

# Specific Oncolytic Effect of a New Hypoxia-Inducible Factor-Dependent Replicative Adenovirus on von Hippel-Lindau-Defective Renal Cell Carcinomas<sup>1</sup>

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## ABSTRACT

Mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene are responsible for a hereditary cancer syndrome characterized by high susceptibility to hemangioblastomas of the retina and central nervous system, pheochromocytomas, and renal cell carcinomas. In agreement with its role as a tumor suppressor, the vast majority of spontaneous clear cell carcinomas of the kidney present loss of heterozygosity at the *VHL* locus. Recently, it has been shown that *VHL* works as the substrate recognition component of an E3 ubiquitination complex that targets the hypoxia-inducible factor (HIF) for proteosomal degradation. Under normal oxygen tension, the half-life of HIF transcription factors is extremely short because of its high degradation rate by the proteasome, resulting in undetectable HIF activity in normal cells. However, in *VHL*-deficient tumor cells, the HIF transcriptional pathway is constitutively activated because of impaired ubiquitination of this transcription factor. To target *VHL*-deficient tumors, we have exploited this feature to develop a conditionally replicative adenovirus (Ad9xHRE1A), the replication of which is HIF dependent. In this new oncolytic adenovirus, the expression of the E1A gene is controlled by an optimized minimal promoter containing HIF recognition elements. Here, we show that the induction of the E1A gene, as well as the viral replication and cytolytic effect of Ad9xHRE1A, are dependent on HIF activity. As a consequence, this virus efficiently kills *VHL*-deficient cells both *in vitro* and *in vivo*, as well as cells growing under hypoxic conditions. These data suggest that Ad9xHRE1A could be used as a highly specific therapy for *VHL*-deficient cancers and probably many other tumors that show extensive hypoxic areas or increased HIF activity by genetic alterations other than *VHL* loss.

## INTRODUCTION

Because oxygen is essential for the survival of most cell types, they respond to changes in oxygen tension by the induction of adaptive responses aimed to restore oxygen supply and maintain energy balance. The response to decreased oxygen is mediated by the activation of a specific set of genes, most of them under the control of the HIFs.<sup>4</sup> HIF transcription factors are heterodimers of a constitutively ex-

pressed  $\beta$  subunit (also known as aryl receptor nuclear translocator) and an oxygen-regulated  $\alpha$  subunit (1). The HIF- $\beta$  subunit, as well as the three different HIF- $\alpha$  subunits identified to date, belong to the basic helix-loop-helix-Per/aryl receptor nuclear translocator/Sim transcription factors. Under normal oxygen tension, HIF- $\alpha$  is undetectable because of a high degradation rate by the proteasome (1). When oxygen is limiting, its half-life increases and HIF- $\alpha$  protein accumulates, allowing its interaction with HIF- $\beta$  and the transcriptional activation of target genes needed for the cellular adaptation to hypoxia (1). The observation that cells deficient for the tumor suppressor gene *VHL* overexpressed HIF target genes such as VEGF led to the identification of *VHL* protein as a critical element of the oxygen sensing pathway. *VHL* protein is the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF for proteosomal degradation. However, *VHL*/HIF- $\alpha$  interaction depends on the hydroxylation of two specific proline residues on HIF- $\alpha$ , a reaction catalyzed by a novel family of 2-oxoglutarate-dependent dioxygenases (1). Because these enzymes require molecular oxygen for their catalytic reaction, it is accepted that they act as the oxygen sensors that directly control HIF stability in response to oxygen variations. As predicted by this model, loss of *VHL* results in HIF- $\alpha$  accumulation and activation of target genes, regardless of the presence or absence of oxygen.

Long before the role of pVHL on HIF regulation was known, the gene encoding for pVHL was identified as a tumor suppressor involved in a rare hereditary cancer syndrome (*VHL* syndrome) characterized by high frequency of hemangioblastomas, pheochromocytomas, and clear cell renal carcinomas. In agreement with its role as a tumor suppressor, *VHL* is lost in the vast majority of sporadic clear cell carcinomas of the kidney. It is currently unclear whether HIF- $\alpha$  up-regulation is the major factor responsible for the generation of a full transformed phenotype after *VHL* loss (2, 3), but it is generally accepted that HIF target genes contribute to tumor progression in *VHL*-negative cells. For example, the induction of VEGF expression by HIF plays a critical role in promoting tumor-mediated angiogenesis, and accordingly, clear cell carcinomas are highly vascularized and metastatic. In addition, it has been recently described that the HIF activation that occurs in *VHL*-deficient cells results in overexpression of metalloproteinases that promote enhanced migration (4).

Current cancer treatments depend on the use of drugs that have serious side effects and are often of limited value for the control of the disease. This is particularly true for clear cell carcinomas of the kidney (5). Thus, the development of new strategies for the treatment of these and other cancers is needed. One of such strategies that is particularly promising is the use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells (CRADs; Refs. 5, 6). In this approach, the initial administration of the virus kills the infected cells, and a new viral progeny is released that can repeat the process and amplify the oncolytic effect until the tumor is eliminated. One method to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as E1A or E4. The promoters of these genes are substituted by regulatory sequences that are preferentially activated in cancer cells. We and others (6, 7) have successfully devel-

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<sup>4</sup> The abbreviations used are: HIF, hypoxia-inducible factor; MOI, multiplicity of infection; HRE, hypoxia response element; Q-RT-PCR, quantitative real-time PCR; VEGF, vascular endothelial growth factor; CRAD, conditionally replicative adenovirus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pfu, plaque-forming unit(s); *VHL*, von Hippel-Lindau; RCC, renal cell carcinoma; X-Gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; pVHL, *VHL* protein; pRb, retinoblastoma protein (product of the RB tumor suppressor gene); HUVEC, human umbilical vascular endothelial cells.

oped CRADs for the targeting of tumors with specific genetic alterations. The constitutive activation of HIF transcription factors that is intrinsically associated to *VHL* loss makes tumors harboring this alteration suitable targets for a CRAD in which viral replication is under the control of HIF. In fact, we have previously developed a series of CRADs in which replication is controlled by hybrid promoters containing estrogen response elements and HREs to target the hypoxic areas of breast tumors (7–9). In one of these viruses (AdEHE2F), the E4 coding region is under the control of an E2F-1-responsive promoter (7). The activity of E2F transcription factors is required for cell cycle progression, and in most tumor cells, the E2F activity is up-regulated because of diverse alterations in the pRb tumor suppressor pathways, which normally have an inhibitory effect on E2F activity. In addition, expression of the adenovirus E1A protein induces E2F activity by sequestering pRb. In this type of CRAD, the regulation of E4 by E2F activity restricts its expression to proliferating cells, including tumors. However, because E1A protein interferes with pRb, E1A induction ensures E2F activation and E4 expression (7, 10). Thus, regulation of E4 region by E2F is not intended as an independent level of regulation but rather as an amplification loop upon E1A activation in tumor cells.

Here, we describe the generation of a new version of HIF-dependent CRAD (Ad9xHRE1A) to target tumor cells with constitutive activation of the HIF pathway such as VHL-deficient renal carcinomas. We have maintained the E2F-1 promoter in the E4 region of this virus for the reasons discussed above. On the other hand, for the control of the E1A region, we have used an optimized minimal promoter because this is the single most important regulatory element for the replication of adenovirus. This is an artificial promoter that contains nine tandem copies of the HRE for binding of HIF. The simplicity of its sequence reduces the chances that other transcription factors will bind to the promoter and abolish its specificity, especially in the context of an adenovirus genome. We have characterized in detail this new CRAD and have found a tight regulation of the E1A expression that closely correlates with its ability to replicate and kill VHL-deficient cancer cells. Finally, we provide data showing antitumor effect of this virus *in vivo*.

## MATERIALS AND METHODS

**Cell Culture and Reagents.** pVHL-deficient 786-O cell clones stably expressing wild-type *VHL* (WT-10), truncated *VHL* (1-115), or empty plasmid (PCR3) were kindly provided by William Kaelin (Dana-Farber Institute, Boston, MA). Parental *VHL*-negative RCC4 cells (herein described as RCC4–) and the corresponding *VHL* stable transfectants (RCC4+) were provided by Dr. Patrick H. Maxwell (Imperial College, London, United Kingdom). Parental *VHL*-negative RCC10 cells and a clone derived by stable transfection of *VHL* (VHL53) were provided by Dr. Karl H. Plate (Johann Wolfgang Goethe University, Frankfurt, Germany). Parental *VHL*-defective UM-RC6 cells and the stable *VHL* transfectant, UM-RC6 3-4 (herein referred as 3-4), were provided by Dr. Michael I. Lerman and Dr. Sergey V. Ivanov (Laboratory of Immunobiology, National Cancer Institute, Frederick, MD). WT-10-PP13 clone derives from WT-10 cells upon stable expression of P402A, P564G mutant HIF-1 $\alpha$ ; it was generated by Silvia Martín *et al.* and will be described in more detail in a manuscript in preparation.<sup>5</sup> All cells were maintained in RPMI 1640 with Glutamax-I (Life Technologies, Inc.). For WT-10, 1-115, PRC3, WT-10-PP13, VHL +53, and RCC4+, G418 sulfate (100 mg/ml; Promega) was added to culture media. HeLa and IMR-90 human fibroblast were maintained in DMEM (Life Technologies, Inc.). In all cases, culture media were supplemented with 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum. Human umbilical vascular endothelial

primary cells, were isolated and grown as previously described (11). Hypoxia (1%) was induced by culture of cells in an *in vivo* 400 hypoxia workstation (Ruskin Technology). Deferoxamine was purchased from Sigma (St. Louis, MO), anti-HIF-1 $\alpha$  from Transduction Laboratories, anti-HIF-2 $\alpha$ /EPAS from Novus Biologicals (Littleton, CO), and anti- $\alpha$ -Tubulina from Sigma.

**Construction of the Ad9xHRE1A Virus.** The virus was constructed by promoter replacement of the E1A and E4 regions after a previously described method (7). Briefly, we amplified the 9XHIF promoter by PCR from the plasmid p9xHIF-Luc (12), using the primers 5'-TCAGTGCTAGCTTC-GAAGCCATATCACATTTGTAGAGG-3' and 5'-CCTAGAGATCTTTC-GAACAAGCTTGACCACACTTCC-3'. These primers introduce *Bst*BI restriction sites flanking the 9XHIF construct for its introduction into the E1A promoter region of the plasmid pSEHE2F. This plasmid contains unique restriction sites flanking the promoter regions of E1A (*Bst*BI sites) and E4 (*I-CeuI* and *SwaI* sites). In the latter region, we maintained the E2F-1 promoter (bp, –218 to +51; Ref. 7) that was obtained by PCR from human genomic DNA using the primers 5'-TACTGTAACATAACGGTCTTAAGG-TAGCGTGGTACCATCCGGACAAAGCC-3' and 5'-TAAGTATTTAAAT-GGCGAGGGCTCGATCCCGC-3'. The new plasmid (named pSHIFE2F) was digested with *PacI* to liberate the modified viral genome. After ethanol precipitation, 10  $\mu$ g were used to transfect 293E4pIX cells growing in 10-cm dishes by the calcium phosphate method. The cells were treated with 1  $\mu$ M dexamethasone until the cytopathic effect was observed. Individual viral plaques were isolated from infected monolayers growing under semisolid medium. The modification in the E1A and E4 promoter regions were verified by PCR using specific primers. The viruses were amplified in A549 cells growing in the presence of 100  $\mu$ M CoCl<sub>2</sub> to mimic hypoxic conditions, purified using CsCl gradients, and desalted in G-50 Sephadex columns. Titration was done after the plaque-forming assay method.

**Western Blot.** The level of HIF-1 $\alpha$ , HIF-2 $\alpha$ /EPAS and  $\alpha$ -tubulin protein expression was determined by immunoblotting. Immunolabeling was detected by enhanced chemiluminescence (Amersham Pharmacia Biotechnology, Piscataway, NJ) and visualized with a digital luminescent image analyzer (Fuji-film LAS-1000 CH).

**Measure of HIF-Dependent Transcriptional Activity.** Cells were cotransfected by lipofection with the HIF-responsive firefly luciferase reporter (12) and a Renilla luciferase expression plasmid at a 40:1 ratio. We used Lipofectamin (Roche Applied Science) for 786-O and UM-RC6 lines and Superfect (Qiagen) for RCC4 and RCC10 lines. Cell lines were transfected as a pool for either 24 h (Lipofectamin) or 10 h (Superfect) in 100-mm culture dishes, after transfection cells were split into 24-well plates and, 20–24 h after plating, submitted to hypoxia (1%) or left at normoxia for 10 h. Finally, cells were harvested and firefly and Renilla luciferase activities were determined using a dual luciferase system (Promega). Firefly luciferase activity was normalized based on the Renilla luciferase activity. The average and SD of triplicate samples for each condition is represented. HeLa cells were always included as a control for reporter inducibility (data not shown).

**Q-RT-PCR.** The levels of VEGF mRNA and E1-A mRNA were determined by Q-RT-PCR. Immediately after treatment, cells were harvested into 1 ml of Ultraspec reagent (Biotecs, Houston, TX). Total RNA was extracted, quantified, and integrity tested by gel electrophoresis. One  $\mu$ g of total RNA from each sample was retrotranscribed to cDNA (Improm-II reverse transcriptase; Promega). One to 3  $\mu$ l of cDNA samples were used as template for amplification reactions carried out with the LC Fast Start DNA master Sybr Green I kit (Roche Applied Science, Mannheim, Germany) following manufacturer instructions. PCR amplifications were carried out in Light Cycler System (Roche Applied Science) and data analyzed with LightCycler software 3 version 3.5.28 (Idaho Technology, Inc.). For analysis purposes, the amplicon for each of the analyzed genes was cloned, and known amounts of the cloned product were used to generate a standard curve. The number of copies of the interest gene in each sample was extrapolated from the corresponding standard curve by the indicated software. For each sample, duplicate determinations were made, and the gene copy number was normalized by the amount of  $\beta$ -actin on the same samples. The primers used in this study are (5'-3'): VEGF-A, forward (TGCCAAGTGGTCCCAG) and reverse (GTGAGGTTT-GATCCGC);  $\beta$ -actin, forward (CCCAGAGCAAGAGAGG) and reverse (gTCCAGACGCAGGATG); and E1A, forward (CTTGTCTATTATCACCG-GAG) and reverse (TCCGTACTACTATTGCATTCT).

<sup>5</sup> S. Martín-Puig, E. Temes, R. Martín, J. Aragones and M. O. Landázuri. Pro564 and Pro402 are essential for the full induction of HIF1 $\alpha$  by hypoxia, manuscript in preparation.

**Adenoviral Infection and Cell Viability Assays.** Cells were plated at  $10^5$  cells/well in 24-well plates before infections. Twenty-four h later, the media were removed and replaced with 2% media. Cells were infected by exposure to adenovirus preparations diluted to the indicated MOIs in 200  $\mu$ l of RPMI supplemented with 2% FCS. After 1 h incubation with frequent gentle shaking, 300  $\mu$ l of fresh media were added, and 48 h after infection, an additional 0.5 ml of fresh media were added to each well. During the course of the experiment, 0.5 ml of media were removed from cultures and replaced with 0.5 ml of fresh media every 48 h.

Cell viability was determined 7–10 days after infections by the MTT assay (Sigma). At the time of analysis, 25  $\mu$ l of Thiazolyl blue (MTT, 5 mg/ml) solution were added to the cell cultures in 0.5 ml of medium. Four h later, media were removed and precipitated MTT salts solubilized into 150  $\mu$ l of 0.04 N HCl diluted in isopropanol. Product formation was quantified by reading solution absorbance at 550 nm.

**Adenovirus Replication Assay.** Cells were plated in 24-well plates, and 24 h later, cells were infected with Ad-5WT or Ad-9xHRE1A at a MOI of 10 pfu/cell. Twenty-four h after infections, cells were washed four times with fresh medium to removed virus excess. Eight days later, the plates were centrifuged at 4°C for 10 min at  $4000 \times g$  to remove floating cells, and cell culture supernatants were collected. Viral titer was calculated by infection of HEK293 cells with 200  $\mu$ l of the supernatants and 300  $\mu$ l of fresh medium supplemented with 2% serum. Infected HEK293 cells were identified 48 after infection by immunostaining against viral protein (hexon) with the Adeno-X Rapid Titer Kit (BD Biosciences Clontech, Palo Alto, CA) following manufacturer instructions.

**Adenoviral Infectivity Assays, X-Gal Staining.** Cells were seeded on 24-well plates at a density of  $2 \times 10^5$  cells/well. Twenty-four h after plating, cells were infected with Adeno-X-LacZ (BD Biosciences Clontech), an adenovirus that expressed the  $\beta$ -galactosidase gene at a MOI of 50 pfu/cell. Forty-eight h after exposure to Adeno-X-LacZ, infected cells were identified on the cell monolayer by fixing for 20 min in a solution containing 50% (w/w) glutaraldehyde in PBS and staining with the  $\beta$ -galactosidase substrate X-Gal. The staining solution contained X-Gal (1 mg/ml), 2 mM  $MgCl_2$ , 4 mM  $K_3Fe(CN)_6$ , and 4 mM  $K_4Fe(CN)_6$  (Sigma) in PBS. The number of blue cells and unstained cells/field was counted, and percentage of infectivity was determined.

**In Vivo Assays in Nude Mice.** The antitumor effect of Ad-9xHRE1A was tested in human tumor xenografts implanted in 6–8-week-old Swiss-nu/nu mice (Charles River, ICO:Swiss-FoxN1nu). A total of  $1.3 \times 10^7$  1-115

(786-O) cells was resuspended in PBS and injected s.c. in the back flanks. When tumors reached a volume of  $\sim 200$  mm<sup>3</sup>, mice were treated by intratumoral injection of Ad-9xHRE1A ( $1.6 \times 10^8$  pfu in 50  $\mu$ l of glycerol/BSA buffer) or glycerol/BSA buffer (100 mM Tris (pH 8.1), 100 mM NaCl, 1% BSA, and 50% glycerol) alone (control group). One day later, treatments were repeated. Tumor volume was measured weekly and calculated using the equation:  $D \times (d)^2/2$ , where  $D$  and  $d$  are the major and minor diameters, respectively. Sixty days after treatments, mice were sacrificed and tumors excised and weighted.

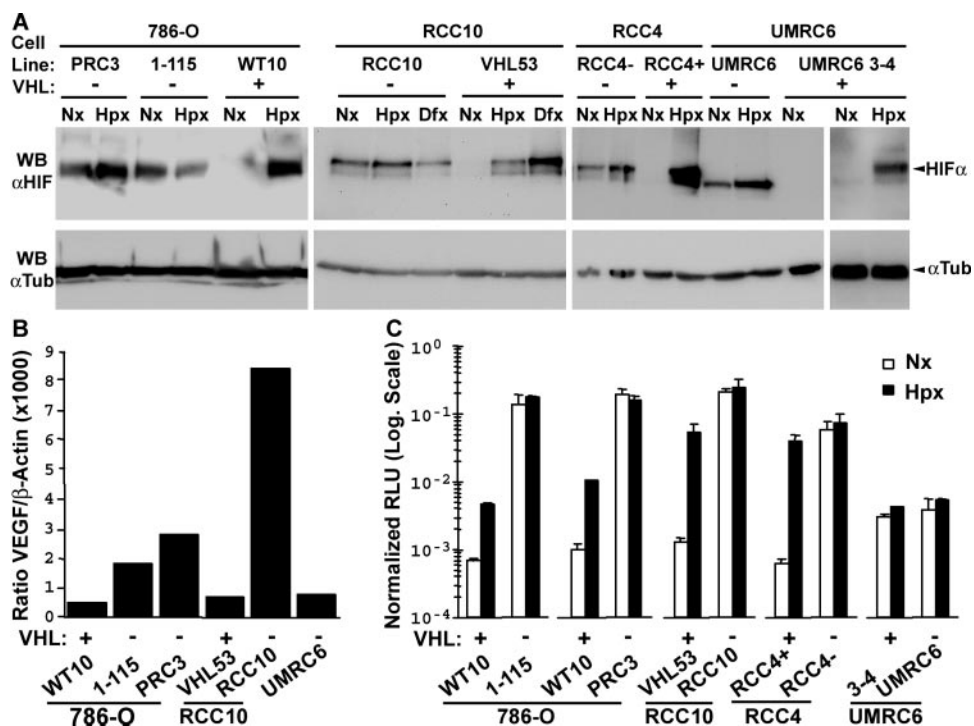
Animals were maintained under specific pathogen-free conditions and treated according to a protocol approved by the Universidad Autonoma de Madrid Animal Care Committee.

**RESULTS**

**Characterization of Clear Cell Renal Carcinoma Cell Lines.**

We chose several different cell lines derived from four independent human clear cell carcinomas of the kidney: 786-O (13, 14); RCC10 (15); UM-RC6 (16, 17); and RCC4 (18) cells. 786-O renal carcinoma cells lack functional pVHL and, as consequence, overproduce HIF-2 $\alpha$  (18) and HIF target genes such as VEGF (19). Several cell lines were obtained by stable transfection of 786-O cells with plasmids encoding wild-type VHL protein (WT-10), truncated inactive VHL protein (1-115), or empty plasmid (PRC3). These cell lines have been widely used for the study of pVHL and HIF- $\alpha$  function (18). Similarly, RCC10, UM-RC6, and RCC4 cells derive from human renal carcinomas lacking functional pVHL and present constitutively elevated HIF-1 $\alpha$  protein under normoxia. Finally, the VHL53, 3-4, and RCC4+ cell lines derive from RCC10, UM-RC6, and RCC4-, respectively, after stable transfection with wild-type VHL gene. As shown in Fig. 1A, all pVHL-defective cell lines (PRC3, RCC10, UM-RC6, and RCC4-) express significant amounts of HIF- $\alpha$  in the presence of oxygen (normoxia) that are not further increased after exposure to hypoxia. In agreement with previous studies (13–18), after restoration of normal VHL function, HIF- $\alpha$  protein was only detected under low oxygen tension (hypoxia) or in the presence of hypoxia mimetic chemical agents such as deferoxamine (Fig. 1A).

Fig. 1. Characterization of the renal cancer cell lines used in this work. A, cells were grown on 6-well plates and transferred to 1% oxygen atmosphere (Hpx, hypoxia) or left at normoxic conditions (Nx) for 12 h. Where indicated, cells were exposed to the hypoxia-mimetic compound deferoxamine (Dfx, 380 $\mu$ M) for the same period of time. After treatments, cellular protein extracts were fractionated by SDS-PAGE, and the level of HIF- $\alpha$  protein was determined by immunoblot. As a control, the level of  $\alpha$ -tubulin on each sample is also shown. In all of the cases, except for 786-O cells, HIF-1 $\alpha$  expression is shown. 786-O cells do not express detectable amounts of HIF-1 $\alpha$ . Therefore, in this case, HIF-2 $\alpha$  was determined instead of HIF-1 $\alpha$ . B, the abundance of VEGF-A mRNA was determined by Q-RT-PCR and represented after normalization by the amount of  $\beta$ -actin. C, cells were transiently transfected with and HRE-driven firefly luciferase reporter plasmid together with a Renilla luciferase expression plasmid and then transferred to Hx (1% O<sub>2</sub>) or left at Nx for 8–10 h. The average firefly luciferase activity, normalized to the Renilla luciferase activity, in duplicate samples is represented. The results shown in A–C are representative of at least two independent experiments.



Importantly, the overexpression of HIF- $\alpha$  under normoxic conditions found in VHL-defective cell lines correlates with the induction of HIF target genes such as VEGF in all cases, except UM-RC6 cell line (Fig. 1B). The unexpected behavior of UM-RC6 cell line regarding VEGF production has been previously noticed (17) and suggests that although HIF- $\alpha$  is overexpressed under normoxia in this cell line (Fig. 1A), it may be nonfunctional for the induction of some target genes such as VEGF.

Next, we investigated the activity of HIF on different cell lines. To this end, we transfected them with a reporter construct that express the firefly luciferase gene under the control of a rat prolactin minimal promoter downstream of nine tandem copies of the HRE found in the VEGF promoter (12, 20). To prevent unspecific expression of the reporter gene, two tandem SV40 polyadenylation signals were included upstream of the promoter control elements (12). Previous experiments with this construct indicate a tight hypoxia-dependent regulation of the reporter gene (12, 20). Fig. 1C shows that the reporter gene expression is elevated under normoxic conditions in all VHL-deficient cells, except UM-RC6, compared with the VHL-competent cells. Moreover, HIF-regulated luciferase expression was induced by hypoxia only in those cell lines expressing functional pVHL. Again the 3-4 cell line, which was generated by stable transfection of VHL into UM-RC6 cells, was exceptional in that hypoxia did not up-regulate HIF activity (Fig. 1C), although expression of pVHL in 3-4 cells restores HIF- $\alpha$  protein regulation by oxygen (Fig. 1A).

Thus, all VHL-deficient cells present deregulated HIF protein expression that results in increased HIF activity, with the exception of the UM-RC6 cell line. Forced expression of pVHL in these renal carcinoma cells restores normal HIF regulation. It is unclear why UM-RC6 cells, despite presenting high basal levels of HIF protein, are unable to induce HRE-dependent reporters or up-regulate VEGF mRNA. Future work will address this issue. Regardless of the mechanism, the deficient induction of HRE-driven genes in this VHL-deficient cell line provides a unique opportunity to test the specificity of HIF-dependent therapeutic strategies.

**Sensitivity of Clear Cell Renal Carcinoma Cells to Adenovirus Infection.** Adenoviruses bind to the surface of cells mainly through the coxsackie adenovirus receptor, and the internalization requires the participation of integrins  $\alpha\beta3$  and  $\alpha\beta5$ . Because loss of pVHL has been related to alterations on integrin function (21), we decided to study whether VHL deficiency affected adenovirus infection. For this purpose, cells were infected with an E1-defective adenovirus expressing the LacZ gene under the control of a cytomegalovirus promoter. After infections, cells were fixed and processed to determine  $\beta$ -galactosidase-expressing cells. As shown in Fig. 2, there is a wide range of sensitivity to adenovirus infection among the different cell lines, WT-10, RCC10, UM-RC6, and HeLa (data not shown) being

the most sensitive ones. However, we found no correlation between VHL status and sensitivity to adenovirus infection (Fig. 2B). It is of note that there is a large difference in the sensitivity to adenovirus infection between the cell lines derived from the same renal cancer cells such as 1-115 and WT-10 (Fig. 2B).

**Generation of Ad9xHRE1A.** For the construction of Ad9xHRE1A, we used an adenoviral backbone designed to facilitate the substitution of E1A and E4 promoters by tumor-specific promoters (7). In the Ad9xHRE1A virus, the E1A coding region is under the control of a minimal promoter containing nine tandem copies of a HRE derived from the VEGF promoter. This artificial sequence was placed upstream of a TATA sequence from the rat prolactin promoter. In addition to the sequence already contained in the adenoviral backbone that insulates the modified E1A promoter (7), we included the SV40 polyadenylation signal to further decrease the possibility of E1A expression from transcripts initiated upstream de HRE- controlled promoter (Fig. 3A). The complete sequence of this region is shown in Fig. 3B. Note that E1A is under the control of a promoter identical to the one used for the reporter assays in Fig. 1C. As in previous CRADs generated by our group (7), the E4 coding region was placed under the control of the E2F-1 promoter (Fig. 3A). In this case, the promoter does not provide a genuine second level of control on viral replication, but it contributes to the attenuation of the virus in normal cells and amplification of viral replication upon E1A induction (7).

To test the efficiency and specificity of the 9xHRE promoter in the control of E1A expression, we infected different cell lines with wild-type or Ad9xHRE1A and determined the level of E1A mRNA by Q-RT-PCR after infection. As shown in Fig. 3C, there are large differences in E1A mRNA expression among the different cell lines infected with wild-type adenovirus. This correlates with the different sensitivity of each cell line to infection (Fig. 2). Importantly, the expression of E1A mRNA upon infection with Ad9xHRE1A in cells with deregulated HIF activity such as 1-115, PCR-3, and RCC10 is similar or even higher than the expression of E1A gene from wild-type adenovirus (Fig. 3C). In contrast, in cells where VHL activity was restored (WT-10) in normal cells (IMR90) or cells with impaired HIF activity (UM-RC6), the expression of E1A upon infection with Ad9xHRE1A is dramatically reduced as compared with infection with the wild-type adenovirus (Fig. 3C). The doses of virus used in this experiment were deliberately high (MOI of 100 pfu/cell). Even at these high viral doses, E1A mRNA levels in VHL-positive cells infected with Ad9xHRE1A were reduced to 10% of the values obtained after infection with wild-type virus (Fig. 3C). Thus, basal E1A expression from Ad9xHRE1A upon infection of nontarget cells is very low. The low expression of E1A in UM-RC6 cells is of particular interest because it suggests that the induction of E1A observed on the other pVHL-deficient cells (1-115, PCR3, and RCC10) is mediated by

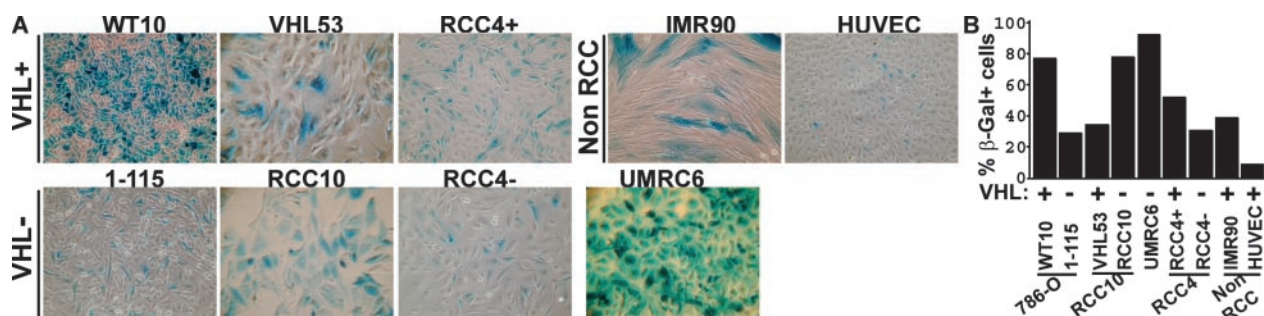


Fig. 2. Susceptibility to adenovirus infection of the different cell lines used in this study. Cells were grown in 24-well plates until confluent and then infected with Ad-LacZ at a MOI of 50 pfu/cell. Forty-eight h after infection, cells were fixed and processed to determine  $\beta$ -galactosidase activity. A representative microphotograph of each cell line is shown. The number of  $\beta$ -galactosidase positive cells in several independent fields was determined, and it is represented as percentage of total number of cells.

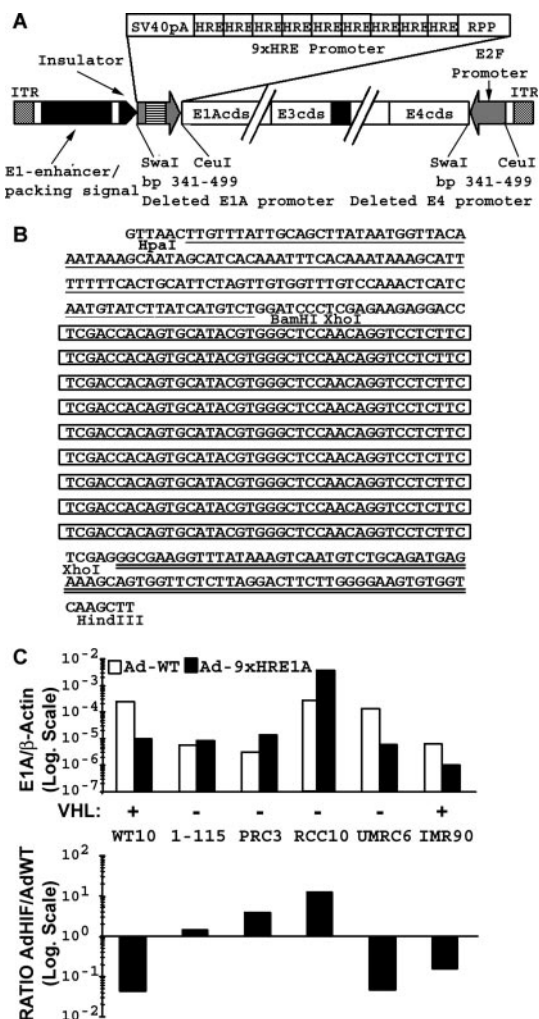


Fig. 3. Characterization of Ad9xHRE1A. *A*, schematic representation of the modifications introduced in the viral genome. An insulator sequence (Insulator) and a polyadenylation signal from SV40 (SV40pA) were placed between the overlapping E1A enhancer/packaging signals and the E1A promoter. The wild-type E1A promoter was substituted by an artificial minimal promoter containing nine HREs upstream of a TATA sequence from the rat prolactin promoter (RPP). The E4 promoter was substituted by the E2F-1 promoter. ITR, inverted terminal repeats; cds, coding sequence. *B*, the complete sequence of the artificial promoter is shown. Underlined sequence corresponds to the polyadenylation signal; double underlined sequence derives from the rat prolactin promoter; the nine HREs from VEGF-A promoter are boxed. The restriction sites are indicated. *C*, cells were infected with Ad9xHRE1A or wild-type adenovirus at a MOI of 100 pfu/cell. Twelve h after infection, RNA was obtained from cells, and the level of E1A mRNA in samples was determined by Q-RT-PCR. The amount of E1A mRNA, normalized by  $\beta$ -actin content, is represented in the top graph. The bottom graph shows the ratio between the normalized E1A expression levels in cells infected with Ad9xHRE1A (AdHIF) and wild-type virus (AdWT). Similar results were obtained in three independent experiments.

the elevated HIF activity and not because of other alterations associated to *VHL* loss.

**Oncolytic Effect of Ad9xHRE1A on RCCs.** Because the expression of E1A from Ad9xHRE1A is tightly regulated and restricted to cells with increased HIF activity, it is expected that its cytolytic effect would be restricted to this type of cells. To verify this hypothesis, we infected a panel of cells with wild-type adenovirus or Ad9xHRE1A at different MOIs and determined the number of viable cells remaining 8–10 days after infection (see “Materials and Methods”). As shown in Fig. 4A, Ad9xHRE1A efficiently eliminates *VHL*-deficient renal cancer cells with increased HIF activity (PRC3, 1-115, RCC10, and RCC4-) as efficiently as wild-type virus. Importantly, Ad9xHRE1A is attenuated in the *VHL*/HIF-defective UM-RC6 cells, although they are highly susceptible to adenovirus infection as demonstrated by the

effect of the wild-type virus (Fig. 4A). In support of the specificity of Ad9xHRE1A, the oncolytic effect of this virus was dramatically reduced as compared with wild-type virus in renal cancer cells where pVHL function had been restored (WT-10, VHL53+, and RCC4+) and tumor cells where the HIF regulation is intact (HeLa cells) or normal cells such as IMR-90 fibroblast and primary HUVEC (Fig. 4A).

In Fig. 4B, we represent the survival of cells infected with the Ad9xHRE1A virus. Each cell type was infected using the MOI that causes >70% mortality when the wild-type adenovirus was used. The results of several independent experiments are represented, demonstrating that the effect of the Ad9xHRE1A virus was highly reproducible. These experiments demonstrate that Ad9xHRE1A is very efficient and specific in the killing of cells with elevated HIF activity. To verify that this selective killing was attributable to the replication and production of competent viral particles, we tested the supernatant

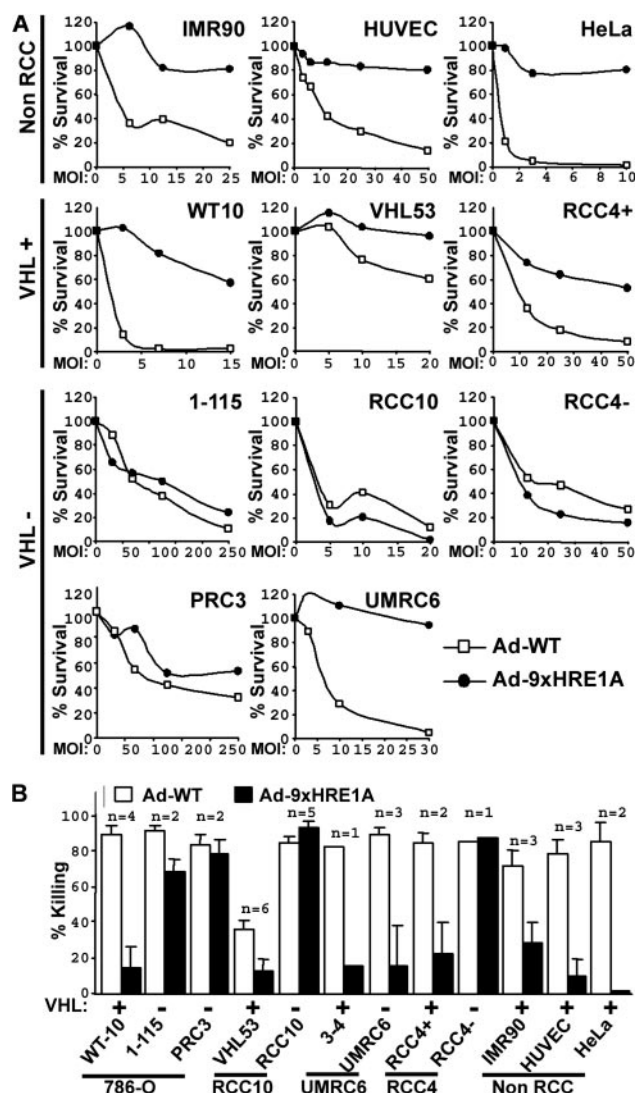


Fig. 4. Oncolytic effect of Ad9xHRE1A on different cell lines. *A*, cells were plated on 24-well plates, and 24 h later, they were infected with Ad9xHRE1A or wild-type adenovirus at the indicated MOIs. The range of MOIs used for each cell line was adjusted according to its susceptibility to adenovirus infection (Fig. 2). Seven to 10 days after infection, the number of surviving cells was assessed by the ability to reduce MTT. The average values of duplicate determinations are represented as percentage of the value obtained for uninfected cultures. The results shown are representative of two to six independent experiments. *B*, the results from *n* independent experiments as the one shown in *A* are represented as the mean  $\pm$  SE. To compare different cell lines, we chose for each case the smallest MOI required to kill >70% of the cells with the wild-type adenovirus.

of infected cultures for the presence of viable viral particles. VHL-deficient cells (RCC10 and UM-RC6) as well as VHL-competent cells (VHL53+) were infected with Ad9xHRE1A or wild-type adenovirus, and 10 days later, culture supernatants were collected and used to infect HEK293 cells. Forty-eight h after exposure to culture supernatants, infected HEK293 cells were identified by immunostaining using antiadenovirus antibodies (hexon protein). As shown in Fig. 5, supernatants of cells infected with wild-type adenovirus contain a large number of infective particles regardless of the cellular source of the supernatant. In contrast, infective viral particles were rescued from RCC10 supernatants infected with Ad9xHRE1A but not from VHL53+ or UM-RC6 cell lines (Fig. 5). Thus, the specific oncolytic effect of Ad9xHRE1A in VHL-deficient cells is because of its restricted replication and amplification in target cells.

**Oncolytic Effect of Ad9xHRE1A against Renal Carcinoma Cells Is Mediated by HIF Activity.** The experiments shown above demonstrate that Ad9xHRE1A replicates in VHL-deficient carcinoma cells but not in VHL-competent cells. Despite the best-characterized consequence of *VHL* loss is deregulation of HIF, it is very likely that other alterations also occur. To prove that Ad9xHRE1A replication in carcinoma cells is attributable to HIF activation and does not depend on other factors, we generated a stable cell line expressing a mutant form of HIF-1 $\alpha$  that is refractory to VHL regulation. Specifically, the proline residues 402 and 564 from human HIF-1 $\alpha$ , required for VHL binding, were mutated to alanine and glycine residues, respectively, and this mutant construct was stably transfected into the VHL-competent WT-10 cell line to generate the WT-10-PP13 cell line. Fig. 6A shows that although both WT-10 and PP13 cells were efficiently eliminated by wild-type adenovirus, only WT-10 but not WT-10-PP13 showed resistance to Ad9xHRE1A infection. This result, together with the lack of effect of Ad9xHRE1A on VHL/HIF-defective UM-RC6 cells, indicate that HIF activity is necessary and sufficient to induce Ad9xHRE1A replication and killing.

These results also suggest that Ad9xHRE1A could be used to target VHL-competent cells under hypoxic conditions. This is of great interest because most solid tumors usually present large areas of hypoxic tissue, whereas HIF is not usually present in detectable amounts in normal tissues under physiological conditions (22–25). To test this possibility, we investigated the effect of Ad9xHRE1A on IMR-90 normal fibroblasts cultured under normoxic or hypoxic (1%

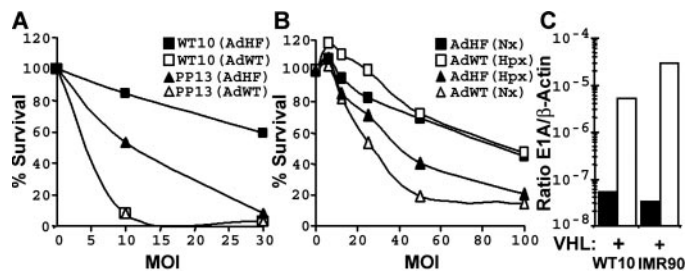


Fig. 6. Active HIF is sufficient to induce E1A expression and cytolysis by Ad9xHRE1A. WT-10 and WT-10-PP13 cell lines (A) or IMR90 fibroblast (B) were plated on 24-well plates, and 24 h later, they were infected with Ad9xHRE1A (*AdHF*) or wild-type (*AdWT*) adenovirus at the indicated MOIs. In the case of IMR90 cells, they were maintained in either hypoxic (1% oxygen, Hpx) or normoxic (Nx) conditions as indicated. Seven to 10 days after infections, the number of surviving cells was assessed by the MTT assay. The average values of duplicate determinations are represented as percentage of the value obtained for uninfected cultures. The experiments shown were repeated once with similar results. C, the human fibroblast IMR90 and the VHL-transfected renal carcinoma cells WT-10 were infected at a MOI of 100 pfu/cell with Ad9xHRE1A and then cultured for 12 h under normoxic (■) or hypoxic (□) conditions. The level of E1A expression was determined by Q-RT-PCR, and it is represented after normalization by the  $\beta$ -actin content.

oxygen) conditions. Unexpectedly, killing of IMR-90 cells by wild-type adenovirus was drastically reduced when cells were grown under hypoxic conditions (Fig. 6B), suggesting that, at least in this cell type, hypoxia interferes with the viral lytic cycle. This effect correlates with reduced expression of E1A mRNA as determined by Q-RT-PCR (data not shown). In contrast with the result obtained with the wild-type virus, Ad9xHRE1A kills IMR-90 cells more efficiently under hypoxic conditions (Fig. 6B). Considering the negative effect of hypoxia on the replication of wild-type adenovirus in this cell type, hypoxia enhances cell killing by Ad9xHRE1A very significantly.

In agreement with these results, hypoxia caused a strong induction (>100-fold) of E1A mRNA production in VHL-competent cells infected with Ad9xHRE1A (Fig. 6C). In conclusion, the absolute requirement of an active HIF for Ad9xHRE1A replication makes it suitable not only for the targeting of VHL-negative renal carcinomas but also for eliminating any other cell type growing under hypoxic conditions.

**Oncolytic Effect of Ad9xHRE1A against RCCs *in Vivo*.** The previous experiments demonstrate that Ad9xHRE1A is very efficient and specific in the elimination of cells with an active HIF transcription factors *in vitro* and suggest that it could be used to eliminate VHL-deficient tumors *in vivo*. To test this possibility, we generated VHL-deficient tumors in immunodeficient nude/nude mice by s.c. injection of 1-115 cells. When tumors reached and approximate volume of 200 mm<sup>3</sup>, animals were treated by intratumor injection of Ad9xHRE1A or vehicle. Treatment was repeated once within 24 h of the first injection and then mice were left untreated for the rest of the experiment. As shown in Fig. 7, four of five tumors treated with Ad9xHRE1A showed a significant reduction of growth as compared with controls. Importantly, two of the four responsive tumors were completely eliminated by Ad9xHRE1A treatment (Fig. 7A). The behavior of the single unresponsive tumor can be explained by a nonefficient intratumor injection because its growth pattern clearly deviates from the rest of the treated group. To confirm the differences in growth of the two tumor groups, 60 days after initial treatment, animals were sacrificed and tumors excised to determine its weight (Fig. 7B). Unfortunately, we were unable to test the effect of Ad9xHRE1A against other VHL-deficient cell lines such as RCC10 *in vivo* because they produced small tumors that regressed spontaneously (data not shown).

Thus, Ad9xHRE1A is able to eliminate VHL-deficient cells not only *in vitro* but also *in vivo*. The effect of Ad9xHRE1A on 1-115 cells *in vivo* is of particular relevance given that this cell line is not very susceptible to adenoviral infection (Fig. 2).

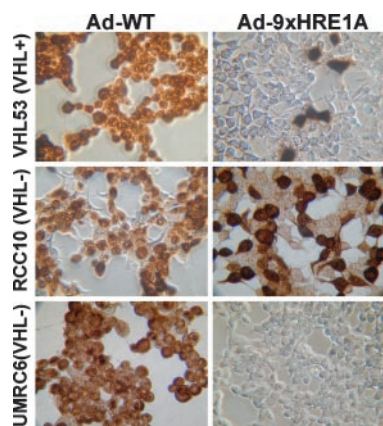


Fig. 5. The oncolytic effect of Ad9xHRE1A is due to viral replication. The indicated cell lines were infected with Ad9xHRE1A or wild-type virus at a MOI of 10 pfu/cell. After infections, cell cultures were extensively washed to eliminate any remaining infective particles in culture. Six to 7 days after infection, cell supernatants were collected and used as viral source to infect HEK293 cells. Forty-eight h after exposure to culture supernatants, HEK293 cells were fixed, and infected cells were visualized by immunostaining against the viral hexon protein. Representative microphotographs of HEK293 after immunostaining are shown. The experiment was repeated once with similar results.

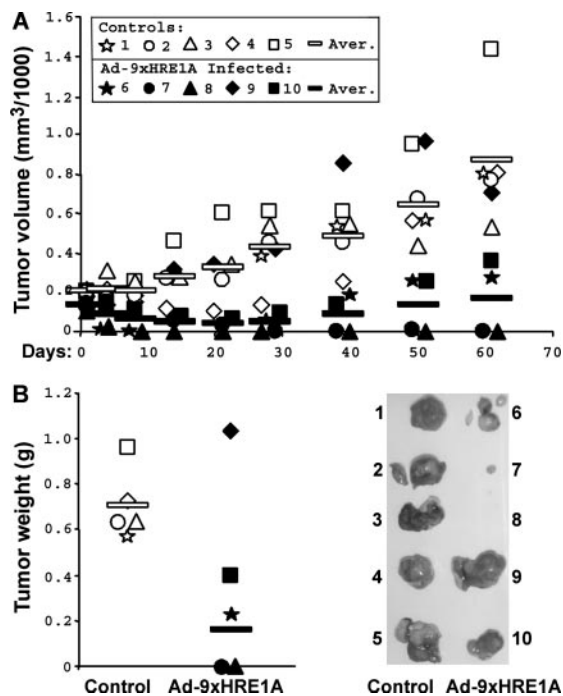


Fig. 7. *In vivo* antitumor effect of Ad9xHRE1A. A, a total of  $10^7$  1-115 (VHL-deficient) cells was s.c. injected into nude mice to generate accessible tumors. When tumors reached 200 mm<sup>3</sup>, mice received intratumor injections of  $1.6 \times 10^8$  pfu of Ad9xHRE1A (mice numbered 6–10) or vehicle alone (mice numbered 1–5) on 2 consecutive days. Then mice were left untreated for 60 days, and tumor growth was measured once/week. Each symbol represents the volume of a single tumor over time. Open symbols are used for the control-treated tumors, and solid symbols represent the tumors in the virus-treated group. The average volume for each animal group is shown (Aver., horizontal bar); animal number 9 was excluded to calculate mean of the virus-treated group. B, sixty days after initial treatment animals were sacrificed and tumors excised to determine its weight. The symbols represent each individual animal/tumor as in A. The average weight for each tumor group is shown (Aver., horizontal bar); animal number 9 was excluded to calculate the mean of the virus-treated group.

## DISCUSSION

Here, we describe the generation and characterization of a new HIF-dependent conditionally replicative adenovirus for the treatment of VHL-deficient tumors. We have previously developed a conditionally replicative adenovirus to target breast tumors in which the E1A expression was driven by a dual estrogen and HIF-responsive promoter (7). However, this is the first description of an adenovirus in which the replication is controlled by HIF alone and the first study of a gene therapy approach to target clear cell renal carcinomas. In addition, the promoter described herein shows no (or extremely low) leaky expression, combined with high inducibility and strict dependence on HIF activity, as compared with the estrogen/hypoxia-inducible promoter previously reported (data not shown). All these characteristics make Ad9xHRE1A particularly well suited for the treatment of clear cell renal carcinomas defective for VHL function. It is of note that control of viral replication is achieved by the regulation of E1A gene expression from an artificial HIF-responsive minimal promoter instead of using the natural occurring promoter from a HIF-target gene. Naturally occurring promoters contain response elements for several different transcription factors, and as a consequence, they have limited value for the control of gene expression in response to a single specific alteration.

Loss or inactivation of *VHL* gene is responsible for the VHL cancer syndrome and the vast majority of sporadic clear cell renal carcinomas. One of the major problems affecting VHL patients is the development of multiple preneoplastic cysts in several organs, including liver, pancreas, and kidney. Another associated problem is the forma-

tion of hemangioblastomas because of the secretion of proangiogenic factors by the VHL-deficient stromal cells. It is likely that a therapy based on an agent such as Ad9xHRE1A could be potentially useful for the treatment of both cyst and hemangioblastomas in these patients. Because in these cases, the target of the therapy will be nonmalignant cells, no selection of cells resistant to therapy is expected. On the other hand, clear cell carcinomas of the kidney are discouragingly refractory to all chemotherapeutic regimens. Therefore, new therapeutic approaches against this disease are of great importance.

Although clear cell renal carcinomas are the most frequent kidney malignance, they comprise only the 2% of all human cancers. Importantly, however, human tumors other than renal carcinomas have been shown to overexpress HIF- $\alpha$  (23, 26, 27). In one study (23), a high proportion of the ovarian and colon carcinomas tested were positive for HIF-1 $\alpha$  and HIF-2 $\alpha$  immunostaining. Despite the molecular mechanism responsible for HIF- $\alpha$  stabilization in these tumors is currently unknown, they are potential targets for Ad9xHRE1A. Finally, we found that hypoxia induced the replication of Ad9xHRE1A in cells with functional HIF regulatory pathways. Because almost all solid tumors present areas of hypoxic tissue where HIF- $\alpha$  protein is induced (28), the potential range of tumor targets for Ad9xHRE1A is wide. In agreement, a recent article (29) describes an HIF-regulated adenovirus for the targeting of hypoxic cells. In this vein, it is important to underline that despite some discrepancies (25), most authors agree that HIF- $\alpha$  protein is not expressed in the vast majority of the tissues under physiological conditions, with the exception of macrophages under some circumstances (22–24). Taking into account that expression of E1A by Ad9xHRE1 is strictly dependent on HIF activity, with very low or none leaky expression, this virus should be highly attenuated in normal cells.

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## Specific Oncolytic Effect of a New Hypoxia-Inducible Factor-Dependent Replicative Adenovirus on von Hippel-Lindau-Defective Renal Cell Carcinomas

Yolanda Cuevas, Rubén Hernández-Alcoceba, Julian Aragonés, et al.

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