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Transforming properties of ovine papillomaviruses E6 and E7 oncogenes

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ABSTRACT

An increasing number of studies suggest that cutaneous papillomaviruses (PVs) might be involved in skin carcinogenesis. However, only a few animal PVs have been investigated regard to their transformation properties. Here, we investigate and compare the oncogenic potential of 2 ovine *Delta* and *Dyokappa* PVs, isolated from ovine skin lesions, *in vitro* and *ex vivo*. We demonstrate that both OaPV4 (*Delta*) and OaPV3 (*Dyokappa*) E6 and E7 immortalize primary sheep keratinocytes and efficiently deregulate pRb pathway, although they seem unable to alter p53 activity. Moreover, OaPV3 and OaPV4-E6E7 expressing cells show different shape, doubling time, and clonogenic activities, providing evidence for a stronger transforming potential of OaPV3 respect to OaPV4. Also, similarly to high-risk mucosal and cutaneous PVs, the OaPV3-E7 protein, constantly expressed in sheep squamous cell carcinomas, binds pRb with higher affinity compared to the E7 encoded by OaPV4, a virus associated to fibropapilloma. Finally, we found that OaPV3 and OaPV4-E6E7 determine upregulation of the pro-proliferative proteins cyclin A and cdk1 in both human and ovine primary keratinocytes. Collectively, results provide evidence for implication of ovine PVs in cutaneous proliferative lesions and skin cancer progression, and indicate sheep as a possible animal model for the study of cutaneous lesions and malignancies.

1. Introduction

The *Papillomaviridae* family includes a diverse group of small, non-enveloped, double-stranded DNA, icosahedral viruses commonly infecting the stratified squamous epithelium of the skin and mucosae of a wide variety of vertebrate species, where they can induce cellular proliferation or persist asymptomatically. About 220 types of human papillomavirus (HPV) have been described, 12 of which (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) are classified as high-risk (HR) carcinogenic human papillomaviruses (Bouvard et al., 2009) by the International Agency for Research on Cancer (IARC).

Yearly, about 528,000 cervical cancer cases caused by HPV infections are estimated accounting for nearly 266,000 deaths (WHO, 2012; Bloem and Ogbuanu, 2017). Mucosal HR HPV infections are also associated with other anogenital malignancies, including vulvar, penile, vaginal, anal cancers (Bloem and Ogbuanu, 2017). Additionally, HPV types 6 and 11 are responsible for up to 90% of anogenital warts (WHO, 2014; Bloem and Ogbuanu, 2017). HPV types belonging to *Betapapillomavirus* have skin tropism and have been initially isolated from individuals suffering by a genetic disorder, *Epidermodysplasia verruciformis* that confers a high susceptibility to beta HPV infection and non-melanoma skin cancer at sun-exposed areas (Bouvard et al., 2009; Tommasino, 2017). Many studies provide evidence for the role of beta HPV types, together with ultra-violet radiations, in skin carcinogenesis also in normal individuals (Tommasino, 2017).

In animals, according to the Papillomavirus episteme (https://pave.niaid.nih.gov), about 71 PV species (belonging to 46 PV genera) have been identified in 75 vertebrate hosts, mostly mammals but also 5 birds, 3 reptiles and, recently, 1 fish (López-Bueno et al., 2016). The majority of viruses have been recovered from healthy epithelia and proliferative lesions of the skin and mucosae but some types are implicated in carcinogenesis. In many cases PVs act in combination with UV-exposition or chemicals, such as the bracken fern quercetin that is implicated in the development of bovine gastrointestinal squamous cell carcinoma (SCC) together with BPV4, (Pennie and Saveria Campo, 1992).

The association between animal PVs and cancer is suggested by the identification of viral DNA and RNA in different malignancies of several host species, such as SCC of cats, dogs (Munday et al., 2017), rabbits

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(Rous and Beard, 1934), bats (Rector et al., 2006), and horses (Scase et al., 2010; Sykora and Brandt, 2017).

This paradigm is reinforced by few *in vitro* studies. Beside the well studied example of *in vitro* proliferative and transforming activities of bovine PVs (Bergman et al., 1988; Corteggio et al., 2013; Nasir and Campo, 2008; Neary and DiMaio, 1989; O'Brien et al., 2001; O'Brien and Campo, 1998), the expression of FcaPV2 genes in feline oral and skin SCCs, and the transforming ability of its E6 and E7 oncoproteins in corrupting p53 and pRb pathways have been recently demonstrated, indicating a possible causative role for FcaPV2 in the development of feline SCC (Altamura et al., 2016).

Ovine papillomaviruses include a number of viruses (OaPV1 to 4) belonging to the two genera *Deltapapillomavirus* and *Dyokappapapillomavirus*. OaPV1, OaPV2, and the recently discovered novel type OaPV4 (Tore et al., 2017) belong to the species *Deltapapillomavirus* 3, while OaPV3 (Alberti et al., 2010) has been included in the *Dyokappapapillomavirus* 1 species. *Deltapapillomavirus* 3 and *Dyokappapapillomavirus* 1 infections are associated to different cellular tropism and clinical outcome. OaPV1/OaPV2, and OaPV4 have been identified in epithelial and cutaneous benign fibropapillomas of merinos and sarda sheep, respectively, and have double tropism for epithelial and dermal cells (Tore et al., 2017). On the contrary, OaPV3 was identified in cases of cutaneous SCC of sarda sheep, and has exclusive epithelial tropism.

The presence of an E5 oncogene and the lack of a pRb binding domain in the E7 of ovine *Deltapapillomaviruses* have been related to the ability to infect fibroblasts and cause fibropapillomas (Narechania et al., 2004). Conversely, OaPV3 genome does not possess an E5 open reading frame and maintains the canonical pRb domain, showing a gene repertoire reminiscent of the epitheliotropic human *Betapapillomavirus* (Alberti et al., 2010), whose carcinogenic activity seems to be restricted to the E6 and E7 oncogenes. To date, *in vitro* studies on transformation properties of ovine papillomavirus are still lacking. Here, we studied the expression of OaPV4 an OaPV3 E6 and E7 oncoproteins in human and ovine keratinocytes, and investigated several features related to the *in vitro* transforming and proliferative activities of these two viruses, chosen as representative of ovine *Delta* and *Dyokappa* genera. Also, expression of early region E6E7 genes is investigated in SCCs of naturally infected sheep.

2. Matherial and methods

2.1. Plasmids and cells

OaPV3-E6E7 full-length open reading frames were amplified with primers OaPV3/E6E7/EcoRI/F (5' GAGAATTCATGGAGGGAAGCCCTC GTAC 3') and OaPV3/E6E7/BamHI/R (5' AAGGATCCCTATGCAGCAC ACGGCGGAC 3'). PCR protocol was profiled according to the Platinum™Pfx DNA Polymerase (Invitrogen, Italy) vendor instructions. The amplified region was digested with BamHI and EcoRI, and cloned into the pLXSN retroviral vector (Clontech, USA) previously digested with the same enzymes. Plasmid pLXSN + OaPV3-E6E7 was this way generated. Plasmid pLXSN + OaPV4-E6E7 was similarly produced with primers OaPV4/E6E7/EcoRI/F (5' GGGAATTCATGCTGAGCAGTAAAT TCCTGG 3') and OaPV4/E6E7/BamHI/F (5' AAGGATCCTCATGGTCG GTTTGCACAGG3').

E6 and E7 genes of both OaPV3 and OaPV4 were amplified, digested with BamHI/EcoRI, and cloned into pGEX-4T1 (pre-digested with the same enzymes) to generate plasmids: pGEX4T + OaPV3-E6, pGEX4T + OaPV3-E7, pGEX4T + OaPV4-E6, and pGEX4T + OaPV4-E7. E6 and E7 of both viruses were also cloned into the pCMV-HA-N EcoRI/XhoI restriction sites to generate plasmids: pCMVHAN + OaPV3-E6, pCMVHAN + OaPV3-E7, pCMVHAN + OaPV4-E6, and pCMVHAN + OaPV4-E7. Plasmid pLXSN +HPV38-E6E7 was provided by the Infection and Cancer Biology (ICB) laboratory at IARC.

NIH/3T3 fibroblasts and Phoenix cells for amphotropic retrovirus production were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). Monolayer cultures of human primary keratinocytes (HPKs) and naturally immortalized keratinocytes (NIKs) were grown as already described (Caldeira et al., 2003) in FAD medium containing: 3 parts of Ham's F-12 (Gibco, Invitrogen), 1 part of DMEM (Gibco, Invitrogen), 2.5% of FBS, hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), insulin (5 μg/ml), cholera toxin (8.4 ng/ml), adenine (24 μg/ml). A feeder layer of mitotically inactivated NIH/3T3, generated by Mitomycin C (Sigma-Aldrich, Italy) treatment, was added every two days to the cultures. All human cell lines were kindly provided by the Infection and Cancer Biology (ICB) laboratory at IARC (Lyon). Primary ovine fibroblasts were provided by: "Laboratorio di Ostretricia e Riproduzione animale (University of Sassari)" and cultured in DMEM supplemented with 10% FBS. Primary lamb keratinocytes (PLKs) were isolated as previously described (Aasen and Izpisúa Belmonte, 2010; Dal Pozzo et al., 2005) from the foreskin of two lambs collected at the slaughterhouse. Briefly, foreskin tissues were deeply rinsed with PBS supplemented with penicillin/streptomycin and cleaned by trimming away any fat and loose fascia. The obtained thin sheets of foreskin tissue were cut into small pieces and incubated in a 0.25% trypsin - EDTA solution (Invitrogen) at 37 °C for 30 min. Trypsinized cells were filtered with a 70 μm pore size filter and centrifuged at 200 g for 10 min. Cellular pellets were resuspended and cultured in PLK medium containing: 1 part of Ham's F12 (Gibco, Invitrogen), 3 parts of DMEM (Gibco, Invitrogen), 10% of FBS, hydrocortisone (0.5 μg/ml), epidermal growth factor (2 ng/ml), transferrin (5 μg/ml), insulin (5 μg/ml), cholera toxin (12.6 ng/ml), adenine (20 μg/ml) and 3,3',5-triiodo-2-thyronine (1.5 ng/ml).

2.2. Immortalization of human and ovine keratinocytes

Phoenix packaging cells were alternatively transfected with pLXSN + OaPV3-E6E7 or pLXSN + OaPV4-E6E7 plasmids carrying the E6E7 region of OaPV3 and OaPV4, respectively. Empty pLXSN was used as control. Transfections were performed using the CalPhos Mammalian Transfection kit (Clontech), according to the manufacturer protocol with minor modifications (25 mM Chloroquine was added to the transfection solution). Forty-eight hours after transfection, high-titre transfected Phoenix cells supernatants were collected and used to infect human (HPKs) and ovine primary keratinocytes (PLKs) to generate the following cell lines: HPKs/OaPV3-E6E7; HPKs/OaPV4-E6E7; HPKs/pLXSN; PLKs1/OaPV3-E6E7; PLKs2/OaPV3-E6E7; PLKs1/OaPV4-E6E7; PLKs2/OaPV4-E6E7; PLKs1/pLXSN; PLKs2/pLXSN. Twenty-four hours after infection, human and ovine transduced keratinocytes were selected for geneticin resistance (G418 disulfate salt, 0.1 mg/ml and 0.2 mg/ml, respectively). Cell growth profiles were designed according to population doubling (PD) levels reached by each cell line at specified time points. For the determination of the PD level, selected cells were seeded and cultured in 25 cm^2 flasks and trypsinized when they reached approximately 80–90% confluence. PD level indicates the number of times cells have doubled since their retroviral transduction, and was calculated taking into consideration the number of passages and the split ratio.

2.3. Colony formation assay

Colony formation assay was performed in duplicate as previously described (Hufbauer et al., 2013). After transduction with empty pLXSN vector or E6E7 genes and selection with G418, 1×10^3 of transduced PLKs were seeded in triplicate in 25 cm^2 flasks. Cells were allowed to grow and proliferate for 15 days. PLK medium was replaced twice a week. Cells were then fixed with a cold solution of methanol:acetic acid (3:1) and stained with 0.5% (w/v) crystal violet in 25% methanol. Cells were counted and the average number of colonies containing more than 50 cells was calculated for each treatment.

2.4. Immunofluorescence

3.5×10^5 PLKs were seeded in SPL Cell Culture borosilicate chamber slides (Euroclone, Italy) and let grow until 70–80% of confluence was reached. Afterwards, cells were washed twice with PBS and fixed in a 1:1 ethanol:acetone solution at −20 °C for 20 min. Ethanol has been allowed to evaporate at room temperature (RT). Fixed cells were incubated with 1% BSA in PBS for 20–30 min in a humid chamber. Monoclonal Anti-pan Cytokeratin FITC conjugated antibodies (clone PCK 26, Sigma-Aldrich, Italy) were diluted 1:50 in 1% BSA/PBS and incubated with cells at RT for 2 h. Before incubation with DAPI (1 µg/ml in MilliQ water) for 1 min slides were washed 3 times with PBS. Cells were finally washed twice before slide assembly in ProLong antifade reagent, and analysed by confocal laser scanning microscopy.

2.5. Immunohistochemistry

Five tissue samples, obtained from 5 sheep SCCs positive to OaPV3, were fixed in 10% paraformaldehyde, dehydrated through ascending grades of alcohols, and embedded in paraffin wax with a HISTO-PRO 200 vacuum tissue processor (Histo-Line, Milan). For immunohistochemistry, 3 µm sections were sliced with a microtome (Leica RM 2245- Nussloch, GmbH) and mounted on positively charged SuperFrost slides (Thermo Scientific, USA). Tissues were dewaxed and rehydrated with Dewax and HIER (heat-induced epitope retrieval) Buffer H pH 8.8 (Thermo Scientific, USA), with an Electric Vegetable Steamer, at 98 °C for 20 min. Slides were cooled down by submersion in bidistilled water at RT for 20 min. The endogenous peroxidase activity was blocked by incubating slides with Dako REAL™ Peroxidase-Blocking Solution (S2023–DAKO, Glostrup, DK) for 30 min. Aspecific antibody binding sites were blocked by incubation with 2% BSA in PBS for 1 h at RT followed by a second incubation with 2.5% Normal Horse Serum (NHS, ImmPRESS Reagent Kit–Vector) for 1 h at RT. Sections were incubated overnight at 4 °C with a 1:1000 dilution of hyperimmune anti-E6 serum, raised against the OaPV3 E6 (Tore et al., 2017), and obtained by immunizing rats with purified recombinant E6 protein expressed in *E. coli*. Afterwards, slides were incubated for 30 min at RT with donkey anti-rat IgG HRP conjugated antibodies (Southern Biotech, USA), previously diluted 1:5000 in ImmPRESS Reagent. Finally, slides were incubated with DAB Peroxidase Substrate (ImmPACT-Vector) until desired stain intensity developed, and lightly counterstained with hematoxylin. All washing steps were performed three times with TBS–0.1% Tween 20 (BiOptica, Milano, Italy). Images of all sample tissues were visualized and obtained with a Nikon Eclipse 80i microscope with a Nikon DS-Fi1 camera (Nikon Instruments Inc., Melville, NY). Normal skin samples obtained from a healthy sheep PCR-negative to PV infection were processed and coupled to lesions during experiments as negative controls.

2.6. GST pull-down and co-immunoprecipitation (CoIP)

For Glutathione S-transferase (GST) pull-down assay, BL21 Rosetta cells were transformed alternatively with pGEX4T + OaPV3-E6, pGEX4T + OaPV3-E7, pGEX4T + OaPV4-E6, pGEX4T + OaPV4-E7 and empty pGEX4T1 plasmids. Transformation was performed by using the TransformAid Bacterial Transformation Kit (Thermo Scientific) and following vendor recommendations. Overnight cultures of positive selected bacterial colonies were diluted 1:10 and grown until OD600 of 0.4. Fusion protein expression was induced by adding 0.1 mM of isopropylthio-β-D-galactopyranoside (UltraPure IPTG-Invitrogen, Italy), and bacteria were harvested 3 h later. Bacteria pellets were re-suspended in NETN buffer (20 mM Tris − HCl pH 8.0; 100 mM NaCl; 1 mM EDTA pH 8.0; 0.5% NP-40; Pierce EDTA-Free Protease Inhibitor Tablet (Thermo Scientific, USA), and sonicated on ice. The insoluble bacterial debris was removed by centrifugation and supernatants containing the fusion proteins were filtered through 0.45 µm filters. Fusion proteins were then purified with Glutathione Sepharose 4B beads (GE Healthcare, UK). Mixtures of cleared bacterial lysates and 75 µl of glutathione beads were incubated at 4 °C for 1–3 hours rocking. After incubation, beads containing the immobilized fusion proteins were recovered by centrifugation, washed 5 times with cold NETN buffer and stored at −20 °C until use. Ten µl of bead-immobilised recombinant proteins were resolved in SDS-PAGE to verify effectiveness of purification, and a BSA curve was included in the gel to estimate purified protein quantification. GST-pulldown was performed using a whole cell lysate of NIKS or primary ovine fibroblasts or PLKs. Before use, cell lysates were pre-cleared through incubation with empty Glutathione Sepharose 4B beads (30 min rocking at 4 °C) to avoid aspecific bindings during pulldown assays. Total protein extracts contained in the pre-cleared cell lysates were quantified using the Pierce BCA Protein Assay kit (Thermo Scientific, USA). Equal amounts (1–2 µg) of bead-immobilised GST/recombinant proteins were mixed with equal amounts of pre-cleared cell lysate (about 600 µg of total protein extract for each pull-down) in a volume of at least 400 µl, and incubated at 4 °C for 1–3 hours with gentle rotation. Beads were collected by centrifugation and washed 10 times with cold NETN buffer. After wash steps, samples were directly resuspended in 10 µl of 4X Laemmli buffer and subjected to western immunoblotting.

For CoIPassays, primary ovine fibroblasts and PLKs were alternatively transfected with pCMVHAN + OaPV3-E6, pCMVHAN + OaPV3-E7, pCMVHAN + OaPV4-E6, pCMVHAN + OaPV4-E7 or empty pCMVHAN by using the TurboFect Transfection Reagent (Thermo Scientific,USA), and following vendor instructions. Forty-eight hours after transfection, cells were harvested. Pelleted cells were resuspended and incubated on ice with IP lysis buffer (20 mM Tris − HCl pH 8.0, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP-40, Pierce EDTA-Free Protease Inhibitor Tablet (Thermo Scientific, USA). After incubation, cell debris was removed by centrifugation and the supernatants containing the protein extracts were collected. Proteins were quantified with the Pierce BCA Protein Assay kit (Thermo Scientific, USA) and equal amounts were used to perform CoIPwith the Pierce HA Tag IP/Co-IP Kit (Thermo Scientific, USA). Immunoprecipitates were eluted at 95 °C in Laemmli buffer and directly subjected to western immunoblotting. Experiments were repeated three times and incubation with the Invitrogen™ HA epitope tag antibody (Thermo Scientific, USA) was used to verify HA binding efficiency in different CoIP experiments.

2.7. Western immunoblotting and antibodies

Equal amounts of total protein extracts were run in single well 10% polyacrylamide gels and transferred into nitrocellulose membranes with a Mini-Trans-Blot Cell (Bio-Rad, USA) at 250 mA for 1 h. After blotting, membranes were blocked with 10% skim milk in PBS-0.05% Tween-20 (PBS-T), mounted in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, USA), and then incubated at 4 °C overnight with the following antibodies: Purified Mouse Anti-Human Retinoblastoma Protein Clone G3-245 (RUO) (BDPharmingen); p53 Antibody (Cell Signalling Technology, 9282); Phospho-Rb (Ser795) Antibody (Cell Signaling Technology, 9301); Phospho-p53 (Ser15) (16G8) Mouse mAb (Cell Signaling Technology, 9286); cyclin A Antibody (H-432) (Santa Cruz biotechnology); Anti-cdc2 (p34) Antibody (Santa Cruz biotechnology); Cdc2 (POH1) Mouse mAb (Cell Signaling Technology,9116); GST Antibody (Cell Signaling Technology, 2622). After incubation with primary antibodies, membranes were washed with PBS-T, incubated with the appropriate HRP-conjugated secondary antibodies (Southern Biotech, USA) for 1 h, washed again, and developed with the Clarity™ western ECL substrate (Bio-Rad, USA) or the Luminata Forte Western HRP substrate (Millipore, USA). Images were acquired with the ChemiDoc XRS + System (Bio-Rad, USA). Densitometric values and normalization to housekeeping gene (see online supplemental material) were calculated with ImageLab 5.2.1 software (Bio-Rad, USA) and then represented graphically as fold changes relative to control cells (transduced with the empty pLXSN). The expression level of the target protein in the control cultures was set as one.

3. Results

3.1. OaPV3 and OaPV4 E6E7 heighten proliferation and prolong lifespan of primary human and ovine keratinocytes

Human primary keratinocytes (HPKs) from three different donors were transduced with pLXSN + OaPV3-E6-E7 and pLXSN + OaPV4-E6-E7 vectors carrying both the E6 and E7 genes of OaPV3 and OaPV4, or with the empty pLXSN. Transduced keratinocytes growth was followed for about 30 days after infection and selection (Fig. 1A). Control keratinocytes (HPKs transduced with empty pLXSN, Fig. 1A) entered a quiescent phase and died within two population doublings. Cells early acquired a flat and enlarged morphology characteristic of arrested cells, and showed features of senescence such as degeneration, irregular shape, intercellular bridges and a high cytoplasm/nucleus ratio (Fig. 1B). On the contrary, HPKs/OaPV3-E6E7 and HPKs/OaPV4-E6E7

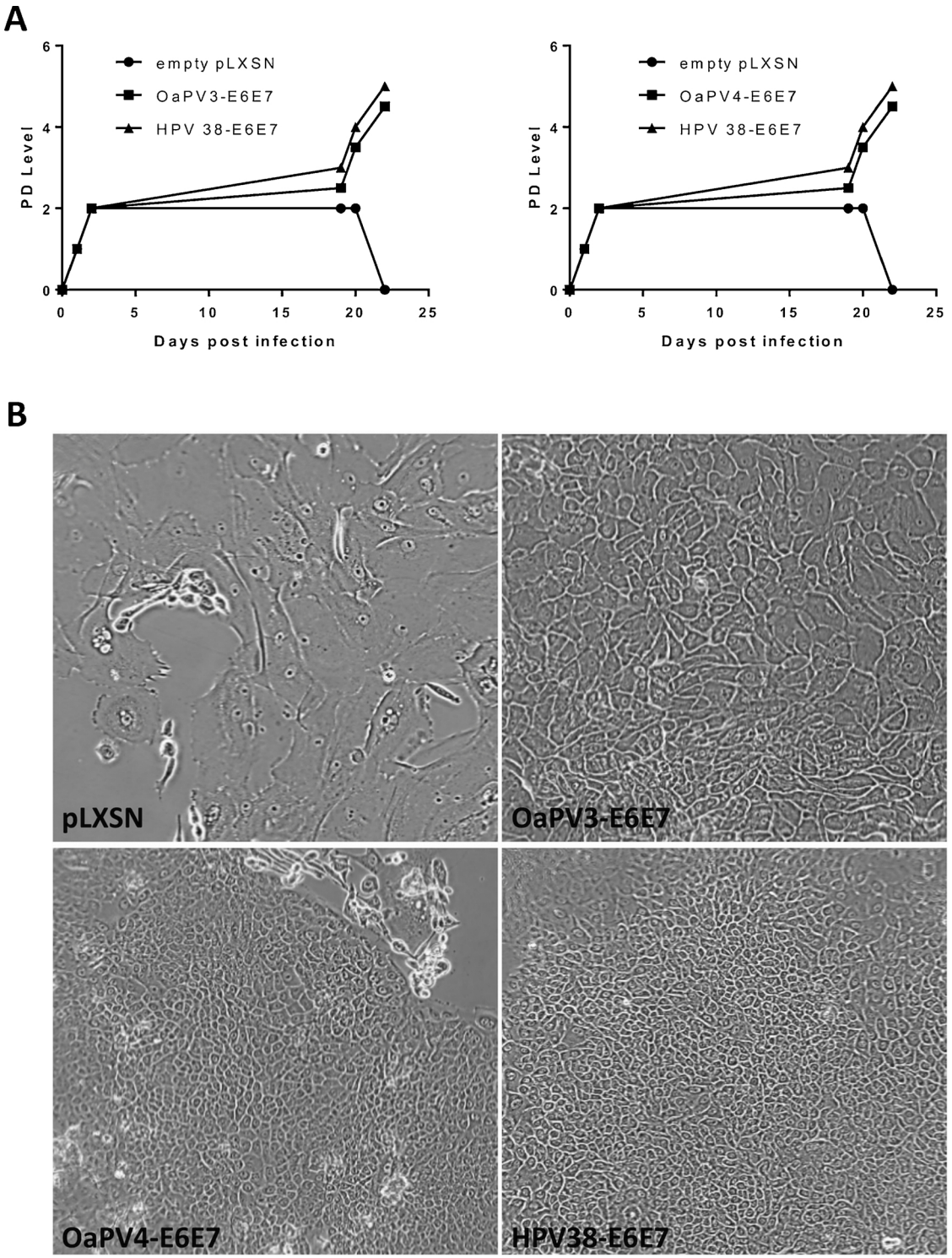

Fig. 1. Effect of OaPV3 and OaPV4 E6E7 on human primary keratinocytes (HPKs) proliferation and morphology. A, growth curves of HPKs from one of the three donors transduced with the indicated recombinant retroviruses. OaPV3-E6E7 and OaPV4-E6E7 expressing cells growth curves overlap. B, cell morphology of transduced HPKs after drug selection. Magnification 10×.

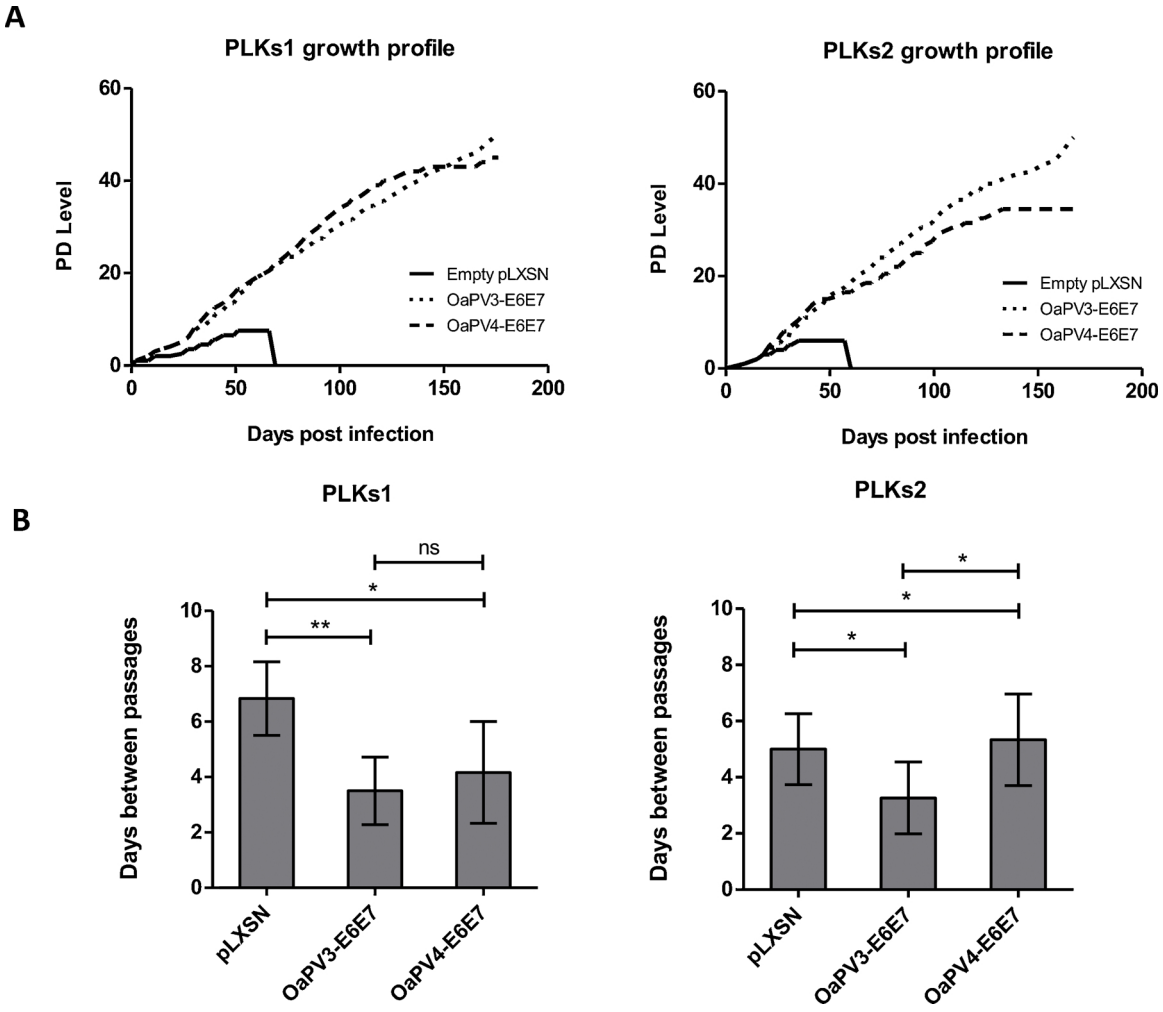

Fig. 2. Effect of OaPV3 and OaPV4 E6E7 on life span and population doubling times of primary lamb keratinocytes (PLKs) obtained from two donors. A, growth curves of PLKs transduced with the indicated recombinant retroviruses, showing the ability of both OaPV3 and OaPV4-E6E7 to increase PLKs lifespan leading to immortalization. B, population doubling (PD) times, calculated as the time length (days) between post-selection passages, demonstrating the fastest proliferative activity of OaPV3-E6E7 expressing PLKs. Results are the mean of 6 values for each experiment. Error bars show standard deviation. p value ≤005.

cell lines continued proliferating and dividing until experiments were voluntarily stopped. In addition to lifespan prolongation, HPKs expressing OaPV3 E6E7 oncogenes acquired a more regular shape with defined borders similarly to what observed when keratinocytes were transduced with HPV38-E6E7, used as a control (Fig. 1B). OaPV4 E6E7 expressing cells, while clearly distinct from control cells, were more heterogeneous in size and shape than the other two populations.

Based on results, similar experiments were repeated by transducing primary lamb keratinocytes (PLKs), which represent the natural target of ovine PVs. Growth profiles of 2 PLKs donors showed that E6E7 expression of both OaPV3 and OaPV4 dramatically prolong PLKs lifespan (Fig. 2A). Indeed PLKs transduced with the empty pLXSN stopped dividing after few population doublings, while OaPV3 and OaPV4 oncogene-expressing PLKs reached a population doubling level ranging from 35 and 50 (about 150–200 days) and they are still proliferating.

Long-term culture for over 50 population doublings revealed that OaPV3-E6E7 expressing PLKs from both donors grew at a constant and very high rate without any apparent crisis even after several freeze-thaw cycles. Conversely, PLKs1/OaPV4-E6E7 reduced their proliferative activity during late passages, and PLKs2/OaPV4-E6E7 stopped dividing and became senescent after 35 population doublings. We speculate that OaPV3-E6E7 expression leads to immortalization of ovine keratinocytes, while OaPV4-E6E7 determines a strong lifespan increase and immortalization to a lesser extent.

Moreover, OaPV4-E6E7 expressing PLKs maintained the typical morphology of PLKs, or of PLKs transduced with the pLXSN empty vector, appearing as heterogeneous cultures of enlarged cells with protrusions and irregular shape. PLKs/OaPV3-E6E7 cells from both donors showed instead an altered stem cell-like morphology appearing smaller, with more regular shape and defined borders (Fig. 3).

An additional evaluation of growth potential conferred by E6E7 genes was provided by looking at the population doubling time (PD time), which corresponds to the number of days each culture took to reach 80–90% confluence (when cultures were split). PLKs/OaPV4-E6E7cell lines always showed a statistically significant shorter PD time compared to control cells PLKs/pLXSN (Fig. 2B). Moreover, OaPV3-E6E7 expressing PLKs showed PDs shorter than OaPV4 even if this last observation was not statistically supported in all donors (Fig. 2B).

3.2. PLKs/OaPV4-E6E7 and PLKs/OaPV3-E6E7 have enhanced clonogenic activity

OaPV3-E6E7 and OaPV4-E6E7 expressing PLKs were tested for proliferation capacity in a clonogenic assay, which evaluates the ability of a single cell to proliferate and form a colony. Compared to control, colony formation assays revealed a 3.2 and 2.5-relative fold increase in number of colonies respectively produced by OaPV3-E6E7 and OaPV4-E6E7 expressing cell lines (Fig. 4A). A statistically significant difference

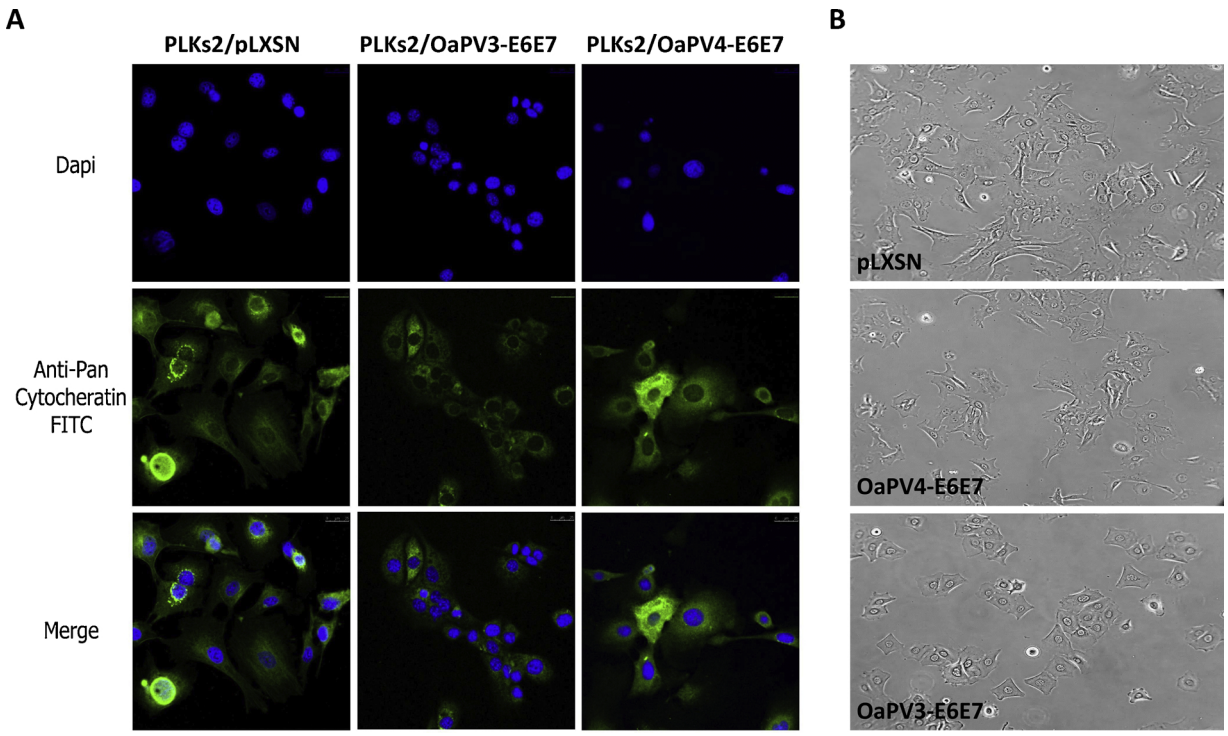

Fig. 3. Cell morphology of PLKs transduced with the indicated recombinant retroviruses. A, immunofluorescence with Anti-pan cytokeratin antibodies showing cell shape modification of primary ovine keratinocytes upon OaPV3 E6E7 expression. Transduced cells appear smaller with higher nucleus/cytoplasm ratio compared to control cells and to PLKs transduced with OaPV4 E6E7. B, cell morphology of transduced PLKs after drug selection as they appear at optical microscope. Magnification 20×.

was also found between OaPV3-E6E7 and OaPV4-E6E7 expressing PLKs, revealing a stronger clonogenic efficiency of OaPV3 respect to OaPV4.

Furthermore, OaPV3-E6E7 expressing keratinocytes generated colonies that mostly resembled *foci* of transformed cells and were constituted by copious, small, packed, and 3-D proliferating keratinocytes. On the other hand, OaPV4-E6E7 expressing colonies were macroscopically and microscopically more similar to control colonies and composed of sparse, flat and large keratinocytes (Fig. 4B, C).

3.3. OaPV3 and OaPV4 E6E7 alter pRb expression

OaPV3-E6E7 and OaPV4-E6E7 were also tested for the ability to alter the expression of p53 and pRb proteins and their phosphorylated counterparts. After retroviral transduction and selection, total proteins were extracted from transduced human and ovine keratinocytes and subjected to western immunoblotting.

Both HPKs and PLKs expressing OaPV3 and OaPV4 E6 and E7 showed increased levels of phospho-pRb (ppRb) in the majority of human and sheep donors (Figs. 5A, B). Since the ppRb form is not able to inhibit the E2F-driven transcription, these results indicate that the viral proteins activate the transcription of E2F-regulated genes. Accordingly, cyclin A and (especially) cdk1 protein levels were upregulated in both human and ovine cells transduced with OaPV3 and OaPV4 oncogenes. Immunoblotting of total protein extracts from transduced HPKs and PLKs never revealed a significant change in p53 and in phospho-p53 expression levels in HPK. It should be pointed out that antibodies against phospho-p53 (ser15) did not work on sheep keratinocytes; therefore, a role of p53 deregulation in promoting cell proliferation can not be ruled out in the natural host.

3.4. OaPV3 E7 binds pRb with the highest avidity

GST-pulldown experiments, carried out in triplicate using naturally immortalized keratinocytes (NIKs), primary lamb keratinocytes (PLKs), or primary ovine fibroblasts protein extracts, produced variable poor results (data not shown), although anti-GST western blots demonstrated comparable GST-binding efficiencies in different lysates and different experiments. When the assay was performed using NIKs, both OaPV3-E7 and OaPV4-E6 seemed able to bind the retinoblastoma tumour suppressor protein (pRb) as expected from *in silico* analysis, since these two oncogenes contain a canonical pRb-binding motif. Unexpectedly, also OaPV4-E7 seemed to bind human pRb even without carrying a classical pRb-binding domain. When GST pull-down assays were repeated using a lysate of ovine cells (either fibroblasts or PLKs) only OaPV3-E7 was able to bind pRb, even if western blotting signals were always very weak. On the contrary, none of the oncogenes associated with human or ovine p53. Incubation of protein extracts with bead-immobilised GST alone, used as control, never produced non-specific binding.

CoIP experiments, carried out in triplicate using transfected primary ovine keratinocytes or fibroblasts protein lysates, confirmed the high pRb binding efficiency of OaPV3-E7, and that E6E7 oncogenes bind p53 at very low efficiency, as suggested by the absence of a detectable p53 signal by western immunoblotting (Fig. 6). Results were reproducible and consistent in both cell lines. Mock cells (not transfected) and cells transfected with the empty pCMV-HA-N carrying the HA tag alone, used as controls, never generated non-specific bindings. The use of HA epitope tag antibodies generated a comparable signal in lanes containing HA-tagged proteins coimmuneprecipitates (data not shown).

3.5. OaPV3 early region gene expression in squamous cell carcinomas

All 5 OaPV3 PCR-positive SCC samples tested positive when probed with the anti OaPV3-E6 serum (Fig. 7). Strong cytoplasmic positivity was only observed in epithelial cells, confirming the tropism of this virus for skin keratinocytes. Uninfected tissues tested always negative to the same serum.

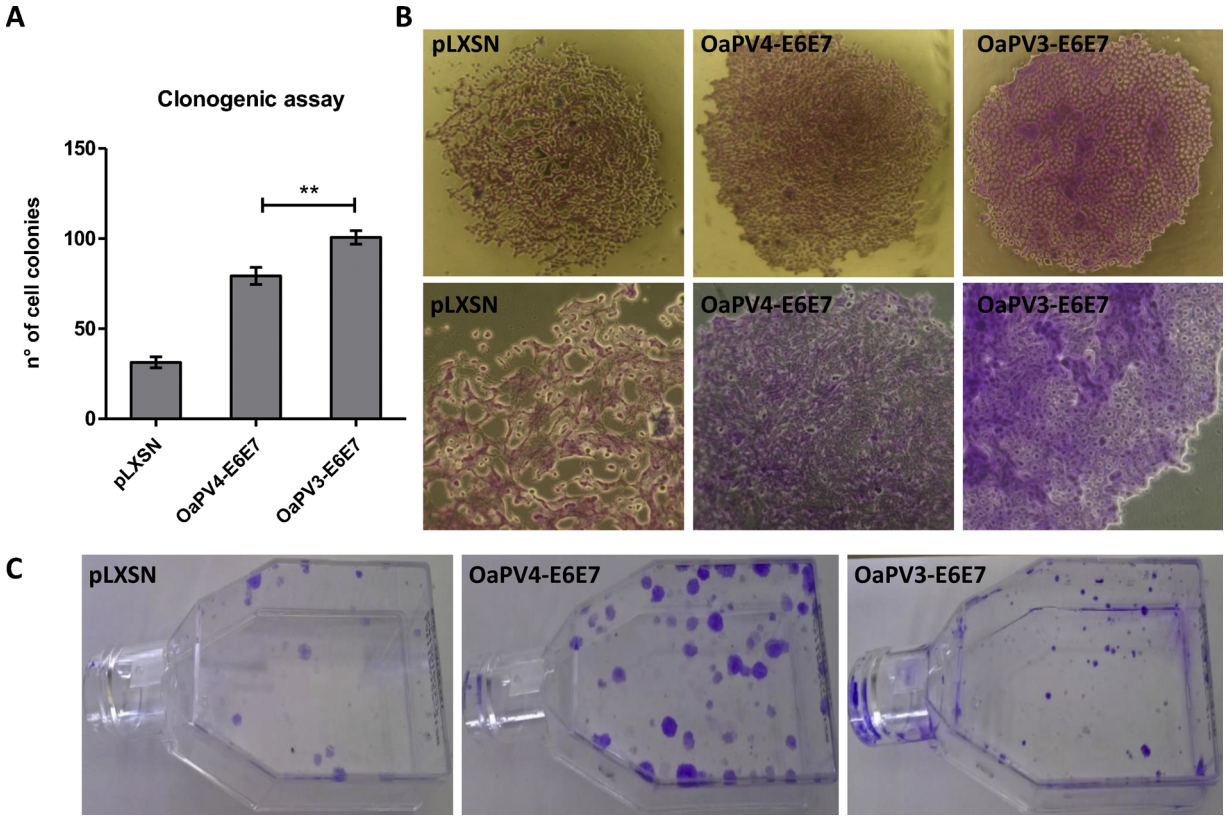

Fig. 4. Clonogenic activities of transduced PLKs. A, number of generated colonies. Error bars show standard deviation. Values are means from two independent experiments. p value ≤ 0.01. B, microscopic aspect of colonies generated by transduced ovine keratinocytes. Magnification 4X (top) and 10x (bottom). C, macroscopic aspect of representative clonogenic assay with transduced PLKs.

4. Discussion

The association virus-cancer has been established for the first time in 1911, when Francis Peyton Rous proved that chicken sarcomas could be transmitted through cell-free tumors extracts. About 20 years later papillomavirus-induced tumors where demonstrated in the cottontail rabbit. Finally, in 2008 Harald zur Hausen was awarded with Nobel Prize for his studies on the association of high-risk HPVs to cervical cancer (zur Hausen, 2009). Although it has been verified that human viruses have been found to cause 10–15% of human cancers worldwide (Moore and Chang, 2010), the degree of associations between animal viruses and cancer remains poorly investigated. Studies on animal viruses represent a valuable tool for clarifying mechanisms of oncogenesis and may contribute to establish animal models for cancer. Transformation properties of animal PVs oncogenes have been investigated in a limited number of viruses, such as the bovine papillomavirus 1 (BPV1), BPV2, BPV4 and the feline papillomavirus FcaPV2 (Altamura et al., 2016; Campo, 2002), even if PVs have been identified in cutaneous malignancies of different animal species, such as dogs, horse, bat, chamois, and sheep.

Here, the transformation properties of ovine PVs E6E7 were investigated *in vitro* and *ex vivo*. Due to their different cellular tropism

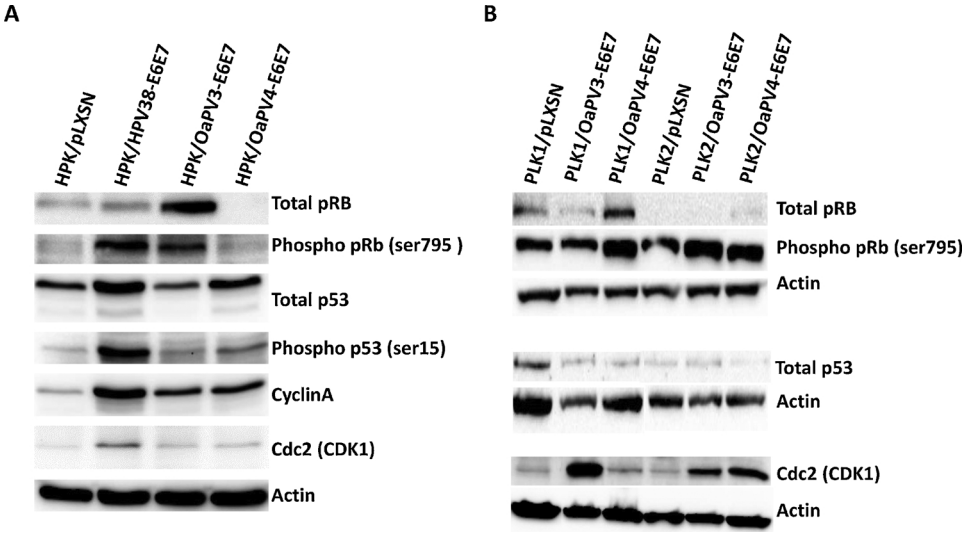

Fig. 5. A, representative western immunoblotting showing deregulation of total and phospho-pRb protein levels and upregulation of cdc2 and Cyclin A in HPKs transduced with the indicated recombinant retroviruses. β-actin was used as loading control. B, total pRb and phosphor-pRb, p53, and cdk1 protein levels in PLKs, transduced with the indicated recombinant retroviruses, are shown in different western immunoblotting. Anti-human phospho p53 and anti-human cyclin A antibodies did not react with the ovine proteins. β-actin, included as a loading control, is shown for each western immunoblotting.

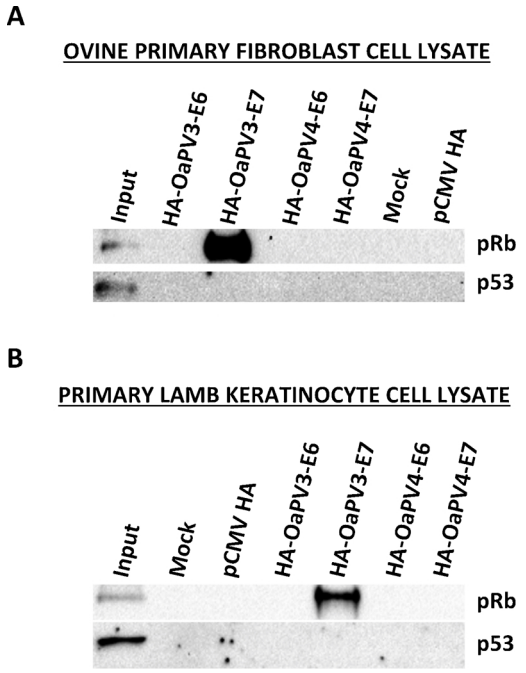

Fig. 6. Western Blot reactivity of co-immunoprecipitated ovine primary fibroblasts (A) and primary lamb keratinocytes (B) transfected with HA-tagged OaPV3 and OaPV4 E6 and E7 with anti-pRb and anti–p53 antibodies. Total cell lysates from non-transfected cells were used as positive control for antibodies reactivity (Input). Co-immunoprecipitates from cells non-transfected or transfected with empty pCMV Ha plasmid were used as negative controls. Anti HA-tag antibody reactivity used as a control to verify HA-tag binding efficiency in different lanes, is not shown.

and associated clinical lesions, the epitheliotropic SCC-associated *Dyokappapapillomavirus* OaPV3 (Alberti et al., 2010) and OaPV4 (Tore et al., 2017), a *Deltapapillomavirus* with mixed cellular tropism isolated from fibropapilloma, were selected as representative of the ovine PVs. First, we demonstrated the expression of the OaPV3-E6E7 early genes in SCCs collected from naturally infected sheep. This confirms previous observations, in which active transcription of early region genes was observed by RT–PCR (Alberti et al., 2010). These data, together with the wide association of OaPV3 to ovine SCC (Vitiello et al., 2017) may suggest a role for OaPV3 oncogenes in skin tumor progression. On the contrary, OaPV4 infects both epithelial and dermal cells, and appears to be related to benign proliferative lesions, such as papillomas and fibropapillomas.

In order to investigate the different pathogenic outcome potentially associated to ovine papillomavirus belonging to *Delta* and *Dyokappa* genera, both human and ovine primary keratinocytes were transduced with the E6E7 early region genes of OaPV4 and OaPV3. Based on results, it appears that both OaPV3 and OaPV4 oncogenes are able to prolong lifespan of human keratinocytes and to immortalize ovine cells. On comparisons, OaPV3 and OaPV4-E6E7 expressing cells showed different shape, doubling time, and clonogenic activities, thus suggesting a stronger transforming potential for OaPV3 respect to OaPV4. Indeed OaPV3-E6E7 expressing cells lost their original cell shape by increasing nucleus/cytoplasm ratio, they doubled faster than OaPV4-E6E7 expressing cells, and they produced an increased number of transformation foci–looking small colonies respect to controls. Conversely, OaPV4-E6E7 expressing cells maintained a shape closer to their original one (epithelioid), they duplicated faster than controls but slower than OaPV3, and generated larger and flat colonies composed by cells resembling the original epithelial cells.

Based on this data we postulated that both OaPV3 and OaPV4-E6E7 proteins are able to immortalize cells, but only OaPV3 may dramatically alter cell functionality and induce cell transformation.

Expression HPV E6 and E7 oncogenes is essential for the initiation and maintenance of cervical cancer (Morrison et al., 2011).

E6 and E7 proteins do not act as typical oncogenes, as they do not directly function as transforming agents, but instead they interact with cellular proteins, among them p53 and pRb, which are well known cellular tumour suppressors.

p53, pRb, and their related pocket proteins are commonly impaired during mucosal alpha High Risk-HPV (HR-HPV) related cancer. The most studied property of HR-HPV E7 oncoproteins is the ability to functionally complement the tumour suppressor pRb promoting its degradation. The ability of HR-HPV to bind pRb with high efficiency is associated with cell transformation and cancer progression (Ghittoni et al., 2010). Indeed, the E7 proteins encoded by the HR-HPV types, such as HPV16 and HPV18, bind pRb with a much higher affinity compared to those encoded by the low-risk type HPVs, such as HPV 6 and HPV 11 (Yim and Park, 2005). Cutaneous HPV-E7 proteins are also able to impair pRb pathway. As an example, HPV38-E7 has pRb binding efficiency comparable to HPV16-E7, and promotes pRb destabilization (Caldeira et al., 2003). Here we demonstrate that OaPV3 and OaPV4-E6E7 expression leads to destabilization of pRb protein levels, but only OaPV3-E7 has the capacity to strongly associate and bind both human and ovine pRb. Our results also suggest that p53 pathway might not be implicated in the pathogenesis of ovine PVs-mediated conditions.

Mitogenic stimuli such as oncogene expression deregulate the expression of cyclins and the related cyclin-dependent kinases (CDKs) leading to the their activation that is necessary for cell cycle progression (Tommasino, 2017). In accordance with this assumption, we found that OaPV3 and OaPV4-E6E7 determine upregulation of the pro-

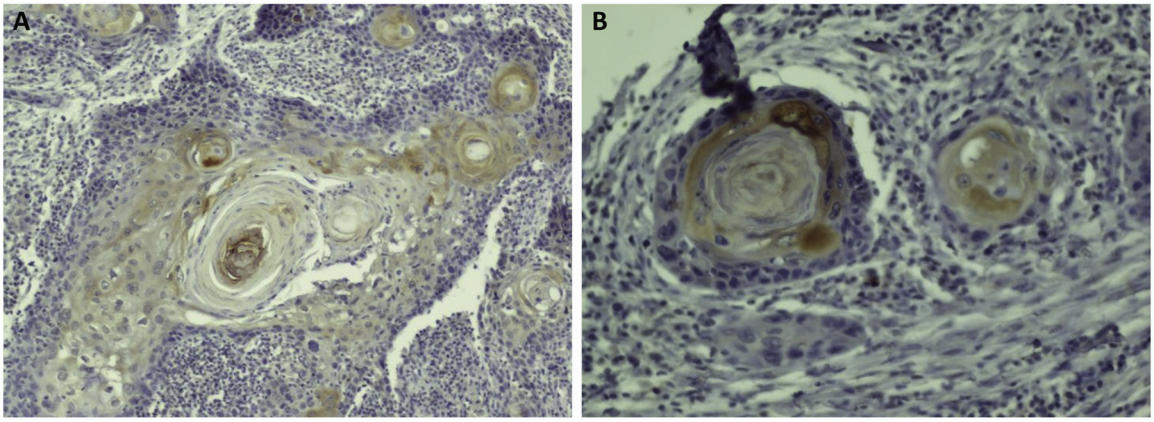

Fig. 7. IHC of histological section showing well differentiate sheep SCC with high cellularity, loss of cell polarity, and spread keratin pearls. A, detection of a diffuse OaPV3 E6 signal in the section. B, keratin pearls with diffuse OaPV3 E6 cytoplasmic signal.

proliferative proteins cyclin A and cdc2 in both human and ovine primary keratinocytes.

Considered together, these findings are hallmarks of cell immortalization and transformation, particularly in OaPV3-E6E7 expressing keratinocytes, and support our hypothesis of a role of OaPV3 in progression of cutaneous squamous cell carcinomas. Further analyses are required to understand whether ovine PV E6 and E7 can associate with cellular factors other than pRb and p53 to overcome cell cycle control signalling pathways and to promote cellular transformation.

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References

Aasen, T., Izpisúa Belmonte, J.C., 2010. Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. Nat. Protoc. 5, 371–382. https://doi.org/10.1038/nprot.2009.241.

Alberti, A., Pirino, S., Pintore, F., Addis, M.F., Chessa, B., Cacciotto, C., Cubeddu, T., Anfossi, A., Benenati, G., Coradduzza, E., Lecis, R., Antuofermo, E., Carcangiu, L., Pittau, M., 2010. Ovis aries Papillomavirus 3: a prototype of a novel genus in the family Papillomaviridae associated with ovine squamous cell carcinoma. Virology 407, 352–359. https://doi.org/10.1016/j.virol.2010.08.034.

Altamura, G., Corteggio, A., Pacini, L., Conte, A., Pierantoni, G.M., Tommasino, M., Accardi, R., Borzacchiello, G., 2016. Transforming properties of Felis catus papillomavirus type 2 E6 and E7 putative oncogenes in vitro and their transcriptional activity in feline squamous cell carcinoma in vivo. Virology 496, 1–8. https://doi.org/10.1016/j.virol.2016.05.017.

Bergman, P., Ustav, M., Sedman, J., Moreno-Lopéz, J., Vennström, B., Pettersson, U., 1988. The E5 gene of bovine papillomavirus type 1 is sufficient for complete oncogenic transformation of mouse fibroblasts. Oncogene 2, 453–459.

Bloem, P., Ogbuanu, I., 2017. Vaccination to prevent human papillomavirus infections: from promise to practice. PLoS Med. 14, 6–11. https://doi.org/10.1371/journal.pmed.1002325.

Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Freeman, C., Galichet, L., Cogliano, V., 2009. A review of human carcinogens–part B: biological agents. Lancet Oncol. 10, 321–322. https://doi.org/10.1016/S1470-2045(09)70096-8.

Caldeira, S., Zehbe, I., Accardi, R., Malanchi, I., Dong, W., Giarrè, M., de Villiers, E.-M., Filotico, R., Boukamp, P., Tommasino, M., 2003. The E6 and E7 proteins of the cutaneous human papillomavirus type 38 display transforming properties. J. Virol. 77, 2195–2206. https://doi.org/10.1128/JVI.77.3.2195-2206.2003.

Campo, M.S., 2002. Animal models of papillomavirus pathogenesis. Virus Res. 89, 249–261. https://doi.org/10.1016/S0168-1702(02)00193-4.

Corteggio, A., Altamura, G., Roperto, F., Borzacchiello, G., 2013. Bovine papillomavirus E5 and E7 oncoproteins in naturally occurring tumors: are two better than one? Infect. Agent Cancer 8, 1. https://doi.org/10.1186/1750-9378-8-1.

Dal Pozzo, F., Andrei, G., Holý, A., Van Den Oord, J., Scagliarini, A., De Clercq, E., Snoeck, R., 2005. Activities of acyclic nucleoside phosphonates against orf virus in human and ovine cell monolayers and organotypic ovine raft cultures. Antimicrob. Agents Chemother. 49, 4843–4852. https://doi.org/10.1128/AAC.49.12.4843-4852.2005.

Ghittoni, R., Accardi, R., Hasan, U., Gheit, T., Sylla, B., Tommasino, M., 2010. The biological properties of E6 and E7 oncoproteins from human papillomaviruses. Virus Genes 40, 1–13. https://doi.org/10.1007/s11262-009-0412-8.

Hufbauer, M., Biddle, A., Borgogna, C., Gariglio, M., Doorbar, J., Storey, A., Pfister, H., Mackenzie, I., Akgül, B., 2013. Expression of betapapillomavirus oncogenes increases the number of keratinocytes with stem cell-like properties. J. Virol. 87, 12158–12165. https://doi.org/10.1128/JVI.01510-13.

López-Bueno, A., Mavian, C., Labella, A.M., Castro, D., Borrego, J.J., Alcami, A., Alejo, A., 2016. Concurrence of iridovirus, polyomavirus, and a unique member of a new group of fish papillomaviruses in lymphocystis disease-affected gilthead sea bream. J. Virol. 90, 8768–8779. https://doi.org/10.1128/JVI.01369-16.

Moore, P.S., Chang, Y., 2010. Why do viruses cause cancer? Highlights of the first century of human tumour virology. Nat. Rev. Cancer 10, 878–889. https://doi.org/10.1038/nrc2961.

Morrison, M.A., Morreale, R.J., Akunuru, S., Kofron, M., Zheng, Y., Wells, S.I., 2011. Targeting the human papillomavirus E6 and E7 oncogenes through expression of the bovine papillomavirus type 1 E2 protein stimulates cellular motility. J. Virol. 85, 10487–10498. https://doi.org/10.1128/JVI.05126-11.

Munday, J.S., Thomson, N.A., Luff, J.A., 2017. Papillomaviruses in dogs and cats. Vet. J. 225, 23–31. https://doi.org/10.1016/j.tvjl.2017.04.018.

Narechania, A., Terai, M., Chen, Z., DeSalle, R., Burk, R.D., 2004. Lack of the canonical pRB-binding domain in the E7 ORF of artiodactyl papillomaviruses is associated with the development of fibropapillomas. J. Gen. Virol. 85, 1243–1250. https://doi.org/10.1099/vir.0.19765-0.

Nasir, L., Campo, M.S., 2008. Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. Vet. Dermatol. 19, 243–254.

Neary, K., DiMaio, D., 1989. Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. J. Virol. 63, 259–266.

O'Brien, V., Campo, M.S., 1998. BPV-4 E8 transforms NIH3T3 cells, up-regulates cyclin A and cyclin A-associated kinase activity and de-regulates expression of the cdk inhibitor p27Kip1. Oncogene 17, 293–301. https://doi.org/10.1038/sj.onc.1201937.

O'Brien, V., Grindlay, G.J., Campo, M.S., 2001. Cell transformation by the E5/E8 protein of bovine papillomavirus type 4. J. Biol. Chem. 276, 33861–33868. https://doi.org/10.1074/jbc.M100958200.

Pennie, W.D., Saveria Campo, M., 1992. Synergism between bovine papillomavirus type 4 and the flavonoid quercetin in cell transformation in vitro. Virology 190, 861–865. https://doi.org/10.1016/0042-6822(92)90926-G.

Rector, A., Mostmans, S., Van Doorslaer, K., McKnight, C.A., Maes, R.K., Wise, A.G., Kiupel, M., Van Ranst, M., 2006. Genetic characterization of the first chiropteran papillomavirus, isolated from a basosquamous carcinoma in an Egyptian fruit bat: the Rousettus aegyptiacus papillomavirus type 1. Vet. Microbiol. 117, 267–275. https://doi.org/10.1016/j.vetmic.2006.06.010.

Rous, P., Beard, J., 1934. A virus-induced mammalian growth with the charactres of tumor (the Shope rabbit papilloma). J. Exp. Med. 60, 701–722.

Scase, T., Brandt, S., Kainzbauer, C., Sykora, S., Bijmholt, S., Hughes, K., Sharpe, S., Foote, A., 2010. Equus caballus papillomavirus-2 (EcPV-2): an infectious cause for equine genital cancer. Equine Vet. J. 42, 738–745. https://doi.org/10.1111/j.2042-3306.2010.00311.x.

Sykora, S., Brandt, S., 2017. Papillomavirus infection and squamous cell carcinoma in horses. Vet. J. 223, 48–54. https://doi.org/10.1016/j.tvjl.2017.05.007.

Tommasino, M., 2017. The biology of beta human papillomaviruses. Virus Res. 231128–231138. https://doi.org/10.1016/j.virusres.2016.11.013.

Tore, G., Cacciotto, C., Anfossi, A.G., Dore, G.M., Antuofermo, E., Scagliarini, A., Burrai, G.P., Pau, S., Zedda, M.T., Masala, G., Pittau, M., Alberti, A., 2017. Host cell tropism, genome characterization, and evolutionary features of OaPV4, a novel Deltapapilloma virus identified in sheep fibropapilloma. Vet. Microbiol. 204, 151–158. https://doi.org/10.1016/j.vetmic.2017.04.024.

Vitiello, V., Burrai, G.P., Agus, M., Anfossi, A.G., Alberti, A., Antuofermo, E., Rocca, S., Cubeddu, T., Pirino, S., 2017. Ovis aries papillomavirus 3 in ovine cutaneous squamous cell carcinoma. Vet. Pathol. 54, 775–782. https://doi.org/10.1177/0300985817705171.

WHO, 2012. GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012.

WHO, 2014. Human papillomavirus vaccines: WHO position paper, October 2014 World Heal. Organ. Wkly. Bull. Epidemiol. Inf. Receiv. 89, 465–492. https://doi.org/10.1186/1750-9378-2-15.

Yim, E.-K., Park, J.-S., 2005. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res. Treat. 37, 319. https://doi.org/10.4143/crt.2005.37.6.319.

zur Hausen, H., 2009. Papillomaviruses in the causation of human cancers–a brief historical account. Virology 384, 260–265. https://doi.org/10.1016/j.virol.2008.11.046.