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# A multimodality reporter gene for monitoring transplanted stem cells

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ABSTRACT

*Introduction:* The aim of this study is to explore the feasibility of a triple-fused reporter gene, termed TGF [herpes simplex virus type 1 thymidine kinase (HSV1-tk), enhanced green fluorescent protein (eGFP) and firefly luciferase (Fluc)], to monitor stem cells using multimodality molecular imaging.

*Methods:* A recombinant adenovirus vector carrying the triple-fused reporter gene (Ad5-TGF) was constructed. Bone marrow mesenchymal stem cells (BMSCs) were transfected with different virus titers of Ad5-TGF [multiplicities of infection (MOIs) were 0, 50, 100, 150, 200 and 250]. The mRNA and protein expressions of HSV1-tk, eGFP and Fluc in the transfected BMSCs were evaluated using polymerase chain reaction and Western blot. After the transfection of the BMSCs with different virus titers of Ad5-TGF (MOIs were 25, 50, 75, 100 and 125), their uptake rates of $^{131}$I-FIAU were measured. Whole-body fluorescence, bioluminescence and micro-positron emission tomography (PET) images were acquired 1 day after the transfected BMSCs were injected into the left forelimb of rats.

*Results:* After the transfection with different titers of Ad5-TGF, the positive transfection rate reached a peak (70%) when the MOI was 100. HSV1-tk, eGFP and Fluc mRNA and protein were detected in the Ad5-TGF-transfected BMSCs, which implies their successful transfection and expression. The BMSCs uptake of $^{131}$I-FIAU increased with the adenovirus titer and incubation time and reached a plateau (approximately 5.3%) after 3 h. Strong signals were observed in the injected left forearms in the fluorescence, bioluminescence and micro-PET images.

*Conclusions:* A triple-fused reporter gene, TGF, can be used as a multifunctional molecular probe for multimodality imaging.



## 1. Introduction

Treating organ damage with stem cell transplantation has become an attractive therapeutic strategy, and promising applications have been reported [1]. Although many techniques can be used to monitor the proliferation, distribution and transplantation of stem cells, the traditional method of histological analysis in vitro is time consuming, labor intensive and invasive. However, close patient evaluation following stem cell transplantation is critical for the success of the therapy.

The advent of molecular imaging was a landmark in the monitoring of stem cell transplantation therapy because it facilitated the molecular evaluation of the biological processes of living tissues and cells [2]. Reporter gene imaging analyzes the radionuclide-labeled substrate or ligand for a specific expression product of an exogenous gene (reporter gene) in target cells. This technique can reveal the location, survival, distribution and differentiation process of the transplanted cell in vivo without causing any damage; therefore, this technique is best suited for clinically monitoring stem cell treatments. Reporter gene imaging is likely to be among the best methods for evaluating stem cell transplantation therapy in vivo. During the last decade, reporter gene imaging techniques have developed considerably. Reporter genes can be divided into two categories: enzyme-based reporter genes and receptor/transporter-based reporter genes. Furthermore, reporter gene imaging is used in radionuclide imaging, magnetic resonance imaging (MRI) and optical imaging [3]. The reporter genes that are used in radionuclide imaging include the herpes simplex virus type 1 thymidine kinase (HSV1-tk) [4,5] and its mutants [6], dopamine receptor type 2 [7], sodium iodide symporter (NIS) [8,9], norepinephrine transporter [10] and estrogen receptor [11]. Reporter genes that are used in MRI include the transferrin

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receptor [12], β-galactosidase [13], tyrosinase [14] and LRP [15], while fluorescent proteins (red and green) [16,17] and luciferase (firefly, Renilla and Gaussia) [18–21] have been utilized for optical imaging.

A fused reporter gene including HSV1-tk, eGFP and Fluc (TGF) has been recently constructed through the protein fusion technique, and its expression products have been used in radionuclide imaging, fluorescence imaging and bioluminescence imaging, which provided feasibility for simultaneous multimodality imaging after transfection [22,23]. This fused gene has been successfully used to track cancer cells and T-cells [22,24] and shows promise in tracking stem cells via plasmid transfection [25,26].

If a multimodality reporter gene could be used to track adenovirus-transfected stem cells, more information could easily be obtained in vitro, ex vivo and in vivo, which would lead to a more efficient understanding of the localization, survival, distribution and differentiation processes of stem cells. The aim of this study was to explore the practicality of monitoring transplanted stem cells via TGF. We transferred TGF into bone marrow mesenchymal stem cells (BMSCs) using adenovirus as a carrier, detected its expression in vitro and performed different imaging modalities in living animals.

## 2. Materials and methods

### 2.1. Ad5-TGF plasmid construction and recombinant adenovirus packaging

TGF was kindly donated by Dr. Sanjiv Sam Gambhir of Stanford University. The plasmid, pDC316-TGF, had a total size of 7214 bp, and TGF had a size of 3900 bp and included Fluc, eGFP and HSV1-tk; CMV was included as the promoter. Ad5-TGF was introduced into recombinant adenovirus by diplasmid transfection. The amplification, purification and titer evaluation of the virus were then performed. The recombinant adenovirus, Ad5-TGF, was obtained in a total volume of 7 ml, and the viral titer was $1.26\times10^{10}$ IU/ml as determined by the 50% tissue culture infective dose method. The amplification and carrier packaging of Ad5-TGF and Ad5-eGFP (used as a control) were completed by Vector Gene Technology Company Ltd. (Beijing, China).

### 2.2. Isolation, cultivation and identification of SD rat BMSCs

SD rats were used as the source rat, and all of the animals in this study were supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The femur and tibia were isolated, and the bone marrow was washed out with Dulbecco's modified Eagle's medium (DMEM)-F12 (Gibco, Langley, USA) culture medium that contained 15% fetal bovine serum (FBS) (Gibco). Next, the bone marrow suspension was inoculated in six-well cell culture plates. The anchorage-dependent cells were isolated and cultured. These primary cells were cultured for two to five generations to obtain pure cultures. The morphological features were then observed. The superficial markers CD44 and CD34 (antibodies from Boster, Wuhan, China) were detected in the BMSCs by immunohistochemistry, and the superficial antigens CD45 and CD90 (antibodies from eBioscience, Santiago, USA) were detected by flow cytometry. The BMSCs were cultured in DMEM-F12 culture medium that contained 15% FBS in an incubator at 37°C with 5% $CO_2$ and saturated humidity.

### 2.3. Ad5-TGF infection of BMSCs and in vitro bioluminescence assay

The BMSCs of generations 3–5 were evenly inoculated in the culture wells. Twenty-four hours after inoculation, the cells of three wells were counted. The culture medium in the remaining wells was removed and washed twice with phosphate-buffered saline (PBS). Next, 300 μl of non-serum-containing OPTI-MEM culture medium (Gibco) was added to each well until it just covered the bottom of the well. The amount of Ad5-TGF that was acquired from each well was calculated according to the cell number and multiplicity of infection (MOI). To promote infection, the cells were cross-blended every 15 min and gently shaken for 2 h in a 37°C incubator. Each well was then supplied with sufficient culture medium that contained 15% FBS and cultured in an incubator at 37°C and 5% $CO_2$ for 48 h. The fluorescent signal of eGFP was observed at a 488-nm wavelength with a fluorescence microscope (Nikon, Osaka, Japan) to detect the positive rate of Ad5-TGF-infected cells at 4, 12, 24, 36, 48, 72, 96, 120, 144 and 240 h. The normal BMSCs without the Ad5-TGF transfection served as a negative control.

### 2.4. MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Forty-eight hours after the Ad5-TGF transfection, the BMSC cell viability was tested with the MTT assay. The cells were inoculated in a 96-well plate and cultured in a moist incubator at 37°C and 5% $CO_2$ for 24 h. The BMSCs were transfected with Ad5-TGF at an MOI of 0, 50, 100, 150, 200 or 250 ($n=4$) for 48 h. Fifty microliters of a fresh 5 mg/ml MTT solution was added into each well, and the plate was incubated in the dark at 37°C for 4 h. The liquid supernatant was then removed, and 200 μl of dimethyl sulfoxide was added into each well. After 10 min of shaking, the plate was read in an enzyme-linked immunosorbent assay reader (Thermo Scientific, Multiskan Spectrum, Finland) at 490 nm. The suppression rates of the different MOIs were calculated.

### 2.5. Polymerase chain reaction (PCR) assay

Total RNA was extracted from the BMSCs after 48 h of Ad5-TGF transfection, and cDNA was obtained by reverse transcription. The Ad5-eGFP-transfected BMSCs and the nontransfected BMSCs were used as two control groups. The expression of HSV1-tk, eGFP and Fluc was tested by PCR. The primer sequences are shown in Table 1. All of the primers were provided by the Invitrogen Corp. (Shanghai, China). The PCR conditions were as follows: a hot start for 5 min at 94°C; 35 cycles that consisted of denaturing for 30 s at 94°C, annealing for 30 s at 56°C and elongation for 60 s at 72°C; followed by a final extension period of 10 min at 72°C.

### 2.6. Western blot

After 48 h of incubation, the Ad5-TGF-transfected BMSCs (MOI=100 IU/cell) were washed three times with cold PBS and then placed in an ice bath for 30 min. The mixture was then transferred into an Eppendorf tube and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined at a 595-nm wavelength by the BCA method. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked by placing the membrane in 5% nonfat, dry milk in TBST (Tris-buffered saline with Tween 20) at 4°C for 1 h. The following antibodies were added to the appropriate membranes at 4°C overnight: anti-eGFP monoclonal antibody (Beyotime, Shanghai, China) at a 1:500 dilution, anti-HSV1-

**Table 1**
Primer sequences for the detection of HSV1-tk, eGFP and Fluc expression by PCR.

| Gene name | Primer sequence | |
|---|---|---|
| HSV1-tk (837 bp) | Forward | 5′-ATGCCCACGCTACTGCGG-3′ |
| | Reverse | 5′-TCAGTTAGCCTCCCCCATCTC-3′ |
| eGFP (717 bp) | Forward | 5′-ATGGTGAGCAAGGGCGAG-3′ |
| | Reverse | 5′-CTTGTACAGCTCGTCCATGCC-3′ |
| Fluc (1641 bp) | Forward | 5′-ATGGAAGACGCCAAAAACATA-3′ |
| | Reverse | 5′-CTTTCCGCCCTTCTTGGC-3′ |