p38 mitogen-activated protein kinase is crucial for bovine papillomavirus type-1 transformation of equine fibroblasts

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Equine sarcoïds represent the most common skin tumours in equids worldwide, characterized by extensive invasion and infiltration of lymphatics, rare regression and high recurrence after surgical intervention. Bovine papillomavirus type-1 (BPV-1) and less commonly BPV-2 are the causative agents of the diseases. It has been demonstrated that BPV-1 viral gene expression is necessary for maintaining the transformation phenotype. However, the underlying mechanism for BPV-1 transformation remains largely unknown, and the cellular factors involved in transformation are not fully understood. Previously mitogen-activated protein kinase (MAPK) signalling pathway has been shown to be important for cellular transformation. This study investigated the role of p38 MAPK (p38) in the transformation of equine fibroblasts by BPV-1. Elevated expression of phosphorylated p38 was observed in BPV-1 expressing fibroblasts due to the expression of BPV-1 E5 and E6. The phosphorylation of the MK2 kinase, a substrate of p38, was also enhanced. Inhibition of p38 activity by its selective inhibitor SB203580 changed cell morphology, reduced the proliferation of sarcoid fibroblasts and inhibited cellular invasiveness, indicating the indispensable role of p38 in BPV-1 transformation of equine fibroblasts. These findings provide new insights into the pathogenesis of equine sarcoïds and suggest that p38 could be a potential target for equine sarcoid therapy.

INTRODUCTION

Equine sarcoïds are the most common fibroblastic skin tumours in equids worldwide (Jackson, 1936; Pascoe & Summers, 1981; Ragland et al., 1970). These tumours can occur as single or multiple lesions in different clinical types including occult, verrucose, nodular, fibroblastic, mixed and malevolent types (Knottenbelt, 2005). Extensive localized infiltration and invasion, rare regression and high recurrence are characteristic of this type of tumour (Knottenbelt, 2005; Martens et al., 2001a; Tarwid et al., 1985). It is now widely accepted that bovine papillomavirus (BPV) types 1 and 2 are the causative agents of sarcoïds. Experimental inoculation of equine skin with BPV induces sarcoïd-like lesions in horses (Ragland & Spencer, 1969). The viral genome is frequently detected in tumours (Ashrafi et al., 2008; Martens et al., 2001a, b; Nixon et al., 2005; Otten et al., 1993) and its proteins are expressed (Borzacchiello et al., 2008; Carr et al., 2001; Nasir & Reid, 1999; Nixon et al., 2005). It is likely that sarcoïd-specific BPV DNA is transmitted between equids by flies (Finlay et al., 2009). The BPV-1 genome isolated from a sarcoïd can transform equine fetal fibroblasts and inhibition of viral gene expression by small interfering RNAs (siRNA) reverts the transformed phenotype of sarcoïd fibroblasts (Yuan et al., 2008a, 2011).

While BPV infection plays a major role in the pathogenesis of sarcoïd, numerous cellular factors have been shown to be important for initiation and progression of tumours. The downregulation of major histocompatibility complex type I (MHC I) by BPV-1 E5 and Toll-like receptor 4 by E2 and E7 is likely to contribute to the persistence of viral infection (Marchetti et al., 2009; Yuan et al., 2010b). The tumour suppressor p53 is abnormally distributed in the cellular cytoplasm of some sarcoïds, which may interfere with sarcoïd cell apoptosis (Nixon et al., 2005). Multiple matrix metalloproteinases (MMP) expression and activities are elevated in sarcoïds, leading to the high invasiveness of sarcoïd fibroblasts (Yuan et al., 2010a). The plateleterived growth factor β receptor (PDGF-βR) is activated in sarcoïds, as is the PDGF-βR–PI3K-AKT signalling cascade (Borzacchiello et al., 2009), suggesting the involvement of growth factor signalling transduction pathways in the cellular transformation of equine fibroblasts.

It has been shown that PDGF-βR activation can trigger the activation of p38 mitogen-activated protein kinase (MAPK) in both rat and guinea pig smooth muscle cells (Pyne & Pyne, 1997; Yamaguchi et al., 2004). The p38 MAPK is involved in many biological processes including cell cycle, cell motility and invasion (Cuenda et al., 1995; Zhao et al., 1999). Sarcoïd fibroblasts that express high...
levels of viral gene expression show enhanced cell proliferation and high invasiveness (Yuan et al., 2008a, 2010a). To determine if p38 is involved in the transformation of sarcoid fibroblasts, we examined p38 expression and explored its functions in equine fibroblasts with or without BPV-1 gene expression.

**RESULTS**

**BPV-1 induces phosphorylation of p38 MAPK in equine fibroblasts**

PDGF-βR is activated in BPV-1-positive equine sarcoids (Borzacchiello et al., 2008, 2009) and it has been shown that activation of PDGF-βR triggers p38 MAPK (p38) phosphorylation and activation in a variety of cells (Pyne & Pyne, 1997; Tangkijvanich et al., 2002; Yamaguchi et al., 2004). This suggests that p38 is activated in equine sarcoid cells expressing BPV-1 proteins (Yuan et al., 2008a). To elucidate this possibility, three sarcoid fibroblast lines EqS01a, EqS02a and EqS04b (EqS) and a normal equine fibroblast line EqPalF were examined for the expression of p38 and its phosphorylated (activated) form (p-p38) by immunoblotting. Both EqS04b and EqS01a contain high copies of BPV-1 DNA and transcripts, while EqS02a has low levels of BPV-1 DNA and transcripts (Yuan et al., 2008a). EqPalF cells are free of BPV-1 DNA (Yuan et al., 2008a). As shown in Fig. 1(a), the expression levels of p38 were constitutively high in both EqPalF and EqS cells. However, the level of p-p38 was significantly higher in EqS04b and EqS01a, but not in EqS02a where p-p38 levels were similar to that in EqPalF cells (Fig. 1a), suggesting that the increased p-p38 levels are due to the levels of expression of BPV-1 protein(s). Our recent study showed that siE6, a BPV-1 E6-targeting siRNA, is able to almost completely knock out E6 expression and drastically reduces E5 and E7 expression in EqS04b cells (Yuan et al., 2011). To corroborate the function of BPV-1 on p38 activation, EqS04b cells treated with siE6 were examined for p38 and p-p38 expression. As seen in Fig. 1(b), although the expression of p38 was not significantly changed, the p-p38 expression was greatly reduced in siE6-treated cells compared with control cells (mock and scramble). Given that siE6 reduces not only E6 but also E5 and E7 mRNAs, it is not possible to state with certainty which viral oncoprotein contributes most to p-p38 upregulation. It is clear, however, that BPV-1 gene activity is responsible for elevated p38 activation.

**BPV-1 E5 and E6 stimulate phosphorylation of p38**

To study the functions of individual BPV-1 proteins, we recently established EqS02aT cells expressing E5, E6 or E7, or containing only empty vector (EqS02a-E5, -E6, -E7 and
BPV-1 induces phosphorylated MAPK-activated protein kinase 2 (MAPKAPK2) expression

MAPKAPK2 (MK2) is one of the substrates of p38 (Rouse et al., 1994; Zarubin & Han, 2005). Given the activation of p38 in sarcoid cells, we next analysed the phosphorylation status of MK2 (p-MK2) in BPV-1-positive or -negative equine fibroblasts by immunofluorescence staining with a p-MK2-specific antibody. The sarcoid EqS04b, 02a and 01a cell lines and the normal EqPalF cells all showed nuclear p-MK2 expression, but both EqS04b and EqS01a showed higher levels of p-MK2 than EqS02a or EqPalF cells (Fig. 3a), reflecting the significantly higher p38 phosphorylation in the former two lines (Fig. 1a). In addition, the high level of viral mRNA expression in EqS04b and EqS01a cells suggests that BPV-1 is associated with the enhanced p-MK2 expression. To corroborate the association, p-MK2 expression was examined in BPV-1 in vitro transformed EqPalF cells (S6-2) (Yuan et al., 2008a). As shown in Fig. 3(b), S6-2 cells showed elevated p-MK2 expression compared with control cells, confirming that the presence of BPV-1 enhances p-MK2 expression.

Inhibition of p38 activity attenuates proliferation of sarcoid fibroblasts

BPV-1 transforms equine fibroblasts resulting in enhanced cell proliferation and cellular invasiveness (Yuan et al., 2008a, 2010a, 2011). Given the activation of p38 by BPV-1, we speculated that p38 activity might be crucial for the transformed phenotype of sarcoid fibroblasts. To elucidate this possibility, the p38 selective inhibitor SB203580 (SB) (Cuenda et al., 1995; Goedert et al., 1997; Lee et al., 1994), shown to inhibit p38 activity in equine neutrophils (Eckert et al., 2009), was used to treat EqS04b cells that are fully transformed and highly invasive (Yuan et al., 2008a, 2010a), followed by cell proliferation and invasiveness assessment. As shown by immunofluorescence and Western blotting (Fig. 4a, b), SB treatment abolished the nuclear expression of p-MK2 in EqS04b cells, demonstrating that MK2 is also a substrate of p38 in equine cells.

The EqS01a, EqS02a, EqS04b and EqPalF lines were incubated with SB under serum-free condition to analyse the consequences of the inhibition of p38 on their transformation status. The treatment caused a more flattened morphology in EqS04b (Fig. 4c) and EqS01a cells (data not shown), while the untreated cells remained spindle-like with a more elongated shape. In contrast the treatment did not change the morphology of EqPalF (Fig. 4c) and EqS02a cells (data not shown). In addition, p38 inhibition by SB drastically inhibited the proliferation of EqS04b (Fig. 5a) and EqS01a (data not shown), whilst EqPalF cells were only slightly attenuated (Fig. 5a) and EqS02a cells were not affected (data not shown). Thus, equine fibroblasts that express high levels of BPV-1 gene expression (EqS01a and EqS04b) appeared highly sensitive to inhibition of p38 activity and those cells (EqPalF and EqS02a) that contain no or low levels of BPV-1 genome and transcripts were less sensitive to p38 inhibition. The EqS02aT (EqS02a-E5, -E6, -E7 and -neo) lines were also incubated with SB and analysed for proliferation. EqS02a-neo (Fig. 5b), -E5 and E7 (data not shown) cells were not affected by SB treatment; however, the expansion of EqS02a-E6 was significantly reduced (Fig. 5b). Recently, we found that E5 and E6 can each enhance proliferation of equine fibroblasts (Yuan et al., 2011). The present observations suggest the interesting possibility that E6 enhances cell proliferation via p38 activation, whilst E5 stimulates cell expansion independently of p38 activity, despite the increase in p-p38 (Fig. 2).
assessed for their invasiveness following SB treatment. The blockage of p38 activity significantly reduced the invasion of EqS04b (Fig. 6a), EqS01a cells (data not shown), EqS02a-E6 (Fig. 6b) and EqS02a-E5 (data not shown) in the Matrigel invasion system. This shows that p38 activity is crucial for the cellular invasion of sarcoid fibroblasts that express BPV-1 gene(s). The reduction of invasiveness was not due to the decrease of cell viability as the treated cells were still viable as shown by their hydrolysis of non-fluorescent Calcein-AM to fluorescent Calcein (Fig. 6a, b, top panel). The inhibition of cell proliferation and invasion by SB strongly indicates that p38 is necessary for the fully transformed phenotype of sarcoid fibroblasts.

Previously, we demonstrated that equine sarcoid fibroblasts express upregulated MMPs including MMP-1 and the upregulation of MMPs contributes to sarcoid cell invasion (Yuan et al., 2010a, 2011). The reduction of cell invasion by p38 MAPK and BPV-1 in equine fibroblasts http://vir.sgmjournals.org 1781

Fig. 3. Detection of phospho-MAPKAPK2 (p-MK2) expression by immunofluorescence in equine fibroblasts. (a) The cells were co-stained with the primary rabbit anti-p-MK2 (Thr334) antibody and secondary Alexa Fluor 488 chicken anti-rabbit IgG for p-MK2 and TRITC-conjugated phalloidin for filamentous actin (F-actin). Magnification, ×400. (b) Co-immunofluorescence staining of p-MK2 and F-actin in empty vector EqPalF cells (neo) or S6-2 cells using antibodies as in (a). Magnification, ×400.

Fig. 4. The inhibition of p38 activity by its inhibitor SB changes the morphology of EqS04b cells. (a) Co-immunofluorescence staining of p-MK2 and F-actin in EqS04b cells treated with 10 μM SB using antibodies as in Fig. 3(a). Magnification, ×400. (b) Expression of p-MK2 and α-tubulin was detected in EqS04b cells treated with 10 μM SB by Western blotting with anti-p-MK2 (Thr334) and anti-α-tubulin antibodies, respectively. (c) Cell morphology of Calcein-AM stained EqS04b and EqPalF cells cultured for 2 days with 10 μM SB, images taken by confocal microscopy. Magnification, ×400.
SB suggests the possible involvement of p38 in the regulation of MMP expression in equine cells. To clarify this, MMP-1 mRNA expression was examined in SB-treated EqS04b cells. As shown in Fig. 6(c), MMP-1 mRNA expression was inhibited by SB in a dose-dependent manner. It has been demonstrated that p38 activity is associated with elevated AP-1 transcription factor expression and activity (Chen & Bowden, 1999; Loesch et al., 2010), and AP-1 plays a major role in the modulation of MMP-1 mRNA expression (Auble et al., 1992). We have recently found that the equine MMP-1 promoter is regulated by AP-1 (Z. Yuan and others, unpublished data). Moreover, Fra-1, one component of AP-1, is upregulated by BPV-1 (Yuan et al., 2008b) and the phosphorylation of c-Jun, another component of AP-1, is increased in BPV-1-positive sarcoid tissues (Borzacchiello et al., 2009). Thus, p38 may modulate MMP-1 expression via AP-1 in equine fibroblasts. To clarify the possibility we next examined Fra-1 and c-Jun proteins in EqS04b cells treated by SB. The expression of p-Fra-1 was detected by immunofluorescence with a p-Fra-1-specific antibody. EqS04b cells showed strong expression of nuclear p-Fra-1, which was abolished by SB treatment (Fig. 6d), indicating that p38 activity is necessary for the accumulation of p-Fra-1. However, the expression of phosphorylated c-Jun appeared not to be affected by SB (data not shown). Thus, the inhibition of MMP-1 transcriptional expression by SB might largely be due to the suppression of p-Fra-1 expression. In addition, the inhibition of sarcoid cell invasion by SB is at least partly attributed to its reduction effect on MMP-1 expression.

**DISCUSSION**

Previously, we demonstrated that BPV-1 infection transforms equine fibroblasts with enhanced cell proliferation and cellular invasiveness (Yuan et al., 2008a, 2010a, 2011). However, the transformation-associated cellular factors remain largely unknown. The present study demonstrates that E5 and E6 activate p38 MAPK by enhancing its phosphorylation, and this is followed by the phosphorylation of MK2 that is a substrate of p38. Inhibition of p38 activity by its chemical inhibitor SB significantly reduced sarcoid fibroblasts proliferation and invasion, indicating that p38 is a crucial cellular factor mediating BPV-1 transformation of equine fibroblasts.

p38 MAPK is involved in inflammation, cell cycle, cell differentiation, cell death, cell motility and invasion (Cuenda et al., 1995; Zhao et al., 1999). The enhancement of p38 phosphorylation (activation) by E5 and E6 proteins is implicated in the mechanism by which BPV-1 transforms equine fibroblasts. It is yet to be elucidated how E5 and E6 stimulate p38 MAPK activation. It may be the downstream effect of the activation of the PDGF β-receptor, possibly by E5 in sarcoids (Borzacchiello et al., 2009).

The p38 activity is essential for the proliferation of sarcoid cells expressing BPV-1. Whereas p38 has previously been considered as a mediator of stress-induced apoptosis (Boosani et al., 2009), it is also associated with enhanced cellular proliferation particularly in breast cancer cells with non-functional p53 (Zhao et al., 1999). We have previously demonstrated that in some sarcoids, p53 expression is perinuclear and its function is abrogated in vitro (Nixon et al., 2005; Yuan et al., 2008a). BPV-1 E6 can inhibit the function of p53 by interaction with its co-activator CBP/p300 (Zimmermann et al., 2000). In this study, we show that inhibition of p38 activity blocks the proliferation of EqS01a and EqS04b cells, in which E6, highly expressed in the two cell lines, is likely to interfere with p53 function (Yuan et al., 2008a). The reduction of cell proliferation in EqS02a-E6 (see Fig. 5b), but not in EqS02a-E5 cells by SB (data not shown), further supports the hypothesis that those cells with malfunctioning p53 are sensitive to p38 inhibition. Whilst the proliferation enhancement by E6 appears to be p38-dependent, E5 may induce a p38-independent pathway for proliferation.

In this study, we show for the first time that p38 activity is crucial for the expression of p-Fra-1 and MMP-1 and
cellular invasion in sarcoid fibroblasts. We have shown that the upregulated MMP expression and activities contribute to the invasiveness of sarcoid fibroblasts (Yuan et al., 2010a). Thus, it appears that at least one way by which p38 regulates cellular invasion is through the regulation of MMPs. The p38 activity has been shown to be critical for

**Fig. 6.** p38 activity is essential for the invasiveness of EqS04b cells. (a, top panel) Inhibition of p38 MAPK activity by SB reduced invasiveness of EqS04b cells in the Matrigel invasion assay. SB was added to the Matrigel and the medium below and above the transwell filter membrane to a final concentration of 10 μM. (a, bottom panel) Relative quantification of invasion of cells in the top panel. The invasion rate of cells treated by SB (10 μM) is relative to that of control cells without inhibitor treatment (0 μM), the value for which was set to 100%. *, P<0.05, versus control cells (0 μM). (b, top panel) Inhibition of p38 by SB reduced invasiveness of EqS02a-E6 cells in the Matrigel invasion assay. (b, bottom panel) Relative quantification of invasion of cells in the top panel. The invasion rate of cells was calculated as in (a). *, P<0.05, versus control cells (0 μM). (c) The expression of equine MMP-1 mRNAs is inhibited by SB in a dose-dependent manner in EqS04b cells. A RQ-PCR was carried out to measure MMP-1 transcripts in cells treated with increasing concentration of SB. Expression levels in SB-treated cells are relative to that without inhibitor treatment (0 μM), the value for which was set to 1. *, P<0.05, versus control (0 μM). (d) SB treatment reduces the expression level of phosphorylated Fra-1 (p-Fra-1) in EqS04b cells demonstrated by immunofluorescence. Cells were stained with a primary rabbit anti-p-Fra-1 (Ser265) antibody and second Alexa Fluor 488 chicken anti-rabbit IgG. Magnification, ×400.
the phorbol myristate acetate-induced MMP-9 expression and in vitro cellular invasion in human cells (Simon et al., 1998) and in H-ras induced in vitro invasion of NIH3T3 cells as well (Behren et al., 2005). Indeed BPV-1 E5 and E6 alone are able to enhance MMP-1 expression and the expression of BPV-1 genes including E5, E6 and E7 is necessary for the elevated Fra-1 expression (Z. Yuan and others, unpublished data). Therefore, the stimulation of equine fibroblast invasiveness by E5 and E6 proteins (Yuan et al., 2011) must be mediated at least partly through the p38-AP-1-MMP-1 signalling cascade.

Additionally, p38 can regulate cell motility. Inhibition of p38 activity prevents actin cytoskeleton reorganization necessary for cell migration (Hedges et al., 1999; Piotrowicz et al., 1998; Rousseau et al., 1997). Since MMPs are not the only factor responsible for the invasiveness of sarcoïd fibroblasts, it is likely that the reduction of sarcoïd cell invasiveness is also due to the inhibition of p38-mediated cell migration by SB. Indeed p38 has been shown to be essential for equine neutrophil migration (Eckert et al., 2009).

Given that AP-1 is largely involved in the modulation of MMP expression (Benbow & Brinckerhoff, 1997; Crawford & Matrisian, 1996) and is important for cellular invasion (Ozanne et al., 2007), the nuclear accumulation of p-Fra-1 – and thus AP-1 activity enhancement – in EqS04b cells must contribute to cellular invasion. The linkage of p-Fra-1 overexpression with p38 activity sheds light on the mechanism of cellular invasion regulated by p38. Many transcription factors have been shown to be phosphorylated and subsequently activated by p38 including the activating transcription factor 2 (ATF-2) that is one component of AP-1 heterodimer complexes (Zarubin & Han, 2005; Zhao et al., 1999). The inhibition of p-Fra-1 expression in EqS04b cells by SB indicates p38 activity is needed for elevated Fra-1 phosphorylation. The elevation is due to enhanced phosphorylation but not expression of Fra-1 since SB treatment showed no effect on Fra-1 mRNA expression in equine fibroblasts (data not shown). Given the drastic inhibition of sarcoïd cell proliferation and invasion by SB, p38 could be a potential target for equine sarcoïd therapy.

**METHODS**

**Cell cultures.** Normal equine embryonic fibroblast line EqPalF, BPV-1 in vitro transformed EqPalF (S6-2), sarcoïd cell lines EqS01a, EqS02a and EqS04b have been described previously (Yuan et al., 2008a). EqS02a-neo, -E5, -E6 and -E7 cells (EqS02aT) were established from stable EqS02a cell lines that express neomycin-resistance gene (neo) or individual BPV-1 genes E5, E6 or E7 as described in our recently published study (Yuan et al., 2011). The EqPalF-neo line, which was EqPalF transfected with the empty vector pcDNA3.1 (Yuan et al., 2011), was also used. All cells were maintained in culture in complete Dulbecco’s modified Eagle’s medium (DMEM) in a 37 °C humidified atmosphere of 5% CO₂ in air. Cells were routinely tested for mycoplasma and were consistently negative.

**Quantitative RT-PCR.** Equine MMP-1 mRNA was quantified by real-time Relative Quantification RT-PCR (RQ-PCR). Real-time PCR primer and probe sets for the MMP-1 gene and the housekeeping gene GAPDH were as described previously (Yuan et al., 2008b). Total RNA extraction, cDNA synthesis and PCR conditions were all as described previously (Yuan et al., 2008a). MMP-1 transcripts were normalized by the endogenous control, GAPDH. The quantification measurements were carried out three times and a Student’s t-test was used to assess the significance of the differences.

**Western blotting.** Cells were washed with PBS and lysed in 50 mM Tris/HCl, pH 7.5, containing 105 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and complete protease inhibitor cocktail (Roche). The protein extracts were cleared by centrifugation, and the supernatants stored at −70 °C. Protein was quantified by the copper sulfate-bicinchoninic acid method and separated by gel electrophoresis. Primary antibody incubation was carried out overnight at 4 °C with rabbit antibodies against p38 MAPK, phospho-p38 (Thr180/Tyr182) or phospho-MAPKAP2 (Thr334) (Cell Signaling Technology) all at 1:1000 dilutions. The p38 MAPK antibody detects all p38α, -β and -γ isoforms. The blots were washed and probed with HRP-linked anti-rabbit antibody (GE Healthcare) at 1:5000 dilutions and protein was detected by enhanced chemiluminescence (Amersham Pharmacia). A rabbit mAb against human z-tubulin (Cell Signaling Technology) was used to control for the quantity of proteins in analysed samples. Densitometry analyses of Western blotting were performed using the software Image J (http://rsb.info.nih.gov/ij/).

**Inhibitors.** The p38 MAPK selective inhibitor SB (Tocris) was dissolved in water as 10 mM. Subconfluent cultured cells were treated with the SB inhibitor at indicated final concentrations or with control solution (water) under indicated serum conditions. For MTT assay with SB, the inhibitor was added to culture medium at a final concentration of 10 μM and medium was changed every 24 h. For invasion assays with SB, the inhibitor was added to the Matrigel and to the medium above and below the transwell filter at a final concentration of 10 μM.

**Immunofluorescence and Calcein-AM staining.** Expression of phosphor-MAPKAPK2 (p-MK2) and phosphor-Fra-1 (p-Fra-1) was detected by immunofluorescence as described previously (Yuan et al., 2008b). Cells were grown on chamber slides for at least 1 day in DMEM–10% FCS, and then stained with primary antibodies against p-MK2 (Thr 334) or p-Fra-1 (Ser 265) (Cell Signaling Technology) and a second Alexa Fluor 488 chicken anti-rabbit IgG (Invitrogen Molecular Probes). TRITC-conjugated phallidin (Sigma) was also added into the p-MK2 staining solution for detecting filamentous actin. Stained cells were mounted with cover glasses using Vectashield (Vector Laboratories), followed by confocal microscopy (Leica TCS 2P2 microscope) for viewing and imaging. EqS04b cells treated with or without SB (10 μM) for 2 days under serum-free condition were stained with 4 μM Calcein AM (BD Biosciences), followed by confocal microscopy.

**Cell proliferation assays.** Assessment of cell proliferation and viability was determined using the Cell Proliferation kit I (Roche) as described previously (Yuan et al., 2008a). Briefly, 5×10⁴ cells were seeded in 24-well tissue culture plates in DMEM containing 10% FCS. MTT assays were performed for cultured cells or culture medium without cells in triplicates every 24 h for a total of 3 days. Spectrophotometric absorbance was measured at 562 nm, and a background reading for medium without cell culture was subtracted. The mean values were plotted as a function of time.

**Invasion/3D migration assays.** Inverse invasion assays were carried out using Matrigel (BD Biosciences) and transwell inserts (Corning Inc.) as described previously (Yuan et al., 2010a). In brief, 2×10⁴ cells were seeded onto each insert filter membrane and incubated for up to
2 days with serum-free DMEM in the bottom chamber and 10% FCS containing medium above the Matrigel. Cells were then stained with 4 μM Calcein AM (BD Biosciences) and viewed by confocal microscopy (Leica TCS SP2 microscope). Cell invasion was measured by scanning optical sections at 10 μm intervals. Only cells in the 20 μm reconstituted Matrigel matrix section and above were considered invasive for quantification purposes. Invasion rate is reported as the percentage of invading cells over the total number of cells. Overall results were determined from three separate assays performed in duplicate.

Transfection of cells with siRNA. EqS04b cells were transfected with siRNA siE6 against BPV-1 E6 gene (siE6), control siRNA (scramble) and transfection complex without any siRNA (mock), respectively. The sequence of siE6 and scrambled siRNA have been described previously (Yuan et al., 2010b). Cells were transfected with 10 nM of siRNA using the Lipofectamine RNAiMAX reagents (Invitrogen) according to the manufacturer’s instructions. Transfections were repeated three times. Transfected cells were cultured for 20 h before harvesting the cells for further analysis.

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