Upregulation of equine matrix metalloproteinase 1 by bovine papillomavirus type 1 is through the transcription factor activator protein-1

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Equine sarcoids 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) activity is necessary for the transformation phenotype of equine fibroblasts. Among the many changes induced by BPV-1, matrix metalloproteinase 1 (MMP-1) upregulation contributes to the invasiveness of equine fibroblasts. However, it is not yet known how BPV-1 proteins regulate equine MMP-1 expression. To elucidate this mechanism, the equine MMP-1 promoter was cloned and analysed. A putative activator protein-1 (AP-1)-binding site was demonstrated to be crucial for upregulated MMP-1 promoter activity by BPV-1. BPV-1 E6 and E7 proteins increased MMP-1 promoter activity, and inhibition of BPV-1 gene expression by small interfering RNA significantly reduced the promoter activity. c-Jun and Fra-1, two components of the AP-1 transcription factor complex, were overexpressed and activated by BPV-1 in equine fibroblasts. Finally, BPV-1 E5, E6 and E7 proteins increased MMP-1 mRNA and protein expression. In conclusion, the expression of MMP-1 can be enhanced by BPV-1 oncoproteins E6 and E7 through the AP-1 transcription factor and by E5 via an indirect mechanism. These findings shed light on the mechanism of BPV-1-mediated equine fibroblast infiltration and indicate that both BPV-1 oncoproteins and AP-1 could be potential targets for equine sarcoïd therapy.

INTRODUCTION

Equine sarcoids are the most common skin tumour 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). They are characterized by extensive localized infiltration and invasion, rare regression and high recurrence (Knottenbelt, 2005; Martens et al., 2001a; Tarwid et al., 1985). Equine sarcoids exhibit six clinical subtypes: occult, verrucose, nodular, fibroblastic, mixed and mal-evolent (Knottenbelt, 2005). Whilst some lesions may remain quiescent for many years, the milder forms of the disease (occult and verrucose) can undergo transformation to the aggressive fibroblastic type, especially following trauma (Ragland et al., 1970; Tarwid et al., 1985). The high recurrence of equine sarcoïds may stem from the high invasiveness of sarcoïd fibroblasts (Yuan et al., 2010a).

Bovine papillomavirus type 1 (BPV-1) and less commonly BPV-2 are the main causes of equine sarcoids. The viral genome is frequently detected in the tumours (Ashrafi et al., 2008; Martens et al., 2001a, b; Nixon et al., 2005; Otten et al., 1993) and the viral proteins are expressed (Borzacchiello et al., 2008; Carr et al., 2001; Nixon et al., 2005).

Tumour cell invasion involves degradation of both basement membranes and stromal extracellular matrix by proteinases including matrix metalloproteinases (MMPs) (Birkedal-Hansen, 1995). In the past decades, MMPs have been demonstrated to play a crucial role in initiating degradation of basement membrane and extracellular matrix to allow tumour cell invasion (Westermarck & Kähäri, 1999). MMP expression and activity are frequently upregulated in tumours (Coussens et al., 2002). Activator protein-1 (AP-1) is the key cellular factor involved in regulation of MMP expression (Lin et al., 1993). Extracellular stimuli including growth factors, cytokines, stress and virus infection can induce MMP expression (Hirata et al., 2003; Lichtinghagen et al., 2003; Pustovrh et al., 2005). Human papillomavirus type 8 (HPV-8) E7 protein has been shown to stimulate the overexpression of MMP-1, MMP-8 and membrane type 1 MMP (MT1-MMP), causing invasion of human keratinocytes (Akgül et al., 2005).
We have previously shown that BPV-1 upregulates MMP-1 expression and activity and is essential for the transformation of sarcoid fibroblasts, including invasiveness (Yuan et al., 2008b, 2010a). However, it remains unclear how BPV-1 proteins regulate MMP-1 expression. Unravelling the mechanism by which BPV-1 proteins regulate MMP-1 expression would improve our understanding of equine sarcoid pathogenesis, thus facilitating diagnosis and treatment of the disease.

In this study, we showed that AP-1 is a crucial cellular factor that mediates the upregulation of MMP-1 expression by BPV-1 proteins in equine fibroblasts.

RESULTS

AP-1 is crucial for upregulation of the equine MMP-1 promoter by BPV-1

We have shown previously that BPV-1 upregulates MMP-1 expression and activity (Yuan et al., 2008b, 2010a), but the underlying mechanism is not yet known. To elucidate the transcriptional regulation of MMP-1 gene expression by BPV-1, the equine MMP-1 promoter was cloned and analysed for its activity in equine fibroblasts. A putative equine MMP-1 promoter (nt −4121 to +47, −4.2 kb; Fig. 1a and Supplementary Fig. S1, available in JGV Online) was amplified, cloned into the luciferase-expressing pGL3-Basic vector (subsequently referred to as MP4.2) and sequenced. The nucleotide sequence of the MP4.2 fragment was consistent with the 5′-flanking sequence (Supplementary Fig. S1) of the MMP-1 gene in the horse genome database (www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9796). Using the program TSEARCH version 1.3, the putative equine MMP-1 promoter was found to contain a typical TATA box at nt −34, one AP-1-binding site at nt −77 and a CAAT box with non-consensus flanking nucleotides at nt −152 (Supplementary Fig. S1). In addition, other known transcription factor-binding sites were also found upstream of the CAAT box (Supplementary Fig. S1).

**Fig. 1.** Equine MMP-1 promoter analysis. (a) Schematic structure of the equine MMP-1 full-length promoter (MP4.2) and its four 5′-truncated forms, MP1.1, MP0.5, MP0.2 and MP0.1. The AP-1-binding site, TATA box and transcription start nucleotide are underlined, with other nucleotides represented by dots; numbers under sequences indicate nucleotide positions. MP0.2m and MP0.2d are two MP0.2 mutants with an inactivated AP-1-binding site. The single nucleotide change in MP0.2m is highlighted in bold. (b) The activity of the MMP-1 promoter is controlled by AP-1. Promoter activities were determined by transient transfection of promoter–luciferase constructs in EqPalF and EqS01a cells. Variation in transfection efficiency was controlled by co-transfecting a Renilla luciferase-expressing plasmid, pBIND. The firefly luciferase activities were corrected against Renilla luciferase activity. Results are means ± SD of three experiments. *, P<0.05; **, P<0.01. P-less, Promoterless pGL3-Basic control; pSV40, pGL3-Promoter vector containing an SV40 promoter.
MP4.2 promoter activity was assessed by determining luciferase activity in transiently transfected normal equine fibroblast EqPalF cells and two equine sarcoïd-derived fibroblast (EqS) lines, EqS01a and EqS04b (Yuan et al., 2008a). EqPalF cells are BPV-1 negative, but both EqS01a and EqS04b are BPV-1 DNA positive and express viral genes (Yuan et al., 2008a). The MP4.2 fragment was able to drive luciferase expression (Fig. 1b) above the background of the promoterless pGL3-Basic control and thus showed itself to be a transcriptional promoter. In addition, the promoter exhibited significantly higher activity in both EqS01a (Fig. 1b) and EqS04b (data not shown) cells than in EqPalF cells, whereas the control simian virus 40 (SV40) promoter, which did not contain an AP-1-binding site, showed similar activity in both types of cell (Fig. 1b). These data indicated that BPV-1 gene expression can upregulate MMP-1 promoter activity and that the upregulation is specific for the MMP-1 promoter. Whilst the activity of the MP4.2 promoter was similar to that of the strong SV40 promoter in EqPalF cells (Fig. 1b), in EqS01a cells the activity of the MP4.2 promoter was approximately fourfold higher than that of the SV40 promoter (Fig. 1b). Thus, the MP4.2 promoter is active in equine fibroblasts, and even more so in sarcoïd cells expressing BPV-1 genes.

To study further the regulation of the equine MMP-1 promoter, a series of deletions containing approximately 1.1, 0.5, 0.2 or 0.1 kb of the 5′-truncated MMP-1 promoter (Fig. 1a) was constructed using the pGL3-Basic vector, designated MP1.1, MP0.5, MP0.2 and MP0.1, respectively. The 0.1 kb truncated promoter (MP0.1) was a basal promoter containing the TATA box at nt −34 but no other known canonical transcription factor-binding sites (Fig. 1a). The other three truncated promoters (MP0.2, MP0.5 and MP1.1) all contained TATA boxes and AP-1 elements (Fig. 1a). As shown in Fig. 1(b), the MP0.1 promoter exhibited minimal activity, only about threefold higher than the promoterless plasmid activity in EqPalF cells. However, inclusion of an AP-1 DNA element in the MP0.2 promoter resulted in a significant increase in activity (Fig. 1b). The 0.5 kb promoter (MP0.5) gave a significantly lower transcriptional activity than MP0.2 in EqS01a cells, indicating the possible presence of negative regulatory elements regulated by BPV-1 (Fig. 1b). When MP1.1 was used, luciferase transcription increased to the level of the full-length promoter in EqS01a cells but not in EqPalF cells, presumably due to unidentified enhancer elements that are upstream of MP0.5 and responsive only in BPV-1-expressing cells (Fig. 1b). To corroborate the positive role of the AP-1 element in regulating the equine MMP-1 promoter, MP0.2 was mutated within the AP-1 element, creating two MP0.2 mutants, MP0.2m and MP0.2d (Fig. 1a). MP0.2m comprised a single nucleotide mutation, whilst MP0.2d comprised a 3 bp deletion. Both mutations removed the significant differences of MP0.2 promoter activities in normal and sarcoïd cells and reduced the promoter activity to levels similar to that of the basal promoter MP0.1 (Fig. 1b), confirming the positive regulatory role of the AP-1-binding site. Taken together, these data indicated that AP-1 is essential for upregulation of MMP-1 promoter activity by BPV-1 in equine fibroblasts.

**BPV-1 E6 and E7 upregulate equine MMP-1 promoter activity**

Equine MMP-1 promoter activity was drastically increased by BPV-1 (Fig. 1b). To elucidate the responsible viral proteins, EqPalF cells were co-transfected with the MP0.2 plasmid and expression vectors with a Myc tag for BPV-1 E5, E6 or E7 (Yuan et al., 2010b), singly or in combination. The successful expression of transfected E5, E6 and E7 constructs was detected, showing similar levels by real-time relative quantification RT-PCR (data not shown) and immunoblotting using an anti-Myc tag antibody (Fig. 2a). When compared with cells co-transfected with the empty vector [stably expressing the neomycin-resistant gene (neo)], E6 and E7 each enhanced the activity of the MP0.2 promoter, but E5 had no effect (Fig. 2b). Additionally, a cooperative effect was observed on the promoter when the three proteins were expressed together (Fig. 2b). To validate these findings further, the MP0.2 plasmid was transfected into EqS02aT cell lines that expressed neo, E5, E6 or E7 (Yuan et al., 2011a). EqS02aT cells are derived from the sarcoïd line EqS02a, which harbours and expresses very low levels of BPV-1 DNA and proteins and is only partially transformed (Yuan et al., 2008a, 2011a). As shown in Fig. 2(c), MP0.2 showed significantly higher activities in E6- and E7- but not in E5-expressing cells when compared with neo cells, confirming the results observed in transiently transfected EqPalF cells. The E6 and E7 constructs were also each co-transfected with the mutant promoter MP0.2m, but neither was able to increase its activity when compared with the neo plasmid (Fig. 2d), further confirming that both viral proteins upregulate MP0.2 activity via the AP-1-binding site.

To corroborate further the positive regulation of E6 and E7 proteins on the MMP-1 promoter, EqS04b cells, which contain high viral load and high levels of viral transcripts (Yuan et al., 2008a), were treated with a small interfering RNA (siRNA) targeting the BPV-1 E6 gene (siE6) (Yuan et al., 2010b, 2011a). The siE6 molecule knocks down BPV-1 E6 transcripts almost completely and causes a reduction in E5 and E7 mRNAs in EqS04b cells (Yuan et al., 2011a). The treated cells were used for promoter construct transfection and luciferase activity assays. As shown in Fig. 3, siE6 treatment decreased the activity of the MP0.2 and MP4.2 promoters by approximately 60 and 80%, respectively, confirming that expression of BPV-1 oncogenes is required for the enhanced promoter activity. In contrast, the basal promoter activity was not changed in siE6-treated cells (Fig. 3), indicating that regulation of MMP-1 transcription by viral proteins takes place upstream of the MP0.1 region. It also appeared that siE6 had a greater effect on the longer MP4.2 promoter than on
the shorter MP0.2 promoter (Fig. 3), suggesting the involvement of other transcription factors besides AP-1. Given the effect of siE6 not only on E6 expression but also on E5 and E7 expression, it was not possible to establish with certainty which of the viral oncoproteins contributes the most to upregulation of the MMP-1 promoter, but the experiments above confirmed the importance of viral proteins in regulation of the equine MMP-1 promoter.

**BPV-1 E5, E6 and E7 upregulate equine MMP-1 expression**

Upregulation of the equine MMP-1 promoter activity by BPV-1 E6 and E7 proteins indicated that MMP-1 expression can be enhanced by these viral proteins. To elucidate the effects of the viral oncoproteins on MMP-1 expression, we examined MMP-1 mRNA and protein expression in transiently transfected EqPalF cells, in which the BPV-1 oncoproteins were expressed at similar levels
(Fig. 2a). Exogenous expression of E5, E6 or E7, either singly or together, increased the expression levels of MMP-1 mRNA and protein in EqPalF cells (Fig. 4a, b). In addition, the introduction of E5, E6 and E7 constructs together induced the highest level of upregulation (Fig. 4a, b), indicating cooperation among the viral proteins. EqS02aT cells were also examined for MMP-1 mRNA expression; this was significantly increased in cells expressing exogenous E5, E6 or E7 in comparison with that in control cells (Fig. 4c), further confirming the observed effects of viral proteins on MMP-1 expression in EqPalF cells. These findings demonstrated that BPV-1 proteins E6, E7 and E5 upregulate expression of the endogenous MMP-1 gene in equine fibroblasts. The upregulation of endogenous MMP-1 expression by E5 was surprising given the inability of E5 to stimulate the MP0.2 promoter, and suggested either that E5 impacts on the activity of the full-length MMP-1 promoter or that it upregulates MMP-1 expression by indirect mechanisms (see below).

BPV-1 gene expression is associated with the accumulation of AP-1

BPV-1 proteins regulate equine MMP-1 promoter activity via the AP-1 DNA element, indicating that BPV-1 may regulate the expression and activity of AP-1 transcription factor. AP-1 transcription factors are leucine zipper proteins that bind to the consensus AP-1 element (5’-TGAG/CTCA-3’) as a dimeric complex formed by Fos family members (c-Fos, Fra-1, Fra-2 and FosB) and/or Jun family members (c-Jun, JunB and JunD) (Angel & Karin, 1991; Karin et al., 1997).

c-Jun, a member of the Jun family, was shown previously to be phosphorylated (activated) in BPV-1-positive equine sarcoid tissues (Borzacchiello et al., 2009). In this study, we examined the expression of c-Jun and phosphorylated c-Jun (p-c-Jun) in equine fibroblast cells. As shown in Fig. 5(a), the three sarcoid lines EqS04b, EqS02a and EqS01a expressed higher levels of c-Jun than EqPalF cells, but the

**Fig. 4.** BPV-1 oncoproteins E5, E6, and E7 regulate equine MMP-1 expression. (a) Relative quantification of equine MMP-1 mRNA expression levels by quantitative RT-PCR. The expression levels of MMP-1 mRNA in EqPalF cells transfected with E5, E6 or E7 sequences, singly or together (E567), are relative to neo cells, the value for which was set to 1. *, P<0.05 versus neo cells. (b) Expression of cytosolic MMP-1 protein and α-tubulin in transiently transfected EqPalF cells was detected by Western blotting with anti-MMP-1 and anti-α-tubulin antibodies. The relative mean densitometry values of the MMP-1 bands in the blot were determined for three separate experiments and are shown in the graph below. The expression levels of MMP-1 protein are relative to neo cells, the value for which was set to 1. *, P<0.05 versus neo cells. (c) Relative expression levels of MMP-1 mRNA in EqS02aT cell lines. Expression is shown relative to neo cells, the value for which was set to 1. *, P<0.05 versus neo cells.
expression of p-c-Jun was only upregulated in EqS04b and EqS01a cells, and not in EqS02a cells (Fig. 5a), which contain low levels of BPV-1 DNA and transcripts (Yuan et al., 2008a), indicating that high levels of viral gene expression might be needed for the activation of c-Jun. To determine the viral protein(s) responsible for the elevated p-c-Jun expression, EqS02aT cells (EqS02a-neo, -E5, -E6 and -E7 cells) were examined for both c-Jun and p-c-Jun expression. As shown in Fig. 5(b), E5, E6 or E7 did not upregulate c-Jun expression, but the level of p-c-Jun was significantly increased by E6. Therefore, E6 is responsible for the stimulation of c-Jun activation in equine fibroblasts.

Previously, we found that BPV-1 upregulated the transcriptional expression of Fra-1 (Yuan et al., 2008b). In this study, we examined the expression of phosphorylated (activated) Fra-1 (p-Fra-1) by immunoblotting and immunofluorescence staining using a specific antibody against p-Fra-1. As shown in Fig. 6(a and b), the three sarcoid fibroblast lines EqS01a, EqS04b and EqS02a expressed high levels of nuclear p-Fra-1, whilst EqPalF cells showed minimal expression of the protein. In addition, EqS04b and EqS01a cells showed higher p-Fra-1 expression levels than EqS02a cells, possibly due to their higher viral gene expression levels (Yuan et al., 2008a). The importance of BPV-1 proteins in the upregulation of p-Fra-1 was confirmed by siE6 treatment, which caused the loss of p-Fra-1 expression in EqS04b cells (Fig. 6c). Thus, p-Fra-1 upregulation appeared to be associated with BPV-1 proteins.

To determine the viral proteins responsible for p-Fra-1 upregulation, EqPalF cells expressing neo, E5, E6 or E7, or E5, E6 and E7 together, and the in vitro BPV-1-transformed equine fibroblast line S6-2 (Yuan et al., 2008a) were examined for Fra-1 transcript and p-Fra-1 protein expression levels. E5, E6 or E7 alone was not able to increase the expression of either Fra-1 mRNA (Fig. 6d) or p-Fra-1 protein (Fig. 6e). However, S6-2 and EqPalF cells
expressing the E5, E6 and E7 proteins together did show significantly increased expression levels of both Fra-1 transcript (Fig. 6d) and p-Fra-1 protein (Fig. 6e) when compared with neo cells. Therefore, it is likely that cooperation among the viral oncoproteins is necessary for the observed p-Fra-1 upregulation. The p-Fra-1 upregulation was possibly due to the increase in Fra-1 mRNA expression levels. The overexpressed p-Fra-1 was shown to accumulate in the nuclei of S6-2 cells (Fig. 6f). Taken together, these data indicated that BPV-1 expression is able to induce the overexpression of activated Fra-1 in equine fibroblasts.
**Fig. 6.** Detection of p-Fra-1 expression in equine fibroblasts. (a) Expression of p-Fra-1 and β-tubulin in equine fibroblasts was detected by Western blotting with anti-p-Fra-1 (Ser265) and anti-β-tubulin antibodies, respectively. The relative mean densitometry values of the p-Fra-1 bands in the blot were determined for three separate experiments and are shown in the graph below. The expression levels of p-Fra-1 in EqS lines (EqS01a, EqS02a and EqS04b) are relative to EqPalF cells, the value for which was set to 1. **, P<0.01 versus EqPalF cells. (b) The cells were stained with primary rabbit anti-p-Fra-1 (Ser265) antibody and secondary Alexa Fluor 488-conjugated chicken anti-rabbit IgG. Magnification ×400. (c) Immunofluorescence staining of p-Fra-1 in control (mock and scramble) and siE6-treated EqS04b cells using the antibodies described in (b). Magnification ×400. (d) Relative quantification of equine Fra-1 mRNA expression levels by quantitative RT-PCR. The expression levels of Fra-1 transcript in EqPalF cells transfected with E5, E6 or E7 sequences, singly or together (E5E7), and in the in vitro BPV-1-transformed S6-2 cells are relative to neo cells, the value for which was set to 1. **, P<0.01 versus neo cells. (e) Expression of p-Fra-1 and β-tubulin was detected in EqPalF cells transfected with the indicated neo or viral constructs and in S6-2 cells by Western blotting with the antibodies described in (a). The relative mean densitometry values of the p-Fra-1 bands in the blot were determined for three separate experiments and are shown in the graph below. The expression levels of p-Fra-1 in cells with viral constructs or in S6-2 cells are relative to neo cells, the value for which was set to 1. **, P<0.01 versus neo cells. (f) Immunofluorescence staining of p-Fra-1 in neomycin-resistant EqPalF cells (neo) or in S6-2 cells using the antibodies described in (b).

The upregulation of AP-1 expression and activity by BPV-1 oncoproteins was consistent with their upregulation of MMP-1 expression.

**DISCUSSION**

Previously, we demonstrated that BPV-1 infection upregulated MMP-1 expression and transformed equine fibroblasts (Yuan et al., 2008a, b). The present study shows that the BPV-1 oncoproteins E5, E6 and E7 each increased equine MMP-1 expression, thus contributing to cellular transformation, and that AP-1 transcription factor is largely involved in the upregulation of equine MMP-1 expression.

AP-1 transcription factor is a large family of dimeric protein complexes formed by homo- and heterodimerization between Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2) and several activating transcription factor family members (Chinenov & Kerppola, 2001; Shaulian & Karin, 2002). It binds to AP-1 DNA motifs found in a wide array of target genes (Auble et al., 1992; Chinenov & Kerppola, 2001) and regulates several cellular functions, including proliferation, differentiation, apoptosis and invasion (Karin et al., 1997; Ozanne et al., 2007). The elevation of AP-1 activity can be oncogenic (Angel & Karin, 1991; Karin & Hawkins, 1996; Smeal et al., 1991). AP-1 plays a major role in regulating the transcriptional expression of MMP genes (such as MMP-1, MMP-3, MMP-7, MMP-9 and MMP-10), the promoter regions of which contain AP-1 DNA elements (Auble et al., 1992; Benbow & Brinckerhoff, 1997; Loesch et al., 2010; Sato et al., 1993; Westermarck & Kähäri, 1999; Yamamoto et al., 1995). These MMP genes can be stimulated by extracellular stimuli such as growth factors, cytokines and viral proteins (Akgül et al., 2005; Behren et al., 2005; Duffy et al., 2003; Kähäri & Saarialho-Kere, 1997; Shapiro, 1998).

A single AP-1 DNA motif (5′-TGAGTCA-3′), which putatively binds the AP-1 transcription factors, was identified at nt −77 in the promoter region of the equine MMP-1 gene (Supplementary Fig. S1). We showed that the proximal AP-1 element was essential for the transcriptional upregulation of equine MMP-1 gene expression by BPV-1 proteins, as mutation of this element dramatically reduced the activity of the MMP-1 promoter. Among the viral oncoproteins, E6 seemed to be the major activator for AP-1 as it was able to activate c-Jun and participate in activation of Fra-1. However, E7 and E5 are important factors as well. The combined effects of E5, E6 and E7 on Fra-1 activation (Fig. 6d, e) and MMP-1 expression (Fig. 4a, b) demonstrated the importance of E5 and E7 as MMP-1 expression regulators. The fact that, like E6, E7 was not able to increase the mutated MP0.2m promoter activity (Fig. 2d) further confirmed the necessity of the AP-1 site for the viral protein’s role in regulating MMP-1 expression. It is likely that E7 acts as a co-activator of AP-1, or it may regulate expression and/or activation of AP-1 subunits other than c-Jun and Fra-1. The upregulation of AP-1 activity by BPV-1 proteins clarifies the mechanism by which BPV-1 stimulates MMP-1 expression and induces cellular invasion (Yuan et al., 2010a).

E5 is the major transforming oncoprotein of BPV-1 (DiMaio & Mattoon, 2001). This oncoprotein does not directly activate MMP-1 promoter activity, which is consistent with its cytoplasmic localization (Burkhardt et al., 1989; Pennie et al., 1993; Surti et al., 1998). However, the observed increase in MMP-1 expression by E5 in this study suggests an indirect modulation. The platelet-derived growth factor β receptor (PDGF-βR) is phosphorylated (activated) in BPV-1 E5-expressing equine sarcoïd (Borzacchiello et al., 2009). It has been shown that BPV E5 interacts physically with phosphorylated PDGF-βR in bovine bladder cancer (Borzacchiello et al., 2006), suggesting that this may also take place in equine fibroblasts that express BPV-1 E5. The activation of PDGF receptor is able to induce p38 mitogen-activated protein kinase (MAPK) activity (Pyne & Pyne, 1997; Yamaguchi et al., 2004), and p38 MAPK is essential for mRNA stability (Pages et al., 2000). Interestingly, five copies of the sequence AUUUA are found in the 3′ UTR of equine MMP-1 mRNA.
BPV-1 E6 is an oncprotein that is able to transform cells. Overexpression of E6 in EqS02a cells that are not fully transformed results in enhanced proliferation and in vitro invasiveness (Yuan et al., 2011a). The cell invasion induced by E6 can be attributed to its role in AP-1 activation, MMP-1 promoter activity enhancement and MMP-1 upregulation.

BPV-1 E7 is a weak transforming oncprotein that binds to cellular p600 and cooperates with E5 and E6 in inducing cell transformation (Corteggio et al., 2011; DeMasi et al., 2005; Huh et al., 2005; Neary & DiMaio, 1989). The regulatory effect of E7 on MMP-1 promoter activity explains its function in increasing MMP-1 expression. Although E7 itself is not sufficient for invasion induction in equine cells (Yuan et al., 2011a), it may contribute to invasion by increasing MMP-1 expression. In humans, the E7 protein of HPV-8 has already been shown to induce overexpression of MMP-1, MMP-8 and MT1-MMP in human adult keratinocytes and to cause invasion of these cells (Akgül et al., 2005). Thus, E7 is an important oncprotein for cellular transformation by papillomaviruses. Previously, both cotton rabbit papillomavirus and HPV-31 E2 proteins were found to transactivate MMP-9 promoter via AP-1-binding sites (Behren et al., 2005). Recently we also found that inhibition of BPV-1 E2 expression caused downregulation of equine MMP-9, indicating that BPV-1 E2 also takes part in the regulation of equine MMP-9 expression. However, this protein appears not to be responsible for the upregulation of equine MMP-1 expression, as neither inhibition (Yuan et al., 2010a) nor overexpression (unpublished data) of E2 protein is able to affect MMP-1 expression level.

The profound effects of BPV-1 proteins on AP-1 and MMP-1 expression suggest that inhibition of BPV-1 proteins may revert the cellular transformation phenotype. Indeed, knockdown of BPV-1 E6 expression (and to a lesser degree E5 and E7 expression) by siE6 inhibited p-Fra-1 expression (Fig. 6c), drastically reduced MMP-1 expression and abolished in vitro sarcoild cell invasion (Yuan et al., 2011a). The fact that AP-1 is drastically elevated and activated in sarcoild fibroblasts suggests that, as a cellular factor, AP-1 might be fundamental to the tumorigenesis and progression of equine sarcoilds. AP-1 expression and activity have been shown to be upregulated in many cancers (Leaner et al., 2009; Matthews et al., 2007; Urakami et al., 1997). Numerous studies have demonstrated that inhibiting AP-1 function has a profound effect on the behaviour of cancer cells and tumours, often interfering with the transformed phenotype (Eferl & Wagner, 2003; Shaulian & Karin, 2002; Vogt, 2001). AP-1 is crucial for cellular invasion (Malliri et al., 1998; Ozanne et al., 2007), and inhibition of AP-1 activity can block cell invasion and metastasis (Leaner et al., 2009). In our recent study (Yuan et al., 2011b), we found that p-Fra-1 expression was associated with p38 MAPK activity, and that inhibition of p38 by the inhibitor SB203580 caused abolition of p-Fra-1 expression, a significant decrease in MMP-1 expression and reversion of the transformed phenotype of equine fibroblasts. This finding supports the necessity of AP-1 for the stimulation of MMP-1 and the transformation of equine fibroblasts by BPV-1. Thus, AP-1 can be a potential therapeutic target for equine sarcoilds. Moreover, the finding of MMP-1 upregulation by BPV-1 E5, E6 and E7 oncproteins improves our understanding of the pathogenesis of equine sarcoilds.

METHODS

Cell cultures. Normal EqPalF, the in vitro BPV-1-transformed S6-2 cell line and sarcoild cell lines EqS01a, EqS02a and EqS04b have been described previously (Yuan et al., 2008a). EqS02a or EqPalF cells that stably express the neomycin-resistant gene (EqS02a-neo or EqPalF-neo) or BPV-1 E5, E6 or E7 (EqS02a-E5, -E6 and -E7, or EqPalF-E5, -E6 and -E7, together referred to as EqS02aT or EqPalFT lines) have been described previously (Yuan et al., 2011a). 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.

Cloning and sequence analysis of the equine MMP-1 promoter. A 5‘-flanking sequence of ~4.2 kb of the equine MMP-1 gene (Supplementary Fig. S1) was identified by a BLAST search of the equine reference genome using the horse MMP-1 coding sequence (GenBank accession no. AF148882). Putative regulatory elements within the sequence were found using the program TFSSEARCH version 1.3 (Yutaka Akiyama, Kyoto University, Japan) and those elements scoring up to 95% are indicated (Supplementary Fig. S1). Subsequently, a DNA fragment of 4168 bp ranging from nt ~4121 to +47 (Supplementary Fig. S1) was amplified by PCR using primer pair EMP4.2F and EMP4.2R (see Supplementary Table S1, available in JGV Online) and EqPalF genomic DNA as input template, using PCR conditions described previously (Yuan et al., 2007a). The amplified fragment of approximate 4.2 kb was cloned upstream of a firefly luciferase gene ORF in the pGL3-Basic vector (Promega) at the KpnI and MluI sites and the construct was designated MP4.2. The cloned MP4.2 fragment was sequenced by cyclic sequencing using oligonucleotide primers corresponding to the 5‘-flanking sequence of the equine MMP-1 gene (Supplementary Fig. S1) at 500 nt intervals.

Promoter assay. To study transcription of the MMP-1 gene, we constructed a series of pGL3-MMP-1 promoter vectors containing 5‘-truncated MMP-1 promoter fragments of 1097, 487, 197 or 123 bp (Fig. 1a) using the method described above for MP4.2 plasmid construction, and these constructs were designated MP1.1, MP0.3, MP0.2 and MP0.1, respectively. All PCR primers used are described in Supplementary Table S1. Inserts were fully sequenced to assure the correctness of insert orientation and sequence. The putative MP0.1 promoter fragment contained a TATA box at nt ~34 but no known transcription factor-binding sites, whilst the three fragments MP0.2, MP0.5 and MP1.1 all contained an AP-1-binding site at nt ~79 (Fig. 1a). To study its effect on gene expression, this AP-1-binding site in
MP0.2 plasmid was changed by PCR-based site-directed mutagenesis using the mutant primers described in Supplementary Table S1 and a QuickChange kit (Stratagene). The AP-1-binding consensus sequence TGAGTCA in MP0.2-LUC was changed to the non-functional TGGGTCGA sequence by point mutation (A→G) or to TGAG by deletion of the three nucleotides TCA. The mutants produced were designated MP0.2m and MP0.2d, respectively. Transient transfections of equine fibroblasts were carried out to determine MMP-1 promoter activities. In addition, a pGL3-Promoter vector (Promega) containing an SV40 promoter (pSV40) was used as a transfection positive control. The promoterless pGL3-Basic vector was also used to determine background firefly luciferase activity. To determine the regulation functions of BPV-1 proteins on MMP-1 promoter activity, the plasmid MP0.2 was introduced into EqS02aT cells expressing E65. The promoterless pGL3-Basic vector was also used to determine firefly luciferase activity. To determine the regulation functions of BPV-1 proteins on MMP-1 promoter activity, the plasmid MP0.2 was introduced into EqS02aT cells expressing E65. The promoterless pGL3-Basic vector was also used to determine firefly luciferase activity. To determine the regulation functions of BPV-1 proteins on MMP-1 promoter activity, the plasmid MP0.2 was introduced into EqS02aT cells expressing E65. The promoterless pGL3-Basic vector was also used to determine firefly luciferase activity.

**Transfection of equine fibroblasts.** The BPV-1 gene expression constructs for E5, E6 or E7 have been described previously (Yuan et al., 2010b). EqPalF cells were transfected with an optimized amount of each expression plasmid (1 μg) or with empty vector pcDNA3.1-mh (neo) using Lipofectamine 2000 and Plus Reagent (Invitrogen) following the supplier’s recommended protocol. Transfected cells were harvested for gene expression analyses at 2 days post-transfection.

**Quantitative RT-PCR for assessment of transcripts.** Equine MMP-1 and Fra-1 mRNAs were quantified by real-time quantitative RT-PCR. The relative quantification RT-PCR primer and probe sets for MMP-1 and Fra-1 gene and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been described previously (Yuan et al., 2008b). Total RNA extraction, cDNA synthesis and PCR conditions were all as described previously (Yuan et al., 2007b). MMP-1 and Fra-1 transcripts were normalized by 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 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 shaking with a mouse mAb against the Myc tag (clone 9E10; Invitrogen) at a 1:1000 dilution or rabbit antibodies against p-Fra-1 (Ser265), c-Jun or p-c-Jun (Ser73) (Cell Signaling Technology), all at 1:1000 dilutions, or with mouse mAb MAB901 (R&D Systems) against human MMP-1 at a final concentration of 1 μg ml−1. The blots were washed and probed with HRP-linked anti-rabbit or anti-mouse antibodies (GE Healthcare) at a 1:5000 dilution and the protein detected by enhanced chemiluminescence (Amersham Pharmacia). A rabbit mAb against human α-tubulin (clone 11H10; Cell Signaling Technology) was used to control for the quantity of proteins in analysed samples. Densitometric analyses of Western blotting were performed using ImageJ software (http://rsh.info.nih.gov/ij).

**Immunofluorescence.** Expression of p-Fra-1 (p-Fra-1) was detected by immunofluorescence as described above. Cells were grown on chamber slides for at least 1–2 days in DMEM/10% FCS, and p-Fra-1 was detected with a primary rabbit anti-p-Fra-1 (Ser265) antibody (Cell Signaling Technology) and a secondary Alexa Fluor 488-conjugated chicken anti-rabbit IgG antibody (Invitrogen Molecular Probes).

**siRNA transfection.** The sequence of the BPV-1 E6 gene-targeted siRNA (siE6) and its related scrambled siRNA have been described previously (Yuan et al., 2010b). EqS04b cells were transfected with 10 nM siRNA using 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 experiments. Mock-treated cells were analysed in parallel.

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