# Optimization of a cationic liposome-based gene delivery system for the application of miR-145 in anticancer therapeutics

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**Abstract.** In order to improve the delivery efficiency of microRNA (miRNA or miR)-145, the present study examined several factors which may affect cationic liposome (CL)-based transfection, including the hydration medium used for the preparation of liposomes, the quantity of the plasmid, the molar ratio of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP)/cholesterol (chol), or DOTAP/chol, and the weight ratio of DOTAP/DNA. In order to enhance the transfection efficiency, protamine was selected as a DNA-condensing agent to form liposome-protamine-DNA (LPD) ternary complexes. An agarose gel retardation assay was used to examine the DNA binding affinity of the CLs. Following transfection, GFP fluorescence images were captured and flow cytometry was performed to determine the transfection efficiency. Furthermore, an MTT assay was performed to determine the cytotoxicity of the liposome complexes. The final optimal conditions were as follows: 5% glucose as the hydration medium, a molar ratio of DOTAP/chol at 3:1 for the preparation of CLs, a weight ratio of DOTAP/protamine/DNA of 3:0.5:1, with 8 $\mu$g plasmid added for the preparation of the LPD complexes. *In vitro*, the LPD complexes exhibited an enhanced transfection efficiency and low cytotoxicity, which indicated that the presented LPD vector enhanced the transfection efficiency of the CLs. The HepG2 cells were found to have the lowest expression levels of miR-145 out of the cell lines tested (A549, BGC-823, HepG2, HeLa, LoVo and MCF-7). Following the transient transfection of the HepG2 cells with miR-145, the results revealed that the overexpression of miR-145 inhibited the proliferation of the HepG2 cells and down-regulated the expression of cyclin-dependent kinase 6 (CDK6), cyclinD1, c-Myc, and Sp1 transcription factor (Sp1). In conclusion, in this study, we optimized a liposome-based delivery system for the efficient delivery of miR-145 into cancer cells. This may provide a foundation for further research into the use of miR-145 in anticancer therapeutics.

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## Introduction

One of the challenges currently facing gene therapy researchers is finding a means of efficiently delivering exogenous genes into cells. There are many factors which affect the efficiency of this process; the gene therapy vector construct plays a key role. Gene delivery systems are broadly classified into viral vectors or non-viral vectors. Non-viral vectors include liposomes, polymeric micelles and other nanoparticles. Compared with viral vectors, non-viral vectors possess several advantages, including low toxicity, biodegradability, ease of synthesis and low immune responsiveness, and therefore non-viral vectors are attracting more research interest (1,2). In particular, cationic liposomes (CLs) composed of N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol (chol), or DOTAP/chol, have been classified as one of the most efficient vectors for plasmid DNA (pDNA) transfection into cells (3,4). However, the molar ratio of DOTAP/chol has an impact on the transfection efficiency. Previous research has indicated that DOTAP/chol liposomes with a molar ratio of 1:1 or 2:1 had a high transfection efficiency (3,5). To achieve a higher transfection efficiency, on the one hand, it is necessary to identify the optimal molar ratio of DOTAP/chol for use in the preparation of liposomes. On the other hand, the hydration medium has an effect on the liposome properties, such as entrapment efficiency and stability (6). According to previous research, hydration media commonly used in the preparation of CLs include 0.9% NaCl, 5% glucose and Tris-HCl (1,4,7). However, a comprehensive study of the effects of different hydration media on CL-based gene delivery systems has not yet

been reported, to the best of our knowledge. In addition, previous studies have demonstrated that CL-mediated gene transfer may be enhanced by the addition of natural polycations which act as DNA-condensing agents and form liposome-polycation-DNA complexes. Natural polycations, such as protamine sulfate (8,9), chitosan (10) and poly-L-lysine (11), have been shown to condense DNA and form nanoparticles which are mechanically stable, of uniform particle size and of controllable morphology. Protamine, which is non-toxic and is composed of natural cationic peptides, may provide unique membrane-translocating and nuclear-localizing activities owing to its abundant amino acid sequence (12).

RNA interference (RNAi) is a process which involves the post-transcriptional silencing of gene expression in molecular biology, and has become more popular as a gene therapy technique and has been applied in the treatment of various diseases. RNAi tools include synthetic short interfering RNA and endogenous microRNA (miRNA or miR) (13). As small non-coding RNAs, miRNAs regulate the expression of multiple proteins at the translation level. miR-145 has been reported to exert potent antitumor effects by targeting multiple genes which are associated with tumor growth, metastasis and invasion. It has been reported that miR-145 is expressed at low levels in breast cancer, colon cancer, lung cancer and other tumor tissues compared with normal tissues (14). As previously demonstrated, by transfecting synthetic miR-145 oligonucleotides into neuroblastoma cells, endometrial stem cells, and bladder cancer cells, the expression of genes, such as insulin-like growth factor receptor I (IGF-IR) and hypoxia-inducible factor 2α (HIF-2α) was downregulated, which resulted in the inhibition of cancer cell growth and invasion (15-17).

In the present study, we evaluated the effects of various factors on the transfection efficiency of DOTAP/chol liposomes, including the hydration medium used for the preparation of the liposomes, the molar ratio of DOTAP/chol, the mass of DOTAP/DNA and CLs combined with protamine, in order to create a non-viral vector with a higher transfection efficiency. Additionally, we found that miR-145 was expressed at low levels in HepG2 cells compared with other cancer cell lines; therefore, we further explored the role of miR-145 in HepG2 cells and its related downstream genes.

## Materials and methods

*Materials*. The plasmid, pCDH-miR-145-GFP, was constructed in our own laboratory. DOTAP was purchased from Corden Pharma (Liestal, Switzerland), and chol and protamine sulfate salt were purchased from Sigma (St. Louis, MO, USA); and Lipofectamine™ 2000 (Lipo 2000) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). The miRcute miRNA isolation kit was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). The primers were synthesized by Sangon Biotech (Shanghai, China).

*Cell culture*. The 293T cells, hepatoma cells (HepG2), cervical cancer cells (HeLa), breast adenocarcinoma cells (MCF-7), lung adenocarcinoma cells (A549), human gastric cancer (BGC-823) and human colorectal cancer cells (LoVo) were all purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 containing 10% fetal bovine serum (FBS) at 37˚C with 5% $CO_2$.

*Construction of gene carriers*

*Preparation of CLs and CL/DNA complexes*. The CLs were prepared using the thin-film dispersion method. Different molar ratios of DOTAP and cholesterol (1:1 to 7:1) were mixed to a total amount of 10 μmol, and the above-mentioned lipids were dissolved in chloroform, and the solvent was then evaporated under a vacuum by rotary evaporation for 30 min at 45˚C in order to form a thin lipid film. The correct amount of hydration medium was then added to the film for 45 min at 50˚C. The multicellular liposomes were obtained following sufficient hydration. Using a handheld extruder, the liposomes were extruded 10 times repeatedly, each through 2 stacks of polycarbonate membranes with progressively decreasing pore sizes of 400, 200 and 100 nm (ME-25S). The resulting CLs were extruded through a 0.22-μm filter for sterilization and stored at 4˚C. The liposomes were diluted with deionized water and the appropriate amount of DNA (pCDH-miR-145-GFP) by mixing in equal volumes and incubated for 20 min at room temperature to form the CL/DNA complexes.

*Preparation of CL-protamine-DNA (LPD) complexes*. Protamine/DNA nanoparticles were prepared by mixing equal volumes of protamine and DNA solution (pCDH-miR-145-GFP) and incubating the resulting mixture for 10 min at room temperature. LPD complexes were prepared by mixing equal volumes of CLs and protamine/DNA nanoparticles and incubating for 20 min at room temperature.

*Single factor tests*. Firstly, to examine the the effects of the hydration medium on the CLs, we used DOTAP/chol (at a molar ratio of 1:1) to prepare the CLs, and hydration medium (deionized water, 0.9% NaCl, 5% glucose, 5% sucrose, 10 mM Tris buffer and 10 mM phosphate buffer). A weight ratio of DOTAP/DNA of 4:1 was used for transfection. Secondly, varying amounts of DNA (2, 4, 6, 8, 10 and 15 μg) were used to compare the relative transfection efficiency, and we selected the DOTAP/chol liposomes (at a molar ratio of 1:1) and the weight ratio of DOTAP/DNA at 4:1 to determine the optimal mass of DNA. Thirdly, to identify the optimal molar ratio of DOTAP/chol, 5% glucose was used as the hydration medium, the molar ratio of DOTAP/chol was 1, 2, 3, 4, 5 and 6, and the mass ratio of DOTAP/DNA was 4:1, the weight of the DNA was 8 μg; under these conditions, we examined the transfection efficiency. Fourthly, we examined the weight ratio of DOTAP/DNA (1, 2, 3, 4, 5, 6, 8 and 10) and used DOTAP/chol liposomes (at a molar ratio of 3:1) to determine the transfection efficiency. Lastly, to examine the weight of protamine in the LPD complexes, we set a weight ratio of DOTAP/protamine/DNA at 3:0.2:1, 3:0.5:1, 3:0.8:1, 3:1:1, 3:1.5:1, 3:2:1 and 3:3:1 for transfection.

*Measurement of particle size and ζ-potential*. The particle size and ζ-potential of the liposomes, lipoplexes and LPD complexes were measured using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) after being dispersed in deionized water.

*Agarose gel retardation assay*. The DNA binding ability of the DOTAP/chol liposomes was evaluated by a gel retardation assay. The lipoplex included 0.5 μg at different molar ratios of DOTAP/chol and a different mass ratio of DOTAP/DNA which

Table I. Sequences of the primers used for RT-qPCR.

| Gene | Forward sequence (5'→3') | Reverse sequence (3'→5') |
|---|---|---|
| CDK6 | CGA ATG CGT GGC GGA GAT C | CCA CTG AGG TTA GAG CCA TC |
| CyclinD1 | GGA TGC TGG AGG TCT GCG AGG AAC | GAG AGG AAG CGT GTG AGG CGG TAG |
| c-Myc | CTT CTC TCC GTC CTC GGA TTC T | GAA GGT GAT CCA GAC TCT GAC CTT |
| Sp1 | GCC TCC AGA CCA TTA ACC TCA GT | GCT CCA TGA TCA CCT GGG GCA T |
| β-actin | GGGACCTGACTGACTACCTC | TCATACTCCTGCTTGCTGAT |
| miR-145 | GTCCAGTTTTCCCAGGAATCCCT | |
| U6 | CTC GCT TCG GCA GCA CA | |

was loaded into individual wells on a 1% agarose gel, and electrophoresis was performed in Tris/Borate/EDTA (TBE) buffer at 120 V for 30 min, followed by staining with ethidium bromide.

*In vitro transfection*. The cells were seeded in a 6-cm dish at a density of approximately $1x10^6$ cells per dish and incubated overnight prior to transfection. The lipoplexes and LPD complexes were transfected into the cells for 6 h with minimum essential medium (MEM) medium in the absence of FBS. The complexes were then removed and added to 10% FBS medium. After 48 h, the GFP expression levels in the transfected cells were analyzed under a fluorescence microscope (Zeiss, Oberkochen, Germany) and using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Lipo 2000 was used as a positive control according to the manufacturer's instructions.

*DNase assay*. Naked DNA, lipoplexes and LPD complexes were added to DNase solution at 37˚C for 30 min and EDTA was used to terminate the reaction. Triton X-100 (1%) was added to disrupt the lipid membranes and subseqently, heparin was added to release the DNA from the protamine/DNA complexes. The resulting products were analyzed by electrophoresis on a 1% agarose gel at 120 V for 30 min.

*Analysis of endogenous miR-145 expression levels in different cancer cell lines by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. According to the instructions of the manufacturer (provider of TRIzol reagent), we extracted the total RNA from the A549, BGC-823, HepG2, HeLa, LoVo and MCF-7 cells. miR-145 was reverse transcribed into cDNA using an miRcute miRNA cDNA kit and miRcute miRNA qPCR detection kits, to measure the expression levels of miR-145. The PCR cycles were as follows: denaturation at 94˚C for 2 min, followed by 40 cycles at 94˚C for 20 sec and 60˚C for 34 sec and snRU6 was used as an internal normalization control as previously described (16). The relative changes in miRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method.

*Cell proliferation assay*. Cell proliferation was measured using by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (16). Following transfection for 48 h, 10,000 HepG2 cells were seeded in 96-well plates and cultured for 24 h. Briefly, MTT solution was added and followed by further incubation for 4 h; DMSO was then added to dissolve the formazan crystals and the optical density was measured at 570 and 630 nm using a microplate reader (Multiskan MK3, Thermo Fisher Scientific Inc.,Waltham, MA, USA).

*Analysis of the downsteam genes regulated by miR-145 by RT-qPCR*. TRIzol reagent was used to extract all RNA which was then reverse transcribed into cDNA using a ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). qPCR was performed using SYBR-Green Real-Time PCR Master Mix (Toyobo). We also searched the TargetScan and miRanda databases in order to predict the binding sites of miR-145 based on different algorithms; genes found on both databases which may affect cell proliferation were selected, including CDK6, cyclinD1, c-Myc and Sp1. The primer sequences of cyclin-dependent kinase 6 (CDK6), cyclinD1, c-Myc, Sp1 transcription factor (Sp1) and β-actin are listed in Table I.

*Statistical analysis*. All experimental results are presented as the means ± standard deviation (SD). Statistical analyses were performed using the Student's t-test. A P-value $<0.05$ was considered to indicate a statistically significant difference.

## Results

*Particle sizes and ζ-potentials of CLs, lipoplexes and LPD complexes*. Particle size and ζ-potential are important physicochemical parameters of nanoparticles, which significantly affect the transfection efficiency *in vitro* or *in vivo* (10). The size and ζ-potential of the lipoplexes and LPD complexes were determined and compared with those of the corresponding lipoplexes. As shown in Fig. 1, differences were observed in the particle size between the lipoplexes and the LPD complexes. Following the addition of protamine, the mean particle size of the complexes decreased; however, the ζ-potential of the complexes remained unaltered. This indicated that protamine had an effect on nanoparticle size by condensing the DNA, whereas it had a limited effect on the ζ-potential of the nanoparticles.

*DNA binding affinity of CLs*. Gel retardation assays are widely used to measure the DNA binding affinity of CLs (1). Our results indicated that with the different weight ratios of DOTAP/DNA ranging from 1 to 7, the migration path of the DNA in the lipoplexes changed (Fig. 2). When the weight ratio was ≥2 in formulation, minimal free DNA was observed in the gel. These results suggested that when the weight ratio of DOTAP/DNA was ≥2, the liposomes and DNA were completely bound and also indicated that the DNA binding affinity of the CLs was unaffected by the hydration medium.

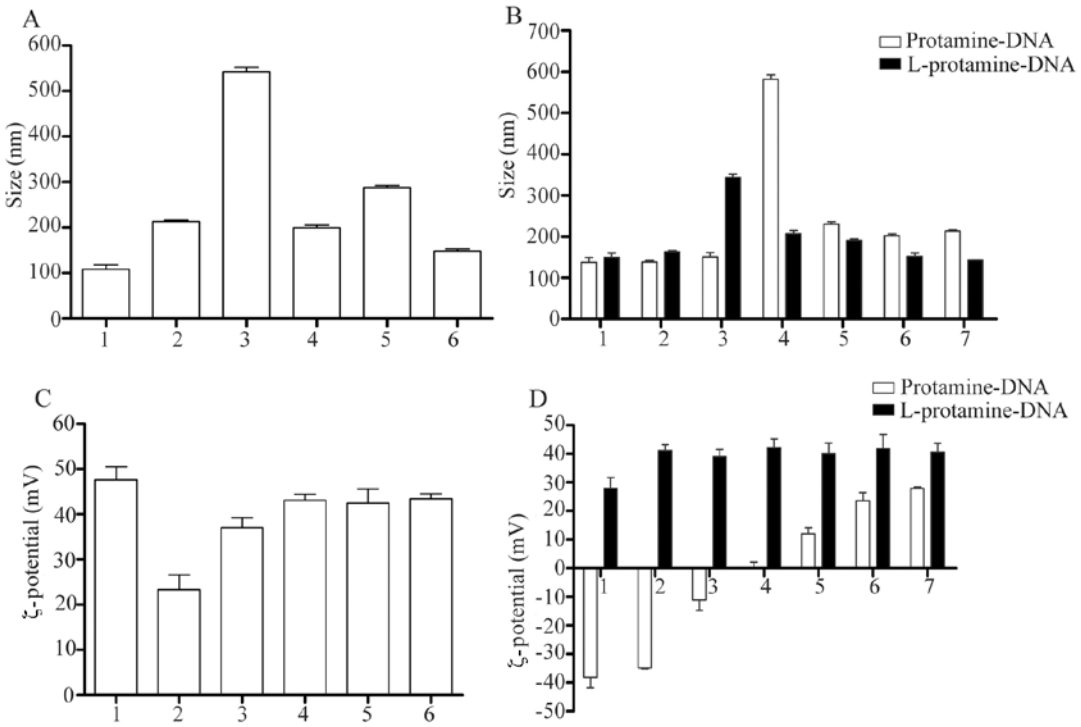


Figure 1. Particle sizes and ζ-potential of nanoparticles. (A and C) The particle sizes and ζ-potential of lipoplexes at different weights of DOTAP/DNA (1-6; the weights of DOTAP/DNA were 1:0, 2:1, 3:1, 4:1, 5:1 and 8:1). (B and D) The particle sizes and ζ-potential of protamine/DNA complexes and LPD complexes (1-7; the weight ratios of DOTAP/protamine/DNA were 3:0.2:1, 3:0.5:1, 3:0.8:1, 3:1:1, 3:1.5:1, 3:2:1 and 3:3:1).

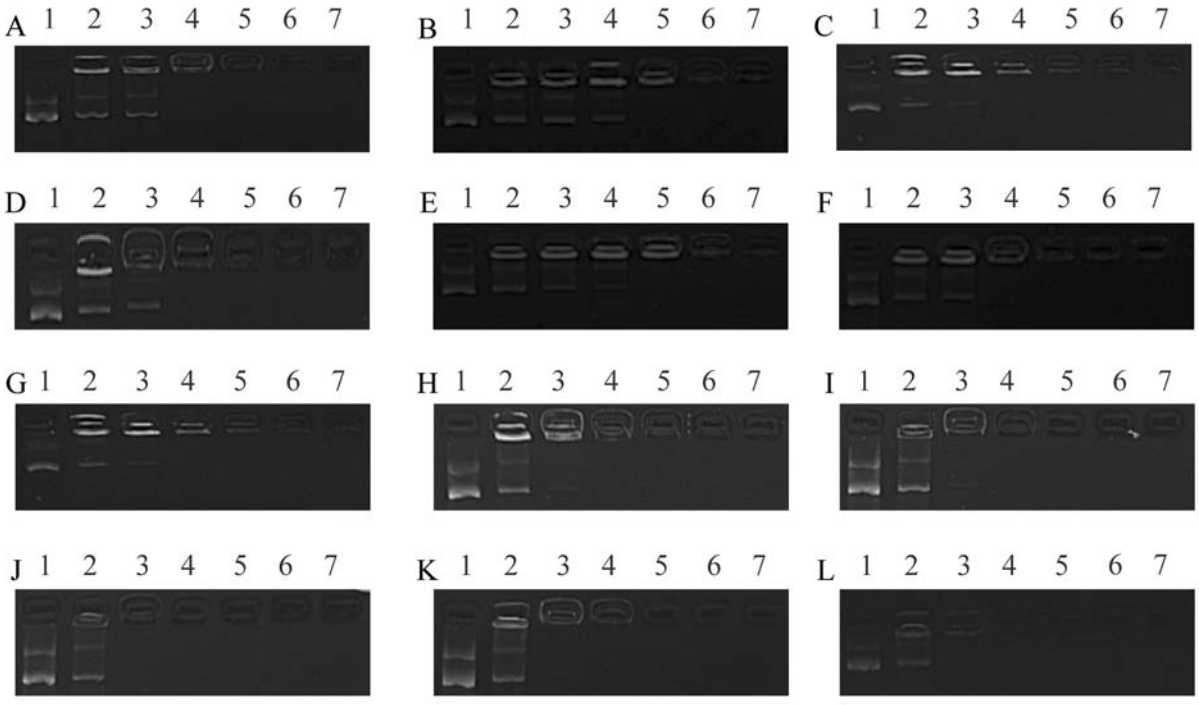


Figure 2. Gel retardation assays. The mass ratio of DOTAP/DNA was 1, 2, 3, 4, 5 and 7 (lanes 2, 3, 4, 5, 6 and 7, respectively). Lanes 1, 0.5 μg plasmid. (A-E) Different hydration medium [DOTAP/cholesterol (chol), 1:1)]; (A) deionized water; (B) 0.9% NaCl; (C) 5% glucose; (D) 10 mM Tris-HCl; (E) 10 mM phosphate buffer; (F) 5% sucrose. (G-L) Different liposomes were complexed with DNA; (G) DOTAP/chol, 1:1; (H) DOTAP/chol, 2:1; (I) DOTAP/chol, 3:1; (J) DOTAP/chol, 4:1; (K) DOTAP/chol, 5:1; (L) DOTAP/chol, 7:1.

*Transfection efficiency of CL/DNA and LPD complexes.* Previous research has indicated that the transfection efficiency of CLs is affected by a number of factors, including the molar ratio of DOTAP/chol and the charge ratio of DOTAP/DNA, and these must be taken into account when determining the optimal transfection protocol *in vitro* (1,23). The present

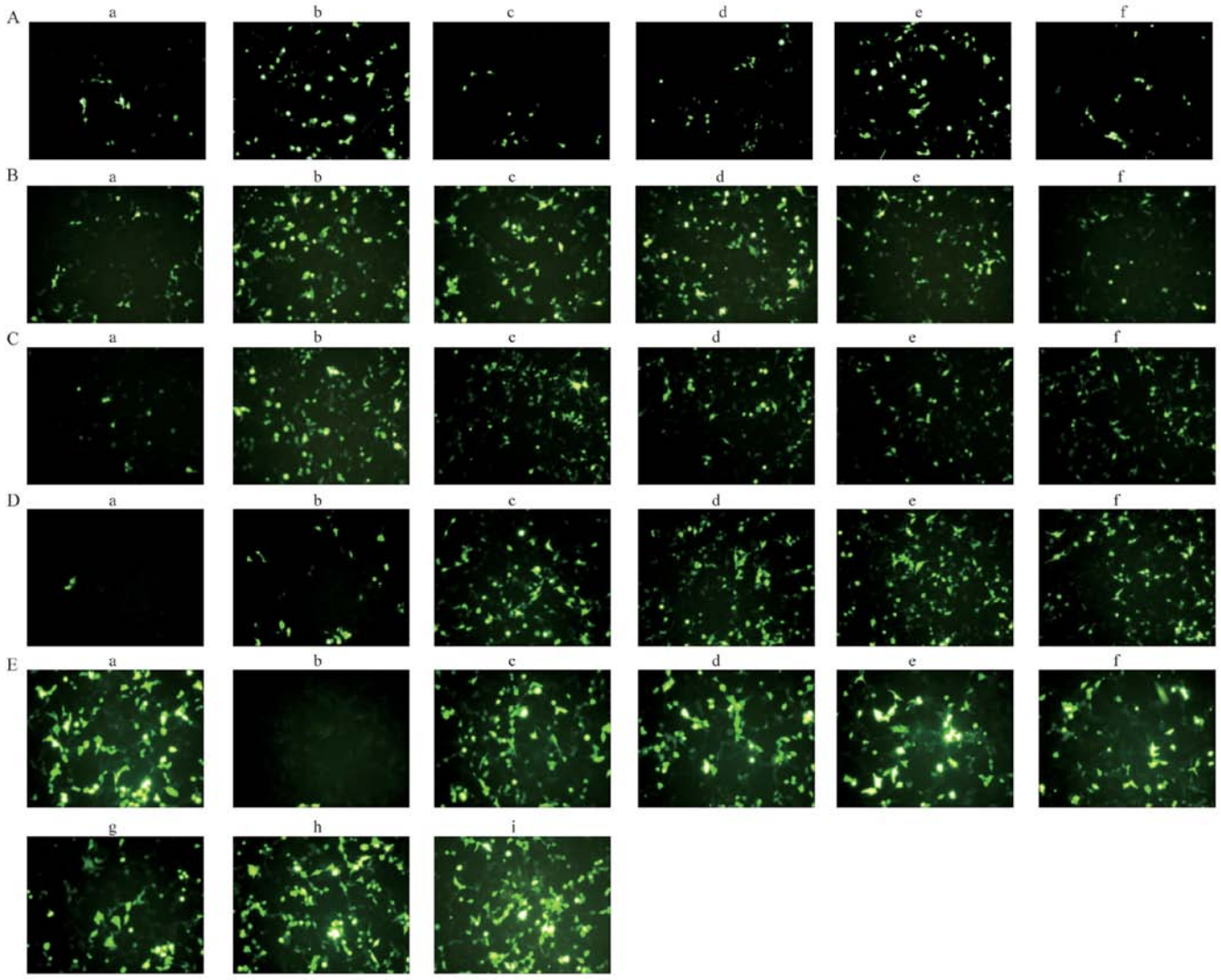


Figure 3. Representative GFP fluorescence images of transfection experiments. The fluorescence intensity of GFP was detected using a fluorescence microscope. (A) DOTAP/cholesterol (chol) liposome (molar ratio of 1:1 and fixed DNA of 8 $\mu$g) in different hydration media [(panels a-f) water, 5% glucose (Glu), 0.9% NaCl, 10 mM Tris-HCl, 10 mM phosphate buffer and 5% sucrose, respectively]; (B) DOTAP/chol liposome (molar ratio of 1:1 and 5% Glu as the hydration medium) and different amounts of DNA [(panels a-f) 2, 4, 6, 8, 10 and 15 $\mu$g, respectively]; (C) DOTAP/chol liposome (fixed DNA of 8 $\mu$g and 5% Glu as the hydration medium) at different molar ratios of DOTAP and chol [(panels a-f) the molar ratio of DOTAP/chol with 1, 2, 3, 4, 5 and 6, respectively]; (D) DOTAP-chol liposome (molar ratio of 3:1, pDNA fixed of 8 $\mu$g) were complexed with DNA at various weight ratios (panels a-f) the weight ratio of DOTAP/DNA with 1, 2, 3, 4, 5 and 6, respectively; (E) protamine combined with liposome at different weight ratios of protamine and DNA (panel a) Lipofectamine™ 2000 (Lipo 2000), (panel b) protamines and DNA complex, (panels c-i) the LPD with different weight ratio of protamines/DNA (0.2, 0.5, 0.8, 1.0, 1.5, 2.0 and 3.0, respectively) and DNA.

study examined these factors (hydration medium, the amount of DNA, the molar ratio of DOTAP/chol, the weight ratio of DOTAP/DNA and the weight of protamine/DNA) in detail. By examining these factors, we determined the optimal conditions to prepare DOTAP/chol liposomes for use in a transfection protocol. To determine the effects of the hydration medium on transfection, water, 5% glucose, 0.9% NaCl, 10 mM Tris-HCl and 10 mM phosphate buffer and 5% sucrose were used. We found that the optimal hydration medium for DOTAP/chol liposomes was 5% glucose (Figs. 3A and 4A). When the hydration medium was changed from deionized water to 5% glucose, the transfection efficiency increased from 6.2 to 20.5%, respectively (Fig. 4A). Furthermore, we investigated the optimal amount of DNA for transfection in a 6-cm dish, by testing different amounts of DNA (2, 4, 6, 8, 10 and 15 $\mu$g). The results revealed that when the amount of DNA increased from 2 to 8 $\mu$g, the transfection efficiency increased from 8.2 to 19.8%, respectively. However, with further increases in the amount of DNA, the transfection efficiency decreased (Figs. 3B and 4B). To examine the optimal molar ratio of DOTAP/chol, we tested different molar ratios of DOTAP/chol (1, 2, 3, 4, 5 and 6) to determine the effects on transfection efficiency. The results revealed that for DNA delivery, the optimal molar ratio of DOTAP/chol was 3:1 (Figs. 3C and 4C). When the ratio of DOTAP/chol was changed from 3:1 to 6:1, the effects of DNA transfection progressively diminished. The effect of changing the mass ratio of DOTAP/DNA (1, 2, 3, 4, 5 and 6) on the transfection efficiency was also examined. The results revealed that the transfection efficiency was highest at a DOTAP/DNA weight ratio of 3 (Figs. 3D and 4D), which also indicated that there is not always a positive correlation between the transfection

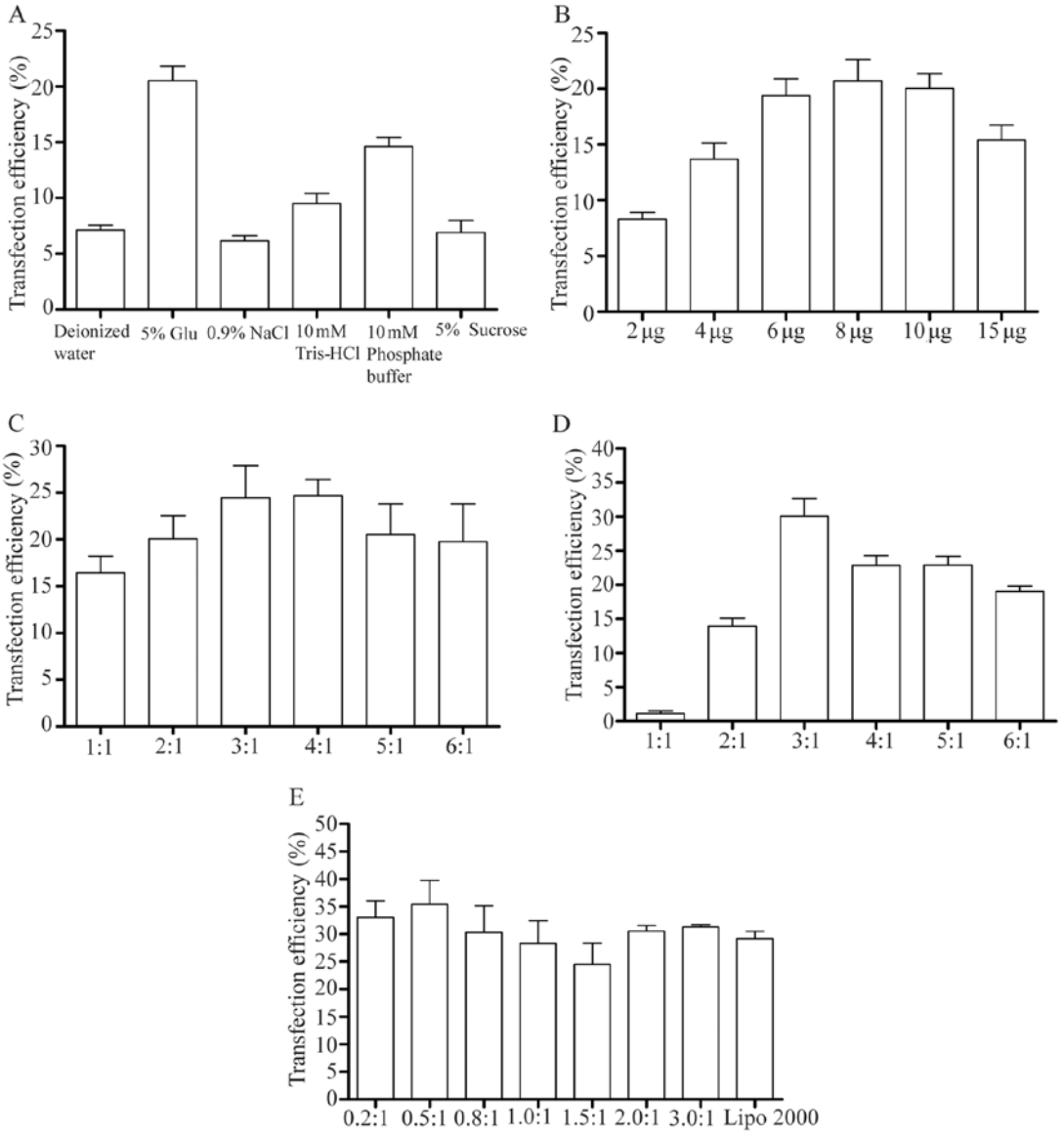


Figure 4. DNA transfection assays. (A) DOTAP/cholesterol (chol) liposome (molar ratio of 1:1 and fixed DNA of 8 $\mu$g) in different hydration media; (B) DOTAP/chol liposome (molar ratio of 1:1 and 5% Glu as the hydration medium) and different amounts of DNA; (C) DOTAP-chol liposome (fixed DNA of 8 $\mu$g and 5% Glu as the hydration medium) at different molar ratios of DOTAP and chol; (D) DOTAP/chol liposomes (molar ratio of 3:1, DNA fixed of 8 $\mu$g) were complexed with pDNA at various weight ratios; (E) protamine combined with liposomes in different weight ratios of protamines and DNA.

efficiency and the mass ratio of DOTAP/DNA. In order to further increase the transfection efficiency, we also considered the DNA condensing agent and the protamine added to the formulation. The weight ratio of the protamine/DNA ranged from 0.2 to 3 (0.2, 0.5, 0.8, 1.0, 1.5, 2 and 3) and these nanoparticles were combined with CLs to form LPD complexes for transfection. The results indicated that the addition of a certain amount of protamine to form the LPD complexes improved the effects of DNA delivery. The highest transfection efficiency was observed with a weight ratio of protamine/DNA of 0.5 (Figs. 3E and 4E). Compared to transfection only with Lipo 2000, the LPD complexes had better transfection efficiency.

*Toxic effects of the complex on 293T cell proliferation*. To determine the toxicity of the liposomes, an MTT assay was performed to evaluate the effects of different gene carriers on 293T cell proliferation. Compared to transfection with Lipo 2000 and the CLs, the lipoplexes and LPD complexes did not exhibit any significant toxic effects (Fig. 5A). The results suggested that these preparations had a good compatibility with the cells. Compared with the cells transfected with the CLs, the viability of the cells transfected with the complexes containing DNA was relatively low; this may perhaps be due to the presence of miR-145 expression, which inhibited cell proliferation.

*DNase assay*. DNA is easily degraded *in vivo* and is a factor which restricts the effectiveness of gene therapy. Thus, we also used DNase to confirm that the liposomes and the LPD complexes encapsulated the DNA in order to prevent DNA degradation. Naked DNA was easily degraded by DNase;

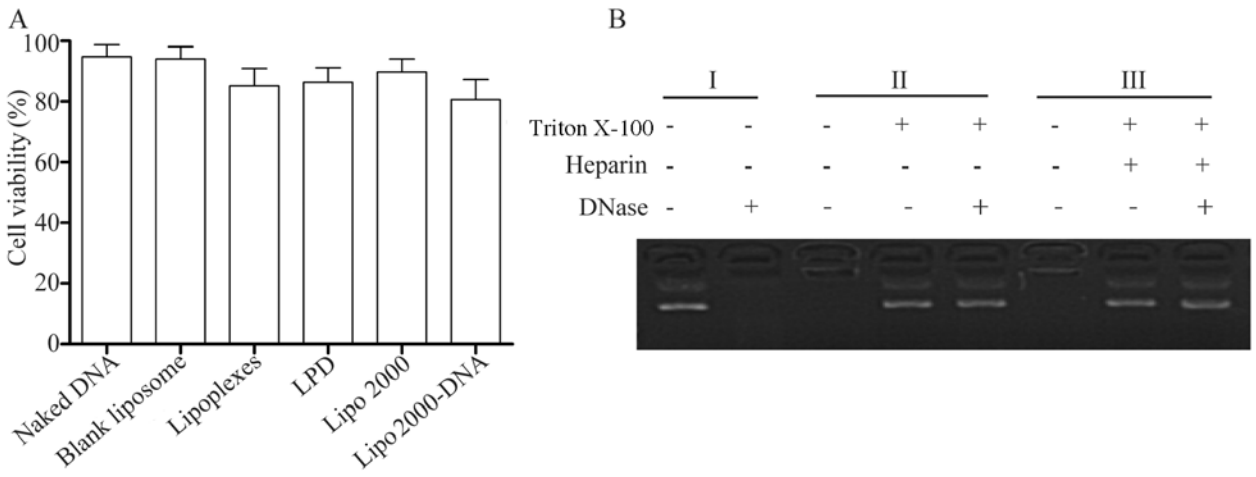


Figure 5. (A) *In vitro* 293T cell survival rates; (B) DNaseI digestion assay. I, naked DNA; II, lipoplexes; III, LPD complexes; each sample contained 0.5 $\mu$g DNA incubated with (+) or without (-) DNaseI at 37˚C for 30 min.

however, in the lipoplexes or the LPD complexes, DNA degradation occurred to a lesser extent (Fig. 5B). These results indicated that the liposomes and protamine had a better encapsulation efficiency and protected the DNA from degradation by DNase.

*Endogenous miR-145 expression levels*. In order to determine the basal expression of miR-145 in different cancer cell lines, we first detected the expression of miR-145 in the A549, BGC-823, HepG2, HeLa, LoVo and MCF-7 cell lines. As shown in Fig. 6A, the majority of the cell lines exhibited a low expression level of miR-145, of which the HepG2 cells had the lowest. Thus, the HepG2 cells were selected for use in the subsequent experiments.

*miR-145 inhibits HepG2 cell proliferation by regulating cell cycle-related genes*. In order to investigate the potential mechanisms responsible for the inhibitory effects of miR-145 in HepG2 cells, we used LPD complexes to deliver miR-145 to the HepG2 cells, with a higher transfection efficiency than Lipo 2000 (Fig. 6B). The results of MTT assay indicated that the delivery of miR-145 significantly inhibited the proliferation of the HepG2 cells (Fig. 6C, $P<0.05$). Following transfection, miR-145 expression was significantly increased in the HepG2 cells by approximately 9-fold compared to the endogenous level of miR-145. RT-qPCR confirmed the overexpression of miR-145 in the HepG2 cells (Fig. 6D). Previous studies have demonstrated that miR-145 regulates certain genes and consequently affects cancer cell proliferation, migration and invasion. We used the TargetScan and miRanda websites to predict the target genes of miR-145. We found that miR-145 combines with the mRNA 3'UTR of the target gene, and found several genes associated with the cell cycle, including CDK6, cyclinD1, the transcription factor, Sp1, and the proto-oncogene, c-Myc. The results of RT-qPCR indicated that the overexpression of miR-145 in the HepG2 cells had a significant effect on the expression levels of these genes (Fig. 6E). RT-qPCR revealed an approximate 39% decrease in the mRNA expression of CDK6 with the overexpression of miR-145 ($P<0.01$), and an approximate 30% decrease in the mRNA expression of cyclinD1. c-Myc mRNA expression was downregulated by approximately 18% with the overexpression of miR-145 ($P<0.05$). Sp1 mRNA expression was downregulated by approximately 35% ($P<0.01$). These data indicate that the inhibitory effects induced by miR-145 may occur through the regulation of the expression of cell cycle-associated genes.

## Discussion

Non-viral gene vectors have been widely used in research and gene therapy as they are considered safe to use, are easily prepared and provide a good targeting efficiency. However, their use as a liposome-mediated gene delivery system for the delivery of DNA non-viral gene vectors is often associated with two main concerns: difficulty in escaping from endosomes and difficulty in entering the nucleus (18). DOTAP/chol liposomes are an efficient gene delivery vector and effectively overcome the above two concerns; however, there is still potential to improve aspects of the design of DOTAP/chol CLs, including optimization of the hydration medium, the mass of DNA and the molar ratio of DOTAP/chol (19). The screening and comparison of different hydration media, with the aim of improving the transfection efficiency, has been rarely reported in the literature, to the best of our knowledge. Water, 5% dextrose, 0.9% NaCl and 10 mM Tris-HCl have been used as hydration media in liposome preparation. In the present study, we compared different hydration media in order to determine whether the selection of hydration media affects the transfection efficiency. The results of the gel retardation assay demonstrated that there were no significant changes to the migration path of the DNA in the presence of the different hydration media, which may suggest that the hydration medium has minimal effect on the DNA binding affinity of CLs. However, the transfection experiments revealed a difference in transfection efficiency among the liposomes prepared with different hydration media; there was a 4-fold increase in the transfection efficiency of CLs prepared with 5% glucose compared with those prepared with either water or 0.9% NaCl (Figs. 3A and 4A).

These findings confirm that the selection of hydration medium has an effect on CL-mediated gene delivery, and suggest that it

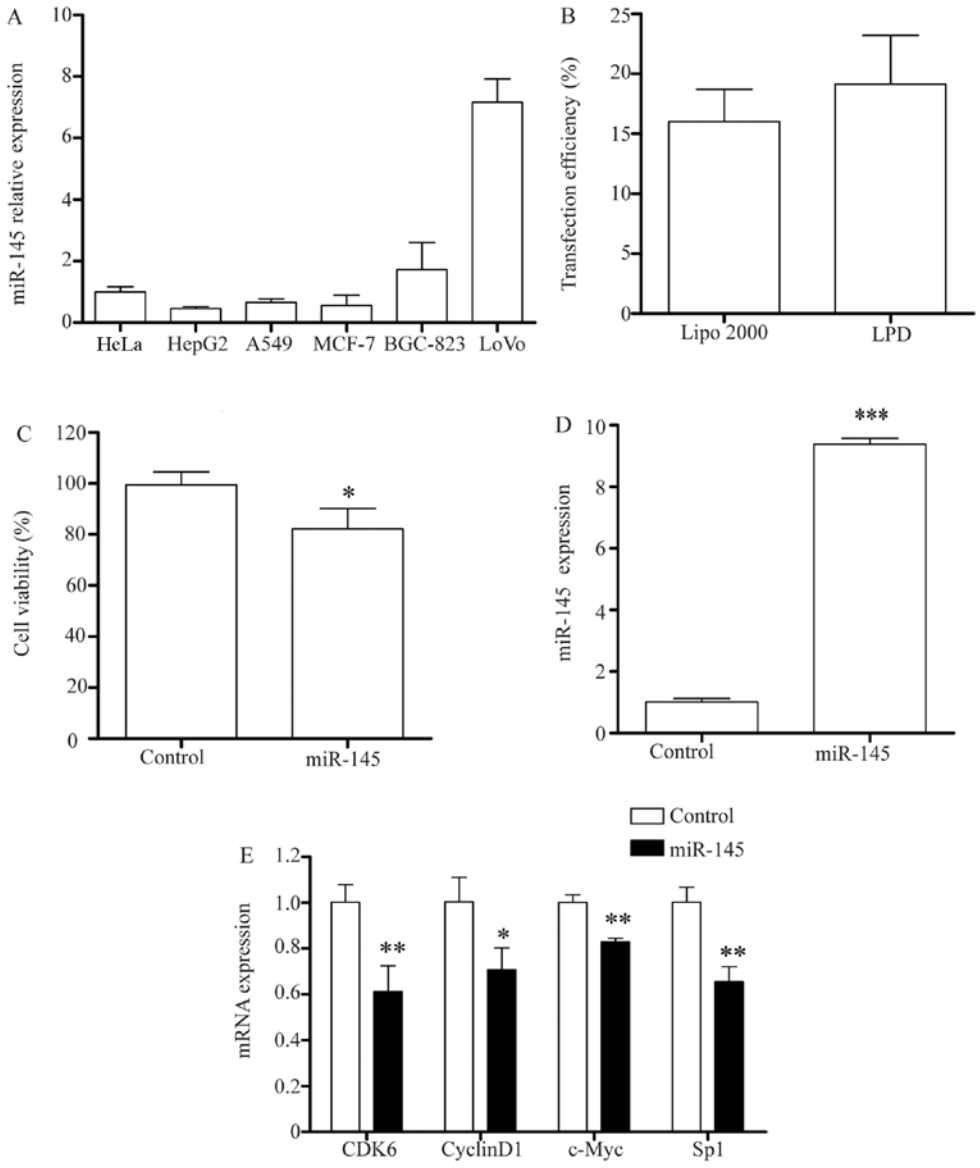


Figure 6. (A) Expression of miR-145 in different cell lines. (B) Transfection efficiency of LPD complexes in HepG2 cells. (C) Following the transfection of HepG2 cells, cell proliferation was determined by MTT assay. (D) RT-qPCR confirmation of miR-145 overexpression 48 h following transfection of HepG2 cells. (E) RT-qPCR analysis of miR-145 gene expression in HepG2 cells. $^{*}$P>0.01 and <0.05; $^{**}$P>0.001 and <0.01; $^{***}$P<0.0001 (all compared to the control group).

should be considered as a key step in liposome preparation. The mechanism responsible for 5% glucose enhancing gene delivery remains unclear; however, we presumed that it not only acted as an isosmotic solution, but may also meet the glucose addiction of cell lines cultured *in vitro*, which may effectively enhance cellular uptake or improve endosomal escape.

In previous research, dioleoylphosphatidylethanolamine (DOPE) or chol were often used as helper lipids. DOPE which may destabilize cell membranes, has been shown to be beneficial for lipid membrane fusion with the cell and endosomal membrane, which enables escape from the endosomes (20). However, this material has great defects in the presence of serum, as liposomes obtained from DOPE would present obstacles as regards to fusion with the cell membrane, thus reducing the transfection efficiency. It has been found that chol does not have a prominent effect on promoting cell fusion; however, chol does not have ionizable groups and does not greatly affect surface charge. In the presence of serum, the carrier holds more positive charges and also maintains its integrity. Moreover, chol promotes phase alteration to form separated domains (21,22). Thus, for *in vivo* transfection or transfection under approximate physiological conditions, chol is a much better helper lipid. The molar ratio of DOTAP/chol in CLs plays a crucial role in transfection. In this study, the results of the gel retardation assay demonstrated that when the weight ratio of DOTAP/DNA exceeded 2, the DNA was totally encapsulated within the CLs which were composed of different molar ratios of DOTAP/chol (Fig. 2G-L). The results also illustrated that chol had a minimal effect on the surface charge of carriers. When the molar ratio of DOTAP/DNA was 3:1, the expression of GFP and the transfection efficiency (measured by flow cytometry), exhibited optimal results (Figs. 3C and 4C). By increasing the mass ratio of DOTAP/DNA, the transfection efficiency increased and then decreased (Figs. 3D and 4D). Thus, we hypothesized that at a low weight ratio of DOTAP/DNA and the surface charge of the lipoplexes were less (Fig. 1C) which may induce the complexes

to enter the cells or the that the escape from endosomes was less. While the high weight ratio of DOTAP/DNA was probably the reason for the DNA to bind firmly with the CLs and enter the cells, the lipoplexes only released small amounts of DNA, resulting in a reduced transfection efficiency. Furthermore, it is possible that the cytotoxicity of lipoplexes increased with the increasing weight ratio of DOTAP/DNA, and this may prove harmful to transfection (23).

Liposome-polycation-DNA complexes are based on the CL/DNA lipoplexes and have been developed as a novel non-viral vector in recent years. Compared with CLs, LPD complexes are more efficient at condensing DNA which significantly improves transfection efficiency, and therefore they are promising clinical gene therapy vectors (24). Protamine is a natural polymer and has received FDA approval for use in clinical treatments. Protamine is a basic protein which is positively charged and arginine-rich, and it was extracted from marine fish sperm. Moreover, protamine has several advantages, such as low antigenicity and a good safety profile (25,26). As a cationic polymer, protamine may be used to condense a long chain of DNA to improve resistance to the radical changes of external forces. Furthermore, it has been found that protamine has a specific nuclear localization signal, since it is rich in amino acids, and therefore it is more beneficial in terms of enabling the complex to enter nuclear translocation (12). Our study proved that protamine improved the transfection efficiency of a CL-based gene delivery system (Figs. 3E and 4E). The mechanism of action of LPD may that a core complex is formed with protamine and DNA, and this complex in conjunction with CLs then forms a LPD nanoparticle with a shell-core structure (9). Moreover, in our study, the size of the nanoparticles decreased following the addition of protamine to form the LPD complexes (Fig. 1). Particle size also plays an important role in gene delivery. The complexes of different sizes enter cells through different endocytic pathways, which consequently affects the transfection efficiency. DNA easily undergoes enzymatic degradation *in vivo*, which is a restriction of gene therapy (27); thus ways of preventing DNA degradation by DNase must be considered when designing systemic gene therapy. In our study, we found that the shell-core construction of LPD complexes not only protected the DNA from enzymatic degradation (Fig. 5B), but that the lipid membrane restricted DNase from touching the encapsulated DNA.

In previous studies, it has been suggested that miR-145 has an anti-tumor ability and targets multiple tumor genes which are involved in cancer cell growth, metastasis and invasion (16,28,29). It has been shown that miR-145 is expressed at low levels in a different types of tumor tissue, such as colon, breast, liver and lung cancer tissue. We selected several cancer cell lines and then observed the endogenous expression of miR-145 (Fig. 6A). It was found that miR-145 had the lowest expression level in the HepG2 cells compared with the other cancer cells. miR-145 as a tumor suppressor, has been shown to inhibit cancer cell growth by targeting c-Myc and p53 in colon and breast cancers (30), IGF-IR in human bladder cancer (17) and HIF-2α in neuroblastoma (15). In our study, our results indicated that miR-145 reduced the mRNA expression of CDK6, cyclinD1, c-Myc and Sp1 in the HepG2 cells, thus indicating that the inhibitory effects of miR-145 on HepG2 cell proliferation may occur through the regulation of the cell cycle-associated genes, which results in anticancer effects.

In conclusion, in this study, we systematically evaluated the various factors which affect the preparation of DOTAP/chol liposomes in order to improve the transfection efficiency. Furthermore, we clarified that protamine improved the transfection efficiency of the CLs. Finally, the present study demonstrated that miR-145 is a potential novel target for anticancer therapeutics.

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