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# Genetic engineering of mesenchymal stem cells by non-viral gene delivery

Weiwei Wang[1], Xun Xu[1], Zhengdong Li[1], Andreas Lendlein[1, 2], Nan Ma[1, 2, 3]

[1] Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany

[2] Helmholtz Virtual Institute − Multifunctional Materials in Medicine, Berlin and Teltow, Germany

[3] Institute of Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

***** *Corresponding author: Nan Ma, Institute of Chemistry and Biochemistry, Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany. E-mail: nan.ma@hzg.de*

**Abstract**

Mesenchymal stem cells (MSCs) are an ideal cell source for tissue engineering and regenerative medicine as they possess self-renewal properties and multilineage differentiation potential. They can be isolated from various tissues and expanded easily through normal cell culture techniques. Genetic modifications of MSCs to further improve their therapeutic efficacy have been widely studied and extensively researched. Compared to viral gene delivery methods, non-viral methods generate less toxicity and immunogenicity and thus represent a promising and effective tool for the genetic engineering of MSCs. In the last decades, various non-viral gene delivery strategies have been developed and some of them have been applied for MSC transfection. This paper gives an overview of the techniques, influencing factors and potential applications of non-viral methods used for the genetic engineering of MSCs.

***Keywords:*** Mesenchymal stem cells, regenerative medicine, non-viral gene delivery, transfection, lipoplex, polyplex

**Abbreviations**

| | |
|---|---|
| Bcl-2 | B-cell lymphoma-2 |
| BDNF | Brain-derived neurotrophic factor |
| COMP | Cartilage oligomeric matrix protein |
| CNTF | Ciliary neurotrophic factor |
| CXCR-4 | C-X-C chemokine receptor type 4 |
| DOPE | 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine |
| DOSPA | 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate |
| DOTAP | N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate |
| DOTMA | N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride |
| GDNF | Glial cell line-derived neurotrophic factor |
| GNAS | Guanine nucleotide binding protein, alpha stimulating |
| NGF | Nerve growth factor |
| PAMAM | Poly(amidoamine) |
| PMMA | Poly(methyl methacrylate) |
| PPAR | Peroxisome proliferator-activated receptor |
| RGD | Arginine-glycine-aspartic acid |
| Runx2 | Runt-related transcription factor 2 |
| SDF-1 | Stromal cell-derived factor-1 |
| TAZ | Transcriptional coactivator with PDZ-binding motif |
| TERT | Telomerase reverse transcriptase |
| TGF | Transforming growth factor |
| VEGF | Vascular endothelial growth factor |

## 1. Introduction

Stem cell therapy has opened up entirely new possibilities for the promising field of tissue repair and regenerative medicine [158]. Mesenchymal stem cells (MSCs), an important type of adult stem cells, have been widely studied and utilized [170]. MSCs are self-renewing cells with the multilineage potential to differentiate into a variety of cell types, such as adipocytes, chondrocytes and osteocytes [135, 148]. MSCs were first identified about 30 years ago by Friedenstein et al. [48] and have ever since been isolated from bone marrow due to their ability to adhere to cell culture plastics [135]. It is now known that MSCs can also be obtained from various other tissues including peripheral blood [195], periosteum [32, 118], umbilical

cord blood [94], synovial membrane [33], pericytes [19], trabecular bone [122, 125], adipose tissue [14, 194], limbal stroma [137], amniotic fluid [75], lung [110], dermis [185] and muscle [15].

Due to several intrinsic advantages, MSCs are considered an ideal cell source for regenerative medicine. As adult stem cells, the utilization of MSCs is not subject to any ethical concerns and up to this day they have been applied to treat diverse human diseases, such as Parkinson's disease, myocardial infarction, cancer, hurler syndrome, spinal cord injury and acute graft-versus-host disease (as summarized by Hodgkinson et al. [65]). MSCs can be easily isolated from a variety of tissue sources and be expanded to a large scale by *in vitro* or *ex vivo* culture without loss of stem cell properties. The bedside applications of MSCs in clinical therapy can be performed in different ways including local transplantation, systemic administration and combination with tissue engineering. After systemic injection, the homing of MSCs to the sites of inflammation and injury occurs endogenously and can be enhanced by different methods [61, 65, 83, 190]. The therapeutic benefits of MSCs are attributed not only to their differentiation to tissue-specific cells, but also to the signalling through paracrine secretion and cell-to-cell contact [144]. Excitingly, they are immune privileged and display immunosuppressive effects, facilitating the allogeneic transplantation of MSCs [107, 120].

Although MSC based therapies benefit from the aforementioned advantages, the therapeutic success is still restricted by certain limitations. The *in vitro* expansion conditions and prolonged cell culture periods can influence the multipotency and phenotypes of MSCs [82, 164]. In some cases, the cultured MSCs may undergo transformation at an early stage and thus lose their therapeutic effect [50]. Furthermore, the clinical benefits after transplantation may also be limited due to the poor survival of MSCs [22, 67] and their low quality in case of age-related functional decline [153]. Most importantly, not all of the transplanted MSCs necessarily differentiate into the desired lineage to repair the damaged tissue. This potential risk was impressively demonstrated by transplanted MSCs into the heart which then differentiated into osteoblasts [18].

To address these questions and enhance the therapeutic efficacy, genetic modification of MSCs is generally perceived as a promising approach. Various viral and non-viral gene delivery methods have been developed in order to deliver genes in an optimal manner for the specific requirement. Although viral methods enabled high transduction efficiency and long-

term gene expression, their application is still limited due to some disadvantages, such as toxicity, immunogenicity, carcinogenicity, poor target cell specificity, high costs and inability to transfer large size genes [8, 16, 154, 169, 187]. Non-viral methods, despite being associated with a relatively low transfection efficiency and transient transgene expression, show a high potential due to advantages including relative safety, ability to transfer large size genes, less toxicity and easiness for preparation. In addition, non-viral vectors can be modified with tissue- or cell-specific ligands for targeting gene delivery [35, 92, 188].

Genetic modification of MSCs offers a promising potential for stem cell based therapies and numerous clinical benefits could be achieved. By delivering the inductive genes, MSCs could be guided to differentiate towards the desired lineages. They could also serve as a gene/drug delivery carrier after genetic modification and transplantation. Furthermore, MSCs could be traced easily both *in vitro* and *in vivo* after genetic modification with fluorescent proteins. Thus, genetic modification can be used as a tool for molecular and biological mechanism studies and therefore accelerate the use of human MSCs in clinical applications.

In this review, we will summarize the currently used non-viral methods for genetic engineering of MSCs, discuss the influencing factors on gene delivery efficiency and outline the potential of genetically engineered MSCs for stem cell based therapies. Moreover, the transfer of mRNA and siRNA into MSCs will be discussed.

## 2. Intracellular barriers of non-viral gene delivery into MSCs

Generally, the non-viral gene delivery into MSCs was performed *in vitro*. Effective gene delivery required that nucleic acid materials could be efficiently internalized by the cells and be transported into the appropriate cellular compartment in which the functionalization of the nucleic acid materials took place.

Passing through the cell membrane is one of the most important barriers for an efficient gene transfer. Although mRNA and siRNA do not need to cross the nuclear envelope, they still need to be transferred into the cytoplasm for functionalization. The electrostatic repulsion exists between plasmid DNA and the cell membrane since both are negatively charged. However, the entry of DNA into cytoplasm can be facilitated either by physical method or by chemical method. Transient holes on the cell membrane produced via physical gene delivery methods such as electroporation and sonoporation enable the free entry of DNA. The gene

delivery carriers such as cationic lipids or cationic polymers involved in chemical methods can form complexes with plasmid DNA via electrostatic interaction. Such complexes present the positive surface charge and can be easily internalized by cells via endocytosis [112].

The transfer of nucleic acid materials in cytoplasm is another barrier. For cationic lipids or cationic polymers mediated gene delivery, after endocytosis, the endosomes containing DNA will transform into digestive lysosomes. Endosomes first mature from “early” to “late” endosomes, and then fuse with lysosomes [104]. DNA in endosomes would eventually be degraded by lysosomal hydrolytic enzymes unless it could escape before the endosomes became mature. It has been demonstrated that the pH-responsive amphipathic peptides or lipid components with acid sensitive bonds can facilitate the DNA escape by rupturing the endosome membrane [98, 182]. Cationic polymers such as polyethylenimine (PEI) can induce the endosome rupture by another mechanism termed “proton sponge effect” [17]. The nucleic acid materials, either released from endosomes or delivered directly to cytoplasm, either in free form or in complexed form, have to be transported to the appropriate sites where they exert their functions. During the cytoplasmic diffusion, the nucleic acid materials suffer the degradation by digestive enzymes in the cytoplasm [93]. However, some results have suggested that the package by polycations could protect DNA from degradation [115].

The nuclear envelope is a crucial barrier for DNA entry into the nucleus. The nuclear envelope has a double-membrane structure and is interrupted by nuclear pore complexes (NPCs). NPCs have a small diameter (~9 nm) and play the role of controlling the transport through the nuclear envelope by allowing the free diffusion of small molecules and restricting the free entry of large marcomolecules [10]. The nuclear uptake of large proteins is mediated by nuclear localization signal (NLS) peptide in an active manner through sequence-specific recognition [176]. Hoare et al. used the peptides containing reiterated motifs of NLS in a cationic lipid mediated gene delivery into MSCs and observed a significantly enhanced transgene expression, presumably due to the improvement of nuclear entry [64]. The dissolution and reorganization of the nuclear envelope during or close to mitosis have been supposed to facilitate the nuclear entry of DNA molecules [36]. As a proof, various types of cells exhibited the dependence of transfection efficiency on cell cycle. The cells in S and G2/M phases showed significantly higher transfection efficiency than that in G1 phase [20, 21, 108, 138]. In addition the culture conditions inhibiting the division of human MSCs could decrease the transfection efficiency [89].

## 3. Techniques for non-viral gene delivery into MSCs

In the last decades, various non-viral gene delivery strategies have been developed and studied thoroughly. In the following paragraph, we will focus on the techniques that have been applied on MSCs, excluding other methods such as needle injection, jet injection, gene gun and hydrodynamic gene transfer. Furthermore, we will present the process and mechanism of gene delivery into MSCs via non-viral methods (Figure 6) as well as summarize the gene transfer techniques (Table 1).

### 3.1 Physical methods

#### 3.1.1 Microinjection

Microinjection is a mechanical process using a micropipette to penetrate the cell membrane and/or the nuclear envelope and inject the nucleic acid materials directly into a single living cell at a microscopic level [31, 179]. It is a relative simple, economic, effective, reproducible and non-toxic method with the potential to transfer large size DNA. However, microinjection is not necessarily suitable to transfer the nucleic acid materials into a large number of cells since it requires the individual manipulation of each cell. Furthermore, the size of the micropipette is a crucial parameter as a micropipette with a large diameter may damage the cells. A successful case of achieving high transfection efficiency (over 70%) without causing significant cell damage has been reported, where a tiny nanoneedle of 200 nm in diameter was used for gene delivery into the nuclei of human MSCs [60]. Strong GFP expression in the transfected human MSCs was observed 24 hours and 48 hours post transfection (Figure 1, [60]). Similarly, Tsulaia et al. performed the nuclear microinjection using a glass needle of 275 nm in diameter and achieved high transgene expression in human MSCs [161].

#### 3.1.2 Electroporation

Electroporation is a widely applied gene delivery method which utilizes the high-voltage electrical currents to create transient nanometer-scale pores on the cell membrane and thus allows the nucleic acid materials to enter into the cells.

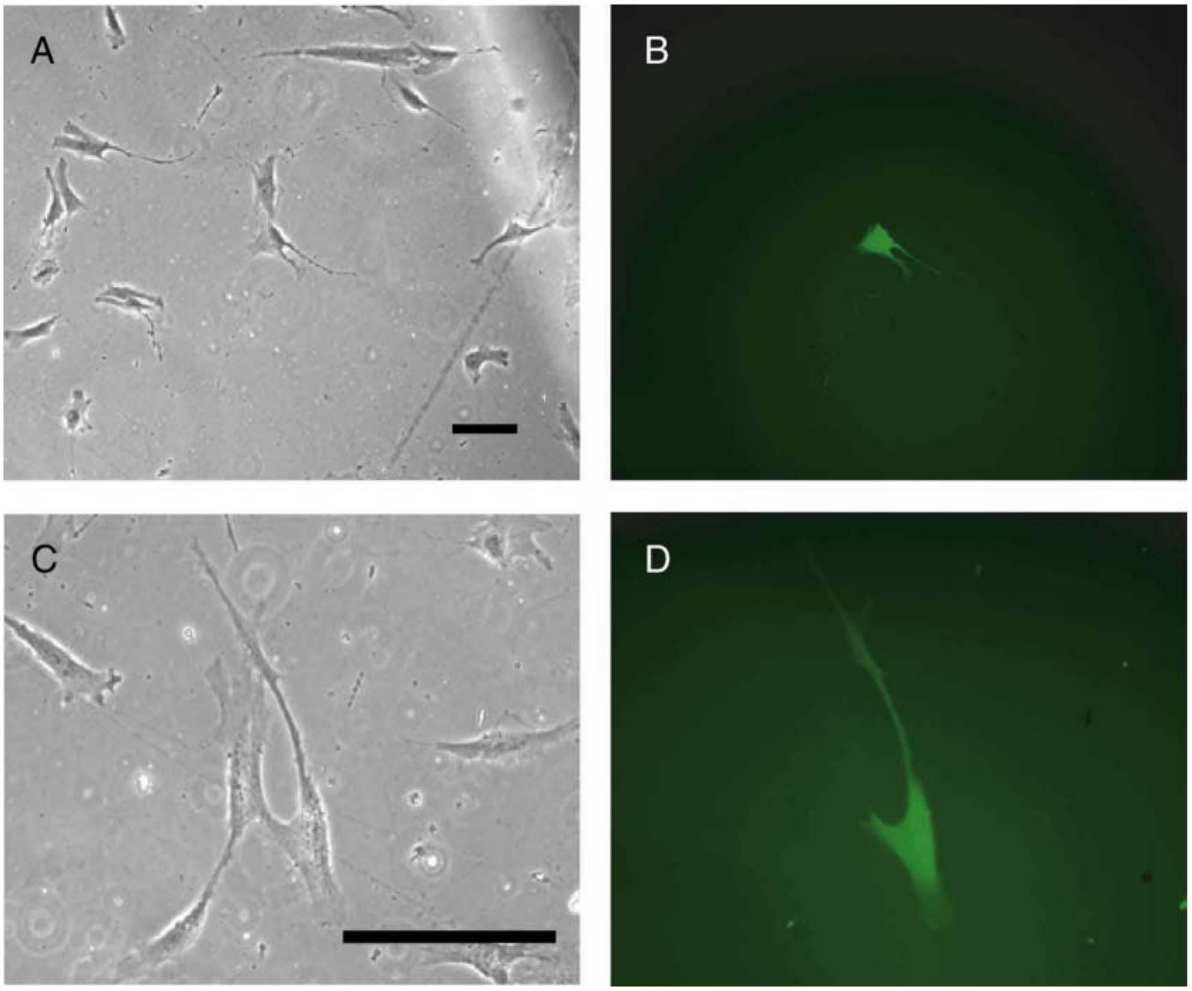


**Figure 1. GFP expression in a human MSC transfected by using a DNA-adsorbed nanoneedle. (A) A human MSC after incubation for 24 hours. (B) The fluorescence image of the human MSC. (C) The human MSC after incubation for 48 hours. (D) The fluorescence image of the human MSC after incubation for 48 hours. Scale bars = 100 µm. Reprinted from [60], Copyright 2008, with permission from Elsevier.**

Electroporation is also known as “microporation” when the transfection was performed at a microscale. An extension of electroporation termed “nucleofection” has been developed, in which the DNA was driven directly into the cell nuclei. Since the transfection of cells is no longer dependent on cell division, nucleofection has been believed to be suitable to transfect nonproliferating and hard-to-transfect primary cells [55]. Electroporation is a highly reproducible technology and its transfection efficiency can reach up to that of viral methods [4]. DNA with large size (100-150 kb) can be efficiently delivered by electroporation [24, 106].

For MSC transfection, nucleofection has been proved to present better transfection results than conventional electroporation [119] and transfection mediated by cationic lipids [3, 59,

113]. The nucleofection efficiency of human bone marrow derived MSCs could reach up to around 70% (Figure 2) [6]. Human umbilical cord blood-derived MSCs transfected with

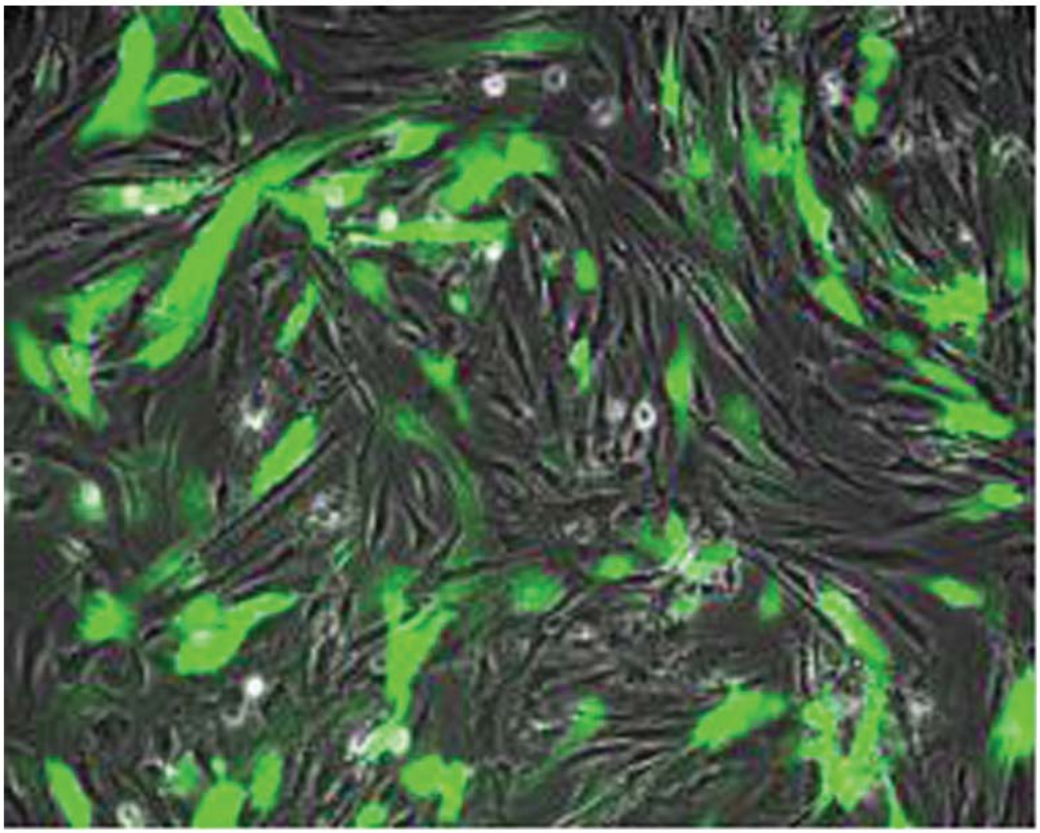

**Figure 2. Transfection efficiency of nucleofection of hMSCs with pEGFP. Bone marrow-derived hMSCs were nucleofected with 5 µg pEGFP. After nucleofection, the hMSCs were transferred immediately into complete growth medium and grown for 24 hours. The cells were replated after nucleofection with pEGFP and observed with a fluorescence microscope. Taken with permission from reference [6]. The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers.**

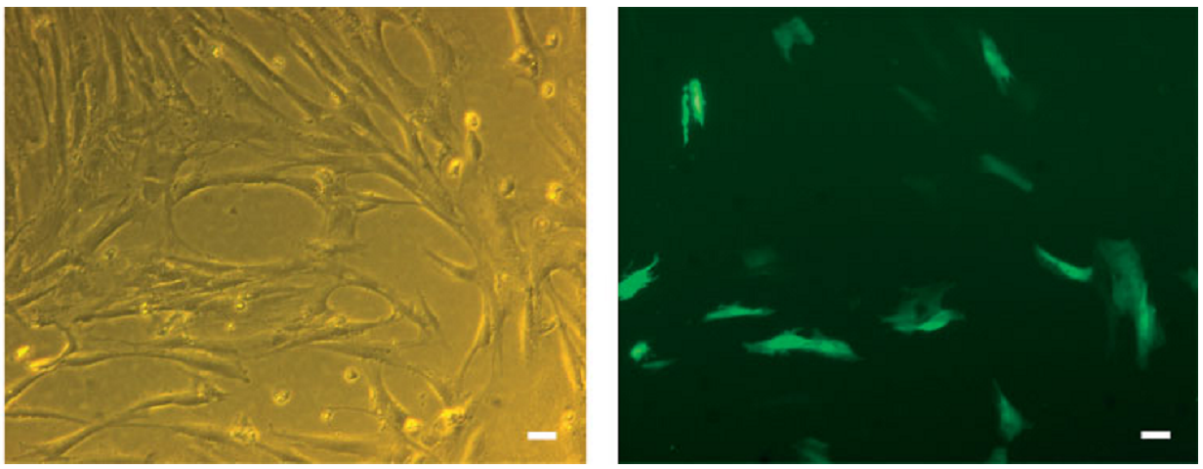

**Figure 3. Phase contrast (left) and fluorescence (right) microscopic view of Lipofectamine 2000 mediated pEGFP-N3 transfer in human MSCs, 24 hours after transfection (scale bars = 50 µm). Taken with permission from reference [66], Copyright©2005 John Wiley & Sons, Ltd.**

microporation exhibited high transfection efficiency (over 80%) while maintaining their immunophenotype, proliferation activity, differentiation potential, and migration ability towards cancer cells [101]. Moreover, it has been shown that the mRNA delivery into MSCs via nucleofection achieved a significantly higher protein expression than the plasmid DNA transfection [178].

The key aspects for influencing the transfection efficiency of electroporation include the electrical current intensity, the electric pulse and the cell type. Ziv et al. investigated the influence of the electrical current on electroporation. They discovered that the electrolytic gas bubble formation in the flow-through system could be avoided by using the alternating current (AC) of electrical pulses instead of the conventional direct current (DC),

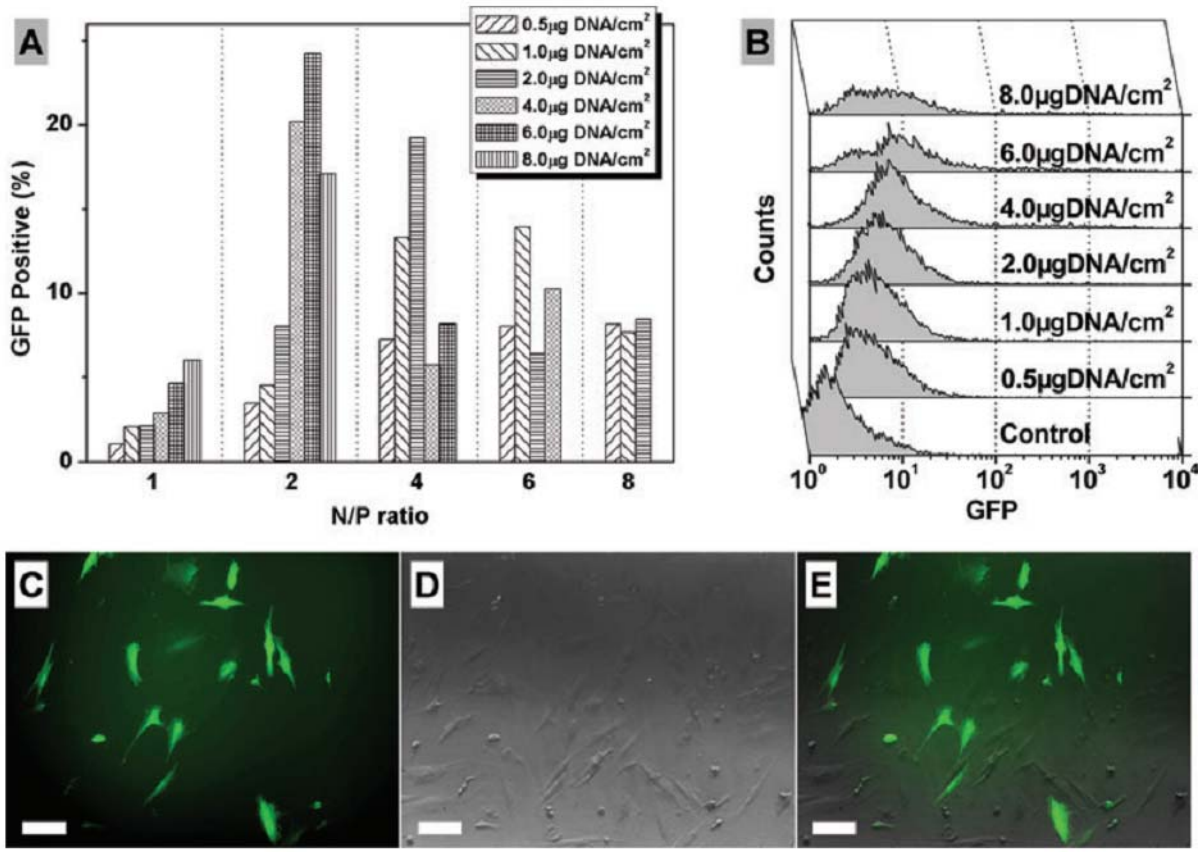


**Figure 4. PEI (branched, molecular weight 25000 Dalton) mediated gene delivery into bone marrow-derived hMSCs. Transfection efficiencies at various N/P ratio and DNA (pEGFP) dosage (A), and the histograms of FACS analysis at N/P ratio 2 and various DNA dosage (B). (C-E): Representative GFP expression of transfected hMSCs at N/P ratio 2 and DNA dosage 6.0 μg/cm$^2$. Fluorescence image (C), phase-contrast image (D) and merged (E) (scale bars = 100 μm). Taken with permission from reference [173], Copyright© 2011 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd.**

resulting in successful gene expression in MSCs [192]. Despite the aforementioned advantages, the high voltage involved in electroporation may lead to cell damage [39, 54, 59]. Other safety issues include the concern that high voltage may influence the stability of genomic DNA of the cells.

### 3.1.3 Sonoporation

Sonoporation, also known as ultrasound-facilitated gene transfer, uses ultrasonic waves to induce cell membrane permeabilization and consequently allows the intracellular entry of nucleic acid materials. With each ultrasonic cycle, part of the energy of the propagating waves is absorbed by the cells and results in the effect on cell membrane. In general, ultrasound was used together with the contrast agents to improve the gene transfer efficiency [40]. Contrast agents are gas-filled microbubbles normally stabilized by surface active molecules such as polymers or phospholipids. When activated by high-energy ultrasonic waves, microbubbles rapidly oscillate, expand, shrink and finally break up [174]. This causes the release of local shock waves and creates transient permeabilization on the membrane of nearby cells. Quite different to electroporation, in which DNA is driven by electric force and moves along the electric field, DNA moving in sonoporation is a completely passive diffusion [85].

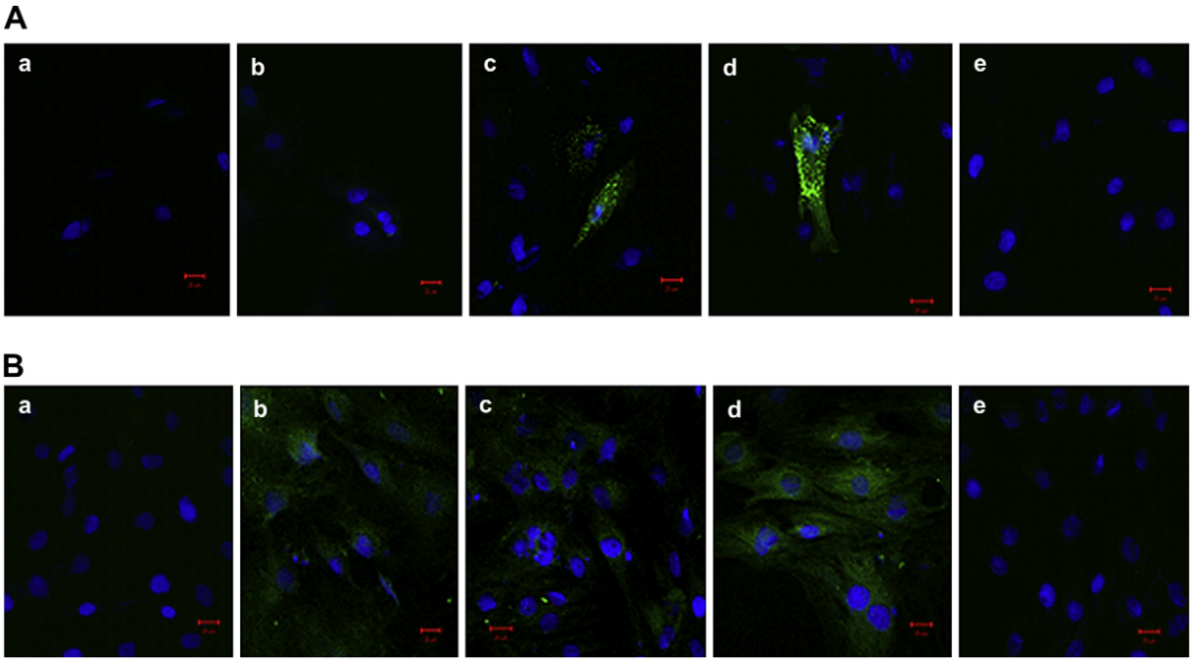


**Figure 5. Rat MSCs transfected with reverse transfection method. Multilayered and gene-functionalized titanium (Ti) films composed of chitosan (Chi) and DNA were prepared by layer-by-layer (LBL) assembly technique. GFP expression of rat MSCs adhered to different substrates after culturing 1 day (A) and 3 days (B) was observed using a confocal laser scanning microscope: (a) cells on TCP; (b) cells on TCP transfected with lipofectamine 2000; (c) cells on Ti film transfected with lipofectamine 2000; (d) cells on Chi/pEGFP-hBMP2 LBL-modified Ti film; and (e) cells on**

**Chi/control DNA LBL-modified Ti film. Reprinted from [72], Copyright 2009, with permission from Elsevier.**

**Table 1. Non-viral techniques applied for gene delivery into MSCs**

| Method | Advantages | Limitations | Carrier | Nucleic acid material | References |
|---|---|---|---|---|---|
| Microinjection | Simple, effective, reproducible, non-toxic, able to transfer large size DNA | Not suitable for a large number of cells | | DNA | [60, 161] |
| Electroporation | Effective, reproducible, able to transfer large size DNA | Cell damage, stability of genomic DNA might be influenced by high voltage | | DNA | [3, 6, 9, 26, 46, 58, 59, 81, 86, 95, 101, 105, 111, 113, 119, 131, 192] |
| | | | | mRNA | [142, 178] |
| Ultrasound-microbubbles | Safe | Low efficiency, cell damage | | DNA | [139] |
| | | | | siRNA | [126] |
| Cationic lipids | Easy to prepare, low cost, effective | Cytotoxicity, low efficiency at the presence of serum component | Lipofectamine™ based transfection reagent (DOSPA/DOPE) | DNA | [1, 12, 27, 42, 64, 66, 116, 150, 165, 168] |
| | | | | mRNA | [141] |
| | | | | siRNA | [66] |
| | | | Lipofectin™ transfection reagent | DNA | [111] |

| | | | | | |
|---|---|---|---|---|---|
| | | | (DOTMA/DOPE) | | |
| | | | Escort™ transfection reagent (DOTAP/DOPE) | DNA | [166] |
| | | | DOTAP/DOPE | mRNA | [141] |
| | | | GenePORTER™ 2 transfection reagent (cationic lipid/DOPE) | DNA | [13, 77, 155] |
| | | | Other lipids | DNA | [52, 66, 175] |
| | | | | siRNA | [66, 140] |
| Cationic polymers | Easy to prepare and chemically modify, low cost, effective | Cytotoxicity, safety concern for undegradable polymers | PEI | DNA | [1, 26, 28, 37, 38, 43, 57, 73, 89, 96, 97, 160, 162, 171, 173] |
| | | | PEI | mRNA | [141] |
| | | | PEI-acetic anhydride | DNA | [70] |
| | | | PEI-palmitic acid | DNA | [76] |
| | | | PEI-hyaluronic acid | DNA | [147] |
| | | | PEI-PLGA nanoparticles | DNA | [87, 130, 184] |
| | | | PEI-silica nanoparticles | DNA | [129] |
| | | | PEI-chitosan shell/PMMA core nanoparticles | DNA | [133] |
| | | | PLL | DNA | [43] |
| | | | PLL-palmitic acid | DNA | [27, 76] |

| | | | | | |
|---|---|---|---|---|---|
| | | | PLGA | DNA | [57, 151] |
| | | | Dendrimer (PAMAM based) | DNA | [89, 143, 149, 150, 165] |
| | | | Dendrimer (PAMAM-hydrophobic chains) | DNA | [146] |
| | | | Dendrimer (PAMAM-MSCs binding peptides) | DNA | [145] |
| | | | Dendrimer (PAMAM-RGD) | DNA | [128] |
| | | | Chitosan | DNA | [29, 72, 121] |
| | | | Spermine-pullulan | DNA | [62, 78, 84, 124, 159] |
| | | | Spermine-dextran | DNA | [71, 78, 79] |
| | | | | siRNA | [117] |
| | | | Spermine-mannan | DNA | [78] |
| | | | Spermine-gelatin | DNA | [69] |
| | | | Peptides | DNA | [80, 88, 127] |
| | | | Cationized *Lycium barbarum* polysaccharides | DNA | [167] |
| | | | poly(β-amino esters)s | DNA | [140, 183] |
| | | | poly(amidoamine)s | DNA | [132] |
| Inorganic nanoparticles | Easy to prepare, low | Relatively low | Hydroxyapatite nanoparticle | DNA | [30] |

| | | | | | |
|---|---|---|---|---|---|
| | cytotoxicity, high stability | efficiency | Magnetic nanoparticles of synthetic hydroxyapatite and natural bone mineral | DNA | [180] |
| Reverse transfection | High efficiency, low cytotoxicity, sustained gene release, long-term transgene expression | Not suitable to transfect cells supposed to be locally injected or systemically administrated | | DNA | [13, 30, 62, 69-73, 77, 80, 84, 96, 121, 124, 155, 160, 162, 171] |

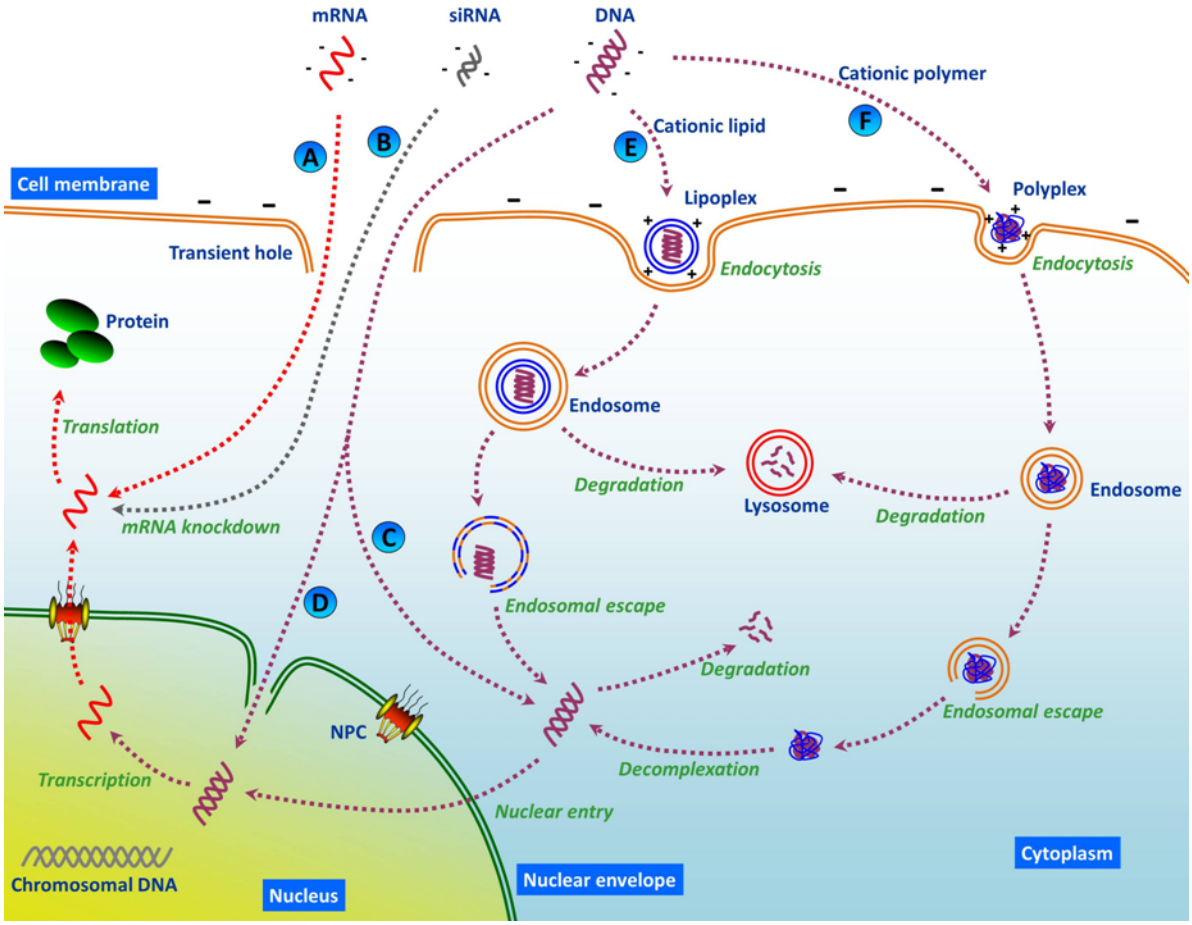


**Figure 6. The process and mechanism of non-viral gene delivery into MSCs. The intracellular entry of the naked nucleic acid materials (DNA, mRNA or siRNA) is restricted by the electrostatic repulsion since both cell membrane and nucleic acid materials are negatively charged. Transient holes on the cell membrane, produced by using physical methods (e.g. microinjection, electroporation and sonoporation (A-C)), allow the free entry of nucleic acid materials into cytoplasm. The intracellular entry can also be facilitated by chemical delivery carriers (e.g. cationic lipids (E) and cationic polymers (F)) that form positively charged complexes with nucleic acid materials (e.g. DNA). The complexes are mainly internalized via endocytosis, and the DNA has to escape from endosomes otherwise it will be degraded when endosomes transform into lysosomes. DNA travelling in cytoplasm exposes itself to the risk of being degraded by cytoplasmic nuclease. Finally, a small fraction of DNA enters into cell nuclei where the DNA functions. DNA can also be directly delivered into cell nuclei via microinjection or nucleofection (D).**

The gene transfer efficiency of sonoporation is influenced by several factors, such as the ultrasound frequency and intensity, the duration of treatment, the DNA amount, the contrast agents and the cell type. Although sonoporation is a safe gene delivery method, the relative low gene transfer efficiency is the major weakness. The utilization of sonoporation to transfect rat bone marrow derived MSCs has been demonstrated by Pu et al., who delivered hVEGF165 gene via an ultrasound-targeted microbubble destruction and optimized the

transfection conditions [139]. Otani et al. delivered siRNA into rat MSCs derived from bone marrow and adipose tissue by combining ultrasound and microbubbles. Although cell damage was observed after the treatment, the significant knockdown of the targeting mRNA was achieved, indicating that sonoporation could serve as a promising technique for RNA interference [126].

### 3.2 Chemical methods

Different from physical methods, in which nucleic acid materials were normally used in their naked form, chemical methods involve chemical carriers to complex and deliver nucleic acid materials into cells. In the last decades various chemical carriers have been prepared and intensively investigated, aiming to get a high transfection performance, such as low toxicity, high efficiency, biodegradability and targeting specificity. Among them, cationic lipids and cationic polymers are the most widely adopted carriers for MSC transfection. Recently, inorganic materials have also been employed as carriers to deliver genes into MSCs.

#### 3.2.1 Cationic lipids

In general, cationic lipid molecules are composed of three parts: hydrophilic head, linker and hydrophobic anchor [25]. According to the number of charges on the hydrophilic head which normally consists of one or more anime groups, cationic lipids can be classified as monovalent or multivalent. Multivalent cationic lipids can increase the transfection efficiency by offering a higher charge density of the membrane of lipid-DNA complexes (lipoplexes) [41]. Hydrophobic anchors are the nonpolar moieties of the cationic lipid molecules that drive the formation of lipoplexes. The hydrophilic head and the hydrophobic anchor are connected by the linker. The linkers determine the biodegradability of the cationic lipids, influence the contact between the positively charged cationic head and the negatively charged nucleic acid materials, and show effect on the cytotoxicity and transfection efficiency [102, 157].

The transfection efficiency could be improved by involving the co-lipid (e.g. DOPE) into the cationic lipid mediated gene delivery [51, 74, 114, 152]. It was assumed that DOPE facilitated the formation of liposomes and the transition of lipoplexes from a bilayer structure to a hexagonal arrangement under the pH value of the endosome level, which might induce the fusion or the destabilization of endosomal membranes [44, 90, 177, 193]. Up to date, the exact mechanisms by which cationic lipids deliver genes are still unclear. So far three aspects are perceived as the main contributions of cationic lipids: to enhance the binding of nucleic

acid materials to the cell membrane, to prevent the degradation of nucleic acid materials in cytoplasm and to facilitate the DNA escape from endosomes. Positively charged lipoplexes are able to bind to cell surfaces via electrostatic interaction and can be internalized in two different ways - by fusion with the cell membrane and by endocytosis [45]. Currently, it is believed that endocytosis is the more suitable approach [134, 186]. After internalization, the lipoplex can destabilize the endosomal membrane especially at the presence of a co-lipid, and can accordingly induce flip-flop of the anionic lipids. The anionic lipids then form charge-neutral ion pairs with cationic lipids, which causes the displacement of DNA from lipoplex and the subsequent DNA release into cytoplasm [182].

Cationic lipids mediated gene delivery has been widely applied for delivering nucleic acid materials including DNA, mRNA, and siRNA into MSCs. Depending on the functions of the delivered genes, lipotransfected MSCs could serve as a gene/drug delivery carrier or could be a guide to differentiate into the desired lineages [116, 140, 166, 168]. Rejman et al. transfected MSCs with mRNA encoding CXCR-4 and observed that the cationic lipids (Lipofectamine$^{TM}$ 2000 or DOTAP/DOPE) mediated delivery obtained around 80% CXCR-4 positive cells, as compared to 40% obtained by cationic polymer (PEI) mediated delivery [141]. Hoelters et al. compared different cationic lipids for transferring DNA and siRNA into human MSCs. The transfected cells maintained the proliferation activity and differentiation capacity into different mesodermal lineages without loss of transgene expression. The representative images of the cells transfected by Lipofectamine$^{TM}$ 2000 are shown in Figure 3. The siRNA delivery resulted in an efficient long-term RNA interference [66]. Cationic lipids are also usable together with cationic polymers. The combination of poly-*L*-lysine (PLL)-palmitic acid with Lipofectamine$^{TM}$ 2000 created an additive effect and increased the transfection efficiency of PLL-palmitic acid [27].

### 3.2.2 Cationic polymers

Cationic polymers typically contain a high density of amine groups, which are protonatable at neutral pH value. When mixed with negatively charged DNA, cationic polymers bind to DNA and form polymer-DNA complexes (polyplexes) through electrostatic interaction. Polyplexes are normally positively charged nanosized particles that can bind to the anionic sites on cell membrane and be internalized by cells via endocytosis. Inside cytoplasm, cationic polymers can protect DNA from degradation and facilitate DNA escape from endosomes.

Among numerous cationic polymers, PEI was the most widely used and investigated one. PEI is able to condense large DNA molecules into homogeneous spherical particles [23, 109]. After internalization, DNA can be well protected by PEI from degradation due to the high charge density of PEI. It is believed that PEI can induce the endosome rupture and thereby release DNA from endosomes into cytoplasm via "proton sponge effect" [17]. Since PEI is partially protonated at neutral pH, its remaining nitrogens can be further protonated at the lower pH in endosome, which will induce the influx of chloride counter ions, cause osmotic pressure within the endosomes and eventually trigger the swelling and the rupture of endosomes [2]. The presence of nitrogens that are protonatable at lower pH value seems crucial to induce the "proton sponge effect". One evidence is PLL which has only primary amine groups and is incapable of further protonation at lower pH value, resulting in less effective transgene expression than PEI in rat bone marrow-derived MSCs [43]. King et al. compared branched PEI, linear PEI (jetPEI$^{TM}$) and dendrimer based vectors (Superfect$^{TM}$) for gene delivery into human MSCs. The comparable transfection efficiencies were obtained using these three polymers 24 hours and 48 hours post transfection [89]. It has been observed that the transfection efficiency of PEI was highly dependent on polymer/DNA ratio (N/P ratio) and polyplex dose (an example is given in Figure 4) [1, 173]. In addition to PEI, a large number of cationic polymers have been developed, studied and used as non-viral gene carriers to deliver nucleic acid materials into MSCs, including natural polymers such as chitosan, dendrimers such as PAMAM and polypeptides such as PLL.

One of the valuable advantages of cationic polymers is that they can be easily modified to improve its transfection performance such as increasing efficiency, reducing cytotoxicity and realizing specific targeting. Compared to the unmodified PLL, the PLL conjugated with palmitic acid exhibited an enhanced capacity for cell binding and cell uptake as well as a significantly higher (~5-fold) gene delivery efficiency [76]. PEI acetylated with acetic anhydride showed an improved transfection efficiency on rat MSCs over the unmodified PEI [70]. The combination of PEI and hyaluronic acid through covalent binding resulted in the significant increase of transfection efficiency and cell viability in human MSCs [147]. Recently, a novel receptor mediated gene delivery has been applied for MSC transfection. The PAMAM dendrimers were functionalized by peptides with high binding affinity to MSCs. Such polymers exhibited a lower cytotoxicity and a higher transfection efficiency than native dendrimers, and showed the potential for targeting transfection of MSCs to minimize unwanted side effects in other tissues [145].

### 3.3 Inorganic Nanoparticles

Inorganic nanoparticles have been used alone or combined with organic carriers to deliver nucleic acid materials into living cells, since they can be loaded with nucleic acid materials via absorption or conjugation and be internalized by the cells. Compared to organic nanoparticles, inorganic nanoparticles hold some advantages including easiness for preparation, low cytotoxicity and high stability. Several types of inorganic nanoparticles have been employed as gene transfer carriers, including calcium phosphate [49], carbon nanotubes [53], magnetic nanobeads [99, 100], silica [11], gold [56] and quantum dots [156]. Hydroxyapatite nanoparticles mediated gene delivery into rat MSCs resulted in a significantly decreased cytotoxicity compared to cationic lipid, although the transfection efficiency was relatively lower [30]. Magnetofection of MSCs has been performed by using magnetic nanoparticles and magnetic force. Magnetofection, as the name implies, is a method that uses magnetic fields to concentrate magnetic particles loaded with nucleic acid materials into the target cells. It has been proved simple, highly efficient and capable of targeting gene transfer both *in vitro* and *in vivo* [99, 136]. Wu et al. prepared magnetic nanoparticles of synthetic hydroxyapatite and natural bone mineral as gene carriers and achieved sustained gene expression in about two weeks. They also investigated the effect of magnetic force on transfection efficiency, observing an enhanced gene expression under the presence of external magnets [180].

### 3.4 Reverse transfection

The conventional gene delivery into adherent cells was normally performed in a two dimensional (2D) cell culture system (e.g. cell culture plate) by adding nucleic acid materials to the pre-seeded cells. Recently, a different gene delivery method termed “reverse transfection” has been developed [191]. The difference between these two methods is the addition order of DNA and the cells. In conventional gene delivery, DNA was added for transfection after the seeded cells had completely attached to the cell culture surface. In reverse transfection, DNA was first loaded into the substrate materials and then the cells were seeded for transfection. The DNA, in free or complexed form, was able to interact with the substrate materials through either specific bindings, such as antigen-antibody and avidin-biotin, or through non-specific bindings, such as hydrophobic interaction, electrostatic binding, and van der Waals force [34, 163]. The MSCs cultured on the chitosan/DNA layer-by-layer modified titanium films have been observed to show a higher transgene expression than those transfected by conventional approaches (Figure 5) [72].

Compared to conventional methods, reverse transfection provides several advantages. Firstly, reverse transfections can be carried out using a three dimensional (3D) scaffold [70, 84], offering a more similar structure to the natural extracellular environment of cells in the body. It was observed that the interactions between cell-cell and cell-matrix are different in 2D and 3D systems, leading to different cellular behavior of MSCs [123]. The 3D transfection showed significantly enhanced efficiency than the 2D transfection [62, 70, 71]. Secondly, reverse transfection allows for a sustained gene release and consequently prolongs the transgene expression [71, 80, 172]. When the substrate materials containing genes are exposed to the cell culture medium *in vitro* or to the body fluid *in vivo*, the genes are released as a result of the swelling, dissolution or degradation of the substrate materials. The release speed is determined by multiple factors, including substrate composition [69], solution type [96], preparation methods and further treatment [84]. Huang et al. implanted poly (lactic-*co*-glycolic acid) (PLGA) sponges containing complexed DNA and human MSCs into the subcutaneous tissue of SCID (Severe Combined Immunodeficiency) mice and found that the transgene expression could be detected for up to 15 weeks, indicating the long-term active transfection by this system [73]. The cell viability can also be improved by sustained release because gradually released DNA complexes may have lower cytotoxicity than those added in one portion. Finally, the reverse transfection is ideal for the implantation of cell-scaffold composites. Compared to conventional methods in which the transfected cells need to be reseeded on scaffold materials for implantation, reverse transfection does not need this reseeding step. The scaffold materials, DNA and MSCs can be implanted together into the tissue. And the DNA incorporated in the scaffolds is capable of long-term *in vivo* transfection. Therefore, reverse transfection offers high potential in tissue engineering and regenerative medicine, especially for to bone regeneration [30, 70, 121, 160] and cartilage repair [62, 77].

Up to this date, several natural and synthesized substrate materials have been utilized for reverse transfection of MSCs including gelatin [84], collagen [13, 70, 77], fibronectin [171], poly (ethylene glycol) [96], PLGA [73] and poly(*L*-lactic acid) [80].

## 4. Factors influencing gene delivery efficiency

Compared to virus mediated gene delivery, the main drawbacks of non-viral gene delivery are the relatively low transfection efficiency and the transient transgene expression. Hence, to apply the non-viral gene delivery for MSC based therapies, it is crucial to make the transfection efficiency and the transgene expression period meet the therapeutic requirements.

Although the delivery methods and the carrier types are most important for transfection activity and therefore have been widely and deeply investigated as we discussed above, there are still some other factors strongly influencing the transfection efficiency of MSCs via non-viral methods. In the following paragraph, we will list and discuss these factors.

### 4.1 Nucleic acid materials

It has been observed that the sequence and structure of plasmid DNA can influence the transgene expression activity. Haleem-Smith et al., transfected human bone marrow-derived MSCs with different plasmids. Using the nucleofection technique, they first compared the transfection efficiency of two plasmids, namely pcDNA5/FRT-GFP and pEGFP-C1. Although both were driven by the cytomegalovirus (CMV) promoter, pcDNA5/FRT-GFP resulted in nearly 2-fold the transfection efficiency and about 10- to 20-fold higher GFP expression per cell than pEGFP-C1, suggesting that the transfection efficiency and the expression level of the transgene are highly dependent on the composition of the plasmid construct. Furthermore, they tested other plasmids containing the collagen type Iα1, collagen type IIα1 or COMP promoter respectively. Compared to CMV, plasmid containing either collagen type Iα1 or COMP promoter showed lower transfection efficiency, due in part to the lower transcription activity of these two promoters. Transfection with the plasmid containing collagen type IIα1 exhibited only a negligible efficiency, mainly because collagen type II is not expressed in the MSCs [58]. Tsulaia et al. discovered that the structure of plasmid DNA plays an important role to regulate transgene expression and that a supercoiled DNA induced higher transfection efficiency (~ 5-fold) than its linearized form after delivery into human MSCs via microinjection [161]. In addition, the influence of the types of the delivered nucleic acid material has been demonstrated that nucleofection of MSCs with mRNA resulted in a significantly higher protein expression than DNA transfection [178].

### 4.2 Receptor mediation

The involvement of receptors is another effective strategy to improve the transfection efficiency. Receptors can enhance the binding of DNA complexes to the cells or nuclei and can consequently facilitate the penetration of DNA complexes through the cell membrane or nuclear envelope. The enhanced transfection efficiency was achieved by using peptides containing reiterated motifs of NLS in a cationic lipid mediated gene delivery [64]. RGD, the integrin-binding peptides being widely used for gene delivery [91, 189], has also been

employed for MSC transfection. However, the effect of RGD on the transfection efficiency is still controversial, as so far different results have been observed [28, 128].

### 4.3 Cells

As a matter of fact, the transfection efficiency of human MSCs is highly dependent on the cell sources. By transfecting human MSCs derived from 30 donors with the mediation of PEI, we observed a variation of transfection efficiency among these MSC samples [173]. The transfection efficiency was not influenced by the age or gender of the donors, but was affected by the cell cycle. MSCs with high percentage of cells in the S-phase exhibited high transfection efficiency, which might be caused by the dissolution and reorganization of the nuclear envelope during or close to mitosis facilitating the nuclear entry of DNA molecules. Similarly, King et al. observed that the culture conditions inhibiting the division of human MSCs decreased the transfection efficiency [89]. These results suggested that using strategies to promote the proliferation of human MSCs may be helpful to enhance the transfection efficiency. Moreover, species have effect on MSC transfection. The MSCs derived from different species (human, rat and rabbit) showed different transfection efficiency [64]. Besides the cell source, the transfection efficiency can also be affected by the cell status including culturing density, passage number, and cell distribution [13, 89, 96].

### 4.4 Properties of the cell growing surface

Both of the chemical and physical properties of the cell growing substrate have been demonstrated to influence the transfection efficiency. Dhaliwal et al. studied the effect of different extracellular matrix (ECM) proteins including collagen I, vitronectin, laminin, collagen IV, fibronectin and ECM gel and their combinations on gene transfer into mouse MSCs. Compared to uncoated surface, the coating of collagen IV, fibronectin and ECM gel resulted in better cell spreading and in an increased transgene expression. In contrast, the coating of collagen I and vitronectin led to less cell spreading and the transgene expression of the cells on collagen I was inhibited. Although the coating of fibronectin resulted in higher transfection efficiency than collagen I, a higher polyplex internalization was found in the cells growing on collagen I. This finding suggests that the polyplexes internalized by cells on fibronectin are likely to be trafficked more efficiently to the cell nuclei than the polyplexes internalized by cells on collagen I [38]. Previous studies also indicated that fibronectin and collagen I mediated the polyplex internalization and transgene expression in MSCs through different endocytic pathways. Fibronectin promoted internalization through clathrin-mediated

endocytosis and this pathway enabled more efficient transfection than caveolae-mediated endocytosis and macropinocytosis [37]. Hence, a possible reason for the influence of ECM proteins on MSC transfection could be that MSCs on different ECM proteins internalized DNA through different pathways, which consequently affected the intracellular trafficking and transgene expression. Furthermore, physical properties of the cell growing surface were also proved to influence the MSC transfection. Cells growing on hydrogels with different stiffness presented different transfection efficiency. When the elastic moduli of the hydrogels was raised from 10 kPa to 670 kPa, the transgene expression level increased more than 2-fold [26]. These results indicated that the cellular microenvironment, where the cells reside, is an important factor for MSC transfection.

### 4.5 Medium movement

Transgene expression of MSCs can also be influenced by the movement of cell culture medium during the transfection procedure. By orbitally shaking the cell culture medium, an increased transfection efficiency of MSCs was observed in both the monolayer transfection and the 3D scaffold mediated reverse transfection [13]. Okazaki et al. compared the 3D scaffold mediated reverse transfections under different medium conditions: static, agitated and stirred. They found that, compared to static conditions, the transfection efficiency was improved by agitating the medium and was further increased by stirring the medium [124]. In another study, cells cultured on a 3D poly (glycolic acid)-reinforced collagen sponge exhibited higher and more sustained transgene expression in the perfused medium than in static or stirred conditions [69]. These findings emphasize the potential benefits of medium movement. Firstly, medium movement can increase the collision efficiency between the DNA complexes and the cells, and thereby enhance the endocytosis [13]. Secondly, medium movement is able to supply oxygen and nutrients to the cells, excrete the cellular waste more efficiently, and consequently improve the cell proliferation. The more rapidly proliferated cells showed higher potential for the nuclear internalization of the DNA [69, 124].

## 5. Benefits from genetic engineering of MSCs

The main aim of genetic engineering of MSCs is to improve the efficacy of MSC based therapy. Various functional nucleic acid materials have been delivered alone or co-delivered into MSCs by different methods in order to achieve the desired results (Table 2). The benefits from genetic modification of MSCs can generally be grouped into the categories listed below.

### 5.1 Differentiation

#### 5.1.1 Osteogenesis

Bone morphogenetic proteins (BMPs) are the most widely used genes to stimulate osteogenesis of MSCs. Hosseinkhani et al. cultured MSCs on a 3D sponge containing BMP-2 gene. After subcutaneous implantation of this cell-scaffold system into the back of rats, homogeneous bone formation was observed [70]. Other studies involving mice models demonstrated that bone formation can also be generated by using MSCs transfected with not only BMP-2 [116], but also BMP-4 [73] or BMP-9 [6]. It was noticed that the synergistic effect of dual genes can further stimulate the bone formation. Six weeks after *in vivo* transplantation, the MSCs transfected with the gene encoding BMP-2 and Runx2 resulted in a higher level of bone formation than those transfected with BMP-2 alone [95]. The co-delivery of GNAS-siRNA or Noggin-siRNA could significantly accelerate the osteogenic differentiation of human adipose-derived stem cells induced by BMP-2 [140].

**Table 2: Benefits achieved via genetic engineering of MSCs**

| Benefits | | Nucleic acid materials | References |
|---|---|---|---|
| Differentiation | Osteogenesis | BMP-2 | [6, 30, 69-72, 116, 143] |
| | | BMP2, Runx2 | [95] |
| | | BMP-2, GNAS-siRNA, Noggin-siRNA | [140] |
| | | BMP-4 | [73] |
| | | BMP-9 | [6] |
| | Chondrogenesis | TGF-β receptors | [81] |
| | | TGF-β1 | [62] |
| | | TGF-β2 | [168] |
| | | SOX-9 | [9, 87] |
| | | SOX Trio | [130, 184] |
| | | BMP-4 | [151] |
| | Adipogenesis | TAZ-siRNA | [117] |
| Angiogenesis | Enhance angiogenesis | VEGF, NGF | [42] |
| | | Adrenomedullin | [79] |
| | Inhibit angiogenesis | Endostatin | [77, 155] |
| Enhance cell survival | | Bcl-2 | [97] |
| | | TERT | [52, 175] |
| | | Adrenomedullin | [79] |
| Cell migration | | CXCR-4-mRNA | [142] |
| | | SDF-1 | [171] |

| | | | |
|---|---|---|---|
| Gene/drug delivery | | BDNF | [101, 150] |
| | | CNTF | [149] |
| | | GDNF | [13, 180] |
| | | Erythropoietin | [88, 113] |
| | | Epidermal growth factor (EGF) | [166] |
| | | Preproinsulin | [86] |
| | | Interleukin 12 | [3] |

### 5.1.2 Chondrogenesis

TGF-β is a well-known chondrogenic differentiation factor. TGF-β binds to the TGF-β type II receptor to form a complex that recruits the TGF-β type I receptor [7, 63]. The co-delivery of the type I and type II TGF-β receptors could increase the expression of the TGF-β receptor signaling in human adipose-derived stromal cells, resulting in an increased chondrogenic differentiation [81]. Human bone marrow-derived MSCs transfected with TGF-β type II produced collagen type II and aggrecan 48 hours after transfection, implicating that TGF-β type II can initiate and enhance the chondrogenic differentiation [168]. SOX-9 is a high-mobility-group domain transcription factor expressed in chondrocyte as well as in other tissues. It plays a crucial role in inducing and maintaining the expression of collagen II which is another important chondrogenic marker. Transfection with SOX-9 gene could promote the chondrogenesis of MSCs both *in vitro* and *in vivo* [9, 87]. Multiple deliveries of SOX-5, SOX-6 and SOX-9 genes (SOX Trio), in which SOX-5 and SOX-6 cooperated with SOX-9, have been proved to induce chondrogenic differentiation of MSCs [130, 184]. Recently, BMP-4 induced MSC chondrogenesis has been reported to improve the cartilage regeneration in the cartilage defect rabbits [151].

### 5.1.3 Adipogenesis

Runx2 and PPARγ are two key transcription factors that drive MSCs to differentiate into osteoblasts and adipocytes, respectively. It has been shown that TAZ functions as a differentiation modulator by coactivating Runx2-dependent gene transcription and repressing PPARγ-dependent gene transcription [68]. The delivery of TAZ-siRNA into MSCs to knockdown the TAZ activity could promote adipogenic differentiation, demonstrating the feasibility of using siRNA to direct the differentiation of MSCs [117].

### 5.2 Angiogenesis

Angiogenesis means the growth of new capillary blood vessels from pre-existing host vessels [47]. It is a key process in reparative and regenerative therapy, since neovascularization is essential to supply the defect area with nutrients, cytokines and functional cells and to dispose metabolic waste products [5]. Compared to untransfected MSCs, the transplantation of MSCs transfected with plasmid DNA encoding adrenomedullin (a peptide able to promote angiogenesis and inhibit apoptosis) into infarcted rat hearts resulted in significantly improved capillary density and cardiac function [79]. Although early-stage angiogenesis may be essential for tissue repair and regeneration, persistence of the vascular network may influence the later transformation/maturation in naturally avascular tissues such as articular cartilage. Based on this concern, the approach to regress vascularization was investigated by transfecting MSCs with plasmid DNA encoding endostatin. The transfected cells were observed to express endostatin, an anti-angiogenic factor, at a therapeutic level without losing their chondrogenic differentiation capacity, suggesting the potential of using anti-angiogenically modified MSCs for avascular tissue repair [77, 155].

### 5.3 Cell survival

The poor survival rate of MSCs after transplantation largely restricts the clinical benefits [22, 67]. Hence, to improve the cell survival it is of great importance for their therapeutic efficacy. After the delivery of hTERT gene into porcine and monkey MSCs, cells showed vigorous proliferation activity with high population doublings (PDs) and prolonged life spans, whereas the phenotype, the differentiation potential and the karyotype remained similar. Compared to the untransfected cells, the apoptotic rate of the transfected MSCs was significantly lower even at high PD [52, 175]. Li et al. transfected MSCs with Bcl-2 gene and found that Bcl-2 overexpression reduced the MSC apoptosis by 32% and enhanced the secretion of VEGF by more than 60% under *in vitro* hypoxic conditions. In contrast to MSCs transfected with control gene, Bcl-2 modified MSCs increased 2.2-fold (4 days), 1.9-fold (3 weeks) and 1.2-fold (6 weeks) of cellular survival *in vivo*, after transplantation into the myocardium of the infarcted rat hearts. Moreover, the transplantation of Bcl-2 modified MSCs resulted in a higher capillary density in the infarct border zone, a smaller infarction size and a remarkable recovery of cardiac function [97].

### 5.4 Cell migration

The therapeutic efficacy of systemically administered MSCs might also be restricted by their poor targeting specificity to the desired sites. The genetic modification of the cells with targeting receptors might solve the problem. The transwell migration experiments showed a significantly increased migration of CXCR-4-mRNA transfected MSCs toward a gradient of SDF-1, suggesting that the overexpression of mRNA mediated chemokine receptor allows for the transient initiation of chemotaxis [142]. We have reported that rat MSCs transfected with SDF-1 gene showed an attraction effect on c-kit+ stem cells, which demonstrated the inherent ability of cytokine modified MSCs to induce the migration of other functional cells to the relevant sites of the transplanted MSCs [171].

### 5.5 Gene/drug delivery

Finally, MSCs transfected with functional genes can also serve as a gene/drug delivery carrier. After the transplantation of transfected MSCs, the expressed proteins can function as therapeutic agents in the target tissue or organs, where the MSCs accumulated via active migration, guided targeting, or local injection. For example, MSCs have been used to deliver anti-tumor agents to specifically targeting tumors, since they possess the capacity to migrate toward cancer tissue [103, 181]. Up to this date, various functional genes have been involved in non-viral transfection of MSCs, such as BDNF, GDNF, and erythropoietin.

## 6. Conclusion

Genetic modification of MSCs via non-viral gene delivery has been widely and thoroughly researched over the past years. Different techniques and various functional genes have been applied and some remarkable results have been observed, indicating the high potential of non-viral gene transfer for improving the therapeutic efficacy of MSC based therapy. However, the relatively poor gene transfer activity including the low transfection efficiency and the short transgene expression period still pose a problem for an effective application of non-viral gene delivery. Further research is necessary and future studies should focus on the aspects of developing new techniques, preparing efficient gene carriers, optimizing transfection conditions, reducing cytotoxicity and cell damage, controlling gene release, and enhancing targeting specificity.

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