Effects of 60 kDa prolactin and estradiol on metabolism and cell survival in cervical cancer: Co-expression of their hormonal receptors during cancer progression

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Received January 16, 2018; Accepted September 18, 2018

DOI: 10.3892/or.2018.6743

Abstract. Estrogens and estrogen receptors (ERs), such as ERα and ERβ, prolactin (PRL) and prolactin receptor (PRLR) have been reported to be involved in the physiopathology of uterine cervical cancer (UCC). The 60 kDa PRL is an isoform of PRL, which is produced by UCC-derived cells. The present study aimed to evaluate the expression of hormonal receptors in different degrees of cervical lesions, and to determine whether 60 kDa PRL and 17β-estradiol (E2) modulated cell survival and metabolism in UCC cells, and in HaCaT cells transduced with human papillomavirus (HPV) 16 and 18 E6/E7 oncogenes. ERα, ERβ, PRLR, Ki67 and B-cell lymphoma 2 expression levels were analyzed in biopsies of precursor lesions and UCC using immunohistochemistry. In addition, HeLa, SiHa and C33A cells, and transduced HaCaT cells, were stimulated with 60 kDa PRL, E2 or a combination of both. Proliferation was evaluated using the xCELLigence platform, apoptosis was analyzed by flow cytometry and cell metabolism was determined using the MTT assay. The results revealed that ERα, ERβ, PRLR and Ki67 expression levels were increased during the progression of cancer. *In vitro*, 60 kDa PRL alone significantly increased proliferation of SiHa cells. Furthermore, E2 alone or in combination with 60 kDa PRL increased the sensitivity of SiHa cells to cisplatin and increased the percentage of apoptosis; in HaCaT cells, these treatment strategies had the opposite effect on cisplatin sensitivity. Treatment with E2 increased mitochondrial activity in HeLa and SiHa cells, and in HaCaT cells transduced with HPV 16 E6/E7 and HPV 18 E6 oncogenes. PRL had a similar effect on HeLa cells, and on HaCaT cells transduced with HPV 18 E6 and HPV 16 E7. The co-expression of these receptors demonstrated the hormonal dependence of UCC. In addition, E2 and the 60 kDa PRL significantly impacted the metabolism, but not the survival, of cells.

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Key words: prolactin, estradiol, cell survival, cervical cancer

Introduction

Uterine cervical cancer (UCC) is a global health problem and one of the most challenging medical issues, due to the complexity of the pathophysiological mechanisms that are involved (1). This complexity limits the ability to determine molecular, biochemical and clinical markers that allow for adequate classification of precursor lesions. At present, clinicians cannot specify the evolution of cancer once the process has been initiated.

Due to the almost constant presence of the human papillomavirus (HPV) in premalignant and malignant lesions of the cervix, this infection is among the most prevalent sexually transmitted diseases. It is well known that variation among

the >100 viruses of the HPV family is a determining factor in the immune response of the host. HPV types 16 and 18 continue to be the principal causal agents; however, the effects of co-infection with several subtypes remain unknown; this phenomenon is currently more frequently observed (2,3). The routine use of cytology for the early diagnosis of dysplastic lesions, as well as the administration of HPV vaccines to women who have not had prior contact with HPV, have not exhibited a significant impact on the mortality index generated by the disease (4-6). Previous studies have revealed that HPV is associated with UCC; however, its presence is insufficient, and it requires cofactors for its permanence and for the initiation of carcinogenesis (7,8).

Numerous studies using transgenic mouse models have significantly demonstrated the requirement of estrogens in HPV-driven UCC (5-9). The cervix, particularly the transformation zone of the uterine ectocervix, which serves as the site of initiation of tumorigenesis, is an estrogen-responsive tissue (10). Epidemiological research has demonstrated the higher risk of developing UCC as a result of a long-term oral contraceptive use and multiple pregnancies (11-13).

Steroid hormones achieve their biological effects through receptors. Current research indicates the existence of two types of nuclear estrogen receptor (ER): ERα and ERβ, of which there are several isoforms (14,15). Investigations using clinical specimens indicated the presence of a differential ER expression pattern in cervical tumor epithelium (9). Recently, the role of stromal ERα in the progression of UCC has gained prominence (16,17).

The signaling complex of prolactin (PRL) and PRL receptor (PRLR), using both endocrine and local paracrine/autocrine pathways, has also been implicated in the pathology of UCC (18-20). Initial studies analyzed the association between PRL/PRLR and the signaling pathways involved in proliferation and apoptosis of UCC cell lines. The expression levels of both PRL and PRLR were revealed to be increased in the cell lines, and are associated with cell survival; in addition, treatment with 200 ng/ml human recombinant PRL (hPRL) exerts a protective effect against etoposide-induced apoptosis (18,19). It has also been revealed that activation of signal transducer and activator of transcription 3 (STAT3) is required for the anti-apoptotic effects of hPRL (20), and PRLR expression is increased in histopathological samples of patients with UCC compared with in normal cervical tissues (20).

Extrapituitary sources of PRL in humans include uterine and brain tissues, lacrimal, sweat and adrenal glands, immune and mammary epithelial cells, skin fibroblasts, and the kidneys and ovaries (21). However, the mechanism by which local PRL contributes to PRL-induced responses is unclear. Larrea *et al* evaluated the production of a 60 kDa PRL isoform by human peripheral mononuclear cells from healthy subjects and patients with systemic lupus erythematosus (SLE); immunoreactivity was preferentially detected in subjects with SLE (22).

Our previous study identified the presence of the 60 kDa PRL in the protein extracts and supernatant of three cell lines derived from UCC (HeLa, SiHa and C33A). Conversely, this isoform was absent in the extracts and supernatant of an immortalized keratinocyte cell line (HaCaT) (18). Following isolation of 60 kDa PRL, its bioactivity was tested. Using the Nb2 cell line as a model system to analyze the effects of hPRL on cell mitogenesis, stimulation with 60 kDa PRL was revealed to increase cell growth (23,24). Local 60 kDa PRL has been revealed to exert bioactive effects and to phosphorylate STAT3 (25).

The association between estrogen and PRL has been reported. Estrogen regulates PRL at the transcriptional level, acting on the specific sequence on its promoter via its function as a regulatory steroid hormone (26). Duan *et al* identified a functional, non-canonical estrogen responsive element (ERE) and an activator protein 1 (AP-1) site located in the PRL distal promoter, and suggested that the effects of ERs, as well as ERE and AP1 transcription factors, on the regulation of autocrine/paracrine PRL in the human breast are critical for the progression of breast cancer (27). Furthermore, the role of cross-regulation of ERα levels by endogenous PRL in increasing estrogen responsiveness in breast cancer cells has been established (28).

Ki67 is a well-known cell proliferation marker, which has been proposed as a useful tool to distinguish between dysplastic and non-dysplastic lesions (29). In addition, the overexpression of B-cell lymphoma 2 (Bcl-2) has been reported in premalignant and malignant lesions of the cervix, and is associated with the development of invasive cervical disease (30,31).

Based on these findings, the present study evaluated the co-expression of ERs and PRLR in UCC samples and precursor lesions. In addition, the association between estrogen and PRL in cancer was analyzed by determining the effects of 17β-estradiol (E2) and 60 kDa PRL stimulation on the cell survival and metabolism of UCC cell lines, and of HaCaT cells transduced with HPV 16 and 18 E6/E7 oncogenes.

Materials and methods

Reagents. A polyclonal antibody against ERα (MC-20; cat. no. sc-542) and monoclonal antibodies against PRL (E-9; cat. no. sc-48383) and ERβ (B-3; cat. no. sc-373853) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Monoclonal antibodies against Ki67 (30-9; cat. no. 790-4286) and Bcl-2 (124 cat. no. 790-4464) were obtained from Ventana Medical Systems, Inc. (Tucson, AZ, USA). Deparaffinization, antigen retrieval, cell conditioning and immunohistochemical staining were conducted using BenchMark ULTRA instrument and BenchMark ULTRA reagents, solutions, kits and accessories from Ventana Medical Systems, Inc. μMACS™ Protein G MicroBeads MultiMACS™ Protein G kit, which was used for the isolation of 60 kDa PRL, was provided by Miltenyi Biotec GmbH (Bergisch Gladbach, German). E2 and MTT were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), and cisplatin (cat. no. 479306) was purchased from Sigma-Aldrich; Merck KGaA.

Cell culture. HeLa, SiHa and C-33A cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The non-tumorigenic keratinocyte HaCaT cell line was kindly provided by Dr Petra Boukamp from the German Cancer Research Center (DKFZ, Heidelberg, Germany). SiHa is a cell line derived from a grade II squamous cell carcinoma of the cervix that expresses HPV 16; this cell line is adherent and has a doubling time of 26 h. HeLa is cell line derived from

an adenocarcinoma of the cervix, which is positive for HPV 18, and has a doubling time of 24 h. The C-33A cell line was used because it is derived from a carcinoma of the cervix and does not express HPV; this cell line has a doubling time of 29 h. The HaCaT cell line is a line of immortalized keratinocytes; this cell line is adherent, HPV-negative and has a doubling time of 29 h.

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of charcoal stripped fetal bovine serum, penicillin G (10,000 U/ml), streptomycin (10,000 μg/ml) and amphotericin B (250 μg/ml) was used to grow all cells. Media, charcoal stripped serum, penicillin G and streptomycin were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Cells were stored in a water-jacketed incubator at 37˚C in an atmosphere containing 5% CO_2; cells were grown without exceeding 80% confluence.

Tissue samples. The present study was approved by the Ethical Investigation and Biosecurity Committee of the University Center of Health Sciences at the University of Guadalajara (Reference Number C.I. 093/13 CUCS; Guadalajara, Mexico). Written informed consent was obtained from all patients included in the present study. Tissue samples were obtained from the Department of Pathology at the Hospital Civil Fray Antonio Alcalde (Guadalajara, Mexico). The sample collection period ran between August 2013 and February 2017. After collection, tissues were fixed in 4% formalin and embedded in paraffin. A total of 52 specimens were examined, which were selected according to tissue integrity, and were classified by experienced pathologists. According to the proportion of epithelial thickness that presents mature and differentiated cells, 13 biopsies were characterized as cervical intraepithelial neoplasia (CIN) I, 12 as CIN II and 12 as CIN III. Of 15 UCC cases, 11 were diagnosed as epidermoid and 4 were characterized as adenocarcinomas. The mean age for patients with CIN was 28.16 years, and was 33 years for patients with cancer.

Automated immunohistochemistry. Serial sections (4 μm) from the formalin-fixed paraffin-embedded blocks were used for the detection of ERα, ERβ, PRLR, Ki67 and Bcl-2 using immunohistochemical methods. ERα, ERβ and PRLR antibodies were used at a concentration of 1:50 in 100 μl of a 1X concentrated solution of Tris-buffered saline with 0.1% Tween-20 (TBS-T); Ki67 and Bcl-2 antibodies were obtained prediluted by Ventana Medical Systems, Inc., and were optimized for use on Ventana staining platforms. Slides were loaded into the BenchMark ULTRA instrument (cat. no. N750-BMKU-FS 05342716001; Ventana Medical Systems, Inc.). Sections were stained with hematoxylin and eosin and underwent Ventana OptiView DAB IHC detection; the OptiView Amplification kit was used in conjunction with the OptiView DAB IHC Detection kit (both Ventana Medical Systems, Inc.) to increase staining intensity. The entire process lasted ~2.5 h. As a negative control, the primary antibody was omitted, and breast cancer tissues obtained from the Department of Pathology at the Hospital Civil Fray Antonio Alcalde (Guadalajara, Mexico) were used as a positive control.

Specimens were analyzed under an optical microscope (Carl Zeiss AG, Oberkochen, Germany) and digital image files were obtained at x40 magnification. For digital evaluation, five fields from each lesion were selected, and for digital analysis, the cell counter function was used on Image-pro Plus 6.0® software (Media Cybernetics, Inc., Rockville, MD, USA), in which the color intensity of positive objects (brown) was manually preset for each pattern (pixel per pixel) based on the hue-saturation-intensity histogram. The samples in which optical density was very high for the expression of the protein were characterized as intense staining, whereas samples in which the expression of the protein was positive, regardless of density, were characterized as positive staining.

Isolation and purification of 60 kDa PRL. The 60 kDa PRL present in the UCC cell supernatants was isolated using a magnetic bead kit (Protein G MicroBeads MultiMACS™), according to the manufacturer's protocol. The presence of correctly separated PRL was determined using polyacrylamide gel electrophoresis and silver nitrate staining.

After isolation, 60 kDa PRL was purified and concentrated using 50 kDa molecular cut-off filters (Amicon® Ultra 0.5 ml centrifugal filters). Filtration at 4˚C was performed under the following conditions: Filtration phase, 14,000 x g for 30 min; recovery phase, 1,000 x g for 2 min. Quantification of 60 kDa PRL was conducted using a Thermo Scientific NanoDrop 2000c Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

Hormone stimulation. E2 was added to cells at a concentration of 10 nM, and the autocrine 60 kDa PRL was added at a concentration of 200 ng/ml. Cisplatin (1 μg/ml) was used as a cell death control, since it is a common chemotherapeutic agent administered to patients with UCC. After applying the stimuli, cells were stored in a water-jacketed incubator at 37˚C in an atmosphere containing 5% CO_2. Cell proliferation and mitochondrial function were evaluated after 72 h. Apoptosis was detected after 48 h of stimulation.

Cell proliferation. The cellular proliferation of HeLa, SiHa and C33A cells was assessed using the xCELLigence platform (Roche Applied Science, Penzberg, Germany), which measures in real time the number of cells attached to the bottom of a modified 96-well plate. Once the optimal conditions to study the behavior of the cell lines were determined, the effects of stimulation with E2 and 60 kDa PRL were analyzed separately or in combination. Briefly, cells at a density of $0.25x10^4$/well were introduced into the xCELLigence reading station, and after 4 h they were stimulated. The proliferation rate was assessed in real time every 30 min over a period of 72 h. HaCaT cells were also included as a negative control of neoplasia. Cisplatin stimulation was used as a cell death control. Three independent experiments were performed with three replicates in each case.

Apoptosis detection. HeLa, SiHa, C33A and HaCaT cells were seeded at a density of $2.5x10^6$ cells/well in 1.5 ml charcoal stripped fetal bovine serum in 6-well plates. After 24 h, they were stimulated with E2, 60 kDa PRL or both. In addition, the effects of these hormones on cisplatin-induced death were evaluated. The percentage of positive cells was determined in 48 h by flow cytometry using an Attune® Acoustic Focusing Cytometer (Thermo Fisher Scientific, Inc.) and the

Annexin V-FLUOS staining kit (cat. no. 11988549001; Roche Applied Science), according to the manufacturer's protocol. Results were analyzed using FlowJo V10-1r5 (FlowJo, LLC, Ashland, OR, USA) and reported as geometric mean fluorescence intensity. Each sample was tested three times in three independent experiments.

MTT assay. MTT reduction is one of the most useful methods for measuring cell metabolism through the evaluation of mitochondrial activity (32). Cells were stimulated with E2, 60 kDa PRL or a combination of both, during 72 h. Following stimulation, MTT was added at a proportion of one-tenth of the final medium volume, and cells were incubated for 4 h at 37˚C in an atmosphere containing 5% CO_2. The supernatant was removed from the well and was replaced with HCl-isopropanol solution, in order to dissolve the formed crystals. The results were analyzed by spectrophotometry and absorbance at 570 nm was measured.

HPV 16 and HPV 18 E6 and E7 cloning. E6 and E7 open reading frames were amplified from genomic DNA obtained from the biopsies of patients infected with HPV 16 or HPV 18 in a previous study (33). Polymerase chain reaction (PCR) was performed using the Expand High Fidelity PCR system kit (cat. no. 11 732 650 001; Roche Applied Science) using the following primer pairs: HPV 16 E6, forward 5'-CAGACATTT TATGCACCAAA-3', and reverse 5' CTCCATGCATGATTA CAGC 3'; HPV 16 E7, forward 5'-TAGAGAAACCCAGCT GTAATCA-3', and reverse 5'-AGGATCAGCCATGGTAGA TTAT-3'; HPV 18 E6, forward 5'-AATACTATGGCGCGC TTTGA-3', and reverse 5'-TTGCCTTAGGTCCATGCATAC T-3'; and HPV 18 E7, forward 5'-CGCAGAGAAACACAA GTATAAT-3' and reverse 5'-GATCAGCCATTGTTGCTT A-3'. The four amplified products were independently cloned into a pGEM-T Easy cloning vector (cat. no. A1360; Promega Corporation, Madison, WI, USA) and were sequenced with M13 forward and reverse primers (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cloned ORF sequences were corroborated with the reference sequences reported in GenBank (HPV 16, accession no. K02718; HPV 18, accession no. AY262282; https://www.ncbi.nlm.nih.gov/genbank/); only HPV 16 E6 exhibited a substitution described in the AF402678 HPV 16 sequence (268T>G). Finally, the ORFs were subcloned into a pLVX-Puro lentiviral expression vector (cat. no. 632164; Clontech Laboratories, Inc., Mountain View, CA, USA) using *Eco*RI restriction.

Lentivirus production and HaCaT cell infection. To produce the lentivirus, Lenti-X 293T cells (cat. no. 632180; Clontech Laboratories, Inc.) were used. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (10,000 U/ml and 10,000 μg/ml, respectively); cells ($4x10^6$) were seeded in a 100 mm tissue culture plate, incubated for 24 h and independently transfected with pLVX-Puro empty vector, pLVX-HPV 16 E6, pLVX-HPV 16 E7, pLVX-HPV 18 E6 or pLVX-HPV 18 E7 vectors, using Lenti-X HTX Packaging system from the Lenti-X Lentiviral Expression system (Clontech Laboratories, Inc.). After 48 h post-transfection, infectious viral particles were collected from the cell supernatant, and the presence of the virus was determined using Lenti-X GoStix (Clontech Laboratories, Inc.). HaCaT keratinocytes were individually infected with 100 μl each viral supernatant, and selection of transduced cells was conducted with 1 μg/ml puromycin. Following RNA extraction from the established cell lines, E6 and E7 expression levels were determined with reverse transcription-quantitative PCR (RT-qPCR).

RNA isolation and RT-qPCR. Total RNA was extracted from the cell lines and biopsies using the RNeasy Plus Mini kit (Cat. 74136; QIAGEN Mexico, S. de R.L. de C.V., San Ángel, Mexico), according to the manufacturer's protocol. RNA was quantified by measuring the absorbance at 260/280 nm. Subsequently, 5 μg total RNA was used to generate cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) primed with Oligo dT, according to the manufacturer's protocol. cDNA was used to confirm E6 and E7 expression using the LightCycler® FastStart DNA Master PLUS SYBR Green I kit on a LightCycler 2.0 instrument (Roche Applied Science), according to the manufacturer's protocol. Primers used to amplify E6 and E7 were the as those used for cloning. To normalize qPCR reactions, β-actin-expression was assessed using the following primer sequences: Forward, 5'-TCCGCAAAGACCTGTACG-3' and reverse, 5'-AAGAAA GGGTGTAACGCAACTA-3'. Thermocycling conditions were as follow: One denaturation step at 95˚C for 10 min, followed by an amplification program of 35 cycles consisting of the following steps: 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 12 sec, followed by a final extenstion step at 72˚C for 10 min. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ algorithm (34).

Statistical analysis. Statistical assessment was conducted using SPSS Statistics 20 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) statistical software. Data obtained from three independent tests were analyzed using one-way analysis of variance with Bonferroni correction in SPSS. The data are presented as the means ± standard error of the mean. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of ERα, ERβ and PRLR in cervical epithelia. To analyze the expression levels of ERα, ERβ and PRLR, immunohistochemistry was performed on premalignant lesions and UCC tissues. The mean age for patients with dysplasia was 28.16 years, and 33 years for patients with cancer. Three squamous cell carcinoma samples (20%) did not express ERα. Of the samples negative for PRLR expression, there were two UCC samples (13.3%), two CIN III samples (16.6%) and three CIN I samples (23.1%). All of the samples were positive for ERβ (Table I).

A total of 10 representative samples from each of the four groups were selected to analyze the co-expression of the receptors, using five similar fields for each lesion. The magnified views

Table I. Expression of ERs, PRLR, Ki67 and Bcl-2 in intraepithelial lesions and in cervical cancer tissues.

	Histopathological diagnosis				
				Invasive carcinom	
Positive protein expression	CIN I	CIN II	CIN III	Adenocarcinoma	Squamous cell carcinoma
ERα (%)	100	100	100	100	80.0
ERβ (%)	100	100	100	100	100
PRLR (%)	76.9	100	83.4	100	86.7
Ki67 (%)	84.6	91.6	100	100	100
Bcl-2 (%)	-	-	83.3	75.0	-
Total (n)	13	12	12	4	11

Bcl-2, B-cell lymphoma 2; ER, estrogen receptor, PRLR, prolactin receptor.

of dysplastic and neoplastic epithelium are presented in Fig. 1A; ERα nuclear expression was most noticeable in the neoplastic tissue. The mean optical densities of ERα positive expression in CIN I (155.7), CIN II (277.2), CIN III (321.0) and cancer (481.0) samples were gradually increased as the lesion progressed toward malignancy; however, there were no significant differences between the CIN III and CIN II groups. Similarly, as shown in Fig. 1B, a progressive increase in the nuclear positive expression of ERβ was observed from CIN I to cancer; these increases were statistically significant in all groups (mean optical densities, 103.7, 206.7, 315.4 and 461.6; $P<0.05$).

As shown in Fig. 1C, in UCC samples, PRLR expression was increased compared with in the dysplastic epithelium and there was a significant increase in positive expression from CIN I to cancer (mean optical densities, 184.2, 420.0, 622.0 and 942.1; $P<0.05$). Immunostaining was mainly diffusely localized in the cytoplasm; however, in five cancer samples (50%) it was also evident at the nuclear level.

Expression of Ki67 and Bcl-2 in cervical epithelia. The present study detected Ki67 and Bcl-2 protein expression, in order to evaluate the proliferative and anti-apoptotic nature of the premalignant lesions and UCC tissues. Nuclear Ki67 positive expression was considerably higher in neoplastic epithelium (mean optical density: 394.10 in UCC vs. 69.0, 153.50 and 193.80 in CIN I, CIN II and CIN III, respectively; $P<0.05$); however, it cannot be confirmed that the expression pattern gradually increased as the degree of dysplasia advanced to cancer, since there were no statistically significant differences between the CIN II and CIN III groups (Fig. 2A).

Only five samples (8.8%) were positive for Bcl-2 (three adenocarcinomas and two CIN III samples), which is an intracellular membrane protein that prevents apoptotic cell death (30). The immunostaining of Bcl-2 was cytoplasmic and was localized predominantly in the basal layer. The images presented in Fig. 2B are examples of the negative Bcl-2 staining detected in most lesions.

Evaluation of cell proliferation in real time. To determine whether exogenous E2 and 60 kDa PRL, individually or in combination, are required for the proliferation of UCC, analyses were performed on HeLa, SiHa, C33A and HaCaT cells using the xCELLigence system RTCA biosensor instrument. The cell index curves revealed several important details about the kinetics of initial adhesion and cell survival. Only stimulation with 60 kDa PRL had a significant effect on SiHa cell proliferation ($P=0.04$; Fig. 3). In addition, in SiHa cells, E2 alone and E2 combined with PRL tended to increase cisplatin-induced death; however, only treatment with a combination of both hormones reached statistical significance ($P=0.03$; Fig. 3). The proliferation of HeLa and C33A cells was not significantly affected in response to any of the hormones. Both SiHa and HeLa cells began to proliferate at ~33 h, whereas C33A cell proliferation began at 11 h. Conversely, in HaCaT cells, E2 and 60 kDa PRL significantly increased the proliferative index ($P=0.015$ for E2, $P=0.003$ for PRL and $P=0.01$ for the combination; Fig. 3). In addition, the combination decreased cisplatin-induced cell death, and proliferation began before 11 h.

Evaluation of apoptosis. Annexin V-FITC/PI staining was examined by flow cytometry to investigate the induction of apoptosis by E2 and 60 kDa PRL in SiHa, HeLa, C33A and HaCaT cells (Fig. 4). Cell apoptosis was defined as Annexin V^+ or Annexin V^+/PI^+.

Hormones did not have a direct impact on the percentage of apoptosis in SiHa and HeLa cells. Only E2 stimulation increased the percentage of apoptosis in SiHa cells in the presence of cisplatin, either when it was used individually ($P=0.006$) or in association with 60 kDa PRL ($P=0.035$). Conversely, C33A cells experienced a significant reduction in the apoptotic index in response to E2 ($P=0.024$) and combination treatment ($P=0.006$). HaCaT cells exhibited similar behavior to C33A in response to E2 ($P=0.007$) and combination treatment ($P=0.006$). However, only in the HaCaT cell line did combination treatment confer a protective effect against the cytotoxic effects of cisplatin ($P=0.01$).

The proportion of necrotic cells in the UCC and HaCaT cell lines was also compared. E2 combined with 60 kDa PRL reduced necrosis in SiHa ($P=0.003$), HeLa ($P=0.04$) and C33A ($P=0.006$) cells. Conversely, the necrosis index in HaCaT cells was not affected by these treatments (data not shown).

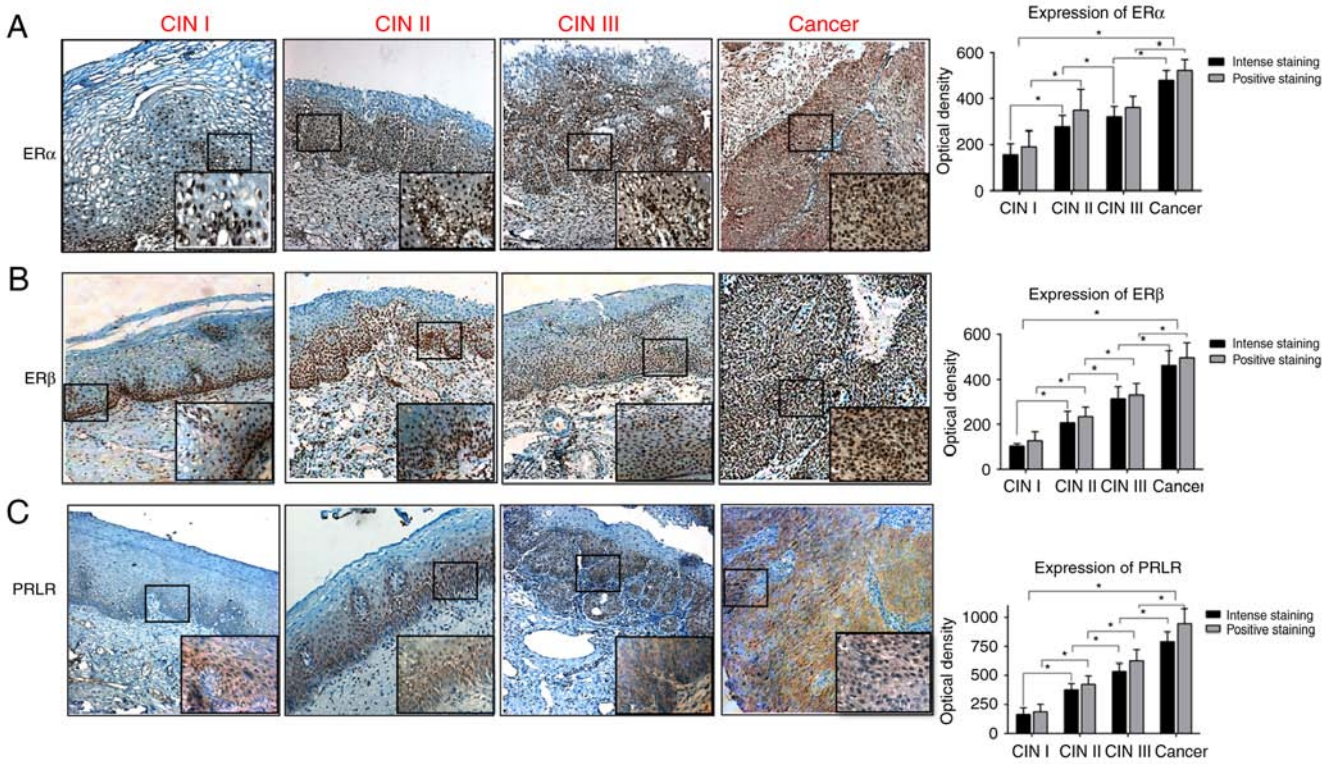

Figure 1. Expression of ERα, ERβ and PRLR in precursor lesions and cervical cancer tissues. (A) ERα, (B) ERβ and (C) PRLR expression levels were detected using immunohistochemistry; brown coloration indicated positive staining. The expression of the receptors increased as the lesion progressed. Left panels, images shown are representative of five fields (magnification, x10; magnification inset images, x40). The small boxes represent the magnified regions. Right panels, epithelial distribution of receptor expression in samples from each pathological grade. Samples in which cellular optical density was very high for the expression of the protein were characterized as intense staining, whereas positive samples regardless of density were characterized as positive staining. *P<0.05 (analysis of variance). CIN, cervical intraepithelial neoplasia; ER, estrogen receptor; PRLR, prolactin receptor.

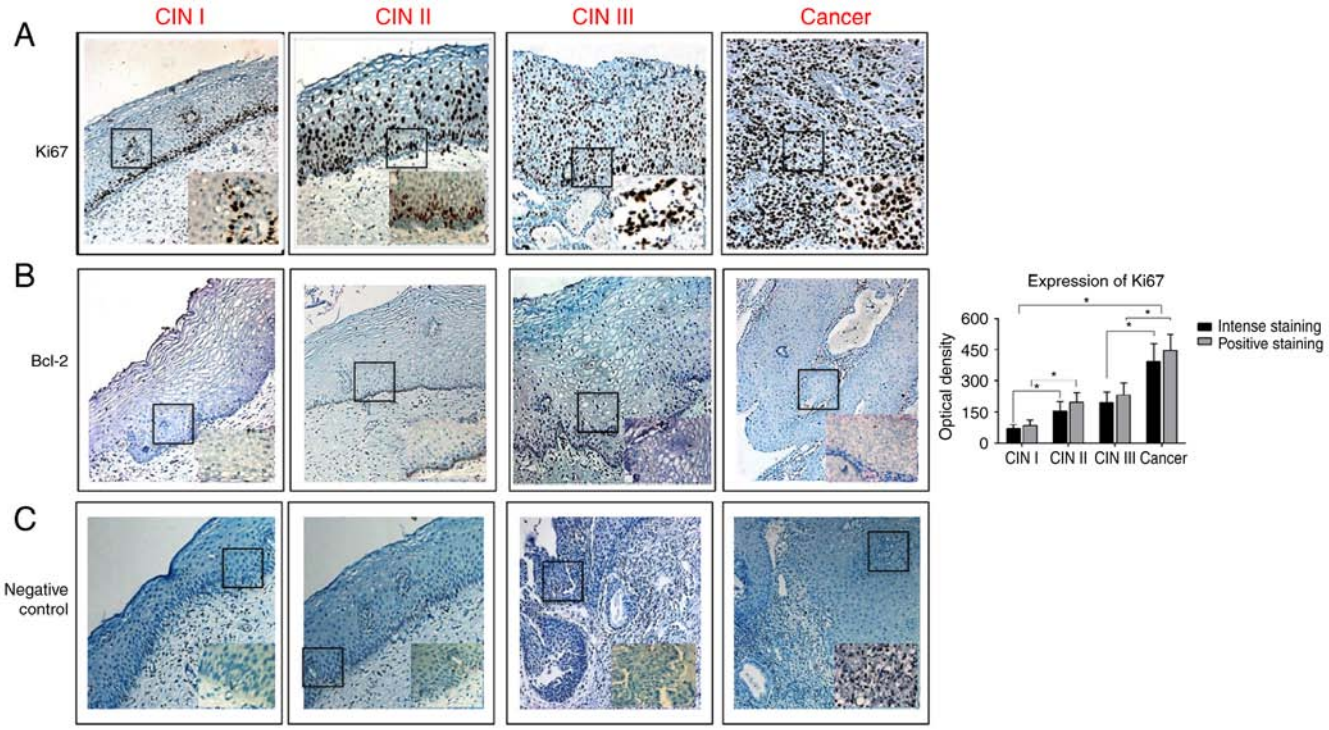

Figure 2. Expression of Ki67 and Bcl-2 in the precursor lesions and cervical cancer tissues. (A) Ki67 and (B) Bcl-2 expression levels were detected using immunohistochemistry; brown coloration indicated positive staining. Only Ki67 expression increased as the lesion progressed. Left panels, images shown are representative of five fields (magnification, x10; magnification inset images, x40). The small boxes represent the magnified regions. (C) Images of the negative control, in which primary antibodies were omitted, are shown. Right panels, epithelial distribution of Ki67 expression in samples from each pathological grade. Samples in which cellular optical density was very high for the expression of the protein were characterized as intense staining, whereas positive samples regardless of density were characterized as positive staining. *P<0.05 (analysis of variance). Bcl-2, B-cell lymphoma 2; CIN, cervical intraepithelial neoplasia.

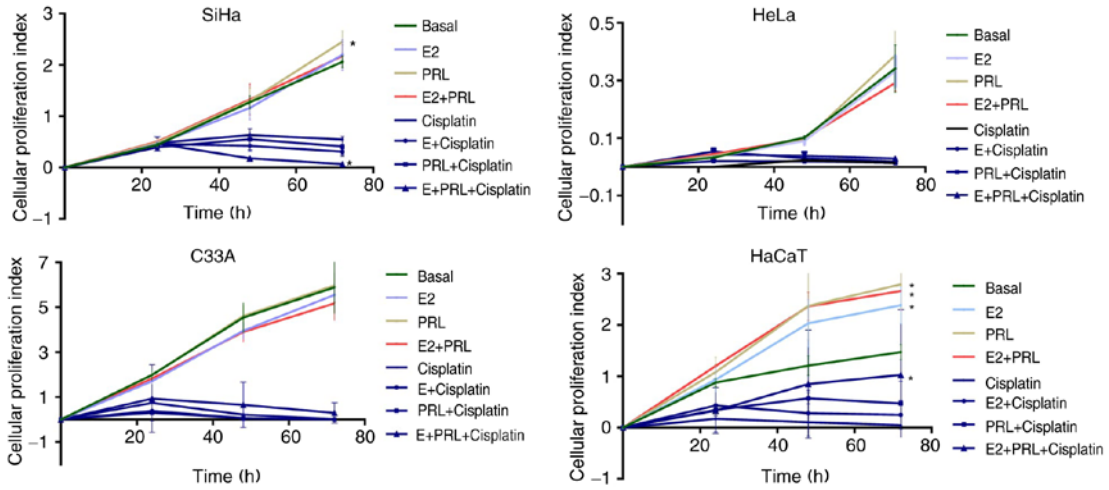

Figure 3. Effects of stimulation with E2 (10 nM) and 60 kDa PRL (200 ng/ml) on the proliferation of cervical cancer cells. The proliferation of SiHa, HeLa, C33A and HaCaT cells, and the effects of the hormones on cisplatin (1 μg/ml)-induced cell death were assessed using the xCELLigence platform for 72 h. Only stimulation with 60 kDa PRL significantly increased proliferation of SiHa cells. In addition, in SiHa cells, E2 alone and E2 combined with 60 kDa PRL decreased the proliferative index in the presence of cisplatin. All results were confirmed in triplicate in three independent experiments under the same experimental conditions. *P<0.05 (analysis of variance). E2, 17β-estradiol; PRL, prolactin.

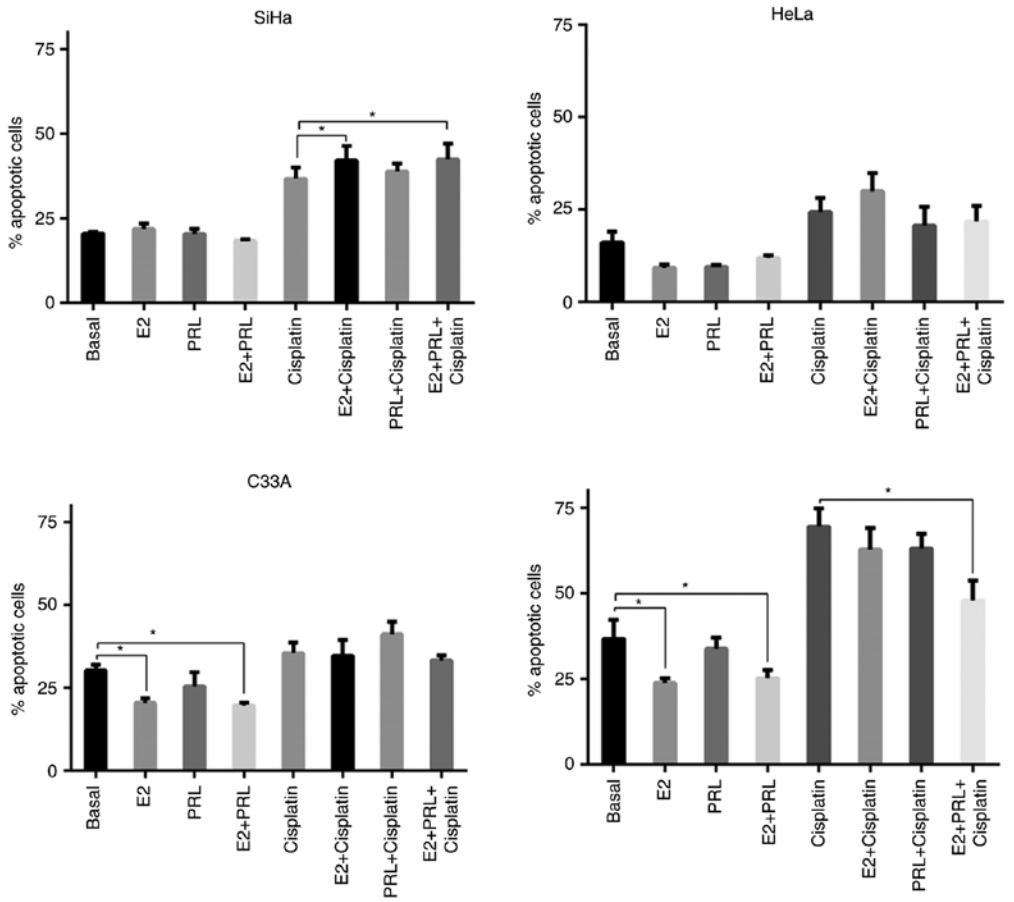

Figure 4. Effects of stimulation with E2 (10 nM) and 60 kDa PRL (200 ng/ml) on cisplatin (1 μg/ml)-induced apoptosis of SiHa, HeLa, C33A and HaCaT cells during 48 h. Only in SiHa cells did E2 alone or E2 combined with 60 kDa PRL increase the percentage of apoptosis in the presence of cisplatin. Annexin V-fluorescein isothiocyanate/propidium iodide staining was examined by flow cytometry. All results were confirmed in triplicate in three independent experiments under the same experimental conditions. Data are presented as the means ± standard error of the mean. *P<0.05 (analysis of variance). E2, 17β-estradiol; PRL, prolactin.

Determination of cellular metabolism. To understand the possible roles of estrogen and 60 kDa PRL in cervical carcinoma, mitochondrial activity was assessed by the MTT assay. The energetic metabolism of HeLa and SiHa cells was significantly increased 72 h after stimulation with E2 (P=0.003 and P=0.052, respectively). The same effect was observed in

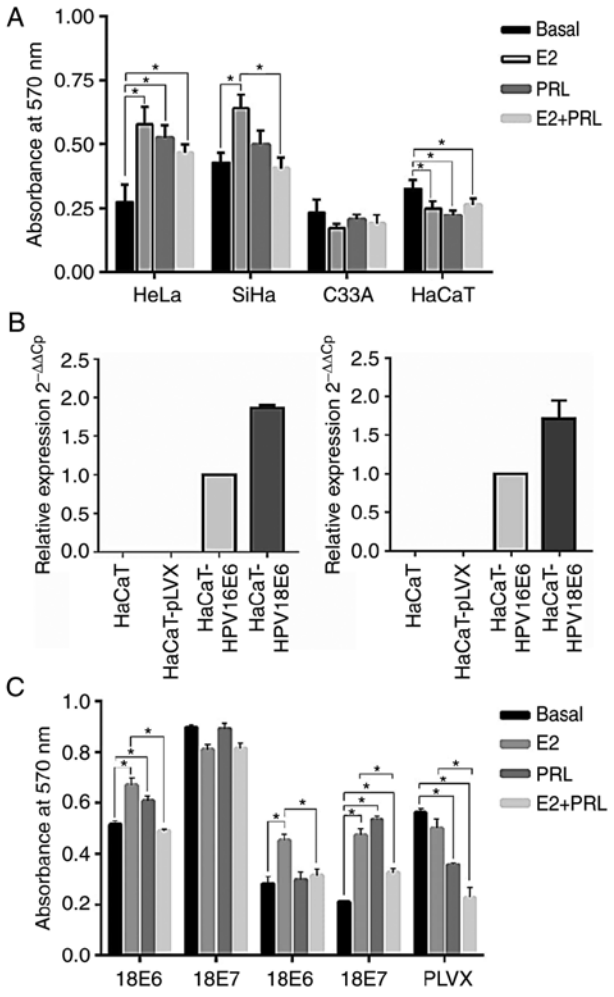

Figure 5. Effects of stimulation with E2 (10 nM) and 60 kDa PRL (200 ng/ml) on the metabolism of cervical cancer cells, and on HaCaT cells transduced with E6/E7 viral oncogenes from HPV 16 and 18 during 72 h. (A) Effect of stimulation on HeLa, SiHa, C33A and control HaCaT cells. Hormones produced an increase in the metabolism of HeLa cells, but only E2 had the same effect on SiHa cells. (B) Evaluation of E6 and E7 oncogene expression in HaCaT cells transduced with pLVX vector alone, E6 or E7 from HPV 16 and HPV 18. Relative expression was calculated using HPV 16 E6 expression as calibrator and β-actin expression as a reference gene; the same approach was used to calculate the relative expression of E7, using E7 from HPV 16 expression as calibrator. (C) Effects of hormonal stimulation on HaCaT cells transduced with E6/E7 viral oncogenes from HPV 16 and HPV 18. Hormones increased cellular metabolism in HaCaT cells transduced with HPV 18 E6 and HPV 16 E7 viral oncogenes. E2 accelerated mitochondrial function in HaCaT cells transduced with HPV 16 E6. Graphs show the results of experiments performed in triplicate, repeated at least three times. *P≤0.05 (ANOVA test). 18 E6 and 18 E7, HaCaT cells transduced with E6 and E7 from HPV 18, respectively; 16 E6 and 16 E7, HaCaT cells transduced with E6 and E7 from HPV 16, respectively; pLVX, HaCaT cells transduced with empty vector; E2, 17β-estradiol; PRL, prolactin.

HeLa cells in response to 60 kDa PRL and combination treatment (P=0.004 and P=0.007, respectively). Treatment of C33A with the hormones had no significant effect, whereas in HaCaT cells it produced a decrease in mitochondrial activity (P=0.033, P=0.02 and P=0.05 for E2, 60 kDa PRL and combination treatment, respectively. Notably, in HeLa and SiHa cells, 60 kDa PRL exhibited a tendency to modulate the effects produced by E2; however, only in SiHa cells did it reach statistical significance (P=0.05). This modulation was not evident in C33A and HaCaT cells (Fig. 5A).

In the present study, HaCaT cells were transduced with the viral oncogenes HPV 18 and 16 E6 and E7 followed by hormonal stimulation. As shown in Fig. 5B, the mRNA expression levels of HPV 16 and 18 E6 and E7 oncogenes were increased in response to transduction. Stimulation with E2 and 60 kDa PRL resulted in increased cell metabolism in HaCaT cells transduced with the HPV 18 E6 viral oncogene (P=0.01 and P=0.042, respectively; Fig. 5C). The modulating effects of 60 kDa PRL on estrogen were evident (P=0.003; Fig. 5C). No change from the baseline was observed in HaCaT cells transduced with the HPV 18 E7.

In HaCaT cells transduced with the E6 and E7 oncogenes of HPV 16, E2 stimulation produced a significant increase in cellular metabolism (P=0.02 and P=0.002; respectively). Only in cells transduced with HPV 16 E7, did 60 kDa PRL exhibit an E2-like effect (P=0.001; Fig. 5C). In addition, modulatory effects of PRL on E2 in HaCaT cells transduced with HPV 16 E6 and E7 were demonstrated (P=0.02 and P=0.001, respectively). Notably, HaCaT transduced only with PLVX viral particles only exhibited a similar behavior to untransduced HaCaT cells; hormonal stimulation decreased cellular metabolism.

Discussion

Previous studies have suggested that estrogen exposure may be a critical factor for the development of UCC (5-10); however, the participation of estrogen and its role in the control of certain processes, such as proliferation, apoptosis, migration and metastasis, remains to be elucidated.

The cervical carcinogenic effects of estrogen can be explained by analyzing its possible synergism with the infection of epithelial cells by HPV, which is the primary risk factor for the development of this type of cancer. HPV 16 contains a transcription enhancer that is activated by cellular elements other than the viral E2 protein, which resides on a 232-bp segment that contains one binding site for steroid receptors (35). In the genome of HPV 16, seven regions of high resemblance to ER sequences have been described (36,37). Epidemiological studies have revealed an elevated risk of UCC among HPV-positive women who had used an oral contraceptive medication containing estrogen (11-13).

In this study, a gradual and significant increase in the expression of ERα and ERβ was detected as the degree of dysplasia progressed to the tumor phenotype. The role of ERs in the pathogenesis of UCC is inconclusive due to a large number of contradictory results. For example, *in vivo* studies have significantly demonstrated the requirement of ERs in HPV-associated cancer of the cervix (6,38,39), whereas the analysis of cervical samples remains open to question (9,17,40).

Chung *et al* (2008) revealed that K14E7 transgenic mice expressing HPV16 E7 develop UCC after prolonged treatment with physiological levels of exogenous estrogen and ERα serves a crucial role in this outcome (38). In addition, it has been observed that inhibition of ERα is effective in treating and preventing UCC in mice (39). In contrast to ERα-sufficient HPV transgenic mice, ERα-deficient HPV transgenic mice fail to develop UCC when treated with estrogen; notably, estrogen-treated ERα-null HPV transgenic mice do not develop any aspect of the progressive disease leading to UCC (6,38).

Immunohistochemistry has also been used to investigate the prognostic significance of ERα; a previous study reported that the positive expression of ERα in UCC is 68.18%, which is significantly higher compared with in the normal cervix (35%) and in chronic cervicitis (50%). Furthermore, ERα in carcinoma was revealed to be associated with HPV infection, the depth of infiltration and the presence of lymphatic metastasis (40). These results demonstrated that cervical neoplastic cells express ERα and suggested that estrogens could regulate some tumor functions via this receptor.

Previous studies have also revealed that ERα in UCC is decreased to undetectable levels, at the protein and mRNA level, compared with the precursor lesions (8,17). Furthermore, another study did not detect ERα protein expression in any of the analyzed invasive cervical carcinoma samples; however, significant mRNA expression levels of ERα were detected in the same samples. In this previous study, immunopositivity for ERβ protein was detected in 70.6% of UCC samples; however, the staining was almost exclusively cytoplasmic (41). ERβ was not detected in UCC by den Boon *et al* (17). Therefore, evidence of the presence of ERs in human cervical neoplasms remains limited in nature and inconclusive.

The physiological interplay between estrogens and PRL has been well tested; however, the impact in cancer has been poorly explored. E2 directly induces extrapituitary PRL gene expression in breast cancer cells, and a functional noncanonical ERE and an AP1 site are located in the PRL distal promoter, which E2 can act (42,43). In the present study, similar to that observed for ERα and ERβ, PRLR expression was increased in UCC. In 10 samples from each group (CIN I, II, III and UCC) ERs and PRLR expression was evidenced in the same histopathological sections, leading to the hypothesis that these receptors are co-expressed.

It has previously been reported that PRLR expression is significantly increased in UCC compared with in normal tissue and precursor lesions (19). The internalization of PRLR and its translocation to the nucleus has been the subject of intense debate, due to conflicting results (44). The present experiment is in favor of the nuclear translocation of PRL complexed with its receptor, due to evidence of the presence of PRLR in the nucleus in 50% of UCC samples.

Ki67 is a nuclear and nucleolar protein that is expressed only in the active phases of the cell cycle (G_1, S, G_2 and M phases) but not in the resting phases (G_0 and early G_1). In the present study, Ki67 expression in the samples was directly associated with severity of the cervical lesions; this finding was similar to the results of a previous study, which detected Ki67 in ~100% of UCC specimens (43). Although overexpression of Ki67 is correlated with high cellular proliferation (45), in a systematic review, Kisser and Zechmeister-Koss (2015) concluded that evaluation of Ki67 could not be recommended for the triage of women with atypical squamous cells of indeterminate significance or low-grade squamous intraepithelial lesions cytology, due to insufficient high-quality evidence (46).

In the present study, low Bcl-2 expression was evidenced. Although it has been suggested that Bcl-2 expression is strongly associated with the development of invasive cervical disease (30,31), other studies have reported that Bcl-2 positivity confers a better 5-year survival rate and prognosis (47). Shukla *et al* (2014) reported that Bcl-2 expression is much lower in UCC compared with in premalignant lesions (48).

Our previous study discovered the presence of a PRL isoform with a molecular weight of ~60 kDa in the protein extracts and supernatants of HeLa, SiHa and C33A cells, which was absent in HaCaT cells (18). Due to the relevance that has recently been conferred to local PRL in the pathophysiology of some tumors, its bioactivity was initially determined (25). Subsequently, cells were stimulated with this PRL isoform, in order to evaluate the effects of its use separately and in combination with E2 on the mechanisms underlying cell survival and metabolism.

The present study revealed that E2 and 60 kDa PRL did not modulate the proliferation of most cell lines derived from UCC; only in SiHa cells did stimulation with 60 kDa PRL have a significant effect. Nair *et al* reported that treatment with 10^{-9} mol/l E2 enhances cellular proliferation of C33A, Caski, HT3 and C4I cells; however, it was suggested that local estrogen formed by high aromatase activity may be responsible for this outcome (49). Differences between the two studies may have been reported due to the different methods used to measure proliferation. In addition, the effects induced by estrogens vary depending on cell type, the used concentration and the time of application. In the present study, SiHa and HeLa cells entered the proliferative phase at ~33 h, whereas in HaCaT cells, proliferation began earlier. In HaCaT cells, treatment with the hormones increased proliferative index.

Previous studies have supported the pro-proliferative, anti-apoptotic and pro-metastatic roles of PRL in the progression of some tumor types. The particular PRL response depends on factors such as the intensity of the expression of PRLR and other molecules acting as collaborators (43,50-52). In UCC, hPRL does not affect proliferation; however, it decreases etoposide-induced cell death; this effect is mediated by the phosphorylation of STAT3 (18,20). Recently, 60 kDa PRL, which was isolated from the supernatant of HeLa cells, was revealed to induce both responses (25).

The combination of E2 and 60 kDa PRL was evaluated in the present study, in order to determine if it has any impact on the mechanisms of programmed cell death. In addition, the present study aimed to determine if the presence of these hormones modifies the response of tumor cells to cisplatin treatment. Only in C33A cells, did the use of E2 separately and with 60 kDa PRL produce a decrease in the percentage of apoptosis. Notably, this cell line is not infected by HPV; therefore, this may explain the different behavior in comparison to HeLa and SiHa cells, in which no effects were shown. Regarding the impact on cisplatin-induced cell death, in SiHa cells, cotreatment with E2 and 60 kDa PRL resulted in increased sensitivity of these cells to the chemotherapeutic agent, whereas in HaCaT cells, this cotreatment had a protective effect.

Previous studies have reported the synergism between estrogen and PRL in the promotion of breast cancer (26-28). Conversely, in the present study, a synergistic effect of these hormones on the progression of UCC through the modulation of proliferation and apoptosis was not identified.

Another critical feature of cancer is the alteration in cellular metabolism to favor the uncontrolled growth of cells (53). HeLa cells treated with E2 and 60 kDa PRL, either individually or in combination, exhibited an increase in mitochondrial

activity. In addition, in SiHa cells, stimulation with E2 had a similar result. In addition, HaCaT cells were transduced with HPV 16 and 18 viral E6 and E7 oncogenes; HaCaT cells transduced with HPV 18 E6 possessed similar behavior to HeLa cells, although E2+PRL had no effect on HaCaT cells transduced with HPV 18 E6. Similar to SiHa cells, E2 stimulation increased the metabolism of HaCaT cells transduced with HPV 16 E6/E7 oncogenes.

The effects of E2 on SiHa and HeLa cells, as well as in HaCaT cells transduced with E6 oncogene of HPV 18 and with E6 and E7 oncogenes of HPV 16 can be explained by the important role of this hormone within mitochondria. Estrogens have been reported to exert direct and indirect effects on mitochondrial function, and co-localization of ERα and/or ERβ with the mitochondria has been reported (52). Estrogens can regulate mitochondrial morphology, biogenesis, bioenergetics and mitochondrial genome transcription, among other functions, in a cell-specific manner (54-56).

The progression of UCC has been associated with an increase in viral oncogene expression in HPV-positive lesions in patients (57). Estrogens can transactivate viral oncogenes; ERα activated by estradiol can bind to responsive elements within the long control region of the HPV genome and can increase E6 and E7 transcription (36,58,59). This can profoundly affect cell biology, for example by inducing metabolic reprogramming. Zeng *et al* demonstrated that E6/E7 expression induces aerobic glycolysis, also known as the 'Warburg effect'. This induction is mediated by c-MYC, which elevates hexokinase-II, the rate-limiting enzyme responsible for the first step in the glycolytic pathway (60). In addition, it has been reported that O-linked GlcNAcylation (O-GlcNAc) and O-GlcNAc transferase are markedly increased in HPV-induced cervical neoplasms and mouse embryonic fibroblasts transduced with HPV 16 oncogene E6 or E6/E7 (61). It has been proposed that O-GlcNAc is a nutrient sensor that modulates some cellular pathways in response to metabolic regulation (62). Hypoxia in cancer has been associated with the metabolic shift towards aerobic glycolysis, and the ability of the E7 oncoprotein to enhance the activity of hypoxia-inducible factor 1α has been demonstrated (63,64). The present results suggested that the relationship of viral oncogenes E6/E7 with estrogen and PRL may influence metabolic pathways in UCC.

The hypothesis that PRL may regulate metabolic genes is more difficult to explain. Previous studies have suggested that PRL regulates citrate production of prostate epithelial cells, and AP-1 mediates PRL regulation of mitochondrial aspartate aminotransferase expression, which is an important protein in the metabolic pathway for citrate production in the prostate (65,66).

Estrogens and PRL can cooperate in various physiological processes; however, they can also have antagonistic functions (67). In the present study, it was revealed that in SiHa and HeLa cells, and in HaCaT cells transduced with the viral oncogene E6 of HPV 18 and the viral oncogenes E6/E7 of HPV 16, 60 kDa PRL tended to negatively modulate the effects of E2 on metabolism. One possible explanation for this result is the differential roles of PRL stimulation on ERα and ERβ transcription; PRL upregulates the mRNA expression levels of both ERs in a context- and dose-dependent manner via the Janus kinase 2-STAT5 pathway. Either STAT5a or STAT5b stimulates ERα transcription, whereas only STAT5b stimulates ERβ (68,69). Different roles have been postulated for both ERs in various cancer models (70). It is unknown if in UCC 60 kDa PRL regulates the effects of estrogen through ERβ. It would be interesting to evaluate in future studies the role of 60 kDa PRL in the modulation of ERs in UCC, and the potential impact on the pathophysiology of the disease.

The MTT assay has been extensively used to measure cell proliferation and survival capacities. In the present study, the different findings obtained from the xCELLIGENCE platform and the MTT assay may seem contradictory. Other studies have also reported differences in cell number determination using metabolism-based assays and other methods (71-73). For example, Wang *et al* demonstrated a significant difference in the determination of the antiproliferative activity of green tea polyphenols when using MTS-based assays in comparison to ATP- and DNA-based methods and the trypan blue assay (73). The present results coincided with those of other studies, with regards to the need for careful selection of the methods used for *in vitro* evaluation of cell proliferation.

As aforementioned, several studies have analyzed the association between estrogens and PRL with regards to cancer progression and metabolism; however, to the best of our knowledge, this is the first study to evaluate this hormonal correlation in UCC, particularly using an isoform of PRL that is secreted by tumor cells.

In conclusion, the present study revealed that ERs and PRLR are co-expressed in UCC and their precursor lesions; in addition, they are associated with the severity of injury. However, the individual and combined stimulation of estrogen and PRL under the conditions used in the present study did not exhibit a significant impact on proliferation/apoptosis in UCC cell lines. However, cellular metabolism, as evaluated through mitochondrial succinate dehydrogenase enzyme activity, was elevated in HeLa cells treated with both hormones individually or in combination, and in SiHa cells stimulated with E2. In the HaCaT cell line transduced with HPV 16 E7 and 18 E6 viral oncogenes, E2 and 60 kDa PRL individually increased cellular metabolism; however, although only in HaCaT cells transduced with HPV 16 E7 did the combination affect metabolism. Furthermore, E2 accelerated mitochondrial function in HaCaT cells transduced with HPV 16 E6.

Some histopathological specimens used in the present study had no information available regarding HPV infection; this information may assist further in interpretation of the immunohistochemical findings. In addition, no information regarding clinical variables was available, which may have allowed for analysis of the correlation between these data and the histopathological expression of hormone receptors. A further prospective study with more appropriate and accurate data should be conducted, in order to understand how HPV infection modulates hormone receptors to initiate and maintain the process of cervical carcinogenesis.

Acknowledgements

The authors would like to thank the histopathology technicians at the Pathological Anatomy Service of the Civil Hospital of Guadalajara Fray Antonio Alcalde (Guadalajara, México) for their assistance.

Funding

The present study was partially supported by the Consejo Nacional de Ciencia y Tecnología (grant no. 222205) and the Fondo de Investigación en Salud-IMSS (grant no. FIS/IMSS/PROT/PRIO/15/046 to LFJ-S).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ARL, ARDA, IGRL and CAI performed the experiments. JRDR and EILP were involved in sample collection and sample processing, and provided immunohistochemical assistance. PCOL and LFJS assisted in flow cytometry data analysis and DNA cloning. JGMB, SDTA, MMB and JFMV assisted in the interpretation of results and statistical analysis, and reviewed and edited the manuscript. ALPS designed the study, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Investigation and Biosecurity Committee of the University Center of Health Sciences at the University of Guadalajara (Reference Number C.I. 093/13 CUCS; Guadalajara, Mexico). Written informed consent was obtained from all patients included in the present study.

Patient consent for publication

Written informed consent was obtained from all patients included in the present study.

Competing interests

The authors declare that they have no competing interests.

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