

# Characterization of antimicrobial peptide II37



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## Expression and Characterization of Antimicrobial Peptide LL37 in Dog Peripheral Blood Endothelial Progenitor Cells *In Vitro*

### Highlights

- The lentivirus vector pGC-FU-LL37-GFP was constructed and characterized
- Dog peripheral blood endothelial progenitor cells (EPCs) were successfully obtained
- Expression of LL37 in was achieved by lentivirus infection.
- A combination of the anti-infection effect of LL37 and angiogenesis of EPCs might be achieved to benefit clinical therapy

### Abstract

**Objective** To construct the lentivirus vector expressing antimicrobial peptide LL37, explore the methodology of obtaining endothelial progenitor cells (EPC) from dog periphery mononuclear cells and optimize the infection approach to establish the EPC-LL37 cell line.

**Materials and Methods** Antimicrobial peptide LL37 was expanded, enzyme digested and constructed into PGC-FU vector. And a total of 20 mL of adult dog blood were drawn from femoral artery and peripheral blood mononuclear cells (PBMC) were obtained using Ficoll density gradient centrifugation. After that, EPCs were generated from PBMC by adding cytokine cocktail (VEGF,  $\beta$ -FGF and other growth factors). Then, the LL37 was introduced into EPCs using lentivirus infection. Finally, we used west-blot to examine the expression of LL37 in EPC. **Results** The pGC-FU-LL37-GFP was constructed successfully. Then the virus was produced and measured titer

was  $2.00 \times 10^8$  TU/mL. EPCs were successfully cultured and confirmed by Dil-acLDL and FITC-UEA-1 staining. An approximately 60% infection efficacy was confirmed by immunofluorescence. After optimization, Furthermore, we successfully observed LL37 expression in EPC via western blot analysis.

**Conclusions** GC-FU-LL37-GFP vector was successfully constructed. EPCs could be generated from dog peripheral blood through density gradient centrifugation and cytokine cultivation. The LL37-expressing EPCs were confirmed by western-blot analysis. A combination of the anti-infection effect of LL37 and angiogenesis of EPCs might be achieved to benefit clinical therapy improvement.

**Keywords:** antimicrobial peptide, LL37, endothelial progenitor cells, lentivirus infection

## Introduction

LL37, the only human member in cathelicidin family, is derived from the last 37 amino acid residues of the C-terminus of human cationic antimicrobial peptide 18 and presents a linear structure without a disulfide bridge(1). LL37 is widely distributed in various bodily fluids and tissues, including epithelial cells and innate immune cells, such as neutrophils(1-2). Along with its potent and broad spectrum antimicrobial activity, LL37 also exhibits an important role in regulating various biological processes, such as cell migration, cytokine production, and angiogenesis(3-5) and it is reported to be involved in wound healing, immune response, and neovascularization in injured tissues(6-8).

However, it is still elusive whether LL37 can be applied as a therapeutic agent in cardiovascular related tissue engineering.

Endothelial progenitor cells (EPCs), first isolated from adult peripheral blood (PB) in 1997 (9), have shown to incorporate into foci of physiological or pathological neovascularization(10-11). The finding that EPCs can home to sites of neovascularization and differentiate into ECs in situ is consistent with “ vasculogenesis” which is a critical process involved in both embryonic neovascularization (12) and postnatal neovascular formation in adult organism (13). The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation. Furthermore, many studies have focus on the valuable capability to translate potential of EPC to improve the clinical applicability in the fight against cardiovascular diseases(14-16).

Accordingly, based on these accumulating lines of evidence, we hypothesized that expression small peptide LL37 on EPC can facilitate angiogenesis and may extent a more powerful function in disease treatment. Therefore, it is necessary to establish the LL37 expressed EPCs. Since our long-term aim is to use a canine model to study the cardiovascular diseases, we described here an in vitro culture method of dog EPCs and a lentivirus mediated expression of LL37 on dog EPC.

## Materials and Methods

### Animals and ethics statement

Five healthy adult male Mongrel dogs, weighted from 10. 0-20. 5 kg, were obtained from experimental animal center of WuhanUniversity school of

medicine. The dogs were then intramuscularly injected with Suminaxin II (Containing haloperidol and dihydroetorphine) to achieve the anesthesia and 20 mL peripheral blood were drawn from femoral artery of the dog. The whole protocol in this study including animal anesthesia, peripheral blood (femoral artery) drawing was all approved by the Medical Ethics Committee of Renmin Hospital Affiliated to Wuhan University. All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Wuhan University and fully complied with guidelines issued National Research Council.

Reagents and equipments GC-FU Vector (GeneChem Company, Shanghai, China), Age I (New England Biolabs [NEB], MA, US), In-Fusion™ PCR Cloning Kit (Clontech, CA, US), Plasmid Kit (Promega, WI, US), Endothelial growth medium (EGM-2) (Clonetics, CA, US), Endothelial cell growth medium supplements (EGM-2MV) (Clonetics, CA, US), Fibronectin-Fn (Sigma, MO, US), Trypsin (Gibco, US), FITC-CD34 and APC-CD133 monoclonal antibody (eBioscience, CA, US), Dil-acetylated low-density lipoprotein/Dil-Ac-LDL (Molecular Probe, US), FITC-Ulex europaeus agglutinin (UEA-1) (Sigma MO, US), .

#### Construction of the recombinant lentiviral vector

LL37 gene was expanded, digested and ligated into the lentivirus vector. Then ligation product was transformed into the competent *E. coli*. After growing on LB agar for 16 hr, bacteria clones were selected and the positive clone was confirmed by restriction enzyme digestion.

#### Generation of recombinant lentivirus

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Three-plasmid system including the recombinant LL37 lentivirus vector and 2 auxiliary packaging vectors was employed in the study. All the three plasmids were performed with medium extraction ready for transfection. Then  $1 \times 10^6$  mycoplasma free 293FT cells were seeded in 60mm Petri dish, incubated overnight and transfected with OPTI-medium (Invitrogen, CA, US) by either  $\text{CaCl}_2$  /BES method or Lipofectamine 2000 (Invitrogen, CA, US) following manufacturer's instructions. Transfection medium was replaced with complete medium 8 hr later. The supernatant was collected and concentrated to high titer at 48 hr after culture. Then the lentiviral titer was measured and standardized to meet the criteria of the following experiments.

Isolation of endothelial progenitor cells ( EPCs )from periphery blood of the dog

EPCs were obtained by mononuclear cells(MNCs) isolation from the peripheral blood of the dogs using a density gradient method according to a previously description. Briefly, 20 mL peripheral blood were harvested from femoral artery of the dogs and diluted with PBS at ratio of 1: 1. Diluted blood was then slowly loaded onto the same volume of Ficoll-Lymphocyte separation medium (TBD Corp, Tianjing, China). After centrifugation at 1500 rpm for 30 min, MNCs were collected from the interface and washed with PBS for 2-3 times. Then the MNCs were resuspended in Endothelial growth medium(EGM-2)(Containing VEGF-A, IGF-1, EGF, FGF-2 and ascorbic acid) supplemented with 20% fetal bovine serum, 100 U/mL penicillin, and 100 ug/mL streptomycin, adjusted to  $4 \times 10^6$  / mL and seeded in 6 well plate. The

non-adherent cells were removed by medium change every 3 or 4 days.

When 80% confluence was reached, the adherent cells were passaged for expansion. The number of EPCs and EPC colonies were counted and the cells were harvested for further study.

#### Characterization of the EPCs and EPC colonies counting

At the 7th day of culture, adherent cells were respectively incubated with Dil-Ac-LDL at 37°C for 3 hours and FITC-UEA-1 for 4 hours. Cells exhibiting double fluorescence after fluorescence microscope examination were identified as EPCs. The cells were incubated with von Willebrand factor (vWF), VE-cadherin, CD34 and CD133 primary antibodies, and followed by corresponding secondary antibodies incubation. Flow cytometry analysis was performed to examine the surface marker expression. The cells in 12 randomly selected high-power (100×) microscope fields were counted and cells or colonies per mm<sup>2</sup> were used as EPC numbers or colonies. The inclusion criteria as follows: Spindle-shaped cells and colonies consisted with multiple thin, flat cells emanating from a central cluster of round cells were included.

#### Transfection EPCs with lentiviral vector pGC-FU-LL37-GFP

EPCs were collected by Trypsin digestion and seeded in 6 well plate at a density of  $5 \times 10^4$  cells per well. The cells were allowed to adhere overnight at 37°C 5% CO<sub>2</sub>. The packaged lentivirus ( $1 \times 10^8$  TU/mL) was added into the culture medium and cells were incubated for 48 h. Medium was changed 24 hrs after infection. The expression of GFP on EPCs was confirmed by using

confocal fluorescence microscope at Day 3. When 80% of the GFP positive cells were observed, the EPCs would then be collected for further analysis of the LL37 expression.

#### Western Blot Analysis of antimicrobial peptide LL37 expression in transgenic EPCs

Total protein from EPCs was mechanically homogenized in ice-cold lysis buffer. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane, then the membranes were respectively incubated overnight with the following primary antibodies: Rabbit anti-Cathelicidin (1: 200 dilution), Mouse Anti-GFP (1: 1000 dilution), Mouse anti-GAPDH (1: 5000 dilution). Then the blots were incubated with the following secondary antibodies: Goat Anti-Rabbit IgG (1: 5000 dilution), Goat anti-Mouse IgG (1: 5000 dilution). Quantification of the bands was carried out using software for densitometric analysis.

#### Statistical analysis

Data were expressed as mean  $\pm$  SEM. Multiple group comparison was performed using one-way ANOVA followed by Fisher's Least Significant Difference (LSD).  $P < 0.05$  was considered as statistically significant.

## RESULTS

#### Characterization of Lentiviral vector pGC-FU-LL37-GFP

We confirmed that the lentiviral vector plasmid pGC-FU was integrated with gene LL37 by PCR (Figure 1) and immunofluorescence (Figure 2). In virus titer



determination, a significant difference was found between  $10^{-4}$  uL group and control group but no significant differences between  $10^{-4}$  uL group and  $10^{-5}$  group uL. Therefore, viral particles were existed in  $10^{-4}$  uL group but not in  $10^{-5}$  group. . Supposing there was at least one viral particle in  $10^{-4}$  uL group, the virus titer was:  $2 \times 10^8$  TU/ml  $(1/(10^{-4}) \times 20 \times 10^4) = 2 \times 10^5$  TU/mL uL =  $2 \times 10^8$  TU/mL), and titer was higher enough to perform the further experiment (Table1).

### EPCs Characterization

Primarily cultured MNCs isolated from dog's peripheral blood were allowed to adhere for 24 hours. The adherent cells with round-shaped morphology were early EPCs and they were varied in size after isolation. After culture for 7 days, EPCs exhibited spindle-shaped morphology. Late EPCs were rounder with a pebble-shaped appearance. After culture for 14 days, the adherent cells were nearly reached 100% confluence (Figure 3A-C). Intaking of the Dil acLDL (Red) and UEA-1 lectin (Green) were observed in the early EPCs, which was shown by color merging (yellow color) of the two markers (Figure 3D-F). These results demonstrated that EPCs were successfully isolated and culture-expanded EPCs did not affect their phenotypes.

### Expression of LL37 in EPCs

A approximated 50% of the infection efficacy of lentivirus was achieved after fluorescence microscopy. (Figure 4). LL37 has a molecular weight about 19 kDa. After fused with GFP, the expected fragment size was about 46 kDa.

And the western blot showed a band between the marker sized 36 and 55 kDa( Figure 5), which was consistent with the deduction.

## DISCUSSION

In this study, we firstly constructed the recombinant lentiviral vector pGC-FU-LL37-GFP. Then EPCs were isolated from the periphery blood of the dog. After EPCs characterization, we used lentivirus to infect the dog EPCs to obtain the EPCs expressing LL37. This LL37 expressing EPCs may be employed as cambium in the canine model of ischemia.

Lentiviral vector is derived from human immunodeficiency virus type 1 (HIV-1), and is commonly used as vehicles for gene delivery by making use of interaction between virus glycoprotein and phosphatidylserine(17). Lentiviral vector mediated gene transfer holds great promise to develop a stable cell line for sustaining exogenous gene expression and provide a valuable alternative method for conventional non-viral plasmid transfection (18). We constructed a lentiviral vector to express the recombinant LL37. And a third generation HIV-2 derived lentivirus based 293 T cells was used in viral production.

The innate immune responses are the first line of defense against invading pathogens and it can also trigger the following antigen-specific adaptive immune responses. Antimicrobial peptides (AMPs) are believed as critical effect molecules in innate immunity, which extend their immunomodulatory activity through direct killing of micro-organisms(19). AMPs can be divided into two categories: defensins and cathelicidins. Both of them are reported to involve in the immunopathogenesis of several

infectious diseases. A broad spectrum of antimicrobial effect was exhibited by AMPs. As the only human cathelicidin antimicrobial peptide, LL37 presented in various bodily fluid and tissue, which make it an attractive candidate for potent peptide antibiotic compounds design to overcome postoperative infection problem after long or large tissue injuries(20) <sup>[13]</sup>. As shown by <sup>14</sup>N solid-state NMR studies, LL37 can be formed into dimeric or trimeric aggregates in solution under specific experimental conditions or in lipid bilayers and proteolytic degradation is significantly decreased in the LL37 aggregates, while other native antimicrobial peptides are highly vulnerable to enzymatic degradation(21). The high resolution 3-dimensional structure, membrane orientation and modes of membrane permeation were revealed to elucidate the antimicrobial and other biological properties of this molecule. (22). LL37 and a series of peptides corresponding to different segments of LL37 exhibited potent lytic activities against microbes, red blood cells, and cancer cells. Here, we successfully constructed pGC-FU-LL37-GFP plasmid confirmed by enzyme digestion.

Neovascularization is a crucial step in the development of reconstructed tissue which will be applied to clinical practice. Although some tissue-engineered products such as skin and cornea substitutes have already been in practice, reconstruction of long or large defective tissues remains impossible(23). An extensive vascular system which can provide oxygen and nutrients is needed as the supportive tissue. In order to achieve the fairly uniform distribution of microvessels inside tissue-engineered organoids, newly formed microvessels should penetrate deep inside the organoids(24). Endothelial progenitor cells (EPCs) are precursor cells that can proliferate

and differentiate into vascular endothelial cells, participating in the reconstruction processes after endothelial injury. After vascular injuries, circulating EPCs can be recruited to the injured vessel and differentiate into vascular endothelial cells, accelerating the repair of the injured vascular endothelium(25). In our study, we successfully isolated and cultured EPCs from dog's peripheral blood, which showed a number of advantages than using the BM to generate the EPCs.

In conclusion, we here tried to generation novel EPC product by combining the features of antimicrobial peptide LL37 and EPCs. We successfully obtained a LL37 expressed EPCs which can be used in the following in vivo study of tissue reconstruction. The results demonstrated that the recombinant lentiviral vector of LL37 can be effectively tranfected into cultured EPCs obtained from the adult dog's peripheral blood. The genetically modified EPCs we constructed might be used toin making up newly materials in tissue engineering and may benefit the vascularization and anti-infection abilitiesin long or large segment defect treatment. However, long-term studies especially animal experiments need to be conducted before applying these cells into human study.