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Biomaterials

Induction of pluripotency in bone marrow mononuclear cells via polyketal nanoparticle-mediated delivery of mature microRNAs

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A R T I C L E   I N F O

A B S T R A C T

Since the successful generation of induced pluripotent stem cells (iPSC) from adult somatic cells using integrating-viral methods, various methods have been tried for iPSC generation using non-viral and non-integrating technique for clinical applications. Recently, various non-viral approaches such as protein, mRNA, microRNA, and small molecule transduction were developed to avoid genomic integration and generate stem cell-like cells from mouse and human fibroblasts. Despite these successes, there has been no successful generation of iPSC from bone marrow (BM)-derived hematopoietic cells derived using non-viral methods to date. Previous reports demonstrate the ability of polymeric micro and nanoparticles made from polyketals to deliver various molecules to macrophages. MicroRNA-loaded nanoparticles were created using the polyketal polymer PK3 (PK3-miR) and delivered to somatic cells for 6 days, resulting in the formation of colonies. Isolated cells from these colonies were assayed and substantial induction of the pluripotency markers Oct4, Sox2, and Nanog were detected. Moreover, colonies transferred to feeder layers also stained positive for pluripotency markers including SSEA-1. Here, we demonstrate successful activation of pluripotency-associated genes in mouse BM-mononuclear cells using embryonic stem cell (ESC)-specific microRNAs encapsulated in the acid sensitive polyketal PK3. These reprogramming results demonstrate that a polyketal-microRNA delivery vehicle can be used to generate various reprogrammed cells without permanent genetic manipulation in an efficient manner.

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1. Introduction

Since the successful generation of induced pluripotent stem cells (iPSC) from adult somatic cells, various methods have been developed for iPSC generation; the primary method is to deliver the ESC-specific transcription factors such as Oct4, Sox2, Klf4, and c-Myc to fibroblasts [1,2]. In comparison to the initial approach for iPSC generation, other studies have utilized alternative factors to attempt to improve the overall efficiency and the potential for clinical use of iPSCs [3–6]. The reprogramming of genes using viral transduction can cause abundant insertional mutagenesis and this type of genome modification limits its use for clinical generation of iPSCs. Recent studies have used removable PiggyBac transposons or episomal systems to obtain virus-free iPSC generation, but DNA constructs are still used, designating a possibility for genomic alternations [7,8]. As an alternate non-genetic approach, the protein or mRNA products of the reprogramming genes have been delivered directly to cells, without the viral DNA at all [9–11]. While these methods of transduction are safer, the delivery of proteins to cells is largely inefficient.

Another significant concern that needs to be resolved before human iPSCs can be utilized for clinical purposes is the generation of safe and functional cell types for therapy [12]. Fibroblasts from the embryo, the mouse tail-tip, and the human dermal fibroblasts have been the most broadly used cell sources for reprogramming, mainly due to relative ease of culture. However, some of these cells are not clinically available or need surgery to obtain them. Thus it is highly desirable to reprogram hematopoietic-lineage cells [13,14] from patients that are easily accessible and less exposed to

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environmental mutagens. There have been reports on successful generation of iPSCs from clinically compatible cells such as blood cells using genetic methods (e.g. viruses and plasmids). However, there are no known reports for non-genetic methods to create an iPSC from hematopoietic cells.

MicroRNAs (miRNAs) are well-characterized regulators of development and differentiation [15,16]. Recent studies have shown that certain miRNAs are crucial supporters of genes that regulate pluripotency and are highly expressed in embryonic stem cells (ESCs) and vital to generate iPSCs [5,17–20]. A recent report demonstrated that viral delivery of both of miR-302s and -367 can facilitate generation of iPSC using mouse and human fibroblasts [19]. Additionally, another study demonstrated that lipofection of synthetic mature miR-302s, -200c, -369-3p and -369-5p, can generate iPSCs using human fibroblasts [20]. However, miRNA-mediated iPSC generation using hematopoietic cells has not been reported yet.

Micro and nanoparticles formulated from polyketals are an exciting drug delivery vehicle as they degrade into the neutral, well tolerated compounds acetone and cyclohexanedimethanol; thereby avoiding the inflammatory problems associated with polyester-based materials. Previously we reported that the hydrolysis kinetics of the polyketal poly (cyclohexane 1,4-diylacetone dimethylene kettle) (PCADK) can be accelerated by increasing its hydrophilic/hydrophobic balance. Using this principle, we were able to generate a polyketal copolymer, termed PK3, which had a hydrolysis half-life of <2 days at pH 4.5 but is stable for several weeks at pH 7.4 [21]. Polyketals can be formulated into both nanoparticles and microparticles, allowing delivery of molecules of different sizes [22,23]. Additionally, recent reports demonstrate that polyketals can be used to deliver siRNA to macrophages, both protecting the payload from serum-induced degradation, and allowing for intracellular release within macrophages [24].

In this study, we created miRNA-loaded polyketal nanoparticles using ion-pairing and the rapidly-hydrolyzing polymer PK3. Encapsulation of miRNAs was efficient and particles contained high levels of miRNAs for transduction. The ability of miRNA-loaded PK3 to reprogram cells was tested in mouse BM-derived mononuclear cells (MNCs).

2. Materials and methods

2.1. Cell culture

Mouse BM-MNCs were isolated from an Oct4-GFP reporter mouse [25]. Terminally differentiated cells have no green fluorescence under this promoter. Cells were cultured in α-Minimal Essential Medium (MEM) or Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), which was supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and macrophage colony stimulating factor (M-CSF, 10 ng/ml). The monocyte–macrophage phenotype was determined using flow cytometry with PE-conjugated anti-mouse CD11b (BD Biosciences, San Jose, CA). Flow cytometric data were analyzed with FlowJo (TreeStar, Inc., Ashland, OR) or CFlow Plus (BD Biosciences, San Jose, CA) using appropriate controls: isotype-matched IgG and unstained controls as described previously [26].

2.2. MicroRNA synthesis

Mature microRNAs which are known to be highly expressed in ESC were chosen for reprogramming (Table 1). Synthetic mouse-specific miRNA-302A, -302B, -302C, -302D, and -367 were synthesized and modified to the 2′-O-methylated form for enhanced stability against nucleases (Integrated DNA Technologies, Inc. Coralville, IA).

2.3. PK3 synthesis

PK3 was synthesized as described previously [21]. Briefly, 1,4-cyclohexanedimethanol and 1,5-pentanediol were dissolved in distilled benzene and heated to 100 °C. Recrystallized p-toluenesulfonic acid (PTSA) was dissolved (~1 mg) in ethyl acetate and added to the benzene solution to catalyze the reaction.

Table 1
Nucleotide sequence of ESC-specific microRNAs.

miRNA	Sequence
302A	UAA GUG CUU CCA UGU UUU GGU GA
302B	UAA GUG CUU CCA UGU UUU AGU AG
302C	UAA GUG CUU CCA UGU UUC AGU GG
302D	UAA GUG CUU CCA UGU UUG AGU GU
367	GAA UUG CAC UUU AGC AAU GGU GA

The polymerization reaction was initiated by the addition of equimolar 2,2-diethoxypropane (DEP). Additional 2,2-dimethoxypropane (DMP) and benzene were subsequently added to the reaction to compensate for loss of volume in the form of ethanol/methanol and the solvent benzene that had distilled off. After 48 h, the reaction was stopped with triethylamine and isolated by precipitation in cold hexanes. The solid polymer was then filtered off, rinsed in hexanes and vacuum dried prior to storage at −20 °C. The average molecular weight of PK3 was 2316 Da as measured by gas permeation chromatography.

2.4. Generation of miRNA-loaded PK3

To generate miRNA-loaded particles, miRNAs were pooled and ion-paired to neutralize negative charges. Pooled miRNAs (1.25 mg) were incubated with an equal molar amount of N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) dissolved in methanol and incubated for 15 min at room temperature. Following incubation, RNAse free water and methylene chloride were added to extract the unreacted and ion-paired miRNA, respectively. Following centrifugation at 750 rpm for 5 min, the methylene chloride fraction was then added to PK3 and sonicated 10 times for 1 s, followed by high-speed homogenization (PowerGen, Fisher Scientific Pittsburgh PA) in 5% polyvinyl alcohol to generate particles. After evaporation of solvent for 4 h at room temperature, particles were spun down, washed several times, and freeze dried for characterization and future use. Particle size was determined by scanning electron microscopy (Zeiss Ultra60 FE-SEM, Carl Zeiss Microscopy GmbH, Munich, Germany) and loading was determined by hydrolyzing the particles in hydrochloric acid and measuring absorbance at 260/280 nm.

2.5. Release kinetics

miRNA-loaded PK3 nanoparticles (10 mg) were suspended in pH 4.5 and pH 7.4 buffer solutions (10 ml). The suspensions were kept at 37 °C under gentle shaking. At specific time points, the suspensions (100 μl) were centrifuged at 10,000 g for 2 min to remove unhydrolyzed particles. The supernatant (3 μl) was subjected to cDNA synthesis using NCode miRNA cDNA synthesis kit (Invitrogen, Carlsbad, CA), which was then conducted to EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit (Invitrogen, Carlsbad, CA) and analyzed by a FAST 7500 Real-Time PCR system (Life Technologies, Grand Island, NY) to quantify the relative concentrations of miRNA released from the PK3 nanoparticles.

2.6. Fluorescence microscopy

Images showing Oct4-GFP expression were taken with a fluorescence microscope Eclipse Ti (Nikon Instrument Inc., Melville, NY). To determine the reprogramming, immunocytochemistry (ICC) of live cells in culture was performed with a PE-conjugated antibody against mouse SSEA-1 (eBioscience, San Diego, CA) in fresh cell culture medium and cells were visualized using a fluorescence microscope.

2.7. qRT-PCR of pluripotency genes

Total RNA was isolated using RNeasy kit (Qiagen Inc. Valencia, CA), according to the manufacturer's protocol. Complementary DNA was synthesized using TaqMan® Reverse Transcription Reagents (Life Technologies, Grand Island, NY). Quantitative real-time PCR was performed using Taqman Universal PCR master mix with Applied Biosystem's 7500/Fast 7500 Real-Time System as described previously [27]. All primers used for PCR analysis are listed in Table 2.

3. Results

3.1. Generation of particles

To generate particles, we employed ion-pairing with DOTAP followed by a single oil/water emulsion as described previously (Fig. 1A). Typical efficiency of ion-pairing was >90% as measured by miRNA extracted to the aqueous phase following separation. After particles were generated, hydrolysis confirmed 8.75 μg of miRNA

Table 2
Real-time RT-PCR primers for mouse pluripotency genes.

Genes	Forward	Reverse	Probe
Oct3/4	CAATGAGAACCTTCAGGAGATATGC	TCAATGCTAGTTCGCTTTCTCTTC	ATCGGAGACCCTGGTGCAGGCC
Sox2	AAACCAAGACGCTCATGAAGAAG	CGCTCGCCATGCTGTTC	ATAAGTACACGCTTCCCGGAGGCT
Nanog	CAGCATCCATTGCAGCTATCC	CTGCCCCACATGGAAAGG	CAGGGCTATCTGGTGAACGCATCTGG

per 1 mg of particle, or roughly 40% encapsulation efficiency. Further, particles were analyzed by SEM and measurements were taken using ImageJ. As the histogram in Fig. 1B demonstrates, particles ranged from 200 to 1000 nm with an average size of 500 ± 175 nm.

3.2. Release kinetics

To determine release rate of miRNAs from PK3, particles were incubated in acidic and neutral pH levels and miR-302a levels in the supernatant were determined by quantitative real-time PCR (qRT-PCR). As the data in Fig. 2 demonstrate, release of miRNA from PK3 was accelerated at pH 5 compared to pH 7. Furthermore, release of miRNA peaked at 24 h, suggesting potential rapid release of miRNA from endosomes or phagosomes within cells.

3.3. Immunocytochemistry for pluripotency

To determine levels of PK3-miR-induced reprogramming of MNC, we used freshly isolated BM-MNCs. Mouse BM-MNCs were isolated from Oct4-GFP mouse, which can provide an easy readout of pluripotency due to the fact that differentiated somatic cells do not express this pluripotent marker. Cells were cultured according to the schematic depicted in Fig. 3. After 5 days of culture, cells were treated with M-CSF for 2 days to induce into a macrophage phenotype (Fig. 3). These cells expressed CD11b, a macrophage marker which was confirmed by flow cytometric analysis (Fig. 3A). The cultured BM-MNCs were then incubated with PK3-miR-particles in media (20 nM, 17 μg PK3/ml) every 48 h for 1 week (Fig. 3B). During treatment with PK3-miR, we also added 1 mM of valproic acid (VPA), an inhibitor of histone deacetylase (HDAC) which was reported to enhance reprogramming efficiency [28].

In the cultured BM-MNCs, Oct4-GFP signal was detected 8 days after PK3-miR nanoparticle treatment and ESC-like colonies appeared. These Oct4-GFP colonies were transferred onto gelatin-coated plates with Mitomycin-C treated mouse embryonic fibroblasts as a feeder cell (Fig. 4). After transfer to the feeder cells, the colonies were maintained in mouse ESC media which was composed of Dulbecco's modified Eagle Media (DMEM) supplemented with 15% fetal calf serum, non-essential amino acids, antibiotics (penicillin and streptomycin), glutamate and mouse leukemia inhibitory factor (LIF, 100 μg/ml). In this culture condition, mouse Oct4-GFP-positive colonies maintained Oct4 expression and grew showing typical mouse ESC-like morphology over the next 3 weeks. These ESC-like colonies were stained positive for SSEA-1, a mouse ESC pluripotency marker (Fig. 5).
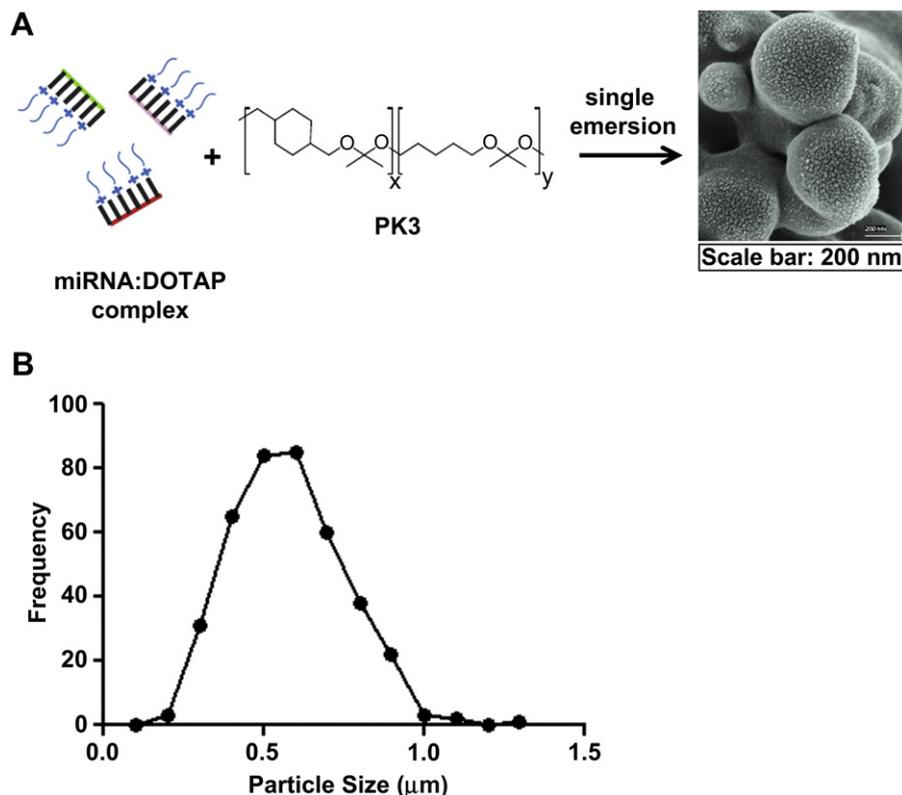
Fig. 1. Preparation and characterization of PK3-miRNA particles. A. Pool of four miRNAs was first complexed with the cationic lipid DOTAP. Next, the miRNA:DOTAP complexes were added to an organic solution containing the PK3 polyketal and via a single emulsion/solvent evaporation procedure, spherical particles of submicron size encapsulating miRNA were generated as shown by the scanning electron micrograph. B. The particle size distribution calculated from electron micrograph images shows PK3-miRNA particles ranging from 0.2 to 1 μm, averaging 500 ± 175 nm.
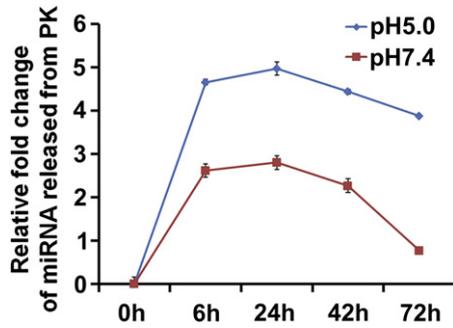
Fig. 2. Release kinetics of PK3-miR (finely ground powder) at pH 5.0, and 7.4 (37 °C). Released miRNAs were measured by qRT-PCR of miR-302a at indicated time points.

3.4. Induction of pluripotency genes

To confirm whether PK3-miR treatment induced pluripotency in the cultured cells at the mRNA level, qRT-PCR for pluripotency-related genes such as *Oct4*, *Sox2* and *Nanog* was performed. BM-MNCs were collected 8 days after PK3-miR treatment and compared with non-treated cells. The results demonstrated that there was significantly increased expression of *Oct4* ($P < 0.01$), *Sox2* ($P < 0.05$), and *Nanog* ($P < 0.01$) as compared to control cells (Fig. 6). These data quantitatively demonstrated that pluripotency genes were induced by incubation with PK3-miR for only 1 week.

We next compared the reprogramming efficiency for iPSC generation between our PK3-miR method and an established non-viral method using Lipofectamine delivery of a plasmid in which all four reprogramming genes were included: OCT4 (O), SOX2 (S), KLF4 (K), and c-MYC (M) [29]. While transfection with this OSKM-expressing episomal plasmid induced undetectable levels of pluripotency gene expression at 8 days, PK3-miR repeatedly induced generation of Oct4-GFP-positive clones during the same period and induced expression of pluripotency genes (Fig. 6). These results suggest that ESC-specific miRNA delivery can more rapidly induce pluripotency

status than transfection with OSKM-expressing plasmid. These results were consistent with previous reports in which miRNA treatment induced faster pluripotency gene expression than a viral transduction method [19].

4. Discussion

While iPSCs have generated tremendous enthusiasm for potential therapeutic applications, generation of these ESC-like cells requires genetic modification of cells. Further, while there have been many reported successes for reprogramming of fibroblasts, the use of macrophages and mononuclear cells [1,2,13], despite their better accessibility presents a challenge due to their phagocytic nature. Recent reports have demonstrated the ability of plasmid-associated nanoparticles to transform fibroblasts into iPSCs [30]; however there are no reports of nanoparticle-mediated reprogramming of hematopoietic cells.

In this report, we successfully encapsulated the ESC-specific microRNAs into the acid sensitive polyketal PK3 particle for reprogramming of cultured BM-MNCs. These particles were made by ion-pairing the miRNA with the positively-charged carrier DOTAP. Ion-pairing in this manner was quite efficient and the conjugate was easily extracted to the organic layer for use in a hydrophobic nanoparticle. While DOTAP may have some toxicity at high levels, the high amount of miRNA per mg of particle allowed uptake with very small concentrations. Moreover, should the concentration of DOTAP be toxic, other carriers exist that exhibit reduced toxicity such as spermidine and some polyethylenimine derivates [31–33]. The miRNA was rapidly released from acidic pH of 5.0, but not in neutral pH of 7.0. Previous reports using the polyketal PK3 demonstrated this similar phenomenon for siRNA, as well as the ability of the nanoparticle to protect the RNA from nucleases [24]. While it is still unknown how the compounds escape the endosome/phagosome, it is thought to be a potential proton sponge effect. Moreover, all studies to date using polyketals have demonstrated efficient cellular uptake and release as
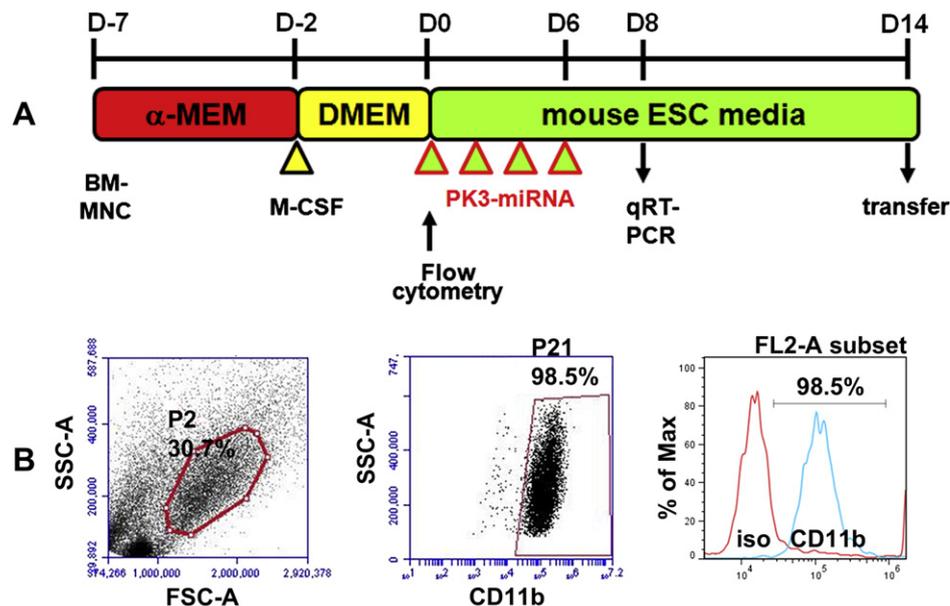

Fig. 3. The PK3-miRNA reprogramming protocol. A. Induction of ESC-like cells from Oct4-GFP BM-MNC using PK3-miR particle. Time course demonstrates the culture schedule, treatment for induction of pluripotency, and check points. Oct4-GFP mouse BM-MNC were isolated and cultured for five days followed by treatment with M-CSF and cultured two more days in DMEM. Then the cells were treated with 20 nM of miRNAs (miR-302s and -367) 4 times every other day as indicated and cultured under mouse ESC media. At day 8, Oct4-GFP-positive, ESC-like colonies emerged and were subjected to qRT-PCR for determination of pluripotency genes. At day 14, colonies transferred on to MEF feeders. B. Flow cytometry results of M-CSF treated BM-MNCs showing CD11b, suggesting a macrophage phenotype.
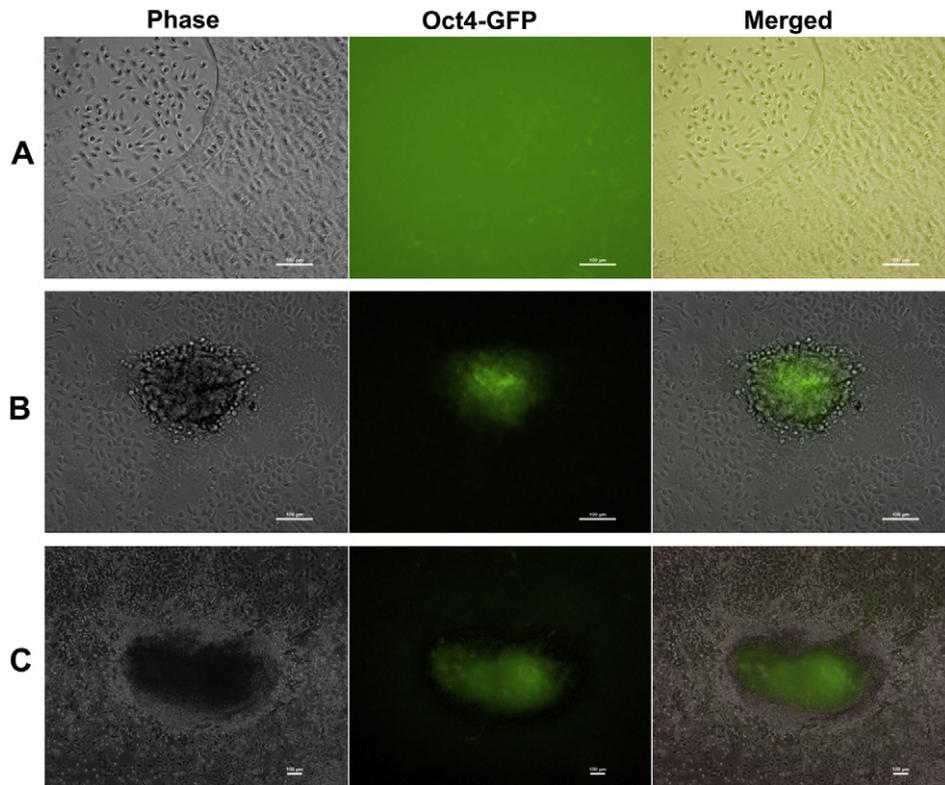
Fig. 4. Detection of Oct4-GFP signal from PK3-miR treated ESC-like mouse colonies. A. Mock-PK3 treated control at day 16. B and C. Expression of GFP signals in the PK3-miR treated cells on feeder cells, which form ESC-like colonies. Pictures taken at day 16 (B) and day 17 (C).
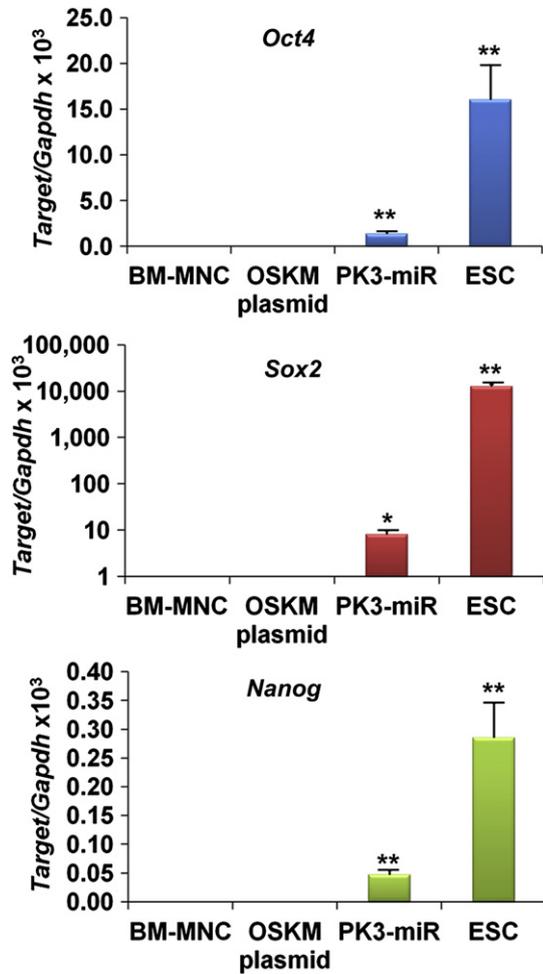
measured by gene knockdown, intracellular protein measurements, or inhibition studies.

After using M-CSF to induce the MNCs into a macrophage phenotype, cells were incubated every 48 h with low amounts of PK3-miRs. As a control, empty nanoparticles were used and no activation of Oct4 expression was seen, nor was there evidence of colony formation. In contrast, cells initially negative for Oct4 had robust expression following one week of treatment, indicating successful reprogramming. Moreover, after transfer to feeder layers, cells maintained expression of *Oct4*, as well as a pluripotency marker SSEA-1. While these particular particles were unlabeled, our preliminary experiments conducted with fluorescent protein-loaded nanoparticles demonstrated rapid uptake in to cultured BM-MNCs (data not shown). These data confirm our previous results showing macrophages rapidly internalize these particles and release contents within the cells [34,35]. Interestingly, while not tested specifically in this study, we have recently generated N-acetylglucosamine containing nanoparticles that increase uptake in nonphagocytic cells [36]. It would be of great interest to see if these modified nanoparticles could transform fibroblasts as well. While interesting, macrophages may be a more clinically useful cell for generating iPSCs due to their ease of collection, culture, and abundance [13,14].
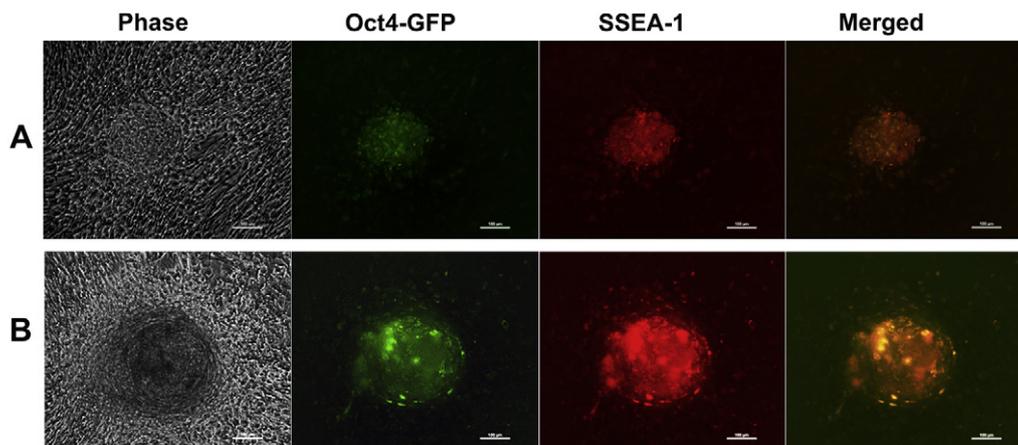
Fig. 5. Morphologies and expression of SSEA-1 of PK3-miR-induced cell colonies. Two representative types of colonies (A and B) were shown which were grown in different culture plates. A colony shown in A is at passage 0 and appeared less mature than the one shown in B which is taken at passage 1. The green fluorescence indicates Oct4-GFP expression and the red fluorescence SSEA-1 staining.

Fig. 6. mRNA expression of pluripotency-related genes in mouse BM-MNCs treated with PK3-miR particle. Uncultured BM-MNCs were used as a negative control. OSKM plasmid was used as a non-viral method control. Mouse ESC was used as a positive control. Data are Mean ± SEM ($n = 3$), $*P < 0.05$, $**P < 0.01$ v.s. BM-MNC.

sufficient to completely reprogram the cells and additional miRNA may be needed. Previously reported generation of iPSC using synthetic miRNAs in mouse and human fibroblasts employed a different set of miRNAs which includes miR-302s, -200c, -369-3p and -369-5p [19]. It could also be possible that the doses of miRNAs were insufficient to trigger the full response due to intracellular degradation. This may be overcome by potentially using locked nucleic acids and future studies will examine this possibility. Finally, the timing of delivery may also have been insufficient. As prior studies demonstrated 1 month of virus treatment to induce pluripotency, we will examine longer treatment periods as well. Interestingly, while we did not generate iPSCs, recent studies have shown the ability to differentiate partially reprogrammed cells into target cells [40,41]. Moreover, miRNAs were used to directly reprogram somatic cells (fibroblasts) to neuronal cells [42] or cardiomyocytes [43]. Thus, it could be possible that our PK3-miR formulation with these miRNAs may be useful for such partial reprogramming-differentiation approach or direct reprogramming into target cells. Finally, a recent report demonstrated that the efficiency of generation of iPSCs using blood-derived cells was increased by p53 shRNA [44] and this may also complement our approach.

5. Conclusion

In this study, we demonstrate the potential of using nanoparticles made from polyketal polymers as a virus-free delivery system of ESC-specific microRNAs to reprogram hematopoietic-lineage cells. Treatment for just 8 days with these particles induced pluripotent-gene and protein expression in cultured BM-MNCs. The use of this well tolerated, easily tunable polymer could open the door for its use both in generating pluripotent stem cells or targeted differentiated cells. This is the report of a nanoparticle-microRNA system to reprogram primary MNCs and may provide an opportunity for use of microRNA as an efficient tool for future in clinical applications in the field of regenerative medicine.

Acknowledgments

To date, there have been only few reports of successful generation of reprogrammed cells from hematopoietic-lineage cells other than hematopoietic stem cells such as CD34$^+$ cells; however these cells exist in low numbers in human and need cytokine treatment for collection [13]. There is wealth of studies outlining the difficulty in transfecting hematopoietic cells and polyketal-based nanoparticles may help overcome this. This is not to say that it is impossible to reprogram these cells, but in fact a recent report demonstrates the use of retroviruses to generate iPSCs from aged bone marrow-derived myeloid cells [37]. Although they were able to generate iPSCs, initial conditions required 1 month of retrovirus treatment (several rounds) to generate a few colonies. On the other hand, there have been a few reports of generating reprogrammed cells using nanoparticles; however fibroblasts were used in most cases [30,38]. Interestingly, one group reported the use of magnetic nanoparticles to introduce plasmids to cells non-virally [30]. Further, in another study, dendrimers were used to introduce pluripotency genes and packaging plasmids into 293 cells to create viruses for reprogramming [39]. Although the authors use nanoparticles to increase the efficiency of iPSC generation, this method still used potentially dangerous viral methods.

While the results of our study were exciting, it should be noted that we did not establish a true iPSC line. This could indicate that the selected combination of miRNAs (302 family and 367) are not

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