

# Cell and gene therapy with reporter gene imaging in myocardial ischemia

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## Abstract

**Objective:** Reporter gene/probe systems have proved to be reliable for monitoring gene/cell therapy. We sought to evaluate whether a reporter gene/probe system, namely the human estrogen receptor ligand binding domain (hERL)/16 $\alpha$ -<sup>18</sup>F fluoro-17 $\beta$ -estradiol (<sup>18</sup>F-FES), could be used for monitoring vascular endothelial growth factor (VEGF) gene expression and response to bone marrow mesenchymal stem cell (MSCs) therapy in ischemic heart disease. **Animals and Methods:** Reporter gene hERL and therapeutic gene VEGF165 were linked through internal ribosome entry site (IRES), and then the recombinant adenovirus vector Adenovirus 5-hERL-IRES-VEGF (Ad5-EIV) was constructed and transfected into MSCs, and named Ad5-EIV-MSCs. Rat myocardial infarction was induced by coronary arterial branch ligation, and Ad5-EIV-MSCs were transplanted by injection into the peripheral myocardium, while non-transfected MSCs transplantation used as controls. Fluorine-18-FDG micro-PET imaging was performed to confirm myocardial infarction 1 day after surgery. Fluorine-18-FES micro-PET/CT images were acquired 2 days after Ad5-EIV-MSCs transplantation. Myocardial specimens were obtained and stained with hematoxylin-eosin (H&E) staining to verify the myocardial infarction. The expression of estrogen receptor (ER) and VEGF was detected using immunohistochemistry (IHC). **Results:** Rat myocardial infarction models were successfully produced and confirmed by H&E staining. Images of <sup>18</sup>F-FDG PET showed obvious reduced or absent uptake of <sup>18</sup>F-FDG on the infarct myocardium, while uniform and well-distribution on the normal myocardium. <sup>18</sup>F-FES micro-PET/CT showed the tracer notable accumulated in the apical region where Ad5-EIV-MSCs were injected with an uptake value of 0.38 $\pm$ 0.09%ID/g, which was much higher than that of surrounding normal myocardium with nearly no uptake of <sup>18</sup>F-FES (0.10 $\pm$ 0.03%ID/g, n=5, P<0.05). In the group of non-transfected MSCs, the apical uptake was similar to that of normal myocardium. Immunohistochemistry studies demonstrated positive expression of both ER and VEGF in the involved region accompanied by active angiogenesis. **Conclusion:** This study confirmed that hERL/<sup>18</sup>F-FES could be used as a reporter gene/probe system for monitoring gene and cell therapy in the ischemic heart disease.

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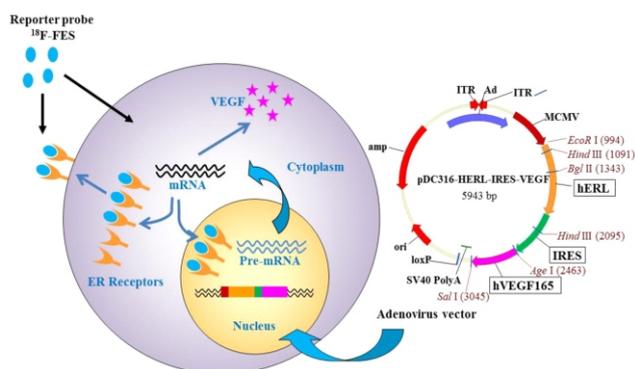
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## Introduction

Ischemic heart disease (IHD) is a leading cause of morbidity and mortality worldwide [1]. Unlike many other tissues in the body, human myocardium has little ability to repair itself after myocardial infarction (MI) [1]. Notwithstanding improved treatment, the morbidity and mortality from IHD remain a concern. Many studies [2-6] have reported the use of bone marrow mesenchymal stem cells (MSCs) for myocardial repair, with additional benefit from gene therapy with the vascular endothelial growth factor (VEGF) gene. Combined stem cell and VEGF gene therapy show great potential to promote myocardial repair and angiogenesis, and may have a profound impact on the morbidity and mortality from IHD [7,8].

Radiolabeled reporter gene/probe imaging is a strategy for monitoring therapeutic gene expression. In our previous research [9], a recombinant adenovirus vector, carrying a reporter gene (human estrogen receptor ligand binding domain, hERL) and a therapeutic gene (vascular endothelial growth factor, VEGF165) through an internal ribosome entry site (IRES), was constructed and named as Ad5-hERL-IRES-VEGF (Ad5-EIV). We confirmed the feasibility of the reporter gene/probe system, hERL/16 $\alpha$ -[<sup>18</sup>F] fluoro-17 $\beta$ -estradiol (<sup>18</sup>F-FES), to monitor gene expression of VEGF from the results of the proof-of-concept studies [9]. Human ERL is part of the estrogen receptor (ER), a human endogenous receptor, fulfilling all the requirements of a reporter gene, in that it lacks of immunogenicity, non-toxic, and small in size. Its corresponding probe, <sup>18</sup>F-FES, is a well-studied positron emission tomography (PET) tracer and bind to ER specifically [10-14]. Thus,

hERL and  $^{18}\text{F}$ -FES comprise a feasible reporter gene/probe imaging system. The schematic diagram of this reporter gene/probe system is shown in Figure 1.



**Figure 1.** Schematic diagram of reporter gene/probe system hERL/ $^{18}\text{F}$ -FES PET/CT for monitoring cell/gene therapy. A recombinant adenovirus vector Ad5-hERL-IRES-VEGF (Ad5-EIV) carries a reporter gene hERL and a therapeutic gene VEGF-165 through the internal ribosome entry site (IRES) as the linker and was transfected into bone marrow mesenchymal stem cells (MSCs). VEGF expression is mainly in cytoplasm, and ER expression is in the nucleus, cytoplasm and cell membrane. The probe,  $^{18}\text{F}$ -FES, binding to ER, can be detected by micro-PET.

In our previous study, we also confirmed that the expression of the two genes correlated well with each other, and the *in vivo*  $^{18}\text{F}$ -FES PET/CT imaging of a rat muscle model confirmed that the reporter gene hERL bound to the reporter probe specifically and could express the quantity of VEGF *in vivo*. In this study, we wanted to further verify if this system could be used to monitor cell / gene therapy in ischemic heart disease.

## Animals and Methods

### Animals

Adult male SD rats were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

### Preparation and identification of Ad5-EIV transfected MSCs

The construction of Ad5-EIV, rat bone mesenchymal stem cell isolation, culture, and *in vitro* virus infection were conducted as described previously, and all related identification and detection are described in our previous study [9, 15, 16]. MSCs between passages three and ten were transfected with Ad5-EIV (viral titers, multiplicity of infection=100), and the adenovirus-infected cells were called Ad-EIV-MSCs [9].

The expression of ER and VEGF in Ad5-EIV-MSCs was detected by immunocytochemistry, and the method was described in a previously published report [17]. Briefly, the cells

were seeded on sterile glass coverslips and grown to semi-confluency, and then fixed in freshly prepared 4% paraformaldehyde-PBS at room temperature for 10 minutes, following by incubating the coverslips in 0.5% Triton X-100 in PBS at room temperature for 5 minutes and blocked the coverslips in 1% BSA for 1 hour at room temperature. A mouse monoclonal antibody to ER $\alpha$  (ER $\alpha$  (1D5): sc-56833, Santa Cruz Biotechnology, CA, USA) at a dilution of 1:200 and a rabbit monoclonal antibody to VEGF (Beyotime, China) at a dilution of 1:200 were used as primary antibodies and incubated overnight at 4°C. Each section was incubated with a rabbit/mouse secondary antibody (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Then sections were developed in 3, 3'-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, and observed under a light microscope at  $\times 200$  magnifications.

### Preparation of myocardial infarction rat model

Myocardial infarction was induced in male SD rats weighing 200–250g. Surgical procedures were performed aseptically under anesthesia with isoflurane inhalation. After performance of a left thoracotomy via the fourth intercostal space, the beating heart was visualized and the anterior descending artery was ligated permanently with 4-0 silk threads. Then, 100  $\mu\text{L}$  of cell suspension, Ad-EIV-MSCs ( $1 \times 10^6$ ) in serum-free DMEM/F12 medium, were injected into the myocardium adjacent to the infarct area using an insulin syringe. The non-transfected MSCs with same volume and same amount of cells were used as control group ( $n=5$  for each group). The pneumothorax resulted by surgery was reduced by expulsion of air, the chest was then closed, and the rats were allowed to recover.

### Micro-PET/CT imaging *in vivo*

The probe  $^{18}\text{F}$ -FDG was synthesized automatically after  $^{18}\text{F}$  was produced by a cyclotron (MINItrace<sup>®</sup>, GE Healthcare, Milwaukee WI, USA), with radiochemical purity higher than 95%.  $^{18}\text{F}$ -FDG micro-PET/CT imaging was performed 1 day after surgery to confirm the site of myocardial infarction.

Fluorine-18-FES was prepared according to the established procedures [18–20]. Briefly, the precursor, 3-methoxymethyl-16 $\beta$ ,17 $\beta$ -epiestriol-Ocyclicsulfone (MMSE, 2.0mg) (Huayi Isotope, Changshu, China) in anhydrous MeCN (1ml), was added to the dried [ $^{18}\text{F}$ ]F<sup>-</sup>, and the mixture was heated at 110°C for 10 minutes, subsequently hydrolyzed using 1.5mL HCl (0.2mol/L) at 110°C for 5 minutes. After cooling to room temperature, 1.5mL (0.2mol/L) NaHCO<sub>3</sub> was added to neutralize the solution. The reaction mixture was passed through a Sep-Pak column and then injected into HPLC for separation of  $^{18}\text{F}$ -FES.  $^{18}\text{F}$ -FES micro-PET/CT scans were performed 2 days after animal surgery.

Positron emission tomography imaging was initiated about 1 hour after intravenous injection of the radioactive probe (about 7.4MBq/animal) via a tail vein. Scanning was performed with a micro-PET/CT scanner (Inveon PET/CT, Siemens Preclinical Solution, Knoxville Tennessee, USA). The animals were anesthetized with 5% isoflurane gas and placed in the prone position on the bed, and then rats were anes-

thetized with 1%-2% isoflurane gas during the time of image acquisitions. Two bed positions were acquired.

Positron emission tomography images were reconstructed with the standard ordered-subset expectation maximization method, and were displayed in transverse, coronal, and sagittal planes. Computed tomography was used for both image fusion and attenuation correction. Regions of interest (ROIs) were drawn on the area where Ad5-EIV-MSCs were injected and adjacent normal myocardium served as background, the uptake was expressed as percent injected dose per gram of tissue (% ID/g).

### Histology examination

The animals were sacrificed by anesthetic overdose, and the hearts were removed and myocardial specimens were prepared. Each specimen was fixed with 10% buffered formalin and embedded in paraffin. A few serial sections were prepared from each specimen. Hematoxylin and eosin (H&E) staining was performed to further confirm the inclusion of infarcted and noninfarcted tissue. To detect the expression of the exogenous genes in the myocardium, immunohistochemical staining of VEGF and ER were performed using the abovementioned procedures.

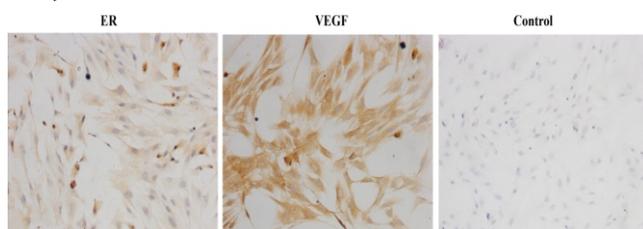
### Statistical analysis

Data are expressed as mean±SD. Significance between two measurements was determined by Student's t-test. P values of less than 0.05 were considered statistically significant.

## Results

### ER and VEGF expression in Ad5-EIV-MSCs

As shown in Figure 2, the expression of ER and VEGF were clearly visualized in Ad5-EIV-MSCs, which suggested successfully transfection of Ad5-EIV in MSCs.

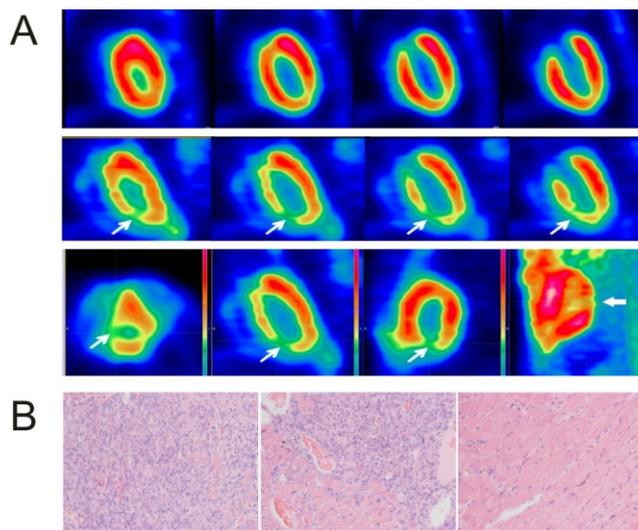


**Figure 2.** Immunocytochemical staining of Ad-EIV-MSCs ( $\times 200$ ). A positive ER staining (left) and stronger positive VEGF staining (middle) can be observed. The control was negative (right).

### $^{18}\text{F}$ -FDG micro-PET imaging on myocardial infarction animal model

$^{18}\text{F}$ -FDG PET imaging is considered a golden standard for evaluation of myocardial viability after myocardial infarction [21]. Therefore, we performed  $^{18}\text{F}$ -FDG PET myocardial imaging to determine whether the myocardial infarction was successful. Obvious reduced or absent uptake of  $^{18}\text{F}$ -FDG was seen on the infarct myocardium, while uniform and well-distribution on the normal myocardium (Figure 3A). H&E sta-

ining also proved myocardial infarction (Figure 3B).



**Figure 3.** A. Representative decay-corrected coronal  $^{18}\text{F}$ -FDG micro-PET images of normal rat (upper row) and myocardial infarction model (middle row). The lower row shows the transaxial, coronal, sagittal and maximum density projection lateral images of the myocardial infarction model, respectively (from left to right). Myocardial infarction regions are indicated by a white arrow, showing obvious reduced or absent uptake of  $^{18}\text{F}$ -FDG. B. HE staining myocardial in-farction area (left), junction zone (middle) and normal myocardium (right).

### $^{18}\text{F}$ -FES micro-PET/CT imaging of reporter gene hERL

On Ad5-EIV-MSCs transplanted myocardial infarction model,  $^{18}\text{F}$ -FES micro-PET/CT image showed the tracer notable accumulated in the apical and anterior region where Ad5-EIV-MSCs were injected (Figure 4A) with the uptake value of  $0.38 \pm 0.09\%$  ID/g, which was much higher than that of surrounding normal myocardium with nearly no uptake of  $^{18}\text{F}$ -FES ( $0.10 \pm 0.03\%$  ID/g,  $n=5$ ,  $P<0.05$ ). In the group of non-transfected MSCs (Figure 4B), the uptake of  $^{18}\text{F}$ -FES on the apical and anterior wall was nearly background and similar to the other parts of the myocardium.

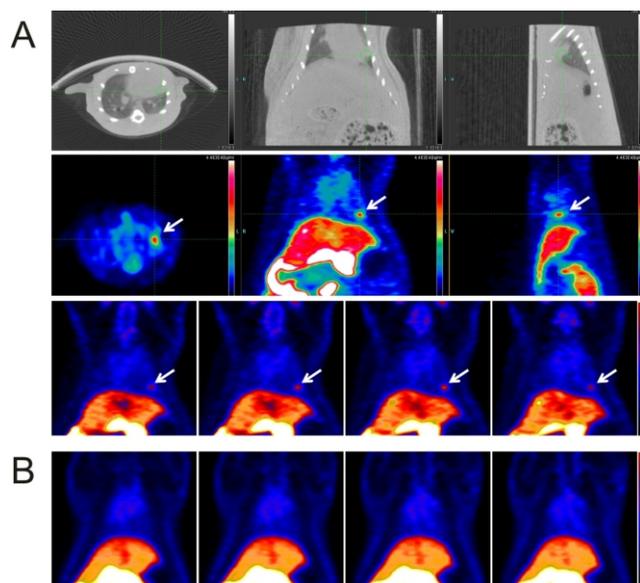
### Immunohistochemical staining

Immunohistochemical staining showed positive expression of both ER and VEGF in the apical and anterior wall, which was consistent with the in vitro cell experiment. No positive immunoreactions were found in the control specimens (Figure 5). VEGF staining was consistent with brisk angiogenesis.

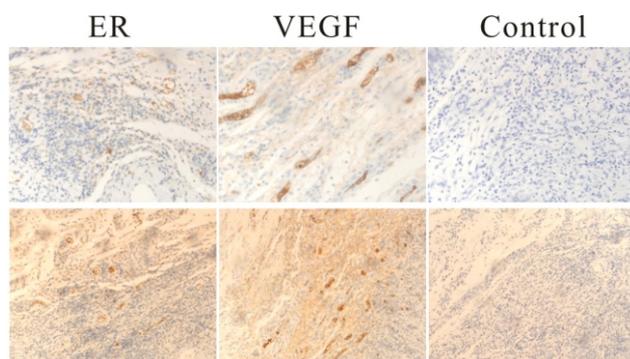
## Discussion

In our previous study, we successfully demonstrated that the recombinant adenovirus vector, Ad5-EIV, can transfer both the reporter hERL gene and therapeutic VEGF gene into MSCs simultaneously. The expression of these two genes correlated well with each other. The in vivo  $^{18}\text{F}$ -FES PET/CT imaging of a rat muscle model confirmed reporter gene hERL product was expressed in vivo and bound to reporter probe specifically [9]. In this study, we directly transplanted Ad5-

EIV-MSCs into infarcted myocardium to further verify the application of this reporter gene/probe system. From the *in vivo* micro-PET imaging, high uptake of  $^{18}\text{F}$ -FES was seen in the Ad5-EIV-MSCs transplanted infarcted myocardium with high target-to-background ratio, while no uptake in MSCs transplanted group. These results suggested that the reporter gene hERL and reporter probe  $^{18}\text{F}$ -FES could be used to monitor gene expression and stem cell viability in ischemic heart disease. To the best of our knowledge, this is the first time this reporter gene system has been employed in myocardial infarction model.



**Figure 4.** A. Representative decay-corrected  $^{18}\text{F}$ -FES micro-PET/CT images of Ad5-EIV-MSCs transplanted rat myocardial infarction model, specific radioactivity accumulation (white arrow) was observed in the apical region where Ad5-EIV-MSCs were injected. CT and corresponding PET images are shown in the upper and middle row with transaxial, coronal, sagittal view. The lower row shows the continuous positive image of coronal view, and the obvious uptake of  $^{18}\text{F}$ -FES was seen on the images (white arrow). B. Coronal  $^{18}\text{F}$ -FES PET images of rat with non-transfected MSCs injected, nearly no uptake of  $^{18}\text{F}$ -FES shows on the heart area.



**Figure 5.** Immunohistochemical staining of apical where Ad5-EIV-MSCs were transplanted (upper:  $\times 400$ , lower:  $\times 200$ ). A positive ER staining (left) and stronger positive VEGF staining and angiogenesis (middle) can be observed. The control was negative (right).

Other reporter genes have also been used in the heart. Reporter gene herpes simplex virus 1 thymidine kinase (HSV1-

sr39tk) and its corresponding reporter probe 9-[4-((18)F)fluoro-3-hydroxymethyl-butyl]guanine ( $^{18}\text{F}$ -FHBG) was first reported to allow imaging of cardiac HSV1-sr39tk reporter gene expression in 2002 [22], with the reporter gene administered by intra myocardial injection of the adenovirus vector. Subsequently, some reporter genes have been transfected into stem cells and then transplanted into myocardium in acute myocardial infarction rat models [23, 24]. Multimodality reporter genes, such as TGF [16], reported by our group, is a triple-fused reporter gene of herpes simplex virus type 1 thymidine kinase (HSV1-tk), enhanced green fluorescence protein (eGFP), and firefly luciferase (FLuc), were used to monitor gene expression and cell viability by PET, fluorescence and bioluminescence imaging. However, three reporter gene needs to be fused into one construct, and the gene fusion processes are difficult. Most importantly, the introduction of exogenous genes into humans is dangerous to some extent [25]. Sodium/iodide symporter (NIS) reporter gene has also been reported; it has the advantage of a more available reporter probe, but the tracer (radioactive technetium or iodine) easily becomes unbound from the cells [26].

In this study, we use hERL as the reporter gene; it is a fragment of the estrogen receptor. There are some advantages of this reporter gene system: no or low ER expressed in myocardium, very low uptake in normal myocardium with low background counts. Moreover, we used MSCs and the VEGF gene for combined therapy, and the expression of ERL and VEGF were positively correlated. As genes are expressed only in viable cells after transfection, our successful PET imaging demonstrated that our transplanted cells were viable.

MSCs are multipotent cells that are capable of differentiating into different cell types [27], including myocardial cells [28, 29]. In addition, MSCs are relatively simple to isolate using standard culture media with bovine serum [30], making them an attractive cellular therapeutic candidate. MSCs have improved heart function in both animal models of acute myocardial injury as well as in clinical studies of patients with heart failure [31]. VEGF is a well-known potent angiogenic factor [32]. In animal models and phase 1 clinical trials, VEGF therapy (delivered as protein, plasmid, or adenovirus) significantly improved myocardial perfusion and function [33]. This combined VEGF gene and MSCs strategy may have a better therapeutic effect than either used alone, which has been demonstrated by Matsumoto et al. (2005) [34]. VEGF-expressing MSCs transplanted into myocardium can differentiate into myocardial cells in the appropriate microenvironment [34]; while the expression of VEGF gene can promote myocardial angiogenesis, which is more conducive to the recovery of myocardial viability. In order to evaluate the expression of therapeutic gene expression and cell viability, noninvasive imaging technology is needed. In this study, a reporter gene and a therapeutic gene were linked with IRES and co-expressed successfully. Reporter gene imaging can provide indirect information of therapeutic gene and cells, providing a good monitoring method. This strategy will have great potential in refractory ischemic cardiomyopathy.

However, there have some drawbacks of our study. Firstly, although adenovirus is a good vehicle for gene transfer with high efficacy, exogenous genes do not express their products

for a long amount of time due to transient transfection [25]. In this regard, lentivirus-mediated transfection allows an exogenous gene to be inserted into the genome of the target cell, allowing gene expression over a longer period of time [35]. Secondly, due to adenovirus transient transfection, we did not monitor gene expression and evaluate recovery of myocardium infarction for a long time. In the next step, we will use  $^{18}\text{F}$ -FES PET and  $^{18}\text{F}$ -FDG PET imaging to evaluate the distribution, duration and expression extent of therapeutic genes, cell viability, as well as therapeutic effect, over a longer time period using lentivirus.

*In conclusion*, in a preliminary proof-of-concept study, we demonstrated the feasibility of using a reporter gene/probe system, hERL/ $^{18}\text{F}$ -FES, for monitoring gene and cell therapy in ischemic heart disease.

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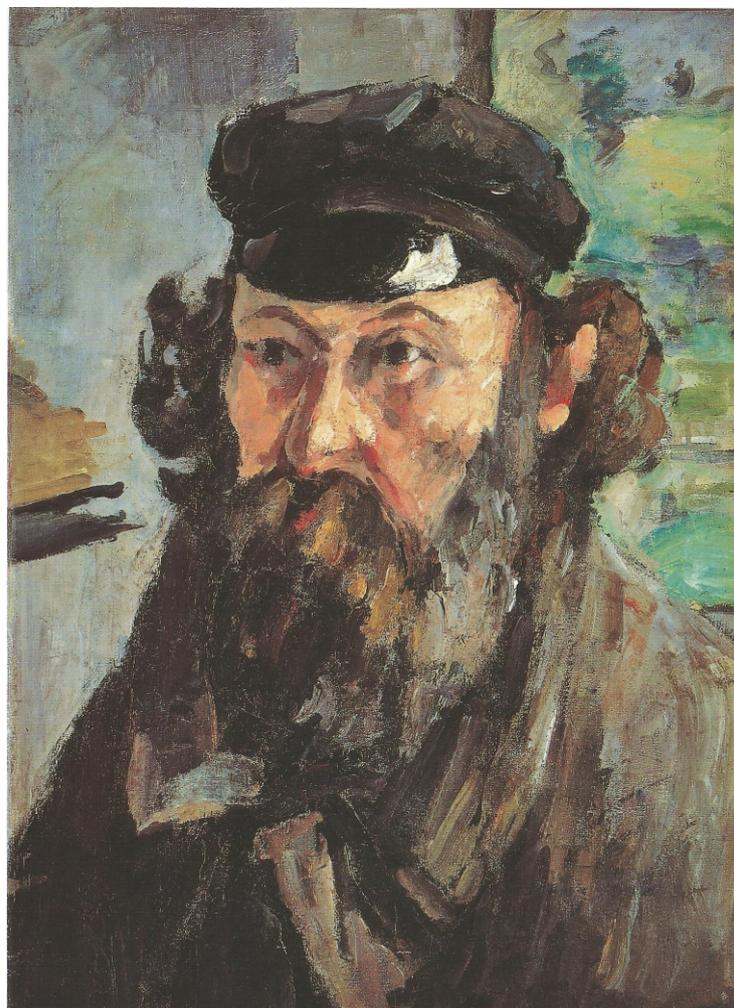
This work was supported by the National Natural Science Foundation of China (No. 30970853, 81371626), and the Clinical Research Physician Program of Tongji Medical College, Huazhong University of Science and Technology (No. 5001530008). We thank Dr. Biao Li and Dr. Sheng Liang at Ruijin Hospital, Shanghai Jiaotong University, for their kind help in preparing  $^{18}\text{F}$ -FES and performing micro-PET/CT imaging.

*The authors of this study declare no conflicts of interest*

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