

High risk HPV types 18 and 16 are potent modulators of oral squamous cell carcinoma phenotypes in vitro

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Abstract

Background

Human papillomavirus (HPV) has been confirmed as the primary etiological factor that transforms cervical epithelia into cancer. The presence of HPV in oral cancers suggests that HPV may play a similar role in transforming the oral epithelia. A high degree of variability in the prevalence of HPV in oral cancers has been found, however, raising questions regarding its role in the transformation and development of oral cancers. The goal of this study was to test our hypothesis that high risk HPV infection will alter the phenotype of an already transformed oral squamous cell carcinoma cell line, CAL27, *in vitro*.

Results

CAL27 cells transfected with HPV18, HPV16, and co-transfected with these high risk HPV types, demonstrated increased proliferation, adhesion to fibronectin, and cell spreading compared with non-transfected CAL27 controls. These differences were not a function of increased cell survival, as no differences in viability were found between HPV-positive and HPV-negative cells. HPV16 transfectants and HPV16/18 co-transfectants proliferated to a similar degree in each treatment, while notable differential responses to cell density and to the presence of serum were observed between HPV18 and the other HPV transfectants.

Conclusions

This study is among the first to explore the role of HPV types 18 and 16 as factors that influence the phenotypic behaviors of an oral squamous cell carcinoma. The finding that HPV18 and HPV16 measurably alter adhesion and proliferative potential of oral cancers is significant, suggesting a multifactorial role of HPV, not only in transforming, but also in modulating, the phenotype and treatment responsiveness of precancerous and cancerous oral lesions. These studies augment the existing evidence that HPV is a significant risk factor in oral cancer and will be helpful in establishing a rubric for generalizing the effects that HPV18 and HPV16 infection may have on various types of oral cancers, which could aid in the development of more effective and accurate prognostic indicators and treatments for oral cancers with concomitant HPV infections.

Background

As many as three dozen HPV types are known to infect mucosal surfaces of the genital tract, with fourteen prevalent high risk HPV types detected in virtually all cases of invasive cervical cancers [1, 2]. HPV16 is the most common HPV strain present in biopsies from women with cervical squamous cell carcinoma (SCC) and is also the most common strain of high risk HPV from biopsies of HPV-positive oral SCC (87%). HPV18 is the second most common high risk HPV strain found in these HPV-positive oral cancers (35%). Other common high risk cervical cancer HPV strains are rarely, or never, detected in oral SCC biopsies [3, 4].

The transformation of cervical SCCs is attributable to the HPV Early (E) genes, which code for proteins that, in addition to promoting viral replication, are capable of binding and inactivating transcription factors with tumor suppressor function, such as

p53 and *Rb*, regulators of cell-cycle checkpoints at the G₁ phase [5-7]. Although this pathway of HPV-induced transformation is well established in cervical SCC, there is some evidence that HPV infection could also function to transform the oral mucosa via these mechanisms [8].

Recent studies have provided further evidence that HPV is an independent risk factor for oral SCC, determining that HPV is found in three times as many precancerous oral mucosa, and almost five times as many oral cancers, compared with normal oral mucosa [4, 9-14]. However, the lower incidence of HPV18 and HPV16 in premalignant lesions, combined with an overall lower prevalence of HPV in oral SCC, suggests that these high risk HPV strains may induce transformation in some subset of oral cancers, while in established oral SCCs (OSCC) they may infect and subsequently act to modulate phenotypes [3].

Much of the literature related to HPV infection and oral cancers involves retrospective analyses of tumor biopsies and epidemiologic studies. While these and other studies have informed our understanding of the roles of HPV in oral cancer, they have not adequately addressed the apparent contradictory evidence that HPV infection may not be causally related to the formation of all, or even most, oral carcinomas. One possibility to explain the detection of HPV DNA in oral cancer biopsies, but not in premalignant oral lesions, may be the inability of varied methods, such as PCR and other detection techniques, to distinguish between HPV infections causally related to cancer development and those that are concomitant, non-causal HPV infections [15, 16]. By examining the effects of HPV infection on transformed OSCC *in vitro*, the etiologic factors that are necessary and sufficient to transform the oral mucosa, and those factors that may only promote proliferative potential in already transformed OSCC, can be more fully elucidated.

Although some studies have demonstrated the transformation of human foreskin and cervical keratinocytes *in vitro* using HPV16 [17, 18], until recently, the role of HPV in already transformed OSCC had not been investigated. We recently determined that an OSCC cell line, transfected with HPV16, exhibited significantly increased proliferation, when compared with non-transfected controls [19]. The increased proliferation was observed even in the absence of serum, and the effects were specific to proliferation, adhesion, and morphology, but not cell viability.

The goal of this study was to examine the effects of HPV 18, as well as HPV 16, both alone and in combination, on the proliferative phenotype of OSCC *in vitro*. Based upon our previous findings that infection of the OSCC cell line, CAL27, with HPV16 led to increased proliferation [19], we hypothesized that infection with high risk HPV18 would result in similar phenotypic alterations of these cells, particularly adhesion, proliferation, and morphology. We further hypothesized that co-infection with HPV16 and HPV18 would yield phenotypic changes similar to those observed for CAL27 infected with only HPV 16. We tested these hypotheses by transfecting CAL27 with the full-length HPV 16 and HPV18 genomes and establishing cellular adhesion and proliferation assays for transfected and non-transfected CAL27.

Our results provide one of the first demonstrations that HPV18 significantly affects the proliferative potential of CAL27 cells *in vitro*. In particular, we have determined that HPV18 and HPV16 transfection, and HPV16/18 co-transfection, produce

measurable differences in adhesion, morphology, and proliferation, compared with non-transfected controls. This study adds to the growing body of evidence that suggests that HPV may significantly alter the phenotype of OSCC *in vitro*. These results should aid in the delineation of possible mechanisms responsible for the development or progression of oral cancers by HPV.

Results

Transfection

To measure the effects of HPV on CAL27 cells *in vitro*, cells were transfected with the high-risk HPV types, HPV16 and HPV18. Total RNA collected prior to, and following, HPV transfection confirmed that CAL27 did not express HPV mRNA transcripts until transfected (Fig. 1). Specifically, our results demonstrated that CAL27 cells did not express HPV18 (Fig. 1A: lane 4), but did express HPV18-specific mRNA following transfection (Fig. 1A: lane 3). The previously-reported HPV18-positive cervical adenocarcinoma cell line, GH354, expressed HPV18 mRNA (Fig. 1A: lane 5).

In addition to HPV18, CAL27 cells were transfected with HPV16 and co-transfected with both HPV18 and HPV16. Our results again confirmed that CAL27 (Fig. 1A: lane 8) and CAL27-mTF (mock transfectants Fig. 1A: lane 6) did not express HPV16 (Fig. 1A: lane 8) until transfection (Fig. 1A: lane 7).

Densitometry measurements of the EtBr band intensities following RE-RT-PCR confirmed transfection efficiency and allowed comparison of HPV expression levels between endogenously-expressed HPV (GH354) and HPV16- or HPV18-transfected CAL27 cells (Fig. 1B). Our results demonstrated that transfection with either HPV16 or HPV18 resulted in expression levels roughly equivalent to the endogenously expressed HPV18 from the GH354 cell line.

Cell Spreading

Based upon our previous observations of increased cell spreading following HPV16 transfection, we hypothesized that HPV18- and HPV16/18-transfected CAL27 cells would exhibit discernable differences in cell spreading. Our results revealed a markedly higher percentage of cell spreading among HPV18- (62%), HPV16/18- (75%) and HPV16-transfected (98%) cells compared to non-transfected controls (30%) or mock transfectants (not shown) in the absence of serum by day 1 (Fig. 2A). The presence of serum (FBS) increased the percentage of cells spread in the non-transfected controls (75%), but was comparatively higher among the HPV transfectants (HPV16: 98%; HPV18: 97%; HPV16/18: 93%) at day 1. By day 3, the non-transfected CAL27 cells had increased in spreading slightly in NS (from 30% to 36%) while in FBS the percent spreading was virtually identical from day 1 to day 3. The HPV transfected cells were all at or near 100% spread by day 3 in both NS and FBS.

Viability

To determine if the morphologic changes to CAL27 cells were due to a change in viability or cell survival, we sought to determine the number of viable cells for each

assay we performed (adhesion and proliferation), particularly in the absence of serum (Figure 2B). Our results found no statistically significant differences in viability between non-transfected CAL27 (73%) and HPV16- or HPV-16/18-transfectants (84% and 81%, respectively) (n=723, p>0.05). A slightly higher initial level of viability was found among HPV18-transfectants (95%); however, measurements by day 2 indicated no remaining differences in viability between transfectants (98-100%) and non-transfectants (97-99%) (n=729, p>0.05) or mock transfectants (not shown).

Morphology

The changes in cellular morphology, specifically the extent of cell spreading, described above, are significant and demonstrable between the non-transfected CAL27 controls and HPV-transfected cells (Figure 3). Striking differences in absolute number and degree of spreading were observed between non-transfected CAL27 cells (Fig. 3A), and the markedly increased and ubiquitously spread HPV18- (Fig. 3B), HPV16- (Fig. 3C) and HPV16/18-transfected cells plated in serum by day 3.

Adhesion

To determine if the HPV-stimulated increases in CAL27 spreading were correlated with any measurable alteration in cellular adherence, 30-minute *in vitro* adhesion assays were performed to compare CAL27 controls with HPV-transfectants. No significant differences in cellular adhesion were found among the HPV-transfectants or between transfected cells and controls in three, separate, independent experiments using the standard adhesion assay format (Fig. 4) (n=24 replicates per condition, p>0.21).

Our previous results using a modification from the standard adhesion assay that minimizes the loss of adherent cells, demonstrated that HPV16 transfection significantly increased CAL27 adhesion to fibronectin (FN) but not to naked plastic (NP) or wells coated with bovine serum albumin (BSA) [19]. Based upon these results, we hypothesized that demonstrable increases in adhesion to FN, but not NP or BSA, would be found among HPV-transfectants (HPV18, HPV16/18) using the adhesion assay modification. These results indicated statistically significant increases in adhesion to FN among HPV-transfectants; HPV18 induced a 58% increase (n=24, p<0.05), HPV16 inducing a 47% increase (n=24, p<0.05), but HPV16/18 induced only a 22% increase which was not significant (n=24, p=.07) (Fig. 4). As expected, no differences in adhesion were found on NP or BSA between any groups.

Proliferation

Our previous studies with OSCC proliferation revealed that CAL27 cells proliferated more rapidly when plated at higher density, even in the absence of serum. Furthermore, transfection of CAL27 with HPV16 further increased CAL27 proliferation at low densities, with this effect further modulated in the presence of serum [19]. Based upon these previous observations, we hypothesized that HPV18- and HPV16/18-transfected CAL27 cells would exhibit increased proliferation, even at low density, and these effects would be amplified in the presence of serum. To provide a baseline for comparison of proliferation, we plated CAL27 cells at high and low density in FBS and NS for three days (Fig. 5A) and confirmed that CAL27 proliferated more rapidly in FBS, with these effects further increased at higher density (n=24, p<0.01).

HPV16

To confirm our previous results, we plated HPV16-transfected CAL27 cells and measured their proliferation over three days (Fig. 5B). In keeping with our previous report, we observed that HPV16-transfectants proliferated more rapidly when plated at high density (HD) than at low density (LD). In addition, the presence of serum (FBS) further increased HPV16-driven proliferation by 2.9-fold at LD and 1.25-fold at HD. All treatment conditions of HPV16-transfected CAL27 cells were statistically distinct from one another (HD-FBS; HD-NS; LD-FBS; LD-NS) (n=24 replicates per condition, p<0.01).

HPV18

To assess the effects of HPV18, HPV18-transfected CAL27 cells were plated at high and low densities, with and without serum, to measure their rates of proliferation (Fig. 5C). As with HPV16, HPV18 stimulated an increase in CAL27 proliferation under each condition that was distinct and significant from non-transfectant controls. HPV18-stimulated proliferation was more strongly influenced by the presence of serum than by density, with HD-FBS proliferating at 1.5-fold the rate of HD-NS, and LD-FBS proliferating at 4.5-fold the rate of LD-NS. Unlike HPV16-transfectants, HPV18-transfectants under LD-FBS conditions proliferated to a greater extent than those at HD-NS (n=24 replicates per condition, p<0.01).

HPV16/18

The combinatorial effects of HPV16 and HPV18 were assessed by plating HPV16/18 co-transfectants at high and low densities, with and without serum, to gauge proliferative responses (Fig. 5D). As with HPV16, HPV16/18-transfectants proliferated more rapidly when plated at high density than low density, with serum further modulating this effect. HPV16/18 proliferation at HD-FBS was 1.8-fold higher than at LD-FBS, while HD-NS was 2.9-fold more than LD-NS. The presence of serum increased HD-proliferation by 30%, and increased proliferation at LD by 210% (n=24 replicates per condition, p<0.05).

HPV comparisons

To test our hypothesis that HPV18- and HPV16/18-transfectants induce an increase proliferation that is comparable to the increases induced by HPV16, we performed side-by-side, three-day proliferation assays under specific conditions (LD-NS; LD-FBS; HD-NS; HD-FBS) (Fig. 6). As expected, HPV-transfected cells demonstrated increased proliferation compared with non-transfected CAL27 cells in each assay and this proliferation was increased at both densities by the presence of serum (Fig. 6A and B). HPV16- and HPV16/18- transfectants in each assay proliferated to a similar degree, which were indistinguishable from one another; however, some differences were observed between HPV18 and the other HPV transfectants.

HPV18-induced proliferation differed from HPV16- and HPV16/18-induced proliferation under two of the specific treatments. First, in the LD-FBS treatment, HPV18-stimulated proliferation was significantly higher than either HPV16- (1.5-fold, p<0.01) or HPV16/18- (1.5-fold, p<0.01), while all HPV strains were significantly higher than CAL27 non-transfectants (n=24 replicates per condition, p<0.01) (Fig. 6A). Second, in the HD-NS treatment (Fig. 6D) HPV18-driven proliferation was significantly lower than that of HPV16 (p<0.01), but not

significantly different from the co-transfectants ($p=0.486$), although all HPV strains were significantly higher than CAL27 ($n=24$ replicates per condition, $p<0.01$).

Responsiveness to serum

To distinguish the effects of serum from the effects of density among HPV strains, we performed a reductive analysis in which we determined the relative-fold increase in proliferation due to serum for each condition. To accomplish this we divided the increase in proliferation between day 1 and day 3 in FBS by the increase in proliferation between day 1 and day 3 in NS, first at LD and then at HD (Fig. 7) (Formula = LD-FBSd3 minus LD-FBSd1 divided by LD-NSd3 minus LD-NSd1). Our results demonstrated that although the HPV strains induced higher absolute proliferation than non-transfected CAL27 cells, the relative-fold increase for all HPV strains clustered tightly, to a similar degree, at HD. Interestingly, HPV18 did not cluster with HPV16 or HPV16/18 at LD but demonstrated a relative-fold increase almost 50% higher under this condition.

Discussion

We recently reported some of the first evidence that HPV16 induced significant phenotypic changes in OSCC behaviors, such as increased adhesion and proliferation, as well as changes in cellular morphology [19]. Based upon these observations, we sought to examine the roles of HPV18, and re-confirm the role of HPV16, in mediating phenotypic alterations in OSCC cells. More specifically, this study expands our analysis of high risk oral HPV strains to include the potential for infection of OSCC with HPV18 and co-infection with HPV16 and HPV18 to alter OSCC phenotypes under specific conditions *in vitro*.

Based upon our prior research findings, we hypothesized that transfection of CAL27 with HPV18, and co-transfection with HPV16 and HPV18, would produce significant phenotypic alterations in OSCC behavior, particularly morphology, adhesion, and proliferation. The introduction of HPV18, HPV16, and HPV16/18 to CAL27 did produce marked increases in cell spreading, which could possibly have been explained by a difference in cell viability. For example, if cells are more likely to survive due to the presence of HPV, they would also be more likely to adhere, spread, and consequently proliferate. Our results, however, conflict with this assumption and no significant differences were observed between HPV transfectants and non-transfectants with respect to viability to explain this phenomenon.

One possible explanation for these results may involve production of, and adherence to, the extracellular matrix (ECM). Prior studies of HPV-positive carcinomas and HPV-transformed epithelia found that these cells have higher expression levels of (ECM), such as fibronectin (FN) [20, 21]. As a result, these carcinomas and transformed epithelia also have increased upregulation of pp125 FAK (focal adhesion kinase), a cytoplasmic signaling molecule that is regulated by, and regulates, cellular adhesion to specific ECM substrates, such as FN [22, 23]. This study found that HPV18, HPV16, and to a lesser extent HPV16/18, increased CAL27 adhesion to FN, but not to naked plastic (NP) or bovine serum albumin (BSA)-coated plates. This increased adhesion of HPV transfected OSCC may help to explain not only adhesive

properties, but also observed morphologic and proliferative changes of OSCC exposed to HPV.

Although all HPV-transfectants exhibited increased spreading, adhesion, and proliferation, our results demonstrated that HPV18 also exhibited treatment-specific behaviors that may be distinguished from those of HPV16- and HPV16/18-transfectants. For example, HPV18-driven proliferation was found to be lower at HD-NS, an *in vitro* condition that approximates the growing tumor environment of an OSCC prior to angiogenesis, or the growth of tumor-specific blood supplies [24]. In fact, several notable studies of cervical cancers found that HPV18 infection exhibits tropism for glandular tissues and is less likely than HPV16 to persist in squamous cell epithelium [2, 25]. Thus, the similarities between oral SCC tissue and cervical SCC tissue may partially explain the propensity for HPV18 to exhibit less of an effect than HPV16 on cellular proliferation under high density conditions.

Prior research has demonstrated that the simultaneous presence of both HPV16 and HPV18 is relatively rare in OSCC, an altogether different viral distribution than is noted with cervical cancers [1, 4]. This study found that HPV16/18 co-transfectants did not adhere or proliferate to the extent of HPV16- or HPV18-transfected cells. CAL27 is an OSCC, a comparatively less hospitable host for HPV18 than salivary adenocarcinomas [26], which may explain the correlation of higher proliferative phenotype associated with HPV16 in HD-NS. That HPV18 drives a comparatively higher proliferative response in LD-FBS conditions may indicate that, although HPV18 is less prevalent, the effects of this strain may influence cellular events following angiogenesis or during metastasis or perhaps in a different tissue type altogether.

Conclusions

The results of this study suggest that HPV types 16 and 18 are factors that influence the phenotypic behaviors of OSCC. We have provided evidence that HPV18 and HPV16 significantly increased spreading and proliferation of the OSCC cell line, CAL27. Furthermore, both HPV18 and HPV16 strongly altered fibronectin-specific adhesion in this cell line, a potential activator of signaling cascades that directly modulate progression through the cell cycle. The finding that HPV18 and HPV16 measurably alter adhesion and proliferative potential is significant, indicating that HPV may have multiple influences on precancerous and cancerous lesions. The results of this study also suggest that HPV18 and HPV16 should be considered high risk oral HPV types. A more detailed analysis including other OSCC cell lines, along with salivary adenocarcinomas, could help to establish a rubric for generalizing the effects that HPV16 or HPV18 infection may have on various types of oral cancer, and could aid in the development of more effective and accurate prognostic indicators and treatments for oral cancers with concomitant HPV infections.

Methods

Cell culture

CAL27 (human oral squamous cell carcinoma) and GH354 (human cervical adenocarcinoma) cell lines were obtained from American Type Culture Collection (ATCC: Manassas, VA). CAL27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine, adjusted to contain 3.7 g/L sodium bicarbonate and 4.5 g/L glucose, with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) solution and 10% fetal bovine serum (FBS) from HyClone (Logan, UT) in 75 cm² BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO₂ in humidified chambers. GH354 cells were also maintained as described with the addition of 20% FBS.

Transfection

CAL27 cells were seeded in T25cm² BD Falcon tissue-culture treated flasks in appropriate media as described above and allowed to achieve 70% confluence. Cells were then transiently transfected by adding 1 µg/mL of the full-length human papilloma virus type 18, cloned into the pBR322 vector (ATCC #45152), or HPV type 16, cloned into the pBluescript SK-vector (ATCC #45113). CAL27 cells were also co-transfected by adding 1µg/mL of HPV18 and 1µg/mL of HPV16. The transfections were performed using the Stratagene Mammalian Transfection Kit (La Jolla, CA) according to the manufacturer's recommended protocol for CaPO₄ transfection. As part of this procedure, CAL27 cells were co-transfected with the G418-resistant control plasmid, pWLneo. Cells were then incubated at 37°C and 5% CO₂ in humidified chambers for 24 hrs, at which time the media was changed and the cells were incubated as described for another 24 hrs. Cells were then split 1:10 and incubated for 24 hrs, at which time they had reached 30% confluence. Transfectants were then selected using G418 antibiotic (Stratagene: La Jolla, CA), added drop-wise to the culture medium at a concentration of 100 µg/mL. After selection, transfected cells were maintained in the same cell culture conditions as described above for CAL27. Mock transfectants of CAL27 (mTF) were also established by performing the aforementioned transfection procedures, but without using virus, control plasmid, or G418.

RT-PCR

RNA was isolated from 1.5 x 10⁷ cells of each of the experimental and control cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) and the procedure recommended by the manufacturer. RT-PCR was performed with the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following primers synthesized by SeqWright (Houston, TX): HPV18 forward primer, ATGGCGCGCTTTGAGGATCC; HPV18 reverse primer, GCATGCGGTATACTGTCTCT; HPV16 forward primer, ATGTTTCAGGACCCACAGGA; HPV16 reverse primer, CCTCACGTCGCAGTAACTGT. One µg of template RNA was used for each reaction. The reverse transcription step ran for 30 min at 47°C, followed by denaturation for 2 min at 94°C. Thirty-five amplification cycles were run, consisting of 20 sec denaturation at 94°C, 30 sec of annealing at 58°C, and 6.5 min of extension at 72°C. Final extension was run for 5 min at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% agarose gels (Cambrex: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY).

Morphology

The number and percent of spreading and non-spreading cells were determined for each of the experimental and control cell lines in the adhesion assays, and for each day and condition in the proliferation assays. To accomplish this, cells were fixed in 50 μL of 10% buffered formalin, and were stained with crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The number and percent of spreading and non-spreading cells were then determined by visual inspection using a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany) and confirmed with digital capture and Adobe Photoshop (San Jose, CA) Image Analysis tools.

Viability

Prior to plating cells for adhesion and proliferation assays, aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). At each time point (day 1-3), several wells were processed using the Trypan stain, and live cells were enumerated using this procedure [27, 28].

Adhesion

Cell adhesion assays of CAL27, CAL27-HPV18, CAL27-HPV16, CAL27-HPV16/18, and CAL27-mTF were performed as previously described [29, 30] in Corning Costar 96-well assay plates (Corning, NY) at a concentration of 1.2×10^5 cells per well (100 μL of 1.2×10^6 cells/mL solution) suspended in serum-free DMEM with no additives. Wells were either uncoated, (NP = naked plastic), or coated with 1% bovine serum albumin (BSA) in 10% non-fat dried milk solution or 20 $\mu\text{g}/\text{mL}$ of fibronectin solution for 1 hr at 25°C. Cells were then plated and allowed to attach for 30 min at 37°C. For the standard adhesion assay, non-adherent cells were removed by suspending the plates upside-down in a rotating tank of 1X PBS for 10 min at room temperature. For the modified adhesion assay, cells were allowed to attach for 30 min at 37°C, and there was no plate suspension step. Cells in both the standard and modified assays were fixed using 50 μL of 10% buffered formalin and subsequently stained using crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The relative absorbance was then measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). Data were analyzed and graphed using Microsoft Excel (Redmond, WA). Three separate, independent replications of these assays were performed.

Proliferation

In vitro proliferation assays of CAL27, CAL27-HPV18, CAL27-HPV16, CAL27-HPV16/18, and CAL27-mTF were performed in the appropriate media that was either supplemented with 10% fetal bovine serum (FBS) or that contained no serum (NS) in Corning Costar 96-well assay plates (Corning, NY). Assays were performed at two concentration; 1.2×10^4 cells per well (low density, LD) and 1.2×10^5 cells per well (high density, HD), and their proliferation was measured over three days. Cultured cells were fixed after 24 hrs (day 1), after 48 hrs (day 2), and after 72 hrs (day 3) using 50 μL of 10% buffered formalin, and were stained using crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The relative absorbance was measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT).

Data were analyzed and graphed using Microsoft Excel (Redmond, WA). Three separate, independent replications of this experiment were performed.

Statistics

The differences between treatments were measured using a *t* distribution, $\alpha=.05$. All samples were analyzed using two-tailed *t* tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed *t* test. As long as the sample size is even moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses [31]. To confirm the effects observed from these experiments, further analysis of the data was facilitated with ANOVA using SPSS (Chicago, IL). Significance for ANOVA was 0.05.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KK conceived, monitored, and coordinated the experimental design. TC, DJ, DJ, and SO carried out the microscopy and *in vitro* assays. Both KK and SO contributed equally to the writing of this manuscript.

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Figures

Figure 1. CAL27 cells expressed HPV *in vitro* only after transfection

(A) RT-PCR using total RNA collected from CAL27 cells demonstrated mock transfectants (mTF: lane 6) and non-transfectant cells did not express HPV16-mRNA (lane 8) or HPV18-mRNA (lane 4). RT-PCR confirmed the expression of HPV18-mRNA (lane 3) and HPV16-mRNA (lane 7). (B) Scanning densitometry measurement of relative endpoint RT-PCR band intensities from endogenous HPV (GH354: A, lane 5) was compared to HPV16, HPV18 and HPV16/18 co-transfectants, with HPV mRNA expression roughly equivalent in all samples.

Figure 2. Relative percentage of spreading cells and cell viability

(A) To determine the qualitative effects of HPV transfection of CAL27 cells, we examined cultured cells and transfectants on day 1 and day 3 to estimate the proportion of spreading and non-spreading cells. Approximately one-third of CAL27 control cells were spread with NS, while nearly three-quarters of HPV18 and 16/18 cells, and nearly all of HPV16 transfectants, were spread under the same conditions by day 1. About three-quarters of CAL27 cells spread with FBS, while nearly all HPV transfected cells were spread by day 1. (B) Using the Trypan viability assay, no statistically significant differences in viability were found between transfected and non-transfected cells.

Figure 3. CAL27 morphology is altered by the presence of HPV

CAL27 cells (A) were cultured in media containing NS (data not shown) or FBS, fixed with formalin and stained with crystal violet. Analysis revealed that HPV18-positive cells (B), HPV16-positive cells (C), and HPV16/18-positive cells (D) increased in number and ratio of spreading to a greater extent than non-transfected (A) or mock-transfected cells (data not shown) under similar conditions.

Figure 4. HPV transfection altered CAL27 adhesion to fibronectin (FN)

Standard 30-minute adhesion assays did not reveal significant differences in adhesion between HPV-transfected and non-transfected CAL27 cells. Significant increases in CAL27 adhesion to FN were observed in modified 30-minute adhesion assays for HPV18- (58%) and HPV16-transfectants (47%) (n=24 per condition, p<0.05), while a more modest increase (not statistically significant) was observed between HPV16/18-transfectants (22%) and CAL27 controls (n=24 per condition, p>0.05).

Figure 5. HPV transfection altered CAL27 proliferation *in vitro*

CAL27 cells (A), HPV16- (B), HPV18- (C), and HPV16/18-transfectants were plated at low density (LD: 10⁴ cells per well) or high density (HD: 10⁵ cells per well) and allowed to proliferate with 10% fetal bovine serum (FBS) or no serum (NS) for three days. All HPV-transfectants proliferated more rapidly than non-transfectants, although HPV16- and HPV16/18-positive cells responded more acutely to the presence of serum than HPV18-positive cells, which displayed a more heightened responsiveness to density than to serum.

Figure 6. Comparisons of HPV-transfected CAL27 cell proliferation

Side-by-side, three-day proliferation assays of CAL27, HPV18-, HPV16-, HPV16/18-transfectants under specific conditions LD-NS (A), LD-FBS (B), HD-NS (C), and HD-FBS (D) were performed. Direct comparisons revealed that HPV-stimulated proliferation was significantly higher than for CAL27 controls and all HPV-induced increases in proliferation were similar under all four conditions, except for HPV18. HPV18-stimulated proliferation was significantly higher than HPV16 or 16/18 at LD-FBS (A) and was significantly lower than HPV16 at HD-NS (D) (n=24 replicates per condition, p<0.01).

Figure 7. HPV-induced proliferative response to serum

The relative increase in proliferation between serum- and no serum-treated cells was determined for each cell type (HPV+/-). Using the formula ((FBSd3-FBSd1)/(NSd3-NSd1)) we assessed the relative-fold increase in proliferation from day 1 to day 3, between the serum and no serum treatment groups. Although a greater absolute level of proliferation was observed with HPV transfected cells, the relative increase

between serum and no-serum treatment among HPV-transfected and non-transfected cells was similar at high density. At low density, however, HPV18 induced a much larger serum-dependent increase in proliferation than either HPV16 or HPV16/18.

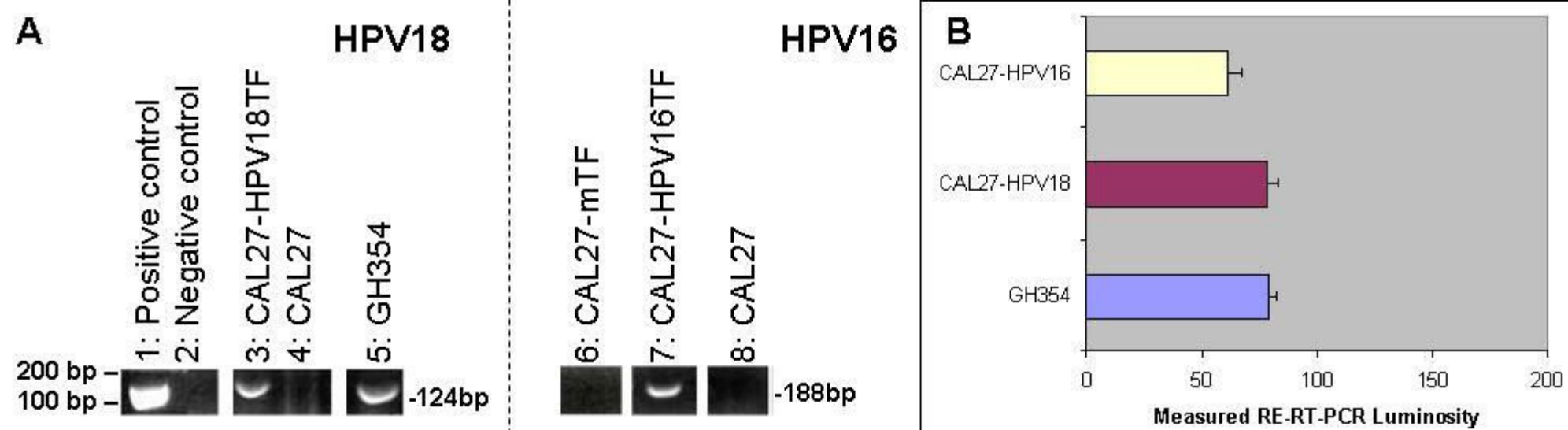


Figure 1

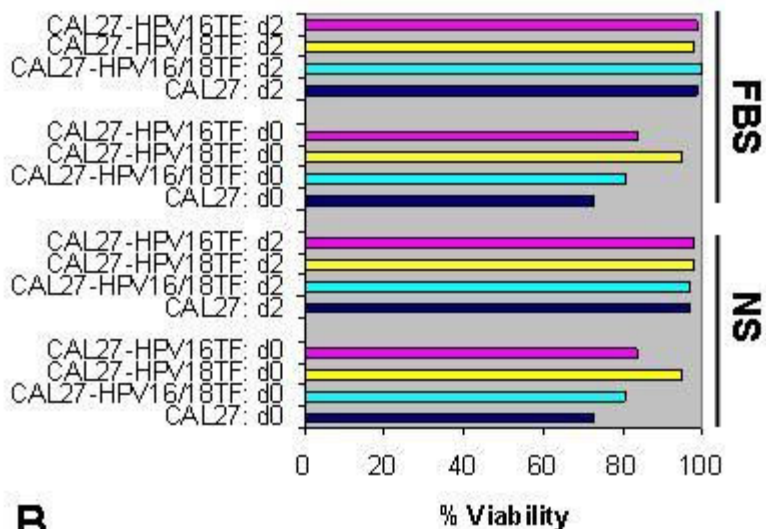
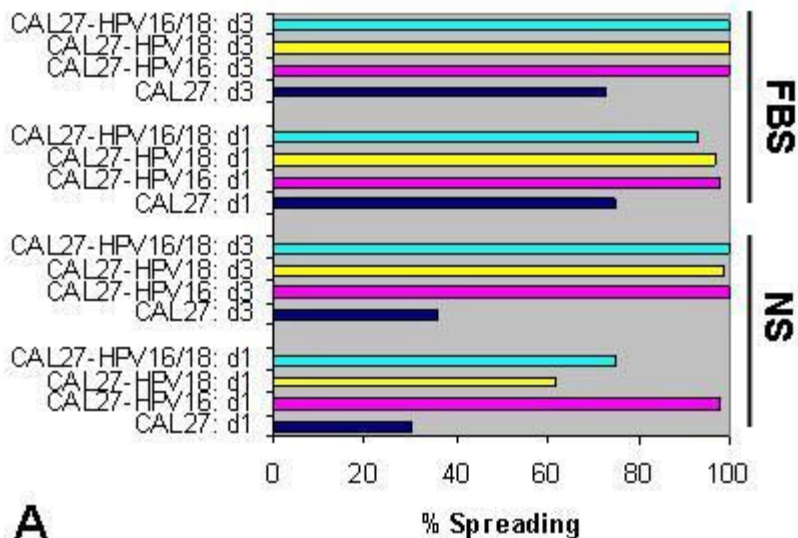


Figure 2

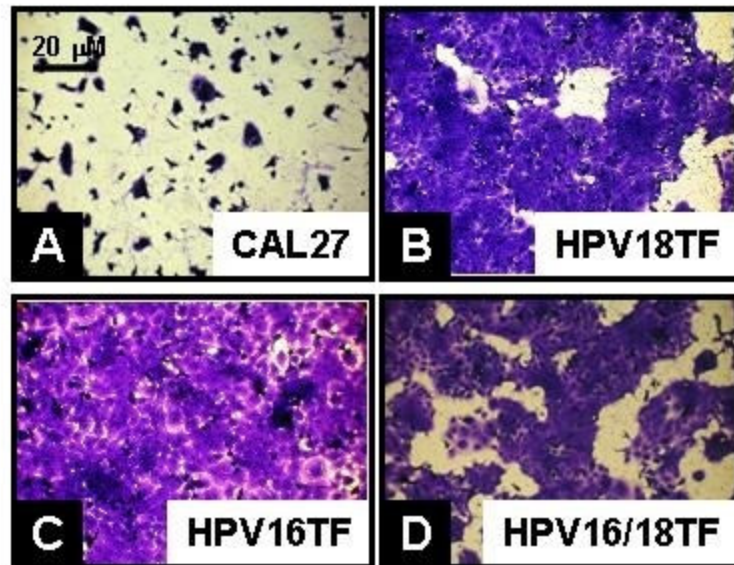


Figure 3

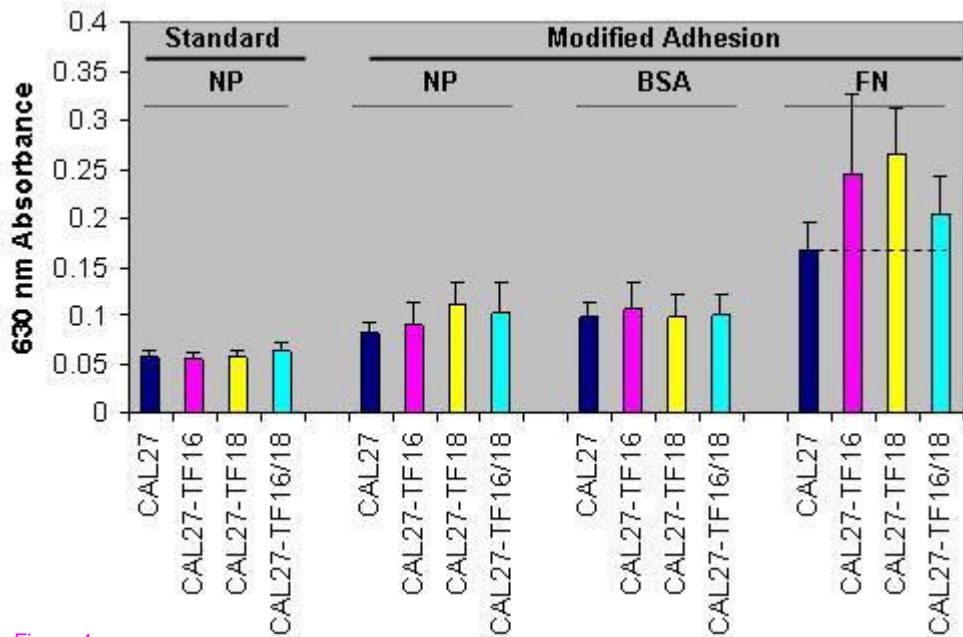
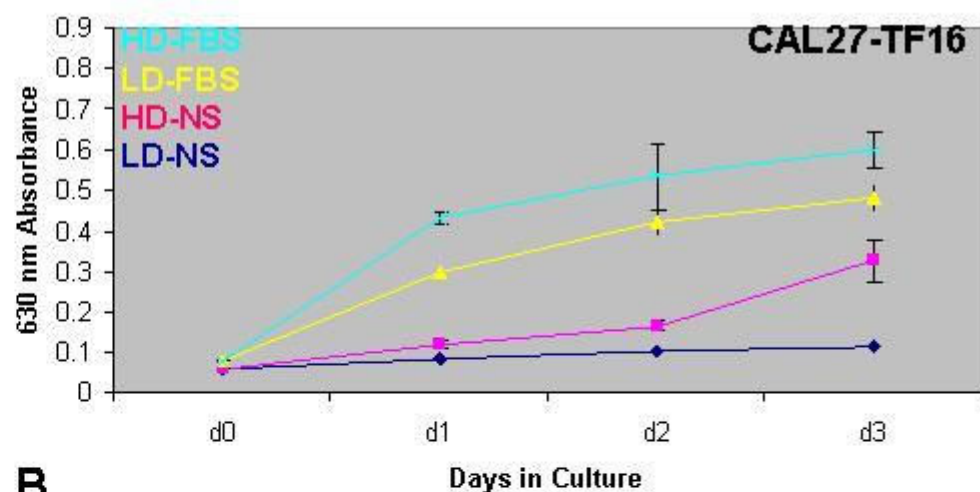
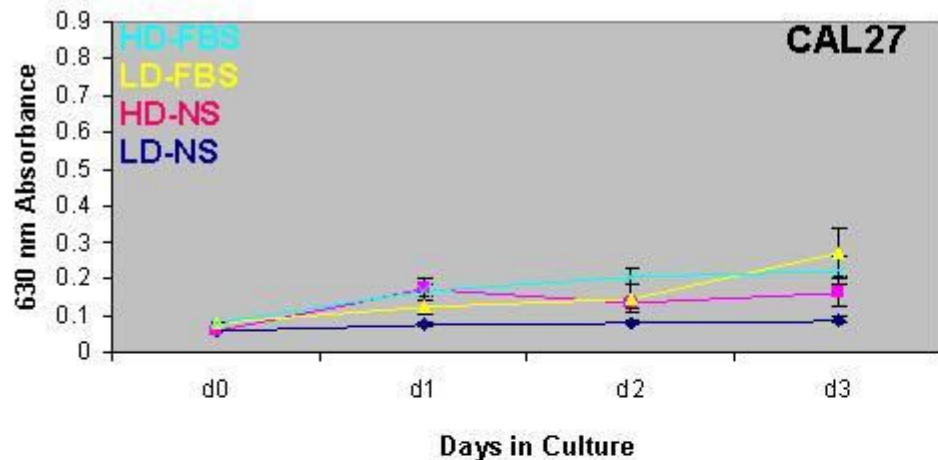
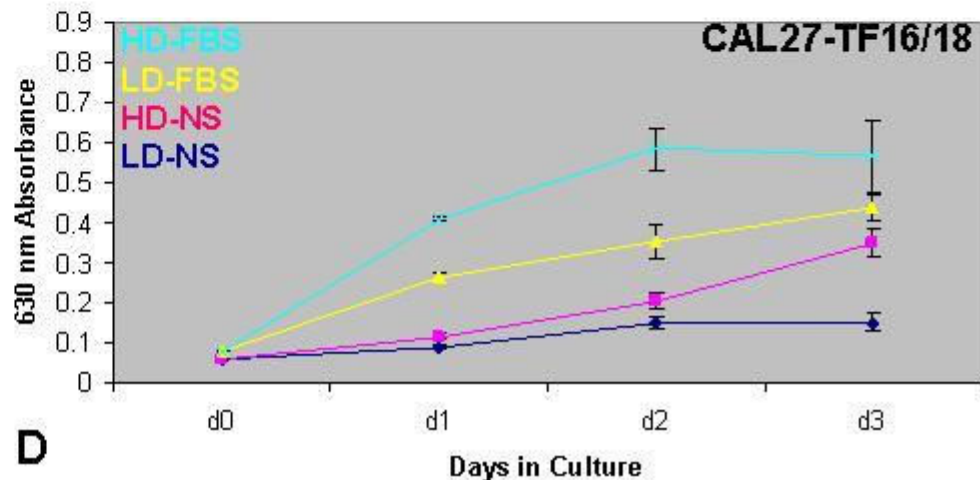
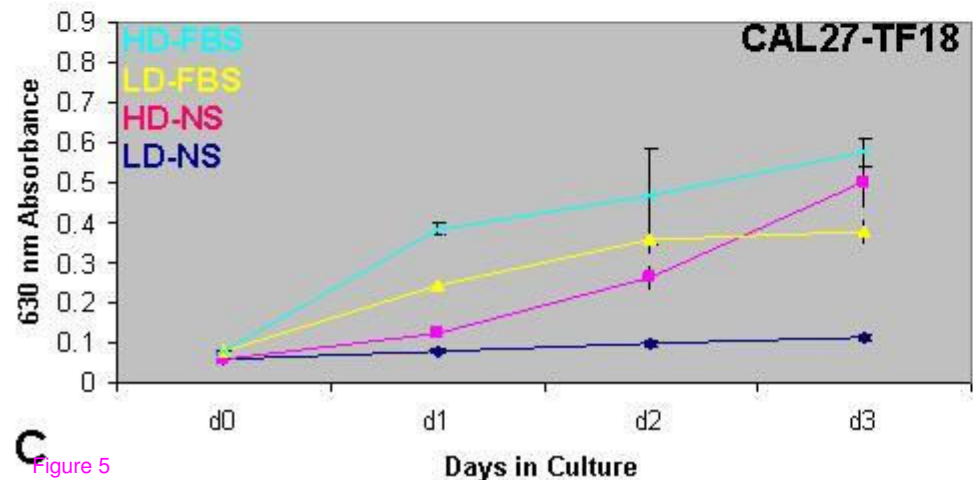


Figure 4



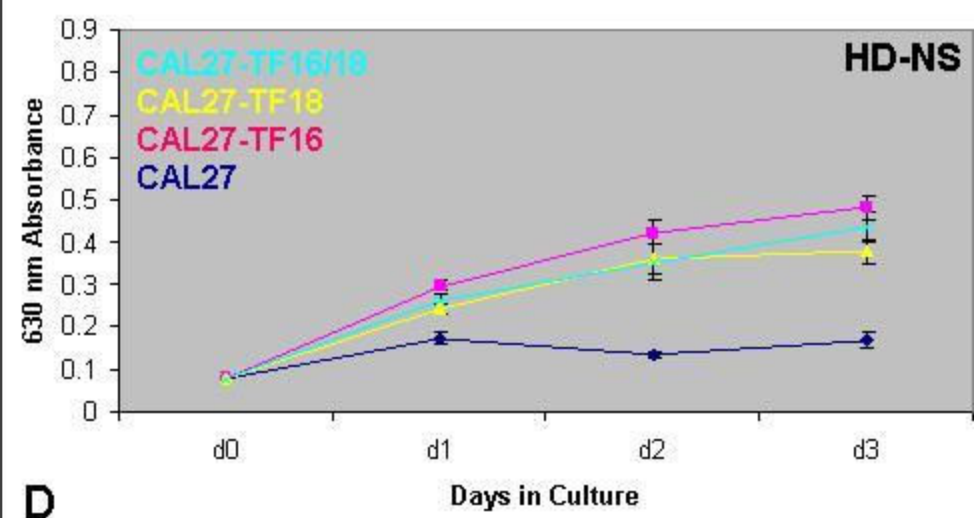
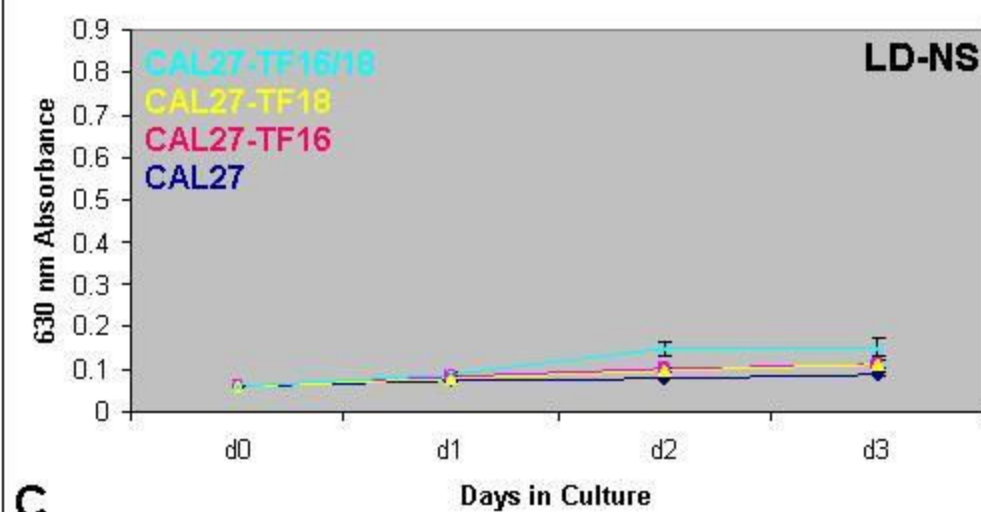
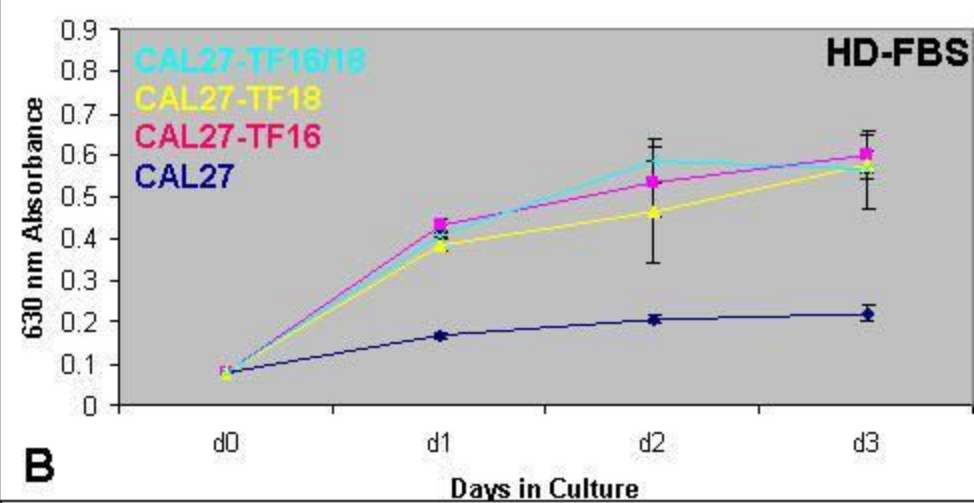
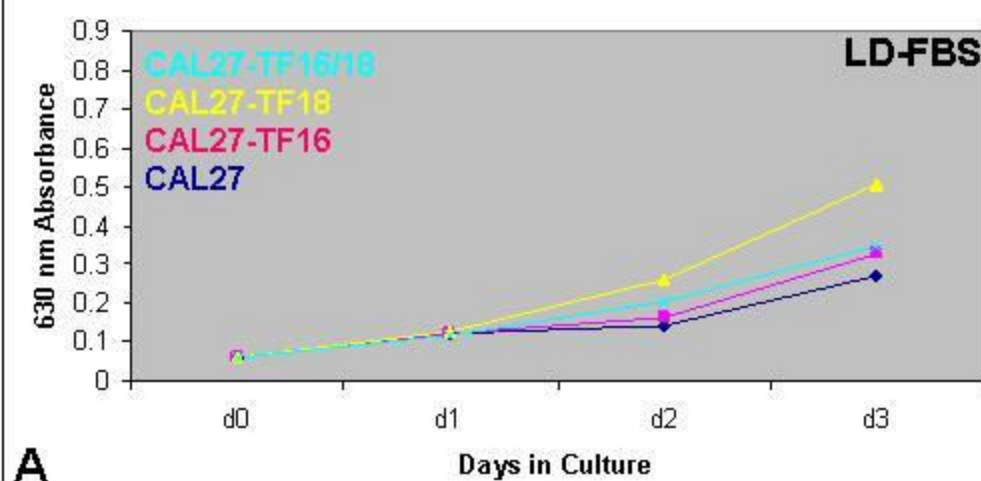
A

B



C

D



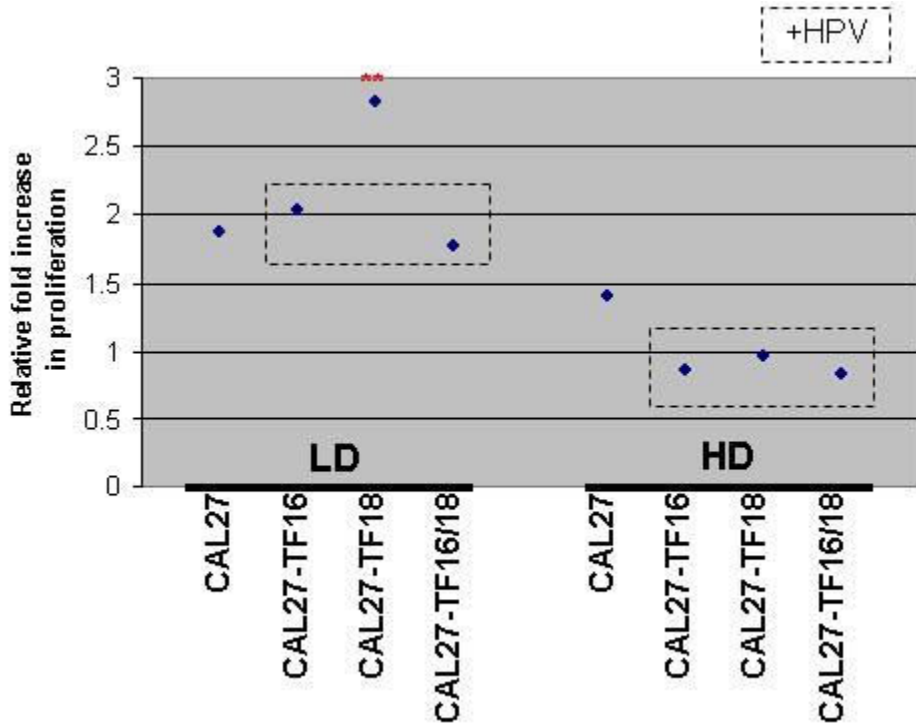


Figure 7