Expert Opinion

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Nuclear targeting of viral and non-viral DNA

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The nuclear envelope presents a major barrier to transgene delivery and expression using a non-viral vector. Virus is capable of overcoming the barrier to deliver their genetic materials efficiently into the nucleus by virtue of the specialized protein components with the unique amino acid sequences recognizing cellular nuclear transport machinery. However, considering the safety issues in the clinical gene therapy for treating critical human diseases, non-viral systems are highly promising compared with their viral counterparts. This review summarizes the progress on exploring the nuclear traffic mechanisms for the prominent viral vectors and the technological innovations for the nuclear delivery of non-viral DNA by mimicking those natural processes evolved for the viruses as well as for many cellular proteins.

Keywords: gene delivery, importin, non-viral vectors, nuclear localization signal, nuclear pore, nuclear transport, transgene expression, viral vectors

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1. Introduction

Delivery of functional DNA into mammalian cells is a fascinating approach for treating critical human diseases precisely and effectively. Among the existing DNA carriers, viral vectors are efficient tools as they possess natural mechanisms to enter cells, escape endosomes and finally transport their DNA into the cytoplasm. However, they also pose serious risks in terms of immunogenicity and carcinogenicity when administered to patients. On the other hand, non-viral synthetic vectors lack these disadvantages, but poor efficiency in transgene delivery limit their implementations in gene therapy programs at present [1-3]. Following endocytosis of a plasmid DNA in the non-viral route, the DNA must be released into the cytosol from the endosomes before nuclear transport for protein expression. However, plasmid DNA disappears with an apparent half-life of 50 – 90 min from the cytoplasm because of the action of cytosolic nucleases [4]. Microinjection of plasmid DNA into the nucleus produced much higher gene expression than microinjection of the same plasmid DNA into the cytosol, indicating that nuclear membrane is one of the major hurdles to gene transfer using a non-viral system [5-13].

In dividing cells, although the nuclear envelope disassembles on a regular basis offering an opportunity for DNA to enter the nucleus, the DNA that is waiting in the cytoplasm for the next cell division is sensitive to degradation by the nucleases. In non-dividing cells, the nuclear membrane always restricts the transport of non-viral DNA into the nucleus.

The nuclear membrane pore (NMP) [5] has an internal channel diameter of ~ 25 nm [14], which generally prevents naked DNA crossing effectively into the nucleus. However, the NMP permits passive transfer of gold particles < 9 – 10 nm in diameter and linear DNA fragments up to ~ 300 bp [15-18] as well as signal-facilitated transport of proteins and small DNA segments (up to ~ 1 kbp) [9,18-21]. Signal-mediated nuclear import is now well established for the proteins possessing

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nuclear localization signal (NLS). While the monopartite NLS resembling that of the SV40 large tumour antigen (KKKRKV) consists of a cluster of basic amino acid residues preceded by a helix-breaking proline residue [22,23], the bipartite NLS representing that of the Xenopus phosphoprotein nucleoplasmin (KRPAATKKAGQAKKKK) is made up of two clusters of basic residues separated by 9 – 12 residues [24]. The basic NLS of a cargo binds in cytoplasm to importin-α, which in turn binds to importin-β responsible for docking the cargo to NMP by specifically binding to a subset of hydrophobic phenylalanine-glycine-rich repeats of NMP proteins [25,26]. Directionality of translocation across the NMP is subsequently determined by the nucleotide-bound state of the small GTPase Ran. Ran binds the cytoplasmic importin/NLS cargo when it is in a GDP-bound form and exchanges its GDP for GTP upon entering the nucleus, allowing complex dissociation. Ran-GTP is then returned to the cytosol in a complex with an exportin/NES (nuclear export signal) cargo and the GTP is hydrolysed to GDP for another round of import [27].

Since viral vectors are very efficient in overcoming the nuclear barrier, an understanding of the mechanism related to the nuclear transport of viral DNA would help to design strategies for smart delivery of non-viral DNA across the nuclear membrane.

2. Nuclear delivery of viral DNA

To replicate and maintain their genomes, most eukaryotic DNA viruses and some RNA viruses have developed highly efficient strategies to gain access to the nucleus of their host cells via NMP. The size and shape of viral nucleocapsids seem to play a key role in determining the principal mechanisms by which viral genomes are translocated through the NMP and delivered into the nucleus of a non-dividing cell [28,29]. Intact virions with a diameter smaller than the maximum diameter of the NMP, such as hepatitis B virus, can either translocate through the NMP by recruiting appropriate nuclear import receptors or undergo conformational changes that allow their outer surface to interact with channel components [29], thus resulting in the release of the genome in the nucleus. On the other hand, virions or subviral particles significantly larger than the maximum diameter of the NMP, have to partially disassemble or uncoat themselves either in the cytosol or after docking at the NMP before translocation of their genomes into the nucleus [30].

2.1 Adenoviruses

Adenoviruses are large non-enveloped DNA viruses (70 – 100 nm in diameter) with an outer protein shell composed of 12 pentonal complexes with each containing a base and a projecting fibre, and 240 hexon subunits. After sequential binding to the cell surface CAR receptors and the fibronectin-specific integrins through the fibre and the penton base protein respectively, the virus is internalized by receptor-mediated endocytosis and rapidly released to the cytosol after lysis of ensosomal membrane. Having reached the cytosol, the adenovirus encounters a viscous, crowded cytosol that acts as a barrier, preventing diffusion to the nucleus. To accomplish intracellular translocation, the viral capsid interacts with cytosolic molecular motor (dynein) which drives capsid motility rapidly along microtubule through the cytosol [31-35]. Cytoplasmic dynein can only generate movement towards the minus end of microtubules, which is usually located adjacent to the nucleus at the microtubule organizing centre (centrosomes) [36]. While some reports indicated that importin and transportin are required for adenovirus nuclear import in permeabilized cells [37,38], other findings suggest that the nuclear traffic is independent of the cytosolic factors and the binding of nuclear pore protein, CAN/Nup214 with the adenovirus capsid [39,40] is implicated in the process with collaborations of the molecular chaperone, Hsc70 and histone H1 required for the disassembly of the capsid and release of the adenovirus genome for final nuclear entry [37,40].

Among other DNA viruses, parvoviruses being only 18 – 24 nm in diameter [41] can easily pass through the NMP without capsid disassembly or deformation by virtue of an NLS present in the minor capsid protein VP1 [42,43], while herpesviruses (120 nm in diameter) dock the nuclear membrane in an NLS-dependent way before the disassembly of capsids, facilitating DNA transport across the NMP [27].

2.2 Retroviruses

Retroviruses are enveloped RNA viruses (~ 100 nm in diameter) consisting of a lipid bilayer, RNA and proteins, including gag proteins as the major components of the viral capsid with about 2000 – 4000 copies per virion, protease to function in proteolytic cleavages during virion maturation, pol proteins responsible for synthesis of viral DNA from RNA and integration into host DNA after infection and env proteins with a role in association with entry of virion into the host cell. Retroviral replication in target cells begins with binding of the virion envelope glycoprotein to the reciprocal cell surface receptor. With most retroviruses, the virion membrane fuses with the cell plasma membrane, releasing the internal core of the virion into the cell cytoplasm. The viral RNA genome in the core is reverse transcribed into cDNA. The completed viral cDNA intermediate in a matured complex termed the preintegration complex (PIC) subsequently translocates into the nucleus and the viral cDNA integrates into the host genome. The ability of incoming retroviral PICs to cross the nuclear membrane differs between lentiviral and simple retroviral vectors. The PICs of the simple murine leukemia virus (MLV) only enter the nucleus during mitosis and, thus, cannot infect non-dividing cells. By contrast, lentiviruses such as human immunodeficiency virus type 1 (HIV-1) infect dividing and non-dividing cells, like terminally differentiated macro-phages [44-46]. The nuclear import of HIV-1 PICs might be attributed not only to the

existence of NLSs in the viral proteins that comprise PICs [47], but also to the other factors including the viral central DNA 'flap' structure formed during reverse transcription [48], the viral CA protein [49,50] and the cellular proteins, importin 7 [51] and nucleoporin [52]. Recently, transportin-SR2 has been shown to faciliate PIC nuclear import after binding to HIV-1 integrase [53,54]. However, the concrete pathway of HIV nuclear entry is debatable [55-58]. Despite the large size of HIV-1 PIC probably exceeding the limit for NMP transport, it is possible that the lentiviral PIC unfolds at the nuclear envelope allowing the viral cDNA to move through NMP in a similar way to adenovirus or herpes virus [27,29].

Among other retroviruses, avian sarcoma virus (ASV) and foamy virus (FV) also enter the nucleus of non-dividing cells. Like HIV-1, an NLS in ASV IN protein is speculated to mediate PIC nuclear import [59-61], while the factors responsible for the nuclear translocation of FV PICs are still unknown.

3. Different approaches for nuclear delivery of non-viral DNA

Synthetic approaches based on liposomes, polymers, peptides and inorganic particles are extremely inefficient compared with the viral vectors in terms of the exogenous protein production per plasmid copy. The major hurdles in a non-viral route is the lack of a nuclear targeting device to facilitate rapid nuclear transport of plasmid DNA. Following endocytosis across the plasma membrane and escape from the endosomes, the non-viral DNA either in free or carrier-bound forms must pass through the cytosol to recognize the NMP for subsequent nuclear translocation. Significant efforts in the last decade have enabled designs of various new approaches for nuclear targeting of non-viral DNA.

3.1 Nuclear delivery of nuclear localization signal-conjugated DNA

Since most of the non-viral DNA carriers, especially liposomes, are known to release DNA in the cytosol after being fused with the endosomal membrane, one fascinating approach is to couple NLSs to plasmid DNA for its nuclear targeting [62]. Crosslinking agents such as cyclo-propapyrroloindole [9], sulpho-MBS (3-maleimidobenzoyl-*N*-hydroxysuccinimide ester) [63] and 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC) [64], have been used to associate NLSs to DNA. Conjugation of an NLS peptide (PKKKRKVEDPYC) via sulpho-MBS to a closed linear plasmid DNA being capable of expressing hepatitis B surface antigen (HbsAg) led to a 15-fold enhancement both in the priming of antibody responses to HbsAg after intramuscular injection and the transfection efficiency *in vitro* [63]. Transgene expression could be increased by 1000-fold in dividing HeLa and 3T3 cells and 10 – 30-fold in non-dividing macrophage and dorsal root ganglion neurons with a single SV40 Tag NLS peptide (PKKKRKVEDPYC) being attached via SMCC to a capped 3.3-kbp CMV luciferase linear DNA fragment (CMVLuc-NLS) [64]. Lipofection of non-dividing aortic endothelial cells with the conjugate of a non-classical NLS, the M9 sequence of heterogeneous nuclear ribonucleoprotein A1 (GNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRN-QGGY) and a 13-residue cationic peptide (for enhancing DNA binding ability) resulted in a 63-fold increase in marker gene expression [65]. In addition, streptavidin-conjugated NLSs have also been successfully coupled to biotinylated DNA [18]. Microinjection of a construct consisting of a biotinylated 900-bp green fluorescent protein (GFP) expression sequence conjugated via streptavidin to a 39-amino-acid peptide (H-CKKKSSSDDEATADSQHSTPP- KKKRKVEDPKDFP-SELLS) containing a functional SV40 large T antigen NLS, resulted in the four-fold increase in GFP expression compared with the construct coupled to a mutant NLS [18]. However, the covalent coupling procedure might reduce the transcriptional activity of DNA owing to the exposure to organic solvent and prevent DNA sequence-specific conjugation [66]. Peptide nucleic acid (PNA) emerged as a smart linker for coupling NLSs to DNA by precisely hybridizing to its target DNA sequence, which is far away from the transcriptional site [67-69]. An increase of up to eight-fold was observed in both the nuclear translocation of fluorescence-marked oligonucleotides and the efficacy of polyethylenimine (PEI)-mediated transfection of Cos-7 cells with the oligonucleotides and the reporter constructs hybridized to PNA-SV40 Tag NLS [69]. PNAs, the synthetic homologues of nucleic acids with the sugar backbone replaced by an uncharged mimic of repeating 2-aminoethyl-glycine units, provide biological stability and access to a variety of chemical modifications [66].

3.2 DNA sequence-specific nuclear delivery

A 366-bp sequence of DNA containing the simian virus 40 (SV40) origin of replication and early promoter has been shown to be necessary for the nuclear entry of plasmid DNA in a variety of non-dividing cultured cell lines [70-74], with the 72-bp repeats of the SV40 enhancer facilitating the maximal transport [72,73]. By contrast, two other strong promoter and enhancer sequences, the human cytomegalovirus (CMV) immediate-early promoter and the Rous sarcoma virus LTR, were unable to direct nuclear localization of plasmids. Since the DNA sequence binds during transcription in the nucleus to a number of general transcription factors that are expressed in most cell types, the SV40 enhancer of cytoplasmic plasmid DNA might be coated with the newly synthesized NLS-containing transcription factors for the subsequent nuclear import using the nuclear transport machinery [75-78]. A specific DNA sequence that provides the binding sites for the transcription factors exclusively expressed in a certain cell type could be used for cell-specific nuclear import of DNA. Thus, a DNA nuclear targeting sequence of smooth muscle gamma actin (SMGA) promoter supported the nuclear

import of plasmid DNA containing the sequence specifically in the smooth muscle cells (SMCs) [75,76]. SMGA promoter is regulated transcriptionally by the complement of positive and negative transcriptional regulators present within SMCs including SRF (serum response factor) and Nkx factors [76].

Recently, the capacity of the NFκB transcription factor to shuttle between the cytoplasm and the nucleus has been exploited to increase plasmid DNA nuclear import [74,79-81]. In the cytoplasm, NFκB interacts with the IκB subunit masking the NLS moiety of NFκB, thus preventing its translocation into the nucleus [82]. Upon stimulation, for example by TNF-α, a cascade of events leads to IκB degradation allowing NFκB to penetrate into the nucleus, where it binds to high-affinity κB sites of 10 bp. Plasmid DNA bearing a 50-bp fragment made of five κB motifs inserted upstream of SV40 or CMV promoters driving the expression of luciferase gene increased both the luciferase activity and the amount of fluorescent plasmids inside the nucleus of various TNF-α-treated cell lines transfected with polyplexes [74].

4. Conclusion

Viral vectors are superior to the non-viral vectors partly as a result of their highly organized structures with various NLSs capable of efficiently recognizing the cellular nuclear transport proteins for delivering their genomic DNAs into the nucleus. Intensive efforts have been made on the nuclear delivery of non-viral DNA by either coupling NLSs to the DNA by different chemical methods or selecting specific DNA sequences with the high binding affinity towards NLS-containing transcription factors. Since the mechanisms of nuclear traffic of the viral particles as well as the nuclear proteins across the NMP have yet to be fully elucidated, more technological advancements on the nuclear traffic of non-viral DNA could be expected in the near future.

5. Expert opinion

Intracellular delivery of an exogenous gene to express a desirable protein could be a very promising approach in clinical medicine for treating both genetic and acquired human diseases. Although the current non-viral vectors promote inadequate transgene expression, they are superior to the viral vector for clinical applications because they have no potential immunogenic or carcinogenic effects. Additionally, the non-viral systems have the capacity for carrying DNA of a wide range of sizes and offer high level of flexibility for targeting any particular cell type for efficient and specific cellular uptake of the DNA. With the help of the proton-sponge effect or membrane disruption, they facilitate effective endosomal escape of DNA either in free or carrier-bound forms. A study using A549 cells revealed that adenovirus vectors demonstrate 70% escape efficiency, whereas lipoplexes demonstrate 53% efficiency [83]. So, the rate-limiting step for transgene expression in the non-viral route is the transport of DNA across the cytosol to cross the nucleus envelope. The mesh-like cytoskeleton inhibits the mobility of the DNA > 2 kb, while oligonucleotides diffuse rapidly throughout the cell [84]. Additionally, the conformation of plasmid DNA affects cytosolic diffusion and subsequent transfection with super-coiled and linearized DNA demonstrating the highest and lowest transfection levels, respectively [85]. However, the adenoviruses and other viruses overcome these barriers by maintaining DNA inside the capsids even after endosomal escape, using microtubules to move towards the nucleus [36] and finally recognizing cellular nuclear transport machinery to dock and cross the NMPs.

In addition to the size and conformation of DNA, other factors that affect the nuclear translocation of non-viral DNA include the specific type of NLSs, the number of NLS peptides conjugated to each DNA fragment and the manner in which they are conjugated [86]. Moreover, the ability of an NLS sequence to mediate nuclear translocation also depends on the vectors with the lipoplexes that release DNA to the cytosol probably being better than the polyplexes that are released into the cytosol along with associated DNA, rendering the DNA-conjugated NLS inactive due to steric, electrostatic or size restrictions [87]. However, the site (cytosol or nucleus) for the release of DNA from the polymer and peptide complexes is still a matter of controversy [86], which demands more investigations for developing an appropriate nuclear targeting device for them.

Coupling a NLS sequence to a plasmid DNA and complexing the conjugate with a non-viral carrier might change the physicochemical properties of the carrier, resulting in the enhancement of transgene expression due to the increase either in cellular uptake or endosomal escape of the DNA other than the increase in nuclear delivery of the DNA [88]. So, the final effect of an NLS sequence on the nuclear delivery of non-viral DNA should be properly interpreted for further development of the area.

Declaration of interest

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