공학석사학위논문

Induced myogenic commitment of human chondrocytes via non-viral delivery of minicircle DNA

미니써클 유전체의 비바이러스성 전달을 통한

인간 연골세포의 근육세포로의 분화 유도

2014년 8월

서울대학교 대학원

화학생물공학부

홍지은

Abstract

Induced myogenic commitment of human chondrocytes via non-viral delivery of minicircle DNA

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Regenerative medicine covers the whole process of regenerating or replacing the human cells, tissues and organs to restore their normal function. The damaged tissues undergo wound healing process, which mostly end up with incomplete repair, and severely damaged tissues are often irreparable or difficult to be expanded. Therefore, cell therapies have widely studied to provide sufficient quantities of specifically differentiated cells. Especially, methods to change cell fate from a terminally-differentiated stage to another cell lineage or to multipotent/pluripotent state have been investigated. Though induced pluripotent stem cells (iPSCs) can be differentiated into the desired state, iPSC technology is currently time-consuming process and it has cell-type dependent reprogramming efficiency. Furthermore, clinical safety and genetic/epigenetic fidelity still remain as major concerns. In order to bypass inducing pluripotent stage, transdifferentiation/direction conversions have recently been developed. Objective of this thesis is to utilize non-viral system to deliver minicircle DNA vector (MCDNA, polycistronic vector containing reprogramming defined factors)

to induce transdifferentiation from human chondrocytes into myogenic cells along with SB-431542, an inhibitor of the activin receptor-like kinase receptors. In order to increase the efficiency of gene delivery into chondrocytes, MCDNA was delivered into the chondrocytes via electroporation followed by poly (β-amino esters) (PBAE) transfection. The transfected cells were sorted by fluorescence-activated cell sorting (FACS), cultured on a Matrigel coated plate, passed onto multi-well plate, and then treated by SB-431542 to induce the myogenic commitment. This study demonstrated that human chondrocytes can be induced to be efficiently transdifferentiated into myogenic cells through the non-viral system.

Keywords: transdifferentiation, poly (β-amino esters), minicircle DNA, SB-431542
Student Number: 2012-20986

Contents

List of Figures and Table

Chapter 1. General Introduction

1.1 Duchenne muscular dystrophy

Muscular dystrophy (MD) is a group of inherited disorders with degeneration of muscle and muscle proteins, which eventually leads to death. Muscular dystrophy can be categorized into nine major types without any effective treatment, and they vary in the strength decline patterns and disease progression speed. The three most common types of MD are Duchenne MD (DMD), myotonic dystrophy and Facioscapulohumeral muscular dystrophy (FSHD). Myotonic dystrophy type 1 and FSHD are the most common type in adults [1, 2], and Duchenne MD is the most common type in young males [3].

Duchenne MD is the first reported one and a fatal X-linked regreessive disease caused by the mutation of dystrophin gene [4], with prevalence of 1 in 3,500 males' births. DMD patients are usually diagnosed before the age of five, start to use wheelchairs by the age of twelve, and most of them don't survive till their 30s. Dystrophin in muscle cells is key component which connects cytoskeleton of a myofiber to the extracellular matrix (ECM) through cell membrane, therefore, a DMD patient without sufficient amount of dystrophin results in instability of muscle fiber membrane, losing muscle strength progressively, and eventually leading to death in young age due to respiratory or heart failure.

Duchenne MD, the most progressive and severe type, has brought an increased interest in regenerative medicine approaches using stem cell based therapies as well as gene therapies. Still there have been no available cure for DMD, current medications are only able to decrease the progression speed and replacing the function of damaged tissues.

Gene therapy approaches are currently in the developmental stage for Duchenne muscular dystrophy. Since DMD is caused by gene mutations or errors in the splicing

system, gene replacement therapies focus on the correction of the gene regions.

There are two products by Prosensa Holding (Prosensa) and GlaxoSmithKline (GSK) and by Sarepta Therapeutics Inc. (Sarepta) that encodes anti-sense oligonucleotides (AON) to target exon 51 [5].

Drisapersen developed by Prosensa and GSK and eteplirsen designed by Sarepta Therapeutics are designed to restore the mutated open reading frame (ORF) of dystrophin gene as they anneal to precursor messenger RNA and replace the splicing system in the right way. In result, these drugs generate short version of functional dystrophin. This technique is called exon-skipping strategies to cure DMD [6],and both of the drugs have progressed to Phase IIB-II clinical trials. Both drugs showed impressive results in animal models [7, 8], and in the phase 1/2A studies [9]. However, drisapersen did not show significant improvement in the patients on the ages of 5-16 years in the phase III trials, but helped the patients aged seven years or younger to walk further in a six-minutes walk test on a 96-week extension trial. Recently, GSK has announced to stop researching on drisapersen and to give the full right to the drug back to Prosensa for the further developmental studies [10].

Clinical studies with eteplirsen demonstrated its treatment efficiency on 12-years-old boys for 24 weeks treatment, resulted in increased expression of dystrophin-positive myofibers [11] and greater increases with extended treatment on 30 mg/kg or 50 mg/kg cohorts. The latest updates for both drugs were reported at conferences and its related press release [12].

Despite of their promising signals, there are few points to be concerned, such as setting of primary clinical endpoint, admitting walking ability of boys who are too young to be tested or who lost the ability already, and other ways to measure the treatment efficiencies.

Gene replacement therapies have also been researched mostly focusing on correction of the mutated sequence. There are adenoviral methods and recombinant adeno-associated virus (rAAV)-based vectors mediated methods [13]. Gene replacement therapies can be applied in much broader ways than the exon-skipping strategies, for the

latter is only applied on the specific patients with the unique region of DMD gene.

All the viral genes of adeno-associated virus(AAV) are replaced with therapeutic genes to improve the titer and stability as well as to prevent its own disadvantages [14]. Due to the length limitation of rAAV vectors, full length of DMD gene (dystrophin gene) cannot be inserted, therefore, only functional parts were recombined to express microdystrophin [15, 16] or minidystrophin [17]. The reports related to rAAV vectors demonstrated the improvement on microdystrophin production and survivability in mouse models [18] and in canine models [19]. Microdystrophin strategy utilized only one rAAV vector, however, delivery of two or three vectors were also applied to include larger domains of DMD gene to generate minidystrophin or whole DMD gene [20]. Replacement of mutated gene into whole or larger region of functional gene showed better improvement in physiological areas than that of the microdystrophin system [21], though it showed low efficiency in the full length replacement [20]. Delivery of multi rAAV vectors was called trans-splicing, for the vectors were overlapped in some domains to construct larger gene regions inside the muscle cells.

The limitation of this strategy is the immune responses in human and large animals [22], so the researcher have investigated several methods to decrease immune rejection such as usage of muscle-specific promoter which resulted in delay of the activation of immune system [22] and which suggested importance of precisely designed protocol for gene therapies [23].

While gene therapies are to prevention of disease progression, cell therapies focus on not only stopping regression but also replacing lost cells. Stem cell based therapy has been considered as a potential strategy to repair segmental necrosis and to generate muscle fiber for lost cell replacement along with gene correction *ex vivo* or *in vitro*. Satellite cell is a muscle-specific stem cell which is located in muscle tissues and which has a self-renewal ability and a potential to be differentiated into the desired state as replacement of injured cell [24-26].

Injection of myoblasts from healthy donors was attempted to induce expression of dystrophin as it had shown improved results in *mdx* mouse model [27]. However, the

immune rejection of transplanted cells occurred and the cells could not spread widely but rapidly reached dead [28-30]. Autologous transplantation also had attention in clinical tests in 2007 [31]. The muscle-derived stem cells expressing myogenic markers such as CD34 or CD133 were transplanted and resulted in improvement in muscle function (e.g.. strength and structures).

Induced pluripotent stem cells (iPSCs) have arisen as an alternative cell source due to its likeness to embryonic stem cells. Patient-specific iPSCs with correction of DMD gene can be the best cell source for autologous transplantation as they are differentiated into the desired state with promising regulatory genes such as MyoD and Pax7 [32-34].

1.2 Non-viral delivery system

Treatment of DMD has been more focused on gene and cell therapies; viral or virus-derived delivery system is mostly used in the clinical research areas, such as correction of mutated gene regions. However, due to the risk of mutagenesis of host genome despite of its higher efficiency, delivery of foreign genes via transduction has been replaced by non-viral methods, such as chemical-based reprogramming, liposomal methods, polymeric gene carrier systems and physical methods. Still, non-viral transfection needs improvement on the transfection efficacy and reduction of cell cytotoxicity with some systems.

Two well-known chemical-based transfection methods are calcium phosphate-based transfection and diethylaminoethyl (DEAE)-dextran transfection. Both can be stable and transient transfection methods, but differ in some views; the inquired amount of delivered plasmid DNA (pDNA) or RNA and effective cell types for each methods. Calcium phosphate-based transfection includes the step of coprecipitation of calcium cation and nucleic acids in phosphate buffered saline and cellular take up of the precipitants. Stimuli such as DMSO treatment or changing the buffer during the transfection steps improved the transfection efficiencies for some cell types [35].

DEAE-dextran transfection on eukaryotic cells includes positively charged chemical and polymeric portions , requires less amount of nucleic acids than chemical-based methods, and can be applied on both adherent or suspended cultured cells [36].

Delivery of exogenous genes with lipids or liposomes construct liposomal vesicles to merge into cell membranes for bilipid layer of the lipoplexes resembles the cell membrane [37]. The transfection efficiency was dependent on transfected cell types, media components such as serum and antibiotics, cell confluency and the length or structure of nucleic acids.Lipofectamine™ is a commercial transfection reagent based on liposomal gene delivery system and developed by Invitrogen™ [38].

Introduction of exogenous genes into mammalian cells with physical methods have also investigated, such as electroporation, opto-perforation, and gene gun. Opto-perforation is a laser-mediated transfection strategies which transport genes into alive cells [39]. Recently, it was reported that selecting specific cells were available for localized delivery of gene and the technology has potentials to be applied into micro- or smaller sized techniques [40, 41]. Delivery of plasmid DNAor RNA by electroporation has been reported without transgene and vector integration into the host cell genome [42]. Also, protein has been delivered into cells to specify cell fate while reprogramming or differentiation as well as to reprogram the cell fate [43, 44]. Electroporation is a physical transfection method that makes temporary pores in cell membranes to deliver exogenous genes or other substances by electrical forces. The electroporation is usually highly efficient for cultured mammalian cells. Gene gun shots biocompatible beads which are coated with exogenous gene into target region of tissues. This technique also aims specific localization as optoporation technique and applied in studies about biomimetic materials and DNA vaccination [45, 46].

Polyplex or lipoplex with genes can be immobilized on scaffolds or substrates to change cell fate or to induce cell differentiation. Cationic polymeric gene carriers are used as a transfection method to encapsulate negatively charged nucleic acids with positively charged polymers. When DNA is delivered into a cell, the cell uptakes the polyplex of cationic polymer and DNA, the endosomal escape is promoted, and DNA is

released in the cytoplasm and transported to the nucleus consequently as cationic polymer degrades. Protein delivery system has been investigated with hydrogels and scaffolds, such as protein-coated scaffold and protein-containing hydrogels [47, 48].

1.3 Transdifferentiation and reprogramming

Since cell therapy demandssufficient quantities of tissues differentiated into the desired states, replacement of damaged muscle cells in DMD patients requires enough number of cells with dystrophin expression. Although multipotent/pluripotent stem cells are normally used because of their self-renewal ability and potency, collecting stem cells from the patient or induction of pluripotency from somatic cells are major barriers for the progression speed is fast and reprogramming of cells is time-consuming.

Cellular reprogramming is defined as a process that the fully differentiated cell crosses all the way back to its pluripotency to have the potential to reach another cell fate which never happens in natural ways. In 2006, it was proven that fully differentiated cells could be induced into the pluripotent stage by some defined factors [49]. Since it covers the full process of de- and re-differentiating, it demands precise steps to analyze the pluripotency including teratoma formation and chimeric mouse generation. To save the time and labor, direct conversion and transdifferentiation have drawn attentions as an alternative.

Transdifferentiation is a method that a fully defined cell type is changed into another cell fate without necessarily inducing the pluripotent stage. Therefore, this method includes the partial dedifferentiation and induced differentiation simultaneously or serially, or direct change of cell fate without any characterized dedifferentiation stages. It has been proven that direct reprogramming is experimentally possible. With defining factors, mouse fibroblasts were directly conversed into cardiomyocytes [50] and into functional neurons [51].

Chapter 2. Preparation of minicircle DNA and Polymeric Gene Carrier

2.1 Introduction

Minicircle DNA is a circular non-viral plasmid DNA vector generated by cis-recombination from a parental plasmid mediated by ΦC31 integrase. Since the host strain of minicircle DNA, ZYCY10P3S2T, has an arabinose-inducible system and the I-Scel endonuclease, induction of MCDNA from the parental plasmid works easier and the residual parental plasmid gets removed gradually (Figure 2). MCDNA encodes the Thomson factors, which encodes Lin28, Nanog, Sox2 and Oct4, to reprogram cells and green fluorescence protein as a reporter. In 2010, human adipose stem cells were transfected with MCDNA and generated human induced pluripotent stem cells (iPSCs) [52].

Poly (beta-amino ester) is produced by esterification of acrylate monomers and amino-alcohol monomers and by end-group modification with diamine to enhance positive charge of the final products (Figure 1). It has been developed for effective non-viral nanoparticle-based gene delivery system [53] and the efficiency is comparable to the viral method in some human cell lines [54].

2.2 Methods

2.2.1 Synthesis of PBAE

1, 4-butanediol diacrylate (C, 411744, Sigma-Aldrich, MO, USA) and 3-amino-1-pentanol (32, 123048, Sigma-Aldrich, MO, USA) were reacted at a 1.2:1.0 molar ratio at 90℃ for 24 hours. Then the end group of C32-Ac polymers were modified with diamine monomers (103; 1,3-diaminopropane, D0114, TCI, Japan, 117; 1,3-pentanediamine, D2252, TCI, Japan, 122; $(PEO)_4$-bis-amine , 17774, Molecular Biosciences, CO, USA) in anhydrous tetrahydrofuran. The end-capping reactions were performed overnight at room temperature using a 1.6-fold molar excess of amine over acrylate end groups. The synthesized polymers were dissolved in anhydrous dimethyl sulfoxide at a concentration of 100 mg/mL and stored at -20℃

2.2.2 Minicircle DNA preparation

To generate MC DNA (SRM100PA-1, System Biosciences, Mountain View, CA, USA), bacteria with parental MC DNA were inoculated at 37℃ for 8 hours in LB broth (L3022, Sigma-Aldrich, Saint Louis, MO, USA) and then grown at 37℃ overnight in Terrific Broth (22711022, Invitrogen™, Carlsbad, CA, USA) supplemented with kanamycin (B5264, Sigma-Aldrich, Saint Louis, MO, USA). The following day MC induction medium (LB broth containing 4% 1N NaOH and 0.02wt% L-(+)-Arabinose (A3256, Sigma-Aldrich, Saint Louis, MO, USA) was added and the working temperature was decreased to 30℃ for 5 hours. The bacteria were harvested and MC DNA was purified with EndoFree Giga Kit (12391, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration of MC DNA was measured and its size was confirmed with restriction enzyme digestion and agarose gel electrophoresis.

2.2.3 Agarose gel electrophoresis

Various weight ratios of MCDNA to PBAEs were compared by agarose gel electrophoresis to see whether DNA was completely surrounded by PBAEs. Agarose powder (16500-500, Invitrogen™, Carlsbad, CA, USA) was added into diluted 1X TAE buffer (15558-026, Invitrogen™, Carlsbad, CA, USA) in 1% (w/v). The agarose solution was boiled in a microwave, poured into a gel casting tray with 5-mm wide comb, and was cooled. Loading dye (R0611, Thermo Scientific, Vilnius, Lithuania) and complexed MCDNA/PBAE nanoparticles were mixed in a ratio of 1 to 5, loaded in wells and run at 110 V for an hour. The gel was soaked in diluted ethidium bromide (L07482, Alfa Aesar, Ward Hill, MA, USA) solution for 15 minutes and was visualized with UV light and the photo was taken with GelDoc-It™ TS 310 machine (97-0255-01, UVP, Upland, CA, USA).

2.2.4 Dynamic light scattering analysis of MCDNA/PBAE

Particle sizes and zeta potentials were measured by dynamic light scattering detector (PSS·Nicomp 380 ZLS, Particle Sizing Systems, Port Richey, FL, USA). PBAE stock solution and MCDNA solution were separately diluted in 25mM sodium acetate buffer (pH 5) to the required concentrations to form nanoparticles at the ratio of 1:30 DNA/PBAE weight ratio in the same condition. 120 μL of polyplex solution (n=3) was mixed by vortexing, incubated for 10 minutes to self-assemble, and the particle size was measured 30 minutes after the complexation. The sample was diluted in 1 mL PBS (10010-049, Gibco, Carlsbad, CA, USA) and zeta-potential was analyzed using the Smoluchosky model for aqueous suspensions. The viscosity of solution and refractive index were assumed the same as those of pure water at 25℃.

2.2.5 Cell culture

Human chondrocytes were maintained in DMEM (11995, Gibco, Grand Island, NY, USA) with 10% FBS (US1520, Biowest, Nuaillé, France), 100 U/mL penicillin-streptomycin (15140, Gibco, Grand Island, NY, USA), 10 mM HEPES (15630, Gibco, Grand Island, NY, USA), 1% MEM NEAA (11140, Gibco, Grand Island, NY, USA), 0.05 mg/mL l-ascorbic acid (A4544, Sigma-Aldrich, Saint Louis, MO, USA) and 0.4 mM l-proline (P5607, Sigma-Aldrich, Saint Louis, MO, USA) at 37℃ with 5% CO_2.

2.2.6 FACS analysis of transfected chondrocytes

After culturing the transfected chondrocytes for 48 hours, the cells were washes with PBS and detached with Accutase (A11105, Gibco® , Carlsbad, CA, USA) for 10 min at 37℃, collected by centrifugation at 1,100 rpm for a minute, and resuspended in PBS. After filtering with 35 μm cell-strainer (352235, BD Falcon, Canaan, CT, USA), the cells were sorted using FACS Aria II. The GFP signal standard was set with untransfected chondrocytes, and the transfected cells with positive GFP signals were sorted and plated on Matrigel (354234, BD Biosciences, MA, USA) coated plate. The sorted chondrocytes were maintained in Essential 8™ medium (A1517001, Gibco, CA, USA) at 37℃ with 5% CO_2.

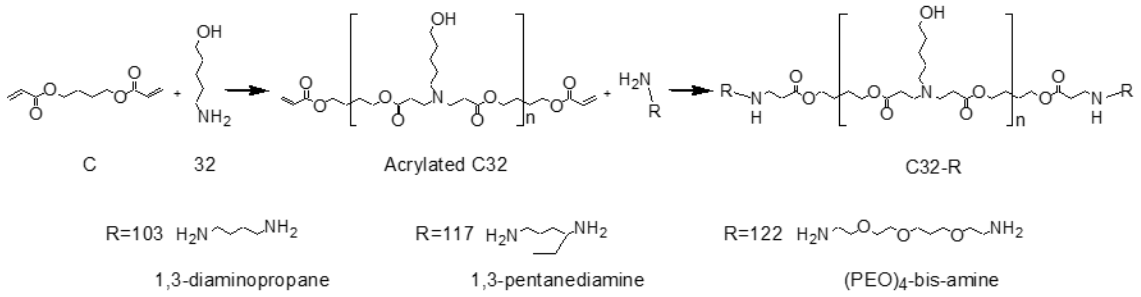

Figure 1. Synthesis of poly (β-amino esters)

1,4-butanediol diacrylate (C) and 3-amino-1-pentanol (32) were reacted to generate acrylated poly (β-amino esters), and three different types of diamines (103, 1,3-diaminopropane; 117, 1,3-pentanediamine; 122, $(PEO)_4$-bis-amine) were added to modify the end-group to increase the positive charge of polymers.

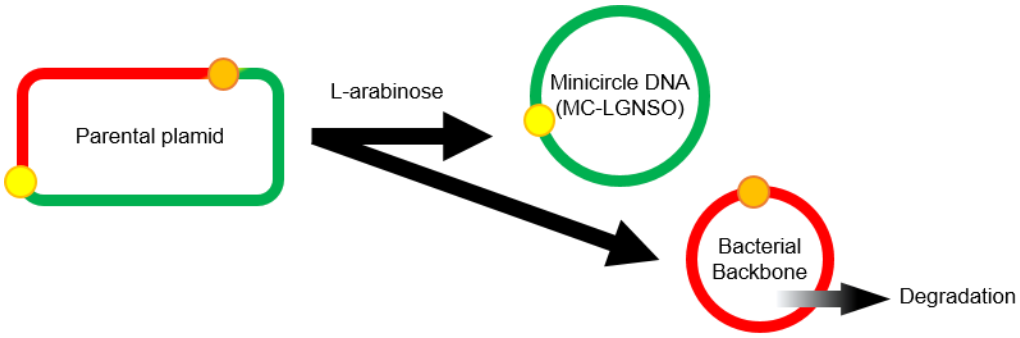

Figure 2. Minicircle DNA preparation

Parental plasmids were multiplied in the host *e. coli.*, and the minicircle DNA was induced by l-arabinose. MC-LGNSO means minicircle DNA with four defined reprogramming factors (Lin28, Nanog, Sox2, and Oct4) and green fluorescence protein (GFP). The bacterial backbone degrades after the induction.

2.3 Results

2.3.1 Preparation of MCDNA

The generation of minicircle DNA was confirmed by restriction enzyme digestion. Nsi1, which cuts the bacterial backbone of parental plasmid, was added to both parental and minicircle DNAs and the products were run on agarose gel (Figure 3). The agarose gel photo showed that parental plasmid had two different sizes that indicates two isoforms- supercoiled and loosen, and that induced minicircle DNA solution still had residual amount of parental plasmid. But the residual parental plasmid or digested parental plasmid were not detected after the digestion step.

2.3.2 Optimization of MCDNA/PBAE weight ratio

Prior to cellular transfection, optimal weight ratio of MCDNA to PBAE was investigated from 1:1 to 1:30. Complexed MCDNA/PBAE nanoparticles were subjected to agarose gel electrophoresis. Due to complexation and stable nanoparticle formation, fully complexed nanoparticles are not influenced electroshift mobility within agarose gel. At 1:1 and 1:5 ratio, the residual MCDNA was run through 1% agarose gel, and MCDNA was slightly remained at the ratio of 1:10. However, at the ratio of 1:30, MCDNA was fully complexed with three types of PBAEs (Figure 4). Therefore, further studies with PBAE were performed at the ratio of 1:30 to completely encapsulate MCDNA with PBAEs.

2.3.3 Properties of MCDNA/PBAE nanoparticles

We further investigated the complexed nanoparticle formation via dynamic light scattering analysis. In figure 5, the sizes of nanoparticles were measured around 200 ~300 nm. MCDNA/C32-122 nanoparticle showed the smallest size among three

polyplexes, and the MCDNA/C32-117 were the largest. Nanoparticles were also analyzed to see how the complexation and surface charges of them differed with PBAE types by dynamic light scattering detector. It is known that the positively charged particles can be easily taken up by cells compared to the negatively charged one [55, 56]. While MCDNA/C32-122 showed the positive surface charge (13.60 ± 0.7 mV), the surface charges of MCDNA/C32-103 and MCDNA/C32-117 were -18.27 ± 6.87 mV and -23.73 ± 3.65 mV. Therefore, we assumed that C32-103 and C32-122 polymers to have higher transfection efficiencies than C32-117 polymer.

2.3.4 Transfection efficiencies of electroporation and MCDNA/PBAE system

Transfection efficiency via electroporation and PBAE was analyzed by flow cytometry. FSC indicates forward scatter that analyze the size of a cell and SSC means side scatter that shows the complexity of a cell. In figure 6, the size and the complexity of electroporated chondrocytes was decreased. The percentage of GFP-positive cells were about 1.98% in average in multiple samples. For the percentage of transfected cells were little, multiple peaks or movement of the peak didn't appear, but long-tailed peak was shown with higher GFP signal tail (Figure 6B). The electroporated cells were also observed by fluorescence microscope. As the amount of delivered MCDNA increases, the cells changed into smaller sizes and circular-like morphologies (Figure 7). Minicircle DNA has reprogramming factors and GFP gene, so it would change cells to have a decrease of cell size and GFP-positive signals.

Transfection using MCDNA/PBAE nanoparticles was less effective than the electroporation according to the results – less than 0.05%. Though MCDNA/C32-122 nanoparticle showed the smallest particle size and positive surface charge, MCDNA/C32-103 polyplex resulted in higher efficiency as MCDNA amount increases (Figure 8).

Since electroporation efficiency was much higher than PBAE transfection, we decided to reprogram chondrocytes with serial steps of transfection, firstly electroporation, GFP-positive cell sorting, and then PBAE transfection (Figure 9).

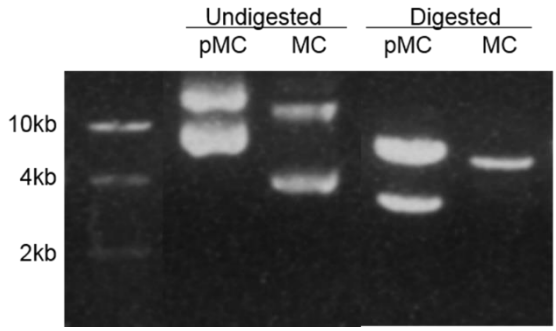

Figure 3. Restriction enzyme digestion of parental and minicircle DNA

Parental plasmid DNA corresponds to 10,040 bp band and minicircle DNA corresponds to 6,136 bp band. Nsi1 cuts only the bacterial backbone of parental plasmid and the parental plasmid is cut into two products with 6,545 bp band and 3,495 bp band. Though residual parental plasmid was shown before Nsi1 digestion on the minicircle DNA bands, it was degraded during the digestion step.

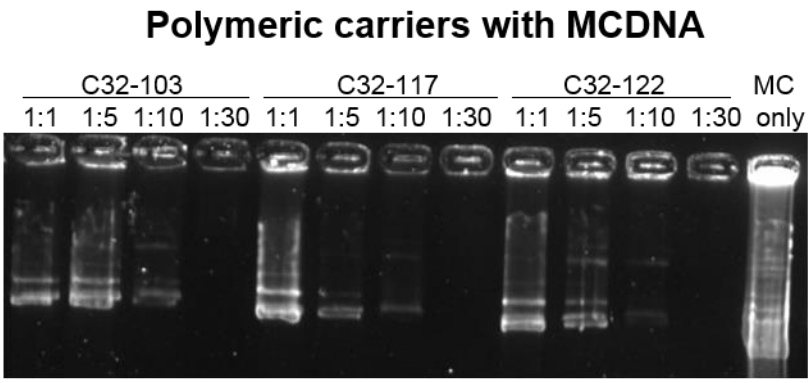

Figure 4. Optimization of MCDNA/PBAE weight ratio

Agarose gel retardation test was performed to check whether MCDNA and PBAE fully complexed. C32-103, C32-117 and C32-122 refers to the three different types of PBAEs, and the ratio of MCDNA to PBAEs were written as 1:1, 1:5, 1:10 and 1:30. Thecomplexation was fully achieved at the ratio of 1:30 without any residual MCDNA.

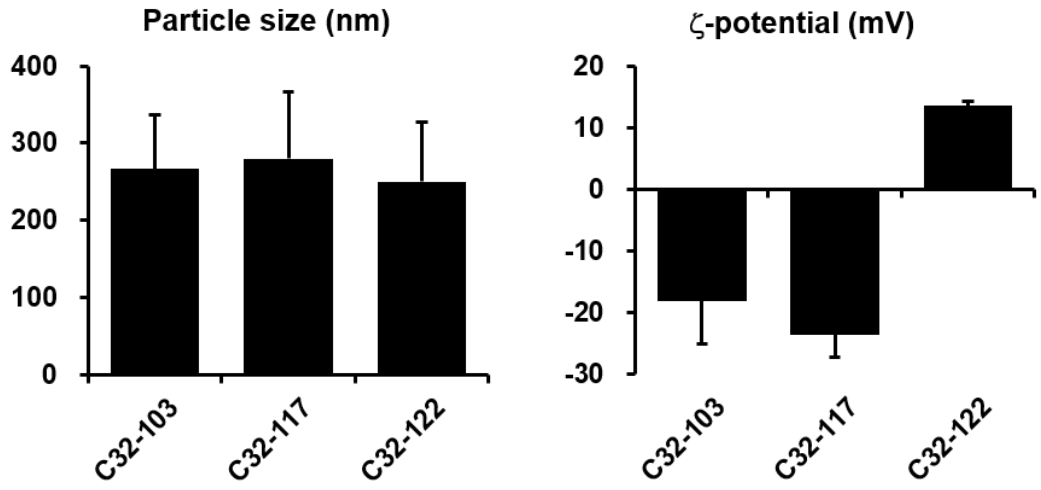

Figure 5. Properties of MCDNA/PBAE nanoparticles

The sizes of nanoparticles were around 250 nm. While MCDNA/C32-122 nanoparticles showed positive surface charge, MCDNA/C32-103 and MCDNA/C32-117 were negatively charged.The particles were small enough for the cellular uptake, and C32-122 represents as the most promising one according to the positive surface charge.

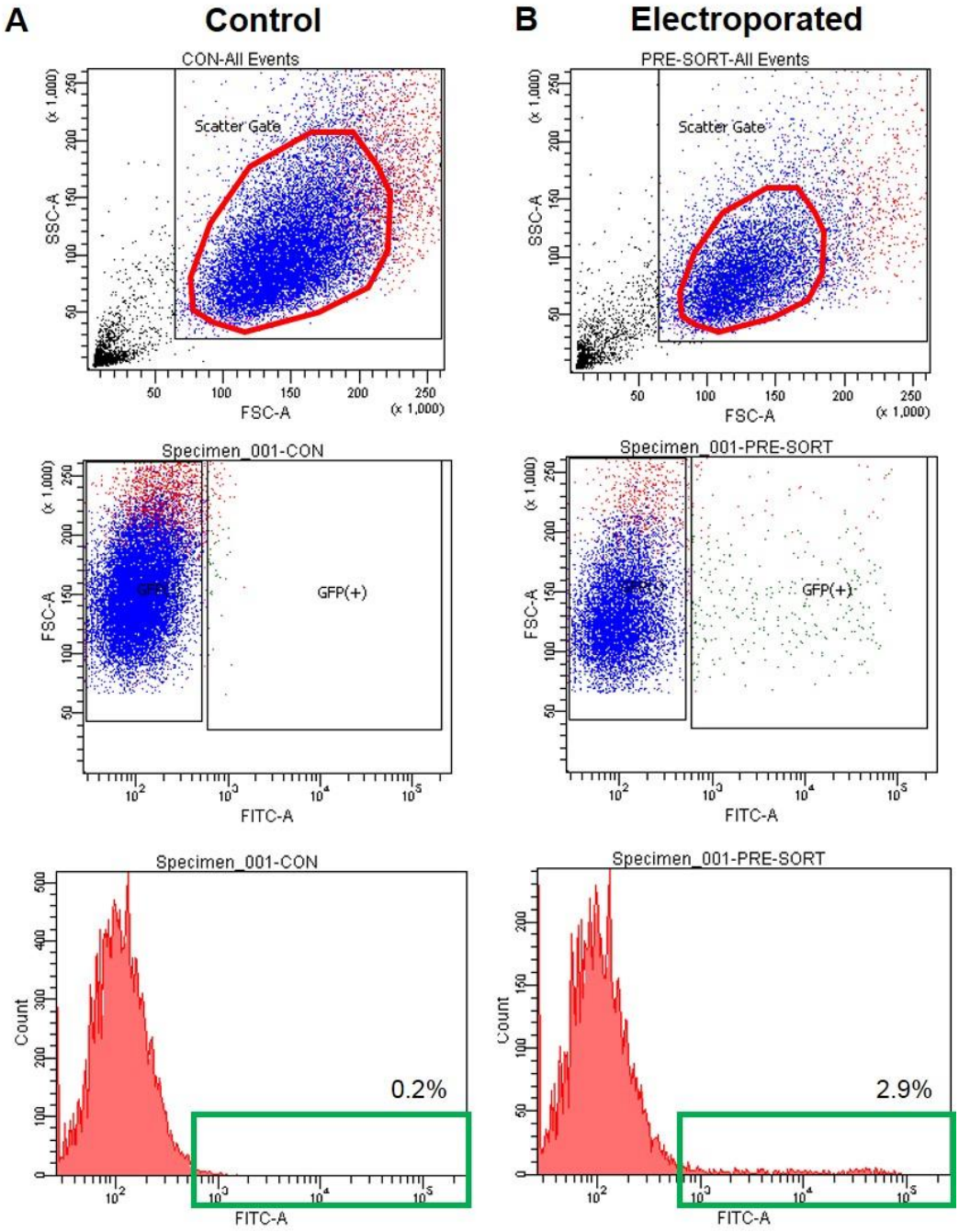

Figure 6. Flow cytometry data of electroporation

Electroporated human chondrocytes were analyzed by flow cytometry. The transfected cells were smaller than the normal chondrocytes and more GFP-positive cells were detected. A, graphs on the left, is the data of normal chondrocytes and B is that of transfected cells.

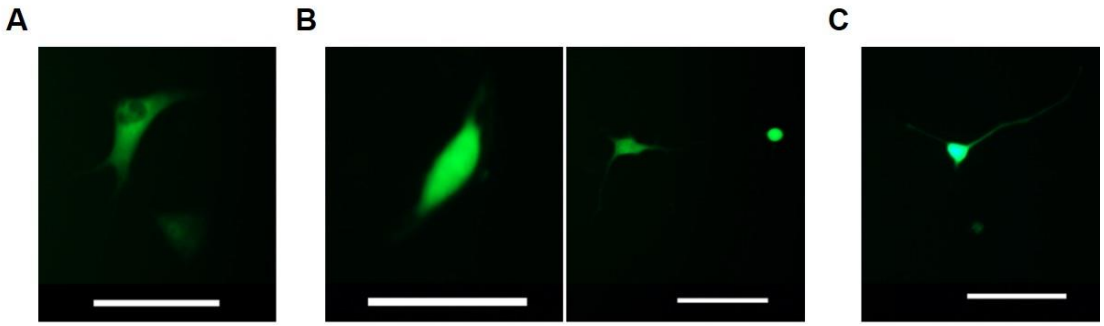

Figure 7. Decrease in cell size and higher cell circularity were observed by fluorescence microscopic image system as more MCDNA was delivered. A, B and C differs in the amount of MCDNA delivered; A, 7.5 μg/ 1M cells; B, 18.75 μg/ 1M cells; C, 37.5 μg/1M cells. Green, GFP. Scale bar=100μm.

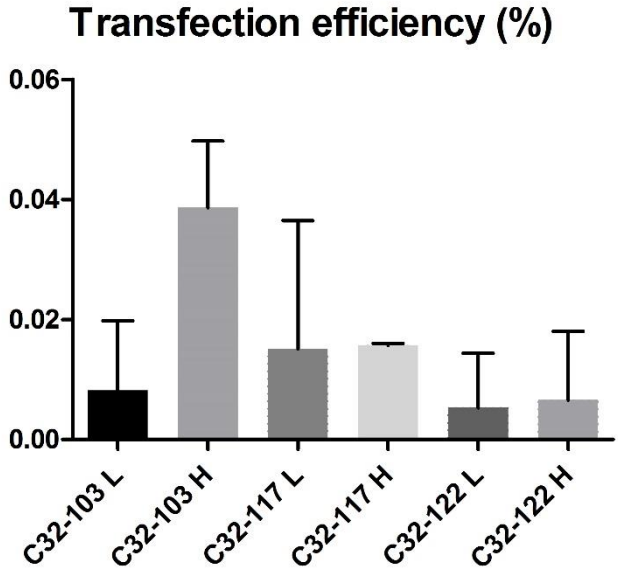

Figure 8. MCDNA/PBAE transfection efficiency

MCDNA was delivered into human chondrocytes using three different types of PBAEs. 'L' refers less amount of MCDNA and 'H' refers more MCDNA.Although MCDNA/C32-122 nanoparticle showed positive surface charge (Figure 5), MCDNA/C32-103 polyplex resulted in higher efficiency as MCDNA amount increases.

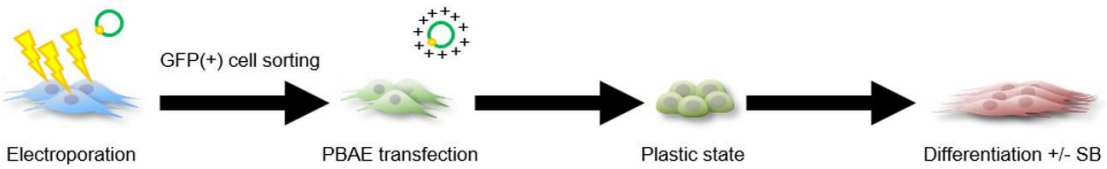

Figure 9. Experimental scheme of transdifferentiation of human chondrocytes

Since electroporation efficiency (~1.98% in ave.) was much higher than PBAE transfection (<0.04%), MCDNA was delivered into human chondrocytes via electroporation followed by cell sorting and PBAE transfection. To induce myogenic differentiation, SB-431542 compound was treated when MCDNA-delivered cells were in the plastic state.

2.4 Discussion

Gaspar *et al.* studied to optimize the biosynthesis process to enhance the yield of minicircle DNA from the host *E. coli*. [57]. For the process includes bacterial growth and minicircle DNA induction steps, the controlled parameters were growth temperature, growth time, concentration of inducer (L-arabinose), induction temperature and duration. This group used minicircle DNA with only GFP gene, and showed that higher yield and purity of minicircle DNA was achieved by controlling those parameters. However, it was reported that residual parental plasmid were slightly present, so it is still needed to develop the methodology of biosynthesis of MCDNA. In this study, the residual parental plasmid were observed before the restriction enzyme digestion step but disappeared after the step. Therefore, the deeper understanding of the mechanism and further studies to enhance the purity will be additionally investigated.

Optimal weight ratio of plasmids to PBAE was previously studied. Sunshine *et al.* found that increasing weight ratio up to 1:50 achieved optimal transfection efficiency but showed increased cell cytotoxicity [58]. Keeney *et al.* studied PBAE-based MCDNA delivery system and reported that less PBAE (1:10) was used to complex stable nanoparticles with MCDNA because of its smaller vector size [59]. Decreasing the amount of PBAE resulted in less cytotoxicity as an additionally advantage. We also sought to optimize the weight ratio of MCDNA to PBAE. Running complexed MCDNA/PBAE samples in agarose gel has shown that 1:30 resulted in complete formation of nanoparticles without remaining free MCDNA.

Cellular uptake of nanoparticles and release of delivered gene are also considered important in gene carrier system. Yue *et al.* studied the effect of nanoparticles' surface charge on cellular uptake and intracellular trafficking with chitosan-based nanoparticles [56]. The positively charged nanoparticles are likely to contact more with cell surface than the negatively charged ones due to their electrostatic interactions. They concluded that positive charge enhanced the cellular uptake of nanoparticle in eight cell lines and that some positively charged nanoparticles could be released from lysosome

while neutrally and negatively charged particles could co-localize with lysosome. In this study, MCDNA/C32-122 nanoparticle had positive surface charge and MCDNA/C32-103 was less negatively charged than MCDNA/C32-117. We further studied to find out the effect of surface charge on the transfection efficacies with three types of PBAE and two different amount of MCDNA.

Mincircle DNA was delivered into human adipose stem cells and fibroblasts using electroporation and Lipofectamine 2000 by Jia *et al.* to generate iPSCs, but the overall reprogramming efficiency was around 0.005% [52]. Borestrőm *et al.* delivered mRNA with an mRNA reprogramming kit to induce pluripotency from human chondrocytes and fibroblasts and the reprogramming and resulted in ~0.1% (human chondrocytes) and ~2% (BJ fibroblasts) [60]. Although reprogramming of other cell lines with MCDNA has widely been studied, its transfection efficiency on chondrocytes has not been reported much. As we examined the delivering efficiency of plasmid vectors into many other cells including HeLa, HEK 293T and HepG2, it was found that human chondrocytes showed much less efficiency than those cell lines. Narhinh *et al.* set improved methodology to generate iPSCs from adult human fibroblasts with serial steps of transfection- electroporation followed by several transfection with Lipofectamine 2000 [61]. We used the similar approach, serial transfection system, to induce plastic state of human chondrocytes as we found out much higher transfection efficiency using electroporation than using PBAE.

Chapter 3. Transdifferentiation of Human Chondrocytes into Myogenic Commitment

3.1 Introduction

During reprogramming steps into iPSCs, plastic intermediate cells also generated during the process, and that changing the duration of overexpression of transcriptional factors and culture conditions may affect the cells in the plastic state [62]. Kurian *et al.* recently reported that developed reprogramming methodology which includes the transition through a plastic intermediate state to convert human fibroblasts to angioblast-like progenitor cells [63]. Also, Efe *et al.* generated cardiomyocytes through a transient, plastic state of mouse embryonic fibroblasts by several transcriptional factors [64].

By delivering MCDNA, we have created partially reprogrammed chondrocytes. For further application in muscular diseases, we have examined whether these plastic cells can transdifferentiate into myoblasts. Myogenic differentiation of plastic chondrocytes was induced with TGF-β inhibitor.

Chondrocytes and myogenic cells are from the same mesodermal lineage, however, they differ from each other since they are differentiated and ended up in the fully differentiated states. Chondrocytes exist only in healthy cartilage and maintains cartilaginous matrix, while myogenic cells are shown in many areas of a body such as heart, lung and muscles. To compare those cells, we analyzed their messenger RNA expression levels on the specific genes and cell surface markers.

3.2 Methods

3.2.1 Generation of plastic state chondrocytes

3.2.2 Myogenic differentiation with SB-431542

For myogenic commitment, the cells in the plastic intermediate stage were maintained in SkGM-2 Medium (CC-3245, Lonza, Walkersville, MD, USA). To compare differentiation ability of SB-431542 compounds, three different media were used; 1) SkGM-2 Medium as negative control condition, 2) DMEM (11995, Gibco, Grand Island, NY, USA) with N-2 supplement (17502, Gibco, Carlsbad, CA, USA) as positive control condition, 3) DMEM with N-2 supplement and 10 μM SB-431542 (S4317, Sigma-Aldrich, Saint Louis, MO, USA) as the differentiation condition (Figure 9).

3.2.2 RT-PCR

Normal chondrocytes, transfected cells and sorted cells were treated with Trizol® reagent and collected in 1.5 mL-tube. Chloroform was added and the tube was shaken vigorously for 15 seconds using hand. After incubating at room temperature for 10 minutes, centrifugation was performed in 15,000 rpm for 20 minutes at 4℃. The clear aqueous phase at the top was transferred to new tube and isopropanol was added and mixed using hand. After incubating at room temperature for 5 minutes centrifugation in 15,000 rpm for 20 minutes at 4℃ was performed to collect RNA as white pellets. RNA pellet was washed with 75% ethanol, dissolved completely in molecular grade water and denatured at 60℃ for 10 minutes. RNA concentration was measured and reverse-transcriptional PCR was performed with cDNA kit (EZ006M, Enzynomics, Korea) according to manufacturer's instructions. PCR with chondrocyte-related markers (Collagen type I, II, and III, aggrecan), and myogenic markers (myod1,

myogenin) was done to observe gene expression levels of the cells. PCR with MC DNA-related primers was also performed to check whether the foreign DNA inserted into host genome.

3.2.3 Immunocytochemistry

Differentiated cells were fixed with 4% paraformaldehyde (6506-4405, Daejung, Korea) for twenty minutes, washed three times with PBS (70011-069, Gibco, Carlsbad, CA, USA) and permeabilized with 0.1% Triton X-100 (X100, Sigma-Aldrich, MO, USA) in PBS. The samples were blocked with 1% BSA (9998, Cell Signaling, MA, USA)in PBS for 30 minutes. Primary antibodies to myosin (ab7784, Abcam, Cambridge, UK) and f-actin (R415, Invitrogen™, Carlsbad, CA, USA) were diluted in 1% BSA/PBS at 1:150 ratio. Secondary antibodies, DyLight™ 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (115-485-003, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor® 594 Phalloidin (A12381, Invitrogen™, Carlsbad, CA, USA) were diluted in 1%BSA/PBS at 1:500 ratio. After blocking, the cells were incubated with antibody working solution for 40 minutes at room temperature, then washed with PBS three times. The diluted secondary antibodies working solution was treated onto the cells for an hour at room temperature in the dark. The cells were washed with PBS three times for 5 minutes each, and DAPI (D9542, Sigma-Aldrich, Saint Louis, MO, USA) staining was performed for five minutes followed by washing step. The samples were observed with fluorescence microscopy.

3.3 Results

3.3.1 Gene expression level differences during transfection

Messenger RNA expression of electroporated chondrocytes were analyzed by RT-PCR. The transfected chondrocytes expressed lower levels of chondrocyte-related genes compared to the normal chondrocytes (Figure 10). Due to the transcriptional factors in minicircle DNA, partial reprogramming of human chondrocytes were achieved and resulted in downregulation of chondrocyte-related genes.

3.3.2 Morphological change

Human chondrocyte (HC)-derived cells were differentiated with three different types of media. In the normal growth medium, HC-derived cells showed non-oriented areas and the least number of multinuclear cells, and took longer time to get slightly oriented. However, in the differentiation media with or without SB-431542 compound, the cells started to get oriented earlier and showed more multinuclear cells. SB-431542 is a drug which is developed by the enterprise GlaxoSmithKline (GSK) as an inhibitor of the activin receptor-like kinase (ALK) receptors [65]. It is also known that this compound suppresses the transforming growth factor-beta1-induced proliferation of osteosarcoma cells in human cells [66]. It was reported that SB-431542 compound promoted myogenesis of pluripotent cells [67-69]. The differentiated cells with SB-431542 compound generated the most number of multinuclear parts than the cells without it (Figure 11).

Myosin, the myogenic cell surface marker, was expressed higher on the cells cultured in the differentiated medium with SB compound comparing to the cells with the growth medium. F-actin staining showed how well the differentiated cell were aligned (Figure 12).

3.3.3 Gene expression level differences during SB-compound treatment

Gene expression levels were analyzed by RT-PCR with myogenic primers, myogenin and myogenic differentiation 1 (MyoD1, MyoD). Myogenic gene expression level was higher in SB-treated cells comparing to the SB-non-treated cells (Figure 13). This showed that SB-431542 compound also promoted myogenesis of the partially reprogrammed chondrocytes.

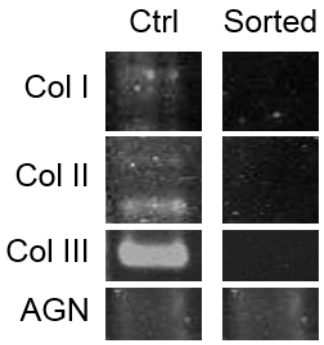

Figure 10. Messenger RNA expression profiling of electroporated chondrocytes

Reverse-Transcriptase PCR was performed to assess the mRNA level of collagene genes. Transfected chondrocytes showed lower levels of chondrocyte-related genes compared to the normal chondrocytes.

Gene	Forward Primers	Reverse Primers
GAPDH	TGT TGT GGA TCT GAC CTG CC	TTC TCA GTG TGG CGG AGA TG
18s	CCC TGT AAT TGG AAT GAG TCC ACT T	ACG CTA TTG GAG CTG GAA TTA C
COL Ia(I)	GCT TCA GGT CCC ATG GGT CC	ACC AAC AGG GCC AGG CTC TC
COL IIA/IIB	GTG AGC CAT GAT TCG CCT CGG	CAC CAG GTT CAC CAG GAT TGC C
COL III	TGG GTT GAC CCT AAC CAA GG	CCC AGT GTG TTT CGT GCA AC
AGN	ATT TCA AGA AGG CGA GGC GT	CTC ACA TAC CTC CTG GTC TGC
MyoD1	AGC ACT ACA GCG GCG ACT C	TAG TAG GCG CCT TCG TAG CA
Myogenin	CAG CTC CCT CAA CCA GGA G	GCT GTG AGA GCT GCA TTC G

Table 1. Human primers used for RT-PCR

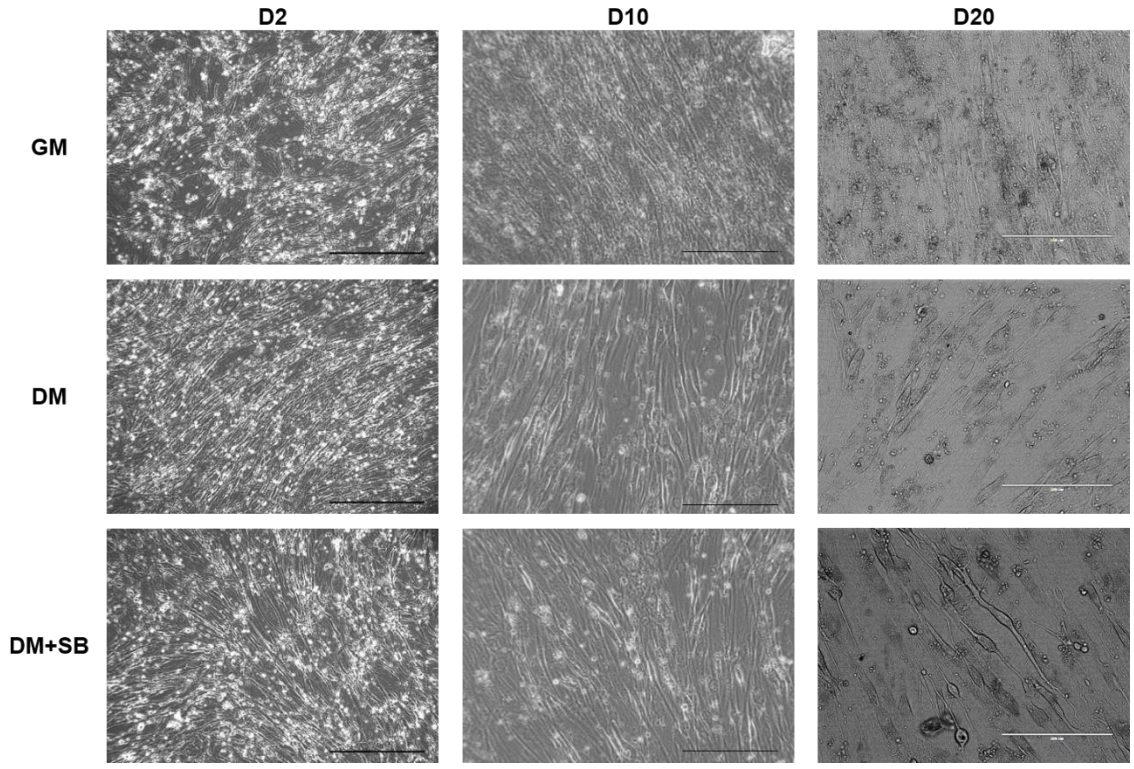

Figure 11. Microscopic images of differentiated cells

HC-derived cells differentiated with three types of media; skeletal muscle cell growth medium, differentiation medium with or without SB-431542 compound. On day 2, cells started to be originted with differentiation media while the cells with growth medium were not oriented. Cells started to get oriented on day 10, and multinuclear cells were observed on day 20. SB-treated cells had more multinuclear cells than the cells without it. **Scale bars are 200 μm on day 2, 100μm on day 10, and 200μm on day 20**.

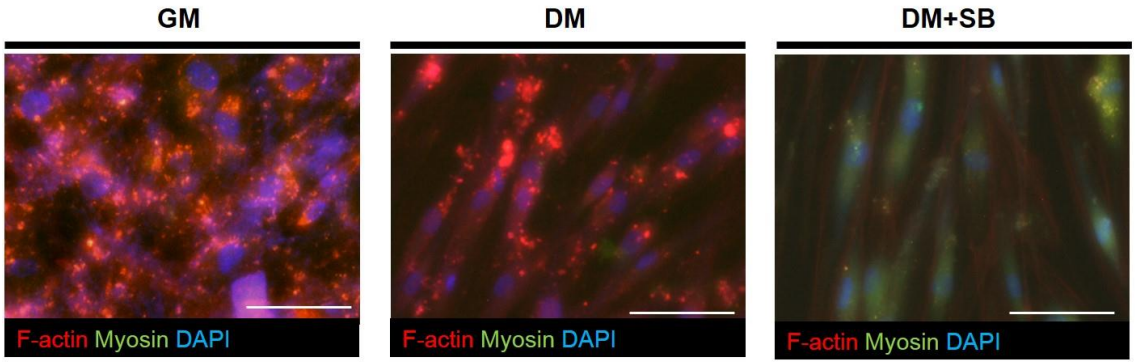

Figure 12. Fluorescence microscopic images of differentiated cells

Human chondrocyte-derived cells were cultured in three different types of media; GM, skeletal muscle growth medium; DM, differentiation medium without SB-431542 compound; DM+SB, differentiation medium with SB-431542 compound. SB-431542 compound induced the cells to be aligned and to express myogenic cell surface markers. The cells were fixed and immunocytochemically dyed after 20 days of SB treatment. Expression of the indicated markers in differentiated cells was visualized. Scale bar = 100 μm.

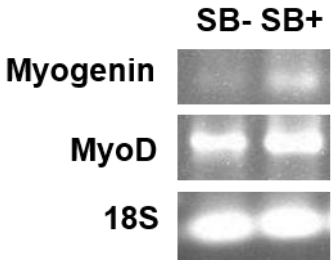

Figure 13. Messenger RNA expression profiling of differentiated cells

SB compound treated cells (SB+) expressed more messenger RNAs of myogenic genes than non-SB compound treated cells (SB-) during the myogenic commitment. Myogenin and MyoD are myogenic markers and 18s is housekeeping gene.

3.4 Discussion

Transdifferentiation has been widely investigated and even applied in clinical areas as reprogramming strategies and methodologies have been developed, since Zhou *et al.* first reported that adult pancreatic cells were reprogrammed into beta cells in vivo using the three viral transcriptional factors among 30 candidate factors [70]. Davis *et al.* first reported that delivery of a single cDNA encoding MyoD could stably transdifferentiate fibroblasts into skeletal myoblasts *in vitro* [71]. Other groups also reported that converting other cells into myogenic cells also achieved with retroviruses [50, 72], lentiviruses [50], micro RNAs [73] and proteins [74]. Although viral reprogramming methods have extensively utilized due to its higher efficiency, it always gave rise to the safety issues so that nonviral methods have also widely been investigated. We also sought for a transdifferentiation process without any viral system, and have used serial transfection of non-integrative MCDNA and chemical treatment on the HC-derived cells. Moreover, partial reprogramming might be sufficient in this case since the chondrocytes and myogenic cells are in the same germ layer, and the partial reprogrammed cells might still retain the epigenetic signatures shared by myogenic cells, thus would not be a hurdle for transdifferentiation.

Characterization of transfected chondrocytes and myogenically committed cells were examined with comparison of messenger RNA expression levels, visualization of morphological changes, and immunocytochemical methods. In the plastic intermediate stage of human chondrocytes, the cells expressed lower levels of the chondrocyte-related markers – type I, II and III collagen (Col Ia(I), Col IIA/IIB, Col III) and aggrecan. For the morphological changes of transfected cells were observed in figure 7, it was assumed that the chondrocyte-related genes might be expressed less in the transfected cells than in the untransfected cells. Expression of three types of collagen genes was decreased in the transfected cells, but the similar aggrecan expression was observed (figure 10). The similar aggrecan expression level might prove that aggarecan could be a putative predictive marker of chondrogenic capacity which was mentioned by Borestrőm *et al.* [60]. The further examination with

additional markers is ongoing to see the expression levels of MCDNA-related genes in the transfected cells and myogenically committed cells.

Knowing the different mRNA expression levels in the transfected cells, myogenic commitment from MCDNA-delivered chondrocytes were performed with SB-431542 compound which is known as an inhibitor of ALK receptor and enhances myogenesis of cells with pluripotency. When the cells were expanded in the skeletal muscle growth medium, the cells started to spread out. As the expanded cells were divided into three different groups, the cells looked much more different from the cells before the induction. With the differentiation medium, the cells started to get aligned and to form multinuclear cells much faster than the cells with the growth medium. SB compound treated cells showed the most amount of multinuclear cells and alignments. Also, the myosin, myogenic protein marker, was expressed the most in the SB-treated HC-derived cells. To see the effect of SB compound on myogenic commitment, mRNA levels of myogenic genes were also examined. Figure 13 showed that SB treated cells expressed the higher myogenic gene level than the nontreated cells, therefore, SB compound also promoted the myogenic commitment of the cells in the plastic intermediate state.

Chapter 4. Conclusions

In this thesis, we sought to induce transdifferentiation of chondrocytes into myogenic cells by overcoming low-transfection efficiency via minicircle DNA and PBAE, followed by using SB-431542 inhibitor after partial reprogramming. Nanoparticles complexed by self-assembly of C32-103 polymer with minicircle DNA were more effective than other two polyplexes for non-viral gene delivery into human chondrocytes though it had negative charge on the surface of nanoparticles. Electroporation of human chondrocytes with minicircle DNA had shown higher efficiency than the nanoparticle-based system, thus chosen for the first transfection method for transdifferentiation of human chondrocytes. After sorting of GFP positive cells, minicircle DNA delivered cells, the nanoparticle-based minicircle DNA delivery was performed with C32-103 polymer which showed the highest efficiency which was observed earlier. Although minicircle DNA delivery did not induce the fully pluripotent cells, our results demonstrated that the chondrocyte-related genes were downregulated after the transfection and characterized the myogenic commitment of chondrocyte-derived cells, thus providing evidence of successful transdifferentiation process.

SB-431542 treated cells generated larger myotubes, expressed higher level of myosin on the cell surface, and had higher mRNA level of myogenin and myod1 than non-treated cells, as it was reported that SB-431542 compound promoted myogenesis of pluripotent cells [67-69]. The differentiation medium, normal DMEM with N-2 supplement, seemed to affect the myogenic differentiation in earlier stage, for chondrocyte-derived cells got oriented without SB-431542 compound two days after the treatment of the medium and generated thin myotubes 20 days after the treatment. Through our results, we here provided the effective experimental strategy to overcome the low-transfection efficiency of chondrocytes and may have shown transdifferentiating potential of chondrocytes, speficifically into myogenic cells.

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국문초록

미니써클 유전체의 비바이러스성 전달을 통한 인간 연골세포의 근육세포로의 분화 유도

재생의학은 인간의 세포나 조직을 재생하거나 대체함으로써 본래의 기능으로 되돌리는 일련의 과정을 포함한다. 세포치료법은 재생의학의 한 분야로서, 손상된 세포나 조직을 대체하기 위해 원하는 분화 단계에 이른 세포를 충분히 공급해주어야 한다. 손상된 세포는 일련의 회복 과정을 거치는데 이는 대부분 불완전한 상태로 재생되며, 극심한 손상일 경우 치료가 불가능하거나 세포가 정상적으로 증식되기 어려워진다. 따라서, 완전히 분화된 세포를 다른 분화 형태의 세포나 다분화능을 가진 세포로 변환시키는 기술이 요구된다. 유도만능줄기세포(iPS cells)의 경우 원하는 상태로 세포를 분화시킬 수 있으나, 현재로서는 다분화능을 유도하기까지 오랜 시간이 걸리며 세포마다 그 유도 효율이 다르게 나타난다. 또한, 임상적 안전성과 유전적 혹은 후생적인 결함 유무 등은 여전히 큰 문제점으로 대두되고 있다. 최근에는 다분화능 유도 과정을 우회하는 교차분화 연구를 통해 이러한 문제점들을 해결하고자 한다. 본 연구는 비바이러스성 전달 시스템을 통해 역분화 인자를 포함한 미니써클 유전체를 인간 연골세포에 전달하고, ALK 수용체를 저해하는 SB-431542 를처리하여최종적으로근육세포로의교차분화를유도하고자하였다. 연골세포로의 유전자 전달 효율을 높이기 위해 미니써클 유전체는 전기천공법과 폴리베타아미노이스터 유전자 전달 시스템의 연속적인 단계를 통해 세포 내로 전달되었다. 형광 이용 세포분류기를 통해 유전자가 전달된 세포를 분류하여 매트리젤이 코팅된 플레이트에서 배양하였고 계대과정을

거친 뒤 SB-431542 물질을 통해 근육세포로 분화될 수 있도록 유도하였다. 본 논문에서는 비바이러스성 전달 시스템을 통해 인간 연골세포가 효율적으로 다른 세포계보로 교차분화될 수 있는 가능성을 보여주고 있다.

주요어: 교차분화, 폴리베타아미노이스터, 미니써클 유전체, SB-431542

학번: 2012-20986