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ORIGINAL ARTICLE

Retrovirus-Mediated Transfection of the Tissue-type Plasminogen Activator Gene Results in Increased Thrombolysis of Blood Clots

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Abstract

Tissue-type plasminogen activator (tPA) is involved in the lysis of blood clots. In this study, we attempted to target thrombolysis and enhance blood clot lysis by generating a construct (pLEGFP-N1-tPA) to integrate tPA gene into the genome of different cell lines. pLEGFP-N1-tPA construct was generated and used to target the tPA gene in different cell lines. The thrombolytic effects mediated by the supernatant from transfected HeLa cells and Linx cells were assessed using plasma thrombus plates. Furthermore, enhanced green fluorescent protein (EGFP), which was fused to the tPA gene in the pLEGFP-N1-tPA construct, was analyzed under the fluorescent microscope to assess tPA localization. We also monitored tPA activity and expression in the transfected cell lines. As part of the study, we successfully generated the pLEGFP-N1-tPA construct. The sequence of this construct was verified and the construct was subsequently used to generate the PT67/pLEGFP-N1-tPA cell line. The pLEGFP-N1-tPA construct was also used to transfect HeLa cells and Linx cells. We observed that supernatants from transfected cells were capable of lysing thrombi. In addition, tPA activity and tPA concentration were elevated in the latter supernatants and tPA was rapidly and stably expressed in the transfected cell lines. These results reveal a potentially important thrombolytic role for tPA-targeted gene therapy following cardiac valve replacement.

Keywords Retroviridae · tPA · Heredity vector · Thrombus · Thrombolysis

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Introduction

Valve replacement is a common treatment strategy for serious valve dysfunctions of the heart including mitral valve stenosis (regurgitation) and aortal valve stenosis (regurgitation) (Ghanta and Kron 2016; Nucifora et al. 2017). Studies have shown that mechanical valves can last for 100 years or even longer. Patients that have had mechanical valves fitted are commonly administered warfarin to aid anti-coagulation of the blood during his or her post-operation rest period. Furthermore, prothrombin time (PT) and international normalize ratio (INR) should be monitored intermittently post-operation. However, due to economic constraints, PT and INR are not always accurately monitored in patients living in developing countries. Inadequate anti-coagulation can lead to thrombus formation (Halldorsdottir et al. 2016) and stroke. Conversely, increases in anti-coagulation can give rise to bleeding (Guglielmetti et al. 2016) and conditions including cerebral hemorrhage and stomach hemorrhage. These complications can lead to health-related issues that can last for a lifetime. In order to combat these issues, we advocate the use of anti-coagulation gene therapy instead of warfarin treatment for patients that have undergone valve replacement surgery. With this in mind we aimed to investigate the effectiveness of replacing mechanical valves in conjunction with localized injection of pLEGFP-N1-tPA-mediated gene therapy. Tissue-type plasminogen activator (tPA) is a lytic agent that can lyse thrombi both in circulating blood and in vitro (Hussain et al. 2016; Fernandes and Umasankar 2016; Tan et al. 2017; Ji et al. 2011). The tPA protein is a serine protein hydrolase composed of 527 amino acids. As part of this analysis, we injected pLEGFP-N1-tPA into the tissue surrounding a newly replaced mechanical valve. Theoretically the injection should result in the integration of the tPA into the host cell genome. Therefore, tPA protein should subsequently be secreted into the blood around the newly fitted valve. In the absence of warfarin administration, a small amount of thrombus will grow on the surface of the metal valve in the beating heart. Secreted tPA around the mechanical valve will activate fibrinogen in the blood resulting in the production of fibrinolysin with concomitant thrombus removal. Following continuous activity by fibrinolysin there should be no thrombus left on the surface of the metal valve. Once tPA circulates out of the heart, it interacts with plasminogen activator inhibitor-1 and related effects are inhibited (Aloni et al. 2016; Liappas et al. 2016; van Overbeek et al. 2016). Thus, tPA is a potentially important target in relation to thrombus formation (Gong et al. 2007, 2010; Tadayon et al. 2015). pLEGFP-N1-tPA is a retroviral gene expression system. It consists of cytomegalovirus (CMV), multi-cloning site (MCS) and enhanced green fluorescent protein (EGFP). EGFP is an easily observed marker with 35-fold greater amplification than green fluorescent protein (GFP) (Condro et al. 2016; Isotani et al. 2016; Bierhuizen et al. 1997). Using molecular methods, exogenous gene such as the tPA gene can be inserted into the MCS of pLEGFP-N1. Subsequently, tPA can be integrated into the genome of host cells following pLEGFP-N1-tPA infection. In theory, exogenous genes can be rapidly and efficiently expressed over long periods using this strategy (Tan and Hu 2013; Mohanlal et al. 2016).

An exogenous gene such as tPA can be fused with the EGFP gene in a retroviral vector. In previous studies we investigated if pLEGFP-N1-tPA targeted thrombolysis in rabbit inferior caval vein and atrium models (Gong et al. 2007, 2010). The latter studies demonstrated that pLEGFP-N1-tPA injected around Dacron patches can effectively lyse thrombi on the patches in rabbit.

In this study, we generated a pLEGFP-N1-tPA construct and cultured a purified packaging cell line PT67/pLEGFP-N1-tPA that resulted in high titre virus. As part of a previous study, we observed thrombolysis in plasma thrombus plates when the endothelial cells of umbilical vein (ECUV) and heart muscle cells (HMC) were infected with pLEGFP-N1-tPA. To investigate if pLEGFP-N1-tPA can cause infections in different cell types, we used pLEGFP-N1-tPA to infect HeLa cells and Linx cells. We subsequently looked for the occurrence of thrombolysis of plasma thrombi. HeLa cells are cervical cancer cells and the Linx cell line was derived from human embryo kidney cells. Supernatant from HeLa cells transfected with pLEGFP-N1-tPA exhibited obvious thrombolysis in plasma thrombus plates. A similar phenomenon was observed in Linx cells infected by pLEGFP-N1-tPA. Furthermore, tPA activity and tPA levels were elevated in supernatants from both cell lines. These results suggest that effective thrombolysis occurred following rapid, efficient and stable tPA expression. This study provides a platform to further investigate the combinatorial effects of tPA gene therapy and cardiac valve replacement to prevent thrombosis.

Materials and Methods

Construction of pLEGFP-N1-tPA

The open reading frame (ORF) of tPA is 1689 bp (Genbank accession number NM-000930). We designed an upstream primer (P_5) as (5′-GCGTCGACACC ATGGATGCAATGAAGAGA-3′) and downstream primer (P_3) as (5′-GACGGA TCCCACGGTCGCATGTTGTCACGAA-3′) based upon the tPA ORF and the MCS of pLEGFP-N1 (Clontech, TAKARA BIO INC., US). The formerly underlined GTCGAC sequence and the latterly underlined GGATCC sequence represent recognition sequences for the restriction enzymes *Sal*I and *Bam*HI, respectively. PBS/tPA (donated by Dr. Xiaobing Liu) was amplified in accordance with instructions from "Molecular Cloning: A Laboratory Manual" (Sambrook and Russell 2001). The tPA gene sequence was amplified by PCR using the PBS/tPA sequence as a template. The following reaction mixture (50 µl) was used for amplification: 0.2 µl PBS/tPA, 1 µl of P_5 (primer), 1 µl of P_3 (primer), 1 µl of dNTPs (10 mM/L), 5 µl of 10× PCR Buffer Pyrobest Mg^{2+} plus (Pyrobest, TAKARA, US), 1 µl of Pyrobest Taq polymerase enzyme and 40.8 µl of deionized water. The amplification reaction was performed in a DNA Engine DYAD™ PCR machine. The PCR reaction conditions were as follows: an initial denaturation step at 95 °C for 7 min; 5 cycles of 95 °C for 2 min, 50 °C for 1 min, 72 °C for 2 min; 25 cycles of 95 °C for 2 min, 65 °C for 1 min, 72 °C for 2 min; and a final extension step of 72 °C for 10 min. The resultant PCR products were

electrophoresed on an agarose gel (1%) and tPA gene product was observed to be a 1689-bp product. pLEGFP-N1 was subsequently transformed into competent *E. coli*-JM109 bacterial cells by electrical 2.5 Kv transforming to generate additional plasmid DNA. Through swaying *E. coli*-JM109 and alkaline lysis, we scavenged more pLEGFP-N1. The extracted plasmid DNA was subsequently digested with *Sal*I and *Bam*HI and the resultant fragments were electrophoresed on an agarose gel (0.6%). The following restriction enzyme digest reaction conditions were used; pLEGFP-N1 40 µl reactive system: 16 µl of deionized water, 4 µl of 10× D buffer (Pyrobest, TAKARA, US), 15 µl of pLEGFP-N1, 3 µl of *Bam*HI, 1.5 µl of *Sal*I, 0.5 µl of BSA. The reaction mixtures were incubated for 6 h at 37 °C. For the tPA amplicon digestion reaction, the following component were added to a 40-µl final reaction mixture: 1 µl of water, 4 µl of 10× D buffer, 30 µl of tPA, 3 µl of *Bam*HI, 1.5 µl of *Sal*I, 0.5 µl of BSA. The reaction mixtures were incubated for 6 h at 37 °C. The resultant tPA and pLEGFP-N1 fragments were ligated using T_4 DNA ligase (16 °C, 12 h) resulting in the recombinant retrovirus vector pLEGFP-N1-tPA. The ligation reaction mixture contained 0.5 µl of T_4 DNA (New England), 5 µl of tPA, 3 µl of pLEGFP-N1, 1 µl of 10× ligation buffer. The ligation mixtures were incubated at 16 °C for 12 h. Finally, the pLEGFP-N1-tPA was transformed into competent *E. coli*-JM109 bacterium. The transformed plasmid was extracted and sequence to confirm the correct sequence was present.

Cell Culture of Purified PT67/pLEGFP-N1-tPA Cell Line

PT67 (Clontech, TAKARA, US) cells were cultured in DMEM (Dulbecco's modified eagle's medium) supplemented with 10% neonate calf serum (NCS). The PT67 cells were grown to 50% confluence in a 60-mm plate. pLEGFP-N1-tPA and pLEGFP-N1 were independently transfected into PT67 cells using Sofast™ a positive ion polymer transfection reagent (Xiamen Sunma Biotechnology Co., Ltd), following the protocols provided by the manufacturer. pLEGFP-N1-tPA contains a neo-resistant gene. Thirty-six hours after transfection, G418 (500 µg/mL) was pipetted into the cell culture medium to select stably transfected cells. A single PT67 cell that strongly expressed EGFP was observed and marked under the fluorescent microscope as it grew into a bundle of cells. We isolated the bundle and fed them continuously to generate a PT67/pLEGFP-N1-tPA cell line that expressed EGFP. No bacterial infection on cells would be stressed while picking out the bundle cells with EGFP by micro moving from 60-mm plate. The cell bundle was initially cultured in a single well of a 96-well plate. The cells were sequentially cultured in a 24-well plate, a 12-well plate, a 6-well plate, 35-mm plate, 60-mm plate, and a 100-mm plate. The purified PT67/pLEGFP-N1-tPA cell line resulted in a high titer of retrovirus. NIH 3T3 cells were used to determine the retrovirus titer. We collected 50 ml of supernatant from the purified PT67/pLEGFP-N1-tPA cells. Following filtration using a 0.45-µm cellulose acetate membrane, the supernatant was stored for future use at 4 °C.

pLEGFP-N1-tPA-Infected HeLa Cells

HeLa cells (Type Cultural Collection Centre of Wu Han University, China) were cultured in DMEM supplemented with 10% neonate calf serum. Upon initial culture, the HeLa cells were maintained in a 24.44-cm^2 bottle. Approximately 8.0×10^5 cells were subsequently passaged into a single well of 6-well plate. Upon reaching 40–50% confluence, the HeLa cells were infected with the supernatant from the purified PT67/pLEGFP-N1-tPA cells. Following infection, the medium was removed and 2.5 mL of supernatant containing polybrene (10 μg/mL) was pipetted into the well. Water was pipetted into separate well of 6-well plate. Both of the afore-mentioned plates placed symmetry in centrifuge were centrifuged for 1 h in a Sigma 11222 centrifuge at 500*g* to improve virus infection. The infected HeLa cells were cultured for 6 h at 37 °C and the supernatant was replaced with 10% NCS + DMEM. After infected 24 h of HeLa, EGFP was observed both in the medium and on HeLa cells membranes when viewed under the fluorescent microscope. The supernatant of HeLa cells/pLEGFP-N1-tPA was collected after culturing for 48 h, 72 h and 96 h and stored at 20 °C until further required (to analyze plasma thrombus formation, tPA activity, tPA concentration and tPA protein expression). The process of infecting HeLa cells was performed seven times in total.

As a control (c), PT67 was infected with pLEGFP-N1. The supernatant of HeLa cells/pLEGFP-N1 was collected to analyze plasma thrombus formation, tPA activity, tPA concentration and tPA protein expression. The infection process was performed 7 times in total. As another control, 10% NCS + DMEM was used as the blank control (bc).

The plasma thrombus plate assay has previously been described in the article "Tissue-type plasminogen activator gene targets thrombolysis in atriums" (Gong et al. 2010). Briefly, 50 μl of concentrated supernatant from HeLa cells/pLEGFP-N1-tPA was pipetted into a single well of the plate. Another well was filled with 50 μl of HeLa cells/pLEGFP-N1 control supernatant or blank 10% NCS + DMEM control fluid. The thrombolysis area was calculated as $((\text{length} + \text{width})/4)^2 \times \pi$. tPA activity was calculated based upon the thrombolysis area and the activity of lumbrokinase. Western blot analysis was performed to check for the presence of an exogenous tPA band.

pLEGFP-N1-tPA-Infected Linx Cells

Linx cells (Joint Lab, Life Science College, Wuhan University, China) were cultured in DMEM supplemented with 10% neonate calf serum. The cells were initially cultured in a 24.44-cm^2 bottle and passaged once every 16 h. After 2 days, the cells had covered one side of the bottle. Next, 8.0×10^5 cells were passaged into a single well of a 6-well plate. When the cells reached 40–50% confluence, they were infected with the supernatant from the purified PT67/pLEGFP-N1-tPA cells. The medium was subsequently removed and 2.5 mL of supernatant plus polybrene (10 μg/mL) was pipetted into the well. One well of another six wells plate was filled with water to balance the plates prior to centrifugation. Both of the afore-mentioned plates

placed symmetry in centrifuge were centrifuged for 1 h at 500*g* in a Sigma 11222 centrifuge. The infected Linx cells were cultured for 6 h at 37 °C. The supernatant was exchanged for 10% NCS + DMEM. The infected Linx cells were fed and passaged for 96 h. At 48 h, 72 h and 96 h, supernatant from the Linx cells/pLEGFP-N1-tPA was collected and stored at 20 °C for future use. This procedure was performed 7 times for Linx cells/pLEGFP-N1-tPA.

For the pLEGFP-N1-transfected PT67 cells, the supernatant of PT67 cells/pLEGFP-N1 was used to infect Linx cells. The supernatant of the Linx cells/pLEGFP-N1 was collected to analyze plasma thrombus formation, tPA activity, tPA concentration and tPA protein expression. This procedure was repeated 7 times for Linx cells/pLEGFP-N1 and as another control, 10% NCS + DMEM was used as a blank control.

A plasma thrombus model plate was generated using the infected cells and Western blot was used to confirm tPA expression.

Statistical Analysis

Through homoscedasticity test, *t*-test of small two group's samples was used to assess the statistical significance of the measured tPA concentration and activity.

Results

Successful Construction of pLEGFP-N1-tPA

We initially performed a test to ensure that PBS/tPA was usable. A PCR was performed to confirm the size of tPA gene. Following agarose gel electrophoresis we observed that the tPA gene was approximately 1689 bp. pLEGFP-N1 was digested with *Sal*I and *Bam*HI to generate a 6891-bp product. pLEGFP-N1-tPA was subsequently generated following the ligation of the digested tPA gene and pLEGFP-N1 fragments using T_4 DNA ligase. The composition of the pLEGFP-N1-tPA was confirmed by sequencing (Figs. 1, 2a–c).

Cell Culture of Purified PT67/pLEGFP-N1-tPA Cell Line

Following the transfection of PT67 cells with pLEGFP-N1-tPA, a single PT67/pLEGFP-N1-tPA cell (Fig. 3) that exhibited strong EGFP was selected. Once the PT67/pLEGFP-N1-tPA cell grew into a bundle, micro-movement was used to isolate PT67 cells that exhibited intense EGFP fluorescence. A purified PT67/pLEGFP-N1-tPA cell line expressing EGFP was successfully cultured and subsequently stored in liquid nitrogen for future use. EGFP was observed in the medium surrounding the PT67/pLEGFP-N1-tPA cells and on the membranes of PT67/pLEGFP-N1-tPA cells. The purified PT67/pLEGFP-N1-tPA cell line gave rise to a high virus titer of 1×10^7 CFU (colony forming units)/mL.

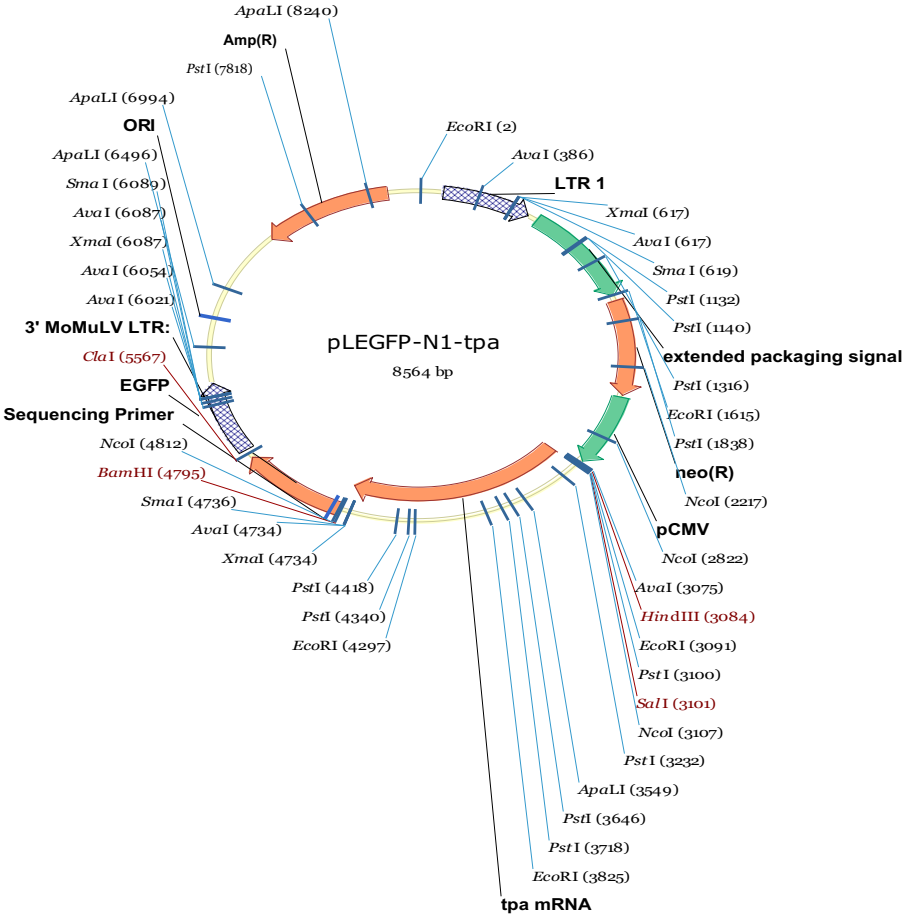

Fig. 1 pLEGFP-N1-tPA diagram

Infection of HeLa Cells with pLEGFP-N1-tPA

pLEGFP-N1-tPA was used to infect HeLa cells and the resultant infection efficiency was 70%. After 24 h EGFP was observed in the medium and on the membranes of HeLa cells when viewed under a fluorescent microscope (Fig. 4a). HeLa cells that were infected with pLEGFP-N1-tPA and expressed EGFP were capable of being passaged for extensive periods. In this study, we passaged the resultant cells for 96 h. Supernatant from HeLa cells/pLEGFP-N1-tPA exhibited obvious thrombolysis in the vitro plasma thrombus plate. Conversely, the supernatant from HeLa cells/pLEGFP-N1 and 10% NCS + DMEM exhibited no thrombolysis in the plasma thrombus plate (Fig. 4c). The tPA activity and tPA concentration in the supernatant from HeLa cells/pLEGFP-N1-tPA were higher than those in the supernatant from HeLa cells/pLEGFP-N1. Furthermore, the tPA activity and concentration were

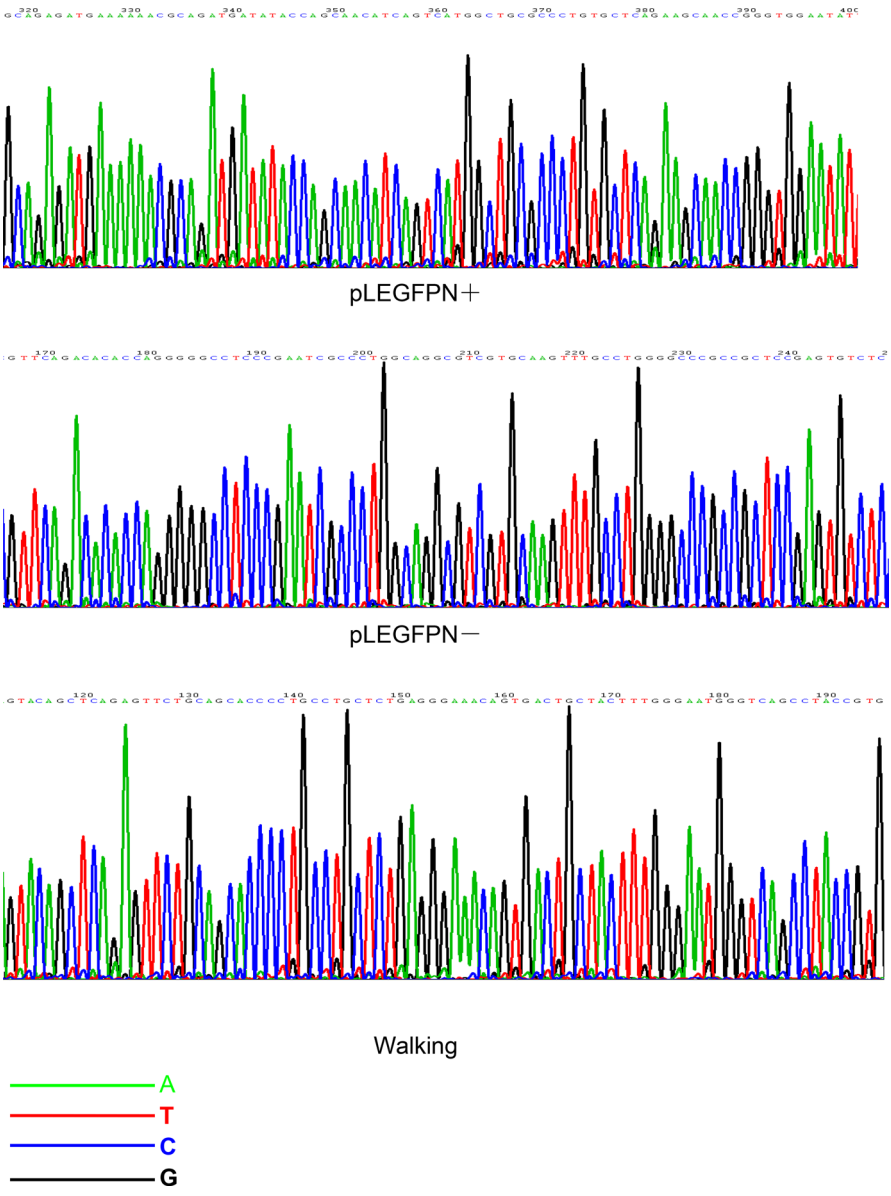

Fig. 2 pLEGFP-N1-tPA sequence confirmation. **a** Sequence of pLEGFP-N1-tPA: pLEGFP-N1+. **b** Sequence of pLEGFP-N1-tPA: pLEGFP-N1−. **c** Sequence of pLEGFP-N1-tPA: walking (middle sequencing. Because pLEGFP-N1-tPA sequence was long, we sequenced pLEGFP-N1+, pLEGFP-N1− and walking)

relatively high and stable in the supernatant of HeLa cells/pLEGFP-N1-tPA at 48 h, 72 h and 96 h (Tables 1 and 2). Western blotting was used to confirm tPA expression in the supernatant from the HeLa cells/pLEGFP-N1-tPA (Fig. 4d).

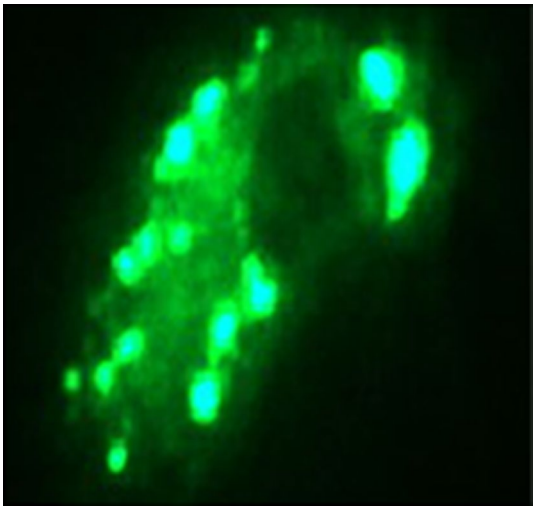

Fig. 3 A single PT67/pLEGFP-N1-tPA cell strongly expressing EGFP was chosen to generate a purified PT67/pLEGFP-N1-tPA cell line

Table 1 tPA activity (U/10^6 cells/24 h) in supernatant from HeLa cells /pLEGFP-N1-tPA at three different time-points ($\bar{x} \pm s$)

Time-points	Times of repeats	tPA activity	*P*
48 h	7	420.16 ± 7.67^{ab}	
72 h	7	417.25 ± 8.20^{c}	> 0.05
96 h	7	422.34 ± 7.93	

tPA tissue-type plasminogen activator

[a](between 48 and 72h), [b](between 48 and 96h), [c](between 72 and 96h), $P > 0.05$, there was no obvious variation in tPA activity in the supernatant from HeLa cells/ pLEGFP-N1-tPA at the three time-points that were analyzed

Table 2 tPA concentration (ng/10^6 cells/24 h) in supernatant from HeLa cells/pLEGFP-N1-tPA and HeLa cells/pLEGFP-N1 ($\bar{x} \pm s$)

Cell type	Times of repeats	48 h	72 h	96 h	*P*
HeLa/pLEGFP-N1-tPA	7	606.27 ± 11.02^{a}	610.22 ± 12.81^{b}	609.75 ± 11.72^{c}	< 0.01
HeLa/pLEGFP-N1	7	50.16 ± 5.32	50.23 ± 5.01	49.78 ± 4.98	

tPA tissue-type plasminogen activator

[a,b,c]Significant variation was observed following comparison of tPA concentration in supernatant from HeLa/pLEGFP-N1-tPA cells and HeLa/pLEGFP-N1 cells at 48 h, 72 h and 96 h, $P<0.01$

Infection of Linx Cells with pLEGFP-N1-tPA

Linx cells were successfully infected with pLEGFP-N1-tPA. The infection efficiency was 70%. After 24 h, EGFP was observed in the medium and on the membranes

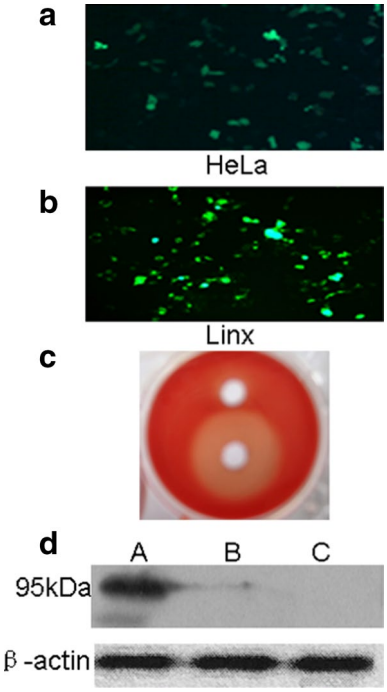

Fig. 4 tPA was successfully transferred into HeLa cells and Linx cells. The transfected cells demonstrated obvious thrombolysis in the plasma thrombus model. **a** pLEGFP-N1-tPA infected HeLa cells after 24 h (note the abundance of EGFP fluorescence). **b** pLEGFP-N1-tPA infected Linx cells after 24 h (once more note the abundance of EGFP fluorescence). **c** The inferior hole of the plasma thrombus model showed a large lysis zone following addition of supernatant of HeLa cells /pLEGFP-N1-tPA or Linx cells/pLEGFP-N1-tPA. No lysis zone was observed for the upper hole following the addition of 10% NCS + DMEM or supernatant from HeLa cells/pLEGFP-N1 or Linx cells/pLEGFP-N1. **d** Western blot analysis confirmed the presence of a 95-kDa exogenous tPA band (tPA, 68kDa + EGFP, 27 kDa). *A* A singular band was observed following analysis of supernatant from HeLa cells /pLEGFP-N1-tPA or Linx cells/pLEGFP-N1-tPA. *B* No tPA band was observed following analysis of supernatant from HeLa cells/pLEGFP-N1 or Linx cells/pLEGFP-N1. *C* No tPA band was observed following analysis of 10% NCS + DMEM. β-actin as utilized as an internal reference

of Linx cells when observed under a fluorescent microscope (Fig. 4b). Linx cells infected with pLEGFP-N1-tPA and expressing EGFP could be passaged in culture for extended periods. For this analysis, the infected Linx/pLEGFP-N1-tPA cells were passaged for 96 h. Supernatant from Linx/pLEGFP-N1-tPA cells exhibited obvious thrombolysis in the plasma thrombus plate, while neither Linx/pLEGFP-N1 cells nor 10% NCS + DMEM showed thrombolysis (Fig. 4c). The tPA levels and associated activity in the supernatant surrounding the Linx/pLEGFP-N1-tPA cells were relatively higher in comparison with the supernatant from the Linx/pLEGFP-N1 cells. Meanwhile, we observed that tPA activity and concentration were high and stable in supernatant from Linx/pLEGFP-N1-tPA cells at 48 h, 72 h and 96 h (Tables 3 and 4). Western blotting was used to confirm the presence of an exogenous tPA band in the supernatant from the Linx/pLEGFP-N1-tPA cells (Fig. 4d).

Table 3 tPA activity (U/10^6 cells/24 h) in supernatant from LinX/pLEGFP-N1-tPA cells at three different time-points ($\bar{x} \pm s$)

Time-point	Times of repeats	tPA activity	*P*
48 h	7	424.26 ± 7.82^{ab}	
72 h	7	421.35 ± 7.69^{c}	> 0.05
96 h	7	423.22 ± 8.13	

tPA tissue-type plasminogen activator

[a](between 48 and 72h), [b](between 48 and 96h), [c](between 72 and 96h), $P > 0.05$, no obvious variation was observed for tPA activity in supernatant from LinX/pLEGFP-N1-tPA cells at the different time-points analyzed

Table 4 tPA concentration in supernatant from LinX/pLEGFP-N1-tPA cells and LinX/pLEGFP-N1 cells ($\bar{x} \pm s$)

Cell type	Times of repeats	48 h	72 h	96 h	*P*
Linx/ pLEGFP-N1-tPA	7	607.28 ± 11.83 [a]	609.17 ± 12.03 [b]	610.23 ± 12.14 [c]	< 0.01
Linx/pLEGFP-N1	7	51.25 ± 5.12	51.07 ± 5.11	52.14 ± 5.23	

tPA tissue-type plasminogen activator

[a,b,c]Obvious variation was observed upon comparison of tPA concentration in supernatant from Linx/pLEGFP-N1-tPA cells and Linx/pLEGFP-N1 cells at 48 h, 72 h and 96 h, $P < 0.01$

Discussion

Previous research demonstrated that when endomembrane of stents was covered with vascular smooth muscle cells that had previously been transfected with constructs containing the tPA gene, high anti-thrombus levels were observed upon transplantation of the stents into animals (Eton et al. 2004). Hussain et al. (2016) investigated whether endovascular therapy combined with intravenous tPA (in appropriately selected patients) administration for large vessel occlusion-related anterior circulation ischemic stroke was superior compared with intravenous tPA administration alone. In a separate study, Ziu et al. (2016) successfully recanalized three patients with cerebral venous sinus thrombosis. The latter procedure was performed by placing a 0.027-in. microcatheter at the proximal portion of the thrombus and infusing 20 mg of alteplase (tPA) dissolved in a liter of normal saline. Alteplase was injected at 100 mL per hour to facilitate an infusion of 2 mg of alteplase per hour for 10 h. Ari et al. (2015) treated seven patients with prosthetic valve thrombosis using a low-dose (25 mg) slow infusion (within 6 h) tPA. If required, thrombolytic therapy (total dose of 150 mg) sessions were repeated until a satisfactory result was achieved. The authors of this study suggested that a low-dose, slow infusion of tPA may be appropriate for bileaflet mitral prosthetic valve thrombosis in relatively stable patients, and may be a better therapeutic option than surgery. Thus, tPA targeted therapy is potentially effective in relation to its anticoagulant effects. pLEGFP-N1 is an amphotropic retroviral system that uses the cytomegalovirus (CMV)

immediate early promoter (Pcmv) and kozak sequence upstream of the EGFP open reading frame to further increase the translation efficiency in eukaryotic cells. Upon expression of EGFP, the exogenous inserted gene is also expressed in infected cells. In the current study, the kozak sequence can also enhance the expression of exogenous tPA within the host cells. Indeed, in a previous study Wang et al. (2013) generated pLEGFP-N1–5HRE-CEAp-TSST-1-linker-CD80TM construct to target tumor cells and the retroviral vector was used for clinical therapy. Furthermore, Everson et al. (2016) suggested that hematopoietic stem cell gene therapy using retroviral vectors is a powerful and promising approach to permanently correct many hematopoietic disorders.

To study tPA targeted treatment of thrombolysis, we generated a pLEGFP-N1-tPA construct. The pLEGFP-N1-tPA construct was subsequently transfected into PT67 package cells. The resultant purified PT67/pLEGFP-N1-tPA cell line resulted in a high viral titer (1×10^7 CFU/mL). When the PT67 cell was grown to 50% confluency in a 60-mm plate, pLEGFP-N1-tPA was transfected into the PT67 cell line. A single PT67 cell that strongly expressed EGFP was observed and marked under the fluorescent microscope. The single cell was permitted to develop into a bundle. The resultant bundle was isolated and cultured continuously to generate the PT67/pLEGFP-N1-tPA packaging cell line. We must ensure that there was no infection when isolating the cell bundle by using a micro-movement technique. A purified PT67/pLEGFP-N1-tPA packaging cell line was cultured for future use. Previous studies have shown that purified packaging cell lines producing high titer retrovirus are important factors in the thorough cleaning of clots. Next, we infected HeLa and Linx cells using supernatant from the PT67/pLEGFP-N1-tPA cell line. Two 6-well plates were subsequently centrifuged for 1 h in a Sigma 11222 centrifuge at 500*g*. A total of 2.5 mL supernatant from the pLEGFP-N1-tPA cell line (we usually pipetted 2 mL into one well of 6-well plate, but we observed 2.5 mL may be more optimal) plus polybrene (10 μg/mL) was pipetted into a single well of a 6-well plate. The addition 2.5 mL into a single well prevents cellular damage after centrifugation. This is since too many cells on the central bottom of one well of the 6-well plate were not covered enough by 2 mL liquid because of the turbulent fluid flow while centrifuging. After centrifuging for one hour, many target cells were damaged. The infection efficiency of target cells was relatively low. Thus, the amount of tPA that was secreted from target cells was limited and there was no obvious thrombolysis in the plasma thrombus plate. However, when 2.5 mL of the packaging virus was pipetted into a single well, all of the cells were sufficiently covered by fluid during centrifugation. The target cells that were infected by pLEGFP-N1-tPA exhibited high infection efficiency. Increased infection efficiency leads to increased secretion of tPA, obvious thrombolysis and thorough thrombus lysis. We also noted that the HeLa and Linx cells should be grown to 40–50% confluency to enhance the infection efficiency. Increased EGFP fluorescence was observed after 24 h of infection. At 48 h, 72 h and 96 h post-infection, there were obvious lysis zones in the plasma thrombus models generated from the supernatant of HeLa/pLEGFP-N1-tPA cells or Linx/pLEGFP-N1-tPA cells. No lysis area was observed for the supernatant from the HeLa/pLEGFP-N1 cells, the Linx/pLEGFP-N1 cells or 10% NCS + DMEM. These results reveal the importance of tPA in this process of thrombolysis and also indicate

the successful transfer of tPA into target cells. Western blot analysis subsequently confirmed the presence of 95-kDa exogenous band for tPA (tPA, 68 kDa + EGFP, 27 kDa). As part of this analysis we observed that the tPA/EGFP fusion protein was stably expressed from early on; associated expression was observed over an extended period. We observed that tPA activity and concentration were elevated at 48 h, 72 h and 96 h. There was very little variation in either tPA activity or concentration at 48 h, 72 h, 96 h ($P > 0.05$). We used an enzyme linked immunosorbent double-antibody sandwich method to measure tPA content. And, we observed significant variation ($P < 0.01$) in tPA concentration between the supernatant of HeLa or Linx cells/pLEGFP-N1-tPA and HeLa or Linx cells/pLEGFP-N1 at 48 h, 72 h and 96 h. In a previous study, we successfully transferred pLEGFP-N1-tPA into target cells such as ECUV (endothelial cell of umbilical vein) cells and heart muscle cells (Gong et al. 2007). We also successfully transferred pLEGFP-N1-tPA into local tissue in an animal inferior vena cava model and in a rabbit left atrium model (Gong et al. 2007, 2010). Both models exhibited successful anti-thrombus activity. These results underpin the potential of tPA-targeted gene therapy in relation to anticoagulation once mechanic valve replacement has been performed.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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