showed that the n[illegible]er of invaded cells decreased remarkably *via* knockdown of PROX1-AS1 *in vitro* (magnificati[illegible]0×). The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$ [illegible]ompar[illegible]th the control cells.

an cancer, servi[illegible]tial biomarker and thera[illegible]rget. O[illegible] silence of lncRNA MN[illegible] ppresses the proliferation and migr[illegible]n of [illegible]r cells[4]. LncRNA Eln-[illegible] fu[illegible] oncogene in the proliferati[illegible]elial OC cells, which is upregulated by estro[illegible]addition, knockdown of lncRNA AFAP1-AS1 [illegible]otes the proliferation and apoptosis of ovarian cancer cells[18].

PROX1 antisense RNA 1 (PROX1-AS1) is a newly explored lncRNA that has been suggested to promote the progression of papillary thyroid carcinoma[19]. Our results showed that PROX1-AS1 expression was significantly upregulated in both ovarian cancer tissues and cells. Multiple lncRNAs have been reported to participate in the development of ovarian cancer, emerging as potential indicators and therapeutic targets. In fact,

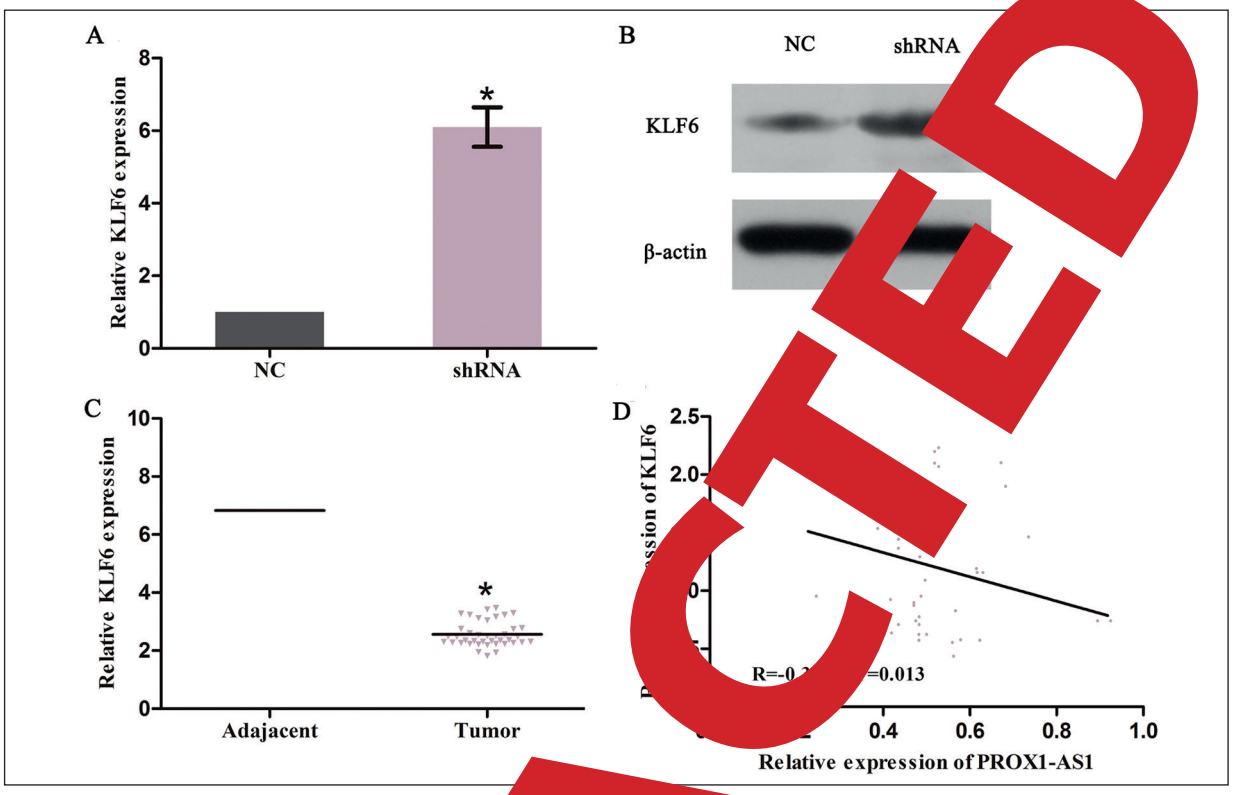

Figure 4. Interaction between PROX1-AS1 and [illegible] in ova[illegible]sues and cells. **A,** RT-qPCR results showed that KLF6 expression was significantly higher in PROX1-[illegible]hRNA [illegible]NA), group compared with NC group. *β-actin* was used as an internal control. **B,** Western blot assay revealed th[illegible] expression of increased in PROX1-AS1 shRNA (shRNA) group compared with NC group. **C,** KLF6 was significa[illegible]nregulated in ovarian cancer tissues when compared with adjacent normal tissues. **D,** The linear correlation betwee[illegible]pression level of KLF6 and PROX1-AS1 in ovarian cancer tissues. The results represented the aver[illegible]e indepe[illegible]periments. Data were presented as the mean ± standard error of the mean. *$p<0.05$.

lncRNA SNHG14 facilita[illegible]s the [illegible]sion of cervical cancer by regul[illegible]g miR-20[illegible]AZ signaling pathway[20]. T[illegible]gh regulation [illegible]epithelial-mesenchymal [illegible] downregulation of lncRNA SPRY4[illegible] ei[illegible]e metastasis of ovarian cancer[21]. LncRNA E[illegible], activated by estrogen, [illegible]ctions as an onc[illegible]ne in epithelial ovarian [illegible]ncer c[illegible][17]. In the present study, PROX1-AS1 [illegible]s firs[illegible]ocked down in ovarian cancer cell[illegible] indic[illegible] that the proliferation of ov[illegible]cer c[illegible] was significantly inhibited after P[illegible] [illegible]as knocked down *in vit*[illegible]over, c[illegible]gration and invasion wer[illegible]rkably inhi[illegible]ted after PROX1-AS1 do[illegible]egula[illegible] above results indicated t[illegible]OX1-AS1 was associated wit[illegible]lopment of ovarian cancer.

Next, [illegible]lored the potential target proteins of P[illegible]-AS1 using bio-informative methods. The results showed that the potential target protein, Krüppel-like factor 6 (KLF6), was significantly downregulated in ovarian cancer tissues. Known as a tumor suppressor, KLF6 takes part in the regulation of a variety of biological processes in many carcinomas. KLF6 is deleted glioblastomas, which is related to poor prognosis of patients by targeting KLF6[22]. KLF6 constrains the progression of hepatocellular carcinoma dissemination by regulating the VAV3-RAC1 signaling axis[23]. Meanwhile, KLF6 accelerates cell proliferation and invasion in epithelial ovarian cancer[24]. In the present study, the mRNA and protein expressions of KLF6 were both significantly upregulated after the knockdown of PROX1-AS1. Moreover, KLF6 expression was negatively correlated with PROX1-AS1 expression in ovarian cancer tissues. All these results suggested that PROX1-AS1 might promote tumorigenesis of ovarian cancer *via* suppressing KLF6.

Conclusions

The above results demonstrated that PROX1-AS1 was highly expressed in both ovarian cancer tissues and cells. Besides, PROX1-AS1 enhanced ovarian cancer cell proliferation, migration, and invasion by targeting KLF6. The novelty of this study was that PROX1-AS1 might contribute to therapy for ovarian cancer as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.
2) Chen Q, Liu X, Xu L, Wang Y, Wang S, Li Q, Huang Y, Liu T. Long non-coding RNA BACE1-AS is a novel target for anisomycin-mediated suppression of ovarian cancer stem cell proliferati invasion. Oncol Rep 2016; 35: 1916-192
3) Siegel RL, Miller KD, Jemal A. Cancer st 2017. CA Cancer J Clin 2017; 67: 7-30.
4) Lv Y, Li H, Li F, Liu P, Zhao X. Long noncoding R MNX1-AS1 knockdown inhibits cell proliferatio and migration in ovarian cance Biother Radiopharm 2017; 32: 91-99.
5) Lalwani N, Prasad SR, Vikra Shan Huettner PC, Fasih N. Hist mo cytogenetic features of ova implications for diagnosis and atme graphics 2011; 31: 625-646.
6) Bast RJ, Hennessy B, GB. The biology var-ian cancer: new o for translation. Nat Rev Cancer 200
7) Cannistra SA. Ca er of the Engl J Med 2004; 351: 25 2529.
8) Li Y, Wu Z, J, Sun L, Lin L, Huang N, Bin J, Liao Y, Liao ong n oding RNA MALAT1 promotes c c can umori nicity and metastasis b sculo mimicry and angiogenesi ett 2 395: 31-44.
9) Li X, M C, Zha ng X, He J, He R, Shao M Kang L, LncRNAAC132217.4, a ated long he -coding RNA, facilitates l squ ll carcinoma metastasis by upgulati ession. Cancer Lett 2017;
10) Chen GH, Li H, He P, Zhang Y, Xu XW. Long g RNA OGFRP1 regulates LYPD3 expressi sponging miR-124-3p and promotes non-small cell lung cancer progression. Biochem Biophys Res Com 5: 578-585.
11) Zhou JF, Wang HG, M Yan W, W Shi YT, Yang XZ. Long no g RNA LINC 0 is highly expressed in cancer a re-dicts favorable prognosi Med rma-col Sci 2018; 22: 0-7715.
12) Peng L, Jiang B n X, Qiu Y, P Huang Y, Zhang C, Zhan in Z, I ao W, De W, Zhang Y, Meng M, L Li C D, Bi X, Li G, Lin DC. Super-enh ed lon coding RNA HCCL5 is a ZEB1 promotes the maligna of he cinoma. Cancer Res 2 79: 572-5
13) Idog Ohashi T, Sas , Nakase H, Tokino T. ding RNA NEAT1 is a transcripti ta and modulates p53-induced tra activati mor-suppressor function. J Cancer 20 2785-2791.
14) Li W, Li H, Zhang L, Hu M, Li F, Deng J, An M, Wu S, Ma R, Lu J, Zhou Y. Long non-coding RNA LINC00672 c tributes to p53 protein-mediated gene sup sion and promotes endometrial cancer che ensitivity. J Biol Chem 2017; 292:
15) g Y, An W, Xu J, Zhang G, Jie J, Zhang Q. The long noncoding RNA-ROR promotes the resistance of radiotherapy for human colorectal cer cells by targeting the p53/miR-145 pathJ Gastroenterol Hepatol 2017; 32: 837-
16) Chiyomaru T, Fukuhara S, Saini S, Majid S, Deng G, Shahryari V, Chang I, Tanaka Y, Enokida H, Nakagawa M, Dahiya R, Yamamura S. Long non-coding RNA HOTAIR is targeted and regulated by miR-141 in human cancer cells. J Biol Chem 2014; 289: 12550-12565.
17) Qiu JJ, Zhang XD, Tang XY, Zheng TT, Zhang Y, Hua KQ. ElncRNA1, a long non-coding RNA that is transcriptionally induced by oestrogen, promotes epithelial ovarian cancer cell proliferation. Int J Oncol 2017; 51: 507-514.
18) Yang SL, Lin RX, Si LH, Cui MH, Zhang XW, Fan LM. Expression and functional role of long non-coding RNA AFAP1-AS1 in ovarian cancer. Eur Rev Med Pharmacol Sci 2016; 20: 5107-5112.
19) Shen Y, Xia E, Bhandari A, Wang X, Guo G. LncRNA PROX1-AS1 promotes proliferation, invasion, and migration in papillary thyroid carcinoma. Biosci Rep 2018; 38. pii: BSR20180862.
20) Ji N, Wang Y, Bao G, Yan J, Ji S. LncRNA SNHG14 promotes the progression of cervical cancer by regulating miR-206/YWHAZ. Pathol Res Pract 2019; 215: 668-675.
21) Yu J, Han Q, Cui Y. Decreased long non-coding RNA SPRY4-IT1 contributes to ovarian cancer cell metastasis partly via affecting epithelial-mesenchymal transition. Tumour Biol 2017; 39: 1393380535.
22) Masilamani AP, Ferrarese R, Kling E, Thudi NK, Kim H, Scholtens DM, Dai F, Hadler M, Unterkircher T, Pla-

TANIA L, WEYERBROCK A, PRINZ M, GILLESPIE GY, HARSH GI, BREDEL M, CARRO MS. KLF6 depletion promotes NF-kappaB signaling in glioblastoma. Oncogene 2017; 36: 3562-3575.

23) AHRONIAN LG, ZHU LJ, CHEN YW, CHU HC, KLIMSTRA DS, LEWIS BC. A novel KLF6-Rho GTPase axis regulates hepatocellular carcinoma cell migration and dissemination. Oncog 4653-4662.

24) ZHANG S, ZHANG JY, LU WANG CH, V LH. MiR-630 promotes lial ovarian cer proliferation and inv targetin F6. Eur Rev Med Pharmac 7; 2 542-4547.