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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*

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ABSTRACT

Felis catus papillomavirus type 2 (FcaPV2) DNA is found in feline cutaneous squamous cell carcinomas (SCCs); however, its biological properties are still uncharacterized. In this study, we successfully expressed FcaPV2 E6 and E7 putative oncogenes in feline epithelial cells and demonstrated that FcaPV2 E6 binds to p53, impairing its protein level. In addition, E6 and E7 inhibited ultraviolet B (UVB)-triggered accumulation of p53, p21 and pro-apoptotic markers such as Cleaved Caspase3, Bax and Bak, suggesting a synergistic action of the virus with UV exposure in tumour pathogenesis. Furthermore, FcaPV2 E7 bound to feline pRb and impaired pRb levels, resulting in upregulation of the downstream pro-proliferative genes Cyclin A and Cdc2. Importantly, we demonstrated mRNA expression of FcaPV2 E2, E6 and E7 in feline SCC samples, strengthening the hypothesis of a causative role in the development of feline SCC.

1. Introduction

Papillomaviruses (PV) are double-stranded DNA viruses that are able to infect both skin and mucosal epithelia in animals and humans, inducing benign and/or malignant hyperproliferative lesions (Munday, 2014a). Recently, a possible role for PV infection has been hypothesized also in the development of skin tumours in the domestic cat (*Felis catus*) (Munday, 2014b). Indeed, feline cutaneous squamous cell carcinomas (SCCs) are frequently associated with DNA of *Felis catus* PV type 2 (FcaPV2), possibly suggesting its contribution to tumour pathogenesis (Munday, 2014b). In addition, a significant proportion of tumours harbouring viral DNA may arise in white-coated old animals at ultraviolet (UV)-exposed sites, indicating UV exposure as a possible co-factor in the neoplastic process, as in their human counterparts (Munday et al., 2011; Thomson, 2007).

Among canonical PV open reading frames, FcaPV2 potentially encodes E2, a transcription factor regulating viral replication and gene transcription, and E6 and E7, which may be putative oncogenes as their products show structural similarities to those of E6 and E7 from high-risk (HR) human PV (HPV) involved in cervical cancer (Lange et al., 2009).

In fact, due to a clear association of these HPV types with human carcinogenesis, especially HPV16 and HPV18, the transforming abilities of their E6 and E7 have been extensively investigated in several *in vitro* and *in vivo* models, highlighting the molecular mechanisms of their carcinogenic properties (Munday, 2014a). The main cellular target of E6 from oncogenic HPV is the p53 tumour suppressor protein (Tommasino, 2014). E6 from HR HPV binds to the p53 protein, leading to its degradation via the proteasome pathway, while cutaneous beta HPV types are able to alter p53

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functions and DNA repair machinery in response to UV irradiation in several *in vitro* and *in vivo* models, leading to the hypothesis of a synergistic action of PV infection and UV exposure in the development of non-melanoma skin cancer (NMSC) (Giampieri et al., 2004; Jackson and Storey, 2000; Marcuzzi et al., 2009; Struijk et al., 2008; Viarisio et al., 2011).

The E7 oncoproteins exert their main transforming activity by impairing the pRb pathway (Tommasino, 2014). HR HPV E7 binds to pRb protein through the pRb binding domain LXCXE, inducing its proteasomal degradation, while cutaneous HPV E7 may similarly degrade pRb or enhance its phosphorylation status; both mechanisms lead to a release of the E2F transcriptional repressor, which in turn activates unscheduled expression of pro-proliferative genes, thus contributing to cancer progression (Cornet et al., 2012; Dyson et al., 1992; Huh et al., 2007; Zerfass et al., 1995).

The biological characteristics of E6 and E7 from several animal PV have been investigated as well (Corteggio et al., 2013; Ganzenmueller et al., 2008). However, no data are available so far regarding the possible transforming properties of FcaPV2 E6 and E7 and the biological activity of the virus *in vivo*. Thus, a role for this virus in the development of feline SCC is far from being proven.

The aim of this work was to establish the first *in vitro* model for the study of the transforming properties of FcaPV2 E6 and E7 and their impact on the p53 and pRb pathways and UVB-induced apoptosis. In addition, to confirm a possible causative role of this emerging oncogenic virus in the development of feline SCC, we determined whether the E6 and E7 oncogenes are expressed in tumour samples. Finally, we assessed the expression of E2 to further demonstrate that the virus is biologically active and is not a simple bystander *in vivo.*

2. Materials and methods

2.1. Cell procedures and UVB treatment

Crandell Rees feline kidney (CRFK) epithelial cells, the NIH/3T3 mouse fibroblast cell line, HaCaT and C33A immortalized human keratinocytes and a Phoenix packaging cell line were purchased from ATCC® cell bank and cultured according to their recommendations. Human foreskin keratinocytes (HFK) from two different donors were obtained and co-cultured with NIH/3T3 as previously described (Bickenbach et al., 1998; Caldeira et al., 2003).

CRFK cells were transfected with FcaPV2 E6E7, E6 and E7 cloned in pCEFL vector using Lipofectamine 2000 (Life Technologies) as reported elsewhere (Valentino et al., 2013) and selected for G418 resistance conferred by the vector. Details of the primers used for cloning and their annealing positions in the open reading frames of cloned genes are shown in Supplementary material 1 and 2, respectively. Expression of transfected genes was assessed by reverse transcription (RT)-polymerase chain reaction (PCR) as described below.

For UVB treatment, cells were irradiated in phosphate-buffered saline (PBS) at 25 mJ/cm^2 using the Bio-Sun irradiation system 365/312 nm (Vilber Lourmat, France) and harvested after 8 h.

2.2. Real-time quantitative PCR

Total RNA was extracted from cells using the Absolutely RNA Miniprep Kit (Agilent Technologies). RT was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), and cDNA was subjected to real-time quantitative PCR (qPCR) for feline p53, Bcl-Xl, Cdc2 and GAPDH using Mesa Green qPCR MasterMix Plus (Eurogentec). Details of primers are summarized in Supplementary material 1. The results were normalized to GAPDH and analysed using the software provided by the Mx3005P qPCR System (Agilent).

RNA extraction and RT from tissue samples were performed as described below, and real-time qPCR for p53 and GAPDH was carried out using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). All the real-time qPCR experiments were performed at least twice in technical duplicate.

2.3. Western blotting procedures and antibodies

Total protein extraction, SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting (WB) were conducted as previously described (Accardi et al., 2006). Primary antibodies against the following proteins were applied overnight at 4 °C at 1:1000 dilution: p53 (Santa Cruz, sc-6243), p21 (Santa Cruz, sc-397), Caspase3 (Cell Signaling, #9662), Bax (Santa Cruz, sc-493), Bak (Santa Cruz, sc-7873), pRb (BD Pharmingen #554136), Cyclin A (Santa Cruz, sc-751), Cdc2 (Santa Cruz, sc-54), GST (Cell Signaling, #2622) and β-actin (Calbiochem, #CP01). Protein band detection and densitometric analysis were performed as reported elsewhere (Pacini et al., 2015). All the WB experiments were performed at least twice, yielding comparable trends.

2.4. GST pull-down assay

FcaPV2 and HPV16 E6 and E7 were cloned in glutathione-*S*-transferase (GST) fusion vector pGEX4T1 or pGEX2T1 using the primers indicated in Supplementary material 1, as previously described (Cornet et al., 2012).

The GST fusion proteins were expressed in competent bacterial strains, purified with Glutathione Sepharose-4B beads (GE Healthcare) and incubated with CRFK, HaCaT and C33A cell lysates as previously reported (Alunni-Fabbroni et al., 2000; Cornet et al., 2012). One-tenth (60 μg) of the total cellular extract (600 μg) used in each GST pull-down assay was loaded on the same gel as a control (input). The molecular complexes were analysed by WB for detection of GST tag, p53 and pRb as described above. The fraction of p53 and pRb bound by viral proteins was estimated by densitometric analysis of pull-down and input bands at the same exposure times, taking into consideration the percentage of total loaded in input lane.

2.5. HFK lifespan assay

FcaPV2 E6E7, E6 and E7 were cloned in pLXSN retroviral vector using the primers detailed in Supplementary material 1, and retroviral supernatants generated by transient transfection of Phoenix cells with the recombinant retroviruses were used to transduce HFK from two different donors, as previously described (Cornet et al., 2012; Pear et al., 1993).

The population doublings (PD) of the obtained cell lines were counted as reported elsewhere (Caldeira et al., 2003).

2.6. Tissue sample collection

Ten normal skin samples (N1–N10) were collected during routine sterilization surgeries at the Veterinary Teaching Hospital of the University of Naples, Italy. One oral SCC (T1), eight cutaneous SCCs (T2–T9) and two non-SCC skin lesions (T10 and T11) were also collected; biopsies were taken from histologically diagnosed lesions of hospitalized cats during normal clinical activities at the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II (T1 and T11) or at private clinics in different regions of Southern Italy with the help of practitioners (T2 and T5–T8); samples T3, T4 and T9 were sent

Table 1.
FcaPV2 DNA detection and mRNA expression in tissue samples. A fragment of L1 gene was amplified by PCR to check for viral DNA. RT-PCR amplifying fragments of E2, E6 and E7 genes was performed to assess gene expression at the mRNA level. Animals' breed, age, anatomical site of sampling and diagnosis are indicated, when known (ESH: European shorthair; UK: unknown; /: not assessable; NA: not assessed; SCC, squamous cell carcinoma).

Sample	Breed	Age (years)	Anatomical site	Diagnosis	FcaPV2 DNA	FcaPV2 mRNA		
					L1	E2	E6	E7
T1	ESH	12	Mandible	SCC	+	+	+	+
T2	ESH	15	Right ear	SCC	+	−	−	−
T3	Siamese	10	UK	SCC	+	+	+	+
T4	ESH	UK	UK	SCC	+	−	−	+
T5	ESH	UK	UK	SCC	+	+	+	+
T6	ESH	UK	UK	SCC	+	+	+	+
T7	ESH	UK	Right ear	SCC	+	+	+	+
T8	ESH	UK	Thorax	SCC	+	NA	NA	NA
T9	Siamese	14	UK	SCC	+	/	/	/
T10	Burmese	3	Left supraorbital region	Epithelial dysplasia	−	NA	NA	NA
T11	ESH	14	Right forepaw	Sarcoma	−	NA	NA	NA
N1	ESH	UK	Abdominal region	Healthy skin	+	+	+	+
N2	ESH	UK	Abdominal region	Healthy skin	+	−	+	−
N3	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA
N4	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA
N5	ESH	UK	Abdominal region	Healthy skin	+	−	−	−
N6	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA
N7	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA
N8	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA
N9	ESH	UK	Abdominal region	Healthy skin	+	−	−	−
N10	ESH	UK	Abdominal region	Healthy skin	−	NA	NA	NA

from the University of Veterinary Medicine of Vienna, Austria, and sample T10 was sent from the Ion Ionescu de la Brad University of Agriculture Sciences and Veterinary Medicine of Iaşi, Romania. Details on animals' breed, age, anatomical sites of sampling and diagnosis are summarized in Table 1. All the samples were collected under local or total anaesthesia without causing any pain or suffering to the animals and immediately stored in RNA*later*® RNA stabilization solution (Life Technologies) at −80 °C.

2.7. PCR and RT-PCR

Total DNA was extracted from tissue samples with the DNeasy Blood and Tissue Kit (Qiagen) and subjected to PCR amplifying a specific fragment of the FcaPV2 L1 gene using the AmpliTaq Gold DNA Polymerase kit (Applied Biosystems). PCR products were purified using the MinElute Gel Extraction Kit (Qiagen) and sent to the CEINGE Advanced Biotechnologies Research Center in Naples for sequencing. The sequences were aligned to the FcaPV2 L1 gene sequence using the Basic Local Alignment Search Tool (NCBI/BLAST).

RNA was obtained using the RNeasy Mini Kit (Qiagen). Sample T9 was not available for RNA extraction. RT to cDNA was performed using the iScript™ cDNA synthesis kit (Bio-Rad). PCR was carried out to amplify specific fragments of the FcaPV2 E2, E6 and E7 genes and feline GAPDH. Details of primers are presented in Supplementary material 1. Randomly chosen samples were sequenced, confirming the identity of the amplicons.

FcaPV2 genome cloned into pBluescript II-KS$^+$ vector (kindly provided by Professor C.E. Lange, Vetsuisse Faculty, Zurich, Switzerland) was used as positive control for the reactions.

2.8. Statistical analysis

For statistical analysis, the independent sample *t*-test ($p < 0.05$ and/or $p < 0.01$) was performed using SPSS 13 software.

3. Results and discussion

Feline SCCs are malignant tumours frequently associated with DNA of FcaPV2, which potentially encodes E6 and E7 putative oncogenes (Munday, 2014b; Thomson, 2007). However, no data are available so far regarding the possible transforming properties of FcaPV2 E6 and E7 and the biological activity of the virus *in vivo*.

E6 and E7 proteins of oncogenic HPV exert their transforming activity mainly by impairing the key cellular pathways regulated by p53 and pRb, respectively (Tommasino, 2014). Thus, FcaPV2 E6E7, E6 and E7 were stably expressed in CRFK epithelial cells (Fig. 1A) and the p53 and pRb pathways investigated. Mucosal HR HPV and cutaneous HPV49 E6 bind to p53, inducing its degradation via the proteasome pathway (Cornet et al., 2012; Scheffner et al., 1990). We performed a GST pull-down assay and demonstrated that FcaPV2 E6 bound to feline p53, suggesting similar properties to those of HR HPV E6 (Fig. 1B). The p53 protein appeared as a double band in both the input and GST-E6 protein lanes, indicating that it was expressed in different post-translational isoforms (Solyakov et al., 2009), which both preserved the ability to form a complex with E6 in our cell systems. Densitometric analysis revealed that E6 mainly bound the upper p53 isoform in feline and human cell lysates (35% and 40%, respectively), with respect to human and feline p53 lower isoform (2%); the biological meaning of this preferential binding of E6 to the slower p53 isoform and the biochemical features of bound p53 remain to be established in future studies. Consistently, we found that p53 protein was significantly downregulated in CRFKE6E7 and CRFKE6 cells but, surprisingly, also in CRFKE7 cells compared with mock cells (CRFKpCEFL) (Fig. 1C and D). Hence, we checked for p53 transcript levels in the cell lines and observed that the presence of E7 but not E6 always correlated with lower mRNA levels, indicating that the two viral oncoproteins can affect p53 by two distinct mechanisms (Fig. 1E). Several cutaneous HPV are known to inhibit UVB-induced cell cycle arrest and apoptosis by impairing p53 transcriptional functions, therefore constituting a possible mechanism contributing to skin carcinogenesis (Accardi et al., 2006; Giampieri et al., 2004; Jackson et al., 2000; Jackson and Storey, 2000; Struijk et al., 2008; Viarisio et al., 2011; White et al., 2014). Here, we showed that UVB-triggered accumulation of p53 and, consistently, p21 were inhibited in CRFKE6E7, CRFKE6 and CRFKE7 cells (Fig. 1F–H). Conversely, the accumulation of the p53 upper band in irradiated CRFKpCEFL cells confirmed that it reflects post-translational modifications, which are known to contribute to

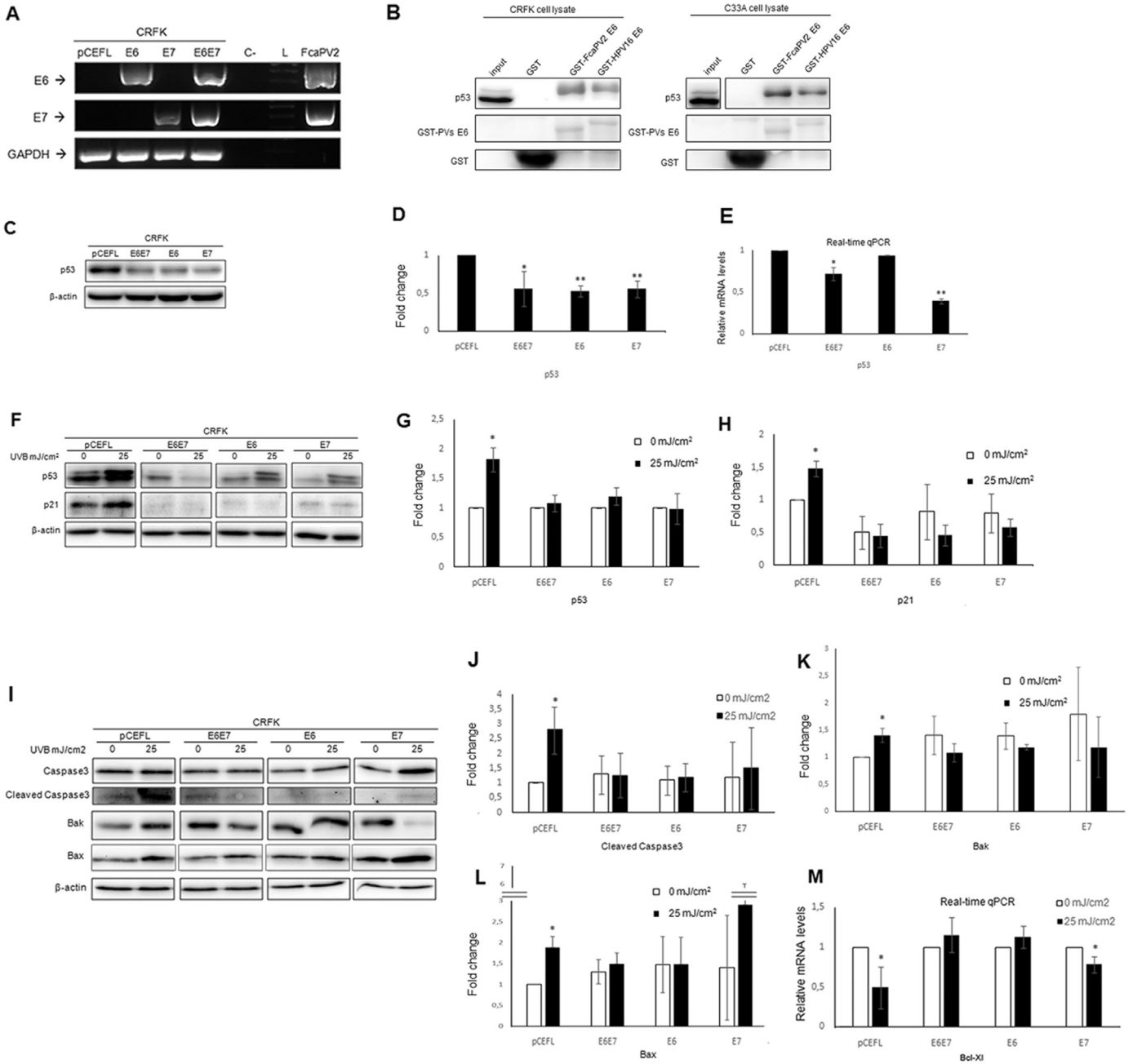

Fig. 1. FcaPV2 E6 binds to feline p53, and both E6 and E7 impair the p53 pathway and UVB-induced apoptosis. (A) RT-PCR gel demonstrating expression of the indicated FcaPV2 oncogenes transfected in CRFK cells. FcaPV2 genome was used as positive control for the amplifications. (L: DNA ladder; C-: no template). (B) A representative GST pull-down assay showing interaction of FcaPV2 E6 with feline p53 in CRFK cell lysate (left panel). The interaction of HPV16 E6 with human p53 in C33A cells was demonstrated as positive control (right panel). The blots are cut as they are shown at different exposure times; the bands were properly aligned basing on the migration with respect to molecular marker loaded onto the gel. (C) WB showing deregulation of p53 protein levels upon expression of FcaPV2 oncogenes in the generated cell lines. β-actin staining was included as a loading control. (D) Mean densitometric values and standard deviations (SD) from independent experiments normalized to β-actin are expressed as fold change relative to CRFK cells expressing empty pCEFL ($^{*}p < 0.05$; $^{**}p < 0.01$). (E) Real-time qPCR data demonstrating downregulation of p53 transcripts in the presence of FcaPV2 E7 oncogene expression. (F) WB analysis of the generated cell lines with or without UVB irradiation, showing inhibition of UVB-triggered accumulation of p53 and p21 upon expression of FcaPV2 oncogenes. (G) Mean densitometric values and SD from independent experiments for p53 are presented as fold change relative to non-irradiated control for each cell line, set equal to 1. In this case, statistical analysis was performed comparing each irradiated cell line with the respective non-irradiated counterpart. (H) Mean densitometric values and SD from independent WB experiments for p21 expressed as fold change relative to CRFK cells expressing empty pCEFL. (I) Representative WB showing inhibition of induction of pro-apoptotic markers after UVB irradiation in cells expressing E6E7, E6 and E7. (J, K, L) Mean densitometric values and SD from independent experiments for Cleaved Caspase3, Bak and Bax are shown as fold change relative to non-irradiated cells selected for empty vector. (M) Real-time qPCR analysis demonstrating inhibition of Bcl-Xl downregulation after UVB treatment by FcaPV2 oncogenes. As for (G), statistical analysis was performed comparing each irradiated cell line with the respective non-irradiated control.

its stabilization and activation upon genomic insult (Solyakov et al., 2009).

In addition, we detected a significant induction of pro-apoptotic markers such as Cleaved Caspase3, Bax and Bak in UVB-treated mock cells but not in cells expressing FcaPV2 oncogenes (Fig. 1I–L). It is worth noting that expression of viral gene products did not result in lower basal levels of Bak and Bax; this may indicate that, unlike those of oncogenic HPV, FcaPV2 E6 and E7 do not target Bax and Bak for protein degradation and act through diverse mechanisms that, rather, block their accumulation (Du

et al., 2004; Liu et al., 2008; Magal et al., 2005; Vande Pol and Klingelhutz, 2013). Consistently, FcaPV2 oncogenes impaired UVB-induced downregulation of the pro-survival gene Bcl-Xl (Fig. 1M). However, the anti-apoptotic activity of E7 was less impressive than that of E6; thus, further studies are needed to better clarify this issue. Considered overall, these findings suggest that FcaPV2 may have evolved different strategies to redundantly impair the p53 pathway by E7 other than by E6, as already demonstrated for HPV16 and HPV38 (Guo et al., 2011; Kim et al., 2013, 2012; Lee et al., 2011; Saidj et al., 2013). However, whether inhibition of apoptosis by FcaPV2 oncogenes is mechanistically dependent upon impairment of p53 remains to be established. Taken together, our data strongly suggest that FcaPV2 oncogenes may synergize with UV exposure in triggering feline skin cancer, as already hypothesized for cutaneous HPV in NMSC (Accardi and Gheit, 2014; Howley and Pfister, 2015).

Next, we characterized the impact of viral oncoproteins on the pRb pathway. E7 of mucosal HR HPV and several cutaneous HPV bind to pRb protein through the pRb binding domain LXCXE and direct it towards proteasomal degradation (Dyson et al., 1992; Huh

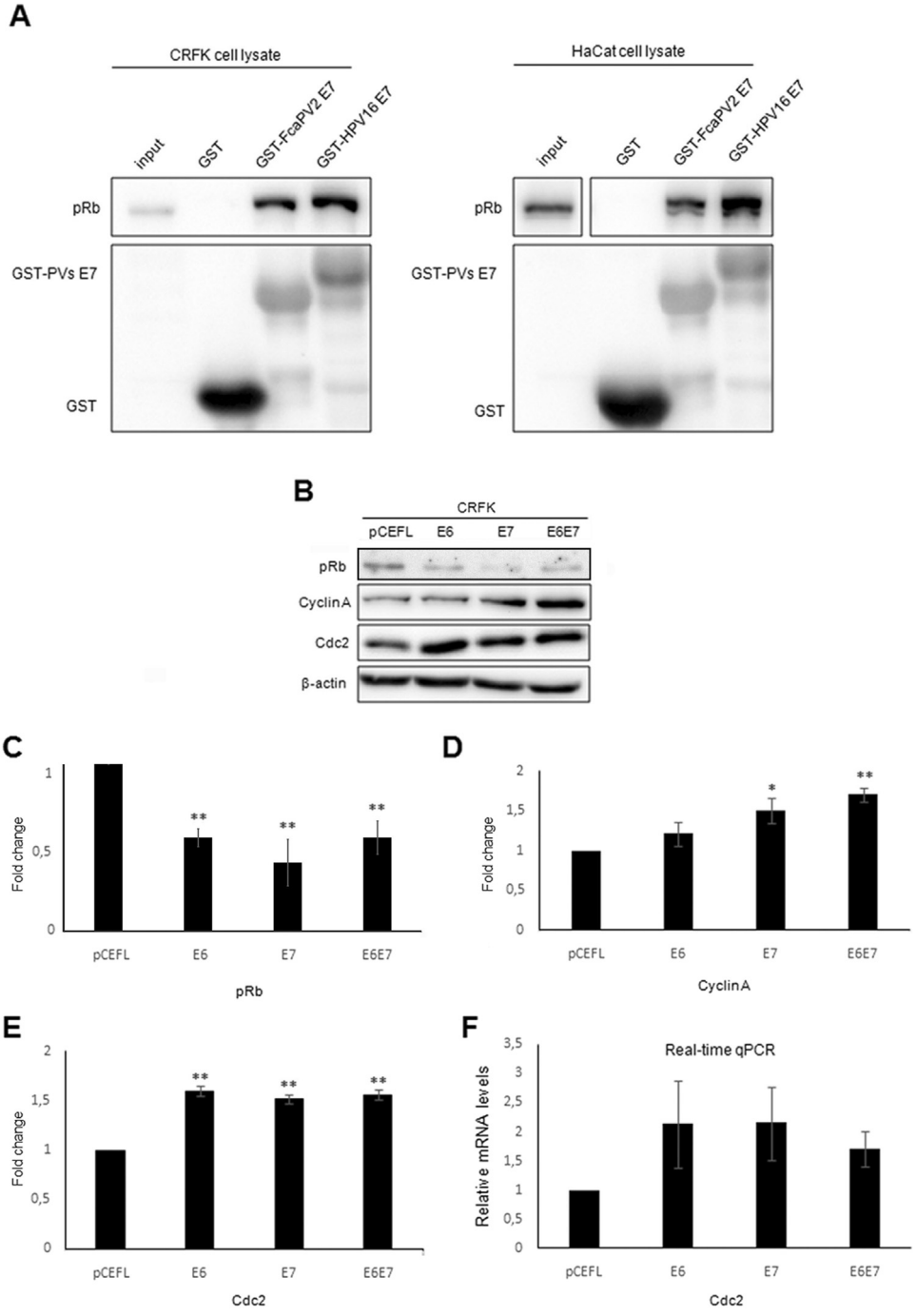

Fig. 2. FcaPV2 E7 binds to feline pRb, and both E7 and E6 alter the pRb pathway. (A) GST pull-down assay showing interaction of FcaPV2 E7 with feline pRb in CRFK cell lysate (left panel). The interaction of HPV16 E7 with human pRb in HaCaT cells was detected as positive control (right panel). Separate scan at different exposure times are shown to clearly appreciate pRb doublet expressed in HaCat cells both in input and pull-down lanes; they were aligned accordingly to their migration relative to the molecular marker. (B) Representative WB showing downregulation of pRb protein levels and upregulation of Cyclin A and Cdc2 upon expression of FcaPV2 oncogenes in CRFK cells. β-actin was included as a loading control. (C, D, E) Mean densitometric values and SD from independent experiments normalized to β-actin are expressed as fold change relative to CRFK cells expressing empty pCEFL ($^{*}p < 0.05$;$^{**}p < 0.01$). (F) Real-time qPCR data confirming Cdc2 upregulation at the mRNA level.

et al., 2007). FcaPV2 E7 contains the LXCXE motif (Lange et al., 2009), and indeed it was able to bind both feline and human total pRb with high efficiency (60% and 100%, respectively). Our data also show that both FcaPV2 E7 and HPV16 E7 complex with the upper isoform of pRb doublet in HaCat cells corresponding to phospho-pRb (Boyer et al., 1996) with an efficacy of 22% and 32%, respectively (Fig. 2A). In addition, we showed that the presence of E7 resulted in lower pRb protein levels, thus suggesting that E7 may similarly degrade pRb (Fig. 2B and C). Interestingly, E6 was also responsible for such an effect when expressed individually (Fig. 2B and C), although no physical interaction with pRb was detected by GST pull-down assay (data not shown). These findings are in accordance with previous studies showing lower pRb expression and increased p16 expression in feline SCCs harbouring FcaPV2 (Munday and Aberdein, 2012), indicating a similar impact of viral oncogenes on pRb protein *in vivo*. Deregulation of pRb leads to the release of the E2F transcriptional repressor, which in turn activates the expression of downstream pro-proliferative genes such as Cyclin A and Cdc2 (Cornet et al., 2012; Dyson et al., 1992; Huh et al., 2007; Zerfass et al., 1995). Consistently, Cyclin A and Cdc2 proteins were upregulated in CRFKE6E7 and CRFKE7 cells, while in CRFKE6 cells Cdc2 but not Cyclin A was overexpressed, suggesting different mechanisms of action by E6 and E7 on the pRb/E2F pathway and cell cycle checkpoints (Fig. 2B–E). Molecular analysis confirmed Cdc2 upregulation at the mRNA level (Fig. 2F). Further studies are needed to understand the mechanism by which FcaPV2 E6 may target the pRb pathway.

Oncogenic human and animal PV are able to efficiently increase the lifespan of human primary keratinocytes (Caldeira et al., 2003; Ganzenmueller et al., 2008). Here, we showed that FcaPV2 E6E7 and FcaPV2 E7 recombinant pLXSN retroviruses increased the lifespan and the proliferative ability of HFK compared with empty retrovirus and FcaPV2 E6 transduced cells (Fig. 3A and B and Supplementary material 3). A similar scenario is reported for HPV16 and HPV38 and, in veterinary oncology, for cottontail rabbit PV, suggesting that E7 and E6 make different contributions to the transforming potential of FcaPV2 as well (Caldeira et al., 2003; Ganzenmueller et al., 2008). Although these results indicate the ability of FcaPV2 oncogenes to deregulate the senescence programme of primary cells, they should be further confirmed in feline keratinocytes.

Having demonstrated the transforming properties of FcaPV2 oncogenes *in vitro*, we investigated whether FcaPV2 was biologically active *in vivo* in a series of feline SCCs, non-SCC skin lesions and normal skin samples (data not shown). We detected the presence of L1 viral gene DNA in 100% of cutaneous SCCs and, for the first time, in one oral SCC, versus 40% of normal skin samples; most importantly, by RT-PCR for E2, E6 and E7 mRNA, the virus was found to be transcriptionally active in 67% of SCC lesions and in only 20% of normal skin samples (Table 1), strengthening the rationale for this work. Expression of E2 suggested that the virus was biologically active and possibly present in episomal form (Munday, 2014a). Thus, we performed rolling circle amplification (RCA) as previously described (Paolini et al., 2015) (data not shown). Circular PV DNA was detected in 1 of 3 analysed SCC samples, indicating that expression of E2 is independent of the physical status of the virus. This is consistent with recent findings showing that HPV genome integration into the host cell does not necessarily disrupt the E2 open reading frame and that E2, E6 and E7 are concomitantly expressed in HPV-driven cancer (Olthof et al., 2015, 2014; Ramirez et al., 2015).

Furthermore, we analysed p53 mRNA levels by real-time qPCR on a subset of tissue samples and found that p53 transcript was downregulated in most of the analysed SCC samples compared with normal skin samples (see Supplementary material 4), confirming the *in vitro* findings; this result reinforces the reliability of

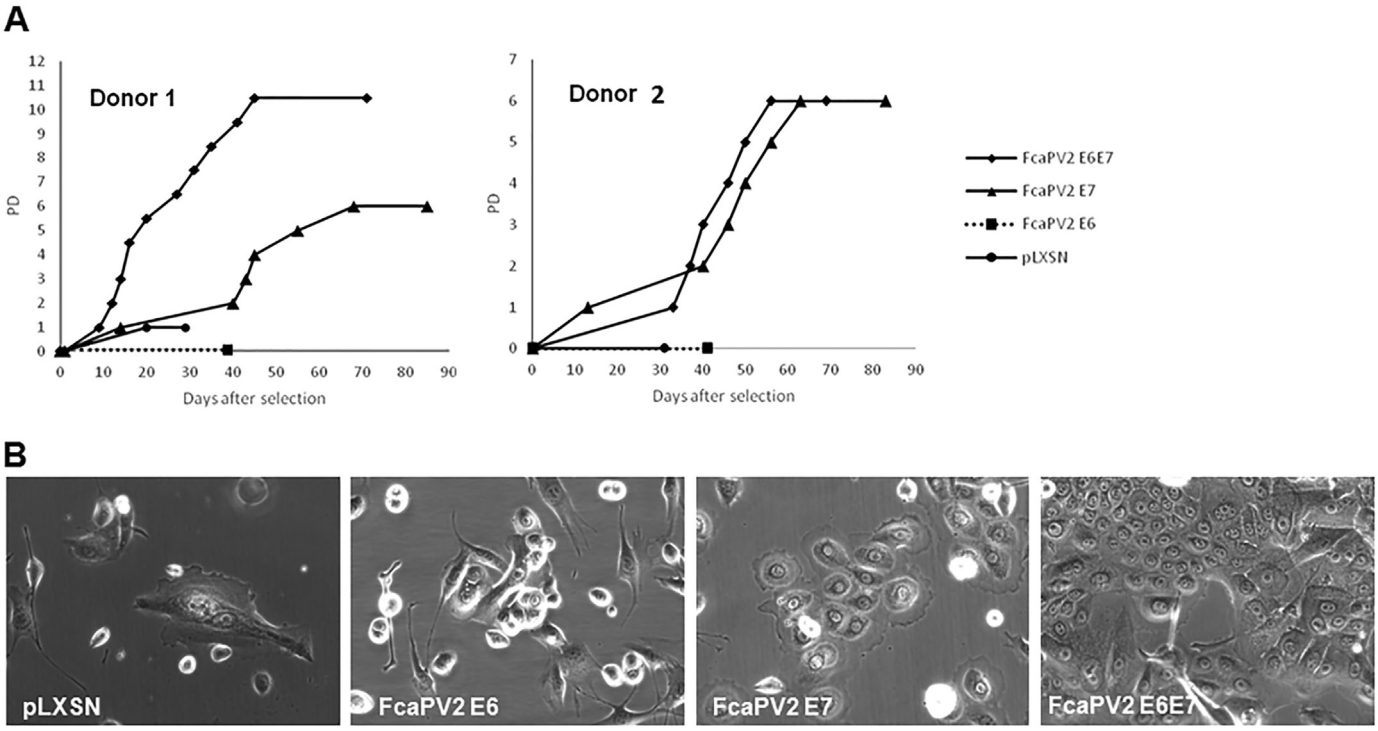

Fig. 3. FcaPV2 prolongs the lifespan of HFK. (A) Growth curves of HFK from two different donors transduced with the indicated recombinant retroviruses, showing the prolonged lifespan and the enhanced proliferative ability conferred by FcaPV2 E6E7 and FcaPV2 E7 compared with control cells (pLXSN). (B) Morphology of the generated cell lines 20 days after transduction. Note the features of cellular senescence in pLXSN and FcaPV2 E6 transduced cells with irregular shape, enlarged cytoplasm and intercellular bridges. Magnification 20 ×.

our experimental model and suggests that FcaPV2 may display transforming activity in the infected epithelia through the molecular activity of both E6 and E7 oncogenes.

4. Conclusions

In this study we characterized, for the first time, the biological properties of FcaPV2 E6 and E7 oncogenes, showing that they display transforming properties in feline cells by impairing the canonical p53 and pRb pathways, possibly also through unreported cross-acting mechanisms on the reciprocal pathways. Therefore, our new *in vitro* model paves the way for future studies to better define the biochemical features and the molecular basis of the transforming ability of FcaPV2 oncoproteins. Finally, expression of E6 and E7 in both oral and skin SCCs *in vivo*, along with the observation that they share several biological properties with mucosal HPV, suggest a mixed tropism of this virus and corroborate the possible causative role of FcaPV2 in the development of feline SCC.

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Authors' contributions: G. Altamura performed most of the experiments *in vitro*; A. Corteggio performed most of the investigations *in vivo*; A. Conte and G.M. Pierantoni significantly contributed to cloning procedures; L. Pacini performed retroviral infections. G. Altamura, M. Tommasino, R. Accardi and G. Borzacchiello conceived the study and drafted the manuscript.

Part of the work reported in this paper was undertaken while the first author was hosted by the International Agency for Research on Cancer. The manuscript was edited by Karen Müller.

The authors declare they do not have any conflicting interests relevant to this study.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.05.017.

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