Original Paper

Lower-Molecular-Weight Chitosan-Treated Polyethyleneimine: a Practical Strategy For Gene Delivery to Mesenchymal Stem Cells

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Key Words

Mesenchymal stem cells • Gene delivery • Safety evaluation • Chitosan • Polyethylenimine

Abstract

Background/Aims: Genetic modification of mesenchymal stem cells (MSCs) is an essential requirement for their use as a delivery vehicle. To achieve higher transfection efficiency and better reproducibility than previously synthesized chitosan (100 kDa)-polyethylenimine (PEI; 1200 Da), we synthesized a low molecular weight PEI (1200 Da)-grafted chitosan (50 kDa) (CP). **Methods:** Safety of CP/DNA or PEI (25 kDa)/DNA was evaluated by an MTT assay using A549 cells or MSCs and a zebrafish embryo model. Effects of CP/DNA on the characteristics of MSCs were evaluated using flow cytometry. Additionally, a pGL3 plasmid was used to investigate the transfection efficiency of PEI (25 kDa), chitosan (100 kDa)-PEI (1200 Da), and CP with different N/P mass ratios on A549 cells and MSCs. Furthermore, CP/pGL3 was used to investigate the effect of serum on transfection, and intracellular transport was assessed by observing the intracellular location of DNA using laser scanning confocal microscopy. In addition, the effect of endocytosis on transfection efficiency was evaluated using A549 cells pre-treated with different inhibitors. Investigations related to analysis of transfection efficiency were all performed using the BCA protein assay to standardize the data. Furthermore, TGF- β 1- and CXCR4-expressing plasmids were applied to evaluate the gene transfer efficiency of CP, including its effects on the osteogenic differentiation and migratory ability of MSCs. **Results:** The safety evaluation demonstrated that CP/DNA had significantly lower toxicity than PEI (25 kDa)/DNA. Additionally, DNA entered MSCs transfected by CP without changing their properties, while the examination of intracellular transport demonstrated that CP/pGL3 was internalized rapidly into MSCs. Furthermore, studies of the internalization mechanism showed that CP/pGL3 complexes entered the cells through caveolae-mediated endocytosis, thereby

suggesting that the CP coating helped DNA enter A549 cells without the requirement for receptors. Compared to PEI (25 kDa), the interference of serum on transfection was reduced significantly with the use of CP in both A549 cells and MSCs. To evaluate the effects of gene delivery using the constructed CP complex and the possibility of obtaining gene-engineered MSCs, TGF- β 1- and CXCR4-expressing plasmids were successfully delivered into MSCs, confirming their ability to induce osteogenesis and change the migratory ability of MSCs, respectively. **Conclusion:** These results demonstrated that CP could be used to deliver genes into MSCs and could potentially be used in gene therapy based on MSCs.

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Introduction

The use of mesenchymal stem cells (MSCs) in tissue engineering and regenerative medicine has great potential because of their self-renewal ability, easy accessibility, and multi-lineage differentiation potential [1]. However, before being administered to a subject, MSCs must be expanded and genetically modified *in vitro* because its distribution in bone marrow is limited and would not provide a powerful therapeutic effect [2, 3].

Successful transfection of MSCs is largely dependent on the efficiency of the vehicle or vector used to deliver a gene with minimal toxicity [4]. Due to safety concerns over the use of viral vectors, non-viral vectors have received greater attention [5] and are now considered a substitute for viral vectors because of their potential advantages [6]. However, further efforts are required to improve their gene transfer ability [7], especially in MSCs. Among the non-viral DNA carriers, cationic polymers have an important position because they can form a complex with DNA through electrostatic interactions. Cationic polymers can be modified by adjusting the constituents of the monomer, chemically modifying the functional groups, or controlling the polymerization conditions. In addition, the composition of cationic polymers can be controlled by conjugation with a targeting ligand or intracellular trafficking enhancer [8].

Polyethylenimine (PEI; 25 kDa) has been shown to be the most effective vector because of its high pH buffering capacity [9]. Nevertheless, its non-degradable properties and high cytotoxicity have restricted its application. Although low molecular weight PEI has lower toxicity, its transfection efficiency was reduced [10]. It was reported that when low molecular weight PEI was combined with high molecular weight degradable linkers, new PEIs could be formed with a suitable molecular weight [11]. As such a linker, chitosan shows low cytotoxicity and high biocompatibility; however, it lacks charge and is insoluble at physiological pH, which has limited its application in gene delivery [12]. Thus, chitosan (100 kDa)-PEI (1200 Da) was developed in our previous study [11]. However, the particle sizes of the chitosan (100 kDa)-PEI (1200 Da)/DNA complexes were not homogeneous, and its poor solubility in water hindered its application. Furthermore, compared to PEI (25 kDa), chitosan (100 kDa)-PEI (1200 Da) has no obvious advantage in providing sufficiently stable transfection efficiency in HeLa, HepG2, and A549 cell lines.

For the construction of a chitosan-PEI carrier with high transfection efficiency and low toxicity, Pimpha et al. prepared chitosan shell-PEI nanoparticles for gene delivery into MSCs [13]. The complex improved the level of gene transfection and extended the time period for expression in MSCs. However, the molecular weight of PEI (750 kDa) is still high and its potential toxicity cannot be ignored.

In the present study, a low molecular weight PEI (1200 Da)-introduced chitosan (50 kDa) (CP) was constructed and characterized. CP had low toxicity *in vitro* and *in vivo* and high transfection efficiency in the presence and absence of serum, making it suitable for biological applications. CP/pGL3 entered the cells through caveolae-mediated endocytosis, which could be helpful for understanding the internalization mechanism of CP. In addition, the osteogenic differentiation properties of MSCs transfected with CP/transforming growth factor (TGF)- β 1 and the *in vitro* migration of MSCs transfected with CP/C-X-C chemokine

receptor type 4 (CXCR4) were investigated. The findings of the present study are expected to lay the foundation for providing potential applications of CP in gene therapy based on the use of stem cells.

Materials and Methods

Materials

Chitosan (50 kDa, 100 kDa) was purchased from Yuhuan Biochemistry Co., Ltd. (Yuhuan, China). PEI (1200 Da, 25 kDa) was purchased from Aldrich Chemical Co., Ltd. (Milwaukee, WI). 1, 1'-Carbonyldiimidazole (CDI) was purchased from Pierce (Rockford, IL). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and trypsin were purchased from Gibco BRL (Gaithersburg, MD). All other chemicals were of analytical grade. A pGL3 plasmid coding for luciferase was kindly provided by the Institute of Infectious Diseases, Zhejiang University. A TGF- β 1-expressing plasmid was purchased from OriGene Co. (Beijing, China). A CXCR4-expressing plasmid was kindly provided by the Department of Immunology, Juntendo University. A Luciferase Assay Kit and Bicinchoninic Acid (BCA) Protein Assay Kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

Animals

Three-week-old male Sprague-Dawley rats (50–60 g) were supplied by the Experimental Animal Center, Zhejiang University, China. All animals were kept in a 12-h light/dark cycle at a temperature of $25 \pm 1^\circ\text{C}$ with free access to food and water. Zebrafish embryos were collected at 6 hours post fertilization (hpf) from the zebrafish aquarium in the Department of Pharmacy, Zhejiang University, and staged according to standard procedures [14]. All experimental procedures were carried out according to the guidelines of Zhejiang University for the welfare of experimental animals.

Cell lines

A549 (human lung carcinoma cell line) cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM (containing 10% FBS) at 37°C and a humidified atmosphere containing 5% CO_2 .

Isolation and culture of MSCs

MSCs were isolated from rat bone marrow cells obtained from the hind femurs of 3-week-old male Sprague-Dawley rats. The cell suspension was placed into a 100-mm dish and cultured at 37°C in a humidified atmosphere containing 5% CO_2 . Second to sixth passage cells at sub-confluence were used for further study.

Synthesis and characterization of CP

CP was constructed as described previously with minor modifications [11]. Briefly, 1 g chitosan powder was dissolved in 50 mL of 0.5% acetic acid (pH 7.0), stirring overnight. CDI was added at a molar ratio of CDI:amine of chitosan = 2:1. PEI (1200 Da) was added dropwise at a molar ratio of PEI:amine of 3:1. Finally, the mixture was dialyzed for 3 days and freeze-dried. The composition of CP was evaluated by ^1H nuclear magnetic resonance (^1H NMR) spectrometry (Avance™ 600; Bruker, Billerica, MA) and elemental analysis.

Determination of buffering capacity

The buffering ability of CP at the drop from pH 10.0 to pH 2.0 was determined, according to the method described by Wang et al. [15]. PEI (25 kDa) and CP were prepared with the pH raised to 10.0 by using 1 M NaOH and were titrated with 0.1 N HCl. The volumes of HCl used were recorded, and pH was measured with a pH meter.

Characterization of CP/DNA complexes

CP/DNA was prepared by combining the CP solution with DNA and incubating at room temperature for 20 min with gentle vortexing. The morphology of CP/DNA complexes was observed under transmission electron microscopy (TEM; JEM-1200EX; JEOL, Tokyo, Japan). Particle sizes, zeta potential, and polydispersity index (PDI) were measured using a laser particle analyzer (Zetasizer 3000HS; Malvern Panalytical, Malvern, UK) with different N/P mass ratios (for the calculation of N/P ratios, 330 Da was used as an average mass per charge for DNA). The effect of CP on DNA condensation (N/P mass ratios from 0.1 to 4) was investigated by electrophoresis on a 1% agarose gel in Tris-acetate buffer at 120 V for 40 min, and viewed on an ultraviolet illuminator to visualize the DNA migration patterns.

Cell viability assays

Cytotoxicity of CP/DNA or PEI (25 kDa)/DNA was determined with an MTT assay. A549 cells or MSCs were seeded into 96-well plates at a density of 7.0×10^3 cells/well. After 24 h, the medium was replaced with fresh medium. The cells were treated with 100 μ L sterile samples at a range of concentrations. After 48 h, 20 μ L MTT solution (5 mg/mL) in phosphate-buffered saline was added to each well, and the plate was incubated for 4 h. The supernatant was removed and 100 μ L DMSO was added to each well. After the formazan crystals had dissolved completely, optical density at 570 nm was determined with a model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA).

Safety evaluation using a zebrafish model

For further safety evaluation *in vivo*, 20 healthy zebrafish embryos were transferred to a 24-well plate by adding 2 mL zebrafish culture medium. Different concentrations of CP/DNA or PEI (25 kDa)/DNA were added to the wells for 96 h at 28.5 °C. To evaluate the toxicity of CP/DNA and PEI (25 kDa)/DNA, the hatching rate of zebrafish embryos at 96 hpf was observed under a stereo microscope (Nanjing Jiangnan Novel Optics Co. Ltd., Nanjing, China).

Effect of CP/DNA complexes on the characteristics of MSCs

The effect of CP/DNA complexes on the characteristics of MSCs was evaluated using surface markers before and after transfection. Surface antigen markers of MSCs were identified using flow cytometry (FC500MCL; Beckman Coulter, Munich, Germany). Cells were detached using trypsin/EDTA, and incubated with the isotype control and specific antibodies, namely, anti-CD34-PerCP (Santa Cruz Biotechnology, Dallas, TX), anti-CD45-PE (BioLegend, San Diego, CA), and anti-CD90-FITC (BioLegend) for 30 min at 4 °C.

Transfection efficiency of the vectors

The pGL3 vector (encoding luciferase) was used to investigate the transfection efficiency of PEI (25 kDa), chitosan (100 kDa)-PEI (1200 Da), and CP with different N/P mass ratios in A549 cells and MSCs. Luciferase assay was carried out according to the manufacturer's instructions (Promega, Madison, WI). Light units (LU) due to luciferase activity were measured using a chemiluminometer (Autolumat LB953; EG&G Berthold, Bad Wildbad, Germany). The results are expressed as relative LU (RLU)/mg cell protein as determined by the BCA protein assay.

Effect of serum on the transfection of CP

To investigate the effect of serum on the transfection of CP/pGL3, A549 cells or MSCs were seeded on 24-well plates (37 °C; 5% CO₂; 4 h). The medium was replaced with fresh medium with or without 10% FBS containing 40 μ L CP/pGL3 or PEI (25 kDa)/pGL3. After 24 h, the samples were removed and the cells were incubated in complete medium. Results are expressed as RLU/mg cell protein as determined by the BCA protein assay.

Intracellular transport of CP/DNA

The pGL3 vector was labeled by a Label IT Tracker Fluorescein Labeling Kit (Mirus, Madison, WI) according to the manufacturer's instructions. MSCs (approximately 1.0×10^4 cells) were cultured in 15-mm glass bottom cell culture dishes. After 24 h, CP/fluorescein-DNA was added to the cells with Opti-MEM. Lysosomes in the transfected cells were labeled by LysoTracker Red (Beyotime). Before being fixed with

paraformaldehyde and stained with 4'-6-diamidino-2-phenylindole, MSCs were treated with CP/DNA for 0.5, 1, 2, 4, and 6 h. The intracellular location of DNA was observed under a laser scanning confocal microscope (IX81-FV1000; Olympus, Tokyo, Japan).

Endocytosis-dependency of cellular uptake

The effect of endocytosis on transfection efficiency was evaluated in A549 cells pre-treated with different inhibitors, as follows: 1) to inhibit the clathrin-mediated endocytosis pathway, 5 µg/mL chlorpromazine (CPZ) was used for 0.5 h [16, 17]; 2) to inhibit caveolae-mediated endocytosis, 10 mM methyl-β-cyclodextrin (MβCD) was used for 0.5 h; and 3) to inhibit macropinocytosis in the culture medium pathway, 20 mM amiloride was used for 15 min [18, 19]. The cells were incubated at 37 °C with CP/pGL3 for 4 h with these inhibitors. Subsequently, the medium was replaced with DMEM containing 10% FBS, 1% L-glutamine, streptomycin (50 U/mL), and penicillin (50 U/mL). The results are expressed as RLU/mg cell protein as determined by the BCA protein assay.

ALP activity assay and calcium node staining

MSCs were seeded on 24-well microplates at a density of 5.0×10^4 cells/well. After 24 h, the medium was replaced with DMEM containing 40 µL CP or CP/TGF-β1 for 4 h. After 14 days, MSCs and MSCs treated with CP or CP/TGF-β1 were lysed and centrifuged at $12,000 \times g$ for 10 min. The supernatant was removed to measure ALP activity according to the manufacturer's instructions. MSCs and MSCs treated with CP or CP/TGF-β1 were stained with 2% alizarin red solution for calcium node staining.

CXCR4 expression

To study the transfection efficiency of CP, we transferred the polymer/CXCR4 linked with enhanced green fluorescent protein (EGFP) into MSCs. MSCs were seeded on 24-well plates at a density of 1.0×10^5 cells/well. After 24 h, each well received 1 µg CXCR4 plasmid in the absence of CP. CP/CXCR4 was diluted in 500 µL Opti-MEM medium for 4 h at 37 °C. The Opti-MEM medium was replaced with DMEM containing 10% FBS, and cultured for 24 h. Green fluorescence was observed under a fluorescence microscope.

In vitro migration of MSCs

The migratory ability of MSCs and MSCs treated with CXCR4 was determined using Transwell plates (Costar, Corning, CA) that were 6.5 mm in diameter with 8-µm pore membranes. The cells (4.0×10^5 cells/mL) were added to the upper chamber. DMEM with 10% FBS or B16F10 cells was added to the lower well of the Transwell plate and cultured at 37 °C overnight. Then, the MSCs on the upper side of the membrane were wiped off, and the migrated MSCs were stained using crystal violet.

Statistical analysis

All experiments were repeated three times, and the results are expressed as the mean and standard deviation. Statistical analyses were performed using non-parametric test. A p value < 0.05 was considered statistically significant.

Results

Synthesis and characterization of CP

The composition of CP was estimated by ^1H NMR (Fig. 1A) and elemental analysis (Fig. 1B). According to ^1H NMR, the proton peak area of CP at $\delta = 3.0$ to 2.5 ppm was largely increased, which indicated that PEI (1200 Da) had been linked with the chitosan (50 kDa) chain. Elemental analysis demonstrated that the nitrogen content of CP was increased compared to chitosan. Therefore, CP was considered to be successfully synthesized. The buffering capacity of the vector should not be negligible for molecules escaping from the endosomes of cells [20]. Fig. 1C shows that CP had a similar buffering capacity as PEI (25 kDa). This shows that CP was suitable for gene transfection.

Characterization of CP/DNA complexes

TEM demonstrated the regular spherical morphology of CP (Fig. 1D). The particle size was 215 nm when the N/P mass ratio was 1 (Fig. 1E). However, at a mass ratio > 2, the complexes were approximately 100 nm in size. When the N/P mass ratio was 1, the zeta potential was negative, as the complex could not form completely. When the N/P mass ratio was increased, the zeta potential of the copolymer increased rapidly to positive values, which is consistent with previous reports [21]. Thus, at a high N/P mass ratio, net electrostatic repulsive forces could prevent aggregation among the complexes. Fig. 1E shows the unsuitable PDI of CP/DNA as the N/P mass ratio increased, revealing that the appropriate N/P mass ratio should

be in the range of 2.5–5. When the N/P mass ratio was 2–4, DNA migration was retarded completely (Fig. 1F), indicating that DNA was bound with CP tightly and completely, so little DNA escaped in this N/P mass ratio range. According to previous studies, CP/DNA has low cytotoxicity and high transfection efficiency in MSCs at an N/P mass ratio of 3 [22]. Therefore, CP/DNA could form the most appropriate positive potential nanoparticles at this ratio (N/P = 3), which was used in the following safety and flow cytometry analyses.

Safety evaluation of non-viral vectors

Fig. 2A–2D shows the cytotoxicity of the copolymer in A549 cells and MSCs. The cytotoxicity of the CP was apparently lower than that of PEI (25 kDa) in A549 cells and MSCs (Fig. 2A and 2C). A similar result was obtained in the comparison of PEI (25 kDa)/DNA and CP/DNA (Fig. 2B and 2D). Furthermore, safety was evaluated *in vivo* by using a zebrafish embryo model. The protocol for this experiment is described in Fig. 2E. Zebrafish embryos were exposed to CP/DNA or PEI (25 kDa)/DNA for 96 h. The results showed that embryo hatching was inhibited in a dose-dependent manner by CP/DNA or PEI (25 kDa)/DNA. The hatching rate of the CP/DNA group at 96 hpf was apparently increased in comparison with

Fig. 1. (A) ^1H NMR spectra of PEI (1200 Da) and CP. (B) Elemental analysis of CP, chitosan (50 kDa), and PEI (1200 Da). (C) The buffering capacities of CP and PEI (25 kDa). (D) TEM of CP (bar = 500 nm). (E) Particle sizes and PDI of CP/DNA complexes at different N/P mass ratios. (F) Agarose gel retardation assay of CP/DNA complexes at different N/P mass ratios.

Fig. 2. *In vitro* cytotoxicity of the copolymer (A) and copolymer/DNA (B) analyzed by the MTT assay in A549 cells at 48 h. *In vitro* cytotoxicity of the copolymer (C) and copolymer/DNA (D) analyzed by the MTT assay in MSCs at 48 h. (E) The protocol for the evaluation of safety using a zebrafish model. (F) The hatching rate of zebrafish embryos exposed to CP/DNA or PEI (25 kDa)/DNA at 96 hpf. Notes: *P<0.05: PEI (25 kDa) group vs. CP group or PEI (25 kDa)/DNA group vs. CP/DNA group.

Fig. 3. MSC surface antigen markers identified with flow cytometric analysis before (A) and after (B) CP/DNA transfection.

the PEI (25 kDa)/DNA group (Fig. 2F), especially at the higher concentrations of 10 and 20 $\mu\text{g}/\text{mL}$, which provides a reference for the safety evaluation of CP.

Effect of CP/DNA complexes on the characteristics of MSCs

The MSCs expressed the surface antigen CD90, but not CD34 or CD45, both before and after transfection with CP/DNA (Fig. 3A and 3B). The level of purity obtained was sufficient because greater than 95% of MSCs were positive for this marker. Therefore, the expression of MSC surface markers was not significantly influenced after transfection with CP/DNA.

Fig. 4. Transfection efficiency of CP/pGL3 with the N/P mass ratio varying from 2.5:1 to 160:1 in A549 cells (C) and MSCs (D), while PEI (25 kDa)/pGL3 was used as a positive control. (E) The transfection efficiency of CP/pGL3 was investigated and compared with chitosan (100 kDa)-PEI (1200 Da)/pGL3 with the N/P mass ratio varying from 2.5:1 to 40:1 in A549 cells. (F) The transfection efficiency of CP/pGL3 was investigated and compared with chitosan (100 kDa)-PEI (1200 Da)/pGL3 with an N/P mass ratio of 40 in MSCs. The effect of serum on the transfection of CP/pGL3 in A549 cells (G) and MSCs (H) compared with PEI (25 kDa)/pGL3. Notes: * $P < 0.05$: PEI (25 kDa)/DNA group vs. CP/DNA group with an N/P mass ratio of 40 in A549 cells or chitosan (100 kDa)-PEI (1200 Da)/DNA group vs. CP/DNA group with the N/P mass ratio varying from 2.5:1 to 40:1 in A549 cells; ** $P < 0.01$: PEI (25 kDa)/DNA or chitosan (100 kDa)-PEI (1200 Da)/DNA group vs. CP/DNA group with an N/P mass ratio of 40 in MSCs.

Transfection efficiency of the vectors

In order to search for the optimum transfection efficiency of CP/pGL3 by adjusting the N/P mass ratio from 2.5:1 to 160:1, we transferred the complex into A549 cells and MSCs, while PEI (25 kDa) was used as control. When the N/P mass ratio reached 5, the transfection efficiency of the complex was similar to that of PEI (25 kDa) in both A549 cells and MSCs (Fig. 4A and 4B). When it reached 40, transfection efficiency was enhanced significantly in comparison with PEI (25 kDa). A better transfection efficiency of CP was observed in A549 cells, with the N/P mass ratio varying from 2.5:1 to 40:1 (Fig. 4C). When the N/P mass ratio

reached 40, the transfection efficiency of CP was higher than that of chitosan (100 kDa)-PEI (1200 Da) in MSCs (Fig. 4D). Thus, this N/P mass ratio was applied to the luciferase assay, the further study of the intracellular transport of CP, and the evaluation of gene delivery efficiency based on CP/TGF- β 1 and CP/CXCR4.

Effect of serum on the transfection of CP

CP induced a slight decrease in pGL3 expression in A549 cells and MSCs, while there was a significant decrease in pGL3 expression in all culture models transfected by PEI (25 kDa) (Fig. 4E and 4F). These results demonstrated that in the presence of serum, the intracellular uptake of PEI (25 kDa) could be inhibited, but such interference was reduced by CP in A549 cells and MSCs.

Intracellular transport of CP/DNA complexes

The intracellular process that CP/DNA undergoes in MSCs was observed by laser confocal fluorescence microscopy. pGL3 was labeled with fluorescein, and lysosomes were labeled with lysosome tracker probes, which can label acidic organelles in live cells at nanomolar concentrations. Recently, LysoTracker has been applied widely in intracellular mechanistic studies [23-25]. Fig. 5A demonstrates the time-dependent changes in CP/DNA complex internalization. After incubation for 1 h, the fluorescein-DNA (green substance) was entrapped in lysosomes (red vesicles) and began to cluster around the nucleus (blue), while the majority of DNA successfully clustered near the nucleus after incubation for 4 h, which demonstrated that CP/DNA was internalized rapidly into MSCs. In contrast, PEI (25 kDa) escapes from endosomes and enters the nucleus more quickly, whereas the DNA carried by chitosan barely enters the nucleus [26, 27]. Therefore, the time required and ability to escape from endosomes and enter the nucleus are very important for both the cytotoxicity and transfection efficiency of vectors. CP escaped from endosomes at a proper combination rate, which enhanced its transfection efficiency, while cytotoxicity was not increased.

Internalization mechanisms of CP

To investigate further the internalization mechanisms of the vector, CP/pGL3 endocytosis in A549 cells was analyzed in the presence of endocytosis inhibitors. In Fig. 5B, DNA internalization was inhibited by M β CD (a lipid-raft inhibitor), but not by CPZ (a clathrin-mediated endocytosis inhibitor) or amiloride (a macropinocytosis-mediated endocytosis inhibitor). Therefore, CP/DNA internalization was only inhibited by M β CD, which selectively extracts cholesterol to organize sphingolipid rafts and has a very close relationship with caveolae-mediated and macropinocytosis-mediated endocytosis [28]. The internalization

Fig. 5. (A) Intracellular transport of CP/pGL3 on MSCs at different time points (0.5, 1, 2, 4, and 6 h) measured by laser confocal microscopy. Blue: nuclei; green: polyplexes; red: lysosomes. Scale bar = 20 μ m. (B) Analysis of the internalization mechanisms of CP/pGL3 in A549 cells. Notes: *P<0.05: M β CD group vs. control or CPZ or amiloride group.

of CP/DNA was inhibited by M β CD, but not completely by amiloride, which demonstrated that CP/DNA entered the cells through caveolae-mediated endocytosis. The clathrin-mediated endocytosis inhibitor CPZ mainly affects receptor-mediated endocytosis [29]. These results indicated that CP could help DNA enter A549 cells without utilizing receptors.

ALP activity assay and calcium node staining

Alizarin red staining demonstrated that the cells formed aggregates or nodules (Fig. 6A). Meanwhile, the osteogenic differentiation of MSCs transfected with CP/TGF- β 1 was evaluated by ALP activity. ALP is an early mineralization-related protein marker for osteogenesis in MSCs. After 14 days of induction, ALP activity was significantly increased by transfection with CP/TGF- β 1 compared to that in naïve MSCs (Fig. 6B). These findings indicated that MSCs transfected with CP/TGF- β 1 successfully differentiated into osteoblasts.

CXCR4 expression

CXCR4 linked with EGFP was delivered to MSCs to assess the transfection efficiency of CP. After 24 h incubation, green fluorescence intensity was clearly increased in MSCs transfected with CP/CXCR4 compared to MSCs (Fig. 7A and 7B), indicating that CP transfected CXCR4 into MSCs efficiently.

In vitro migration of MSCs

CXCR4 overexpression was applied to increase the homing ability of systemically delivered MSCs toward 10% FBS or B16F10 cells. The *in vitro* migration of MSCs-CXCR4 and MSCs was investigated (Fig. 7C), which confirmed that MSCs overexpressing CXCR4 have better tropism than naïve MSCs toward 10% FBS or B16F10 cells. Moreover, it was demonstrated that CXCR4 could be transfected into MSCs efficiently by CP.

Fig. 6. Effects of CP/TGF- β 1 on osteogenic differentiation of MSCs. (A) Calcium node staining. Scale bar = 100 μ m. (B) ALP level of MSCs cultured with CP/TGF- β 1 for 14 days. Notes: *P<0.05: CP/TGF- β 1 group vs. blank or CP group.

Discussion

Polymer aggregation on cell surfaces potentially leads to the cytotoxicity of PEI, which would impair important membrane functions. Besides, the high positive charge of the polymer can damage the cell membrane. The cationic polymers may disturb critical intracellular processes. Particularly, the primary amine could disrupt the function of protein kinase C by disturbing its kinase activity [30, 31]. Furthermore, high molecular weight PEI is significantly more toxic than low molecular weight PEI [32, 33]. Therefore, we chose low molecular weight PEI (1200 Da) and degradable chitosan to synthesize the copolymers. The zebrafish embryo model was applied to investigate whether CP influenced embryonic development. The use of zebrafish has grown in popularity as an *in vivo* model for the screening of nanobiomaterials [34]. The value of this model relies on its biological similarity with more complex organisms, the fact that its genome shares more than 70% of genes with humans, and its high fecundity [35-37]. Nowadays, using zebrafish embryos to test the toxicity of nanoparticles has been highlighted in several studies [38-40]. Our evaluation of safety *in vitro* and *in vivo* laid the foundation for the use of CP/DNA complexes for gene delivery to MSCs.

Under the interference of serum, the transfection efficiency of most cationic polymers is decreased, because of the presence of negatively charged serum proteins that combine competitively with polycations to decrease the combination of cationic polymers with DNA [41]. Although PEI (25 kDa) could be the most effective non-viral vector because of its high pH buffering capacity [9], its high cytotoxicity and non-degradable properties have hampered its application. Furthermore, its high positive charge combines with negatively charged serum proteins and decreases transfection efficiency. When considering the advantages of PEI and chitosan, both have a very low molecular weight, lack charge, and have low cytotoxicity [12, 32, 33], CP has similar buffering capacity as PEI (25 kDa) (Fig. 1C), while it has a lower positive charge. These factors could reduce the interference of serum on the transfection ability of CP.

MSCs are multipotent cells that can be induced to differentiate along many tissue-specific pathways, including bone (osteoblasts), fat cells (adipocytes), and cartilage (chondrocytes) [42]. However, before being administered to a subject, MSCs must be expanded and genetically modified *in vitro*, because only a few MSCs are found in bone marrow and would not provide a powerful therapeutic effect [3]. Therefore, the application of gene therapy largely depends on the efficacy of gene transfection into MSCs. To evaluate the efficiency of gene delivery using CP and the feasibility of obtaining gene-engineered MSCs, TGF- β 1- and CXCR4-expressing plasmids were successfully delivered into MSCs, confirming their ability to induce osteogenesis and change the migratory ability of MSCs, respectively. TGF- β 1 is a growth factor that plays an important role in the osteogenic differentiation of MSCs [43].

Fig. 7. CXCR4 linked with EGFP was used to examine CXCR4 expression in MSCs before (A) and after (B) CP/CXCR4 transfection. After 24 h incubation, green fluorescence was observed under a fluorescence microscope. Scale bar = 200 μ m. (C) The migratory ability of MSCs was determined using Transwell plates before and after being transfected with CP/CXCR4. Scale bar = 100 μ m.

MSCs transfected with CP/TGF- β 1 successfully differentiated into osteoblasts (Fig. 6). Furthermore, CXCR4 overexpression was used to enhance the homing ability of MSCs. CXCR4 and its ligand, SDF-1, have been studied widely in MSC migration because they are expressed in a large number of cells and tissues [44]. Many studies have demonstrated that the SDF-1/CXCR4 axis plays an important role in stem cells [45, 46]. Besides, it has been reported that CXCR4 modification does not influence the proliferation or phenotype of MSCs [47]. Similar to naïve MSCs, MSCs transfected with CXCR4 have a typical fibroblast-like structure, and their viability and proliferation do not change after being cultured for 6 days [48]. Above all, the successful transfection of two plasmids confirmed the potential of CP for gene therapy based on MSCs.

Conclusion

Chitosan (50 kDa)-PEI (1200 Da) (CP) was synthesized, and its structure, nitrogen content, and buffering capacity were characterized. CP/DNA showed much lower toxicity than PEI/DNA in both A549 and MSC culture models as well as in a zebrafish embryo model, and the CP/DNA complex did not change the properties of MSCs. CP/pGL3 exhibited a higher transfection efficiency than PEI/pGL3 in both A549 cells and MSCs, while the interference of serum was reduced significantly. Investigation of its intracellular transport demonstrated that CP/pGL3 was internalized rapidly into MSCs. Internalization mechanism studies showed that CP/pGL3 complexes entered the cells through caveolae-mediated endocytosis. Moreover, MSCs transfected with CP/TGF- β 1 successfully differentiated into osteoblasts, as demonstrated by both ALP activity and mineralization. Furthermore, the *in vitro* migration of MSCs expressing CXCR4 demonstrated better tropism than naïve MSCs. These results indicated that CP could be used to import genes into MSCs and could potentially be used in gene therapy based on stem cells.

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Disclosure Statement

The authors declare no conflict of interests.

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