Establishment and characterization of equine fibroblast cell lines transformed in vivo and in vitro by BPV-1: Model systems for equine sarcoids

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Abstract

It is now widely recognized that BPV-1 and less commonly BPV-2 are the causative agents of equine sarcoids. Here we present the generation of equine cell lines harboring BPV-1 genomes and expressing viral genes. These lines have been either explanted from sarcoid biopsies or generated in vitro by transfection of primary fibroblasts with BPV-1 DNA. Previously detected BPV-1 genome variations in equine sarcoids are also found in sarcoid cell lines, and only variant BPV-1 genomes can transform equine cells. These equine cell lines are morphologically transformed, proliferate faster than parental cells, have an extended life span and can grow independently of substrate. These characteristics are more marked the higher the level of viral E5, E6 and E7 gene expression. These findings confirm that the virus has an active role in the induction of sarcoids and the lines will be invaluable for further studies on the role of BPV-1 in sarcoid pathology.

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Keywords: Bovine papillomavirus type 1; Equine; Sarcoid; Cell line; Transformation

Introduction

Equine sarcoids are locally invasive, fibroblastic skin tumors and represent the most common skin tumor in equids worldwide (Jackson, 1936; Ragland et al., 1970; Pascoe and Summers, 1981). Many studies have reported an association with bovine papillomavirus (BPV) infection based on the presence of BPV-1/2 DNA sequences in the majority of sarcoid tumors (Otten et al., 1993; Bloch et al., 1994; Reid et al., 1994; Martens et al., 2001; Chambers et al., 2003b). Papillomaviruses belong to a large family of animal and human papillomaviruses that normally infect epithelial cells causing hyperproliferative lesions including warts, papilomas or condylomas. Some types of PVs, including BPV types 1 and 2, can also infect fibroblasts and induce benign fibropapillomas. PVs are normally strictly species-specific and the equine sarcoid represents the only documented case of natural cross-species PV infection (Lancaster et al., 1979; Gorman, 1985). We and others have shown that many of the BPV viral genes are expressed in sarcoids (Nasir and Reid, 1999; Carr et al., 2001; Chambers et al., 2003a), with the highest levels detected in nodular sarcoids (Bogaert et al., 2007) supporting active papillomavirus infection. The viral genome is maintained episomally (Amtmann et al., 1980; Yuan et al., 2007a), and while genome copy number can vary, infection in the horse is maintained at a high genome copy number (Yuan et al., 2007a).

In addition we have shown that sarcoids are associated with BPV-1 variant sequences (Chambers et al., 2003b; Nasir and Reid, 2006; Nasir et al., 2007). In this paper we report the establishment and characterization of equine fibroblast cell lines originating from sarcoid tumor biopsies and from equine fibroblasts transfected with full-length BPV-1 DNA isolated from a sarcoid.

Results

Establishment of EqPalFs harboring BPV-1 genomes and sarcoid fibroblast cell lines

The full-length 8 kbp BPV-1 genome was isolated from three sarcoid DNA samples (S5, S6, S11) using RCA. The genomes
were purified, recircularized and used to transfect primary equine fetal palate cells, EqPalF cells. BPV-1 genomes from S5 and S6 were selected for transfection since both of these viral genomes comprise the most frequent BPV-1 variants (E5, LCR) in our bank of sarcoids (Chambers et al., 2003b; Yuan et al., 2007a; Nasir et al., 2007) (Table 1). Similarly, BPV-1 from a bovine papilloma was used to transfect EqPalFs. None of the cells transfected with control plasmids (pcDNA3.1, pEGFPC3) survived G418 selection. The transfections with the 'bovine' BPV-1 were performed twice in triplicate, however the cells were unable to survive. Cells transfected with BPV-1-S11 were also unable to survive. In contrast, cells transfected with BPV-1-S5 or -S6 grew out after 3 weeks of G418 selection and cells transfected with BPV-1-S6 grew faster than cells transfected with -S5. Three clones of S6-transfected cells (S6-1, S6-2 and S6-3) were isolated and grown.

Three naturally occurring sarcoid tumors were explanted into cultured cells and these were termed EqS01a, EqS02a, and EqS04b. PCR analysis and DNA sequencing confirmed that all three tumors harbored BPV-1 variant genomes, as listed in Table 1.

**Morphology**

Cell morphology was assessed by light microscopy (Fig. 1). The S6 and EqS cells grew as monolayers and exhibited a fairly uniform elongated spindle shape morphology compared to EqPalF cells. In both cell types the cells were tightly packed and showed loss of contact inhibition. Since BPV-1 E5 has been shown to be able to disrupt the actin cytoskeleton contributing to the transformed phenotype (Tsirimonaki et al., 2006), we examined the actin cytoskeleton of BPV-1 equine fibroblasts. EqPalF, S6-1, S6-2 and EqS04b cell lines were stained with TRITC-conjugated phalloidin which stains the actin filaments. As shown in Fig. 2, no obvious differences in the actin cytoskeleton were observed in the BPV-1 transformed cells compared to EqPalF control cells.

**Analysis of status and load of viral genomes**

To determine the status of the viral genomes, DNA, digested with HindIII, which cuts the BPV-1 genome once, was subjected to Southern blot analysis using a $\alpha^{32}$P labeled BPV-1 probe. As shown in Fig. 3A, in EqS cells, a single band of approximately 8 kbp was detected indicating that these cells contain episomal BPV-1 whole genomes. In EqS01a cells, a smaller band was also evident suggesting that these cells may also contain a BPV-1 variant genome of a smaller size. Similarly, Southern blot analysis of the S6 cells showed that S6 cells harbor episomal BPV-1 genomes. To further confirm these data, RCA which amplifies circular DNA was performed on the same DNA samples. The results confirm the presence of 8 kbp episomal genomes (Fig. 3B). They also show the presence of the smaller band in EqS01a cells. To determine the BPV-1 viral load in each cell line, DNA was isolated from early passage (p5) cells and subjected to Absolute Quantitative (AQ) PCR. As shown in Fig. 3C, EqS04b cells harbor the highest number of BPV genomes per cells (8 g.e.) and EqS02a cells the lowest (1 g.e.). The remaining cell lines all contain 2 BPV genomes per cell.

**Cells harboring a high viral load show a high level of viral oncogene expression**

No BPV-1 mRNA was detected in EqPalF cells. In contrast, both early and late viral BPV-1 gene transcripts could readily be detected by RT-PCR in EqS and S6 cells. The PCR amplification profile for S6-2 cells is shown in Fig. 4A. To determine the

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**Table 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>E5 variant</th>
<th>LCR variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5</td>
<td>Sw I</td>
<td>LCR II</td>
</tr>
<tr>
<td>S6</td>
<td>Sw I</td>
<td>LCR II</td>
</tr>
<tr>
<td>S11</td>
<td>Sw IV</td>
<td>LCR V</td>
</tr>
<tr>
<td>Cell line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6-1, -2, -3</td>
<td>Sw I</td>
<td>LCR II</td>
</tr>
<tr>
<td>EqS01a</td>
<td>B III</td>
<td>LCR II</td>
</tr>
<tr>
<td>EqS02a</td>
<td>Sw I</td>
<td>LCR IV</td>
</tr>
<tr>
<td>EqS04b</td>
<td>Sw I</td>
<td>LCR VII*</td>
</tr>
</tbody>
</table>

*E5 and LCR variants are described in Chambers et al. (2003b), Yuan et al. (2007a,b) and Nasir et al. (2007). LCR VII represents a novel variant and comprises the nucleotide changes detected in LCR IV and additional substitutions: G7266A, C7836G, and A7882T.
variation of BPV-1 gene expression in the 6 cell lines, the expression of BPV-1 oncogenes E5, E6 and E7 was assessed by relative quantitative (RQ) RT-PCR. EqS02a cell line was arbitrarily assigned as the calibrator since this cell line showed the lowest levels of oncogene expression (Fig. 4B). The highest levels of E5, E6 and E7 oncogene expression were detected in EqS04b cells. These cells contain the highest viral load (8 BPV genomes per cell). The lowest levels of E5 expression were detected in EqS01a cells which contain 2 genomes per cell and the lowest levels of E6 and E7 expression were detected in EqS02a cells harboring one BPV genome per cell.

Cells with high levels of BPV-1 oncogene expression show enhanced cell proliferation

The proliferation potential of cells was assessed by MTT assays. As shown in Fig. 5A, all BPV-1 expressing cell lines showed a growth advantage compared to the control EqPalF cells. To further examine the effect of BPV-1 oncogene expression on cell proliferation, the population doubling times were determined and are presented in Fig. 5A. In agreement with the MTT assays, EqPalF cells exhibited the longest P.D. time (57.8 h). In contrast, the P.D. time was much shorter for all BPV-1 expressing cells. Cells with the highest levels of oncogene expression, EqS04b, S6-1 and S6-2 exhibited shorter P.D. times (26.7, 26.2 and 24.0 h, respectively). Conversely, cells with low viral load and relatively low levels of oncogene expression (EqS02a, EqS01a and S6-3) exhibited longer P.D. times of 37.2, 30.7 and 32.6 h, respectively. EqPalF, S6-2 and EqS01a cells were analyzed for the expression of the proliferation marker, Ki67. As shown in Fig. 5B, both S6-2 and EqS01a exhibited greater Ki67 positive staining, of 21 (±1.8)% and 23 (±2.5)% respectively, compared to EqPalF cells in which 12(±2.1)% of cells were stained positively.

**BPV-1 expressing equine fibroblasts show anchorage independent growth**

To assay for anchorage independent growth, primary EqPalF, EqS and S6 cells were cultured in medium containing methylcellulose for 14 days. ConBPV-1 cells were used as a control.
positive control (Jarrett et al., 1990). As detailed in Table 2, the control EqPalF cells failed to form foci. In contrast, all cell lines, with the exception of EqS01a cells, were able to grow in methylcellulose media. The efficiency of focus formation was highest in EqS04b cells, similar to the ConBPV-1 control cells, and lowest in EqS02a cells. The S6 cells all showed similar efficiency of focus formation but the efficiency was much lower than the positive control cells.

The S6 and EqS cells were also assessed for growth in serum free medium. None of the cell lines examined, including the ConBPV-1 control cells, were able to grow in the absence of serum (data not shown). In the presence of 1% serum, only the EqS cells were able to grow, although their growth was slower than in the presence of 10% serum (Fig. 6).

**BPV-1 can extend the life span of transformed cells but is not able to immortalize cells**

Several of these cell lines (S6-2, EqS01a, EqS02a and EqS04b) have now been maintained long term. Control EqPalFs showed a limited life span of approximately 13 passages (~19 P.D.) whereas S6-2 and EqS01a were able to grow to passage 21 (~33 P.D.) and 30 (~39 P.D.), respectively, followed by growth arrest. This suggests that BPV-1 extends the life span of equine fibroblasts but is not able to immortalize them. However, the sarcoid lines EqS02a and EqS04b have gone through 16 passages (~34 P.D.) and 24 passages (~60 P.D.) respectively and at present show no signs of growth arrest.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Focus formation per 10,000 cells</th>
</tr>
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<tbody>
<tr>
<td>EqPalF</td>
<td>0</td>
</tr>
<tr>
<td>ConBPV1</td>
<td>138.0±14.1</td>
</tr>
<tr>
<td>S6-1</td>
<td>10.4±1.1</td>
</tr>
<tr>
<td>S6-2</td>
<td>11.8±3.1</td>
</tr>
<tr>
<td>S6-3</td>
<td>8.4±1.7</td>
</tr>
<tr>
<td>EqS01a</td>
<td>0</td>
</tr>
<tr>
<td>EqS02a</td>
<td>0.9±0.7</td>
</tr>
<tr>
<td>EqS04b</td>
<td>67.0±25.4</td>
</tr>
</tbody>
</table>

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Fig. 4. (A) RT-PCR analysis of BPV-1 gene expression in S6-2 cells. (B) RQ-RT-PCR analysis of BPV-1 E5, E6 and E7 oncogene expression in sarcoid derived fibroblasts (EqS01a, EqS04b, EqS02a) and BPV-1 transformed EqPalFs cells (S6-1, S6-2, S6-3). Expression is shown relative to the calibrator EqS02a, the value for which has been set to 1.

Fig. 5. (A) Cell proliferation and population doubling (P.D.) times of BPV-1 transformed EqPalF (S6-1, S6-2, S6-3) and sarcoid-derived fibroblasts (EqS01a, EqS04b, EqS02a). (B) Ki67 immunoreactivity in EqPalF, S6-2 and EqS01a cells.
BPV-1 expressing fibroblasts show perinuclear/cytoplasmic p53 expression

Several studies have shown that p53 expression is cytoplasmic/perinuclear in sarcoid in vivo (Johnston et al., 1996; Martens et al., 2000; Nixon et al., 2005; Bogaert et al., 2007). We have also previously demonstrated that the transactivation function of p53 is abrogated in sarcoid cells (Nixon et al., 2005). To determine whether the cell lines derived in this study also express cytoplasmic p53, p53 protein expression was evaluated in EqS01a, S6-2 and control EqPalFs. As demonstrated in Fig. 7, both S6-2 and EqS01a cells show almost total perinuclear/cytoplasmic localization of the p53 protein confirming previous in vivo observations.

Discussion

The involvement of BPV-1 in the induction of equine sarcoïds is now almost universally accepted. Little however is known about the molecular pathology underlying the tumor and the mechanisms used by BPV-1 to induce the tumor. The present study was aimed at the generation of cell lines from sarcoïds and of cell lines transformed by BPV-1 that could help the elucidation of the role of the virus in transformation and serve as in vitro model systems for future studies.

Generation of equine cell lines

We have developed three sarcoïd-derived cell lines (EqS01a, EqS02a and EqS04b) from three independent tumor biopsies which express and maintain episomal variant BPV-1 genomes. All three of the cell lines contain variant BPV-1 genomes. We therefore confirm yet again that sarcoïd, and their derivative cell lines, are associated with variant BPV-1 genomes (also see below). To our knowledge, these are the first established cell lines harboring variant BPV-1 derived from sarcoïds, in addition to our previously described sarc-1 cell line (Nixon et al., 2005). A previous report described a sarcoïd cell line MC-1. However, the virus associated with MC-1 cells and their derivatives was a non-oncogenic replication defective virus presumed to be an endogenous equine retrovirus and a causative relationship between this virus and equine sarcoïds was not established (Cheevers et al., 1982).

In addition, we have developed equine cell lines transformed by the most common variant BPV-1 genome found in sarcoïds. All these cell lines, whether explanted from sarcoïds or transformed in vitro, show enhanced proliferation and increased life span. The cell lines show the typical morphology of transformed fibroblasts and five out of six lines are also capable of growth in an anchorage independent fashion. Only the equine sarcoïd cells however can efficiently grow in low serum, suggesting that additional events are needed for the onset of serum independence in the S6 cells.

We have been repeatedly unable to generate equine cell lines transformed by BPV-1 isolated from bovine warts, with which we have successfully transformed both primary bovine fibroblasts (from conjunctiva or palate) and established mouse cells (NIH 3T3 and C127) (Jarrett et al., 1990; Campo and Spanidios, 1983; unpublished). This point deserves particular attention. We have previously shown that the BPV-1 genome found in sarcoïds is seldom identical to “bovine” wild-type BPV-1, most frequently showing numerous nucleotide variations in genes, both synonymous and non-synonymous, and in the LCR promoter/enhancer region (Chambers et al., 2003b; Yuan et al., 2007a; Nasir and Reid, 2006; Nasir et al., 2007). We have also shown that the variations in the LCR and in the E2 gene lead to increased function of these viral elements in equine

Fig. 6. Cell proliferation measurements of BPV-1 transformed EqPalF (S6-1, S6-2, S6-3) and sarcoïd-derived fibroblasts (EqS01a, EqS02a, EqS04b) in 1% serum, assessed by the MTT assay.

Fig. 7. p53 immunoreactivity using CM-1 anti-p53 antibody in BPV-1 transformed EqPalF (S6-2) and sarcoïd derived fibroblasts (EqS01a) and control EqPalF cells. ×400 magnification.
cells compared to bovine cells (Nasir et al., 2007). We now report that this enhanced function of viral variants is not confined to individual genes/elements but encompasses the whole genome, leading to the establishment of transformed cell clones, the better studied of which (S6-1 to 3) show similar characteristics to those of cell lines explanted from sarcoids. Furthermore, in this study, cells transfected with BPV-1-S11 were unable to survive. S11 comprises the rare LCR variant V and the rare E5 variant IV (Nasir et al., 2007). Only cells transfected with S5 and S6 BPV-1 genomes were able to survive. Both these BPV-1 genomes contain the most common LCR variant II and the most common E5 variant Swiss I (Nasir et al., 2007). These results strongly support our previous conclusions that variants of BPV-1 circulate at a higher frequency in equids than in bovids and show that the most common sarcoïd BPV-1 variants are better suited to transformation of equine cells.

Maintenance and expression of viral genomes

In all cell lines the BPV-1 genome is episomal and in more than one copy, with the exception of EqS02a which only has one copy.

The viral early and late genes are transcribed in all cell lines. Perhaps not surprisingly the levels of BPV-1 RNA are proportional to the levels of viral genomes. This is in full agreement with the results of Bogaert et al. (2007) in sarcoïd tumors in vivo.

The early proteins E1 and E2 are absolutely necessary for replication and maintenance of episomal viral DNA (Piirsoo et al., 1996), and accordingly E1 and E2 RNAs are present in all cell lines.

E5, E6 and E7 are the oncoproteins of BPV-1, with E5 and E6 having major roles and E7 enhancing their actions (Howley and Lowy, 2007). The role of the papillomavirus E5 protein has been reviewed recently (Suprynowicz et al., 2005). Briefly E5 transforms cells primarily through its interaction with, and activation of, the PDGF receptor, and interaction with, and disabling of, the 16k subunit of the vacuolar ATPase. In our equine cell lines, the expression of E5 correlates positively with cellular growth: the higher the levels of E5 RNA, the shorter the cells doubling time (Figs. 4b, 5a). Furthermore, like in bovine cells (Ashrafi et al., 2002; Marchetti et al., 2002; Marchetti et al., 2006; Araibi et al., 2004, 2006; Tsirimonakni et al., 2006) E5 up-regulates kinases and down-regulates MHC class I also in equine cells and in sarcoïds (in preparation), confirming its pivotal role in establishment of infection and cell transformation.

The expression of BPV-1 E6 and E7 genes is similar. It is known that BPV-1 E6 is a transcriptional regulator (Lamberti et al., 1990) and additionally binds to both a calcium binding protein (Chen et al., 1995) and to paxillin (Tong and Howley, 1997). This last interaction leads to disruption of the actin cytoskeleton contributing to the transformed phenotype. The E5 proteins of BPV-1 and BPV-4 also lead to disruption of the actin cytoskeleton in BovPalF cells (Tsirimonakni et al., 2006). However, this study shows that expression of BPV-1 in EqPalFs does not appear to have any discernible effect on the actin cytoskeleton.

BPV-1 E7, like all E7 proteins from ungulate fibropapillomaviruses, does not have a p105Rb binding site (Narechania et al., 2004) and its precise role in aiding transformation has been elusive. A recent report has shown that BPV-1 E7 prevents apoptosis due to detachment from substrate (anoikis) and enhances the ability of the transformed cells to grow in an anchorage independent fashion (DeMasi et al., 2007). In our equine cell lines, the ability to grow independently of substrate is roughly proportional to the levels of E5, E6 and E7 RNAs. EqS04b cells with the highest amount of E5, E6 and E7 RNA grow in agar with a similar efficiency to the control bovine ConBPV-1 cells.

As bovine PalF cells transformed by BPV-1 E5 are capable of anchorage independence (Tsirimonakni et al., 2006), at the moment it is not possible to say which of the BPV-1 oncoproteins is responsible for this cell transformation trait and to what extent.

The late gene L1 is expressed in the cell lines. Expression of L1, the major structural capsid protein, has been observed in sarcoïds although mature virions have not yet been found (Barthold and Olson, 1978, Sundberg et al., 1984). The late (structural) RNAs of papillomavirus are subjected to post-transcriptional control and require the differentiation of keratinocytes for their stability and translation into proteins (Graham, in press). The situation in sarcoïds, EqS and S6 cells therefore resembles the situation in the fibroblastic portion of BPV-1 induced fibropapillomas, where late RNAs can be transcribed but not translated.

The tumor suppressor p53 is disabled in transformed equine cells

One obstacle to cell transformation is the array of tumor suppressor proteins which the cell employs to resist transformation. It is well known that papillomavirus disables numerous tumor suppressors, particularly p105Rb and p53 (reviewed in Boulet et al., 2007). It has been shown that E6 from high-risk human papillomaviruses degrades p53 (Scheffner et al., 1990) but BPV-1 E6 has been reported not to be able to bind human p53 in vitro (Werness et al., 1990) or degrade p53 in mouse C127 cells (Schiller et al., 1984) although it does so in human mammary cells (Band et al., 1993). Surprisingly the interaction between BPV-1 E6 and p53 has not been investigated in the virus natural host cells, either bovine or equine.

In the equine cell lines examined here, whether explanted from sarcoïds or transformed in vitro, p53 is not degraded but stabilized, is almost totally cytoplasmic as previously reported by us and by others (Johnston et al., 1996; Martens et al., 2000; Nixon et al., 2005, Bogaert et al., 2007), and not functional, as already described (Nixon et al., 2005). It is to be noted that p53 function in sarcoïd cells is not lost through mutations as the p53 gene is wild-type in the tumor (Bucher et al., 1996; Nasir et al., 1999), but probably because of the almost total mis-location of the protein. It remains to be seen if the cytoplasmic localization of p53 in sarcoïd cells is due to BPV-1 E6. Also the status of other tumor suppressor genes
remains to be established, although we know that apoptosis-inducing genes are down-regulated in cell lines and in sarcoilds (in preparation).

Great advancements have been made in the understanding of equine sarcoilds but much still needs to be elucidated. The availability of our panel of transformed equine cells presents the best opportunity for a thorough analysis of sarcoild pathogenesis.

Methods and materials

Pathologic samples

Samples of equine fetal palate tissue were collected from an aborted fetus at surgery. Sarcoild tumors were collected at surgery/post mortem. All tissues were taken with informed owner consent. The diagnosis of equine sarcoild was confirmed by histopathological analysis following hematoxylin and eosin staining.

Generation and establishment of equine palate fibroblasts (EqPalFs) and equine sarcoild (EqS) fibroblasts

Tissue samples from fetal palate and sarcoilds were finely dissected and washed with Hanks Buffered Salt Solution (HBSS) (Invitrogen Ltd, Paisley, UK) and incubated overnight at 4 °C in 0.25% trypsin/HBSS. After removal of the trypsin solution, samples were incubated at 37 °C for 30 min followed by the addition of warm Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 1.25 μg/ml fungizone, Ampotericin B and Ciproxin (Sigma, UK), 1% non-essential amino acids and 1% essential amino acids (complete DMEM). The cell suspensions were passed through a 100 μm nylon cell strainer (BD Falcon™) and centrifuged. Resuspended cells were then seeded into T25 flask at 1×10⁶ cells per flask in 10 ml of complete DMEM and incubated in a 37 °C humidified atmosphere of 5% CO₂ in air until cells reached confluence. Cells were passaged by trypsinization and this stage was regarded as the first passage. Three transfected clones were named EqPalFS6-1, EqPalFS6-2 and EqPalFS6-3 (these cell lines will be referred to as S6-1, S6-2 and S6-3, respectively).

Southern blot hybridization

Southern blot analysis was performed using standard protocols (Sambrook et al., 1989). Briefly, approximately 10 μg total cell DNA samples was digested with a single cutter, HindIII, and subjected to 0.8% agarose gel electrophoresis. The DNA was then transferred to nitrocellulose Hybond N+ membranes (GE Healthcare, UK). Membranes were hybridized with a BPV-1 probe for 18 h at 60 °C, washed, air-dried and exposed at −80 °C to x-ray films. The probe was labeled using Prime-it II Random Primer Labeling Kit (Stratagene, La Jolla, USA) with the Exo(−) Klenow enzyme and [α-³²P] dCTP (GE Healthcare, UK).

RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Crawley, UK). Following DNase I treatment, cDNA was synthesized using SuperScript First-Strand Synthesys System (Invitrogen). PCR reactions were performed using Platinum Taq DNA polymerase (Invitrogen) on 2 μl cDNA samples in a total volume of 20 μl with specific primers for BPV-1 early gene E1, E2, E5, E6 or E7, or the late gene L1 (Supplementary data). Samples were initially denatured at 94 °C for 4 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. To ensure the absence of genomic DNA, RT-PCRs were also performed on RNA samples in the absence of reverse transcriptase.
Real-time Absolute Quantitative PCR for viral load assessment

BPV-1/2 viral load was determined by real-time Absolute Quantitative PCR (AQ-PCR) using a BPV-1/2 E7 primer probe set as described previously (Yuan et al., 2007a). Briefly, AQ-PCR was performed on 0.1 μg DNA samples on a 7500 Real Time PCR System (Applied Biosystems, Warrington, UK) with an amplification profile of 2 min at 50 °C, 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 45 s at 60 °C. A serial dilution of the pBPV-1-S6 plasmid was used to generate a standard curve and equine GAPDH primer and probe set (Supplementary data) was used as an internal control for normalization of input DNA amount. Reactions were performed in triplicate. 0.1 μg of input equine DNA was regarded as 32,680 genome equivalents of equine cells based on the fact that a diploid equine cell contains approximately equal. The 2^−ΔΔCt method (Livak and Schmittgen, 2001) was used to compare the relative expression levels of BPV-1 E5, E6 and E7 genes among cell lines.

Real-time relative quantitative PCR for BPV-1 E5, E6 and E7 expression

Real-time Relative Quantitative PCR (RQ-PCR) was performed on 2 μl cDNA samples (0.1 μg) using E5, E6 and E7 specific primer probe sets (Supplementary data). PCR reaction mixes contained 0.2 μM forward and reverse primers, 0.1 μM probe, 25 μl of 2X Platinum Quantitative PCR SuperMix-UDG (Invitrogen, UK), 1 μl ROX Reference Dye and water to a final volume of 50 μl. Reactions were performed on a 7500 Real Time PCR System (Applied Biosystems) with an amplification profile of 2 min at 50 °C, 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 45 s at 60 °C. GAPDH primer probe set (Supplementary data) was used as an endogenous control and all reactions were performed in triplicate. RQ-PCR using serial dilutions of template verified that the efficiencies of amplification of BPV-1 E5, E6, E7 and GAPDH, the internal reference, were approximately equal. The 2^−ΔΔCt method (Livak and Schmittgen, 2001) was used to compare the relative expression levels of BPV-1 E5, E6 and E7 genes among cell lines.

Cell proliferation assays

Assessment of cell proliferation and viability was determined using the Cell Proliferation Kit I (Roche). Briefly, 4 × 10^4 cells were seeded in 24-well tissue culture plates in DMEM containing 10% FCS. MMT assays were performed in triplicate every 24 h for a total of 7 days. Spectrophotometric absorbance at 570 nm and 660 nm was measured, and the difference between A570 and A660 was plotted as a function of time. To determine cell population doubling times during the exponential growth phase, 4 × 10^4 cells were seeded in 24-well plates in 2 ml completed DMEM supplemented with 10% FCS and cell number was counted every 24 h in triplicate wells. The mean values were plotted on a log scale against time on a linear scale. Population doubling (P.D.) was calculated using the formula P.D. = 3.32(log 10 number of cells harvested – number of cells seeded) (Scott et al., 2004).

Anchorage independence and low serum growth assays

To assess anchorage independence growth, 2.0 × 10^5 cells were added to 15 ml Methocel medium (1.0% methylcellulose, 30% FCS in complete F10 Ham’s medium) and transferred to a bacterial grade 100 mm Petri dish and allowed to grow for 2 weeks. Triplicates of each cell line were examined. Fetal bovine conjunctiva cells transformed with BPV-1 (ConBPV-1) were used as a positive control (Jarrett et al., 1990). The average efficiency of colony formation was calculated as number of colonies/number of cells. To assess growth in low serum, cells were cultivated in 24-well plates as described above using complete DMEM but supplemented with either serum free or 1.0% FCS. MTT assays were carried out as described above to evaluate viability and proliferation of cells.

p53 and Ki67 immunocytochemistry

Cells were grown on coverslips in 6-well plates and expression of ki67 and p53 investigated using a two-step immunohistochemical technique using Dako EnVision kit (K4007 for p53 and K4011 for Ki-67) following the manufacturer’s instructions. Briefly, cells were fixed in acetone, rinsed with wash buffer and the endogenous biotin activity quenched by a 5-minute application of H2O2 solution. Coverslips were incubated for 1 h at room temperature in the presence of primary antibody (CM-1 p53 antibody (Novocastra Laboratories, UK) diluted 1:500 in 10 mM Tris Buffer Saline (TBS) and Ki-67 antibody (Dako, Ely, UK) diluted 1:200 in 10 mM TBS. Coverslips were then rinsed in 0.5% Tween20 in 10 mM TBS and incubated with the appropriate labeled polymer-HRP anti-mouse or anti-rabbit solution supplied with the Dako EnVision kit (Dako, UK) for 30 min. Cells were then stained with 3,3-diaminobenzidine and counterstained with hematoxylin. The coverslips were washed and mounted on a glass slide. For negative controls, sections were incubated with a non-related serum instead of the primary antibody.

Cytoimmunofluorescence

Cells were grown on glass coverslips for at least 2 days in DMEM-10% FCS, and F-actin was labeled with tetramethylrhodamine-5-isothiocyanate TRITC-conjugated phalloidin. Images were viewed, processed and photographed using a Leica DMIRE2 confocal microscope and Leica Confocal Software Lite Version (Germany).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.11.037.
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