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The HPV16 E7 oncoprotein increases the expression of Oct3/4 and stemness-related genes and augments cell self-renewal

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ABSTRACT

Oct3/4 is a transcription factor involved in maintenance of the pluripotency and self-renewal of stem cells. The E7 oncoprotein and 17β-estradiol (E_2) are key factors in cervical carcinogenesis. In the present study, we aimed to investigate the effect of the HPV16 E7 oncoprotein and E_2 on the expression pattern of Oct3/4, Sox2, Nanog and Fgf4. We also determined whether the E7 oncoprotein is associated with cell self-renewal. The results showed that Oct3/4, Sox2, Nanog and Fgf4 were upregulated by the E7 oncoprotein *in vivo* and *in vitro* and implicate E_2 in the upregulation of these factors *in vivo*. We also demonstrated that E7 is involved in cell self-renewal, suggesting that the HPV16 E7 oncoprotein upregulates Oct3/4, Sox2, Nanog and Fgf4 expression to maintain the self-renewal capacity of cancer stem cells.

1. Introduction

Persistent infection with high-risk human papillomaviruses (HR-HPVs) is associated with more than 99% of cervical cancer (CC) cases (Paavonen, 2007). Among the HR-HPVs, HPV16 is the most prevalently detected in CC (Paavonen, 2007). HR-HPVs encode three oncogenes, *E5*, *E6* and *E7* (Leechanachai et al., 1992; Münger et al., 1989), and among these, E7 appears to be the main driver of cervical carcinogenesis (Riley et al., 2003). Although the E7 oncoprotein is best known for its ability to bind to and degrade the pRb tumor suppressor protein (Münger et al., 2001), it has been associated with other cellular proteins that play a role in regulating the cell cycle, transcriptional repression, and tumor suppression (Avvakumov et al., 2003; Bernat et al., 2003; Münger et al., 2001). In mice, the combination of HPV16 E7 and exogenous estrogen (E_2) is sufficient to induce high-grade cervical dysplasia and CC (Chung et al., 2008; Herber et al., 1996; Riley et al., 2003) and is responsible for alterations in the expression of many cellular genes (Cortés-Malagón et al., 2013).

In recent years, several studies conducted in cell lines (Ji and Zheng, 2010; Li et al., 2015; Wang et al., 2013b) and cervical tumors (Ji and Zheng, 2010; Li et al., 2015; Wang et al., 2013b; Ye et al., 2008) have found the overexpression of certain stem cell-associated nuclear transcription factors, such as the octamer-

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binding transcription factor 3/4 (Oct3/4), Sox2 and Nanog, in CC. Oct3/4 (also known as POU5F1) is a nuclear transcription factor of the POU-homeodomain family that is involved in various aspects of embryonic stem cells (ESCs). Oct3/4 was recently proposed to act as a multi-functional factor during cancer development because it promotes tumor growth in ESCs (Gidekel et al., 2003) and causes epithelial dysplasia in somatic cells by interfering with cell differentiation (Hochedlinger et al., 2005; Liu et al., 2011). Liu et al. (2011) reported that HR-HPV (HPV16/18) infection may be associated with Oct3/4 mRNA and protein upregulation in the process of cervical carcinogenesis and that viral infection inhibits the differentiation of cervical stem cells (SCs).

Recent studies determined that CC contains a subpopulation of stem-like cancer cells expressing the Oct3/4 protein (Feng et al., 2009; Li et al., 2015; López et al., 2012a); however, the participation of the HPV16 E7 oncoprotein in the mediation of these effects has not been explored. In the present study, we aimed to investigate the effects of the HPV16 E7 oncoprotein and estrogen in modulating the expression pattern of Oct3/4 and stemness-related genes (Nanog, Sox2 and Fgf4) in a mouse model of HPV-associated cervical carcinogenesis (K14E7 transgenic mice). In addition, using a cell line expressing the HPV16 E7 oncoprotein, we determined whether the E7 oncoprotein is involved in increasing the self-renewal ability of cells. Our results reveal that Oct3/4, Sox2, Nanog and Fgf4 are upregulated by the HPV16 E7 oncoprotein or/and estradiol *in vivo* and indicate that the HPV16 E7 oncoprotein might be a critical factor for cell self-renewal that acts via the overexpression of Oct4, Nanog, Sox2 and Fgf4.

2. Materials and methods

2.1. Cells

Primary human keratinocytes (PHKs) derived from foreskins of two-year-old donors were grown at 37 °C in a humidified atmosphere with 5% CO_2 in DMEM: F12 media supplemented with 0.18 mM adenine (Sigma®), 0.1 μg/mL hydrocortisone (Sigma®), 4 μg/mL insulin (Gibco®), 20 ng/mL recombinant epidermal growth factor (PeproTech®), 5% fetal bovine serum (Gibco®) and an antibiotic-antimycotic mixture (Invitrogen®). PHKs express cytokeratin-5, −10 and −14, present low vimentin expression and do not express decorin. The cells were transfected with the E7 oncogene using the Attractene reagent (QIAGEN®, Valencia, CA, USA) according to the manufacturer's recommended protocol. Cells were seeded onto six-well plates (3×10^5 cells/well), and the next day, the cells were transfected with a mixture of 4.5 μl of Attractene/well and 1.2 μg of DNA/well. To obtain a stable cell line, the transfected cells were selected and maintained in growth media containing 200 μg/mL geneticin (G418; Invitrogen®, Carlsbad, CA, USA).

The Saos-2 osteosarcoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line was maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cells were transfected with the E7 oncogene using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To obtain a stable cell line, the transfected cells were selected for two weeks in growth media containing 1200 μg/mL geneticin (G418; Invitrogen, Carlsbad, CA, USA). For maintenance of the selected clones, the cells were grown continuously in medium containing 800 μg/mL G418.

2.2. Culture of tumor spheres

To examine the sphere-forming capacity of cells expressing the HPV16 E7 oncoprotein, cells from sub-confluent (~80%) cultures were resuspended in a serum-free tumor sphere culture medium (MammoCult, StemCell Technologies, Inc., Canada) supplemented with MammoCult Proliferation Supplements. The sphere culture media was changed every 48 h. The spheres were sedimented, and the formed tumor spheres were examined and counted in at least nine different fields under a microscope. A "sphere" was defined as more than 15 cells, and the numbers of spheres that formed after 1, 3, and 5 days of culture were counted. The experiments were repeated at least three times.

2.3. Transgenic mice

K14E7 transgenic mice have been described previously (Herber et al., 1996; Riley et al., 2003), and male K14E7 mice were backcrossed onto the FVB/n background. Female mice were used as heterozygotes in these experiments. The mice were housed in a pathogen-free barrier facility according to the guidelines of the American Association for Laboratory Animal Care (AALAC). All of the experiments and procedures were approved by the Research Unit for Laboratory Animal Care Committee (UPEAL-CINVESTAV-IPN, Mexico; NOM-062-ZOO-1999).

2.4. Hormone treatment

The mice were treated as previously described (Riley et al., 2003). Briefly, one-month-old virgin female transgenic (K14E7) and non-transgenic (NT) mice received a subcutaneous (s.c.) implant in the dorsal skin of continuous-release pellets that deliver 0.05 mg of 17β-estradiol over a period of 60 days (Innovative Research of America, Sarasota, FL, USA). The mice were treated with the hormone for six months. All of the mice in this study that were not treated with E_2 (K14E7 transgenic and NT mice) were in the estrous phase of the estrous cycle. Untreated NT mice were used as controls.

2.5. Total RNA extraction

After sacrifice by cervical dislocation and thoracotomy, cervical tissue was rapidly removed and immediately frozen (−70 °C) for total RNA extraction. The total RNA (large- and small-sized RNAs) from the collected cervical tissues (six female mice from each experimental group) and from cultured cells was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommended protocol. The RNA concentration was spectrophotometrically assessed by measuring the absorbance at 260/280 nm.

2.6. Quantification of mRNA by real-time quantitative PCR (RT-qPCR)

The total RNA (1 μg) was reverse transcribed into cDNAs with oligo(dT) primers and the Superscript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for RT-PCR according to the manufacturer's instructions. Quantitative real-time PCR was performed with an Applied Biosystems 7300 system (Foster City, CA, USA) using SYBR Green (SYBR Green PCR Reagents Kit, USA) and the protocol provided by the manufacturer.

The 25-μl reaction volume consisted of 12.5 μl of SYBR Green PCR Master Mix containing Taq DNA polymerase, reaction buffer, dNTP mix, 1 mM $MgCl_2$ (final concentration), and SYBR Green I dye as well as 0.5 μM of each primer, 5 μl of the template (500 ng of template per reaction) and ultrapure water. All primer sequences and product sizes are described in Table 1. The expression levels of