Carcinogenesis Advance Access published November 23, 2009

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Effects of HPV-16 E5, E6 and E7 Proteins on Survival, Adhesion, Migration and Invasion of Trophoblastic Cells

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Abstract:

Amongst high-risk human papillomaviruses (HPV), HPV-16 infection is the most prevalent causative factor for cervical cancer. Beside other mucosal targets, HPV-16 was reported to infect the placenta and to replicate in trophoblastic cells. Since these cells share invasive properties of tumoral cells, they represent an ideal model to investigate several oncogenic processes. In the present work, we analyzed the impacts of HPV-16 E5, E6 and E7 oncoproteins on the trophoblastic model. Our results showed that E5 impaired the viability of trophoblastic and cervical cell lines but E6 and E7, favouring cell growth, neutralised the E5 cytotoxic effect. In addition, E5 decreased the adhesiveness of trophoblastic cells to the tissue culture plastic and to endometrial cells similarly as previously described for E6 and E7. E5 and E6 plus E7 increased also their migration and their invasive properties. Cells expressing HPV-16 early proteins under the control of the LCR endogenous promoter displayed growth advantage and were also more motile and invasive compared to control cells. Interestingly, the E-cadherin was down regulated in trophoblastic cells expressing E5, E6 and E7. NF-κB and AP-1 activities were also enhanced. In conclusion, HPV-16 early proteins enhanced trophoblastic growth and intensify the malignant phenotype by impairing cell adhesion leading to increased cellular motile and invasive properties. HPV-16 E5 participated, with E6 and E7, in these changes by impairing E-cadherin expression, a hallmark of malignant progression.

Introduction

Human papillomaviruses (HPVs) are a group of small DNA viruses with epitheliotropic cutaneomucosal tropism. The genital infections are commonly cleared within two years but a subset of HPVs termed “high risk” may persist, and in rare cases lead to pre-malignant lesions and to invasive carcinoma. The high risk HPV-16 is the most common sexually transmitted HPV types. Infection with HPV-16 is the cause of 50 to 70% of cervical cancers, the second most frequent cause of death by cancer among females worldwide (Reviewed in [1]). The oncogenic effect of HPV16 is commonly attributed to the E6 and E7 viral proteins that abrogate the cell-cycle checkpoints by inactivating tumor suppressors, such as p53 and pRb, respectively, and targeting a plethora of other key host cellular proteins involved in apoptosis and malignant cellular transformation [2].

Briefly, E6 induces p53 degradation through its association with the cellular protein E6-AP via the ubiquitin proteolytic pathway [3]. Several *in vitro* and *in vivo* studies reported other E6/E6AP targets. Among those, PDZ-motif-containing proteins inducing cell polarity loss [4-7], Bak, FADD and procaspase 8 interfering with apoptosis [8,9], and NFX1 and hTERT contributing to immortalization [10].

E7 induces the degradation of pRB and its “pocket” family members via the proteasome pathway thereby releasing the transcription factor E2F that controls numerous cellular processes [11]. One cyclin-dependent kinase inhibitor, p16, which prevents the phosphorylation of pRb family members, is overexpressed when pRb is inactivated by E7 [12]. E6 and E7 can subvert the immune system by impairing, among others, the expression of adhesion molecules [13-15]. Deregulation of

adhesion molecules is not only a strategy for immune evasion but also a prelude to early carcinogenesis and metastatic processes [16]. E6 and E7 have been reported to cooperate with ErbB-2 in head and neck carcinogenesis by downregulating E-cadherin expression [17]. Extracellular matrix degradation by metalloproteinases is dramatically increased in invasive carcinoma expressing E6 and E7 [18]. All together, these data suggest that E6 and E7 oncoproteins are the main determining factors of the malignant phenotype in HPV-induced cancers.

Recent findings have implicated the HPV-16 E5 protein as an important mediator to oncogenic transformation. The epidermal growth factor receptor (EGFR) is a potential target of E5. The association of E5 with the 16 kDa proton-ATPase subunit could alter the endosomal acidification and lead to a subsequent increase of phosphorylated EGFR at the cell surface [19,20]. E5 enhances the immortalization potential of E6 and E7, and stimulates the proliferation in cooperation with E7 [21,22]. Transgenic mice studies showed that E5 alters the growth and differentiation of stratified epithelia and induces epithelial tumor [23]. E5 confers to cells the property to grow in an anchorage-independent manner [24]. Significant morphological changes of the cells and reorganization of the actin cytoskeleton were attributed to the transforming potential of E5: inducing koilocyte formation in cooperation with E6 [25], changing the lipid composition of the membranes [26,27], inhibiting gap junctional intercellular communication [28,29] and increasing cell motility [30].

Surprisingly, recent studies reported that HPV could infect the human placenta and that various HPV types could complete their entire life cycle in a trophoblastic cell line [31,32]. The trophoblast is known to share similar properties with malignant tumoral cells including invasive and migratory capacities [33]. BeWo cells, known as

“trophoblastic-like cells”, share many morphologic, metabolic, and functional characteristics with trophoblast cells of the normal first trimester placenta [34]. In the present work, we used the BeWo cells as a model to study the effects of HPV-16 E5, E6 and E7 oncoproteins on trophoblastic growth, adhesion, migration and invasion and we investigated whether the expression of the E-cadherin was altered.

Materials and methods

- **Plasmid constructs**

The HPV-16 Early ORF constructs used in this study were engineered to express the Flag tag (LMDYKDDDDKAAA) at their N terminus to facilitate immunological detection. The pEF6-Flag expression vector was kindly provided by Dr M. Kalai (IPH, Belgium). The HPV-16 cloned DNA plasmid template was a kind gift from Pr. E-M. De Villiers (DKFZ, Germany). HPV-16E5 coding sequences were amplified by PCR using primers 5'-AAGGCGGCCGCTATGACAAATCTTGATACTGC-3' and 5'-ATGCTCTAGACATTATGTAATTAAAAAGCG-3'. The two ORF in tandem HPV-16E6/E7 was amplified using 5'-AGCGCGGCCGCTATGCACCAAAAGAGAACTGC-3' and 5' ATGCTCTAGAGATTATGGTTTCTGAGAACAG-3'. PCR products were first sub-cloned into the TOPO-TA vector (Invitrogen), and then inserted into *Not*I/*Xba*I restriction site of pEF6-Flag vector. All plasmid constructions were verified by DNA sequencing. pHPV-16-Early and pLCR-GFP were kindly provided by C. Weyn (ULB, Belgium). pEF6-LacZ (Invitrogen) was used as a negative control. peGFP-NLS was provided by Dr. X. Saelens (VIB, Belgium) and pGL4-Luc-Renilla plasmid was a kind gift of Pr. C. Van Lint (ULB, Belgium). pAP-1-Luc and pNF-κB-Luc were kindly provided by Dr. Yves Jacob (Pasteur Institute, France).

- **Cell culture and transfections**

BeWo and JEG-3 choriocarcinoma cell lines were kindly offered by Pr. A. Pötgens (Aachen, Germany) and Pr. R. Pijnenborg (KUL, Belgium), respectively. C33a

cervical cell lines were purchased from ATCC (American Type Culture Collection). HEC-1A and RL-95 endometrial cell lines were a kind gift from Dr. A. Coosmans (KUL, Belgium). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 1 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. For transient transfection experiments, BeWo and JEG-3 cells were transfected in 6 well plates (6 X 10^5 cells/well) using 3 µg DNA according to the manufacturer's protocol (JET-PEI, PolyPlus; ExGen 500, Fermentas). C33a cells were transfected using 3 µg DNA by the phosphate calcium method [35]. The transfection efficiency was assessed by transfecting separately or co-transfecting 30 ng of peGFP-NLS and was typically 40-60% for BeWo cells. For stable transfections, cells were transferred (2.5 X 10^5 cell/ml) 24 h post-transfection into 60 mm Ø dish, and incubated for 14 days with blasticidin 6.25 µg/ml (Invitrogen). Due to the lack of sequence coding for the blasticidin resistance in the pHPV-16Early and pLCR-GFP plasmids, both constructs (2.7 µg) were cotransfected in stable transfections with pEF6-LacZ (300 ng).

- **RNA extraction and quantitative real time RT-PCR**

Transiently transfected cells were washed with CellScrub™ buffer (Genlantis) in order to remove the extracellular plasmids. Total cellular RNA was extracted using the Nucleospin RNA II Total RNA extraction kit including DNase-I digestion (Macherey-Nagel). An additional stringent DNase-I reaction was performed using TurboDNaseI (Ambion-Applied Biosystems). First-strand cDNAs were generated by reverse transcription from 1 µg of total RNA in a total volume of 20 µl using the

RevertAid H Minus First strand cDNA synthesis kit containing random hexamers and H minus M-MuLV Reverse transcriptase (Fermentas). Quantitative expression of the HPV-16E5, HPV-16E6, HPV-16E7 and beta-galactosidase (*LacZ*) genes was analyzed by real-time PCR using the iCycler iQ system and the iQ Sybr Green Supermix (Bio-Rad). cDNAs (100 ng) were amplified in 25 µl total volume PCR reaction with specific primers under the following cycle conditions: 95˚C for 3 min, followed by 45 cycles of 95˚C for 30 sec and 59˚C for 30 sec. Gene expression was normalized using values obtained for the housekeeping GAPDH gene. The primers used for the amplification: GAPDH, 5' GAAGGTGAAGGTCGGAGTC and 5' GAAGATGGTGATGGGATTTC [36]; 16-E5, 5' CGTCCGCTGCTTTTGTCTGTGTCTACATAC and 5' CACCTAAACGCAGAGGCTGCTGTTATCCAC; 16-E6, 5' GGGAATCCATATGCTGTATGTG and 5' GCTGTTCTAATGTTGTTCC; 16-E7, 5' CAGCTCAGAGGAGGAGGATG and 5' CGCACAACCGAAGCGTAGAGTCACAC; Beta galactosidase (*LacZ*); 5' GCAGCAGTGATGATCCAGAACT and 5' CGTAGGTCACAGATGAGCCAATAA. A standard curve was generated for each gene. A dissociation curve was performed after each experiment to confirm that a single product was amplified. The quantification of transfected transcripts (E5, E6, E7 and *LacZ*) was assessed by calculating the $2^{(-\Delta CT)}$ where ($\Delta C_T = C_{T\ tranfected\ gene} - C_{T\ House\ keeping\ gene}$).

- **Western blotting**

Various protein extraction protocols were optimized in order to detect and retrieve the viral proteins from distinct sites inside the cells. The wild type HPV-16 N terminal flagged E5 protein was detected by western blotting in transiently transfected BeWo cells using the Triton X114 protocol recommended for hydrophobic membrane associated proteins [37]. Forty-eight hours after transfection, cells were washed twice in ice cold PBS then lysed during 1 hour at 4˚C in 500 µl ice-cold Triton X-114 lysis buffer (Triton X-114 1%, Tris HCl 10 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, protease inhibitors from Roche Applied Science). After centrifugation, supernatants were incubated 3 min at 37˚C and centrifuged 1 min at 14000 *g* for phase separation. The lower detergent phase was re-extracted by adding 10 volumes of the Triton X114 lysis buffer and analyzed. HPV-16 E6 was extracted using the Triton X-100 protocol [38]. Briefly, cells were washed twice in ice cold PBS, then extracted with a lysis buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 and protease inhibitors (Roche). After incubation on ice, lysates were centrifuged at 14,000 *g* for 10 min at 4°C. All fractions were stored at -80˚C and quantified using Bradford reagent. Protein extracts (70 µg total protein/lane) or total lysates were subjected to 12.5 % or 10 % SDS-PAGE and transferred to nitrocellulose membranes. Blocking, antibody incubation steps, and washing of the membrane were performed in PBS supplemented with 3 % skimmed milk and 0.05 % Tween 20. Blots were incubated with the anti-Flag HRP-conjugated antibody (Sigma) or with mouse anti-E-Cadherin antibody (BD Transduction Laboratories). Membranes incubated with primary antibodies were consequently incubated with HRP-conjugated anti-mouse secondary antibodies (Amersham Pharmacia Biotech). Blots were incubated with mouse anti-β-Actin antibody to control equal loading (MP-Biomedicals).

Immunoreactive proteins were visualized using chemiluminescence, captured on hypersensitive film (Amersham Pharmacia Biotech).

- Immunofluorescence confocal microscopy

Forty-eight hours after transfection, BeWo and JEG-3 cells were fixed for 5 min with Immunohistofix (Bio-Rad) at room temperature followed by 6 min with 100% methanol at -20°C. After two washes with PBS, the samples were saturated for 1 h with PBS containing 0.5% gelatin and 0.25% bovine serum albumin and stained for 1 h with a 1/10 dilution of an anti-Flag mouse monoclonal immunoglobulin G (IgG) (C-M2, Sigma). The samples were then washed three times with PBS containing 0.2% gelatin and incubated for 1 h with a 1/200 dilution of the secondary antibody: Alexa-488-coupled goat anti-mouse IgG (Molecular Probes). The samples were then washed three times in PBS with 0.2% gelatin and mounted for analysis on a Zeiss LSM510 laser scanning confocal microscope.

- **Cell adhesion assay**

BeWo cells (6 X 10^5) were transiently co-transfected with 30 ng pGL4-Luc-Renilla plasmid and 2.77 µg pEF6-Flag-HPV-16E5 or 2.77 µg pEF6-LacZ as described above. Forty-eight hours post transfection, cells were detached, resuspended in the medium, and counted. Bewo cells (8 X 10^5) from each transfection were lysed in order to assess input luciferase activity. The same number of cells was put in culture with or without RL-95 and HEC-1A endometrial cell lines already attached to the

support up to 95% confluence. After 2 h incubation, the attached cells were washed and processed using the Luciferase Assay Kit (Promega). The percentage of adhesiveness was calculated by normalizing luciferase activity founded in the attached cells to the input of luciferase activity.

- **Cytotoxicity and viability assays**

The LDH release was measured using the Cytotoxicity Detection Kit (Roche) in cells supernatants. Thirty hours after transient transfection or 24 h after media refreshments of stably transfected cell clones, crystal violet staining was used to assess the cellular confluence after stable transfections. Cells were fixed with ice-cold methanol and stained for 15 min with crystal violet solution (0.1% in MQ water). After 3 washes, dishes were dried at room temperature. Cell staining was eluted in 1 ml of acetic acid 10% and measured at 540 nm. In stable transfection, LDH values were normalized to the crystal coloration so that the cytotoxicity level could not be biased by the cell amount.

- **Luciferase assay**

One hundred ng pAP-1-Luc or 50 ng pNF-κB-Luc luciferase reporter plasmids were co-transfected with, respectively, 700 ng of pHPV-16 E5 or 750 ng of pHPV-16 E6/E7 and 30 ng pGL4-Luc-Renilla, into BeWo cells (plated in 24 wells plates at 7 X 10^4 cells/well on the day before). Forty-eight hours after transfection, cells were harvested and luciferase assays were performed according to the protocol of the Dual Luciferase Assay system (Promega). Firefly Luciferase activity was normalized to

Renilla Luciferase activity to correct for transcription efficiency in each reaction.

- **Cell migration and invasion assays**

In both migration and invasion assays, 100 µl of BeWo cells (10^6 cells/ml) in DMEM supplemented with 0.1 % BSA were seeded in the upper compartment of the Boyden chamber. The same cell number was seeded in 24 wells plate without inserts as an input normalization control. The lower compartment was filled with 600 µl of DMEM 1% BSA supplemented with 10% FCS. The 2 compartments were separated by a porous filter (8 µm pore). For invasion assay, the filter was coated using 25 µg of Matrigel (BD). The chambers were incubated for 24 h at 37°C, then fixed in ice-cold methanol and stained with crystal violet (0.1 %). The cells on the upper surface of the filter were scrapped with a cotton swab. Migration and invasion were quantified by elution of the crystal violet in the lower surface of the filter and in the control wells by adding 50 µl of acetic acid 10 % and measuring absorbance at 540 nm. The percentage of migration or invasion was calculated by normalizing absorbance from the lower surface of the filter to the absorbance from the control wells without filter.

- **Statistics**

Results were obtained from at least 3 independent experiments with using different batches of DNA. Data are presented as means ± SD of triplicates from a representative experiment. Triplicates from all experiments were averaged before statistical analysis. Student's two tailed *t*-test was used for statistical analysis and a P value < 0.05 was considered as statistically significant.

Results

HPV-16 early proteins expression in trophoblastic cells

To analyze the impact of HPV-16 E5, E6 and E7 early proteins on the physiology of trophoblastic cells, we first generated plasmids for the expression of N-terminally flagged E5 or simultaneous expression of both N-terminally flagged E6 and unflagged E7 in tandem under the control of the ubiquitous human elongation factor alpha promoter (hEF-1α). The expression of LacZ, HPV-16 E5, E6 and E7 genes were confirmed in transiently transfected BeWo cells by quantitative real time RT-PCR (Figure 1A). Figure 1B, shows that Flag-E5 protein formed monomers (11 kDa), dimers and multimers. In confocal microscopy analysis, HPV-16 E5 was observed in internal membrane compartments of trophoblastic cells (Figure 1C), as previously reported in other cell types [24,39]

HPV-16 E6 was retrieved from transiently transfected BeWo cells using the Triton X-100 lysis protocol, as previously described [38] and detected by immunoblotting (Figure 1B). The 18 kDa band corresponds to the predicted size of HPV-16 E6 [40].

The expression of mRNA able to express the unflagged E7 oncogene from the tandem pHPV-16E6/E7 construction was confirmed by quantitative real time RT-PCR (Figure 1A). These results showed that HPV-16 E5, E6 are expressed in transfected trophoblastic cells (Figure 1A); moreover the presence of E7 mRNA suggested that the E7 protein could also potentially be expressed in those cells.

The HPV-16 E6 and E7 bypass the HPV-16 E5 cytotoxic effect, maintain and promote viability of trophoblastic and cervical cells

We investigated the effect of the expression of HPV-16 early proteins on the viability of the choriocarcinoma BeWo cell line and of the HPV negative cervical carcinoma C33a cell line. Cells were stably transfected either with the control plasmid pEF6-LacZ, with pHPV-16E5, with pHPV-16E6/E7 or with a combination of both pHPV-16E5 and pHPV-16E6/E7 plasmids. As shown in Figure 2A, HPV-16 E5 induced two times more LDH than LacZ control in both BeWo and C33a cells. HPV-16 E6 and E7, on the other hand, despite their lower expression (Figure 1A), not only promoted viability but could also counteract E5 cytotoxic effect. The crystal violet staining showed that the number and the size of E5 colonies were smaller than LacZ, E6/E7 or E5/E6/E7 colonies in both cell types. Moreover, we noticed that expression of the E5 mRNA transcripts were not anymore detectable after 5 passages in stable transfection (data not shown), on the opposite to the expression of the other transcripts (E6, E7, lacZ). The impact of E5, E6 and E7 expression was also studied under endogenous viral regulation by stably transfecting the pHPV-16Early plasmid in which the expression of the entire HPV-16 early region is under the control of the viral LCR

(Long Control Region). The expression of all HPV-16 transcripts was confirmed by quantitative real time RT-PCR (Weyn et *al.* in preparation). BeWo and C33a cells transfected with pHPV-16Early were compared to cells transfected by pLCR-GFP, a control plasmid in which the expression of the Green Fluorescent Protein gene (GFP), is also under the control of the LCR. As shown in Figure 2B, pHPV-16Early stably transfected cells exhibited no cytotoxicity, with greater colonies number than GFP controls (Figure 2B). These results confirmed that physiologically regulated HPV-16 Early proteins promote the growth of both trophoblastic and cervical cells.

The AP-1 and NF-κB pathways are stimulated by HPV-16 E5 and E6/E7 oncoproteins

According to the viability/cytotoxicity results, we analyzed whether the activity of AP-1 and NF-κB transcription factors was modified in trophoblastic cells expressing either HPV-16 E5 or E6/E7. Compared to LacZ expressing cells, AP-1 activity was 18 and 60 times higher in cells co-transfected with pHPV-16 E5 or pHPV-16 E6/E7, respectively (Figure 3A). On the other hand, cotransfection with pHPV-16E5 induced a 4 fold increased in NF-kB acticvation while a 3 fold induction was obtained when cotransfecting pHPV-16 E6/E7 (Figure 3B). Thus, HPV-16 E5 or E6/E7 might influence cellular growth via differential activation of AP-1 and NF-κB.

HPV-16 E5 affects the adhesion of trophoblastic cells

You et *al.* [41] reported that HPV-16 E6/E7 enhanced the malignant phenotype of trophoblastic cells and reduced their adhesion to endometrial cells. Little is known concerning HPV-16 E5 involvement in malignant phenotype and its ability to alter adhesion capacities. In the present work, we analyzed the effect of HPV-16 E5 on the

adhesiveness of BeWo trophoblastic-like cells to the tissue culture plastic and to the RL-95 and HEC-1A endometrial-like cells, a model mimicking trophoblast cells attachment to the endometrial bed. As shown in Figure 4, BeWo cells transfected with pHPV-16E5 were less prone to attach to the plastic substrate or to the RL-95 and HEC-1A endometrial-like cells, compared to LacZ control. These results suggest that HPV-16 E5 reduces adhesiveness of trophoblastic cells to the tissue culture plastic and to endometrial cells. HPV-16 E5, like E6 and E7, affects trophoblast adhesion.

HPV-16 E5 and E6/E7 increase chemotaxic and invasive properties of trophoblastic cells

In regards to the impact of the HPV-16 E5 on BeWo cells adhesion, we analyzed the effect of HPV-16 E5 or E6/E7 expression on BeWo cells motility and invasiveness. Compared to the pEF6-LacZ transfected control, pHPV-16E5 transfected cells migrated and invaded slightly more efficiently, while E6/E7 transfected cells migrated and invaded even up to two times more easily, (Figure 5A and 5B). Similar results were observed with stably transfected BeWo cells (data not shown). Thus, E5, like E6/E7 is able to increase the trophoblast malignant phenotype by triggering cellular migration and invasion.

When HPV-16 early proteins were expressed under the control of the viral LCR (pHPV-16Early), cell migration and invasion were also significantly enhanced compared to control cells (Figure 5C and 5D). The number of cells colonies obtained by transfecting pHPV-16Early or pLCR-GFP was lower as the plasmid allowing blasticidin resistance was cotransfected in a ratio 1 for 10. The expression of HPV-16 E5, E6, E7 under the control of the endogenous original promoter (LCR) or an

ubiquitous promoter (EF-1α) increases motile and invasive properties of trophoblastic cells.

The trophoblastic cells expressing HPV-16 E5, E6/E7 and E5/E6/E7 have defective expression of E-cadherin

Several reports showed that the cell-cell adhesion molecule E-cadherin expression is altered during HPV carcinogenesis. Therefore, the expression of the E-cadherin protein was analyzed by western blotting in total lysates of BeWo cells transiently or stably transfected with pEF6-LacZ, pHPV-16 E5, pHPV-16 E6/E7 or co-transfected with pHPV-16 E5 and pHPV-16 E6/E7 plasmids. Interestingly, the E-cadherin expression was decreased not only in BeWo cells expressing HPV-16 E6/E7 but also in BeWo cells expressing HPV-16 E5 (tested alone or in the concomitant presence of HPV-16 E6 and E7; Figure 6A and 6B). These data suggest that the HPV-16 E5, E6 and E7 downregulate E-cadherin protein expression in trophoblastic cells.

Discussion

The involvement of E5, E6 and E7 oncoproteins in each stage of the multi-step carcinogenesis remains poorly understood and the list of new HPV tissue tropism is still lengthening (Reviewed in [1]). Recently, our group and others revealed that multiple types of HPV could infect and replicate in human trophoblastic cells [31,32]. In the present study, we explored the impacts of HPV-16 E5 and E6/E7 on human trophoblastic cell lines. The trophoblast represents a suitable model to study tumorigenesis, since it behaves in a way similar to malignant tumoral cells but obeys to rigorous spatial-temporal regulation of growth, migration and invasion during the different stages of pregnancy. BeWo cells, baptized “trophoblastic-like cells” share many morphologic, metabolic, and functional characteristics with primary human trophoblasts of the normal first trimester placenta [34,42]. The aim of this study was to evaluate the effects of HPV-16 E5, E6 and E7 oncoproteins on human trophoblasts and to identify new molecular targets.

We showed that HPV-16 E5 and E6/E7 genes products can be expressed in BeWo transfected cells. The flagged HPV-16 E6 protein was shown to be properly expressed from the E6/E7 ORF and the expression of the non-flagged E7 was confirmed by quantitative real time RT-PCR. It is known that detection of the small hydrophobic E5 protein is not easy since a considerable proportion of codons within the HPV 16 E5

ORF are inefficiently translated in human cells [43]. Many groups used SV40 transformed cell lines, optimized E5 codons or the tet-off systems for E5 overexpression [19,43,44]. As observed on Western blot, in an multimerized profile the wild type flagged HPV-16 E5 protein was expressed in BeWo cells. The persistence of multimers under reducing conditions could be attributed to the huge amount of the hydrophobic E5 protein concentrated in the detergent phase during the extraction procedure, favouring protein aggregation [45]. Meanwhile, the localization of this protein was observed in internal cell membranes, as previously reported [45].

We observed that expression of HPV-16 E5 induced a cytotoxic effect on transfected cells. On the contrary, the expression of HPV-16 E6/E7 did not cause any cytotoxicity and could abrogate the E5 induced cytotoxicity, even though coexpression of E6 and E7 was at least 4 times lower than the expression of E5, according to the quantitative real time RT-PCR data. Similar results were observed after stable transfections or transient transfections, on two choriocarcinoma cell lines and on one cervical cell line. These observations suggest that an extended HPV-16 E5 expression alters cell viability.

Although HPV-16 E5 was reported to induce proliferation [46], an extended HPV-16 E5 expression was reported to be lethal in various cell lines [44,47]. Kabsch et al. observed that HPV-16 E5 protein sensitizes human keratinocytes and mouse fibroblasts to apoptosis induced by osmotic stress [48]. By contrast, the same group showed that E5 expressing cells were less sensitive to Fas and TRAIL induced apoptosis [9]. Bravo et al. also discussed a putative correlation between the E5 altered lipid composition in keratinocytes membranes and apoptosis [26]. Kivi et *al.* observed

that microarray analysis could only be performed within the first 24 hours since prolonged expression would be lethal to HaCat cells [30]. Codon optimized HPV-16 E5 expression also reduced *in vitro* life span of keratinocytes [24,43]. Furthermore, several studies reported that E5 expression is usually transient during HPV productive cycle, weak, restricted to undifferentiated cells and that the E5 ORF is often deleted when HPV DNA is integrated [49,50]. HPV-16 E6/E7 proteins are known to be the major oncoproteins and the most relevant to malignant progression of HPV-transformed cells. In the present work, we demonstrated that HPV-16 E6/E7 were not only able to maintain and enhance cell growth for long-term expression in both stably transfected trophoblastic and cervical cell lines but also to reduce E5 cytotoxicity. The positive effect of HPV-16 E6/E7 on trophoblastic cell growth confirmed previous reports [41] but is in discrepancy with the study by Gomez et al. [41]. This latter group transfected a SV40 T antigen immortalized trophoblastic cell line with the complete HPV-16 genome. Surprisingly, on the opposite to many studies and unexpected for a non-lytic virus, they observed a strong reduction of cell viability in the selected transfected cells and a reduced invasive phenotype. Although they detected HPV DNA sequences and L1 protein expression only 7 and 15 days after transfection, respectively, they did not study early proteins expression which makes interpretation uneasy.

It is difficult to assess which molecular signals could be involved in the growth advantage induced by HPV-16 early proteins, especially in trophoblastic cells because those cells are able to produce virtually all cytokines [51]. As we detected a higher transcriptional activity of the AP-1 family transcriptional factors when co-transfecting pHPV-16E5, or even more when co-transfecting pHPV-16 E6/E7, it is possible that

AP-1 activation could play a role in association with other signalling pathways. Meanwhile, the transcriptional activity of NF-κB was significantly higher in cells expressing HPV-16 E5 than those expressing E6/E7, suggesting that AP-1/NF-κB balance could be important to promote viability.

Previous reports showed that HPV-16 E6/E7 decreases the trophoblast attachment to endometrial cells and might alter trophoblast implantation [41]. In the present report, we showed that HPV-16 E5 affects trophoblast adhesion to tissue culture substrate and to endometrial cells. Data reporting the impact of E5 on cell adhesion are relatively scarce. E5 was reported to alter adhesion signaling [30] and to impair gap junction-mediated cell-cell communication by dephosphorylating [28] or downregulating connexin 43 [29]. It is well known that the major oncoproteins E6/E7 affect adhesiveness of several cell types and enhance their malignant phenotype (Reviewed in [52]). The defect in adhesiveness of trophoblastic cells expressing HPV-16 E5 suggests that E5, like E6/E7 might contribute to malignancy and lead to potential abnormal implantation of the early embryo because of trophoblasts inappropriate spreading.

Subsequently, according to the lower attachment of E5 or E6/E7 transfected cells to various supports, we observed that HPV-16 E5 and in a larger measure E6/E7 enhance the trophoblast motility and invasiveness through the extracellular matrix. The expression of either HPV-16 E6 or E7, separately, gave similar results as E6/E7 expressed in tandem (data not shown).

E-cadherin impairment represents the hallmark of malignancy and is strongly associated to a poor prognostic for many tumors [54]. It is not only affecting cell-cell

adhesion, but also cytoskeleton rearrangement, cell adhesion to the extracellular matrix and subsequently migration and invasion [15]. E-cadherin is a Ca^{2+}-dependant cell-cell adhesion molecule connecting cells by direct E-cadherin contact with one another. The cytoplasmic interactions of this transmembrane molecule with the cytoskeleton is believed to be essential in conferring appropriate mechanical properties to cell-cell adhesion [53]. In the present work, we noticed a significant E-cadherin protein down regulation in trophoblastic cells expressing HPV-16 E6 or E7, separately (data not shown) or in tandem, but also in cells expressing HPV-16 E5 alone or in presence of HPV-16 E6/E7. Our results are in agreement with previous publications, reporting that E6 [15] and E7 [14] could reduce E-cadherin expression. It is tempting to suggest that the reduction of adhesion of the BeWo cells expressing HPV-16 E5 on endometrial cells could be, at least partly, due to the E-cadherin downregulation. However, the reduction of adhesion of these cells to the plastic flask might involve other mechanisms.

The transition from epithelial and adhesive cells to mesenchymal and motile cells is an important step during development and tumor progression. A key event in this process is the functional loss of E-cadherin. The NF-κB signalling pathway has also been reported to be a critical mediator of this transition. A direct link between the loss of the E-cadherin expression and the activation of NF-κB activity was reported by different groups [55,56]. The induction of snail transcription factor, an inhibitor of the E-cadherin gene transcription, could be one of the signalling mediators between these two observations [57,58].

It is worth noting that lack of cell adhesion to matrix has in most non-transformed

cells an adverse effect on cell survival, inducing a specific type of apoptosis, known as anoikis. Disruption of E-cadherin is mainly associated with this process. However, activation of AP-1 and NF-κB transcriptional activities, have been involved in the acquisition of anoikis resistance during the epithelial mesenchymal transition process [59]. We speculate that partly because of a differential NF-κB versus AP-1 activation balance, E5 could be less favourable to cell survival compared to E6 and E7. Our results and a recent publication suggest [60] that the activation of both NF-κB and AP-1 by HPV-16 E5 might contribute to carcinogenesis.

In conclusion, our results showed that HPV-16 oncoproteins alter the main human trophoblast functions by affecting its growth and adhesion and by increasing its migratory and invasive properties. The downregulation of E-cadherin expression in trophoblastic cells by the HPV-16 E5, E6 and E7 early proteins could at least partially explain their reduced adhesiveness and subsequent enhanced migratory abilities.

Funding:

Belgian Fund for Scientific Research (FNRS/FRSM-convention 3.4568.09); S Boulenouar was supported by grants from “Les Amis de l’Institut Pasteur de Bruxelles (ASBL) ”, from the “Fondation Rose et Jean Hoguet” and from the “Télévie”.

Acknowledgements:

We thank Dr. E-M. De Villiers for pHPV-16 DNA template, Dr. Michael Kalai for pEF6-Flag vector, Dr. Carine Van Lint for the pGL4-luciferase, Dr. X. Saelens for peGFP-NLS, Pr. A. Pötgens for the BeWo cells, Dr Pijnenborg for the JEG-3 cells, Dr. A. Coosmans for endometrial cells and Dr. C. Pequeux and Dr. A. Allaoui for providing us technical and equipment support, respectively.

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Legends to figures:

Fig. 1. Expression of Flag- HPV-16 E5, E6 and E7 gene products in trophoblastic cells. (A) Real time RT-PCR detection of the transcripts LacZ, E5, E6 and E7 in transiently transfected BeWo cells with the negative control pEF6-LacZ (3 µg), pHPV-16 E5 (3 µg), pHPV-16 E6/E7 (3 µg) or a combination of pHPV-16 E5 (2.7 µg) and pEF6-HPV-16 E6/E7 (0.3 µg). (B) Immunoblot of Flag-HPV-16 E5 and E6 proteins expression in transiently transfected BeWo cells with pHPV-16 E5 and pHPV-16 E6/E7. (C) Immunofluorescence confocal microscopy showing Flag-HPV-16 E5 localisation within transiently transfected BeWo and JEG-3 cells. Data show one experiment representative of three independent experiments.

Fig. 2. HPV-16 E5 on the opposite to HPV-16 E6/E7, alters viability of stably transfected cells (A) but the whole HPV-16 early region favour the viability of the cells (B). BeWo and C33a were stably transfected with the indicated constructs. At the end of the selection, LDH was measured in cells supernatants and the stable clones were colored by the crystal violet method. Each bar represents the mean ± SD of triplicates from a representative experiment. Statistical analysis on all experiments (n=5) showed only a statistically significant difference ($P < 0.05$) between pHPV-16E5 transfected cells and the control lacZ transfected cells and between pHPV-16Early transfected cells and their respective control, pLCR-GFP transfected cells.

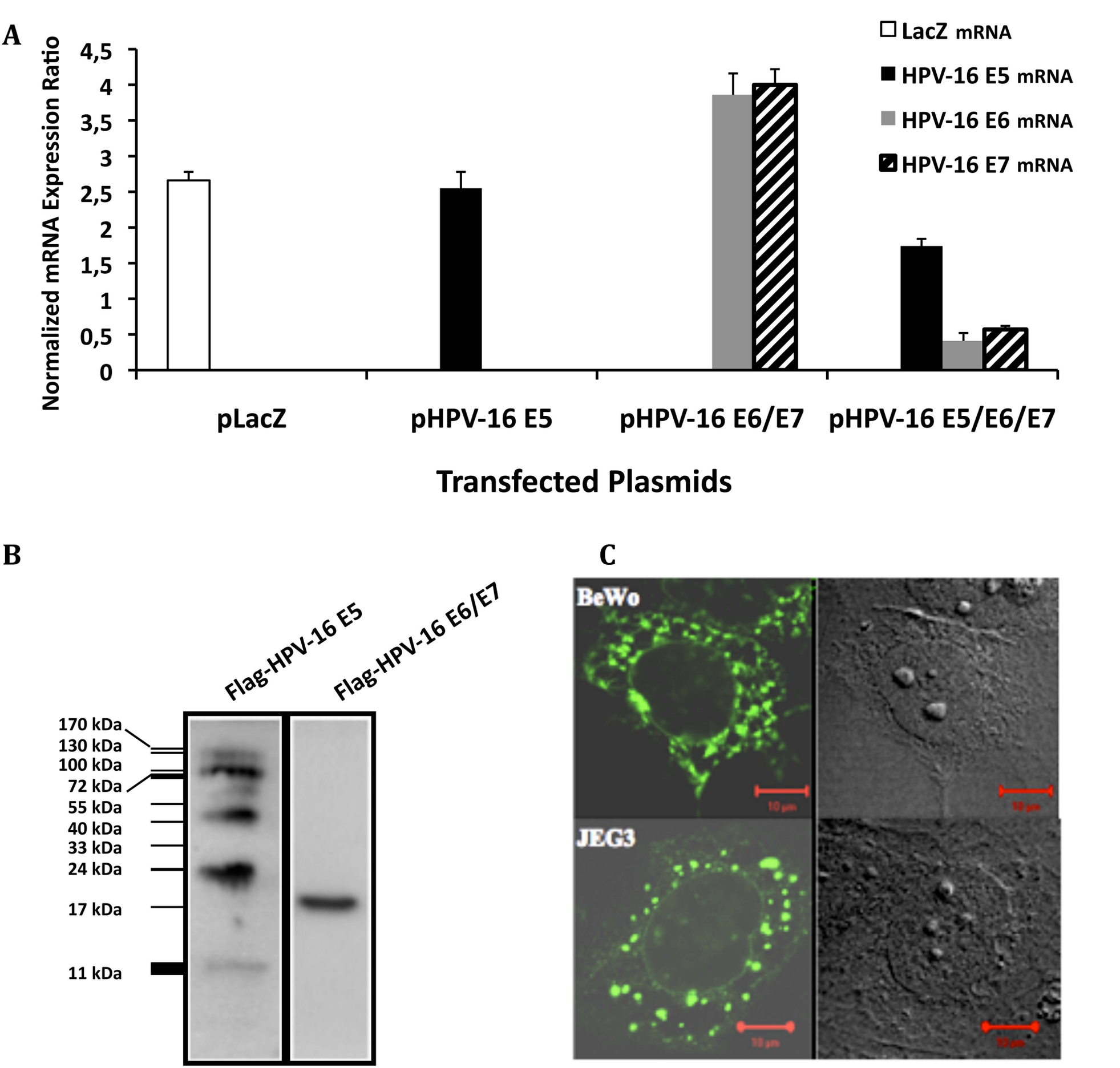
Fig. 3. The AP-1 (A) and NF-κB (B) pathways are differentially activated by HPV-16 E5 and E6/E7 oncoproteins. pAP-1-Luc or pNF-κB-Luc luciferase reporter plasmids were co-transfected in BeWo cells with pHPV-16 E5 or pHPV-16 E6/E7, and with pGL4-Luc-Renilla . Cell extracts were harvested 48 h later. For each extract, Firefly luciferase activity was normalized to the Renilla luciferase activity to correct for transfection efficiency. Each bar represents the mean ± SD of triplicates from a representative experiment. In all AP-1 (n=3) and all NF-*k*B trials (n=3), the difference between pHPV-16 E5 or pHPV-16 E6/E7 transfected cells versus pLacZ transfected cells was statistically significant ($P < 0.05$) as well between pHPV-16 E5 and pHPV-16 E6/E7 transfected cells ($P < 0.05$).

Fig. 4. HPV-16 E5 reduces adhesiveness of trophoblastic cells to endometrial cells and to the plastic substrate. BeWo cells were transiently cotransfected with pGL4-Luc-Renilla plasmid and either with pEF6-Flag-HPV-16 E5 or with pEF6-LacZ. Forty eight hours post-transfection, transfected cells were added to 90% confluent RL-95 or HEC-1A endometrial cells and further incubated for 2 h. Percentage of adhesiveness was calculated by normalizing the Renilla Luciferase activity measured in the attached cells to the input. Each bar represents the mean ± SD of triplicates from a representative experiment out of four experiments. A statistical significance ($P < 0.05$) was observed when analysis the data from the four combined experiments.

Fig. 5. HPV-16 early proteins enhance migration and invasion of trophoblastic cells. BeWo cells were transiently transfected and 24 hr later suspended in 0.1% BSA supplemented DMEM and seeded in the upper compartment of a Boyden chamber,

with the lower compartment filled with 1 % BSA supplemented DMEM. For invasion assay, the filter was coated with Matrigel (BD). Cell confluence was measured by crystal violet cells staining. The percentage of migration or invasion was calculated by normalizing absorbance results from the lower surface of the filter to the absorbance results from the control wells without filter. Each bar represents the mean ± SD of triplicates from a representative experiment. Statistical analysis on 5 independent trials showed significant results ($P < 0.05$).

Fig. 6. HPV-16 E5, E6, E7, E6/E7 and E5/E6/E7 decrease E-cadherin expression in trophoblastic cells. BeWo cells were transiently (right panel) or stably (left panel) transfected with the indicated plasmids. E-cadherin was detected in total protein extracts by western blot. β-Actin served as loading control. Data show one experiment representative of five independent experiments.


A
LacZ mRNA
HPV-16 E5 mRNA
HPV-16 E6 mRNA
HPV-16 E7 mRNA
Normalized mRNA Expression Ratio
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
pLacZ
pHPV-16 E5
pHPV-16 E6/E7
pHPV-16 E5/E6/E7
Transfected Plasmids
B
Flag-HPV-16 E5
Flag-HPV-16 E6/E7
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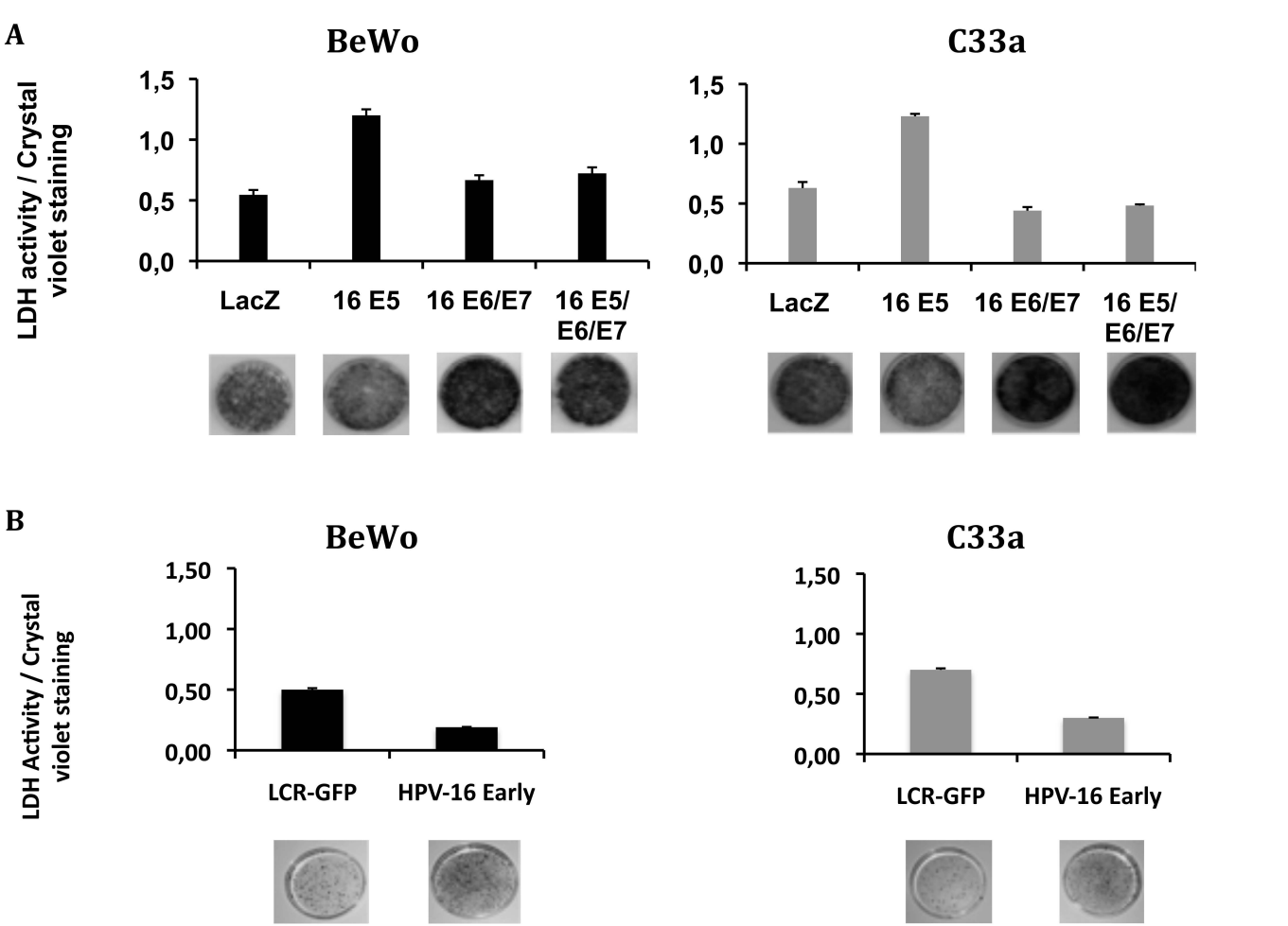
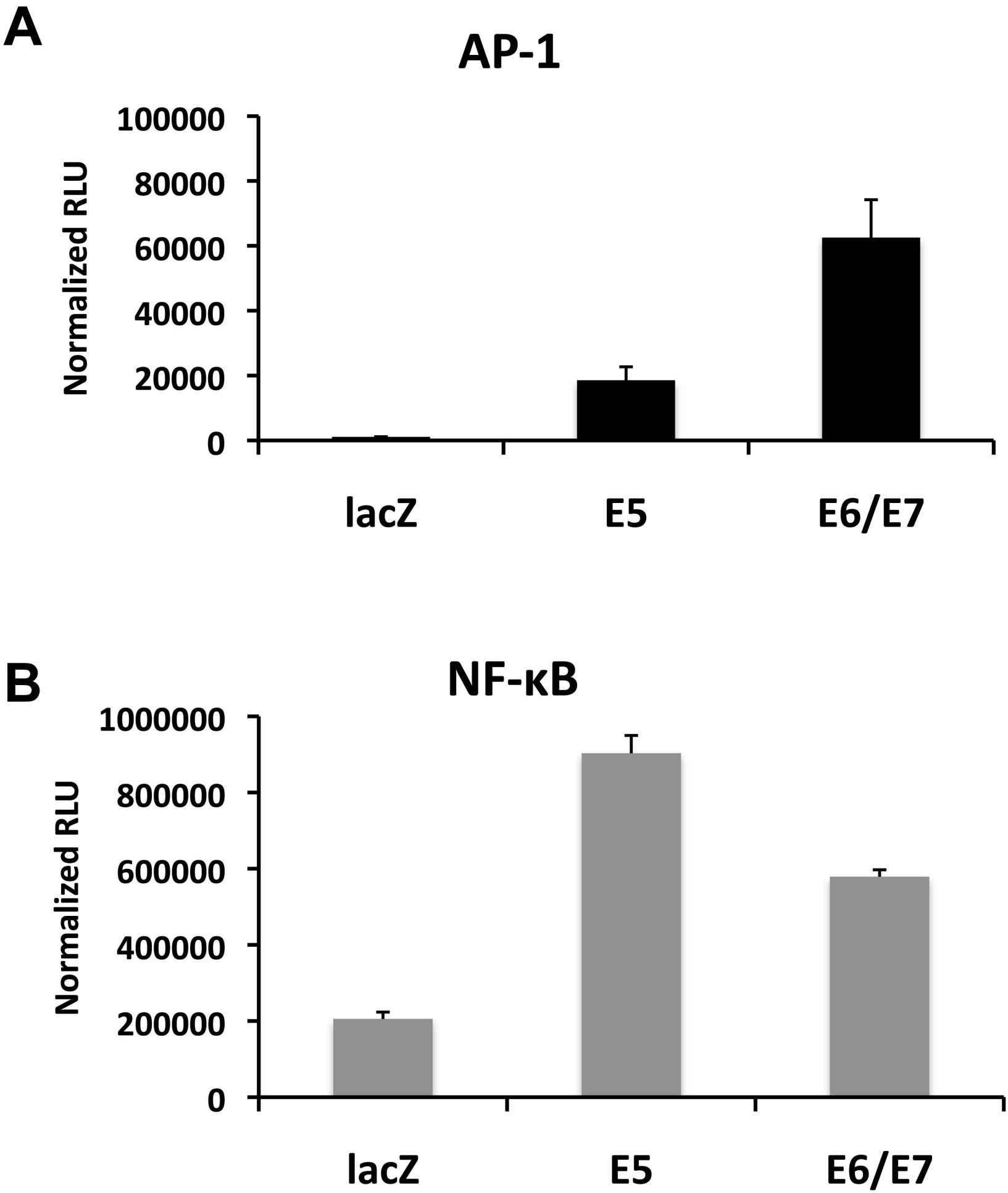
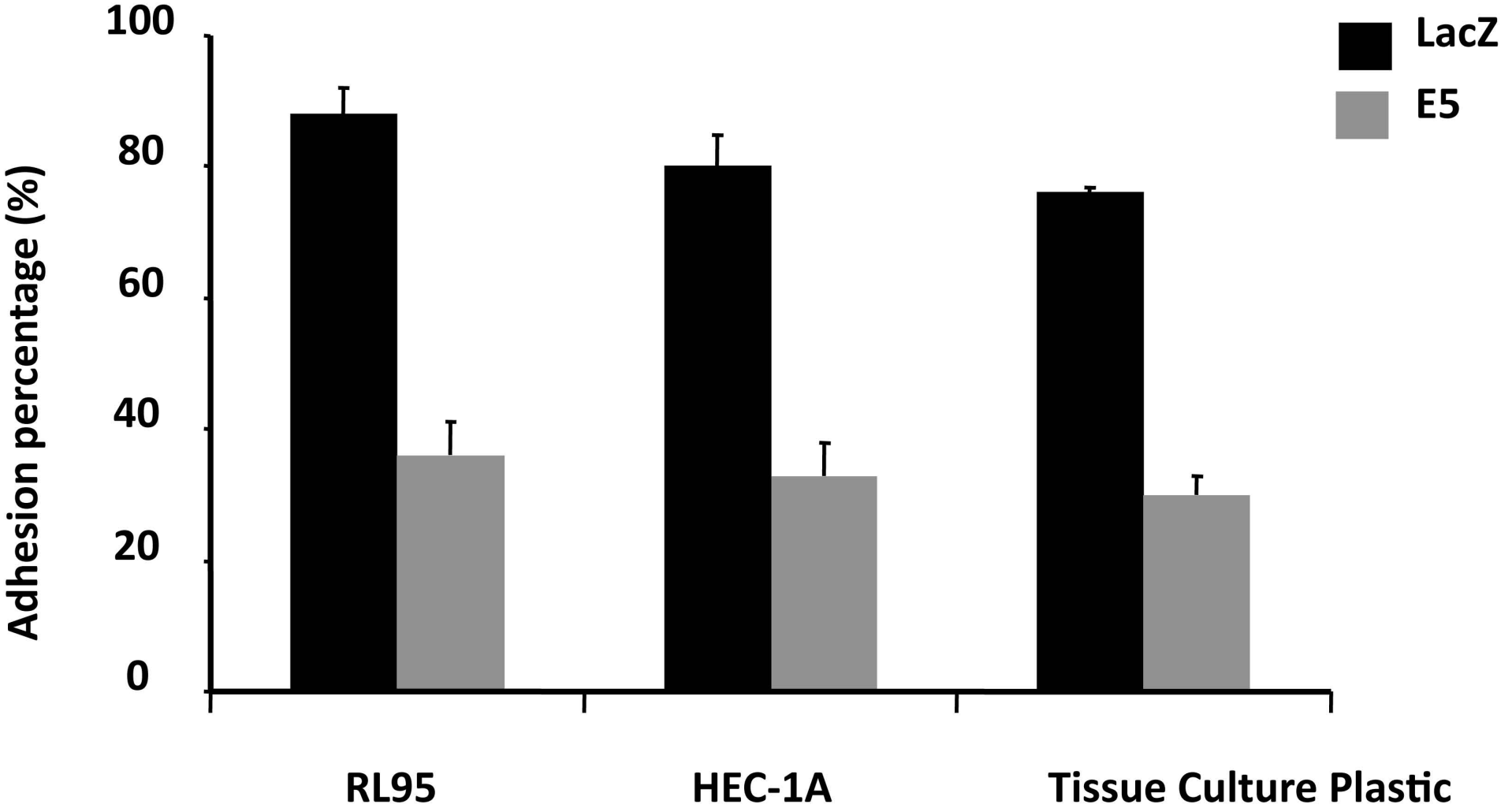
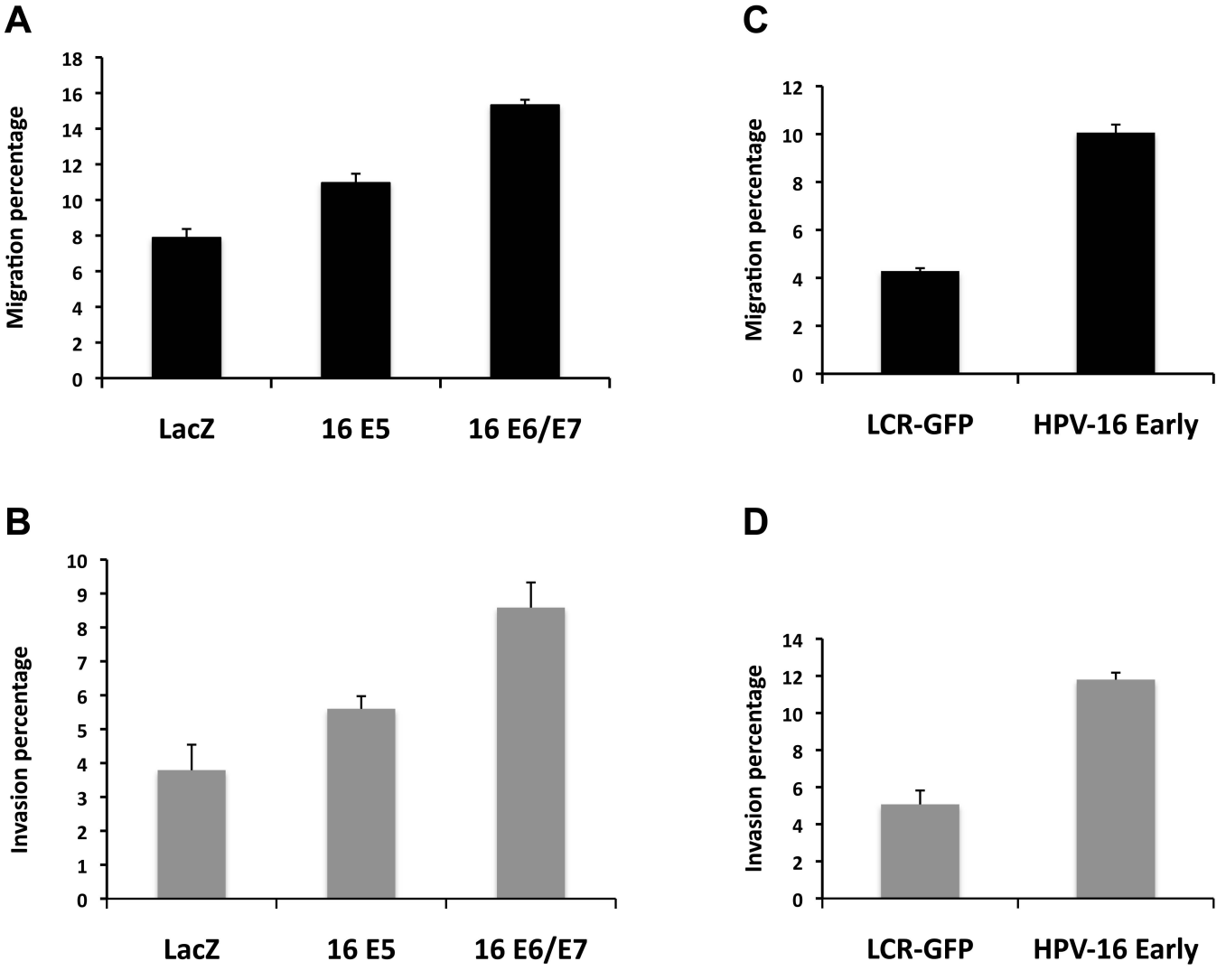
130 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
C
BeWo
JEG3


A
BeWo
C33a
LDH activity / Crystal violet staining
1,5
1,0
0,5
0,0
LacZ
16 E5
16 E6/E7
16 E5/ E6/E7
B
BeWo
C33a
LDH Activity / Crystal violet staining
1,50
1,00
0,50
0,00
LCR-GFP
HPV-16 Early


A
AP-1
Normalized RLU
100000
80000
60000
40000
20000
0
lacZ
E5
E6/E7
B
NF-κB
Normalized RLU
1000000
800000
600000
400000
200000
0
lacZ
E5
E6/E7


100
80
60
40
20
0
Adhesion percentage (%)
LacZ
E5
RL95
HEC-1A
Tissue Culture Plastic


A
Migration percentage
18
16
14
12
10
8
6
4
2
0
LacZ
16 E5
16 E6/E7
B
Invasion percentage
10
9
8
7
6
5
4
3
2
1
0
LacZ
16 E5
16 E6/E7
C
Migration percentage
12
10
8
6
4
2
0
LCR-GFP
HPV-16 Early
D
Invasion percentage
14
12
10
8
6
4
2
0
LCR-GFP
HPV-16 Early

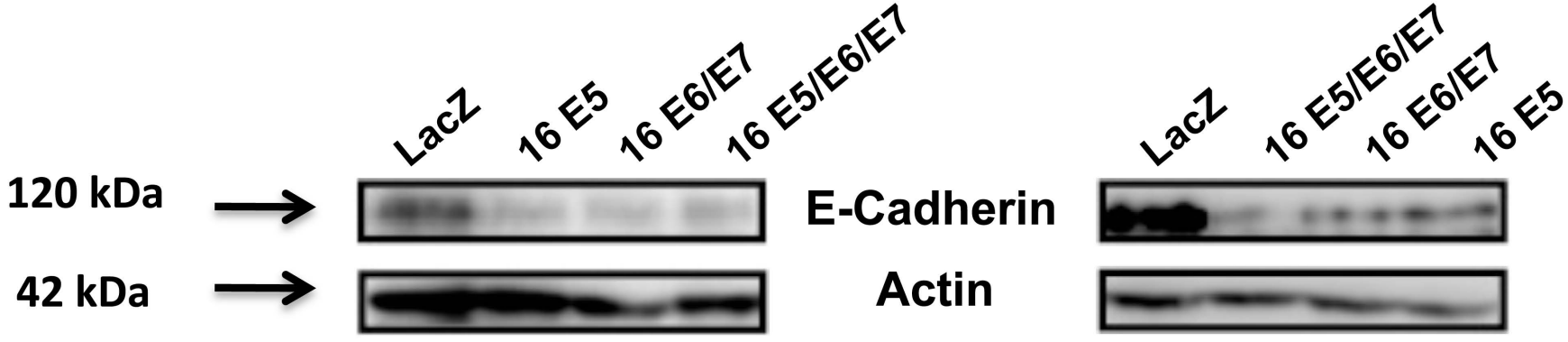

LacZ
16 E5
16 E6/E7
16 E5/E6/E7
120 kDa
E-Cadherin
42 kDa
Actin
LacZ
16 E5/E6/E7
16 E6/E7
16 E5

Fig. 1. Boulenouar et *al.*

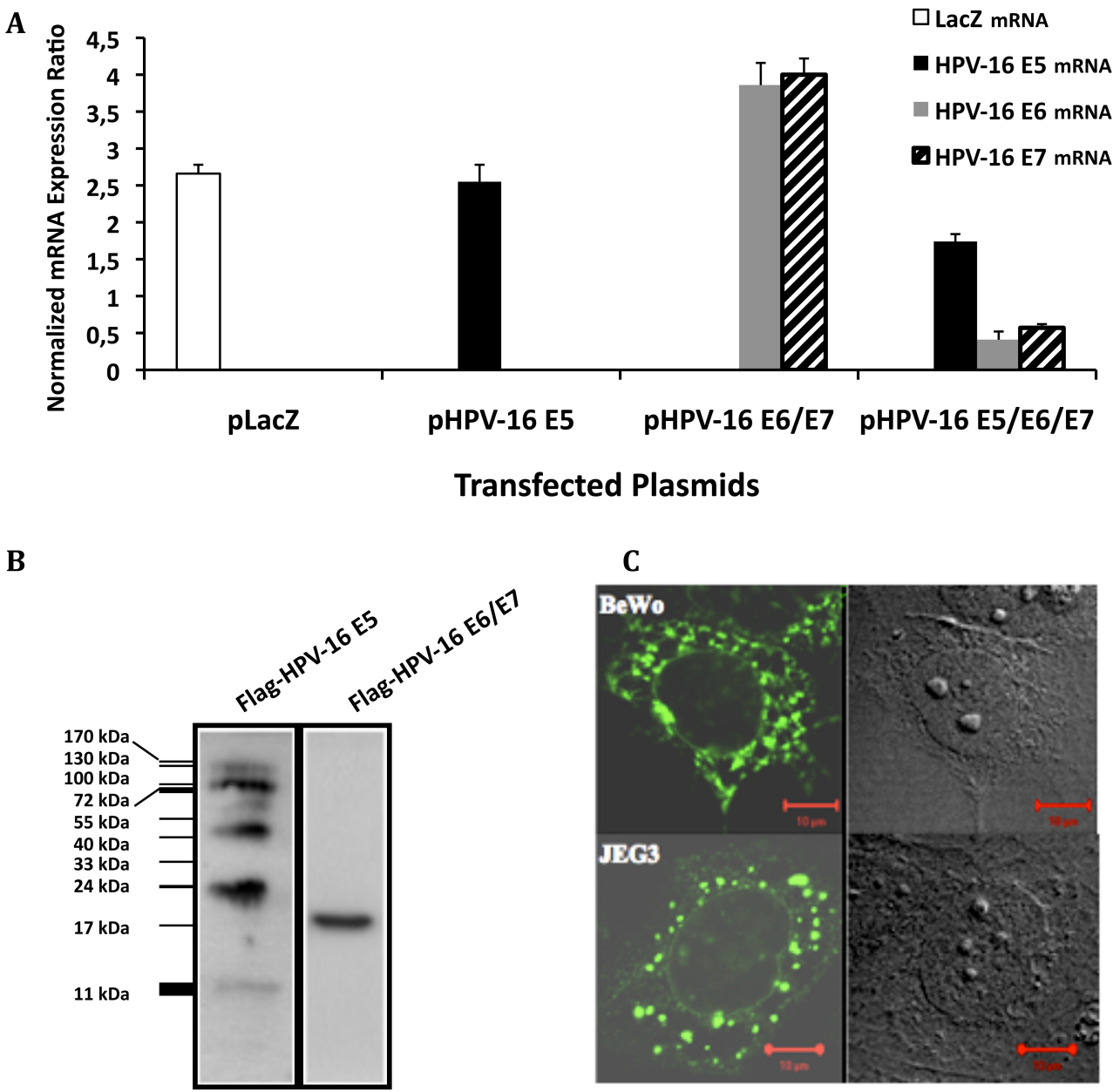


Fig. 2. Boulenouar et *al.*

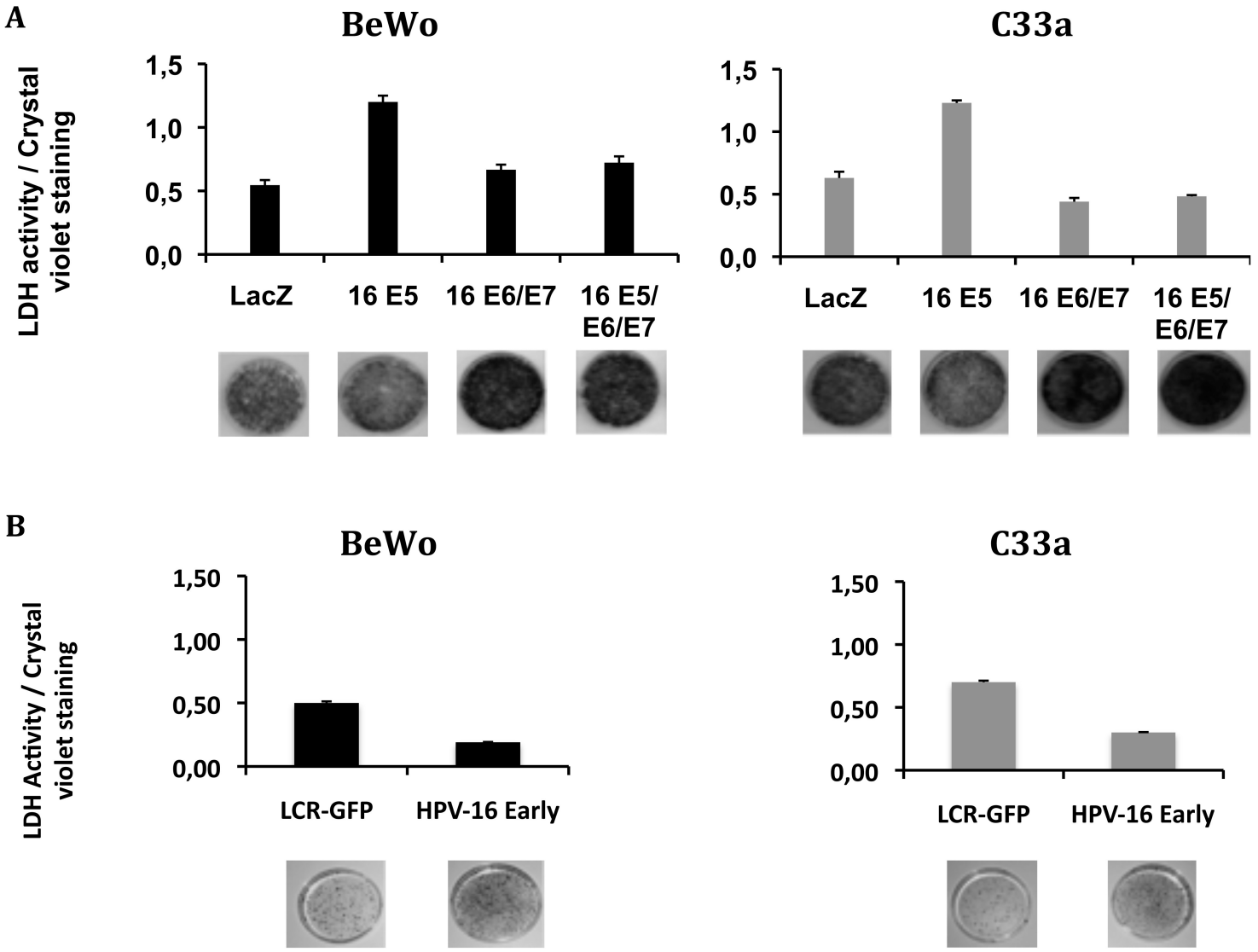


Fig. 3. Boulenouar et *al.*

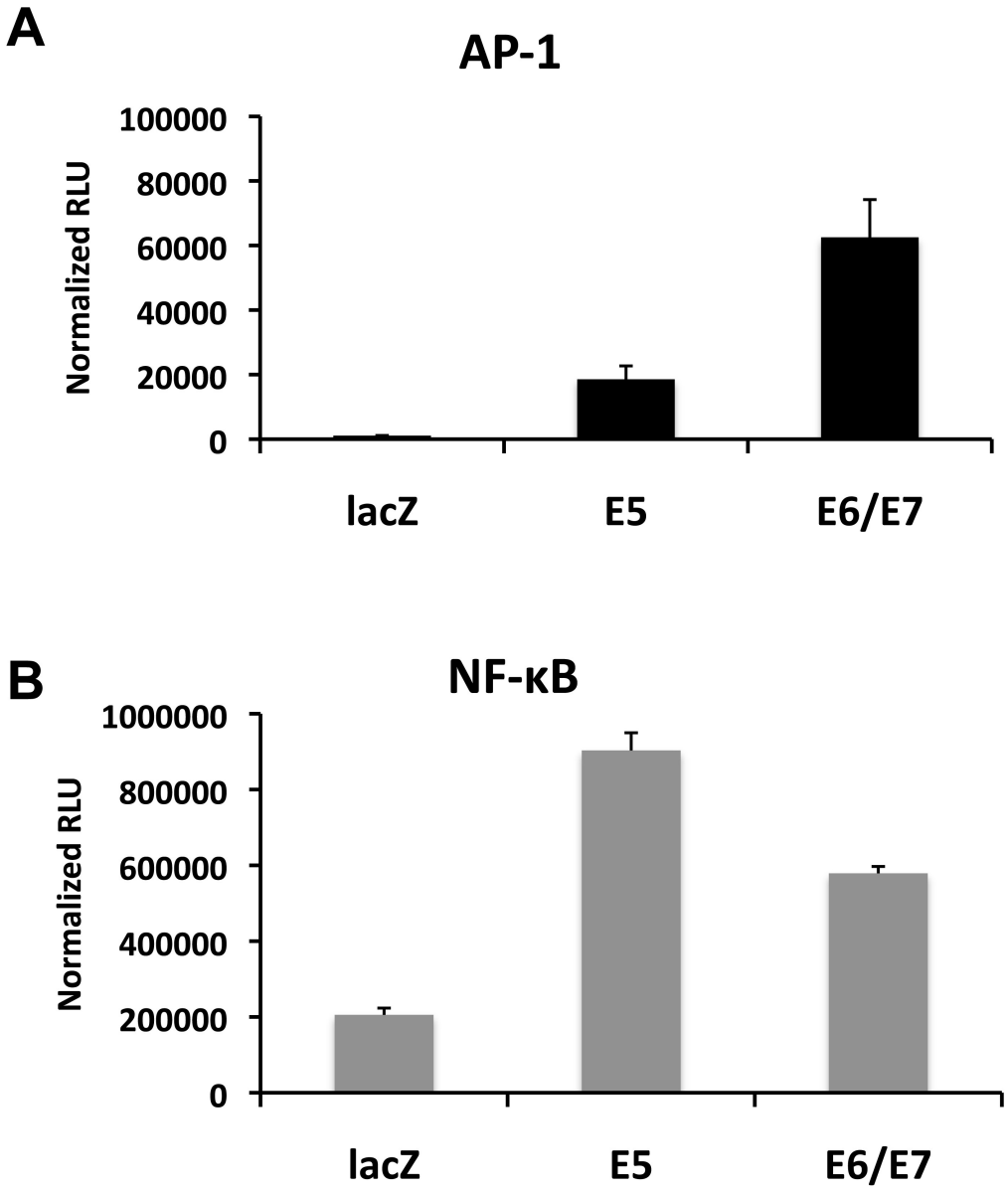


Fig. 4. Boulenouar et *al.*

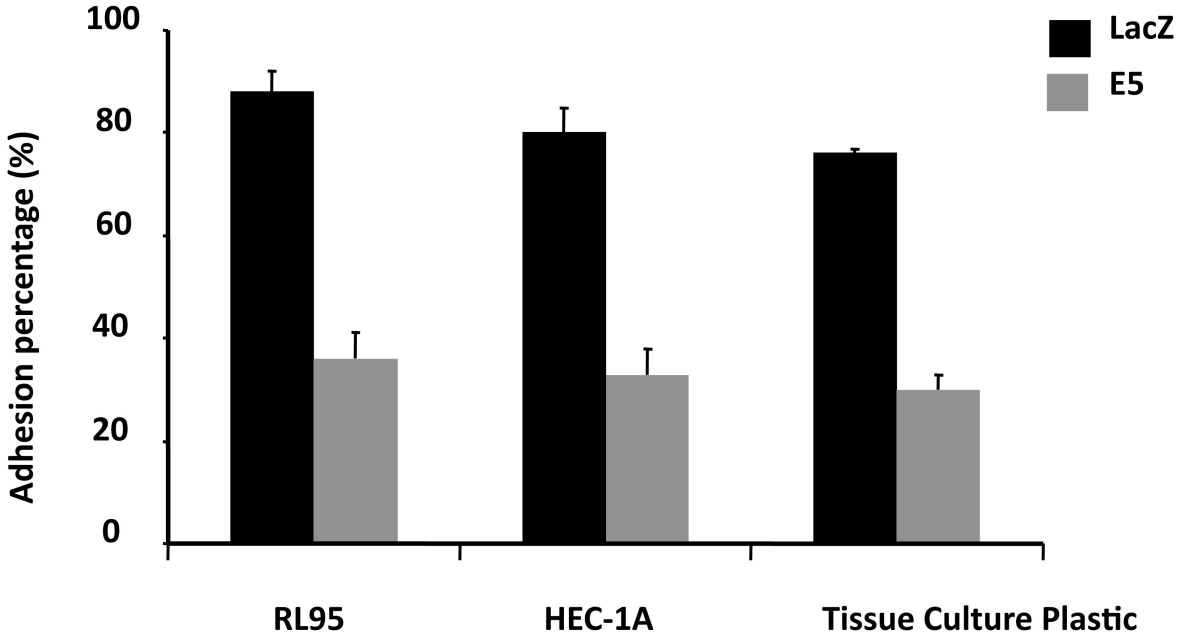


Fig. 5. Boulenouar et *al.*

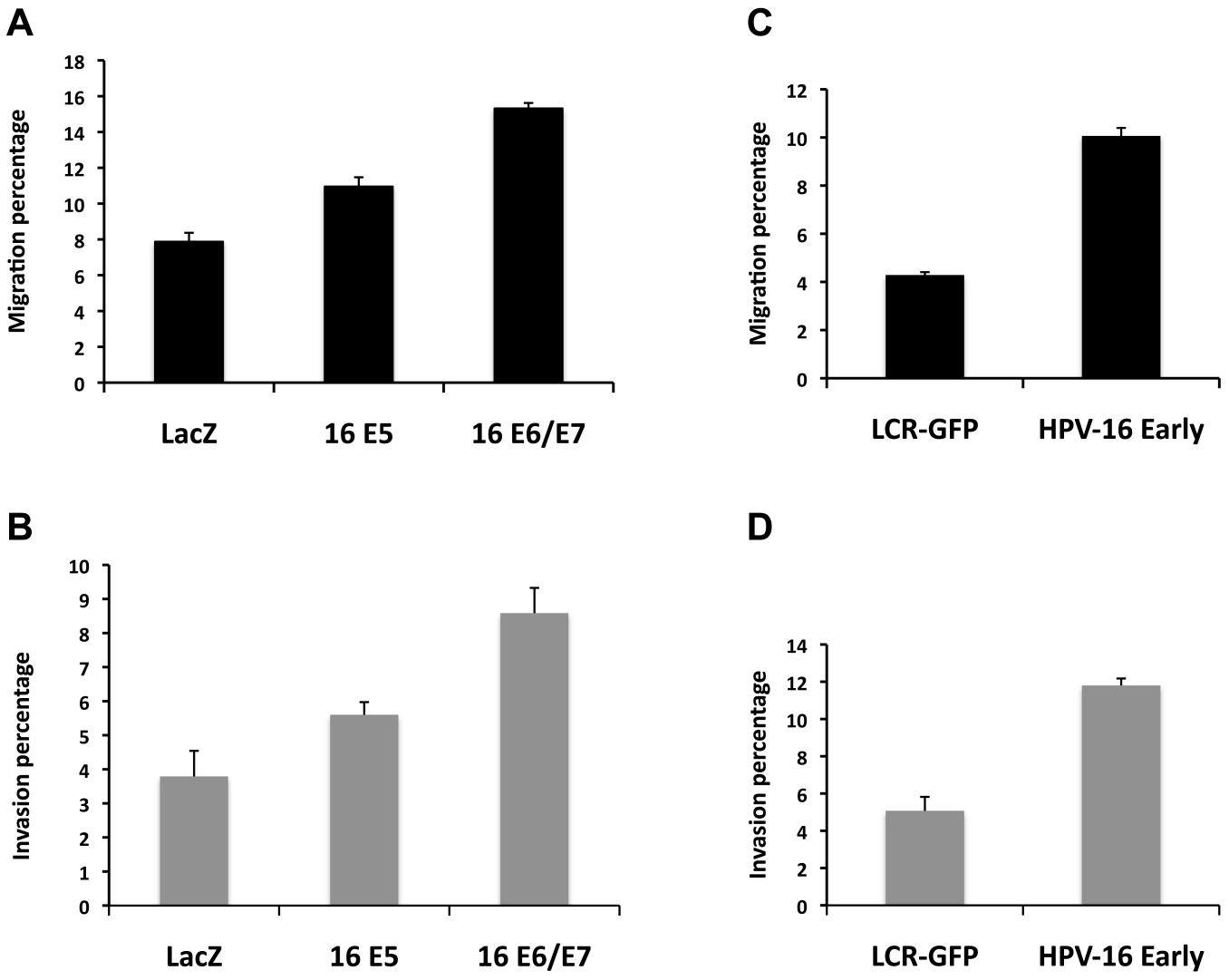
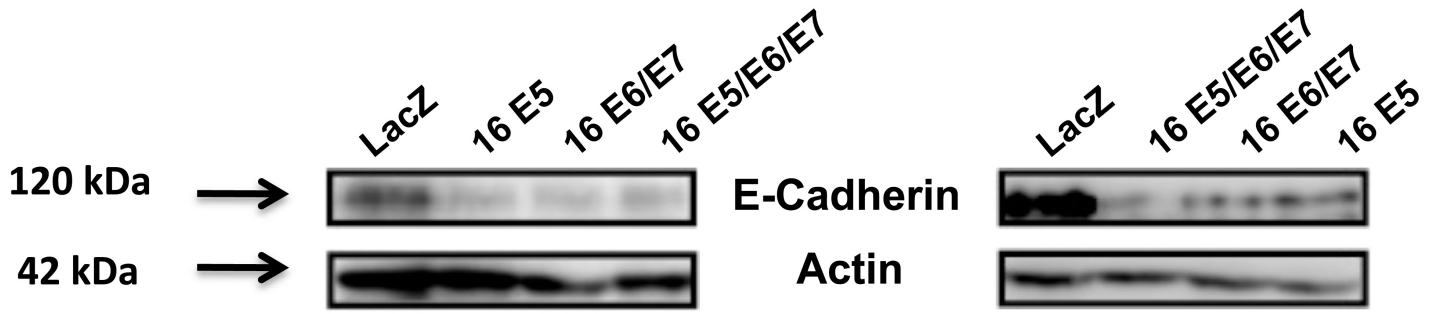



Fig. 6. Boulenouar et *al.*