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ARTICLE

Noninvasive Imaging of Enhanced Prostate-Specific Gene Expression Using a Two-Step Transcriptional Amplification-Based Lentivirus Vector

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Noninvasive evaluation of gene transfer to specific cells or tissues will allow for long-term, repetitive monitoring of transgene expression. Tissue-specific promoters that restrict the expression of a transgene to tumor cells play a vital role in cancer gene therapy imaging. In this study, we have developed a third-generation HIV-1-based lentivirus vector carrying a prostate-specific promoter to monitor the long-term, sustained expression of the firefly luciferase (*fl*) reporter gene in living mice. The *fl* gene in the transcriptionally targeted vector is driven by an enhanced prostate-specific antigen promoter in a two-step transcriptional amplification (TSTA) system. The efficiency of the lentivirus (LV-TSTA)-mediated gene delivery, cell-type specificity, and persistence of gene expression were evaluated in cell culture and in living mice carrying prostate tumor xenografts. *In vivo* bioluminescence imaging with a cooled charge-coupled device camera revealed significantly high levels of *fl* expression in prostate tumors. Injection of LV-TSTA directly into the prostate of male nude mice revealed efficient and long-term *fl* gene expression in the prostate tissue for up to 3 months. These studies demonstrate the significant potential of TSTA-based lentivirus vectors to confer high levels of tissue-specific gene expression from a weak promoter, while preserving cell-type specificity and the ability to image noninvasively the sustained, long-term expression of reporter genes in living animals.

Key Words: lentivirus vector, prostate-specific expression, bioluminescence imaging, two-step transcriptional amplification, reporter gene expression

INTRODUCTION

The success of gene therapy protocols in clinical trials largely depends on efficient gene transfer to target cells. A wide variety of viral and nonviral vectors have been developed and studied for efficiency of transduction and sustained expression of the delivered transgene. Viral vectors are useful vehicles for the delivery of foreign genes into target cells and retroviral vectors are popular due to their ability to integrate into the host cell genome. Retroviral vectors are attractive tools for human gene therapy applications and are largely used in clinical trials for gene therapy [1]. However, a major limitation is their inability to transduce nondividing cells. Among retroviral vectors, lentiviruses have the unique ability to transduce quiescent cells. They have been used for delivery, integration, and expression of transgenes into different cell types, such as myocytes and neurons [2,3]. Also, when pseudotyped with the envelope glycoprotein from vesicular stomatitis virus glycoprotein (*VSV*-G), they can be concentrated to high titers [4,5]. Lentiviruses are highly efficient gene transfer vectors for *in vivo* delivery to achieve stable, long-term expression of transgenes in the target tissue. Much emphasis has been devoted to generating viruses that demonstrate high transduction efficiency and are safe to use. However, due to the pathogenic nature of

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HIV, lentiviruses have continued to raise concerns about biosafety. Therefore, modifications have been under way to refine these vectors by preventing the formation of replication-competent recombinant virus [6].

One of the modifications involves the generation of self-inactivating (SIN) HIV vectors. Self-inactivation is based on introducing a deletion into the U3 region of the 3′ LTR of the DNA used to produce the vector RNA. During reverse transcription, this deletion is transferred to the 5′ LTR of the proviral DNA [7]. The development of SIN lentiviral vectors has addressed the safety concerns to a significant point and has provided the necessary impetus to drive these vectors forward toward clinical use [8–10]. Further improvements in transduction efficiency and transgene expression have been achieved by incorporating a central polypurine tract (cPPT) and a posttranscriptional regulatory element (PRE) into lentivirus vectors [11]. Incorporation of these elements leads to higher transduction efficiency and increased transgene expression and allows for reduced levels of virus administration *in vivo* combined with a higher safety profile.

The use of lentivirus-based vectors *in vivo* is especially significant for tumor-specific targeting. Targeting transgene expression to a specific cell/tissue type presents a considerable impediment for gene therapy researchers. Promoters such as cytomegalovirus (CMV), which are known to be active in a wide variety of cell types, are of limited use. Gene therapy protocols will greatly benefit from the use of tissue-specific promoters. However, many of the known tissue-specific promoters are poor activators of transcription. We have been exploring ways to achieve high levels of transcriptional activity from weak promoters, while maintaining cell-type specificity using a two-step transcriptional amplification strategy (TSTA). Using this strategy, we have previously demonstrated that firefly luciferase (*fl*) gene expression driven by an enhanced PSA promoter can be noninvasively imaged in living mice using a cooled charge-coupled device (CCD) camera and D-Luciferin [12]. We further optimized the TSTA system by varying the number of VP16 activation domains and Gal4 binding sites [13]. We inserted the TSTA system into an adenovirus vector, which was used to evaluate the role of androgen receptor in prostate cancer progression [14]. The TSTA system has thus emerged as a very powerful tool to amplify target gene expression using a relatively weak, tissue-specific promoter.

In this study, we report the development of a TSTA-based lentivirus vector (LV) to monitor noninvasively the prostate-specific expression of *fl* reporter gene in living mice. Transduction efficiency and tissue specificity of LV-TSTA were evaluated in cell culture and in a tumor xenograft model. Localized transgene expression in the prostate was monitored over a period of a few months by noninvasive, repetitive imaging of living mice using bioluminescence imaging.

RESULTS

Construction of the LV-TSTA System

We employed a modified chimeric prostate-specific promoter (PSEBC) to drive the expression of a GAL4-VP2 fusion protein [13,15]. The GAL4-VP2 in turn binds to the five Gal4 binding sites placed upstream of a TATA minimal promoter driving the *fl* reporter gene in a single vector (Fig. 1A). *Note that GAL4 refers to the transactivator and Gal4 refers to the binding sites upstream of the reporter gene*. This transcriptional amplification strategy results in significant amplification of *fl* reporter gene expression from a relatively weak promoter. A single fragment comprising the two expression cassettes was cloned into the third-generation HIV-1-based SIN lentivirus vector (Fig. 1B). We first assessed the ability of LV-TSTA to mediate *fl* expression in cell culture in the absence and presence of androgen. Next, we studied the tumor-specific *fl* expres-

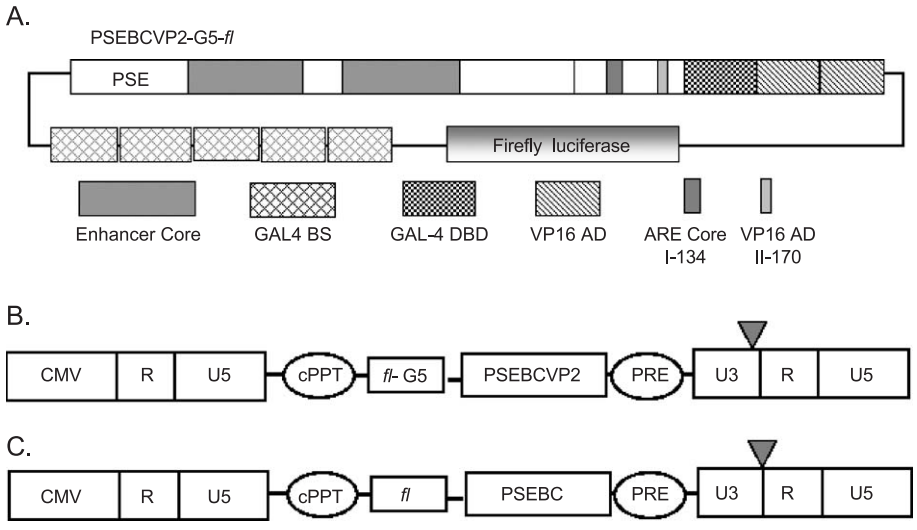

FIG. 1. Schematic illustration of the single vector carrying the TSTA system. (A) The prostate-specific promoter is placed upstream of the GAL4-VP16 fusion protein carrying two VP16 activation domains. The reporter component of the system comprises five Gal4 binding sites positioned upstream of the adenovirus E4 TATA minimal promoter driving the *fl* reporter gene. (B, C) The expression cassettes carrying the TSTA and the one-step system were each inserted into an HIV-1-based third-generation SIN lentivirus vector.

sion in prostate cancer xenografts of male SCID mice using a cooled CCD camera. A plasmid carrying the one-step system (PSEBC-*fl,* abbreviated as PBC-*fl*) was also cloned into the lentivirus vector and used in tumor xenograft experiments (Fig. 1C). Further, we evaluated the ability of LV-TSTA to induce and sustain long-term *fl* expression in the prostate of living mice.

A Lentivirus Vector Carrying the TSTA System Demonstrates a Six-to Sevenfold Induction of FL Activity in Prostate Cancer Cells in the Presence of Androgen

To determine the levels of *fl* gene expression driven by the TSTA-based lentivirus vector, we transduced LNCaP cells with LV-TSTA in the absence and presence of androgen. Forty-eight hours following transduction, we observed FL activity in the cell lysates containing androgen to be six-to sevenfold greater than that observed in the absence of androgen ($P < 0.05$) (Fig. 2A; note that *fl* refers to the gene and FL to the enzyme). The activity increases with time up to 72 h. To confirm further that the augmented PSA promoter-mediated FL activity is restricted to prostate cancer cells, four nonprostate cell lines were transduced with LV-TSTA in the presence of androgen. All four cell lines show FL activity that is at least three orders of magnitude lower than the activity in LNCaP cells (Fig. 2B). The TSTA-based lentivirus vector thus achieves high, cell-type-specific expression of the *fl* gene in the presence of androgen.

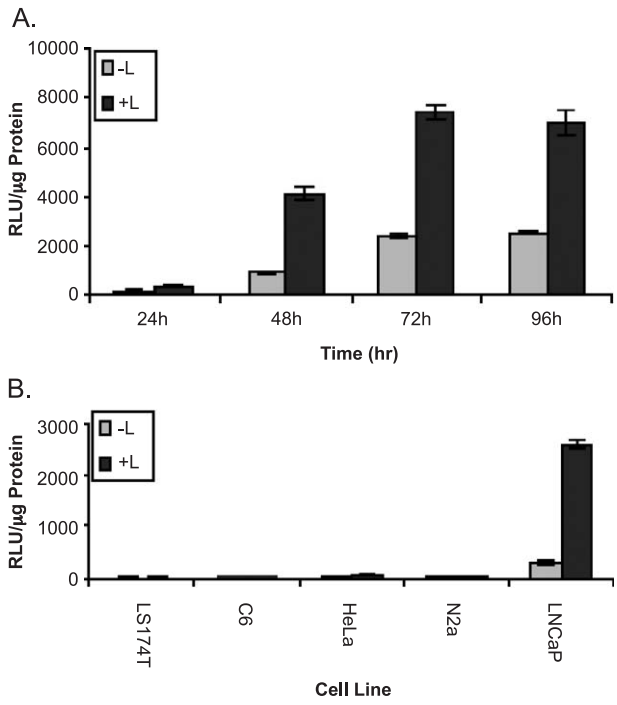

FIG. 2. LV-TSTA-mediated FL enzyme activity in cell culture. (A) LNCaP cells were transiently transduced with LV-TSTA in the absence and presence of synthetic androgen. At the indicated time points, the cells were harvested and assayed for FL enzyme activity using a luminometer. The FL activity is represented as relative light units (RLU) normalized to the micrograms of protein. The error bars represent the standard errors for triplicate measurements. (B) For cell-type specificity experiments, N2a, C6, LS174T, and 293 cells were transduced with LV-TSTA and the cells were harvested after 48 h and assayed for FL enzyme activity.

LV-TSTA-Mediated *fl* Reporter Gene Expression Is Significantly Greater Than *fl* Expression Driven by LV-PBC Vector

To demonstrate the efficacy of the LV-TSTA imaging system, we compared the *fl* reporter gene expression levels driven by LV-TSTA and LV-PBC vectors in male SCID mice carrying LAPC-9 tumor xenografts. The LV-PBC lentivirus contains the augmented PSA promoter, PBC, driving the *fl* gene directly. The LV-TSTA system, on the other hand, contains PBC driving GAL4VP2, which in turn activates the *fl* gene. We injected equal titers of the two viruses (as measured by p24 ELISA) into separate groups of mice. Optical CCD camera imaging of mice injected with LV-TSTA on days 4, 7, 11, and 14 revealed elevated levels of *fl* gene expression in the tumor (5×10^6 photons (p)/s/cm^2/sr on day 14, Fig. 3). The intensity of the bioluminescence signal in the tumor was 125-fold greater at all time points than the corresponding signal intensity using LV-PBC vector (4×10^4 p/s/cm^2/sr on day 14). These results illustrate the strong capability of the TSTA system to amplify the reporter gene expression from the prostate-specific promoter.

LV-TSTA System-Mediated *fl* Gene Expression Is Restricted to Prostate Tumor Cells *in Vivo*

To determine whether the *fl* gene expression driven by the prostate-specific promoter is restricted to prostate cells *in vivo,* we established androgen-dependent LAPC-9 tumor xenografts in male SCID mice. We subsequently injected the tumors with LV-TSTA and imaged the mice every 3 to 4 days until day 18 (Fig. 4A). We detected *fl* gene expression in the tumor from day 3 and observed it to persist strongly until day 18 (bioluminescence signal ranged from 4.9×10^4 to 5.9×10^5 p/s/cm^2/sr). In a second set of experiments, we studied the androgen responsiveness of the tumor by castrating the mice on day 7 following virus injection and subsequently imaging the mice on days 7 and 11 after castration. We observed a significant drop in the bioluminescence signal following castration ($P < 0.05$) and the decrease was maintained until day 11 after castration, indicating the high androgen-responsive nature of the tumor (the decrease in bioluminescence intensity was 3-fold on day 7 and 10-fold on day 11 following castration, Fig. 4B). We measured serum PSA levels in the mice before and after castration and they were in conjunction with the observed bioluminescence signal (Fig. 4C). LAPC-9 tumors do not regress following castration. The decrease in *fl*

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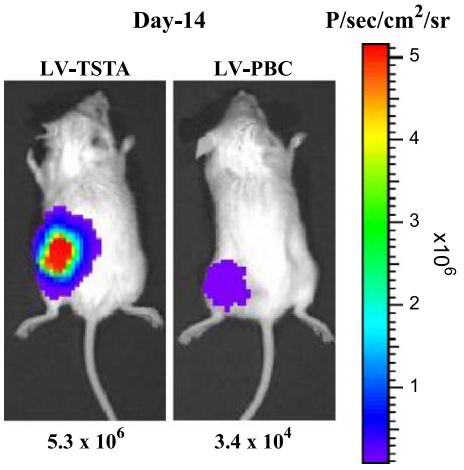

FIG. 3. Comparison of *fl* reporter gene expression using lentivirus carrying the one-step and two-step constructs. Six-week-old male SCID mice were implanted with LAPC-9 tumors in the bottom left flank. When the tumor size reached 0.6 mm in diameter, equal titers of LV-PBC and LV-TSTA were injected directly into the tumor sites in two separate mice. The mice were imaged using the CCD camera on days 4, 7, 10, and 14 following virus injection. The *fl* gene expression is observed to be significantly greater in LV-TSTA mice than in the LV-PBC mice on all days (5×10^6 p/s/cm^2/sr vs 4×10^4 p/s/cm^2/sr on day 14, respectively). Three mice were studied in each category.

expression observed using the LV-TSTA system is the result of androgen deprivation. In other studies using an adenovirus vector carrying the TSTA system (Ad-TSTA-*fl*), we have observed *fl* gene expression to be significantly reduced following castration (25-fold). In contrast, *fl* gene expression in mice injected with an adenovirus carrying the CMV promoter (Ad-CMV-*fl*) remained persistent before and after castration (unpublished results). Following the 18-day scan, we sacrificed the animals, harvested the organs, and assayed them for FL activity. FL activity was seen predominantly in the tumor, with other organs, including the prostate, displaying basal levels of expression ($P < 0.01$, data not shown). We demonstrated the cell-type specificity of LV-TSTA *in vivo* using a nonprostate tumor xenograft model. We injected mice carrying C6 tumors with LV-TSTA in the tumor and imaged them on days 7, 8, and 14. All mice showed background levels of *fl* expression indicative of the highly cell-type-specific nature of LV-TSTA (data not shown).

Direct Delivery of LV-TSTA into the Prostate of Male Nude Mice Results in Sustained, Long-Term Expression of *fl* Reporter Gene

We next investigated the ability of LV-TSTA to deliver and regulate long-term expression of *fl* gene *in vivo*. We delivered LV-TSTA directly to the prostate by intraprostatic injection. CCD camera imaging repeated over a period of several weeks showed a significantly high level of *fl* expression, primarily in the prostate, that showed persistence with time (2×10^5 p/s/cm^2/sr, Fig. 5A). Analysis of FL activity in various organs at different times revealed that gene expression was confined mainly to the prostate tissue with all other organs showing background levels of expression (Fig. 5B). Three mice from each experimental group underwent surgical castration and were subsequently reimaged on days 3 and 7 following the procedure. The bioluminescence signal following castration showed a considerable drop as expected, indicative of the high androgen dependency of the prostate-specific promoter (data not shown). To determine the biodistribution of LV-TSTA vector following systemic administration, we harvested different organs of mice injected 2 weeks previously with the lentivirus vector and assayed them for FL activity. The highest FL activity was found in the spleen followed by liver. All other organs showed basal levels of FL activity (data not shown).

DISCUSSION

The success of gene therapy approaches lies in the development of tools for efficient gene transfer, prolonged expression, and monitoring of gene expression. Major challenges facing the gene therapy community include the availability of specific tumor targeting approaches and sensitive imaging modalities to monitor the sustained expression following gene delivery. Therefore, the use of tissue-specific promoters for targeting tumor cells *in vivo* is highly warranted. We have earlier demonstrated that the transcriptional activity of a weak prostate-specific antigen promoter can be significantly enhanced using the TSTA system [12]. We further optimized the promoter activity by duplicating the enhancer core and increasing the number of VP16 activation domains [13]. We also successfully cloned the effector and reporter constructs into a single vector, which resulted in significant enhancement in the transcriptional activity of the weak, prostate-specific antigen promoter while maintaining tissue specificity. Recently, we have used an adenovirus-based TSTA system to report on the role of androgen receptor signaling in cancer progression from androgen-dependent to -independent phases [14] and further described the characterization of adenovirus vectors for imaging and therapy [16].

In this study, we report the *in vivo* bioluminescence imaging of *fl* reporter gene expression using a transcriptionally targeted lentivirus vector carrying the TSTA system. A fragment carrying the two transcriptional units was cloned into a third-generation HIV-based SIN lentivector containing a posttranscriptional regulatory element and a central polypurine tract. This novel vector contains an enhanced prostate-specific promoter linked to the GAL4-VP16 fusion protein and five Gal4 binding sites positioned upstream of the *fl* reporter gene. Using this vector, we have demonstrated the ability to (i) transduce LNCaP cells efficiently, while maintaining

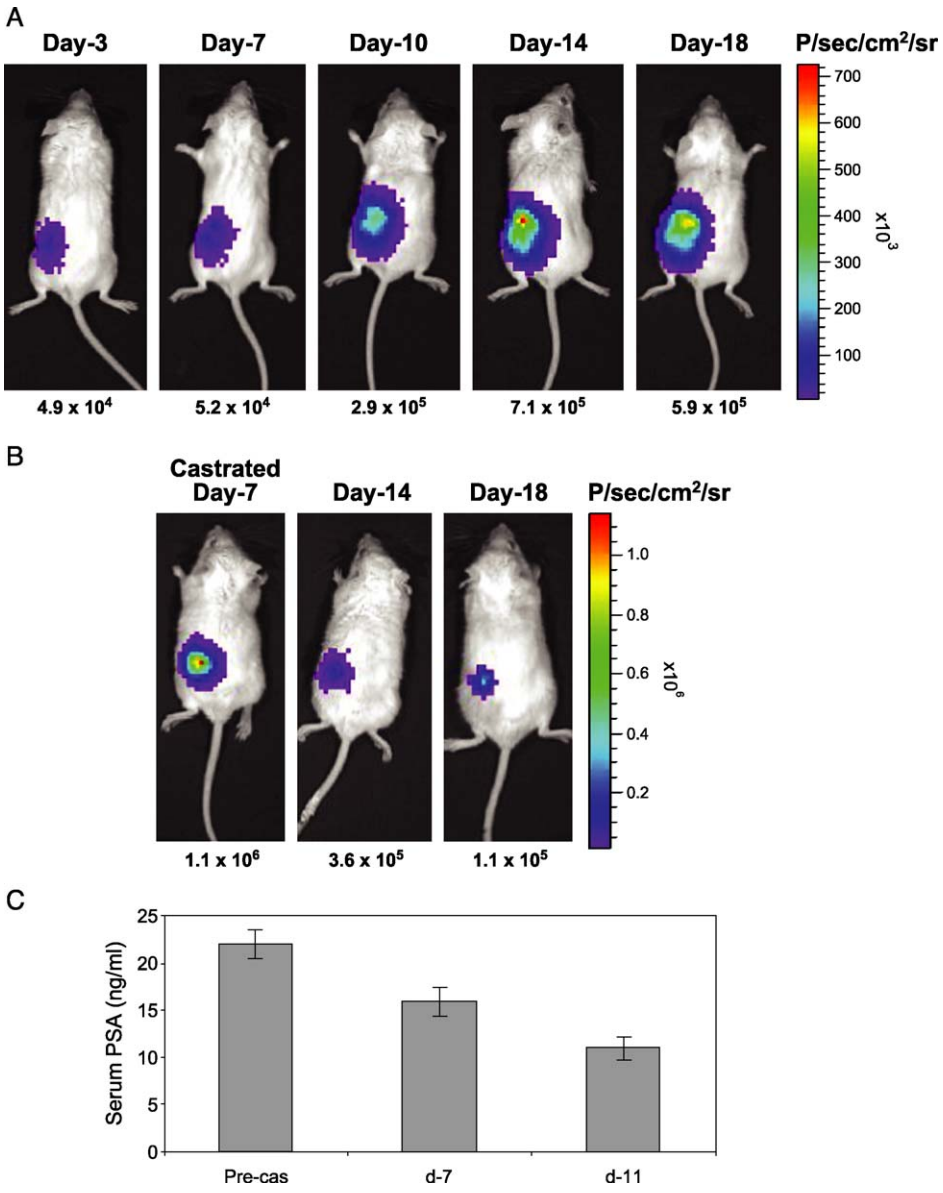

FIG. 4. LV-TSTA-mediated tumor-specific expression of *fl* reporter gene in male SCID mice. (A) Six-week-old male SCID mice were implanted with LAPC-9 tumor xenografts in the bottom left flank. When the tumor size reached 0.5–0.6 cm in diameter, LV-TSTA (50 μl) was injected directly into the tumor. The mice were imaged using the optical CCD camera on days 3, 7, 10, 14, and 18 using D-Luciferin as the substrate (150 mg/kg). A significantly high level of *fl* gene expression was observed in the tumor, which continued to persist till day 18. Analysis of *ex vivo* FL enzyme activity in different organs following imaging showed that the activity was restricted to the tumor, with all other organs showing basal levels of FL activity (data not shown). (B) Effects of androgen withdrawal on *fl* gene expression. Three mice in each experimental group underwent surgical castration on day 7 and were subsequently imaged on days 7 and 11 following the procedure. Androgen withdrawal resulted in a considerable decline in the bioluminescence signal indicative of the high androgen dependency of the prostate-specific promoter. (C) Serum PSA measurements in castrated LAPC-9 tumor mice. The values are in ng/ml and error bars indicate the standard errors for triplicate measurements.

cell-type specificity, (ii) mediate tissue-specific gene expression efficiently in living mice, and (iii) monitor noninvasively the sustained, long-term expression of the reporter gene in living animals.

The utility of LV-TSTA was first demonstrated in cell culture, in which LNCaP cells showed a six- to eightfold induction of FL activity in the presence of androgen. The LV-TSTA system also maintained a high degree of cell-type specificity as observed by the minimal levels of FL activity in four nonprostate cell lines. To demonstrate the high potency of the TSTA system to amplify reporter gene expression *in vivo*, we compared *fl* gene expression in LAPC-9 tumor-bearing mice following administration of LV-TSTA and LV-PBC lentivirus directly into the tumors. In the LV-PBC vector, the expression of the *fl* gene is directly under the control of the enhanced PSE promoter in a one-step system. The bioluminescence signal observed using LV-PBC was over 100-fold greater than the signal observed using the TSTA system. The tissue specificity of the LV-TSTA-mediated *fl* expression was further confirmed *in vivo* by optical imaging of mice carrying LAPC-9 tumor xenografts. Noninvasive imaging of SCID mice injected with LV-TSTA directly into the tumor showed *fl* gene expression primarily in the tumor while all other organs showed basal levels of gene expression. The androgen dependency of the prostate-specific promoter was confirmed by visualizing decreasing *fl* signal following androgen withdrawal by surgical castration.

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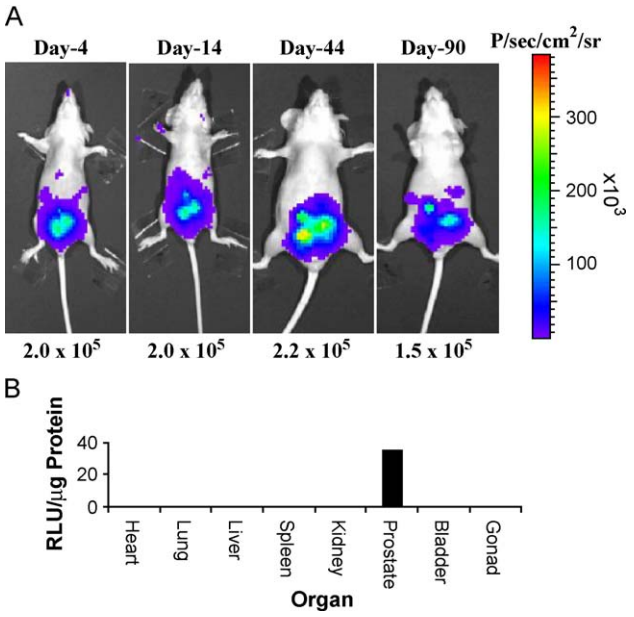

FIG. 5. *In vivo* optical imaging of mice following direct intraprostatic delivery of LV-TSTA. (A) Six-week-old male nude mice were injected with LV-TSTA in the dorsal lobe of the prostate (20 μl in each lobe, see Materials and Methods). The mice were imaged using the CCD camera 2–4 days after virus injection. Subsequent imaging was performed every 3–4 days until day 21 and then once a week until day 90. By day 4, a high level of bioluminescence signal was observed in the prostate (2.2×10^5 p/s/cm^2/sr). The *fl* expression showed strong persistence with time. (B) Analysis of FL activity *ex vivo* following CCD imaging. The mice were sacrificed at different time points after virus injection and the organs harvested and assayed for FL activity. Shown here are values from a mouse on day 80 following virus injection, indicating *fl* expression primarily in the prostate, with basal levels of expression in all other organs.

Mice injected with LV-TSTA in the tumor did not display any signal in the prostate even at late time periods. The lack of signal in the prostate does not necessarily imply that the virus is not leaky. The absence of bioluminescence signal may be due to insufficient amounts of virus leaking out of the tumor and reaching the prostate. The high levels of cell-type-specific amplification attained by the TSTA system thus allow for repetitive, quantitative imaging of gene expression using *in vivo* bioluminescence imaging.

We further demonstrated the utility of LV-TSTA to monitor long-term reporter gene expression in the tissue of interest by delivering the lentivirus directly into the prostate of living mice. Bioluminescence imaging of male nude mice following intraprostatic injection resulted in sustained, long-term expression of the *fl* gene in the prostate, for as long as 3 months. FL enzyme activity assays during and at the end of the imaging period indicated the gene expression to be confined mainly to the prostate, with other organs showing basal levels of reporter gene expression. The *in vivo* bioluminescence signal was down regulated by blocking androgen availability, indicative of androgen dependency of the prostate-specific promoter. Studies to evaluate the effects of androgen supplementation following castration are currently under way and will constitute a part of a subsequent report. In a parallel study, we have recently reported the development and imaging of a transgenic mouse model carrying the *fl* reporter gene in a TSTA system [17]. Using this model, we have shown that the enhanced prostate-specific promoter mediates strong androgen-dependent *fl* expression primarily in the dorsolateral and ventral lobes of the prostate, with minimal levels of expression in the heart. The other accessory sex tissues such as the coagulating gland and the seminal vesicles show basal levels of *fl* expression. The LV-TSTA system thus demonstrates excellent tissue specificity both in the xenograft model and in the intraprostatic delivery model. Optical imaging following systemic administration of LV-TSTA did not lead to any detectable signal in the prostate. However, *ex vivo* analysis of FL activity after 2 weeks showed activity predominantly in the spleen, with low levels of expression in the liver. These results are consistent with previous reports suggestive of the presence of an extensive network of vascular sinuses lined by macrophage-like cells in the spleen and liver that are thought to be responsible for preferential uptake of the vector [18]. The absence of signal in the prostate may be attributed to the viral dose, which may well be below the lower limit of detection in the CCD camera. Significantly higher doses may be warranted to obtain a detectable bioluminescence signal in the prostate tissue. Alternatively, radiolabeling the viral particles with ^{99m}Tc or ^{124}I will allow the monitoring of virus biodistribution *in vivo* following systemic injection. Recently, we have described the noninvasive imaging of ^{99m}Tc-adenovirus-mediated reporter gene expression in living subjects [19].

Earlier work in the field of prostate-specific targeting involved the use of adenoviral vectors [20,21]. High levels of gene expression were achieved, but the tissue specificity was considerably decreased. Increasing the viral dose would be detrimental due to the toxicity associated with higher doses. Therefore, the design of an ideal vector for tumor-specific targeting should aim to address cell-type specificity and the ability to achieve prolonged expression. With newer and safer lentiviruses being developed for use in gene therapy, lentivirus vectors appear to be most suitable for the delivery and sustained expression of transgenes *in vivo*. We have recently reported the noninvasive imaging of lentivirus-mediated reporter gene expression in living mice [22]. The study involved the monitoring of both optical and PET reporter gene expression driven by a CMV promoter. Major limitations of using a constitutive promoter (e.g., CMV) include the inability to control gene expression and potential toxicity. Therefore, transcriptional targeting using lentivirus vectors offers a significant advantage in this regard. The use of targeted lentivirus vectors combined with bioluminescence imaging offers the feasibility of monitoring

efficient, long-term, tissue-specific, and sustained gene expression in living subjects. Yu *et al.* have reported prostate-specific targeting using lentivirus vectors, but their transducing plasmid contains the prostate-specific promoter placed directly upstream of an enhanced green fluorescent protein [23]. The present study is the first to demonstrate the use of a TSTA-based lentivirus vector for noninvasive monitoring of reporter gene expression. We have demonstrated previously that *fl* gene expression driven by the TSTA system is significantly greater than the one-step system (PBC) [12,13]. This clearly illustrates the strong amplifying ability of the GAL4-VP16-carrying TSTA system, which can be used with any weak promoter to achieve robust levels of amplification of the gene of interest without compromising tissue specificity.

In conclusion, we have demonstrated the ability to achieve transcriptional targeting using a novel prostate-specific, TSTA-based lentivirus vector. This system combines the advantages of using significantly improved and safer lentivirus vectors with a powerful amplification strategy to noninvasively image prostate-specific reporter gene expression in living animals. In addition to substituting the promoter with other weak promoters, the reporter gene cassette can also be modified to include multiple reporter genes or a reporter gene and a therapeutic gene, leading to multimodality imaging approaches for long-term, tissue-specific, noninvasive monitoring of gene expression [24]. Such a vector system should be well suited to the noninvasive evaluation of gene expression and disease progression and to predict the success of therapeutic intervention in prostate cancer gene therapy.

Materials and Methods

Cell lines and culture conditions. The human prostate cancer cell line LNCaP was grown in RPMI 1640, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 1 mg/ml streptomycin under 5% carbon dioxide atmosphere. N2a (rat neuroblastoma) cells were grown in Dulbecco's minimum essential medium (D-MEM) with 10% fetal bovine serum and 100 units/ml penicillin. C6 (rat glioma) and 293T cells (human kidney) were cultured in minimal essential medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. LS174T (colon carcinoma) cells were cultured in D-MEM/Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 200 mM L-glutamine.

Construction of lentivirus. The third-generation self-inactivating lentivirus vector, pTK134, was a kind gift from T. Kafri (University of North Carolina, Chapel Hill). The fragment PSEBCVP2-G5-*fl* was excised from the pGL3 backbone of the single vector with *Sal*I and cloned into pTK134 between the *Sal*I and the *Xho*I restriction sites. Several clones with the correct orientation were tested for their FL activity and the fragment with the highest activity was then used to generate the lentivirus.

Production and concentration of lentivirus. Twenty-four hours prior to transfection, 293T cells were seeded 1:6 in 10-cm^2 dishes. The cells were transfected using a modified calcium phosphate transfection protocol [7]. The following amounts of DNA were used per transfection: 6 μg SIN vector, 6 μg ΔNRF, and 3 μg VSV-G envelope plasmid. Twelve hours after transfection, the medium was removed, the cells were washed with PBS, and fresh medium was added. Conditioned medium was collected at 24 h. The virus was concentrated by centrifugation with a 0.45-μm filter (Whatman, Clifton, NJ, USA) at 20,000 rpm for 2 h at 4°C. The pelleted virus was resuspended in 600 μl and was stored frozen in aliquots at −80°C. For experiments involving direct gene delivery into the prostate, the virus was further concentrated to 200 μl and stored in frozen aliquots at −80°C. The viral p24 antigen concentration was determined by using a Coulter HIV-1 p24 antigen assay. The number of transducing units per milliliter of supernatant was determined by limiting dilution analysis in LNCaP cells. The cells were transduced by adding serial dilutions of LV-TSTA in a 48-well plate in the presence of Polybrene (8 μg/ml). Transduced cells were harvested and assayed for FL activity in a luminometer. The titers of LV-TSTA and LV-PBC were determined to be 1×10^5 infectious units (IU)/500 ng HIV-1 p24 and 1×10^3 IU/485 ng HIV-1 p24, respectively. Vector particles were concentrated 200-fold by ultracentrifugation and stored at −80°C. The infectious units for LV-TSTA ranged from 2×10^7 to 4.5×10^7 IU/ml and LV-PBC ranged from 2×10^5 to 3×10^5 IU/ml.

Transduction of LNCaP cells with LV-TSTA. Twenty-four hours prior to transduction, LNCaP cells were plated in six-well dishes in RPMI containing charcoal-stripped serum. Transient transductions with LV-TSTA were performed in the absence and presence of synthetic androgen (Methyltrienolone, R1881, 1 nM). At 24, 48, 72, and 96 h following transduction, the medium was removed and the cells were washed with PBS. The cells were lysed in Passive Lysis Buffer (1×; Promega, Madison, WI, USA) and harvested. FL activity in the cell lysates was measured using a luminometer (TD 20/20; Turner Instruments, Sunnyvale, CA, USA) following the standard assay protocol from Promega. Triplicate observations were made at each time point and the relative light units were normalized to the micrograms of protein in each well. For cell-type-specificity experiments, four nonprostate cell lines, N2a, C6, LS174T, and 293T cells were transduced with LV-TSTA and the cells were harvested after 48 h and assayed for FL activity.

Tumor xenografts. Human prostate tumor xenografts (LAPC-9, androgen dependent) were established in 4-to 6-week old male SCID mice. The tumor cells were kindly provided by Dr. Charles Sawyer's laboratory at UCLA. The tumors were extracted from donor mice, minced into 0.2-mm cubes, bathed in Matrigel, and implanted subcutaneously into the bottom left flank. When the tumors reached 0.5–0.6 mm in diameter, LV-TSTA (50 μl) was injected directly into the tumor site. The virus was allowed to distribute throughout the tumor for 2–4 days, following which the mice were anesthetized (ketamine:xylazine, 4:1) and imaged using the CCD camera with D-Luciferin as the substrate. The mice were imaged repeatedly every 3–4 days for 18 days. Three mice from each group were castrated on day 7 following virus injection and subsequently imaged on days 7 and 11 following castration. Blood samples were obtained from tail-vein incisions of mice before and after castration. Serum PSA levels were determined by an enzymatic immunoassay kit (American Qualex, San Clemente, CA, USA). To compare *fl* gene expression driven by the TSTA system (LV-TSTA) and the enhanced prostate-specific promoter alone (LV-PBC), individual LAPC-9 tumor-bearing mice were injected with the lentivirus in the tumor. The mice were subsequently imaged in the CCD camera on days 4, 7, 11, and 14. Three mice carrying nonprostate tumor xenografts (C6 rat glioma) were injected with LV-TSTA in the tumor. The mice were imaged in the CCD camera with D-Luciferin on days 5, 8, and 14.

Direct delivery of LV-TSTA into the prostate of male nude mice. For the delivery of LV-TSTA directly into the prostate of male nude mice, the animals were anesthetized using ketamine and xylazine. A small, 1-cm vertical midline incision in the lower abdomen was made into the peritoneum. The prostate was exposed using the seminal vesicles as a marker. Twenty microliters of LV-TSTA concentrated stock (2×10^7 TU/ml) was injected into each of the two dorsal prostate lobes. The abdominal wall and skin were closed with fine surgical sutures. The mice were

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imaged using the CCD camera 2 days after virus injection. They were subsequently imaged every 3 to 4 days for 21 days and thereafter once every week until day 90. Three mice from each group were surgically castrated 14 days after virus injection and subsequently imaged on days 3 and 7 following the procedure.

Systemic administration of LV-TSTA. LV-TSTA in phosphate-buffered saline was injected via the tail vein into three naïve SCID mice (100 μl, 4×10^5 IU). The mice were imaged in the CCD camera with D-Luciferin (150 mg/kg) on days 3 and 6. On day 6, a second aliquot of LV-TSTA was injected intravenously and the mice were subsequently imaged on days 10 and 14. Following the 2-week imaging period, the mice were sacrificed, and the organs were harvested and assayed for FL activity.

In vivo CCD camera imaging. Animal care and euthanasia were performed with the approval of the University of California Animal Research Committee. Male nude or SCID mice were anesthetized with ketamine:xylazine (4:1). The mice were injected with D-Luciferin (150 mg/kg body weight; Xenogen, Alameda, CA, USA) 10 min prior to imaging. The animals were placed in a light-tight chamber and a gray-scale reference image was obtained under low-level illumination. Photons emitted from within the animal and transmitted through the tissues were collected using a cooled CCD camera (Xenogen IVIS, Xenogen Corp., Alameda, CA, USA). The acquisition time ranged from 1 to 5 min. Images were obtained and quantified as described previously [25].

Analysis of ex vivo FL activity. Mice carrying LAPC-9 tumors were sacrificed following CCD camera imaging. The organs including the tumor were harvested and homogenized in Passive Lysis Buffer (4 ml/g tissue). The samples were freeze-thawed once at −80°C, and the homogenate was centrifuged at 14,000 rpm for 5 min. FL activity was measured using a luminometer. The relative light units were normalized to micrograms of protein in the sample. Mice that were injected with LV-TSTA directly in the prostate were also sacrificed at different time points and the organs harvested and assayed for FL activity as described above.

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