The role of human papillomavirus type 16 E6/E7 oncoproteins in cervical epithelial-mesenchymal transition and carcinogenesis

YA-MIN CHENG[1,3*], CHENG-YANG CHOU[1], YI-CHIANG HSU[4,5], MING-JENN CHEN[6] and LIH-YUH C. WING[2*]

Departments of [1]Obstetrics and Gynecology, and [2]Physiology, [3]Institute of Clinical Medicine, College of Medicine, National Cheng Kung University; [4]Graduate Institute of Medical Science and [5]Innovative Research Center of Medicine, College of Health Sciences, Chang Jung Christian University, Tainan; [6]Division of Traumatology, Department of Surgery, Chi Mei Medical Center, Tainan, Taiwan, R.O.C.

Received August 19, 2011; Accepted November 29, 2011

DOI: 10.3892/ol.2011.512

Abstract. Cervical cancer is the most common malignancy in females worldwide. This study investigated the prevalence of the E6/E7 oncoproteins of human papillomavirus (HPV) type 16, which are important in fibroblast growth factor (FGF) 2- and 4-induced epithelial-mesenchymal transition (EMT) and cervical tumorigenesis. We investigated the functional interaction between HPV16 E6/E7-transfected Cx cells (CxWJ cells) and treatment with FGF2 and 4, according to the expression of α-smooth muscle actin (α-SMA), vimentin and E-cadherin protein as well as cell growth and invasive ability. The results showed the upregulation of α-SMA and vimentin and the downregulation of E-cadherin protein expression in CxWJ cells. HPV16 E6/E7 infection partially repressed proliferation, but not the invasive ability of FGF2 or FGF4 stimulation in cervical cancer cells (CxWJ cells). These data provide evidence of a functional interaction between HPV16 E6/E7 and FGFs 2 and 4, suggesting that cooperative stimulation of HPV E6/E7 and FGFs activated in human cervical cancer cells is required to completely overcome the oncogenic function associated with the development of cervical epithelial-mesenchymal transition and tumorigenesis.

Introduction

Cervical cancer is one of the most common forms of cancer in females and a leading cause of mortality among gynecological malignancies (1). In Taiwan, cervical cancer is the sixth most common cancer in females (2). The majority of females diagnosed with this cancer exhibit an advanced, widely disseminated malignancy and poor survival rate (3). Accumulating evidence has demonstrated that oncogenic types of human papillomavirus (HPV) are important in the development of precursors of cervical cancer (4). However, only a small fraction of females infected with HPV develop the disease, indicating the contribution of other factors to the progression of lesions in invasive cervical cancer (5).

Fibroblast growth factors (FGFs) are a family of structurally related polypeptides, comprising at least 23 members, commonly identified as the classical FGFs. The classical designations, acidic FGF and basic FGF, are now known as FGF1 and FGF2, respectively (6). FGFs are possible candidates for mitogenic factors (7). FGFs share between 35 and 50% amino acid sequence homology, acting as mediators in a diverse range of developmental and physiological processes, both *in vitro* and *in vivo* (8). FGF receptors (FGFRs) are tyrosine kinases possessing three extracellular immunoglobulin-like domains, a transmembranous region and a cytoplasmic split tyrosine kinase domain, which is activated upon FGF ligand binding (9). FGFs and their receptors are involved in the development of several human cancers (10). Altered protein expression levels of one or more of these receptors and ligands have been identified in cancer of the lung (11), kidney (12), colon (13), head and neck (14), breast (15) and prostate (16). FGF2 is a ubiquitous multifunctional regulator involved in the proliferation and differentiation of a broad spectrum of mesodermal cells (17). FGF4, which is expressed in the vicinity of the posterior endoderm in the gastrula and early somite stage embryos, exhibits broad anterior-posterior patterning activity in the gut endoderm. Specifically, FGF4 promotes posterior and inhibits anterior endoderm cell fate (18). The study of cervical cancer provides a good model for assessing the effect of this microenvironment on epithelial-mesenchymal transition (EMT) (19). Little is known about the role of FGFs in cervical cancer regarding the maintenance of normal cells and the progression to carcinogenesis (20).

In this study, we investigated whether HPV16 E6/E7 transfection contributes to FGF2- and 4-induced tumorigenesis in human malignant cervical cancer cells. The aim of the experiments was to develop the scientific basis required to provide technological support for cervical cancer therapy.

Correspondence to: Dr Ya-Min Cheng, Department of OB/GYN, College of Medicine, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan 704, Taiwan, R.O.C.
E-mail: chengym0687@gmail.com

*Contributed equally

Key words: epithelial-mesenchymal transition, cervical cancer, human papillomavirus, fibroblast growth factors

Materials and methods

Cell culture. Normal cervical epithelial cells (Epi) and cervical cancer cells were obtained from patients with benign uterine neoplasm, admitted to the Department of Obstetrics and Gynecology of the National Cheng Kung University (NCKU) Medical Center, Taiwan, to undergo surgery. The patients underwent total abdominal hysterectomy. Specimens were removed from only typical and clinically clear-cut (Grade II) cases.

Prior written informed consent was obtained from the patients and all procedures had been reviewed and approved by the ethics board at NCKU in adherence to the Declaration of Helsinki.

Cervix tissue was dissected following surgery and immersed in a culture medium for the preparation of normal cervical epithelial cells. The Cx cell is a cervical cancer cell line established by Professor Chou (21). The cells were obtained from a 48-year-old Taiwanese female with squamous cell carcinoma of the uterine cervix, characterized as HPV-negative and p53-mutation-negative. The CxWJ cell was established using a stable clone from Cx cells transfected with HPV16 E6 and E7 (1). Stroma (Str) and SiHa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained on culture dishes, in RPMI-1640 (Cx and CxWJ) or DMEM (Str and SiHa) supplemented with 10% (v/v) FBS. The cells were cultured in an incubator with an atmosphere of 95% air and 5% CO_2 at 37˚C.

Immunoblotting. Total cell lysate (30 and 50 μg) was separated using SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in PBS containing 0.1% Tween-20 (PBST) at room temperature for 1 h. The blots were incubated with primary antibody (E-cadherin, α-SMA, vimentin, β-actin) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2 h, washed with three exchanges of PBST for 30 min, incubated for 1 h with secondary anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase and then washed with three exchanges of PBST for an additional 30 min. The proteins were visualized using a chemiluminescence detection kit (ECL; Amersham Corp., Arlington Heights, IL, USA).

Invasive assay. The invasive assay of cells through type IV collagen was performed as previously reported (22). Briefly, modified Boyden chambers containing polycarbonate filters with 8-μm pores (Becton-Dickinson, Boston, MA, USA) were coated with 0.25 mg/ml type IV collagen (Sigma, St. Louis, MO, USA). Following incubation for 48 h, invaded cells were stained with 0.1% crystal violet solution and photographed using a QImaging Retiga EXi digital camera (Burnaby, BC, Canada) under a Leica DMIRE2 microscope. The number of invaded cells was then counted and subjected to statistical analysis.

Cell proliferation assay. Cells were seeded in culture plates at 5000 cells/well. Different cell wells were treated with 0, 25 and 100 μM FGFs (FGF2 and 4) for 24 h. The number of cells was determined microscopically using a hemocytometer. MTS dye (1 mg/ml) was added to each well for an additional 4 h following treatment. The reaction was stopped by the addition of sodium dodecyl sulfate and optical density was measured at 492 nm using a multi-well plate reader (Powerwave XS, BioTek, Winooski, VT, USA). Background absorbance of the medium was subtracted in the absence of cells. The samples were assayed in at least triplicate, and the mean was calculated for each experiment. Each assay was carried out in triplicate and the results are expressed as the mean (±SEM).

Statistical analysis. Data are shown as the mean (±SEM) of at least three separate experiments. Statistical analysis was performed using the t-test, with significance set at $P<0.05$.

Results

EMT is expressed between the Cx and CxWJ cells. Using an Olympus CKX41 microscope, the cell lines (Epi, Cx and CxWJ) were found to exhibit changes in cell morphology. In Fig. 1A, a microscopic examination revealed Cx cells with polygonal epithelial cell characteristics and CxWJ cells in the shape of a spindle. The expression of epithelial-mesenchymal cell markers was determined using western blotting. Compared with the Cx cells, the expression of E-cadherin protein was downregulated in CxWJ cells and Str cells (Fig. 1B). The expression of α-SMA and vimentin protein was upregulated in CxWJ cells. A similar pattern was observed in Str and SiHa cells. Taken together, these results suggest the occurrence of EMT between the Cx and CxWJ cells. HPV16 E6/E7 transfection therefore induces EMT in cervical epithelial cells.

HPV E6/E7 transfection repressed the number of CxWJ cells compared with Cx cells. To investigate the proliferation of FGF (FGFs 2 and 4) ligands in cervical cancer cell lines (Cx and CxWJ), the number of cells (Fig. 2A) and MTS analysis were used to monitor the reduction percentage of R-tetrazolium into R-formazan, which was quantified using a spectrophotometer at 492 nm (Fig. 2B). Fig. 2A shows that the number of Cx and CxWJ cells increased following treatment with the FGF2 and 4 ligands ($P<0.05$ vs. 0 ng/ml group). Transfection with HPV16 E6/E7 reduced cell growth in CxWJ cervical cancer cells ($P<0.01$ Cx vs. CxWJ cells FGFs 0 ng/ml). Similar results were observed with the 25 and 100 ng/ml treatment of FGFs (2 and 4). HPV E6/E7 transfection repressed the number of CxWJ cells compared with Cx cells ($P<0.05$ vs. Cx cells).

Treatment with FGF ligand (50 ng/ml) also enhanced cell growth in Cx and CxWJ cells ($P<0.05$ vs. SF group; Fig. 2B). As shown in Fig. 2B, the proliferation of CxWJ cells decreased following transfection with HPV16 E6/E7 ($P<0.05$ vs. Cx cells). Taken together, these results indicate that HPV16 E6/E7 transfection is important in the duplication of cervical epithelial cells.

FGF2 and 4 induce invasive activity via HPV16 E6/E7 transfection. To examine the potential invasive activity of FGFs in HPV16 E6/E7-transfected cervical cancer cells, we employed modified Boyden chambers and a digital camera attached to a microscope to identify the activity of Cx and CxWJ cells. Invasive ability increased significantly in CxWJ cells following ligand stimulation with FGF2 (Fig. 3B; $P<0.05$ vs. SF) and FGF4 (Fig. 3D; $P<0.05$ vs. SF), but not in Cx cells (Fig. 3A and C). Treatment with FGFs enhanced the invasive

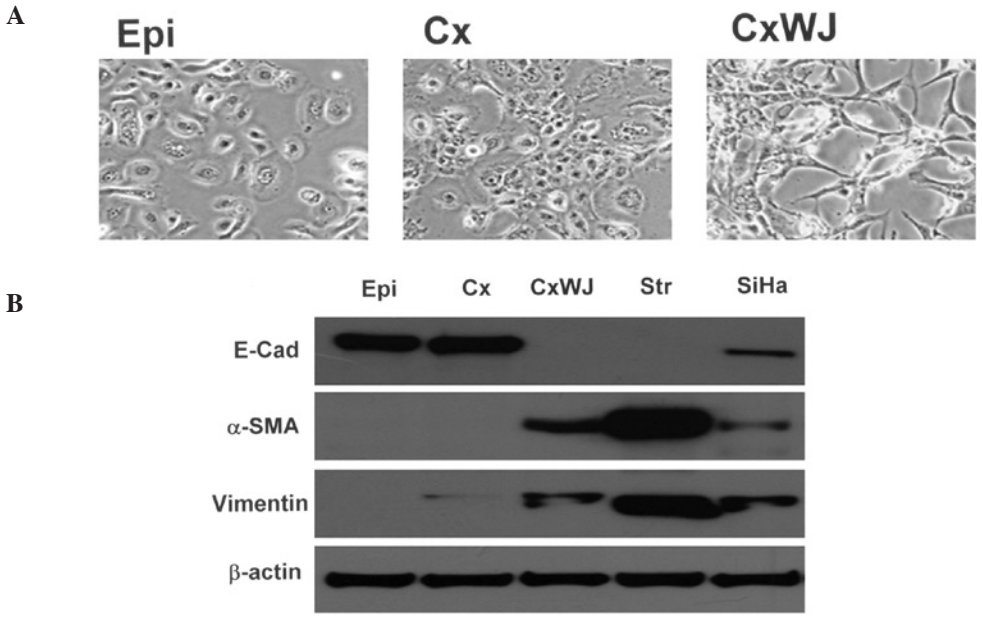

Figure 1. Microscope analysis and expression of EMT cell markers. (A) Morphology of cell lines (Epi, Cx and CxWJ) as observed by microscope. (B) EMT cell markers (E-cadherin, α-SMA and vimentin) were determined using western blotting. EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; Str, stroma; Epi, epithelial cells.

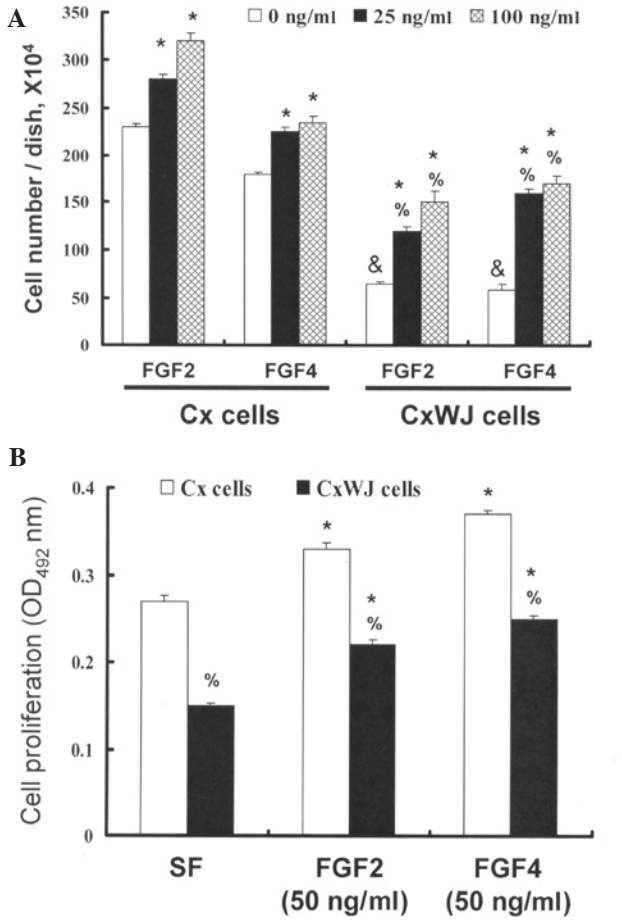

Figure 2. Proliferation effects of transfection with HPV16 E6/7 and FGF ligands in cervical cancer cells (Cx and CxWJ). (A) Number of cells, and (B) MTS assay were used to determine the proliferation of cervical cancer cells. Data were shown as the mean (±SEM) of at least three separate experiments. Statistical analysis was performed via a t-test, with significance set at $^{*}P<0.05$ for the control group (0 ng/ml) and $^{\%,\&}P<0.05$ for the Cx cell group. FGF, fibroblast growth factor; SF, serum-free.

ability of CxWJ cells compared with Cx cells (P<0.05 CxWJ vs. Cx cells). FGF ligand stimulated invasive ability in CxWJ cells (P<0.05 50 vs. 10 ng/ml) in a dose-dependent manner. The results in Fig. 3 show that FGF2 and 4 induce invasive activity via HPV16 E6/E7 transfection.

Discussion

Cervical cancer remains a fatal disease despite the development of various advanced forms of treatment (23). Following human papillomavirus (HPV) infection, cervical epithelial cells develop from premalignant cervical lesions to malignant invasive cancer via a multi-step process.

E-cadherin, the major cadherin molecule expressed by epithelial cells, maps to chromosome 16q22 (4) and serves as a mediator in the adhesion of epithelial cells. The role of E-cadherin as a tumor invasion suppressor in epithelial cells is well supported (24). Loss of the gene expression of E-cadherin has been shown to disrupt E-cadherin-mediated intercellular adhesion and is frequently associated with high-grade tumors, infiltrative growth and lymph node metastasis in a variety of human malignancies. The expression of E-cadherin in transgenic mice has also been associated with the development of invasive carcinoma from well-differentiated adenoma. In this study, we found that HPV16 E6/E7 transfection repressed the expression of E-cadherin protein in CxWJ cells. HPV infection may thus play a significant role in E-cadherin-mediated cervical cancer malignancy.

During the development of tumors, the stroma surrounding the fibroblasts and myofibroblasts express α-smooth muscle actin (α-SMA) constituting the 'desmoplastic reaction' in the process of invasion and metastasis. Vimentin is a developmentally regulated intermediate filament protein found in cells of mesenchymal origin. It is frequently co-expressed with other members of the intermediate filament family including

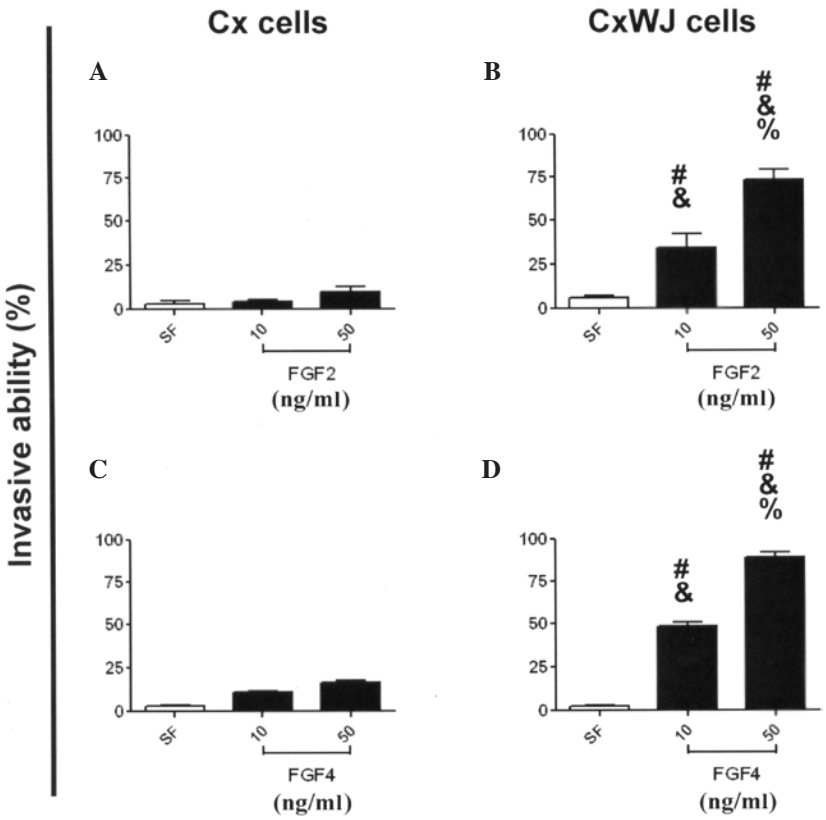

Figure 3. Effects of FGF and HPV16 E6/7 transfection on the invasive ability of cervical cancer cells. (A and C) Cx cells and (B and D) CxWJ cells were evaluated for invasive ability following culturing with FGFs 2 and 4 for 48 h. Cells treated with serum-free (SF) medium were used as the control. Data were presented as the mean (±SEM) of at least three separate experiments. Statistical analysis was determined using the t-test, with significance set at $^{\&}P<0.05$ for the SF control group, $^{\#}P<0.05$ for the Cx cells group and $^{\%}P<0.05$ for the 10 ng/ml group. FGF, fibroblast growth factor.

cytokeratins and neoplasms such as melanoma and breast carcinoma (25,26). Vimentin has been used as a sarcoma tumor marker to identify mesenchyme (27). The α-SMA and vimentin play a key role in the progression of tumors. Our results reveal that HPV16 E6/E7 transfection enhances the expression of α-SMA and vimentin protein in CxWJ cells. HPV infection may be associated with α-SMA and the vimentin-induced metastasis of cervical cancer cells.

In conclusion, our results indicate that HPV16 E6/E7 transfection of CxWJ cells causes them to respond differently to FGF ligand stimulation and increase invasive ability. Further study regarding changes in the epithelium and mesenchymal characteristics should be conducted to elucidate the role of HPV16 E6/E7 and FGF alteration in cervical carcinogensis.

Acknowledgements

The authors would like to express their appreciation for the funding support provided by the National Cheng Kung University Hospital.

References

1. Hsu KF, Wu CL, Huang SC, *et al*: Conditionally replicating E1B-deleted adenovirus driven by the squamous cell carcinoma antigen 2 promoter for uterine cervical cancer therapy. Cancer Gene Ther 15: 526-534, 2008.
2. Lang HC and Wu SL: Lifetime costs of the top five cancers in Taiwan. Eur J Health Econ: Mar 27, 2011 (Epub ahead of print).
3. Chen CC, Lin JC, Jan JS, Ho SC and Wang L: Definitive intensity-modulated radiation therapy with concurrent chemotherapy for patients with locally advanced cervical cancer. Gynecol Oncol: Apr 22, 2011 (Epub ahead of print).
4. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC and Castle PE: Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst 103: 368-383, 2011.
5. Mühlen S, Behren A, Iftner T and Simon C: Influence of HPV16 E2 and its localisation on the expression of matrix metalloproteinase-9. Int J Oncol 37: 337-345, 2010.
6. Chen GJ and Forough R: Fibroblast growth factors, fibroblast growth factor receptors, diseases, and drugs. Recent Pat Cardiovasc Drug Discov 1: 211-224, 2006.
7. Ucuzian AA, Brewster LP, East AT, Pang Y, Gassman AA and Greisler HP: Characterization of the chemotactic and mitogenic response of SMCs to PDGF-BB and FGF-2 in fibrin hydrogels. J Biomed Mater Res A 94: 988-996, 2010.
8. Dorey K and Amaya E: FGF signalling: diverse roles during early vertebrate embryogenesis. Development 137: 3731-3742, 2010.
9. Caverzasio J and Thouverey C: Activation of FGF receptors is a new mechanism by which strontium ranelate induces osteoblastic cell growth. Cell Physiol Biochem 27: 243-250, 2011.
10. Knights V and Cook SJ: De-regulated FGF receptors as therapeutic targets in cancer. Pharmacol Ther 125: 105-117, 2010.
11. Pardo OE, Latigo J, Jeffery RE, *et al*: The fibroblast growth factor receptor inhibitor PD173074 blocks small cell lung cancer growth in vitro and in vivo. Cancer Res 69: 8645-8651, 2009.
12. Tsimafeyeu I, Demidov L, Stepanova E, Wynn N and Ta H: Overexpression of fibroblast growth factor receptors FGFR1 and FGFR2 in renal cell carcinoma. Scand J Urol Nephrol 45: 190-195, 2011.
13. Katoh M and Katoh M: FGF signaling network in the gastrointestinal tract. Int J Oncol 29: 163-168, 2006.
14. Yura Y, Yoshioka Y, Yamamoto S, *et al*: Enhancing effects of fibroblast growth factor on the proliferation of salivary gland carcinoma cells and salivary gland carcinogenesis. J Oral Pathol Med 30: 159-167, 2001.

15. Manuvakhova M, Thottassery JV, Hays S, *et al*: Expression of the SNT-1/FRS2 phosphotyrosine binding domain inhibits activation of MAP kinase and PI3-kinase pathways and antiestrogen resistant growth induced by FGF-1 in human breast carcinoma cells. Oncogene 25: 6003-6014, 2006.
16. Schwertfeger KL: Fibroblast growth factors in development and cancer: insights from the mammary and prostate glands. Curr Drug Targets 10: 632-644, 2009.
17. Vodyanik MA, Yu J, Zhang X, *et al*: A mesoderm-derived precursor for mesenchymal stem and endothelial cells. Cell Stem Cell 7: 718-729, 2010.
18. Morrison GM, Oikonomopoulou I, Migueles RP, *et al*: Anterior definitive endoderm from ESCs reveals a role for FGF signaling. Cell Stem Cell 3: 402-415, 2008.
19. Hardy KM, Yatskievych TA, Konieczka J, Bobbs AS and Antin PB: FGF signalling through RAS/MAPK and PI3K pathways regulates cell movement and gene expression in the chicken primitive streak without affecting E-cadherin expression. BMC Dev Biol 11: 20, 2011.
20. Katoh M: Network of WNT and other regulatory signaling cascades in pluripotent stem cells and cancer stem cells. Curr Pharm Biotechnol 12: 160-170, 2011.
21. Chou CY, Chen YH, Tzeng CC, Cheng YC, Chang CF and Chen TM: Establishment and characterization of a human-papillomavirus negative, p53-mutation negative human cervical cancer cell line. Cancer Lett 102: 173-181, 1996.
22. Benton G, Kleinman HK, George J and Arnaoutova I: Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells. Int J Cancer 128: 1751-1757, 2011.
23. Hasimu A, Ge L, Li QZ, Zhang RP and Guo X: Expressions of Toll-like receptors 3, 4, 7, and 9 in cervical lesions and their correlation with HPV16 infection in Uighur women. Chin J Cancer 30: 344-350, 2011.
24. Simionescu C, Mărgăritescu C, Stepan A, Georgescu CV, Niculescu M and Muntean M: The utility of p16, E-cadherin and Ki67 in cervical squamous intraepithelial lesions diagnosis. Rom J Morphol Embryol 51: 621-626, 2010.
25. De Chiara A, Losito S, Terracciano L, Di Giacomo R, Iaccarino G and Rubolotta MR: Primary plasmacytoma of the breast. Arch Pathol Lab Med 125: 1078-1080, 2001.
26. Sun H, Qin M, Xiao Y, Yang F, Ni W and Liu S: Haemangiomas, leiomyosarcoma and myeloma caused by subgroup J avian leukosis virus in a commercial layer flock. Acta Vet Hung 58: 441-451, 2010.
27. Zhu QS, Rosenblatt K, Huang KL, *et al*: Vimentin is a novel AKT1 target mediating motility and invasion. Oncogene 30: 457-470, 2011.