Human Gene Therapy

DOI: 10.1089/hum.2017.226

Current Status of Nonviral Vectors for Gene Therapy in China

Li Liu[a], Jingyun Yang[a], Ke Men, Zhiyao He, Min Luo, Zhiyong Qian, Xiawei Wei[*]

Laboratory for Aging Research and Nanotoxicology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, 610041, PR China

[a]These authors contributed equally to this work.

*Corresponding author: Xiawei Wei, No. 17, Block 3, Southern Renmin Road, Chengdu, Sichuan 610041, PR China; Phone: +8618081954096; E-mail: xiaweiwei@scu.edu.cn

Co-author contact information: No. 17, Block 3, Southern Renmin Road, Chengdu, Sichuan 610041, PR China

Li Liu, E-mail: lilyscu1990@163.com

Jingyun Yang, E-mail:qwyz002@outlook.com

Ke Men, E-mail:mendingbob@hotmail.com

Zhiyao He, E-mail: heyaode@163.com

Min Luo, E-mail: minluo_scu@163.com

Zhiyong Qian, E-mail: anderson-qian@163.com

Short title: Nonviral Vectors for Gene Therapy in China

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Abstract

With the growing interest in application of nonviral vectors for drug delivery, diagnosis, and imaging, progress has been made in the field of nonviral vector gene therapy in China. Nanobiotechnology studies are important to the development plan of China in 21st century research priorities; the National 973 Plan and "Strategic Priority Research Program" classify nanobiotechnology as a special project and give priority to supporting its development. From 2000 to 2017, many articles on nonviral vector gene therapy were published, and cancer gene therapy is one of the most active and important gene therapy research fields. Although the use of nonviral vectors for gene therapy faces enormous problems, Chinese scientists have also begun to realize both the challenges and opportunities that lie ahead. However, a nonviral gene therapy drug has yet to be approved for sale. Therefore, more work is needed in this field. The present review examines the progress and challenges of nonviral gene therapy in China. Efforts to optimize nonviral vectors with structural modifications and gene delivery systems are described and clinical translation challenges are highlighted.

Keywords: gene therapy, nonviral vectors, nanomaterial, gene delivery

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Introduction

Gene therapy, the introduction of genetic material into target cells for therapeutic benefit, is a crucial strategy for the treatment of many diseases,[1] including cancer,[2] monogenic,[3] cardiovascular,[4] and neurodegenerative diseases. Gene therapy has shown remarkable therapeutic benefits, excellent safety, and was considered to have "returned to center stage" in recent years.[5] Current gene transfection methods include viral vector systems for gene transduction,[6] direct microinjection systems,[7] and nonviral vector systems; nonviral vectors are thought of as chemical methods or nanocarriers.[8] Gene therapy research has made amazing progress in 2017. Adenovirus-associated virus serotype 2-mediated gene therapy has been approved by the Food and Drug Administration for the treatment of inherited retinal dystrophy due to *RPE65* gene deletion. The remarkable benefits that gene therapies provide to patients were first highlighted by retinal gene therapy.[9] Clinical trials on adenovirus-associated viral vector-mediated gene therapies in hemophilia B are also currently ongoing.

With the emergence and vigorous development of nanotechnology, increasing attention has been paid to the research of gene vectors based on nanomaterials in China.[10,11] Nearly 100 Chinese research institutes, including universities, are working on nonviral vectors, and nearly 3000 research papers have been published each year. Of them, nearly 60% concerned nonviral gene therapies according to PubMed. Nonviral gene carriers have the advantage of being relatively simple preparations as their structures are readily modified, have good biocompatibility, are generally small in size, facilitating travel through target cells and higher gene transfer efficiency, and effectively protect exogenous gene cargo.[12]

However, one major disadvantage to nonviral vectors is that their administration causes cationic polymers and liposomes to induce immune responses.[13-15] This effect has drawn public attention, and the Chinese Food and Drug Administration has not yet approved any human gene therapy products for sale. Furthermore, success in clinical trials has been limited owing to numerous technical barriers. Although many problems have not been

overcome, studies on nonviral carriers are still rapidly developing.[16] In the present review, we focus on the current research and challenges of using nonviral vectors for gene therapy in laboratory and clinical studies in China.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Types of nonviral vectors in gene delivery

Naked plasmid DNA is often weak due to its degradation by nucleases, inefficient delivery to cells, and lack of tissue specificity, all of which make it difficult to effectively deliver genes to target cells.[17] Thus, identifying safe and effective gene targeting vectors is key. Gene delivery vectors are broadly divided into four classes, including plasmid, phage, viral, and nonviral vectors.[18] Nonviral vectors are simple in theory but complex in practice and are widely used in gene therapy.[19] Nonviral vectors are divided by their species of origin, which include bacteria, bacteriophages, virus-like particles, erythrocyte ghosts, and exosomes.[20] Nanomaterials, such as calcium phosphates, lipids, and cationic polymers, including chitosan, polyethylenimine, polyamidoamine dendrimers, and poly(lactide-co-glycolide) [PLGA], have been widely used for *in vivo* and *in vitro* gene delivery.[8] The materials used as carriers for nucleic acid delivery include liposomes, polymers, micelles, etc.[21] Here, we summarize the progress related to nonviral gene delivery vectors in recent years in China.

1. Liposomes or lipid-based nanoparticles

Cationic lipids, such as 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride, are widely used. Liposomes are formed by bilayers of phospholipids, which are amphiphilic molecules with a hydrophilic head group facing outward towards water and the hydrophobic tails facing inward forming a spherical molecule ranging in diameter from 25-1000 nm.[22, 23] Liposomes can be used for transport of genes or drug preparations into target cells via membrane fusion.[24, 25]

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Liposomes or lipid-based nanoparticles have been widely used in gene therapy and drug delivery systems because of their adaptability to cargo sizes.[26, 27] However, due to the low transfection efficiency and toxicity of cationic lipids, their application has been limited. Many groups have conducted further investigations to increase gene transfection efficiency and reduce the toxicity of cationic lipid-based nanocarriers mainly through structural modification of the lipids. The surface properties of liposomes, including surface charge, polyethylene glycol (PEG)ylation, and ligand modification, can significantly affect gene delivery efficiency.[28-30] In recent years, chemical modification has been widely used to improve targeting and reduce the toxicity of liposomes. For example, mannosylated and peptide-based liposomes have been used for targeted gene therapy, and the circulation time of PEGylated liposomes is prolonged.[28, 31-34] Modification by crosslinkers to conjugate bovine serum albumin protein to the surface of liposomes has also been reported.[35] Dual-modified liposomes with photolabile-caged cell-penetrating and asparagine-glycine-arginine peptide molecules attached to the liposome surface have bene shown to enhance tumor cell uptake of small interfering RNA (siRNA).[36] Cationic liposomes comodified with ligands of the asialoglycoprotein receptor and β-sitosterol-β-D-glucoside resulted in more efficient transfection but no enhanced cytotoxicity.[37] There are many reports on the chemical modification of liposomes, and Chinese scientists have made many contributions to the development of high efficiency and low toxicity gene delivery vectors.

2. Cationic polymers

Cationic polymers have been used for gene delivery for at least five decades.[38-41] Polymeric nanomaterials, such as polyethylenimine (PEI),[42] chitosan,[43-45] poly(amino-co-ester)s, poly(L-lysine), poly[2-(dimethylamino) ethyl methacrylate],[46, 47] and polyamidoamine (PAMAM), have been widely used for gene delivery. PEI is one of the most widely used and effective nonviral vectors. PEI has a highly positive charge and can adsorb negatively charged DNA molecules. Chitosan is a natural polymer resulting from partially deacetylated chitin after the formation of an amino polysaccharide.[48] PAMAM can combine with the DNA surface via electrostatic interactions,[49] and PLL is a cationic

polypeptide.[50] Cationic polymers as gene vectors for transfection can enhance DNA stability and effectively protect against DNA degradation enzymes.

Although many studies have been conducted on the application of cationic polymers for gene therapy in mouse models,[51-53] the toxicity of these materials is still a subject of public concern. Most cationic polymers have serious acute toxicity, such as cytotoxicity, necrosis, and erythrocyte aggregation, which is mainly due to their poor biocompatibility[54] and are difficult to degrade under physiological conditions.[55] Due to toxicity and stability concerns, the potential application of nanocarrier systems *in vivo*, particularly cationic liposomes, has received considerable skepticism.[56] Chinese nanocarrier scientists have also conducted detailed studies of cationic polymer toxicity. The strong cell-cationic carrier interaction is an important inducer of nonspecific cytotoxicity.[57] For example, PAMAM nanoparticles have been shown to trigger acute lung failure in mice,[58, 59] and PAMAM dendrimers also induce autophagy in neurotoxicity and hepatotoxicity.[60-62] Moreover, PLL-DNA complexes do not quickly release from endosomes, leading to low transfection efficiency and cytotoxicity.

3. PLGA

PLGA is able to deliver drugs and genes efficiently[63] and is expected to be used as a new tool for the treatment of many nervous system diseases,[64-67] as well as in bone tissue engineering.[68] PLGA has also been used in microRNA (miRNA) delivery in hepatic carcinoma[69] and siRNA[70]. However, PLGA drug release and acid degradation products often limit its clinical application.[71] PLGA shows cytotoxicity[72] and lung toxicity in human bronchial Calu-3 cells.[73]

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

4. Other nanomaterial-based therapeutic gene delivery systems

Other nanomaterials (e.g., inorganics), including silicon,[74] iron oxide,[75, 76] calcium sulfate,[77, 78] and nanogold particles,[79, 80] are currently being used as gene vectors.

Nanoparticle-nucleic acid complexes

Current gene therapy is not limited to DNA delivery.[1] Other nucleic acid materials, such as siRNA and miRNA, have also been incorporated into gene therapy programs. Oligonucleotide therapeutics are widely used in gene therapies and may have meaningful clinical productivity due to their advantages.[81] SiRNA are a powerful tool for gene silencing via RNA interference.[82] Nonviral miRNA delivery systems provide a perspective on the future of miRNA-based therapeutics.[83] In recent years, many nonviral vector-based gene therapies have been performed in China, and many animal model studies have demonstrated that nanomaterial-based gene therapy is extremely powerful.

Nonviral vectors have been used in gene delivery, and the complexes they form with nucleic acids are defined as “lipoplexes” or “polyplexes.”[84] The kinetics of the nanoparticle-nucleic acid complex formation process can be divided into three distinct stages: binding or adsorption, where the nucleic acid binds to the surface of the cationic vesicles; transport of the adsorbed nucleic acid aggregate by vesicles to form larger complexes; and adjustment of the nucleic acid-lipid organization. The rate of the process depends on the lipid characteristics, such as membrane fluidity and rigidity.[85] The different types of gene materials that can be complexed with nonviral vectors are summarized in FIGURE 1.

Advances in clustered regularly interspaced short palindromic repeats (CRISPR)/caspase-9 (Cas9) delivery

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Recently, newly developed CRISPR/Cas9 gene editing technology has been demonstrated to be a powerful tool for targeted genetic modification, offering an alternative therapeutic strategy for genetic diseases.[86] By making double-stranded DNA breaks at targeted DNA sites, the CRISPR/Cas9 system specifically induces different types of insertions or deletions via NHRJ or homology-directed repair, facilitating gene mutation, knock-in, deletion, inversion, etc.[87, 88] In particular, with exogenous templates, precise modifications at target loci could generate homology-directed repair, enabling the introduction of desired genomic changes.[89] This development suggests CRISPR/Cas9-mediated gene therapy may be a promising new approach which aims to repair disease-causing alleles at precise chromosomal locations.[90] However, due to the relatively large size of the CRISPR/Cas9 encoding system (i.e., Cas9 encoding plasmids and their protein counterparts), the gene editing efficiency of this technique is greatly limited, and related therapeutic efforts may be challenging.[91] As a result, developing an optimized CRISPR/Cas9 delivery system with high efficiency is greatly needed.

Compared to viral vectors, nonviral gene delivery systems share several advantages owing to their flexible backbone and adjustable surface (FIGURE 2). In recent years, extensive efforts have been made by Chinese researchers to investigate lipid and/or polymeric nanovectors for CRISPR/Cas9 delivery. For example, inspired by viral vectors, Li *et al.* from Sichuan University (Chengdu, PR China) reported constructing a multifunctional polymer-based "core-shell" artificial virus (RRPHC) for delivery of the CRISPR/Cas9 system.[92] This Cas9-delivering artificial "virus" was comprised of a core of fluorinated polymer 33 bound with the CRISPR/Cas9 system and a versatile multifunctional RGD-R8-PEG-HA (RRPH) shell. The polymeric virus demonstrated strong transport ability of the CRISPR/Cas9-encoding plasmid system without an additional nuclear localization signal and was found to induce higher targeted gene disruption efficacy than Lipofectamine 3000. RRPHC delivered the

Cas9-human mutT homolog 1 system successfully, showing effective disruption of the mutT homolog 1 gene *in vivo* with minimum side effects.

To facilitate further clinical development of CRISPR/Cas9 therapeutics, Zhen *et al.* developed a flexible aptamer-liposome chimera vector for the delivery of the CRISPR/Cas9 system.[93] In their work, aptamer-liposome-CRISPR/Cas9 chimeras were prepared through post-insertion of aptamer-lipid conjugates into liposome-CRISPR/Cas9 complexes, which combined the advantageous properties of efficient delivery and increased flexibility. Within this system, the RNA aptamer specifically binds to prostate-specific membrane antigen (PSMA) on prostate cancer cells, and cationic liposomes are used to deliver the therapeutic CRISPR/Cas9 system that targets survival genes, such as polo-like kinase 1. The prepared aptamer-liposome-CRISPR/Cas9 chimeras showed significant cell type binding specificity with remarkable gene silencing ability, promoting conspicuous regression of prostate cancer both *in vitro* and *in vivo*. This work provided a universal strategy for cell type-specific CRISPR/Cas9 delivery, which is critical for safety issues during clinical application.

Aptamer-based, cell-specific CRISPR/Cas9 delivery systems have also been investigated for other cancer types. To apply CRISPR/Cas9-based therapy to highly aggressive pediatric osteosarcomas, Liang *et al.* developed an aptamer-functionalized PEG-PEI-cholesterol (PPC) lipopolymer based on an osteosarcoma cell-specific aptamer LC09.[94] In this system, the CRISPR/Cas9 plasmids targeting vascular endothelial growth factor A (VEGFA) were complexed with the PPC lipopolymer, forming a nano-sized delivery system. Facilitated by the LC09 aptamer, the prepared PPC lipopolymer selectively distributed CRISPR/Cas9 in both orthotopic and lung metastatic osteosarcomas, leading to effective VEGFA genomic editing. As a promising therapeutic target, VEGFA is highly expressed in osteosarcoma.[95] This protein not only contributes to angiogenesis within the tumor microenvironment, but also acts as an autocrine survival factor.[94] In their study, the PPC-mediated gene editing targeted by the CRISPR/Cas9 system efficiently decreased VEGFA expression and secretion,

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

thereby inhibiting orthotopic osteosarcoma malignancy and lung metastasis with no detectable toxicity.

In addition to cancer therapy, nanocarrier-based CRISPR/Cas9 delivery has also been applied for the treatment of infectious disease. For example, hepatitis B virus (HBV) covalently-closed circular DNA (cccDNA) is considered a major barrier to eradication of this virus and is resistant to currently available therapies.[96] Although previous attempts have been made to deliver CRISPR/Cas9 via hydrodynamic injection of the plasmids, vector-based *in vivo* delivery methods targeting HBV cccDNA are still lacking. For this issue, Jiang *et al.* developed LLNs based on the previously characterized TT polymers.[97] These TT polymers are a class of N^1,N^3,N^5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide derivatives with strong siRNA and mRNA delivery capacity. In their study, the TT-based lipid-like nanoparticles demonstrated highly efficient Cas9 mRNA and single guide RNA delivery to liver tissue and successfully targeted HBV cccDNA and the proprotein convertase subtilisin/kexin type 9 gene *in vivo*, providing CRISPR/Cas9-based therapeutic strategies for treating HBV and hypercholesterolemia. Although Cas9 mRNA and single guide RNA are rapidly degraded in mice, this work still provides a temporarily controllable strategy for *in vivo* genome editing, as well as a potential method for treating a wide range of liver-related diseases.

Additionally, other forms of nonviral delivery systems have also been evaluated for CRISPR/Cas9 system delivery. Genome-wide CRISPR libraries have played important roles in indicating critical genes associated with the growth and metastasis of human cancers.[98, 99] However, *in vivo* applications are severely limited due to the use of lentiviral vectors for gene delivery. Xu *et al.* from China Agricultural University (Beijing, PR China) examined the piggyBac (PB) transposon as an alternative vehicle for delivering a guide RNA library for *in vivo* screening.[100] The PB transposon has been widely recognized as a natural nonviral gene vector that can induce stable chromosomal integration and persistent gene expression in vertebrate cells. By constructing CRISPR libraries in the PB transposon, these authors

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

successfully conducted *in vivo* genome-wide screening in mice and identified several new genes which mediate liver tumorigenesis, suggesting a simple and nonviral choice for the *in vivo* delivery of CRISPR libraries.

Advances in plasmid DNA delivery

Folate receptor-α is overexpressed in most types of human colorectal and ovarian carcinoma and has been developed and applied in novel target nanomaterials as a potential and effective cancer therapy target. He *et al.* developed folate-modified liposomes delivering interleukin (IL)-12, IL-15, or human telomerase reverse transcriptase promoter-driven matrix protein genes for the targeted immunotherapy of colon and ovarian cancer.[101-103] In their study, compared to the normal liposome, all folate receptor-α-targeted lipoplexes loaded with various genes significantly suppressed tumor growth and production of malignant ascites, suggesting that folate receptor-α-modified liposomes may be safe and efficient nanomaterials for targeted colon and ovarian cancer therapy.

Gong *et al.* also developed a novel multifunctional "core-shell" ternary nanoparticle-based gene delivery system, which was extremely efficient in gene penetration and expression in the nucleus and highly effective in multiple tumor models.[92, 104, 105] This well-tailored ternary nanoparticle has a "shell" composed of RRPH, which can simultaneously target CD44 and integrin αvβ3 receptors overexpressed on most malignant tumors and tumor vascular endothelial cells, while the "core" is comprised of a mixture of the gene with fluorinated polymers, which facilitate escape of the condensed gene from the endosome and promote gene penetration into the nucleus. This ternary nanoparticle has been demonstrated to be an efficient system for mutT homolog 1-Cas9 delivery in SKOV3 ovarian cancer, pro-apoptotic mouse tumor necrosis factor-related apoptosis inducing ligand gene-Cas9 delivery in B16-OVA melanoma, and tumor necrosis factor-related

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

apoptosis inducing ligand gene delivery in HCT116 colorectal cancer. These antitumor results indicate that these novel RRPH ternary complex nanoparticles might be a promising gene delivery system for the targeted gene therapy of various tumors.

Carbon nanotubes are also used for gene delivery but are limited by their hydrophobic nature. Kong *et al.*[106] at Zhejiang University (Hangzhou, PR China) demonstrated a novel gene delivery vector through the noncovalent binding of chemically modified PEI-cholesterol to single-walled carbon nanotubes via a hydrophobic interaction, which enhanced the transfection efficiency and antitumor effect when combined with pTP53. PAMAM dendrimers have also been explored as nonviral gene carriers due to their specific size, shape, and surface characteristics, which are suitable for gene delivery. Yin *et al.*[107] developed a novel activated endothelial growth factor (EGF)-dendriplex system prepared via self-assembly by EGF and activated PAMAM dendrimer and plasmid DNA complexes. This system was demonstrated to be safe and effective in delivering genes to EGF receptor-positive cells *in vitro* and *in vivo*.

Advances in siRNA delivery

Want *et al.* elaborated three types of hydrophobized PEG-blocked cationic polymers with different distributions of the hydrophobic segments in the polymer chains as siRNA vectors. These cationic polymers formed nano-sized micelles spontaneously and incorporated siRNA into complex micelles well. The polymer composed of PEG, the cationic monomer aminoethyl methacrylate, and 2-(diisopropylamino)ethyl methacrylate showed good siRNA binding capacity, formed stable siRNA-micelle complexes of less than 100 nm in diameter, mediated good gene silencing efficiency, had an inhibitory effect on tumor cell growth *in vitro*, and exhibited better liver gene silencing effects *in vivo*. Therefore, the distribution of the hydrophobic segments in the amphiphilic cationic

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

polymer chains should be seriously considered in the design of siRNA vectors.[108] Liu *et al.* modified PAMAM with phospholipids and loaded siRNA targeting the multidrug resistance (MDR)-1 gene for reverse MDR in human breast cancer MCF-7/ADR cells. Phospholipid-modified PAMAM-siMDR1 dendriplexes enhanced the cellular uptake of siMDR1, exhibited higher gene silencing efficiency, decreased p-glycoprotein expression, increased cellular accumulation of doxorubicin, and inhibited tumor cell migration. Moreover, the phospholipid-modified PAMAM-siMDR1 dendriplexes worked synergistically with paclitaxel for treating MDR, leading to increased cellular apoptosis and cell phase regulation. Therefore, these phospholipid-modified dendriplexes show great promise in reversing drug-resistance *in vitro*.[109] In addition, anti-EGF receptor antibody-modified PAMAM has also been shown to deliver siMDR1 to overcome MDR.[110]

Qian *et al.* developed M2-like tumor-associated macrophage (TAM) dual-targeting nanoparticles, which were utilized to specifically block the survival signal of M2-like TAMs and deplete cells from melanoma tumors by loading anti-colony stimulating factor-1 receptor siRNA. The dual-targeting fusion peptide displayed a synergistic effect between the two targeting units *in vitro*. The dual-targeting nanoparticle-siRNA complexes showed higher affinity for M2-like TAMs than tissue-resident macrophages in the liver, spleen, and lung after administration to tumor-bearing mice. Compared with control treatment groups, the dual-targeting nanoparticle-siRNA complexes resulted in dramatic elimination of M2-like TAMs (52%), decreased tumor size (87%), and prolonged survival. Additionally, immunosuppressive IL-10 and transforming growth factor-β production were inhibited, and expression of immunostimulatory cytokines (IL-12 and interferon-γ) was increased in the tumor microenvironment. Moreover, these dual-targeting complexes downregulated expression of exhaustion markers (programmed cell death protein-1 and T-cell immunoglobulin and mucin domain-containing-3) on infiltrating $CD8^+$ T-cells and stimulated their secretion of interferon-γ. Therefore, these dual-targeting nanoparticles with RNA interference provide a potential clinical strategy for molecular-targeted cancer immunotherapy.[111]

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Zhang *et al.* developed a targeting system involving dioleoyl trimethylammonium propane-based cationic liposomes attached to six repetitive sequences of aspartate, serine, and serine for delivering siRNAs specifically to bone formation surfaces. To manage metabolic skeletal disorders, an osteogenic siRNA that targets casein kinase-2 interacting protein-1 (encoded by pleckstrin homology domain-containing O1) was encapsulated into the targeting cationic liposomes. The *in vivo* systemic delivery of pleckstrin homology domain-containing O1 siRNA into rats using the liposome-siRNA system resulted in selective enrichment of the siRNAs in osteogenic cells and subsequent depletion of pleckstrin homology domain-containing O1. Furthermore, this approach markedly promoted bone formation, enhanced the bone microarchitecture, and increased bone mass in both healthy and osteoporotic rats according to bioimaging analysis. These results indicate that this liposome is a promising targeted delivery system for RNA interference-based bone anabolic therapy.[112]

Advances in miRNA delivery

An RNA aptamer targeting PSMA was used to modify atelocollagen to produce PSMA-targeting vectors, and miRNA (miR-15a and miR-16-1) was loaded onto the RNA aptamer-atelocollagen nanoparticles. The anticancer effect of nanoparticles *in vivo* was investigated using the survival times of a mouse model of human prostate cancer bone metastasis. The anticancer efficacy of miRNA-RNA aptamer-atelocollagen nanoparticles was superior to those of other treatments *in vivo*. This PSMA-targeted miRNA delivery system might allow for the selective killing of prostate cancer cells in bone metastatic foci.[113]

In particular, miR-34a has been incorporated into a solid lipid nanoparticle for cancer stem cell therapy. This miR-34a-nanoparticle system has been shown to induce B16F10-CD44$^+$ cell apoptosis and inhibit cell migration by negatively regulating the cell surface protein CD44. Moreover, this nanoparticle formulation increased miR-34a accumulation in the lungs *in vivo* and extended the persistence of miR-34a in tumor sites. Therefore, the miR-34a-nanoparticle system was also effective in inhibiting B16F10-CD44$^+$ tumor development

and tumorigenicity. Shi *et al.* developed a solid lipid nanoparticle that represents a promising vector for miRNA delivery.[114]

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Toxicity of nonviral vectors for gene therapy

Here, we summarize the barriers and challenges to systemic delivery of exogenous nucleic acids and discuss strategies for overcoming obstacles in the *in vivo* delivery of nucleic acids by using nonviral vectors. First, the toxicity of cationic nanocarriers is a cause for public concern,[15, 115-118] as the toxicity of cationic liposomes has been made clear.[119] The toxicity of cationic liposome carriers mainly depends on their cationic properties which have different effects on different structures. For cationic lipids, their cytotoxicity is mainly dependent on the structure of hydrophilic groups, such as quaternary ammonium molecules, which are more toxic than tertiary amines, and cationic polymers consistently induce immune responses. For example, chitosan has been shown to stimulate a dendritic cell immune response by activating cytoplasmic DNA sensors cyclic GMP-AMP synthase and stimulator of interferon genes and inducing type I interferons.[120] Our team has also reported on the release of damage-associated molecular pattern molecules after cellular necrosis induced by cationic liposomes,[13] as well as on immune responses and related regulatory mechanisms. Furthermore, Wei *et al.* focused on the mechanisms of cationic nanocarrier-induced cellular necrosis. They showed that cationic nanocarriers could interact with the cation-binding site of Na^+/K^+-ATPases, and cellular necrosis could be inhibited by ouabain, which occupied ouabain-binding sites at a markedly low concentration. The complex structures of Na^+/K^+-ATPase-DOTAP and Na^+/K^+-ATPase-ouabain-DOTAP were calculated and are shown in FIGURE 3.

There are many barriers to overcome in the process of nonviral vector gene delivery. Complex biological barriers, such as mucus and blood-brain barriers,[122, 123] are major obstacles to preventing and treating disease.[121] Most scholars have proposed that the

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

main barriers to nanocarriers of gene transfer systems include (1) macrophages, which are widely distributed in tissues and body fluids and can rapidly identify and remove lesions, senescent cells, and foreign material invading the body; (2) nanoparticle-DNA complexes which interact with the cell, leading to membrane nucleus formation and rapid lysosomal engulfment of the nucleus; and (3) lysosomes that can degrade gene materials in cells, thereby affecting gene expression. Surface modification of nanomaterials with antibodies,[124, 125] peptides,[126, 127] lactoferrin,[128] mannose acid,[129] and folic acid [130] has been shown to improve transfection efficiency and targeting, while reducing the side effects of other tissues. Furthermore, lipid PEGylation, other structural/chemical group modifications, and/or controlling the number of free liposomes can reduce toxicity and improve transfection efficiency of nonviral vector gene therapies.[131, 132]

Conclusion

Scientists face three major barriers to the use of nonviral vectors in gene delivery systems: (1) helping nonviral transporters escape the reticuloendothelial system; (2) avoiding the phagocytosis by lysosomes; and (3) preventing plasmid DNA degradation in the cytoplasm of target cells. In addition, avoiding induction of an immune response and acute toxicity when developing gene delivery systems is also a major challenge. Although there are still many problems associated with gene delivery systems, prospect of applying nonviral vector gene therapy remains promising.

Acknowledgments

This work was supported through grants from the National Key Research and Development Program of China (No. 2016YFA0201402) and the Natural Science Foundation of China (No. 81602492).

Author disclosure

The authors have no conflicts of interest to disclose.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

References

1. Cheever TR, Berkley D, Braun S et al. Perspectives on Best Practices for Gene Therapy Programs. Hum Gene Ther 2015;26:127-133

2. Husain SR, Han J, Au P et al. Gene therapy for cancer: regulatory considerations for approval. Cancer Gene Ther 2015;22:554-563.

3. Boudes PF. Gene therapy as a new treatment option for inherited monogenic diseases. Eur J Intern Med 2014;25:31-36.

4. Mason D, Chen Y-Z, Krishnan HV et al. Cardiac gene therapy: Recent advances and future directions. J Control Release 2015;215:101-111.

5. Naldini L. Gene therapy returns to centre stage. Nature 2015;526:351-360.

6. Katz MG, Fargnoli AS, Williams RD et al. Gene Therapy Delivery Systems for Enhancing Viral and Nonviral Vectors for Cardiac Diseases: Current Concepts and Future Applications. Hum Gene Ther 2013;24:914-927.

7. Michaelis M, Sobczak A, Weitzel JM. In Vivo Microinjection and Electroporation of Mouse Testis. Journal of visualized experiments : J Vis Exp 2014:51802.

8. Jin L, Zeng X, Liu M et al. Current progress in gene delivery technology based on chemical methods and nano-carriers. Theranostics 2014;4:240-255.

9. Aguirre GD. Concepts and Strategies in Retinal Gene Therapy. Invest Ophthalmol Vis Sci 2017;58:5399-5411.

10. Davidson S, Truong H, Nakagawa Y et al. A microinjection technique for targeting regions of embryonic and neonatal mouse brain in vivo. Brain Res 2010;1307:43-52.

11. Shi J, Kantoff PW, Wooster R et al. Cancer nanomedicine: progress, challenges and opportunities. Nat Rev Cancer 2017;17:20-37.

12. Nishiyama N, Kataoka K. Current state, achievements, and future prospects of

polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther 2006;112:630-648.

13. Wei X, Shao B, He Z et al. Cationic nanocarriers induce cell necrosis through impairment of Na(+)/K(+)-ATPase and cause subsequent inflammatory response. Cell Res 2015;25:237-253.

14. Schlegel A, Largeau C, Bigey P et al. Anionic polymers for decreased toxicity and enhanced in vivo delivery of siRNA complexed with cationic liposomes. J Control Release 2011;152:393-401.

15. Knudsen KB, Northeved H, Kumar Ek P et al. In vivo toxicity of cationic micelles and liposomes. Nanomedicine 2015;11:467-477.

16. Liang XJ. Nanotechnology and cancer nanomedicine. Biotechnol Adv 2014;32:665.

17. Farris E, Brown DM, Ramer-Tait AE et al. Micro- and nanoparticulates for DNA vaccine delivery. Exp Biol Med 2016;241:919-929.

18. Ginsburg DS, Calos MP. Site-Specific Integration with φC31 Integrase for Prolonged Expression of Therapeutic Genes. Adv Genet . (Academic Press). 2005; pp. 179-187.

19. Ramamoorth M, Narvekar A. Non viral vectors in gene therapy- an overview. J Clin Diagn Res 2015;9:GE01-06.

20. Seow Y, Wood MJ. Biological Gene Delivery Vehicles: Beyond Viral Vectors. Mol Ther 2009;17:767-777.

21. Peddada LY, Garbuzenko OB, Devore DI et al. Delivery of antisense oligonucleotides using poly(alkylene oxide)–poly(propylacrylic acid) graft copolymers in conjunction with cationic liposomes. J Control Release 2014;194:103-112.

22. Modi S, Anderson BD. Bilayer Composition, Temperature, Speciation Effects and the Role of Bilayer Chain Ordering on Partitioning of Dexamethasone and its 21-Phosphate. Pharm Res 2013;30:3154-3169.

23. Huang Y-Y, Sharma SK, Dai T et al. Can nanotechnology potentiate photodynamic

therapy? Nanotechnol Rev 2012;1:111-146.

24. van der Meel R, Fens MH, Vader P et al. Extracellular vesicles as drug delivery systems: lessons from the liposome field. J Control Release 2014;195:72-85.

25. Zhang T-Y, Huang B, Wu H-B et al. Synergistic effects of co-administration of suicide gene expressing mesenchymal stem cells and prodrug-encapsulated liposome on aggressive lung melanoma metastases in mice. J Control Release 2015;209:260-271.

26. Zulliger R, Conley SM, Naash MI. Non-viral therapeutic approaches to ocular diseases: an overview and future directions. J Control Release 2015;219:471-487.

27. Wang Y, Huang L. Composite Nanoparticles for Gene Delivery. Adv Genet 2014;88:111-137.

28. Xia Y, Tian J, Chen X. Effect of surface properties on liposomal siRNA delivery. Biomaterials 2016;79:56-68.

29. Buyens K, De Smedt SC, Braeckmans K et al. Liposome based systems for systemic siRNA delivery: Stability in blood sets the requirements for optimal carrier design. J Control Release 2012;158:362-370.

30. Li Y, Liu R, Shi Y et al. Zwitterionic poly(carboxybetaine)-based cationic liposomes for effective delivery of small interfering RNA therapeutics without accelerated blood clearance phenomenon. Theranostics 2015;5:583-596.

31. Bai J, Liu Y, Sun W et al. Down-regulated lysosomal processing improved pegylated lipopolyplex-mediated gene transfection. J Gene Med 2013;15:182-192.

32. Gao J, Yu Y, Zhang Y et al. EGFR-specific PEGylated immunoliposomes for active siRNA delivery in hepatocellular carcinoma. Biomaterials 2012;33:270-282.

33. Xu H, Wang KQ, Deng YH et al. Effects of cleavable PEG-cholesterol derivatives on the accelerated blood clearance of PEGylated liposomes. Biomaterials 2010;31:4757-4763.

34. Xu H, Deng Y, Chen D et al. Esterase-catalyzed dePEGylation of pH-sensitive vesicles modified with cleavable PEG-lipid derivatives. J Control Release 2008;130:238-245.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

35. Chen T, Wang R, Lu T et al. Modification of Liposomes with Proteins by Dansyl-labeled Heterobifunctional Crosslinker. J Biomater Appl 2010;26:117-125.

36. Yang Y, Yang Y, Xie X et al. Dual-modified liposomes with a two-photon-sensitive cell penetrating peptide and NGR ligand for siRNA targeting delivery. Biomaterials 2015;48:84-96.

37. Zhang Y, Rong Qi X, Gao Y et al. Mechanisms of co-modified liver-targeting liposomes as gene delivery carriers based on cellular uptake and antigens inhibition effect. J Control Release 2007;117:281-290.

38. Wagner E. Chapter Eight - Polymers for Nucleic Acid Transfer—An Overview. Adv Genet. L Huang, D Liu and E Wagner, eds. (Academic Press). 2014; pp. 231-261.

39. Chen C-K, Jones CH, Mistriotis P et al. Poly(ethylene glycol)-block-cationic polylactide nanocomplexes of differing charge density for gene delivery. Biomaterials 2013;34:9688-9699.

40. Yang C, Li H, Goh SH et al. Cationic star polymers consisting of α-cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. Biomaterials 2007;28:3245-3254.

41. Bishop CJ, Kozielski KL, Green JJ. Exploring the role of polymer structure on intracellular nucleic acid delivery via polymeric nanoparticles. J Control Release 2015;219:488-499.

42. Xia W, Dechao N, Chen H et al. Polyethyleneimine-Based Nanocarriers for Gene Delivery. Curr Pharm Des 2015;21:6140-6156.

43. Ragelle H, Vandermeulen G, Préat V. Chitosan-based siRNA delivery systems. J Control Release 2013;172:207-218.

44. Corbet C, Ragelle H, Pourcelle V et al. Delivery of siRNA targeting tumor metabolism using non-covalent PEGylated chitosan nanoparticles: Identification of an optimal combination of ligand structure, linker and grafting method. J Control Release 2016;223:53-63.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

45. Ragelle H, Riva R, Vandermeulen G et al. Chitosan nanoparticles for siRNA delivery: Optimizing formulation to increase stability and efficiency. J Control Release 2014;176:54-63.

46. Liu X, Yin H, Zhang Z et al. Functionalization of lignin through ATRP grafting of poly(2-dimethylaminoethyl methacrylate) for gene delivery. Colloids Surf B Biointerfaces 2015;125:230-237.

47. Tanasienko IV, Yemets AI, Finiuk NS et al. DMAEM-based cationic polymers as novel carriers for DNA delivery into cells. Cell Biol Int 2015;39:243-245.

48. Martinez LR, Mihu MR, Han G et al. THE USE OF CHITOSAN TO DAMAGE CRYPTOCOCCUS NEOFORMANS BIOFILMS. Biomaterials 2010;31:669-679.

49. Mastorakos P, Kambhampati SP, Mishra MK et al. Hydroxyl PAMAM dendrimer-based gene vectors for transgene delivery to human retinal pigment epithelial cells. Nanoscale 2015;7:3845-3856.

50. Patil ML, Zhang M, Minko T. Multifunctional Triblock Nanocarrier (PAMAM-PEG-PLL) for the Efficient Intracellular siRNA Delivery and Gene Silencing. ACS Nano 2011;5:1877-1887.

51. Yang X-Z, Dou S, Wang Y-C et al. Single-Step Assembly of Cationic Lipid–Polymer Hybrid Nanoparticles for Systemic Delivery of siRNA. ACS Nano 2012;6:4955-4965.

52. Chen C-J, Wang J-C, Zhao E-Y et al. Self-assembly cationic nanoparticles based on cholesterol-grafted bioreducible poly(amidoamine) for siRNA delivery. Biomaterials 2013;34:5303-5316.

53. Gao L-Y, Liu X-Y, Chen C-J et al. Core-Shell type lipid/rPAA-Chol polymer hybrid nanoparticles for in vivo siRNA delivery. Biomaterials 2014;35:2066-2078.

54. Shi J, Choi JL, Chou B et al. Effect of Polyplex Morphology on Cellular Uptake, Intracellular Trafficking, and Transgene Expression. ACS Nano 2013;7:10612-10620.

55. Lee YS, Kim SW. Bioreducible polymers for therapeutic gene delivery. J Control

Release 2014;190:424-439.

56. Rehman Zu, Zuhorn IS, Hoekstra D. How cationic lipids transfer nucleic acids into cells and across cellular membranes: Recent advances. J Control Release 2013;166:46-56.

57. Xue HY, Narvikar M, Zhao J-B et al. Lipid Encapsulation of Cationic Polymers in Hybrid Nanocarriers Reduces Their Non-Specific Toxicity to Breast Epithelial Cells. Pharm Res 2013;30:572-583.

58. Sun Y, Guo F, Zou Z et al. Cationic nanoparticles directly bind angiotensin-converting enzyme 2 and induce acute lung injury in mice. Part Fibre Toxicol 2015;12:4.

59. Li C, Liu H, Sun Y et al. PAMAM nanoparticles promote acute lung injury by inducing autophagic cell death through the Akt-TSC2-mTOR signaling pathway. J Mol Cell Biol 2009;1:37-45.

60. Wang S, Li Y, Fan J et al. The role of autophagy in the neurotoxicity of cationic PAMAM dendrimers. Biomaterials 2014;35:7588-7597.

61. Li Y, Zhu H, Wang S et al. Interplay of Oxidative Stress and Autophagy in PAMAM Dendrimers-Induced Neuronal Cell Death. Theranostics 2015;5:1363-1377.

62. Li Y, Zeng X, Wang S et al. Inhibition of autophagy protects against PAMAM dendrimers-induced hepatotoxicity. Nanotoxicology 2015;9:344-355.

63. Mohammad R, Mahboubeh E, Maryam H. Current Strategies in the Modification of PLGA-based Gene Delivery System. Curr Med Chem 2017;24:728-739.

64. De Laporte L, Lei Yan A, Shea LD. Local gene delivery from ECM-coated poly(lactide-co-glycolide) multiple channel bridges after spinal cord injury. Biomaterials 2009;30:2361-2368.

65. Thomas AM, Shea LD. Polysaccharide-modified scaffolds for controlled lentivirus delivery in vitro and after spinal cord injury. J Control Release 2013;170:421-429.

66. Walthers CM, Seidlits SK. Gene delivery strategies to promote spinal cord repair. Biomark Insights 2015;10:11-29.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

67. Zhang C, Wang YS, Wu H et al. Inhibitory efficacy of hypoxia-inducible factor 1alpha short hairpin RNA plasmid DNA-loaded poly (D, L-lactide-co-glycolide) nanoparticles on choroidal neovascularization in a laser-induced rat model. Gene Ther 2010;17:338-351.

68. Qu X, Cao Y, Chen C et al. A poly(lactide-co-glycolide) film loaded with abundant bone morphogenetic protein-2: A substrate-promoting osteoblast attachment, proliferation, and differentiation in bone tissue engineering. J Biomed Mater Res A 2015;103:2786-2796.

69. Cai C, Xie Y, Wu L et al. PLGA-based dual targeted nanoparticles enhance miRNA transfection efficiency in hepatic carcinoma. Sci Rep 2017;7:46250.

70. Liu P, Yu H, Sun Y et al. A mPEG-PLGA-b-PLL copolymer carrier for adriamycin and siRNA delivery. Biomaterials 2012;33:4403-4412.

71. Zeng W, Liu Z, Li Y et al. Development and characterization of cores–shell poly(lactide-co-glycolide)-chitosan microparticles for sustained release of GDNF. Colloids Surf B Biointerfaces 2017;159:791-799.

72. Esmaeili F, Dinarvand R, Ghahremani MH et al. Cellular cytotoxicity and in-vivo biodistribution of docetaxel poly(lactide-co-glycolide) nanoparticles. Anticancer Drugs 2010;21:43-52.

73. Mura S, Hillaireau H, Nicolas J et al. Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. Int J Nanomedicine 2011;6:2591-2605.

74. Liu Y, Lou C, Yang H et al. Silica Nanoparticles as Promising Drug/Gene Delivery Carriers and Fluorescent Nano-Probes: Recent Advances. Curr Cancer Drug Targets 2011;11:156-163.

75. Kievit FM, Veiseh O, Fang C et al. Chlorotoxin Labeled Magnetic Nanovectors for Targeted Gene Delivery to Glioma. ACS Nano 2010;4:4587-4594.

76. Li L, Jiang W, Luo K et al. Superparamagnetic Iron Oxide Nanoparticles as MRI contrast agents for Non-invasive Stem Cell Labeling and Tracking. Theranostics 2013;3:595-615.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

77. Gu J, Wang T, Fan G et al. Biocompatibility of artificial bone based on vancomycin loaded mesoporous silica nanoparticles and calcium sulfate composites. J Mater Sci Mater Med 2016;27:64.

78. Rincon MY, VandenDriessche T, Chuah MK. Gene therapy for cardiovascular disease: advances in vector development, targeting, and delivery for clinical translation. Cardiovasc Res 2015;108:4-20.

79. Cai L, Li J, Zhang X et al. Gold nano-particles (AuNPs) carrying anti-EBV-miR-BART7-3p inhibit growth of EBV-positive nasopharyngeal carcinoma. Oncotarget 2015;6:7838-7850.

80. Jin L, Zeng X, Liu M et al. Current Progress in Gene Delivery Technology Based on Chemical Methods and Nano-carriers. Theranostics 2014;4:240-255.

81. Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. Nat Biotechnol 2017;35:238-248.

82. Hong CA, Nam YS. Functional Nanostructures for Effective Delivery of Small Interfering RNA Therapeutics. Theranostics 2014;4:1211-1232.

83. Zhang Y, Wang Z, Gemeinhart RA. Progress in MicroRNA Delivery. J Control Release 2013;172:962-974.

84. Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. Eur J Pharm Sci 2010;40:159-170.

85. Dan N, Danino D. Structure and kinetics of lipid-nucleic acid complexes. Adv Colloid Interface Sci 2014;205:230-239.

86. Men K, Duan X, He Z et al. CRISPR/Cas9-mediated correction of human genetic disease. Sci China Life Sci 2017:1-11.

87. Cong L, Ran FA, Cox D et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819-823.

88. Song B, Fan Y, He W et al. Improved hematopoietic differentiation efficiency of

gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. Stem Cells Dev 2015;24:1053-1065.

89. Mali P, Yang L, Esvelt KM et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823-826.

90. Savic N, Schwank G. Advances in therapeutic CRISPR/Cas9 genome editing. Transl Res 2016;168:15-21.

91. He Z-Y, Men K, Qin Z et al. Non-viral and viral delivery systems for CRISPR-Cas9 technology in the biomedical field. Sci China Life Sci 2017:1-10.

92. Li L, Song L, Liu X et al. Artificial virus delivers CRISPR-Cas9 system for genome editing of cells in mice. ACS Nano 2016;11:95-111.

93. Zhen S, Takahashi Y, Narita S et al. Targeted delivery of CRISPR/Cas9 to prostate cancer by modified gRNA using a flexible aptamer-cationic liposome. Oncotarget 2017;8:9375.

94. Liang C, Li F, Wang L et al. Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma. Biomaterials 2017;147:68-85.

95. Yang J, Yang D, Sun Y et al. Genetic amplification of the vascular endothelial growth factor (VEGF) pathway genes, including VEGFA, in human osteosarcoma. Cancer 2011;117:4925-4938.

96. Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015:gutjnl-2015-309809.

97. Jiang C, Mei M, Li B et al. A non-viral CRISPR/Cas9 delivery system for therapeutic gene targeting in vivo. Cell Res 2017.

98. Koike-Yusa H, Li Y, Tan E-P et al. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat Biotechnol 2014;32:267-273.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

99. Wang T, Wei JJ, Sabatini DM et al. Genetic screens in human cells using the CRISPR-Cas9 system. Science 2014;343:80-84.

100. Xu C, Qi X, Du X et al. piggyBac mediates efficient in vivo CRISPR library screening for tumorigenesis in mice. Proc Natl Acad Sci U S A. 2017;114:722-727.

101. Liang X, Luo M, Wei X-W et al. A folate receptor-targeted lipoplex delivering interleukin-15 gene for colon cancer immunotherapy. Oncotarget 2016;7:52207.

102. Luo M, Liang X, Luo S-T et al. Folate-modified lipoplexes delivering the interleukin-12 gene for targeting colon cancer immunogene therapy. J Biomed Nanotechnol 2015;11:2011-2023.

103. He Z-Y, Deng F, Wei X-W et al. Ovarian cancer treatment with a tumor-targeting and gene expression-controllable lipoplex. Sci Rep 2016;6:23764.

104. Li L, Li X, Wu Y et al. Multifunctional Nucleus-targeting Nanoparticles with Ultra-high Gene Transfection Efficiency for In Vivo Gene Therapy. Theranostics 2017;7:1633.

105. Li L, Song L, Yang X et al. Multifunctional “core-shell” nanoparticles-based gene delivery for treatment of aggressive melanoma. Biomaterials 2016;111:124-137.

106. Kong F, Liu F, Li W et al. Smart Carbon Nanotubes with Laser-Controlled Behavior in Gene Delivery and Therapy through a Non-Digestive Trafficking Pathway. Small 2016;12:6753-6766.

107. Yin Z, Liu N, Ma M et al. A novel EGFR-targeted gene delivery system based on complexes self-assembled by EGF, DNA, and activated PAMAM dendrimers. Int J Nanomedicine 2012;7:4625-4635.

108. Wang C, Du L, Zhou J et al. Elaboration on the Distribution of Hydrophobic Segments in the Chains of Amphiphilic Cationic Polymers for Small Interfering RNA Delivery. ACS Appl Mater Interfaces 2017;9:32463-32474.

109. Liu J, Li J, Liu N et al. In vitro studies of phospholipid-modified PAMAM-siMDR1 complexes for the reversal of multidrug resistance in human breast cancer cells. Int J

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Pharm 2017;530:291-299.

110. Li J, Liu J, Guo N et al. Reversal of multidrug resistance in breast cancer MCF-7/ADR cells by h-R3-siMDR1-PAMAM complexes. Int J Pharm 2016;511:436-445.

111. Qian Y, Qiao S, Dai Y et al. Molecular-Targeted Immunotherapeutic Strategy for Melanoma via Dual-Targeting Nanoparticles Delivering Small Interfering RNA to Tumor-Associated Macrophages. ACS Nano 2017;11:9536-9549.

112. Zhang G, Guo B, Wu H et al. A delivery system targeting bone formation surfaces to facilitate RNAi-based anabolic therapy. Nat Med 2012;18:307-314.

113. Hao Z, Fan W, Hao J et al. Efficient delivery of micro RNA to bone-metastatic prostate tumors by using aptamer-conjugated atelocollagen in vitro and in vivo. Drug Deliv 2016;23:874-881.

114. Shi S, Han L, Gong T et al. Systemic delivery of microRNA-34a for cancer stem cell therapy. Angew Chem Int Ed Engl 2013;52:3901-3905.

115. Roursgaard M, Knudsen KB, Northeved H et al. In vitro toxicity of cationic micelles and liposomes in cultured human hepatocyte (HepG2) and lung epithelial (A549) cell lines. Toxicol In Vitro 2016;36:164-171.

116. Hwang T-L, Hsu C-Y, Aljuffali IA et al. Cationic liposomes evoke proinflammatory mediator release and neutrophil extracellular traps (NETs) toward human neutrophils. Colloids Surf B Biointerfaces 2015;128:119-126.

117. Hwang T-L, Sung CT, Aljuffali IA et al. Cationic surfactants in the form of nanoparticles and micelles elicit different human neutrophil responses: A toxicological study. Colloids Surf B Biointerfaces 2014;114:334-341.

118. Hwang T-L, Fang C-L, Al-Suwayeh SA et al. Activated human neutrophil response to perfluorocarbon nanobubbles: Oxygen-dependent and -independent cytotoxic responses. Toxicol Lett 2011;203:172-180.

119. Lv H, Zhang S, Wang B et al. Toxicity of cationic lipids and cationic polymers in gene

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

delivery. J Control Release 2006;114:100-109.

120. Carroll EC, Jin L, Mori A et al. The Vaccine Adjuvant Chitosan Promotes Cellular Immunity via DNA Sensor cGAS-STING-Dependent Induction of Type I Interferons. Immunity 2016;44:597-608.

121. Ross KA, Brenza TM, Binnebose AM et al. Nano-Enabled Delivery of Diverse Payloads Across Complex Biological Barriers. J Control Release 2015;219:548-559.

122. Yao H, Wang K, Wang Y et al. Enhanced blood–brain barrier penetration and glioma therapy mediated by a new peptide modified gene delivery system. Biomaterials 2015;37:345-352.

123. Zhang F, Xu CL, Liu CM. Drug delivery strategies to enhance the permeability of the blood-brain barrier for treatment of glioma. Drug Des Devel Ther 2015;9:2089-2100.

124. Li J-R, Shi L, Deng Z et al. Nanostructures of Designed Geometry and Functionality Enable Regulation of Cellular Signaling Processes. Biochemistry 2012;51:5876-5893.

125. Deng Z, Weng IC, Li J-R et al. Engineered Nanostructures of Antigen Provide an Effective Means for Regulating Mast Cell Activation. ACS Nano 2011;5:8672-8683.

126. Jin S, Li S, Wang C et al. Biosafe Nanoscale Pharmaceutical Adjuvant Materials. J Biomed Nanotechnol 2014;10:2393-2419.

127. Jiang D, England CG, Cai W. DNA Nanomaterials for Preclinical Imaging and Drug Delivery. J Control Release 2016;239:27-38.

128. Bi C, Wang A, Chu Y et al. Intranasal delivery of rotigotine to the brain with lactoferrin-modified PEG-PLGA nanoparticles for Parkinson's disease treatment. Int J Nanomedicine 2016;11:6547-6559.

129. Lopez AI, Kumar A, Planas MR et al. Biofunctionalization of silicone polymers using poly(amidoamine) dendrimers and a mannose derivative for prolonged interference against pathogen colonization. Biomaterials 2011;32:4336-4346.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

130. Wang X, Li J, Wang Y et al. HFT-T, a Targeting Nanoparticle, Enhances Specific Delivery of Paclitaxel to Folate Receptor-Positive Tumors. ACS Nano 2009;3:3165-3174.

131. Chan C-L, Majzoub RN, Shirazi RS et al. Endosomal Escape and Transfection Efficiency of PEGylated Cationic Lipid–DNA Complexes Prepared with an Acid-Labile PEG-Lipid. Biomaterials 2012;33:4928-4935.

132. Wang Y, Miao L, Satterlee A et al. Delivery of Oligonucleotides with Lipid Nanoparticles. Adv Drug Deliv Rev 2015;87:68-80.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Figure legends

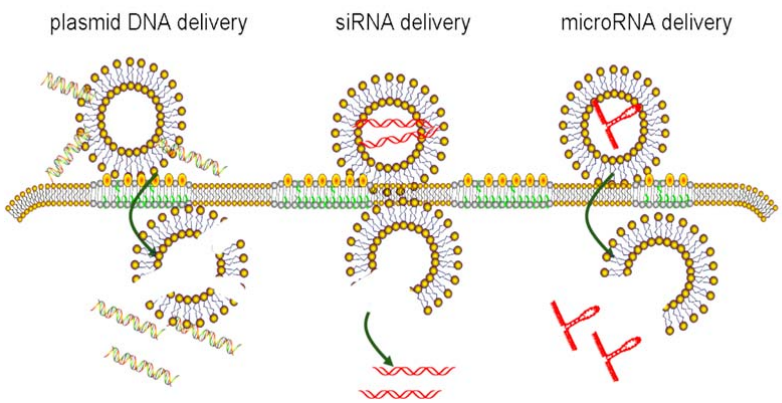

Figure 1 Non-viral delivery systems of gene delivery systems. Three examples of non-viral nucleic acid delivery are shown, each complexed to a cationic liposome: plasmid DNA, siRNA and miRNA

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

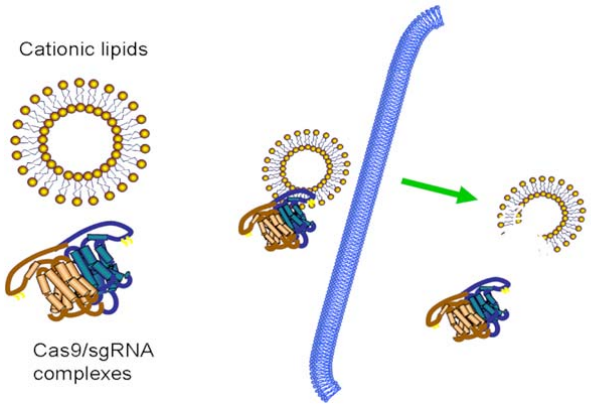

Figure 2 Non-viral gene delivery systems of CRISPR/Cas9 gene-editing systems. The example shown depicts a pre-loaded ribonucleoprotein, consisting of the Cas9 enzyme with sgRNA already bound. This Cas9-sgRNA complex then interacts with cationic lipids, which facilitate cell entry.

Human Gene Therapy
The Current Status of Nonviral Vectors for Gene Therapy in China (DOI: 10.1089/hum.2017.226)

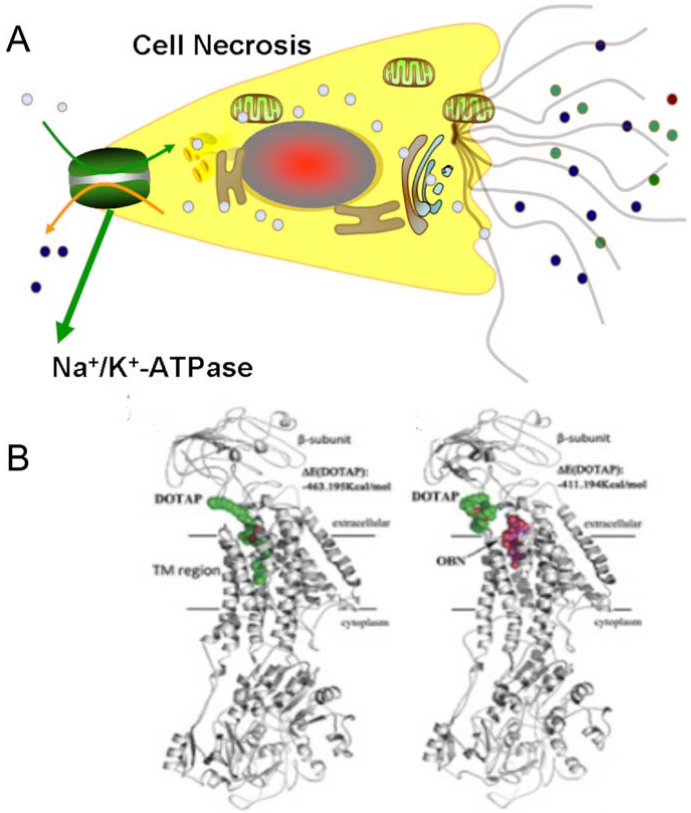

Figure3 (A) Cationic nanocarrier-induced cell necrosis. Cationic nanocarriers could interact with the cation-binding site of Na^+/K^+-ATPase. (B) The complex structures of Na^+/K^+-ATPase-DOTAP and Na^+/K^+-ATPase-ouabain/DOTAP were calculated (Xiawei Wei et al. Cell Res 2015; 25:237-253).