

E6-regulated overproduction of prostaglandin E2 may inhibit migration of dendritic cells in human papillomavirus 16-positive cervical lesions

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Abstract. Continuous human papillomavirus (HPV) infection is a critical cause of cervical lesions; however, the specific mechanism is currently not clear. E6 is one of the most important oncoproteins associated with HPV, which regulates synthases in the production of prostaglandin E2 (PGE₂). Notably, PGE₂ has been reported to be upregulated in cervical lesions. An insufficient number of mature dendritic cells (DCs), which is unable to cause an effective immune response, is an important cause of cervical lesions. Therefore, this study explored the possible causes of HPV16-positive cervical lesions by identifying the relationship between E6, PGE₂ and DCs. Firstly, the distribution and status of DCs in clinical biopsy specimens and animal models were analyzed with immunohistochemistry and flow cytometry, which demonstrated that the migratory ability of DCs was inhibited in HPV16-positive cervical lesions. Furthermore, using immunohistochemistry, western blotting and ELISA, it was revealed that as the degree of cervical lesions increased, the expression of PGE₂ and its synthases increased. Subsequently, as determined using Transwell and 3D migration assays, it was revealed that a high concentration of PGE₂ inhibited the migration of DCs, which may explain the phenomenon observed in cervical lesions. Notably, E6 was identified to regulate PGE₂ expression. The *in vivo* experiments indicated that E6 may increase the expression levels of PGE₂ in cervical lesions, which could eventually

induce inhibition of the migration of DCs. In conclusion, the present study suggested that E6 regulated overproduction of PGE₂, which may induce inhibition of DC migration in HPV16-positive cervical lesions.

Introduction

Dendritic cells (DCs) serve a critical role in the immune system, and initiate and control the immune response. The main function of DCs is to detect foreign antigens and present them to T cells. After being activated by injury or inflammatory stimuli, DCs migrate to draining lymph nodes and activate T cells to differentiate, thus initiating the immune response (1,2). Therefore, the migration of DCs to draining lymph nodes is essential in mediating anti-tumor immunity. According to previous reports, the number of CD83-positive DCs, which present foreign antigens, is increased in cervical intraepithelial neoplasia (3,4) and significantly decreased in draining lymph nodes (5). These changes in DCs may contribute to the immune evasion of tumor cells. Therefore, it is important to elucidate the mechanisms underlying this phenomenon.

As a cyclooxygenase (COX)-induced product of arachidonic acid released from membrane phospholipids, prostaglandin E2 (PGE₂) modulates various pathological and physiological processes. Numerous studies have demonstrated that PGE₂ is closely associated with the development of various malignant lesions (6-8). Furthermore, in cervical lesions, PGE₂ expression is higher compared with in normal tissues (9-11). Additionally, certain studies have revealed that PGE₂ modulates the migration of DCs (12,13). Therefore, PGE₂ may be associated with changes in DCs in cervical lesions.

The development of cervical lesions has been linked to infection with certain high-risk types of human papillomavirus (HPV) (14). The most prevalent type is HPV16, which accounts for >50% of cervical malignancy cases. The constitutive expression of E6 oncoprotein is one of the major risk factors for the development of high-grade lesions, which is required for the onset and maintenance of the malignant phenotype (15). Notably, E6 has been reported to induce decreased migration of Langerhans cells and their precursor-like cells,

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one type of which is DCs (16,17). Furthermore, the transcription of COX-2 has been reported to be regulated by E6 (18); the COX pathway is associated with the production of bioactive prostanoids, including PGE₂. Therefore, the production of PGE₂, which affects the migration of DCs in cervical lesions, may be regulated by E6.

The present study used murine bone marrow DCs to determine the effects of PGE₂ on the migration of DCs, which may be associated with changes to DCs in cervical lesions. Furthermore, the association between E6 oncoprotein and the upregulated expression of PGE₂ in HPV16-positive cervical lesions was investigated. The results obtained in the present study may improve the understanding of immune evasion in cervical lesions. Notably, the biological implications of these findings may provide a novel perspective in the immunological surveillance of various malignant lesions.

Materials and methods

Samples. Cervical biopsy specimens (age range, 20–80 years) were collected between January 2012 and December 2017 from the Department of Obstetrics and Gynecology, Daping Hospital, Army Medical University (Third Military Medical University; Chongqing, China). Only HPV16-positive samples were selected from samples obtained for clinical HPV typing for subsequent experiments. According to the cytological and histological evaluation of fresh specimens, cervical disease status was categorized as normal squamous epithelium (n=27), low-grade squamous intraepithelial lesion (LSIL; n=25), high-grade squamous intraepithelial lesion (HSIL; n=21) and squamous carcinoma (SCC; n=15). The present study was approved by the Ethics Committee of Daping Hospital, Army Medical University (Third Military Medical University). The participants provided written informed consent, and this study was conducted in accordance with the Declaration of Helsinki.

Cell culture. All animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University. DCs were isolated from mouse bone marrow as previously described with slight modifications (19,20). Balb/C mice (age, 6–8 weeks; weight, 20–25 g; n=80) were provided by the Experimental Animal Center of Daping Hospital. Mice were maintained in a specific pathogen-free environment at 22±2°C with 55±5% humidity under a 12-h light/dark cycle. Food and water were provided *ad libitum*. Briefly, the mice were sacrificed by cervical dislocation, the femurs and tibias were collected, bone marrow cells were flushed out with a 1-ml syringe and the red cells were removed using erythrocyte lysis fluid (Beyotime Institute of Biotechnology). The remaining cells were cultured with RPMI-1640 medium supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; Peprotech, Inc.) and IL-4 (Peprotech, Inc.). On day 3, non-adherent granulocytes, as well as B and T lymphocytes, were gently removed and fresh media were added. The immature DCs were collected on day 6. On day 7, the DCs were stimulated with 1 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) for 24 h at 37°C in an atmosphere containing 5% CO₂. Mature DCs were collected on

day 8. Morphological and phenotypic tests were performed using microscopy and flow cytometry to confirm the successful isolation of DCs. Based on Baratelli *et al* (21), PGE₂ (Sigma-Aldrich; Merck KGaA) was added to the culture media of DCs at a final concentration of 0, 5 or 10 µg/ml for 24 h.

Immunohistochemistry. Cervical biopsy specimens were sequentially fixed in 4% paraformaldehyde for ≥24 h at room temperature, dehydrated and embedded in paraffin, then cut into 7-µm sections. After dewaxing and rehydration, heat-mediated antigen retrieval was performed with 1 mM EDTA (pH 9.0) in a pressure boiler for 10 min. Microsomal PGE synthase (mPGES)-1 (1:100; cat. no. 160140; Cayman Chemical Company), mPGES-2 (1:100; cat. no. 160145; Cayman Chemical Company) and cytosolic PGE synthase (cPGES) (1:100; cat. no. 18219; Cayman Chemical Company), CD83 (1:50; cat. no. bs-4826R; BISS) and CD1a (prediluted; 1 drop; cat. no. ZA-0544; OriGene Technologies, Inc.) were incubated with the sections overnight at 4°C. After washing with PBS (pH 7.4), sections were incubated with appropriate secondary antibodies (cat. no. PV-9000; OriGene Technologies, Inc.) for 20 min at room temperature, according to the manufacturer's protocol. Color reaction was developed by incubation with the DAB detection system (cat. no. ZLI-9019; OriGene Technologies, Inc.) for 10 sec at room temperature, and the sections were counterstained with hematoxylin for min at room temperature. After sequential dehydration using graded ethanol and xylene, the sections were mounted and covered with a coverslip. The sections were observed under a light microscope (CTR6000; Leica Microsystems, Inc.). The images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Western blot analysis. Western blotting was performed to determine protein expression levels. The lysates of DCs and tissue samples preserved in liquid nitrogen were prepared using lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology). The concentration in all samples was measured using the BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.), and the protein samples (30–50 µg) were separated by SDS-PAGE on 10% gels. Proteins were then transferred to nitrocellulose membranes and blocked in PBS (containing 5% BSA and 0.1% Tween-20; both from BBI Life Sciences Corporation). Subsequently, blots were incubated with antibodies against mPGES-1 (1:200; cat. no. 160140; Cayman Chemical Company), mPGES-2 (1:200; cat. no. 160145; Cayman Chemical Company) and cPGES (1:1,000; cat. no. 18219; Cayman Chemical Company), E6 (1:200; cat. no. sc-1584; Santa Cruz Biotechnology, Inc.), tubulin (1:1,000; cat. no. 11224-1-AP; ProteinTech Group, Inc.), or GAPDH (1:2,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc.) overnight at 4°C. Following incubation with horseradish peroxidase-conjugated goat anti-mouse (1:1,000; cat. no. SA00001-1; ProteinTech Group, Inc.), goat anti-rabbit (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) or donkey anti-goat (1:1,000; cat. no. A0181; Beyotime Institute of Biotechnology) secondary antibody at room temperature for 1 h, immunoreactivities were detected using enhanced chemiluminescence substrate (cat. no. 34577; Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using

Table I. Nucleotide sequences of the primers used for reverse transcription-PCR.

Gene	Sense primer	Antisense primer
HPV16 E6	5'-AATGTTTCAGGACCCACAGG-3'	5'-ACTGTTGCTTGCAGTACACACAT-3'
GAPDH	5'-ATCAAGAAGGTGGTGAAGCAG-3'	5'-GCCAAATTCGTTGTACATACC-3'

HPV16, human papillomavirus 16.

ImageJ software (Image J bundled with 64-bit Java 1.6.0_20; National Institutes of Health) with protein expression levels normalized to tubulin or GAPDH.

Reverse transcription-PCR analysis. Total RNA was extracted from tissue samples preserved in liquid nitrogen and DCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized using HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper) (cat. no. R233-01; Vazyme), following the manufacturer's protocol. PCR was conducted using 2X Taq Plus Master Mix II (Dye Plus) (cat. no. P213-01, Vazyme). Semi-quantitative PCR was conducted as follows: Initial denaturation at 95°C for 3 min, followed by 26 cycles at 95°C for 15 sec, 59°C for 30 sec and 72°C for 30 sec. The primers used for PCR are listed in Table I. Electrophoresis was performed using 1% agarose gel and 4S GelRed (1:10,000; BBI Life Sciences Corporation), and the images were captured using ChemiDOC XRS system (Bio-Rad Laboratories, Inc.). Densitometric analysis was performed using ImageJ software (Image J bundled with 64-bit Java 1.6.0_20; National Institutes of Health) with mRNA expression levels normalized to GAPDH.

Cell transfection. The double-stranded E6-specific small interfering RNA (siRNA) sequences and control siRNA were prepared and annealed by Neuron Biotech Co. [OBiO Technology (Shanghai) Corp., Ltd.]. Two sequences were used for further experiments: siRNA-1, 5'-GCAATGTTTCAGGACCCACAG-3'; siRNA-2, 5'-GCACAGAGCTGCAAACAACTA-3'. SiHa (5x10⁵ cells/well; Cell Bank of the Chinese Academy of Sciences) were seeded in 6-well plates and transfected with 100 nmol/l siRNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h. Subsequently, the medium was replaced with DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS for a further 24 h, and cells were subsequently used for other experiments.

Transwell migration assay. The lower chambers of 24-well Transwell plates (Merck KGaA) were filled with 600 µl serum-free medium containing C-C motif chemokine ligand 19 (CCL19; 100 ng/ml; Peprotech, Inc.). Following treatment with different concentrations of PGE₂ (0, 5 and 10 µg/ml), cultured supernatants of SiHa cells (positive control) or cultured supernatants of siRNA-transfected SiHa cells at 37°C for 24 h, DCs (1x10⁵ cells/well) resuspended in 100 µl serum-free medium were deposited in the upper chambers of the Transwell plates and allowed to migrate for 3 h at 37°C in an atmosphere

containing 5% CO₂. Since there were almost no residual cells on the chamber membrane, the method of counting cells in the lower compartment was used, instead of image acquisition. The number of migrated DCs harvested from the lower chambers was counted using a hemocytometer.

3D migration assay. DCs (45 µl; 0.45x10⁶ cells/ml) were embedded into a collagen matrix (final concentration of collagen matrix, 1.7 mg/ml; BD Biosciences) in migration chambers (Electron Microscopy Sciences). The remaining space of the chambers was filled with medium containing 200 ng/ml CCL19. Migration of DCs was recorded by bright-field time-lapse video microscopy at 37°C using an inverted microscope (Carl Zeiss AG) fitted with x10 objectives and AxioCam cameras; recording started 10 min after injection. Cells were imaged at a frame rate of 2 min up to 61 frames. Computer-assisted cell tracking was performed with custom-written software (ImageJ bundled with 64-bit Java 1.6.0_20; National Institutes of Health). The average speed was calculated as step length (µm) per minute for each cell. A total of 30 randomly selected cells were included in one experiment.

Flow cytometry. Cultured DCs were harvested and their phenotypes evaluated by fluorescence-activated cell sorting (FACS) analysis. Cells were blocked for 15 min at 4°C with PBS containing 0.5% BSA, and were then incubated with respective antibodies for 30 min at 4°C. After being washed twice with PBS, the cells were resuspended in 200 µl PBS. The antibodies included phycoerythrin-conjugated anti-mouse CD40 (1:100; cat. no. 12-0401; eBioscience; Thermo Fisher Scientific, Inc.), CD80 (1:100; cat. no. 12-0801; eBioscience; Thermo Fisher Scientific, Inc.), CD86 (1:100; cat. no. 12-0862; eBioscience; Thermo Fisher Scientific, Inc.), major histocompatibility complex II (MHCII; 1:100; cat. no. 12-5321; eBioscience; Thermo Fisher Scientific, Inc.) and C-C chemokine receptor type 7 (CCR7; 1:100; cat. no. 12-1971; eBioscience; Thermo Fisher Scientific, Inc.). Same-species and same-isotype IgG (1:100, cat. no. 12-4031; 1:100, cat. no. 12-4888; 1:100, cat. no. 12-4321; eBioscience; Thermo Fisher Scientific, Inc.) was used as an isotype control, which was used as the template to conduct gating. FACS analysis was performed on a FACSCalibur flow cytometer using CellQuest Pro software (version 6.0; BD Bioscience).

PGE₂ measurement. PGE₂ concentration in cervical biopsy specimens and animal tumor tissues was determined using the PGE₂-enzyme immunoassay kit (cat. no. 500141; Cayman Chemical Company), according to the manufacturer's protocol.

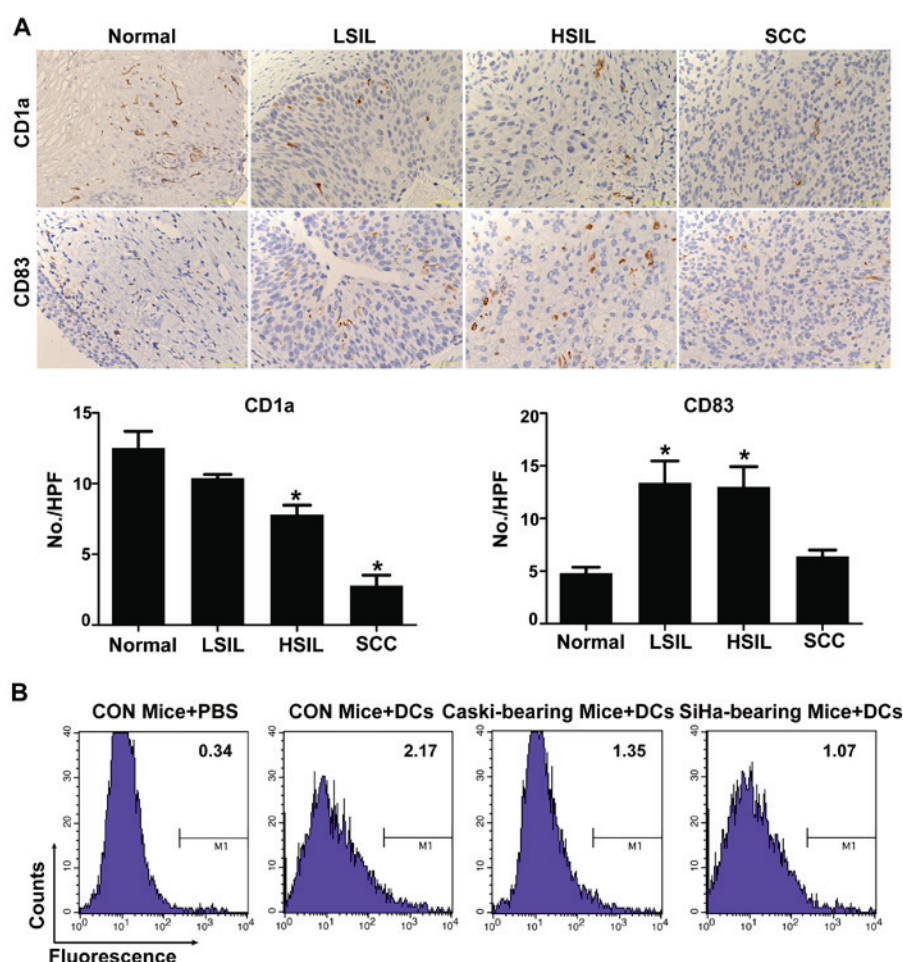


Figure 1. Expression of DCs in human papillomavirus 16-positive cervical lesions. (A) CD1a and CD83 immunostaining in cervical biopsy specimens. Original magnification, x200. Semi-quantitative evaluation of CD1a and CD83 expression was performed in normal squamous epithelium (n=4), LSIL (n=5), HSIL (n=5) and SCC (n=5). *P<0.05 vs. Normal. (B) Preliminary verification in animal experiments. Qtracker™ 705-labeled DCs were injected into control mice, CaSki-bearing mice and SiHa-bearing mice. Popliteal lymph nodes were collected and the number of labeled DCs was detected by flow cytometry. DCs, dendritic cells; HSIL, high-grade squamous intraepithelial lesion; HPF, high-power field; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous carcinoma.

In vivo migration assay. Female Balb/C-nu mice (n=8/group; age, 4–6 weeks; weight, 20–25 g; Experimental Animal Center of Daping Hospital) were housed in exhaust-ventilated closed system cages in a specific pathogen-free environment. The animals were maintained at 22±2°C with 55±5% humidity under a 12-h light/dark cycle. Food and water were provided *ad libitum*. SiHa and E6-siRNA transfected SiHa cells were cultured with DMEM supplemented with 10% FBS at 37°C in a 5% incubator containing CO₂. CaSki cells (Cell Bank of the Chinese Academy of Sciences) were cultured with RPMI-1640 medium supplemented with 10% FBS at 37°C in a 5% CO₂ incubator. Each mouse was injected with 2.5×10⁵ cells into the left footpad to create tumors. After ~3 weeks, when the tumor size reached ~10 mm², 2×10⁶ treated DCs were injected intratumorally. The injected DCs were labeled with Qtracker™ 705 Cell Labeling kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Animals injected with PBS were used as controls. After 48 h, the mice were sacrificed and the popliteal lymph nodes were made into single cell suspensions using nylon mesh and a 1-ml syringe. Subsequently, 20,000 cells were counted by flow cytometry, the labeled DCs were detected and the positive rate was calculated.

In addition, for the detection of labeled DCs, the popliteal lymph nodes of mice were dissected 48 h post-DC injection, then embedded in Tissue-Tek OCT compound (Sakura FineTek Japan) and frozen in liquid nitrogen. Cryosections (8 μm) were cut using a cryostatic microtome (Leica Microsystems GmbH). Sections were mounted onto slides, dried and frozen at -20°C before use. The slides were then fixed with acetone (15 min, 4°C) and counterstained with DAPI. After washing, the slides were mounted in 50% glycerol (in PBS) and examined using confocal microscopy. All animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University. All efforts were made to minimize animal suffering and reduce the number of animals used.

Statistical analysis. The experiments were repeated three times. Histogram and scatter graphs were generated using GraphPad Prism 5 software (GraphPad Software, Inc.) and were presented as the mean ± standard deviation. Differences between multiple groups were analyzed by one-way ANOVA followed by Dunnett's post hoc test using IBM SPSS Statistics 19 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

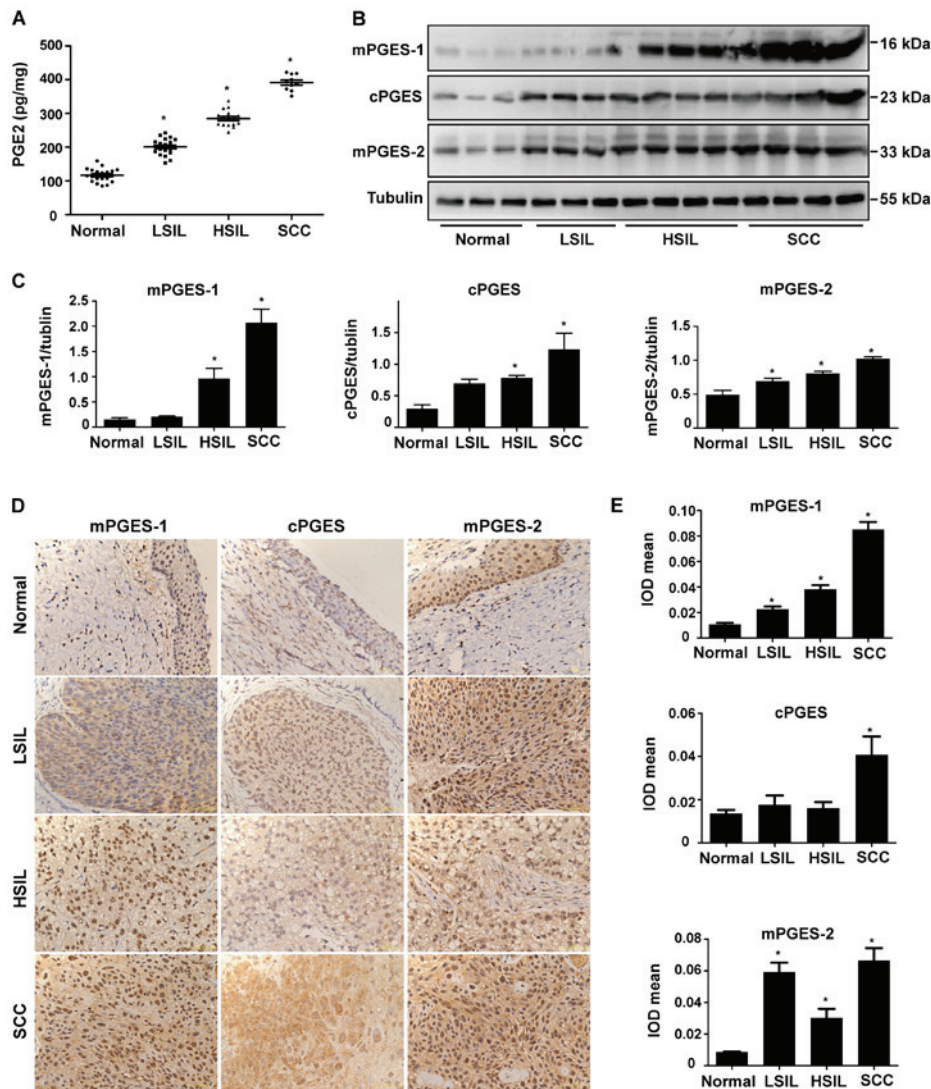


Figure 2. Expression of PGE₂ in human papillomavirus 16-positive cervical lesions. (A) Detection of PGE₂ expression in cervical biopsy specimens by ELISA. Evaluation of PGE₂ expression was performed in normal squamous epithelium (n=20), LSIL (n=20), HSIL (n=16) and SCC (n=10). (B) Detection of mPGES-1, mPGES-2 and cPGES expression in cervical biopsy specimens by western blotting. (C) Semi-quantitative evaluation of mPGES-1, mPGES-2 and cPGES expression. (D) mPGES-1, mPGES-2 and cPGES immunostaining in cervical biopsy specimens. Original magnification, x200. (E) Semi-quantitative evaluation of mPGES-1, mPGES-2 and cPGES expression was performed in normal squamous epithelium (n=5), LSIL (n=5), HSIL (n=5) and SCC (n=5). *P<0.05 vs. Normal. cPGES, cytosolic PGE synthase; HSIL, high-grade squamous intraepithelial lesion; IOD, integrated optical density; LSIL, low-grade squamous intraepithelial lesion; mPGES, microsomal PGE synthase; PGE₂, prostaglandin E₂; SCC, squamous carcinoma.

Results

Status of DCs in HPV16-positive cervical lesions. The distribution of DCs in cervical biopsy specimens was investigated. Immature DCs were characterized by CD1a expression. Mature DCs, which capture foreign antigens to initiate the immune response, expressed CD83. According to Fig. 1A, all HPV16-positive samples demonstrated reduced expression of CD1a and increased expression of CD83, compared with normal tissues. CD1a expression was significantly reduced in the HSIL and SCC groups (P<0.05), whereas CD83 expression was significantly upregulated in the LSIL and HSIL groups (P<0.05). CCR7 expression was also detected; however, not only DCs, but also cervical cells expressed CCR7 and it was difficult to detect the expression of CCR7 on DCs only (data not shown). These results indicated that, after capturing foreign antigens, the majority of DCs may be

trapped in the lesion site and unable to migrate to the draining lymph nodes. The most likely explanation of this phenomenon was that the migration ability of DCs was suppressed. Therefore, an *in vivo* experiment was performed to investigate this possibility. To mimic the microenvironment of HPV16-positive lesions, immunodeficient mice were injected with two HPV16-positive cell lines: SiHa and CaSki. Labeled DCs were subsequently injected intratumorally. Control mice not bearing tumors were directly injected with DCs into the footpad and served as the control group. To investigate the migration of DCs, the labeled cells in the draining lymph nodes were collected and counted. The cell numbers from the two tumor-bearing groups were reduced compared with the control group (Fig. 1B). These findings indicated that the migration of DCs was inhibited in HPV16-positive cervical lesions. However, the mechanism underlying the decreased migration of DCs requires further investigation.

Expression of PGE₂ in HPV16-positive cervical lesions. The expression of PGE₂ in cervical biopsy specimens was investigated. As depicted in Fig. 2A, the expression of PGE₂ was gradually up-regulated in LSIL, HSIL and SCC samples, which was accompanied by the development of disease. All HPV16-positive lesions demonstrated significant upregulation compared with normal tissues ($P < 0.05$). Furthermore, the expression levels of PGE₂ synthases were detected, which are required for PGE₂ production. Western blotting and immunohistochemistry were used to detect the expression levels of three isoforms of PGE₂ synthases: mPGES-1, cPGES and mPGES-2. As demonstrated in Fig. 2B-E, the expression levels of all three PGE₂ synthases were increased in HPV16-positive lesions compared with normal tissue. Particularly, the SCC group exhibited the strongest expression ($P < 0.05$). These findings suggested that PGE₂ and PGE₂ synthases may be upregulated in HPV16-positive cervical lesions.

Effect of PGE₂ on the migration of DCs. The effect of PGE₂ on the migration ability of DCs was investigated. PGE₂ was added to the culture media of DCs at a final concentration of 0, 5 or 10 $\mu\text{g/ml}$. The cultured supernatant of SiHa cells was applied to treat the DCs. The migration of DCs was determined using a Transwell migration assay. As depicted in Fig. 3A, the migration of DCs was significantly decreased in response to PGE₂ in a dose-dependent manner ($P < 0.05$). Furthermore, after being treated with SiHa supernatant, DCs exhibited declined migration ability as well. A 3D migration assay was subsequently performed to validate these results (Fig. 3B); the results were in line with the Transwell migration assay. Therefore, PGE₂ may inhibit the migration of DCs in cervical lesions.

Expression of CCR7 on DCs is affected by PGE₂. As shown in Fig. 4, DCs exhibited marked upregulation of all co-stimulatory molecules after being stimulated with LPS. However, the phenotypic features of immature and mature DCs remained unchanged, following PGE₂ exposure. Since CCR7 is required for the migration of DCs, the surface expression of CCR7 was also detected. CCR7 expression was markedly downregulated on immature and mature DCs following PGE₂ exposure. This result indicated that PGE₂ affected the migration ability of DCs.

E6 regulates PGE₂ expression in HPV16-positive cervical lesions. Due to its central role in the development of HPV16-positive cervical lesions, the effect of the E6 oncoprotein on PGE₂ expression was investigated. Firstly, E6 expression was observed in cervical biopsy specimens. As demonstrated in Fig. 5A, E6 was undetectable in normal samples. Conversely, the expression of E6 gradually increased from low to high-grade lesions, which was parallel to disease development and progression. The highest expression was observed in SCC samples. E6-specific siRNA was subsequently used to knock down E6 expression in SiHa cells. As depicted in Fig. 5B, two siRNAs were used to knock down E6 expression, with siRNA-1 exhibiting a stronger effect. The cultured supernatant of siRNA-transfected SiHa cells was collected and used to treat DCs in a Transwell migration assay. As shown in Fig. 5C, following treatment with siRNA-transfected supernatant, DCs exhibited improved migration ($P < 0.05$). Furthermore,

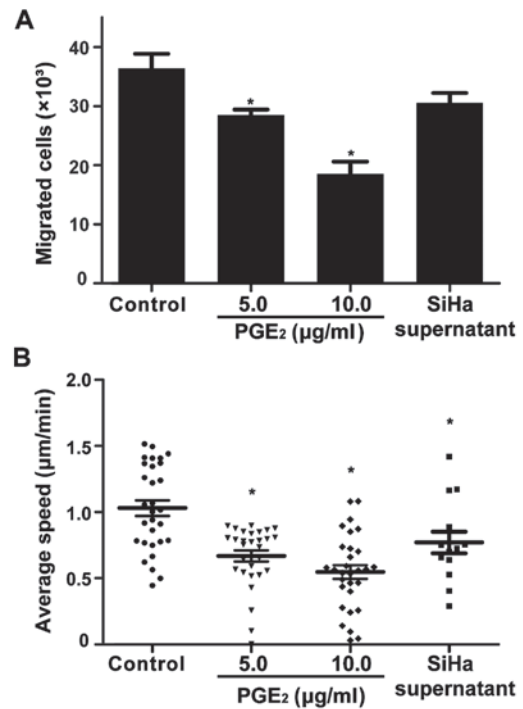


Figure 3. Effect of PGE₂ on the migration of DCs. (A) Migration of DCs, as determined by Transwell migration assay. DCs were treated with PGE₂ or SiHa cell supernatant for 24 h. Data are representative of three independent experiments and are shown as means \pm standard deviation. (B) DC migration was determined by 3D migration assay. DCs were treated with PGE₂ or cell supernatant for 24 h. Average speed was quantified. Data are representative of three independent experiments and are shown as means \pm standard deviation. * $P < 0.05$ vs. Control. DCs, dendritic cells; PGE₂, prostaglandin E₂.

siRNA-transfected SiHa cells were used to perform an *in vivo* migration assay on immunodeficient mice. The E6-knockdown SiHa cells and control SiHa cells were applied to create tumors. The labeled DCs were injected in the same way as aforementioned. The labeled cells in the draining lymph nodes were collected and counted to investigate the migration ability of DCs. As shown in Fig. 6A and B, the numbers of DCs were significantly increased in both siRNA-transfected groups compared with the control group ($P < 0.05$). This indicated that the migration ability of DCs was improved following E6 knockdown. Furthermore, the concentration of PGE₂ in tumors was measured. As shown in Fig. 6C, tumors created by the E6 knockdown SiHa cells produced significantly less PGE₂ compared with normal cells ($P < 0.05$). These results indicated that E6 regulated the production of PGE₂ in HPV16-positive cervical lesions, which affected the migration ability of DCs.

Discussion

E6 is broadly expressed in different stages of cervical lesions, and is responsible for host cell transformation and disease development (22,23). The present study revealed that E6 expression was markedly higher in late cervical lesions than in early cervical lesions; this result was in line with existing reports (24,25). Following infection with HPV, DCs undergo a phenotypic conversion from a tissue resident, antigen-capturing cell to a highly migratory antigen-presenting cell, in a process known as maturation. Previous studies have demonstrated that

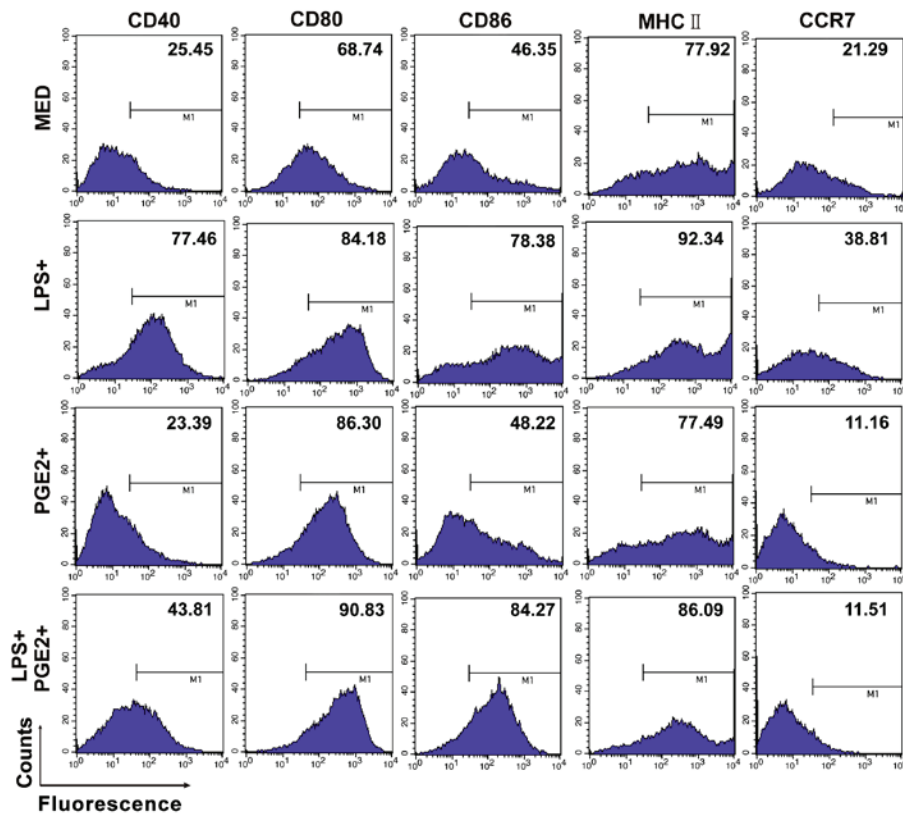


Figure 4. Effect of PGE₂ on the expression of surface markers of DCs. DCs were cultured with or without LPS (1 μ g/ml) and PGE₂ (5 μ g/ml) for 24 h. Surface markers of DCs were analyzed by flow cytometry. The numbers (upper right) indicated the percentage of positive cells. Representative images of three experiments are presented. CCR7, C-C chemokine receptor type 7; DCs, dendritic cells; LPS, lipopolysaccharide; MHCII, major histocompatibility complex II; PGE₂, prostaglandin E2.

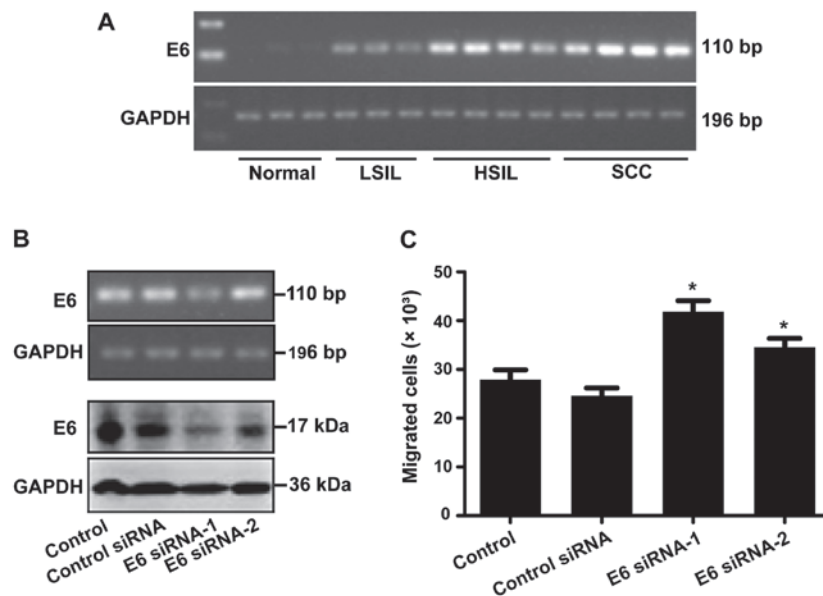


Figure 5. E6 affects the migration of DCs. (A) Detection of E6 expression in cervical biopsy specimens by PCR. (B) Expression of E6 in SiHa cells transfected with siRNA detected by PCR and western blotting. Representative images of three experiments are presented. (C) Detection of DC migration using a Transwell migration assay. DCs were treated with the cultured supernatant of siRNA-transfected SiHa cells. Data are representative of three independent experiments and are shown as means \pm standard deviation. * P <0.05 vs. Control. DCs, dendritic cells; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous carcinoma; siRNA, small interfering RNA.

HPV regulates the differentiation, function and distribution of DCs in infected sites of cervical epithelial lesions, which allows for evasion of immune surveillance (26,27). HPV

may therefore promote tumor progression by reducing the number of DCs and attenuating immune surveillance. The present study revealed that the number of CD1a-positive DCs

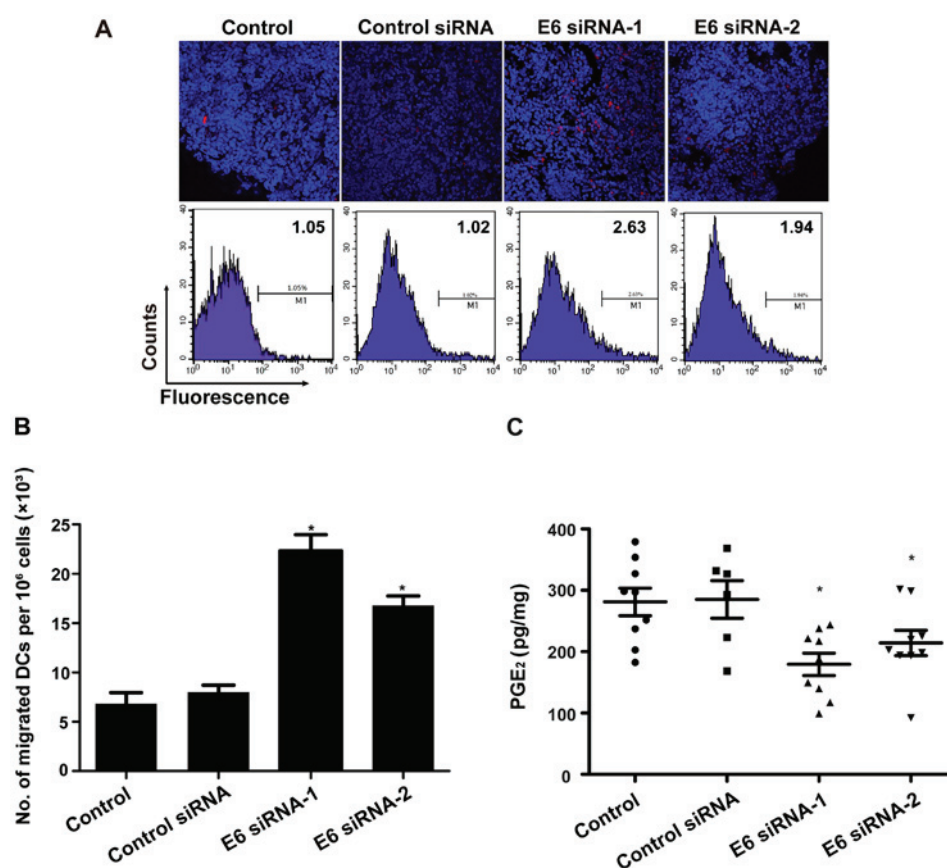


Figure 6. E6 regulates PGE₂ expression and affects the migration of DCs *in vivo*. (A and B) Qtracker™ 705-labeled DCs were injected into groups of mice (n=8/group), which were treated with different SiHa cells to create tumors. After 48 h, three mice in each group were sacrificed, and the popliteal lymph nodes were collected, snap frozen and cryosectioned. The number of positive DCs was detected by confocal microscopy; original magnification, x400. Red staining indicated Qtracker™ 705-labeled DCs; blue staining indicated nuclei. The remaining mice were sacrificed, the popliteal lymph nodes were made into single cell suspensions and the number of positive DCs was detected by flow cytometry. Data are shown as means ± standard deviation. (C) Tumor tissues were collected from each group (control group, n=9; control siRNA group, n=6; E6 siRNA-1 group, n=9; E6 siRNA-2 group, n=9) and detection of PGE₂ concentration was performed by ELISA. Data are shown as means ± standard deviation. *P<0.05 vs. Control. DCs, dendritic cells; PGE₂, prostaglandin E₂; siRNA, small interfering RNA.

was negatively associated with the degree of cervical lesion, which was consistent with the findings of a previous study (3). However, it also revealed that the number of CD83-positive DCs was significantly increased in early lesions, but decreased in late lesions. It was speculated that in early lesions, DCs matured by capturing antigens and accumulated at the lesion sites. In late lesions, DCs that captured antigens may not migrate in time. Therefore, the migration of DCs to the lymph nodes after capturing antigens in early lesions may be a critical step in the immune response. Khaiboullina *et al* (28) observed the variation and expression of HPV-related proteins in DCs by transfecting the HPV18 gene. The results revealed that the mRNA expression levels of E6 and E7 were upregulated in transfected cells. In addition, the migration ability of transfected DCs was reduced, which was accompanied with a decreased ability to produce cytokines, which may inhibit and delay the immune response of viral antigens. Notably, knockdown of E6 expression restored the migration ability of DCs in the current study; however, the mechanism by which E6 inhibited the migration of DCs was not clarified.

As an inflammatory factor, PGE₂ has been related to carcinogenesis in several types of cancer, including cervical cancer (29). PGE₂ stimulates carcinogenesis by promoting angiogenesis (30), increasing proliferation of cancer cells (31),

suppressing apoptosis of cancer cells (32) and inducing immune tolerance (33). The activities of phospholipase A₂, COX and PGE₂ synthases are essential to the biosynthesis of PGE₂. A previous study demonstrated that COX-2 is rapidly induced by oncogenes, growth factors, cytokines and tumor promoters, and serves an important role in the development of cancer (34). Furthermore, COX-2 regulates tumor growth by binding to its corresponding receptors through various downstream prostate products, instead of acting as a signal transduction kinase. It has been reported that HPV16 and COX-2 serve a synergistic role in cervical carcinogenesis. In addition, certain studies have demonstrated that the expression of COX-2 is significantly upregulated in HPV16E6/E7-positive cervical cancer tissues and cell lines (18,35). Prostaglandin levels in cervical cancer tissue have been reported to be significantly higher than those in adjacent tissues, and high expression of COX-2 is positively correlated with prostaglandin levels (11). E6 may promote the synthesis of COX-2 by activating the epidermal growth factor receptor/→RAS/→MAPK/→activator protein 1 signaling pathway (18). The present study revealed that with the development of cervical lesions, the concentration of PGE₂ also increased. The *in vivo* experiments using SiHa cells revealed that the concentration of PGE₂ in tumors was significantly downregulated following E6 knockdown

compared with in the control group. This indicated that E6 may exert a positive regulatory effect on the synthesis of PGE₂.

As an inducible synthase, mPGES-1 has been identified to mainly interact with COX-2, whereas cPGES is not inducible and mainly interacts with COX-1. Conversely, the role of mPGES-2 is not clear (36). It has been confirmed that COX-2/mPGES-1 expression is upregulated in cervical pre-cancerous and invasive cancer tissues (37). According to Mattila *et al* (38), with the disease development of human glioma, the expression of these three synthases is gradually increased. The results obtained in the present study demonstrated that cPGES and mPGES-2 were also expressed during the development of cervical lesions, and were significantly upregulated in invasive cancer tissues. However, more samples and further investigation is required to identify the specific synthase that serves the predominant role in the pathogenesis of cervical lesions.

PGE₂ acts on neighboring tissues to maintain the micro-environment in a steady state. PGE₂ exerts its functions by activating four receptors: EP1-EP4. Notably, EP2 and EP4 are expressed during the entire life cycle of myeloid DCs (39). PGE₂ regulates the functions of DCs by: i) Regulating the expression of CCR7 on the surface of DCs to affect chemotaxis (40,41); ii) regulating the intracellular calcium flux and migration-related signaling pathways (42,43); iii) regulating the expression of MHCs, co-stimulatory molecules and markers of maturation in DCs, as well as the ability of DCs to activate T-cell immune responses (44); and iv) regulating the expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase in DCs, which affect the basement membrane and allow the migration of DCs (21). Due to the important role of DCs in specific cellular immunity, the impact and mechanism of PGE₂ are attracting increased attention. However, there remain controversial conclusions. Some studies have revealed that PGE₂ enhances the migration and antigen-presenting ability of DCs (12,13); therefore, PGE₂ is often used as an important component of cytokine cocktails that stimulate the maturation of DCs *in vitro*. Conversely, other studies, including the present study, have demonstrated that PGE₂ inhibits the migration of DCs (21,45,46). These differences may be explained by the fact that the concentration and state of PGE₂ used by the researchers differed, which may cause inconsistent results.

Notably, the role of PGE₂ requires further investigation. The present results are unable to fully elucidate the specific mechanism underlying regulation of DC migration. This is one of the limitations of the present study. In addition, the receptors that mediate the effect of PGE₂ induced by cervical lesions are unclear and the specific signaling pathway associated with the effects of PGE₂ on migration of DCs remains unclear. Therefore, we are currently collecting more clinical samples to validate the results obtained in this study. In addition, the expression of key molecules in the signaling pathway induced by PGE₂ will also be verified in these samples. In the current study, the expression of CCR7 was slightly decreased when the migration of DCs was inhibited by PGE₂. This finding indicated that there may be other mechanisms underlying the regulatory effects of PGE₂ on the migration of DCs, such as those affecting formation of the cytoskeleton and focal adhesion. Furthermore, immune evasion of cervical lesions is

a complex process; inhibited migration of mature DCs may be one effect of immune evasion. It may regulate immature DCs to keep dormant and remain in a steady state. Furthermore, it may induce the production of regulatory DCs, thereby inhibiting T-cell proliferation and keeping T cells less reactive, which eventually induces immune tolerance. All of these are issues we aim to focus on in our future study.

Similar to what was reported in previous studies, the present study revealed that, in HPV16-positive cervical lesions, the expression of PGE synthase was significantly upregulated, resulting in the overproduction of PGE₂ (18). Therefore, the use of drugs to suppress PGE₂ synthesis may reduce the risk of cervical lesions. In the treatment of cervical cancer, patients with upregulated expression of COX-2 have a worse prognosis than patients who do not, irrespective of whether radiotherapy or chemotherapy is administered (47,48). Therefore, COX-2 inhibitors, such as ibuprofen and aspirin, may reduce the risk of cervical cancer recurrence (29). However, COX-2 is the main rate-limiting enzyme, and not a terminal enzyme, in the PGE₂ synthesis pathway. COX-2 affects a number of downstream signaling pathways and is likely to have a double effect. Therefore, the traditional treatment strategy using COX-2 as a therapeutic target is not ideal. The abnormal balance of the PGE synthase-PGE₂-EPs signaling pathway as downstream target of COX-2 may inhibit the migration of DCs and lead to immune evasion of cervical cancer cells. Restoring the balance of the PGE synthase-PGE₂-EPs signaling pathway may serve as a novel therapeutic strategy for cancer.

In conclusion, the present study revealed that a high concentration of PGE₂ inhibited the migration of DCs in HPV16-positive cervical lesions. Furthermore, the production of PGE₂ was mediated by the oncoprotein E6. Notably, these findings may improve the understanding of immune evasion in cervical lesions, and contribute to the treatment of cervical cancer and other types of cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JG and JHan designed this study. JHuang and GD performed the majority of the experiments and were major contributors in

writing the manuscript. QZ and YC assisted with the experiments and data analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Daping Hospital, Army Medical University (Third Military Medical University). All participants signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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