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RESEARCH PAPER

Long non-coding RNA ARAP1-AS1 promotes the proliferation and migration in cervical cancer through epigenetic regulation of DUSP5

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ABSTRACT
Emerging reports have indicated that long non-coding RNAs (lncRNAs) play pivotal roles in multiple cancers, containing cervical cancer. LncRNA ARAP1 antisense RNA 1 (ARAP1-AS1) was previously identified as a tumor-promoter in bladder cancer. However, the expression profile and possible modulation mechanism of ARAP1-AS1 in cervical cancer need to be further studied. In the current research, ARAP1-AS1 was discovered to exhibit a high level in cervical cancer cells. Besides, the knockdown of ARAP1-AS1 repressed cell proliferative and migratory capacities in cervical cancer, as detected by loss-of-function assays including CCK-8, EdU, colony formation, and transwell assays. Additionally, dual-specificity phosphatase 5 (DUSP5), lowly expressed in cervical cancer cells, was found to be negatively modulated by ARAP1-AS1. In subsequence, mechanism experiments proved that ARAP1-AS1 recruited enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) to regulate DUSP5 expression. Finally, rescue assays certified the oncogenic function of ARAP1-AS1/EZH2/DUSP5 axis in cervical cancer. This research probed the expression level and regulatory mechanism of ARAP1-AS1 underlying cervical cancer, which might shed novel lights into the exploration on cervical cancer treatment.

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KEYWORDS
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Introduction

As one of the most lethal tumors, cervical cancer occurs frequently and the incidence has been increased in younger patients.[1–3] Although 5-y survival rate of cervical cancer patients at early stage is above 80%, the prognosis of advanced patients is still unsatisfying.[4,5] It is noteworthy that efficient agents are lack for patients with advanced and recurrent cervical cancer. Therefore, exploring the potential biomarker is necessary for the improvement of therapeutic effect.

In recent decades, accumulating evidence has indicated that long non-coding RNAs (lncRNAs) function as vital molecules under the tumorigenesis and development of human cancers, including cervical cancer.[6–9] LncRNAs are a class of RNA molecules with a length of longer than 200 nucleotides (nt) and lack the capacity in protein-coding.[10] Previous studies have confirmed that lncRNAs mediate gene expression at transcriptional level or post-transcriptional level. For instance, SP1-induced LINC00511 promotes the progression of glioma via targeting miR-124-3p/CCND2 axis.[11] LncRNA XLOC_006390 accelerates tumorigenesis and metastasis in cervical cancer by sponging miR-331-3p and miR-338-3p.[12] Besides, ELK1-activated lncRNA MIR100HG indicates poor prognosis and enhances osteosarcoma progression through epigenetically downregulating LATS1 and LATS2.[13] LncRNA ARAP1 antisense RNA 1 (ARAP1-AS1) is a newly identified lncRNA that promotes bladder cancer progression through cooperating with miR-4735-3p/NOTCH2 axis.[14] However, its functional role and mechanism in cervical cancer need to be further clarified.

Dual-specificity phosphatase 5 (DUSP5) is one ERK specific phosphatase and has been reported to suppress tumor progression.[15,16] For example, lncRNA CRNDE promotes cell proliferation via epigenetically silencing DUSP5/CDKN1A expression in colorectal cancer.[17] DUSP5 downregulation correlates with paclitaxel resistance and poor prognosis in basal-like breast cancer.[18] P68 RNA helicase promotes invasion of glioma cells through negatively regulating DUSP5.[19] However, whether DUSP5 exerts functions in cervical cancer is scarcely known.

The purpose of the present study is to interrogate the expression pattern and modulatory mechanism of ARAP1-AS1 in cervical cancer. Our results revealed that ARAP1-AS1 was upregulated in cervical cancer and exacerbated cell proliferative and migratory capacities. Importantly, we found that ARAP1-AS1 epigenetically silenced DUSP5 expression to accelerate cell proliferation and migration by recruiting enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2).

Materials and methods

Cell culture and transfection plasmids

Human normal cervical epithelial cell (HcerEpic) and cervical cancer cell lines (CaSki, C33A, HeLa, and SiHa) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin was utilized for cell cultivation.

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Cells were cultivated at 37°C in an incubator with 5% CO_2. For transfection, SiHa and HeLa cell lines at the confluence of 50%-80% were collected and treated in triplicate with Lipofactamine2000 (Invitrogen, Carlsbad, CA, USA) for 48 h based on the protocol. The shRNAs specific to ARAP1-AS1 (shARAP1-AS1#1/2/3), EZH2 (shEZH2), and DUSP5 (shDUSP5), together with the EZH2 overexpression plasmids (pcDNA3.1/EZH2) were simultaneously constructed by GenePharma (Shanghai, China). Nonspecific shRNAs and empty pcDNA3.1 vector were taken as negative control, respectively.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA in SiHa and HeLa cells were extracted using TRIzol reagent (Invitrogen) as per the user guide. The RNAs were reversely transcribed into cDNA using the Reverse Transcription Kit (Vazyme, Chongqing, China). The amplification condition of the real-time PCR was as following: 95°C for 30 s, 5 s for 40 cycles, and 60°C for 35 s. The abundance of transcript was determined by SYBR PremixEx Taq (Vazyme). Expression levels of genes were normalized to GAPDH. Data were calculated on basis of the sample threshold cycle (Ct) values of three different replications.

Microarray analysis

Total RNA was extracted by using Trizol RNA extraction kit (Invitrogen life technologies) and quantified by Nano Drop ND-1000. Besides, standard denaturing agarose gel electrophoresis was utilized to assess RNA integrity. The expression profile of mRNAs in SiHa cells under transfection of shARAP1-AS1#1/2/3 or sh-NC was detected by Arraystar Mouse LncRNA Microarray V3.0. The cells preparations and microarray hybridization were implemented via the Agilent Gene Expression Hybridization Kit (Agilent Technology, USA). Feature Extraction software (Agilent Technologies, version 11.0.1.1) analyzed the acquired array images.

Cell viability assay

The assessment of SiHa and HeLa cell viabilities was dissected by use of cell counting kit-8 (CCK-8) solution (Solarbio, Billerica, MA, USA). Transfected cells were seeded in 96-well plate at a density of 6×10^4 cells/well overnight. Then, cells were treated with 0.2 mg/ml CCK-8 reagent at room temperature (RT) for an incubation of extra 4 h. The absorbance at the test wavelength of 450 nm was measured on a microplate reader (Bio-Tek, Winooski, VT, USA).

5-ethynyl-2′-deoxyuridine (EdU) staining

The Cell-Light™ EdU Imaging Kit from RiboBio (Sichuan, China) was applied for assessing proliferative ability of SiHa and HeLa cells in line with instruction. In short, cells in 48-well plates (3×10^3 cells/well) were incubated with 100 μL of EdU (50 μM). Afterward, cells in 4% paraformaldehyde were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min. Images were acquired by IX70 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) at 100 × magnification.

Colony formation assay

In colony formation assay, trypsinized cells were suspended in DMEM medium (Gibco) added with 10% FBS. Then, cells were plated in six-well dishes and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for about 2 weeks. PBS, methanol and 0.1% crystal violet (1 mg/mL) were utilized to wash, fix, and stain cell colonies, respectively. Colonies containing more than 50 cells were randomly selected and calculated.

Transwell migration assay

Transwell chambers of 8-μm-pore size were bought from Corning Costar (Cambridge, MA, USA). 2×10^4 transfected SiHa or HeLa cells were starved in 150 μL of serum-free DMEM on the upper chamber, and 700 ml of complete medium containing 20% FBS was put into the lower chamber. After 24 h of migration, the migrated cells in the lower surface were fixed with 4% methanol and stained with 0.1% crystal violet (Beyotime, Shanghai, China; Cat # C-3886). Photomicrographs were obtained and counted in five randomly selected fields under Olympus IX73 microscope (Olympus) at a magnification of 100×. Each procedure was repeated for more than 2 times.

Luciferase reporter assay

DUSP5 promoter sequences were sub-cloned into downstream of firefly luciferase reporter in pGL3 promoter vector. Co-transfection in SiHa and HeLa cells was conducted by pGL3-DUSP5 promoter, pRL-TK-Renilla plasmid (Promega, Madison, WI, USA) and shARAP1-AS1#2 or shNC for 48 h. Dual-Luciferase Assay System (Promega) was applied in triplicate for determining the final luciferase intensity.

Chromatin immunoprecipitation (ChIP)

For ChIP assay, the available ChIP Assay Kit from Upstate Biotechnology (Lake Placid, NY, USA) was used in accordance with the standard method. SiHa and HeLa cells were incubated in 1% formaldehyde for 20 min at RT. After cross-linking, DNA fragments were obtained with size of 200–800 bp, and then immunoprecipitated with 2 μg of anti-EZH2, 2 μg anti-H3K27me and 2 μg anti-IgG overnight at 4°C with rotation. 30 microliters of protein A/G-agarose beads were added for 2 h at 4°C. The final chromatin was purified using Fragment DNA purification kit (Intron Biotechnology) prior to detection by qRT-PCR. Biological triplicate was conducted.

RNA immunoprecipitation (RIP)

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit was acquired commercially from Millipore (Bedford, MA, USA) and utilized following the specification. SiHa and HeLa cells were re-suspended in RIP lysis buffer with anti-EZH2 or the control anti-IgG antibody. The magnetic bead and

Proteinase K were then added into each group. At length, the precipitated RNAs were isolated and purified, followed by qRT-PCR analysis.

Subcellular fractionation assay

Cytoplasmic and nuclear RNAs were separated, extracted, and purified through the Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Canada) following the instructions of the manufacturer. The expression levels of ARAP1-AS1, GAPDH, and U1 were detected by qRT-PCR.

Western blot

The complete proteome was extracted in lysis buffer (Intron Biotechnology, Seoul, Korea). Concentration of protein was determined by utilizing a BCA protein assay kit (Solarbio). After electrophoresis on 15% SDS-PAGE, the protein was transferred onto polyvinylidene fluoride membrane (Amersham, Munich, Germany). Following blocking with 5% nonfat milk at RT for 2 h, the immunoblot was probed with primary antibodies of EZH2 (ab186006; 1:2000; Abcam, Cambridge, MA, USA), DUSP5 (ab232333; 1:2000; Abcam) and GAPDH (ab181602; 1:2000; Abcam) all night at 5°C. Membranes were washed with Tris-buffered saline containing Tween-20 (TBST) thrice and incubated with Anti-IgG secondary antibodies (1:4000; Abcam) at RT for 2 h. Blots were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Data are expressed as the mean ± SD for at least three repeats and analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (San Diego, IL, USA). Statistical comparisons were performed by Student's t-test and one-way analysis of variance. Statistical significance was defined as p value less than 0.05.

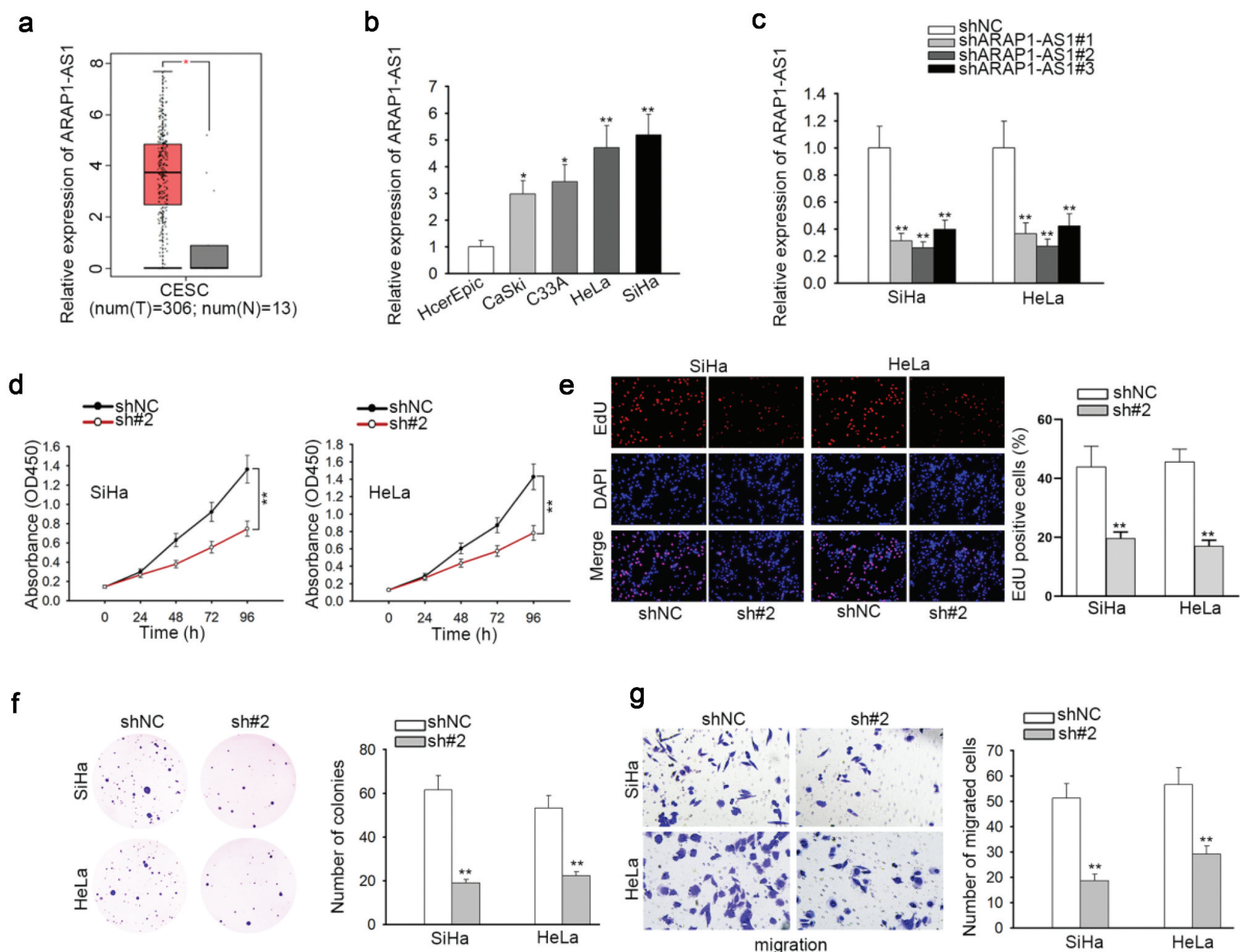

Figure 1. **ARAP1-AS1 depletion hindered cervical cancer cell proliferation and migration.** (**A**) ARAP1-AS1 expression in cervical and endocervical cancers (CESC) tissues was obtained from TCGA dataset. (**B**) ARAP1-AS1 expression in cervical cancer cell lines and normal cell line HcerEpic was detected by qRT-PCR. (**C**) The specific shRNAs were used to silence ARAP1-AS1 expression in SiHa and HeLa cell lines. (**D–F**) The proliferative ability of SiHa and HeLa cells was measured by CCK-8, EdU and colony formation assays. (**G**) Cell migration was determined by transwell assay after silencing ARAP1-AS1. Data are the means ± SD of triplicate determinants (*P < .05, **P < .01).

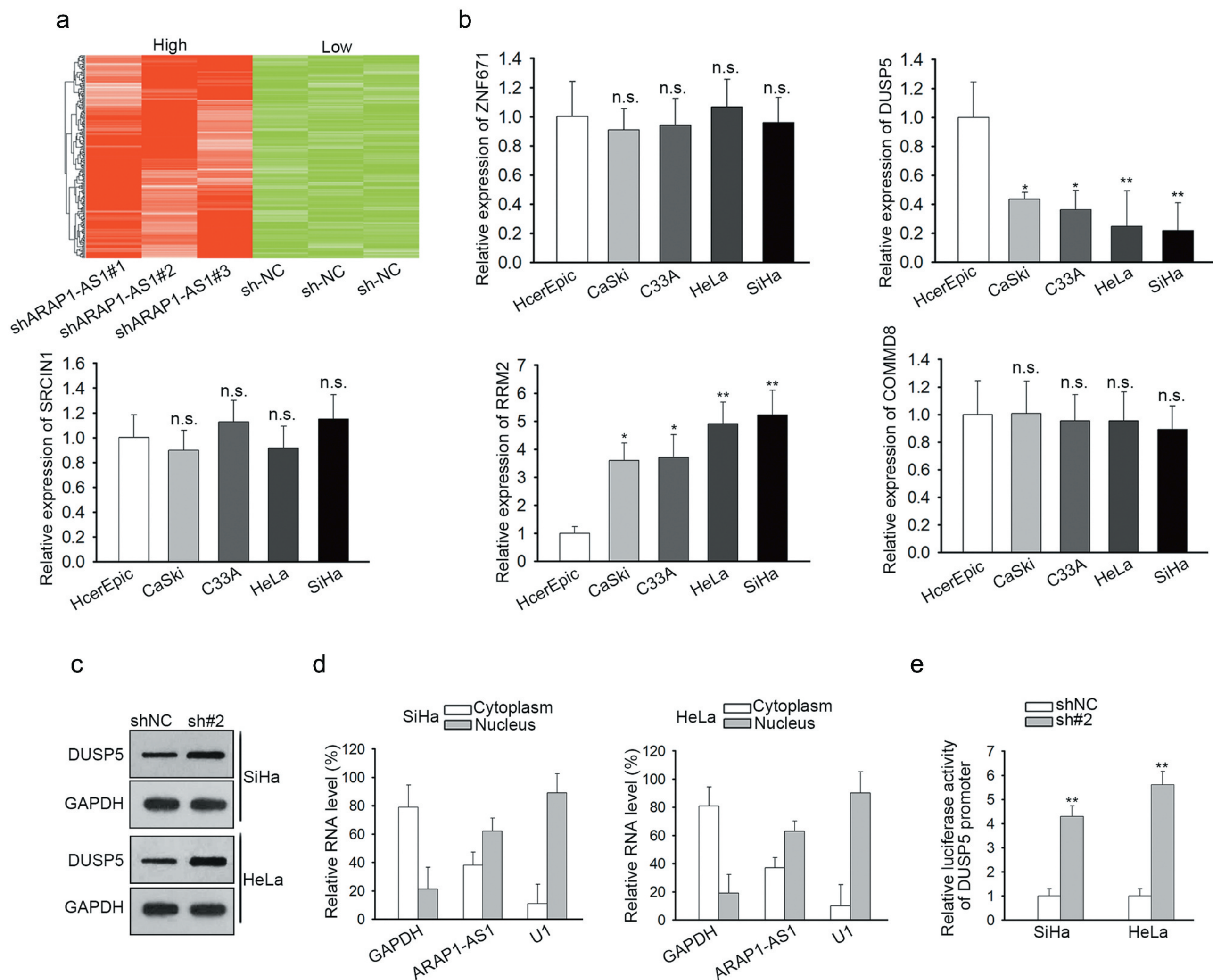

Figure 2. **DUSP5 expression was negatively affected by ARAP1-AS1**. (**A**) The heatmap exhibited the mRNAs upregulated by ARAP1-AS1 silence and five upregulated mRNAs with the most significance (ZNF671, DUSP5, SRCIN1, RRM2 and COMMD8) were selected. (**B**) qRT-PCR tested the levels of five mRNAs in cervical cancer cells and HcerEpic cells. (**C**) The protein level of DUSP5 was assessed upon ARAP1-AS1 depletion in SiHa and HeLa cells. (**D**) The distribution of ARAP1-AS1 in SiHa and HeLa cells was detected via subcellular fractionation assay. (**E**) The luciferase activity of DUSP5 promoter in SiHa and HeLa cells transfected with shARAP1-AS1#2 or shNC was evaluated using dual-luciferase reporter assay. Data are the means ± SD of triplicate determinants (*P < .05, **P < .01, n.s. presented no significance).

Results

ARAP1-AS1 silencing repressed cell proliferation and migration in cervical cancer

The cancer genome atlas (TCGA) dataset (https://portal.gdc.cancer.gov/) presented that lncRNA ARAP1-AS1 expressed at a high level in cervical and endocervical cancers (CESC) tissues (Figure 1A). To confirm the expression status of ARAP1-AS1 in cervical cancer, cervical cancer cell lines (CaSki, C33A, HeLa, and SiHa) and normal cervical epithelial cells (HcerEpic) were used for qRT-PCR. The results manifested that compared to HcerEpic cell line, ARAP1-AS1 was remarkably upregulated in cervical cancer cells, especially in SiHa and HeLa cells (Figure 1B). To explore the function of ARAP1-AS1 in cervical cancer, we designed loss-of-function assays. Thus, ARAP1-AS1 expression was stably knocked down by transfecting shARAP1-AS1#1/2/3 in SiHa and HeLa cells (Figure 1C), and shARAP1-AS1#2 with optimal efficiency was applied for the subsequent experiments. Subsequently, CCK-8 results showed that cell viability was impaired by ARAP1-AS1 silencing (Figure 1D). Moreover, EdU assay demonstrated that cell proliferation was hampered when ARAP1-AS1 was knocked down (Figure 1E). Additionally, colony formation ability was also restrained in cells transfected with shARAP1-AS1#2 (figure 1F). In transwell assay, ARAP1-AS1 silencing exerted inhibitory impact on the migratory ability of SiHa and HeLa cells (Figure 1G). These data suggested that ARAP1-AS1 was upregulated in cervical cancer and hastened the proliferation and migration of cervical cancer cells.

ARAP1-AS1 regulated DUSP5 expression through affecting the activity of DUSP5 promoter

Subsequently, the mechanism of ARAP1-AS1 in cervical cancer was probed. Firstly, differentially expressed mRNAs under ARAP1-AS1 inhibition was analyzed through microarray analysis and the heatmap was plotted (Figure 2A). Then, the top five upregulated mRNAs in ARAP1-AS1

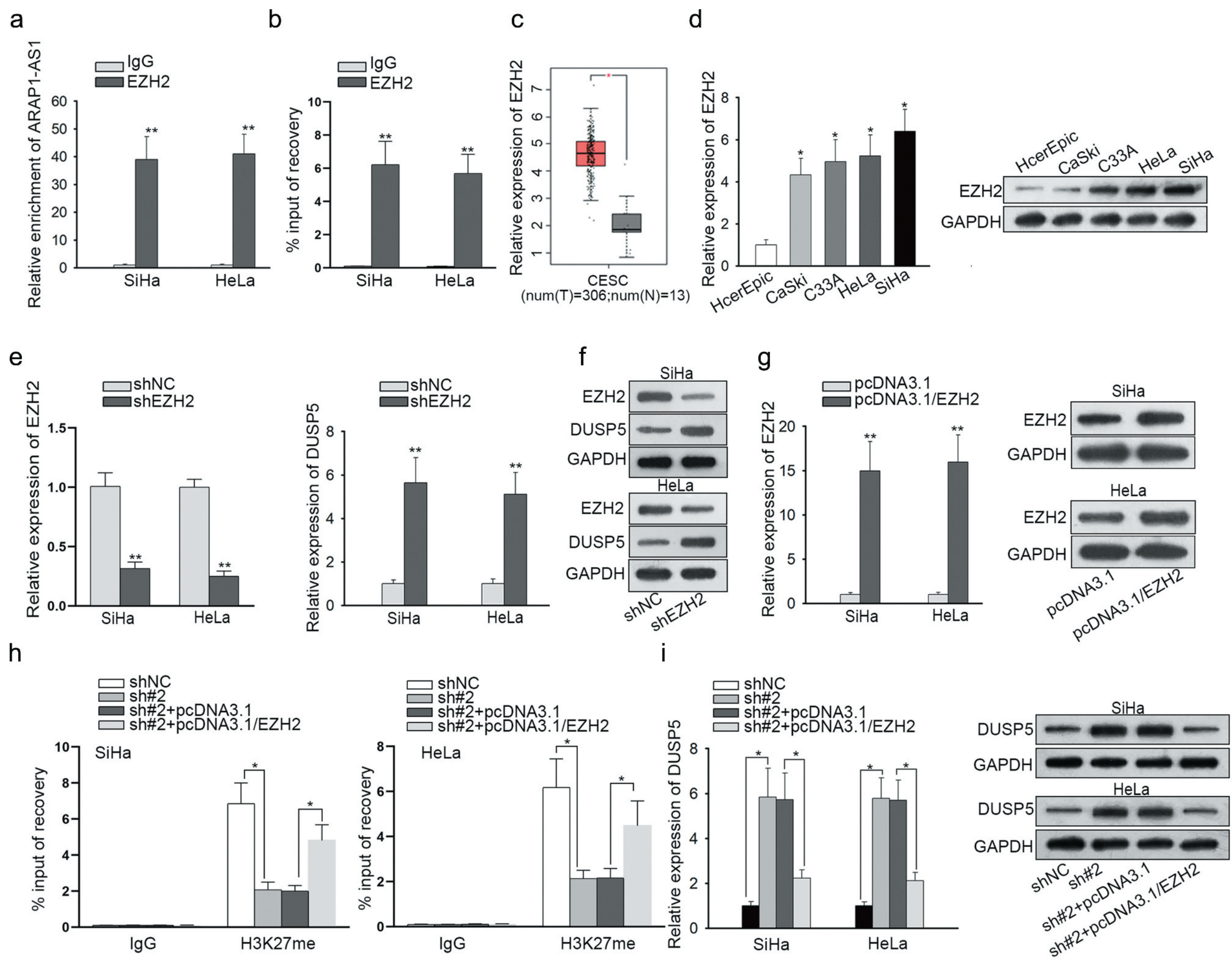

Figure 3. ARAP1-AS1 epigenetically regulated DUSP5 expression through recruiting EZH2. (**A**) RIP assay was applied to verify the combination between EZH2 and ARAP1-AS1. (**B**) The interaction of EZH2 with DUSP5 promoter was confirmed by ChIP assay. **C.** EZH2 expression was predicted at a high level in CESC tissues by TCGA dataset. (**D**) EZH2 mRNA and protein levels were tested in cervical cancer cells and HcerEpic cell line, as measured by qRT-PCR and western blot. (**E** and **F**) EZH2 knockdown efficiency and DUSP5 mRNA and protein expressions upon EZH2 knockdown were estimated. (**G**) The transfection efficacy of pcDNA3.1/EZH2 was determined by qRT-PCR and western blot. (**H**) ChIP-qRT-PCR was used to detect the binding of H3K27me to DUSP5 promoter in SiHa and HeLa cells transfected with indicated plasmids. (**I**) The mRNA and protein levels of DUSP5 were tested in the groups of shNC, shARAP1-AS1#2, shARAP1-AS1#2+ pcDNA3.1 and shARAP1-AS1#2 + pcDNA3.1/EZH2. Data are the means ± SD of triplicate determinants (*P < .05, **P < .01).

silenced cells were selected for further analysis. Expressions of these mRNAs were detected in cervical cancer cells by qRT-PCR, and results showed that only DUSP5 was lowly expressed in cervical cancer cells in comparison with HcerEpic cells (Figure 2B). Moreover, western blot analysis measured that protein level of DUSP5 was increased by silencing ARAP1-AS1 (Figure 2C). Subsequently, we investigate the relationship between ARAP1-AS1 and DUSP5. Before the investigation, the distribution of ARAP1-AS1 in SiHa and HeLa cells was determined by subcellular fractionation assay. As shown, ARAP1-AS1 was mainly expressed in the nucleus of cervical cancer cells (Figure 2D). Strikingly, we discovered that the luciferase activity of DUSP5 promoter was strengthened upon ARAP1-AS1 downregulation (Figure 2E). These findings illustrated that ARAP1-AS1 negatively modulated DUSP5 at transcriptional level in cervical cancer.

ARAP1-AS1 epigenetically silenced DUSP5 via recruiting EZH2

Former researches demonstrated that lncRNAs could affect mRNAs expressions through epigenetic regulation.[20,21] Considering the results above, we assumed that ARAP1-AS1 might regulate DUSP5 through EZH2-mediated methylation. Besides, we searched UCSC genome browser (http://genome.ucsc.edu/) and found out the potential regulation of EZH2 on DUSP5. Then, RIP and ChIP assays were performed, and the results displayed the notable enrichment of ARAP1-AS1 or DUSP5 promoter in anti-EZH2 group, hinting the interaction between EZH2 and ARAP1-AS1 or DUSP5 promoter (Figure 3A-B). According to TCGA database, EZH2 expression was found at a high level in CESC tissues (Figure 3C). Further, the upregulated mRNA and protein levels of EZH2 in cervical cancer cells were confirmed by qRT-PCR and western blot analyses (Figure 3D). In addition, DUSP5 mRNA and protein

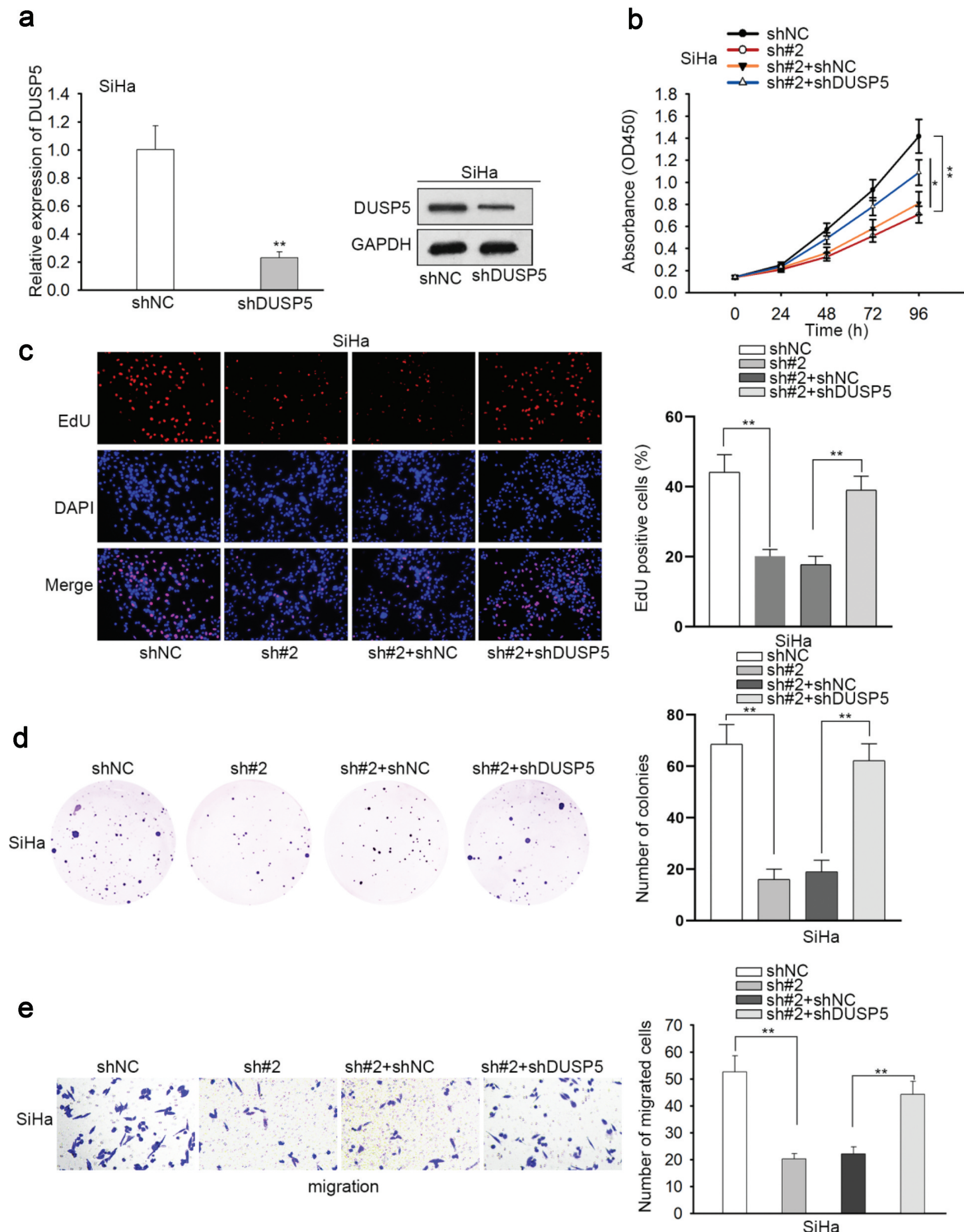

Figure 4. ARAP1-AS1 facilitated cervical cancer cell proliferation and migration by epigenetically regulating DUSP5. (A) The mRNA and protein levels of DUSP5 in shDUSP5 transfected SiHa cells were evaluated by qRT-PCR and western blotting. (**B**–**D**) Cell proliferation was evaluated by CCK-8, EdU and colony formation assays in the groups of shNC, shARAP1-AS1#2, shARAP1-AS1#2+ shNC and shARAP1-AS1#2+ shDUSP5. (**E**) Cell migration was assessed by transwell assay in the groups of shNC, shARAP1-AS1#2, shARAP1-AS1#2+ shNC and shARAP1-AS1#2+ shDUSP5. Data are the means ± SD of triplicate determinants (*$P < .05$, **$P < .01$).

expressions were both increased in response to EZH2 down-regulation (Figure 3E-F). Next, we overexpressed EZH2 and observed that the binding affinity of H3K27me to DUSP5 promoter impaired by shARAP1-AS1#2 was recovered by co-transfection of pcDNA3.1/EZH2 (Figure 3G-H). Finally, qRT-PCR and western blotting analyses showed that DUSP5 mRNA

and protein levels increased by ARAP1-AS1 depletion were restored after EZH2 expression was elevated (Figure 3I). To sum up, ARAP1-AS1 epigenetically silenced DUSP5 by recruiting EZH2.

ARAP1-AS1 accelerated the proliferation and migration by silencing DUSP5 in cervical cancer

To further validate the regulatory mechanism of ARAP1-AS1 in cervical cancer, rescue assays were designed and carried out. Firstly, the transfection efficacy of shDUSP5 was validated through qRT-PCR and western blot (Figure 4A). The data from CCK-8, EdU, and colony formation assays illustrated that cell proliferation repressed by ARAP1-AS1 inhibition was reversed when DUSP5 was downregulated (Figure 4B-D). Transwell assay demonstrated that ARAP1-AS1 downregulation restrained cell migration but this inhibition was counteracted by silencing of DUSP5 (Figure 4E). To be concluded, ARAP1-AS1 silenced DUSP5 to drive the proliferation and migration of cervical cancer cells.

Discussion

Mounting evidence has emerged that dysregulated lncRNAs may be effective diagnostic, therapeutic, or prognostic biomarkers implicated in initiation and progression of cervical cancer.[22,23] For instance, LINC00152 promotes cell proliferation of cervical cancer via miR-216b-5p/HOXA1 axis.[24] LncRNA AB073614 boosts the proliferation and represses apoptosis in cervical cancer by repression of RBM5.[25] LncRNA PCAT6 is a prognostic marker in cervical cancer and mediates cell growth and metastasis through Wnt/β-catenin pathway.[26] Considering the unsatisfactory effect of cervical cancer treatment, the investigations of more lncRNAs involving in the progression of cervical cancer appear to be extremely imperative.

LncRNA ARAP1-AS1 has been demonstrated to be highly expressed and exhibit oncogenic property in bladder cancer through miR-4735-3p/NOTCH2 axis.[14] In this research, the high level of ARAP1-AS1 in cervical cancer was also observed and loss-of-function experiments revealed that ARAP1-AS1 repression inhibited the proliferation and migration in cervical cancer. Our study illustrated the carcinogenic role of ARAP1-AS1 in cervical cancer.

DUSP5, dramatically upregulated by ARAP1-AS1 inhibition, was known as a tumor repressor in many other tumors,[17–19] but its function was seldom researched in cervical cancer. We explored its expression in cervical cancer cells and discovered that DUSP5 was lowly expressed in cervical cancer cells. Besides, ARAP1-AS1 was mainly localized in the nucleus of cervical cancer cells. Also, the activity of DUSP5 promoter could be affected by ARAP1-AS1. These findings provoked us to further investigate the relationship between ARAP1-AS1 and DUSP5.

Based on above data, we hypothesized that ARAP1-AS1 might involve in epigenetic regulation. Numerous works have explained the epigenetic regulation in various cancers. For example, lncRNA DUXAP8 promotes cell growth in pancreatic carcinoma by epigenetic silence of CDKN1A and KLF2.[27] LncRNA SNHG3 boosts the malignancy of glioma via downregulating KLF2 and p21.[28] LncRNA LBCS restrains self-renewal and chemoresistance in bladder cancer by epigenetically silencing SOX2.[29] EZH2 is a main effector participating in the epigenetic regulation. In order to validate our assumption, mechanism experiments were performed. It was affirmed that ARAP1-AS1 could silence DUSP5 expression by recruiting EZH2. Eventually, rescue assays validated that ARAP1-AS1 promoted the cellular progression of cervical cancer via epigenetically silencing DUSP5.

In conclusion, our research interrogated the expression pattern and regulatory mechanism of ARAP1-AS1 underlying cervical cancer development, which might provide deeper insights into the exploration of treatment for patients with cervical cancer.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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