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Effects of LncRNA Lnc-LIF-AS on cell proliferation, migration and invasion in a human cervical cancer cell line

Weiguo Song[a,1], Juan Wang[a,1], Hanyuan Liu[a,1], Chenchen Zhu[a], Fei Xu[a], Lili Qian[b], Zhen Shen[b], Jing Zhu[b], Shuai Yin[c], Jiwei Qin[c], Liang Chen[d], Dabao Wu[b], Björn Nashan[c], Ge Shan[d,*], Weihua Xiao[d,*], Ying Zhou[a,*]

[a] Department of Obstetrics and Gynecology, Anhui Provincial Hospital Affiliated to Anhui Medical University, Hefei 230001, China
[b] Department of Obstetrics and Gynecology, The First Affiliated Hospital of University of Science & Technology of China, Anhui Provincial Hospital, Hefei, Anhui Province 230001, China
[c] Organ Transplantation Center, The First Affiliated Hospital of University of Science & Technology of China, Anhui Provincial Hospital, Hefei 230001, China
[d] Hefei National Laboratory for Physical Sciences at Microscale, the CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences, University of Science and Technology of China, Hefei 230027, China

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ABSTRACT

This study explored the effect of LncRNA Lnc-LIF-AS on cell proliferation, migration and invasion in the human cervical cancer (HCC) cell line SiHa. SiHa cells had the lowest expression of Lnc-LIF-AS in the 4 human cervical cancer cell lines (SiHa, ME-180, C-33A and HeLa) and were transfected and divided into the SiHa/con (transfected with pMIGRI) cell group, SiHa/Lnc-LIF-AS (transfected with pMIGRI-Lnc-LIF-AS) cell group, and SiHa/Lnc-LIF-AS-DN (transfected with pMIGRI-Lnc-LIF-AS-DN, in which the sequences overlapping with LIF mRNA was deleted) cell group. Overexpression of Lnc-LIF-AS could promote the proliferation, colony formation, invasion and migration in SiHa and ME-180 cells. And the low expression of Lnc-LIF-AS suppress the proliferation, colony formation invasion and migration in HeLa cells when the Lnc-LIF-AS expression has been suppressed. In the SiHa/Lnc-LIF-AS cells group, the cell cycle was mainly halted in the S phase and overexpression of Lnc-LIF-AS had no effect on the apoptosis of SiHa cells. Overexpression of Lnc-LIF-AS could promote the secretion of LIF in SiHa cells, and the supernatant from SiHa/Lnc-LIF-AS cells could promote cell proliferation in the SiHa/con cells. The STAT3 inhibitor could inhibit cell proliferation in the SiHa/Lnc-LIF-AS cells. The expression level of Lnc-LIF-AS in cervical cancer tissues was higher than that in normal tissues and the expression level of Lnc-LIF-AS was positively correlated with the level of LIF. In the SiHa/con and SiHa/Lnc-LIF-AS-DN cell groups, there were no significant differences in cell proliferation, cell migration and cell invasion. The overexpression of Lnc-LIF-AS can promote cell proliferation, migration and invasion in cervical cancer cells, and the core function domain of this lncRNA was located in the overlapping a 3′-UTR base sequence of LIF mRNA.

1. Introduction

Cervical cancer is the third most common cancer in women in worldwide, with an estimated 530,000 new cases and 275,000 deaths per year [1–3]. Current studies have found that nearly all cases of human papillomavirus (HPV) infection were detected in 99.8% cervical cancer patients[4]; in particular, HPV-16 and HPV-18, which cause approximately 70% of all cervical cancers worldwide were detected most often[5]. It has been reported that the E6 and E7 oncogenes of high-risk HPV were associated with the development and the maintenance of the malignant phenotype of cervical cancer [6]. These viral oncogenic proteins affect the expression of critical cell cycle regulators, such as the tumor suppressor protein p53 [3] and retinoblastoma protein (pRb) [7], respectively. However, evidence indicates that isolated HPV infection is insufficient to cause cervical cancer, the development of tumors is a complex process involving the activation of oncogenes and the inactivation of tumor suppressor genes [8]. Therefore, it is particularly important to study the pathogenesis of cervical cancer.

Long noncoding RNAs (LncRNAs), newly identified members of the

* Corresponding authors.
E-mail addresses: shange@ustc.edu.cn (G. Shan), xiaow@ustc.edu.cn (W. Xiao), caddie1234@gmail.com (Y. Zhou).
[1] **Note:** Weiguo Song, Juan Wang and Hanyuan Liu contributed equally to the present study.

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Table 1
List of primers used in this study.

Transcript	Primer sequence(5′-3′)	Product size (bp)
GAPDH(HUMAN)	F:GGGAAACTGTGGCGTGAT R:GAGTGGGTGTCGCTGTTGA	299
Lnc-LIF-AS for qPCR	F:CCCTTTACCTCTGAGCAATCC R:GCATAGGTGGGGGCAGTTAG	111
Lnc-LIF-AS for plasmid construction	F:CTAGGCGCCGGAATTAGATCTAGTGTCCCTCCCAGCCTGTG R:GCGGAATTCGTTAACCTCGAGTCTACTCTTGGCTGGCTCTCCG	1345
Lnc-LIF-AS-DN for plasmid construction	F:AGATCCGCTAGCGCTACCGGTAGTGTCCCTCCCAGCCTGTG R:TTATCTAGATCCGGTGGATCCTCTCCGGAGTGTACTGATGT	1252
Lnc-LIF-AS for probe	F:TAATACGACTCACTATAGGGACCTCTGAGCAATCCATCTC R: GAATCTACTCTTGGCTGGCT	241
miR-579-3p	F: ACACTCCAGCTGGGTTCATTTGGTATAAACC R: TGGTGTCGTGGAGTCG	23
miR-644b-3p	F: ACACTCCAGCTGGGTTCATTTGCCTCCCAG R: TGGTGTCGTGGAGTCG	22
miR-340-5p	F: ACACTCCAGCTGGGTTATAAAGCAATGAGA R: TGGTGTCGTGGAGTCG	22
miR-1202	F: ACACTCCAGCTGGGGTGCCAGCTGCAGTG R: TGGTGTCGTGGAGTCG	21
miR-3972	F: ACACTCCAGCTGGGCTGCCAGCCCCGTTCC R: TGGTGTCGTGGAGTCG	22
Lnc-LIF-AS siRNA	GCACAGGAGCTGACACTTA	24

Notes: GAPDH: glyceraldehyde phosphate dehydrogenase; F: forward; R: reverse.

noncoding RNA family [9], are more than 200 nucleotides in length [10], lack protein-coding ability [11] and are thought to be the "noise" of genomic transcription [12–14]. LncRNA is usually transcribed by RNA polymerase II [15], is formed by the fragmentation and modification of the precursor RNA [16] and has no biological function [17]. However, accumulating studies indicate that it can target the local gene [18] and the distal gene [19] and mainly regulates gene expression at the transcription and posttranscription levels [20]; furthermore, changes in their tissue- or cell-specific expression and/or their primary or secondary structures are thought to promote or inhibit cell proliferation, metastasis and invasion [21]. Recently, a large number of LncRNAs have been shown to be involved with tumorigenesis, indicating that the different expression of specific LncRNAs could be the indicators of early cancer diagnosis [22,23]. The expression of PCGEM1 in prostate cancer was upregulated, and the repression of PCGEM1 could arrest the proliferation of prostate cancer cells [24]. HOTAIR (HOX transcript antisense intergenic RNA) is reported to be overexpressed in breast cancer, is required for the viability of breast cancer cells and is correlated to tumor invasiveness and metastasis [25]. MEG3 is a tumor suppressor lncRNA that is highly expressed in normal brain tissue and is downregulated in gliomas [26]. HULC, a 1.6-kb oncogenic lncRNA, is overexpressed in hepatocellular carcinoma (HCC) and is associated with HCC susceptibility in hepatitis B virus carriers [27]. In cervical cancer, the downregulation of LncRNA UCA1 inhibits the proliferation and invasion of cervical cancer cells through miR-206 expressions [28]; LncRNA SNHG20 overexpression can promote cell proliferation and invasion via the miR-140-5p-ADAM10 axis in cervical cancer [29], and the LncRNA PVT1 contributes to the cervical cancer phenotype and is associated with poor patient prognosis [30].

In our previous study, we found that ENST00000447565 (Lnc-LIF-AS, an LIF natural antisense lncRNA) regulates the stability of LIF mRNA by overlapping a 3′-UTR of LIF mRNA, which might contribute to the inhibition of the degradation of LIF mRNA mediated by other RNAs. Thus far, the function or relative information about Lnc-LIF-AS is not known. In this study, we aimed to investigate the effects of Lnc-LIF-AS on the biological behaviors of cervical cancer cells.

2. Materials and methods

2.1. Cell lines

The cervical cancer cell lines SiHa, ME-180, C-33A, HeLa, 293T, and HaCat were purchased from ATCC. SiHa, C-33A, HeLa and 293T were inoculated in a 60-mm culture dish with DMEM (HyClone, Logan, UT, USA), HaCat was inoculated with MEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and ME-180 was inoculated with Macoy's 5A medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 units/ml penicillin and 100 mg/ml streptomycin (HyClone) and cultured in an incubator at 37°Cwith 5% CO_2 and saturated humidity. After cells grew along the dish wall, the medium was changed every 1–2 days and 0.25% trypsin (Sigma-Company, USA) was used for digestion and subculture [31].

2.2. Construction of SiHa/Lnc-LIF-AS and/Lnc-LIF-AS-DN stable cell lines

The pMIGRI-Puro-Lnc-LIF-AS plasmid was constructed by inserting a full-length of human Lnc-LIF-AS cloned by RT-PCR into the BglIIto XhoI site of pMIGRI-Puro (Addgene #27490, USA). The pMIGRI-Puro-Lnc-LIF-AS-DN plasmid was also constructed by inserting a full-length of human Lnc-LIF-AS-DN (with deletion the overlapping sequences with LIF mRNA) cloned by the RT-PCR into the BglIIto XhoI site of pMIGRI-Puro. pMIGRI-Puro-Lnc-LIF-AS, pMIGRI-Puro-Lnc-LIF-AS-DN and the control plasmid with their packaging vectors pCL-10A1 were co-transfected to the 293T cells. Then, the virus supernatant was used to infect SiHa cells. SiHa/Lnc-LIF-AS stable cell lines, SiHa/Lnc-LIF-AS-DN stable cell lines and SiHa/con cells were generated by selection with 2 μg/ml puromycin (Merck)for 2 weeks as described previously [32].

2.3. Fluorescence in situ hybridization (FISH)

Probe sequences are shown in Table 1. RNA probes were generated with a Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific), with the corresponding insertion in the T vector as a template, and then labeled with Alexa Fluor546, by using a ULYSIS Nucleic Acid Labeling Kit (Invitrogen), which added a fluor on every G in the probe to amplify the fluorescence intensity. The cell membrane was disrupted on ice for 20 min with 1 × PBS and 1% Triton X-100, and then the slides were washed with 1 × PBS and 0.4% Tween-20 for 10 min. Fixed cells and RNA probes were denatured at 80 °C for 10 min and then incubated at 37 °C for 12 h with 30 ng/μl human Cot-1 DNA (Life Technologies), 20% dextran sulfate, 500 ng/μl yeast total RNA (Ambion) and 500 ng/μl salmon sperm DNA (Sigma). Slides were washed with 2 × SSC at 37 °C for 10 min. The sections were subsequently stained

with 4′, 6-diamidino-2-phenylindole (DAPI, Beyotime, China) for nuclear visualization for 10 min. All fluorescence images (630×) were captured using a fluorescence microscope (Nikon, Japan).

2.4. miRNA analysis and cell transfection

The miRNAs that might be related to the sequence that overlaps with the 3′-UTR base sequence of LIF mRNA in Lnc-LIF-AS were predicted by miRtarget and miRbase software and selected for future study. A density of 3×10^5 SiHa cells per well were plated in 6-well and cultured overnight. Transfection was performed when the cell density was approximately 80% and changed to normal medium without 100 units/ml penicillin and 100 mg/ml streptomycin 1 h before transfection. Approximately 20 μmol/L of control reagent, miRNA stimulator (RiboBio, China), plasmid and siRNA (GenePharma, China) were transfected to cells according to the instructions of the manufacturer of Lipofectamine 2000 (Invitrogen, USA). After 8 h of transfection, the medium was discarded, and the cells were further cultured in DMEM with 10% FBS for 24 h. Then cells were collected to detect the expression of Lnc-LIF-AS and LIF [33].

2.5. Colony formation assay

The number of all cells per hole was strictly counted and cells were kept in uniform distribution. For SiHa/con, SiHa/Lnc-LIF-AS and SiHa/Lnc-LIF-AS-DN cells, 100 and 300 cells were split into 6-well plates separately and were run in triplicate. Cells were allowed to grow for 2 weeks in 5% CO_2 incubators before being stained with 0.5% Crystal Violet Staining Solution (Solarbio). The quantitation of the colony formation assays was described in a histogram. The results represent mean values of two duplicate experiments, and error bars show S.D.

2.6. Scratch, migration and invasion assays

For scratch assays, 400,000 cells, including SiHa/con, SiHa/Lnc-LIF-AS and SiHa/Lnc-LIF-AS-DN cells, were plated in 6-well plates. After the cells were attached, cell monolayers were scraped by a middle pipet tip consistently and washed with PBS to gently remove cell debris. All cells were cultured in 1% FBS in DMEM. Photos were taken during the subsequent 24 h and 48 h to monitor scratch closure. For the transwell migration assay, a cell suspension containing 4×10^5/mL cells was prepared in serum- free media, 1 mL of media containing 10% fetal bovine serum was added to the lower chamber, and then 500 μl of prepared cell suspension was added to each insert (Millipore# PIEP30R48, pore size: 8 μm). For the transwell invasion assay, 300 μl of warm serum-free media was added to the interior of the inserts and allowed to rehydrate the ECM layer for 1 h at room temperature. Then, 2×10^4 cells were plated into the transwell inserts (Chemicon #ECM550, pore size: 8 μm). All the steps were strictly followed by the transwell migration and invasion assay kit. After 24 h, cells that did not migrate were removed by scratching the upper side of the membrane with a cotton swab before fixation in 4% methanol for 5 min at room temperature. Cells were then stained with crystal violet staining solution for 5 min. The percentage of migration was determined by calculating the sum of the area of total migrated cells on the entire membrane by using ImageJ software.

2.7. Tumorigenicity in mice

Animal experiments were performed as described previously [34]. Five-week-old female nude mice (Experiment Animal Center of Shanghai, China) (each group, n = 5) were subcutaneously injected with 6×10^6 SiHa/con, or SiHa/Lnc-LIF-AS or SiHa/Lnc-LIF-AS-DN cells in 0.1 mL PBS containing 20% Matrigel. The growth of solid tumors of SiHa/con, or SiHa/Lnc-LIF-AS or SiHa/Lnc-LIF-AS-DN cells after injection was measured every five days for up to 30 days. All of the animals were sacrificed to remove the tumors for analysis. The tumors were assessed as described in previous reports [34]. The use of mice was approved by the Animal Care and Use Committee of USTC University.

2.8. Statistical evaluation

SPSS16.0 software was used for all statistical analyses. Data of all the experiments were presented as the mean ± standard deviation, which were replicated at least three times. Student's two-tailed *t*-test or analysis of variance (ANOVA) were used to assess the statistical significance of the difference. A *P*-value < 0.05 was considered statistically significant.

The other materials and methods are found in supplementary materials.

3. Results

3.1. Expression of Lnc-LIF-AS in cervical cancer cell lines

In a previous study, we reported that ΔNp63α can inhibit the levels of LIF mRNA by direct transcription regulation and decrease LIF mRNA stability by suppressing the expression of Lnc-LIF-AS (ENST00000447565) [32]. There have been no reports on the function of Lnc-LIF-AS. Here, we first checked the expression status of Lnc-LIF-AS in the cervical cancer cell lines SiHa, ME-180, C-33A and HeLa. The expression of Lnc-LIF-AS was detected by qRT-PCR. As shown in Fig. 1A, the expression of Lnc-LIF-AS in the 293T and HaCat cell lines were quite low. In the SiHa, ME-180, C-33A and HeLa cell lines, the expression of Lnc-LIF-AS was increased. The expression of Lnc-LIF-AS was highest in the C-33A cell line and lowest in the SiHa cell line compared with the HaCat cell lines. Thus, SiHa cells were selected for all subsequent experiments. The FISH was conducted to clarify the location of Lnc-LIF-AS in 293T, HaCat, SiHa, ME-180, C-33A and HeLa cells, and the results showed that Lnc-LIF-AS is mainly located in the cytoplasm (Fig. 1B).

3.2. Lnc-LIF-AS promotes cell proliferation in cervical cancer cells

To test the effect of Lnc-LIF-AS on cell growth, we established SiHa/Lnc-LIF-AS, which overexpressed the Lnc-LIF-AS gene, and a control cell line SiHa/con. The relative expression levels of Lnc-LIF-AS in these cell lines were confirmed using qRT-PCR analyses. The results showed that the expression of Lnc-LIF-AS in SiHa/Lnc-LIF-AS cells was significantly increased compared with that in SiHa/con cells ($P < 0.0001$) (Fig. 2A). We also detected the expression of Lnc-LIF-AS in the ME-180/Lnc-LIF-AS (transfected with pMIGRI-Lnc-LIF-AS) cells group and the results indicated that the expression of Lnc-LIF-AS in ME-180/Lnc-LIF-AS cells was significantly increased compared with that in ME-180/con cells ($P < 0.0001$) (Fig. 2I). qRT-PCR analysis of the expression of Lnc-LIF-AS in the HeLa/con (transfected with control plasmid) cells group and HeLa/siLnc-LIF-AS (transfected with siRNA) cells group. The results showed that the expression of Lnc-LIF-AS in HeLa/siLnc-LIF-AS cells was obviously decreased than that in HeLa/con cells (Fig. 2L).

The RTCA assay was conducted to detect the proliferation of SiHa cells after stable overexpression of Lnc-LIF-AS. As shown in Fig. 2B, the proliferation of SiHa/Lnc-LIF-AS cells was obviously increased compared with the SiHa/con cells. The number of SiHa/Lnc-LIF-AS cell colonies was significantly increased compared with the numbers in the SiHa/con cells (Fig. 2C). Flow cytometry of PI-stained cells was used to detect the cell cycle distribution of SiHa cells with Lnc-LIF-AS overexpression. We found that the proportion of SiHa cells in the G1 phase among the SiHa/con cells group and SiHa/Lnc-LIF-AS cells group were (81.35 ± 1.38)% and (64.27 ± 1.43)%, respectively (Fig. 2D). In the SiHa/Lnc-LIF-AS cells group, the cell cycle was primarily halted at the S

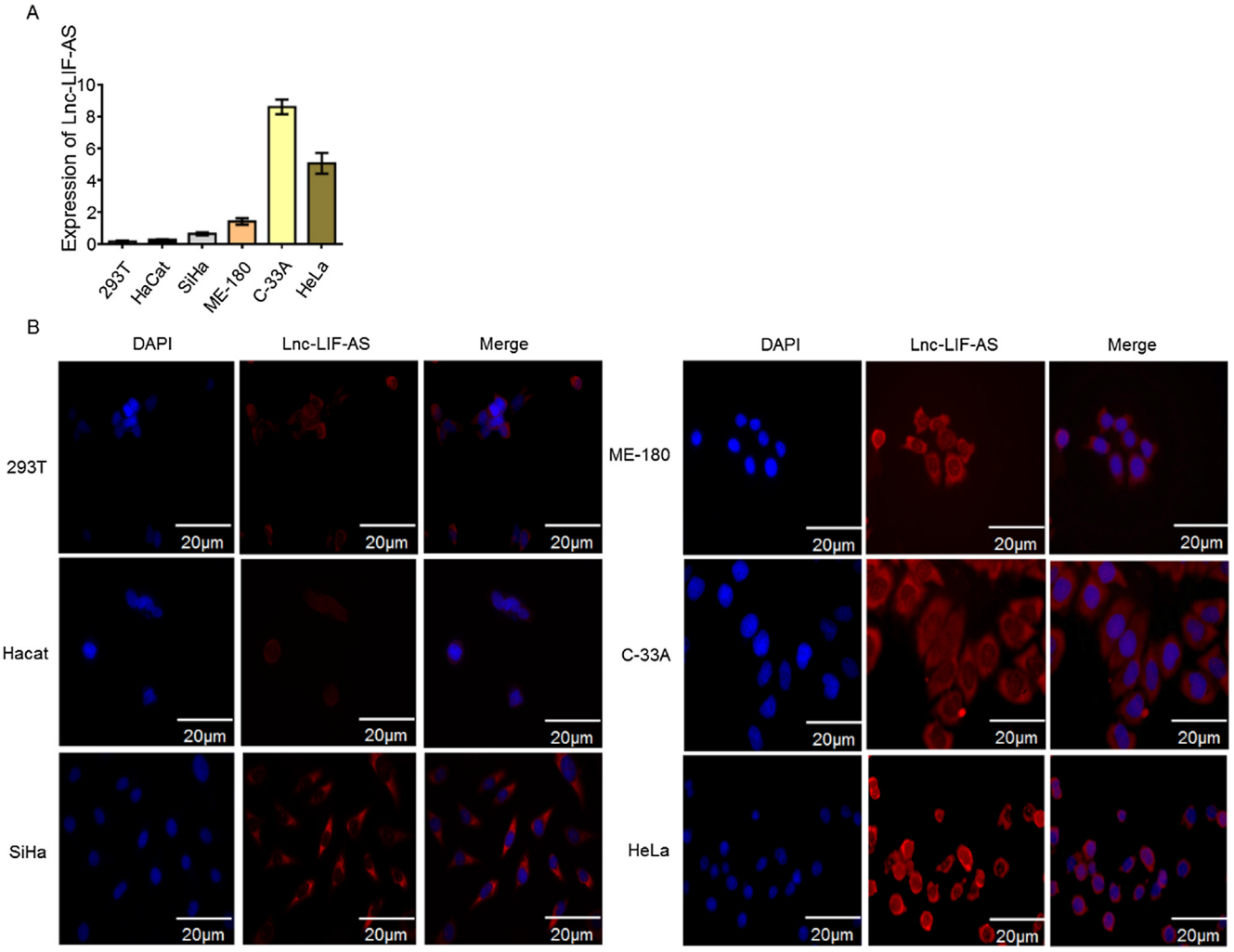

Fig. 1. Expression levels of Lnc-LIF-AS in cervical cancer cells. **(A)** qRT-PCR analysis of the expression of Lnc-LIF-AS in the 293T, HaCat, SiHa, ME-180, C33A and HeLa cell lines. **(B)** The expression location of Lnc-LIF-AS in 293T, HaCat, SiHa, ME-180, C-33A, HeLa cells detected by FISH. Scale bar, 20 μm.

phase, while the proportion of cells in the G1 phase decreased. The proliferation of SiHa cells with Lnc-LIF-AS overexpression was increased compared with that in the SiHa/con cells group ($P < 0.05$) (Fig. 2D). A flow cytometry apoptosis assay indicated that there was no significant difference in cell apoptosis in SiHa/con and SiHa/Lnc-LIF-AS cells: (2.41 ± 1.32)% and (2.18 ± 1.51)%, respectively ($P > 0.05$) (SFig. 1A). Western blot also showed that the overexpression of Lnc-LIF-AS had no effect on the expression of the apoptosis-related proteins Caspase-3, Caspase-7 and Caspase-9 (Fig. 1B).

Western blot was used to detect the expression level of P-STAT3 and LIF. The results also showed that the overexpression of Lnc-LIF-AS promotes the expression of P-STAT3 and LIF in SiHa cell lines (Fig. 2E). An ELISA kit was used to detect the expression of LIF and the results showed that the secretion of LIF in SiHa/Lnc-LIF-AS cells was increased compared with SiHa/con cells (Fig. 2F). We added the supernatant from SiHa/Lnc-LIF-AS cells to SiHa/con cells and found that it can promote cell proliferation in SiHa/con cells (Fig. 2G). We also found that the STAT3 inhibitor can inhibit cell proliferation in SiHa/Lnc-LIF-AS cells (Fig. 2H).

We also test the effect of Lnc-LIF-AS on cell growth in ME-180 and HeLa cells. The results have showed that the overexpression of Lnc-LIF-AS promotes the proliferation (Fig. 2J) and colony formation (Fig. 2K) in ME-180 cells. And the low expression of Lnc-LIF-AS suppress the proliferation (Fig. 2M) and colony formation (Fig. 2N) in HeLa cells when the Lnc-LIF-AS expression has been suppressed.

3.3. Lnc-LIF-AS promotes cell migration and invasion in cervical cancer cells

To study the role of Lnc-LIF-AS in cell migration, we performed scratch assays. As shown in Fig. 3A, after 48 h of cultivation, the healing rates after scratching the SiHa cell monolayers in the SiHa/con cells group was (68.84 ± 2.57)%. The healing rate of the scratching of SiHa cells in the SiHa/Lnc-LIF-AS cells group was (91.84 ± 1.54)%, which was significantly higher than that in the SiHa/con cells group ($P < 0.05$) (Fig. 3A). After we found that Lnc-LIF-AS can promote cell migration, we further examined the function of Lnc-LIF-AS in cell invasion. The number of cells migrating to the bottom of the Transwell through the Matrigel in the SiHa/con cells group was 17.42 ± 11.37. The number of cells that migrated to the bottom of the Transwell through the Matrigel in the SiHa/Lnc-LIF-AS cells group was 74.36 ± 12.14, which was significantly higher than that in the SiHa/con cells group ($P < 0.001$) (Fig. 3B).

We also test the effect of Lnc-LIF-AS on cell migration and invasion in ME-180 and HeLa cells. The results have showed that the overexpression of Lnc-LIF-AS promotes the migration (Fig. 3C) and invasion (Fig. 3D) in ME-180 cells. And the low expression of Lnc-LIF-AS suppress the migration (Fig. 3E) and invasion (Fig. 3F) in HeLa cells when the Lnc-LIF-AS expression has been suppressed.

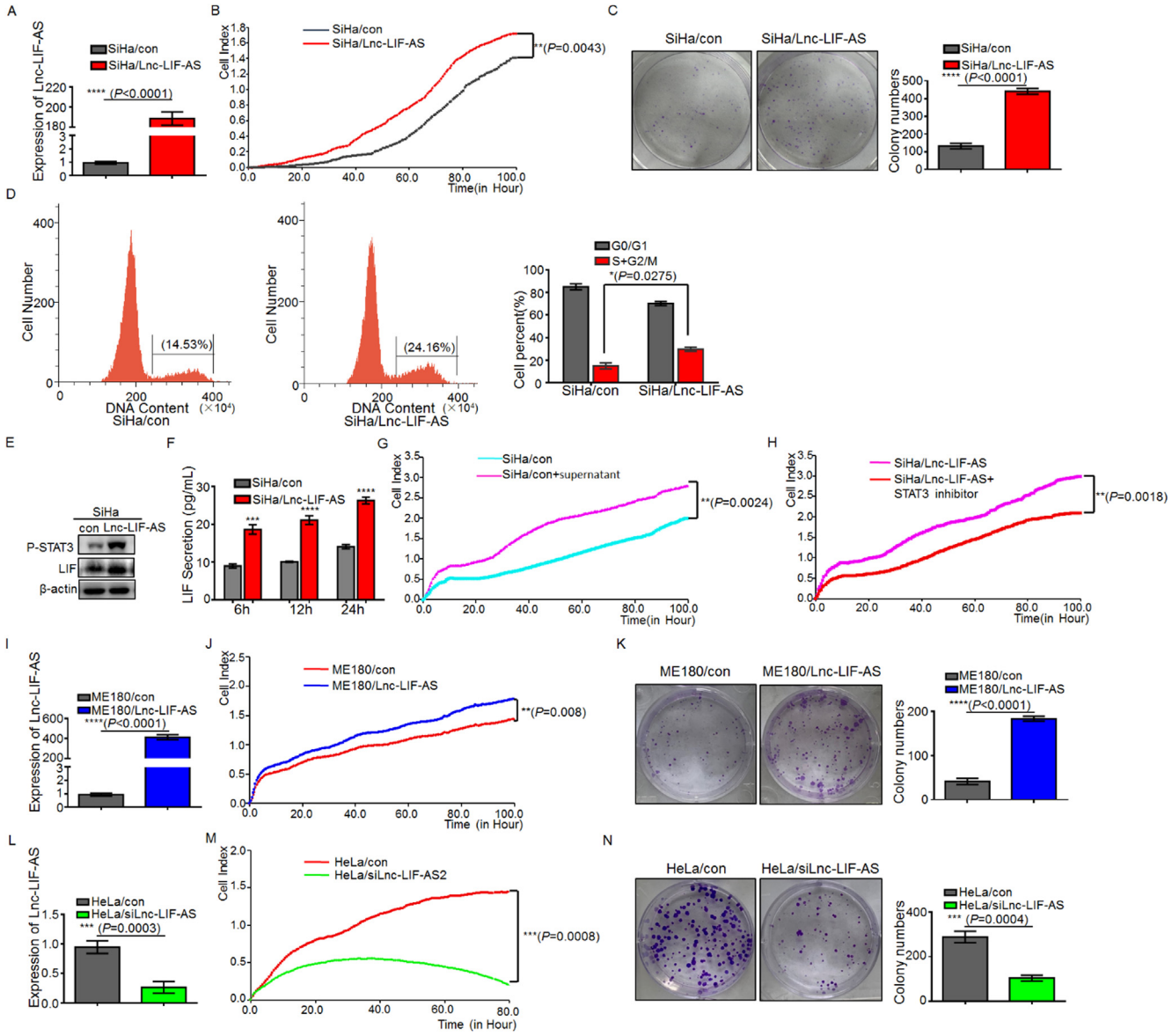

Fig. 2. The overexpression of Lnc-LIF-AS promotes proliferation of cervical cancer cells. (**A**) qRT-PCR analysis of the expression of Lnc-LIF-AS in the SiHa/con (transfected with pMIGRI) cells group and SiHa/Lnc-LIF-AS (transfected with pMIGRI-Lnc-LIF-AS) cells group. **** refers to $P < 0.0001$ compared with SiHa/con cells group. **(B)** Cell proliferation (as detected by RTCA assay) of SiHa cells among the two groups. ** refers to $P < 0.01$ compared with SiHa/con cells group. **(C)** Lnc-LIF-AS overexpression can promote cell proliferation detected by colony formation assay. ** refers to $P < 0.01$ compared with SiHa/con cells group. **(D)** The overexpression of Lnc-LIF-AS increases the proportion of cells in S phase and decrease the proportion of cells in G1 phase in SiHa cells that detected by using flow cytometry with PI staining. **(E)** The expression level of P-STAT3 (Y705) and LIF detected by Western blot assay. **(F)** The secretion level of LIF in SiHa/con cells group and SiHa/Lnc-LIF-AS cells group. *** refers to $P < 0.001$ and **** refers to $P < 0.0001$ compared with SiHa/con cells group. **(G)** Cell proliferation (as detected by RTCA assay) of SiHa/con cells after stimulated by supernatant from SiHa/Lnc-LIF-AS cells. ** refers to $P < 0.01$ compared with SiHa/con cells group. **(H)** Cell proliferation (as detected by RTCA assay) of SiHa/Lnc-LIF-AS cells after added STAT3 inhibitor. ** refers to $P < 0.01$ compared with SiHa/Lnc-LIF-AS cells group. **(I)** qRT-PCR analysis of the expression of Lnc-LIF-AS in the ME-180/con (transfected with pMIGRI) cells group and ME-180/Lnc-LIF-AS (transfected with pMIGRI-Lnc-LIF-AS) cells group. **** refers to $P < 0.0001$ compared with SiHa/con cells group. **(J)** Cell proliferation (as detected by RTCA assay) of ME-180 cells with Lnc-LIF-AS overexpression. ** refers to $P < 0.01$ compared with ME-180/con cells. **(K)** Colony numbers of the ME-180/con cells group and ME-180/Lnc-LIF-AS cells group. **** refers to $P < 0.0001$ compared with ME-180/con cells group. **(L)** qRT-PCR analysis of the expression of Lnc-LIF-AS in the HeLa/con (transfected with control plasmid) cells group and HeLa/siLnc-LIF-AS (transfected with siRNA) cells group. *** refers to $P < 0.001$ compared with HeLa/con cells group. **(M)** Cell proliferation (as detected by RTCA assay) of HeLa cells with the Lnc-LIF-AS expression has been suppressed. *** refers to $P < 0.001$ compared with HeLa/con cells group. **(N)** Colony numbers of the HeLa/con cells group and HeLa/siLnc-LIF-AS cells group. *** refers to $P < 0.001$ compared with HeLa/con cells group.

3.4. Lnc-LIF-AS promotes tumor growth in vivo

To evaluate the role of Lnc-LIF-AS in regulating tumor growth in vivo, we transplanted SiHa/con and SiHa/Lnc-LIF-AS cells into five-week-old female athymic nude mice. Cells (6×10^6) were transplanted sub-cutaneously into the right flank (n = 5 per group) and tumor growth was monitored for 30 days (Fig. 4E). On day 30, mice were euthanized and tumors were collected. Some representative mice bearing tumors and final tumor sizes are shown in Fig. 4A, B. Tumor sizes and weights in the SiHa/Lnc-LIF-AS group were significantly higher when compared with the SiHa/con group ($p < 0.05$) (Fig. 4B, D). We next examined the levels of Lnc-LIF-AS in tumors derived from mice using qRT-PCR. As expected, Lnc-LIF-AS expression levels were high in SiHa/Lnc-LIF-AS cells group (Fig. 4C). The promotional effect of

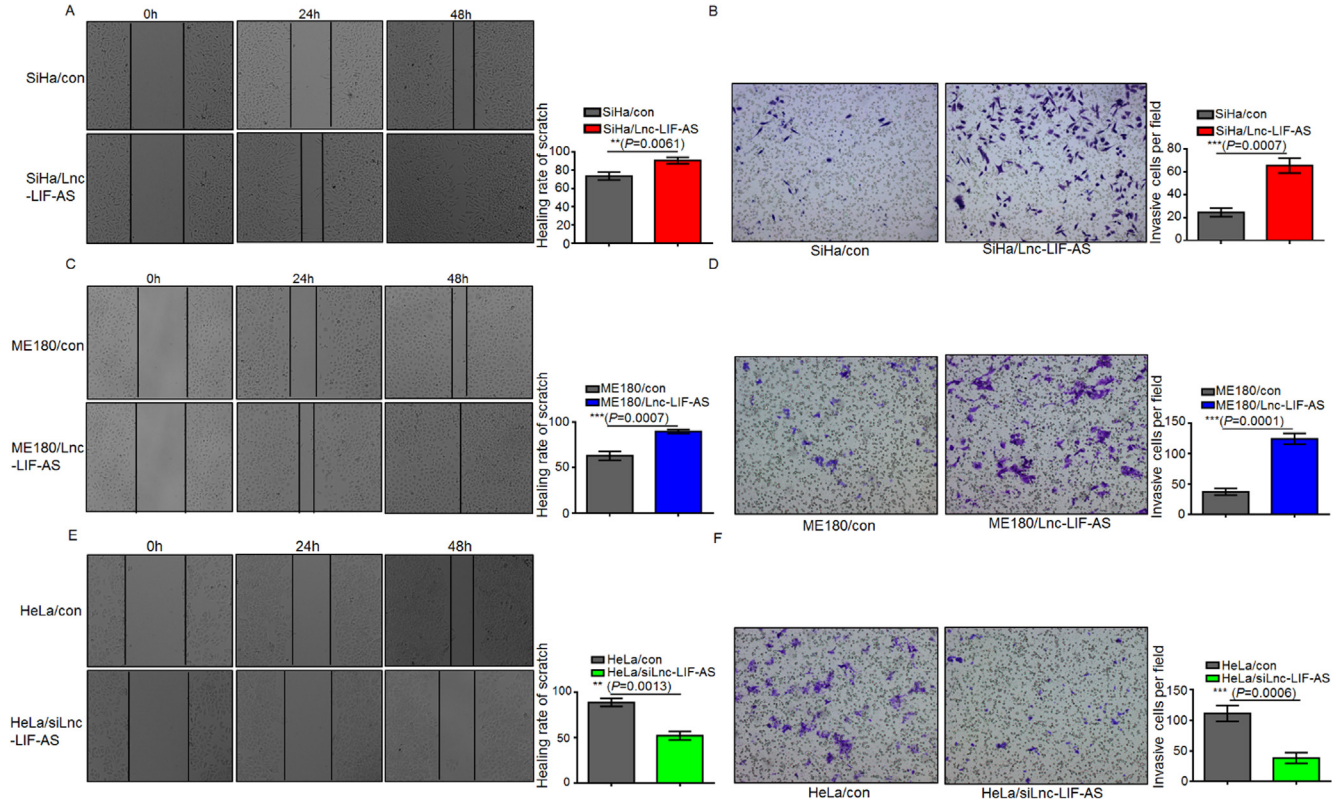

Fig. 3. The overexpression of Lnc-LIF-AS promotes the migration and invasion of cervical cancer cells in vitro. **(A)** Cell migration ability and the wound healing rate of SiHa cells after stably transfected with pMIGRI and pMIGRI-Lnc-LIF-AS was measured by the wound healing assay. ** refers to $P < 0.01$ compared with SiHa/con cells group. **(B)** Cell invasion capacity of SiHa cells after stable transfection was measured by the transwell assay. *** refers to $P < 0.001$ compared with SiHa/con cells group. **(C)** Cell migration ability and the wound healing rate of ME-180 cells with Lnc-LIF-AS overexpression. *** refers to $P < 0.001$ compared with ME-180/con cells group. **(D)** Cell invasion capacity of ME-180 cells with Lnc-LIF-AS overexpression. *** refers to $P < 0.001$ compared with ME-180/con cells group. **(E)** Cell migration ability and the wound healing rate of HeLa cells with the Lnc-LIF-AS expression has been suppressed. *** refers to $P < 0.001$ compared with HeLa/con cells group. **(F)** Cell invasion capacity of HeLa cells with the Lnc-LIF-AS expression has been suppressed. ** refers to $P < 0.01$ compared with HeLa/con cells group.

Lnc-LIF-AS on tumor growth was further assessed by an immunohistochemical analysis of the expression of proliferation-associated antigen Ki67, CD34 and VEGF. As expected, the tumors overexpressing Lnc-LIF-AS showed extremely increased expression of Ki67, CD34 and VEGF compared to that in control tumors (Fig. 4F).

Then we detected the expression of Lnc-LIF-AS and LIF in clinical tissues. As shown in Fig. 6A, the expression of Lnc-LIF-AS in 15 cervical cancer tissues was higher than that in normal tissues. Besides, correlation analysis of 15 cases of cervical cancer revealed that Lnc-LIF-AS expression was positively correlated with LIF expression ($P < 0.05$) (Fig. 6B).

3.5. Overexpression of Lnc-LIF-AS-DN does not affect the proliferation, migration and invasion of SiHa cells

To study the regulation mechanism of Lnc-LIF-AS, we established the SiHa/Lnc-LIF-AS-DN, which overexpressed the Lnc-LIF-AS gene with a deletion of overlapping sequences with LIF mRNA and a control cell line SiHa/con. The qRT-PCR results showed that the expression of Lnc-LIF-AS-DN in SiHa/Lnc-LIF-AS-DN cells was significantly increased compared with that in SiHa/con cells ($P < 0.0001$) (Fig. 5A). The number of SiHa/Lnc-LIF-AS-DN cells colonies showed no significant difference when compared with numbers in the SiHa/con cells ($P > 0.05$) (Fig. 5B). Additionally, the RTCA results indicated that the proliferation of SiHa/Lnc-LIF-AS-DN cells showed no significant difference compared with the SiHa/con cells ($P > 0.05$) (Fig. 5C). For scratch assays, in the SiHa/Lnc-LIF-AS-DN cells group and SiHa/con cells group, the healing rates showed no obvious difference: (84.34 ± 1.32)% and (82.46 ± 2.25)%, respectively ($P > 0.05$) (Fig. 5D). For the transwell invasion assay, in the SiHa/Lnc-LIF-AS-DN cells group and SiHa/con cells group, there was no clear difference in the number of cells that migrated to the bottom of the transwell through the Matrigel: 74.24 ± 9.46 and 77.32 ± 5.78, respectively ($P > 0.05$) (Fig. 5E). In the SiHa/con and SiHa/Lnc-LIF-AS-DN cells group, the percentage of the cell cycle halted at the S phase was (16.23 ± 1.32) % and (16.60 ± 1.76) %, respectively. The proliferation of SiHa cells with Lnc-LIF-AS-DN overexpression showed no significant difference compared with that in the SiHa/con cells group ($P > 0.05$) (Fig. 5F). And the results of tumorigenicity in mice also showed that the overexpression of Lnc-LIF-AS-DN did not affect proliferation of SiHa cells in vivo ($P > 0.05$) (Fig. 5G, SFig. 2A, B).

3.6. Overexpression of miR-579-3p and miR-664-3p promote the expression of the mRNA of Lnc-LIF-AS and LIF in SiHa cells

To further study the regulation mechanism of Lnc-LIF-AS in SiHa cells, the miRNAs that might be related to the sequence that overlaps with the 3′-UTR base sequence of LIF mRNA in Lnc-LIF-AS were predicted by miRtarget and miRbase software and selected for future study (Fig. 7A). The results showed that the overexpression of miR-579-3p and miR-664-3p could promote the expression of the mRNA of Lnc-LIF-AS and LIF in SiHa cells (Fig. 7B), while the overexpression of miR-340-5p, miR-1202 and miR-3972 had no effects on the expression of the mRNA of Lnc-LIF-AS and LIF in SiHa cells(Fig. 7C).

4. Discussion

Cervical cancer is the fourth most prominent cause of death from

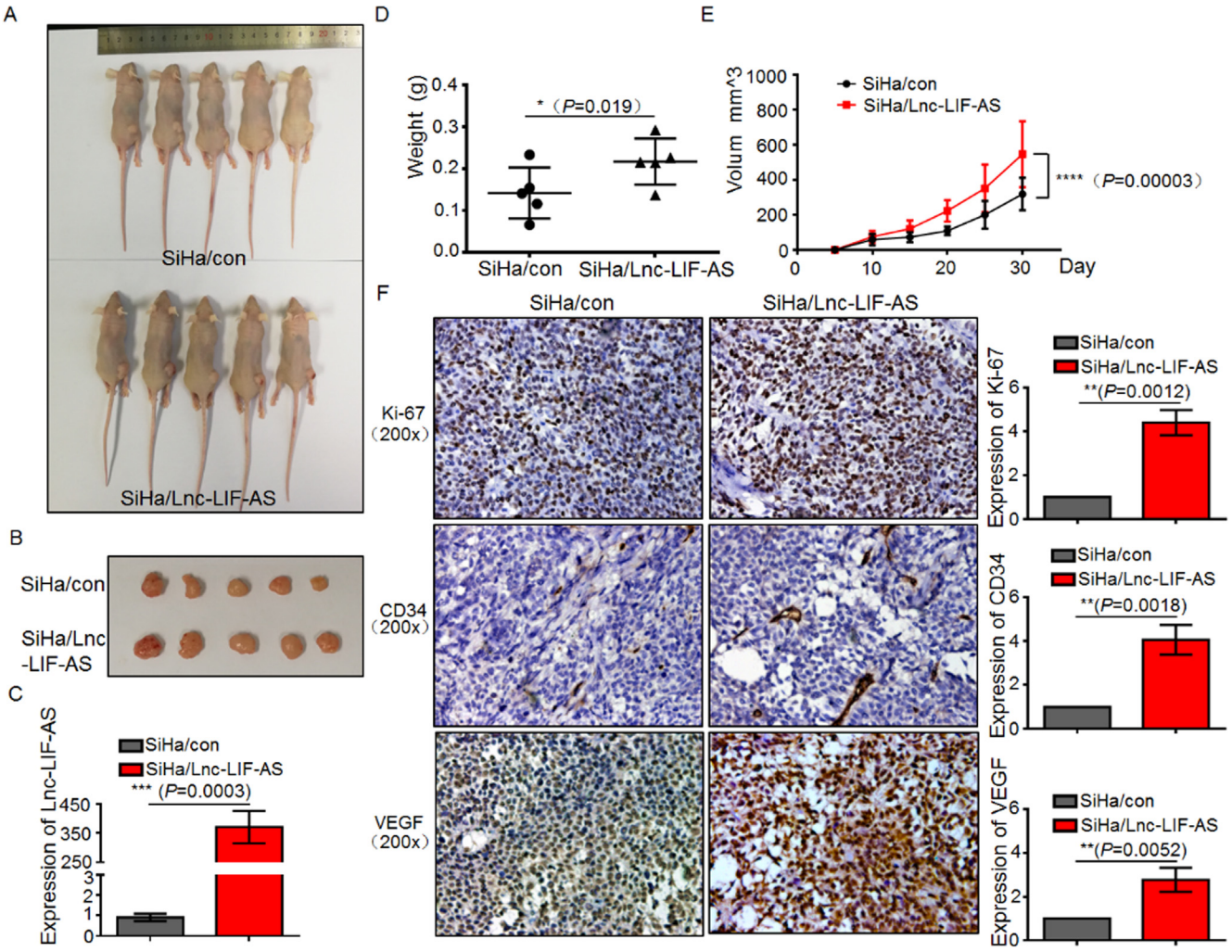

Fig. 4. The overexpression of Lnc-LIF-AS promotes tumor growth in vivo. (**A**, **B**) Images of the xenograft tumors among the two groups after injection for up to 30 days. (**C**) The relative expression levels of Lnc-LIF-AS in tumor tissues were detected by qRT-PCR. *** refers to $P < 0.001$ compared with SiHa/con cells group. (**D**) The tumor weight distribution in the two groups. * refers to $P < 0.05$ compared with SiHa/con cells group. (**E**) Tumor growth curves in the two groups. The tumor volume was measured every five days after 1 week of injection. **** refers to $P < 0.0001$ compared with SiHa/con cells group. (**F**) The overexpression of Lnc-LIF-AS promotes the expression of tumor proliferation-associated antigen Ki-67, CD34 and VEGF (tested by immunohistochemistry (×200 magnification). ** refers to $P < 0.01$ compared with SiHa/con cells group.

gynecological malignancy in women [35,36], and its development is a multistep and multifactor process[37] in which the abnormal expression of genes may play a critical role. However, to date, its pathological mechanism is not yet clear [38,39]. Therefore, it is necessary to find new potential biomarkers for diagnosis, treatment, and prognosis to improve the effect of clinical treatment for cervical cancer [40]. Recently, LncRNA has received increasingly more attention and has become a hot topic in tumor research [41]. Dysregulation of LncRNAs have been widely reported in human cervical cancer, and LncRNAs play critical roles in tumor occurrence, invasion, metastasis and recurrence [39,42,43].

Karin and colleagues identified that IL-6 can be a tumor-promoting cytokine by causing inflammation and STAT3 activation [44]. STAT3 is recognized as a true oncogene, and the constitutive tyrosine phosphorylation of STAT3 on position 705 (Y705) is observed in a large number of tumors [45,46]. STAT3 is the main transcription factor activated by IL-6-type cytokines such as IL-6, oncostatin M (OSM), and leukemia inhibitory factor (LIF) and can activate Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling [47–50]. As a kind of IL-6-type cytokine, LIF can lead to the deregulated activation of IL-6 signaling, promoting an increase of its cellular functions and constitutive STAT3 activation [51]. So Lnc-LIF-AS can promote cell proliferation by increasing the expression of LIF and P-STAT3.

In the previous study, we showed that ΔNp63α can inhibit the levels of LIF mRNA by direct transcription regulation and decrease LIF mRNA stability by suppressing the expression of Lnc-LIF-AS [32]. We also found that a high level of LIF in cervical cancers was related to poor patient survival, and the decrease of ΔNp63α weakened the differentiation of cervical cancerous cells. These results suggest that ΔNp63α may form a complex network in the regulation of cervical cancer differentiation [32]. However, the function or related information of Lnc-LIF-AS in cervical cancer remains unclear. In our study, we found that the expression of Lnc-LIF-AS markedly promoted the proliferation, migration and invasion of SiHa, ME-180 and HeLa cells, while the overexpression of Lnc-LIF-AS has no effect on the apoptosis of SiHa cells. By constructing SiHa/con and SiHa/Lnc-LIF-AS-DN cells, we have made it clear that Lnc-LIF-AS plays a role in cervical cancer by overlapping a 3′-UTR base sequence of LIF mRNA. In our previous study, we found that Lnc-LIF-AS regulates the stability of LIF mRNA by overlapping a 3′-UTR of LIF mRNA and promoting the expression of LIF. We also found that the secretion of LIF in SiHa/Lnc-LIF-AS cells was increased compared with the control cells. The supernatant from SiHa/Lnc-LIF-AS cells can promote cell proliferation in the SiHa/con cells,

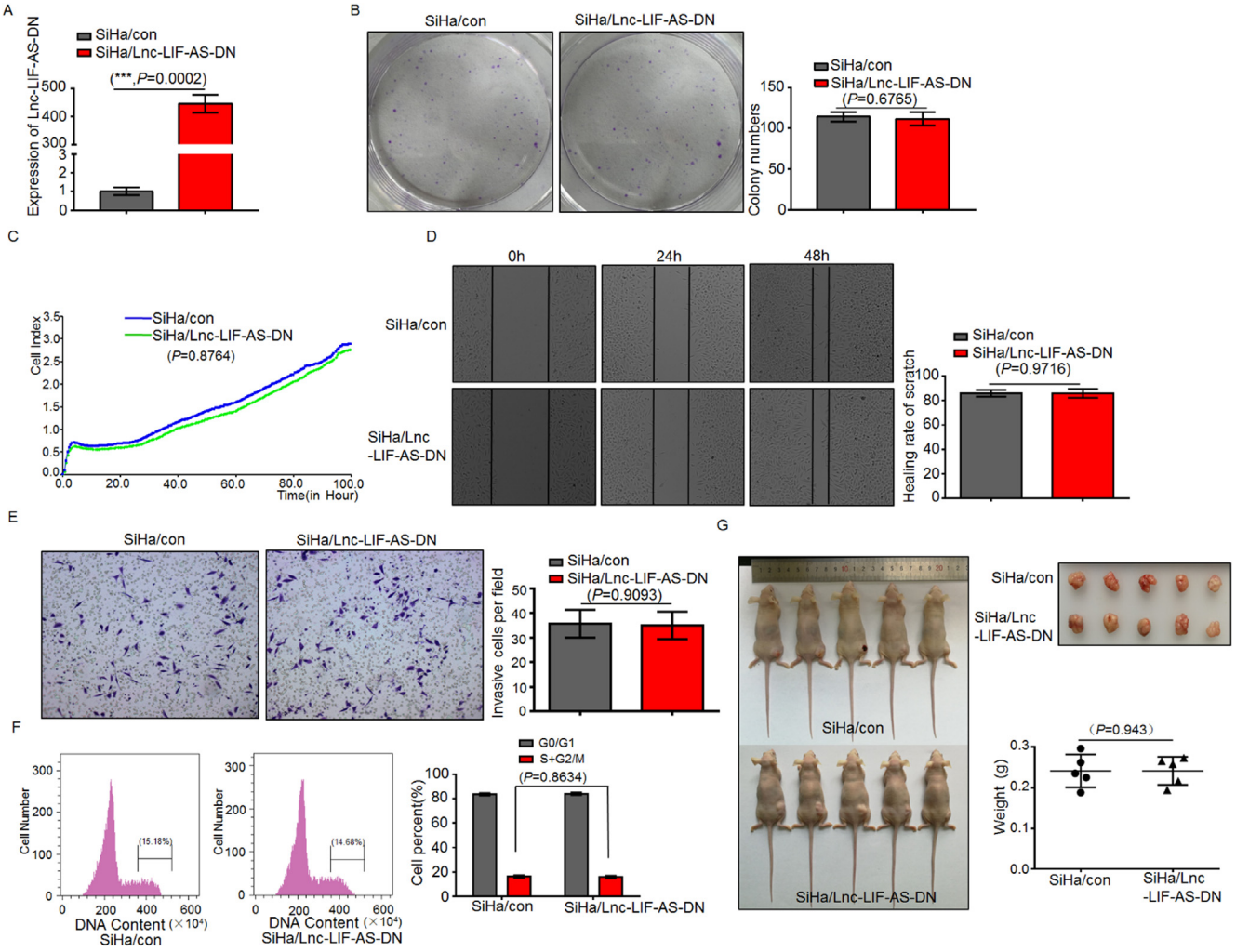

Fig. 5. The overexpression of Lnc-LIF-AS-DN does not affect the proliferation, migration and invasion of SiHa cells. **(A)** The expression of Lnc-LIF-AS-DN was detected using qRT-PCR in the SiHa/con (transfected with pMIGRI) cells group and SiHa/Lnc-LIF-AS-DN (transfected with pMIGRI-Lnc-LIF-AS-DN) cells group. *** refers to $P < 0.001$ compared with SiHa/con cells group. **(B, C)** Lnc-LIF-AS-DN overexpression has no effects on cell proliferation of SiHa cells after stable transfection was measured by colony formation assay and RTCA assay. **(D)** Lnc-LIF-AS-DN overexpression has no effects on cell migration capacity of SiHa cells after stable transfection was measured by the wound healing assay. **(E)** Lnc-LIF-AS-DN overexpression has no effects on cell invasion capacity of SiHa cells after stable transfection was measured by the transwell assay. **(F)** The overexpression of Lnc-LIF-AS-DN has no effects on cell cycle of SiHa cells after stable transfection that detected by using flow cytometry with PI staining. **(G)** The results of tumorigenicity in mice showed that the overexpression of Lnc-LIF-AS-DN did not affect proliferation of SiHa cells in vivo.

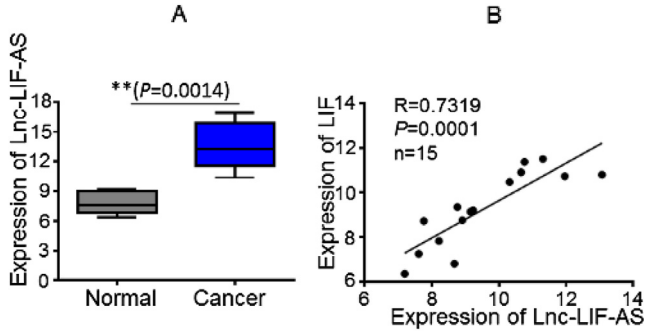

Fig. 6. The expression level of Lnc-LIF-AS in normal tissues and cervical cancer tissues. **(A)** The expression level of Lnc-LIF-AS in normal tissues and cervical cancer tissues. ** refers to $P < 0.01$ compared with normal tissues. **(B)** The relationship between Lnc-LIF-AS and LIF in cervical cancer tissues.

and the STAT3 inhibitor can inhibit cell proliferation in SiHa/Lnc-LIF-AS cells. The clinical samples of cervical cancer showed that Lnc-LIF-AS was highly expressed in cervical cancer tissues and positively correlated with the expression of LIF. Through miRtarget and miRbase software, we predicted miRNAs that might be related to the deletion sequences of Lnc-LIF-AS, and we selected miR-1202, miR-3972, miR-579-3p, miR-664-3p and miR-340 for future study. We found that the overexpression of miR-579-3p and miR-664-3p can promote the expression of the mRNA of Lnc-LIF-AS and LIF, but the specific mechanism is still unclear.

Taken together, the present study revealed that Lnc-LIF-AS might be an oncogene in cervical cancers. The overexpression of Lnc-LIF-AS promotes the proliferation, migration and invasion of cervical cancer cells, and the core function domain of this lncRNA was located in the overlapping a 3′-UTR base sequence of LIF mRNA. Finally, miR-579-3p and miR-664-3p might be related to the regulation of LIF and Lnc-LIF-AS. We believe that these findings support Lnc-LIF-AS as a potential new biomarker or therapeutic target for cervical cancer.

5. Conclusion

The overexpression of Lnc-LIF-AS can promote cell proliferation, migration and invasion in cervical cancer cells, and the core function

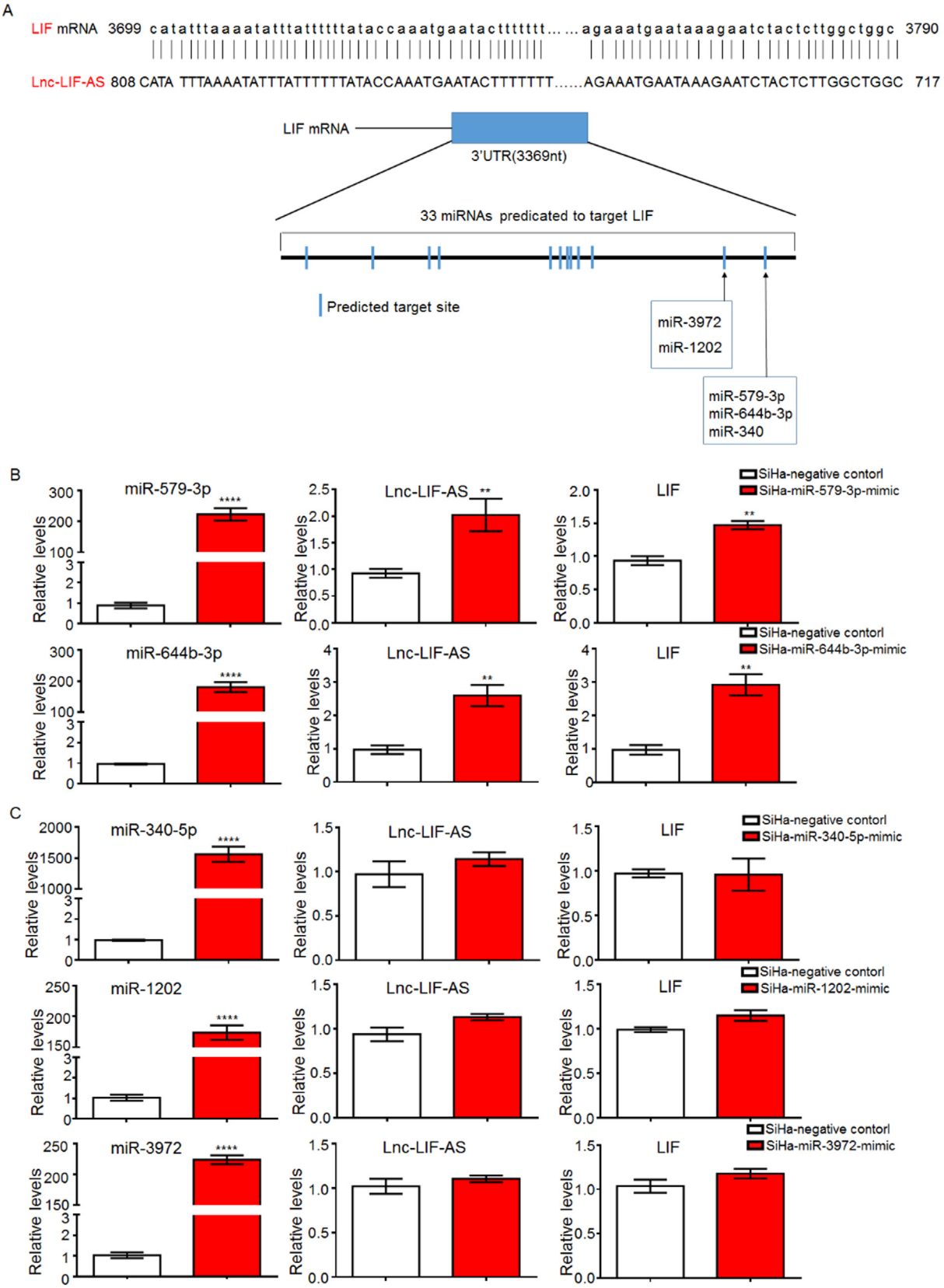

Fig. 7. The miRNAs that might be related to the sequence that overlaps with the 3′-UTR base sequence of LIF mRNA in Lnc-LIF-AS were predicted by miRtarget and miRbase software and some miRNAs could promote the expression of the mRNA of Lnc-LIF-AS and LIF. **(A)** The sequence that overlaps with the 3′-UTR base sequence of LIF mRNA in Lnc-LIF-AS and sites that likely to related to some miRNAs predicted by miRtarget and miRbase software. **(B)** The miR-579-3p and miR-644b-3p could promote the expression of the mRNA of Lnc-LIF-AS and LIF. **(C)** The miR-340-5p, miR-1202 and miR-3972 had no effects on the expression of the mRNA of Lnc-LIF-AS and LIF.

domain of this lncRNA was located in the overlapping a 3′-UTR base sequence of LIF mRNA.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.05.004.

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