

Bovine Delta papillomavirus E5 oncoprotein interacts with TRIM25 and hampers antiviral innate immune response mediated by RIG-I-like receptors

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Abstract

Persistent infection and tumourigenesis by papillomaviruses (PVs) require viral manipulation of various of cellular processes, including those involved in innate immune responses. Herein, we showed that bovine PV (BPV) E5 oncoprotein interacts with a tripartite motif-containing 25 (TRIM25) but not with Riplet in spontaneous BPV infection of urothelial cells of cattle. Statistically significant reduced protein levels of TRIM25, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) were detected by Western blot analysis. Real-time quantitative PCR revealed marked transcriptional downregulation of RIG-I and MDA5 in E5-expressing cells compared with healthy urothelial cells. Mitochondrial antiviral signalling (MAVS) protein expression did not vary significantly between diseased and healthy cells. Co-immunoprecipitation studies showed that MAVS interacted with a protein network composed of Sec13, which is a positive regulator of MAVS-mediated RLR antiviral signalling, phosphorylated TANK binding kinase 1 (TBK1), and phosphorylated interferon regulatory factor 3 (IRF3). Immunoblotting revealed significantly low expression levels of Sec13 in BPV-infected cells. Low levels of Sec13 resulted in a weaker host antiviral immune response, as it attenuates MAVS-mediated IRF3 activation. Furthermore, western blot analysis revealed significantly reduced expression levels of pTBK1, which plays an essential role in the activation and phosphorylation of IRF3, a prerequisite for the latter to enter the nucleus to activate type 1 IFN genes. Our results suggested that the innate immune signalling pathway mediated by RIG-I-like receptors (RLRs) was impaired in cells infected with BPVs. Therefore, an effective immune response is not elicited against these viruses, which facilitates persistent viral infection.

Bovine *Delta* papillomavirus E5 oncoprotein interacts with TRIM25 and hampers antiviral innate immune response mediated by RIG-I-like receptors

Running title: E5 oncoprotein impairs RLR-mediated host antiviral defence

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Abstract – Persistent infection and tumourigenesis by papillomaviruses (PVs) require viral manipulation of various of cellular processes, including those involved in innate immune responses. Herein, we showed that bovine PV (BPV) E5 oncoprotein interacts with a tripartite motif-containing 25 (TRIM25) but not with Riple in spontaneous BPV infection of urothelial cells of cattle. Statistically significant reduced protein levels of TRIM25, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) were detected by Western blot analysis. Real-time quantitative PCR revealed marked transcriptional down-regulation of RIG-I and MDA5 in E5-expressing cells compared with healthy urothelial cells. Mitochondrial antiviral signalling (MAVS) protein expression did not vary significantly between diseased and healthy cells. Co-immunoprecipitation studies showed that MAVS interacted with a protein network composed of Sec13, which is a positive regulator of MAVS-mediated RLR antiviral signalling, phosphorylated TANK binding kinase 1 (TBK1), and phosphorylated interferon regulatory factor 3 (IRF3). Immunoblotting revealed significantly low expression levels of Sec13 in BPV-infected cells. Low levels of Sec13 resulted in a weaker host antiviral immune response, as it attenuates MAVS-mediated IRF3 activation. Furthermore, western blot analysis revealed significantly reduced expression levels of pTBK1, which plays an essential role in the activation and phosphorylation of IRF3, a prerequisite for the latter to enter the nucleus to activate type 1 IFN genes. Our results suggested that the innate immune signalling pathway mediated by RIG-I-like receptors (RLRs) was impaired in cells infected with BPVs. Therefore, an effective immune response is not elicited against these viruses, which facilitates persistent viral infection.

Keywords: bovine papillomavirus E5 oncoprotein; melanoma differentiation-associated gene 5 (MDA5); mitochondrial antiviral-signalling (MAVS) protein; retinoic acid-inducible gene I (RIG-I), Tripartite motif containing 25 (TRIM25).

1. Introduction

Pattern recognition receptors (PRRs) are responsible for sensing the presence of pathogens, including viruses, since they recognise conserved features of microbes known as pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). Four different classes of PRRs have been identified: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

RLRs are PRRs expressed both in professional and in various non-professional immune cells, including epithelial cells (Takeuchi and Akira, 2010). RLRs play a major role in triggering and modulating antiviral immunity by detecting exogenous viral RNAs (Loo and Gale, 2011; Yoneyama et al., 2015). The RLR family is composed of retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RLRs localise to the cytosol, and their expression is maintained at low levels in resting cells but is greatly increased after virus infection (Loo and Gale, 2011; Chow et al., 2018). The activation of RIG-I and MDA5 is regulated by multiple ubiquitin E3 ligases of the tripartite motif (TRIM) proteins such as TRIM containing 25 (TRIM25) and Riplet (Oshiumi et al., 2013). RIG-I and MDA5 sense viral RNAs through the mitochondrial antiviral signalling (MAVS) protein (Seth et al., 2005). Although the majority of MAVS is present on the outer mitochondrial membrane (OMM), a small proportion is located in the mitochondria-associated membranes (MAMs) as well as in the peroxisomes (Goubau et al., 2013). RIG-I and MDA5 harbour caspase activation and recruitment domains (CARDs), and they bind to and activate MAVS via CARD-CARD interactions, triggering polymerisation of MAVS into prion-like structures required for antiviral signalling (Hou et al., 2011; Banoth and Cassel, 2018). Activation

of MAVS on mitochondria and MAMs results in stimulation of the kinases TBK1 and IKK and, consequently, of the transcription factors IRF3, IRF7, and NF- κ B for the induction of genes encoding type I and type III interferon and pro-inflammatory cytokines (Onoguchi et al., 2007; Chow et al., 2018; Ablasser and Hur, 2020). LGP2 lacks antiviral signalling activity. LGP2 has been proposed to be an accessory protein important for regulating RIG-I and MDA5 signalling (Chow et al., 2018). Indeed, LGP2 interacts with MAVS in microsomes, blocking RIG-I/MAVS. After virus infection, LGP2 is rapidly released from MAVS and redistributed to mitochondria, which correlates with IRF3 activation (Esser-Nobis et al., 2020).

Besides RNA ligands from RNA viruses, RLRs recognise DNA ligands from DNA viruses as well as those derived from bacteria (Chow et al., 2018). RLRs are known to detect herpesviruses, adenoviruses, and poxviruses (Goubau et al., 2013; Chow et al., 2018; Huo et al., 2019). Recently, it has been shown that high-risk human papillomaviruses (HPVs) can downregulate RLR expression, thus creating a cellular milieu suitable for their persistence (Albertini et al., 2018).

Bovine papillomaviruses (BPVs), a heterogeneous group of species-specific viruses distributed worldwide (IARC, 2007), comprise 29 types assigned to five genera (<https://pave.niaid.nih.gov/>; Yamashita-Kawanishi et al. 2020). Bovine δ PVs are known to infect epithelial and mesenchymal cells and are unique among BPVs to show cross-species transmission and infection (IARC, 2007). Besides skin tumours, bovine δ PVs play a very important role in the bladder carcinogenesis of large ruminants, such as cattle and buffaloes (Campo et al., 1992; Roperto et al., 2013). Bovine δ PVs show their transforming activity through the E5 protein, a highly conserved oncoprotein, believed to be the major δ PV oncoprotein (DiMaio and Petti, 2013). E5 forms dimers and displays pathogen activity via numerous pathways in the absence of other viral genes (DiMaio and Petti, 2013). E5 can bind to the activated form of platelet-derived growth factor β receptor (PDGF β R) (Borzacchiello et al., 2006; Roperto et al., 2013). E5 can also act via the calpain3 pathway and/or it binds to the subunit D of the V₁-ATPase proton pump (Roperto et al., 2010b; Roperto et al., 2014). E6 and E7 are less studied δ PV oncoproteins.

We aimed to investigate the interaction between E5 oncoprotein and E3 ubiquitin ligase TRIM25 and the downregulation of multiple downstream effectors of the host antiviral response pathway mediated by the RLRs in a spontaneous model of bovine papillomavirus disease.

2. Materials and Methods

2.1 Ethics statement

In this study, animal experiments were not performed. All the samples were collected post-mortem from slaughterhouses, and therefore, no ethical approval was required.

2.2 Animal samples

Bladder mucosa samples from 15 cows clinically suffering from chronic enzootic hematuria were collected from public slaughterhouses after bladder neoplasms had been identified during mandatory post-mortem examination. Bladder mucosa samples from 10 healthy cows were also collected. Animals from both groups were 3-5 years old. All bladder samples were immediately subdivided and either fixed in 10% buffered formalin for microscopic investigation or frozen in liquid nitrogen and stored at -80°C for subsequent molecular biology analysis.

2.3. Antibodies

Rabbit antibodies against RIG-1, MDA5, IRF3, phospho-IRF3, TBK1, phospho-TBK1, and TRIM25, were obtained from Cell Signaling Technology (Leiden, Netherlands). Rabbit antibody anti-RNF135 (RIPLET) was purchased from Sigma-Aldrich (MO, USA). Mouse anti-MAVS, anti-Sec13, and β -actin antibodies were purchased from Santa Cruz Biotechnology (TX, USA). Rabbit polyclonal anti-E5 serum recognising the C-terminal 14 amino acids of the BPV E5 oncoprotein was kindly gifted provided by Prof. DiMaio (Yale University, New Haven USA).

2.4. RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from bladder samples from 15 cows suffering from chronic enzootic hematuria and 10 healthy cows using the RNeasy Mini Kit (Qiagen, NW, DE), according to the manufacturer's instructions. Genomic DNA was removed from the RNA preparations using RNase-free DNase Fermentas Life Sciences (Thermo Fisher Scientific, MA, USA). A total of 1 µg RNA was used to generate single-stranded cDNA, using the QuantiTect Reverse Transcription Kit (Qiagen NW, DE), according to the manufacturer's instructions. PCR was performed with a specific primer set designed using Primer3, an online tool, for BPV-2 E5, BPV-13 E5, bovine RIG-I, MDA5, and TRIM25 genes. The following primers were used: *BPV-2 E5* ORF forward 5'-CACTGCCATTTGTTTTTTC-3', reverse 5'-GGAGCACTCAAAATGATCCC-3'; *BPV-13 E5* ORF forward 5'-CACTGCCATTTGGTGTCTT-3', reverse 5'-AGCAGTCAAAATGATCCCAA-3'; *RIG-I* forward 5'-AGGAAAAGATTCGCCAGATACAGA-3'; reverse 5'-ATGGCATTCTCCACCACTC-3'; *MDA5* forward 5'-TGAAGCAGGGTAAGAGAGC-3'; reverse 5'-TCAGACTCTGTACTGCCTTCAC-3'; *TRIM25* forward 5'-CGGAGCTCCTGGAGTATGTG-3'; reverse 5'-TAGTTCAGGGATGCGTCAGC-3'. Conditions for PCR were as follows: 94 °C for 5 min, 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s.

2.5. Sequence analysis

PCR products, obtained by RT-PCR, were purified using the QIAquick PCR Purification Kit 131 (Qiagen NW, DE) and were subjected to bidirectional sequencing using the Big Dye-Terminator v1.1 Cycle 132 Sequencing Kit (Applied Biosystems, CA, USA), according to the manufacturer's recommendations. Dye terminators from 133 sequences were removed using a DyeEx-2.0 Spin Kit (Qiagen), and sequences were run on a SeqStudio 134 Genetic Analyzer (Thermo Fischer Scientific, CA, USA). Electropherograms were analysed using Sequencing Analysis v5.2 and Sequence Scanner v1.0 softwares (Thermo Fischer Scientific, CA, USA). The sequences were analysed using the BLAST program.

2.6. Real-time reverse transcription PCR (RT-PCR).

To perform real-time RT-PCR analysis, total RNA and cDNA from diseased and healthy urinary bladder samples were generated, as described above. Real-time PCR was performed with a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using iTaq Universal SYBR(r) Green Supermix (Bio-Rad). Each reaction was performed in triplicate, and the primers used for RIG-I, MDA5, and TRIM 25 were the same as those used for RT-PCR. The PCR thermal profile was as follows: 95 degC for 10 min, 40 cycles of 94 degC for 15 s, and 58 degC for 30 s, followed by a melting curve. Relative quantification (RQ) was calculated using the CFX Manager software, based on the equation $RQ = 2^{-\Delta\Delta Cq}$, where Cq is the quantification cycle to detect fluorescence. Cq data were normalised to the bovine β -actin gene (forward: 5'-TAGCACAGGCCTCTCGCCTTCGT-3', reverse 5'-147 GCACATGCCGAGCCGTTGT-3').

2.7. Western blot analysis

Healthy and diseased bovine urothelial samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 400 mM NaCl, 1 mM 151 ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 1.7 mg/mL aprotinin, 50 mM 152 NaF, and 1 mM sodium orthovanadate). Protein concentration was measured using the Bradford assay (Bio-Rad). For western blotting, 50 µg protein lysate was heated at 90 °C in 4X premixed Laemmli sample buffer (Bio-Rad), clarified by centrifugation, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (GE Healthcare, UK). Membranes were blocked with Tris-buffered saline and 0.1% Tween 20 (TBST)- containing 5% bovine serum albumin (BSA) for 1 h at room temperature. The membranes were subsequently incubated overnight at 4 °C with primary antibodies, washed three times with TBST, incubated for 1 h at room temperature with goat anti-rabbit or goat anti-mouse (Bio-Rad) HRP- conjugated secondary antibody, diluted at 1:5,000 in TBST containing 5% BSA, and washed three times with TBST. Immunoreactive bands were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and ChemiDoc XRS Plus (Bio-Rad). Images were acquired using Image Lab Software version 2.0.1.

2.8. Immunoprecipitation

Total protein extracts from normal and pathological bladders, obtained as previously described, were immunoprecipitated. Protein samples (1mg) were incubated with anti-TRIM25 or anti-rabbit IgG (isotype), anti-Riplet or anti-rabbit IgG (isotype), and anti-MAVS or anti-mouse IgG antibodies (Bethyl Laboratories, Inc., TX, USA) for 1 h at 4 °C with gentle shaking. Thereafter, the samples were centrifuged at 1,000g for 5 min at 4 °C and incubated with 30 µL of Protein A/G-Plus Agarose (sc-184 2003) (Santa Cruz Biotechnology) overnight at 4 °C. The immunoprecipitates were washed four times in complete lysis buffer and separated on polyacrylamide gels. Subsequently, the proteins were transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature (25 °C) in TBST with 5% BSA, and then incubated with primary antibodies overnight at 4 °C. After three washes in TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. Chemiluminescent signals were developed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and were detected using the ChemiDoc XRS gel documentation system (Bio-Rad).

2.9. Statistical analysis

Results are presented as the mean \pm standard error (SE). Data were assessed by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons of means using the GraphPad PRISM software version 8 (GraphPad Software, San Diego, CA, USA). A p-value [?] 0.05 indicated statistical significance.

3. Results

3.1 Virological findings

It is well known that bovine δ PVs are the most important infectious agents involved in the etiopathogenesis of the majority of bovine urothelial tumours (Campo et al., 1992; Roperto et al., 2010a). E5 oncoprotein expression is correlated with the transformation of both mesenchymal and epithelial cells to form benign and malignant tumours (Supryniewicz et al., 2006). Therefore, we attempted to verify whether the δ PV E5 oncoprotein was expressed in the examined samples. First, we detected E5 oncoprotein transcripts by RT-PCR, the sequencing of which showed 100% identity with BPV-2 and BPV-13 sequences deposited in GenBank (Accession numbers M20219.1 and JQ798171.1, respectively) (Supplemental Fig. S1). Furthermore, Western blot analysis revealed the expression of E5 oncoprotein, which showed that abortive infection, a prerequisite for immune evasion and cell proliferation, takes place (Fig. 1).

3.2 Expression of tripartite motif-containing 25 (TRIM25) and Riplet proteins.

As many viruses, including human papillomavirus, have E3 ubiquitin ligases as their targets (Ban et al., 2018), we wondered whether the bovine δ PV E5 oncoprotein might interact with some ligases involved in the antiviral innate immune response mediated by RLRs, the ubiquitination of which appears to be a key post-translational modification. However, the molecular mechanisms of ubiquitin-mediated RIG-I and MDA5 activation remain to be fully understood (Shi et al., 2017; Oshiumi, 2020).

Many studies have reported that TRIM25 and Riplet are two essential E3 ubiquitin ligases for RIG-I signalling as they are known to ubiquitinate and activate RLRs (Oshiumi et al., 2013; Oshiumi, 2020).

Therefore, we investigated these two ligases by performing co-immunoprecipitation studies using anti-TRIM25 and anti-Riplet antibodies. The assay revealed the presence of E5 oncoprotein in anti-TRIM25 immunoprecipitates only, suggesting that the E5 oncoprotein of bovine δ PVs interacts with TRIM25 but not with Riplet (Fig. 2). Our results are in line with *in vitro* studies performed on cells experimentally infected with HPV18, which showed that TRIM25 but not Riplet was a target of viral E6 oncoprotein (Chiang et al., 2018). We then investigated the expression levels of these two ligases. Western blot analysis of total extracts detected unmodified levels of Riplet expression (Fig. 3) and a statistically significant reduction in the expression of TRIM25 (Fig. 4). To understand whether the marked reduction in TRIM25 expression levels could be attributed to transcriptional events and/or increased protein degradation, we investigated the presence of TRIM25 transcripts by RT-PCR. Sequencing of the obtained cDNA amplicons showed 100% identity with bovine TRIM25 sequences deposited in GenBank (Accession number: NM_001100336.1) (Supplemental Fig. S2). Then, we performed a real-time PCR analysis on cDNA using specific primers for bovine

TRIM25. This molecular assay did not show any variation in transcript expression in cells infected with bovine δ PVs compared with cells from clinically normal cattle (Fig. 5). These results suggest that bovine δ PVs interfere at the protein level rather than at the transcriptional level in reducing TRIM25 expression.

3.3. Expression levels of RIG-I and MDA5 and their downstream effectors

Expression of RLRs is ubiquitous and is typically maintained at low levels in resting cells, but is greatly increased after virus infection (Loo and Gale, 2011). Therefore, we decided to investigate RLR expression during spontaneous BPV infection.

We detected reduced expression levels of both RIG-I and MDA5 by Western blot analysis in urothelial cells infected by bovine δ PVs compared with urothelial cells from clinically normal cattle (Fig. 6). We assumed that the levels of these proteins could be due to transcriptional reduction. Using specific primers for bovine RIG-I and MDA5, we carried out a real-time PCR. Sequencing of the transcript amplicons revealed cDNA fragments showing 100% identity with bovine RIG-I and MDA5 sequences deposited in GenBank (Accession numbers: XM_002689480.6 and XM_010802053.2, respectively) (Supplemental Fig. S3). Real-time PCR of cDNA revealed a statistically significant reduction in both RIG-I and MDA5 transcripts in δ PV-positive cells compared with δ PV-negative cells (Fig. 7). These results suggest that, like HPVs, bovine δ PVs may interfere at the transcriptional level rather than at the protein level in reducing RIG-I and MDA5 expression to prevent their antiviral activities.

RIG-I and MDA5 interact with a mitochondrial adaptor, the mitochondrial antiviral signalling (MAVS) protein (Yoneyama et al., 2015; Oshiumi, 2020). It remains unclear how MAVS acts as a scaffold to assemble the signalosome in RLR-mediated antiviral signalling (Chen et al., 2018). Western blot analysis of MAVS expression revealed unmodified protein expression levels in both δ PV-infected and healthy cells (data not shown). Our results are in line with experimental data showing that the expression levels of MAVS did not significantly vary in cells in which the E6 oncoprotein of HPV18 was shown to act as a RIG-I transcriptional repressor (Albertini et al., 2018). We then performed co-immunoprecipitation studies using an anti-MAVS antibody. Western blot analysis performed on the immunoprecipitates detected the presence of RIG-I and MDA5 as well as TRIM25, phosphorylated TANK-binding protease 1 (pTBK1), phosphorylated interferon regulatory factor 3 (IRF3), and Sec13, which is believed to be a positive regulator of MAVS (Chen et al., 2018) (Fig. 8). Western blot analysis performed on total extracts revealed a statistically significant reduction in the expression levels of Sec13 in δ PV-infected cells compared with cells from clinically normal cattle (Fig. 9), which suggests that MAVS activation might be compromised in cells spontaneously infected with bovine δ PVs. MAVS subsequently phosphorylates and activates TBK1 and IRF3, via an unknown mechanism, which results in the production of interferons as well as proinflammatory factors (Fang et al., 2017). Western blot analysis performed on anti-MAVS immunoprecipitates revealed the presence of pTBK1 and pIRF3, which suggests that MAVS forms a complex with pTBK1 and pIRF3 and plays a critical role in driving and coordinating synergistic functional activities of these downstream components. Moreover, we investigated the expression levels of TBK1 and IRF3 in total extracts by immunoblotting, which revealed statistically significant reduced levels of both proteins in cells infected with bovine δ PVs compared with healthy cells (Fig. 10). Furthermore, western blot analysis revealed statistically significant reduced expression levels of pTBK1 (Fig. 11). TBK1 is activated via phosphorylation (Liu et al., 2015), which in turn phosphorylates and activates IRF3. Subsequently, IRF3 enters the nucleus to activate type 1 IFN (Fitzgerald et al., 2003; Fang et al., 2017). Altogether, our results suggest that the transcriptional downregulation of RIG-I and MDA5 in cells infected with bovine δ PVs is responsible for an aberrant downstream signalling pathway, including TBK1/IRF3, which may lead to the impairment of the host antiviral response. Because of downregulated RLRs, an adequate innate immune response is not elicited against spontaneous bovine δ PV infection, thus leading to persistent infection in the cells.

4. DISCUSSION

This study provides novel mechanistic insights into the role of E5 oncoprotein in dysregulating the host antiviral innate immune response in a spontaneous model of bovine papillomavirus disease. Our study

showed, for the first time, that the E5 oncoprotein of bovine δ PVs interacts with TRIM25, a key player in antiviral immunity (Koliopoulos et al., 2018), to hamper innate immune signalling pathway mediated by RIG-I and MDA5. These results are of interest as there are very limited, controversial *in vivo* studies based on the role of TRIM25 in RLR activation, which remains elusive (Hayman et al., 2019; Wang and Hur, 2020).

E5 oncoprotein did not appear to influence the transcriptional activity of TRIM25; therefore, it is conceivable that E5 oncoprotein enhanced TRIM25 proteasomal degradation, which may hinder the activation of RIG-I and MDA5. It is well known that TRIM25 ubiquitinates and activates RLRs in a dose-dependent manner (Gack et al., 2007). Our results appear to be corroborated by experimental studies that showed that HPV oncoproteins could enhance the proteasomal degradation of TRIM25 (Chiang et al., 2018). Furthermore, our study suggested the existence of multiple evasion mechanisms based on bovine δ PV-mediated inhibition of key components of the RLR pathways. Indeed, E5-expressing cells showed a marked reduction in the transcriptional activity of both RIG-I and MDA5. Reduced RIG-I and MDA5 mRNA levels detected by real-time PCR suggested that some proteins of bovine δ PVs could downregulate the transcriptional activity of RIG-I and MDA5, which allowed δ PVs to impair the innate antiviral response, a prerequisite for persistent infection. Our results appeared to be strengthened by experimental data from *in vitro* studies in which HPV oncoproteins have been shown to act as transcriptional repressors of RIG-I and MDA5 to impair the viral host response during persistent infection (Reiser et al., 2011; Albertini et al., 2018). RLRs catalyse the conversion of MAVS fibrils to prion-like aggregates. Although MAVS activation is a complex, multistep process, this conformational change of MAVS is essential for the recruitment of downstream signalling molecules (Hou et al., 2011). Not much is known about the mechanism(s) of how MAVS functions in antiviral signalling pathways (Chen et al., 2018); therefore, the activation mechanism of MAVS downstream pathways remains elusive (Zhu et al., 2019). In our study, MAVS expression levels did not vary significantly. Many viruses block RLR-mediated immune signalling thus inhibiting host antiviral response without modifying MAVS expression levels (Zhang et al., 2020). It is conceivable that in our spontaneous model of PV infection, the marked reduction in the expression levels of RIG-I and MDA5 may be responsible for the loss of conformational changes thus compromising the activation of MAVS, which is necessary for activating and propagating the antiviral signalling cascade. In addition, we found reduced expression levels of Sec13, which may contribute to further attenuation of MAVS downstream signalling. It has been suggested that Sec13 facilitates MAVS aggregation and ubiquitination and is thus required for RLR-MAVS-related antiviral responses (Chen et al., 2018). It has been shown that Sec13 expression correlates with MAVS activation. Indeed, the overexpression of Sec13 increases MAVS activation, whereas Sec13 downregulation attenuates MAVS activation (Chen et al., 2018). *In vitro* studies have shown that MAVS may serve as a scaffold to facilitate the interaction of TBK1 with IRF3 (Liu et al., 2015). MAVS has been shown to activate the transcription factor IRF3 through TBK1 (Fang et al., 2017). We found a marked reduction in the expression levels of total and phosphorylated TBK1, which may result in perturbation of IRF3 activation as TBK1 plays a crucial role in allowing efficient IRF3 phosphorylation in the IFN-producing pathways that require MAVS as the adaptor protein (Fang et al., 2017). Many viruses inhibit RIG-I/MAVS signalling by blocking TBK1 phosphorylation (Darlympe et al., 2015). It is conceivable that the E5 oncoprotein of bovine δ PVs is a key player involved in the downregulation of TBK1 activation. Low expression of TBK1 has been shown to markedly reduce IFN1 induction (Seth et al., 2005) and proinflammatory macrophage (M1) polarisation (Stone et al., 2019). Furthermore, we found reduced expression levels of IRF3, which may hamper their interaction network, a critical step in the production of IFNs (Ding et al., 2014; Liu et al., 2015).

Bovine δ PVs must escape innate immune surveillance to establish persistent infections and viral proteins may manipulate this process through several mechanisms. This study showed that similar to human PVs, bovine PVs perturb the RLR-mediated innate immune signalling pathway through the viral E oncoprotein, which is encoded in the early stages of PV infection. This perturbation results in an abnormal host antiviral response, which allows PVs to continue their infectious cycle leading to persistent viral infection. Bovine δ PVs reduce the levels of the DNA sensors that can recognise BPVs, which can hamper pTBK1 signalling as well as the production of IFNs, similar to human PVs (Hong and Laimins, 2017). IFN production plays

a crucial role in the immune response against PV infection as IFNs promote the clearance of latent PV episomes in persistently infected cells (Westrich et al., 2017) and/or rapid reduction in PV episome copies per cell (Herdman et al., 2006). It has been suggested that basal cells in the initial infection usually contain low levels (around 100 copies per cell) of human and bovine PV episomes (Turek et al., 2002; Groves and Colemans, 2015). Animal cells that fail to resolve their infection and retain oncogene expression for years can facilitate tumourigenesis by BPVs (Doorbar, 2006).

In conclusion, bovine δ PVs must escape innate immune surveillance to establish persistent infections, and viral proteins manipulate this process through several mechanisms. Despite the importance, molecular mechanisms for many bovine δ PV oncoproteins remain poorly characterised, in part due to challenges in identifying their substrates. Therefore, further investigations aimed to clarify the functional role of viral oncoproteins at the intersection of immune evasion and aberrant proliferation of cells persistently infected by bovine δ PVs, warrant future research.

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Conflict of interests

The authors have no conflict of interests to declare

Data availability statement

All data supporting this manuscript are reported and can be found in our paper

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Figure legends

Fig. 1. (A) Western blot analysis of E5 protein from three healthy and four representative diseased bladder mucosa samples. Results of Western blot analysis are representative of three independent experiments.

Fig. 2. Immunoprecipitation assay using anti-TRIM25 and anti-Riplet antibodies in healthy and pathological bladder samples. Western blot analysis revealed that TRIM25 only interacted with E5 protein. Panels A and B show representative data from three independent experiments

Fig. 3. (A) Western blot analysis of total Riplet protein in healthy and diseased bladder samples. (B) Densitometric analysis of total Riplet protein relative to the β -actin protein level. Panels A and B show representative data from three independent experiments.

Fig. 4. (A) Western blot analysis of TRIM25 in 10 normal and 15 diseased bladder samples. (B) Densitometric analysis was performed by comparing the protein expression level of TRIM25 with that of β -actin.

TRIM25 protein level was significantly lower in pathological bladder mucosa samples than in healthy samples. The calculations were based on two independent determinations. The values are expressed as a percentage of the average values for the healthy samples (**p [?] 0.01).

Fig. 5: (A) Western blot analysis of RIG-1 and MDA5 in normal and pathological bovine urinary bladder samples. (B) Densitometric analysis was performed by comparing the protein expression levels of total RIG-1 and MDA5 with those of β -actin. RIG-1 and MDA5 protein levels were significantly reduced in the infected mucosa samples compared with the healthy samples (* p [?] 0.05).

Fig. 6. Real-time RT-PCR analysis of RIG-I and MDA5 mRNA levels in 10 healthy and 15 pathological bladder samples. RIG-I and MDA mRNA expressions were significantly reduced in diseased bladder samples compared with normal bladder samples (*** p [?] 0.001). Data are expressed as the mean \pm S.E.M. of three independent experiments performed in triplicate.

Fig. 7. Real-time RT-PCR of TRIM25 mRNA levels in 10 healthy and 15 pathological bladder samples. Data are expressed as the mean \pm S.E.M. of independent experiments performed in triplicate.

Fig. 8. Immunoprecipitation using an anti-MAVS antibody in healthy and diseased bladder samples. Western blot analysis revealed that MAVS interacted with RIG-I, MDA5, TRIM25, phosphorylated TBK1 (pTBK1), phosphorylated IRF3 (pIRF3) and Sec13. Immunoprecipitation panel shows representative data from three independent experiments.

Fig. 9. (A) Western blot analysis of total Sec13 protein performed in healthy and pathological bladder mucosa samples. (B) Densitometric analysis was performed by comparing the protein expression level of total Sec13 with that of β -actin. Sec13 protein level was significantly reduced in the pathological bladder mucosa samples (** p [?] 0.01). Panels A and B show representative data from three independent experiments.

Fig. 10. (A) Western blot analysis of IRF3 and TBK1 in normal and diseased bovine bladders. (B) Densitometric analysis was performed by comparing the protein expression levels of total TBK1 and IRF3 with those of β -actin. IRF3 and TBK1 protein levels were significantly reduced in the neoplastic bladder mucosa samples compared with healthy samples (*** p[?]0.001 and * p [?] 0.05, respectively). Panels A and B show representative data from three independent experiments.

Fig. 11. (A) Western blot analysis of phosphorylated TBK1 (pTBK1) in the total lysate in healthy and pathological samples. (B) Densitometric analysis of pTBK1 protein was performed relative with β -actin protein levels. The calculations were based on three independent determinations. The values for the latter are expressed as percentages of the average values for the healthy samples (* p[?]0.05). Panels A and B show representative data from three independent experiments.

Supplemental Fig S1. (A) Real-time RT-PCR analysis of BPV-2 and BPV-13 E5 mRNA expression in healthy and pathological bovine bladder samples. Lane MW: DNA molecular weight marker (100-base pair (bp) ladder); lanes 2 – 4: three representative diseased bladder samples; lane 5: healthy bladder sample; lane C: no template control (no cDNA added). (B) The amplicon sequences showed 100% identity with BPV-2 E5 and BPV-13 E5 sequences deposited in GenBank (Accession numbers: M20219.1 and JQ798171.1, respectively). Electrophoretic representative data were obtained from three independent experiments.

Supplemental Fig S2. (A) TRIM25 cDNA amplification by PCR in normal and pathological bovine urinary bladder samples compared with β -actin. Lane 1: molecular weight marker (DNA marker ladder); lanes 2-5: four representative diseased bladder samples; lanes 6-9: healthy bladder samples; in the last channel: negative control (RNA without reverse transcriptase subjected to PCR analysis). (B) The lower part of the figure shows the alignment of the sequences, which revealed 100% identity with bovine TRIM25 transcript sequences deposited in GenBank (Bos taurus tripartite motif containing 25 (TRIM25), mRNA: NM_001100336.1).

Supplemental Fig S3. (A) RIG-I and MDA5 cDNA amplification by PCR in normal and neoplastic bovine urinary bladder samples compared with β -actin. Lane 1: molecular weight marker (DNA marker ladder);

lanes 2-5: four representative diseased bladder samples; lanes 6-9: healthy bladder samples; in the last channel: negative control (RNA without reverse transcriptase subjected to PCR analysis).

(B) The lower part of the figure shows the alignment of the sequences, which revealed 100% identity with bovine RIG-I and MDA5 transcript sequences deposited in GenBank (Bos taurus DExD/H-box helicase 58 (DDX58), transcript variant X1, mRNA: XM_002689480.6; Bos taurus interferon induced with helicase C domain 1 (IFH1), mRNA: XM_010802053.2).









