

Transforming properties of ovine papillomaviruses E6 and E7 oncogenes

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3 **Transforming properties of ovine papillomaviruses E6 and E7**  
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5 **oncogenes**  
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62  
63 **Abstract**  
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65 An increasing number of studies suggest that cutaneous  
66 papillomaviruses (PVs) might be involved in skin carcinogenesis. However,  
67 only a few animal PVs have been investigated regard to their transformation  
68 properties. Here, we investigate and compare the oncogenic potential of 2  
69 ovine *Delta* and *Dyokappa* PVs, isolated from ovine skin lesions, *in vitro* and  
70 *ex vivo*. We demonstrate that both OaPV4 (*Delta*) and OaPV3 (*Dyokappa*) E6  
71 and E7 immortalize primary sheep keratinocytes and efficiently deregulate  
72 pRb pathway, although they seem unable to alter p53 activity. Moreover,  
73 OaPV3 and OaPV4-E6E7 expressing cells show different shape, doubling  
74 time, and clonogenic activities, providing evidence for a stronger transforming  
75 potential of OaPV3 respect to OaPV4. Also, similarly to high-risk mucosal and  
76 cutaneous PVs, the OaPV3-E7 protein, constantly expressed in sheep  
77 squamous cell carcinomas, binds pRb with higher affinity compared to the E7  
78 encoded by OaPV4, a virus associated to fibropapilloma. Finally, we found  
79 that OaPV3 and OaPV4-E6E7 determine upregulation of the pro-proliferative  
80 proteins cyclin A and cdk1 in both human and ovine primary keratinocytes.  
81 Collectively, results provide evidence for implication of ovine PVs in  
82 cutaneous proliferative lesions and skin cancer progression, and indicate  
83 sheep as a possible animal model for the study of cutaneous lesions and  
84 malignancies.  
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123 **Introduction**  
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125 The *Papillomaviridae* family includes a diverse group of small, non-  
126 enveloped, double-stranded DNA, icosahedral viruses commonly infecting the  
127 stratified squamous epithelium of the skin and mucosae of a wide variety of  
128 vertebrate species, where they can induce cellular proliferation or persist  
129 asymptotically. About 220 types of human papillomavirus (HPV) have been  
130 described, 12 of which (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) are  
131 classified as high-risk (HR) carcinogenic human papillomaviruses (Bouvard *et*  
132 *al.*, 2009) by the International Agency for Research on Cancer (IARC).  
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142 Yearly, about 528,000 cervical cancer cases caused by HPV infections  
143 are estimated accounting for nearly 266,000 deaths (WHO, 2012; Bloem and  
144 Ogbuanu, 2017). Mucosal HR HPV infections are also associated with other  
145 anogenital malignancies, including vulvar, penile, vaginal, anal cancers  
146 (Bloem and Ogbuanu, 2017). Additionally, HPV types 6 and 11 are  
147 responsible for up to 90% of anogenital warts (WHO, 2014; Bloem and  
148 Ogbuanu, 2017). HPV types belonging to *Betapapillomavirus* have skin  
149 tropism and have been initially isolated from individuals suffering by a genetic  
150 disorder, *Epidermodysplasia verruciformis* that confers a high susceptibility  
151 to beta HPV infection and non-melanoma skin cancer at sun-exposed areas  
152 (Bouvard *et al.*, 2009, Tommasino 2017). Many studies provide evidence for  
153 the role of beta HPV types, together with ultra-violet radiations, in skin  
154 carcinogenesis also in normal individuals (Tommasino 2017).  
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170 In animals, according to the Papillomavirus episteme  
171 (<https://pave.niaid.nih.gov>), about 71 PV species (belonging to 46 PV genera)  
172 have been identified in 75 vertebrate hosts, mostly mammals but also 5 birds,  
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183 3 reptiles and, recently, 1 fish (López-Bueno *et al.*, 2016). The majority of  
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185 viruses have been recovered from healthy epithelia and proliferative lesions of  
186  
187 the skin and mucosae but some types are implicated in carcinogenesis. In  
188  
189 many cases PVs act in combination with UV-exposition or chemicals, such as  
190  
191 the bracken fern quercetin that is implicated in the development of bovine  
192  
193 gastrointestinal squamous cell carcinoma (SCC) together with BPV4, (Pennie  
194  
195 and Campo, 1992).  
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198 The association between animal PVs and cancer is suggested by the  
199  
200 identification of viral DNA and RNA in different malignancies of several host  
201  
202 species, such as SCC of cats, dogs (Munday *et al.*, 2017), rabbits (Rous and  
203  
204 Beard, 1934), bats (Rector *et al.*, 2006), and horses (Scase *et al.*, 2010;  
205  
206 Sykora and Brandt, 2017).  
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209 This paradigm is reinforced by few *in vitro* studies. Beside the well  
210  
211 studied example of *in vitro* proliferative and transforming activities of bovine  
212  
213 PVs (Bergman *et al.*, 1988; Corteggio *et al.*, 2013; Nasir and Campo, 2008;  
214  
215 Neary and DiMaio, 1989; O'Brien *et al.*, 2001; O'Brien and Campo, 1998), the  
216  
217 expression of FcaPV2 genes in feline oral and skin SCCs, and the  
218  
219 transforming ability of its E6 and E7 oncoproteins in corrupting p53 and pRb  
220  
221 pathways have been recently demonstrated, indicating a possible causative  
222  
223 role for FcaPV2 in the development of feline SCC (Altamura *et al.*, 2016).  
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226 Ovine papillomaviruses include a number of viruses (OaPV1 to 4)  
227  
228 belonging to the two genera *Deltapapillomavirus* and  
229  
230 *Dyokappapapillomavirus*. OaPV1, OaPV2, and the recently discovered novel  
231  
232 type OaPV4 (Tore *et al.*, 2017) belong to the species *Deltapapillomavirus* 3,  
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234 while OaPV3 (Alberti *et al.*, 2010) has been included in the  
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243 *Dyokappapapillomavirus* 1 species. *Deltapapillomavirus* 3 and  
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245 *Dyokappapapillomavirus* 1 infections are associated to different cellular  
246 tropism and clinical outcome. OaPV1/OaPV2, and OaPV4 have been  
247 identified in epithelial and cutaneous benign fibropapillomas of merinos and  
248 sarda sheep, respectively, and have double tropism for epithelial and dermal  
249 cells (Tore *et al.*, 2017). On the contrary, OaPV3 was identified in cases of  
250 cutaneous SCC of sarda sheep, and has exclusive epithelial tropism.  
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254 The presence of an E5 oncogene and the lack of a pRb binding domain in the  
255 E7 of ovine *Deltapapillomaviruses* have been related to the ability to infect  
256 fibroblasts and cause fibropapillomas (Narechania *et al.*, 2004). Conversely,  
257 OaPV3 genome does not possess an E5 open reading frame and maintains  
258 the canonical pRb domain, showing a gene repertoire reminiscent of the  
259 epitheliotropic human *Betapapillomavirus* (Alberti *et al.*, 2010), whose  
260 carcinogenic activity seems to be restricted to the E6 and E7 oncogenes. To  
261 date, *in vitro* studies on transformation properties of ovine papillomavirus are  
262 still lacking. Here, we studied the expression of OaPV4 an OaPV3 E6 and E7  
263 oncoproteins in human and ovine keratinocytes, and investigated several  
264 features related to the *in vitro* transforming and proliferative activities of these  
265 two viruses, chosen as representative of ovine *Delta* and *Dyokappa* genera.  
266 Also, expression of early region E6E7 genes is investigated in SCCs of  
267 naturally infected sheep.  
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303 **Matherial and methods**  
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307 *Plasmids and cells*  
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309 OaPV3-E6E7 full-length open reading frames were amplified with  
310 primers OaPV3/E6E7/EcoRI/F (5'  
311 GAGAATTCATGGAGGGAAGCCCTCGTAC 3') and OaPV3/E6E7/BamHI/R  
312 (5' AAGGATCCCTATGCAGCACACGGCGGAC 3'). PCR protocol was  
313 profiled according to the Platinum<sup>TM</sup>Pfx DNA Polymerase (Invitrogen, Italy)  
314 vendor instructions. The amplified region was digested with BamHI and  
315 EcoRI, and cloned into the pLXSN retroviral vector (Clontech, USA)  
316 previously digested with the same enzymes. Plasmid pLXSN+OaPV3-E6E7  
317 was this way generated. Plasmid pLXSN+OaPV4-E6E7 was similarly  
318 produced with primers OaPV4/E6E7/EcoRI/F (5'  
319 GGGAATTCATGCTGAGCAGTAAATTCCTGG 3') and  
320 OaPV4/E6E7/BamHI/F (5' AAGGATCCTCATGGTCGGTTTGCACAGG3').  
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335 E6 and E7 genes of both OaPV3 and OaPV4 were amplified, digested  
336 with BamHI/EcoRI, and cloned into pGEX-4T1 (pre-digested with the same  
337 enzymes) to generate plasmids: pGEX4T+OaPV3-E6, pGEX4T+OaPV3-E7,  
338 pGEX4T+OaPV4-E6, and pGEX4T+OaPV4-E7. E6 and E7 of both viruses  
339 were also cloned into the pCMV-HA-N EcoRI/XhoI restriction sites to generate  
340 plasmids: pCMVHAN+OaPV3-E6, pCMVHAN+OaPV3-E7,  
341 pCMVHAN+OaPV4-E6, and pCMVHAN+OaPV4-E7. Plasmid  
342 pLXSN+HPV38-E6E7 was provided by the Infection and Cancer Biology (ICB)  
343 laboratory at IARC.  
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363 NIH/3T3 fibroblasts and Phoenix cells for amphotropic retrovirus  
364 production were cultured in Dulbecco's Modified Eagle Medium (DMEM)  
365 supplemented with 10% of fetal bovine serum (FBS). Monolayer cultures of  
366 human primary keratinocytes (HPKs) and naturally immortalized keratinocytes  
367 (NIKs) were grown as already described (Caldeira *et al.*, 2003) in FAD  
368 medium containing: 3 parts of Ham's F-12 (Gibco, Invitrogen), 1 part of  
369 DMEM (Gibco, Invitrogen), 2.5% of FBS, hydrocortisone (0.4 µg/ml),  
370 epidermal growth factor (10 ng/ml), insulin (5 µg/ml), cholera toxin (8.4 ng/ml),  
371 adenine (24 µg/ml). A feeder layer of mitotically inactivated NIH/3T3,  
372 generated by Mitomycin C (Sigma-Aldrich, Italy) treatment, was added every  
373 two days to the cultures. All human cell lines were kindly provided by the  
374 Infection and Cancer Biology (ICB) laboratory at IARC (Lyon). Primary ovine  
375 fibroblasts were provided by: "Laboratorio di Ostetricia e Riproduzione  
376 animale (University of Sassari)" and cultured in DMEM supplemented with  
377 10% FBS. Primary lamb keratinocytes (PLKs) were isolated as previously  
378 described (Aasen and Izpisúa Belmonte, 2010; Dal Pozzo *et al.*, 2005) from  
379 the foreskin of two lambs collected at the slaughterhouse. Briefly, foreskin  
380 tissues were deeply rinsed with PBS supplemented with  
381 penicillin/streptomycin and cleaned by trimming away any fat and loose  
382 fascia. The obtained thin sheets of foreskin tissue were cut into small pieces  
383 and incubated in a 0.25% trypsin - EDTA solution (Invitrogen) at 37°C for 30  
384 min. Trypsinized cells were filtered with a 70 µm pore size filter and  
385 centrifuged at 200g for 10 min. Cellular pellets were resuspended and  
386 cultured in PLK medium containing: 1 part of Ham's F12 (Gibco, Invitrogen), 3  
387 parts of DMEM (Gibco, Invitrogen), 10% of FBS, hydrocortisone (0.5 µg/ml),  
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423 epidermal growth factor (2 ng/ml), transferrin (5 µg/ml), insulin (5 µg/ml),  
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425 cholera toxin (12.6 ng/ml), adenine (20 µg/ml) and 3,3',5-triiodo-2-thyronine  
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427 (1.5 ng/ml).  
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### 432 *Immortalization of human and ovine keratinocytes*

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434 Phoenix packaging cells were alternatively transfected with  
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436 pLXSN+OaPV3-E6E7 or pLXSN+OaPV4-E6E7 plasmids carrying the E6E7  
437  
438 region of OaPV3 and OaPV4, respectively. Empty pLXSN was used as  
439  
440 control. Transfections were performed using the CalPhos Mammalian  
441  
442 Transfection kit (Clontech), according to the manufacturer protocol with minor  
443  
444 modifications (25 mM Chloroquine was added to the transfection solution).  
445  
446 Forty-eight hours after transfection, high-titre transfected Phoenix cells  
447  
448 supernatants were collected and used to infect human (HPKs) and ovine  
449  
450 primary keratinocytes (PLKs) to generate the following cell lines:  
451  
452 HPKs/OaPV3-E6E7; HPKs/OaPV4-E6E7; HPKs/pLXSN; PLKs1/OaPV3-  
453  
454 E6E7; PLKs2/OaPV3-E6E7; PLKs1/OaPV4-E6E7; PLKs2/OaPV4-E6E7;  
455  
456 PLKs1/pLXSN; PLKs2/pLXSN. Twenty-four hours after infection, human and  
457  
458 ovine transduced keratinocytes were selected for geneticin resistance (G418  
459  
460 disulfate salt, 0.1 mg/ml and 0.2 mg/ml, respectively). Cell growth profiles  
461  
462 were designed according to population doubling (PD) levels reached by each  
463  
464 cell line at specified time points. For the determination of the PD level,  
465  
466 selected cells were seeded and cultured in 25 cm<sup>2</sup> flasks and trypsinized  
467  
468 when they reached approximately 80-90% confluence. PD level indicates the  
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470 number of times cells have doubled since their retroviral transduction, and  
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483 was calculated taking into consideration the number of passages and the split  
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485 ratio.  
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#### 489 *Colony formation assay*

492 Colony formation assay was performed in duplicate as previously  
493 described (Hufbauer et al., 2013). After transduction with empty pLXSN vector  
494 or E6E7 genes and selection with G418,  $1 \times 10^3$  of transduced PLKs were  
495  
496 seeded in triplicate in 25 cm<sup>2</sup> flasks. Cells were allowed to grow and  
497  
498 proliferate for 15 days. PLK medium was replaced twice a week. Cells were  
499  
500 then fixed with a cold solution of methanol:acetic acid (3:1) and stained with  
501  
502 0.5% (w/v) crystal violet in 25% methanol. Cells were counted and the  
503  
504 average number of colonies containing more than 50 cells was calculated for  
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506 each treatment.  
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#### 513 *Immunofluorescence*

515  $3.5 \times 10^5$  PLKs were seeded in SPL Cell Culture borosilicate chamber  
516  
517 slides (Euroclone, Italy) and let grow until 70-80% of confluence was reached.  
518  
519 Afterwards, cells were washed twice with PBS and fixed in a 1:1  
520  
521 ethanol:acetone solution at -20°C for 20 min. Ethanol has been allowed to  
522  
523 evaporate at room temperature (RT). Fixed cells were incubated with 1% BSA  
524  
525 in PBS for 20-30 min in a humid chamber. Monoclonal Anti-pan Cytokeratin  
526  
527 FITC conjugated antibodies (clone PCK 26, Sigma-Aldrich, Italy) were diluted  
528  
529 1:50 in 1% BSA/PBS and incubated with cells at RT for 2h. Before incubation  
530  
531 with DAPI (1µg/mL in MilliQ water) for 1 min slides were washed 3 times with  
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543 PBS. Cells were finally washed twice before slide assembly in ProLong  
544 antifade reagent, and analysed by confocal laser scanning microscopy.  
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### 549 *Immunohistochemistry*

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552 Five tissue samples, obtained from 5 sheep SCCs positive to OaPV3,  
553 were fixed in 10% paraformaldehyde, dehydrated through ascending grades  
554 of alcohols, and embedded in paraffin wax with a HISTO-PRO 200 vacuum  
555 tissue processor (Histo-Line, Milan). For immunohistochemistry, 3  $\mu$ m  
556 sections were sliced with a microtome (Leica RM 2245- Nussloch, GmbH) and  
557 mounted on positively charged SuperFrost slides (Thermo Scientific, USA).  
558 Tissues were dewaxed and rehydrated with Dewax and HIER (heat-induced  
559 epitope retrieval) Buffer H pH 8.8 (Thermo Scientific, USA), with an Electric  
560 Vegetable Steamer, at 98°C for 20 min. Slides were cooled down by  
561 submersion in bidistilled water at RT for 20 min. The endogenous peroxidase  
562 activity was blocked by incubating slides with Dako REAL™ Peroxidase-  
563 Blocking Solution (S2023 - DAKO, Glostrup, DK) for 30 min. Aspecific  
564 antibody binding sites were blocked by incubation with 2% BSA in PBS for 1 h  
565 at RT followed by a second incubation with 2.5% Normal Horse Serum (NHS,  
566 ImmPRESS Reagent Kit – Vector) for 1 h at RT. Sections were incubated  
567 overnight at 4 °C with a 1:1000 dilution of hyperimmune anti-E6 serum, raised  
568 against the OaPV3 E6 (Tore et al., 2017), and obtained by immunizing rats  
569 with purified recombinant E6 protein expressed in *E. coli*. Afterwards, slides  
570 were incubated for 30 min at RT with donkey anti-rat IgG HRP conjugated  
571 antibodies (Southern Biotech, USA), previously diluted 1:5000 in ImmPRESS  
572 Reagent. Finally, slides were incubated with DAB Peroxidase Substrate  
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603 (ImmPACT-Vector) until desired stain intensity developed, and lightly  
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605 counterstained with hematoxylin. All washing steps were performed three  
606  
607 times with TBS–0.1% Tween 20 (BiOptica, Milano, Italy). Images of all sample  
608  
609 tissues were visualized and obtained with a Nikon Eclipse 80i microscope with  
610  
611 a Nikon DS-Fi1 camera (Nikon Instruments Inc., Melville, NY). Normal skin  
612  
613 samples obtained from a healthy sheep PCR-negative to PV infection were  
614  
615 processed and coupled to lesions during experiments as negative controls.  
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618

### 619 620 *GST pull-down and Co-Immunoprecipitation (CoIP)*

621  
622 For Glutathione S-transferase (GST) pull-down assay, BL21 Rosetta  
623  
624 cells were transformed alternatively with pGEX4T+OaPV3-E6,  
625  
626 pGEX4T+OaPV3-E7, pGEX4T+OaPV4-E6, pGEX4T+OaPV4-E7 and empty  
627  
628 pGEX4T1 plasmids. Transformation was performed by using the  
629  
630 TransformAid Bacterial Transformation Kit (Thermo Scientific) and following  
631  
632 vendor recommendations. Overnight cultures of positive selected bacterial  
633  
634 colonies were diluted 1:10 and grown until OD600 of 0.4. Fusion protein  
635  
636 expression was induced by adding 0.1 mM of isopropylthio- $\beta$ -D-  
637  
638 galactopyranoside (UltraPure IPTG-Invitrogen, Italy), and bacteria were  
639  
640 harvested 3 hours later. Bacteria pellets were re-suspended in NETN buffer  
641  
642 (20 mM Tris-HCl pH 8.0; 100mM NaCl; 1mM EDTA pH 8.0; 0.5% NP-40;  
643  
644 Pierce EDTA-Free Protease Inhibitor Tablet (Thermo Scientific, USA), and  
645  
646 sonicated on ice. The insoluble bacterial debris was removed by  
647  
648 centrifugation and supernatants containing the fusion proteins were filtered  
649  
650 through 0.45  $\mu$ m filters. Fusion proteins were then purified with Glutathione  
651  
652 Sepharose 4B beads (GE Healthcare, UK). Mixtures of cleared bacterial  
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663 lysates and 75µl of glutathione beads were incubated at 4°C for 1 to 3 hours  
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665 rocking. After incubation, beads containing the immobilized fusion proteins  
666  
667 were recovered by centrifugation, washed 5 times with cold NETN buffer and  
668  
669 stored at -20°C until use. Ten µl of bead-immobilised recombinant proteins  
670  
671 were resolved in SDS-PAGE to verify effectiveness of purification, and a BSA  
672  
673 curve was included in the gel to estimate purified protein quantification. GST-  
674  
675 pulldown was performed using a whole cell lysate of NIKS or primary ovine  
676  
677 fibroblasts or PLKs. Before use, cell lysates were pre-cleared through  
678  
679 incubation with empty Glutathione Sepharose 4B beads (30 min rocking at  
680  
681 4°C) to avoid aspecific bindings during pulldown assays. Total protein extracts  
682  
683 contained in the pre-cleared cell lysates were quantified using the Pierce BCA  
684  
685 Protein Assay kit (Thermo Scientific, USA). Equal amounts (1 to 2 µg) of  
686  
687 bead-immobilised GST/recombinant proteins were mixed with equal amounts  
688  
689 of pre-cleared cell lysate (about 600 µg of total protein extract for each pull-  
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691 down) in a volume of at least 400 µl, and incubated at 4°C for 1-3 hours with  
692  
693 gentle rotation. Beads were collected by centrifugation and washed 10 times  
694  
695 with cold NETN buffer. After wash steps, samples were directly resuspended  
696  
697 in 10 µl of 4X Laemmli buffer and subjected to western immunoblotting.  
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700  
701 For ColPassays, primary ovine fibroblasts and PLKs were alternatively  
702  
703 transfected with pCMVHAN+OaPV3-E6, pCMVHAN+OaPV3-E7,  
704  
705 pCMVHAN+OaPV4-E6, pCMVHAN+OaPV4-E7 or empty pCMVHAN by using  
706  
707 the TurboFect Transfection Reagent (Thermo Scientific,USA), and following  
708  
709 vendor instructions. Forty-eight hours after transfection, cells were harvested.  
710  
711 Pelleted cells were resuspended and incubated on ice with IP lysis buffer (20  
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713 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NP-40, Pierce  
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723 EDTA-Free Protease Inhibitor Tablet (Thermo Scientific, USA). After  
724 incubation, cell debris was removed by centrifugation and the supernatants  
725 containing the protein extracts were collected. Proteins were quantified with  
726 the Pierce BCA Protein Assay kit (Thermo Scientific, USA) and equal  
727 amounts were used to perform CoIP with the Pierce HA Tag IP/Co-IP Kit  
728 (Thermo Scientific, USA). Immunoprecipitates were eluted at 95°C in Laemmli  
729 buffer and directly subjected to western immunoblotting. Experiments were  
730 repeated three times and incubation with the Invitrogen™ HA epitope tag  
731 antibody (Thermo Scientific, USA) was used to verify HA binding efficiency in  
732 different CoIP experiments.  
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#### 747 *Western Immunoblotting and antibodies*

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749 Equal amounts of total protein extracts were run in single well 10%  
750 polyacrylamide gels and transferred into nitrocellulose membranes with a  
751 Mini-Trans-Blot Cell (Bio-Rad, USA) at 250 mA for 1 h. After blotting,  
752 membranes were blocked with 10% skim milk in PBS-0.05% Tween-20 (PBS-  
753 T), mounted in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, USA),  
754 and then incubated at 4°C overnight with the following antibodies: Purified  
755 Mouse Anti-Human Retinoblastoma Protein Clone G3-245 (RUO)  
756 (BDPharmingen); p53 Antibody (Cell Signalling Technology, 9282); Phospho-  
757 Rb (Ser795) Antibody (Cell Signaling Technology, 9301); Phospho-p53  
758 (Ser15) (16G8) Mouse mAb (Cell Signaling Technology, 9286); cyclin A  
759 Antibody (H-432) (Santa Cruz biotechnology); Anti-cdc2 (p34) Antibody  
760 (Santa Cruz biotechnology); Cdc2 (POH1) Mouse mAb (Cell Signaling  
761 Technology, 9116); GST Antibody (Cell Signaling Technology, 2622). After  
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783 incubation with primary antibodies, membranes were washed with PBS-T,  
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785 incubated with the appropriate HRP-conjugated secondary antibodies  
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787 (Southern Biotech, USA) for 1 h, washed again, and developed with the  
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789 Clarity™ western ECL substrate (Bio-Rad, USA) or the Luminata Forte  
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791 Western HRP substrate (Millipore, USA). Images were acquired with the  
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793 ChemiDoc XRS+ System (Bio-Rad, USA). Densitometric values and  
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795 normalization to housekeeping gene (see online supplemental material) were  
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797 calculated with ImageLab 5.2.1 software (Bio-Rad, USA) and then  
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799 represented graphically as fold changes relative to control cells (transduced  
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801 with the empty pLXSN). The expression level of the target protein in the  
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803 control cultures was set as one.  
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843 **Results**  
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845 *OaPV3 and OaPV4 E6E7 heighten proliferation and prolong lifespan of*  
846 *primary human and ovine keratinocytes*  
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849 Human primary keratinocytes (HPKs) from three different donors were  
850 transduced with pLXSN+OaPV3-E6-E7 and pLXSN+OaPV4-E6-E7 vectors  
851 carrying both the E6 and E7 genes of OaPV3 and OaPV4, or with the empty  
852 pLXSN. Transduced keratinocytes growth was followed for about 30 days  
853 after infection and selection (Fig. 1A). Control keratinocytes (HPKs  
854 transduced with empty pLXSN, Fig. 1A) entered a quiescent phase and died  
855 within two population doublings. Cells early acquired a flat and enlarged  
856 morphology characteristic of arrested cells, and showed features of  
857 senescence such as degeneration, irregular shape, intercellular bridges and a  
858 high cytoplasm/nucleus ratio (Fig. 1B). On the contrary, HPKs/OaPV3-E6E7  
859 and HPKs/OaPV4-E6E7 cell lines continued proliferating and dividing until  
860 experiments were voluntarily stopped. In addition to lifespan prolongation,  
861 HPKs expressing OaPV3 E6E7 oncogenes acquired a more regular shape  
862 with defined borders similarly to what observed when keratinocytes were  
863 transduced with HPV38-E6E7, used as a control (Fig. 1B). OaPV4 E6E7  
864 expressing cells, while clearly distinct from control cells, were more  
865 heterogeneous in size and shape than the other two populations.  
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885 Based on results, similar experiments were repeated by transducing  
886 primary lamb keratinocytes (PLKs), which represent the natural target of ovine  
887 PVs. Growth profiles of 2 PLKs donors showed that E6E7 expression of both  
888 OaPV3 and OaPV4 dramatically prolong PLKs lifespan (Fig. 2A). Indeed  
889 PLKs transduced with the empty pLXSN stopped dividing after few population  
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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-E6E7 cell lines always showed a statistically significant shorter PD time compared to control cells PLKs/pLXSN (Fig. 2B). Moreover, OaPV3-E6E7 expressing PLKs showed

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963 PDs shorter than OaPV4 even if this last observation was not statistically  
964 supported in all donors (Fig. 2B).  
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970 *PLKs/OaPV4-E6E7 and PLKs/OaPV3-E6E7 have enhanced clonogenic*  
971 *activity*  
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974 OaPV3-E6E7 and OaPV4-E6E7 expressing PLKs were tested for  
975 proliferation capacity in a clonogenic assay, which evaluates the ability of a  
976 single cell to proliferate and form a colony. Compared to control, colony  
977 formation assays revealed a 3.2 and 2.5-relative fold increase in number of  
978 colonies respectively produced by OaPV3-E6E7 and OaPV4-E6E7  
979 expressing cell lines (Fig. 4A). A statistically significant difference was also  
980 found between OaPV3-E6E7 and OaPV4-E6E7 expressing PLKs, revealing a  
981 stronger clonogenic efficiency of OaPV3 respect to OaPV4.  
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991 Furthermore, OaPV3-E6E7 expressing keratinocytes generated  
992 colonies that mostly resembled *foci* of transformed cells and were constituted  
993 by copious, small, packed, and 3-D proliferating keratinocytes. On the other  
994 hand, OaPV4-E6E7 expressing colonies were macroscopically and  
995 microscopically more similar to control colonies and composed of sparse, flat  
996 and large keratinocytes (Fig. 4B, 4C).  
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1006 *OaPV3 and OaPV4 E6E7 alter pRb expression*  
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1008 OaPV3-E6E7 and OaPV4-E6E7 were also tested for the ability to alter  
1009 the expression of p53 and pRb proteins and their phosphorylated  
1010 counterparts. After retroviral transduction and selection, total proteins were  
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1023 extracted from transduced human and ovine keratinocytes and subjected to  
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1025 western immunoblotting.  
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1028 Both HPKs and PLKs expressing OaPV3 and OaPV4 E6 and E7  
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1030 showed increased levels of phospho-pRb (ppRb) in the majority of human and  
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1032 sheep donors (Figs 5A, 5B). Since the ppRb form is not able to inhibit the  
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1034 E2F-driven transcription, these results indicate that the viral proteins activate  
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1036 the transcription of E2F-regulated genes. Accordingly, cyclin A and  
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1038 (especially) cdk1 protein levels were upregulated in both human and ovine  
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1040 cells transduced with OaPV3 and OaPV4 oncogenes. Immunoblotting of total  
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1042 protein extracts from transduced HPKs and PLKs never revealed a significant  
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1044 change in p53 and in phospho-p53 expression levels in HPK. It should be  
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1046 pointed out that antibodies against phospho-p53 (ser15) did not work on  
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1048 sheep keratinocytes; therefore, a role of p53 deregulation in promoting cell  
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1050 proliferation can not be ruled out in the natural host.  
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#### 1055 *OaPV3 E7 binds pRb with the highest avidity*

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1058 GST-pulldown experiments, carried out in triplicate using naturally  
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1060 immortalized keratinocytes (NIKs), primary lamb keratinocytes (PLKs), or  
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1062 primary ovine fibroblasts protein extracts, produced variable poor results (see  
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1064 online supplemental material), although anti-GST western blots demonstrated  
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1066 comparable GST-binding efficiencies in different lysates and different  
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1068 experiments. When the assay was performed using NIKs, both OaPV3-E7  
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1070 and OaPV4-E6 seemed able to bind the retinoblastoma tumour suppressor  
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1072 protein (pRb) as expected from *in silico* analysis, since these two oncogenes  
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1074 contain a canonical pRb-binding motif. Unexpectedly, also OaPV4-E7 seemed  
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1083 to bind human pRb even without carrying a classical pRb-binding domain.  
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1085 When GST pull-down assays were repeated using a lysate of ovine cells  
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1087 (either fibroblasts or PLKs) only OaPV3-E7 was able to bind pRb, even if  
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1089 western blotting signals were always very weak. On the contrary, none of the  
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1091 oncogenes associated with human or ovine p53. Incubation of protein extracts  
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1093 with bead-immobilised GST alone, used as control, never produced non-  
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1095 specific binding.  
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1098 ColP experiments, carried out in triplicate using transfected primary  
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1100 ovine keratinocytes or fibroblasts protein lysates, confirmed the high pRb  
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1102 binding efficiency of OaPV3-E7, and that E6E7 oncogenes bind p53 at very  
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1104 low efficiency, as suggested by the absence of a detectable p53 signal by  
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1106 western immunoblotting (Fig. 6). Results were reproducible and consistent in  
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1108 both cell lines. Mock cells (not transfected) and cells transfected with the  
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1110 empty pCMV-HA-N carrying the HA tag alone, used as controls, never  
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1112 generated non-specific bindings. The use of HA epitope tag antibodies  
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1114 generated a comparable signal in lanes containing HA-tagged proteins  
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1116 coimmunoprecipitates (data not shown).  
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#### 1123 *OaPV3 early region gene expression in squamous cell carcinomas*

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1125 All 5 OaPV3 PCR-positive SCC samples tested positive when probed  
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1127 with the anti OaPV3-E6 serum (Fig. 7). Strong cytoplasmic positivity was only  
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1129 observed in epithelial cells, confirming the tropism of this virus for skin  
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1131 keratinocytes. Uninfected tissues tested always negative to the same serum.  
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## 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 were 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 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

1201 collected from naturally infected sheep. This confirms previous observations,  
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1203 in which active transcription of early region genes was observed by RT-PCR  
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1207 (Alberti *et al.*, 2010). These data, together with the wide association of OaPV3  
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1209 to ovine SCC (Vitiello *et al.*, 2017) may suggest a role for OaPV3 oncogenes  
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1211 in skin tumor progression. On the contrary, OaPV4 infects both epithelial and  
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1213 dermal cells, and appears to be related to benign proliferative lesions, such as  
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1215 papillomas and fibropapillomas.  
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1218 In order to investigate the different pathogenic outcome potentially  
1219 associated to ovine papillomavirus belonging to *Delta* and *Dyokappa* genera,  
1220 both human and ovine primary keratinocytes were transduced with the E6E7  
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1222 early region genes of OaPV4 and OaPV3. Based on results, it appears that  
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1224 both OaPV3 and OaPV4 oncogenes are able to prolong lifespan of human  
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1226 keratinocytes and to immortalize ovine cells. On comparisons, OaPV3 and  
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1228 OaPV4-E6E7 expressing cells showed different shape, doubling time, and  
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1230 clonogenic activities, thus suggesting a stronger transforming potential for  
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1232 OaPV3 respect to OaPV4. Indeed OaPV3-E6E7 expressing cells lost their  
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1234 original cell shape by increasing nucleus/cytoplasm ratio, they doubled faster  
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1236 than OaPV4-E6E7 expressing cells, and they produced an increased number  
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1238 of transformation foci-looking small colonies respect to controls. Conversely,  
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1240 OaPV4-E6E7 expressing cells maintained a shape closer to their original one  
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1242 (epithelioid), they duplicated faster than controls but slower than OaPV3, and  
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1244 generated larger and flat colonies composed by cells resembling the original  
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1246 epithelial cells.  
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1263 Based on this data we postulated that both OaPV3 and OaPV4-E6E7  
1264 proteins are able to immortalize cells, but only OaPV3 may dramatically alter  
1265 cell functionality and induce cell transformation.  
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1269 Expression HPV E6 and E7 oncogenes is essential for the initiation  
1270 and maintenance of cervical cancer (Morrison *et al.*, 2011).  
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1273 E6 and E7 proteins do not act as typical oncogenes, as they do not  
1274 directly function as transforming agents, but instead they interact with cellular  
1275 proteins, among them p53 and pRb, which are well known cellular tumour  
1276 suppressors.  
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1282 p53, pRb, and their related pocket proteins are commonly impaired  
1283 during mucosal alpha High Risk-HPV (HR-HPV) related cancer. The most  
1284 studied property of HR-HPV E7 oncoproteins is the ability to functionally  
1285 complement the tumour suppressor pRb promoting its degradation. The ability  
1286 of HR-HPV to bind pRb with high efficiency is associated with cell  
1287 transformation and cancer progression (Ghittoni *et al.*, 2010). Indeed, the E7  
1288 proteins encoded by the HR-HPV types, such as HPV16 and HPV18, bind  
1289 pRb with a much higher affinity compared to those encoded by the low-risk  
1290 type HPVs, such as HPV 6 and HPV 11 (Yim and Park, 2005). Cutaneous  
1291 HPV-E7 proteins are also able to impair pRb pathway. As an example,  
1292 HPV38-E7 has pRb binding efficiency comparable to HPV16-E7, and  
1293 promotes pRb destabilization (Caldeira *et al.*, 2003). Here we demonstrate  
1294 that OaPV3 and OaPV4-E6E7 expression leads to destabilization of pRb  
1295 protein levels, but only OaPV3-E7 has the capacity to strongly associate and  
1296 bind both human and ovine pRb. Our results also suggest that p53 pathway  
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1323 might not be implicated in the pathogenesis of ovine PVs-mediated  
1324 conditions.  
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1327 Mitogenic stimuli such as oncogene expression deregulate the  
1328 expression of cyclins and the related cyclin-dependent kinases (CDKs)  
1329 leading to the their activation that is necessary for cell cycle progression  
1330 (Tommasino, 2017). In accordance with this assumption, we found that  
1331 OaPV3 and OaPV4-E6E7 determine upregulation of the pro-proliferative  
1332 proteins cyclin A and cdc2 in both human and ovine primary keratinocytes.  
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1335 Considered together, these findings are hallmarks of cell immortalization and  
1336 transformation, particularly in OaPV3-E6E7 expressing keratinocytes, and  
1337 support our hypothesis of a role of OaPV3 in progression of cutaneous  
1338 squamous cell carcinomas. Further analyses are required to understand  
1339 whether ovine PV E6 and E7 can associate with cellular factors other than  
1340 pRb and p53 to overcome cell cycle control signalling pathways and to  
1341 promote cellular transformation.  
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1745 **Figure legends**  
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1750 **Figure 1:** Effect of OaPV3 and OaPV4 E6E7 on human primary keratinocytes  
1751 (HPKs) proliferation and morphology. A, growth curves of HPKs from one of  
1752 the three donors transduced with the indicated recombinant retroviruses.  
1753 OaPV3-E6E7 and OaPV4-E6E7 expressing cells growth curves overlap. B,  
1754 cell morphology of transduced HPKs after drug selection. Magnification 10X.  
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1762 **Figure 2:** Effect of OaPV3 and OaPV4 E6E7 on life span and population  
1763 doubling times of primary lamb keratinocytes (PLKs) obtained from two  
1764 donors. A, growth curves of PLKs transduced with the indicated recombinant  
1765 retroviruses, showing the ability of both OaPV3 and OaPV4-E6E7 to increase  
1766 PLKs lifespan leading to immortalization. B, population doubling (PD) times,  
1767 calculated as the time length (days) between post-selection passages,  
1768 demonstrating the fastest proliferative activity of OaPV3-E6E7 expressing  
1769 PLKs. Results are the mean of 6 values for each experiment. Error bars show  
1770 standard deviation. p value  $\leq 0,05$   
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1783 **Figure 3:** Cell morphology of PLKs transduced with the indicated recombinant  
1784 retroviruses. A, immunofluorescence with Anti-pan cytokeratin antibodies  
1785 showing cell shape modification of primary ovine keratinocytes upon OaPV3  
1786 E6E7 expression. Transduced cells appear smaller with higher  
1787 nucleus/cytoplasm ratio compared to control cells and to PLKs transduced  
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1803 with OaPV4 E6E7. B, cell morphology of transduced PLKs after drug selection  
1804 as they appear at optical microscope. Magnification 20X.  
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**Figure 4:** Clonogenic activities of transduced PLKs. A, number of generated  
1810 colonies. Error bars show standard deviation. Values are means from two  
1811 independent experiments. p value  $\leq 0.01$ . B, microscopic aspect of colonies  
1812 generated by transduced ovine keratinocytes. Magnification 4X (top) and 10x  
1813 (bottom). C, macroscopic aspect of representative clonogenic assay with  
1814 transduced PLKs.  
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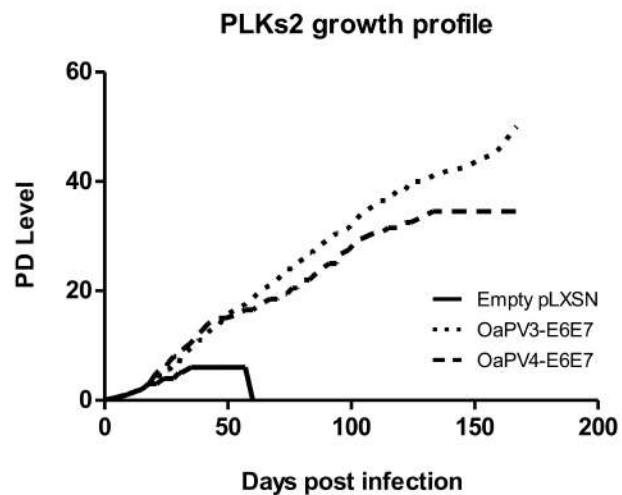
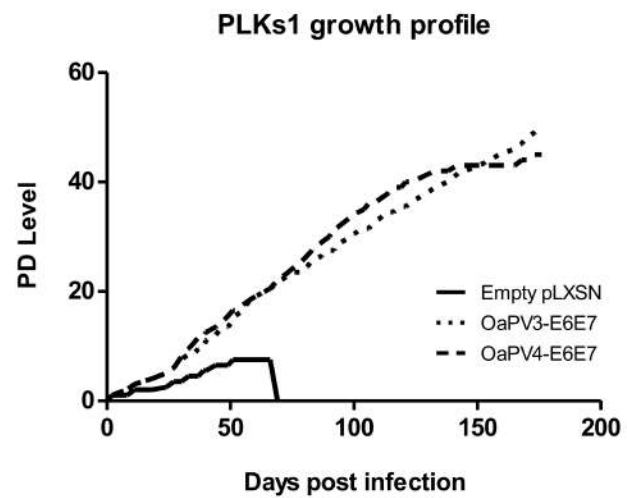
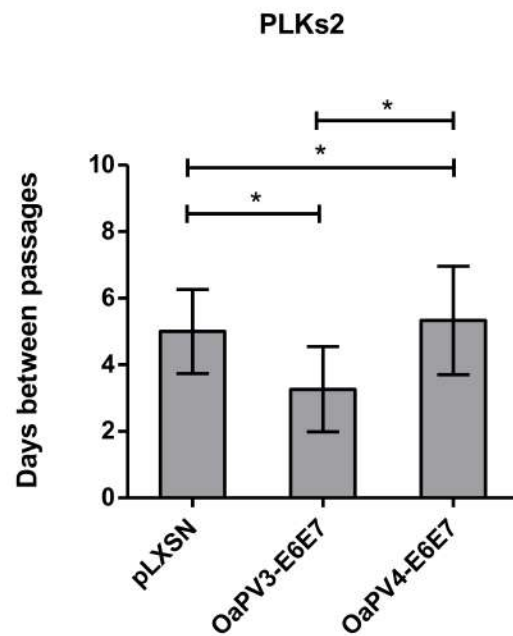
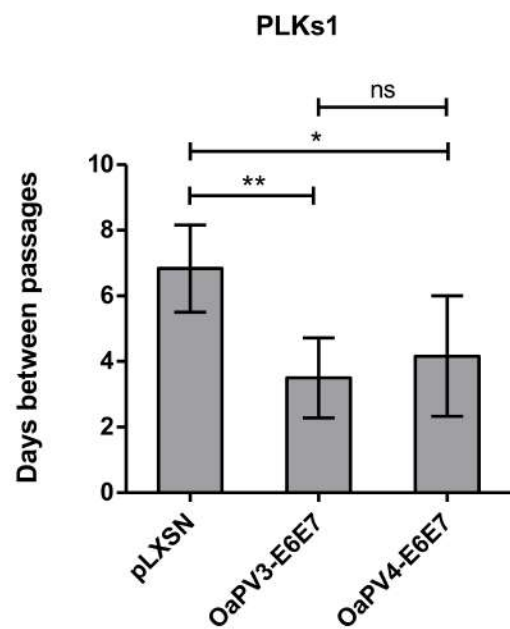
**Figure 5:** A, representative western immunoblotting showing deregulation of  
1824 total and phospho-pRb protein levels and upregulation of cdc2 and Cyclin A in  
1825 HPKs transduced with the indicated recombinant retroviruses.  $\beta$ -actin was  
1826 used as loading control. B, total pRb and phosphor-pRb, p53, and cdk1  
1827 protein levels in PLKs, transduced with the indicated recombinant  
1828 retroviruses, are shown in different western immunoblotting. Anti-human  
1829 phospho p53 and anti-human cyclin A antibodies did not react with the ovine  
1830 proteins.  $\beta$ -actin, included as a loading control, is shown for each western  
1831 immunoblotting.  
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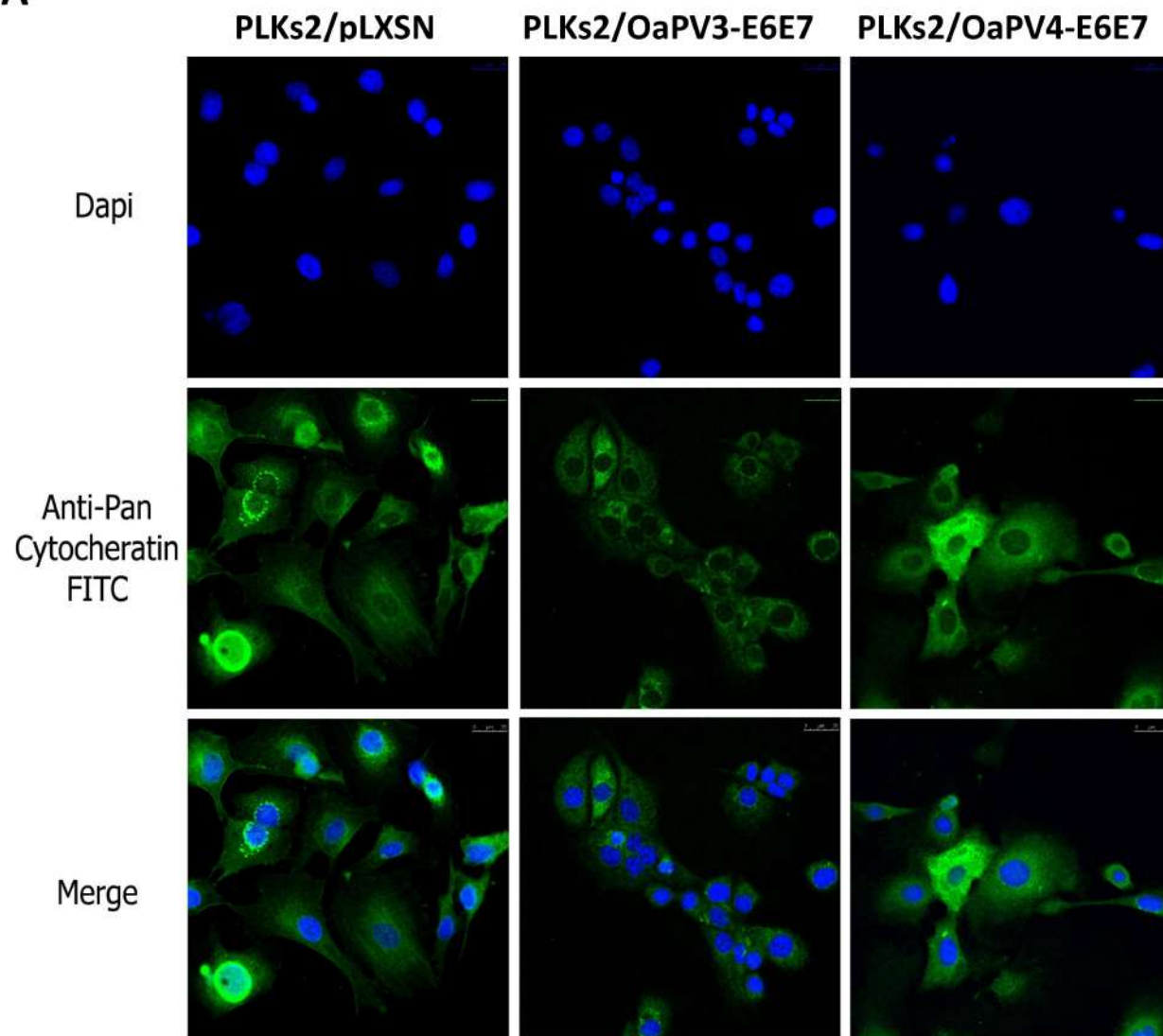
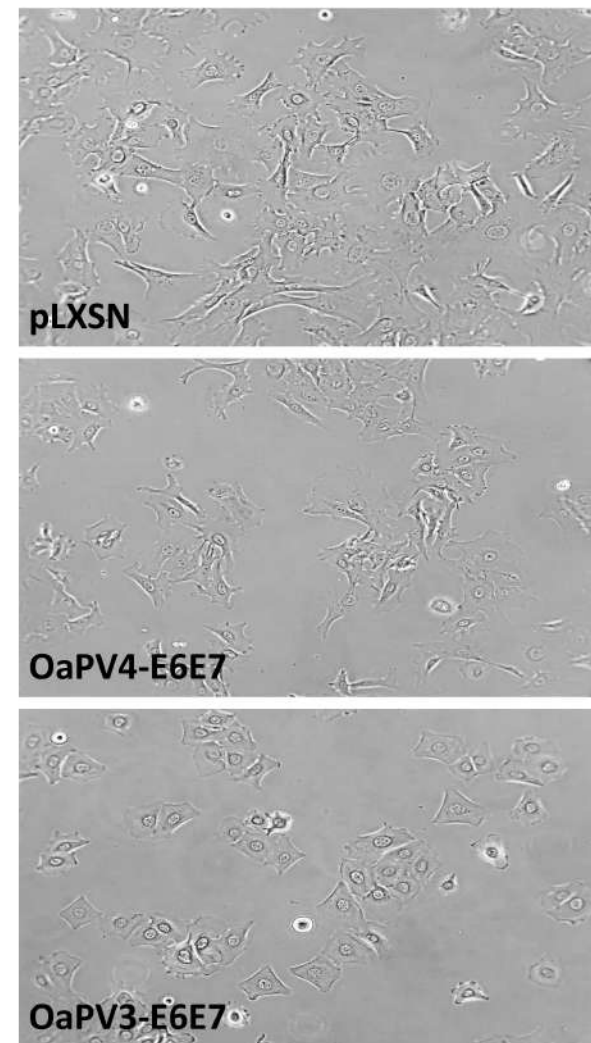
**Figure 6:** Western Blot reactivity of co-immunoprecipitated ovine primary  
1846 fibroblasts (A) and primary lamb keratinocytes (B) transfected with HA-tagged  
1847 OaPV3 and OaPV4 E6 and E7 with anti-pRb and anti-p53 antibodies. Total  
1848 cell lysates from non-transfected cells were used as positive control for  
1849 antibodies reactivity (Input). Co-immunoprecipitates from cells non-transfected  
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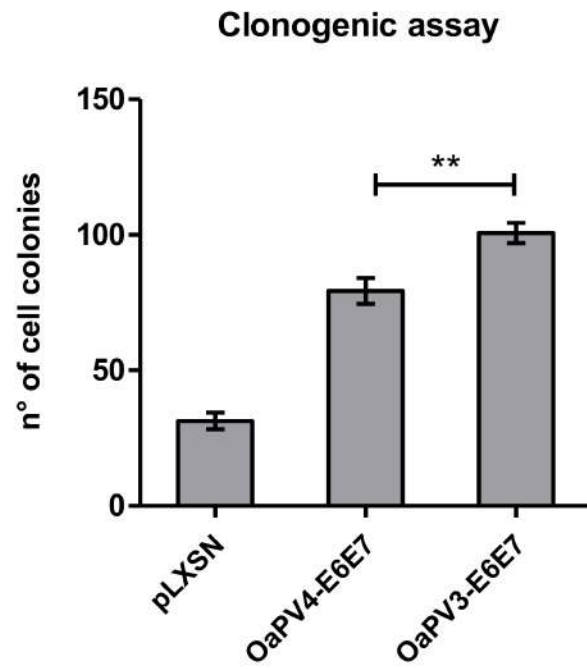
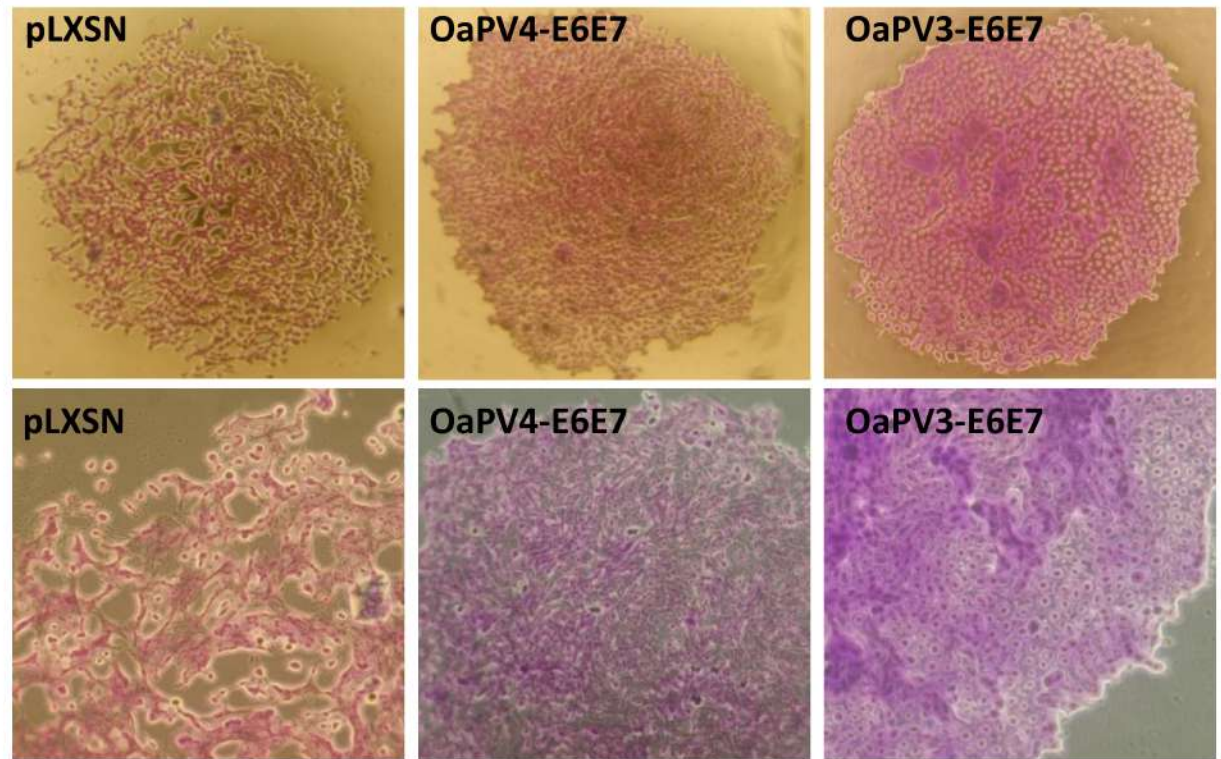
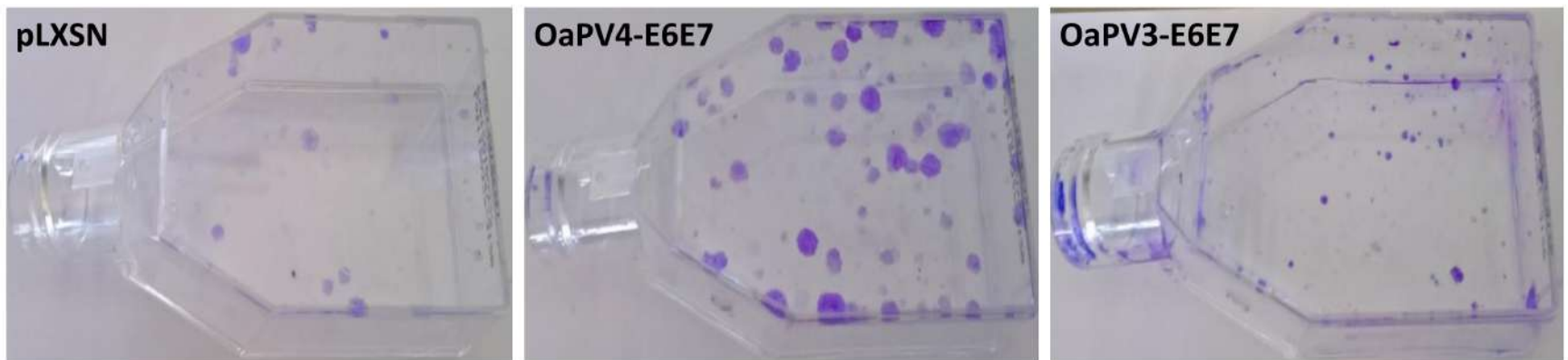


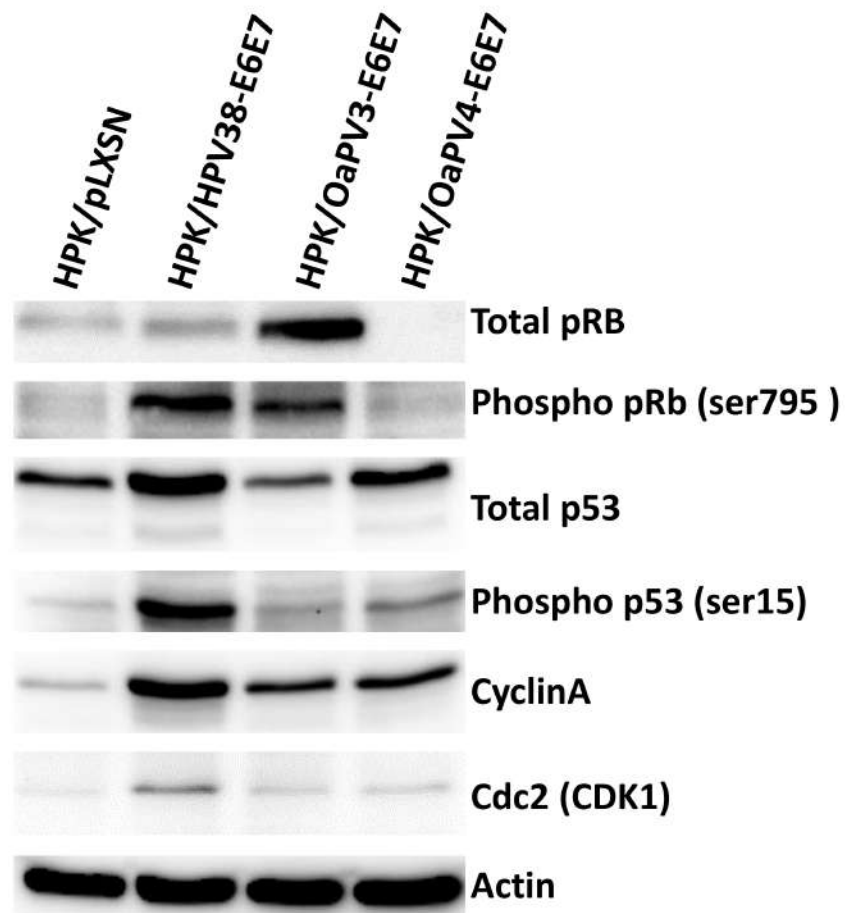
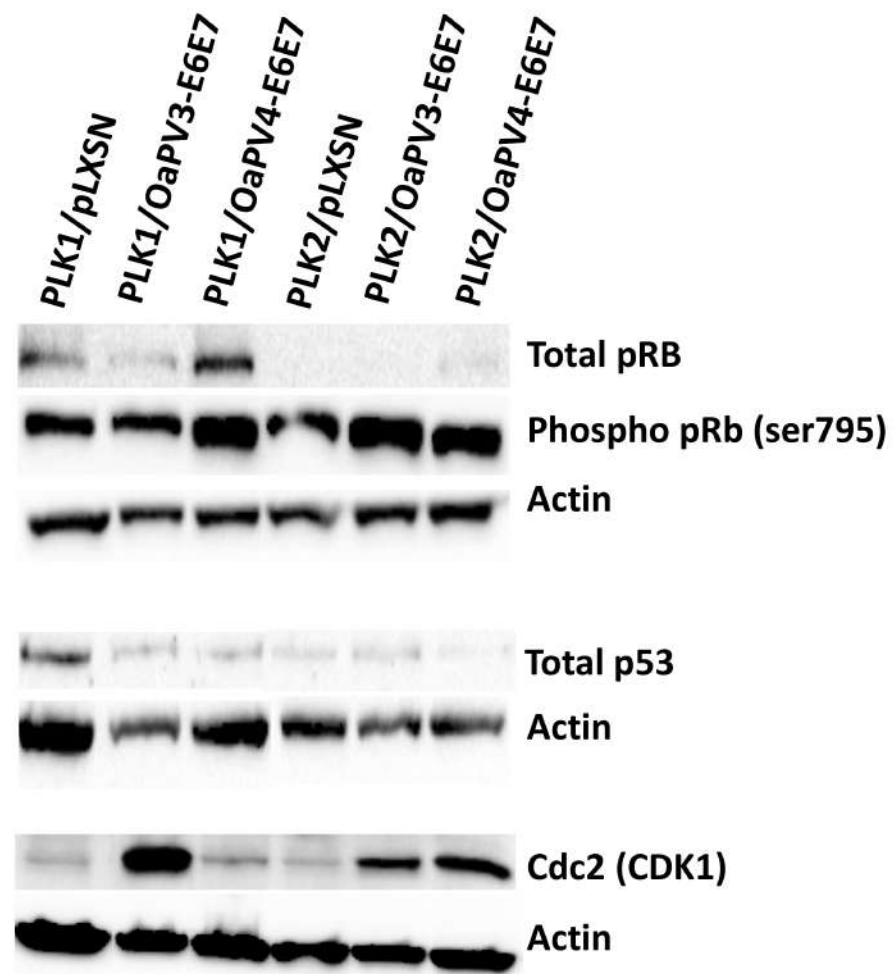
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1863 or transfected with empty pCMV Ha plasmid were used as negative controls.  
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1865 Anti HA-tag antibody reactivity used as a control to verify HA-tag binding  
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1867 efficiency in different lanes, is not shown.  
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1872 **Figure 7:** IHC of histological section showing well differentiate sheep SCC  
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1874 with high cellularity, loss of cell polarity, and spread keratin pearls. A,  
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1876 detection of a diffuse OaPV3 E6 signal in the section. B, keratin pearls with  
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1878 diffuse OaPV3 E6 cytoplasmic signal.  
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**A****B**

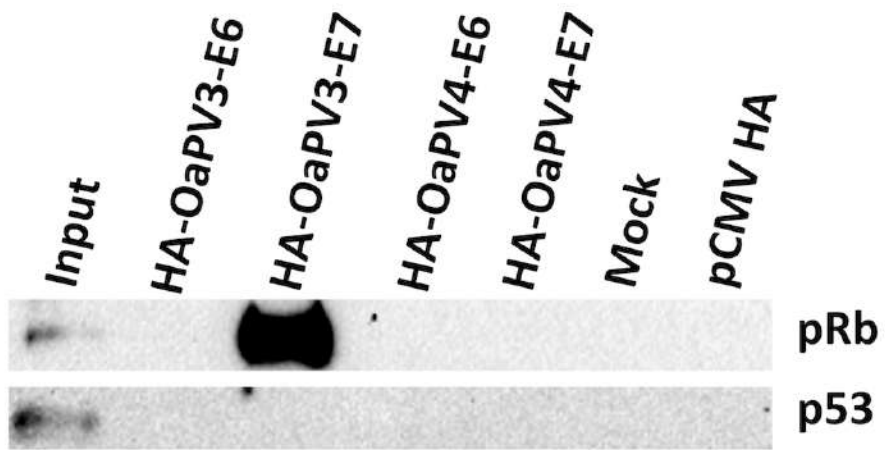
**A****B**

**A****B****C**

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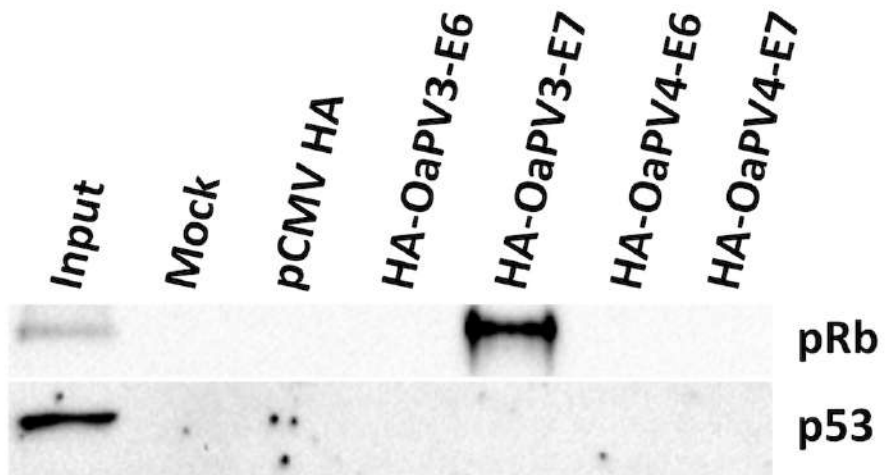
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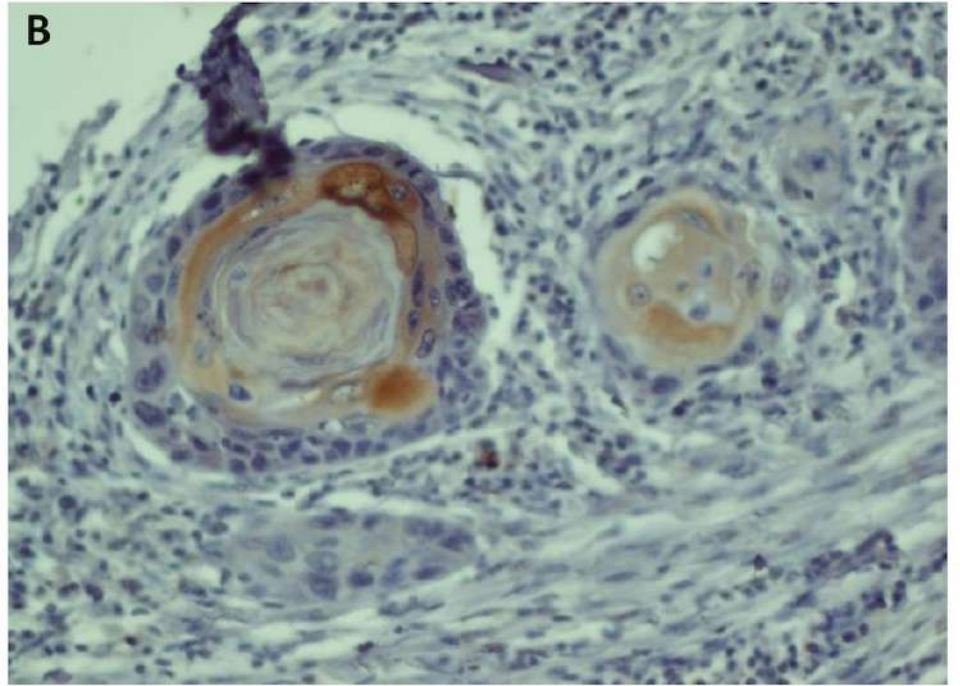
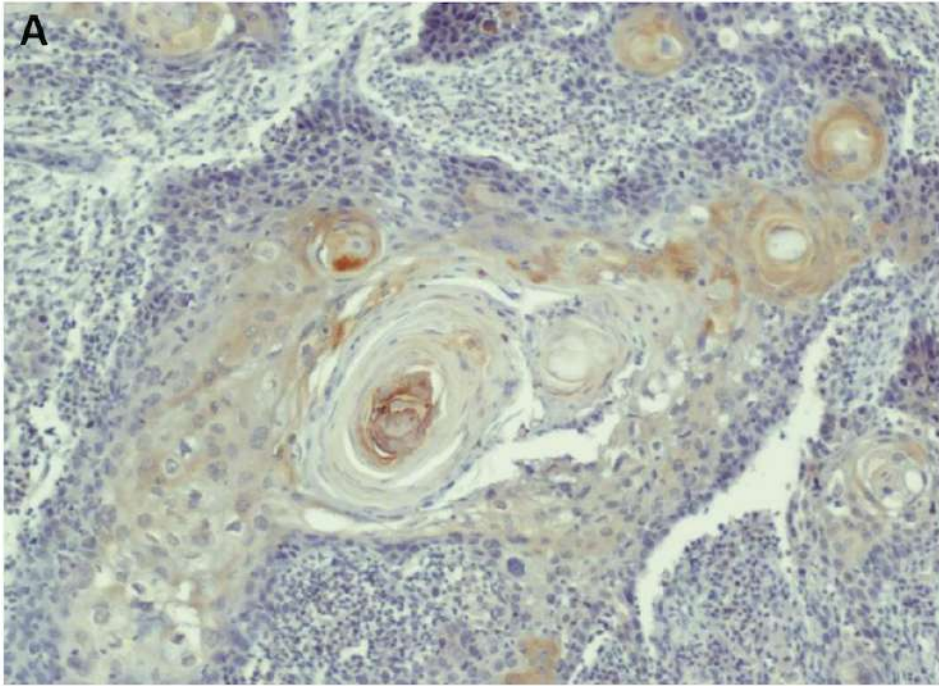
**OVINE PRIMARY FIBROBLAST CELL LYSATE**

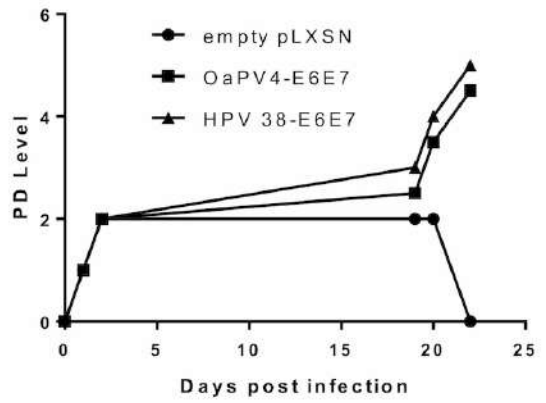
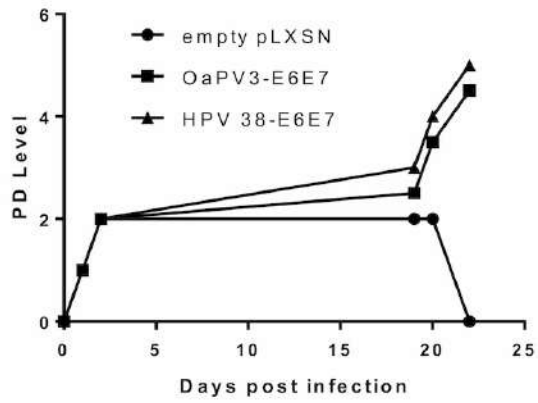


**B**

**PRIMARY LAMB KERATINOCYTE CELL LYSATE**





**A****B**