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ORIGINAL ARTICLE

Using cationic polyurethane-short branch PEI as microRNA-driven nano-delivery system for stem cell differentiation

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Abstract

Background: Non-viral gene delivery, such as using biodegradable polyurethane short-branch polyethylenimine (PU-PEI), has been considered a potentially safer gene delivery system in comparison to conventional virus systems.

Methods: The polycationization of DNA complexes protects DNA from nuclease degradation, and these DNA complexes are nanoscale in size to enter the cell through endocytosis.

Results: Due to the net positive surface charge of the cell, these polyplexes efficiently bind to the cell through electrostatic interactions with negatively charged membrane components. Cationic PU-PEI has been shown to be non-cytotoxic and has a high transfection efficiency, making it a practical gene delivery material in diseases.

Conclusion: We developed a PU-PEI nanomedicine-based platform to efficiently deliver microRNA in promoting differentiation capacity of stem cells, especially on induced pluripotent stem cells.

Keywords: Induced pluripotent stem cells; Non-viral gene delivery; Polyurethane short-branch polyethylenimine

1. INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by inhibiting mRNA translation or enhancing mRNA degradation. There are several miRNAs that have been reported to be involved in cardiovascular development. For example, miR-1 and miR-133, which are derived from a common precursor transcript, regulate the differentiation and proliferation of cardiomyocytes in mice and are involved in cardiac cell fate decisions during cardiogenesis.[1–3] Non-viral gene delivery, such as polymer-based gene delivery system, has presented the characteristics of biocompatible and biodegradable abilities *in vitro* and *in vivo*. Polyurethane short-branch polyethylenimine (PU-PEI) has been considered to be potentially safer delivery system in comparison to conventional virus systems.[4] The polycations/DNA complexes can protect DNA from nuclease degradation, and are nanoscale in size and small enough to enter the cell through endocytosis. In addition, cationic polymers can provide a pH-buffering ability and behave as a "proton sponge" which assists the escape of the complexes from lysosome and improves the transfection efficiency.[5] Cationic PU-PEI has been shown to be non-cytotoxic and has a high transfection efficiency,[6,7] making it a promising *in vivo* gene delivery material in disease models. Herein we developed a PU-PEI nanomedicine-based platform to efficiently deliver microRNA in promoting differentiation capacity of stem cells, including induced pluripotent stem cells (iPSCs).

2. METHODS

2.1. iPSC culture and *in vitro* myocardiogenic induction

Murine iPSCs were generated from mouse embryonic fibroblasts (MEFs) derived from 13.5-day-old embryos of C57/B6 mice. The iPSCs were reprogrammed by transduction with retroviral

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vectors encoding four transcription factors (Oct-4/Sox2/Klf4/c-Myc) or three transcription factors (Oct-4/Sox2/Klf4) as previously described, with some modifications.[8] The mouse iPSCs were transfected with pCx-EGFP to constitutively express green fluorescence and were maintained and differentiated *in vitro* as described previously.[9] Every 3 to 4 days, colonies were detached with 0.2% collagenase IV (Invitrogen), dissociated into single cells using 0.025% trypsin (Sigma-Aldrich) and 0.1% chicken serum (Invitrogen) in PBS, and replated onto MEFs. For embryoid body (EB) formation, iPSCs were dissociated into a single-cell suspension using 0.25% trypsin-EDTA and were plated onto nonadherent culture dishes in DMEM with 15% FBS, 100 mM MEM nonessential amino acids, 0.55 mM 2-mercaptoethanol, and antibiotics at a density of 2×10^6 cells/100 mm plate. After 4 days in floating culture, EBs were transferred onto gelatin-coated plates and maintained in the same medium for 24 hours. EBs were then assigned for in vitro hepatocyte differentiation using a two-step procedure as previously described, with some modifications.[10]

2.2. Synthesis of polyurethane short-branch polyethylenimine

L-lysine-diisocyanate 0.145 g (a) N,N'-bis-(2-hydroxyethyl)-piperazine 0.1024 g (b) were respectively dissolved in 1 mL anhydrous DMF solvent and mixed in a three-neck reaction flask under a dry nitrogen purge, heated at 60°C and allowed to react for 12 hours using a 0.5 wt% dibutyltin dilaurate catalyst. Then an excess amount of methanol (4 mL) was slowly added into the reaction mixture until no unreacted isocyanate was detected. The polyurethane was precipitated and purified in ethyl ether and dried at 40°C under vacuum. The polymers were characterized by FT-IR and ^{1}H NMR.^{1}H-NMR (400 MHz, DMSOd_6, ppm) δ: 2.50-2.71 (-N_2(CH_2CH_2)$_2$), 2.99, 3.9 (-NCH_2CH_2O-), 3.12 (-NHCH($COOCH_3$)CH_2-), 1,21-1.81 (6H, -CH($COOCH_3$)$CH_2CH_2CH_2CH_2$-), 2.90 (-CH_2CH_2NH-), 3.67 (-$NHCOOCH_3$), 3.4 (-$COOCH_3$), 8.01 (-NHCH($COOCH_3$)CH_2-), 3.51 (-$CH_2NHCOOCH_3$). PU-sbPEI was synthesized using the aminolysis reaction of polyurethane (c) and small branch PEI (MW = 600) (sbPEI) in scheme 1. First, 0.1 g polyurethane was dissolved in 1 mL of anhydrous DMF and 0.6 g sbPEI (d) was dissolved in 0.5 mL MeOH with 1mL Et3N. Two solutions were mixed slowly and allowed to react at 45°C for at least 48 hours. The polymer was precipitated in an excess amount of anhydrous ethyl ether. Purification was performed by re-dissolving the above polymer in 3 mL MeOH and precipitating in 4 mL three times before vacuum-dried at 40°C. The polymer (PU-sbPEI) (e) was further characterized by FTIR and^{1}H NMR. PU-sbPEI:^{1}H-NMR(400 MHz, D_2O, ppm)δ:2.48(-N_2(-CH_2-CH_2)$_2$), 2.91(-NCH_2CH_2O-), 3.99(-NCH_2CH_2O-), 4.35(-NHCH(CO-)CH_2-),2.84,1.32,1.53(-$CHCH_2CH_2CH_2$-), 2.91(-CH_2CH_2NH-), 3.56(-O-CH_3), 3.8(-$CONHCH_2$-), 2.48, 3.14(PEI:-CH_2-CH_2-), 3.51(-$CH_2NHCOCH$-), 8.01(-NHCH (CONH-)CH_2-), 0.95(PEI:-NH-).

2.3. miR-181a overexpression by polyurethane short-branch polyethylenimine

The microRNA-181a expression plasmid (pMiR-181a) was amplified by PCR and cloned into the pLV vector. pMiR-181a was diluted in opti-MEM to a final concentration of 800 ng/μL. PU-PEI was diluted in opti-MEM with a final concentration of 2.4 μg/μL. The pMiR-181a plasmid and PU-PEI were mixed in a 1:1 ratio to a final concentration of 400 ng/μL of plasmid DNA and 1.2 μg/uL of PU-PEI to form DNA-PU-PEI complexes. The complexes were then incubated at room temperature for 30 minutes. The miR-181a expression levels were further confirmed by Taqman microRNA amplification systems with pre-made miR-181a-specific primers. Cells were grown to approximately 70% confluency prior to transfection. The complexes were added directly to cells and were removed 6 hours post-transfection. Forty-eight hours later, cells were harvested and subjected to further experiments. The oligonucleotide sequences were as follows: microRNA Scramble: 5′-CATTAATTGTCGGACAACTCAAT-3′; miR-181a sequence: 5′- ACCAUCGACCGUUGAUUGUACC -3′.

2.4. Statistical Analysis

The results are expressed as mean ± SD. Statistical analyses were performed using the *t*-test for comparing two groups, and one-way or two-way analysis of variance, followed by Bonferroni's test, was used to detect differences among three or more groups. The correlation between expression levels and age were analyzed by the Pearson's correlation coefficient and unpaired Student t-test. Results were considered statistically significant at $p < 0.05$. All analyses were performed using SPSS 12.0.

3. RESULTS

3.1. Recruitment of miR-181a during cardiac differentiation

Recent studies have shown that iPSCs are capable of differentiation into functional cardiomyocytes after incubation in cardiac differentiation medium. We generated mouse iPSCs by overexpressing four genes Oct4/Sox2/Klf4/c-Myc (mRe-7 iPSC clone). The mRe-7 clone stained positively for alkaline phosphate and exhibited embryonic stem cell (ESC)-like morphology (Fig. 1A). Additionally, mRe-7 cells were capable of differentiation into different lineages using specific protocols. We next evaluated the capacity of mRe-7 cells to differentiate into cardiomyocyte-like cells. The expression of cardiac-specific markers in iPSC-derived cardiomyocytes was compared with that in primary cultures of mouse fetal hepatocytes (Fig. 1B). After switching the mRe-7-derived embryoid bodies (EBs) into cardiac differentiation medium for 6 days, the expression of stemness genes (*Oct4* and *Nanog*) declined, and cardiac-specific genes, including *Gata4*, *Mlc-2a*, *Mlc-2v*, *cTnI*, *cTnt*, *Hcn4*, and *α-MHC*, were gradually upregulated (Fig. 1B). Immunofluorescence staining further revealed the expression of ANP within the cytosol and the expression of gap junction connexin 43 (Cx43) at the cell junctions in mRe-7-derived cardiomyocyte-like cells at 2 weeks after induction of differentiation (Fig. 1C). We also detected cardiac troponin expression in the myofilaments of mRe-7-derived cardiomyocyte-like cells (Fig. 1C). As the enxt step, we performed profiling of miRNA expression in the process of cardiac differentiation from iPSCs by miRNA microarray analysis. We examined and compared the miRNA expression patterns in ESCs and the mRe-7 iPSC clone, different stages of differentiation of mRe-7-derived cardiomyocyte-like cells (days 7, 14, and 21), fetal cardiomyocytes, and adult cardiomyocytes. The profiles of the differentially expressed miRNAs are displayed in Figure 1D. miRNAs upregulated or downregulated more than two-fold upon cardiac differentiation as compared to original mRe-7 iPSCs and ESCs were further assessed. Remarkably, a subset of three miRNAs was characterized by similarly high expression levels in both day 14 and day 21 mRe-7-derived cardiomyocytes and fetal/adult cardiomyocytes as compared to ESCs and iPSCs (Fig. 1D). The expression of these three miRNAs (miR-181a, miR-148a, and miR-152) was further validated by qRT-PCR. Among these miRNAs, miR-181a expression was significantly elevated along the cardiac differentiation time course, and its expression was even higher than that in adult cardiomyocytes (Fig. 1E). Taken together, our data indicated that miR-181a expression was the most highly elevated during cardiac differentiation from iPSCs, therefore, we hypothesized that miR-181a might play pivotal roles in regulating differentiation to cardiac-specific lineages.

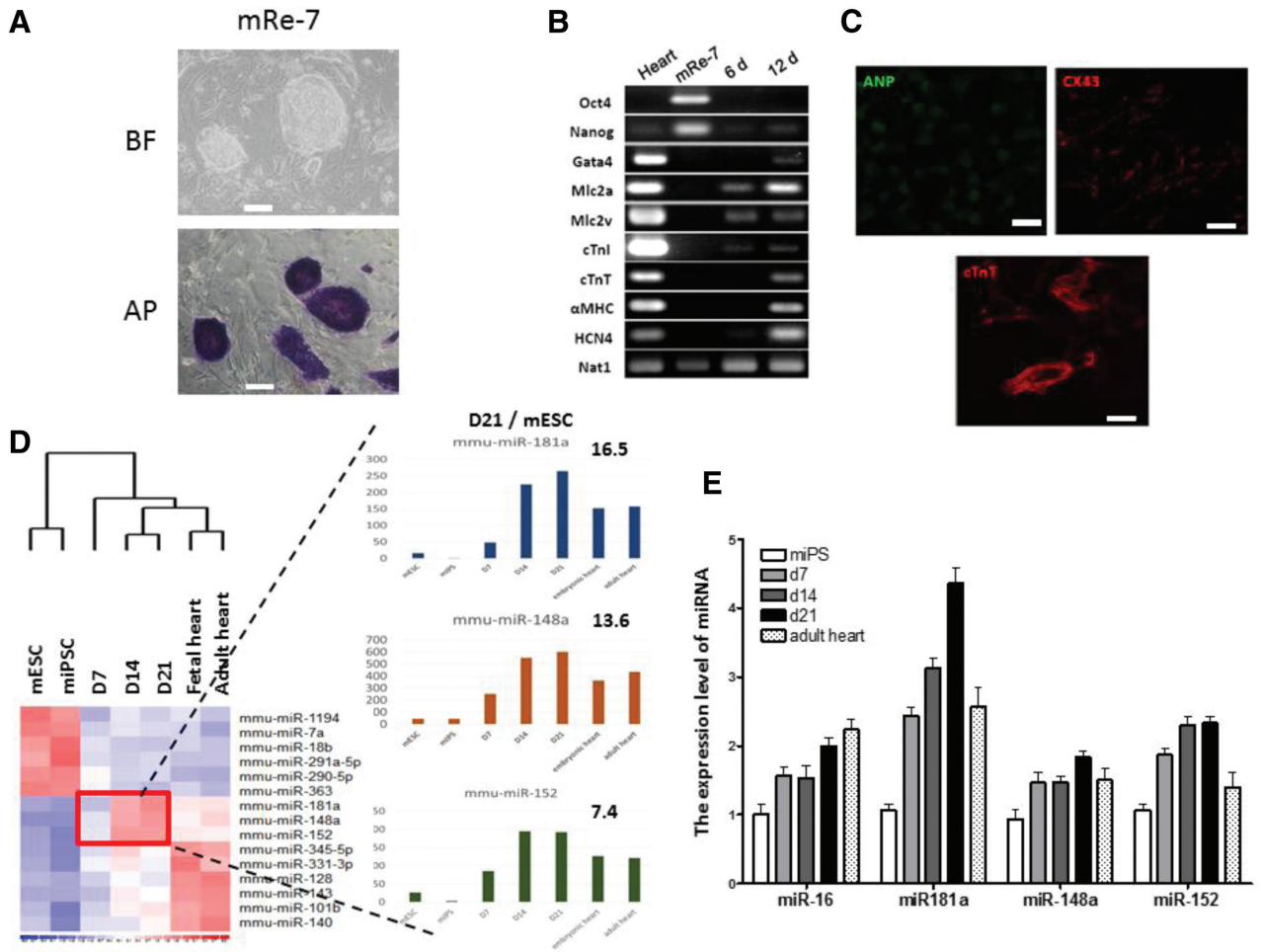

Fig. 1 Upregulation of miR-181a in iPSC-derived cardiomyocytes. A, mRe-7 iPSC clone was generated by overexpressing four genes Oct4/Sox2/Klf4/c-Myc (mRe-7 iPSC clone). Both mRe-7 clone and Yamanaka's iPSC clone (miPSC clone) were stained positive for alkaline phosphate (ALP) and exhibited an embryonic stem cell (ESC)-like morphology. Bar = 50 μm. B, Six or 12 days after cardiomyogenic induction of mRe-7 cells, the mRNAs of stemness genes (Oct-4 and Nanog) and cardiogenic-related genes (Gata4, Mlc2a, Mlc2v, cTnI, cTnt, α-MHC, and HCN4) were detected by RT-PCR. Heart tissue of 8-week-old C57/B6-mice was used as positive control. C, Immunofluorescence staining further revealed the expression of atrial natriuretic peptide (ANP) within the cytosol and the expression of gap junction connexin 43 (CX43) at the cell junctions, and the expression of cardiac troponin (cTnT) in the myofilament of mRe-7-derived cardiomyocyte-like cells (Bar = 50 μM). D, Using microRNA array, the expression profiling of microRNA in mouse embryonic stem cells (mESCs), mRe-7 cells, fetal heart, and adult heart, mRe-7-derived differentiated cells at post-differentiation 8, 14, and 21 days. was analyzed and shown in the hierarchical heat map. E, The miRNA expression levels of miR16, miR-181a, miR-148a, and miR-152 miPSCs, in iPSC-differentiated cells for 8 days, 14 days, 21 days, and adult heart were detected by quantitative real-time RT-PCR. Data shown here are the mean ± SD of three independent experiments.

3.2. Enhanced cardiac differentiation/maturation by polyurethane short-branch polyethylenimine-mediated miR-181a delivery

Recent evidence has demonstrated a crucial role of miR-181a in promoting mammalian myoblast differentiation, indicating a functional link between this miRNA and the complex process of mammalian skeletal myogenic differentiation.[11,12] PU-PEI has been reported to be non-cytotoxic and highly efficient transfection agent.[6,7] In the process of transfection, it gets degraded, releasing the cargo DNA, thus allowing its expression (Fig. 2B). We hypothesized that miR-181a may play an important role in the processes of cardiac differentiation and maturation, therefore, stable miR-181a-overexpressing mRe-7 iPSCs were generated using the PU-PEI delivery system (Fig. 2A) to tranfect cells with plasmid vectors encoding EGFP and miR-181a. An empty vector-delivery control cells were produced in parallel. Following the formation of EBs and induction of cardiac differentiation, the microscopically visualized GFP signal revealed that the PU-PEI-miR-181a complex was successfully delivered to more than 90% of the mRe-7-derived EBs (Fig. 2C, left panel). At 3 weeks post-differentiation, the miR-181a transfection efficiency was confirmed by quantitative-RT-PCR, and the miR-181a expression level in the cells increased to 3.5-fold compared to the level in the control vector-transfected cells (Fig. 2C, right panel). Concomitantly with miR-181a overexpression, several cardiac precursor markers, including transcription factors (Mef2c, Tbx5, Tbx20, and Myoc/d), myofilament proteins (α-MHC, Actn2, and Mlc2v), the ANP gene *Nppa*, the pacemaker channel *Hcn4* gene, and the calcium channel *Ryr2* gene were also significantly overexpressed (Fig. 2D). These findings suggested that upregulation of miR-181a may promote the differentiation capability/maturation of iPSCs towards cardiogenic lineages.

4. DISCUSSION

The adult heart contains cardiac stem cells that can be differentiated into mature cardiomyocytes and are therefore a candidate source for cardiac cell transplantation therapy.[13] However, there are

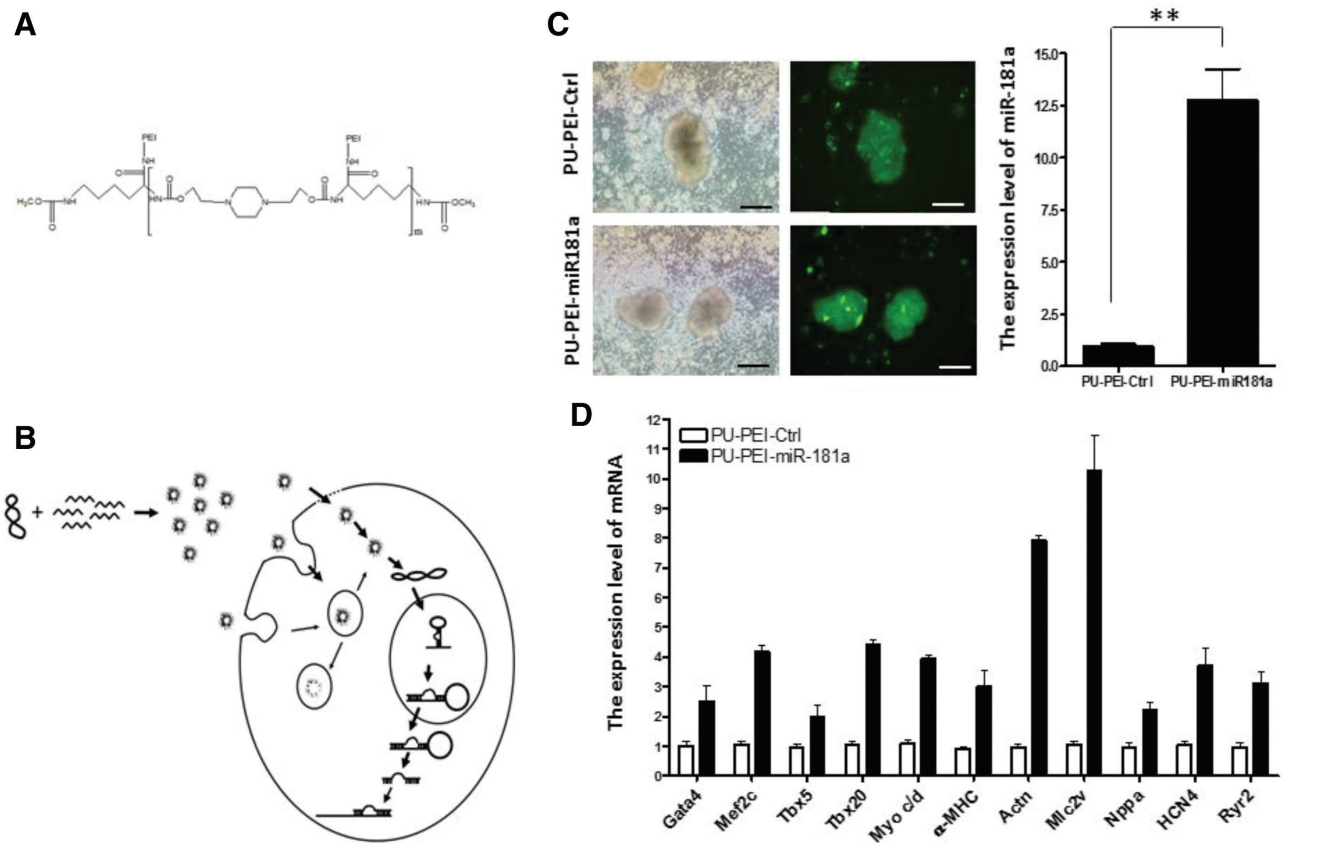

Fig. 2 Polyurethane short-branch polyethylenimine (PU-PEI) synthesis and PU-PEI-mediated delivery of miR-181a into iPSC cells. A, Synthesis of PU-PEI. B, Transfection steps of PU-PEI into iPSCs. C, Phase contrast photographs of morphology in PU-PEI control vector-transfected iPSCs (upper panel) and PU-PEI-miR-181a-transfected iPSCs (lower panel). The expression level of miR-181a before and after transfection was measured by RT-PCR method (right panel). D, The mRNA expression levels of Gata4, Mef2c, Tbx5, Tbx20, Myo c/d, α-MHC, Actin, Mlc2v, Nppa, HCN4, and Ryr2. *$p < 0.05$. Data shown here are the mean ± SD of three independent experiments.

insufficient numbers of somatic stem cells in adult organs, and the differentiation capacity of somatic stem cells is limited. Moreover, the damaged cardiomyocytes or myocardial scars, which are the target areas for myocardial regeneration, may lack the signals for cardiomyogenic differentiation to generate functional cardiomyocytes for clinical cell therapy. However, it has been estimated that over 99% of implanted cells die within 1 week after transplantation.[13] Poor viability of implanted cells may partly explain the reasons for the marginal improvement of cardiac function. Thus, cell therapies aiming to replace the lost cardiomyocytes are currently limited by the inability to deliver large numbers of cells that resist graft cell death. Strategies to enrich cells with cardiomyogenic differentiation potential and to enhance the engraftment of transplanted cells are required. Nanomedicine-based gene delivery, one of non-viral gene delivery systems, is potentially safer than viral-based delivery. In summary, we demonstrated that PU-PEI is the efficient polymer-based non-viral gene delivery systems. Therefore, our data indicated that non-viral system may be a crucial method for gene delivery in stem cell differentiation.

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