Treatment of prostate cancer with Ad5/3Δ24hCG allows non-invasive detection of the magnitude and persistence of virus replication *in vivo*

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Abstract

Hormone refractory metastatic prostate cancer is a deadly disease that currently lacks curative treatments. Conditionally replicating adenoviruses (CRAds) are promising new agents against cancer due to their innate capability to cause oncolysis of tumor cells. Their antitumor effect is determined in part by their capacity for infecting cancer cells. However, the respective primary receptor, the coxsackie-adenovirus receptor (CAR), is variably expressed in many cancer types. We created Ad5/3Δ24hCG, a novel CRAd retargeted to the adenovirus serotype 3 receptor, which has been reported to be highly expressed in tumors. Furthermore, we added a transgene for the β-chain of human chorionic gonadotropin (hCGβ), whose expression was tightly coupled to virus replication. Ad5/3Δ24hCG was found effective in killing prostate cancer cells, and oncolysis was seen in concordance with hCGβ production. In a s.c. *in vivo* model of hormone refractory prostate cancer, Ad5/3Δ24hCG treatment resulted in statistically significant tumor growth inhibition. Moreover, i.v. injection of Ad5/3Δ24hCG prolonged the survival of mice with hormone refractory prostate cancer metastatic to the lung. Detection of hCGβ in serum samples confirmed viral replication *in vivo*. Infection of human clinical samples of cancerous and normal prostatic tissue resulted in effective hCGβ production in cancer tissue, whereas it remained low in nonmalignant tissue, suggesting cancer-specific replication. These results suggest that Ad5/3Δ24hCG is a potent virus for the treatment of hormone refractory prostate cancer *in vitro* and *in vivo*. These preclinical data set the stage for translation into clinical studies. [Mol Cancer Ther 2007;6(2):742–51]

Introduction

Prostate carcinoma is the most common cancer in men, and the incidence is rapidly increasing (1). In 2005, the incidence of new cases in the United States was 232,090 (2). Surgery, radiotherapy, and brachytherapy are potentially curative treatments for local prostate carcinoma, when watchful waiting is not an option. However, ~35% of cases recur or are detected when metastatic (3). Hormonal therapies are usually effective initially, but given enough time, hormone refractory disease eventually emerges (4, 5). This is a common and deadly disease; it lacks curative options; and it is the main cause of prostate cancer–related mortality, which amounted to an estimated 30,350 cases in the United States in 2005 (2). The only treatment with a demonstrated survival benefit is docetaxel, which lengthens the life of patients with a mean of less than 3 months, despite often grueling side effects (6). Therefore, additional and novel treatments are needed. One such approach could be oncolytic viruses.

With regard to oncolytic viruses, most preclinical and clinical data exist for conditionally replicating adenoviruses (CRAd; ref. 7). The antitumor effect of CRAds is mediated as a direct result of viral replication in target tissue, and their tumor selectivity is based on engineered or natural features that use the genetic differences between normal and tumor cells (8). For example, tumor-specific promoters can be used (9) or deletions that abrogate replication in normal cells but are transcomplemented in tumor cells (8). One such modification is the deletion of 24 bp from the constant region 2 of *E1A* (10, 11). This deletion has been shown to reduce replication in normal cells while continuing to allow effective oncolysis of tumor cells deficient in the Rb/p16 pathway (10, 11). This region of E1A is responsible for the binding of Rb for release of E2F, which is critical for the synthesis of S phase components and for the activation of other adenoviral genes (12). The Rb/p16 pathway is one of the central growth control pathways. Consequently, dysfunction of

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this pathway may be required for the carcinogenic process to progress (13). Therefore, tumor cells, including prostate cancer cells, universally display aberration of this pathway (14, 15).

Adenovirus serotype 5 (Ad5) has been widely used as a platform for construction of adenoviral gene therapy agents because of its high capacity for transduction of dividing and nondividing cells. In addition, it can express high transgene levels, and its genome remains episomal. Moreover, its genome is relatively well understood, which facilitates the construction of recombinant viruses (8). The degree and localization of expression of the primary coxsackie-adenovirus receptor (CAR) is a major factor determining the amount of subsequent gene expression and oncolysis (16–18). Switching of the fiber knob of Ad5 to the Ad3 knob retargets the virus to the serotype 3 receptor (8, 16). The Ad3 receptor is distinct from CAR (19), and contrary to CAR, it is highly expressed on many types of cancers and not down-regulated during carcinogenesis (16). Cluster of differentiation 46 might be one but perhaps not the only receptor for Ad3 (20). In prostate cancer, CAR has been shown to be expressed to a variable and often low degree (21, 22). In particular, clinical prostate cancer specimens frequently feature low CAR (23).

Traditionally, 15 years has been required for full clinical evaluation of new candidate anticancer agents. However, this process could be expedited, if there were ways to obtain comprehensive longitudinal pharmacokinetic and efficacy data from early phase trials. With regard to oncolytic viruses, one possibility is the use of secretory marker peptides, in which production can be tightly coupled to virus replication (24–26). This can be achieved by the use of adenoviral E3 region gene promoters. E3 products are normally responsible for interactions with host defense mechanisms. E3gp19K prevents MHC class I molecules from being transported to the cell surface, which results in slower recognition of infected cells by cytolytic T cells. However, this function may be superfluous in tumor cells, which are often deficient in MHC presentation. Furthermore, it has been suggested that deletion of 6.7K/gp19K does not reduce the replicativity of the virus (27). Moreover, the deletion of 6.7K/gp19K could enhance the recognition of tumor cells by CTLs, which could be advantageous from an immunotherapeutic point of view.

Human chorionic gonadotropin β-chain (hCGβ) is a secreted molecule without known biological effects, but it is routinely measured for pregnancy testing, and therefore, accurate and clinically approved tests are readily available. We have previously shown that transgenes can be inserted into the E3gp19K region for expression about 8 h postinfection, which corresponds to physiologic E3gp19K production (26). Moreover, we saw tight coupling of protein production to the magnitude and duration of virus replication. Here, our hypothesis was that we can develop a new CRAd, which is capsid modified for effective transduction of prostate cancer cells and whose persistence and magnitude of replication can be measured noninvasively by detection of hCGβ.

Materials and Methods

Cell Culture

Hormone refractory DU-145, PC-3, and hormone-sensitive LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). PC-3MM2 cells are a highly metastatic hormone refractory subline of PC-3 (courtesy of Isaiah J. Fidler, MD Anderson Cancer Center, Houston, TX). PC-3 and LNCaP cells were cultured in RPMI 1640 supplemented with 10% FCS (PromoCell GmbH, Heidelberg, Germany), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Sigma, St. Louis, MO). PC-3MM2 cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L sodium pyruvate (Sigma). DU-145 cells were cultured according to supplier's recommendations. Human transformed embryonal kidney cell line 293 was purchased from Microbix (Toronto, Ontario, Canada), and human lung adenocarcinoma cell line A549 from the American Type Culture Collection. 911 cells are courtesy of Dr. van der Eb (University of Leiden, the Netherlands). These cells were cultured in the recommended conditions.

Viruses

Construction of Ad5/3Δ24hCG. *hCGβ* was inserted into the *E3* region of pAdEasy-1.5/3Δ24 using a strategy that replaces the *gp19K* reading frame but retains most of *E3* intact (26). pAdEasy-1.5/3Δ24 is a pAdEasy-1–derived plasmid containing a chimeric 5/3 fiber and a 24-bp deletion in the constant region 2 of E1A. Briefly, the *Spe*I (27,082) to *Nde*I (31,089) fragment from the Ad5 genome was inserted into the multicloning site of pGEM5Zf+ (Promega, Madison, WI), resulting in a plasmid pTHSN, which was further digested with *Sun*I/*Mun*I creating a 965-bp deletion in the *E3* region (*6.7K* and *gp19K* deleted). A 498-bp cDNA encoding the β subunit of hCGβ was amplified by a PCR reaction that generated *Sun*I/*Mun*I restriction enzymes sites flanking the gene; hCGβ(S): 5′-ACG TCG TAC GAT GGA GAT GTT CCA GGG GCT G-3′, hCGβ(AS): 5′-TGT GCA ATT GTT ATT GTG GGA GGA TCG GGG T-3′. This fragment was inserted into *Sun*I/*Mun*I-digested pTHSN. pAdEasy-1.5/3Δ24hCGβ was generated by homologous recombination in *Escherichia coli* between *Fsp*I-linearized pTHSN-hCGβ and *Srf*I-linearized pAdEasy-1.5/3Δ24. The Ad5/3Δ24hCG virus genome was released by *Pac*I digestion and transfection into 911 cells. The virus was propagated on A549 cells, and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm, and standard plaque assay on 293 cells was done to determine infectious particles. For other viruses, see Table 1.

Cell Killing *In vitro*

Prostate cancer cells were seeded at 10^4 cells per well on 96-well plates and incubated overnight. Monolayers in quadruplicates were infected for 1 h at 37°C in 50–70 μL of growth medium with 2% FCS. Cells were washed once, and 100 μL of growth medium with 5% FCS was added. Cells were incubated at 37°C, and cell viability was measured

Table 1. Features of adenoviruses used in the study

Name	E1 region	E3 region	Fiber knob	VP/pfu ratio*	Reference
Ad300WT	Wild type	Wild type	Wild type	8.6	ATCC
Ad5luc1	Deleted, luciferase transgene inserted	Deleted	Wild type	25.6	(35)
Ad5/3luc1	Deleted, luciferase transgene inserted	Deleted	Ad3	4.8	(16)
Ad5lucRGD	Deleted, luciferase transgene inserted	Deleted	RGD-4C motif in HI loop	53	(36)
Ad5/3Δ24	24-bp deletion in Rb binding constant region 2	Wild type	Ad3	15.9	(29)
Ad5-Δ24RGD	24-bp deletion in Rb binding constant region 2	Wild type	RGD-4C motif in HI loop	54	(37)
Ad5/3Δ24hCG	24-bp deletion in Rb binding constant region 2	hCGβ cDNA replaces *6.7K/gp19K*	Ad3	15	This paper

*Viral particles/plaque-forming units.

4 to 8 days after infection using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), i.e., the 3-(4,5,dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay. For crystal violet assays, prostate cancer cells were seeded at 5×10^4 cells per well on 24-well plates. Cells were incubated overnight and infected for 1 h at 37°C in 250 μL of growth medium with 2% FCS. Cells were washed, 1 mL of growth medium with 5% FCS was added, and cells were incubated at 37°C. Three to five days later, cells were fixed with 10% formalin and stained with 1% crystal violet. For both MTS and crystal violet, internal positive and negative controls ensured validity and optimal duration of the assay. To obtain a maximal dynamic range, reactions were stopped before complete cell killing was seen with the fastest virus.

Correlation of hCGβ Levels and Virus Replication

PC-3MM2 cells were seeded at 5×10^4 cells per well on 24-well plates and incubated overnight. Monolayers were infected with Ad5/3Δ24hCG at 100 viral particles per cell for 1 h at 37°C in 250 μL of growth medium with 2% FCS. Cells were washed once, and 1.5 mL of growth medium with 5% FCS was added. At 24, 48, and 72 h, growth media and cells were harvested. hCGβ levels were analyzed from the media, and cells were subsequently freeze-thawed thrice. After centrifugation, the supernatant was titered on 293 cells by tissue culture infectious dose 50 ($TCID_{50}$) assay.

hCGβ Measurement

***In vitro* Assays.** During the crystal violet experiment, 30 μL of growth media was collected from each well at 6, 24, 48, and 72 h and at 96 and 120 h if cells were still viable. Samples were frozen at −20°C and analyzed for the cumulative amount of secreted hCGβ. hCGβ was quantitated as previously described (28).

***In vivo* Assays.** For the s.c. model, 5 days after the last virus injection, blood samples were taken from the great saphenous vein of two mice per group and at day 10 from three mice per group. For the intrapulmonary model, blood samples were taken from the great saphenous vein of 10 mice per group at baseline and then every 4 days beginning on day 8. Samples were centrifuged, and the serum was collected for hCGβ analysis.

Tissue Samples

Fresh prostate tissue was obtained with signed informed consent from patients undergoing prostatectomy at the Helsinki University Central Hospital. The tissue samples were divided into 200-mg pieces estimated to contain 50,000 cells each. Samples were homogenized mechanically, divided into duplicates and infected for 1 h at 37°C in 500 μL of growth medium with 2% FCS. Cells were washed once, and 5 mL of growth medium with 20% FCS was added. For hCGβ measurement, 100 μL of growth medium was collected at 12, 24, 48, and 72 h postinfection and analyzed as described above. Internal positive and negative controls were used to validate the experiment. Histopathologic analysis of tissue samples was done by an experienced pathologist.

Animal Experiments

S.c. tumors ($n = 5$ mice and 10 tumors per group) were established by injecting 5×10^6 PC-3MM2 cells into the flanks of 6-week-old male NMRI/nude mice (Taconic, Ejby, Denmark). Mice received intratumoral injections of 3×10^8 viral particles on days 5, 6, and 7 after cell inoculation. Tumor size was measured every other day in two dimensions, and a formula (length × width^2 × 0.5) was used to calculate tumor volume. Mean volume for each group ± SE was plotted versus the day after the first viral injection. As required by animal regulations, mice were killed when the tumor size reached 1 cm in any diameter.

Intrapulmonary tumors ($n = 10$ per group) were established by injecting 1.5×10^6 PC-3MM2 cells into the left lung of NMRI/nude mice. Tumors were allowed to develop for 5 days. Mice were given four i.v. injections of 2×10^{10} viral particles on days 5, 6, 10, and 11 after cell inoculation. Plasma samples were harvested at baseline and then every 4 days beginning on day 8. On days 4, 9, 12, and 18, chest radiographs were obtained from Ad5/3Δ24hCG- and mock-treated mice using a Linac set at 45 kV, 10 mAs (Linac LLC, Albequerque, NM). Mice were monitored daily. Animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

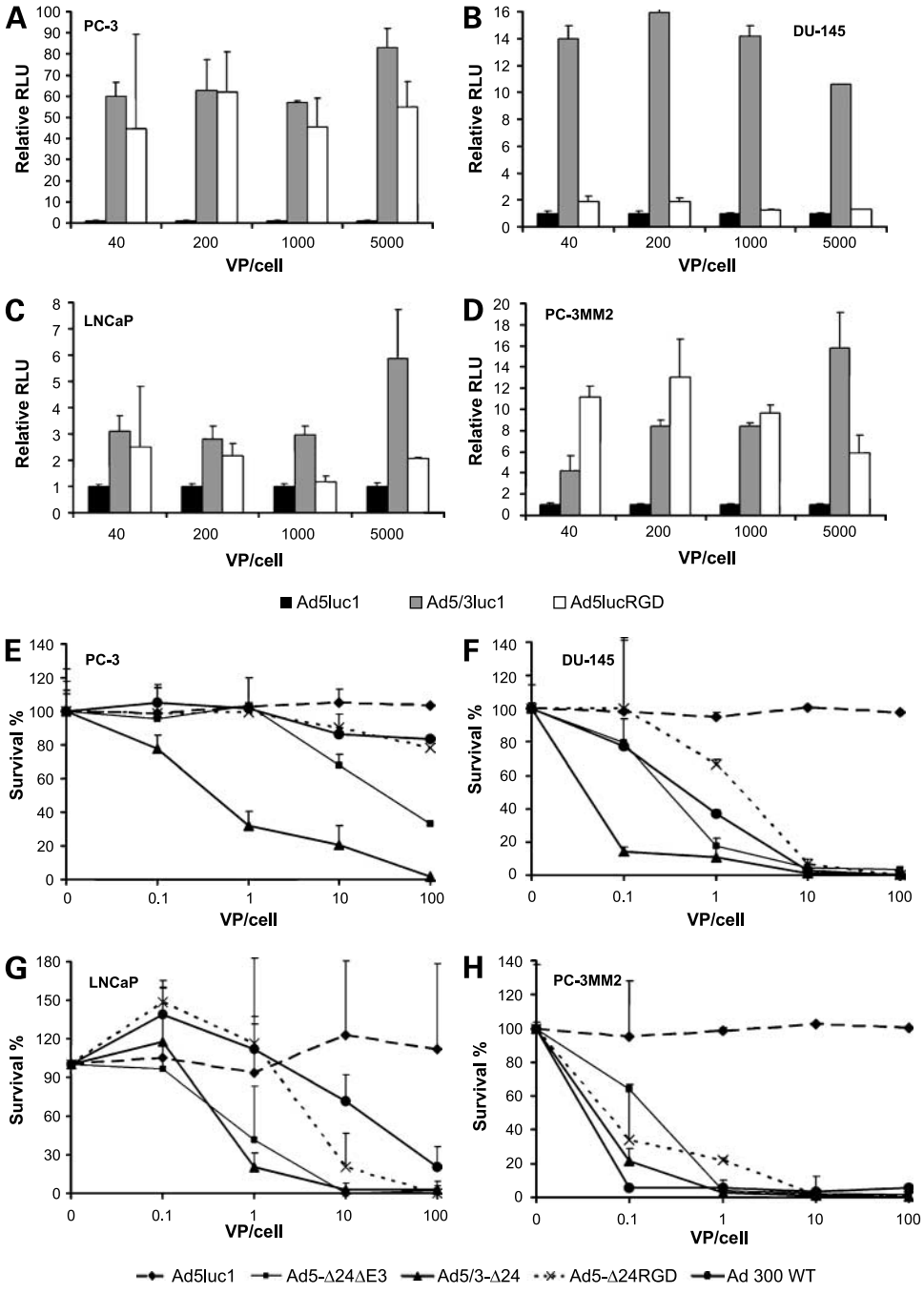

Figure 1. Transductional targeting for increasing gene transfer into prostate cancer cells. **A-D,** cell lines were infected with replication-deficient luciferase expressing Ad5luc1, and isogenic capsid-modified viruses Ad5/3luc1 and Ad5lucRGD. Ad5luc1 has a wild-type Ad5 capsid, whereas Ad5/3luc1 and Ad5lucRGD feature capsids modified with the serotype 3 knob or RGD-4C in the HI loop, respectively. Luciferase activity was measured after 24 h and is expressed as relative light units (*RLU*) normalized for total protein concentration. Data are expressed relative to Ad5luc1, which was set as 1. **E-H,** Ad5/3Δ24 and Ad5Δ24RGD are isogenic oncolytic viruses with capsids modified with the serotype 3 knob or RGD-4C in the knob HI loop, respectively. Ad5Δ24ΔE3 has a wild-type Ad5 capsid. All of these replicate selectively in tumor cells dysfunctional in the Rb/p16 pathway. Wild-type adenovirus (Ad300WT) was used as a positive control. MTS assays were used to compare the mitochondrial activity (i.e., viability) of infected cells. Effective killing by Ad5/3Δ24 identified 5/3 chimerism as a useful approach for construction of novel agents. Virus dose is expressed as particles per cell. *VP*, viral particles. *Bars,* SD.

Statistical Analysis

Mean size, SD, and SE were calculated using Microsoft Excel. For comparison of tumor size in the s.c. tumor model, we constructed a repeated-measures linear model with PROC MIXED (SAS Version 9.0, Cary, NC), which treated the within-group effect of time as a continuous variable and the treatment group as a fixed effect. This method models the changes in tumor size over time and allows a single preplanned comparison between groups to include all time points. The effects of treatment group, time, and the interaction of treatment group and time were evaluated by F tests. The differences in predicted treatment means at the study completion (day 9) were compared with an unadjusted t test. Survival analyses were conducted by plotting Kaplan-Meier curves, and the comparisons between groups were done pairwise by the log-rank procedure with SPSS 11.5 (SPSS Inc., Chicago, IL). A P value of <0.05 was considered statistically significant.

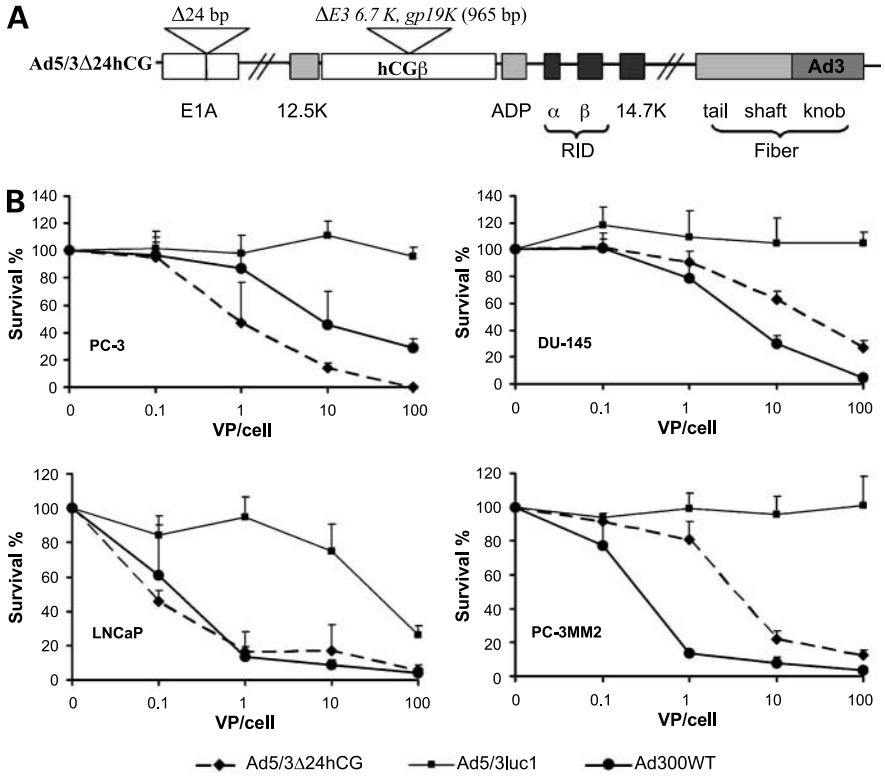

Figure 2. A, schematic illustration of Ad5/3Δ24hCG, a novel virus that has a 24-bp deletion in the constant region 2 of the adenoviral E1A gene. This defect reduces replication in quiescent normal cells but continues to allow effective oncolysis of tumor cells with a defective Rb/p16 pathway. hCGβ is expressed from the native E3 promoter, therefore allowing tight coupling of expression to virus replication. hCGβ is a secretory protein that can be quantitated as a measure of virus replication. The virus contains a chimeric fiber with the knob domain of Ad3 in the Ad5 capsid. **B,** killing of prostate cancer cells by Ad5/3Δ24hCG *in vitro*. Cells were infected with Ad5/3Δ24hCG, Ad5/3luc1 (replication-deficient control) or Ad300WT, a wild-type adenovirus (positive control). Cell viability was measured with the MTS assay. Virus dose is expressed as particles per cell. *Bars,* SD.

Results

Retargeting Adenovirus to the Serotype 3 Receptor Increases Transduction of Prostate Cancer Cells *In vitro*

Due to reported variable expression of CAR on prostate cancer cells, our first goal was to determine if capsid modification can increase transduction. Infection of hormone refractory PC-3 cells with Ad5/3luc1 and Ad5lucRGD at 5,000 viral particles per cell resulted in up to 83- and 55-fold higher luciferase expression than with Ad5luc1, the control virus with a serotype 5 capsid

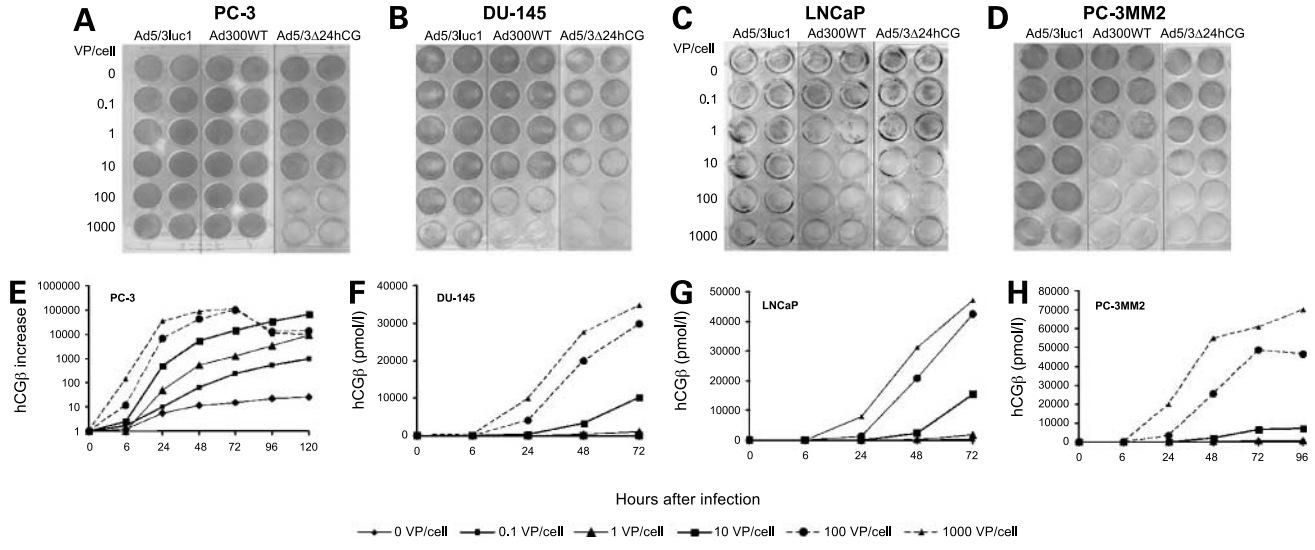

Figure 3. Oncolysis and hCGβ production mediated by Ad5/3Δ24hCG. **A-D,** cells were infected with Ad5/3Δ24hCG, Ad5/3luc1 (replication-deficient control) and Ad300WT, a wild-type adenovirus (positive control). When cell killing was nearly complete with the fastest virus within each experiment, live cells were stained with crystal violet. **E-H,** quantitation of hCGβ production was used as a measure of virus replication. During the crystal violet experiment, 30-μL samples of the growth media were collected and analyzed for hCGβ. The cumulative concentration of hCGβ was plotted as a function of time. Kinetic coupling to oncolysis and dose-dependent increase in hCGβ suggest tight association of hCGβ production with virus replication. Virus dose is expressed as particles per cell.

(Fig. 1A). With PC-3MM2 cells, Ad5/3luc1 was up to 16-fold more effective in luciferase delivery than Ad5luc1 (Fig. 1D). In addition, with DU-145 and LNCaP cells, gene delivery mediated by Ad5/3luc1 was higher than that of Ad5luc1 (Fig. 1B and C). Although Ad5lucRGD was also better than Ad5luc1 in some lines, Ad5/3luc1 was most effective overall. In cell lines with reasonable CAR expression (e.g., LNCaP), the advantage of capsid modification was less dramatic.

Interestingly, the effect of capsid modifications seemed to be most dramatic at small viral particles per cell. This suggests that nonspecific mechanisms of infection such as pinocytocis, MHCα, and HSPG-mediated delivery (common to each of the viruses) have a more important role with higher doses, whereas specific receptor-mediated delivery predominates at lower concentrations.

Ad5/3Δ24 Mediates Effective Oncolysis of Prostate Cancer Cells

To test if increases in transduction translated into an increased oncolytic effect, prostate cancer cell lines were infected with replication-competent adenoviruses with the respective capsid modifications (Fig. 1E-H). Ad5/3Δ24 displayed higher (PC-3, DU-145) or similar (LNCaP, PC-3MM2) cell killing when compared with Ad5-Δ24ΔE3 or Ad5-Δ24RGD (control viruses with an Ad5 capsid or RGD-4C in the fiber HI loop). This suggested that retargeting of oncolytic adenoviruses to the Ad3 receptor can be effective for the killing of prostate cancer cells.

Construction and Characterization of Ad5/3Δ24hCG

We constructed a novel virus containing the Ad3 knob in the Ad5 fiber shaft and a 24-bp deletion in the constant region 2 of *E1A* (Fig. 2A). To facilitate noninvasive detection of virus persistence and replication, a 498-bp cDNA encoding for hCGβ was inserted into the 6.7K/gp19K locus of an otherwise intact E3 region. The absence of wild-type E1A, the presence of the 24-bp deletion in E1A, and the presence of the Ad3 knob in the fiber were confirmed with PCR as described (29). The hCGβ insert and about 700 bp flanking it on both sides were sequenced. Virus production yielded 4.2×10^{12} viral particles/mL, and the ratio of viral particles to infectious particles was 15.

To determine the cell killing capacity of Ad5/3Δ24hCG, prostate cancer cells were infected in comparison to a wild-type (Ad300WT) and a replication-deficient adenovirus (Ad5/3luc1). Ad5/3Δ24hCG was more (PC-3) or similarly (DU-145, LNCaP) effective in cell killing in comparison to Ad300WT (Fig. 2B). With PC-3MM2 cells, Ad5/3Δ24hCG was less effective than Ad300WT at some doses. These data indicated that Ad5/3Δ24hCG is capable of mediating oncolysis of prostate cancer cells *in vitro*.

hCGβ Secretion by Ad5/3Δ24hCG during Replication and Oncolysis

In the crystal violet cell killing experiment, the efficacy of Ad5/3Δ24hCG was correlated to hCGβ production. With PC-3 cells, Ad5/3Δ24hCG exhibited a two log difference in cell killing compared with Ad5/3luc1 and Ad300WT (Fig. 3A). In DU-145 cells, the difference to Ad300WT was one log, and in LNCaP and PC-3MM2 cells, Ad300WT was more effective than Ad5/3Δ24hCG. Growth media collected from these wells showed increasing amounts of hCGβ in concurrence with oncolysis (Fig. 3E-H). The most distinct increase was seen at 10, 100, and 1,000 viral particles per cell, consistent with the cytopathic effect mediated by Ad5/3Δ24hCG. Plateau concentrations of 35,000 pmol/l in DU-145 cells and 112,000 pmol/l in PC-3 cells were reached at 72 h. With PC-3, cell death occurring at 1,000 viral particles per cell reduced the production of hCGβ. The non-hCGβ-secreting control viruses did not induce hCGβ production (data not shown), which confirms that virus replication or oncolysis per se does not result in hCGβ production. Further showing that hCGβ production is coupled to virus replication, marker peptide concentrations correlated closely with the production of new virions (Fig. 4A).

Antitumor Effect of Ad5/3Δ24hCG and Plasma hCGβ Profile *In vivo*

The antitumor efficacy of Ad5/3Δ24hCG was tested in a murine model of hormone refractory prostate cancer. Ad5/3Δ24hCG treatment resulted in statistically significant tumor growth inhibition ($P < 0.05$) when compared with the untreated group (Fig. 4B). In comparison to positive controls, similar antitumor efficacy was seen. Plasma hCGβ was analyzed on days 5 and 10 after the last viral injection. All mice treated with Ad5/3Δ24hCG had high plasma hCGβ levels at both time points (Fig. 4C), whereas mice treated with the other viruses showed only baseline levels. Interestingly, a higher hCGβ concentration was detected on day 5 than on day 10, suggesting rapid early virus replication.

hCGβ Production in Clinical Samples of Malignant and Nonmalignant Prostatic Cells

Ad5/3Δ24hCG-induced production of hCGβ was tested in fresh samples of malignant and nonmalignant prostatic cells. Infection of malignant prostate tissue with 500 and 5,000 viral particles per cell resulted in higher hCGβ production after 24 h than was observed in the nonmalignant tissue sample. The difference in hCGβ production was even more pronounced at 48 and 72 h postinfection, resulting in 21 pmol/l versus 83 pmol/l at 5,000 viral particles per cell (Fig. 4D) and 1.4 pmol/l versus 16 pmol/l at 500 viral particles per cell, $P < 0.05$ (Fig. 4E).

Antitumor Efficacy in an Orthotopic Model of Hormone Refractory Prostate Cancer Metastatic to the Lung

To examine the antitumor efficacy of Ad5/3Δ24hCG in an orthotopic model, intrapulmonary hormone refractory prostate cancer metastases were established in mice. They then received two i.v. injections of Ad5/3Δ24hCG, Ad5/3Δ24, or no virus in two cycles. Chest radiographs were

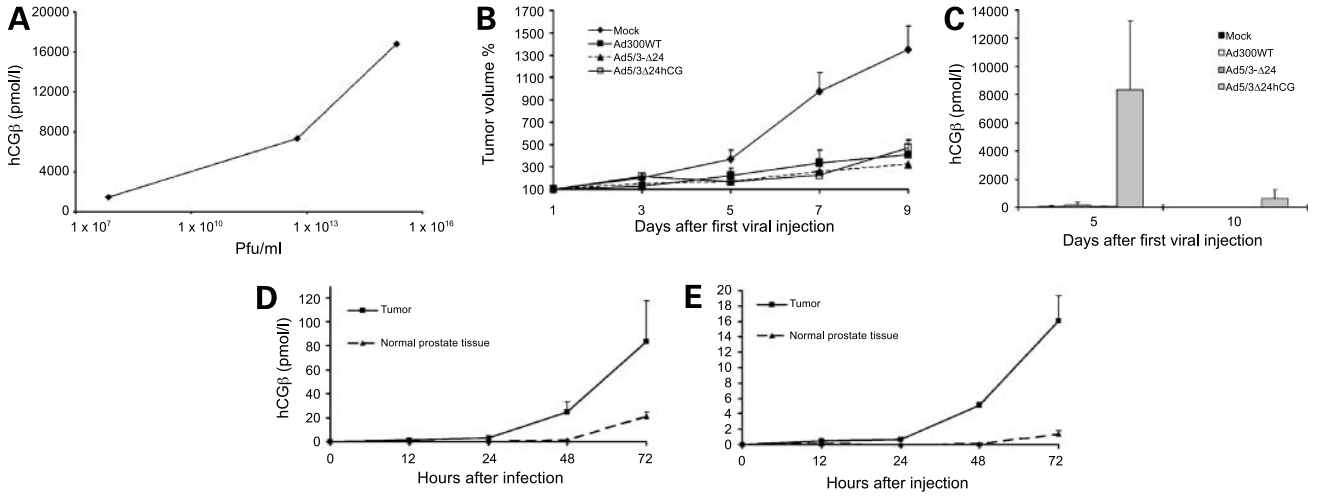

Figure 4. **A,** correlation of hCGβ production to viral replication. PC-3MM2 cells were infected with 100 viral particles per cell of Ad5/3Δ24hCG. The production of hCGβ into growth media at 24, 48, and 72 h after infection was plotted versus production of functional virions. **B** and **C,** antitumor effect of Ad5/3Δ24hCG *in vivo*. **B,** to model aggressive hormone refractory prostate cancer, tumors were established by injecting 5×10^6 PC-3MM2 cells into the flanks of male nude mice. Tumors were injected daily for 3 d with 3×10^8 viral particles of Ad5/3Δ24hCG, Ad300WT (a wild-type adenovirus), Ad5/3Δ24 (a positive control oncolytic adenovirus), or with vehicle only. Tumor size is presented relative to the initial tumor size. *Bars,* SE. Tumors treated with Ad5/3Δ24hCG were significantly smaller ($P < 0.05$) than untreated tumors. **C,** noninvasive quantitation of virus replication *in vivo* by measurement of serum hCGβ. On days 5 and 10 after the last virus injection, blood samples were collected and analyzed for hCGβ. **D** and **E,** analysis of Ad5/3Δ24hCG replication on clinical samples of benign and cancerous prostate. **D,** samples were infected with 5,000 virus particles per cell, and the cumulative amount of secreted hCGβ was measured. **E,** infection with 500 viral particles per cell. The difference in production of hCGβ between cancerous and normal prostatic tissue was statistically significant ($P < 0.05$) and suggests more effective replication of the virus in tumor tissue versus normal prostate tissue. *Bars,* SD.

taken on days 4, 9, 12, and 18 after cell inoculation to study tumor-associated congestion (Fig. 5). Congestion increased rapidly in mock-treated mice but not so in mice responding to Ad5/3Δ24hCG.

There was no increase in hCGβ from baseline levels in mice given Ad5/3Δ24 or no virus (data not shown). In contrast, all mice in the Ad5/3Δ24hCG group had increased plasma hCGβ levels after virus treatment (Fig. 6A-J). Three patterns of response were seen. Six mice responded to both virus injection cycles, seen as two increases in hCGβ production (Fig. 6A-F). Two mice had an initial response, but failed to respond a second time (Fig. 6G and H), whereas two mice did not seem to respond much (Fig. 6I and J). Despite the aggressive behavior of the model, administration of Ad5/3Δ24hCG resulted in statistically significant survival ($P = 0.002$) when compared with the untreated mice. In addition, Ad5/3Δ24 treatment (the positive control) gave a survival benefit ($P = 0.0061$) in comparison to untreated mice (Fig. 6K). Ad5/3Δ24hCG and Ad5/3Δ24 were equally effective, and no toxicity to mice was seen after virus injections. All mice eventually died because of their tumor.

Discussion

In this study, we evaluated the infectivity of prostate cancer cells with adenoviruses featuring a CAR, an αvβ-integrin, or a serotype 3 receptor-targeted capsid, and the latter was found most useful. Correlation of infectivity to oncolytic potential was confirmed with the respective CRAds (Fig. 1). Therefore, we constructed Ad5/3Δ24hCG, a novel CRAd, which replicates selectively in cells dysfunctional in the Rb/p16 pathway and effectively infects prostate cancer cells (Fig. 2A). Consequently, effective oncolysis of prostate cancer cells was seen (Figs. 2B and 3A-D). Moreover, effective hCGβ production was seen in concordance with virus replication (Figs. 3E-H and 4A). The new virus was effective in suppressing the growth of prostate cancer tumors in mice, and effective virus replication *in vivo* was confirmed by the detection of hCGβ in serum (Fig. 4B and C).

We were able to obtain fresh clinical samples of both malignant and normal prostate tissue, and higher hCGβ production was seen in the former (Fig. 4D-E), suggesting tumor-specific replication of the virus. The low-level production of hCGβ seen also in normal prostate tissue at 72 h may reflect acclimatization of the sample to *in vitro* conditions and subsequent unphysiologic cellular division (replicating cells phosphorylate Rb). Alternatively, a low level of E3 promoter activity might be present in infected cells even in the absence of virus replication.

hCGβ production in Ad5/3Δ24hCG is controlled by the native E3 promoter. This results in coupling of hCGβ production to viral replication, and hCGβ production begins about 8 h after infection (26). As hCGβ assays are routinely used for pregnancy diagnosis (30), sensitive and inexpensive tests are readily available. Both intact hCG and hCGβ

are normally secreted by placental trophoblasts during early pregnancy. Intact hCG stimulates progesterone production in the corpus luteum and maintains pregnancy. However, free hCGβ has no known biological function (31). The use of hCGβ as a marker is appealing also because it is secreted into urine (28), which facilitates noninvasive monitoring of virus replication through urine sampling, although it has been disputed how well serum and urine hCGβ correlate (32).

hCGβ levels are usually low or undetectable in men (28). However, low level expression of hCGβ has been detected in some prostate adenocarcinomas (33), but the frequency has been low (9%). In this study, hCGβ production was very low in prostate cancer cells lines (0 viral particles per cell curves in Fig. 3E-H). Therefore, the increase in hCGβ in the serum of treated mice was probably caused by virus replication. A lower level of hCGβ on day 10 in comparison to day 5 in the s.c. tumor model suggests effective initial dissemination of the virus, with subsequent killing of tumor cells. On day 10, there may not have been many tumor cells remaining, and therefore, lower hCGβ levels were seen.

An orthotopic model of aggressive, hormone refractory prostate cancer metastatic to the lung was developed. Following treatment with Ad5/3Δ24hCG, responses could be detected with chest X-ray (Fig. 5), and measurements of serum hCGβ suggested virus replication (Fig. 6). Interestingly, a second increase in hCGβ was seen in most mice, which suggested that despite theoretically continuous replication, tumors were not cured with the first cycle of injections. However, abrogation of replication was not due to resistance to the virus because a second increase in hCGβ production was seen. Instead, it seems likely that intratumoral barriers were responsible for the attenuation of the effect. Most importantly, treatment increased the survival of mice, despite the aggressive nature of the model. Further improvements in survival might be possible with the optimization of the schedule, combination with chemo- or radiation therapy, or arming the CRAd with an antitumor transgene.

These findings show that improved means for longitudinal analysis of viral kinetics and persistence can help detect and dissect *in vivo* obstacles that otherwise might be difficult to analyze. If such approaches could be tested in clinical trials, translational bench-to-bedside and back-development might be expedited. Measurements of blood or urine samples would be convenient and patient friendly.

In summary, we have developed a novel oncolytic adenovirus, which shows efficacy in preclinical models of prostate cancer. Previously, favorable results have been

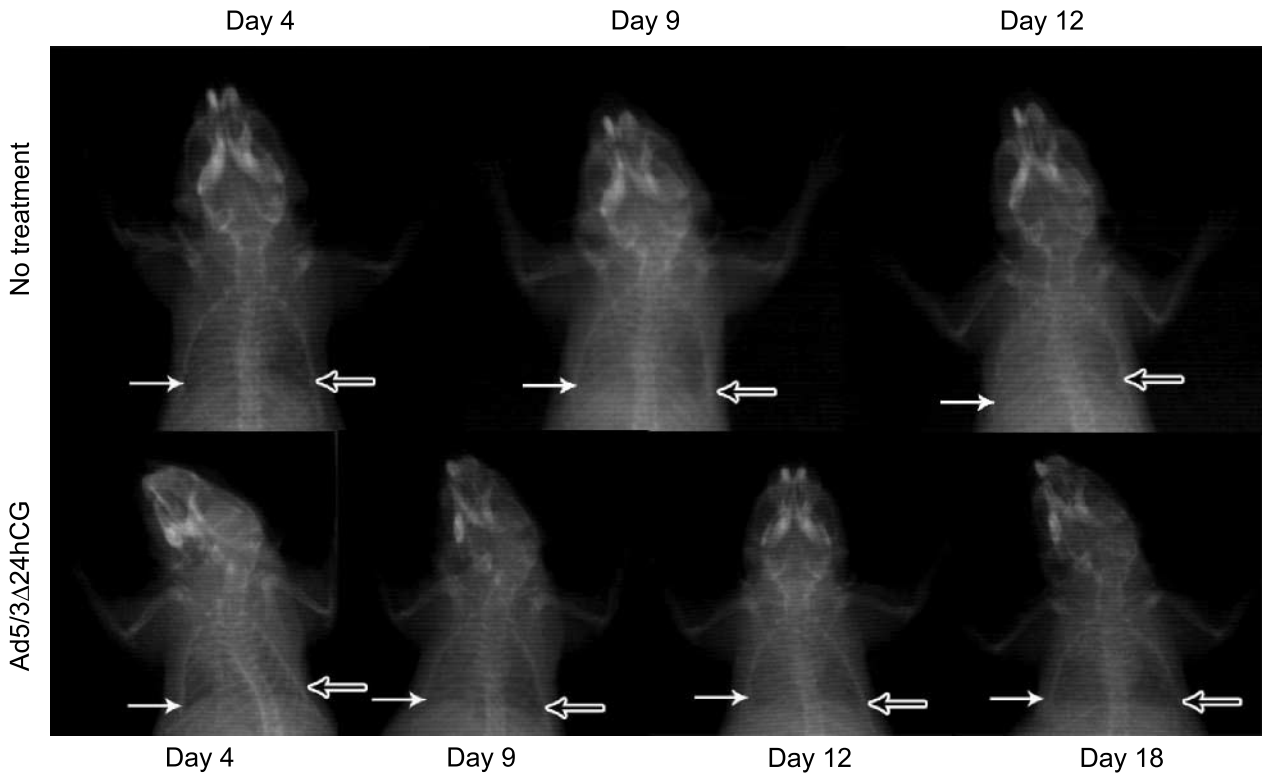

Figure 5. Chest radiographs (mouse viewed from above) of Ad5/3Δ24hCG-treated and untreated mice with lung metastatic hormone refractory prostate cancer. *Top row,* tumor progression in an untreated mouse. The left lung (*white arrows*) becomes increasingly congestive as the tumor progresses. Congestion can be seen by comparing the transparency of the tumor-bearing left lung to the right lung without tumor (*black arrows*). *Bottom row,* congestion in the left lung reduces following tumor response and mouse (Fig. 6A) was still alive on day 18.

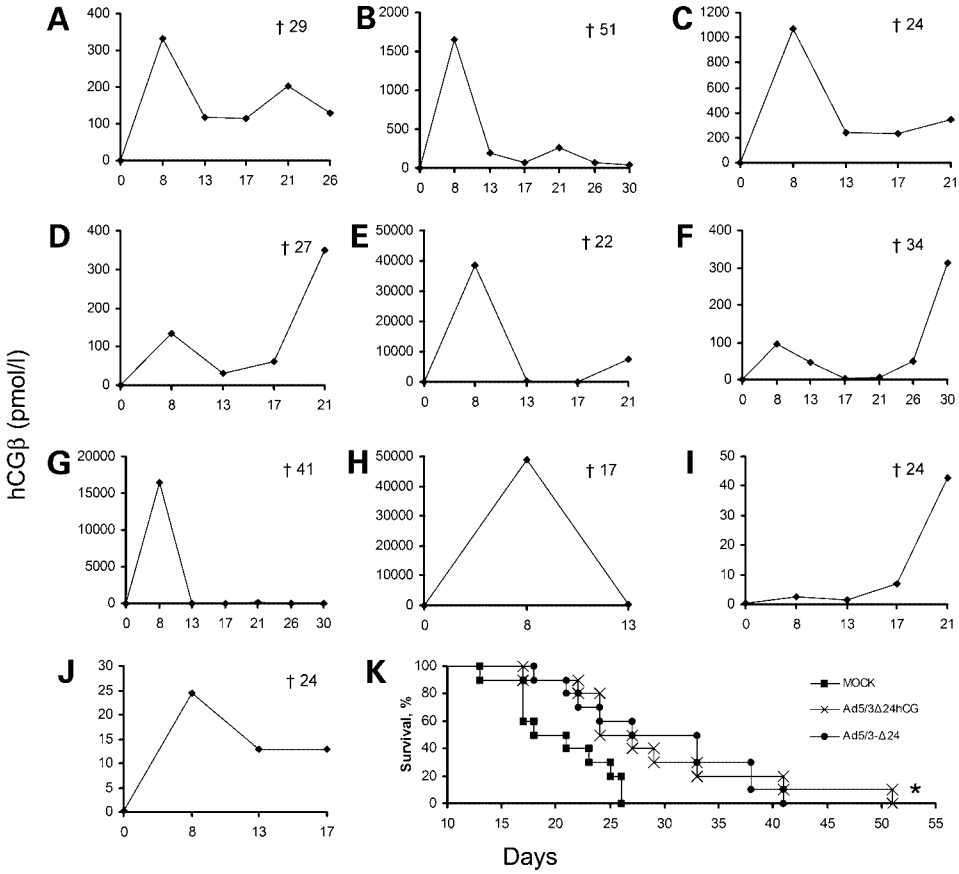

Figure 6. Systemic antitumor efficacy in an orthotopic model of aggressive lung metastatic hormone refractory prostate cancer. **A-J,** hCGβ production *in vivo* after i.v. injection of viruses on days 5, 6, 10, and 11. All Ad5/3Δ24hCG-injected mice are shown, and three patterns of response were seen. **A-F,** six mice responded to both virus injection cycles, seen as two increases in hCGβ production. **G** and **H,** two mice had an initial response, but failed to respond a second time. **I** and **J,** low or no response, seen as low production of hCGβ. †, time of death (days post-cell-inoculation). **K,** survival of mice treated with Ad5/3Δ24hCG, Ad5/3Δ24 (positive control), or no virus. Survival of Ad5/3Δ24hCG-treated mice was significantly longer than those of mock treated ($P < 0.002$).

obtained in preliminary toxicity and biodistribution data using related agents with identical capsids (34), which facilitates formal studies in preparation for putative clinical trials.

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