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# Monitoring of prostate cancer growth and metastasis using a PSA luciferase report plasmid in a mouse model

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## ABSTRACT

**Objective:** To construct a PSA luciferase report plasmid and monitor the growth and metastasis of prostate cancer after emasculation in SCID mice. **Methods:** PSA promoter sequence and luciferase gene were amplified by PCR and subsequently inserted into pZsGreen1-1 vector to construct pPSA-FL-Luc vector. LNCaP cells that were stably transfected with pPSA-FL-Luc were used to establish a SCID mouse xenograft model. Then, the growth and metastasis of prostate cancer were monitored via living imaging. **Results:** We successfully constructed a PSA luciferase plasmid, pPSA-FL-Luc. DHT enhanced luciferase activity in a concentration-dependent manner in 293T cells with pPSA-FL-Luc transfection. Prostate cancer SCID mouse model was established with pPSA-FL-Luc transfected LNCaP cells. In tumor bearing mice with or without emasculation, pPSA-FL-Luc plasmid was applied to monitored tumor growth and metastasis based on bioluminescence imaging. **Conclusions:** We construct a pPSA-FL-Luc plasmid, which stably expresses luciferase and can be applied to monitor tumor development in a prostate SCID mouse model.

## 1. Introduction

In USA, human prostate cancer is the most common carcinoma in males, and the second leading cause of cancer related mortality in men<sup>[1–3]</sup>. Recent years, the frequency of prostate cancer is increasing in China<sup>[4]</sup>. Prostate specific antigen (PSA) that is secreted by human prostate epithelial cells is a specific protein. Altering of PSA level in serum indicates initiation, development and metastasis of prostate cancer and predicts prognosis for tumor patients<sup>[5–7]</sup>. Recently, PSA functions as a main sensitive biomarker for clinical diagnosis, treatment and prognosis. PSA is mainly expressed in prostate cells and tissues and keeps a low concentration in serum under normal condition. During

prostate cancer initiation, PSA is released into the blood due to cancer cell proliferation and rupture, which leads to a significant increase of PSA level in serum. Thus, elevated serum PAS level is a critical marker for clinical diagnosis of prostate cancer<sup>[8–11]</sup>. Human prostate epithelial cells specifically express PSA. Accordingly, detecting the level change of PSA in human prostate epithelial cells is more convenience and accurate for predicting development, metastasis and prognosis of prostate cancer.

Luciferase, as a classic report molecule, is widely used to label cells and tissues. Living imaging technology is used to directly monitor cells and tissues change in living animals based on bioluminescence imaging<sup>[12,13]</sup>. Therefore, luciferase report gene was applied to prostate cancer in our study. Promoter sequence of PSA was inserted into luciferase reports gene plasmid to construct a pPSA-FL-Luc plasmid. We established a tumor bearing SCID mouse model using LNCap cells with pPSA-FL-Luc transfection. Tumor growth and metastasis were tracked using bioluminescence imaging in mice. Our date indicate that PSA luciferase report plasmids are useful for monitoring growth and metastasis of prostate cancer in mice.

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## 2. Materials and methods

### 2.1. Cell lines

The human prostate cancer cell line, LNCaP, and human embryonic kidney cell line, 293T (the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China), were cultured in complete RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) with 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin (Sigma, St-Louis, MO, USA) in a humidified containing of 5% CO<sub>2</sub> incubator at 37 °C.

### 2.2. Expression constructs

PGL3-Basic vector and pRL-TK vector were obtained from Promega Co. (Madison, WI, USA). pZsGreen 1-1 vector and pMD 18-T vector were purchased from Clontech Co. (Mountain View, CA, USA) and Takara Co. (Takara Bio, Shiga, Japan). PSA promoter cassette was amplified from genomic DNA, which was isolated from LNCaP cells, using forward primer CAC ATT GTT TGC TGC ACG TTG and reverse primer AGC TTG GGG CTG GGG AGC C. The length of PCR product was 655 bps. The PCR product connected to a pMD 18-T vector to form pMD 18-PSA. The linear skeleton of pZsGreen1-1 and PSA promoter fragment were collected after pZsGreen1-1 and pMD 18-PSA vectors cutting with *Sac* I and *Hind* III. T4 DNA ligase connected PSA promoter fragment to linear skeleton to construct a eukaryotic expression vector, which carried the PSA promoter and was named as pPSA-ZsGreen. The full length of firefly luciferase (FL-Luc) gene was cloned from pGL3-Basic vector, each incorporating *Bam*H I and *Not* I restriction site. The FL-Luc fragment and pPSA-ZsGreen vector were cut by *Bam*H I and *Not* I. T4 DNA ligase connected FL-Luc fragment to pPSA-ZsGreen linear skeleton to construct a luciferase eukaryotic expression plasmid, which was started by PSA and named as pPSA-FL-Luc.

### 2.3. Detection of PSA luciferase report plsmid

The human embryonic kidney cells, 293T, were seeded in 12 well plates after counting. When the cell confluence reached to 50%, pPSA-FL-Luc and pRL-TK plasmids were transfected into 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 hours transfection, 293T cells were treated with 0.1 nmol/L, 1.0 nmol/L, 10.0 nmol/L and 50.0 nmol/L 100.0 nmol/L dihydrotestosterone (DHT), respectively, for 48 hours. Total proteins were isolated from 293T cells and the expression of fluorescence was detected using a Dual Luciferase Reporter Gene Assay Kit (Promega, USA).

### 2.4. Cell tranfection and selection

LNCaP cells were seeded in 24 well plates and treated with different G418 (Sigma, USA) concentration (400  $\mu$ g/mL, 600  $\mu$ g/mL, 800  $\mu$ g/mL, 1 000  $\mu$ g/mL and 1 200  $\mu$ g/mL). The lowest concentration that killed all LNCaP cells after 10 to 14 days treatment was selected for further studies. LNCaP cells were transfected with pPSA-FL-Luc using lipofectamine 2000. After 24 hours transfection, cells were cultured in medium with G418 and the medium was changed every 3 days. Cell colonies were selected and seeded in 96 well plates for luciferase activity detection after 2 weeks treatment. The positive cells were further cultured in medium with G418 to obtain LNCaP/Luc/PSA cell line, which was stably expressed luciferase report gene.

### 2.5. In vivo experiments

SCID mice (SHANGHAI SLAC LABORATORY ANIMAL CO. LTD, Shanghai, China) were used to establish a SCID mouse xenograft model.  $2 \times 10^6$  LNCaP/Luc/PSA cells were inoculated subcutaneously into the flank of the SCID mouse. At week 8 after implantation, tumor nodes were explanted and divided into small pieces (1 mm×1 mm×1 mm). Then, the xenograft tumor tissues were injected into the capsule of prostate in SCID mice under anesthesia. After 6 weeks, mice were injected with 150  $\mu$ L D-luciferin (15 mg/mL, Biotium, Hayward, CA, USA) via intraperitoneal injection followed by isoflurane for anesthetization. *In vivo* xenogen imaging was performed to observe the location and intensity of fluorescence in SCID mice. SCID mice were divided into two groups according to with or without testicular castration. 1, 3 and 5 weeks after resection, tumor growth and metastasis were compared between two groups based on bioluminescence imaging. All animal protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

### 2.6. Statistical analysis

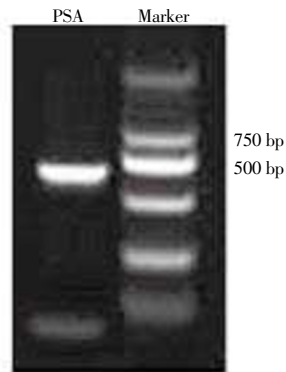
Results are expressed as Mean±SD. Significance was established, with SPSS statistical package for Windows Version 19.0 (SPSS, Chicago, IL, USA), using ANOVA when appropriate. Difference were considered significant when  $P < 0.05$ .

## 3. Results

### 3.1. Amplification of PSA promoter cassette

PSA promoter cassette was amplified from genomic DNA, which was isolated from LNCaP cells, using proper primers.

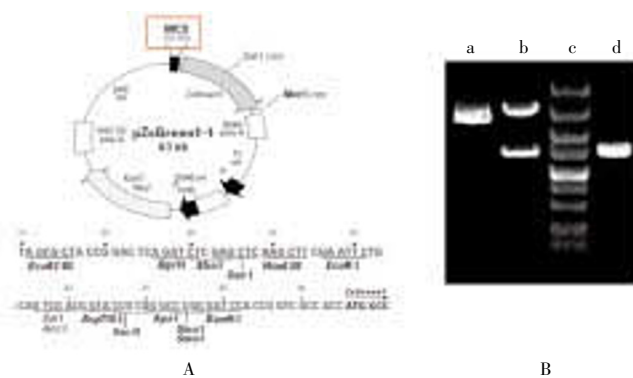
As shown in Figure 1, the length of PCR product was between 500 bps and 750 bps, which were consistent with the length of PSA promoter sequence (655 bps).



**Figure 1.** PCR result of PSA promoter sequence. There is a fragment between 500 bps and 750 bps, which was consistent with PSA promoter sequence.

**3.2. pPSA-FL-Luc plasmid construction**

PCR products were subcloned into pMD 18-T vector. Sequencing was used to confirm the correctness of PSA promoter sequence. PSA promoter cassette was connected to pZsGreen 1-1vector to construct pPSA-ZsGreen (Figure 2A). Furthermore, the full length of firefly luciferase (FL-Luc) gene was cloned from pGL3-Basic vector. The FL-Luc cassette was further moved into pPSA-ZsGreen using *BamH* I and *Not* I, resulting in pPSA-FL-Luc (Figure 2B).

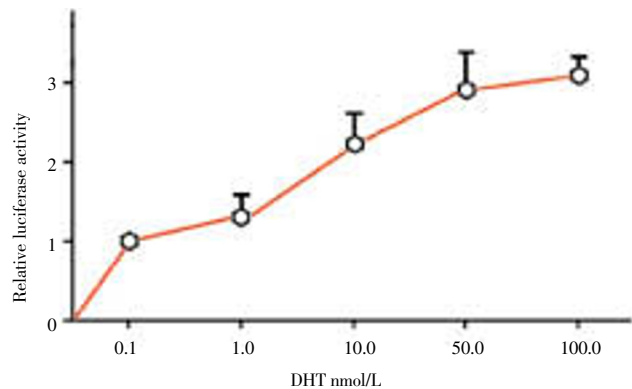


**Figure 2.** Construction of pPSA-FL-Luc plasmid. A) pPSA-FL-Luc was identified by electrophoresis gel. a) pPSA-FL-Luc recombinant plasmid. b) Enzyme-digested product of pPSA-FL-Luc. c) DL5000 Marker. d) PCR amplification product of FL-Luc.

**3.3. Functional identification of pPSA-FL-Luc**

293T cells were seeded in 12 well plates and transfected with pPSA-FL-Luc and pRL-TK plasmids, respectively, using lipofectamine 2000. Transfected 293T cells were treated

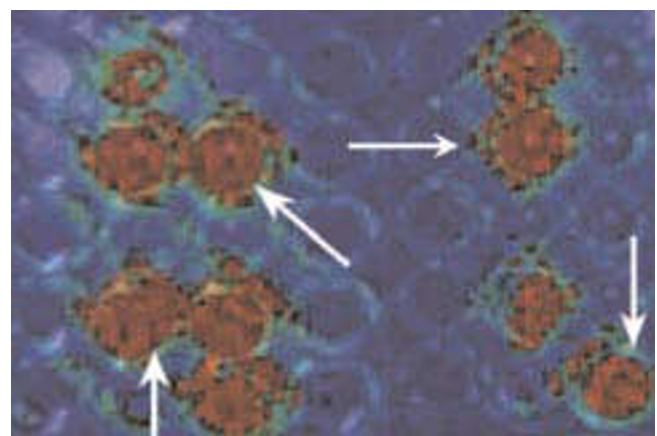
with different concentration of DHT (0.1 nmol/L, 1.0 nmol/L, 10.0 nmol/L, 50.0 nmol/L and 100.0 nmol/L). After 48 hours treatment, total proteins were isolated from 293T cells and the expression of fluorescence was detected using a commercial kit. We found that luciferase activity was enhanced by DHT in a concentration-dependent manner, the overall comparison and the difference between each two groups were significant ( $P < 0.05$ , Figure 3). Our data indicated that the pPSA-FL-Luc plasmid had a normal function.



**Figure 3.** Luciferase activities in cells treated by DHT at different concentration. Luciferase activity was enhanced by DHT in a concentration-dependent manner.

**3.4. LNCaP/Luc/PSA cell line screening**

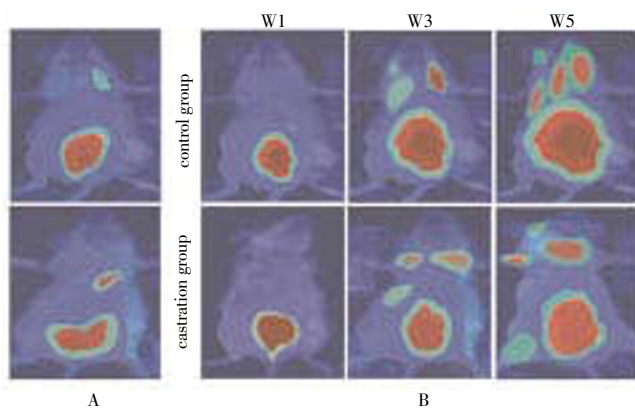
pPSA-FL-Luc plasmids were transfected into prostate cell line, LNCaP. After 2 weeks selection by G418, cell clones were selected and cultured in 96 well plates. D-luciferin was added into each well and selected cells were expressed hyperfluorescence (Figure 4).



**Figure 4.** LNCaP cells were expressed fluorescence. LNCaP cells that were transfected with pPSA-FL-Luc plasmids were selected by G418 for 2 weeks. White arrow indicated LNCaP/Luc/PSA cells.

### 3.5. Hormone sensitivity–prostate cancer mouse model was treated with castration

A SCID mouse xenograft model was established with LNCaP/Luc/PSA cells. SCID mice were injected with D–luciferin via intraperitoneal injection. *In vivo* xenogen imaging showed that the tumor located in the prostate of mice according to fluorescence (Figure 5A). Furthermore, bioluminescence imaging indicated that testicular castration slowed down tumor growth and metastasis in SCID mice (Figure 5B).



**Figure 5.** Bioluminescence imaging of SCID mice.

A) Bioluminescence imaging indicated that tumors were located in prostate of SCID mice. B) According to the intensity of fluorescence, testicular castration inhibited tumor growth and metastasis in SCID mice.

## 4. Discussion

Prostate cancer is one of the most common malignant cancers in males. The incidence of prostate cancer is increasing along with age. Tumor metastasis is the leading cause of poor prognosis for patients with prostate cancer<sup>[14,15]</sup>. One of the critical causes for prostate cancer initiation is androgen oversecretion<sup>[16–18]</sup>. As *PSA* gene expression is regulated by androgen<sup>[19,20]</sup>, monitoring of prostate cancer growth and metastasis using a *PSA* luciferase report plasmid based on bioluminescence imaging may be a novel and useful method.

*PSA* gene locates on the long arm of chromosome 19 and consists with 5 exons and 4 introns. Its promoter contains a TATA–box, a GC–box and an androgen response element (ARE). In this study, Genomic DNA that was isolated from LNCaP cells was used as templates for *PSA* promoter sequence amplification. After gel electrophoresis, the fragment was retrieved and subcloned into a pMD 18–T

vector. Next, FL–Luc cDNA was amplified from pGL3–Basic vector. Finally, pZsGreen 1–1 vector was used as a skeleton, *PSA* promoter cassette following FL–Luc cDNA were inserted into vector to construct a p*PSA*–FL–Luc report plasmid. To verify the correctness of p*PSA*–FL–Luc plasmid, 293T cells were transfected with p*PSA*–FL–Luc plasmids and treated with different concentration of DHT to start luciferase gene expression. Our results found that DHT increased luciferase activity in a concentration–dependent manner and the p*PSA*–FL–Luc plasmid was approved by these results.

After expression vector construction, LNCaP cells were transfected with p*PSA*–FL–Luc plasmids and selected by G418 for 2 weeks. Alive cells were chosen and cultured in 96 well plates. Under bioluminescent system, LNCaP cells were expressed hyperfluorescence after fluorescein substrate incubation, suggesting that we got an LNCaP/Luc/PSA cell line. Next, LNCaP/Luc/PSA cells were implanted into the flank of SCID mice via subcutaneous injection. After tumor formation, tumor nodes were divided into small pieces and transplanted into prostate of SCID mice via subcapsular injection to form an in situ tumor model. 6 weeks after model establishment, D–luciferin was injected into SCID mice via intraperitoneal injection. *In vivo* xenogen imaging showed that the tumor located in the prostate of mice according to fluorescence. Furthermore, SCID mice, which underwent testicular castration, exhibited a slower tumor growth and less metastasis as compared with control mice under bioluminescence imaging. As compared with traditional method for tumor detection, *in vivo* xenogen imaging is a potent method, which can directly, sensitively and constantly track tumour progression<sup>[21,22]</sup>.

In conclusion, we construct a *PSA* luciferase report plasmid and subsequently establish an LNCaP/Luc/PSA cell line. LNCaP/Luc/PSA cells are used to establish an in situ prostate cancer model in SCID mouse. *In vivo* xenogen imaging monitors tumor growth and metastasis of prostate cancer in SCID mice with or without testicular castration based on the location and intensity of fluorescence.

### Conflict of interest statement

We declare that we have no conflict of interest.

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