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LncRNA PROX1-AS1 promotes proliferation, invasion, and migration in prostate cancer *via* targeting miR-647

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Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) act as an important role in many diseases. In this research, lncRNA PROX1-AS1 was explored to identify how it functioned in the development of prostate cancer (PC).

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect PROX1-AS1 expression in PC patients. Then, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, colony formation assay, and transwell assay were performed to identify its function in PC cells. Furthermore, the potential mech[illegible] was also explored using mechanism ass[illegible]

RESULTS: PROX1-AS1 expression leve[illegible]as significantly higher in PC tissue samples an[illegible]l lines. Results of MTT assay, colony formation[illegible]say, and transwell assay showed that cell pro[illegible]eration and invasion were inhi[illegible]rough th[illegible] silence of PROX1-AS1 in PC [illegible], w[illegible]cell proliferation and invasion we[illegible]romot[illegible]hrough the overexpression of P[illegible]-AS1 i[illegible]cells. Furthermore, the expres[illegible]f miR[illegible] regulated *via* the sil[illegible]e [illegible]AS1 in PC cells, while the expr[illegible]on of [illegible]7 was down-regulated *via* the [illegible]expressio[illegible]ROX1-AS1 in PC cells. Fur[illegible]chanism as[illegible]howed that miR-647 w[illegible]target of PRO[illegible]1-AS1 in PC. Correlati[illegible]nalys[illegible]ed that miR-647 expression w[illegible]negatively [illegible]ted with PROX1-AS1 exp[illegible]on in PC tissue[illegible]

CON[illegible]SIONS: Results a[illegible]ove suggested that [illegible]X1-AS[illegible]uld enhance cell proliferatio[illegible] invas[illegible]of PC [illegible]ells by sponging miR-647 [illegible]e appl[illegible]as a novel target for the trea[illegible]of PC.

[illegible]rds:
L[illegible] noncod[illegible]NA, PROX1-AS1, Prostate cancer, [illegible]647.

Introduction

[illegible]state cancer (PC), as one of the most com[illegible]mo[illegible]ncers in males, is the third highest cause of cancer-related deaths in developed countries worldwide[illegible]o the aging [illegible]ulation, increased [illegible]ene[illegible]d the use of prostate-specific antigen (PSA)[illegible]um for screening and diag[illegible]he prevalen[illegible]PC, accounting for 27[illegible]al cancer cases, [illegible] increasing over the [illegible] decades and approximately 233,000 new [illegible]es were annu[illegible] diagnosed with PC. Among [illegible], 29,480 ca[illegible]end with deaths[2]. However, m[illegible]PC patie[illegible]are diagnosed at advanced stag[illegible]the lack of specific and sensitive meth[illegible]for early PC screening. Therefore, [illegible]veral studies have been carried out to help to [illegible]d the molecular and biological mecha[illegible]sm [illegible]he progression of PC.

Long noncoding RNAs (lncRNAs) are a subgroup of non-coding RNAs without the ability of encoding proteins. Numerous studies have discovered that lncRNAs emerge as an important role in tumorigenesis with the potential to regulate gene expression. LncRNA LINP1 promotes repair of DNA double-strand breaks and enhances the sensitivity of cancer cells to radiotherapy in breast cancer[3]. The knockdown of lncRNA NEAT1 promotes the development of PC by disturbing the cell cycle and inhibiting cell proliferation[4]. The upregulation of lncRNA LINC01510 is negatively associated with the prognosis in patients with colorectal cancer, which may serve as a potential independent prognostic biomarker[5]. LncRNA AC132217.4 promotes tumor metastasis in oral squamous cell carcinoma *via* the regulation of IGF2 expression[6].

Recently, the function of lncRNA PROX1-AS1, as a novel lncRNA, has caught much attention. However, few studies have uncovered the function of PROX1-AS1 in PC. Our work aims to identify whether PROX1-AS1 participates in the progression of PC and the potential underlying mechanism.

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Patients and Methods

Tissue Samples

50 PC patients were collected in this research and they underwent surgical resection at The First People's Hospital of Yulin. Before the surgery, no radiotherapy and chemotherapy treatment were performed in any patient. After surgical resection, all the tissue samples were immediately snap-frozen in liquid nitrogen. This investigation was approved by the Ethics Committee of The First People's Hospital of Yulin. Signed written informed consents were obtained from all participants before the study.

Cell Culture

The PC cell lines (LNCaP, DU145, and 22Rv1) and normal human prostate epithelial cell line (P69) were bought from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL p[enicil]lin, and 100 µg/mL streptomycin in a hu[midified] incubator at 37°C with 5% CO_2.

Cell Transfection

Lipofectamine 2000 reage[nt] (Invitroge[n], Carlsbad, CA, USA) was uti[lized] [t]ransient-ly transfect PC cells wi[th] [n]egati[ve] control, complementary deoxyrib[o]nucleic [a]cid (cDNA) oligonucleotides ag[ainst] PRO[X1-AS1] (si-PROX1-AS1) or lent[ivirus] [v]ector [o]f PROX1-AS1 (PROX1-AS1) resp[ectiv]ely, whi[ch] [w]ere provided by GenePharma [Co.] (Shanghai, China). [Cel]ls were collected for f[urther] [e]xperiments [after] incubated for 24 [h].

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

According to the manufacturer's instructions, total R[NA] was extracted from ti[ssue]s and cells were separated by [Tri]zol reagent (Invitrogen, Carlsbad, CA, USA). By using a Reverse Transcription Kit (TaKaRa, Dalian, China), RNA was reverse transcribed to cDNA for Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). By using SYBR Green (TaKaRa, Dalian, China), RT-qPCR was performed to determine the expression of PROX1-AS1 in tumor and non-malignant tissues with a normalizing control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for RT-PCR are the following: PROX1-AS1 primer: forward: 5′-CTAGTTAPCAGGGPCAPCAC-3′; reverse: 5′-AACAGAGAGPCGTGGAAC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5′-CACC[...]CCTC-CACCTTTG-3′ and reverse: 5′-CCAC[...]CT-GTTPCTGTAG-3′. The thermal cycle was as follows: 30 sec at 95°C, 5 sec at 95°C, 40 cycles at 95°C, and 35 sec at [...]

Cell Proliferation Assay

Cell Proliferation Reagent WST-1 (WST; Roche, Basel, Switzerland) was used to detect cell proliferation. The transfected cells were seeded at a density of 2×10^3 cells/well in 200 µL culture medium in 96-well plates. Following the manufacturer's protocol, the cell proliferation was assessed every 24 h.

Colony Formation Assay

Cells were placed in a 6-well plate for 10 days. Cells were treated with 10% formaldehyde for 30 min. Then, 0.5% crystal violet was used for staining for 5 min. The analysis was performed with Image-Pro Plus 6.0 (Media Cybernetics, Silver Springs, MD, USA).

Transwell Assay

5×10^4 cells in 200 µL serum-free DMEM were transformed to the top chamber of an 8 µm pore size insert (Millipore, Billerica, MA, USA) coated with 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The bottom chamber was added with DMEM and FBS. 48 h later, the top surface of chambers was wiped by a cotton swab and immersed for 10 min with precooling methanol. Next, they were stained in crystal violet for 30 min. Three fields were used to count the data for invasion membrane.

Luciferase Assay

The 3′-UTR of PROX1-AS1 was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3′-UTR. Site-direction mutagenesis of the miR-647 binding site in PROX1-AS1 3′-UTR as mutant (MUT) 3′-UTR was conducted through quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Then, they were used for transfection of PC cells. The luciferase assay was conducted on the dual luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The differences between the two groups were compared by the Student's *t*-test. The statistical significance was defined as $p<0.05$.

Results

PROX1-AS1 Expression Level in PC Tissues and Cells

PROX1-AS1 expression was monitored by RT-qPCR in 50 PC patients' tissues and three PC cell lines. PROX1-AS1 was remarkably higher expressed in PC tissue samples compared with adjacent tissues (Figure 1A). The PROX1-AS1 expression level was higher in PC cells than in P69 (Figure 1B).

Cell Proliferation and Invasion were Repressed Via Knockdown of PROX1-AS1 in LNCaP PC Cells

The LNCaP PC cell line was selected for the knockdown of PROX1-AS1, transfection efficiency of which was detected by RT-qPCR (Figure 2A). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay showed that cell growth ability of LNCaP cells was inhibited *via* the knockdown of PROX1-AS1 (Figure 2B). Moreover, colony formation assay also revealed that the number of colonies was significantly reduced by the knockdown of PROX1-AS1 in LNCaP cells (Figure 2C). Furthermore, transwell assay also showed that the number of invaded cells was reduced after the knockdown of PROX1-AS1 in LNCaP cells (Figure 2D).

Cell Proliferation and Invasion were Enhanced Via Overexpression of PROX1-AS1 in DU145 PC Cells

DU145 PC cell line was selected for overexpression of PROX1-AS1. Transfection efficiency of which was detected by RT-qPCR (Figure 3A). The MTT assay showed that the cell growth ability of DU145 cells was promoted *via* the overexpression of PROX1-AS1 (Figure 3B). Moreover, colony formation assay also revealed that the number of colonies was significantly increased *via* the overexpression of PROX1-AS1 in DU145 cells (Figure 3C). Furthermore, transwell assay also showed that the number of invaded cells was increased after the overexpression of PROX1-AS1 in DU145 cells (Figure 3D).

The Interaction Between MiR-647 and PROX1-AS1 in PC

Starbase v2.0 (http://starbase.sysu.edu.cn/mirRNA.php) was used to find the miRNAs that contained complementary base with PROX1-AS1. As miR-647 was a tumor suppressor, it was selected from these microRNAs that interacted with PROX1-AS1 (Figure 4A). RT-qPCR assay showed that the expression of miR-647 was higher in sh-PROX1-AS1 group than in control group, while the expression of miR-647 was lower in PROX1-AS1 lentivirus group than in control

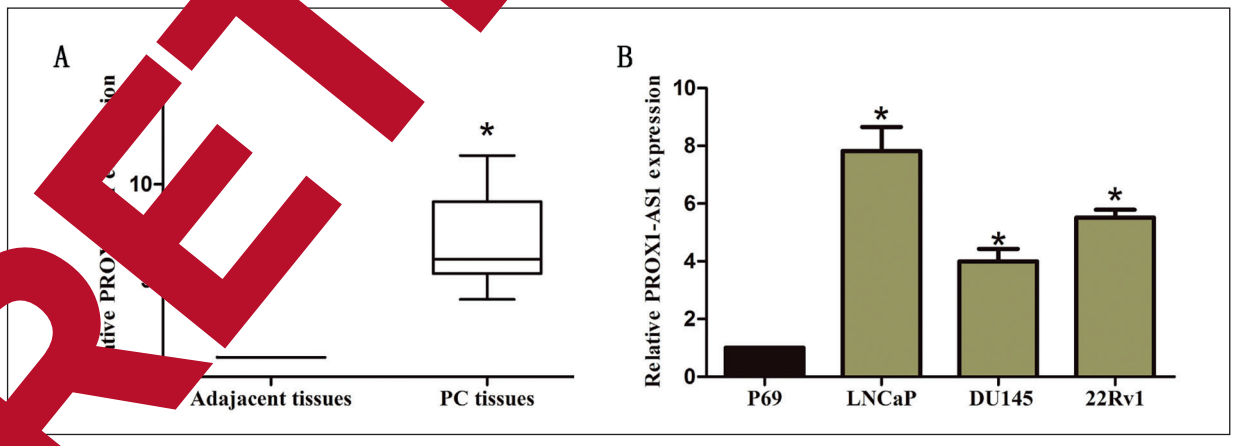

Figure 1. The expression levels of PROX1-AS1 in PC tissues and cell lines. **A,** PROX1-AS1 expression was significantly increased in the PC tissues compared with adjacent tissues. **B,** Expression levels of PROX1-AS1 were determined in the human PC cell lines and P69 by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

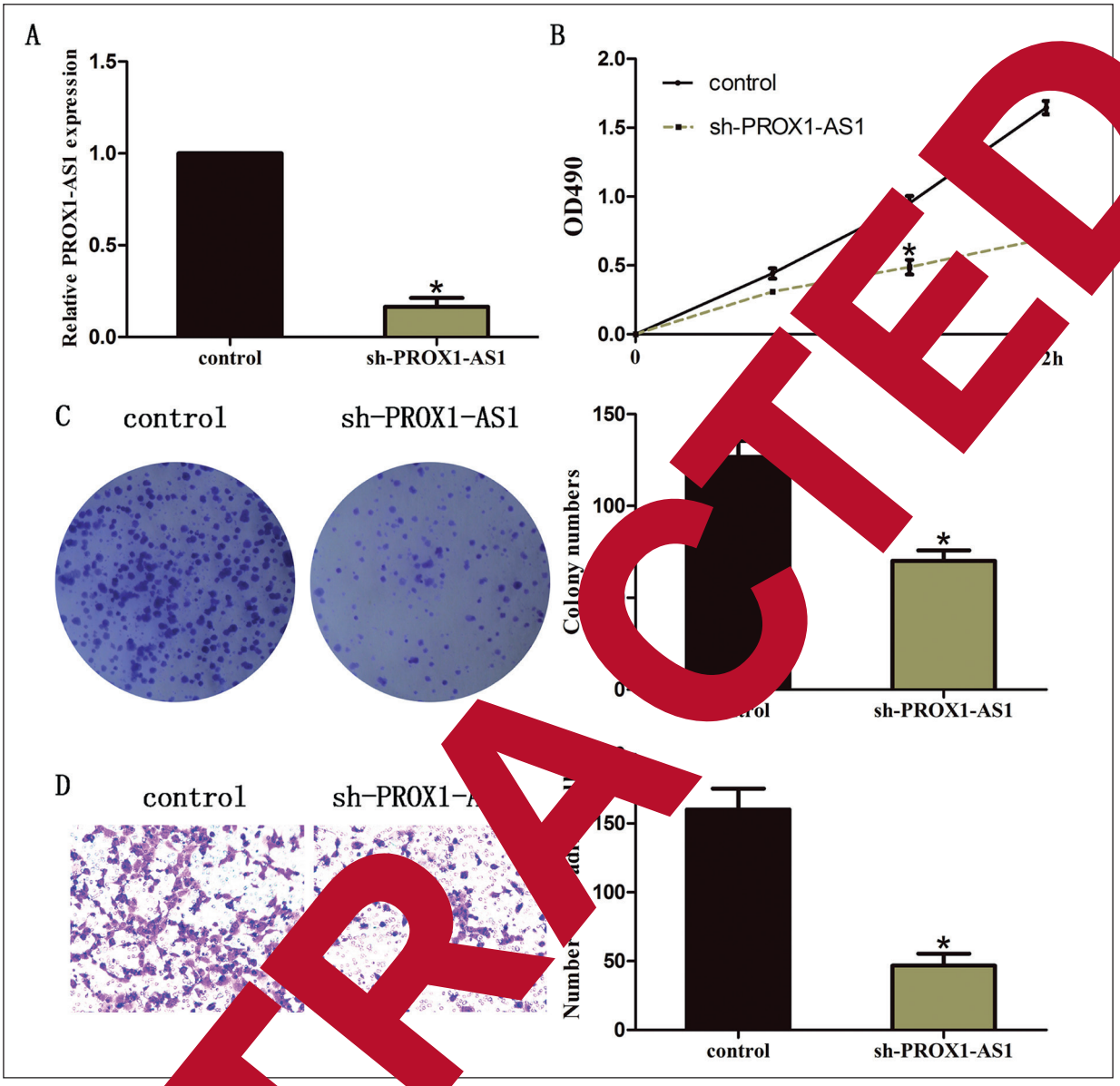

Figure 2. Fun[illegible]al ass[illegible]wed silenced PROX1-AS1 inhibited PC cell proliferation and invasion. **A,** PROX1-AS1 expression in [illegible]C cells transfe[illegible]h sh-PROX1-AS1 and control vector was detected by RT-qPCR. GAPDH was used as an internal [illegible]ol. **B,** MTT assay [illegible]d that silence of PROX1-AS1 significantly repressed cell proliferation in PC cells. **C,** Colony f[illegible]ion assay showed that t[illegible]umber of colonies was significantly decreased *via* silence of PROX1-AS1 in PC cells (magni[illegible]on: 10×). Transwell assay showed that the number of invaded cells was significantly decreased *via* knockdown of PR[illegible]-AS1 in [illegible]ells (magnification: 40×). *$p<0.05$, as compared with the control cells.

(Fig[illegible]4B and 4C). Furthermore, the luc[illegible]se assa[illegible]aled that co-transfection of PRO[illegible]AS1-WT and miR-647 largely decreased th[illegible]tivity, while co-transfection of P[illegible]X1-AS1 MUT and miR-647 had no effect the luciferase activity either (Figure 4D). We [illegible]r found the negative correlation between mi[illegible] and PROX1-AS1 expression level in PC tissues (Figure 4E).

Discussion

Numerous lncRNAs have been reported to serve as oncogenes or tumor-suppressors in the progression of PC. For example, lncRNA ZEB1-AS1 epigenetically modulates the progression of PC by regulating the expressions of ZEB1 and downstream molecules[7]. By targeting miR-198 and promoting the MAPK1 signaling pathway, lncRNA SChLAP1

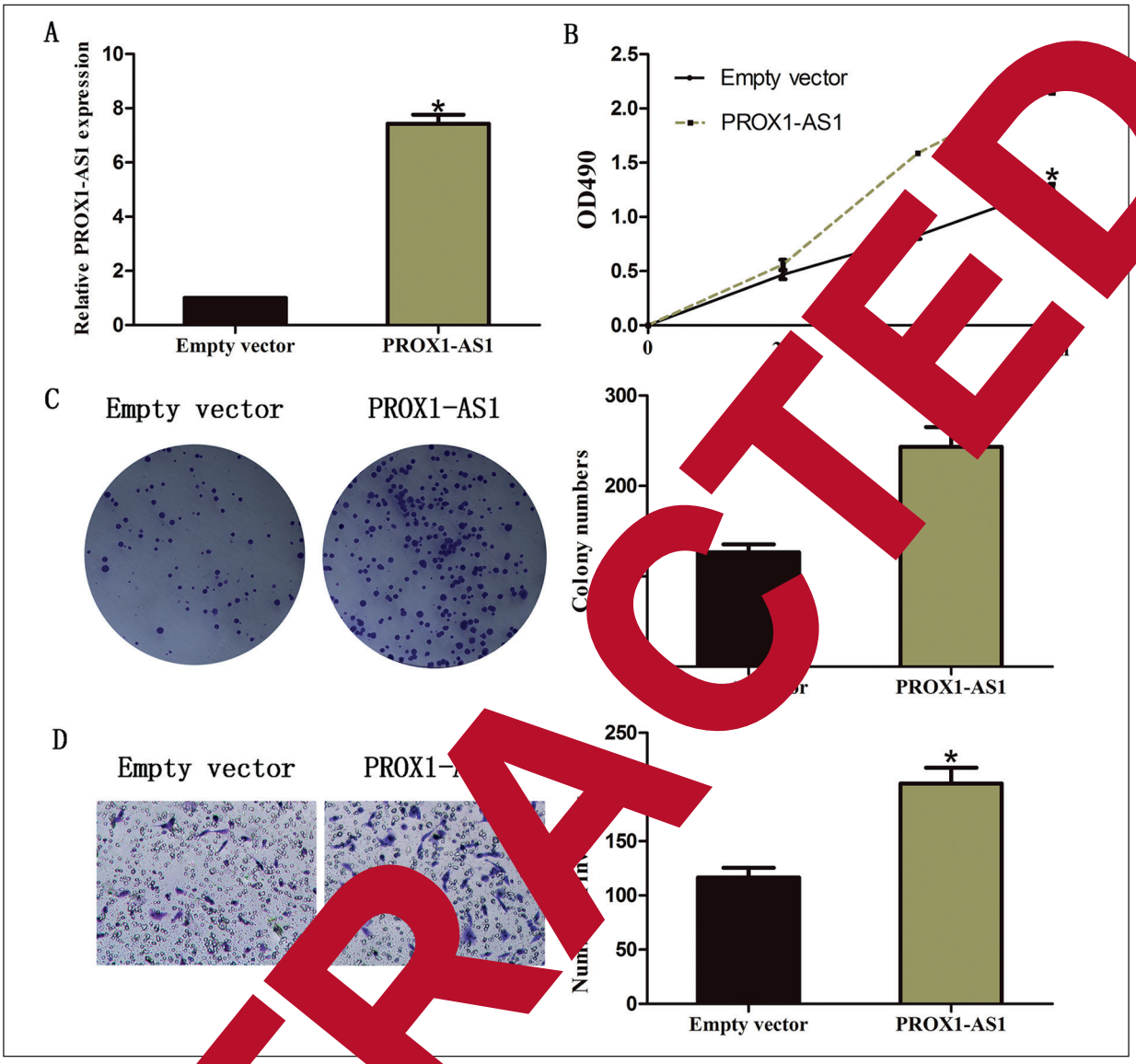

Figure 3. Function showed overe PROX1-AS1 promoted PC cell proliferation and invasion. **A,** PROX1-AS1 expression in PC ed with PROX AS1 lentivirus (PROX1-AS1) and empty vector was detected by RT-qPCR. GAPDH was us an inte trol. **B,** MTT assay showed that overexpression of PROX1-AS1 significantly promoted cell proliferation PC cells. **C,** Co mation assay showed that the number of colonies was significantly increased *via* silence of PROX1- PC cells (magni 10×). **D,** Transwell assay showed that the number of invaded cells was significantly increase overexpression of PRO AS1 in PC cells (magnification: 40×). *$p<0.05$, as compared with the control cells.

promot rolifer and metastasis in PC[8]. NA L R erates the invasion of PC ce silenc MP2/3 expression[9]. Andro-gen- ced lncR A SOCS2-AS1 enhances cell gr esses cell apoptosis in PC[10]. lcRNAs have recently been found to be involved in the development, differentiation, and ration, as well as cell cycle regulation an grammed cell death of malignant tumor. PROX1 antisense RNA 1 (PROX1-AS1) is a newly explored lncRNA in papillary thyroid carcinoma[11]. Our work showed that PROX1-AS1 was higher expressed in PC tissues and cell lines. Besides, the cell proliferation and invasion of PC cells were repressed *via* knockdown of PROX1-AS1, while the cell proliferation and invasion of PC cells were promoted *via* the overexpression of PROX1-AS1. Above results suggested that PROX1-AS1 promoted proliferation and aggressiveness of PC *in vitro*.

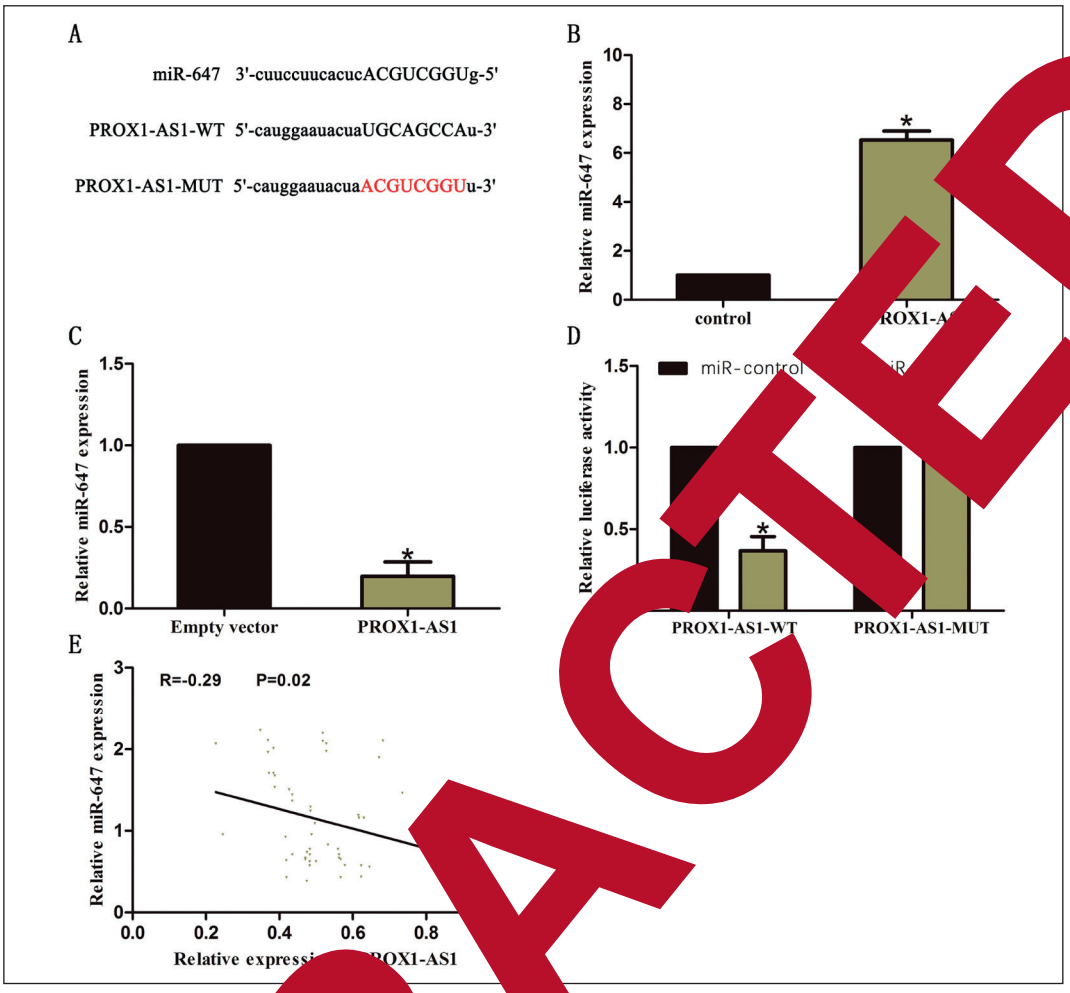

Figure 4. The interaction betwe[illegible]R-64[illegible] PROX1-A[illegible] PC. **A,** The binding sites of miR-647 on PROX1-AS1. **B,** MiR-647 expression was increa[illegible] sh-PRO[illegible]S1 group co[illegible]pared with control vector group. **C,** MiR-647 expression was decreased in PROX1-AS1 len[illegible](PROX[illegible]pared with empty vector group. **D,** Co-transfection of miR-647 and PROX1-AS1-WT strongly d[illegible]d [illegible]ucifera[illegible]tivity, while co-transfection of miR-control and PROX1-AS1-WT did not change the lucife[illegible]activi[illegible]ear correlation between the expression level of miR-647 and PROX1-AS1 in PC tissues. The results re[illegible]t the averag[illegible]ee independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05

The int[illegible]tion betwe[illegible]RNAs and microRNA[illegible]s been widely ex[illegible]d recently. For exam[illegible]functi[illegible]g as a ceRNA of microRNA[illegible] lncR[illegible]UICLM enhances colorectal can[illegible]ta[illegible]s *via* [illegible]lating of ZEB2[12]. By spongi[illegible]-27b-3[illegible]cRNA KCNQ1OT1 fa[illegible]es c[illegible]li[illegible]ion and cell invasion in th[illegible]gressio[illegible]n-small cell lung cancer by upre[illegible]ating HSP[illegible]0AA1[13]. LncRNA LINC00052 de[illegible]ion and invasion of hepatocellu[illegible]arcinom[illegible] cells through the upregulation of [illegible]341L3, which is modulated by miR-452-5p[14]. [illegible]rgeting miR-221/SOCS3, lncRNA GAS5 sup[illegible]es cell proliferation, cell metastasis and gemcitabine resistance in pancreatic cancer[15].

MiR-647, predicted as the possible target microRNA of PROX1-AS1 through bioinformatics analysis, has been reported to regulate cell tumorigenic phenotypes in PC including suppressing cell proliferation, inducing cell cycle arrest and apoptosis[16] and inhibiting cell invasion and metastasis[17]. Therefore, we detected miR-647 expression and PROX1-AS1 expression in PC tissues. Results showed that the miR-647 expression in PC tissues was negatively related to PROX1-AS1 expression. We further found that miR-647 expression could be suppressed by the knockdown of PROX1-AS1 in PC cells, and that miR-647 expression could be promoted by the overexpression of PROX1-AS1 in PC

cells. Besides, luciferase assay indicated that miR-647 could directly bind to PROX1-AS1, and the expression of miR-647 was negatively associated with PROX1-AS1 in PC tissues. All the results above suggested that PROX1-AS1 might promote the progression of PC by sponging miR-647.

Conclusions

We identified that PROX1-AS1 could enhance PC cell proliferation and aggressiveness by sponging miR-647 *in vitro*, indicating that PROX1-AS1 might act as a candidate target for therapy of PC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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