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The Transfection Efficiency Improvement of hrDNA Targeting Vectors With NLS Peptide*

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Abstract Human ribosomal gene (hrDNA) targeting vectors (10～14 kb) constructed by the group are novel non-viral vectors which could specifically integrate into the ribosomal loci and characterized by their security and stable expression of therapeutic genes. However, the low transfection efficiency handicapped their clinical application. Although nuclear localization signals (NLS) could facilitate the nuclear entry of non-viral vectors and improve the transfection efficiency, the expression of therapeutic gene varied dramatically with the coupling methods and the type of chemistry used. The hrDNA vectors were conjugated by succinimidyl-[4-(psoralen-8-yloxy)]butyrate (SPB) with Simian Virus 40 NLS (SV 40 NLS) peptide through electrostatic interaction efficiently, which could protect the plasmid DNA(pDNA) from degradation of DNase. The polyethylenimine (PEI), which is an economical and low toxic polymer and wildly used *in vivo* gene therapy, was employed to transfect the primary human dermal fibroblasts (HDF). When conjugated with NLS peptide, the 12 kb hrDNA emerged in the nucleus within 60 min under the view of confocal microscopy. The GFP fluorescence analysis by flow cytometry showed that the transfection efficiency was increased to 4～5 folds. In conclusion, an effective procedure was developed to improve the non-viral transfection efficiency and promise the preclinical trial of hrDNA vectors.

Key words nuclear localization signal peptide, human rDNA vectors, transfection efficiency, human dermal fibroblasts, gene therapy, polyethylenimine
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Non-viral vectors are more advantageous than viral vectors for the security, ease of preparation and mild immunogenicity, however, the transient expression of therapeutic gene hindered their clinical utilization. The human ribosomal gene (hrDNA) targeting vectors developed by our group could integrate into the ribosomal DNA (rDNA) loci at a relative high recombination frequency (10^{-5}～10^{-4}) [1] and express the therapeutic genes stably. We have obtained the site-specific integrated cell lines, such as HT1080[1,2] and HL7702[3]. And the integrated FⅧ gene of hemophilia A could stably express for years. Human dermal fibroblasts (HDF) is one of the ideal targeting cells for *ex vivo* therapy because of its convenient acquisition and culture[4], however, the low transfection efficiency by hrDNA vectors greatly impede its preclinical trial.

Nuclear membrane is the most important barrier for the nuclear entry of exogenous DNA. Micromolecules (40～60 ku) of less than 9 nm in diameter could enter into the nuclear passively independent of energy while the macromolecules such as nucleophilic protein must be mediated by the nuclear localization signal (NLS) with energy [5]. Generally, the plasmid DNA (pDNA) could not enter into the nucleus until the membrane collapsed during cell division. Therefore, most of the pDNA translocated into the cytoplasm would be destroyed by the nuclease in either lysosome or cytoplasm prior to its entry into the nucleus and less than 1% pDNA overcame the barrier eventually[6]. The result could be worse when the size of pDNA is larger than 10 kb (> 700 ku) which could be sequestered in the cytoplasm for a longer time[7] and the transfection

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efficiency declines dramatically[8]. Therefore, sequestration of the cytoplasm and nuclear membrane might contribute to the low transfection efficiency of hrDNA vectors which are 10～14 kb in size, especially in the primary or non-dividing cells.

Recently, many kinds of NLS peptide of nucleophilic protein from virus are recruited to facilitate the nuclear uptake of pDNA [9], such as the short basic amine acids-rich peptide PKKKRKV of T antigen of Simian Virus 40 (SV40) [10], YGRKKRRQRRR [11] of the trans-acting activator of transcription(TAT) domain of human immunodeficiency virus-1 (HIV-1) and the GNQSSNFGPMKGGNF - GGRSSGPYGGGGQYFAKPRNQGGYGGC [12, 13] of M9 derived from the heterogeneous nuclear ribonucleoprotein (hnRNP) A1. However, the transfection enhancement of NLS varied dramatically with the coupling methods and the type of chemistry used[14]. NLS peptide coupled the pDNA either through the electrostatic interaction or covalent conjugation. Although the pDNA conjugated by NLS by covalent bond could be accumulated into the nucleus efficiently, the transfection efficiency of the therapeutic gene was not enhanced significantly [15, 16]. Whereas, the NLS coupled with pDNA *via* weak electrostatic interaction might be useless for the dissociation of the NLS from pDNA [9]. SPB (succinimidyl-[4-(psoralen-8-yloxy)]butyrate) that is a kind of DNA intercalating reagent could easily conjugate to α-amine of the first amine acid of NLS (termed SPB-NLS) during the peptide synthesis. The feature that SPB do not interfere with the gene expression makes it an ideal coupling agent between NLS and pDNA [17]. Therefore, we investigated whether the NLS could improve the transfection efficiency of hrDNA vectors in primary HDF.

1 Materials and methods

1.1 Materials

DMEM (Dulbecco's modified Eagle's medium) with high glucose, FBS (Fetal Bovine Serum), P/S (Penicillin-Streptomycin) were purchased from Gibco and pEGFP-N1, Lysosensor Green DND189 were obtained from Invitrogen. The hrDNA vectors pHrneo(10 kb), pHrnGFP(12 kb) were constructed by our group. SV40 NLS SPB-GGGPKKKRKV (4.4 mg, molecular mass: 1 325.6, purity was higher than 96%) was synthesized by Shanghai Science Peptide Biological Technology Co. LTD (Shanghai, China). Briefly, the N-Hydroxysuccimide group of SPB(50 mg, obtained from Pierce) substituted the α-amine group of the first Glycine at N-terminal and then the other amino acids were subsequently coupled according to the peptide synthesis protocol of the company. Branched PEI (25 ku), HEPES and Hoechst33258 were bought from Sigma, DNase Ⅰ was obtained from Takara and Cy3 Label IT Tracker Kit was from Mirus.

1.2 Agarose gel electrophoresis mobility shift assay

One microgram pDNA was dissolved in 20 μl HEPES solution (20 mmol/L, pH7.4), then mixed with NLS peptide (NLS/DNA molar ratio = 1×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 respectively) and incubated for 10 min before running electrophoresis on 0.6% agarose gel.

1.3 DNase Ⅰ protection assay

SPB-NLS/pHrnGFP (molar ration = 1×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 respectively, pHrnGFP= 1 μg) complexes prepared same as 1.2 were digested by DNase Ⅰ at 37℃ for 3 min or 10 min separately. Reactions were stopped by heating it at 80℃ for 10 min. Then electrophoresed the digestion products of SPB-NLS/ pHrnGFP complex on 1% agarose gel with the coiled pDNA as control.

1.4 Isolation and culture of HDF

Primary HDF was isolated from human foreskin of 3 different person discarded in the surgery with permission. Briefly, the corium was diced to less than 1 mm × 1 mm pieces after trimming away the subcutaneous fat and cutex with sterile scalpel. The pieces then were scratched on the bottom of 25 mm^2 flask for 12 h to adhere at 37℃, 5% CO_2 incubator. At the end of adhesion, 5 ml Amino-Max Ⅱ (Gibco, Invitrogen) each flask was added to maintain cell culture. 1～2 weeks later, confluent HDF (termed passage 0) migrated from the corium species was digested with 0.25% trypsin (0.02% EDTA) and transferred to new flasks to culture in DMEM supplemented by 10% FBS. HDF of passage 3～4 was used for transfection experiment.

1.5 Analysis of transfection efficiency with flow cytometry

HDF was seeded into 12-well plate (5×10^4/well) 24 h before the transfection. The transfection solution was prepared as follow: briefly, 2 μg plasmid was dissolved in 100 μl HEPES(20 mmol/L, pH7.4), mixed with NLS(NLS: pHrnGFP molar ratio = 5×10^3, 1×10^4, 2×10^4, 4×10^4, separately) and incubated for 10 min at

room temperature. At the end of incubation, added 2.08 μl PEI(1 g/L, N/P = 8) into the solution, mixed immediately and left to further incubate for 30 min. After incubation, the transfectation solution was added to cells for 48 h.

The HDF was analyzed by Flow Cytometry (BD FACScalibur) 48 h after the PEI transfection. HDF transfected with pHrneo was used as the control to define the natural fluorescence limitation and thus to evaluate the transfection efficiency. Ten thousands individual cells each sample were used for statistical analysis with BD cytometry software.

1.6 Investigation of plasmid subcellular location with confocal microscopy

Plasmid was labeled by Cy3 (Cy3 ∶ pHrnGFP = 0.25 ∶ 1, *v* ∶ *w*) using the Cy3 Label IT Tracker Kit following the manufacturer's recommended protocol prior to transfection. HDF was seeded on cover slides 24 h before the transfection (4×10^4 cells in each 24-well-plate well). During the transfection, Lysosensor Green DND 189 (1 μmol/L) was added for 30 min to indicate the low-pH lysosome under the microscopy view. At the end of 60 min transfection, HDF was fixed by 4% paraformaldehyde and counterstained with Hoechst33258. Then the subcellular location of plasmid was investigated with Confocal Microscopy (Leica TCS SP5).

1.7 Nuclear pore complex blockage assay

Nucleocytoplasm transport can be inhibited specifically by wheat germ agglutinin (WGA) [18]. pHrnGFP was labeled by Cy3 using the Cy3 Label IT Tracker Kit following the recommended protocol prior to transfection. WGA (100 mg/L) was added to the culture media during the 2 h-transfection. At the end of transfection, HDF was fixed by 4% paraformaldehyde and counterstained with Hoechst33258.

1.8 MTT cytotoxicity assay

Cytotoxicity of NLS to HDF cells was estimated by the MTT assay. Briefly, HDF was seeded in 96-well culture plate (4×10^3 cell/well) 24 h before transfection. PEI-NLS/pHrnGFP complex (NLS ∶ pHrnGFP molar ration at 0, 1×10^3, 5×10^3, 1×10^4, 2×10^4, 4×10^4 respectively) was added into wells for 48 h. At the end of transfection, aspirated the media, added 20 μl MTT dye (5 g/L) to each well and further incubated for 4 h at 37℃, 5% CO_2 condition. At the end of incubation, 100 μl DMSO was added to each well to dissolve the insoluble purple formazan dye. The cell viability was determined by a Microplate Reader (Bio-rad MT550) at 570 nm relative to control.

1.9 Statistical analysis

The experimental data were compared by SPSS (13.0) using One-Way analysis of variance and individual group differences were considered significant when $P < 0.05$.

2 Results

2.1 NLS decreased electrophoresis mobility of plasmid in agarose gel

The electrophoresis mobility of plasmid in the agarose gel was retarded when conjugated with NLS for the increased molecular mass as well as the declined net charge of the complex (Figure 1). The retardation was not obviously until the molar ratio of NLS ∶ pHrnGFP increased to 5×10^3. And as the molar ratio increased to 2×10^4, the NLS/pHrnGFP complex was totally immobilized in the gel which might indicate that the plasmid was capsulated by NLS thoroughly.

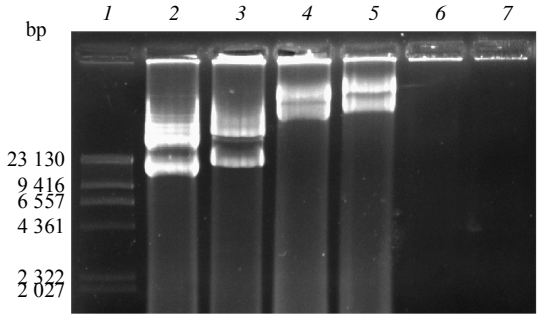

Fig. 1 Agarose gel electrophoresis mobility shift assay
1: λ/*Hin*dⅢ Marker; *2*: pHrnGFP plasmid; *3*～*7*: Denoted that the molar ratio of SPB-NLS/pHrnGFP were 1×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 respectively.

2.2 NLS protection assay

Plasmid translocated into cells could be degraded by nucleases of the lysosome and cytoplasm [6]. The protection capability of SPB-NLS to plasmid from nuclease was emulated with DNase Ⅰ which is a commercial analogue of endogenous nuclease. The pattern of DNase Ⅰ digestion showed that NLS (molar ratio > 2×10^4) could protect the plasmid from degradation for a short time (Figure 2). When the digestion time increased to 10 min, the plasmid was completely diced into fragments less than 500 bp (Figure 3) which indicated the protection ability of NLS to plasmid DNA was limited.

2.3 NLS improved the transfection efficiency significantly

Compared transfection efficiency of pHrnGFP/

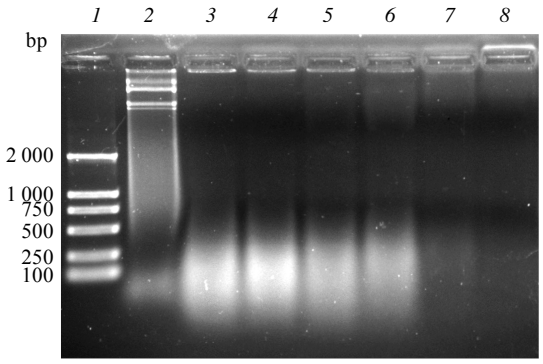

Fig. 2 DNase Ⅰ protection assay (digested for 3 min)

1: DL2000 marker; *2*: Coiled pHrnGFP plasmid; *3*: Digestion product of pHrnGFP; *4*～*8*: Demonstrate the digestion product of NLS/pHrnGFP complex at molar ratio 1×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 respectively.

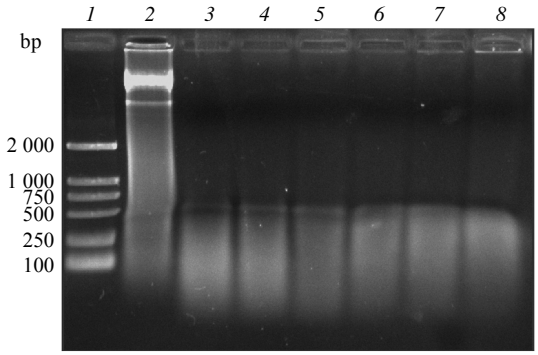

Fig. 3 DNase Ⅰ protection assay (digested for 10 min)

1: DL2000 marker; *2*: Coiled pHrnGFP plasmid; *3*: Digestion product of pHrnGFP; *4*～*8*: Demonstrate the digestion product of NLS/pHrnGFP complex at molar ratio 1×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 respectively.

NLS complex at different molar ratio as shown in Figure 4, a significant difference was observed when the molar ratio increased to 2×10^4. And the transfection efficiency was improved 4～5 folds, ranging from 3% to 4%. However, the transfection efficiency was slightly declined when the molar ratio boosted 2-fold to 4×10^4. This phenomenon might due to the stereospecific blockade of transcription factors by excessive NLS peptide[16].

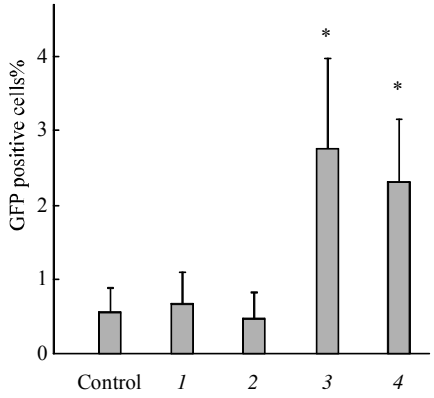

Fig. 4 Comparison of transfection efficiency of pHrnGFP/NLS at different molar ratio

Control inferred that transfection efficiency of pHrnGFP conjugated without NLS; *1*, *2*, *3*, *4* denoted that transfection efficiency of pHrnGFP conjugated with NLS at molar ratio = 5×10^3, 1×10^4, 2×10^4, 4×10^4 respectively. (values are $\bar{x}\pm s$, $^*P<0.05$).

2.4 NLS facilitated the nuclear entry of plasmid

The translocation of plasmid into nucleus promoted by NLS was confirmed by the confocal microscopy. Compared with plasmid without NLS

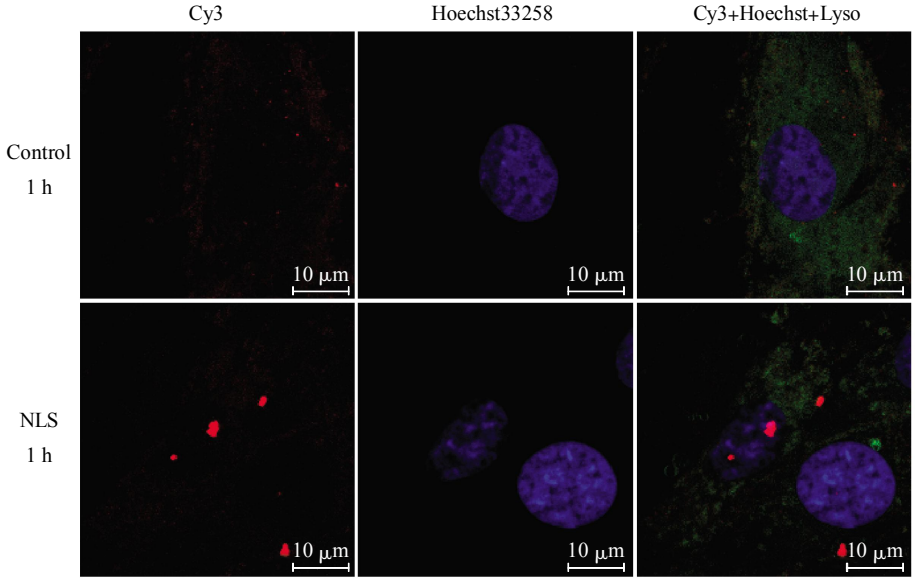

Fig. 5 Subcellular localization of pDNA

Control(upper panel) indicated HDF 1 h post-transfected with pHrnGFP; NLS(lower panel) referred to HDF 1 h post-transfected with NLS/pHrnGFP at molar ratio= 2×10^4.

conjugation, more red signals of Cy3 which indicated the presence of plasmid were observed in nucleus of the cells transfected with NLS/pHrnGFP complex 1h post-transfection(Figure 5). And we also found that the distribution pattern of plasmid indicated by red did not matched with the lysosome referred by green (stained with pH-sensitive dye Lysosensor Green DND189), which verified the "proton sponge" [19] of PEI facilitated the release of plasmid to cytoplasm from endosome or lysosome. The bigger and brighter red dots of NLS group showed that NLS might promote more plasmid into nucleus by condensing more plasmid together.

2.5 NLS facilitated nucleus uptake of plasmid through NPC

WGA inhibits the nulceocytoplasm transport by cross-linking the nuclear pore complex (NPC) specifically [18]. We found that the pDNA was totally excluded from the nucleus when the NPC was blocked by WGA during transfection (Figure 6), which confirmed the fact that NLS/pDNA complex was imported into nucleus through NPC [20] and the peptide we recruited was functional as nuclear localization signal.

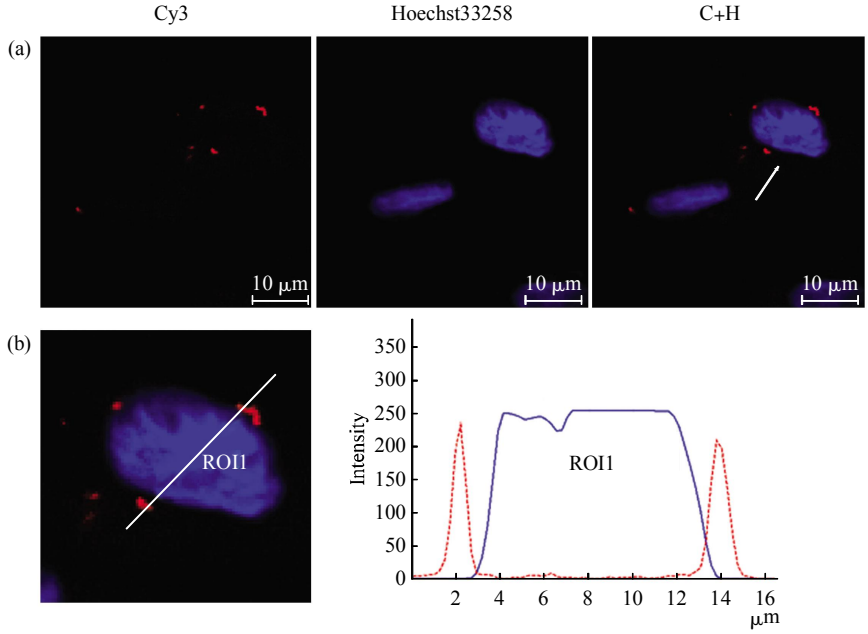

Fig. 6 WGA blockage of nuclear entry

(a) HDF 2h post-transfected with NLS/pHrnGFP complex. (b) Analysis of fluorescence intensity of cell indicated by yellow arrow in A (ROI1) (blue line referred to fluorescence intensity of Hoechst33258 while red dashed line denoted fluorescence of Cy3).

2.6 MTT cytotoxicity

Cytotoxicity of NLS/pHrnGFP was measured by MTT assay as increasing the amount of NLS as shown in Figure 7. At the end of 48 h incubation, the viability of HDF was slightly declined when the molar ratio of NLS/pHrnGFP was less than 1×10^4 compared to those in the absence of NLS. We presumed that the toxicity was mainly attributed to PEI when the amount of NLS was low. A significant difference was observed when the molar ratio of NLS/pHrnGFP increased to 2×10^4 which might due to the DNA damage by excessive intercalating molecule SPB[21].

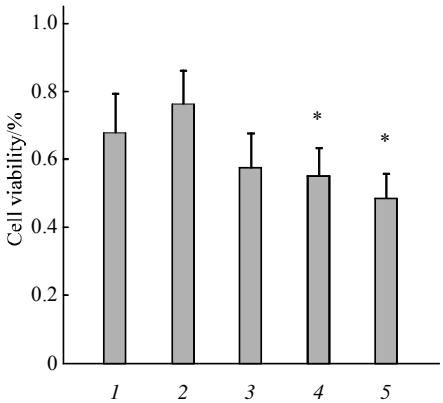

Fig. 7 Cytotoxicity of transfection

1: Cell viability of HDF transfected with pHrnGFP without conjugation of SPB-NLS; *2*～*5*: Indicated the cell viability of HDF transfected with pHrnGFP conjugated with SPB-NLS at molar ratio of 5×10^3, 1×10^4, 2×10^4, 4×10^4 respectively. (values are $\bar{x} \pm s$, $^*P < 0.05$).

3 Discussion

NLS could facilitate the nuclear entry of plasmid DNA by binding to the receptors of the nuclear pore complex and the exact mechanisms are still unclear. Some groups found exogenous DNA could not be translocated into nucleus even when conjugated with NLS[22, 23]. While others verified the nuclear uptake of NLS-conjugated plasmid, there was no transfection enhancement of gene coupled with NLS covalently[16]. These studies showed that the effect of NLS on transfection efficiency fluctuated wildly as the coupling methods and chemistry varied. Even employed by the same strategy of conjugation between NLS and plasmid, the consequence of Zanta[10] and MA van der Aa[22] came out differently which might caused by trivial experimental setup. In our study, we achieved the nuclear entry of 10 kb plasmid within 60 min as well as the transfection enhancement up to 4～5 folds in the presence of SPB-NLS peptide. And we also confirmed the repeatability of our procedure in HeLa cells (unpublished data). Since the PEI is an economical and low toxic reagent which is wildly used in *in vivo* gene therapy, the method we developed might be beneficial for its application in preclinical research. Recently, many site-specific coupling approaches have been developed to avoid the transcription inhibition of therapeutic gene caused by NLS conjugation, such as PNA (peptide nuclear acids)[24], TFO (triple helix formation oligonucleotide)[25], DNA recognition domain of restriction enzyme[26]. All these methods are not wildly used because of their disadvantage of high cost and laborious preparation. The conjugation procedure we developed is fast-to-use, economical and convenience, therefore, we suggest that the SPB-NLS could be generally employed to improve the transfection efficiency of the non-viral gene delivery.

It is still challenging to unveil the mechanisms that how NLS could facilitate the nuclear entry of plasmid in spite of many researchers focused on nucleosytoplasm transport[27]. In the present study, we found that there might be different mechanisms of how NLS contributed to the enhancement of the transfection efficiency. Firstly, NLS might prevent the plasmid from nuclease degradation so as to extend its half-life in the cytoplasm or endosome prior to nuclear entry. Secondly, the condensation of plasmid by NLS could accumulate more plasmid inside the micelles formed by PEI to increase copies of plasmid translocated into cells. We also found part of plasmid was detained in the cytoplasm which might be due to the inert of NLS when degraded or dissociated with plasmid. The exact mechanisms of the NLS need to be further explored.

Compared to their viral counterpart, the biggest drawback of non-viral vectors is low transfection efficiency, especially in primary and non-dividing cells. And the transfection efficiency declined dramatically when the size of plasmid is up to 10 kb[8]. Therefore, it is difficult for the genetic modification of primary cells as the two long homologous arms of gene targeting vector extended its size over 10 kb. The procedure developed in the present study might accelerate the preclinical research of hrDNA vectors, which is 10～14 kb in size. On the other hand, the DNA intercalating reagent SPB could cause double strand breaks (DSB) of DNA when exposed to UVA (ultraviolet A radiation) to invoke the homologous recombination repair pathway[28, 29]. Therefore, the SPB-NLS might also increase the recombination frequency of gene targeting vectors when combined with UVA treatment.

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核定位信号肽提高核糖体区打靶载体转染效率的研究 *

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摘要 核糖体区打靶载体(10～14 kb)是中南大学医学遗传学国家重点实验室构建的一种具有定点整合能力的非病毒载体，具有安全性好及长期稳定表达的特点，但是较低的转染率成为其应用于临床的主要障碍．核定位信号肽可以促进非病毒载体进入细胞核，从而提高其转染效率．但是核定位信号肽提高外源基因表达的能力却严重受到其与 DNA 偶联方式及偶联剂的影响．采用偶联剂 SPB 可以通过静电作用将核糖体区打靶载体与 SV40 核定位信号肽有效结合，并且可以防止其被 DNase 降解．应用聚乙烯亚胺转染人原代皮肤成纤维细胞后，激光共聚焦显微镜观察显示，核定位信号肽可以在 60 min 内携带 12 kb 的质粒 DNA 进入细胞核．转染后 48 h，流式细胞仪检测 GFP 表达，结果显示转染率提高了 4～5 倍．聚乙烯亚胺是一种毒性小，而且价格低廉的高分子转染试剂，广泛被应用于体内基因治疗的研究中，上述研究将会促进核糖体区打靶载体在临床基因治疗中的应用．

关键词 核定位信号肽，核糖体区打靶载体，转染效率，原代皮肤成纤维细胞，基因治疗，聚乙烯亚胺
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