Long non-coding RNA, steroid receptor RNA activator (SRA), induces tumor proliferation and invasion through the NOTCH pathway in cervical cancer cell lines

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Abstract. Contemporary research has focused on the function of long non-coding RNAs (lncRNAs) in carcinogenesis. However, the involvement of the lncRNA, steroid receptor RNA activator (SRA), in cervical carcinogenesis remains to be elucidated. In the present study, we investigated the biofunctional consequences of lncRNA SRA knockdown *in vitro*. To verify the role of lncRNA SRA in cell proliferation, migration, and invasion, lncRNA RNA interference was utilized to knock down lncRNA SRA expression in cervical cancer cell lines, resulting in our discovery that lncRNA SRA knockdown inhibited cell proliferation, cell migration and tumor invasion in the cervical cancer cell lines. Additionally, *in vitro* experiments using the lncRNA SRA-knockdown cervical cancer cell lines revealed that lncRNA SRA is a strong inducer and modulator of the expression of genes related to epithelial-mesenchymal transition and the NOTCH signaling pathway. In conclusion, our findings demonstrated that lncRNA SRA is highly correlated with cancer progression and cervical cancer cell proliferation and migration. Furthermore, these results indicate that lncRNA SRA may be a potential therapeutic target and prognostic marker for cervical malignancy.

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Abbreviations: CCK-8, Cell Counting Kit-8; EMT, epithelial-mesenchymal transition; lncRNA, long non-coding RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; siNC, negative-control siRNA; siRNA, small interfering RNA; SRA, steroid receptor RNA activator

Key words: steroid receptor RNA activator, SRA, long non-coding RNA, invasion, migration, cervical cancer

Introduction

Cervical cancer is the fourth leading cause of cancer-related mortality in women worldwide (1). Widespread employment of screening programs has reduced the incidence and mortality associated with this cancer. However, it remains a major public health issue, particularly concerning advanced cases (1). Accordingly, recent research has focused on identifying tumor-specific markers capable of predicting the biological behavior of cervical cancers with respect to cell motility and invasion (2).

Non-coding RNAs (ncRNAs) are potential key factors influencing gene regulation and cancer cell phenotypes (3,4). Recently, more than 3,000 human long intervening coding RNAs and most long ncRNAs (lncRNAs) have been found to correlate the activity associated with DNA-binding proteins, including chromatin-modifying complexes (5) that epigenetically modify the expression of various genes (6). Additionally, transcription of lncRNAs modulates gene expression in response to external oncogenic stimuli or DNA damage (7).

Human lncRNA, steroid receptor RNA activator (SRA), was shown to co-activate several human sex hormone receptors and was found to be highly associated with several types of cancers including ovarian and breast cancer (8-11). In addition, lncRNA SRA functionally interacts with several proteins, including nuclear receptors, such as the retinoic acid receptor, as well as proteins involved in myogenic differentiation (12,13). Although lncRNA SRA is also correlated with the progression of other malignancies, its role in cervical cancer has not yet been fully elucidated.

The present study investigated the expression and molecular function of SRA in cervical cancer cell lines and elucidated the role of SRA in the metastatic progression of cervical cancer.

Table I. Primer sequences used in the present study.

Gene	Primer sequence		Product size (bp)
	Forward (5'-3')	Reverse (5'-3')	
SRA	CTCCCTTCTTACCACCACCA	TGCAGATACACAGGGAGCAG	217
MMP-9	CGCTACCACCTCGAACTTTG	GCCATTCACGTCGTCCTTAT	196
MMP-2	GGATGATGCCTTTGCTCG	ATAGGATGTGCCCTGGAA	487
VEGF	TTGCTGCTCTACCTCCAC	AAATGCTTTCTCCGCTCT	419
E-cadherin	ATTCTGATTCTGCTGCTCTTG	AGTAGTCATAGTCCTGGTCCT	421
β-catenin	TGCAGTTCGCCTTCACTATG	ACTAGTCGTGGAATGGCACC	162
N-cadherin	CCCAAGACAAAGAGACCCAG	GCCACTGTGCTTACTGAATTG	140
Vimentin	TGGATTCACTCCCTCTGGTT	GGTCATCGTGATGCTGAGAA	111
Snail	GAGGCGGTGGCAGACTAG	GACACATCGGTCAGACCAG	178
NOTCH1	GCCGCCTTTGTGCTTCTGTTC	CCGGTGGTCTGTCTGGTCGTC	300
HES1	TCAACACGACACCGGATAAA	TCAGCTGGCTCAGACTTTCA	111
P300	GACCCTCAGCTTTTAGGAATCC	TGCCGTAGCAACACAGTGTCT	301

SRA, steroid receptor RNA activator; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

Materials and methods

Cell lines. Squamous cell cervical carcinoma (SiHa) cells and epidermoid cell cervical carcinoma (CaSki; originally derived from a small-bowel mesentery metastasis) cells and human cervical cancer cell lines [American Type Culture Collection (ATCC) Manassas, VA, USA], were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% (v/v) fetal bovine serum and penicillin/streptomycin. Cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Only cells that had been passaged <20 times were utilized in the experiments.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). We extracted total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into first-strand cDNA using a reverse transcription reagent kit (Invitrogen). The cDNA template was amplified by qRT-PCR using the SYBR-Green Real-Time PCR kit (Toyobo Co., Ltd., Osaka, Japan) on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). We used *U6* as the internal standard for all quantifications. We analyzed the relative gene expression using the $2^{-\Delta\Delta Ct}$ method, and the results are expressed as the extent of change relative to control values. All qRT-PCR experiments were repeated at least three times. Primers used for the PCR reactions are listed in Table I.

Small interfering RNA (siRNA) transfection. We obtained siRNA for SRA lncRNA and negative-control siRNA (siNC) from Genolution Pharmaceuticals (Seoul, Korea). Cells (5x10⁴ cells/well) were seeded into 6-well plates and transfected with 10 nM siRNA in phosphate-buffered saline using the G-Fectin kit (Genolution Pharmaceuticals) according to the manufacturer's instructions. The siRNA-transfected cells were used in the *in vitro* assays 48 h after transfection. The target sequence for the siSRA was, 5'-CTCCCTTCTTACCACCACCA-3'. Experiments were repeated at least three times.

Plasmid constructs and generation of stable cell lines. Full-length human SRA-transcript cDNA was amplified by PCR and inserted into the pLenti6/V5-D-TOPO vector using the ViraPower lentiviral expression system (Invitrogen) according to the manufacturer's instructions. We then transfected the plasmid into 293FT cells for packaging, with the resultant lentivirus used to infect the desired cell lines. Stably SRA-transfected cells were selected in medium containing blasticidin (Invitrogen).

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was utilized to evaluate cell proliferation. Cells (2x10³ cells/well) were seeded into 96-well flat-bottomed plates containing 100 μl of complete medium per well. The cells were incubated overnight to allow for cell attachment and recovery, and subsequently transfected with siNC or siSRA for 24, 48, 72 or 96 h. An aliquot of 10 μl of CCK-8 solution was supplemented into each well, followed by incubation for 2 h. Absorbance was measured at 450 nm to estimate the number of viable cells in each well. The assay was performed in triplicate.

Matrigel invasion assay. We performed a Matrigel invasion assay using the BD Biocoat Matrigel invasion chamber (pore size, 8-μm; 24-wells; BD Biosciences, Bedford, MA, USA) according to the manufacturer's protocol. Cells (5x10⁴) were seeded in the upper chamber, which contained serum-free medium, and complete medium was added to the lower chamber. The Matrigel-invasion chamber was incubated at 37°C under 5% CO_2 for 48 h. Non-invading cells were removed from the upper chamber using cotton-tipped swabs. Cells that had invaded through the pores onto the lower side of the filter were stained (Diff-Quik; Sysmex, Kobe, Japan) and counted

using a hemocytometer. The assay was repeated at least three times.

Wound-healing migration assay. We evaluated cell migration using a wound-healing assay. Approximately 5×10^5 cells were seeded into 6-well culture plates containing serum-enriched medium and allowed to grow to 90% confluence in complete medium. The serum-containing medium was eliminated, and cells were serum-starved for 24 h. When the cells reached 100% confluence, an artificial homogenous wound was made by scratching the monolayer using a sterile 200-μl pipette tip. Following the scratching, cells were washed with serum-free medium. Images of cells migrating into the wound were captured at 0, 24 and 48 h using a microscope. Three independent experiments were performed in triplicate.

Western blot analysis. We used radioimmunoprecipitation assay buffer to extract proteins, and a Pierce BCA protein assay kit (both from Thermo Fisher Scientific, Waltham, MA, USA) was used to measure protein concentration. Proteins were boiled with 2X sample buffer, subsequently resolved on 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat dried milk in 1X Tris-buffered saline containing 0.1% Tween-20 (pH 7.6) at room temperature for 1 h, the membranes were incubated with primary antibodies at 4˚C overnight under continual agitation. The following primary antibodies were used: rabbit anti-human vascular endothelial growth factor (VEGF) (1:500), rabbit anti-human matrix metalloproteinase (MMP)-2 (1:500) (both from Abcam, Cambridge, UK, USA), rabbit anti-human MMP-9 (1:1,000), rabbit anti-human E-cadherin (1:1,000), rabbit anti-human β-catenin (1:1,000) (all from Cell Signaling Technology, Danvers, MA, USA), mouse anti-human vimentin (1:1,000; Sigma-Aldrich, St. Louis, MO, USA), mouse anti-human Snail (1:1,000), rabbit anti-human SOX-2 (1:1,000), rabbit anti-human Nanog (1:1,000), rabbit anti-human Oct4 (1:1,000) (all from Cell Signaling Technology), and mouse anti-human β-actin (1:5,000; Sigma-Aldrich). We visualized the proteins using an enhanced chemiluminescence system (Amersham, Little Chalfont, UK), and band intensities were quantified using a luminescent image analyzer (LAS-4000 Mini; Fujifilm, Uppsala, Sweden).

Statistical analysis. IBM SPSS version 23 for Windows (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The Kolmogorov-Smirnov test was used to validate standard normal-distributional assumptions. Statistical significance was determined using the Fisher's exact-test, Pearson's Chi-square test and Student's t-test. Mean differences were considered significant at P<0.05. Results are presented as the means ± standard deviation.

Results

SRA expression is elevated in cervical cancer cells. We performed qRT-PCR to estimate the expression levels of SRA in five different cell lines, one of which was derived from human embryonic kidney cells (HEK293), whereas the other four were derived from human cervical cancers. We
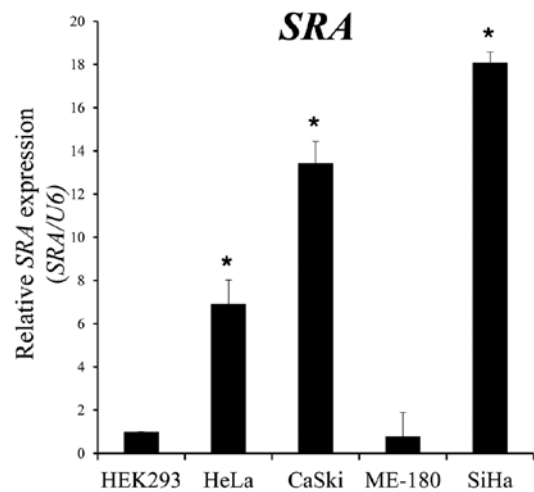

Figure 1. SRA expression in various cell lines. SRA lncRNA expression was evaluated by qRT-PCR, with *U6* as an internal control. *P<0.05 vs. non-tumor control. lncRNA, long non-coding RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SRA, steroid receptor RNA activator.

observed that SRA expression levels were significantly higher in epithelioid cervical carcinoma (HeLa), CaSki and SiHa cells as compared with levels observed in epidermoid cervical carcinoma cells (ME-180) (Fig. 1).

Knockdown of SRA decreases cell proliferation in cervical cancer cells. To examine the functional role of SRA in cervical cancer cells, siRNA was utilized to downregulate SRA expression in the CaSki and SiHa cells, which showed significantly higher levels of SRA expression. We evaluated the knockdown efficiency of siSRAs, and verified that siSRA transfection was more efficient in silencing SRA as compared with siNC (Fig. 2A and C). We then performed a CCK-8 assay to determine the effect of SRA knockdown on cell proliferation. Our results showed that siRNA-mediated knockdown of SRA in CaSki and SiHa cells substantially decreased cell proliferation (Fig. 2B and D). These findings indicated that SRA is involved in the proliferation of cervical cancer cells.

SRA knockdown inhibits cervical cancer cell migration and invasion. Wound-healing and Matrigel-invasion assays were performed to determine whether SRA plays a role in cervical cancer cell migration and invasion. In the wound-healing assay, siRNA-mediated knockdown of SRA inhibited cell migration in the CaSki and SiHa cells (Fig. 3A and D). Moreover, SRA-knockdown inhibited cell invasion in the CaSki and SiHa cells according to the results of the Matrigel-invasion assay (Fig. 3B and E). The extent of inhibition upon SRA knockdown was quantified by qRT-PCR (Fig. 3C and F).

SRA knockdown inhibits MMP-9, MMP-2 and VEGF expression in cervical cancer cells. To elucidate the molecular mechanisms underlying the inhibitory effect of SRA knockdown on cell migration and invasion, we explored the relationship between SRA expression and MMP-2, MMP-9 and VEGF expression. We observed that siSRA treatment decreased levels of *MMP-2*, *MMP-9* and *VEGF* mRNA in the CaSki cells (Fig. 4A) as well as MMP-2, MMP-9 and
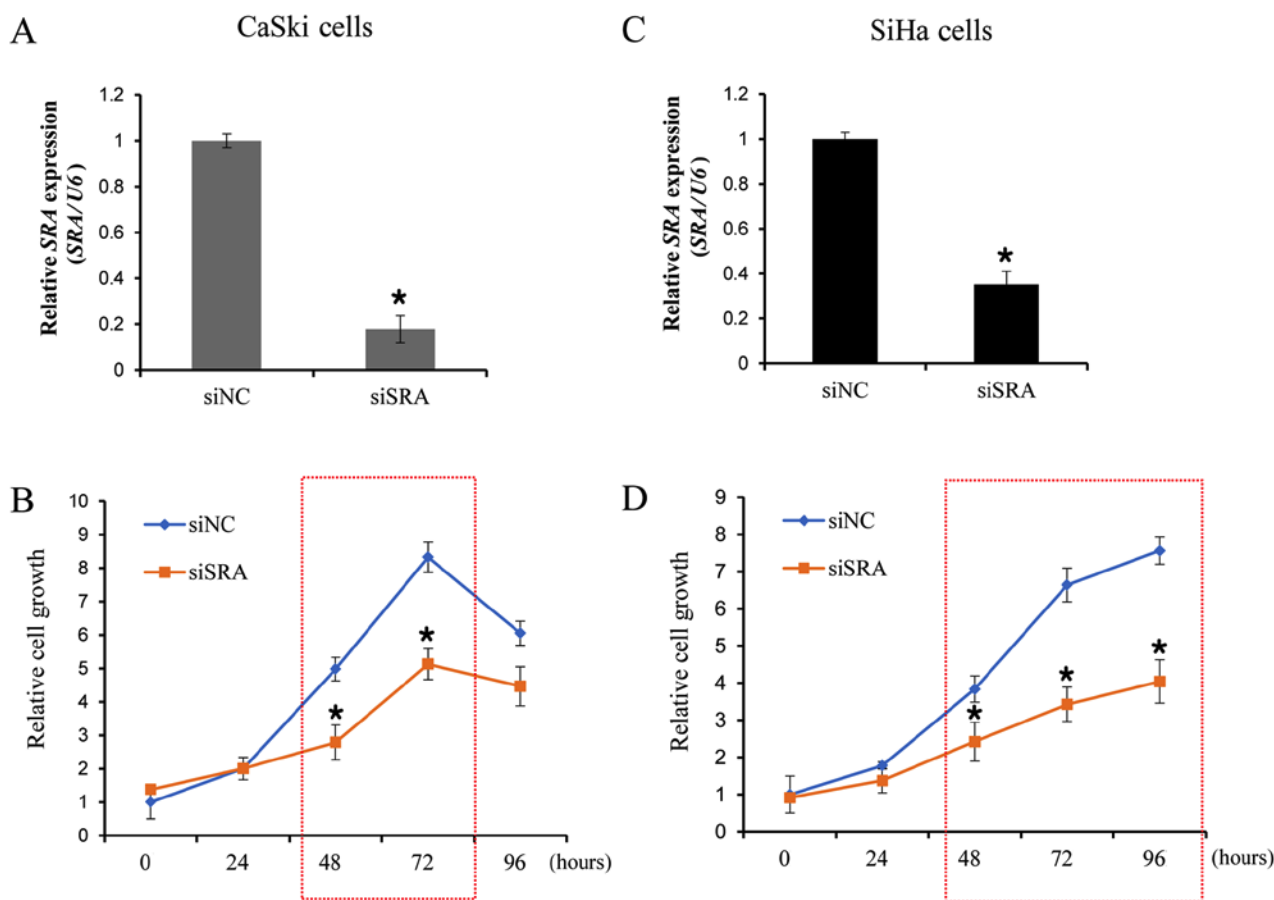
Figure 2. SRA knockdown inhibits the proliferation of cervical cancer cells. (A and C) Cells were transfected with SRA-specific siRNA (siSRA) and negative-control siRNA (siNC), and knockdown efficiency was determined by qRT-PCR analysis in CaSki and SiHa cells. (B and D) The proliferation capacity of cervical cancer cells transfected with siSRA and siNC was determined using the Cell Counting Kit-8 assay. Bars indicate means ± standard deviation of three independent experiments; *P<0.05 vs. siNC. CaSki, epidermoid cell cervical carcinoma cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SiHa, squamous cell cervical carcinoma cells; SRA, steroid receptor RNA activator.

VEGF protein levels (Fig. 4B). These findings indicated that SRA promoted cervical cancer cell migration and invasion via upregulation of MMP-9, MMP-2 and VEGF expression.

SRA knockdown downregulates expression of genes related to epithelial-mesenchymal transition (EMT) and the NOTCH-signaling pathway in cervical cancer cells. Since EMT is crucial to cell migration and invasion, we examined whether SRA is required for EMT. EMT was monitored using qRT-PCR and western blotting following siRNA-mediated knockdown of SRA in CaSki cells. SRA knockdown resulted in increased E-cadherin expression and decreased β-catenin and vimentin expression (Fig. 5A and B). Additionally, the EMT-mediating transcription factor Snail and Twist were downregulated in the siSRA-transfected cells relative to levels observed in the siNC-transfected cells (Fig. 5A and B). These results suggested that SRA may be involved in the dysregulation of EMT-related genes in cervical cancer cells, thereby promoting migration and invasion.

To elucidate the mechanism by which SRA promotes a malignant phenotype in cervical cancer cells, we assessed the status of important signaling cascades controlled by NOTCH in the SRA-knockdown cells. SRA knockdown in the CaSki cells resulted in downregulation of NOTCH1, HES1 and p300 expression [both mRNA (Fig. 6A) and protein (Fig. 6B)]. These

data indicated that SRA promoted tumor growth *in vitro* via the EMT and NOTCH signaling pathways.

Discussion

lncRNAs are transcripts of >200 nucleotides lacking protein-coding capability (7). Numerous lncRNAs are capped, spliced and polyadenylated similar to their protein-coding counterparts and exhibit tissue-specific expression patterns (14). Furthermore, lncRNAs are essential for the regulation of chromatin structure, gene expression and translational control (15). The mounting list of functionally characterized lncRNAs implies that these transcripts are critical to various physiological processes (16). Therefore, these findings suggest that modified lncRNA expression may affect cancer development and progression (17). However, little is known concerning the regulatory roles of lncRNAs and their relevance to malignant diseases.

Recently, lncRNAs have become the focus of intense research due to their critical roles in malignant processes, including tumorigenesis, drug resistance and metastasis (18-20). The present study explored the molecular function of SRA expression in cervical cancer cell lines. Our findings showed that downregulated SRA expression was correlated with decreased cell growth, migration and invasion
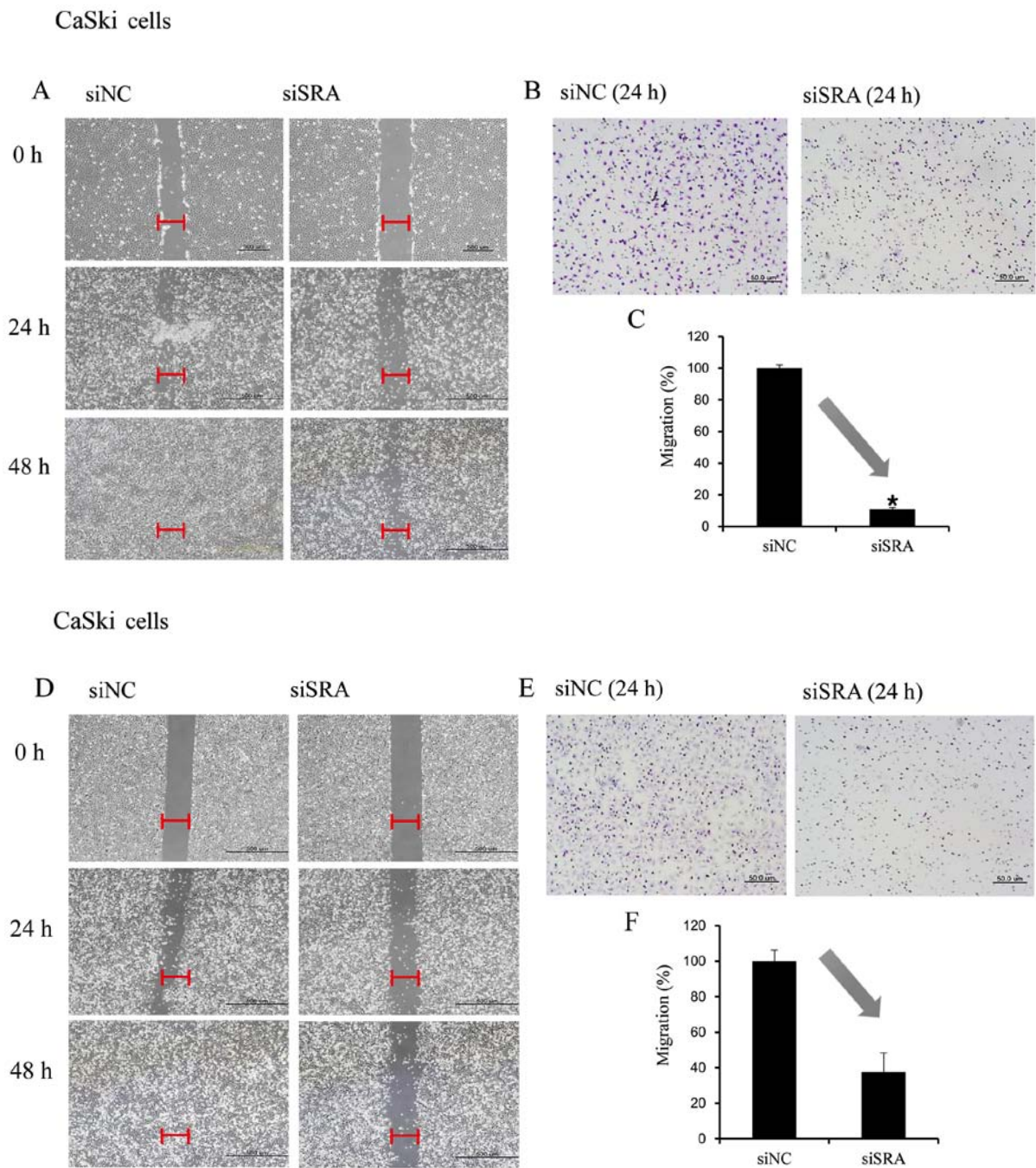
Figure 3. SRA lncRNA promotes cell migration and invasion. (A and D) Wound-healing assay was used to determine migration ability of the siSRA-transfected CaSki and SiHa cells (magnification, x200). (B and E) Matrigel-invasion assay was used to determine invasion after 24 h in siSRA-transfected CaSki and SiHa cells. Each assay was performed in triplicate. Data are presented as means ± standard deviation. (C and F) siRNA-mediated knockdown of SRA in CaSki and SiHa cells analyzed using qRT-PCR; *P<0.05 vs. control. CaSki, epidermoid cell cervical carcinoma cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SiHa, squamous cell cervical carcinoma cells; siSRA, SRA-specific siRNA; SRA, steroid receptor RNA activator.

in cervical cancer cells. This effect of SRA on tumor progression may be mediated by genes involved in cell migration, invasion, EMT and the NOTCH signaling pathway, as well as genes that encode VEGF, MMP-9, MMP-2, E-cadherin, β-catenin, vimentin, Snail, NOTCH1, p300 and HES1. Our findings suggested that SRA could potentially represent a novel biomarker and therapeutic target for cervical cancer.

We discovered that knockout of lncRNA SRA expression decreased cervical cancer cell proliferation, migration and invasion. Specifically, the expression levels of MMP-2, MMP-9 and VEGF were significantly lower in cells with

siRNA-mediated SRA knockdown. MMP-2 and MMP-9 degrade basement-membrane collagen at the site of local invasion, thereby promoting tumor-cell invasion and metastasis, which in turn leads to decreased survival rates in various types of cancers (21,22). Moreover, tumor angiogenesis plays a decisive role in tumor growth, invasion and metastasis (23), and the angiogenic factor, VEGF, a major target of many anti-cancer medications, plays a pivotal role in tumor angiogenesis and increases the ability of malignant cells to migrate and invade Matrigel membranes (24). Our findings suggested that SRA stimulated oncogenic activity in cervical cancer cells
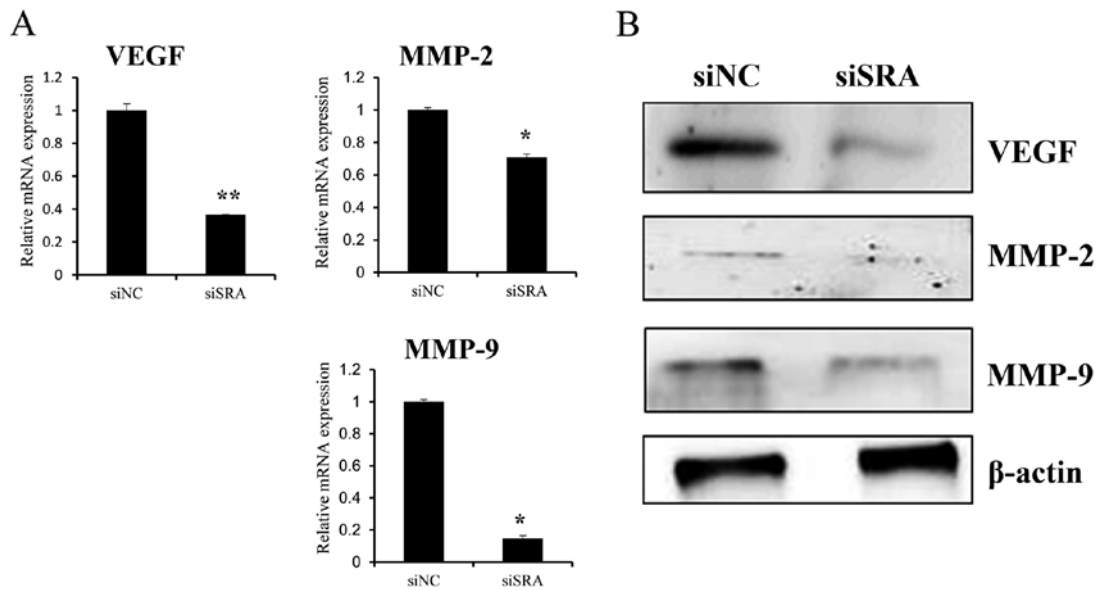
Figure 4. SRA knockdown decreases MMP-9, MMP-2 and VEGF expression in cervical cancer cells. (A) *VEGF*, *MMP-2* and *MMP-9* mRNA expression levels were analyzed by qRT-PCR. (B) Protein lysates were obtained from siSRA- and siNC-transfected CaSki cells 48-h post-transfection. VEGF, MMP-2, and MMP-9 protein levels were analyzed by western blotting; *P<0.05 vs. siNC. MMP, matrix metalloproteinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siNC, negative-control siRNA; siSRA, SRA-specific siRNA; SRA, steroid receptor RNA activator; VEGF, vascular endothelial growth factor.
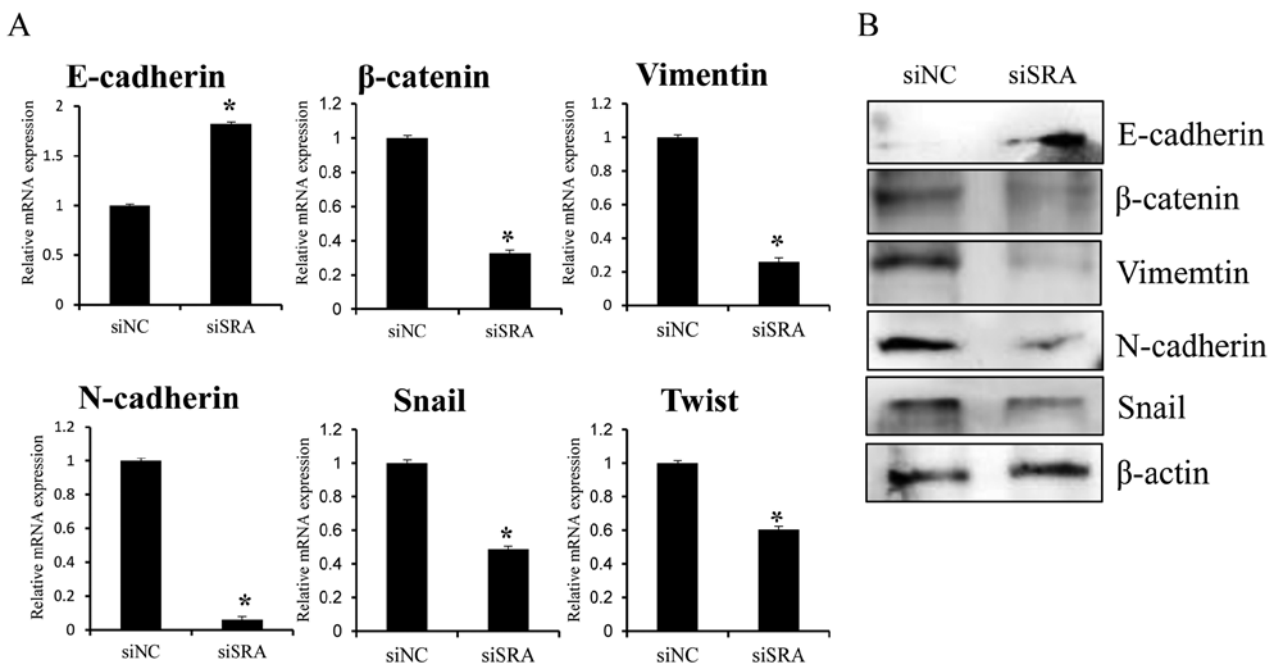

Figure 5. Effect of SRA knockdown on EMT-related genes in CaSki cells. CaSki cells were transfected with siSRA and siNC for 48 h. *E-cadherin*, *β-catenin*, *vimentin*, *N-cadherin*, *Snail* and *Twist* mRNA expression and protein levels were analyzed by (A) qRT-PCR and (B) western blotting, respectively; *P<0.05 vs. siNC. CaSki, epidermoid cell cervical carcinoma cells; EMT, endothelial-to-mesenchymal transition; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siNC, negative-control siRNA; siSRA, SRA-specific siRNA; SRA, steroid receptor RNA activator.

by promoting aggressive and metastatic characteristics via upregulating MMP-2, MMP-9 and VEGF expression.

We found that knockout of lncRNA SRA in cervical cancer cells reduced cell migration and invasion, possibly by preventing EMT induction. We analyzed the following EMT-related genes in the present study: *E-cadherin*, *β-catenin*, *vimentin*, *N-cadherin*, *Snail* and *Twist*. EMT is a well-characterized process by which epithelial cells lose their cell polarity and cell-to-cell adhesion and acquire migratory and invasive properties to become mesenchymal stem cells (25), processes that occur during metastasis in many carcinomas (25). Loss of E-cadherin is considered a crucial event in EMT, whereas N-cadherin promotes transendothelial migration, which causes diminished intercellular connection between two adjacent endothelial, thereby allowing cancer cells to slip through (26). Additionally, β-catenin weakens
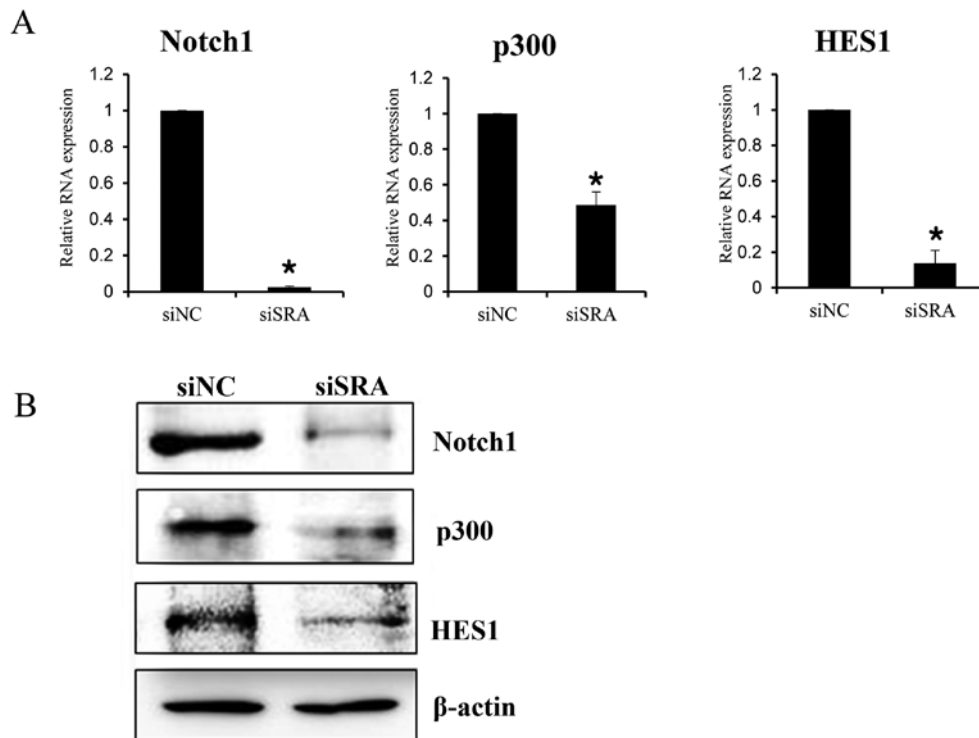
Figure 6. SRA regulates the NOTCH signaling pathway. (A) *NOTCH1*, *p300* and *HES1* mRNA levels were determined by qRT-PCR in siSRA- and siNC-transfected CaSki cells. Each assay was performed in triplicate. Data are presented as means ± standard deviation; *P<0.05 vs. control. (B) Protein lysates were obtained from siSRA- and siNC-transfected CaSki cells. NOTCH1, p300 and HES1 protein levels were analyzed using western blotting. CaSki, epidermoid cell cervical carcinoma cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siNC, negative-control siRNA; siSRA, SRA-specific siRNA; SRA, steroid receptor RNA activator.

cell-to-cell adhesion between epithelial cells, and promotes a more mobile and loosely associated mesenchymal phenotype (27). Furthermore, enhanced expression of transcription factors, such as Snail and Twist, is associated with loss of intercellular adhesion (28), and vimentin constitutes a major component of the cytoskeleton in mesenchymal cells, with its upregulation promoting EMT induction (29).

In the present study, we also investigated whether the NOTCH signaling pathway was compromised upon SRA knockdown. A clear relationship was detected between decreased levels of NOTCH1, HES1 and p300 proteins and SRA knockdown in CaSki cells. The NOTCH signaling pathway represents a highly conserved cell-signaling mechanism (30) that contributes to tumor progression, including invasion, metastasis, EMT and angiogenesis (31,32). NOTCH1 is a regulatory transmembrane receptor that plays a crucial developmental role in cell-fate determination and pattern formation (30). Several studies reported that NOTCH1 induces anoikis resistance, inhibits p53 activity, upregulates Myc expression, and abrogates the growth-inhibitory effects of TGF-β in cervical cancer cells (33-35). Additionally, HES1 influences the maintenance of stem and progenitor cells as a member of the NOTCH-signaling pathway (36) and p300, a transcriptional co-activator in the NOTCH1 pathway, functions as a histone acetyltransferase and regulates transcription via chromatin remodeling. Furthermore, p300 also plays a crucial role in cell proliferation and differentiation (37).

Based on our results, we hypothesized that lncRNA SRA functions as a key regulator of various signaling mechanisms involved in EMT establishment and NOTCH signaling. Our data provide evidence, for the first time, that lncRNA SRA promotes EMT and NOTCH signaling in cervical cancer cells, potentially contributing to cervical cancer growth, invasion and migration. The clinical impact of lncRNA SRA expression and its potential therapeutic value in the treatment of advanced cervical cancer patients needs to be evaluated in future studies.

In conclusion, these findings showed that lncRNA SRA is correlated with motility and invasiveness in cervical cancer cells. Furthermore, lncRNA SRA promoted cervical cancer progression by inducing cell migration and invasion via upregulation of MMP-2, MMP-9, VEGF, as well as genes related to EMT and the NOTCH signaling pathway. Therefore, lncRNA SRA constitutes a potential therapeutic target and prognostic marker for cervical cancer.

Acknowledgements

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