

ORIGINAL ARTICLE

Potent antitumor activity of Oct4 and hypoxia dual-regulated oncolytic adenovirus against bladder cancer

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Most solid tumors undergo hypoxia, leading to rapid cell division, metastasis and expansion of a cell population with hallmarks of cancer stem cells (CSCs). Tumor-selective replication of oncolytic adenoviruses may be hindered by oxygen deprivation in tumors. It is desirable to develop a potent oncolytic adenovirus, retaining its antitumor activity even in a hypoxic environment. We have previously generated an Oct4-dependent oncolytic adenovirus, namely Ad9OC, driven by nine copies of the Oct4 response element (ORE) for specifically killing Oct4-overexpressing bladder tumors. Here, we developed a novel Oct4 and hypoxia dual-regulated oncolytic adenovirus, designated AdLCY, driven by both hypoxia response element (HRE) and ORE. We showed that hypoxia-inducible factor (HIF)-2 α and Oct4 were frequently overexpressed in hypoxic bladder cancer cells, and HIF-2 α was involved in HRE-dependent and Oct4 transactivation. AdLCY exhibited higher cytolytic activities than Ad9OC against hypoxic bladder cancer cells, while sparing normal cells. AdLCY exerted potent antitumor effects in mice bearing human bladder tumor xenografts and syngeneic bladder tumors. It could target hypoxic CD44- and CD133-positive bladder tumor cells. Therefore, AdLCY may have therapeutic potential for targeting hypoxic bladder tumors and CSCs. As Oct4 is expressed in various cancers, AdLCY may be further explored as a broad-spectrum anticancer agent.

Gene Therapy (2015) 22, 305–315; doi:10.1038/gt.2014.122; published online 15 January 2015

INTRODUCTION

Hypoxia is a common feature of growing solid tumors. Adaptation to low oxygen condition in cells results in the transcriptional activation of more than 100 genes that regulate key aspects of tumorigenesis, including angiogenesis, metabolism, proliferation, invasion and metastasis.¹ Promoters containing hypoxia response element (HRE) can be transactivated by hypoxia and drive-related gene expressions, leading to defective vasculogenesis and abnormal metabolic activity when tumors progress. Hypoxia-inducible factor (HIF)-1, which is an oxygen-sensitive transcriptional activator, primarily mediates this response. HIF-1 consists of two subunits, namely an oxygen-regulated subunit HIF-1 α (or its paralogs HIF-2 α and HIF-3 α) and a constitutively expressed subunit HIF-1 β .² HIF-1 α is degraded via the ubiquitin-proteasome pathway in normoxia. Whereas the HIF-1 α subunit becomes stable and regulates the expression of target genes in hypoxia. Furthermore, unusual overexpression of HIF-1 α has been found in various cancers. HIF-1 α preferentially induces genes encoding glycolytic enzymes, whereas HIF-2 α induces genes involved in tumor invasion, such as matrix metalloproteinases and the initiation of cancer stem cell (CSC) factors.^{3–5} HIF-2 α activates signaling pathways such as Oct4 and Notch, which control the self-renewal and multipotency of CSCs.⁶ All of the altered characteristics of tumors may impair effective cancer treatment.

Replication-selective oncolytic adenoviruses are an attractive strategy for cancer therapy because they are able to infect, replicate in and lyse tumor cells. Viruses can be modified in various ways to improve their selectivity and therapeutic efficacy. First, viruses have mutated genes that are essential for viral replication in non-tumor cells but can be selectively compensated by specific cellular mutations in cancer cells.⁷ Second, the enhancer or promoter region of the *E1A* gene, which is required for adenovirus replication, can be modified with tumor- or tissue-specific promoters.^{8–12} On these modifications, oncolytic adenoviruses are capable of replicating and lysing tumor cells while sparing non-tumor cells. These viruses have shown acceptable anti-tumor activity and overall safety in various cancers, including bladder cancer.^{13–15} In the past decade, numerous clinical trials have been conducted to assess the potential of oncolytic viruses for cancer therapy.¹⁶ An oncolytic vaccinia virus, Pexa-Vec (formerly known as JX-594), was engineered to selectively replicate in cells with alterations of the RAS pathway and to express human granulocyte-macrophage colony-stimulating factor (hGM-CSF). Pexa-Vec was employed in clinical trials for the treatment of hepatocellular carcinoma (phase I) and colorectal cancer (phase II).¹⁷ An oncolytic herpes simplex virus type 1, Talimogene laherparepvec (T-VEC), which was manipulated to express hGM-CSF, has been tested for the treatment of unresected Stage IIIB, IIIC or IV melanoma. Results from the clinical trials indicated that these oncolytic viruses hold promise as anticancer agents.^{18,19}

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Received 29 April 2014; revised 8 October 2014; accepted 25 November 2014; published online 15 January 2015

Oct4 is a transcriptional factor that is a key regulator of pluripotency and self-renewal in embryonic stem cells and is also expressed in bladder cancer.^{20,21} We have demonstrated that Oct4 expression reflects tumor progression and regulates motility of bladder cancer cells.¹¹ We generated an E1B 55-kDa-deleted adenovirus, designated Ad9OC, which is driven by nine copies of the Oct4 response element (ORE) ligated to a human cytomegalovirus minimal (CMVmini) promoter.¹⁰ In addition to Ad9OC, we have also generated another oncolytic adenovirus, named AdWS4, under the control of the Oct4 promoter.¹¹ These two Oct4-regulated oncolytic adenoviruses can specifically kill bladder cancer cells overexpressing Oct4 and exert potent antitumor activity in animal tumor models.^{10,11}

Limited viral replication is one of the major obstacles to reaching a highly therapeutic effect.²² A level of hypoxia similar to that found within solid tumors reduces the replication of adenoviral vectors by reducing E1A expression and hence oncolytic potentials.^{23,24} Hypoxia has been exploited to drive the replication of oncolytic adenoviruses aiming at increasing therapeutic efficacy for solid tumors exhibiting significant areas of hypoxia.^{25–27} Currently designed oncolytic adenoviruses may require additional modifications to target tumor cells in hypoxic regions. To overcome such drawbacks, in the present study, we generated a new hypoxia-activated oncolytic adenovirus, designated AdLCY, which contains a dual hypoxia/Oct4-responsive promoter composed of the CMVmini promoter ligated with six copies of the HRE and nine copies of the ORE.

Bladder cancer is the most common cancer in the urinary system in the United States.²⁸ Progression to or presentation with muscle-invasive disease usually worsens the survival rate of patients and requires more aggressive therapy. Oct4 and Sox2,

which are stemness markers expressed in bladder cancer, have been implicated to be responsible for proliferation and differentiation of CSCs and are correlated to disease prognosis.^{29,30} We found that using HRE/ORE segments to transcriptionally regulate adenoviral replication in Oct4-overexpressing cancer cells increased viral replication and oncolytic activities in hypoxic environments, thereby improving antitumor activity against bladder cancer. As Oct4 is expressed in a broad spectrum of cancer and tumor hypoxia increases malignant progression and metastasis,^{1,31} Oct4 and hypoxia dual-regulated oncolytic adenoviruses may be broadly applicable.

RESULTS

Combination of the CMVmini promoter with 6×HRE and 9×ORE increased its transcriptional activity in hypoxic bladder cancer cells. We first used quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis to examine Oct4 mRNA levels in various human and murine bladder cancer cells under normoxic and hypoxic conditions. Levels of Oct4 mRNA expression were higher in hypoxic than in normoxic conditions in all the cells tested (Figure 1a). In human bladder cancer cells, hypoxia induced Oct4 mRNA expression by 4- to 23-folds. Accordingly, higher levels of Oct4, HIF-1α and HIF-2α proteins were also detected in these cell lines under hypoxia than under normoxia (Figure 1b). In the murine MBT-2 cell line and its two sublines, hypoxia also induced Oct4 mRNA expression, albeit at lower levels of induction compared with those in human bladder cancer cells (Figure 1a). Regarding protein expression levels, MBT-2 and MBT-2-LM7 cells under hypoxic conditions expressed higher levels

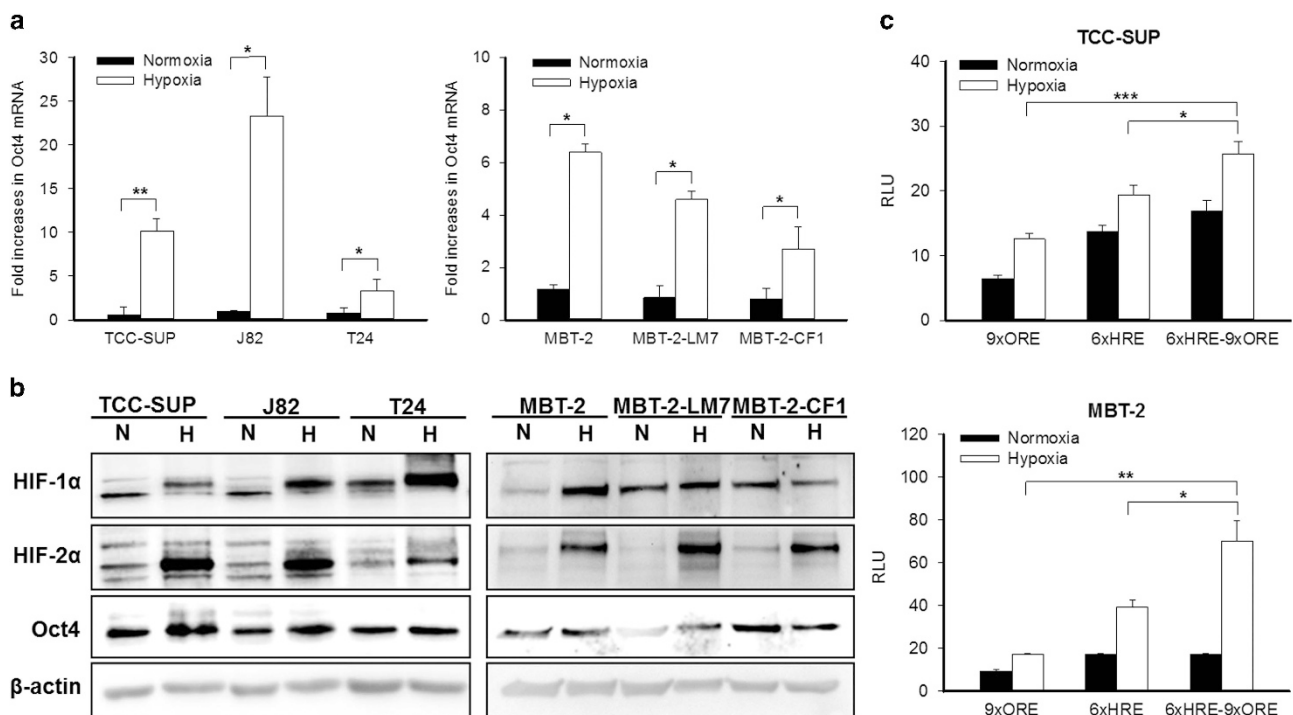


Figure 1. The CMVmini-6×HRE-9×ORE promoter was highly responsive to endogenous Oct4 and HIFs in hypoxic human and murine bladder cancer cells. **(a)** Expression of Oct4 mRNA in bladder cancer cells under normoxic and hypoxic conditions for 48 h, as determined by quantitative real-time RT-PCR. **(b)** Detection of HIF-1α, HIF-2α and Oct4 expression in bladder cancer cells after exposure to normoxia (N) or hypoxia (H) for 48 h. The expression of β-actin served as the loading control. **(c)** Determination of promoter activities. TCC-SUP and MBT-2 cells were transfected with single dual-luciferase reporter constructs, which contained the CMVmini promoter ligated with either 6×HRE or 9×ORE, or both to drive firefly luciferase, as well as the CMV promoter to drive *Renilla* luciferase, and then exposed to normoxia or hypoxia for 48 h. Promoter activities were determined by a dual-luciferase reporter assay. The ratio of firefly luciferase activity to *Renilla* luciferase activity was expressed as relative light units (RLU) ($n=4$). Values are the mean \pm s.e.m. of the mean. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

of Oct4, HIF-1 α and HIF-2 α proteins than those under normoxic conditions (Figure 1b). However, only HIF-2, but not Oct4 and HIF-1 α proteins, was elevated following hypoxic induction (Figure 1b). We next used three different reporter constructs to examine the promoter activities of the CMVmini promoter ligated with either 6 \times HRE or 9 \times ORE, or both in hypoxic or normoxic TCC-SUP and MBT-2 cells. As shown in Figure 1c, activities of the three promoters increased when TCC-SUP (upper panel) and MBT-2 (lower panel) cells were under hypoxic conditions. Moreover, the CMVmini promoter ligated with both 6 \times HRE and 9 \times ORE exerted higher transcriptional activity than that conjugated with either 6 \times HRE or 9 \times ORE. Collectively, these results indicate that the CMVmini-6 \times HRE-9 \times ORE promoter was highly responsive to endogenous Oct4 and HIFs in hypoxic cells.

Knockdown of HIF-2 α suppressed Oct4 expression as well as Oct4- and hypoxia-dependent transactivation in bladder cancer cells

As Oct4 has been identified as a HIF-2 α -specific target gene,⁵ we next tested whether silencing HIF-2 α expression reduces Oct4 expression and thereby decreases the transcriptional activity of the CMVmini-9 \times ORE promoter in hypoxic tumor cells. TCC-SUP and MBT-2 cells that have been transfected with a reporter construct containing the CMVmini-9 \times ORE promoter were transfected with short hairpin RNA (shRNA) constructs specific to HIF-2 α (shHIF-2 α) or green fluorescent protein (GFP) (shGFP) for 24 h and then exposed to hypoxia or normoxia for additional 24 h. Knockdown of HIF-2 α expression resulted in decreased Oct4 expression in TCC-SUP (Figure 2a, left panel) and MBT-2 (Figure 2b, left panel) cells under hypoxic conditions. Furthermore, the transcriptional activity of the CMVmini-9 \times ORE was also down-regulated in hypoxic TCC-SUP (Figure 2a, right panel) and MBT-2 (Figure 2b, right panel) cells after transduction with shRNA specific to HIF-2 α . These results confirmed that HIF-2 α is involved not only in HRE-dependent transactivation, but also regulates Oct4 transactivation. Given that the transcriptional activity of the CMVmini-6 \times HRE-9 \times ORE promoter was higher than that of the CMVmini-9 \times ORE promoter (Figure 1c), we generated AdLCY, which is an Oct4 and hypoxia dual-regulated oncolytic adenovirus, by adding six copies of the HRE upstream of the CMVmini-9 \times ORE for driving adenovirus E1A gene expression in the context of the E1B 55-kDa-deleted adenovirus Ad9OC.¹⁰

AdLCY exerted higher tumor-selective cytolytic activity against hypoxic bladder cancer cells than Ad9OC

The Oct4-dependent oncolytic adenovirus Ad9OC, which was derived from the E1B-55-kDa-deleted oncolytic adenovirus Ad5WS1, was constructed under the transcriptional control of 9 \times ORE-CMVmini promoter.³² To further construct an Oct4 and hypoxia dual-regulated oncolytic adenovirus, the promoter of Ad9OC was replaced with 6 \times HRE and 9 \times ORE-CMVmini promoter to generate AdLCY (Figure 3a). The cytolytic effects of AdLCY and Ad9OC on TCC-SUP cells were compared (Figure 3b). While Ad9OC induced similar degrees of cytopathic effects (CPE) in cells regardless of hypoxic exposure, the degree of CPE induced by AdLCY was \sim 10-fold greater under hypoxic than normoxic conditions in both TCC-SUP and MBT-2 cells, as assessed by crystal violet staining. Furthermore, complete cytolysis was seen in TCC-SUP and MBT-2 cells infected with AdLCY at doses as low as 1 multiplicity of infection (MOI) under hypoxic conditions. In marked contrast, AdLCY failed to induce evident CPE in normal epithelial cells, including human SV-HUC-1 and murine NMuMG cells, even at high MOIs.

Efficient adenovirus infection requires the coxsackievirus and adenovirus receptor (CAR), a major receptor of adenovirus serotype 5 for virus attachment and internalization to enter host cells.^{33–35} To determine the infectivity of AdLCY in bladder cancer cells, we tested the expression of CAR by flow cytometric analysis

(Figure 4a). Our results revealed that CAR expression was detected in both human bladder cancer cells (82.45%, TCC-SUP) and normal urothelial cells (34.63%, SV-HUC-1). Murine bladder cancer cells (MBT-2) and normal murine mammary gland epithelial cells (NMuMG) also expressed CAR (9.68 and 19.04%, respectively), albeit at lower levels than human cells. To assess the susceptibility of cells to adenoviral infection, we used replication-defective adenoviral vectors expressing GFP (AdGFP) to infect these cells and detect GFP expression after 48 h. Figure 4b shows that all of the four cell lines could be infected by AdGFP, with human cells more susceptible than murine cells, indicating that both human and murine non-tumor and tumor cells could be infected by adenoviruses. Furthermore, cell susceptibility to adenoviral infection was positively correlated with the surface expression level of CAR. The tumor-specific cytolysis observed in AdLCY was governed by dual-regulation of Oct4 and hypoxia. We used the colorimetric WST-1 assay to compare the survival of bladder cancer and normal cells after infection with AdLCY or Ad9OC. As shown in Figure 4c, AdLCY exerted higher cytolytic activities against TCC-SUP and MBT-2 cells under hypoxic than normoxic conditions. Of note, under hypoxic conditions, AdLCY was more effective than Ad9OC against both cancer cells. Furthermore, TCC-SUP cells were more sensitive to AdLCY- and Ad9OC-induced cytolysis than MBT-2 cells. Although murine MBT-2 cells expressed low levels of CAR, they were also susceptible to adenovirus infection, and AdLCY and Ad9OC could replicate in the cells and induce cytolysis. These results are in accordance with our previous findings that MBT-2 cells could produce adenoviral late proteins, indicative of productive replication, after infection with oncolytic adenoviruses, albeit to a lesser extent than human tumor cells.¹⁰ Although normal human SV-HUC-1 and murine NMuMG cells could be infected by AdGFP (Figure 4b), they were resistant to cytolysis induced by either AdLCY or Ad9OC (Figure 4c). Taken together, these results indicate that AdLCY selectively killed bladder cancer cells, in particular hypoxic tumor cells, while sparing normal cells.

AdLCY had higher antitumor activity than Ad9OC in mice bearing human bladder tumor xenografts or syngeneic bladder tumors

Compared with Ad9OC, AdLCY was more effective in inhibiting tumor growth (Figure 5a) and prolonging survival (Figure 5b) in mice bearing human TCC-SUP tumor xenografts. Similar antitumor effects of AdLCY were also observed in the MBT-2 syngeneic tumor model (Figures 5c and d). We next used immunohistochemical staining to examine the *in vivo* targeting efficacy of AdLCY in TCC-SUP tumor-bearing mice (Figure 5e). Thirty-one days after tumor cell injection, HIF-1 α was detected abundantly, indicating the size of hypoxic regions within tumors became larger along with tumor growth. Compared with tumors infected with AdLacZ, which is a replication-defective adenoviral vector encoding β -galactosidase, adenoviral proteins were detected abundantly in AdLCY- and Ad9OC-infected tumors, indicative of productive replication of both viruses in hypoxic bladder tumors. Notably, more AdLCY targeted tumor tissue in hypoxic regions, especially in cells that expressed the CSC markers CD44 and CD133. Collectively, these results indicate that the oncolytic efficacy of AdLCY was superior to that of Ad9OC through more efficacious targeting of hypoxic tumors and CSCs.

AdLCY targeted more CD44- and CD133-positive bladder tumor cells than Ad9OC

CD44 and CD133 are major CSC markers, and numbers of CD44- and CD133-positive tumor cells usually increase in hypoxic tumor tissues. When TCC-SUP cells were infected with AdLacZ, which was replication-defective, and exposed to hypoxic conditions, the percentages of CD44- and CD133-positive cells rose from 75.92 to 88.10% (Figure 6a) and from 2.32 to 13.23% (Figure 6b),

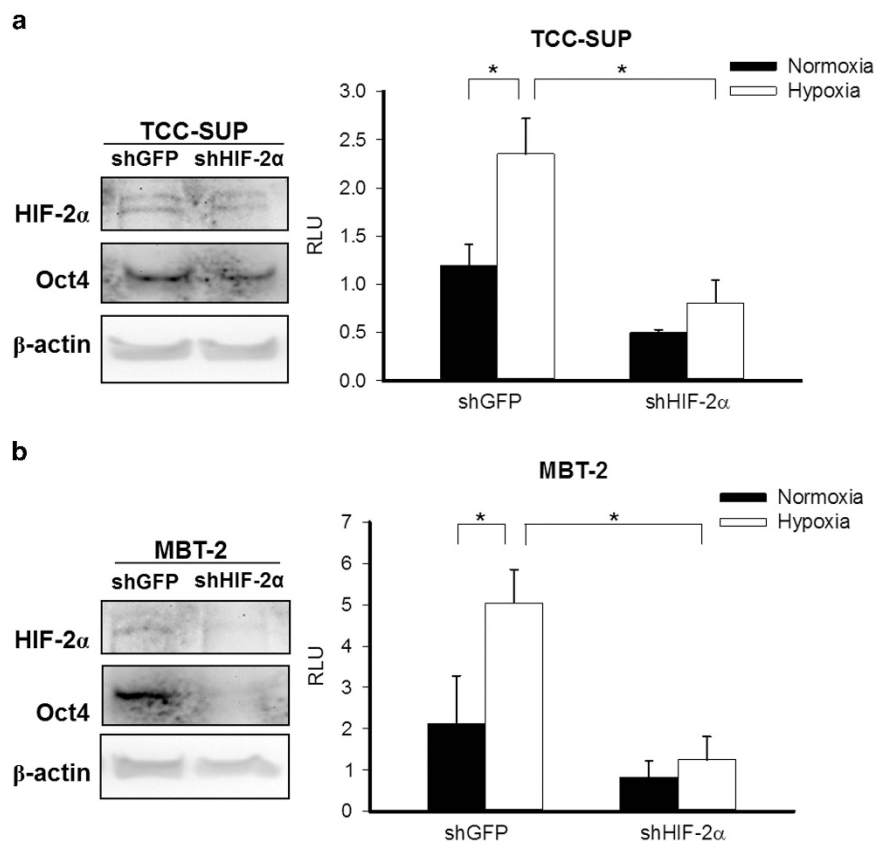


Figure 2. Knockdown of HIF-2 α suppressed Oct4 expression as well as Oct4- and hypoxia-dependent transactivation in bladder cancer cells. TCC-SUP and MBT-2 cultured in six-well plates were transfected with 5 μ g of shRNA constructs specific to HIF-2 α or GFP. After being cultured for 3 days, the cells were exposed to hypoxia or normoxia for 48 h, followed by immunoblot analysis. Expression of HIF-2 α and Oct4 in HIF-2 α knockdown TCC-SUP (**a, left**) and MBT-2 (**b, left**) cells was detected. The expression of β -actin served as the loading control. In another set of experiments, cells transfected with shRNA constructed as described above for 24 h were re-seeded in 24-well plates and cultured overnight, followed by transfection with 1 μ g of pFRL2-9 \times ORE-CMVmini, which drives firefly luciferase by the CMVmini promoter ligated with 9 \times ORE and drives *Renilla* luciferase by the CMV promoter contained in the plasmid backbone. After 24 h, the cells were exposed to hypoxia or normoxia for 48 h. Transcriptional activities of the CMVmini promoter ligated with 9 \times ORE in TCC-SUP (**a, right**) and MBT-2 (**b, right**) cells under normoxia or hypoxia were determined by a dual-luciferase reporter assay. The ratio of firefly luciferase activity to *Renilla* luciferase activity was expressed as relative light units (RLU) ($n=4$). Values are the mean \pm s.e.m of the mean. * $P < 0.05$.

respectively. These data indicate that hypoxia induced the formation of much more CD133-positive cells than CD44-positive cells. Both Ad9OC and AdLCY targeted hypoxic tumor cells. After viral infection at an MOI of 1, the percentages of surviving CD44-positive cells (AdLCY: 32.21% versus Ad9OC: 48.99%) (Figure 6a) and CD133-positive cells (AdLCY: 3.18% versus Ad9OC: 6.35%) (Figure 6b) decreased. CSCs that display self-renewal capability in tumors are responsible for the re-growth and metastasis of tumors after anticancer therapy. We next examined the self-renewal capability of TCC-SUP cells after infection with various adenoviruses *in vitro* by the soft-agar colony formation assay (Figure 6c, upper panel) and quantified numbers of colonies (Figure 6c, lower panel). Consistent with the results shown in Figure 6a, cells infected with AdLaZ, which was non-cytolytic, produced more colonies under hypoxia than under normoxia, indicating that hypoxia was responsible for altering cellular phenotypes by increasing self-renewal capability. Cells infected with either Ad9OC or AdLCY produced smaller colonies than those infected with AdLaZ. Notably, cells infected with AdLCY produced less colonies under hypoxic than under normoxic conditions. Moreover, under normoxic conditions, cells infected with either AdLCY or Ad9OC also produced less colonies than those infected with AdLaZ. These results indicate that AdLCY and Ad9OC could suppress the self-renewal capability of TCC-SUP cells either under hypoxic or normoxic conditions. As Ad9OC was transcriptionally regulated by the ORE, but not by the HRE, it was

anticipated that no significant differences were found in the numbers of colonies obtained from cells infected with Ad9OC and grown in hypoxic and normoxic conditions. Collectively, these results suggest that, under hypoxic conditions, AdLCY suppressed the self-renewal capability of bladder cancer cells through more effective killing of CD44- and, in particular, CD133-positive tumor cells. Immunofluorescence staining of tumor tissue sections also indicates that AdLCY was more efficacious than Ad9OC at targeting tumors and hypoxic CSCs (Figure 6d). Immunofluorescence double staining shows that AdLCY targeted CD133-positive cells in tumors. CD133 and adenovirus hexon proteins were colocalized in some areas within tumor tissue.

DISCUSSION

Oxygen deprivation often occurs during tumorigenesis, and it usually changes the microenvironment within tumors. HIFs, such as HIF-1 α and HIF-2 α , are associated with resistance to chemotherapy and radiotherapy. Along with tumor progression, these transcriptional factors activate a series of regulatory programs, enhance metastasis and initiate the generation of CSCs. These changes therefore become a major challenge for successful cancer treatment.³⁶⁻³⁸ We previously showed that downregulation of HIF-1 α expression inhibited lung tumor growth through an anti-angiogenesis mechanism.³⁹ Nevertheless, cytolytic effects of oncolytic viruses are usually attenuated in a hypoxic

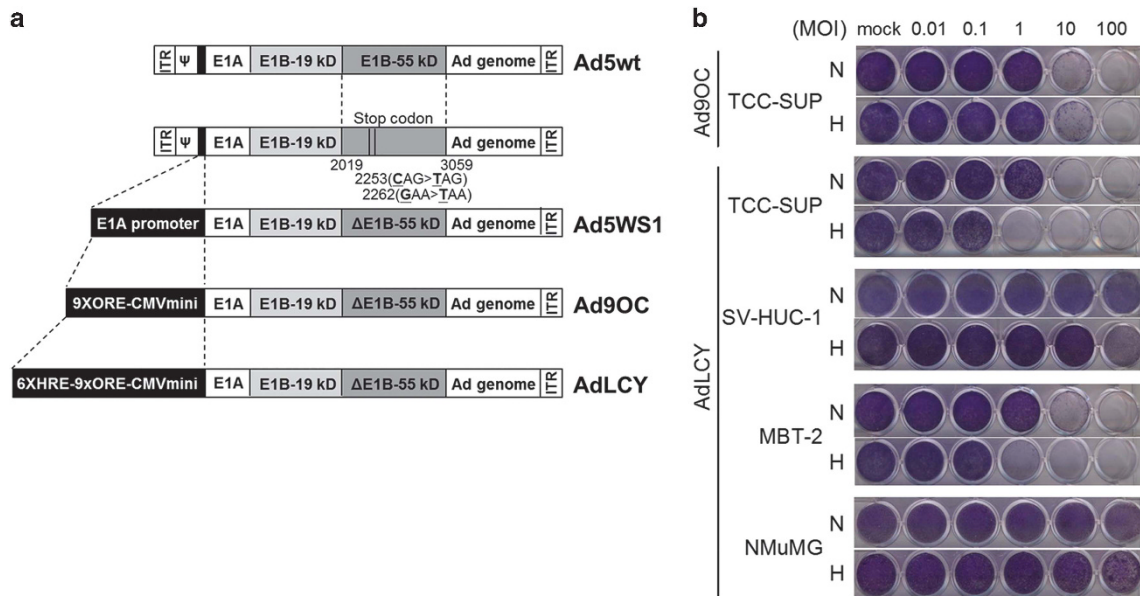


Figure 3. AdLCY displayed potent cytolytic activity against hypoxic bladder cancer cells while sparing normal cells. **(a)** Schematic diagram showing the genome organization of three recombinant adenoviruses, Ad5WS1, Ad9OC and AdLCY, as well as wild-type adenovirus type 5 (Ad5wt). Two point mutations were made at bases 2253 (C-T) and 2262 (G-T) of the *E1B-55 kD* gene (nucleotide 2019–3059) of Ad5 to generate premature translation stop codons, resulting in Ad5WS1, an E1B-55 kD-deleted adenovirus driven by the *E1A* promoter. ITR, inverted terminal repeat; ψ , packaging signal. Numbers indicate nucleotide positions of Ad5. The *E1A* promoter was replaced with 9 \times ORE-CMVmini and 6 \times HRE-9 \times ORE-CMVmini promoters to generate Ad9OC and AdLCY, respectively. **(b)** Virus-induced CPE. Bladder cancer (TCC-SUP and MBT-2) and normal epithelial (SV-HUC-1 and NMuMG) cells were infected with increasing MOIs of AdLCY or Ad9OC, or were mock-infected under normoxia (N) or hypoxia (H) for 48 h. Cells were then cultured in normoxic conditions for 5 days, and the CPE was visualized by crystal violet staining.

environment. In the present study, to increase their cytolytic effects, we added six copies of HRE to the regulatory region of Ad9OC, which is regulated by Oct4,¹⁰ and generated a novel Oct4 and hypoxia dual-regulated AdLCY. We found that an oncolytic virus driven by six copies of the HRE was highly responsive to hypoxia. We have previously shown that reporter constructs carrying six copies of the HRE conferred higher hypoxic responsiveness than those carrying three copies, and that a saturation effect occurred with more than six copies, indicating six copies of the HRE upstream of the CMVmini promoter resulted in maximal hypoxia-inducible gene expression.⁴⁰ Transcriptional activity was even higher in a hypoxic environment when oncolytic adenoviruses were driven by the combination of 6 \times HRE and 9 \times ORE than when they were under the control of either 6 \times HRE or 9 \times ORE alone. Notably, AdLCY had no cytolytic effects on non-tumor cells. We showed that CAR was expressed on the cell surface of both human normal urothelial cells and murine mammary gland epithelial cells. Nevertheless, based on our previous⁹ and current studies, AdLCY failed to replicate in and consequently lyse these non-tumor cells, despite being able to infect them, as evidenced by GFP expression after infection with AdGFP. The tumor specific cytolysis observed in AdLCY was governed by dual-regulation of Oct4 and hypoxia. As HREs are present on the Oct4 promoter, HIF-2 α can activate the expression of Oct4.⁵ Furthermore, HIF-2 α and Oct4 bind directly to the 6 \times HRE and 9 \times ORE, respectively, thus enhancing the replication capability of AdLCY and allowing the virus to efficiently target tumor cells. This dual-regulated mechanism may be attributed to a greater oncolytic activity of AdLCY in comparison with Ad9OC.

During tumor invasion, hypoxia-associated factors switch cells from an HIF-1 α -dependent to an HIF-2 α -dependent signaling pathway and promote the formation of CSC characteristics.⁴¹ One of the essential stemness markers activated by HIF-2 α is Oct4,⁵ which acts with other stem cell factors, such as Lin28, to increase CSC proliferation.⁴² CSCs are a subpopulation of tumor cells that

augment tumorigenesis in specific tumor microenvironments. Self-renewal and differentiation are two hallmark traits of CSCs. The existence of highly tumorigenic CSCs might be responsible for the initiation and progression of numerous aggressive epithelial cancers into locally invasive, metastatic and incurable disease states.^{43,44} CSCs have also been identified in human bladder cancer tissues.^{45,46} Potential therapeutic targets to eradicate CSCs are being developed and showing promising results against locally advanced and metastatic cancers,⁴⁷ including the inhibition of the HIF pathway.⁴⁸ Consistent with these findings, our results show that HIF-2 α shRNA inhibited the expression of Oct4 and its transactivational activity (Figure 2).

In the present study, while in hypoxic J82 and TCC-SUP cells, Oct4 mRNA was induced 23- and 10-folds, respectively, and their Oct4 protein expression was significantly elevated, Oct4 mRNA was only induced 5-fold and Oct4 protein expression was slightly increased in hypoxic T24 cells (Figures 1 and 2). As HIF-2 α can activate Oct4 signaling pathways,⁶ lower expression of HIF-2 α in T24 cells in response to hypoxia might account for lower Oct4 gene expression. Of note, unlike Oct4 expression patterns in parental MBT-2 cells, expression of Oct4 in MBT-2-CF1 cells was increased at the mRNA, but not at the protein level under hypoxia. MBT-2-CF1 was a subline derived from MBT-2 cell line by *in vivo* passages of MBT-2 cells in our laboratory. MBT-2-CF1 cells exhibited increased tumorigenic potential in mice. Therefore, genetic alterations in MBT-2-CF1 cells during *in vivo* passages may have occurred and account for the discrepant expression of Oct4. However, additional study is needed to further characterize MBT-2-CF1 cells.

In the present study, we also found that AdLCY targeted CD44- and CD133-positive cells. CD44 and CD133 are both transmembrane proteins and surface markers for CSCs. CD44 generally acts as a specific receptor of hyaluronic acid, promotes cell migration and is usually highly expressed in almost all cells. A gene ontology analysis showed that more than one-third of the CD133-upregulated genes

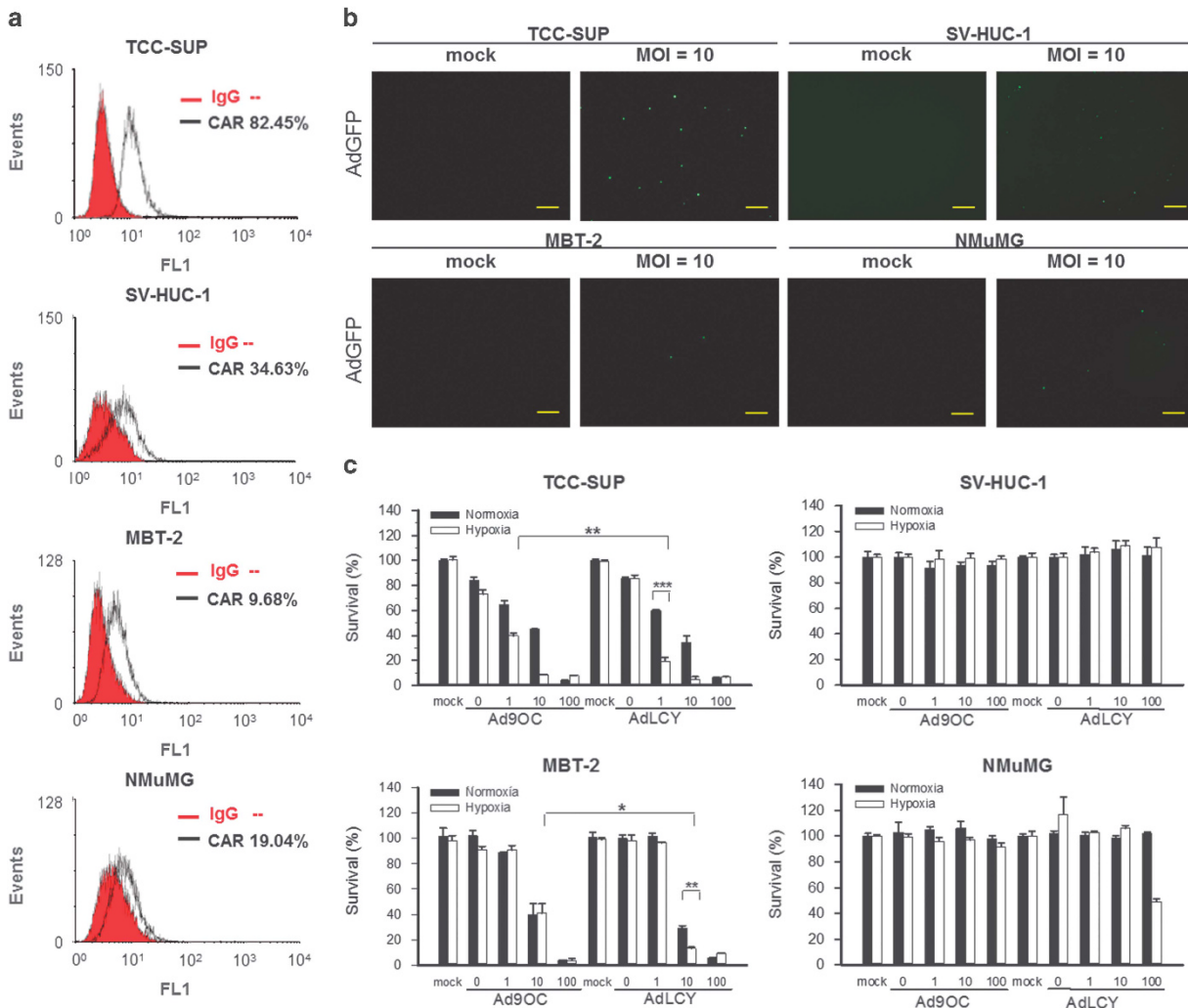


Figure 4. AdLCY exhibited higher tumor-selective cytolytic effect than Ad9OC on bladder cancer cells. **(a)** Analysis of surface expression of CAR on bladder cancer and normal epithelial cells by flow cytometry. Black lines denote cell percentages expressing CAR. Red zones represent background fluorescence on the cells. **(b)** Detection of AdGFP-infected cells. Various cells were infected with AdGFP at an MOI of 10 or mock-infected for 48 h. Cells expressing GFP were observed under fluorescence microscopy. Representative images in each group are shown ($\times 40$ magnification; scale bar = 500 μm). **(c)** Cytotoxic effects of AdLCY and Ad9OC. Bladder cancer and normal epithelial cells were infected with increasing MOIs of AdLCY or Ad9OC, or were mock-infected under normoxia (N) or hypoxia (H) for 48 h. Cells were then cultured in normoxic conditions for 5 days, and cell survival was determined by the WST-1 assay. Values are the mean \pm s.e.m. of the mean. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

are involved in the cell cycle process and a certain number of CD133-downregulated genes are involved in the immune response.⁴⁹ The CD133-related gene expression signature identifies aggressive glioblastoma subtypes as well as high-grade bladder and breast cancers.⁴⁹ Therefore, cancers originating from distinct tissue types may share some fundamental properties at the stem-cell level. For these reasons, CD133 provides a pivotal molecular target for cancer treatment, especially for more invasive tumors. However, the sparseness of CD133-positive cells in bladder cancer tumors makes targeting their cells challengeable. It was shown that overexpression of HIF-1 α and HIF-2 α activates the CD133 promoter through ETS proteins.⁵⁰ Therefore, multiple onco-targets (including Oct4 and CD133) of hypoxia-inducible factors suggest that HIFs are master regulators of stemness properties of cancer-initiating cells and also serve as potential therapeutic targets. As a hypoxic-dependent oncolytic virus, AdLCY may be more efficacious than Ad9OC at targeting CSC cells.

AdLCY can replicate in a hypoxic environment. Tumors are not capable of growing beyond a certain size without angiogenesis

because of the limited diffusion of oxygen. When the volume of a solid tumor is $> 1 \text{ mm}^3$, tumor cells would be faced with hypoxia.⁵¹ An *in vivo* ^{31}P nuclear magnetic resonance (NMR) spectroscopy has estimated that $\sim 4\%$ of the hypoxic cell fraction occurred in tumors $< 100 \text{ mm}^3$, and the fraction increased to 40% when tumor volume grew to 250 mm^3 .⁵² For these reasons, in our animal model, when visible and palpable nodules (mean tumor volume: $41.51 \pm 7.70 \text{ mm}^3$; range: $8.81\text{--}117.07 \text{ mm}^3$) developed at all injection sites on day 21, we injected the tumors with oncolytic adenoviruses on days 21, 23 and 25. We found that, compared with injections of saline and Ad9OC, intratumoral injections of AdLCY significantly retarded tumor growth in mice with subcutaneous TCC-SUP tumors. Mouse survival after AdLCY treatment was also significantly prolonged. The mean tumor volume on day 26 was 185.03 mm^3 in the saline-treated control group. When hypoxic areas expanded within tumors at this stage, tumoricidal effects of hypoxia-independent oncolytic adenoviruses might be attenuated. The emergence of CSCs following hypoxia promotes cancer progression and metastasis. Indeed, we also found metastatic nodules in the lungs at day 61

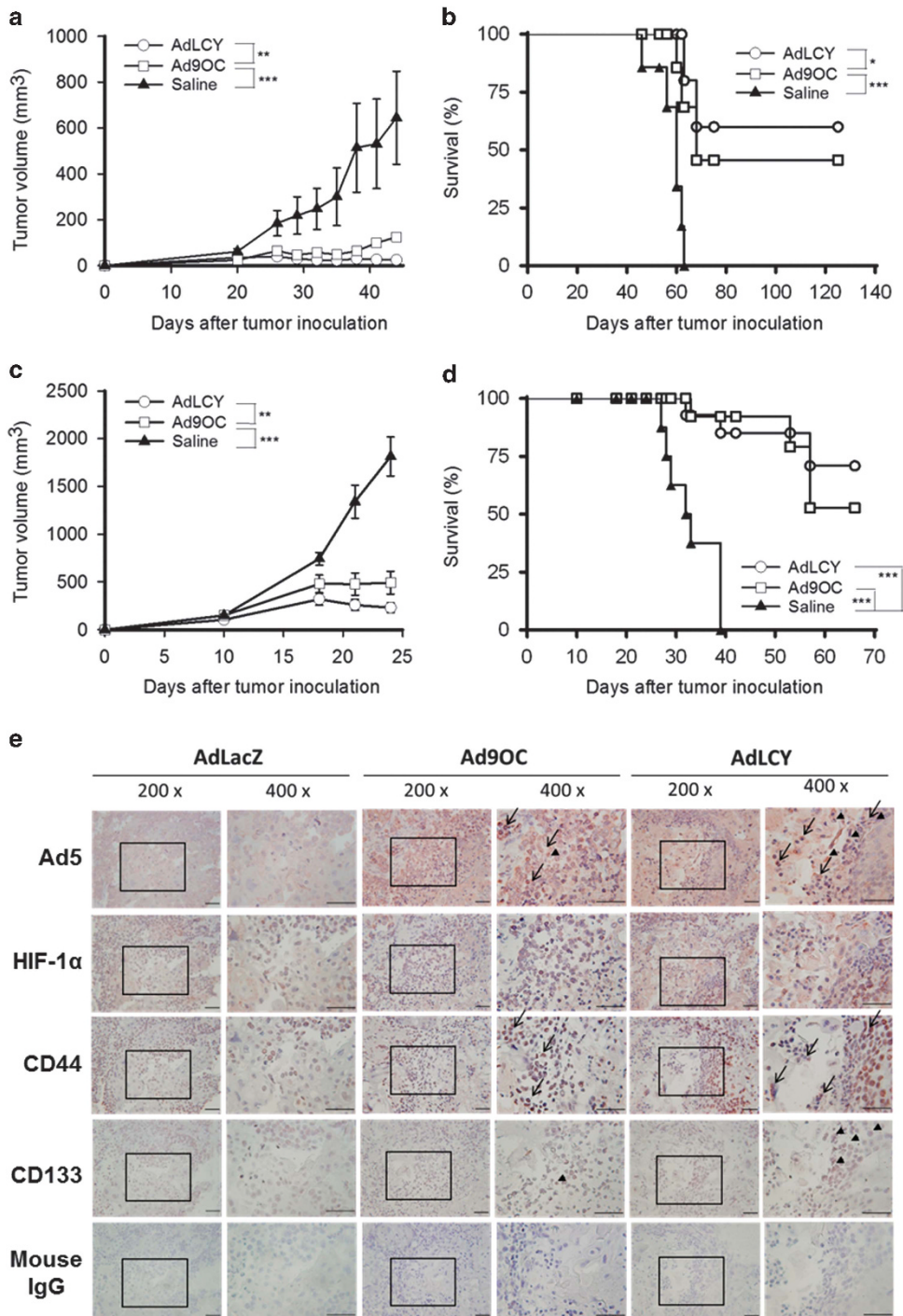


Figure 5. AdLCY had higher oncolytic activity than Ad9OC against TCC-SUP xenografts and syngeneic MBT-2 tumors. **(a, b)** Groups of NOD/SCID mice ($n=5-6$) were subcutaneously inoculated with TCC-SUP cells (5×10^6) on day 0, and intratumorally treated with 5×10^8 PFU of AdLCY or Ad9OC, or with saline on days 21, 23 and 25. Tumor volumes **(a)** and Kaplan–Meier survival curves **(b)** were determined. **(c, d)** Groups of C3H/HeN mice ($n=8$) were subcutaneously inoculated with MBT-2 cells (5×10^6) on day 0, and intratumorally treated with 5×10^8 PFU of AdLCY or Ad9OC, or with saline on days 11, 13 and 15. Tumor volumes **(c)** and Kaplan–Meier survival curves **(d)** were determined. Values are the mean \pm s.e.m. of the mean. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. **(e)** NOD/SCID mice were inoculated with TCC-SUP cells and then treated with AdLCY, Ad9OC or AdLacZ as described in **a**, tumors were excised on day 31, and serial formalin-fixed paraffin sections of tumor tissues were immunohistochemically stained for adenovirus type 5 (Ad5) proteins, HIF-1 α , CD44 and CD133. Representative images in each group are shown (scale bar = 50 μ m). Images shown at $\times 400$ magnification correspond to the boxed areas in the images shown at $\times 200$ magnification. Colocalization of Ad5 with CD44 and with CD133 is indicated by arrows and arrowheads, respectively. Background staining was determined by substituting isotype control IgG for the primary antibody.

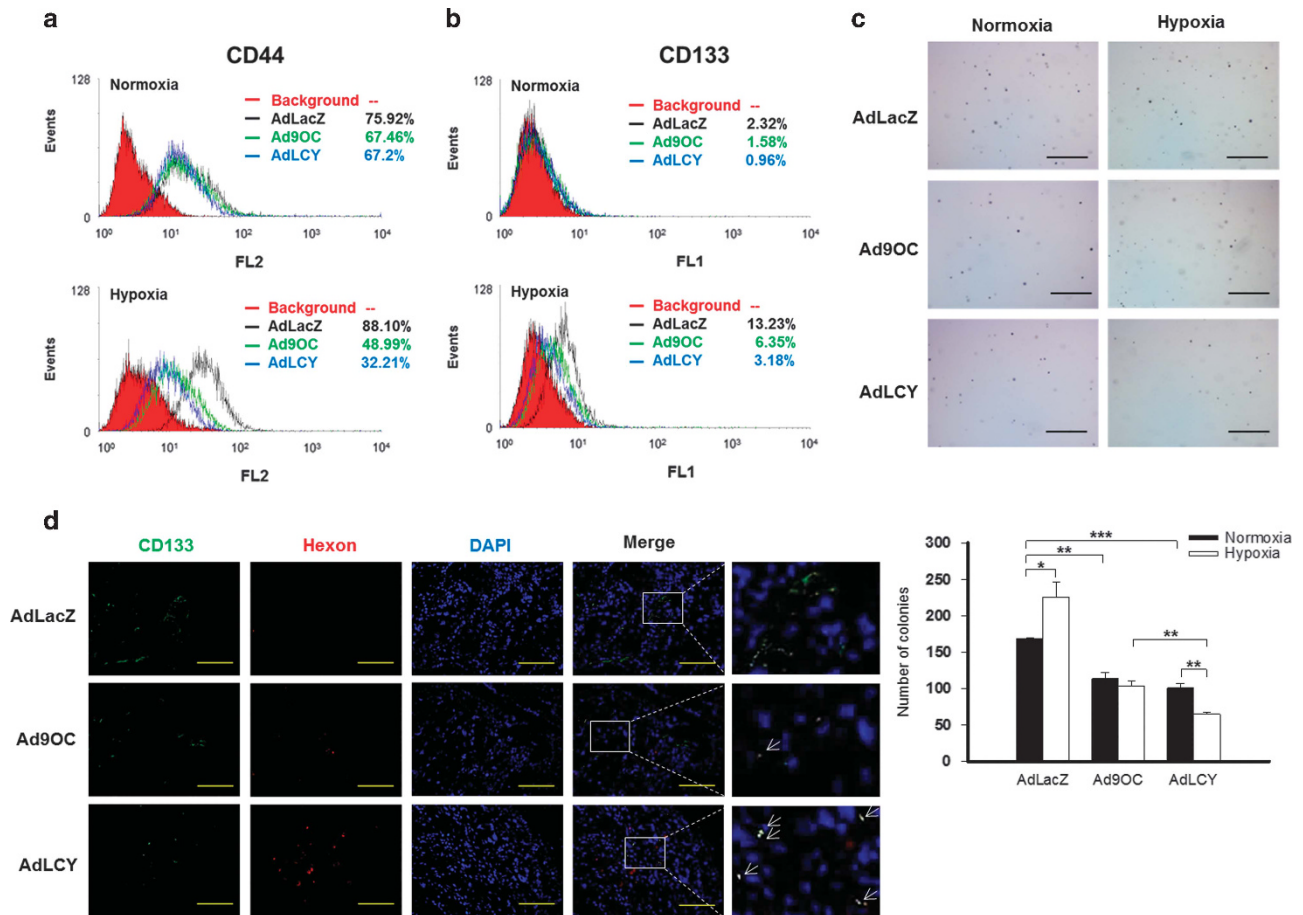


Figure 6. AdLCY targeted more CD44- and CD133-positive bladder tumor cells than Ad9OC. **(a, b)** Detection of cells expressing CD44 and CD133 after viral infection. TCC-SUP cells were infected with AdLCY, Ad9OC or AdLacZ at an MOI of 1 for 5 days. Intensities of CD44 **(a)** and CD133 **(b)** expression on surviving cells after viral infection were analyzed by flow cytometry. Black, green and blue lines denote cell percentages after AdLacZ, Ad9OC and AdLCY infection, respectively; red zones represent background fluorescence on the cells. **(c)** Self-renewal capability of bladder cancer cells after viral infection under hypoxic or normoxic conditions. TCC-SUP cells infected with various adenoviruses at an MOI of 1 were cultured under hypoxic or normoxic conditions for 48 h. Cells were then grown on soft agar, and cell colonies were observed and counted after 24 days. Representative images for colonies grown in soft agar in each group are shown ($\times 40$ magnification; scale bar = 500 μm , upper panel). The number of colonies in each group was quantified (lower panel). Each group was performed in triplicate. Results are representative of three independent experiments. Values are the mean \pm s.e.m. of the mean. $***P < 0.001$; $**P < 0.01$; $*P < 0.05$. **(d)** Immunofluorescence double staining showing targeting of AdLCY to CD133-positive cells in bladder tumors. NOD/SCID mice bearing TCC-SUP tumors ($n = 5$) were intratumorally treated with 5×10^8 PFU of AdLCY, Ad9OC or AdLacZ on days 21, 23 and 25, and then killed on day 31. Representative images in each group are shown ($\times 400$ magnification; scale bar = 50 μm). Magnified images correspond to the boxed areas in the merged images. Colocalization of Ad5 hexon proteins with CD133 is indicated by arrows. The nucleus was counterstained with DAPI.

after tumor cell inoculation in our subcutaneous bladder tumor models. Therefore, more effective targeting of AdLCY to CSCs and tumor cells in a hypoxic environment may account for its potent antitumor effects. However, most of the CSC models that have been studied rest on experimental foundations. Different characteristics of CSCs within tumors reflect different cancer types and hosts used.⁵³ Thus, further investigation of the characteristics of CSCs that increase in hypoxic areas of different tumors during tumor progression would be of great help in designing ideal oncolytic adenoviruses with potent antitumor activity.

In conclusion, we have developed AdLCY, an Oct4 and hypoxia dual-regulated oncolytic adenovirus, which is potent and efficacious for treating hypoxic bladder tumors in animal models. As hypoxia and CSCs are hallmarks of tumor growth and progression, AdLCY may be broadly exploited for treating a wide range of primary and metastatic tumors.

MATERIALS AND METHODS

Cells and mice

Human bladder cancer (TCC-SUP, J82 and T24) and murine bladder cancer (MBT-2) cell lines, as well as immortalized normal human urothelial cell line (SV-HUC-1) and normal murine mammary gland epithelial cell line (NMuMG), have been previously described.¹⁰ MBT-2-LM7 (lung-metastatic MBT-2 clone 7) was derived from a metastatic lung nodule after subcutaneous implantation of MBT-2 tumors in C3H/HeN mice.¹⁰ A subline of the MBT-2 cell line, designated MBT-2-CF1, was derived from *in vivo* passages of parental MBT-2 cells, which resulted in increased tumorigenicity in C3H/HeN mice. All cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 2 mm L-glutamine, 10% cosmic calf serum (Hyclone, Logan, UT, USA) and 50 mg ml⁻¹ gentamicin. Female NOD/SCID mice (6–8 weeks old) were purchased from the Laboratory Animal Center of National Cheng Kung University. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University.

Hypoxic conditions for cell culture

Hypoxia was achieved using a hypoxia chamber (GasPak EZ Anaerobe Container System; Becton Dickinson, Sparks, MD, USA) with three sachets (GasPak EZ Anaerobic System Sachets; Becton Dickinson) and one anaerobic indicator strip (GasPak EZ Anaerobic System with indicator; Becton Dickinson). The container was then incubated at 37 °C. After 2.5 h, the percent of oxygen was less than or equal to 1%. The *in vitro* studies were then carried out in the chamber, and the anaerobic indicator strip was used to monitor the percentage of oxygen during all experiments performed to confirm hypoxic conditions.

Analysis of promoter activities

DNA fragments containing nine copies of tandemly repeated sequences of ORE (9×ORE) and the CMVmini promoter derived from pTRE vector (Clontech, Mountain View, CA, USA) were placed upstream of the luciferase gene of the single dual-luciferase reporter plasmid pFRL2⁵⁴ to generate pFRL2-9×ORE-CMVmini.¹⁰ The pFRL2 plasmid carries firefly luciferase gene driven by the tested promoter cloned upstream of the gene as well as *Renilla* luciferase gene driven by the CMV promoter. Rather than using a test reporter plasmid and a control reporter plasmid to normalize transfection efficiency in reporter assays, pFRL2 plasmid can be used alone to reduce the problems of copy-number variation and leaky transcription. Downstream of the CMV promoter is a chimeric intron. Transfection studies have demonstrated that the presence of an intron flanking a cDNA insert frequently increases the level of gene expression. The internal control *Renilla* luciferase is a 36-kDa monomeric protein that does not require post-translational modification for its activity. The DNA fragment encompassing six copies of tandemly repeated sequences of HRE (6×HRE) was excised from p6×HRECAT3-CMVmini by *KpnI* and *SacI* digestion. This restricted fragment was then subcloned into *KpnI*- and *SacI*-restricted pFRL2-9×ORE-CMVmini. The DNA fragment of 9×ORE that had been excised from pFRL2-9×ORE-CMVmini by *KpnI* digestion was inserted into the *KpnI*-restricted pFRL2-6×HRE-CMVmini to generate pFRL2-6×HRE-9×ORE-CMVmini. Cells (1×10^5) were transfected with 0.8 µg of pFRL2-9×ORE-CMVmini, pFRL2-6×HRE-CMVmini or pFRL2-6×HRE-9×ORE-CMVmini using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, the cells were placed in a hypoxia chamber or cultured for 48 h at 37 °C. The cell lysates were then collected, and their firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay (Promega, Madison, WI, USA). The firefly luciferase activity was normalized to *Renilla* luciferase activity, and the relative luciferase units (RLU) were expressed as a fold activation relative to the basal level under normoxia.

RNA interference of HIF-2α

For HIF-2α knockdown experiments, pLKO.1-puro-based lentiviral vectors including stem-loop cassettes encoding shRNA targeting human *HIF-2α* gene (TRCN000003804) and murine *HIF-2α* gene (TRCN0000082307), as well as *GFP* gene (TRCN0000072184), were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan.

Construction of recombinant adenoviruses

The AdEasy system was used to generate AdLCY, an E1B-55-kDa-deleted adenovirus under the control of the CMVmini promoter ligated with 6×HRE and 9×ORE.⁵⁵ To insert a multiple cloning site on the backbone of an E1B-55-kDa adenovirus, we have constructed pAd5YS plasmid previously.¹⁰ The DNA fragment encompassing the 6×HRE-9×ORE-CMVmini promoter was excised from pFRL2-6×HRE-9×ORE-CMVmini by *NotI* and *BglII* digestion, and cloned into the *NotI/BamHI* sites of pAd5YS, resulting in pAd5YS-6×HRE-9×ORE-CMVmini. Subsequently, the 6×HRE-9×ORE-CMVmini fragment was excised by *BsrGI* and *MfeI* digestion and cloned into pShuttle to generate pShuttle-6×HRE-9×ORE-CMVmini. AdLCY was generated as previously described.¹⁰ Ad9OC, which is an E1B-55-kDa-deleted adenovirus driven by the 9×ORE-CMVmini promoter, and AdLacZ, a replication-defective adenoviral vector encoding β-galactosidase, have been previously described.¹⁰ AdGFP, a replication-defective adenoviral vector encoding GFP under the control of the CMV promoter, has been previously described.⁵⁶ All recombinant adenoviruses were propagated, purified using CsCl density gradient centrifugation and quantified by the plaque assay.

Analysis of CPE and cell viability

For monitoring CPE, bladder cancer cells (TCC-SUP and MBT-2) and normal epithelial cells (NMuMG and SV-HUC-1) were infected with either AdLCY or Ad9OC at different MOIs or left uninfected under normoxic or hypoxic conditions for 48 h. Cells were then cultured in normoxic conditions for 5 days. The CPE was visualized by fixing and staining the remaining monolayer with 10% formalin/0.05% crystal violet. Cell survival was also assessed with a colorimetric WST-1 assay (Dojindo Laboratories, Tokyo, Japan).

Quantitative real-time RT-PCR analysis

Trizol (Invitrogen) was used to isolate total RNA from cells according to the manufacturer's protocols. Total RNA (2 µg) was reverse-transcribed into cDNA using a Verso cDNA synthesis kit (Thermo Fisher Scientific, Barrington, IL, USA). Quantitative real-time PCR was performed using a SmartCycler System (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions. Each reaction contained 50 ng cDNA, SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), and 5 µmol of each forward and reverse primer. Two oligonucleotide primer pairs specific to Oct4 (forward, 5'-CGAAAGAGAAAGCGAACCAGTATC-3'; reverse, 5'-TTACAGAACCACACTC GGACCAC-3') and GAPDH (forward, 5'-GCCATCACTGCCACCCAG-3'; reverse, 5'-TCTTACTCCTTGGAGGCCATGT-3') were used. Normalization was performed using human glyceraldehyde-3-phosphate dehydrogenase GAPDH as an internal control, and relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.⁵⁷

Immunoblot analysis

Total cell lysates were collected and subjected to immunoblot analysis using rabbit antibodies against HIF-1α (Novus Biologicals, Littleton, CO, USA) and HIF-2α (Novus Biologicals), and mouse monoclonal antibody against Oct4 (C-10; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) were used as the secondary antibodies, where appropriate. The blots were reprobed with β-actin (Sigma-Aldrich, St Louis, MO, USA) which served as the loading control.

Animal studies

TCC-SUP cells (5×10^6) were inoculated subcutaneously into the right flank of NOD/SCID mice on day 0. Groups of 5-6 mice were intratumorally treated with 5×10^8 plaque forming units (PFU) of AdLCY or Ad9OC, or with saline on days 21, 23 and 25. All the mice were monitored for tumor growth and survival. A tissue caliper was used to measure the tumors twice a week in two perpendicular axes, and the tumor volume was calculated as (length of tumor) × (width of tumor)² × 0.45. The time of animal death was recorded. All moribund mice, which were killed, were also recorded as dead.

Immunohistochemistry

To detect tumor targeting of AdLCY and Ad9OC, TCC-SUP tumor-bearing mice were treated with AdLCY, Ad9OC or AdLacZ and killed on day 31. Serial formalin-fixed paraffin sections of tumor tissues were stained with rabbit anti-adenovirus type 5 antibody (Abcam, Cambridge, UK), rabbit anti-mouse HIF-1α antibody (Abcam), mouse anti-CD44 monoclonal antibody (8E2; Cell Signaling, Danvers, MA, USA) and mouse anti-human CD133/1 monoclonal antibody (AC133; Miltenyi Biotec, Bergisch Gladbach, Germany). After they had been sequentially incubated with appropriate secondary antibodies (Jackson ImmunoResearch) for 2 h at room temperature with aminoethylcarbazole as the substrate chromogen (Invitrogen Zymed Laboratories, Camarillo, CA, USA), the slides were counterstained with hematoxylin.

Flow cytometry

Bladder cancer cells (TCC-SUP and MBT-2) and normal epithelial cells (SV-HUC-1 and NMuMG) (1×10^6 per analysis) were incubated on ice with anti-CAR (RmcB, American Type Culture Collection, Manassas, VA, USA) monoclonal antibody for 1 h, followed by incubation for 30 min on ice with FITC-conjugated goat anti-mouse antibody. Isotype immunoglobulin G was used as a matched control. TCC-SUP cells were infected with AdLCY, Ad9OC or AdLacZ at an MOI of 1 for 5 days. Subsequently, the cells (1×10^6 per analysis) were stained either with the phycoerythrin (PE)-conjugated anti-CD44 antibody (IM7; eBioscience, San Diego, CA, USA) for 1 h at room

temperature or with the mouse anti-human CD133/1 monoclonal antibody (AC133; Miltenyi Biotec) for 2 h at 4°C followed by incubation with the fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Baltimore, MD, USA). The cells were washed twice with the staining buffer and suspended in DMEM-based buffer for flow cytometric analysis. These samples were then analyzed with a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Colony formation assay

TCC-SUP cells infected with various adenoviruses at an MOI of 1 were cultured under hypoxic or normoxic conditions for 48 h. Subsequently, cells were thoroughly dissociated with 0.1% trypsin to prepare single-cell suspension. Each well of 24-well plates was covered with a basal layer of 300 µl of 0.5% agarose containing 50% complete medium (DMEM with 10% cosmic calf serum (Hyclone, Logan, UT, USA)). After the medium was solidified, 300 µl of 0.35% agarose containing 2000 cells and 50% complete medium in triplicate was added to each well. These wells were then covered with 200 µl of complete medium and the plates were incubated at 37°C for 29 days. Numbers of colonies were then counted using a cytometer (Celigo Cytometer; Cytellect, San Diego, CA, USA) according to the manufacturer's instructions.

Immunofluorescence double staining

Tumor sections were fixed in 10% formalin and stained with an anti-human CD133/1 (AC133; Miltenyi Biotec) monoclonal antibody and subsequently incubated with a fluorescein-conjugated secondary antibody IgG. After being washed three times with PBS, they were stained with an anti-adenovirus type 5 hexon antibody (Abcam) and subsequently incubated with a fluorescein-conjugated secondary IgG antibody. The nucleus was counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

Statistical analysis

All values are means ± s.e.m. of the mean. Statistical significance between groups was assessed using Student's *t*-test. Tumor volumes were compared using two-way ANOVA. The survival analysis was performed using the Kaplan-Meier survival curve and the log-rank test. Any *P* value of < 0.05 is regarded statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Bert Vogelstein (John Hopkins School of Medicine) for generously providing plasmids for AdEasy System and Richard A. Hodin (Harvard Medical School) for generously providing pFRL2 plasmid. This work was supported by grants from the National Science Council (NSC 97-3112-B-006-005-001), Taiwan.

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