Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 90–104
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Review article

Non-viral gene transfection technologies for genetic engineering of stem cells

Jun-ichiro Jo, Yasuhiko Tabata *

Department of Biomaterials, Kyoto University, Kyoto, Japan

Received 4 January 2007; accepted in revised form 20 April 2007
Available online 21 July 2007

Abstract

The recent rapid progress of molecular biology together with the steady progress of genome projects has given us some essential and revolutionary information about DNA and RNA to elucidate various biological phenomena at a genetic level. Under these circumstances, the technology and methodology of gene transfection have become more and more important to enhance the efficacy of gene therapy for several diseases. In addition, gene transfection is a fundamental technology indispensable to the further research development of basic biology and medicine regarding stem cells. Stem cells genetically manipulated will enhance the therapeutic efficacy of cell transplantation. In this paper, the carrier and technology of gene delivery are briefly overviewed while the applications to the basic researches of biology and medicine as well as regenerative medical therapy are introduced. A new non-viral carrier and the cell culture system are described to efficiently manipulate stem cells.

Keywords: Non-viral gene carrier; Genetic engineering; Stem cells; Drug delivery system; Regenerative medical therapy

1. Introduction

Recent rapid progress of genome sciences and molecular biology has enabled to genetically analyze biological phenomena and promote the basic researches of gene biology and medicine. Genetic manipulation is one of the biomedical technologies and methodologies to replace a defective gene, correct a mutational gene, and induce an intrinsic healing potential [1,2]. Among them, gene therapies with genetic drugs for intractable diseases, such as genetic diseases, malignant tumors, and autoimmune diseases, have been clinically attempted [1,3–6]. On the other hand, the phenomenon of RNA sequence-specific gene suppression (RNA interference) has been discovered in mammalian cells [7]. Genetic analysis and therapeutic trials by use of the small interference RNA (siRNA) have been greatly paid attention [8–11]. Efficient usage of the RNA-related mechanism will be able to artificially enhance or suppress the level of gene expression. In this circumstance, it is indispensable to develop the technology and methodology for safe and efficient gene delivery which play an important role in the research tool to identify and investigate the genes contributing to the biological function of cells, as well as in enhancement of in vivo efficacy in gene therapy or the transplantation therapy with cells genetically engineered.

The gene delivery system is generally divided into two categories in terms of gene transfection carrier materials: viral [12–15] and non-viral [16–20] systems. For the viral system, the vector of retrovirus, lentivirus, adenovirus, and adenoassociated virus has been used to be potentially efficient, although there still remain some drawbacks to be resolved for the clinical applications. Some scientific trials have been performed to tackle the cytotoxicity and immunogenicity of virus itself and the possible mutagenesis

* Corresponding author. Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81 75 751 4121; fax: +81 75 751 4646.
E-mail address: yasuhiko@frontier.kyoto-u.ac.jp (Y. Tabata).

0939-6411/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2007.04.021

of cells transfected [21]. On the contrary, the non-viral system, such as naked plasmid DNA and plasmid DNA complexed with cationized carriers, is rather safe and has no limitation in the molecular size of DNA applied. However, the low transfection efficiency in vitro and in vivo is one major drawback for the research and therapeutic applications. Therefore, several synthetic materials, including calcium phosphate [22–24], cationized liposomes [25–27], and cationized polymers like poly-L-lysine [27–30] and polyethylenimine (PEI) [31–39], have been tried to molecularly design for not only the enhancement of cell internalization and transfection but also efficient delivery of plasmid DNA to the target site and the consequently high level expression. For successful gene therapy, it is necessary to consider the body distribution of gene injected pharmacokinetically and pharmacodynamically and achieve the efficient delivery of gene to the target organ and tissue [40–43]. Since generally gene expression by the non-viral system is transient, this system is not suitable to carry out the basic research of cells and treat diseases for which gene expression is required over several weeks or more. It has been well recognized that several viruses can integrate stably into the host genome, resulting in stable and long-term gene expression. However, the integration into the host genome possibly causes mutagenesis of cells transfected [21]. It is reported that non-viral gene carrier also can prepare cells stably expressed through an antibiotic selection [44]. However, the procedure is not practical because it takes a long time period to perform it and optimize the conditions. Therefore, to tackle the expression period issue, the controlled release inside the cells or in the tissue will be promising. Drug delivery system (DDS) technology and methodology are practically useful to modify and regulate the level and time period of gene expression.

In this paper, the DDS technology and methodology to enhance the in vitro and in vivo efficiency of gene expression are overviewed, while several examples of non-viral gene delivery and the gene expression data are introduced to emphasize importance of gene delivery technology in the basic researches of stem cells as well as the therapeutic applications.

2. Classification of non-viral gene delivery systems

2.1. Physical stimuli

Physical stimuli have been demonstrated to enhance the transfer of plasmid DNA into cells by positively carrying the DNA to the proximity of cell membrane and/or by temporarily microdisrupting the cell membrane. Based on this idea, several physical methods have been investigated to enhance gene expression in vivo by using the electric field (electroporation) [45–50], hydrodynamic pressure [51–54], ballistic pressure (gene gun) [55,56], magnetic field [57], and ultrasound [58–65]. In vivo electroporation of plasmid DNA has been attempted for a variety of tissues, such as the skin, kidney, lung, liver, skeletal and cardiac muscles, joints, spinal cord, brain, retina, and cornea [47–50]. Electrical field applied can enhance the permeability of cell membrane, resulting in increasing the internalization of plasmid DNA into the cell. Indeed, in vivo electroporation dramatically increases the local level of gene expression compared with that of free plasmid DNA injection. However, the tissue damage associated with electroporation always limits the efficiency of gene transfection [66]. Another physical stimulus is pressure, which is applied for the vascular delivery of gene, called ''hydrodynamic gene delivery'', improves in vivo transfection with a more widespread distribution than the local injection, particularly in the liver and skeletal muscle [51–54]. A rapid injection of plasmid DNA at an extremely high volume leads to highly efficient transfection in the liver. Zhang et al. have demonstrated that the hydrodynamic gene delivery results in a transient decrease in the heart function, but simultaneously a rapid increase in the venous pressure that leads to the fenestrae enlargement in the liver sinusoids [51]. This enabled the plasmid DNA to physically enhance the internalization into hepatocytes, thereby improving the efficiency of gene expression. When a large volume of plasmid DNA solution was injected into the femoral artery of non-human primates for a short time period, the plasmid DNA uptake and the expression were more efficient than those of the direct intramuscular injection [54]. Recently, Liang et al. have demonstrated that the hydrodynamic method achieves the high level expression of a dystrophin plasmid DNA in skeletal muscles for a mouse model of muscular dystrophy [67]. However, the practical application of this method to treat the Duchenne muscular dystrophy is still limited in terms of the low efficiency of plasmid DNA uptake by muscle cells and short time period of gene expression. In addition, there are still technological problems to be resolved for the clinical application of this method, such as the injection of a large volume of plasmid DNA with and the consequent tissue damage.

Bombardment of tissues by plasmid DNA-coated gold microspheres (gene gun) has been effective in gene expression only for superficial tissues, such as the skin. This approach allows the plasmid DNA to mechanically penetrate through the cell membrane into the cytoplasm or even the nucleus [55,56]. For the plasmid DNA coupled to paramagnetic nanoparticles, magnetic fields could provide the force necessary to assist the gene transfer. It has been demonstrated that the application of a strong magnetic field (magnetofection) after the local injection of plasmid DNA-coated paramagnetic nanoparticles enhanced the in vivo gene transfer to the gastrointestinal tract and the vasculature of ear [57]. However, since the level of gene expression is relatively low, further technical improvement of the method by combination with non-viral delivery systems is required for more efficient gene expression.

Recently, ultrasound (US) irradiation has been widely applied aiming at the efficacy improvement of therapeutic and diagnostic substances. It has been demonstrated that US irradiation could enhance the transfection efficiency of

plasmid DNA both in vitro and in vivo [59–65,68]. US irradiation generally generates the acoustic cavitation that can form transient pores at the cell membrane to accelerate the cellular uptake of plasmid DNA. It is recognized that this cavitation enables a plasmid DNA to deliver into the cytoplasm. Microbubbles of US contrast agents can act as "cavitation nuclei" and facilitate the generation of further cavitation. Taniyama et al. reported that transient pores were formed at the cell membrane in vitro immediately after US irradiation in the presence of Optison™, albumin-coated octa-fluoropropane gas microbubbles [59]. A combination of US irradiation with Optison [69,70] enhanced the transfection efficiency of plasmid DNA for the skeletal muscle [59], blood vessel [60], kidney [71], and spinal cord [72].

On the other hand, Unger et al. reported that the gene expression of plasmid DNA complexed with a cationic liposome was enhanced by US exposure [73]. Thus, combination with US irradiation is an important and useful strategy to modulate the expression level of plasmid DNA delivered with the non-viral system. We have experimentally confirmed the enhancing effect of US irradiation on the transfection efficiency of plasmid DNA combined with non-viral vectors in both in vitro [74,75] and in vivo [58,76]. As the non-viral carrier, gelatin derivatives prepared by the chemical introduction of different amine compounds, such as ethylenediamine (Ed), spermidine (Sd), and spermine (Sm), were used to allow plasmid DNA to form a complex with the gelatin derivatives. Following transfection of luciferase plasmid DNA complexed with the gelatin derivatives into rat gastric mucosal (RGM-1) cells with or without US exposure, gene expression was assessed to evaluate the effect of the type of gelatin derivatives on the gene transfection [74]. The cells incubated with the complex exhibited significantly stronger luciferase activity than that of free plasmid DNA while the activity was further enhanced by US irradiation. Among the amine derivatives of gelatin, the Sm derivative was the most effective in enhancing the level of gene expression because of the buffering ability superior to those of the Ed and Sd derivatives. In vivo experiments have confirmed that US irradiation significantly enhanced the gene expression of plasmid DNA [58,76]. These findings strongly indicate that US is a promising combinational tool to enhance the expression level of plasmid DNA complexed with the non-viral carriers.

2.2. Polymer-based gene carriers

Polymer-based gene carriers can be divided into three categories in terms of the apparent molecular size. The carrier with different size ranges has a size-dependent material character and can be used considering the characteristic in different cases required (Fig. 1).

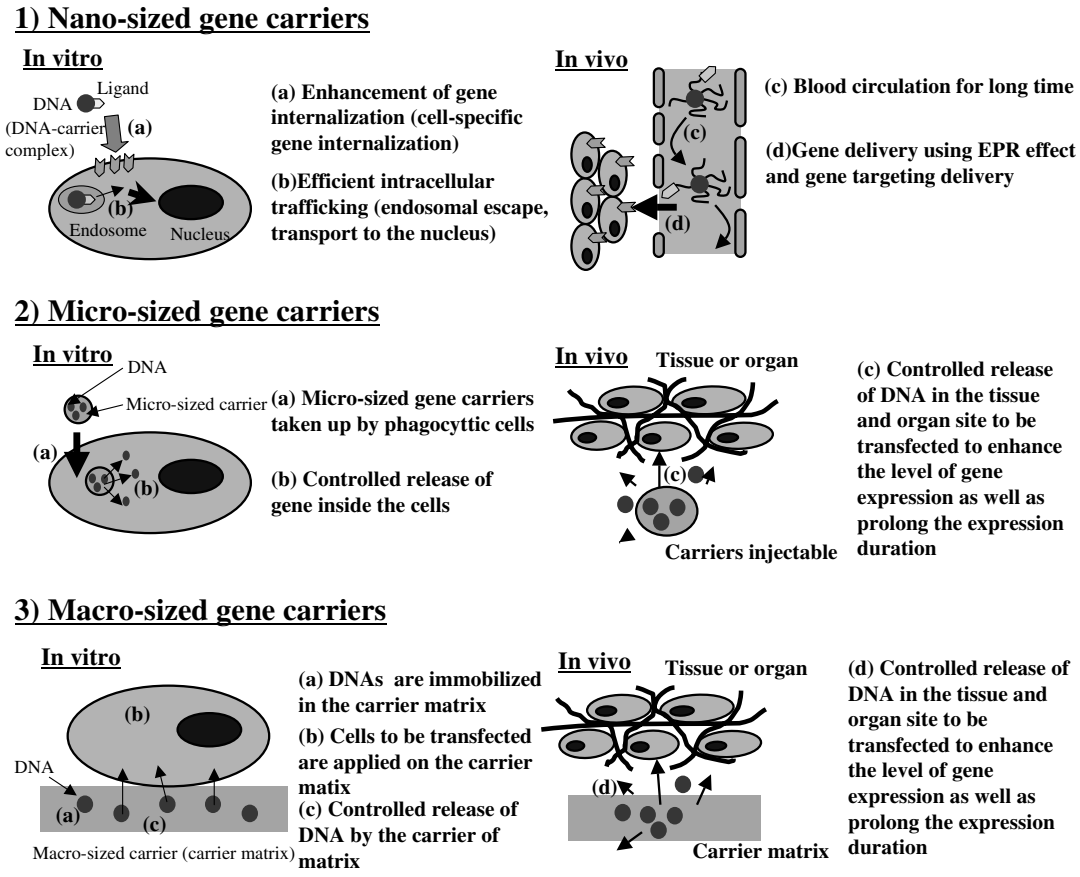

Fig. 1. Classification of non-viral gene carriers in terms of the carrier size.

2.2.1. Nano-sized gene carriers

Nano-sized gene carriers are mainly used for transit gene expression in vitro and the in vivo targeting of gene expression.

2.2.1.1. Nano-sized gene carriers for in vitro gene expression. Many research or review papers have pointed out several problems to be improved for efficient gene expression in vitro. Firstly, it is difficult to allow a naked DNA itself to internalize and transfect into cells, only when the DNA is applied into the cell culture medium. The plausible reason is the molecular size and surface charge of DNA itself. DNA is a polyanion of phosphate group-repeated chain and has an expanded molecular structure due to the intramolecular repulsion force of negative charge at the physiological pH. On the other hand, the membrane of cells is also negatively charged. Therefore, it is well recognized that DNA cannot interact with the cell membrane due to the electrostatic repulsion and consequently hardly be internalized and transfected. To enhance the internalization of DNA into cells, the DNA-carrier complex should have an appropriate molecular size and surface charge which are suitable to the interaction with the cell membrane and subsequently internalization into cells [53]. As one trial to enhance the cell internalization of DNA, covalent coupling of the non-viral carrier with the ligand for cell surface receptors, such as folate [77–80], transferrin [81–83], mono- or oligosaccharides [84–89], peptides [90–94], and proteins [81–83,95–98], has been attempted. Secondly, efficient intracellular trafficking ways, such as efficient endosomal escape and transport to the nucleus after DNA internalization, must be regulated for efficient gene expression. Considering the normal intracellular trafficking, the DNA-carrier complex internalized via the endocytosis pathway is carried into the endosomal compartment, followed by the subsequent lysosomal degradation. Therefore, the carrier should be designed to allow to effectively escape the DNA-carrier complex from the endosome into the cytoplasm. For example, when a carrier was covalently linked with a peptide capable to disrupt the lysosome membrane under an acidic condition where lysosomal enzymes biologically function, it effectively enhanced the level of gene expression [99]. It is reported that the carrier covalently linked with functional groups having a buffering capacity to accelerate the endosomal escape, so-called "proton sponge effect", enhanced the gene expression [100]. It is demonstrated that the carrier covalently linked with a peptide of nuclear localization signal (NLS) enables gene to positively transport to the nucleus [101].

Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through an α-1,6 linkage. The hydroxyl groups of pullulan can be chemically modified with ease to give it various chemical, physical, and biological properties. The carboxylmethyl pullulan of negative charge is a promising drug carrier which can prolong the drug lifetime in the bloodstream [102]. Pullulan derivatives with cholesteryl groups self-aggregated to form stable monodispersed nanoparticles of hydrogel which can incorporate water-soluble proteins, such as bovine serum albumin, chymotrypsin, and insulin [103–106]. Hydrogel microspheres prepared by the crosslinking of pullulan containing quaternary ammonium groups are tested as a bile acid sorbent [107]. On the other hand, pullulan has an inherent ability to accumulate in the liver [108] at higher amounts via the asialoglycoprotein receptor than other water-soluble polymers [109,110]. It is demonstrated that chemical conjugation with this pullulan enabled interferon (IFN) to target to the liver and consequently induce the IFN-specific enzyme thereat [111]. Since pullulan is a non-ionic polymer and cannot electrostatically interact with plasmid DNA, spermine was coupled covalently to allow pullulan to cationize by a *N*,*N*′-carbonyldiimidazole (CDI) activation method. Spermine is one of the polyamines present in the body, and cationization with spermine converted gelatin to a non-viral carrier of plasmid DNA with higher transfection efficiency than that with other amine compounds [76]. The cationized pullulan derivative was complexed with a plasmid DNA and applied to HepG2 cells for in vitro gene transfection. The level of gene expression depended on the molecular weight of pullulan and the extent of spermine introduced. Pre-treatment of cells with asialofetuin decreased the level of gene expression by the complexes. These findings indicate that the cationized pullulan derivative is a promising non-viral carrier of gene which is internalized in a receptor-mediated fashion [112].

2.2.1.2. Nano-sized gene carriers for in vivo gene expression. Since DNA in the naked form is readily degraded and denatured by nucleases present in the body [113], it is necessary to protect DNA from the nuclease hydrolysis by the complexation with a carrier [114–116]. Additionally, after administration into the body, generally the rapid uptake of drug-carrier complex by the mononuclear phagocyte system (MPS) takes place, which prevents the drug injected from delivering to the site of action other than the MPS tissue and organ. One of the effective ways to tackle this problem is the surface modification of the drug-carrier complex with poly(ethylene glycol) (PEG) or PEG-like polymers. It is well known that the cationized polymer modified with PEG electrostatically interacts with DNA to obtain complexes with a core-shell micelle structure. The polymer micelles of plasmid DNA from poly-L-lysine (PLL), poly(ethylenimine) (PEI), and poly(di-methylaminoethyl methacrylate) (PAMA) showed the blood circulation for longer time periods than free plasmid DNA [117]. Nano-sized carriers modified with PEG or PEG-like polymers enabled DNA to accumulate in the tumor or inflammation site, because of the anatomical vascular characteristics, such as the leaky vasculature and the lack of lymph vessels, so-called enhanced permeability and retention (EPR) effect [118,119]. Pronectin F+, an artificial protein with repeated RGDS sequences, was cationized and modified with PEG. When intravenously injected to mice carrying a

subcutaneous mass of Meth-AR-1 fibrosarcoma, the complex of PEG-introduced cationized Pronectin F+ and plasmid DNA enhanced the level of gene expression in the tumor to a significantly high extent compared with the complex of PEG-free cationized Pronectin F+ and free plasmid DNA [120].

On the other hand, the cationized pullulan derivative was complexed with a plasmid DNA and intravenously injected for in vivo gene transfection. The liver level of gene expression by the pullulan derivatives depended on the extent of spermine introduced. When a plasmid DNA coding the NK4 of hepatocyte growth factor (HGF)/scatter factor antagonist was complexed with the cationized pullulan and intravenously injected to mice 1 day before RLmale1 tumor cell inoculation, the complex significantly prolonged the survival period of tumor-bearing mice for a longer time period, while the number of tumor cells grown in the liver and the glutamic pyruvate transaminase (GPT) level were low compared with those of plasmid DNA injection. These findings indicate that the liver targeting of NK4 plasmid DNA by complexation with the spermine–pullulan significantly enhanced the expression level specifically in the liver, resulting in augmented suppression effect on tumor growth [121].

In addition, we have demonstrated that the anti-fibrotic activity of a plasmid DNA of TGF-β receptor siRNA expression vector in a mouse model of renal fibrosis was significantly enhanced by the complexation of a cationized gelatin and the intrarenal injection [122]. The injection of plasmid DNA-cationized gelatin complex significantly decreased the level of TGF-β receptor and α-smooth muscle actin over-expression, the collagen content of fibrotic mice kidney, and the fibrotic area of renal cortex, in contrast to the free plasmid DNA injection.

2.2.2. Micro-sized gene carriers

Micro-sized gene carriers are mainly used for the controlled release of gene in the organ or tissue and in the inside of cells.

Biodegradable polymers have been employed for the controlled release of plasmid DNA to enhance the level of gene expression as well as prolong the expression duration. The controlled release technology enables the DNA to increase and prolong the local concentration over an extended time period around the tissue injected. The biodegradable polymers are formulated into various shapes, such as microspheres and sheets. The biodegradable polymers of microsphere shape can be used as micro-sized gene carriers of injectability. Several researches have been reported on the controlled release of DNA from the microspheres of biodegradable poly(lactic acid)-poly(ethylene glycol) [123], poly(2-aminoethyl propylene phosphate) [124], poly(β-(4-aminobutyl)-L-glycolic acid) [125], and gelatin [122,126–128]. We have prepared cationized gelatin by chemically introducing amine residues to the carboxyl groups of gelatin. The cationized gelatin prepared was crosslinked by various concentrations of glutaraldehyde to obtain cationized gelatin hydrogels with different in vivo degradabilities as the release carrier of DNA. The cationized gelatin hydrogels can be easily formulated into a microsphere type. The hydrogel microspheres of cationized gelatin incorporating a plasmid DNA not only enhanced the level of gene expression to a significantly greater extent than the plasmid DNA injected in the solution form, but also prolonged the duration of gene expression. The time profile of plasmid DNA release was in good accordance with that of hydrogel microsphere degradatation [126,128]. The plasmid DNA is immobilized and biologically stabilized in hydrogel microspheres by the complexation with the cationized gelatin. When the hydrogel microspheres incorporating plasmid DNA are injected, with the degradation of hydrogel to generate water-soluble cationized gelatin fragments, the controlled release of plasmid DNA-cationized gelatin complexes is achieved to enhance the concentration of plasmid DNA around the injection site, resulting in increased efficacy of gene transfection. For the hydrogel system, the plasmid DNA release is driven only by the degradation of release carrier, which is quite different from the conventional mechanism reported so far where the release of plasmid DNA is governed by the DNA diffusion through the water phase in the hydrogel. The controlled release technology promoted the biological activity of an anti-tumor plasmid DNA [127,129] of NK4 which is a protein composed of the NH_2-terminal hairpin and the subsequent four-kringle domains of HGF. It is known that the NK4 is an HGF antagonist and inhibits the bioactivity of HGF for tumor, such as tumor metastasis and angiogenesis. The subcutaneous injection of hydrogel microspheres incorporating the NK4 plasmid DNA into nude mice with ascitic AsPC-1 tumor cells significantly prolonged the mice survival compared with that of NK4 plasmid DNA in the solution form. On the other hand, matrix metalloproteinase-1 (MMP-1) digestion allows a fibrotic tissue to convert the tissue to healing-inducible state where the natural process of tissue regeneration can function to heal fibrosis. Cationized gelatin microspheres incorporating a MMP-1 plasmid DNA were injected into the subcapsule of mouse kidney in advance, and then the mice received streptozotocin (STZ) to induce diabetic renal disease. It is reported that the advanced lesion of STZ-induced diabetic kidney mimics some findings of early-stage clinical diabetic nephropathy. It has been shown that renal fibrosis was histologically suppressed by the application of cationized gelatin microspheres incorporating MMP-1 plasmid DNA, which was in contrast to that of free MMP-1 plasmid DNA. On the contrary, the administration of empty cationized gelatin microspheres was not effective and the tissue appearance was similar to that of the saline-administered control group [130]. It is concluded that the controlled release is a promising technology to enable plasmid DNA to enhance the in vivo biological effects. The cationized gelatin hydrogel microspheres can be also applied to the controlled release of small interfering RNA (siRNA) that silences the func-

tions of RNA in the sequence-specific manner. The cationized gelatin hydrogel microspheres incorporating a siRNA of heat shock protein 47 (HSP47), which is a collagen-specific molecular chaperone, were retrogradedly injected via the ureter of unilateral ureteral obstruction (UUO) mice. Injection of the cationized gelatin hydrogel microspheres incorporating HSP47 siRNA achieved the controlled release at the injected site and improved the therapeutic effect compared with that of free HSP47 siRNA [131]. The cationized gelatin hydrogel microspheres incorporating a siRNA of vascular endothelial growth factor (VEGF), which is a key molecule in vasculogenesis as well as angiogenesis, were subcutaneously injected to tumor-bearing mice. Controlled release of the VEGF siRNA enabled to inhibit the expression of VEGF mRNA and secretion of VEGF protein in the tumor cells, resulting in suppression of tumor growth, which is contrast to that of free VEGF siRNA [132].

Moreover, it has been recognized that gelatin microspheres are taken up by phagocytic cells. For example, when given to human endothelial progenitor cells (EPC) of phagocytic property, the cationized gelatin microspheres incorporating plasmid DNA were internalized and consequently achieved the controlled release of plasmid DNA inside EPC [133]. This intracellular release technology could enhance the level of gene expression as high as or higher than that of an adenoviral vector [134].

2.2.3. Macro-sized gene carriers

Macro-sized gene carriers are mainly used for the controlled release of DNA in the tissue and organ sites to be transfected and the field of gene-based tissue engineering. In the macro-sized gene carrier systems, genes are immobilized in the biodegradable polymer matrix, followed by the application of cells to be transfected and the implantation or injection in vivo. Several researches have been reported on the controlled release of DNA by the carrier of matrix type prepared from biodegradable polymers, such as poly(D,L-lactic acid-co-glycolic acid) (PLGA) [135–141], poloxamer [116], poly(ethylene-co-vinyl acetate) [142], silk-elastinlike polymer [143], atelocollagen [144,145], denatured collagen-PLGA [146], and gelatin [126,128]. Mooney et al. have reported that the in vivo sustained release of a plasmid DNA encoding platelet-derived growth factor (PDGF) from a PLGA matrix carrier enhanced the deposition of extracellular matrix and blood vessel formation [135,136]. The controlled release of plasmid DNA with an atelocollagen minipellet has been investigated to demonstrate the enhancement of gene expression level and the consequent therapeutic effects with some disease model animals [144,145]. We have explored cationized gelatin hydrogel sheets as the macro-sized gene carrier in vivo. Generally, gelatin is not degraded by simple hydrolysis, but by proteolysis. The water content of hydrogel is one of the factors affecting the crosslinking extent of hydrogels; the higher the water content of hydrogels, the smaller their crosslinking extent. Hydrogel with smaller crosslinking extents or higher water contents is more susceptible to enzymatic digestion, resulting in faster degradation. For example, a cationized gelatin hydrogel with a water content of 98.3 wt% was degraded with time to completely disappear in the femoral muscle of mice 14 days after implantation. The time periods of complete degradation for the cationized gelatin hydrogels with water contents of 97.4 and 99.7 wt% were 21 and 7 days, respectively [128]. This indicates that in vivo degradation of gelatin hydrogels can be controlled by their water content. When plasmid DNA was incorporated into the cationized gelatin hydrogel with different water contents and implanted into the mouse muscle, the remaining plasmid DNA decreased with time although the time profile depended on the type of hydrogel. The plasmid DNA remained in the muscle for longer time periods as the water content of hydrogels used became lower. The time profile of plasmid DNA remaining was well correlated with that of hydrogel remaining, irrespective of the hydrogel water content. This finding indicates that as expected, plasmid DNA was released from the cationized gelatin hydrogel as the hydrogel was degraded in vivo. It is likely that the plasmid DNA molecules ionically complexed with the cationized gelatin cannot be released from the cationized gelatin hydrogel unless hydrogel degradation takes place to generate water-soluble cationized gelatin fragments. Based on this release mechanism, it is conceivable that the plasmid DNA molecules are released from the hydrogels as a complex with degraded gelatin fragments of positive charge. If the plasmid DNA-cationized gelatin complex has a positive charge, the charge will be able to assist the cell internalization of plasmid DNA because the complex easily interacts ionically with the cell surface of negative charge. In addition, this complex prevents the plasmid DNA from the enzymatic degradation by DNase. It is well recognized that polyionic complexation effectively suppresses the DNase degradation of plasmid DNA [114–116]. Taken together, it is likely that the plasmid DNA is biologically stabilized by the incorporation into the hydrogel and the controlled release enhances the local concentration of plasmid DNA around cells to be transfected, resulting in increased efficiency of gene transfection. It has been shown that the time period of gene expression induced by lacZ plasmid DNA incorporated in hydrogel was significantly longer than that of lacZ plasmid DNA in the solution form. It is possible that the extended release technology enables the plasmid DNA to maintain the concentration at the implanted site for a longer time period, resulting in prolonged gene transfection.

2.3. Liposome-based gene carriers

Cationized liposomes have been investigated most extensively as the non-viral gene carrier for efficient gene delivery [147–149]. The cationized liposomes are composed of various kinds of lipids, such as quaternary ammonium detergents, the cationized derivatives of cholesterol and

diacylglycerol, and the lipid derivatives of polyamines. Complexation with the cationized liposome converts the charge of plasmid DNA positive to permit the interaction with the negative charge of cell membrane, resulting in enhancing gene transfection into cells. It has been demonstrated that dioleoylphosphatidylethanolamine (DOPE) of a natural lipid is capable of facilitating the endosomal escape of plasmid DNA [150]. The mixture of the DOPE with other cationized lipids has been commercially available as lipofection reagents. In addition, it has been demonstrated that the lipid composed of cholesterol and *N*-[1-(2,3-dimyristyloxy)propyl]-*N*, *N*′-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide show a destabilizing property of endosome membrane [151]. The liposome can be prepared easily to include large-sized DNA [152] and has been applied to in vitro gene transfection. However, the liposome is not always suitable for in vivo gene delivery [153]. This is because the liposome injected intravenously tends to be mainly accumulated in the lung [154]. To overcome the issue of body distribution, modification of the liposome with cell-selective moieties has been attempted experimentally [42,155,156]. Kawakami et al. demonstrated that liposomes with the galactose and mannose residues on the surface were designed and demonstrated to deliver plasmid DNA selectively to liver parenchymal and liver non-parenchymal cells, respectively, in a receptor-mediated fashion, in contrast to the liposome without sugar residues [157–159].

A liposome with a fusigenic nature combined with the hemagglutinating virus of Japan (HVJ) designed [160]. The HVJ-liposome is prepared by the molecular fusion of liposome with HVJ which is inactivated by ultraviolet irradiation and expresses two glycoproteins of hemagglutinin–neuraminidase (HN) protein and fusion (F) protein on the surface [161]. The HN protein binds to a receptor of sialic acid and degrades the receptor through its neuraminidase activity. The F protein is cleaved by various proteases to generate a hydrophobic fusion peptide. Since the HVJ-liposome is internalized into cells in a membrane fusion–mediated fashion, it has a characteristic property to avoid the lysosomal degradation of plasmid DNA incorporated prior to the delivering to the cytoplasm [162]. The HVJ-liposome exhibits gene expression superior to the conventional liposome, but the level is not always as high as expected [162]. As the second generation, a HVJ-envelope (HVJ-E) vector, which is the HVJ particle itself without the liposome fusion, has been developed. The HVJ-E is a promising carrier to incorporate plasmid DNA as well as an antitumor agent [163], small interfering RNA (siRNA) [163], and magnetic beads [164]. It has been reported that the level of gene expression by HVJ-E conjugated with the cationized gelatin for tumor cells in vitro and that of liver cells in vivo after intravenous injection were enhanced to a significantly higher extent compared with those of HVJ-E alone [165].

Recently, to overcome the barrier of cell membrane and intracellular organella membranes against the efficient gene expression, multifunctional envelope type nano device (MEND) has been proposed by Harashima and co-workers [166]. The MEND is composed of a lipid envelope and DNA-polycation complexes incorporated in the envelope. In addition, ligands for specific cells and membrane fusion peptides with a pH-responsive property can be introduced on the surface of lipid envelope. It has been reported that the MEND modified with transferrin [167] and octaarginine [168] was internalized into cells in a receptor-mediated endocytotic and a non-endocytotic fashion, respectively, to demonstrate the enhanced gene expression.

3. Applications of non-viral gene carriers to the basic biology and medicine of stem cell biology and regenerative medical therapy

Since stem cells possess high potentials of proliferation and differentiation into different cell lineages, they have been widely investigated for their clinical applications to tissue repair and regeneration [169]. Compared with embryonic stem (ES), adult stem cells can be harvested from patients themselves and the ethical barrier for clinical applications is low. cells. Based on this situation, some clinical experiments of cell-based tissue regeneration have been conducted with adult stem cells, such as mesenchymal and neural stem cells. In addition to the inherent multipotentials of stem cells, it is no doubt that combinational use of humoral active factors provides an appropriate environment which enables the cells to enhance more efficiently their ability for tissue regeneration. Since gene transfection can genetically engineer stem cells in terms of the biological functions as well as the proliferation and differentiation abilities [170], it is necessary to develop efficient gene transfection technologies usable as a tool for the basic research of stem cell biology and medicine. In addition, stem cells genetically engineered by genes to activate and improve the biological functions can be used for cell transplantation therapy (gene-cell hybrid therapy) (Fig. 2). Recent research results about the non-viral carrier of cationized polysaccharide are introduced aiming at the enhancement of in vitro and in vivo gene expression in the following section.

3.1. In vitro gene transfection for stem cells by cationized polysaccharides

We have explored a cell-specific gene carrier of polysaccharides which can be recognized by the cell surface receptors of sugar-recognition for enhanced gene expression.

Pullulan, dextran, mannan, and amylopectin were used as the starting polysaccharides of gene transfection carrier. To cationize the polysaccharide for polyionic complexation with plasmid DNA, spermine was introduced to the hydroxyl groups of polysaccharide by a CDI activation method. Complexation with the polysaccharide derivatives enabled a plasmid DNA to enhance the expression level of cells to a significantly higher extent compared with those of LipofectAmine™ and Superfect™ commercially available,

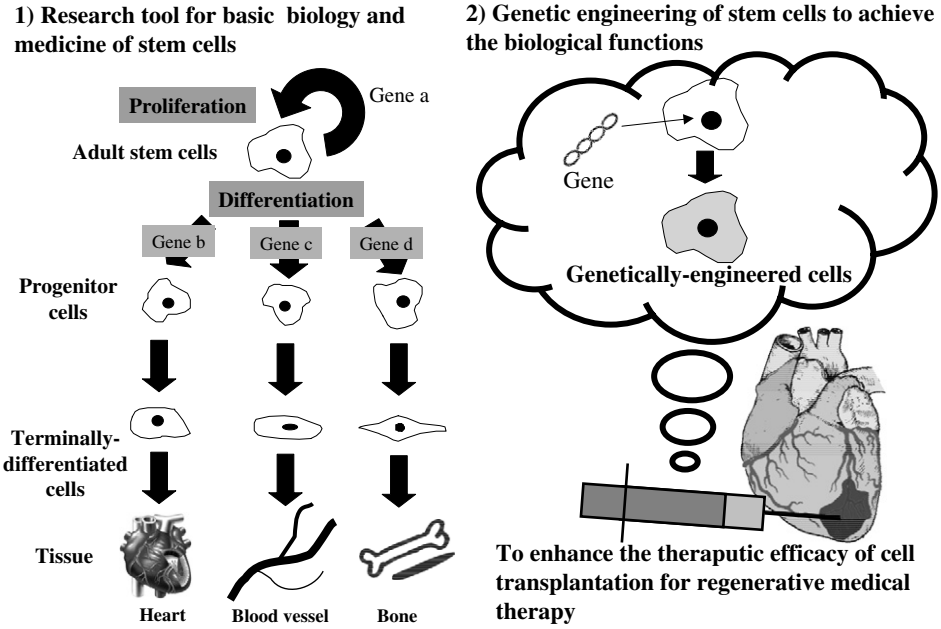

Fig. 2. Application of non-viral gene transfection technology to stem cell biology and regenerative medical therapy.

while the enhanced gene expression depended on the polysaccharide type. The complex of spermine–pullulan and plasmid DNA showed the highest level of gene expression among the plasmid DNA complexes with other cationized polysaccharides. An inhibition assay with asialofetuin, which is a ligand of the sugar-recognizable cell surface receptors, revealed that the blockage of cell receptors suppressed the level of gene expression. These findings suggest the possibility that the plasmid DNA complex with the cationized polysaccharide derivative is selectively internalized into cells through a sugar-specific receptor of cell surface, resulting in the enhanced gene expression. The similar enhancement of gene expression by the cationized polysaccharide was observed for other cells, such as embryonic stem cells, mesenchymal stem cells, and adipo-derived stromal cells (unpublished data). The cationized polysaccharide can be also applied to the transfection of siRNA that can silence gene expression in a sequence-specific manner to demonstrate the enhance siRNA effect (unpublished data).

3.2. New technologies of gene transfection to genetically engineer stem cells

A new non-viral method of gene transfection was designed to enhance the level of gene expression for rat mesenchymal stem cells (MSC). Pullulan was cationized by the chemical introduction of spermine to prepare cationized pullulan of non-viral carrier (spermine–pullulan). The spermine–pullulan was complexed with a plasmid DNA of luciferase and coated on the surface of culture substrate together with Pronectin® of artificial cell adhesion protein. MSC were cultured and transfected on the complex-coated substrate (reverse transfection) and the level and duration of gene expression were compared with those of MSC transfected by culturing in the medium containing the plasmid DNA–spermine–pullulan complex (conventional method). The gene expression was enhanced and prolonged by the reverse transfection method to a significantly greater extent compared with that of the conventional method. The reverse method permitted the transfection culture of MSC in the presence of serum, in marked contrast to the conventional method, which gave cells a good culture condition, resulting in lower cytotoxicity. The reverse transfection could be carried out for a three-dimensional (3-D) culture substrate, the non-woven fabric of polyethylene terephtalate (PET) coated with the complex and Pronectin while the cell culture was developed by bioreactor systems, such as an agitated and stirring culture method. The level and duration of gene expression for MSC were significantly enhanced by the two bioreactor methods compared with those of the static method (Fig. 3). It is possible that the medium circulation improves the culture conditions of cells in terms of oxygen and nutrition supply and wastes, excretion, resulting in enhanced gene expression. These results strongly demonstrated that comparable to the research and development of non-viral carriers themselves, it is important to improve the technology and methodology of cell culture which give cells good conditions to maintain their vital and biological functions as well as efficiently enhance gene transfection [171].

Gene transfection in combination with three-dimensional (3-D) tissue constructs has been reported as a new trial [172,173]. An in vitro culture system which is composed of plasmid DNA-impregnated 3-D scaffold and a perfusion culture method has been developed to enhance the expression of plasmid DNA for MSC [174–176]. MSC were cultured in the collagen sponge reinforced with

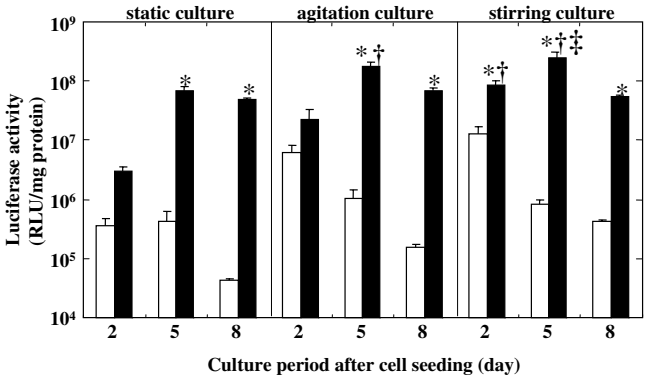

Fig. 3. Time course of luciferase expression level of MSC transfected by the conventional (open columns) and reverse methods (solid columns) in the static, agitation, and stirring cultures in the PET non-woven fabric: (open columns) the plasmid DNA-cationized pullulan complex in the absence of FCS and (solid columns) the complex in the presence of FCS. $^{*}P < 0.05$ versus the level in the presence of FCS by the conventional method at the corresponding time. $^{\dagger}P < 0.05$ versus the level in the absence of FCS by the reverse method in the static culture at the corresponding time. $^{\ddagger}P < 0.05$ versus the level in the absence of FCS by the reverse method in the agitation culture at the corresponding time.

poly(glycolic acid) (PGA) fiber which incorporates the complexation of a non-viral vector and a plasmid DNA encoding bone morphogenetic protein (BMP)-2 gene. The level of in vitro gene expression was enhanced by combination of a perfusion culture method to a significantly greater extent than that of the conventional static culture method. Since the 3-D culture substrate has a large surface area available for cell attachment and the subsequent proliferation compared with the 2-D tissue culture plate, cells can be proliferated in the 3-D matrix at higher rates and for longer time periods than those in the 2-D one. Moreover, the perfusion culture method can supply nutrients and oxygen to the cells proliferated in the 3-D matrix more efficiently than the static culture method, while harmful metabolic products and wastes generated from cells can be excluded more efficiently. Our previous results demonstrated that the proliferation of MSC was greatly influenced by the culture methods and significantly enhanced by the perfusion culture method compared with the static method. The better the cell proliferation, the higher the level of gene transfection for the cells [177]. It is concluded that the extent of plasmid DNA transfected is greatly influenced by that of cell proliferation.

Macro-sized gene carriers are used for the field of microarrays to analyze or discover new genes contributing to the proliferation and differentiation of cells. The recent rapid development of molecular biology together with the steady progress of genome projects has given us many libraries of DNA and RNA and enabled to elucidate biological phenomena at a genetic level. Under these circumstances, it gets more and more important to develop high throughput screening systems. Microarray technology enables to analyze many genes functionally unknown all at once. The complex of gene and non-viral carrier is arrayed onto a glass microscope slide, followed by application of cells to be transfected. The functions of genes unknown are rapidly analyzed by fluorescence or immunofluorescence etc [145,178–182].

3.3. Technology to genetically engineer stem cells for generative medical therapy

The controlled release system with the cationized gelatin is effective in genetically manipulating the biological function of stem cells as described above. The cationized gelatin microspheres incorporating plasmid DNA were readily taken up by the cells of phagocytic nature and could achieve the sustained release of plasmid DNA inside the cells. Interestingly, this intracellular release enabled the plasmid DNA to enhance the level of gene expression significantly higher than that of virus system [133]. This system will break through the virus-related problems that good therapeutic results of cells genetically engineered by virus vectors cannot be applied to the clinical medicine. Here, we introduced a new therapeutic concept for cell-based gene delivery (gene-cell hybrid therapy). This concept worked very well to therapeutically treat pulmonary hypertension for which there is no effective clinical treatment at present. Endothelial progenitor cells (EPC) of phagocytic nature were isolated and incubated with cationized gelatin microspheres incorporating plasmid DNA of angiogenic adrenomedullin to genetically engineer the biological function through the transfection of plasmid DNA. When the gene-engineered EPC were injected intravenously into monocrotaline (MCT)-induced pulmonary hypertension model rats, superior therapeutic effect was observed to that of original EPC injection. The EPC can be naturally recruited as a natural carrier of gene to the ischemic site of disease. The benefits of gene-cell hybrid therapy are due to inherent ability of EPC to phagocytose cationized gelatin microspheres capable of plasmid DNA release and the positive migration, that is natural targeting, to the sites of injured endothelium. When incubated with cationized gelatin microspheres incorporating green fluorescent protein (GFP) plasmid DNA and the GFP plasmid DNA solution, EPC, not monocytes/macrophages, were strongly transfected to express the GFP protein by the former, in remarked contrast to the latter. Although the Rhodamine B isothiocyanate-labeled DNA molecules were mainly distributed to the cytoplasm rather than nucleus, the DNA molecules incorporated in cationized gelatin microspheres were continuously released in the cytoplasm of EPC after phagocytosis and the cationized gelatin–DNA complexes released were transferred to the nucleus. This is because the microspheres incorporating plasmid DNA enhanced the level of DNA expression. There are several possible reasons why the DNA release was effective. It is possible that polyion complexation with cationized gelatin prevents the plasmid DNA from the enzymatic degradation in the cytoplasm. Moreover, the GFP-expressing EPC intravenously administered were incorporated into pulmonary arterioles and capillaries in MCT rats and differentiated into matured

endothelial cells. Taken together, it is highly possible that as expected, the EPC injected circulate in the blood and target to the pulmonary endothelia injured in MCT rats. Thus, EPC serves not only as a vehicle for gene delivery to injured pulmonary endothelia, but also as a tissue-engineering tool in restoring intact pulmonary endothelium. The injection of EPC genetically modified by the plasmid DNA of adrenomedullin significantly improved the therapeutic efficacy in the pulmonary hypertension compared with that of original EPC [133].

MSC are being expected as one of cell sources usable for cardiac reconstruction because of their differentiation potential and ability to supply growth factors. However, the therapeutic potential of MSC is often hindered by the poor viability at the transplanted site. Therefore, as one trial to overcome this issue, a non-viral carrier of cationized polysaccharide is introduced to genetically engineer MSC for activation of the biological functions. Spermine-introduced dextran of cationized polysaccharide (spermine–dextran) was internalized into MSC by way of a sugar-recognizable receptor to enhance the expression level of plasmid DNA. When genetically engineered by the spermine–dextran complex with plasmid DNA of adrenomedullin (AM), MSC secreted a large amount of AM which is an anti-apoptotic and angiogenic peptide (Figs. 4a and b). Transplantation of AM gene-engineered MSC significantly improved cardiac functions after myocardial infarction compared with that of MSC alone (Fig. 4c). Thus, this genetic engineering technology by the non-viral spermine–dextran is a promising strategy to enhance the therapeutic efficacy of MSC for ischemic heart disease [183].

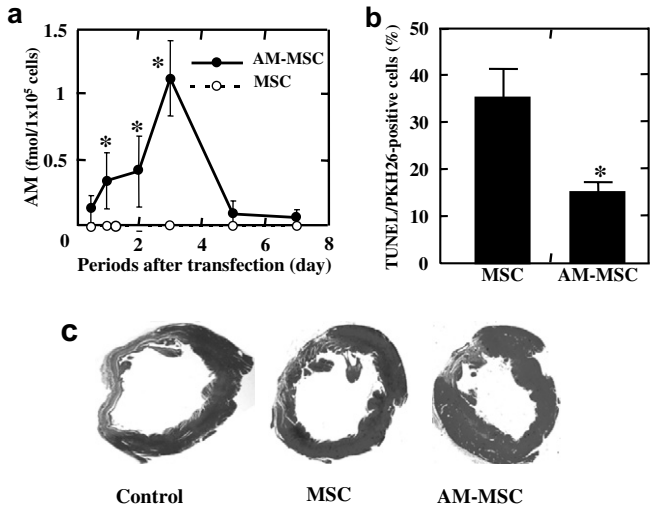

Fig. 4. (a) Time course of AM secreted from MSC following the transfection by spermine–dextran–AM plasmid DNA complexes (AM-MSC). $*P < 0.05$ versus the level of normal MSC at the corresponding time period. The level of AM secretion increased after 1-day incubation is the high level lasted for at least 3 days after gene transfection. (b) In vivo anti-apoptotic effects of AM-MSC. Quantitative analysis of in vivo TUNEL assay for AM-MSC and MSC. AM-MSC were rarely positive by TUNEL staining. $*P < 0.05$ versus the MSC. (c) Representative photographs of myocardial sections stained with Masson trichrome.

4. Conclusion

For gene transfection and expression, various carriers of viral and non-viral systems have been investigated and developed. Both the carriers have advantages and disadvantages for gene delivery. Depending on the applications, one can choose them considering the characteristic features. However, in case the carrier is used for the genetic manipulation and their medical therapy, from the viewpoint of the handling ease and clinical acceptance, the non-viral delivery system is practically preferable. So far, several non-viral vectors have been explored to enhance the level of gene expression as high as that of viral vectors. In addition, their poor selectivity for cells and tissues is another research point to be improved. As one trial to answer the selectivity issue, it is necessary to introduce DDS technology and methodology into the non-viral gene carrier system more positively. In near future, gene transfection technology is going to get more and more important not only for the basic research of medicine and biology, but also for the medical applications, such as gene, cell transplantation, and regenerative medical therapies. It has been reported that siRNA may modify the biological functions and differentiation fate of stem cells. It is possible that DDS technologies and methodologies enable siRNA-mediated biological manipulation of stem cells for efficient cell transplantation therapy. In addition, comparable to the research and development of non-viral carrier materials, it is important to improve the technology and methodology of cell culture for gene transfection. It is likely that better culture conditions make cells to maintain their vital and biological functions even in gene transfection, resulting in efficiently enhanced gene expression. As the genetic manipulation technology of stem cells is still in its infancy, it will take a time to become well established. Increasing significance of delivery technology and methodology in future will further help progress of stem cells biology and medicine as well as the medical applications. We will be happy if this review stimulates readers' interest in the idea and research field of stem cells engineering to assist understanding of gene delivery technology importance in genetic manipulation of stem cells.

References

[1] T.A. Ratko, J.P. Cummings, J. Blebea, K.A. Matuszewski, Clinical gene therapy for nonmalignant disease, Am. J. Med. 115 (2003) 560–569.
[2] A. Mountain, Gene therapy: the first decade, Trends Biotechnol. 18 (2000) 119–128.
[3] J. Nemunaitis, T. Fong, J.M. Robbins, G. Edelman, W. Edwards, R.S. Paulson, et al., Phase I trial of interferon-gamma (IFN-gamma) retroviral vector administered intratumorally to patients with metastatic melanoma, Cancer Gene Ther. 6 (1999) 322–330.
[4] G.G. Vecil, F.F. Lang, Clinical trials of adenoviruses in brain tumors: a review of Ad-p53 and oncolytic adenoviruses, J. Neurooncol. 65 (2003) 237–246.
[5] S.R. Riddell, M. Elliott, D.A. Lewinsohn, M.J. Gilbert, L. Wilson, S.A. Manley, et al., T-cell mediated rejection of gene-modified

HIV-specific cytotoxic T lymphocytes in HIV-infected patients, Nat. Med. 2 (1996) 216–223.
[6] R.M. Blaese, K.W. Culver, A.D. Miller, C.S. Carter, T. Fleisher, M. Clerici, et al., T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years, Science 270 (1995) 475–480.
[7] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[8] I.R. Gilmore, S.P. Fox, A.J. Hollins, S. Akhtar, Delivery strategies for siRNA-mediated gene silencing, Curr. Drug Deliv. 3 (2006) 145–147.
[9] C.X. Li, A. Parker, E. Menocal, S. Xiang, L. Borodyansky, J.H. Fruehauf, Delivery of RNA interference, Cell Cycle 5 (2006) 2103–2109.
[10] F.L. Tan, J.Q. Yin, Application of RNAi to cancer research and therapy, Front. Biosci. 10 (2005) 1946–1960.
[11] T. Tuschl, A. Borkhardt, Small interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy, Mol. Interv. 2 (2002) 158–167.
[12] P.D. Robbins, S.C. Ghivizzani, Viral vectors for gene therapy, Pharmacol. Ther. 80 (1998) 35–47.
[13] G. Duisit, A. Salvetti, P. Moullier, F.L. Cosset, Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines, Hum. Gene Ther. 10 (1999) 189–200.
[14] Y.S. Kim, H.K. Lim, K.J. Kim, Production of high-titer retroviral vectors and detection of replication-competent retroviruses, Mol. Cells 8 (1998) 36–42.
[15] K.W. Peng, S.J. Russell, Viral vector targeting, Curr. Opin. Biotechnol. 10 (1999) 454–457.
[16] P.L. Felgner, G.M. Ringold, Cationic liposome-mediated transfection, Nature 337 (1989) 387–388.
[17] T. Nishi, K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, et al., High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation, Cancer Res. 56 (1996) 1050–1055.
[18] V. Toncheva, M.A. Wolfert, P.R. Dash, D. Oupicky, K. Ulbrich, L.W. Seymour, et al., Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers, Biochim. Biophys. Acta 1380 (1998) 354–368.
[19] D. Oupicky, C. Konak, K. Ulbrich, M.A. Wolfert, L.W. Seymour, DNA delivery systems based on complexes of DNA with synthetic polycations and their copolymers, J. Control. Release 65 (2000) 149–171.
[20] M. Nishikawa, L. Huang, Nonviral vectors in the new millennium: delivery barriers in gene transfer, Hum. Gene Ther. 12 (2001) 861–870.
[21] R.E. Donahue, S.W. Kessler, D. Bodine, K. McDonagh, C. Dunbar, S. Goodman, et al., Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer, J. Exp. Med. 176 (1992) 1125–1135.
[22] F.L. Graham, A.J. van der Eb, A new technique for the assay of infectivity of human adenovirus 5 DNA, Virology 52 (1973) 456–467.
[23] E.T. Schenborn, V. Goiffon, Calcium phosphate transfection of mammalian cultured cells, Methods Mol. Biol. 130 (2000) 135–145.
[24] A. Maitra, Calcium phosphate nanoparticles: second-generation nonviral vectors in gene therapy, Expert Rev. Mol. Diagn. 5 (2005) 893–905.
[25] S.A. Audouy, L.F. de Leij, D. Hoekstra, G. Molema, In vivo characteristics of cationic liposomes as delivery vectors for gene therapy, Pharm Res. 19 (2002) 1599–1605.
[26] M. Voinea, M. Simionescu, Designing of 'intelligent' liposomes for efficient delivery of drugs, J. Cell Mol. Med. 6 (2002) 465–474.
[27] M.C. Pedroso de Lima, S. Neves, A. Filipe, N. Duzgunes, S. Simoes, Cationic liposomes for gene delivery: from biophysics to biological applications, Curr. Med. Chem. 10 (2003) 1221–1231.
[28] J.W. Nah, L. Yu, S.O. Han, C.H. Ahn, S.W. Kim, Artery wall binding peptide-poly(ethylene glycol)-grafted-poly(L-lysine)-based gene delivery to artery wall cells, J. Control. Release 78 (2002) 273–284.
[29] J.H. Lee, H.G. Klein, Cellular gene therapy, Hematol. Oncol. Clin. North Am. 9 (1995) 91–113.
[30] M. Molas, R. Bartrons, J.C. Perales, Single-stranded DNA condensed with poly-L-lysine results in nanometric particles that are significantly smaller, more stable in physiological ionic strength fluids and afford higher efficiency of gene delivery than their double-stranded counterparts, Biochim. Biophys. Acta 1572 (2002) 37–44.
[31] D. Fischer, T. Bieber, Y. Li, H.P. Elsasser, T. Kissel, A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. 16 (1999) 1273–1279.
[32] P. Marschall, N. Malik, Z. Larin, Transfer of YACs up to 2.3 Mb intact into human cells with polyethylenimine, Gene Ther. 6 (1999) 1634–1637.
[33] P. Campeau, P. Chapdelaine, S. Seigneurin-Venin, B. Massie, J.P. Tremblay, Transfection of large plasmids in primary human myoblasts, Gene Ther. 8 (2001) 1387–1394.
[34] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, et al., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[35] M.A. Gosselin, W. Guo, R.J. Lee, Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine, Bioconjug. Chem. 12 (2001) 989–994.
[36] S. Ferrari, E. Moro, A. Pettenazzo, J.P. Behr, F. Zacchello, M. Scarpa, ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo, Gene Ther. 4 (1997) 1100–1106.
[37] J.L. Coll, P. Chollet, E. Brambilla, D. Desplanques, J.P. Behr, M. Favrot, In vivo delivery to tumors of DNA complexed with linear polyethylenimine, Hum. Gene Ther. 10 (1999) 1659–1666.
[38] L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, et al., Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, J. Gene Med. 3 (2001) 362–372.
[39] S. Brunner, E. Furtbauer, T. Sauer, M. Kursa, E. Wagner, Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation, Mol. Ther. 5 (2002) 80–86.
[40] N.G. Rainov, C.M. Kramm, Vector delivery methods and targeting strategies for gene therapy of brain tumors, Curr. Gene Ther. 1 (2001) 367–383.
[41] M. Pooga, U. Langel, Targeting of cancer-related proteins with PNA oligomers, Curr. Cancer Drug Targets 1 (2001) 231–239.
[42] M.A. Gosselin, R.J. Lee, Folate receptor-targeted liposomes as vectors for therapeutic agents, Biotechnol. Annu. Rev. 8 (2002) 103–131.
[43] T.J. Wickham, Ligand-directed targeting of genes to the site of disease, Nat. Med. 9 (2003) 135–139.
[44] A. Jimenez, J. Davies, Expression of a transposable antibiotic resistance element in Saccharomyces, Nature 287 (1980) 869–871.
[45] L.M. Mir, P.H. Moller, F. Andre, J. Gehl, Electric pulse-mediated gene delivery to various animal tissues, Adv. Genet. 54 (2005) 83–114.
[46] D.J. Wells, Gene therapy progress and prospects: electroporation and other physical methods, Gene Ther. 11 (2004) 1363–1369.
[47] P. Bigey, M.F. Bureau, D. Scherman, In vivo plasmid DNA electrotransfer, Curr. Opin. Biotechnol. 13 (2002) 443–447.
[48] J. Gehl, Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research, Acta Physiol. Scand. 177 (2003) 437–447.
[49] J.M. McMahon, D.J. Wells, Electroporation for gene transfer to skeletal muscles: current status, BioDrugs 18 (2004) 155–165.
[50] C. Bloquel, E. Fabre, M.F. Bureau, D. Scherman, Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications, J. Gene Med. 6 (Suppl. 1) (2004) S11–S23.

[51] G. Zhang, X. Gao, Y.K. Song, R. Vollmer, D.B. Stolz, J.Z. Gasiorowski, et al., Hydroporation as the mechanism of hydrodynamic delivery, Gene Ther. 11 (2004) 675–682.
[52] N. Kobayashi, M. Nishikawa, Y. Takakura, The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels, Adv. Drug Deliv. Rev. 57 (2005) 713–731.
[53] F. Liu, M. Nishikawa, P.R. Clemens, L. Huang, Transfer of full-length Dmd to the diaphragm muscle of Dmd(mdx/mdx) mice through systemic administration of plasmid DNA, Mol. Ther. 4 (2001) 45–51.
[54] G. Zhang, V. Budker, P. Williams, V. Subbotin, J.A. Wolff, Efficient expression of naked dna delivered intraarterially to limb muscles of nonhuman primates, Hum. Gene Ther. 12 (2001) 427–438.
[55] N. Yang, R. Horn, Evidence for voltage-dependent S4 movement in sodium channels, Neuron 15 (1995) 213–218.
[56] Z. Cui, R.J. Mumper, Microparticles and nanoparticles as delivery systems for DNA vaccines, Crit. Rev. Ther. Drug Carrier Syst. 20 (2003) 103–137.
[57] F. Scherer, M. Anton, U. Schillinger, J. Henke, C. Bergemann, A. Kruger, et al., Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo, Gene Ther. 9 (2002) 102–109.
[58] H. Hosseinkhani, Y. Tabata, Ultrasound enhances in vivo tumor expression of plasmid DNA by PEG-introduced cationized dextran, J. Control. Release 108 (2005) 540–556.
[59] Y. Taniyama, K. Tachibana, K. Hiraoka, M. Aoki, S. Yamamoto, K. Matsumoto, et al., Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle, Gene Ther. 9 (2002) 372–380.
[60] Y. Taniyama, K. Tachibana, K. Hiraoka, T. Namba, K. Yamasaki, N. Hashiya, et al., Local delivery of plasmid DNA into rat carotid artery using ultrasound, Circulation 105 (2002) 1233–1239.
[61] P. Schratzberger, J.G. Krainin, G. Schratzberger, M. Silver, H. Ma, M. Kearney, et al., Transcutaneous ultrasound augments naked DNA transfection of skeletal muscle, Mol. Ther. 6 (2002) 576–583.
[62] G. Danialou, A.S. Comtois, R.W. Dudley, J. Nalbantoglu, R. Gilbert, G. Karpati, et al., Ultrasound increases plasmid-mediated gene transfer to dystrophic muscles without collateral damage, Mol. Ther. 6 (2002) 687–693.
[63] Q.L. Lu, H.D. Liang, T. Partridge, M.J. Blomley, Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage, Gene Ther. 10 (2003) 396–405.
[64] S. Chen, R.V. Shohet, R. Bekeredjian, P. Frenkel, P.A. Grayburn, Optimization of ultrasound parameters for cardiac gene delivery of adenoviral or plasmid deoxyribonucleic acid by ultrasound-targeted microbubble destruction, J. Am. Coll. Cardiol. 42 (2003) 301–308.
[65] R. Bekeredjian, S. Chen, P.A. Frenkel, P.A. Grayburn, R.V. Shohet, Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart, Circulation 108 (2003) 1022–1026.
[66] A.C. Durieux, R. Bonnefoy, T. Busso, D. Freyssenet, In vivo gene electrotransfer into skeletal muscle: effects of plasmid DNA on the occurrence and extent of muscle damage, J. Gene Med. 6 (2004) 809–816.
[67] K.W. Liang, M. Nishikawa, F. Liu, B. Sun, Q. Ye, L. Huang, Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA, Gene Ther. 11 (2004) 901–908.
[68] H. Hosseinkhani, T. Aoyama, O. Ogawa, Y. Tabata, Ultrasound enhances the transfection of plasmid DNA by non-viral vectors, Curr. Pharm. Biotechnol. 4 (2003) 109–122.
[69] T. Li, K. Tachibana, M. Kuroki, M. Kuroki, Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in mice–initial results, Radiology 229 (2003) 423–428.
[70] S.V. Pislaru, C. Pislaru, R.R. Kinnick, R. Singh, R. Gulati, J.F. Greenleaf, et al., Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities, Eur. Heart J. 24 (2003) 1690–1698.
[71] H. Koike, N. Tomita, H. Azuma, Y. Taniyama, K. Yamasaki, Y. Kunugiza, et al., An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney, J. Gene Med. 7 (2005) 108–116.
[72] M. Shimamura, N. Sato, Y. Taniyama, H. Kurinami, H. Tanaka, T. Takami, et al., Gene transfer into adult rat spinal cord using naked plasmid DNA and ultrasound microbubbles, J. Gene Med. 7 (2005) 1468–1474.
[73] E.C. Unger, T.P. McCreery, R.H. Sweitzer, Ultrasound enhances gene expression of liposomal transfection, Invest. Radiol. 32 (1997) 723–727.
[74] T. Aoyama, H. Hosseinkhani, S. Yamamoto, O. Ogawa, Y. Tabata, Enhanced expression of plasmid DNA-cationized gelatin complex by ultrasound in murine muscle, J. Control. Release 80 (2002) 345–356.
[75] H. Hosseinkhani, T. Aoyama, O. Ogawa, Y. Tabata, Ultrasound enhancement of in vitro transfection of plasmid DNA by a cationized gelatin, J. Drug Target. 10 (2002) 193–204.
[76] H. Hosseinkhani, T. Aoyama, S. Yamamoto, O. Ogawa, Y. Tabata, In vitro transfection of plasmid DNA by amine derivatives of gelatin accompanied with ultrasound irradiation, Pharm. Res. 19 (2002) 1471–1479.
[77] J. Sudimack, R.J. Lee, Targeted drug delivery via the folate receptor, Adv. Drug Deliv. Rev. 41 (2000) 147–162.
[78] Y. Lu, P.S. Low, Folate-mediated delivery of macromolecular anticancer therapeutic agents, Adv. Drug Deliv. Rev. 54 (2002) 675–693.
[79] X.B. Zhao, R.J. Lee, Tumor-selective targeted delivery of genes and antisense oligodeoxyribonucleotides via the folate receptor, Adv. Drug Deliv. Rev. 56 (2004) 1193–1204.
[80] K.A. Mislick, J.D. Baldeschwieler, J.F. Kayyem, T.J. Meade, Transfection of folate–polylysine DNA complexes: evidence for lysosomal delivery, Bioconjug. Chem. 6 (1995) 512–515.
[81] E. Wagner, M. Cotten, R. Foisner, M.L. Birnstiel, Transferrin–polycation–DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells, Proc. Natl. Acad. Sci. USA 88 (1991) 4255–4259.
[82] Z.M. Qian, H. Li, H. Sun, K. Ho, Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway, Pharmacol. Rev. 54 (2002) 561–587.
[83] R. Kircheis, L. Wightman, A. Schreiber, B. Robitza, V. Rossler, M. Kursa, et al., Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application, Gene Ther. 8 (2001) 28–40.
[84] M. Hashida, M. Nishikawa, F. Yamashita, Y. Takakura, Cell-specific delivery of genes with glycosylated carriers, Adv. Drug Deliv. Rev. 52 (2001) 187–196.
[85] C. Bies, C.M. Lehr, J.F. Woodley, Lectin-mediated drug targeting: history and applications, Adv. Drug Deliv. Rev. 56 (2004) 425–435.
[86] Y.H. Choi, F. Liu, J.S. Choi, S.W. Kim, J.S. Park, Characterization of a targeted gene carrier, lactose-polyethylene glycol-grafted poly-L-lysine and its complex with plasmid DNA, Hum. Gene Ther. 10 (1999) 2657–2665.
[87] R.I. Mahato, S. Takemura, K. Akamatsu, M. Nishikawa, Y. Takakura, M. Hashida, Physicochemical and disposition characteristics of antisense oligonucleotides complexed with glycosylated poly(L-lysine), Biochem. Pharmacol. 53 (1997) 887–895.
[88] T. Ferkol, J.C. Perales, F. Mularo, R.W. Hanson, Receptor-mediated gene transfer into macrophages, Proc. Natl. Acad. Sci. USA 93 (1996) 101–105.
[89] N.R. Chowdhury, C.H. Wu, G.Y. Wu, P.C. Yerneni, V.R. Bommineni, J.R. Chowdhury, Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis in vivo, Prolonged

persistence in cytoplasmic vesicles after partial hepatectomy, J. Biol. Chem. 268 (1993) 11265–11271.
[90] R.J. Parkes, S.L. Hart, Adhesion molecules and gene transfer, Adv. Drug Deliv. Rev. 44 (2000) 135–152.
[91] P. Erbacher, J.S. Remy, J.P. Behr, Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway, Gene Ther. 6 (1999) 138–145.
[92] S.L. Hart, R.P. Harbottle, R. Cooper, A. Miller, R. Williamson, C. Coutelle, Gene delivery and expression mediated by an integrin-binding peptide, Gene Ther. 2 (1995) 552–554.
[93] M. Colin, M. Maurice, G. Trugnan, M. Kornprobst, R.P. Harbottle, A. Knight, et al., Cell delivery, intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells, Gene Ther. 7 (2000) 139–152.
[94] R.M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, et al., Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle, Nucleic Acids Res. 32 (2004) e149.
[95] B.A. Sosnowski, A.M. Gonzalez, L.A. Chandler, Y.J. Buechler, G.F. Pierce, A. Baird, Targeting DNA to cells with basic fibroblast growth factor (FGF2), J. Biol. Chem. 271 (1996) 33647–33653.
[96] T. Blessing, M. Kursa, R. Holzhauser, R. Kircheis, E. Wagner, Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery, Bioconjug. Chem. 12 (2001) 529–537.
[97] R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, et al., Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery, Gene Ther. 4 (1997) 409–418.
[98] N. Shimizu, J. Chen, S. Gamou, A. Takayanagi, Immunogene approach toward cancer therapy using erythrocyte growth factor receptor-mediated gene delivery, Cancer Gene Ther. 3 (1996) 113–120.
[99] E.K. Gaidamakova, M.V. Backer, J.M. Backer, Molecular vehicle for target-mediated delivery of therapeutics and diagnostics, J. Control. Release 74 (2001) 341–347.
[100] T. Niidome, L. Huang, Gene therapy progress and prospects: nonviral vectors, Gene Ther. 9 (2002) 1647–1652.
[101] R. Tachibana, H. Harashima, Y. Shinohara, H. Kiwada, Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors, Adv. Drug Deliv. Rev. 52 (2001) 219–226.
[102] K. Masuda, M. Sakagami, K. Horie, H. Nogusa, H. Hamana, K. Hirano, Evaluation of carboxymethylpullulan as a novel carrier for targeting immune tissues, Pharm. Res. 18 (2001) 217–223.
[103] T. Nishikawa, K. Akiyoshi, J. Sunamoto, Macromolecular complexation between bovine serum albumin and the self-assembled hydrogel nanoparticle of hydrophobized polysaccharides, J. Am. Chem. Soc. 118 (1996) 6110–6115.
[104] K. Akiyoshi, T. Nishikawa, S. Shichibe, J. Sunamoto, Stabilization of Insulin Upon Supramolecular Complexation with Hydrophobized Polysaccharide Nanoparticle, Chem. Lett. (1995) 707–708.
[105] K. Akiyoshi, S. Deguchi, H. Tajima, T. Nishikawa, J. Sunamoto, Self-Assembly of Hydrophobized Polysaccharide—Structure of Hydrogel Nanoparticle and Complexation with Organic-Compounds, Proc. Japan Acad. Ser. B-Phys. Biol. Sci. 71 (1995) 15–19.
[106] K. Akiyoshi, T. Nishikawa, Y. Mitsui, T. Miyata, M. Kodama, J. Sunamoto, Self-assembly of polymer amphiphiles: Thermodynamics of complexation between bovine serum albumin and self-aggregate of cholesterol-bearing pullulan, Colloids Surf. A–Physicochem. Eng. Aspects 112 (1996) 91–95.
[107] M. Nichifor, D. Cristea, G. Mocanu, A. Carpov, Aminated polysaccharides as bile acid sorbents: in vitro study, J. Biomater. Sci.-Polym. Ed. 9 (1998) 519–534.
[108] T. Yamaoka, Y. Tabata, Y. Ikada, Body distribution profile of polysaccharide after intravenous administration, Drug Deliv. 1 (1993).
[109] Y. Kaneo, T. Tanaka, T. Nakano, Y. Yamaguchi, Evidence for receptor-mediated hepatic uptake of pullulan in rats, J. Control. Release 70 (2001) 365–373.
[110] T. Tanaka, Y. Fujishima, S. Hanano, Y. Kaneo, Intracellular disposition of polysaccharides in rat liver parenchymal and nonparenchymal cells, Int. J. Pharm. 286 (2004) 9–17.
[111] K. Xi, Y. Tabata, K. Uno, M. Yoshimoto, T. Kishida, Y. Sokawa, et al., Liver targeting of interferon through pullulan conjugation, Pharm. Res. 13 (1996) 1846–1850.
[112] J.I. Jo, T. Ikai, A. Okazaki, M. Yamamoto, Y. Hirano, Y. Tabata, Expression profile of plasmid DNA by spermine derivatives of pullulan with different extents of spermine introduced, J. Control. Release 118 (2007) 389–398.
[113] L. Yu, H. Suh, J.J. Koh, S.W. Kim, Systemic administration of TerplexDNA system: pharmacokinetics and gene expression, Pharm. Res. 18 (2001) 1277–1283.
[114] P.M. Mullen, C.P. Lollo, Q.C. Phan, A. Amini, M.G. Banaszczyk, J.M. Fabrycki, et al., Strength of conjugate binding to plasmid DNA affects degradation rate and expression level in vivo, Biochim. Biophys. Acta 1523 (2000) 103–110.
[115] I. Moret, J. Esteban Peris, V.M. Guillem, M. Benet, F. Revert, F. Dasi, et al., Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum, J. Control. Release 76 (2001) 169–181.
[116] S. Park, K.E. Healy, Nanoparticulate DNA packaging using terpolymers of poly(lysine-g-(lactide-b-ethylene glycol)), Bioconjug. Chem. 14 (2003) 311–319.
[117] Y. Kakizawa, K. Kataoka, Block copolymer micelles for delivery of gene and related compounds, Adv. Drug Deliv. Rev. 54 (2002) 203–222.
[118] T.N. Palmer, V.J. Caride, M.A. Caldecourt, J. Twickler, V. Abdullah, The mechanism of liposome accumulation in infarction, Biochim. Biophys. Acta 797 (1984) 363–368.
[119] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release 65 (2000) 271–284.
[120] H. Hosseinkhani, Y. Tabata, PEGylation enhances tumor targeting of plasmid DNA by an artificial cationized protein with repeated RGD sequences, Pronectin, J. Control. Release 97 (2004) 157–171.
[121] J. Jo, M. Yamamoto, K. Matsumoto, T. Nakamura, Y. Tabata, Liver targeting of plasmid DNA with a cationized pullulan for tumor suppression, J. Nanosci. Nanotechnol. 6 (2006) 2853–2859.
[122] T. Kushibiki, N. Nagata-Nakajima, M. Sugai, A. Shimizu, Y. Tabata, Delivery of plasmid DNA expressing small interference RNA for TGF-beta type II receptor by cationized gelatin to prevent interstitial renal fibrosis, J. Control. Release 105 (2005) 318–331.
[123] C. Perez, A. Sanchez, D. Putnam, D. Ting, R. Langer, M.J. Alonso, Poly(lactic acid)-poly(ethylene glycol) nanoparticles as new carriers for the delivery of plasmid DNA, J. Control. Release 75 (2001) 211–224.
[124] J. Wang, P.C. Zhang, H.Q. Mao, K.W. Leong, Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier, Gene Ther. 9 (2002) 1254–1261.
[125] Y.B. Lim, S.O. Han, H.U. Kong, Y. Lee, J.S. Park, B. Jeong, et al., Biodegradable polyester, poly[α-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier, Pharm. Res. 17 (2000) 811–816.
[126] Y. Fukunaka, K. Iwanaga, K. Morimoto, M. Kakemi, Y. Tabata, Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation, J. Control. Release 80 (2002) 333–343.
[127] T. Kushibiki, K. Matsumoto, T. Nakamura, Y. Tabata, Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin, Gene Ther. 11 (2004) 1205–1214.
[128] T. Kushibiki, R. Tomoshige, Y. Fukunaka, M. Kakemi, Y. Tabata, In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents, J. Control. Release 90 (2003) 207–216.
[129] T. Kushibiki, K. Matsumoto, T. Nakamura, Y. Tabata, Suppression of the progress of disseminated pancreatic cancer cells by NK4 plasmid DNA released from cationized gelatin microspheres, Pharm. Res. 21 (2004) 1109–1118.

[130] T. Aoyama, S. Yamamoto, A. Kanematsu, O. Ogawa, Y. Tabata, Local delivery of matrix metalloproteinase gene prevents the onset of renal sclerosis in streptozotocin-induced diabetic mice, Tissue Eng. 9 (2003) 1289–1299.
[131] M. Miyazaki, Y. Obata, K. Abe, A. Furusu, T. Koji, Y. Tabata, et al., Gene transfer using nonviral delivery systems, Perit. Dial. Int. 26 (2006) 633–640.
[132] G. Matsumoto, T. Kushibiki, Y. Kinoshita, U. Lee, Y. Omi, E. Kubota, et al., Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma, Cancer Sci. 97 (2006) 313–321.
[133] N. Nagaya, K. Kangawa, M. Kanda, M. Uematsu, T. Horio, N. Fukuyama, et al., Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells, Circulation 109 (2003) 889–895.
[134] H. Kasahara, E. Tanaka, N. Fukuyama, E. Sato, H. Sakamoto, Y. Tabata, et al., Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia, J. Am. Coll. Cardiol. 41 (2003) 1056–1062.
[135] W.L. Murphy, D.J. Mooney, Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices, J. Periodontal. Res. 34 (1999) 413–419.
[136] L.D. Shea, E. Smiley, J. Bonadio, D.J. Mooney, DNA delivery from polymer matrices for tissue engineering, Nat. Biotechnol. 17 (1999) 551–554.
[137] D. Wang, D.R. Robinson, G.S. Kwon, J. Samuel, Encapsulation of plasmid DNA in biodegradable poly(D,L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery, J. Control. Release 57 (1999) 9–18.
[138] Y. Capan, B.H. Woo, S. Gebrekidan, S. Ahmed, P.P. DeLuca, Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA, Pharm. Res. 16 (1999) 509–513.
[139] D. Luo, K. Woodrow-Mumford, N. Belcheva, W.M. Saltzman, Controlled DNA delivery systems, Pharm. Res. 16 (1999) 1300–1308.
[140] M.L. Hedley, Formulations containing poly(lactide-co-glycolide) and plasmid DNA expression vectors, Expert Opin. Biol. Ther. 3 (2003) 903–910.
[141] J.H. Jang, L.D. Shea, Controllable delivery of non-viral DNA from porous scaffolds, J. Control. Release 86 (2003) 157–168.
[142] H. Shen, E. Goldberg, W.M. Saltzman, Gene expression and mucosal immune responses after vaginal DNA immunization in mice using a controlled delivery matrix, J. Control. Release 86 (2003) 339–348.
[143] Z. Megeed, J. Cappello, H. Ghandehari, Controlled release of plasmid DNA from a genetically engineered silk-elastinlike hydrogel, Pharm. Res. 19 (2002) 954–959.
[144] T. Ochiya, S. Nagahara, A. Sano, H. Itoh, M. Terada, Biomaterials for gene delivery: atelocollagen-mediated controlled release of molecular medicines, Curr. Gene Ther. 1 (2001) 31–52.
[145] T. Ochiya, Y. Takahama, S. Nagahara, Y. Sumita, A. Hisada, H. Itoh, et al., New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet, Nat. Med. 5 (1999) 707–710.
[146] I. Perlstein, J.M. Connolly, X. Cui, C. Song, Q. Li, P.L. Jones, et al., DNA delivery from an intravascular stent with a denatured collagen-polylactic-polyglycolic acid-controlled release coating: mechanisms of enhanced transfection, Gene Ther. 10 (2003) 1420–1428.
[147] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2005) 145–160.
[148] S. Simoes, A. Filipe, H. Faneca, M. Mano, N. Penacho, N. Duzgunes, et al., Cationic liposomes for gene delivery, Expert Opin. Drug Deliv. 2 (2005) 237–254.
[149] C.R. Dass, Lipoplex-mediated delivery of nucleic acids: factors affecting in vivo transfection, J. Mol. Med. 82 (2004) 579–591.
[150] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, Biochim. Biophys. Acta 1235 (1995) 289–295.
[151] A. El Ouahabi, M. Thiry, V. Pector, R. Fuks, J.M. Ruysschaert, M. Vandenbranden, The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids, FEBS Lett. 414 (1997) 187–192.
[152] G.D. Schmidt-Wolf, I.G. Schmidt-Wolf, Non-viral and hybrid vectors in human gene therapy: an update, Trends Mol. Med. 9 (2003) 67–72.
[153] L. Huang, S. Li, Liposomal gene delivery: a complex package, Nat. Biotechnol. 15 (1997) 620–621.
[154] S. Kawakami, Y. Ito, S. Fumoto, F. Yamashita, M. Hashida, Enhanced gene expression in lung by a stabilized lipoplex using sodium chloride for complex formation, J. Gene Med. 7 (2005) 1526–1533.
[155] S. Kawakami, F. Yamashita, K. Nishida, J. Nakamura, M. Hashida, Glycosylated cationic liposomes for cell-selective gene delivery, Crit. Rev. Ther. Drug Carrier Syst. 19 (2002) 171–190.
[156] P. Sapra, P. Tyagi, T.M. Allen, Ligand-targeted liposomes for cancer treatment, Curr. Drug Deliv. 2 (2005) 369–381.
[157] S. Kawakami, J. Wong, A. Sato, Y. Hattori, F. Yamashita, M. Hashida, Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice, Biochim. Biophys. Acta 1524 (2000) 258–265.
[158] S. Kawakami, S. Fumoto, M. Nishikawa, F. Yamashita, M. Hashida, In vivo gene delivery to the liver using novel galactosylated cationic liposomes, Pharm. Res. 17 (2000) 306–313.
[159] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, Gene Ther. 7 (2000) 292–299.
[160] Y. Kaneda, Y. Saeki, R. Morishita, Gene therapy using HVJ-liposomes: the best of both worlds? Mol. Med. Today 5 (1999) 298–303.
[161] Y. Okada, Sendai Virus-induced Cell Fusion, Academic Press, San Diego, Methods Enzymol. 221 (1993) 18–41.
[162] Y. Kaneda, Y. Tabata, Non-viral vectors for cancer therapy, Cancer Sci. 97 (2006) 348–354.
[163] M. Ito, S. Yamamoto, K. Nimura, K. Hiraoka, K. Tamai, Y. Kaneda, Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin, J. Gene Med. 7 (2005) 1044–1052.
[164] N. Morishita, H. Nakagami, R. Morishita, S. Takeda, F. Mishima, B. Terazono, et al., Magnetic nanoparticles with surface modification enhanced gene delivery of HVJ-E vector, Biochem. Biophys. Res. Commun. 334 (2005) 1121–1126.
[165] H. Mima, R. Tomoshige, T. Kanamori, Y. Tabata, S. Yamamoto, S. Ito, et al., Biocompatible polymer enhances the in vitro and in vivo transfection efficiency of HVJ envelope vector, J. Gene Med. 7 (2005) 888–897.
[166] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery, Pharmacol. Rev. 58 (2006) 32–45.
[167] T. Kakudo, S. Chaki, S. Futaki, I. Nakase, K. Akaji, T. Kawakami, et al., Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system, Biochemistry 43 (2004) 5618–5628.
[168] I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, et al., Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement, Mol. Ther. 10 (2004) 1011–1022.
[169] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, et al., Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[170] T. Asahara, C. Kalka, J.M. Isner, Stem cell therapy and gene transfer for regeneration, Gene Ther. 7 (2000) 451–457.
[171] A. Okazaki, J.I. Jo, Y. Tabata, A reverse transfection technology to genetically engineer adult stem cells, Tissue Eng. 13 (2007) 245–251.
[172] W.H. Zimmermann, C. Fink, D. Kralisch, U. Remmers, J. Weil, T. Eschenhagen, Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes, Biotechnol. Bioeng. 68 (2000) 106–114.

[173] K. Partridge, X. Yang, N.M. Clarke, Y. Okubo, K. Bessho, W. Sebald, et al., Adenoviral BMP-2 gene transfer in mesenchymal stem cells: in vitro and in vivo bone formation on biodegradable polymer scaffolds, Biochem. Biophys. Res. Commun. 292 (2002) 144–152.
[174] H. Hosseinkhani, M. Yamamoto, Y. Inatsugu, Y. Hiraoka, S. Inoue, H. Shimokawa, et al., Enhanced ectopic bone formation using a combination of plasmid DNA impregnation into 3-D scaffold and bioreactor perfusion culture, Biomaterials 27 (2006) 1387–1398.
[175] H. Hosseinkhani, Y. Inatsugu, Y. Hiraoka, S. Inoue, H. Shimokawa, Y. Tabata, Impregnation of plasmid DNA into three-dimensional scaffolds and medium perfusion enhance in vitro DNA expression of mesenchymal stem cells, Tissue Eng. 11 (2005) 1459–1475.
[176] H. Hosseinkhani, Y. Inatsugu, Y. Hiraoka, S. Inoue, Y. Tabata, Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic Acid) fiber, Tissue Eng. 11 (2005) 1476–1488.
[177] Y. Xie, S.T. Yang, D.A. Kniss, Three-dimensional cell-scaffold constructs promote efficient gene transfection: implications for cell-based gene therapy, Tissue Eng. 7 (2001) 585–598.
[178] J. Ziauddin, D.M. Sabatini, Microarrays of cells expressing defined cDNAs, Nature 411 (2001) 107–110.
[179] R.Z. Wu, S.N. Bailey, D.M. Sabatini, Cell-biological applications of transfected-cell microarrays, Trends Cell Biol. 12 (2002) 485–488.
[180] K. Honma, T. Ochiya, S. Nagahara, A. Sano, H. Yamamoto, K. Hirai, et al., Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions, Biochem. Biophys. Res. Commun. 289 (2001) 1075–1081.
[181] T. Yoshikawa, E. Uchimura, M. Kishi, D.P. Funeriu, M. Miyake, J. Miyake, Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown, J. Control. Release 96 (2004) 227–232.
[182] E. Uchimura, S. Yamada, L. Uebersax, T. Yoshikawa, K. Matsumoto, M. Kishi, et al., On-chip transfection of PC12 cells based on the rational understanding of the role of ECM molecules: efficient, non-viral transfection of PC12 cells using collagen IV, Neurosci. Lett. 378 (2005) 40–43.
[183] J.I. Jo, N. Nagaya, Y. Miyahara, M. Kataoka, M. Harada-Shiba, K. Kangawa, et al., Transplantation of genetically engineered mesenchymal stem cells improves cardiac function in rats with myocardial infarction: benefit of a novel nonviral vector, cationized dextran, Tissue Eng. 13 (2007) 313–322.