

Therapeutic Improvement of a Stroma-Targeted CRAd by Incorporating Motives Responsive to the Melanoma Microenvironment

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We have previously designed a conditionally replicative oncolytic adenovirus (CRAd) named Ad-F512 that can target both the stromal and the malignant melanoma cell compartments. The replication capacity of this CRAd is driven by a 0.5-Kb SPARC promoter fragment (named F512). To improve CRAd's efficacy, we cloned into F512 motives responsive to hypoxia (hypoxia-responsive element (HRE)) and inflammation (nuclear factor kappa B) to obtain a chimeric promoter named κ BF512HRE. Using luciferase as a reporter gene, we observed 10–15-fold increased activity under hypoxia and 10–80-fold induction upon tumor necrosis factor- α addition. We next constructed a CRAd (Ad- κ BF512HRE) where E1A activity was under κ BF512HRE regulation. Treatment of nude mice harboring established tumors made of a mix of SB2 melanoma cells and WI-38 fibroblasts with Ad- κ BF512HRE led to the complete elimination of tumors in 100% of mice (8/8). Moreover, Ad-5/3- κ BF512HRE, a viral variant pseudotyped with a chimeric 5/3 fiber, exerted a strong lytic effect on CAR-negative melanoma cells and was highly effective *in vivo* on established tumors made of melanoma cells and WI-38 fibroblasts, leading to the complete elimination of 4/5 tumors. These results indicate that this improved stroma-targeted oncolytic adenovirus can override the resistance of melanoma tumors and might become of significant importance for melanoma therapeutics.

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INTRODUCTION

Although melanoma accounts for only 3–5% of all skin cancers, it is responsible for approximately 75% of all deaths from skin cancer worldwide and disproportionately targets young individuals, especially women (Wu *et al.*, 2003; Lens and Dawes, 2004; Thompson *et al.*, 2005). The standard therapy for the metastatic disease remained decarbazine with transient response rates of <10%, although interleukin-2

treatment seems to benefit a select group of patients, although with significant associated toxicity (Schadendorf *et al.*, 2009). Two promising new agents, ipilimumab, a humanized mAb that blocks the T-cell surface protein CTLA-4, and the inhibitor of the BRAF gene, vemurafenib, seem to benefit <20% of patients and are limited by a relatively short duration that averages 6 months (Hodi *et al.*, 2010; Chapman *et al.*, 2011).

The use of oncolytic viruses for tumor targeting appears as a potential powerful approach for the treatment of advanced stages of cancer combined or not with radiotherapy or chemotherapy (Garber, 2006; Alemany, 2007; Choi *et al.*, 2012). Direct treatment of accessible melanoma lesions with a replicative herpes simplex virus expressing granulocyte-macrophage colony-stimulating factor (Oncovex-GMCSF) resulted in a 28% objective response in a phase II clinical trial encouraging the initiation of a phase III study (Senzer *et al.*, 2009). Oncovex-GMCSF exhibits a strong antitumor lytic activity and a highly attenuated neurovirulence due to a mutation in the ICP34.5 gene (Senzer *et al.*, 2009), although it was not specifically designed to attack cancer cells nor the supportive stroma. Few conditionally replicative oncolytic adenoviruses (CRAds) whose replication is driven by tumor-specific promoters and hence replicate mainly in the cancer niche (Hogg *et al.*, 2010) have also entered clinical trials with promising therapeutic effects, although none have included

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Abbreviations: CRAd, conditionally replicative oncolytic adenovirus; DPE, downstream promoter element; FBS, fetal bovine serum; HRE, hypoxia-responsive element; INR, initiator sequence; NF- κ B, nuclear factor kappa B; PBS, phosphate-buffered saline; TNF- α , tumor necrosis factor- α

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melanoma patients yet (Dent *et al.*, 2010). The lack of strong and specific responsive promoters and the difficulties to disseminate inside the tumor mass owing to the presence of stromal barriers hampered the therapeutic efficacy of oncolytic vectors (Cairns *et al.*, 2006). We have recently designed a stroma-targeted CRAAd, Ad-F512, whose replication is driven by a 0.5-kB SPARC promoter fragment named F512. The rationale underlying this was that SPARC is expressed in most stromal cells in close contact with the malignant compartment (Podhajcer *et al.*, 2008). In human melanomas, SPARC is additionally expressed by the malignant cells themselves, indicating that the CRAAd could eventually replicate in both cell compartments. Indeed, Ad-F512 exhibited therapeutic efficacy *in vivo* on human melanoma tumors xenografted in nude mice (Lopez *et al.*, 2009). However, established melanomas made of a mix of human melanoma cells and human fibroblasts were highly resistant to viral attack (Lopez *et al.*, 2009).

In the present study, we decided to make use of specific characteristics of the melanoma microenvironment and designed an improved version of Ad-F512. For this purpose, we incorporated in the 0.5-kB SPARC promoter motives responsive to hypoxia and inflammation; this triple chimeric promoter enabled viral replication in the stromal and malignant cell compartment, and showed an enhanced response to hypoxia and inflammation. This improved adenovirus also showed enhanced *in vivo* capacity to eliminate resistant melanomas made of malignant cells mixed with human fibroblasts xenografted in nude mice.

RESULTS

Analysis of the F512-SPARC promoter motifs

Before adding genetic motives responsive to hypoxia and inflammation, we performed a detailed study of the F512-SPARC promoter motifs to establish whether its potency or specificity can be improved. On the basis of *in silico* analysis and previous studies (Hafner *et al.*, 1995), we observed the presence of two putative transcription initiator sequences (INRs) and a downstream promoter element (DPE) in addition to a TATA-like motif (TCTAAA). By using primer extension analysis on RNA extracted from A375N melanoma cells, we confirmed that SPARC mRNA transcription initiates from both the INR1 and the INR2 sites of the endogenous promoter at a ratio of 1: 2 (Figure 1a). Similar results were observed in cervical cancer HeLa cells, although F512 transcriptional activity was extremely low (data not shown). The existence of the DPE and the TATA-like elements, and the fact that both INRs were active, led us to consider that F512 could be a functional dual promoter where the DPE element could initiate transcription through INR1 and the TATA-like sequence might work with INR2 (Figure 1a). We generated seven mutated versions of F512 containing the consensus TATA box, DPE, or the null version of both (Figure 1b) placed upstream of luciferase. Replacing the TATA-box-like element with a nonsense sequence (TATA-null) reduced F512 activity to <20% of the activity of the native sequence (Figure 1b). Interestingly, replacing the native TATA-like box sequence by the consensus one also diminished F512 activity to 50% of the

levels of the native sequence (Figure 1b). Mutational studies that rendered an INR2-null or DPE-null sequence led to 50% inhibition of F512 transcriptional activity (Figure 1b). Neither a DPE element that was mutagenized to obtain the other well-characterized DPE sequence (DPE-mut1) (Juven-Gershon and Kadonaga, 2010) nor an INR1-null sequence induced significant changes in F512 activity (Figure 1b). Thus, F512 works as a dual promoter using the TATA-like and DPE sequences to initiate transcription, although the TATA-like/INR2 motives seem to be the predominant one in terms of F512 transcriptional activity. As all the modifications that we performed resulted in lower levels of F512 activity, we decided to move forward with the wild-type version of F512.

Incorporation to F512 of genetic elements responsive to melanoma microenvironment

We constructed different plasmids that included six (101 bp) or twelve (202 bp) copies of the nuclear factor kappa B (NF- κ B)-responsive element obtained from the HIV long terminal repeat sequence (Adriaansen *et al.* 2007; Khoury *et al.*, 2007) combined or not with three copies of the hypoxia-responsive element ($3 \times$ HRE) obtained from the mouse VEGF promoter (from -889 to -939 bp) (Shima *et al.*, 1996). Both elements were cloned at different positions with respect to F512, and the activity was assessed through luciferase expression assays.

The dual chimeric promoters that included $3 \times$ HRE inside (F512-HRE) or upstream (HRE-F512) of F512 exhibited 6- and 7-fold increased activity in A375N SPARC-positive human melanoma cells compared with F512 under hypoxia, indicating that placement of $3 \times$ HRE inside F512 did not affect its transcriptional activity (Figure 2a). The dual chimeric promoters containing 6 (6 κ BF512) or 12 (12 κ BF512) copies of the NF- κ B-responsive element exhibited 9- and 17-fold induction, respectively, upon tumor necrosis factor- α (TNF- α) addition, indicating a superior activity of the latter (Figure 2b). No TNF- α effect on cell viability was observed under these conditions (data not shown).

Thus, we constructed a triple chimeric promoter where the 12 κ BRE motif was placed upstream of, and the HRE inside, F512. This triple chimeric promoter (κ BF512HRE) exhibited 21-, 7-, and 22-fold increased activity in the presence of TNF- α , hypoxia, or TNF- α + hypoxia, respectively, compared with the activity of F512 in normoxia (Figure 2c). Although no additive effect was observed, the whole data indicate that the triple chimeric promoter would be able to respond to low oxygen tension and inflammatory conditions.

Activity of the chimeric promoter in an adenoviral context

The next step was to prepare a nonreplicative adenovirus where luciferase activity was driven by κ BF512HRE (Ad- κ BF512HRE-Luc) (Figure 3a). Ad- κ BF512HRE-Luc exhibited 30–35, 10–20, and 30–35 increased fold activity in melanoma cells in the presence of TNF- α , hypoxia, or TNF- α + hypoxia, respectively, compared with Ad-F512 under the same conditions (Figure 3b). It is noteworthy that the chimeric promoter exhibited at least 60-fold increased activity in A375N and SB2 cells compared with the activity observed

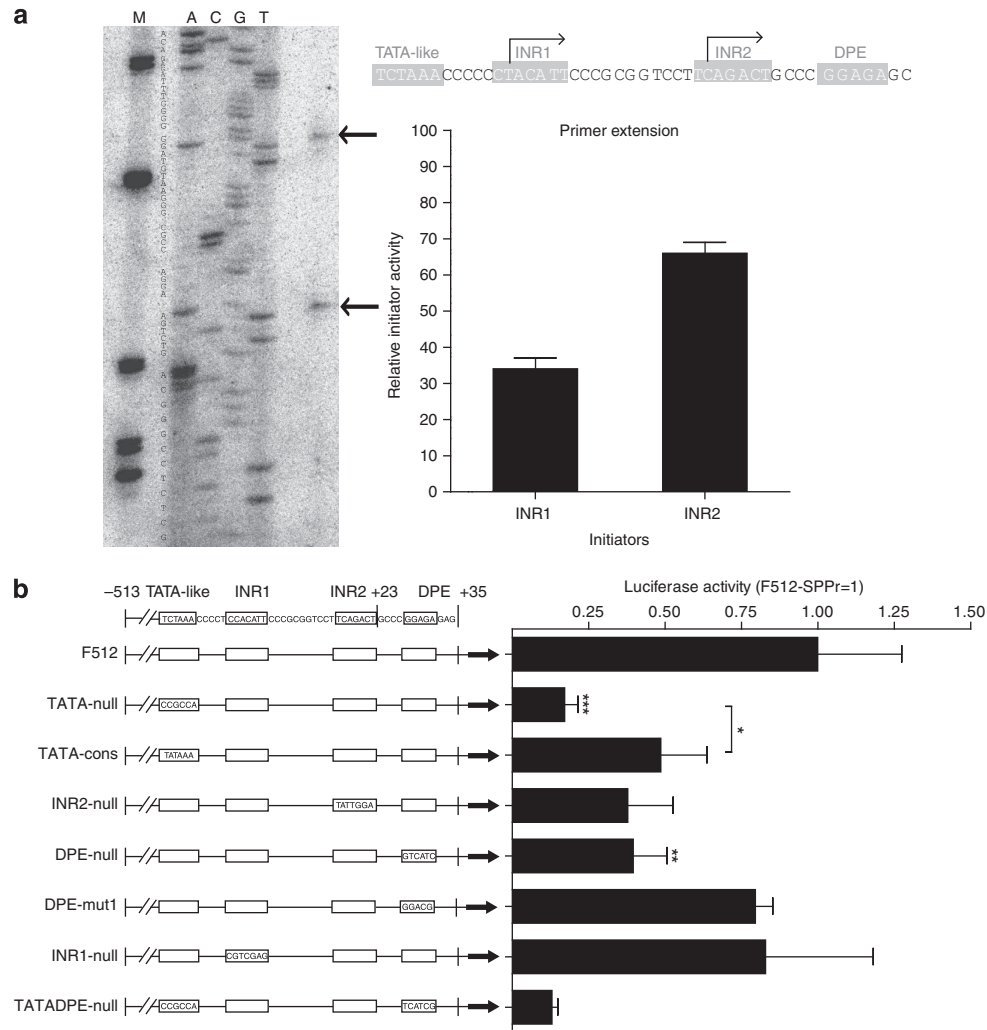


Figure 1. Transcriptional activity of mutated versions of the SPARC promoter. (a) Primer extension analysis of mRNA transcribed from the endogenous SPARC promoter region of A375N melanoma cells. The sequence and the main initiation sites are shown (see arrows). The location of each element (initiator sequence (INR), TATA-like, and downstream promoter element (DPE)) and the relative activity of each initiation site is shown (right panel). Note that there is a T in the INR1 of **a** (obtained from A375N cells) compared with a C in the INR1 sequence of **b** (obtained from human lymphocytes). (b) Luciferase activity of the different mutated versions of the F512-SPARC promoter. The bars represent mean \pm SD ($n = 3$). Data were analyzed by one-way analysis of variance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

in IIB-MEL-LES cells. Ad-kBF512HRE-Luc was also highly active in WI-38 human fetal fibroblasts, reaching 84-, 14-, and 55-fold increased activity in the presence of TNF- α , hypoxia, or TNF- α + hypoxia, respectively, compared with Ad-F512-Luc (Figure 3c). Overall, the data indicate that kBF512HRE responded to hypoxia and inflammatory conditions in an adenoviral context.

Construction of a CRAd driven by the kBF512HRE chimeric promoter

Thus, a CRAd named Ad-kBF512HRE, whose replication is driven by the kBF512HRE promoter, was constructed by inserting the insulator-kBF512HRE promoter cassette into the E1a region to regulate the expression of the *E1a* gene (Figure 4a). We evaluated its replication capacity by assessing the adenoviral genome copy number increase after 72 hours. Ad-kBF512HRE exhibited an enhanced replication capacity

under TNF- α + hypoxia in the two melanoma cell lines, although its overall activity was much higher in SB2 melanoma cells compared with A375N cells (Figure 4b, c, and e). The higher replication and lytic capacity of Ad-kBF512HRE in SB2 cells compared with A375N cells was probably related to the highest CAR levels observed in the former cells that allowed higher infectivity by the CRAd (see Supplementary Figure S1 online).

Ad-kBF512HRE also showed 2.5-fold enhanced replication capacity in WI-38 fibroblast cells under TNF- α + hypoxia, although the overall activity was lower than the one observed in SB2 cells (Figure 4d). Of note was the inhibitory effect exerted by TNF- α on Ad-F512 replication in SB2 cells that was not observed with the new CRAd carrying the chimeric promoter (Figure 4b). This effect was not related to TNF- α itself, as it showed no direct inhibitory activity on cell growth (data not shown).

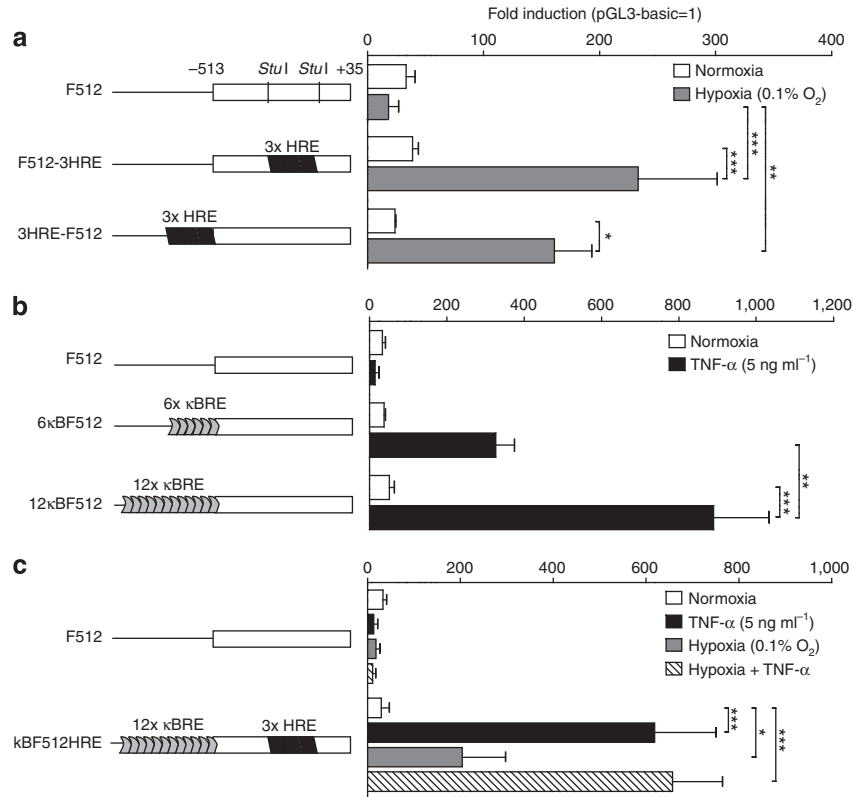


Figure 2. Addition of nuclear factor kappa B (NF-κB) and hypoxia-responsive element (HRE) enhanced the transcriptional activity of the F512-SPARC promoter. A375N melanoma cells were transfected with F512 or chimeric promoters containing (a) HRE, (b) NF-κB responsive elements (κBRE), or (c) both. Cells were placed under (a) hypoxia, (b) tumor necrosis factor-α (TNF-α), or (c) both. Values are expressed relative to the activity of the promoter-less vector pGL3-basic. Data expressed as the mean ± SD ($n = 3$) were analyzed by one-way analysis of variance followed by the Bonferroni test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Ad-kBF512HRE exhibited improved antitumor activity in human melanoma xenografts

We next evaluated Ad-kBF512HRE antitumor activity *in vivo* in a mixed tumor model of SB2 melanoma cells and WI-38 fibroblasts that was described as partially resistant *in vivo* to Ad-F512 activity (Lopez *et al.*, 2009). Established tumors were highly heterogenous in terms of hypoxic areas regardless of whether they were made of malignant cells alone or combined with WI-38 fibroblasts (Supplementary Figure S2a and b online). It was also noteworthy that, in mixed tumors, malignant cells accommodated mainly at the periphery of the tumor mass, whereas fibroblasts were more evenly distributed (Supplementary Figure S2c online). In initial experiments, we observed that administration of five intratumoral doses of 1×10^{10} vp of Ad-F512 to mice bearing mixed SB2/WI-38 tumors did not improve mice survival compared with three doses of the virus, and <40% of mice benefited from both treatment schemes (Supplementary Figure S2d–f online). In clear contrast, intratumoral administration of five doses of Ad-kBF512HRE to mice bearing mixed SB2/WI-38 tumors led to the complete elimination of tumors in 100% of mice in two independent experiments, compared with <40% tumor elimination in mice treated with Ad-F512 (Figure 5). Mostly important, tumors did not recur even after more than 100 days of follow-up. Hexon staining confirmed viral replication inside the tumor mass (Supplementary Figure S2g online).

Fiber exchange highlights the improved therapeutic efficacy of the CRAd based on the chimeric promoter

In clear contrast with the results obtained with SB2 tumors, administration of five doses of Ad-kBF512HRE to nude mice bearing IIB-MEL-LES tumors had no effect on tumor growth (Figure 6a). Flow-cytometer analysis demonstrated that IIB-MEL-LES cells expressed neglectable levels of CAR receptor while they expressed high levels of CD46, one of the receptors of adenovirus type 3 (Supplementary Figure S1 online).

Therefore, we engineered Ad-kBF512HRE with a chimeric 5/3 fiber to redirect viral entry. This pseudotyped CRAd, Ad-5/3-kBF512HRE, exhibited 405-, 230-, and 145-fold increased replication capacity on IIB-MEL-LES cells in the presence of TNF-α, hypoxia, and TNF-α + hypoxia, respectively, compared with Ad-kBF512HRE under the same conditions (Figure 6b). This increased replication was accompanied by an enhanced lytic activity (Figure 6c). Fiber pseudotyping also improved viral lytic capacity on WI-38 fibroblast, as these cells expressed higher levels of CD46 than CAR (Supplementary Figure S1 online and data not shown).

On the basis of the *in vitro* data, we further assessed the *in vivo* efficacy of Ad-5/3-kBF512HRE on CAR-negative, CD46-positive, IIB-MEL-LES-established melanoma xenografts. Administration of five doses of 1×10^{10} vp of Ad-5/3-kBF512HRE on mice bearing established IIB-MEL-LES melanomas led to the complete elimination of tumors in 2/4

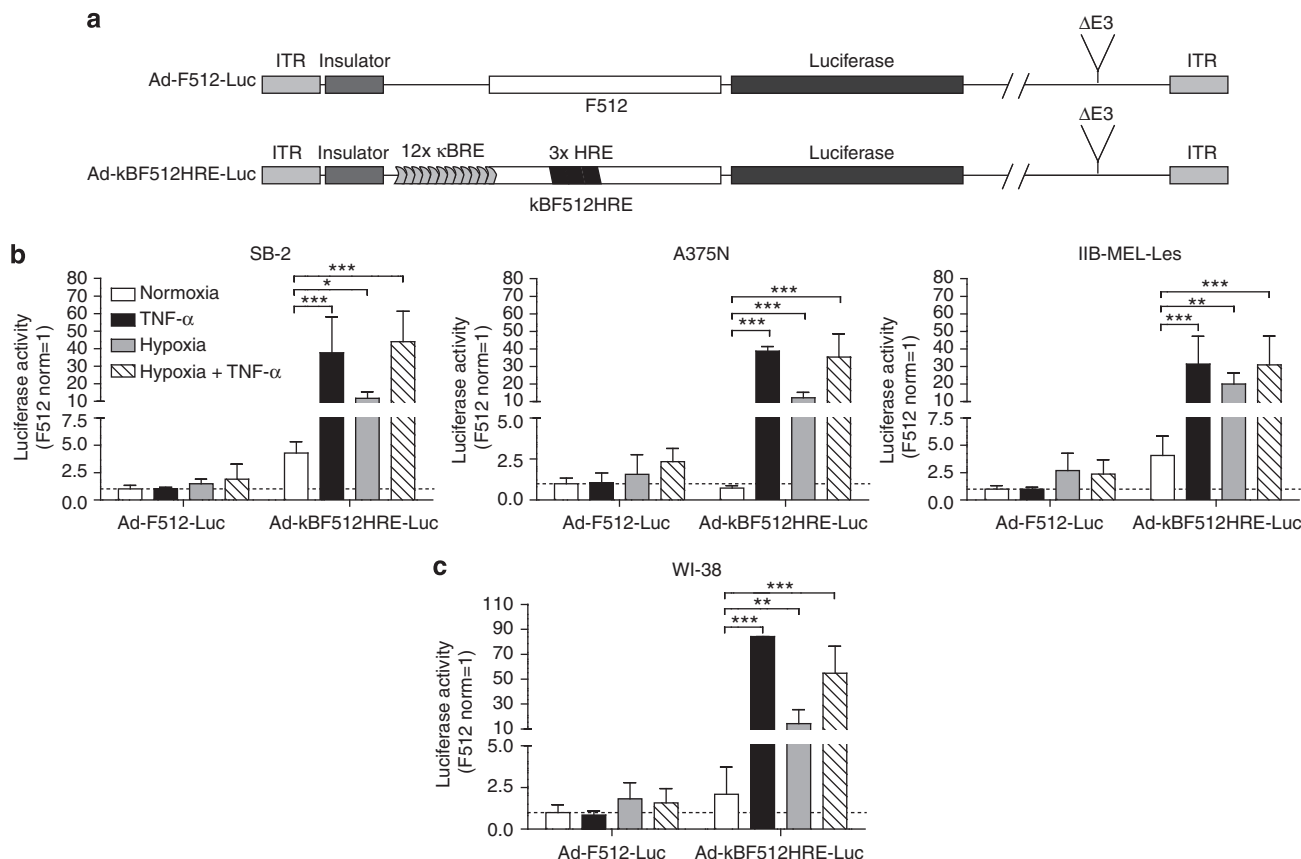


Figure 3. Transcriptional activity of the F512-SPARC promoter combined with NF-κB and hypoxia-responsive element (HRE) in an adenoviral context.

(a) Scheme representing Ad-F512-Luc and Ad-kBF512HRE-Luc reporter adenoviruses (NF-κB responsive elements (κBRE)). Luciferase assay on (b) melanoma cells (SB2, A375N, and IIB-MEL-Les) and (c) human fetal fibroblasts (WI-38). Cells transduced with the different viruses were placed under normal oxygen levels (21% O₂), hypoxia (0.1% O₂), tumor necrosis factor-α (TNF-α) (5 ng ml⁻¹), or hypoxia + TNF-α. Error bars represent the mean ± SD (*n* = 3). Data are expressed relative to the activity of Ad-F512-Luc vector and were analyzed by one-way analysis of variance followed by the Bonferroni test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. ITR, inverted terminal repeat.

mice and a strong delay in tumor growth in one additional mouse (Figure 6d), with a statistically significant increase in survival rate (Figure 6d, insert).

Next, we established whether Ad-kBF512HRE retargeting through pseudotyping with the 5/3 chimeric fiber can also render mixed tumors made of IIB-MEL-LES cells and WI-38 fibroblasts sensitive to oncolytic attack. Treatment of mice bearing established tumors made of IIB-MEL-LES and WI-38 cells with five doses of 1×10^{10} vp of Ad-5/3-kBF512HRE led to the complete tumor elimination in 4/5 mice, a strong delay in tumor growth in 1 additional mouse, and a significant increase in the overall survival (Figure 6e). The effect of the non-pseudotyped CRAd did not differ significantly from phosphate-buffered saline (PBS) (Figure 6d and e).

DISCUSSION

Here we show that incorporation of motives responsive to hypoxia and inflammation to a stroma-targeted conditionally replicative adenovirus greatly improved the therapeutic efficacy of the virus, leading to the elimination of human melanomas established in nude mice. Virus retargeting following engineering of a chimeric 5/3 adenovirus serotype fiber to target melanoma tumors devoid of CAR expression

confirmed the increased potency of this improved oncolytic adenovirus.

To enhance viral replication, we first accomplished a deep study of the F512-SPARC promoter fragment. Mutational analysis demonstrated that both the TATA-like and the DPE sequences were active and that the native sequences of both motifs exhibited better activity than the consensus one. Thus, the F512-SPARC promoter is, to our knowledge, the first native promoter in which both the TATA and the DPE sequences are transcriptionally active, suggesting that F512 is a mixed-mode promoter (Juven-Gershon and Kadonaga, 2010). Further efforts to establish whether these alternate initiation sites could be associated with specific cell types did not succeed. Thus, the possibility that F512 will be active in different cell types depending on the availability of the TATA-like or the DPE-related transcription factors warrants further investigation.

Further to the molecular analysis of the F512 promoter, we incorporated motives responsive to hypoxia and inflammation that are hallmarks of more aggressive melanomas and have been shown to correlate with bad prognosis (Bedogni and Powell, 2009). Few reports described the incorporation of genetic responsive elements to promoters for enhanced

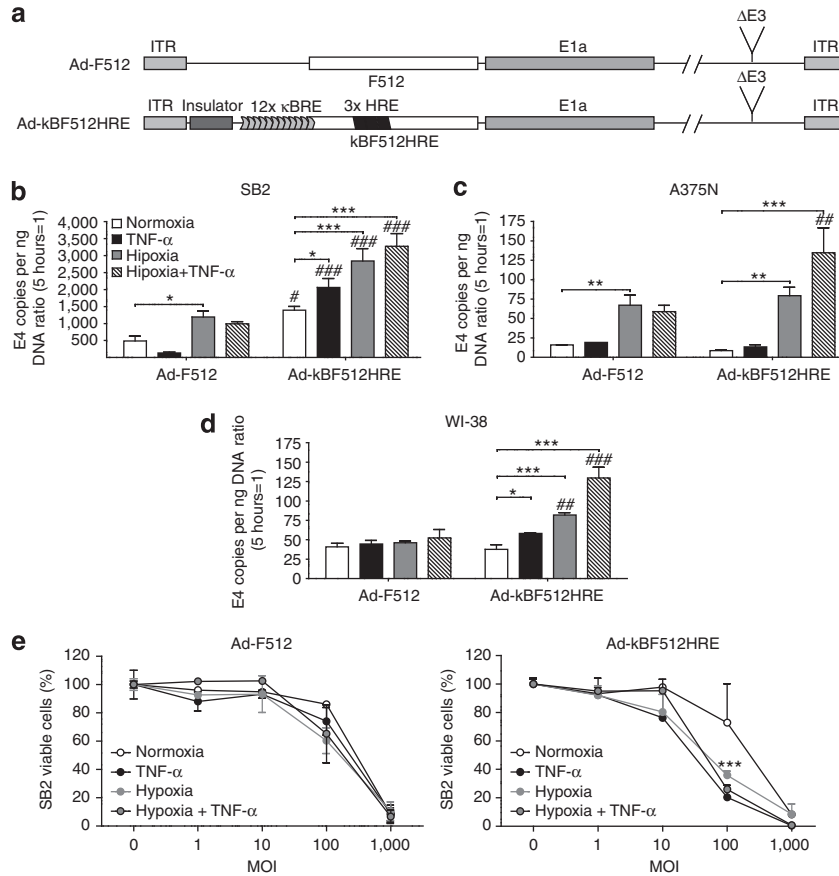


Figure 4. Replication capacity and *in vitro* lytic activity of Ad-kBF512HRE. (a) Scheme representing the different conditionally replicative oncolytic adenoviruses (CRAds). (b–d) Cells were infected with 500 vp per cell and after 5 hours were placed under different conditions as depicted. Five and 72 hours later, cells were collected and their DNA was isolated to assess E4 gene copies. E4 data at 72 hours were normalized with the data at 5 hours. Error bars represent the mean ± SD (n = 3). (e) *In vitro* lytic activity of the CRAds on SB2 melanoma cells. Data were normalized to uninfected cells under the same experimental conditions and analyzed by one-way analysis of variance followed by the Bonferroni test. *P < 0.05, **P < 0.01, and ***P < 0.001. *Relative to normoxia; #Relative to Ad-F512 under the same experimental conditions. ITR, inverted terminal repeat; MOI, multiplicity of infection.

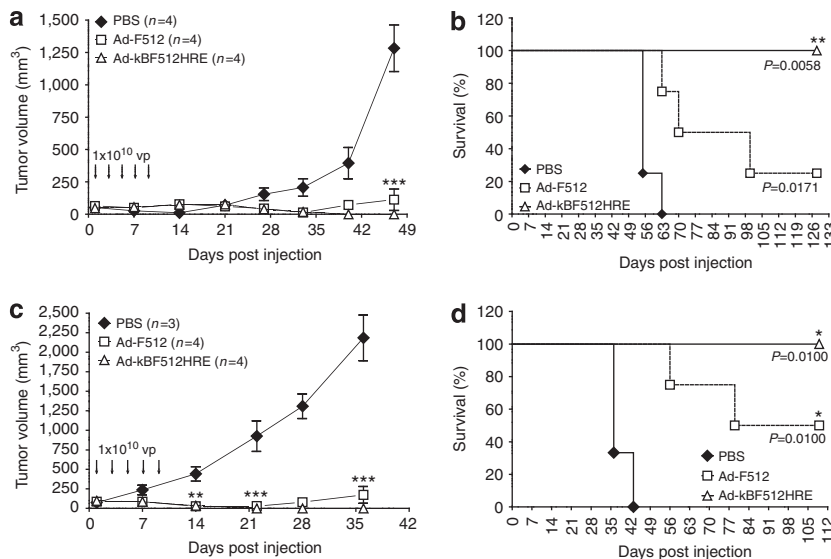


Figure 5. *In vivo* therapeutic efficacy of Ad-kBF512HRE on established tumors. Tumor growth (a, c) and Kaplan–Meier survival curves (b, d) corresponding to mice harboring tumors made of a mix of CAR-positive SB2 melanoma cells and WI-38 human fibroblasts. For further details see the Materials and Methods section. *P < 0.05, **P < 0.01, and ***P < 0.001. PBS, phosphate-buffered saline.

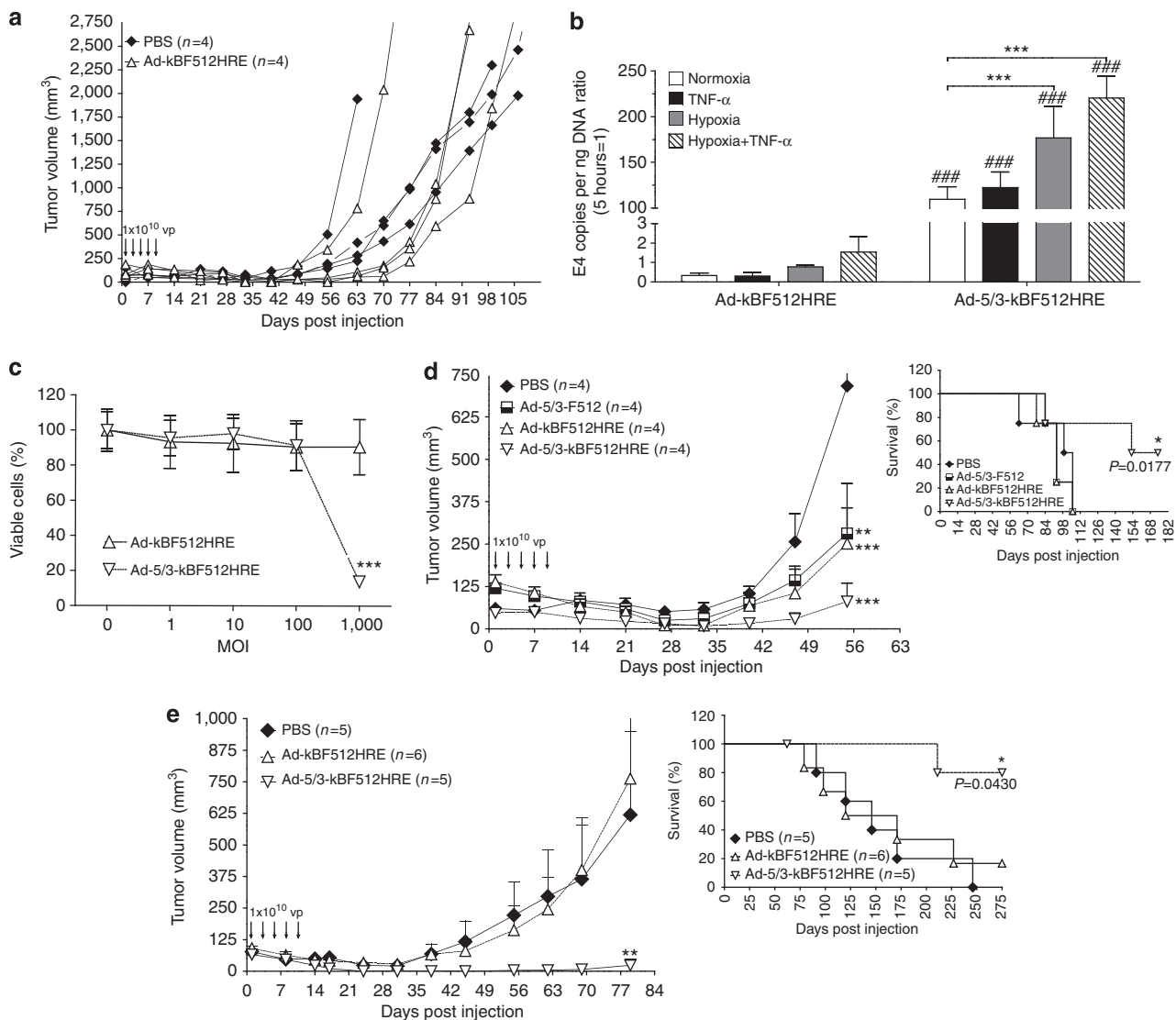


Figure 6. *In vivo* therapeutic efficacy of Ad-5/3-kBF512HRE on CAR-negative tumors. *In vivo* growth of subcutaneous tumors made of CAR-negative IIB-MEL-LES melanoma cells (a, d) or a mix of IIB-MEL-LES and WI-38 fibroblasts (e) treated intratumorally with five administrations of the viruses or phosphate-buffered saline (PBS). Kaplan–Meier curves are included (d, e). For further details, see the Materials and Methods section. (b) Conditionally replicative oncolytic adenovirus (CRAd) replication in IIB-MEL-LES cells assessed by E4 gene DNA copies. Error bars represent the mean \pm SD ($n = 3$). *Relative to normoxia; #relative to Ad-kBF512HRE under the same experimental conditions. (c) *In vitro* lytic activity of the CRAds carrying native fiber 5 or the chimeric fiber 5/3 on IIB-MEL-LES cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. MOI, multiplicity of infection; TNF- α , tumor necrosis factor- α .

transcriptional regulation of therapeutic genes (Yew *et al.*, 2001; Greco *et al.*, 2002; Lee *et al.*, 2002; Liu *et al.*, 2004; Lee *et al.*, 2006; Guo *et al.*, 2007; Poulsen *et al.*, 2008), but only one study attempted to combine NF- κ B and HRE elements in a plasmid that conferred only a slight enhanced activity to the KDR promoter when assessed in primary murine endothelial cells (Modlich *et al.*, 2000). Here we observed that both in a plasmid and in an adenoviral context the triple chimeric promoter increased its transcriptional activity greatly, leading to enhanced viral efficacy under hypoxia and upon TNF- α addition in human melanoma cells and fetal fibroblasts. Moreover, the oncolytic virus carrying this triple chimeric promoter showed enhanced *in vivo* therapeutic efficacy both on tumors made of melanoma cells alone and on tumors made

of a mix of melanoma cells and stromal cells. Although we were unable to observe an *in vitro* synergistic effect when the replication/lytic capacity of Ad-kBF512HRE was assessed in the presence of hypoxia + TNF- α , we can envisage an *in vivo* positive loop because hypoxia has been shown to enhance transient changes in NF- κ B levels (Vaupel, 2010). Pseudotyping this virus with a chimeric fiber 5/3 overrides the natural resistance of a melanoma cell line devoid of CAR receptors such as IIB-MEL-LES. This is consistent with previous studies showing that some human fresh melanoma might not express CAR (Rivera *et al.*, 2004). Thus, assessment of viral receptor expression in fresh melanoma samples before treatment can help predict viral success in a potential clinical trial.

The tumor microenvironment involves the malignant cells themselves, fibroblasts, endothelial cells, and inflammatory cells. This microenvironment is characterized by low levels of oxygen tension and increased proinflammatory conditions that might enhance NF- κ B responsiveness (Melnikova and Bar-Eli, 2009). New blood vessel formation by endothelial cells and enhanced remodeling of the extracellular matrix by neighboring fibroblasts to create either new tracks or barriers help tumors develop beyond an initial avascular growth and protect malignant cells from the attack of therapeutic drugs. Moreover, the dynamic cross-talk between malignant melanoma cells and surrounding stromal cells is a key event for allowing dissemination (van Kempen *et al.*, 2008). Melanoma and inflammatory cells induce fibroblast proliferation through the release of platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor- β , whereas fibroblasts produce a series of growth factors such as IGF1, hepatocyte growth factor, basic fibroblast growth factor, and transforming growth factor- β that can support melanoma cell proliferation (Hsu *et al.*, 2002; Hendrix *et al.*, 2003).

Recent studies have shown encouraging studies looking at combining the oncolytic adenovirus H101 with an small interfering RNA against Bcl2 in different human uveal melanoma cells lines (Huang *et al.*, 2012). A couple of studies have also shown that a CRAAd carrying IL-24 helped in inducing apoptosis of melanoma cells following radiotherapy (Chai *et al.*, 2012; Jiang *et al.*, 2012). Thus, the possibility of acting on human melanoma tumors with an oncolytic virus that can target both the stromal and the malignant compartment, respond to melanoma microenvironment, and whose infectivity can be tailored according to cell surface expression of viral receptors can be of significant importance for melanoma therapeutics.

MATERIALS AND METHODS

Cell lines

See Supplementary Material online.

Plasmids and recombinant adenoviruses

See Supplementary Material online.

Primer extension

A measure of 1 μ g of poly(A) RNA extracted from A375N melanoma cell line using PolyATtract mRNA Isolation System (Promega, Madison, WI, USA) was annealed with 1 pmol of [γ - 32 P] end-labeled reverse primer (5'-TCTCCAGCGGTTCCATCTTCCAGC-3'). The reverse transcripts were generated according to the protocol of Primer Extension System-AMV Reverse Transcriptase (Promega), using 55 $^{\circ}$ C as annealing temperature for primer and RNA binding. The same primer was used for sequencing with fmol DNA Cycle Sequencing System (Promega) according to the supplier's protocol using 55 $^{\circ}$ C as melting temperature. The extension products from primer extension and sequencing were analyzed by 8 M urea-6% polyacrylamide gel electrophoresis. The products were detected by gel drying and exposure to phosphorimaging screens (Molecular Dynamics, Madison, WI) for quantitative analysis using a Storm scanner and ImageQuant software (Storm 860 by Molecular Dynamics, now GE Healthcare, Madison, WI).

Luciferase assays and E4 real-time PCR

See Supplementary Material online.

Flow cytometry

Cells at 80% confluence were washed with PBS, detached with 5 mM EDTA, resuspended in DMEM/F12 containing 10% fetal bovine serum (FBS), washed again with PBS, and resuspended at a concentration of 5×10^6 per ml in cold PBS containing 10% FBS and 1% sodium azide (PBS/FBS/NaAz). For further details see Supplementary Material online.

In vitro and in vivo assays and ethics statement

All experiments were approved by the Institutional Animal Care and Use Committee of the Fundación Instituto Leloir (Protocol no. 300P). The Fundación Instituto Leloir has an approved Animal Welfare Assurance as a foreign institution with the Office of Laboratory Animal Welfare, Number A5168-01.

Six- to eight-week old male athymic N:NIH (S)-nu mice (obtained from the animal facility of the Faculty of Veterinary, University of La Plata) were subcutaneously injected on the left flank with 5×10^6 melanoma cells mixed or not with 2×10^6 WI-38 fibroblasts. When the average tumor volume reached 100 mm 3 , mice received 1×10^{10} vp/mouse of CRAAd or vehicle (PBS) administered intratumorally on days 1, 3, and 7 (three doses) or 1, 3, 5, 7, and 9 (five doses). Sequential doses were administered in different areas of the tumor, trying to avoid leakage. Tumor size was estimated once a week with caliper measurements (volume = $0.52 \times (\text{width})^2 \times \text{length}$). Mice were followed up and then killed when tumors reached approximately 2 cm 3 ; in several cases, data are presented as tumor growth of individual mice, whereas in other cases, data are presented as average tumor volume. All animals under study received food and water *ad libitum*. Survival rates were calculated with the Kaplan–Meier method, and multiple comparisons of survival were evaluated by the Bonferroni-corrected method. A *P*-value 0.05/*K* (where *K* = number of comparisons) was considered statistically significant. Data analysis was performed with the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Material is linked to the online version of the paper at <http://www.nature.com/jid>

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