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Expression of the immediate early IE180 protein under the control of the hTERT and CEA tumor-specific promoters in recombinant pseudorabies viruses: Effects of IE180 protein on promoter activity and apoptosis induction

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ABSTRACT

Since the pseudorabies virus (PRV) genome encodes for a single immediate-early protein, IE180, we reasoned that this strong transactivating protein could represent a key regulatory switch that could be genetically manipulated in order to alter its tropism towards cancer cells. We therefore initiated studies to test whether the human telomerase reverse transcriptase (hTERT) and carcinoembryonic antigen (CEA) tumor promoters could functionally replace the IE180 promoter. We show that both promoters can functionally substitute the IE180 promoter in plasmid constructs and recombinant viruses, and observed that IE180 differentially auto-regulated each promoter tested, with PRV IE180 negatively regulating the hTERT promoter but positively hyper-activating the CEA promoter. Interestingly, we also observed that the recombinant PRV-TER and PRV-CEA viruses preferentially replicated in diverse cancer cell lines compared to control non-cancer cells, and the PRV-CEA was capable of additionally inducing a profound apoptotic phenotype which we correlated to the overexpression of IE180.

Introduction

The *Herpesviridae* family is divided into the three subfamilies *alpha-*, *beta-*, and *gammaherpesvirinae*. The *alphaherpesvirinae* subfamily includes herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), pseudorabies virus (PRV), equine herpesvirus (EHV1), and bovine herpesvirus 1 (BHV1) (Pellet and Roizman, 2007). Productive infection of cells by members of the *alphaherpesvirinae* subfamily is typified by a short reproductive cycle that can be subdivided into three temporally regulated phases, immediate-early (IE or α), early (E or β), and late (L or γ). IE genes are the first viral genes expressed at the mRNA level after infection, and they are expressed even in the presence of inhibitors of cell protein synthesis. This suggests that only host factors are required for their expression. Expression of E genes during infection is dependent on IE proteins while expression of L genes is dependent on DNA replication (Roizman et al., 2007). HSV-1 has six IE genes: α0, α4, α22, α27, α47 and US1.5, which encode infected-cell proteins (ICPs) 0, 4, 22, 27, 47 and US1.5 respectively. ICP4 is the major viral regulatory protein essential for the replicative cycle of HSV-1 (Roizman et al., 2007). In comparison, PRV has only one IE gene which encodes for the IE180 protein (Ihara et al., 1983; Mettenleiter, 2000; Tombácz et al., 2009). Both ICP4 and IE180 therefore represent critical viral transactivator proteins necessary for replication initiation via regulation of viral gene expression, either positively or negatively (Ihara et al., 1983; Leopardi et al., 1995). In addition, these proteins also show a high level of autoregulation and have been shown to influence not only gene expression of other viruses but also the promoters of mammalian cells (Green et al., 1983; Wu and Berk, 1988; Yuan et al., 1989; Wong et al., 1997; Martin et al., 1990).

Oncolytic virotherapy is an emerging field in which replicative competent modified viruses are used to treat diseases, such as cancer. HSV-1 has emerged as a very promising candidate in oncolytic virotherapy because of our indepth knowledge and understanding of its genome and due to the fact that HSV-1 exerts a generally moderate pathogenicity in humans (Campadelli-Fiume et al., 2011). PRV has also been suggested as an oncolytic agent for human tumors as it shares the same advantages described for HSV-1, but at the same time has additional inherent advantages including the absence of virulence, recombination and seroprevalence in the human population

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(Sawitzky, 1997; Boldogköi et al., 2002; Prieto et al., 2002; Boldogköi and Nogradi, 2003; Wollmann et al., 2005). In general, the success of oncolytic therapy is directly linked to the capacity of the virus in question to specifically replicate in cancer cells. The most established strategy for post-entry restriction of gene expression or viral oncolysis is transcriptional regulation (Dorer and Nettelbeck, 2009). For transcriptional regulation of oncolytic virus replication, promoters of essential viral genes have been replaced with specific cellular promoters that are only active or hyperactive in cancer cells. For example, PRV recombinant mutants carrying glycoprotein D and HSV-1 thymidine kinase genes under transcriptional control of the human HER-2/neu promoter have been studied in bladder cancer cells (Shiau et al., 2007). Due to the strict temporal regulation of herpes virus replication, tight transcriptional regulation can be theoretically achieved by placing an essential IE protein (e.g. ICP4 and IE180) under the control of tumor-specific promoters; however, it cannot be ignored that IE proteins themselves can auto regulate both viral and mammalian promoters. Thus, an important initial step that needs to be examined, even before constructing herpes-based oncolytic vectors, is the effect that such gene modifications can have on viral replication and/or cellular functions (e.g. apoptosis).

In the present study we initiated experiments to primarily and directly address the aforementioned caveat: can a tumor specific promoter control IE180 expression and does IE180 in turn auto regulate these promoters. CEA is a cell membrane-bound glycoprotein and a member of the immunoglobulin supergene family. Initially believed to be an oncofetal antigen, it has also been observed to be expressed in normal adult tissue (Hammarstrom, 1999). hTERT, the catalytic subunit of telomerase, is repressed in somatic tissues but is up regulated in more than 90% of human cancers (Shay and Bacchetti, 1997; Liu et al., 2004). Utilizing these two promoters, we analyzed the capacity of both mammalian promoters to control viral IE expression and at the same time determined the capacity of the PRV IE180 and HSV-1 ICP4 proteins to autoregulate these promoters. Herein we show that PRV IE180 protein expression can be governed by CEA and hTERT human tumor-specific promoters, as both promoters can functionally substitute the IE180 promoter in PRV-CEA and PRV-TER recombinant viruses. Surprisingly, we observed differences between promoter activity, the capacity of viral IE proteins to differentially autoregulate the promoters tested and a specific apoptotic phenotype induced by IE180 when under the control of the CEA promoter. These data therefore demonstrate the capacity of herpes IE proteins to auto regulate their own expression regardless of the promoter driving their transcription, and at the same time show a previously unrecognized pro-apoptotic capacity associated with IE180 overexpression, a property that could be potentially exploited to target cancer cells if developing PRV-based oncolytic vectors.

Results

Analysis of EGFP expression under the control of the hTERT and CEA promoters

The behavior of the hTERT and CEA promoter constructs was first analyzed by assessing, in parallel, the expression of IE180 protein levels and its activity using EGFP as a surrogate marker of gene transactivation. Plasmids containing the hTERT and CEA promoters, including mRNA leader regions of 62 nt and 110 nt, respectively, driving the IE180-coding sequence were constructed and the ability of the tumor promoters to drive IE180 expression was assayed by western blot analysis (Fig. 1, panel A), and their activity was determined by assessing the transactivation of the gG promoter in the presence of the IE180 protein via the expression of

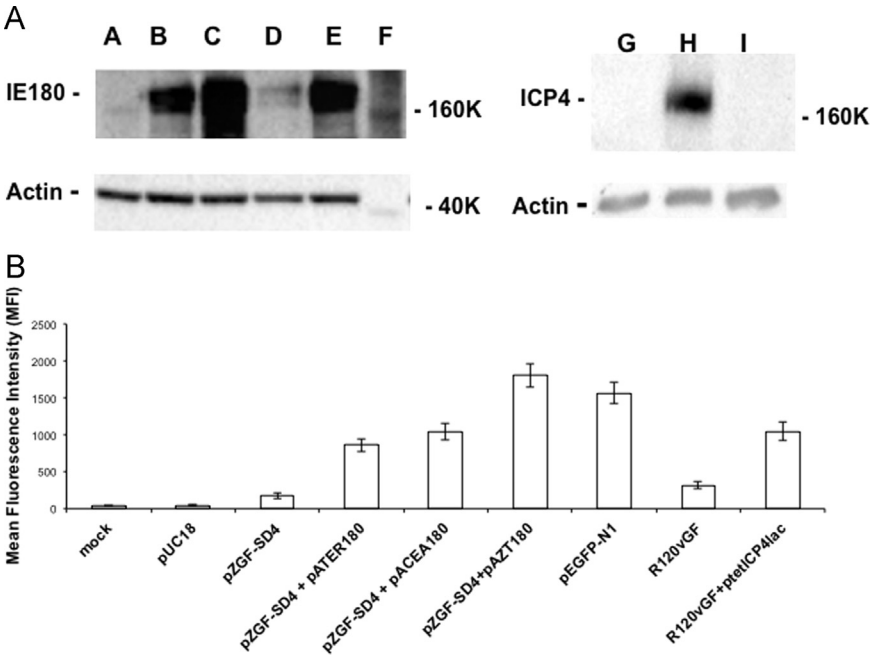

Fig. 1. hTERT and CEA promoter-mediated IE180 expression. Panel A. Hela Tet-Off cells were mock transfected (lanes A and G) or transfected with 5 μg of pRIE180 (lane B), pAZT180 (lane C), pATER180 (lane D), pACEA180 (lane E), ptetICP4lac (lane H) or pEGFPN1 (lane I) plasmids. Protein lysates were collected at 48 h posttransfection in RIPA buffer and Western blot analysis was performed for IE180, HSV-1 ICP4 or Actin, the latter serving as a loading control. Novex sharp pre-stained protein standard (Life Technologies) (lane F) was included. Panel B. hTERT and CEA promoter-mediated IE180 expression were additionally evaluated in parallel transactivation assays. Cotransfection of pATER180 (4 μg), pACEA180 (4 μg), and pAZT180 (4 μg), with pZGF-SD4 (1 μg), produces transactivation of the gG promoter resulting in increased EGFP expression. EGFP protein expression was determined by flow cytometry 24 h posttransfection. pEGFP-N1 (1 μg) and pUC18 (1 μg) were included as transfection controls. The activity of HSV-1 ICP4 protein expressed by ptetICP4lac plasmid was studied by viral transactivation in HeLa Tet-Off cells transfected with 5 μg of ptetICP4lac DNA and infected with R120vGF virus (MOI of 0.05 $TCID_{50}$/cell), and EGFP protein expression was determined by flow cytometry 24 h p.i. Each value represents the mean and standard deviation (error bars) from three independent experiments.

EGFP when these plasmids were cotransfected with pZGF-SD4 into HeLa Tet-Off cells (Fig. 1, panel B). IE180 protein expression from the CEA promoter with the plasmid pACEA180 (Fig. 1, panel A, lane E) was 2.3 fold higher than that achieved with the endogenous IE180 promoter using the plasmid pRIE180 (Fig. 1, panel A, lane B), while the plasmid pAZT180 that expresses PRV IE180 under the control of the tetracycline-responsive promoter (Fig. 1, panel A, lane C) expressed IE180 4.5 fold more than the plasmid pRIE180 (Fig. 1, panel A, lane B). In contrast, while IE180 expression under the control of hTERT promoter with the plasmid pATER180 (Fig. 1, panel A, lane D) was functional, IE180 protein levels were 5.6 fold less than that achieved with the endogenous IE180 promoter using the plasmid pRIE180 (Fig. 1, panel A, lane B). The activity of IE180 expressed in Hela Tet-Off cells was next assayed by transactivation of the PRV gG promoter using the plasmid pZGF-SD4, which expresses EGFP under control of PRV gG promoter (Muñoz et al., 2010). The transactivation of the gG promoter in the presence of the IE180 protein, via the expression of EGFP, was quantified by flow cytometry (Fig. 1, panel B). The expression of EGFP under the control of the CMV-IE promoter (plasmid pEGFPN1) was 9.3 fold higher than when under the control of the gG promoter (plasmid pZGF-SD4). The transactivation of EGFP expression under the control of gG promoter, however, increased 5.2 fold when IE180 was produced under the control of the hTERT promoter (plasmid pATER180) and 6.3 fold under the control of the CEA promoter (plasmid pACEA180), which correlates well with the robust IE180 protein expression observed using the CEA promoter (Fig. 1, panel A, lane E) compared to the hTERT promoter (Fig. 1, panel A, lane D). When compared to the tetracycline-responsive promoter (Ptet) (plasmid pAZT180) (Fig. 1, panel A, lane C and panel B), however, the production of IE180 protein and EGFP transactivation with the two tumor-specific promoters in Hela Tet-Off cells was lower (Fig. 1, panel A, lanes D, E and panel B).

Effect of PRV-IE180 and HSV-1-ICP4 proteins on the activity of the hTERT and CEA tumor-specific promoters

To examine the role of PRV-IE180 on the activity of the hTERT and CEA promoters, and to compare the activity of IE180 with that of the HSV-1-ICP4 protein, EGFP expression assays were performed with reporter plasmids containing the hTERT promoter fused to the EGFP reporter gene (pZTERGF) (Fig. 2, panel A) or the CEA promoter fused to the EGFP reporter gene (pACEA-FG) (Fig. 2, panel B). The expression levels of EGFP were measured by flow cytometry analysis. Each of these reporter plasmids was co-transfected into Hela Tet-Off cells alone or with plasmids that express PRV-IE180 (pAZT180) or HSV-1-ICP4 (ptetICP4lac) proteins under the control of the tetracycline-responsive promoters. ICP4 expression by the ptetICP4lac plasmid was confirmed by western blot (Fig. 1, panel A, lane H) and its activity validated by positive transactivation of the ICP4 deficient GFP-expressing virus R120vGF (Fig. 1, panel B). Compared to control samples, the presence of the HSV-1 IE protein ICP4 increased EGFP expression from the hTERT promoter 2.5 fold (Fig. 2, panel A) and 3.2 fold from the CEA promoter (Fig. 2, panel B). Interestingly, the presence of the PRV IE180 increased EGFP expression from the CEA promoter 4.8 fold (Fig. 2, panel B), but reduced EGFP expression from the hTERT promoter 3.0 fold (Fig. 2, panel A). Importantly, this inhibitory phenotype could be reproduced in a parallel assay where IE180 was expressed from its own viral promoter using the plasmid pRIE180 (Fig. 3). Specifically, while IE180 transactivated the PRV DNA polymerase (pol) (UL30 gene) (p30GF plasmid) and gG (pZGF-SD4 plasmid) promoters by 7.2 fold, and 6.6 fold, respectively, IE180 again reduced hTERT promoter activity by 2.4 fold (Fig. 3). These results demonstrate that the IE180 protein represses the hTERT promoter but hyper-activates the CEA promoter. In addition, these data are in concordance with the IE180 western blot results observed with the pATER180 and pACEA180 plasmids (Fig. 1, panel A) in which IE180 protein expression driven by pACEA180 (Fig. 1, panel A, lane E) was 2.3 fold higher compared to levels achieved with the endogenous viral IE180 promoter, using the plasmid pRIE180 (Fig. 1, panel A, lane B), while IE180 protein expression driven by the hTERT promoter, with the plasmid pATER180 (Fig. 1, panel A, lane D), was 5.6 fold less compared to pRIE180 (Fig. 1, panel A, lane B).

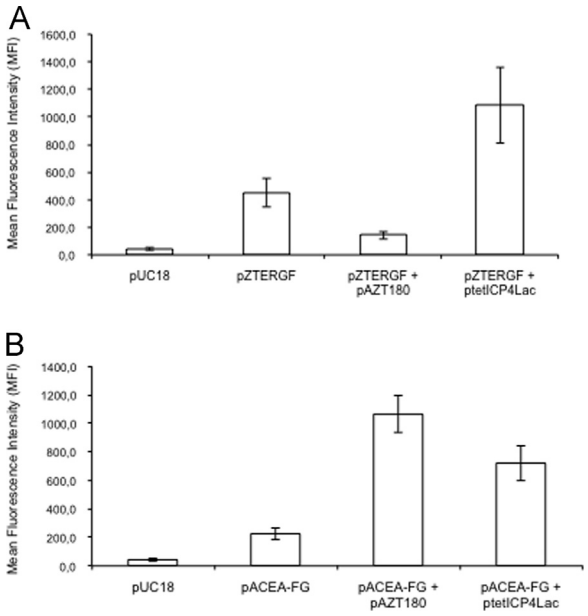

Fig. 2. Induction of EGFP expression from the hTERT and CEA promoters mediated by PRV IE180 and HSV-1 ICP4 proteins. Panel A. The influence of PRV IE180 or HSV-1 ICP4 on the activation of the hTERT promoter (plasmid pZTERGF, 2 μg) was studied in Hela Tet-Off cells by co-transfection with the plasmid pAZT180 (2 μg), which expresses IE180, or with ptetICP4lac (2 μg), which expresses ICP4. Plasmid pZTERGF expresses EGFP under the control of the hTERT promoter. EGFP protein expression was determined by flow cytometry 24 h posttransfection. Each value represents the mean and standard deviation (error bars) from three independent experiments. Panel B. The influence of PRV IE180 or HSV-1 ICP4 on the activation of the CEA promoter (plasmid pACEA-FG (2 μg) was studied in Hela Tet-Off cells by co-transfection with the plasmid pAZT180 (2 μg), which expresses IE180, or with ptetICP4lac (2 μg), which expresses ICP4. Plasmid pACEA-FG expresses EGFP under the control of CEA promoter. EGFP protein expression was determined by flow cytometry 24 h posttransfection. Each value represents the mean and standard deviation (error bars) from three independent experiments.

Expression of PRV IE180 under the control of hTERT and CEA tumor promoters in recombinant pseudorabies viruses

We next assessed the replication of recombinant PRV viruses in which the hTERT or CEA human tumor promoters were used to drive IE180 expression in lieu of its endogenous viral promoter. The transfection of PBAC90 with the hTERT-IE180 cassette of pOTER180-N or the CEA-IE180 cassette of pOCEA180-N into U2OS cells resulted in the production of PRV-TER and PRV-CEA viruses, respectively. Replacement of the PRV IE180 promoter with the hTERT or CEA promoters in the PRV recombinants was confirmed by PCR characterization of packaged viral DNA from PRV-TER, PRV-CEA, the parental virus vBecker2 and PBAC90 (Fig. 4, panel A and B). Specific primers for each promoter P180-S (P_{IE180}), TERT-2D (P_{hTERT}), CEA-S (P_{CEA}) and 180-AS (coding sequence of IE180 gene) were used for amplification (Table 1). The specificity of the amplification products was confirmed by DNA size resolution on a 1% agarose gel: 1201 bp for P_{IE180}-IE180, 710 bp for PhTERT-IE180 and 636 bp for P_{CEA}-IE180 cassettes and 523 bp for DNA polymerase (UL30), which was used as a reference (Fig. 4, panel B).

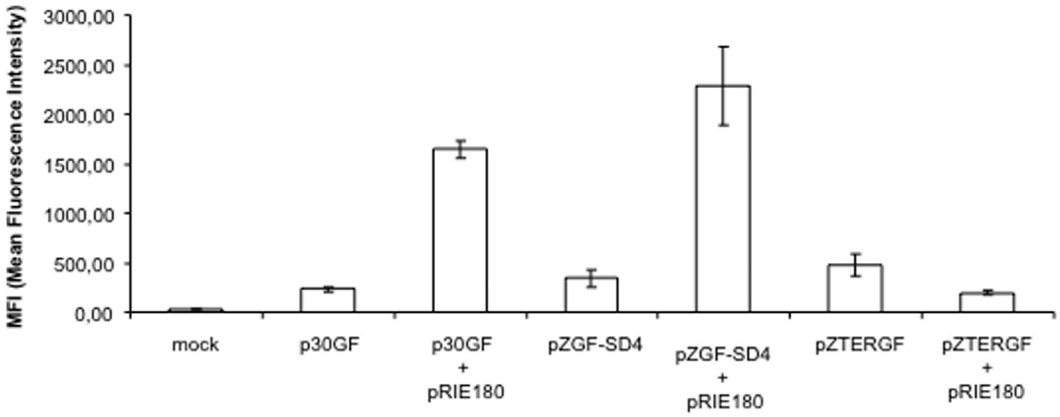

Fig. 3. Induction of EGFP expression from the hTERT promoter mediated by PRV IE180. The influence of PRV IE180, expressed under the control its own promoter (plasmid pRIE180), on the activation of the hTERT promoter (plasmid pZTERGF, 2 μg), PRV gG promoter (plasmid pZGF-SD4) and DNA polymerase (UL30) promoter (plasmid p30GF, 2 μg) was evaluated by co-transfection of pRIE180 (2 μg) with pZTERGF (2 μg) or pZGF-SD4 (2 μg) plasmids in Hela Tet-Off cells. EGFP protein expression was determined by flow cytometry 24 h posttransfection. Each value represents the mean and standard deviation (error bars) from three independent experiments.

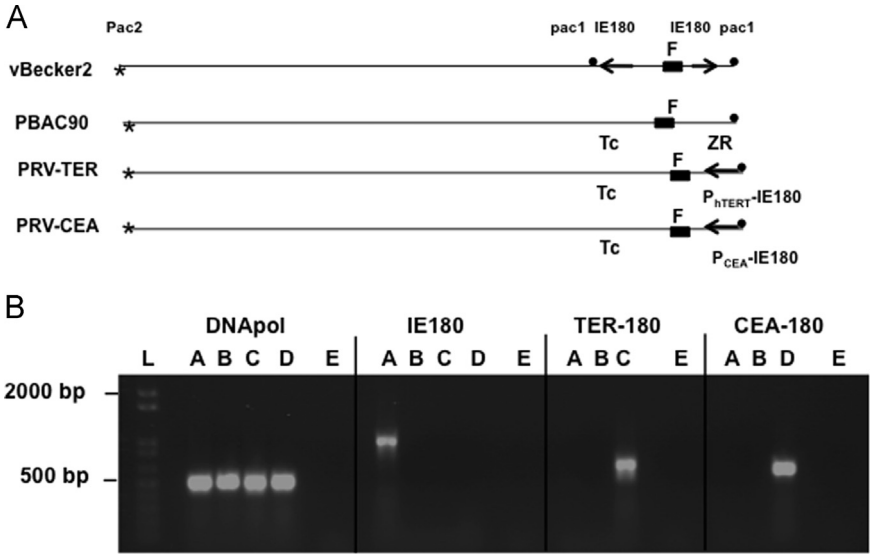

Fig. 4. Characterization of PRV-TER and PRV-CEA recombinant viruses. Panel A. Schematic diagram of the linear genomes of PBAC90 and the recombinant viruses vBecker2, PRV-TER, PRV-CEA, indicating IE180 gene copies (arrows), the *pac*1 (solid circle) and *pac*2 (bold star) packaging sequences, insertion of F BAC replicon (F) (solid rectangle) and genes conferring antibiotic resistance, tetracycline (Tc) and Zeocin (ZR). Panel B. PCR amplification of the viral DNA polymerase (UL30) (DNA size 523 bp), IE180 (Pr-cs) (DNA size 1201 bp), PhTERT-IE180cs (DNA size 710 bp), P_{CEA}-IE180cs (DNA size 636 bp) cassettes from vBecker2 (lane A), PBAC90 (lane B), PRV-TER (lane C) and PRV-CEA (lane D). Negative PCR control without DNA (lane E). PCR was carried out using specific primers P180-S, TERT-2D, CEA-S and 180-AS for the IE180 cassette and DNApol-D and DNApol-R primers for the UL30 gene. A 1 Kbp DNA ladder was used as DNA size control (Invitrogen) (lane L). PCR products were separated by gel electrophoresis in 1% agarose gel.

Specific fragments could be amplified from the PRV-TER (Fig. 4, panel B, lane C), PRV-CEA (Fig. 4, Panel B, lane D) and vBecker2 (Fig. 4, Panel B, lane A) by using specific primers for each promoter but not from the PBAC90 (Fig. 4, Panel B, lane B).

IE180 mRNA expression from these recombinant viruses was then analyzed by quantitative real-time RT-PCR (RT-qPCR) in U2OS cells infected at a MOI of 0.1 $TCID_{50}$/cell. Total RNA was harvested at 12 h and 24 h post-infection (p.i.) and mRNA expression was analyzed by RT-PCR and qPCR by using the IE180 specific primers q180-S and q180-AS (Table 1). β-actin was used as reference cell gene for RTqPCR analyses (Table 1). At 12 h p.i. IE180 mRNA expression in U2OS cells after PRV-TER infection was reduced by 14% compared to cells infected with vBecker2, while at 24 h p.i. a 14.1 fold increase in IE180 mRNA expression was observed (Fig. 5, panel A). In contrast, IE180 mRNA expression in U2OS cells infected with PRV-CEA increased 31.7 fold at 12 h p.i. compared to cells infected with vBecker2 and 32.5 fold at 24 h p.i. (Fig. 5, panel A). This IE180 mRNA overexpression by PRV-CEA at 12 h and 24 h p.i. parallels the IE180 protein detection observed by western blot (Fig. 5, panel B, lanes E and J). The 12 h p.i. results are in concordance with the transfection-based studies detailed above (Figs. 1 and 2) where the presence of IE180 induces the activity of the CEA promoter but reduced the activity of the hTERT promoter. At 24 h p.i., however, the down-regulation of the hTERT promoter by the IE180 protein reversed but the overall levels of IE180 mRNA (Fig. 5, panel A) and protein (Fig. 5, panel B) were consistently lower than those achieved with PRV-CEA.

Characterization of PRV-TER and PRV-CEA: Analysis of apoptosis induction

To characterize the growth of PRV-TER and PRV-CEA in vitro, we determined the yield of progeny virus in non-cancer cells (FP7, human lung fibroblast cell line) and cancer cells (U2OS, human osteosarcoma cell line; Hela, human cervix carcinoma cell line; and HT29, human colon adenocarcinoma cell line). Viral yield of wild-type PRV vBecker2 harvested from FP7 and U2OS cells were similar and in the order of 6–7 logs $TCID_{50}$ (Fig. 6, panel A). In contrast, infection of FP7 control cells with PRV-TER and PRV-CEA yielded titers of 2.7 and 3.1 log, respectively, approximately 1000

Table 1
Oligonucleotide primers and size of DNAs amplified by PCR and RT-PCR. The primers were constructed in promoter (Pr), mRNA leader region (L) or coding sequence (cs) of the genes.

Gene	primer	Sequence	PCR DNA size (bp)
UL30 (cs)	DNApol-D	5′-GTGGAGGTCAACTGCACG-3′	523
	DNApol-R	5′-GCGACGGCGTACATGTCC-3′	
UL30 (Pr)	PRPol-S	5′-CTCGAGCAGCGCCAGCAGG-3′	398
	PRPol-AS	5′-CCATGGCTCGTCCCACG-3′	
IE180 (Pr-cs)	P180-S	5′-TCTGATTGGCTCGCTAGC-3′	1021
	180-AS	5′-GGCCGAAGAGGAGATCCTCG-3′	
hTERT (Pr)	TERT-2D	5′-CGCGTCCGGACCTGGAGG-3′	553
	TERT-2R	5′-GCGCCATGGCGGGGGTGG-3′	
CEA (Pr)	CEA-S	5′-CTCGAGCCACCTCTGTCACC-3′	482
	CEA-AS	5′-CCATGGTCTCTGCTGTCTGC-3′	
hTERT(Pr)-IE180 (cs)			
	TERT-2D	5′-CGCGTCCGGACCTGGAGG-3′	710
	180-AS	5′-GGCCGAAGAGGAGATCCTCG-3′	
CEA(Pr)-IE180 (cs)			
	CEA-S	5′-CTCGAGCCACCTCTGTCACC-3′	636
	180-AS	5′-GGCCGAAGAGGAGATCCTCG-3′	
GAPDH (cs)	GAPDH-S	5′-CCACCCATGGCAAATTCC-3	595
	GAPDH-AS	5′-TCTAGACGGCAGGTCAGG-3	
qPCR			
IE180 (cs)	q180-S	5′-CGCCCTTCGAGACCAGCGT-3′	122
	q180-AS	5′-GAAGCCGACGATCTCCACCGC-3′	
hTERT (L)-IE180 (cs)			
	L-hTERT	5′-TGCTGCGCACGTGGGAAGC-3′	207
	180-AS	5′-GGCCGAAGAGGAGATCCTCG-3′	
CEA (L)-IE180 (cs)			
	L-CEA	5′-AGCTCTTCTCCACAGAGG-3′	208
	180-AS	5′-GGCCGAAGAGGAGATCCTCG-3′	
Hu β-actin	Huß-actin For	5′-GCGAGCACAGAGCCTCGCCTT-3′	125
	Huß-actin Rev	5′-CATCATCCATGGTGAGCTGGCGG-3′	

fold less virus compared to infected U2OS cancer cells, where the viral yields were 5.8 and 4.4 log, respectively (Fig. 6, panel A), suggesting that the replication of the recombinant PRV viruses is more efficient in cancer cells. To determine whether the replicative potential of PRV-TER and PRV-CEA is indeed greater in cancer cells, we measured the viral yields of these recombinant viruses in two additional cancer cell lines. Viral yield of vBecker2 harvested from FP7, Hela and HT29 were similar across cell lines, in the order of 6–7 log $TCID_{50}$ and comparable to the levels determined in U2OS cells (Fig. 6, panel B). In contrast, but similar to what was observed in U2OS cells, infection of the Hela and HT29 cancer cell lines with PRV-TER or PRV-CEA yielded titers in the order of 2–3 logs $TCID_{50}$ higher than in FP7 cells (Fig. 7, panel B). The infection of FP7 cells with PRV-TER or PRV-CEA yielded titers of 2.7 log and 3.1 log, respectively, at least 1000-fold less virus compared to the parental virus vBecker2 which yielded a titter of 6.3 log $TCID_{50}$ (Fig. 6, panels A and B).

Interestingly, while PRV-CEA replicated more efficiently in cancer cells versus non-cancer cells, PRV-CEA viral yield in U2OS cells was 21 fold less than PRV-TER. We also observed that U2OS cells infected with PRV-CEA were morphologically different than U2OS cells infected with vBecker2 or PRV-TER. While vBecker2 or PRV-TER induced typical cytopathic effect, PRV-CEA-infected U2OS cells showed pronounced cytoplasmic blebbing (Fig. 6, panel B), a phenotype consistent with induced apoptosis (Allen et al., 1997). Thus, we reasoned that the lower replication of PRV-CEA compared to vBecker2 or PRV-TER may be a causative effect of apoptosis induction. To test this hypothesis, we studied apoptosis induction in U2OS cells infected with viruses vBecker2, PRV-TER or PRV-CEA at a MOI of 0.2 $TCID_{50}$/cell. After 24 h p.i., apoptosis was assessed by annexin-V-FITC staining followed by flow cytometric analysis. The percentage of late apoptotic cells with vBecker2 and PRV-TER viruses were 0.77% and 0.51% respectively (Fig. 7, panels

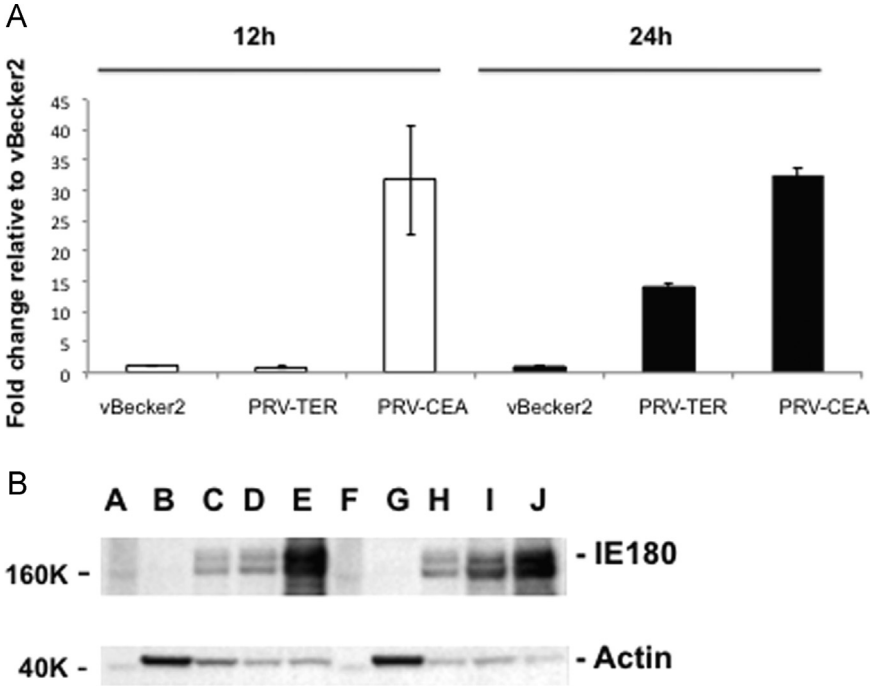

Fig. 5. IE180 expression under the control of hTERT and CEA promoters in PRV-TER-and PRV-CEA-infected cells. Panel A. IE180 mRNA expression. U2OS cells were infected with PRV-TER, PRV-CEA and vBecker2 at a MOI of 0.1 $TCID_{50}$/cell, cells were harvested at 12 h (open bar) and 24 h p.i. (solid bar) and RNA was isolated and purified. IE180 mRNA was quantified by RTqPCR using IE180 specific primers and values are normalized to β-actin mRNA values. IE180 mRNA expression in U2OS cells after infection with vBecker2 at 24 h p.i. was increased 8.93 fold in relation to 12 h p.i. Each value represents the mean and standard deviation (error bars) from two independent experiments. Panel B. Expression of IE180 protein by PRV-TER and PRV-CEA recombinant viruses. U2OS cells (lane B and G) were infected with vBecker2 (lane C and H), PRV-TER (lane D and I)) and PRV-CEA (lane E and J) at a MOI of 0.1 $TCID_{50}$/cell and IE180 protein expression was determined 12 and 24 h p.i. Novex sharp pre-stained protein standard (Life Technologies) (lane A and F) was included.

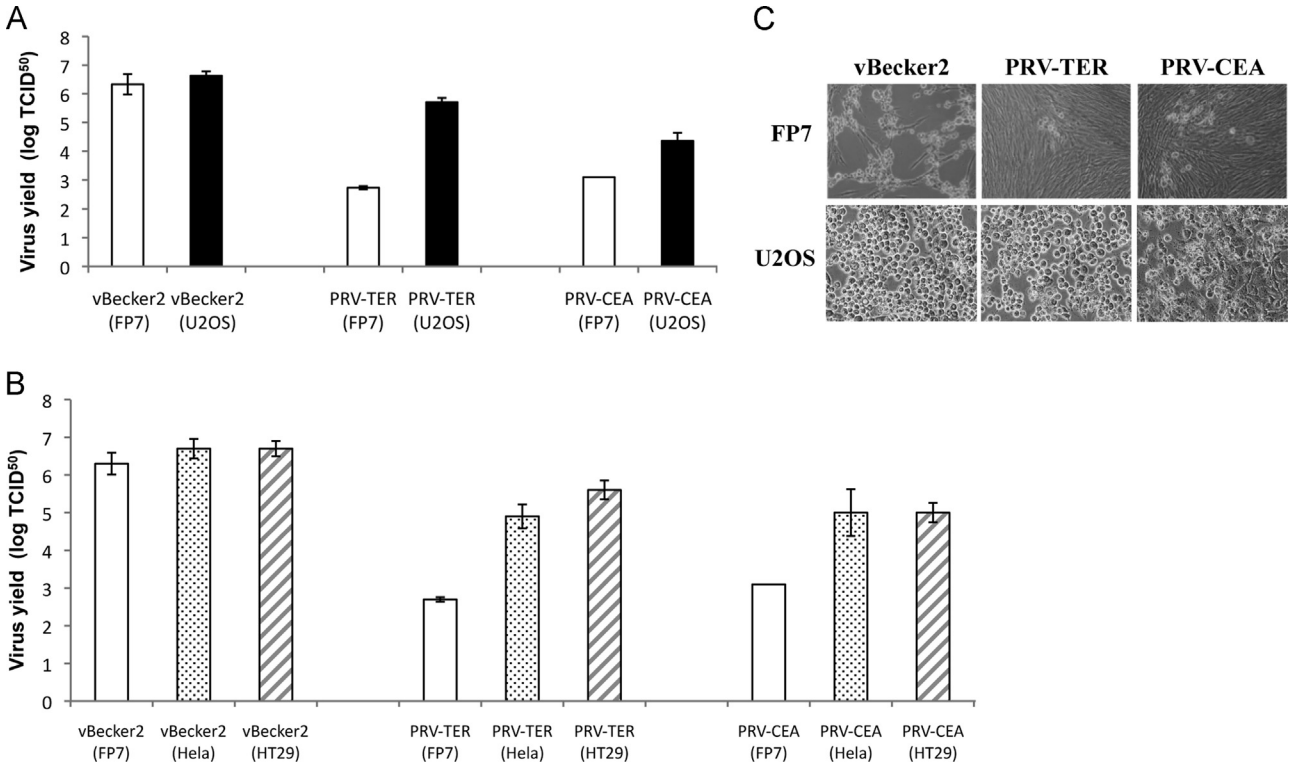

Fig. 6. Wild-type PRV vBecker2, PRV-TER and PRV-CEA viral yields in infected mammalian cells. FP7 and U2OS cells were infected at a MOI of 0.2 $TCID_{50}$/cell, washed to remove unadsorbed virus, and incubated for 48 h at 37 °C. Virus yield was determined from total lysates of infected cells. Each value represents the mean and standard deviation (error bars) from three independent experiments (panel A). Virus yields determined, as described above, in Hela and HT29 cells (panel B). Infected and uninfected U2OS cells were examined by optical microscopy at 48 h p.i. (panel C).

A and B), while the percentage of the early apoptotic cells was 1.77% for PRV-TER and 1.36% for vBecker2. Interestingly, and in line with our morphological observations, the percentage of late apoptotic cells after PRV-CEA virus infection reached 3.55% compared to 0.25% in mock-noninfected cells (Fig. 7, panels A and B). While these results suggest apoptotic induction in PRV-CEA infected cells, we also assessed poly-(ADP ribose) polymerase (PARP) cleavage in infected cells, an additional indicator of apoptosis. The cleavage of PARP is catalyzed by caspase-3 in the later events of apoptosis and is related to depletion of NAD and ATP during apoptosis (Boulares et al., 1999). The presence of a cleaved PRAP protein product of 89 kDa (Fig. 7, panel C) is a well-known apoptotic hallmark. As expected, we observed cleaved PRAP in U2OS cells infected with PRV-CEA at 12 h (Fig. 7, panel C, lane E) and 24 h (Fig. 7, panel C, lane J) p.i., which directly correlated with IE180 expression (Fig. 5, panel B, lanes E and J respectively). These results show that over-expression of IE180 in U2OS cells infected at 12 h p.i. with PRV-CEA (Fig. 5 panel A, and panel B, lane E) and not vBecker2 (Fig. 5, panel B, lane C) or PRV-TER (Fig. 5, panel B, lane D) clearly results in apoptosis (Fig. 7, panel C, lane E), which is in line with studies where the IE180 protein was suggested as a possible inducer of apoptosis (Tomioka et al., 2008, 2013). Importantly, this apparent IE180-mediated apoptotic phenotype was additionally confirmed in PK15-IE180 cells, which stably express IE180 under the control of the tetracycline-responsive promoter (Ptet) (Oyibo et al., 2014). In the presence of doxycycline, the expression of IE180 induced early and late apoptosis at 48 h post induction in 1.78% and 4.63% of cells, respectively, compared to 0.75% and 1.29% in mock non-induced cells (Fig. 8, panels A and B). In addition, the induction of IE180 expression at 48 h, in the presence of doxycycline, was confirmed by western blot analysis (Fig. 8, panel C, lane C) and its activity confirmed by transactivation of the gG promoter of plasmids pZGF-SD4 and pOGF-SD4 (Fig. 8, panel D).

Discussion

IE180 has a high level of similarity to the IE proteins of other alphaherpesviruses such as ICP4 (HSV-1), IE140 (VZV, Varicella-zoster virus), IE1 (EHV1, equine herpesvirus 1) and p180 (BHV-1, bovine herpesvirus 1) (Vlcek et al., 1989). Moreover, all of these viral gene products are multifunctional proteins that share similar functional domains. They have been divided into five colinear regions based on their predicted amino acid sequences, with a high level of similarity in regions 2 and 4 and little, if any, in regions 1, 3 and 5 (Cheung, 1989; Wu and Wilcox, 1991). The auto-regulatory domain, the nuclear localization signal (NLS) and the transactivation domain (TAD) are located in regions 2 and 4. We previously described that the IE180 protein of PRV can down-regulate the transactivation of the HSV-1 ICP4 promoter (Gómez-Sebastian and Tabares, 2004) and some viral DNA replication was detected in the presence of IE180 when 293T cells were infected with HSV-1 d120 strain deficient in ICP4 (Gómez-Sebastian and Tabares, 2004). This indicates that complementation between the IE180 protein of PRV and the ICP4 protein of HSV-1 occurs to some extent, at least up to the early phase of viral replication. The IE proteins ICP4 and IE180 stimulate transcription from both cellular and viral genes; however no promoter sequences have been identified that are uniquely involved in transcriptional stimulation (Abmayr et al., 1988). Thus, the different behavior of ICP4 and IE180 in the activation and repression of the hTERT promoter, respectively, could be related to interactions with cell factors involved in transcription, such as TFIID. ICP4 can activate transcription from cellular promoters such as human ubiquitin B

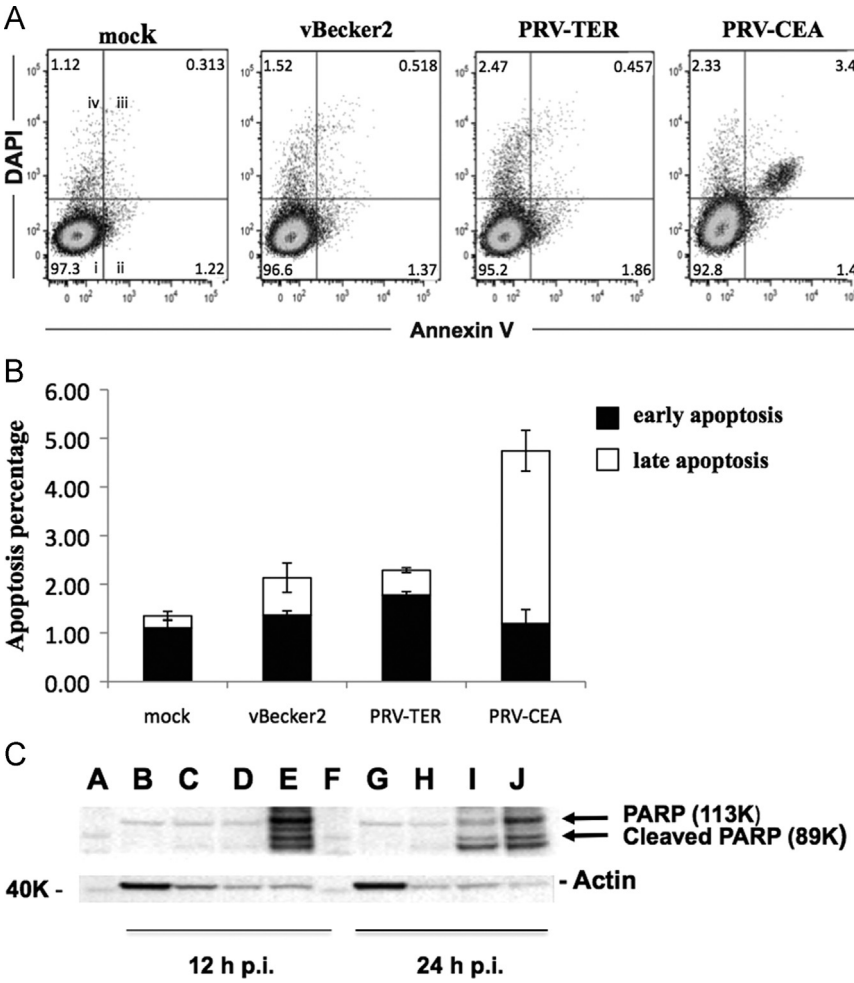

Fig. 7. PRV-CEA virus infection induces apoptosis in U2OS cells. Mock-and vBecker2, PRV-TER and PRV-CEA infected (MOI of 0.2 $TCID_{50}$/cell) cells were stained with annexin V-FITC and DAPI and analyzed by flow cytometry. Percentage of live cells (i), percentage of early apoptotic cells (ii), late apoptotic cells (iii) and percentage of dead cells (iv) are shown (Panel A). Percentages of early (solid bar) and late (open bar) apoptotic cells are summarized. Each value represents the mean and standard deviation (error bars) from three independent experiments (Panel B). In addition, apoptosis was detected by caspase activation resulting in PARP cleavage in U2OS infected cells 12 h and 24 h p.i. by western blot analysis using a purified mouse anti-human PARP (Panel C). Novex sharp pre-stained protein standard (Life Technologies) (lane A and F) was included.

(Kemp and Latchman, 1988) and cytokine vascular endothelial growth factor-A (VEGF-A) (Wuest et al., 2011). Analogously, the role of IE180 as a potent transcriptional activator has been established and, like most typical cellular activators, IE180 contains a separate domain for DNA-binding and another for transactivation (Martin et al., 1990). IE180 can activate transcription from cellular promoters such as beta-globin and topoisomerase I (Green et al., 1983; Wong et al., 1997); however, in our study we observed that it represses the hTERT promoter but hyper-activates the CEA promoter. Further studies are still needed to dissect this differential repressive/activating capacity at the molecular level, but in the context of developing recombinant viruses, our results highlight the importance of conducting promoter activity studies when swapping IE promoters out for cellular promoters, such as tumor specific promoters. The ICP4 and IE180 gene products are the major regulatory proteins for the replicative cycle of HSV-1 and PRV, respectively, affecting viral gene expression either positively or negatively, including auto regulation (Ihara et al., 1983; Leopardi et al., 1995). This auto regulation can be interchanged between both viruses (Gómez-Sebastian and Tabares, 2004) and seems to play an important role in viral replication. In our study, the substitution of the IE180 promoter in vBecker2 with the CEA promoter produced a significant overexpression of IE180 at 12 h p.i. due to hyperactivation of the CEA promoter by the PRV IE180 protein. This resulted in viral yields 48 h p.i. of 2 logs less than vBecker2, which we attributed to the induction of apoptosis via a IE180-dependent mechanism although overexpression of IE180 may also have an inhibitory effect on early protein 0 (EP0) and large latency transcript (LLT) expression (Ou et al., 2002) with may additionally diminish viral replication. EP0 activates PRV gene transcription in combination with the IE180, enhances the synthesis of infectious virus (Ono et al., 1998) and its mRNA expression is detected in inverse relationships to IE180 mRNAs expression (Tombácz et al., 2009), thus over expression of IE180 may also affect alternate viral-specific mechanisms that are currently under investigation.

In contrast to PRV-CEA, when the IE180 promoter in vBecker2 was replaced with the hTERT promoter, which is repressed by IE180 protein, viral production increased approximately one log compared to PRV-CEA, but unlike the CEA promoter, the hTERT promoter exhibited IE180-dependent autoregulation, resulting in less efficient virus production compared to vBecker2. Interestingly, at later time points p.i. there was an apparent loss of hTERT auto down-regulation in U2OS cells infected with PRV-TER, which we hypothesize could be related to other viral processes and kinetic events. Nonetheless, our data show that the exchange of viral promoters for cellular promoters is feasible but at the same time subject to numerous regulatory checkpoints, both viral and cellular, and these processes should be taken into consideration and evaluated when constructing recombinant PRV vectors. The effects of replacing the IE180 promoter in PRV with

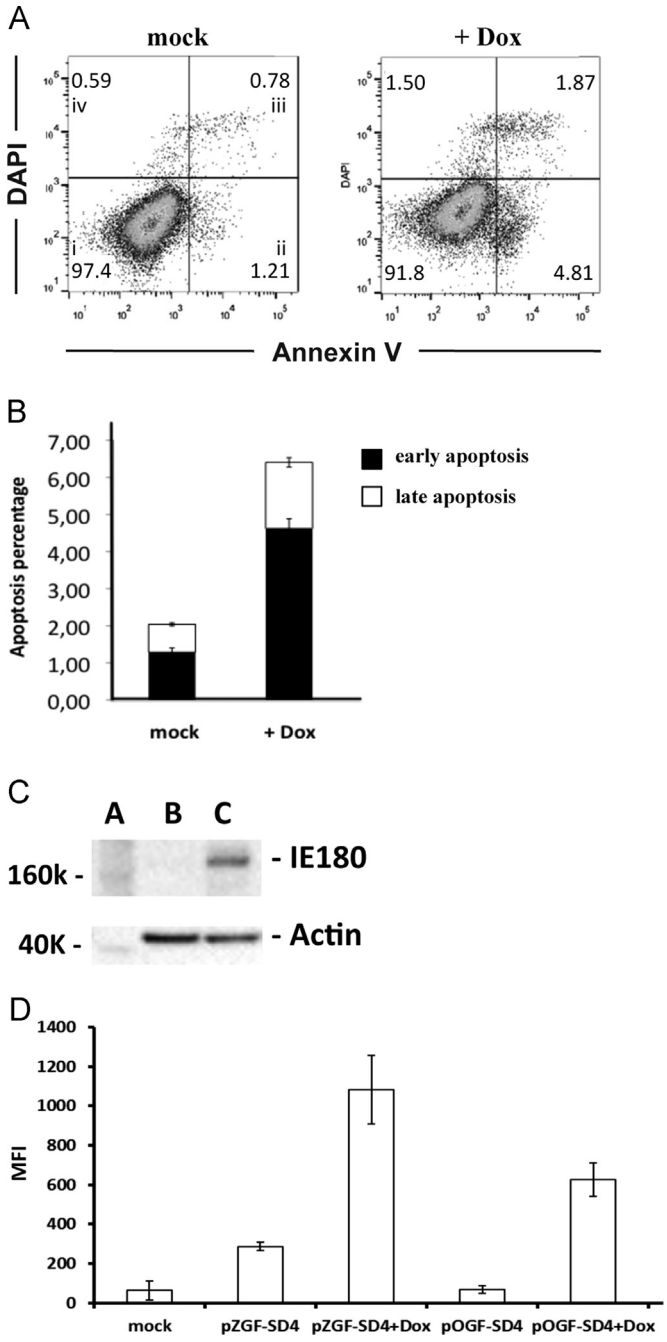

Fig. 8. IE180 protein induces apoptosis of PK15-IE180 cells. PK15-IE180 cells were cultured in presence or absence of 2 μg/ml doxycycline to induce IE180 expression. Panel A. Cells were washed in annexin buffer and directly analyzed by flow cytometry after the addition of annexin V-FITC and DAPI. Percentage of live cells (i), percentage of early apoptotic cells (ii), late apoptotic cells (iii) and the percentage of dead cells (iv) are shown. Panel B. Columns represents the mean from two independent experiments of early (solid bar) and late (open bar) apoptotic cell percentages. Panel C. Protein lysates of PK15-IE180 cells in the presence (lane B) or absence (lane A) of 2 μg/ml doxycycline were collected at 48 h postinduction and Western blot analysis was performed with 30 μg of protein for PRV IE180 and Actin, an internal cellular loading control. Panel D. Transactivaton of the gG promoter on PK15-IE180 cells. PK15-IE180 cells were transfected with pZGF-SD4 or pOGF-SD4 plasmids in the presence or absence of 2 μg/ml doxycycline. EGFP protein expression was determined by flow cytometry 48 h posttransfection and is expressed as fluorescence intensity (MFI). Each value represents the mean and standard deviation (error bars) from three independent experiments.

the tissue-specific promoters of the bovine cytokeratin IV gene (CKIV), the bovine cytokeratin VIb gene (CKVIB) or the inducible promoters drosophila heat-shock gene HSP70 or the tetracycline-responsive promoter (Ptet) have previously been studied (Glazenburg et al., 1992, 1995; Muñoz et al., 2011), and the authors concluded that the replicative ability and virulence of pseudorabies virus can be influenced by altering control of IE gene expression, as may be happening after infection with the recombinant PRV-CEA virus.

While not the immediate focus of this study, the results presented here also suggest that PRV-CEA may represent a putative oncolytic vector. First, the IE180 promoter can be successfully replaced with the CEA tumor promoter, IE180 mRNA is expressed to high levels from this promoter and most importantly, IE180 does not negatively auto regulate the CEA promoter as is the case with the hTERT promoter. Second, PRV-CEA replication was more efficient in the cancer cell lines U2OS, Hela and HT29 compared to control FP7 human fibroblasts. This significant difference would suggest that PRV-CEA preferentially replicates in cancer cells, where CEA promoter activity is presumably hyper activated due to the cellular environmental context. It would be interesting to see if this apparent specificity for cancer cells holds up across a larger panel of cancer cell lines from different tumor types. Lastly, and in line with the hyper activation of the CEA promoter in U20S cells resulting in increased IE180 mRNA, we observed a clear induction of apoptosis in cancer cells. This phenotype was clearly due to IE180 over expression since a similar phenotype was observed when IE180 was conditionally over expressed in PK15-IE180 porcine cells. These results are in concordance with studies performed in transgenic mice expressing PRV IE180, where it was suggested that IE180 over expression induced apoptosis although it was not officially demonstrated (Tomioka et al., 2008, 2013). It is interesting that over expression of IE180 induces apoptosis, while other viral immediate-early proteins, like ICP4 have been proposed to have anti-apoptotic functions (Leopardi and Roizman, 1996; Peri et al., 2011). Indeed IE180 suppresses phosphorylation of translation initiation eIF2a factor (Van Opdenbosch et al., 2012), which could play a role in its capacity to induce apoptosis (Jiang and Wek, 2005). Nonetheless this apparent pro-apoptotic function may represent an added advantage in the context of PRV-based oncolytic vectors, in which PRV could be genetically manipulated to preferentially initiate a lytic replication cycle in tumor cells via CEA promoter transcriptional regulation and at the same time induce apoptosis of cancer cells a “missile and warhead” approach. PRV has also been put forth as a suitable oncolytic agent for human tumors due to the absence of virulence, although PRV replicates efficiently in human cell lines (Sawitzky, 1997; Fernández et al., 1999; Boldogköi et al., 2002; Prieto et al., 2002; Boldogköi and Nogradi, 2003; Wollmann et al., 2005; Shiau et al., 2007). In our studies, PRV-TER and PRV-CEA recombinants showed significant attenuation in human cells as its replication was at least 1000-fold less compared to the parental virus vBecker2. Thus, there still remains room for significant improvement of PRV-CEA as a putative oncolytic virus. For example, further studies aimed at improving biosafety [e.g. increasing ganciclovir sensitivity of these viruses by incorporating HSV-1 TK gene into PRV viral genome (Gomez-Sebastián and Tabarés, 2004) and increasing cancer cell specificity are still needed and are in progress.

Conclusions

In the present study we show that PRV IE180 protein expression can be governed by CEA and hTERT human tumor-specific promoters, as both promoters can functionally substitute the IE180 promoter in PRV-CEA and PRV-TER recombinant viruses. IE180 showed negative regulation on the hTERT promoter but positive hyperactivation of the CEA promoter. PRV-TER and PRV-CEA viruses preferentially replicated in several human cancer cell

lines compared to control non-cancer cells, and PRV-CEA was also capable of inducing apoptosis which we correlated to the overexpression of IE180. These data therefore support the further development of PRV-based oncolytic vectors and at the same time show a previously unrecognized pro-apoptotic capacity associated with IE180 overexpression, a property that could be additionally exploited to target cancer cells.

Material and methods

Viruses and cells

The Hela human cervix carcinoma cell line, HT29 human colon adenocarcinoma cell line, U2OS human osteosarcoma (osteoblast) cell line and the FP7 human lung fibroblast cell line were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal calf serum. HeLa Tet-Off cells (Clontech) were grown in DMEM containing 10% fetal bovine serum supplemented with 100 μg/ml Geneticin (Life Technologies). PK15-IE180-expressing cells (Oyibo et al., 2014) were grown in DMEM containing 10% fetal bovine serum and supplemented with 500 μg/ml Geneticin to select for cells possessing the PRV IE180 transgene. When necessary, 2 μg/ml doxycycline (Clontech) was added to the medium to induce expression of the IE180 gene. The parental virus vBecker2 was generated by transfection of pBecker2 plasmid (Smith and Enquist, 2000) into Hela Tet-Off cells. The recombinant HSV-1 R120vGF virus expresses EGFP and is deficient in ICP4 (Bello-Morales et al., 2014) and was generated in E5 cells as per standard protocols.

Cloning of hTERT and CEA tumor promoters

DNA fragments of hTERT and CEA promoters upstream of the initiating ATG codon were amplified by PCR and characterized by DNA sequencing performed by an automated DNA sequencing system from Applied Biosystem. The core hTERT promoter sequence (Horikawa et al., 1999) (nucleotides-543 to +6 from the ATG initiation codon) was amplified from Hela cell DNA by PCR (Muñoz et al., 2011) using primers TERT-2D and TERT-2R (Table 1) and cloned into pGEM-Teasy (Promega), giving rise to the plasmid pGTER1P. The hTERT promoter generated by PCR was mutated to include an *Nco*I restriction site at the ATG initiation codon (primer hTERT-2R). The core CEA promoter (nucleotides-474 to +4 from the ATG initiation codon) (Schrewe et al., 1990) was amplified from human amnion cell DNA by PCR (Muñoz et al., 2011) using primers CEA-S and CEA-AS (Table 1) and cloned into pGEM-Teasy (Promega), giving rise to the plasmid pGCEA-A. The CEA promoter generated by PCR was mutated to include an *Nco*I restriction site at the ATG initiation codon (primer CEA-AS).

Plasmid constructs

Plasmids used in transient expression assays included the following: (i) pRIE180-expresses PRV IE180 under the control of the natural IE180 promoter (Muñoz et al., 2010). (ii) pAZT180-expresses PRV IE180 under the control of the tetracycline-responsive promoter (Ptet) (Muñoz et al., 2011). (iii) ptetICP4lac-expresses HSV-1 ICP4 under the control of the tetracycline-responsive promoter (Ptet). The ICP4 coding sequence was amplified by PCR and cloned into pUHD10-1 (Gossen and Bujard, 1992) blunted at the *Eco*RI and *BamH*I sites. The tetO-ICP4 fragment was then excised by *Xho*I/*Hind*III digestion and cloned into *the Hind*III site of pHSVpuc (Geller et al., 1993). (iv) pZGF-SD4-includes the promoter-regulatory region of the gG gene, the EGFP-coding sequence, and the SV40 polyadenylation signal, flanked by the PRV homologous recombination regions (Muñoz et al., 2010). (v) p30GF – was constructed by replacing the gG promoter (*Xho*I-*Nco*I fragment, *Nco*I includes translation initiation ATG codon) in pSD4-AG with the UL30 promoter (*Xho*I-*Nco*I fragment, *Nco*I site includes the translation initiation ATG codon of UL30). The *Xho*I-*Nco*I 400 bp fragment of the UL30 promoter was amplified by PCR using primers PRPol-S and PRPol-AS (Table 1). (vi) pATER180 – expresses IE180 under the control of the hTERT promoter (P_{TER}) and was constructed by replacing the tetracycline-responsive promoter (Ptet) (*Eco*RI blunt-*Nco*I fragment) in pAZT180 with the hTERT promoter (*Hind*III blunt-*Nco*I fragment of pGTER1P). (vii) pACEA180 – was constructed by replacing the tetracycline-responsive promoter (Ptet) promoter (*Eco*RI blunt-*Nco*I fragment) in pAZT180 with the CEA promoter (*Xho*I blunt-*Nco*I fragment of pGCEA-A). (viii) pZTER-GF – was constructed by replacing the gG promoter (*Stu*I-*Nco*I fragment) in pZGF-SD4 with the hTERT promoter (*Spe*I-*Nco*I fragment of pGTERT1P). (ix) pACEA-FG – was constructed by replacing the gG promoter (*Stu*I-*Nco*I fragment) in pZGF-SD4 with the CEA promoter (*Xho*I-*Nco*I fragment of pGCEA-A. (x) pOZPAC-GF – was obtained by inserting the *Not*I fragment of pORIPAC-GF (Prieto et al., 2002), which includes the EGFP expression cassette, in the *Not*I site of the pOZPAC plasmid (Muñoz et al., 2011). (xi) pOZPACGF-N – was obtained from pOZPAC-GF, eliminating the *Nde*I site of the pUC18 vector by blunting the *Nde*I site followed by religation. (xii) pOTER180-N and pOCEA180-N – were obtained by inserting pATER180 and pACEA180 *Ase*I-*Mlu*I fragments in the pOZPACGF-N plasmid digested with *Ase*I-*Mlu*I obtaining pOTER180-N and pOCEA180-N, respectively. The p180FG *Sph*I-*Hinc*II fragment contains the EGFP expression gene under the control of the PRV IE180 promoter (Muñoz et al., 2010).

Recombinant PRV viruses

The PRV-TER recombinant virus was made by cotransfecting the *ApaL*I-*Kpn*I fragment of pOTER180-N (1.5 μg) together with PBAC90 DNA (Muñoz et al., 2011) (4 μg) into U2OS cells using Lipofectamine 2000 (Invitrogen), as per the manufacter's protocol. Positive foci of infection were detected one-week post-transfection. Analogously, the PRV-CEA recombinant virus was made by cotransfecting the *ApaL*I-*Kpn*I fragment of pOCEA180-N (1.5 μg) together with PBAC90 DNA (Muñoz et al., 2011) (4 μg) into U2OS cells using Lipofectamine 2000 (Invitrogen). Positive foci of infection were detected one-week post-transfection. Stocks of virus were produced by propagation in U2OS cells. PBAC90 is a non-infectious PRV BAC deficient in the IE180 gene (Muñoz et al., 2011).

Flow cytometric measurement of EGFP expression

A total of 6×10^5 HeLa Tet-Off cells were seeded into 12.5 cm^2 flasks. Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen), as per the manufacter's protocol, harvested 24 h posttransfection, fixed with paraformaldehyde, washed once in PBS, resuspended in 0.7 ml of 1X PBS, and analyzed with a Flow Cytometer (Cytomics FC500 MPL, Beckman Coulter). The fluorescent EGFP protein (excitation: 488 nm, emission: 508 nm) produced was used to monitor the efficiency of expression by fluorescence microscopy or flow cytometry. The mean fluorescence intensity (MFI) was measured.

Western blot analysis

Extracts from transiently transfected Hela Tet-Off cells or U2OS cells infected with recombinant viruses were lysed in RIPA buffer (Sigma) supplemented with a Complete, Mini, EDTA-free protease cocktail inhibitor (Roche) and analyzed by 12% PAGE. Prot-

eins were transferred to a Hybond ECL nitrocellulose membrane (Amersham) in 20 mM Tris–HCl (pH 8.3), 150 mM glycine, 20% methanol and 0.02% SDS (15 V; 30 min) using a Transblot cell (Bio-Rad). The membrane was blocked in PBS with 3% BSA (4 °C, overnight) and then incubated with rabbit polyclonal anti-IE180 (Gómez-Sebastian and Tabares, 2004) (1:200), anti-β-actin (Thermo Scientific) (1:1000), or mouse monoclonal anti-ICP4 (Abcam, Cambridge, UK) (1:2000) in blocking solution with 0.05% Tween 20 (4 °C, overnight) with constant rocking. The membrane was washed extensively in PBS with 0.05% Tween 20 and incubated for 1 h with IgG anti-mouse peroxidase (1:4000; Sigma) or IgG anti-rabbit peroxidase (1:5000; Sigma) for mouse monoclonal or rabbit polyclonal antibodies, respectively. Blots were developed with ECL detection reagents (Amersham) and exposed to Kodak X-OMAT AR film. Densitometric analysis was performed using Adobe Photoshop CS4 where Actin served as a normalization control.

Isolation of viral and cellular DNA

Packaged viral DNA was isolated from the cytoplasmic cell fraction (Prieto et al., 2002). Total DNA from infected cells was isolated by SDS-proteinase treatment (QF Amp DNA Micro, Qiagen).

Analysis of nucleic acids by PCR and RT-PCR

Total RNA from U2OS cells infected with PRV recombinants at a multiplicity of infection (MOI) of 0.1 $TCDI_{50}$/ml was isolated using the RNeasy mini kit (Qiagen) or acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and treated with 4 U/ml DNAse (DNA-free kit, Applied Biosystems, Ambion). No DNA contamination was found in any of the RNA preparations by specific PCR amplification of the cellular GAPDH gene. Reverse-transcription PCR (RT-PCR) was carried out with 2.0 μg of RNA using the First Strand cDNA Synthesis Kit (Roche) to amplify viral IE180 and cellular GAPDH mRNAs, the latter serving as an internal control, using the specific primers listed in Table 1. The RT-PCR program consisted of: 1 cycle (10 min, 65 °C) of RNA denaturation, 30 min at 55 °C for retrotranscription and 5 min at 85 °C for RT inactivation. The PCR program consisted of: 1 cycle (15 min, 95 °C) of DNA denaturation and Taq DNA polymerase activation, 35 cycles (1 min, 95 °C) of denaturation, 1 min at 53 °C (50 °C for GAPDH) for annealing, and 1 min at 72 °C for elongation, and a final cycle (10 min, 72 °C) for further elongation. PCR products were analyzed by 1% agarose gel electrophoresis, and the specificity of the amplification products was confirmed by the size of the DNA products (Table 1).

Quantitative Real-Time PCR (RTqPCR)

One μg of RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). RTqPCR was performed to amplify the viral IE180 and cellular β-actin mRNA, the latter serving as an internal control, using the specific primers for each mRNA listed in Table 1. The RTqPCR reactions were performed with a Real-Time PCR StepOnePlus™ instrument (Applied Biosystems) using FAST-SYBR Green (Applied Biosystems) according to the manufacturer's protocol. The reaction mixture consisted of 1X FAST SYBR Green (Applied Biosystems), sense and antisense primers each at 0.25 μM, and 15 ng cDNA. Thermal cycling consisted of an initial 10 min denaturation step at 95 °C followed by 40 cycles of denaturation (15 s at 95 °C) and annealing/extension (1 min at 60 °C).

Evaluation of apoptosis

Apoptosis was measured by flow cytometric analysis using the AnnexinV-FITC (BD) staining kit as per the manufacturer's instructions. Uninfected and infected U2OS cells were trypsinized after 24 h p.i., washed with PBS and incubated with Annexin V-FITC and DAPI solution (Bender MedSystems, Burlingame) in the dark for 20 min. Afterwards, cells were treated with DAPI and samples were analyzed by flow cytometry with a FACSCANTO II cytometer (Becton Dickinson, Mountain View) and data processed using BD FlowJo 8.7 software. The amount of early apoptosis and late apoptosis was determined as the percentage of Annexin V^+/$DAPI^-$ and Annexin V^+/$DAPI^+$, respectively (Miranda-Lorenzo et al., 2014). In addition, apoptosis produces caspase activation, which leads to poly(ADP ribose) polymerase (PARP) cleavage, which was detected in U2OS infected cells 12 and 24 h p.i. by western blot analysis using a purified mouse anti-human PARP antibody (Cell Signalling, US) (1:1000). Bound anti-PARP was detected using an anti-mouse peroxidase secondary antibody (1:4000; Sigma).

Acknowledgments

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