Arch Gynecol Obstet
DOI 10.1007/s00404-014-3471-6

GYNECOLOGIC ONCOLOGY

Pentoxifylline attenuates HPV-16 associated necrosis in placental trophoblasts

Sarah S. Chen · Barry S. Block · Philip J. Chan

Received: 8 May 2014 / Accepted: 10 September 2014

Abstract
Purpose Human papillomaviruses (HPV) have been associated with placental inflammation resulting in high risk preterm birth. The premise for this study was that treatment with anti-inflammatory pentoxifylline would inhibit HPV-mediated placental cell death. The objectives were: (1) to study the effects of HPV-16 exposure on trophoblast cells and (2) to evaluate pentoxifylline in preventing the damaging effects of HPV-16.
Methods Mouse embryos (1-cell) were cultured (G1plus medium, 72 h, 37 °C, 5 % CO2-in-air), divided into four groups at blastocyst-stage and incubated to implantation stage. Implanted trophoblasts were exposed to either HPV-16 (group 1), concomitant HPV-16 and 3 mM pentoxifylline (group 2), 3 mM pentoxifylline only (group 3) or control medium (group 4) and further incubated for 24 h. HPV-16 were from SiHa cell lysates. Trophoblast structural integrity was assessed by morphometric analysis while apoptosis and necrosis were detected using dual-stain fluorescence assay.
Results Trophoblast outgrowth was reduced by 90 % ($P < 0.05$) in HPV-16 presence ($629 \pm 265\ \mu^2$, mean + SEM) when compared with controls ($6{,}456 \pm 795\ \mu^2$). Pentoxifylline attenuated the effects of HPV-16 ($4{,}308 \pm 362\ \mu^2$). Nuclear size of HPV-16 infected trophoblasts was smallest among the groups ($P < 0.005$). HPV-16 decreased cell viability and increased necrosis but not apoptosis. Pentoxifylline prevented HPV-16 associated necrosis in trophoblasts.
Conclusions HPV-16 decreased nuclear size and trophoblast outgrowth but these effects were attenuated by the phosphodiesterase inhibitor, pentoxifylline. The action of HPV-16 on trophoblasts was increased cell necrosis suggesting that HPV-16 pathogenesis involved either cAMP inhibition and/or activated TNF pathways.

Keywords HPV · Trophoblasts · Placenta · Necrosis · Pentoxifylline

S. S. Chen · B. S. Block · P. J. Chan (✉)
Department of Gynecology and Obstetrics, Loma Linda University School of Medicine, 11370 Anderson St., Suite 3950, Loma Linda, CA 92354, USA
e-mail: pchann@yahoo.com

Introduction

Human papillomaviruses (HPV) are classified Group 1 human carcinogens (IARC, International Agency for Research on Cancer) extensively studied for their role in cervical cancer. HPV are double-stranded DNA viruses with a well-defined affinity for epithelial cells. The high risk HPV serotypes are recognized as the causative agents in nearly all cervical cancers. HPV types 16 and 18 are responsible for 50–70 % of all cases [1]. In addition, the viruses are extremely prevalent. It has been estimated that women have up to a 79 % lifetime risk of becoming infected with the virus [2]. The overall prevalence in the United States was estimated to be 26.8 % in a study published in 2007 [3]. This very common virus has been well studied in its relationship to cervical cancer, but it is less well known for its ability to infect placental tissue.

Weyn and colleagues [4] presented evidence of HPV-16 in transabdominally obtained placental cells, refuting the idea that contamination occurs during passage via the vaginal route. HPV has also been recovered more frequently in placentas obtained from patients with spontaneous preterm labor and spontaneous abortions than from term or elective abortions [5–9]. Gomez et al. [5] showed

Published online: 17 September 2014

that HPV infection of trophoblasts induces cell death, and reduces cell invasion, possibly placental invasion into the uterine wall. They showed that infection by HPV induces pathological sequelae that are associated with placental dysfunction and spontaneous preterm delivery.

Preterm delivery is the major cause of neonatal morbidity and mortality, with an estimate of 14.9 million babies born preterm worldwide in 2010. Preterm birth is the single largest direct cause of neonatal deaths, directly resulting in 35 % of the world's 3.1 million deaths a year [10]. Intrauterine infection and inflammation are risk factors for adverse neurological outcome. Chronic amniotic fluid inflammation may cause preterm labor. Bacteria has been considered to be the main trigger, but viral infections also appear to be involved [11]. Recently, HPV DNA positive test results have been associated with abnormal placental findings, premature rupture of membrane and preterm birth [12–14]. However, more studies of HPV pathogenesis specifically in the placenta are still warranted.

Pentoxifylline is a competitive non-selective phosphodiesterase inhibitor that increases intracellular cyclic AMP and has been routinely employed for circulatory diseases for more than 20 years [15, 16]. We hypothesized that treatment with anti-inflammatory pentoxifylline would inhibit HPV-mediated cell necrosis. Recently, cultured trophoblast cells were used as a model for HPV infectivity studies [17]. In this study, the objectives were: (1) to determine the effects of HPV-16 exposure on cultured trophoblast cells and (2) to evaluate the role of pentoxifylline in preventing the damaging effects of HPV-16 on the trophoblasts.

Materials and methods

SiHa cell lysate procedure

The preparation of SiHa cell lysates was as previously reported [17]. SiHa cells (ATCC, American Type Culture Collection, Manassa, VA) derived from human cervical cell carcinoma contained 1–2 copies of HPV-16 located on chromosome 13 [18]. The SiHa cells were cultured in Eagle Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA), supplemented with 10 % fetal bovine serum (Invitrogen), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma Chemical Co). The cells were passaged and processed after reaching 80 % confluence [19]. The SiHa cells were placed in 15 mL centrifuged tubes and centrifuged at $300\times g$ for 10 min. The supernatants were decanted and the pellets of cells combined in a microfuge tube containing 0.4 mL of G-2 plus version 5 medium (G-2 v5, VitroLife, Engleswood, CO). The cells were lysed by using a sterile glass rod as a pestle to crush the cells and release the HPV-16 gene fragments. The resultant cell extract was placed in cryovials and stored frozen at −23 °C. The lysates were warmed to 37 °C prior to their use in the mouse embryo experiments. The lysates were previously shown to have transformative properties [17].

Culture of pronuclear stage mouse embryo to the implantation stage

Cryopreserved 1-cell mouse fertilized oocytes in cryo straws were obtained from a commercial supplier (Embryotech Laboratories Inc., Haverhill, MA) and stored in liquid nitrogen until use. The straws were thawed as recommended by the supplier and the contents expelled into a droplet of G-1 v5 medium (G-1 v5, VitroLife, Engleswood, CO) in a petri dish. The 1-cell embryos were washed by passage through two additional droplets of G-1 v5 medium. The washed embryos were pooled together at the center well of a double-well culture dish (Falcon 3037, Becton–Dickinson, Franklin Lakes, NJ) containing 1 mL of G-1 v5 medium. Water was placed on the outer moat for humidity. The cell culture dish was placed in an incubator and incubated at 37 °C, 5 % CO2 in air mixture. After 3 days of incubation, the embryos were randomly divided into 4 groups and placed into four culture dishes containing G-2 v5 medium. The G-2 v5 medium has more nutrients to support blastocyst growth in vitro. Embryos that had not developed to the early blastocyst stage were not used. The embryos were further incubated (an additional 2 days) until they reached the implanted stage.

The implanted embryos in each dish were exposed to either thawed SiHa cell lysate (10 μL) containing HPV-16 (Group 1), concomitant SiHa lysate and 3 mM pentoxifylline (group 2), 3 mM pentoxifylline only (group 3) or control medium (group 4) and further incubated for 24 h. Trophoblast nuclear size and cell area were assessed by morphometric analyses as described below.

Dual fluorescence stain analysis

The dual fluorescence stain method [20] was used to distinguish viable, apoptotic or necrotic trophoblast cells in each implanted embryo. One drop of bisbenzimide (5 μL of 10 μM, Hoechst 33342, Sigma Chemical Co., St. Louis, MO) was added into each culture dish containing the implanted embryos incubated in 1 mL culture medium. After 1 min, 5 μL of propidium iodide (32 μM, Sigma Chemical Co., St. Louis, MO dissolved in saline) was added to the same culture dish. After 1 min, the culture medium with the stains was discarded and pre-warmed culture medium (0.5 mL) was added to each dish. The culture dish was placed on the stage of an epifluorescence

UV-microscope set at magnification of 500× (Nikon Optiphot, Nikon Instruments, Melville, NY) and images of the fluorescent colored cells in each embryo captured on a digital camera. The percentages of viable, apoptotic and necrotic trophoblast cells were determined for each embryo. In this study, a viable cell was defined as the capacity of the cell to exclude the fluorescent dyes and possessed a clear coloration. In contrast, an apoptotic (completely blue color) or necrotic (pink-red color) cell was identified by the cell capacity to absorb bisbenzimide or propidium iodide stain respectively.

Trophoblast morphology stain procedure

Trophoblast cells and inner cell mass nuclei and cytoplasm were stained using a sequential Fast Green FCF and Rose Bengal (Spermac Stain Enterprises Inc., Republic of South Africa) staining procedure [21]. Basically, the procedure involved rinsing the culture dishes with the implanted embryos in culture medium followed by fixation in 4 % formalin (5 min). The fixative was rinsed off with water and Spermac stain A (Rose Bengal mixture) was pipetted into each culture dish to permit staining for 2 min. Stain A was rinsed off and stain B (Pyronin Y mixture) pipetted into the dishes for 1 min staining. This was followed by rinsing and staining with the final stain C (Fast Green FCF—Janus Green mixture) for 1 min. The culture dishes were rinsed in water, air-dried and stored in a dark drawer until the time of analyses.

Spectrophotodensitometry

Cell dimensions were measured using a spectrophotodensitometric method which facilitated the determination of embryo growth and migration. The Spermac-stained inner cell mass and trophoblast cells were located on each slide using the Nikon Diaphot inverted microscope (400× magnification) and the images digitized and recorded. The pre-analytical phase included using Adobe Photoshop software to standardize each image. The image outline of each nucleus or the entire trophoblast cell was traced onscreen and measurements determined using the Adobe Photoshop histogram function. The recorded data was entered into Microsoft Excel spreadsheets and analyzed.

Statistical analysis

Data from the morphometric analyses of cell dimensions were presented as mean ± standard error of the mean (SEM). For each treatment group, the mean dimension of the trophoblast nucleus or cell area was calculated and tested using ANOVA and significance of means compared using the two-tailed Student's *t* test. The numbers of viable, apoptotic or necrotic trophoblast cells for each treatment group were tested using the two-tailed Mantel–Haenszel Chi-square test statistics (OpenEpi.com, Open Source Epidemiologic Statistics for Public Health, Atlanta, GA). A value of $P < 0.05$ was considered significant.

Results

The trophoblast cells of implanted embryos exposed to HPV-16 demonstrated cell shrinkage (Fig. 1) and were 90 % smaller in size (629 ± 265 μ^2, mean ± SEM) when compared with control trophoblasts (6,456 ± 795 μ^2). Pentoxifylline attenuated the cell shrinkage effects of HPV16 (4,308 ± 362 μ^2) (Table 1). Trophoblast cell shrinkage has been reported to be associated with F-actin cytoskeleton disruption while cell expansion correlated with migratory or invasive activity [22]. When the antioxidant pentoxifylline was present during HPV-16 exposure, the trophoblasts remained significantly larger than the trophoblasts in the HPV-16 exposed group.

Similarly, the nuclear size of HPV-16 exposed trophoblasts were 13-fold smaller (56.5 ± 38.4 μ^2) than control trophoblasts (800.91 ± 120.5 μ^2). The addition of pentoxifylline attenuated the dramatic nuclear shrinkage in the HPV-16 exposed trophoblasts (430.9 ± 29.8 μ^2). A large nuclear size in the trophoblast cell was indicative of endoreduplication required for rapid differentiation and intensive growth [23]. Pentoxifylline treatment by itself resulted in a lesser twofold reduction in nuclear dimension. Overall, the nuclear size of an HPV16-infected trophoblast was the smallest among all the treated trophoblast cells ($P < 0.005$).

The presence of HPV-16 decreased cell viability through cellular necrosis (Table 2). When pentoxifylline was present in the HPV-16 treated trophoblasts, cell viability was comparable to the control trophoblasts and cellular necrosis was negligible. The pentoxifylline treatment did not affect trophoblast viability as shown in the pentoxifylline only group. In addition, cellular apoptosis but not necrosis was detected in the pentoxifylline only group. There were no differences in the percentages of apoptotic trophoblast cells in the four treatment groups after 24 h of culture (Table 2).

Discussion

Human papillomavirus (HPV) are classified Group 1 human carcinogens implicated in some miscarriages [5–8]. HPV expressed oncogenes induce placental inflammation resulting in high risk preterm birth. HPV infection rates in

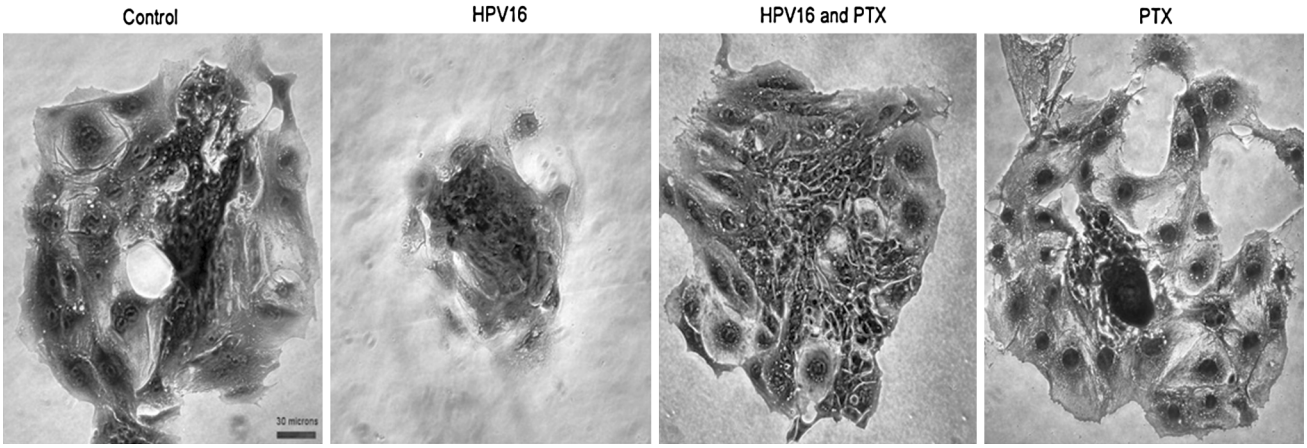

Fig. 1 Implanted mouse trophoblasts cells after 24 h exposure (at 37 °C, 5 % CO_2 in air) to either: *1* control medium, *2* HPV16 SiHa lysate, 3 HPV16 SiHa lysate and 3 mM pentoxifylline (PTX) or *4* 3 mM pentoxifylline (PTX) only

Table 1 Comparison of the area (mean ± SEM) of each trophoblast nucleus or each trophoblast size after 24 h exposure (at 37 °C, 5 % CO_2 in air) to either: (1) control medium, (2) HPV16 SiHa lysate, (3) HPV16 SiHa lysate and 3 mM pentoxifylline or (4) 3 mM pentoxifylline only

Treatment group	Number of cells (*n*)	Size of nucleus (μ^2)	Size of trophoblast (μ^2)
Control	17	800.91 ± 120.5	6,456.6 ± 795.5
HPV16 SiHa lysate	38	56.5 ± 38.4[a]	629.3 ± 265.9[a]
HPV16 SiHa lysate and 3 mM pentoxifylline	81	430.9 ± 29.8[a, b]	4,308.1 ± 362.0[a, b]
3 mM pentoxifylline only	73	496.4 ± 11.3[a, b]	3,876 ± 86.9[a, b]

[a] Different from the control group ($P < 0.05$)

[b] Different from the HPV16 SiHa lysate group ($P < 0.05$)

Table 2 Comparison of the percentages of live, apoptotic and necrotic trophoblast cells after exposure to either: (1) control medium, (2) HPV16 SiHa lysate, (3) HPV16 SiHa lysate and 3 mM pentoxifylline or (4) 3 mM pentoxifylline only

Treatment group	Total cells (*n*)	# Viable cells (%)	# Apoptotic cells (%)	# Necrotic cells (%)
(1) Control group	114	80 (70.2)	26 (22.8)	8 (7.0)
(2) HPV16 SiHa lysate	86	37 (43.0)[a]	18 (20.9)	31 (36.0)[a]
(3) HPV16 SiHa lysate and 3 mM pentoxifylline	62	42 (67.7)[b]	19 (30.6)	1 (1.6)[b]
(4) 3 mM pentoxifylline	170	131 (77.1)[b]	39 (22.9)	0 (0.0)[a, b]

Bisbenzimide and propidium iodide dual-stain epifluorescence analyses were performed on the cells in each group. Incubation was at 37 °C, 5 % CO_2 in air for 24 h

[a] Different from the control group ($P < 0.05$)

[b] Different from the HPV16 SiHa lysate group ($P < 0.05$)

spontaneous abortions of the first and second trimesters have been reported to be in the 50–70 % range [5, 6]. When the placentas from the spontaneous abortions and preterm deliveries cases were compared with those from elective abortions and term births, a higher percentage of HPV infection was detected [5]. The origin of the HPV found in the placental cells was postulated to be circulating cell-free HPV DNA in the blood. Indeed, using the sensitive QIAamp circulating nucleic acid kit procedure and TaqMan technology, Mazurek and colleagues provided evidence of the circulating cell-free HPV-16 DNA in blood plasma [24]. The findings suggested that HPV was involved in the early termination of pregnancy but the mechanism of action remained elusive. Recent evidence suggested that the compromised placenta characterized by impaired trophoblast cell adhesion and trophoblast cellular necrosis [8, 9, 17, 25] resulting from HPV-associated inflammatory response as one of the etiologies of preterm delivery or pregnancy loss.

Trophoblast cells are placental cells that participate in embryonic implantation and form critical cellular layers to facilitate fetal and maternal blood interactions in the pregnant uterus. Impaired cellular functions in trophoblasts or in BeWo ('trophoblastic-like cells') choriocarcinoma cell lines have been linked to HPV-16 expressed oncoproteins E5, E6 and E7 [26–29]. Imprinting studies [30, 31] showed that the HPV oncoproteins caused pathogenesis

through hypermethylation of the SOX2 gene leading to gestational trophoblastic diseases such as hyatidiform mole and choriocarcinoma [32].

In the present study, HPV-16 exposure significantly reduced trophoblastic outgrowth and cell size. Trophoblast cell shrinkage has been reported to be associated with F-actin cytoskeleton disruption [22]. Furthermore, HPV-16 exposure caused nuclear shrinkage suggesting disruption of trophoblast endoreduplication and differentiation [23]. Dual-stain fluorescence analyses indicated that the damaging effects were due to HPV-associated cellular necrosis. However, when pentoxifylline was administered to the HPV-exposed trophoblasts, the negative effects of HPV-16 were reversed and the trophoblasts remained viable even in the presence of the virus. Given that pentoxifylline functioned as a phosphodiesterase inhibitor that increased cyclic AMP and inhibited tumor necrosis factor (TNF or TNF-α), the results here suggested that the necrosis of trophoblast cells by HPV involved inhibition of cyclic AMP and activation of the TNF pathway [33, 34]. A recent study provided support for this premise [35, 36]. It is recognized that the HPV oncogenes also affect other pathways involving p53 and Rb genes and these have been reviewed [37]. Nevertheless, the end result of HPV exposure was the demise of the placental trophoblast cells which were essential for the continuance of pregnancy. Further studies are needed to corroborate the present findings of pentoxifylline treatment to abrogate HPV-related pathogenesis in the placenta.

Acknowledgments The SiHa cells were kindly donated by Drs. Duerksen-Hughes and her laboratory team, the Department of Basic Sciences in the Division of Biochemistry at Loma Linda University, CA, USA.

Conflict of interest We declare that we have no conflict of interest.

References

1. Munoz N, Castellsagué X, de Gonzalez AB, Gissmann L (2006) HPV in the etiology of human cancer. Vaccine 24:1–10
2. Syrjänen K, Syrjänen S (1990) Epidemiology of human papilloma virus infections and genital neoplasia. Scand J Infect Dis Suppl 69:7–17 (Review)
3. Dunne EF, Unger ER, Sternberg M, McQuillan G, Swan DC, Patel SS, Markowitz LE (2007) Prevalence of HPV infection among females in the United States. JAMA 297:813–819
4. Weyn C, Thomas D, Jani J, Guizani M, Donner C, Van Ryssel-berge M, Hans C, Bossens MYE, Fontaine V (2011) Evidence for human papillomavirus in the placenta. J Infect Dis 203:341–343
5. Gomez LM, Ma Y, Ho C, McGrath CM, Nelson DB, Parry S (2008) Placental infection with human papillomavirus is associated with spontaneous preterm delivery. Hum Reprod 23:709–715
6. Sarkola ME, Grénman SE, Rintala MA, Syrjnen KJ, Syrjnen SM (2008) Human papillomavirus in the placenta and umbilical cord blood. Acta Obstet Gynecol Scand 87:1181–1188
7. You H, Liu Y, Agrawal N, Prasad CK, Edwards JL, Osborne AF, Korourian S, Lowery CL, Hermonat PL (2008) Multiple human papillomavirus types replicate in 3A trophoblasts. Placenta 29:30–38
8. Manavi M, Czerwenka KF, Schurz B, Knogler W, Kubista E, Reinold E (1992) Latent cervical virus infection as a possible cause of early abortion. Gynakol Geburtshilfliche Rundsch 32:84–87
9. Hermonat PL, Han L, Wendel PJ, Quirk JG, Stern S, Lowery CL, Rechtin TM (1997) Human papillomavirus is more prevalent in first trimester spontaneously aborted products of conception compared to elective specimens. Virus Genes 14:13–17
10. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, Kinnery M, Lasn J, Born Too Soon Preterm Birth Action Group (2013) Born too soon: the global epidemiology of 15 million preterm births. Reprod Health 10 Suppl 1:S2
11. Vrachnis N, Vitoratos N, Iliodromiti Z, Sifakis S, Deligeoroglou E, Creatsas G (2010) Intrauterine inflammation and preterm delivery. Ann N Y Acad Sci 1205:118–122
12. Zuo Z, Goel S, Carter JE (2011) Association of cervical cytology and HPV DNA status during pregnancy with placental abnormalities and preterm birth. Am J Clin Pathol 135:260–265
13. Cho G, Min KJ, Hong HR, Kim S, Hong JH, Lee JK, Oh MJ, Kim H (2013) High-risk human papillomavirus infection is associated with premature rupture of membranes. BMC Pregnancy Childbirth 6:173. doi:10.1186/1471-2393-13-173
14. McDonnold M, Dunn H, Hester A, Pacheco LD, Hankins GD, Saade GR, Costantine MM (2014) High risk human papillomavirus at entry to prenatal care and risk of preeclampsia. Am J Obstet Gynecol 210:138.e1–138.e5
15. Harris E, Schulzke SM, Patole SK (2010) Pentoxifylline in preterm neonates: a systematic review. Paediatr Drugs 12:301–311
16. Lauterbach R, Rytlewski K, Pawlik D, Hurkała J, Wójtowicz A, Bręborowicz G, Szymankiewicz M (2012) Effect of pentoxifylline, administered in preterm labour, on the foetal-placental circulation and neonatal outcome: a randomized, prospective pilot study. Basic Clin Pharmacol Toxicol 110:342–346
17. Hong LJ, Oshiro BT, Chan PJ (2013) HPV-16 exposed mouse embryos: a potential model for pregnancy wastage. Arch Gynecol Obstet 287:1093–1097
18. Meissner JD (1999) Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. J Gen Virol 80:1725–1733
19. Tungteakkhun SS, Filippova M, Neidigh JW, Fodor N, Duerksen-Hughes PJ (2008) The interaction between human papillomavirus type 16 and FADD is mediated by a novel E6 binding domain. J Virol 82:9600–9614
20. Rowland SC, Jacobson JD, Patton WC, King A, Chan PJ (2003) Dual fluorescence analysis of DNA apoptosis in sperm. Am J Obstet Gynecol 188:1156–1157
21. Chan PJ, Corselli JU, Jacobson JD, Patton WC, King A (1999) Spermac stain analysis of human sperm acrosomes. Fertil Steril 72:124–128
22. Han J, Li L, Hu J, Yu L, Zheng Y, Guo J, Zheng X, Yi P, Zhou Y (2010) Epidermal growth factor stimulates human trophoblast cell migration through Rho A and Rho C activation. Endocrinology 151:1732–1742
23. Hinck L, Thissen JP, Pampfer S, De Hertogh R (2003) Effect of high concentrations of glucose on differentiation of rat trophoblast cells in vitro. Diabetologia 46:276–283
24. Mazurek AM, Fiszer-Kierzkowska A, Rutkowski T, Składowski K, Pierzyna M, Scieglińska D, Woźniak G, Głowacki G, Kawczyński R, Małusecka E (2013) Optimization of circulating cell-free DNA recovery for KRAS mutation and HPV detection in plasma. Cancer Biomark 13:385–394

25. Wang J, Mayernik L, Armant DR (2007) Trophoblast adhesion of the peri-implantation mouse blastocyst is regulated by integrin signaling that targets phospholipase C. Dev Biol 302:143–153
26. Boulenouar S, Weyn C, Van Noppen M, Moussa Ali M, Favre M, Delvenne PO, Bex F, Noël A, Englert Y, Fontaine V (2010) Effects of HPV-16 E5, E6 and E7 proteins on survival, adhesion, migration and invasion of trophoblastic cells. Carcinogenesis 31:473–480
27. You H, Liu Y, Carey MJ, Lowery CL, Hermonat PL (2002) Defective 3A trophoblast-endometrial cell adhesion and altered 3A growth and survival by human papillomavirus type 16 oncogenes. Mol Cancer Res 1:25–31
28. You H, Liu Y, Agrawal N, Prasad CK, Chiriva-Internati M, Lowery CL, Kay HH, Hermonat PL (2003) Infection, replication, and cytopathology of human papillomavirus type 31 in trophoblasts. Virology 316:281–289
29. Whiteside MA, Siegel EM, Unger ER (2008) Human papillomavirus and molecular considerations for cancer risk. Cancer 113:2981–2994
30. Terra AP, Murta EF, Maluf PJ, Caballero OL, Brait M, Adad SJ (2007) Aberrant promoter methylation can be useful as a marker of recurrent disease in patients with cervical intraepithelial neoplasia grade III. Tumori 93:572–579
31. Long BR, Erickson AE, Chapman JM, Barbour JD, Vu BA, Ho EL, Lanier LL, Sauer MM, Carvalho KI, Nixon DF, Kallas EG (2010) Increased number and function of natural killer cells in human immunodeficiency virus 1-positive subjects co-infected with herpes simplex virus 2. Immunology 129:186–196
32. Li AS, Siu MK, Zhang H, Wong ES, Chan KY, Ngan HY, Cheung AN (2008) Hypermethylation of SOX2 gene in hydatidiform mole and choriocarcinoma. Reprod Sci 15:735–744
33. Basile JR, Zacny V, Munger K (2001) The cytokines tumor necrosis factor-alpha (TNF-alpha) and TNF-related apoptosis-inducing ligand differentially modulate proliferation and apoptotic pathways in human keratinocytes expressing the human papillomavirus-16 E7 oncoprotein. J Biol Chem 276:22522–22528
34. Jin L, Sturgis EM, Zhang Y, Huang Z, Song X, Li C, Wei Q, Li G (2013) Association of tumor necrosis factor-alpha promoter variants with risk of HPV-associated oral squamous cell carcinoma. Mol Cancer 12:80. doi:10.1186/1476-4598-12-80
35. Sorimachi K, Waring P, Hapel AJ, Fukasawa I, Kaneko Y, Masawa N (2013) Characterisation of apoptosis in myb-transformed hematopoietic cell (MTHC-A) lines: TNF-α-induced apoptosis and prevention by cAMP. J Clin Exp Hematop 53:115–120
36. Desaintes C, Goyat S, Garbay S, Yaniv M, Thierry F (1999) Papillomavirus E2 induces p53-independent apoptosis in HeLa cells. Oncogene 18:4538–4545
37. Mighty KK, Laimins LA (2014) The role of human papillomaviruses in oncogenesis. Recent Results Cancer Res 193:135–148 (Review)