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Original Articles

The high-risk HPV oncogene E7 upregulates miR-182 expression through the TGF-β/Smad pathway in cervical cancer

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ABSTRACT

Accumulating experimental evidence has shown that the aberrant expression of microRNAs (miRNAs) is involved in the development and progression of human cervical cancer. Previously, we identified miR-182 as an oncomiRNA in cervical cancer. However, the mechanism by which miR-182 is regulated and the interaction between human papillomavirus (HPV) and miR-182 in cervical cancer development remains unknown. In the present study, we explored the link between HPV E7 and miR-182 and verified that high-risk HPV E7 upregulated miR-182 expression through TGF-β/Smad4 signaling pathway in cervical cancer. By contrast, low-risk HPV E7 did not affect the expression of TGF-β and miR-182. Mechanistically, as high-risk HPV E7 bound to pRb, E2F was released from the complex and bound to the TGF-β promoter region, resulting in TGF-β overexpression. Furthermore, the Smad4 signaling pathway was activated upon TGF-β overexpression, which led to an interaction between Smad4 and the miR-182 promoter region, subsequently inducing the upregulation of miR-182 in both cervical cancer cells and the surrounding normal cells. In conclusion, this newly identified high-risk HPV E7/TGF-β/miR-182 regulatory network might inform the development of specific therapeutic strategies for cervical cancer.

1. Introduction

Although a preventive vaccine is available, cervical cancer remains the third leading malignancy among women worldwide and kills approximately 260,000 patients annually, approximately 20% of whom are from China [1]. Cervical cancer is readily managed in early stages by surgery, and radiation and chemoradiation therapy are available for advanced stages. However, radiation and chemoradiation often cause severe adverse effects, possibly even infertility and early menopause [2,3]. In addition, treatments in advanced stages are always less effective than early interventions [4]. Therefore, a full understanding of the molecular mechanisms underlying cervical cancer progression is necessary for early detection and effective interventions.

HPV is one of the most important etiological factors contributing to

Abbreviations: miRNAs, microRNAs; HPV, human papillomavirus

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the pathogenesis of cervical cancer. HPVs can be grouped into high-risk and low-risk subtypes. The high-risk HPV oncogenes E6 and E7 are closely related to cellular transformation and immortalization [5]. The E6 gene product binds to and induces the degradation of the p53 tumor suppressor gene to block apoptosis, and the E7 gene product targets and binds to the tumor suppressor gene pRb, consequently promoting entry into S phase to immortalize cells [6]. However, evidence suggests that HPV infection alone is insufficient to induce malignant changes and that other host genetic variations are important for the pathogenesis of cervical cancer [7].

MiRNAs are single-stranded noncoding RNAs with a length of ~22 nucleotides and function via base pairing with complementary sequences within mRNA molecules [8]. MiRNAs are involved in various biological processes, including development, proliferation, apoptosis and differentiation [9,10]. Recent studies indicate that miRNAs can function as oncogenes by inhibiting endogenous tumor suppressor genes or can act as tumor suppressors by repressing cellular oncogenes [11–14]. Previously, two upregulated and nine downregulated miRNAs, relative to their expression in microdissected normal cervical epithelium, were consistently found in cervical cancer cell lines; and importantly, miR-182 was the most highly upregulated miRNA, and increased expression levels of miR-182 were significantly correlated with advanced stages of primary cervical carcinoma [10]. To address whether dysregulated miR-182 expression is associated with genomic alterations, we performed Agilent high-density array comparative genomic hybridization (aCGH) in 7 cervical cancer cell lines and identified 26 chromosomal copy number variations (CNVs). In our investigation of the genomic presence of miRNAs in these 26 regions of genomic aberration, miR-182 was not located within any of these regions [10]. Thus, unfortunately, whether HPV genes interact with miR-182 remains unclear.

Our recent array study showed that transforming growth factor beta (TGF-β) was amplified and overexpressed (> 2-fold) in the cervical cancer group relative to its transcription and expression in the normal group [15]. TGF-β is a multifunctional regulatory protein that controls proliferation, differentiation, and other functions [16]. A higher level of the TGF-β protein was observed in E7-positive squamous cancer cases than in E7-negative cases. In *in vitro* experiments, both the mRNA and protein levels of TGF-β were higher in the cervical cell lines containing high-risk HPV genomes than in cell lines not containing HPV genomes [17]. As the biogenesis of miRNAs is usually regulated by the TGF-β signaling pathway [18], the expression level of miR-182 might also be influenced by TGF-β. Therefore, in the present study, we proposed that high-risk HPV E7 may upregulate miR-182 expression via the TGF-β pathway in cervical cancer.

In this study, we first examined whether high-risk HPV E7 could induce the upregulation of miR-182 via the regulation of TGF-β *in vitro* and then explored the molecular mechanism by which E7 regulates TGF-β in cervical cancer. The pathway downstream of TGF-β/miR-182 in cervical cancer was also explored and validated *in vivo*. Furthermore, a coculture system was used to study the cervical cancer "microenvironment", where cancer cells induced surrounding normal cells to overexpress TGF-β and miR-182. The results of the present study may thus provide new insights into the development and progression of cervical cancer.

2. Materials and methods

2.1. Cell lines

Three human cervical cancer cell lines (HeLa, SiHa and C33A) and two normal cell lines (HSF and hCEC) were obtained from ATCC (USA) and ScienCell (USA). Among the cervical cancer cells, HeLa cells are HPV18-positive, SiHa cells are HPV16-positive, and C33A cells are HPV-negative. HSF cells are normal fibroblasts. HeLa, SiHa, C33A and HSF cells were cultured in full MEM medium at 37 °C in a humidified atmosphere with 5% CO_2. Normal hCECs generated from the ectocervix of premenopausal women aged 35–45 years, were grown and maintained in cervical epithelial cell medium supplemented with growth supplement (CerEpiCGS).

2.2. Tissue specimens

Six cervical carcinoma specimens were collected from the Department of Obstetrics and Gynecology, Guangzhou Panyu Central Hospital and used for IHC or ISH. All protocols were approved by the research ethics committees of Panyu Central Hospital, and signed written consent forms were obtained from each patient. The information of cervical specimens was listed in Supplementary Table 1.

2.3. Cell transfection

DNA fragments of HPV 16 and HPV18 E7 were amplified by PCR from SiHa and HeLa cell genomic DNA. DNA fragments of E7 from other HPV subtypes were obtained from ATCC (USA). The PCR products were purified by a PCR Purification Kit (TaKaRa, China) and ligated to the pcDNA3.1 vector. After construction, the E7 DNA sequence was validated by sequencing. The siRNAs against each target were designed online with the siDESIGN Center and synthesized by RiboBio (China). Vectors, empty vectors, siRNA and nonsense siRNA were transfected using the transfection reagent Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The siRNAs sequences and knock down efficiency were listed in Supplementary Table 2.

2.4. Establishment of the cervical tumor xenograft mice model

Six-week-old female BALB/c athymic mice were bred and maintained by the Laboratory Animal Services Center of the Chinese University of Hong Kong. Mice were anesthetized by ketamine (75 mg/kg)/xylazine (10 mg/kg), and 2×10^6 HeLa cells in Matrigel were injected into the back flank of the mice by subcutaneous injection. Ten days post tumor inoculation, the animals were subjected to siRNA treatment by microneedle injection. All experimental protocols were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and the Government of the Hong Kong SAR.

2.5. qRT-PCR

Cervical cancer cells and cervical tumor xenografts were collected 24 h after siRNA treatment for RNA extraction. Then, 100 ng of RNA was reverse transcribed to cDNA using a First Strand cDNA kit (TaKaRa) following the manufacturer's protocol. qRT-PCR was performed by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California). GAPDH was used as the endogenous control for mRNA expression; U6, for miR-182 expression. Relative gene expression levels were calculated by the Ct method. The primer sequences were listed in Supplementary Table 3.

2.6. Western blotting

Cells were collected, washed with PBS and then resuspended in RIPA lysis buffer to extract total protein. Fifty micrograms of protein was loaded on denaturing SDS-PAGE gels. Immunodetection of TGF-β was performed using an anti-TGF-β (1:500; Santa Cruz Biotechnology, USA) monoclonal antibody. Enhanced chemiluminescence was used for detection. The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer (Bio-Rad) and normalizing the band densities to the densities of the corresponding tubulin bands (tubulin monoclonal antibody, 1:1000; Santa Cruz Biotechnology). Quantitative analysis of the Western blotting results was performed using professional image analysis software

(ImageJ, NIH, USA).

2.7. ELISA

The concentration of TGF-β in the medium was determined by a TGF beta-1 Human ELISA Kit (Cat. No. BMS249-4, Thermo Fisher Scientific) according to the manufacturer's instructions. The OD value at a wavelength of 450 nm was detected using a microplate reader (Tecan Trading AG, Switzerland).

2.8. ChIP-PCR

HeLa cells (2×10^6) were seeded in a 100-mm culture dish and treated with 1% formaldehyde to cross-link proteins to DNA. The cell lysates were sonicated to shear the DNA to sizes of 200–1000 bp. Equal aliquots of chromatin supernatants were separated and incubated with 1 μg of anti-Smad4 or anti-E2F antibodies or an anti-IgG antibody (negative control; Millipore) overnight at 4 ˚C with rotation. After the reverse cross-linking of protein/DNA complexes to free DNA, PCR was performed using specific primers according to the binding sequences in the TGF-β or miR-182 promoter regions predicted by software. Sequencing was used to validate the PCR products.

2.9. Luciferase assays

Total DNA was extracted from the HeLa cells and purified. The purified DNA was used as the template for real-time PCR with Ex Taq DNA polymerase. Agarose gel electrophoresis was performed, the gels were cut, and the PCR fragments of the E2F/Smad4 binding sites were extracted with a gel extraction kit (TaKaRa). The vector pGL4.17 [luc2/Neo] (Promega) and the genes obtained above were incubated in Buffer 3.1 (NEB) with NheI and XhoI. The purified DNA was incubated overnight with T4 DNA ligase. The ligation product was added to competent cells (*E. coli*). Then, we selected a stable clone of plasmid DNA and prepared a miniprep for sequencing. Cell transfections were carried out with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega). For each experimental condition, the luciferase activity was normalized to Renilla luciferase activity.

2.10. IHC

Clinical tumor tissues were frozen in OCT cryomolds (Sakura, Japan), sectioned (8 μm thickness) with a cryostat (Leica Corp., CRYOCUT 1800) at −20 ˚C and mounted onto glass slides (Sail Brand, Cat. No. 7105) at room temperature. The specimens were deparaffinized and hydrated in ethanol, and antigen retrieval was conducted in Tris/EDTA buffer (pH 9.0) for 20 min at 95 ˚C. The primary anti-TGF-β antibody was diluted in 1% BSA solution and incubated overnight at 4 ˚C, followed by incubation with the secondary antibody and streptavidin-horseradish peroxidase complex. 3,3-Diaminobenzidine tetrahydrochloride was used as the chromogen, and the slides were counterstained with hematoxylin.

2.11. ISH

LNA-modified oligonucleotide probes (Exiqon) were used to detect the spatial expression of hsa-miR-182 in cervical carcinoma. The sections were first deparaffinized with xylene 3 times for 5 min each. After the sections were washed individually with 100% ethanol (twice for 5 min), 70% ethanol (for 5 min), 50% ethanol (for 5 min), 25% ethanol (for 5 min), DEPC-treated H_2O (for 1 min) and PBS (twice for 5 min), they were deproteinated via proteinase K (3 μg/ml) digestion at 37 ˚C for 1 h. Then, the sections were washed once with 0.2% glycine in PBS for 30 s, twice with PBS for 30 s each and fixed in 10% formalin for 10 min. After 2 rinses in PBS, the sections were placed in hybridization buffer and incubated at room temperature for 2 h. Then, the sections were placed in 200 μl of hybridization mix (hybridization buffer containing 20 nM miRNA detection probe) and covered with Nescofilm in a humidified chamber at room temperature overnight. Then, the sections were rinsed twice in 2 × SSC (Invitrogen) at 37 ˚C, 3 times in 50% formamide with 2 × SSC at room temperature for 30 min and 5 times in PBST (PBS + 0.1% Tween 20) at room temperature for 5 min. After 1 h in blocking buffer (2 mg/ml BSA in PBST) at room temperature, the sections were incubated with the antibody (1:1000 anti-DIG-AP Fab fragments in antibody diluent (Zymed, Cat. No. 003218)) in a humidified chamber at 4 ˚C overnight. Subsequently, the sections were washed 5 times in PBST for 5 min each and 3 times in TBS for 5 min each. Then, the LNA-ISH staining was developed by a light-sensitive color reaction solution at 37 ˚C in a humidified chamber for 75 min. Then, the sections were washed 3 times in PBST for 5 min each. After air drying, the sections were mounted with Histomount. The LNA-ISH images were acquired under a microscope (LEICA).

2.12. Coculture experiments

HeLa cells and hCECs were cocultured in 24-well flat-bottom plates. HeLa cells were seeded on the bottom of the wells at a density of 5×10^4 cells/ml. hCECs (5×10^3) were seeded on the permeable membrane of 10-mm tissue culture inserts, which were placed in the fibroblast-containing wells. Cells were cultured in DMEM/F12 supplemented with 10% FCS depleted of steroids by dextran-charcoal treatment (FCDCS). E7 siRNA or vector was added to the HeLa cells. Twenty-four hours later, hCECs were collected for the assessment of TGF-β and miR-182 expression levels.

2.13. Statistical analysis

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data are expressed as the means ± SEMs, and differences between groups were analyzed by Student's *t*-test and one-way ANOVA or by the nonparametric Mann-Whitney test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. High-risk HPV E7 induces the overexpression of miR-182 via the regulation of TGF-β in vitro

3.1.1. High-risk HPV E7 upregulates TGF-β at mRNA level

To determine the effect of high-risk HPV E7 on the expression level of TGF-β, we manipulated the expression levels of E7 in cervical cancer cell lines. Upregulation of HPV E7 in cervical cell lines was achieved via transfection with E7 overexpression vectors, while downregulation of HPV E7 was performed by transfection with E7 siRNAs (see Supplemental Fig. 1). As shown in Fig. 1A–E, overexpression of high-risk HPV E7 (from HPV16, 18, 31, 52 and 58) significantly increased the expression levels of TGF-β mRNA, while knockdown of high-risk HPV E7 expression by siRNA transfection attenuated the enhancement effects of high-risk HPV E7 overexpression. Consistent with these findings, similar results were observed in HPV-negative human skin fibroblast (HSF) cells (Fig. 1F–J). However, neither overexpression nor knockdown of low-risk HPV E7 (from HPV6b and 11) affected the expression levels of TGF-β mRNA in C33A or HSF cells (Fig. 1K–N). Furthermore, in HPV-positive cell lines, including SiHa and HeLa, transfection with high-risk HPV E7 siRNA suppressed the expression of TGF-β mRNA (Fig. 1O–P). Collectively, these results indicated that high-risk HPV E7 promoted the expression of TGF-β in cervical cancer cells.

3.1.2. High-risk HPV E7 upregulates TGF-β at protein level

The quantitative real-time polymerase chain reaction (qRT-PCR)

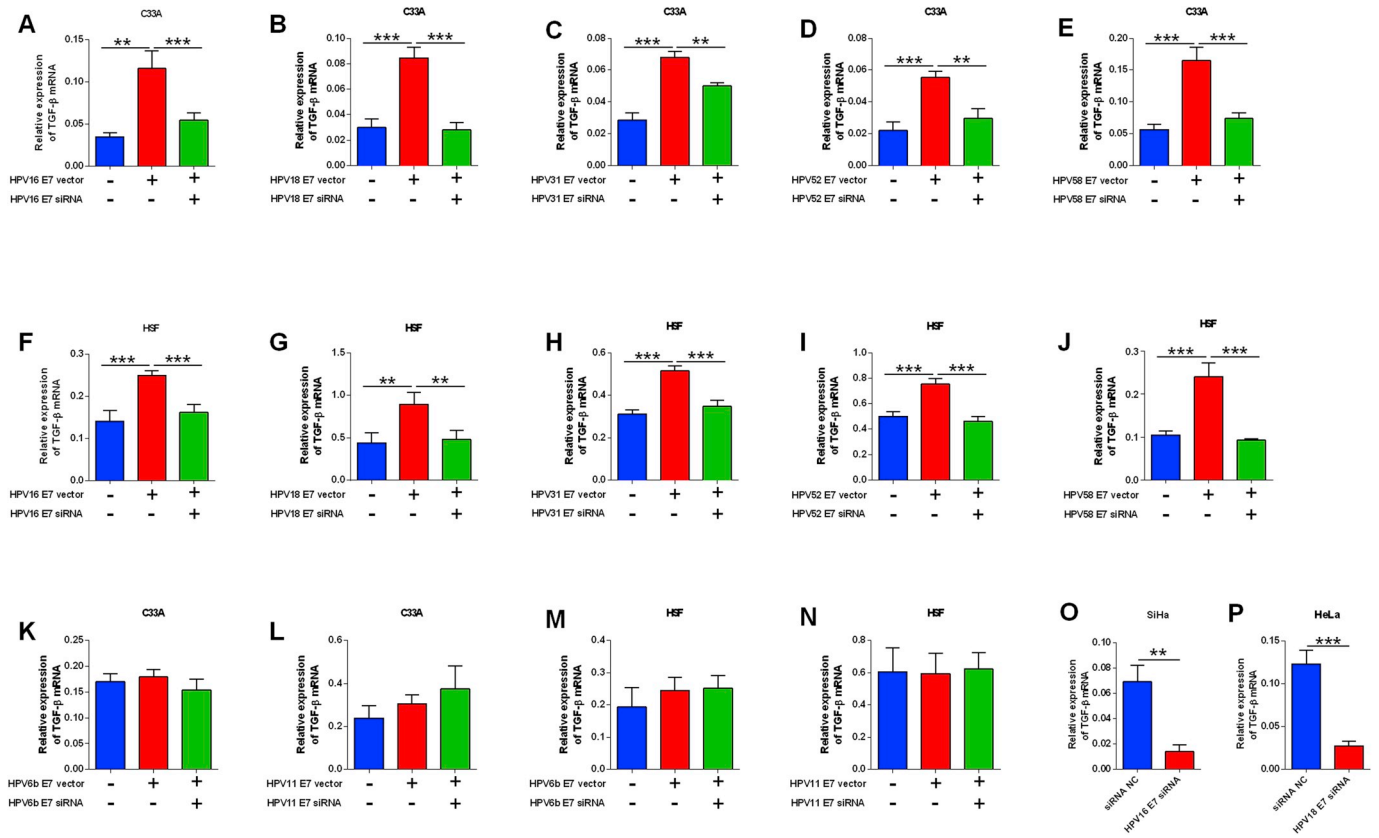

Fig. 1. High-risk HPV E7 upregulated TGF-β mRNA levels. A-E, qRT-PCR analysis of TGF-β mRNA levels in C33A cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and the corresponding siRNAs. **F-J,** qRT-PCR analysis of TGF-β mRNA levels in HSF cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and the corresponding siRNAs. **K-L,** qRT-PCR analysis of TGF-β mRNA levels in C33A cells after transfection with vectors expressing HPV6b and 11 E7 and the corresponding siRNAs. **M-N,** qRT-PCR analysis of TGF-β mRNA levels in HSF cells after transfection with vectors expressing HPV6b and 11 E7 and the corresponding siRNAs. **O,** qRT-PCR analysis of TGF-β mRNA levels in SiHa cells after transfection with HPV16 E7 siRNA. **P**, qRT-PCR analysis of TGF-β mRNA levels in HeLa cells after transfection with HPV18 E7 siRNA. N = 3 per group. Unpaired *t*-test or one-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

results were then further confirmed by Western blotting. HPV18 E7 induced a dramatic increase in the protein expression of TGF-β, which was restored by silencing either HPV18 E7 or TGF-β (Fig. 2A and B). HPV6b E7, however, failed to affect the TGF-β expression level (Fig. 2C and D). Because active TGF-β is released into the extracellular matrix, the protein levels of TGF-β in the cell culture medium were also assessed. At 48 h post transfection, the ELISA results showed that HPV18 E7 slightly increased the TGF-β protein levels in the medium and that this increase was impaired by transfection with HPV18 E7 siRNA or TGF-β siRNA (Fig. 2E and F).

3.1.3. High-risk HPV E7 upregulates miR-182 through TGF-β

Next, the effects of high-risk HPV E7 and TGF-β on the expression level of miR-182 were explored. Overexpression of high-risk HPV E7 markedly increased the expression level of miR-182, while knockdown of either high-risk HPV E7 or TGF-β attenuated the enhancement effects of high-risk HPV E7 overexpression (Fig. 3A–E). Consistent with these findings, similar results were observed in HSF cells (Fig. 3F–J). However, low-risk HPV E7 did not affect the expression levels of miR-182 in C33A and HSF cells (Fig. 3K–N). E7 silencing suppressed the expression of miR-182 in SiHa and HeLa cells, and TGF-β knockdown produced similar effects (Fig. 3O–P). Additionally, TGF-β protein overexpression notably increased the expression of miR-182 in these four cell lines (see supplemental Figure). These data indicated that high-risk HPV E7 promotes the expression of miR-182 through the upregulation of TGF-β expression in cervical cancer cells.

3.2. Molecular mechanism by which high-risk HPV E7 regulates TGF-β expression

3.2.1. Prediction of the binding of E2F and TGF-β promoter region

High-risk HPV E7 binds to the Rb protein and leads to pRb degradation, releasing E2F from the pRb-E2F complex. E2F controls the expression of S phase-specific genes and drives cells through the G1 phase into the S phase of the cell cycle [19]. By using the ConSite program, we identified E2F as a potential transcription factor for TGF-β (with a transcription factor score (TF score) cutoff value of 90%), with a binding sequence of TTTGCCGC on the sense strand.

3.2.2. The interaction between TGF-β and E2F

We first explored the link between E2F and TGF-β. The qRT-PCR results showed that silencing E2F attenuated the enhanced TGF-β expression induced by high-risk HPV E7 overexpression in C33A and HSF cells (Fig. 4A–J). In SiHa and HeLa cells, knockdown of E2F suppressed the expression of miR-182 (Fig. 4K–L). Chromatin from HeLa cells was immunoprecipitated using an antibody against E2F. Genomic DNA fragments bound to E2F were analyzed by PCR using random primers designed to include the predicted binding site (TTTGCCGC) in the TGF-β promoter region. The PCR bands suggested that E2F bound at the predicted site. Moreover, no genomic DNA was immunoprecipitated from the IgG controls, demonstrating that E2F indeed interacted with the TGF-β promoter. The binding sequence was further verified by sequencing (Fig. 4M − N). In addition, a luciferase reporter assay showed that transfection of HeLa cells with pGL4.17 [luc2/Neo] expressing the TGF-β promoter increased the relative luciferase activity (Fig. 4O).

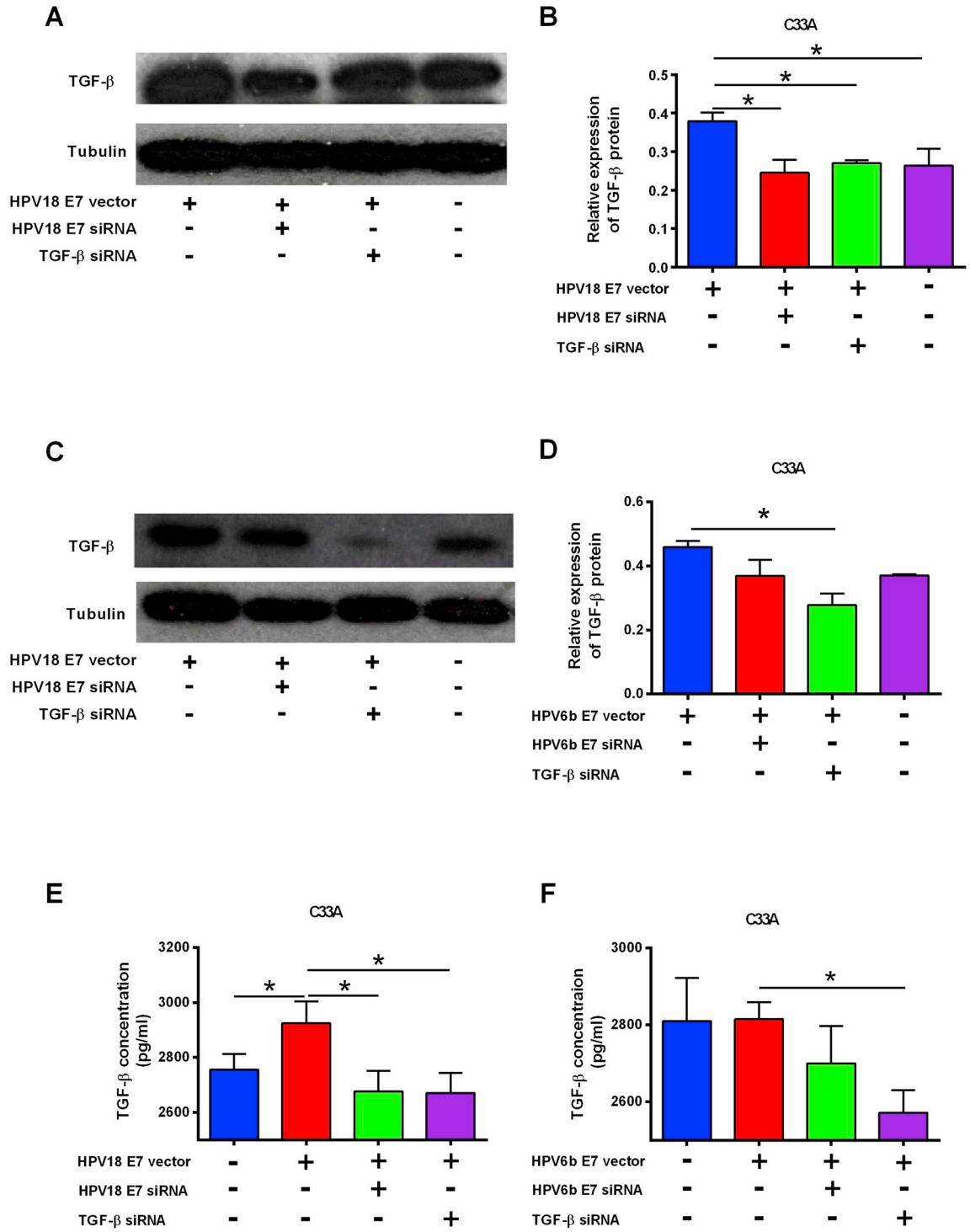

Fig. 2. HPV18 E7 upregulated TGF-β protein levels, while HPV6b E7 did not affect TGF-β protein levels. A-B, The bands and quantitative analysis of TGF-β protein expression levels in C33A cells after transfection with HPV18 E7 vector, HPV18 E7 siRNA and TGF-β siRNA and in control cells. **C-D**, The bands and quantitative analysis of TGF-β protein expression levels in C33A cells after transfection with HPV6b E7 vector, HPV6b E7 siRNA and TGF-β siRNA and in control cells. **E**, ELISA of TGF-β protein concentrations in C33A culture medium after transfection with HPV18 E7 vector, HPV18 E7 siRNA and TGF-β siRNA and in control cells. **F**, ELISA of TGF-β protein concentrations in C33A culture medium after transfection with HPV6b E7 vector, HPV6b E7 siRNA and TGF-β siRNA and in control cells. N = 3 per group. One-way ANOVA: $^{*}P < 0.05$.

However, cotransfection with pGL4.17 [luc2/Neo] expressing the TGF-β promoter and E2F siRNA significantly decreased the relative luciferase activity in HeLa cells compared with that in cells transfected with the nonsense siRNA (Fig. 4P). Taken together, these findings strongly supported the hypothesis that TGF-β expression is upregulated by high-risk HPV E7 through E2F.

3.3. Molecular mechanism by which TGF-β regulates miR-182 expression through Smad4

3.3.1. Prediction of the binding of Smad4 and miR-182 promoter region

The major signaling pathway of all TGF-β family members is activated through ligand binding to a cell-surface receptor complex and a

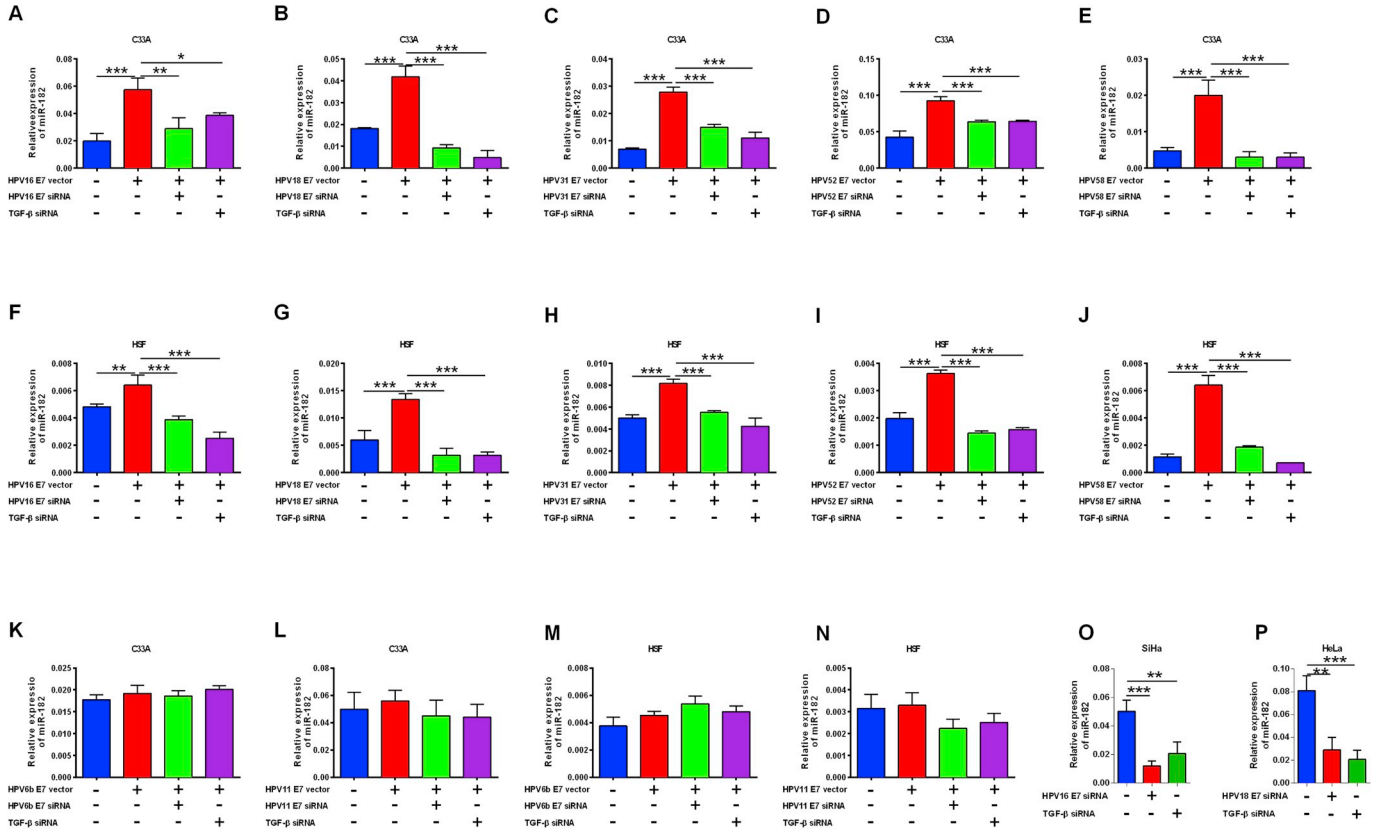

Fig. 3. High-risk HPV E7 upregulated miR-182 expression through TGF-β. A-E, qRT-PCR analysis of miR-182 levels in C33A cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and the corresponding siRNAs as well as with TGF-β siRNA. **F-J**, qRT-PCR analysis of miR-182 levels in HSF cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and the corresponding siRNAs as well as with TGF-β siRNA. **K-L**, qRT-PCR analysis of miR-182 levels in C33A cells after transfection with vectors expressing HPV6b and 11 E7 and the corresponding siRNAs as well as with TGF-β siRNA. **M-N**, qRT-PCR analysis of miR-182 levels in HSF cells after transfection with vectors expressing HPV6b and 11 E7 and the corresponding siRNAs as well as with TGF-β siRNA. **O**, qRT-PCR analysis of TGF-β mRNA levels in SiHa cells after transfection with HPV16 E7 siRNA as well as with TGF-β siRNA. **P**, qRT-PCR analysis of TGF-β mRNA levels in HeLa cells after transfection with HPV18 E7 siRNA as well as with TGF-β siRNA. N = 3 per group. One-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

group of intracellular signaling intermediates known as receptor-activated Smads (R-Smads). Phosphorylated Smads translocate to the nucleus, where they function as transcription factors. Smad4 is a central mediator in TGF-β superfamily signaling [20]. Based on the prediction of the PROMO or TFSEARCH programs, Smad4 was found to be a potential transcription factor for miR-182, with a binding sequence of GACCTCGC on the sense strand.

3.3.2. The interaction between Smad4 and miR-182

In addition, qRT-PCR, ChIP-seq and luciferase reporter assays were used to explore the interaction between Smad4 and miR-182. As shown in Fig. 5A-5L, knockdown of Smad4 via siRNA transfection downregulated miR-182 expression in cervical cancer cells and HSF cells expressing high-risk HPV E7 proteins. ChIP-seq results confirmed the binding between Smad4 and the promoter region of miR-182, with GACCTCGC as the binding site (Fig. 5M − N). The luciferase reporter assay showed that transfection with pGL4.17 [luc2/Neo] expressing the miR-182 promoter increased the luciferase activity in HeLa cells, and that cotransfection of this vector with Smad4 siRNA decreased the luciferase activity compared with that in the control group (Fig. 5O–P). In conclusion, these results demonstrated that TGF-β upregulates miR-182 expression through Smad4.

3.4. Validation of the high-risk HPV E7/TGF-β/miR-182 pathway in vivo

In the preliminary experiments, we found that HPV18 E7 siRNA treatment for three times with three days interval suppressed the *in vivo* tumor growth (see Supplementary Fig. 2). In this study, a nude mouse xenograft model was employed to validate the high-risk HPV E7/TGF-β/miR-182 pathway *in vivo*. Ten days after inoculation, tumors were treated with siRNA and were harvested for qRT-PCR 24 h after transfection. HPV18 E7 silencing downregulated the expression level of TGF-β; silencing either TGF-β or E2F had similar effects. However, knockdown of HPV18 E7, TGF-β or E2F caused a significant decrease in miR-182 expression levels (Fig. 6A and B). Thus, these *in vivo* data were consistent with the *in vitro* results.

3.5. Cervical cancer cells induce surrounding normal cells to express TGF-β and miR-182

3.5.1. Spatial expression of TGF-β and miR-182 in primary cervical carcinoma and adjacent normal cells

Furthermore, the spatial expression of TGF-β and miR-182 in primary cervical carcinoma and adjacent normal cells infected with high-risk HPV were examined. The TGF-β immunohistochemistry (IHC) signal is represented by the brown color, and the hsa-miR-182 locked nucleic acid *in situ* hybridization (LNA-ISH) signal is represented by the purple color. The immunohistochemical staining for TGF-β was strongly positive in the region of the carcinoma, in contrast to the markedly less-intense staining in the adjacent normal stromal (20 ×). Similarly, at the same magnification (20 ×), LNA-ISH showed stronger positive staining for miR-182 in the carcinoma than in the adjacent normal stromal (Fig. 6C and D). These results indicated that the expression levels of TGF-β and miR-182 are elevated in tumor cells.

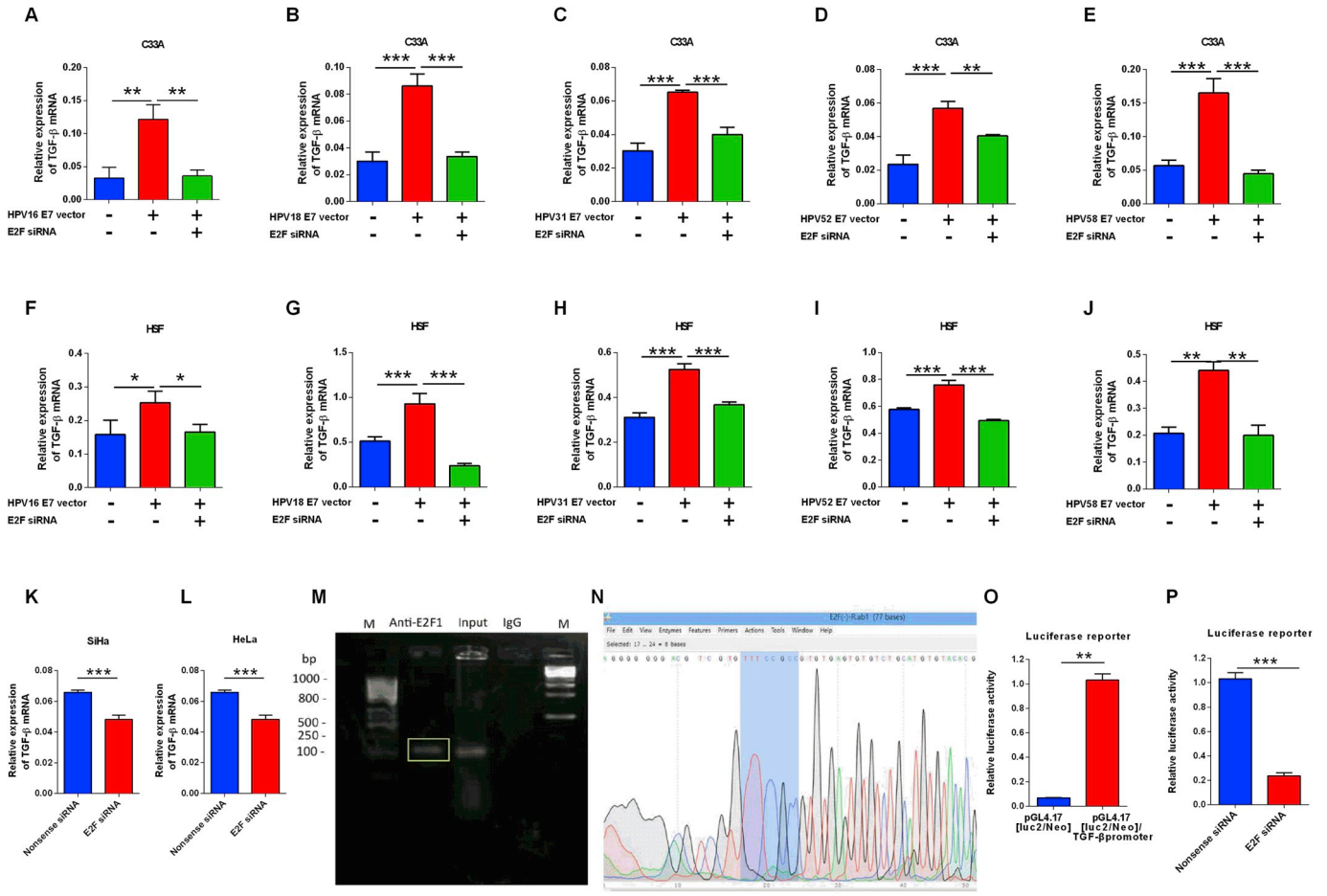

Fig. 4. Mechanism by which high-risk HPV E7 regulates TGF-β expression. A-E, qRT-PCR analysis of TGF-β mRNA levels in C33A cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and E2F siRNA. **F-J**, qRT-PCR analysis of TGF-β mRNA in HSF cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and E2F siRNA. **K**, qRT-PCR analysis of TGF-β mRNA levels in SiHa cells after transfection with E2F siRNA. **L**, qRT-PCR analysis of TGF-β mRNA levels in HeLa cells after transfection with E2F siRNA. **M-N**, ChIP-PCR fragment containing the E2F binding site in the TGF-β promoter region and the sequencing result for the binding site. **O**, HeLa cells were transfected with the luciferase reporter vector pGL4.17 [luc2/Neo] containing the TGF-β promoter sequence, which increased the luciferase activity compared with that in cells transfected with the control pGL4.17 [luc2/Neo] vector. **P**, HeLa cells were cotransfected with the luciferase reporter vector pGL4.17 [luc2/Neo] containing the TGF-β promoter and with E2F siRNA, which decreased the luciferase activity compared with that in cells transfected with nonsense siRNA. N = 3 per group. One-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5.2. Cervical cancer cells induce surrounding normal cells to express TGF-β and miR-182

Previously, we observed that miR-182 was predominantly localized in the cytoplasm and elevated in cervical carcinoma regions compared with its expression in the normal cervical epithelium [10]. In this study, the cervical epithelium adjacent to the carcinoma exhibited a stronger positive signal than the epithelium distant from the carcinoma region (Fig. 6E and F). To address this distance-dependent effect, a coculture system was used. The expression levels of TGF-β and miR-182 in human cervical epithelial cells (hCECs) cocultured with HeLa cells were higher than those in hCECs cocultured with hCECs. Moreover, in the HeLa-hCEC coculture model, E7 silencing in HeLa cells induced a decrease in the TGF-β and miR-182 levels in hCECs. In the hCEC-hCEC coculture system, however, enhanced E7 expression exhibited only a non-significant trend toward increasing the TGF-β/miR-182 expression levels (Fig. 6G and H). These data indicated that cervical cancer cells may induce increased expression of cytokines or oncomiRs in surrounding normal cells.

4. Discussion

We recently reported two consistently upregulated miRNAs in cervical cancer, miR-182 and miR-183 [10]. The sequences of these miRs are located in the same genomic cluster and have been reported to be upregulated in other types of cancers and tissues [21–24]. We discovered that miR-182 was aberrantly expressed in cervical cancer tissue and functioned as an oncomiRNA. Moreover, miR-182 alteration is associated with cervical cancer pathogenesis by disrupting cell proliferation through targeting FOXO1 [10]. However, whether HPV interacts with miR-182 and the mechanism by which this virus regulates miR-182 expression remain unknown. In a later study, we perform microarray analyses with clinical samples and found that TGF-β was amplified and overexpressed in the cervical cancer group [15], suggesting that TGF-β may be a key player in the regulation of miRNA by HPV. Thus, in this study, we elucidated the interplay between high-risk HPV E7 and miR-182 in cervical cancer and discovered that high-risk HPV E7 upregulated miR-182 expression through the TGF-β/Smad4 pathway.

HPVs can be grouped into high-risk and low-risk subtypes based on their association with cervical cancer and precursor lesions. HPV 16, 18, 31, 52 and 58 are the most prevalent subtypes in China, and HPV 16 and 18 are responsible for approximately 70% of all cases [25]. As a viral oncoprotein, high-risk HPV E7 binds to the tumor suppressor protein pRb with high affinity, while low-risk HPV E7 exhibits a low binding affinity [26]. First, we examined the effects of high-risk HPV E7 and low-risk HPV E7 on the expression levels of TGF-β and miR-182. As

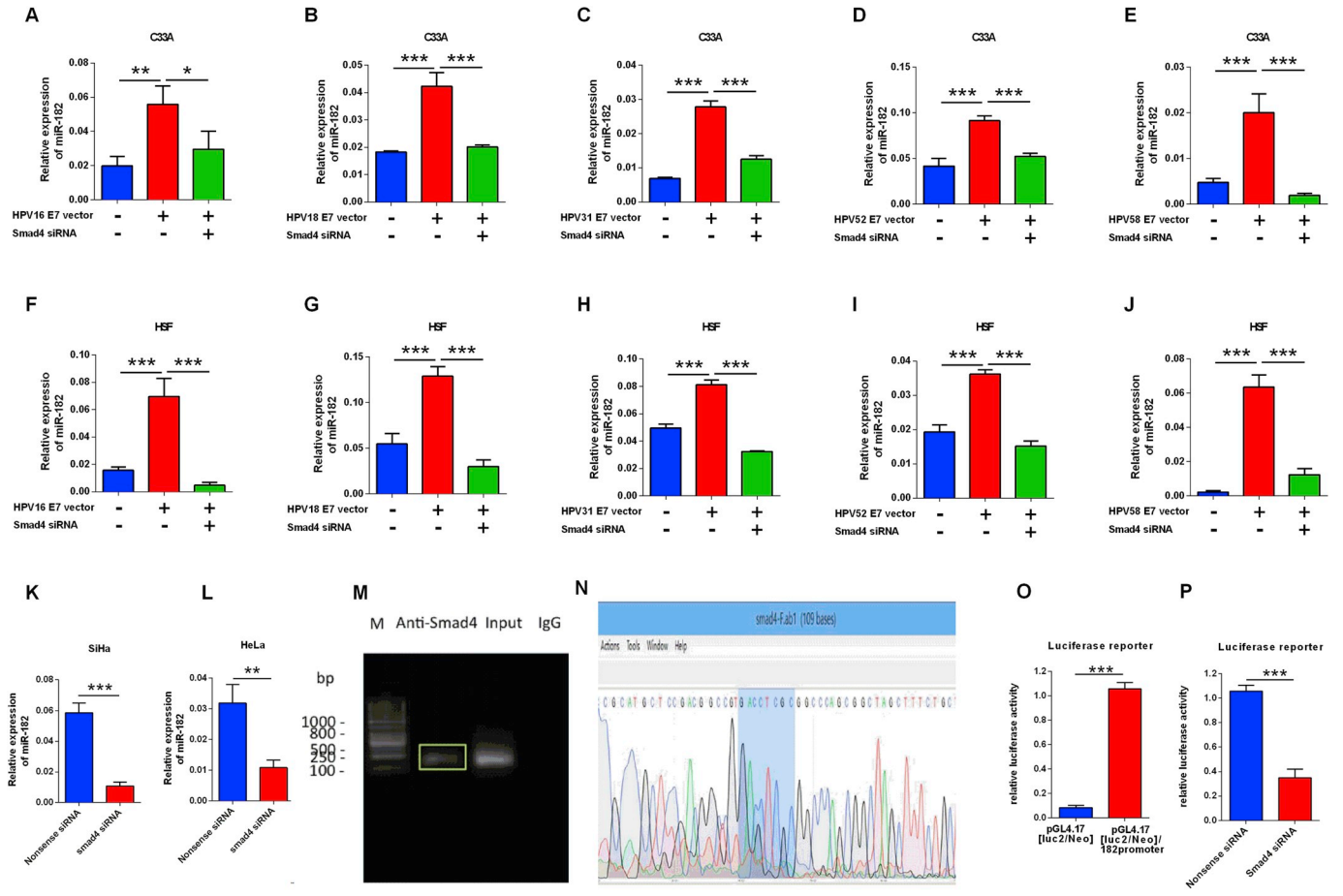

Fig. 5. Mechanism by which TGF-β regulates miR-182 expression. A-E, qRT-PCR analysis of miR-182 levels in C33A cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and Smad4 siRNA. **F-J,** qRT-PCR analysis of miR-182 in HSF cells after transfection with vectors expressing HPV16, 18, 31, 52, and 58 E7 and E2F siRNA. **K,** qRT-PCR analysis of TGF-β mRNA levels in SiHa cells after transfection with E2F siRNA. **L,** qRT-PCR analysis of TGF-β mRNA levels in HeLa cells after transfection with E2F siRNA. **M-N,** ChIP-PCR fragment containing the E2F binding site in the TGF-β promoter region and the sequencing result for the binding site. **O,** HeLa cells were transfected with the luciferase reporter vector pGL4.17 [luc2/Neo] containing the miR-182 promoter, which increased the luciferase activity compared with that in cells transfected with the control pGL4.17 [luc2/Neo] vector. **P,** HeLa cells were cotransfected with the luciferase reporter vector pGL4.17 [luc2/Neo] containing the miR-182 promoter and with Smad4 siRNA, which decreased the luciferase activity compared with that in cells transfected with nonsense siRNA. N = 3 per group. One-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expected, only high-risk E7 significantly increased the expression levels of TGF-β and miR-182; low-risk E7 failed to affect the expression of TGF-β and miR-182. Additionally, knockdown and overexpression of TGF-β affected miR-182 expression. Next, the molecular mechanism by which high-risk E7 regulates miR-182 through the TGF-β pathway was investigated.

TGF-β signaling has been shown to play multiple important roles in cancer progression [27]. In normal cells, TGF-β induces cell cycle arrest at G1 phase to suppress cell proliferation and induce cell differentiation or apoptosis; in cancer cells, components of the TGF-β signaling pathway are mutated or exhibit epigenetic event-induced expression loss [18]. The proliferation of cancer cells and surrounding stromal cells (fibroblasts) induces increased production of TGF-β, which in turn acts on the surrounding stromal cells, immune cells, endothelial and smooth muscle cells, causing immune suppression and angiogenesis and increasing the invasiveness of the cancer. TGF-β acts as a tumor suppressor in early stages but plays an oncogenic role in advanced stages and multiple miRNAs contribute to these processes in relation to TGF-β signaling [28]. In a study by Xu, a higher level of TGF-β1 was observed in E7-positive squamous cancer cases than in E7-negative cases [17]. However, the mechanism underlying this finding remains unknown. Here, we started by predicting the transcription factors of TGF-β and found that E2F was a potential transcription factor with a high score. Importantly, pRb binds to E2F, preventing E2F from interacting with the cell's transcription machinery, and E7 binds to the pRb protein and leads to pRb degradation and thus E2F release [19]. We hypothesized that high-risk HPV E7 regulates TGF-β through E2F, and our qRT-PCR results showed the link between E2F and TGF-β. ChIP-PCR and luciferase assays confirmed the binding of the E2F protein to the TGF-β promoter region. The downstream events involve TGF-β and miR-182. In the TGF-β signaling pathway, Smad proteins mediate the maturation of a subset of miRNAs or regulate the transcription of miRNAs with a SMAD-binding element in the promoter [29]. Based on the prediction of an online program, Smad4 is a potential transcription factor for miR-182. qRT-PCR was then used to explore the relationship between Smad4 and miR-182. ChIP-PCR and luciferase assays confirmed the presence of the Smad4-binding element in the miR-182 promoter region. Collectively, our findings indicated that the complete mechanism operates by the binding of high-risk HPV E7 to pRB and the release of E2F from the complex; E2F then binds to the promoter region of TGF-β and induces the overexpression of TGF-β. With the secretion of TGF-β to the extracellular space, the TGF-β/Smad pathway is activated, and Smad4 binds to the promoter region of miR-182 to upregulate miR-182 expression (Fig. 7). Furthermore, we used a mouse xenograft model to validate the high-risk HPV E7/TGF-β/miR-182 pathway *in vivo*.

In addition, the primary cervical carcinoma staining and coculture results raised a new uncertainty: cancer cells operate intercellularly to drive cancer development; indeed, we observed that cervical cancer

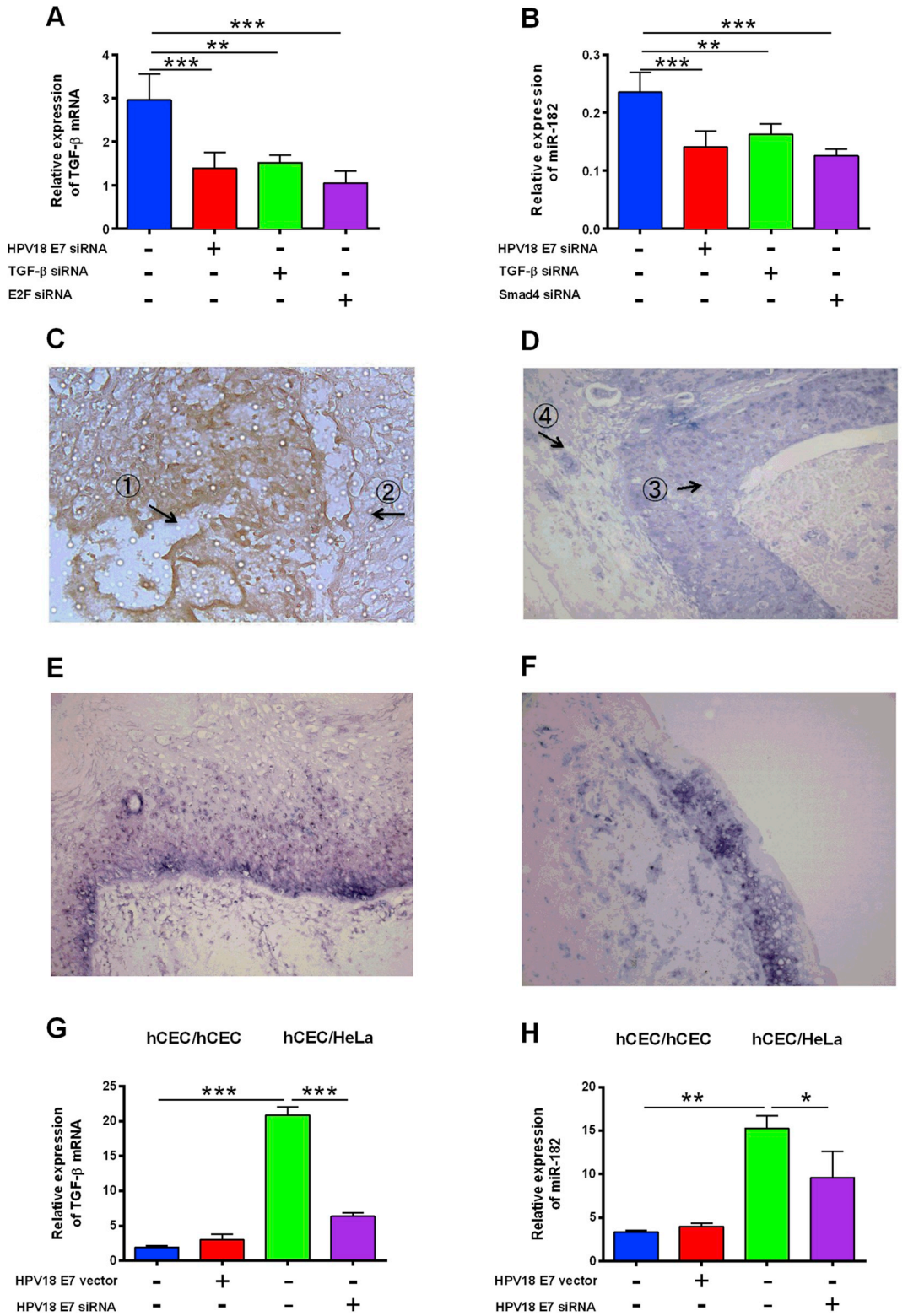

Fig. 6. Validation of the high-risk HPV E7/TGF-β/miR-182 pathway *in vivo* and cervical cancer cells induce surrounding normal cells. A, qRT-PCR analysis of TGF-β mRNA levels in HeLa xenografts after injection with HPV18 E7 siRNA, TGF-β siRNA and E2F siRNA. **B**, qRT-PCR analysis of miR-182 levels in HeLa xenografts after injection with HPV18 E7 siRNA, TGF-β siRNA and Smad4 siRNA. N = 4 per group. **C**, Representative image of IHC for TGF-β in a cervical carcinoma sample. ① indicates tumor cells and ② indicates stromal cells (20carc**D**, Representative image of LNA-ISH for miR-182 in a cervical carcinoma sample. ③ indicates tumor cells and ④ indicates stromal cells (20 ×). **E-F**, The cervical epithelium adjacent to the carcinoma exhibited a stronger positive signal than the epithelium distant from the carcinoma region. **G**, qRT-PCR analysis of TGF-β mRNA levels in hCECs cocultured with hCECs transfected with the HPV18 E7 vector or HeLa cells transfected with HPV18 E7 siRNA. **H**, qRT-PCR analysis of miR-182 levels in hCECs cocultured with hCECs transfected with the HPV18 E7 vector or HeLa cells transfected with HPV18 E7 siRNA). N = 3 per group. One-way ANOVA: $^{**}P < 0.01$, $^{***}P < 0.001$.

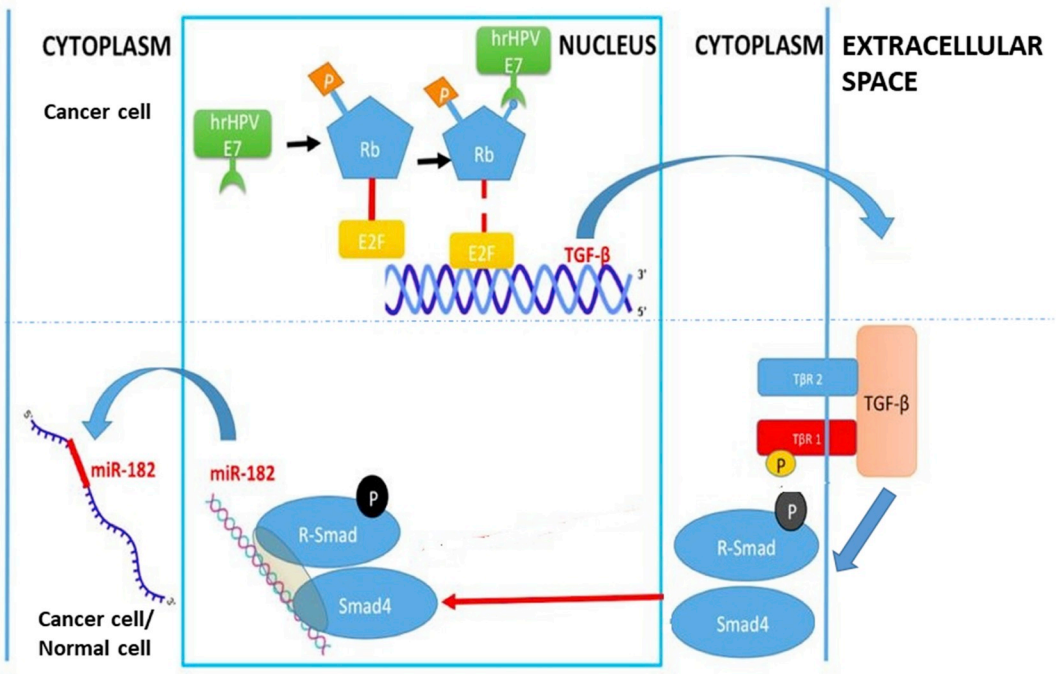

Fig. 7. Schematic of the E7/TGF-β/miR-182 pathway in cervical cancer. High-risk HPV E7 binds to pRB. E2F is released from the complex and binds to the promoter region of TGF-β, inducing the overexpression of TGF-β. With the secretion of TGF-β to the extracellular space, the TGF-β/Smad pathway is activated, and Smad4 binds to the promoter region of miR-182 to upregulate miR-182 expression.

cells induced surrounding normal cells to express higher levels of TGF-β and miR-182. The exact mechanism is unknown, but high-risk HPV genes may reprogram or alter gene expression in the nearby stroma. Thus, the cancer microenvironment and the feedback pathway activity or immune escape caused by TGF-β signaling is worthy of investigation.

The strength of this study is that we have included five high-risk and two low-risk HPV subtypes, which is comprehensive. In addition, study about the interaction between HPV and miRNA in cervical cancer development is scant and this may be the first one. However, this project has remaining weaknesses. Although we documented the functional role and regulatory mechanisms of miR-182 in cervical cancer cells found in the previous [10] and present studies, the scientific importance of the regulatory network in cells surrounding regions of cervical cancer is still unclear, and future studies should investigate this topic to further uncover the effects of HPV oncogenes on the cervical cancer microenvironment. Furthermore, high-risk HPVs are also responsible for anal, oropharyngeal and vaginal cancers [30], and we are curious whether this pathway functions in these other types of cancers.

Finally, we must mention the therapeutic and prognostic value of the E7/TGF-β/miR-182 pathway. Previously, we surveyed miR-182 expression in 17 primary cervical carcinoma and 8 normal cervical epithelium samples from age-matched controls. This analysis confirmed the significant upregulation of miR-182 expression in cervical carcinoma. Moreover, the association analysis results suggested that increased expression of miR-182 is correlated with advanced stages of primary cervical carcinoma. In a mouse xenograft model, inhibition of miR-182 reduced the size of HeLa xenograft tumors in mice [10]. Nevertheless, miR-182 expression has been reported to be upregulated in epithelial ovarian cancer, melanoma and breast tumor cells [31–33]. In glioma, miR-182 can be used as a prognostic marker for progression and patient survival [34]. Considering the results of these studies, miR-182 could be a new and independent prognostic indicator for evaluating the clinical outcomes of cancer patients, and miR-182 inhibition might be a target for gene therapy. In addition, understanding TGF-β/Smad signaling is paramount in cancer treatment. However, as this pathway plays the opposite role in cancer progression, its application will be challenging. The last but most important factor is high-risk HPV E7. Cervical cancer is mainly caused by HPV, and the HPV oncogene is exogenous. Therefore, siRNA-mediated knockdown of the E7 gene may represent a more suitable and safer strategy for the management of cervical cancer than commonly used radiation or chemoradiation therapy. Indeed, we developed several strategies for both the systemic [35] and local delivery of siRNAs that exhibited good safety profiles in several animal models [36]. Therefore, targeting HPV E7, other HPV oncogenes or even human oncogenes may represent a good strategy for the treatment of cervical cancer or cervical intraepithelial neoplasia.

In conclusion, we demonstrated that high-risk HPV E7 upregulates miR-182 expression through the TGF-β/Smad4 pathway. Understanding the precise mechanism in cervical cancer will not only advance the knowledge of cervical cancer progression but also allow the development of specific and effective therapeutic strategies or biomarkers for predicting clinical outcomes.

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Conflicts of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.06.015.

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