Different contribution of bovine papillomavirus type 1 oncoproteins to the transformation of equine fibroblasts

ZhengQiang Yuan, Elizabeth A. Gault, M. Saveria Campo and Lubna Nasir

Equine sarcoids represent the most common skin tumours in equids worldwide, characterized by localized invasion, rare regression and high recurrence following surgical intervention. Bovine papillomavirus type 1 (BPV-1) and less commonly BPV-2 are now widely recognized as the causative agents of the disease. Fibroblasts isolated from sarcoids are highly invasive. Invasion is associated with a high level of viral gene expression and matrix metalloproteinase upregulation. However, it remains unclear to what extent BPV-1 proteins are involved in the transformation of equine cells. To address this question, the individual viral genes E5, E6 and E7 were overexpressed in normal equine fibroblasts (EqPalF cells) and in the immortal but not fully transformed sarcoid-derived EqS02a cell line. The proliferation and invasiveness of these cell lines were assessed. E5 and E6 were found to be responsible for the enhanced cell proliferation and induction of increased invasion in EqS02a cells, whilst E7 appeared to enhance cell anchorage independence. Knockdown of BPV-1 oncogene expression by small interfering RNA reversed the transformed phenotype of sarcoid fibroblasts. Together, these observations strongly suggest that BPV-1 proteins play indispensable roles in the transformation of equine fibroblasts. These data also suggest that BPV-1 proteins are potential drug targets for equine sarcoid therapy.

INTRODUCTION

Equine sarcoi ds are the most common skin tumours in equids worldwide (Jackson, 1936; Pascoe & Summers, 1981; Ragland et al., 1970) with reported prevalence rates ranging from 12.9 to 67 % of all equine tumours (Lavach et al., 1985). Six clinical types of sarcoid are recognized including occult, verrucous, nodular, fibroblastic, mixed and malignant types (Knottenbelt, 2005). Equine sarcoi ds rarely regress, are notoriously difficult to treat and are associated with a high recurrence rate following surgical intervention (Knottenbelt, 2005; Martens et al., 2001a; Tarwid et al., 1985). The high recurrence may be associated with the invasiveness of sarcoi d fibroblasts (Yuan et al., 2010a), which allows cells to infiltrate into tumour-surrounding healthy tissues. Bovine papillomavirus (BPV) DNA has also been detected in apparently healthy skin away from the tumour site (Carr et al., 2001), and tumour recurrence is most frequent when surgical margins are positive for BPV DNA (Martens et al., 2001a). After excision of primary tumours, disseminated tumour cells may be able to invade the surgical margins under the chemoattractant stimulation of the chemokines and growth factors produced during wound healing. Thus, prevention of sarcoi d cell invasion in combination with surgical excision may reduce the risk of tumour recurrence.

BPV type 1 (BPV-1) and less commonly BPV-2 are now widely recognized as the causative agents of equine sarcoi ds. This is based on the fact that (i) BPV-1/2 DNA is detected in the majority of sarcoi d tumours (Chambers et al., 2003b; Martens et al., 2001a, b; Otten et al., 1993; Reid et al., 1994), (ii) BPV genes are expressed in sarcoi ds (Borzacchiello et al., 2008; Carr et al., 2001; Chambers et al., 2003a; Nasir & Reid, 1999), (iii) experimental inoculation of equine skin with BPV induces sarcoi d-like lesions in horses (Ragland & Spencer, 1969) and (iv) BPV-1 DNA can transform primary equine fibroblasts in vitro (Yuan et al., 2008a). BPV-1 is a non-enveloped virus with a dsDNA genome of approximately 8 kb, and the virus normally infects cattle, causing papillomas of skin or mucosa, which generally regress in the host. Occasionally, papillomas may undergo malignant transformation giving rise to squamous cell carcinomas of the bladder and alimentary tract (Campo, 2006). BPV-1 encodes three oncoproteins, E5, E6 and E7. E5 is the major transforming protein, followed by E6. E7 cooperates with E5 and E6 to induce transformation. E5 transforms cells by activation of
the platelet-derived growth factor (PDGF) β-receptor (Petti & DiMaio, 1994; Suprynowicz et al., 2005), and E6 contributes to transformation by its interaction with the focal adhesion protein paxillin (Tong et al., 1997). The transformation function of E7 has been shown to correlate with its ability to bind to p600 (DeMasí et al., 2005).

Tumour cell invasion involves degradation of both basement membranes and stromal extracellular matrix by proteinases including matrix metalloproteinases (MMPs), serine proteases and cathepsins (Birkedal-Hansen, 1995). In the last few decades, MMPs have been demonstrated to play a crucial role in initiating degradation of basement membranes and extracellular matrix to allow tumour cell invasion (Westermarck & Kähäri, 1999). Human papillomavirus (HPV) proteins appear to play an important role in stimulating MMP expression and inducing invasion in human cells. The E7 protein of HPV-8 is able to promote the overexpression of MMP-1, MMP-8 and membrane type 1 MMP, and causes invasiveness of human keratinocytes (Akgül et al., 2005). E6/E7 of HPV-16 induces invasiveness of human breast cancer cells (Yasmeen et al., 2007). Additionally, BPV-1 gene expression upregulates MMP expression and induces invasion (Yuan et al., 2010a). However, little is known about which BPV-1 oncoproteins are involved in transformation and invasion of equine fibroblasts. In this study, we investigated the individual roles of E5, E6 and E7 in cell transformation of equine fibroblasts.

**RESULTS**

**Equine sarcoid fibroblasts are telomerase positive**

EqPalF cells are primary cells explanted from the palate tissue of an abortive horse embryo and are negative for BPV (Yuan et al., 2008a). These cells have a limited lifespan. EqS02a cell line is an immortalized but not fully transformed fibroblast line explanted from a sarcoid tumour (Yuan et al., 2008a, 2010a), with low levels of BPV viral genomes and viral transcripts (Yuan et al., 2008a). EqS04b cell line is a fully transformed sarcoid fibroblast line with high levels of BPV viral genomes and transcripts (Yuan et al., 2008a, 2010a). Like EqS02a cells, EqS04b cells are immortal (Yuan et al., 2008a). To investigate the basis of immortality in these cell lines, we assessed them for telomerase activity. As shown in Fig. 1, both cell lines were positive for telomerase activity, suggesting that activation of telomerase underlies the immortality of these two cell lines. In contrast, EqPalF cells were negative for telomerase activity, in agreement with their limited lifespan.

**BPV-1 E5 and E6 induce full transformation of immortal equine fibroblasts**

Expression of BPV-1 genes is crucial for the transformation of equine fibroblasts (Yuan et al., 2008a). However, it remains unclear which viral proteins are involved in cell transformation. To study the individual roles of BPV-1 oncoproteins in cell transformation, we introduced Myc-tagged BPV-1 sequences for the E5, E6 and E7 genes in EqS02a. The established cell lines were designated EqS02a-E5, -E6 and -E7, respectively. As shown in Fig. 2(a), E5, E6 and E7 were all successfully expressed, although at different levels from those in EqS04b. In contrast, no BPV-1 transcripts could be detected in the control EqS02a line expressing a neomycin-resistance gene (EqS02a-neo). Expression of viral proteins was confirmed using the anti-Myc tag antibody 9E10 (Fig. 2b). The viral gene expression constructs and empty vector were also transfected into the primary fibroblast EqPalF cells and the established cell lines designated EqPalF-E5, -E6, -E7 and -neo. The generation of stable transfectants in EqPalF cells required large amounts of plasmid and pooling of all selected cells, as the cells did not survive clonal selection. The introduced viral genes were expressed in EqPalF cells at levels similar to those in transfected EqS02a cells (data not shown).

The introduction of either E5 or E6 sequences changed the morphology of EqS02a cells. When cultured in 10% serum-containing medium, EqS02a-E5 and -E6 cells both exhibited an elongated and spindle-like shape, similar to the morphology of the fully transformed EqS04b cells (Fig. 2c). In contrast, EqS02a-E7 and -neo cells both showed a flatter and shorter shape, similar to the parental cells. Overexpression of the individual viral sequences did not cause changes in the morphology of EqPalF cells (data not shown). This suggested that BPV-1 E5 and E6 can morphologically transform immortal equine fibroblasts.

All stable cell lines (EqS02a-neo, -E5, -E6 and -E7 and EqPalF-neo, -E5, -E6 and -E7) were all successfully expressed, although at different levels from those in EqS04b. In contrast, no BPV-1 transcripts could be detected in the control EqS02a line expressing a neomycin-resistance gene (EqS02a-neo). Expression of viral proteins was confirmed using the anti-Myc tag antibody 9E10 (Fig. 2b). The viral gene expression constructs and empty vector were also transfected into the primary fibroblast EqPalF cells and the established cell lines designated EqPalF-E5, -E6, -E7 and -neo. The generation of stable transfectants in EqPalF cells required large amounts of plasmid and pooling of all selected cells, as the cells did not survive clonal selection. The introduced viral genes were expressed in EqPalF cells at levels similar to those in transfected EqS02a cells (data not shown).

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**Fig. 1**. Equine sarcoid fibroblasts are telomerase positive. TRAP assays (see Methods) were performed to assess telomerase activity in EqPalF, EqS02a and EqS04b cells. For each cell line, both active (− heat) and inactivated (+ heat) samples were assessed. An absorbance difference of >0.2 between the two samples indicated telomerase activity.
conditions (0.1% FBS), EqS02a-E5 and -E6 cells continued growing, albeit slowly, whereas EqS02a-E7 cells died and EqS02a-neo cells could not grow (Fig. 3b). Thus, E5 and E6 may stimulate growth factor-independent proliferation in immortal equine fibroblasts.

EqPalF-neo, -E5 and -E7 cells showed a limited lifespan of approximately four passages (~7 PDs), at which point the cells became enlarged and underwent growth arrest. In contrast, the EqPalF-E6 cells were able to grow beyond ten passages (~15 PDs) without any signs of growth arrest. At passage 3, EqPalF cell lines were assessed for their viability and proliferation capacity. As shown in Fig. 3(c), EqPalF-E6 cells showed good proliferation, whilst EqPalF-neo and -E7 cells hardly grew and EqPalF-E5 cells stopped proliferating. These data showed that E6 is able to extend the lifespan of EqPalF cells.

**BPV-1 E6 and E7 enhance anchorage-independent growth**

Previously, EqS02a cells were found to form foci with very low efficiency in methylcellulose medium, possibly due to the very low BPV-1 gene expression levels in these cells (Yuan et al., 2008a). To determine the effect of BPV-1 oncoproteins on anchorage-independent growth, the cell lines EqS02a-neo, -E5, -E6 and -E7 were cultured in methylcellulose medium. As shown in Fig. 4, EqS02a-E6 and -E7 cells showed increased focus formation (28.5 ± 2.1 and 17.9 ± 1.8 foci per 10,000 cells, respectively) in comparison with that of control EqS02a-neo cells, which failed to form any foci. EqS02a-E5 cells showed a small increase in focus formation (1.3 ± 1.5 foci per 10,000 cells). EqS04b cells, which express all E6, E7 and E5 oncoproteins, showed a higher focus formation efficiency (68 ± 26.3 foci per 10,000 cells), suggesting possible synergistic effects of E6, E7 and E5. This observation indicated that BPV-1 E6 and E7 are largely involved in anchorage independence of equine sarcoid fibroblasts.

**BPV-1 E5 and E6 contribute to cell invasion**

We have shown previously that BPV-1 proteins are associated with the invasiveness of sarcoid fibroblasts (Yuan et al., 2010a). To identify the viral proteins
responsible for this phenotype, the transfected EqS02a and EqPalF cell lines were tested in Matrigel invasion/3D migration assays. As shown in Fig. 5(a), E5 and E6 both induced significant invasion of EqS02a cells, whereas E7 had no effect. Neither extension of the assay time nor an increase in cell numbers led to invasion of EqS02a-E7 cells, indicating that the invasion of EqS02a-E5 and -E6 cells was not due to the proliferation advantage of the latter two lines (data not shown). None of the EqPalF cell lines expressing individual viral genes were invasive, although the in vitro BPV-1-transformed S6-2 cells (Yuan et al., 2008a) showed an invasive phenotype (Fig. 5b). Thus, BPV-1 E5 and E6 are essential for the invasiveness of sarcoid fibroblasts.

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**Fig. 3.** BPV-1 E5 and E6 enhance proliferation of immortal equine fibroblasts. Cell proliferation was assessed by an MMT assay in EqS02a cell lines expressing a neomycin-resistance gene or BPV-1 E5, E6, or E7 gene (a), stable EqS02a lines under low-serum conditions (0.1% FBS) (b) and EqPalF lines expressing a neomycin-resistance or BPV-1 E5, E6 or E7 gene (c).

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**Fig. 4.** BPV-1 E6 and E7 enhance anchorage-independent growth. EqS02a-neo, -E5, -E6 and -E7 cell lines and EqS04b cells were grown in 1.0% methylcellulose medium. Typical foci are indicated by circles. Phase-contrast images were produced by light microscopy. Magnification, ×200.
Knockdown of BPV-1 oncogene expression reverts the transformed phenotype of sarcoid fibroblasts

The above observations strongly suggested that the BPV-1 proteins E5, E6 and E7 are differentially crucial for the growth and invasion of sarcoid fibroblasts. Thus, inhibition of these viral proteins was expected to impair cell growth and invasion. To determine this, small interfering RNA (siRNA) molecules targeting E5, E6 or E7 (siE5, siE6 and siE7, respectively; Yuan et al., 2010b) were introduced into EqS04b cells and the effects on viral gene expression, MMP expression, cell proliferation and invasion were studied. EqS04b cells were chosen for this study as these cells express BPV-1 genes at high levels and are highly invasive (Yuan et al., 2008a, 2010a). siE6 transfection efficiently inhibited over 95 % of E6 mRNAs (Fig. 6a). Expression of the other transforming genes, E5 and E7, was also reduced significantly by 89 and 88 %, respectively. The BPV-1 genome is transcribed into multiple polycistronic RNAs that are subject to constitutive and alternative splicing (Jia & Zheng, 2009), and this complex pattern of mRNA synthesis may explain why treatment with siE6 caused knockdown of additional viral mRNAs. We have shown previously that the invasive phenotype of sarcoid fibroblasts is mediated partly by MMPs (Yuan et al., 2010a); therefore, MMP expression was also evaluated. MMP-1, -2 and -9 transcripts were significantly decreased by siE6 treatment (Fig. 6b) and the secretion of MMP-2 and MMP-9 proteins was reduced in siE6-treated cells in comparison with control cells (Fig. 6c). Furthermore, invasion of EqS04b cells was completely eliminated by siE6 treatment (Fig. 6d). siE6 treatment also caused a significant attenuation of cell growth (Fig. 6e). Thus, siE6 efficiently knocked down BPV-1 gene expression and reversed the transformed phenotype of sarcoid fibroblasts. Both siE5 and siE7 also significantly inhibited BPV-1 gene expression, albeit with a lower efficiency than siE6 (see Supplementary Fig. S1, available in JGV Online, for siE7 treatment; siE5 data not shown). However, these two siRNAs decreased cell
Fig. 6. Knockdown of BPV-1 gene expression reverts the transformed phenotype of sarcoid fibroblasts. (a) Relative quantification of BPV-1 gene (E5, E6 and E7) mRNA expression levels in scrambled siRNA- and siE6-transfected EqS04b cells. Expression was relative to mock-transfected cells (without any siRNA), the values for which were set to 1. *, P<0.05. (b) Relative quantification of MMP gene (MMP-1, -2 and -9) mRNA expression levels in scrambled siRNA- and siE6-transfected EqS04b cells. Expression was relative to mock-transfected cells, the values for which were set to 1. *, P<0.05. (c) The upper panel shows a representative gelatin zymogram of MMP-2 and -9 proteins secreted by EqS04b cells transfected with...
Recently, we showed that expression of the BPV-1 genome DISCUSSION of sarcoid fibroblasts. high-efficiency knockdown of BPV-1 oncogene transcripts efficient at inhibiting their respective mRNAs, it was the additional Fig. S1). Therefore, although siE5 and siE7 were due to their small effect on MMP expression (Supple- mental). The present study demonstrated that, in equine immortal fibroblasts, (i) telomerase enzyme is activated, (ii) E5 and E6 oncoproteins enhance proliferation, (iii) E5 and E6 both contribute to the induction of invasiveness, (iv) E6 and E7 enhance cell anchorage-independent growth and (v) inhibition of E6 expression reverses cell proliferation and invasion.

**Equine sarcoid fibroblasts in vitro are immortal**

In this study, we showed that equine sarcoid fibroblasts are positive for telomerase activity. Telomerase is a ribonu- cleoprotein reverse transcriptase capable of synthesizing terminal TTAGGG telomeric repeats onto the ends of chromosomes (Morin, 1989). Telomerase is considered to be essential for attainment of cellular immortality by overcoming telomeric erosion and is commonly detected in cancers (Broccoli et al., 1995; Harley et al., 1994; Kim et al., 1999; Shay & Bacchetti, 1997). The high-risk HPV E6 protein can activate telomerase (Klingelhutz et al., 1996).

However, it is not known whether BPV proteins can activate telomerase, although BPV-associated bovine cancers are positive for telomerase (Borzacchiello et al., 2005). The immortal sarcode fibroblasts EqS02a and EqS04b are telomerase positive. However, the contribution of BPV proteins to the immortalization process remains to be elucidated, given that telomerase is activated in EqS02a cells, which express almost undetectable levels of viral genes. It is likely that genetic mutation(s) contribute to telomerase induction in this particular fibroblast line. The absence of telomerase activity in some sarcooids (Argyle et al., 2003) is at variance with the present findings. Whilst there may be several explanations for this, it is possible that the growth of sarcoid fibroblasts in culture requires/induces telomerase activation, as previously observed in immortalized equine cells (Argyle et al., 2003).

**BPV proteins E5 and E6 are transforming for immortal equine fibroblasts**

We showed that BPV-1 proteins E5 and E6 play important roles in the full transformation of equine immortal fibroblasts. It has been proven that E5 transforms cells through its interaction with, and activation of, the PDGF β-receptor (Petti & DiMaio, 1994; Suprynnowicz et al., 2005). The activation of PDGF β-receptor initiates mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K) signalling cascades leading to enhanced cell proliferation and motility (Choudhury et al., 1997; DeMali et al., 1997; Kim et al., 2007; Singh et al., 2007; Tanaga et al., 2004). Equine sarcooids were recently shown to express increased levels of activated PDGF β-receptor bound to the 85 kDa regulatory subunit (p85) of PI3K (Borzacchiello et al., 2008, 2009). So far, it remains unknown whether BPV-1 E5 is also responsible for the activation of PDGF β-receptor in equine cells. Borzacchiello et al. (2006) showed that E5 physically interacts with phosphorylated PDGF β-receptor in bovine bladder cancer, and this suggests that the same may take place in sarcooids. The stimulation of cell proliferation by E5 in this study supports this.

Our data strongly suggest that E5 transformation requires immortalization. E5 transformed immortal EqS02a cells but failed to do so with the primary EqPalF cells. These observations were in agreement with our previous study, which showed that E5 enhanced the proliferation of EqPalF cells previously immortalized by human telomerase reverse transcriptase (Marchetti et al., 2009).

Increasing evidence shows that BPV-1 E6 plays an important role in cell transformation. Expression of BPV-1 E6 by itself can lead to transformation of mouse C127 cells (Neary & DiMaio, 1989; Schiller et al., 1984). Full transformation of bovine fibroblasts by BPV E5 (then called E8) needs E6 (Pennie et al., 1993). The oncoprotein inhibits the function of p53 through interaction with, and inhibition of, the p53 co-activator CBP/p300 (Zimmermann et al., 2001). Our finding that E6 induces anchorage-independent growth is consistent with previous observations that E6 contributes to the anchorage-inde- nendent growth of transformed cells by disrupting focal
adhesion (Tong et al., 1997). In addition, the lifespan extension of EqPalF cells and the transformation of EqS02a cells by BPV-1 E6 strongly support the fundamental functions of the viral protein in the transformation of equine fibroblasts.

E5 and E6 are the major oncoproteins of BPV-1. BPV-1 E7 oncoprotein is weakly transforming but enhances anchorage-independent growth of murine C127 cells induced by BPV-1 E5 or E6 (Boh1 et al., 2001; Neary & DiMaio, 1989). This was supported by our observation that E7 by itself did not stimulate cell proliferation or invasiveness, but enhanced focus formation of EqS02a cells in semi-solid methylcellulose medium (Fig. 4). HPV E7 has been reported to inhibit DNA synthesis and cause cell apoptosis (Park et al., 2000). This finding is further supported by our observation that expression of BPV-1 E7 induced EqS02a cell death under low-serum conditions.

BPV-1 E5 and E6 can induce invasiveness of sarcoid fibroblasts

Invasiveness of sarcoid fibroblasts is associated with BPV-1 gene expression (Yuan et al., 2010a). In this study, we demonstrated that BPV-1 E5 and E6 viral proteins are both responsible for the induction of invasion. The facts that E5 and E6 can induce invasion in EqS02a cells, but failed to do so in primary EqPalF fibroblasts, suggested that E5 and E6 need immortalization of cells for the induction of invasion. We have shown that the mechanism of invasion induction by BPV-1 in equine fibroblasts is at least partially due to the upregulation of MMPs including MMP-1 (Yuan et al., 2010a). However, it is likely that BPV-1 also induces cell invasion by modulating expression of other invasion-associated enzymes (Birkedal-Hansen, 1995), as the broad MMP inhibitor GM6001 only partially inhibits sarcoid cell invasion (Yuan et al., 2010a). It also remains to be elucidated whether BPV-1 can induce a protease-independent mode of cell invasion (amoeboid invasion) (Friedl & Wolf, 2003; Sahai & Marshall, 2003). To our knowledge, this is the first report demonstrating that BPV-1 proteins E5 and E6 are involved in cell invasion. Several HPV viral proteins have been shown to be involved in cell invasion. The high-risk HPV-8 E7 causes invasion of keratinocytes associated with upregulation of MMPs (Akgul et al., 2005). HPV-16 E6/E7 promote invasion of breast cancer cells (Yasmeen et al., 2007). Our data therefore provide a new basis for understanding the mechanisms by which BPV-1 induces equine sarcomas.

Inhibition of viral oncogene expression reverts the transformed phenotype

The main challenge in the clinical management of equine sarcomas stems from the high rate of recurrence of up to 50% (Martens et al., 2000; Tarwid et al., 1985). The recurrence of sarcomas could be caused by localized invasion of BPV DNA-containing fibroblasts that are highly invasive (Yuan et al., 2010a). Indeed, tumour recurrence is more frequent when surgical margins are positive for BPV DNA (Martens et al., 2001a). Given the fact that BPV-1 proteins exert profound effects on cell proliferation and invasion, we speculated that inhibition of BPV-1 gene expression would change the transformed phenotype of sarcoid fibroblasts and that it might be possible to prevent tumour recurrence by blocking cell invasion. Recently, we found that knockdown of BPV-1 E2 expression by introduction of siRNA against the E2 gene (siE2) gradually decreased viral load and viral gene expression and caused a concomitant reduction in cell invasiveness and apoptosis (Gobeil et al., 2009; Yuan et al., 2010a). However, the siE2 treatment required a long period (up to several weeks) with repeated doses, to attain effects. In addition, the inhibition of cell invasiveness by siE2 appeared incomplete (Yuan et al., 2010a), which would restrict its clinical application. To find faster-acting and more effective siRNAs, we examined other viral gene-targeting siRNAs against E5, E6 and E7 expression. Whilst both siE5 and siE7 showed limited effects on cell proliferation and invasion inhibition, siE6 showed very promising results by working quickly and efficiently. Almost-complete degradation of E6 transcripts, significant inhibition of cell proliferation and complete inhibition of cell invasiveness by siE6 were seen in EqS04b cells 2 days after siE6 transfection. Interestingly, the introduction of siE6 not only caused degradation of E6 mRNAs but also led to the decrease of other viral gene transcripts. siE5 and siE7 also showed similar viral gene knockdown patterns. It is known that BPV-1 transcripts are generally intron-containing bicistronic or polycistronic products, which need a further RNA-splicing process to form mature individual viral mRNAs (Jia & Zheng, 2009; Lambert et al., 1988). Theoretically, siRNAs targeting any transcriptional region of the BPV-1 genome would cause degradation not only of the targeted gene transcript but also of the linked pre-transcripts. Therefore, a single siRNA may simultaneously reduce the expression of several viral mRNAs. Although the observed low specificity of BPV-1 gene-targeting siRNAs may cause uncertainty in the investigation of viral gene functions, it might be ideal for antiviral therapy in sarcomas due to their multiple knockdown effects. It remains unclear why siE6 in particular worked better for inhibition of cell proliferation and invasiveness than the other viral gene-targeting siRNAs. The very high level of knockdown efficiency for viral transcripts (~90%) by siE6 might be the reason. siE7 and siE5 designed in this study showed a lower knockdown efficiency for viral gene expression (~80%) than siE6, which appeared not to be enough for successful inhibition of cell proliferation, MMP gene transcription and cell invasiveness.

Several studies have shown that subcutaneous or intratumoral injections of siRNAs targeting various cancer-associated genes, including targeting HPV expression, show therapeutic benefits (Fujii et al., 2006; Gu et al., 2008; Jonson et al., 2008). The remarkable effects brought about by siE6 strongly indicate its promise for sarcoi
tions, provided the same efficacy could be reached in vivo. A clinical trial is warranted for validation of the efficacy of siE6 for in vivo application.

METHODS

Cell cultures. Normal EqPalF, BPV-1-transformed S6-2 cells and sarcoid cell lines EqS02a and EqS04b have been described previously (Yuan et al., 2008a). All cells were maintained in culture in complete DMEM in a 37 °C humidified atmosphere of 5 % CO2 in air. Cells were routinely tested for mycoplasma and were consistently negative.

Telomerase activity assay. A TRAP<sub>ZZE</sub> ELISA Telomerase Detection kit (TRAP assay; Chemicon International) was used to detect telomerase activity in cells. Sample preparation and assays were performed as recommended by the supplier in the protocol provided. In brief, cultured cells were harvested for protein extraction and 1.5 μg total cellular protein was used for each assay. Each protein sample was also heated at 85 °C for 10 min and used as a background control. TRAP assays were performed in triplicate for each sample and the absorbance of samples was measured at 450 and 690 nm. The value for each sample was computed as absorbance (units)=A<sub>450</sub>−A<sub>690</sub>. An absorbance difference of >0.2 between heated and non-heated samples was regarded as an indication of positive telomerase expression in cells.

Transfection of equine fibroblasts and generation of stable cell lines expressing individual BPV-1 genes. To determine the effects of BPV-1 proteins on transformation of equine fibroblasts, we introduce individual BPV-1 genes into two equine fibroblast lines, EqPalF and EqS02a (Yuan et al., 2008a). EqPalF cells are negative for BPV-1 DNA, whilst EqS02a cells contain low levels of BPV-1 genomes and transcripts (Yuan et al., 2008a). Neither cell line is invasive (Yuan et al., 2010a).

Myc-tagged BPV-1 E5, E6 or E7 expression constructs have been described previously (Yuan et al., 2010b). EqPalF and EqS02a cells were transfected with viral gene constructs or with empty vector pcDNA3.1-mH using Lipofectamine 2000 (Invitrogen) following the supplier’s recommended protocol. Transfected cells were selected in the presence of 200 μg geneticin (G418; Invitrogen) ml<sup>−1</sup>. G418-resistant colonies were pooled, and polyclonal populations were grown and used in all experiments to avoid clonal heterogeneity. The pooled cells were grown to confluency and then passaged by trypsinization. This step was regarded as the first passage. All transfected cells were maintained in culture in DMEM containing 200 μg G418 ml<sup>−1</sup> and 10 % FBS (Invitrogen).

Quantitative RT-PCR for BPV and MMP mRNA expression. A real-time absolute quantification RT-PCR was used to assess the expression of BPV-1 genes E5, E6 and E7 in cells, and the BPV-1 genome containing plasmid pBPV-1-S6 (Yuan et al., 2008a) was used to generate standard curves. Equine MMP-1, -2 and -9 mRNA expression and BPV-1 E5, E6 and E7 gene transcripts in siRNA-transfected cells were quantified by real-time quantitative RT-PCR. Real-time PCR primer and probe sets for the MMP genes, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the BPV-1 genome have been described previously (Gobeil et al., 2009; Yuan et al., 2007, 2008a, b, 2010a). Total RNA extraction, cDNA synthesis and PCR conditions were all as described previously (Gobeil et al., 2009; Yuan et al., 2008a). MMP and BPV-1 transcripts were normalized to the endogenous control, GAPDH. All quantification measurements were carried out three times and Student’s t-test was used to assess the significance of the differences.

Western blotting. Cells were washed 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). Protein extracts were cleared by centrifugation and the supernatants stored at −70 °C. The protein was quantified and separated by gel electrophoresis. Primary antibody incubation was carried out with a mouse mAb against the Myc tag (clone 9E10; Invitrogen) at 1:1000 dilutions. The blots were washed and probed with HRP-linked anti-mouse antibody (GE Healthcare) and the protein detected by enhanced chemiluminescence (Amersham Pharmacia). A rabbit mAb against human β-tubulin (Cell Signaling Technology) was used as a control for the quantity of proteins in analysed samples.

Cell proliferation and anchorage-independent growth assays. Assessment of cell proliferation and viability was carried out using a Cell Proliferation kit I (Roche) as described previously (Yuan et al., 2008a). Briefly, 5 × 10<sup>4</sup> cells were seeded in 24-well tissue culture plates in DMEM containing 10 % FBS, MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed every 24 h for a total period of 6 days in triplicate. Spectrophotometric absorbance at 562 nm was measured and a background reading of DMEM without cell culture was subtracted. The mean values were plotted as a function of time. To assess anchorage-independent growth, cells were added to Methocel medium and grown in bacterial-grade Petri dishes for 2 weeks as described previously (Yuan et al., 2008a). EqS04b cells that showed a high efficiency of colony formation were used as a positive control (Yuan et al., 2008a). The mean efficiency of colony formation was calculated as number of colonies/number of cells.

Invasion/3D migration assays. Inverse invasion assays were carried out using Matrigel (BD Biosciences) and Transwell inserts (Corning) as described previously (Hennigan et al., 1994; Yuan et al., 2010a). In brief, 2 × 10<sup>5</sup> 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 % FBS-containing medium above the Matrigel. Cells were then stained with 4 μM calcine acetoxyethyl ester (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 section and above were considered invasive for quantification purposes. Invasion rate is reported as the percentage of invading cells/total number of cells. Overall results were determined from three separate assays performed in duplicate.

BPV-1 gene-targeting siRNA transfection. The sequences of BPV-1 E5-, E6- and E7-targeting siRNAs and scrambled siRNAs have been described previously (Yuan et al., 2010b). EqS04b cells were transfected with siRNAs at a final concentration of 10 nM using Lipofectamine RNAiMAX regents (Invitrogen) according to the manufacturer’s instructions. As a control, cells were also mock treated in parallel. Each transfection was repeated three times. Transfected cells were cultured for 20 h before harvesting the cells for the invasion assay, MTT assay or gene expression assessment.

Zymography. MMP-2 and MMP-9 proteins were assessed by gelatin zymography (Kleiner & Stetler-Stevenson, 1994; Yuan et al., 2010a). Equine fibroblasts were grown to confluency and then maintained in serum-free medium for 24 h. Conditioned medium was concentrated 25-fold using an Amicon Ultra Centrifugal Filter Device (Millipore) and equal amounts of medium (10 μl) were loaded onto a 10 % zymogram (gelatin) gel (Invitrogen) for electrophoresis. The gel treatment, staining, zymogram scanning and image analysis have all been described previously (Yuan et al., 2010a).

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REFERENCE


