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RESEARCH ARTICLE

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The Use of Cell-penetrating Peptide for Delivery of Recombinant Transcription Factor DNA into Primary Human Fibroblast

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Background: Reprogrammed cell therapy has not been applied for clinical purposes due to the malignancy issue. The aim of this study was to design the recombinant vector of the transcription factors and analyze the effectiveness of cell-penetrating peptide delivering system for human primary fibroblast transfection to avoid the malignancy issue.
Materials and methods: The constructions of CCAT/enhancer binding protein alpha (*CEBPA*), hepatocyte nuclear factor 4 alpha (*HNF4A*), nuclear receptor subfamily 1 group I member 2 (*NR1I2*) were confirmed with DNA digestion and sequencing. Breast reduction (BRED) and palate (PAL) tissue were used as human primary fibroblast sources. The transcription factors were delivered into BRED and PAL with recombination of avian leukosis sarcoma virus (ALSV), human immunodeficiency virus (HIV) matrix, and regulator of expression of virion proteins (Rev) (ALMR), tagged with enhanced green fluorescence protein (eGFP). Post-transfection cells were then cultivated with optimized medium. Gene expression was measured with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).
Results: Gene expression levels of *CEBPA*, *HNF4A*, *NR1I2*, glutamate-ammonia ligase (*GLUL*), albumin (*ALB*), and cytochrome P450 (*CYP*) were increased. Transfection with ALMR, which were more efficient in BRED than PAL fibroblasts may have the advantage in autologous cell therapy for elderly patients.
Conclusion: Transfection of transcription factors to human primary fibroblast may be performed by using constructions of plasmid as designed in this study.

Keywords: *recombinant plasmid, hepatocyte-like cells, primary fibroblasts, recombinant peptide, cell reprogramming, autologous cells therapy*

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Introduction

Cell reprogramming is one of the recently discovered techniques[1], which is expected to solve the difficulty of obtaining sufficient numbers of hepatocyte-like cells for cirrhosis therapy.[2,3] Primary hepatocytes are highly differentiated, hence *in vitro* propagation of these cells is not possible. Other cell sources, such as fibroblasts are easier to be obtained from a patient and can potentially be converted into hepatocytes.[4] However, hepatocyte-like cells have not been applied for clinical purposes due to the malignancy issue[5] that may occur due to the use of viral vectors for delivering exogenous transcription factors and the presence of the pluripotent stage. The use of viral transduction could lead to the integration of the viral vector into the chromosomes, causing genetic changes and triggering proliferation of cancer cells. In addition, the remnants of undifferentiated pluripotent cells might become malignant if they advertently infiltrate the patient's vascular system.[6,7]

Due to several safety issues, we designed a cell reprogramming strategy to obtain autologous hepatocyte-like cells, hence malignancy issues can be avoided and reprogrammed cells can be used for clinical purposes.[6] Human primary fibroblasts, which are derived from biomedical waste, were used to produce hepatocyte-like cells in this study. These cells have several advantages, including easy to obtain and process, has minimal obstruction regarding the ethical issues, has the ability to regenerate quickly, and are potential for autologous transplantation.[8,9] Several crucial stages in this study are patient's tissue biopsy, fibroblast isolation, transfection of transcription factor, determination of hepatocyte induction medium composition and incubation time, and characterization of hepatocyte-like cells (Supplementary 1).

In this study, recombination of avian leukosis sarcoma virus (ALSV), human immunodeficiency virus (HIV) matrix and regulator of expression of virion proteins (Rev) (ALMR) peptide was used as a transcription factor delivery system. This recombinant peptide, 7.1 kDa in size, has been reported as a cell-penetrating peptide. Previous study has investigated its unique characteristics, such as the ability to bind biomolecules (peptides, oligonucleotides, and proteins) and deliver extra biomolecules into the nucleus. It has been reported that ALMR can overcome cellular barriers, such as cell membranes, endosome compartments, and nuclear membranes, hence expression of transgene protein could occur even in non-dividing cells.[10] The aim of this study was to design the recombinant vector for the transfection of transcription factors and analyze the effectiveness of ALMR peptide for reprogramming human primary fibroblast to hepatocyte-like cells. ALMR peptide has the potential to substitute virus delivery systems in cell reprogramming, hence the safety of cell therapy can be more enforced.

We avoided sex-determining region Y (*SRY*)-box trenscription factor 2 (*SOX2*), Krüppel-like factor 4 (*KLF4*), octamer-binding transcription factor-4 (*OCT-4*), and cellular-myelocytomatosis oncogene (*C-MYC*) transcription factors which would convert fibroblast into pluripotent cell.[11,12] Instead, we used a cocktail of transcription factors containing CCAAT/enhancer binding protein alpha (*CEBPA*), hepatocyte nuclear factor 4 alpha (*HNF4A*), and nuclear receptor subfamily 1 group I member 2 (*NR1I2*), that has been reported to have the ability to convert mouse fibroblasts into hepatocyte-like cells.[13]

DNA transcription factors are easier to be prepared with minimal laboratory handling compared to protein and RNA.[14] Using *in silico* method, the constructions were designed to be propagated in prokaryotic cells and expressed in mammalian cells. Prior to cell reprogramming, ALMR capability was confirmed with pcDNA3.1-enhanced green fluorescent protein (eGFP) in Chinese hamster ovary, subcloned K1 cell line (CHO-K1). Investigation results of several transcription factor ratios were then compared in human primary fibroblast cells. Moreover, lipofectamine, a common commercial transfection reagent, has been reported to increase the transfection efficiency of plasmid DNA into *in vitro* cell cultures.[15,16] The addition of lipofectamine increases transfection efficiency, suggesting that it might help biomolecules to penetrate the nuclear envelope.[17] The efficiency of transfection by lipofectamine system in human primary fibroblast was investigated and compared to the ALMR system.

Materials and methods

Construction of Recombinant Plasmids

Recombinant plasmids containing genes encoding *CEBPA* (1,084 bp), *HNF4A* (1,360 bp), and *NR1I2* (1,312 bp) transcription factors were designed. pcDNA3.1(+) (Invitrogen, Waltham, MA, USA) was used as a vector of transcription factors. *CEBPA*, *HNF4A*, and *NR1I2* sequences were obtained from http://www.ncbi.nlm.nih.gov/, RefSeq human nucleotide database. All sequences were compiled and arranged into one FASTA file. DNA sequences

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alignment was performed with Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Research Center for Genomics and Bioinformatics, Tokyo, Japan) and BioEdit version 7.1 (Ibis Biosciences, Carlsbad, CA, USA) for codon optimization. Amino acid function was checked using Uniprot website (http://www.uniprot.org). Kozak sequence 5'cgccRccATGG was then added in the beginning of gene sequences as ribosome attachment.

Each synthetic DNA was cloned in pcDNA3.1 flanked with *Nhe*I and *Hind*III restriction enzymes (New England Biolabs, Ipswich, MA, USA). Nebcutter version 2.0 (http://nc2.neb.com/NEBcutter2) was used to ensure that there were no similar restriction sequences found along synthetic DNA strands. DNA2.0 Gene Design & Synthesis (ATUM, Newark, CA, USA) software was used to measure GC percentage. The construction of recombinant plasmid pcDNA3.1-*CEBPA*, pcDNA3.1-*HNF4A*, and pcDNA3.1-*NR1I2* were shown in Figure 1. The plasmids were then synthesized by Genescript's company partner in Indonesia.

Escherichia coli Top 10 was used as host for recombinant plasmid amplification (cloning). *E. coli* was cultured in Luria Bertani (LB) broth containing 100 µg/µL ampicillin. Competent cells were prepared by inducing *E. coli* with 100 mM $MgCl_2$ and 100 mM $CaCl_2$. pcDNA3.1-*CEBPA*, pcDNA3.1-*HNF4A*, and pcDNA3.1-*NR1I2* were transformed to competent cells using heat shock transformation procedure with the ratio of recombinant plasmid:competent cells volume was 1:10 . The constructions were then confirmed with DNA digestion using *Nhe*I and *Hind*III and sequencing analysis. Three colonies were selected for each gene. Isolation of plasmid was performed

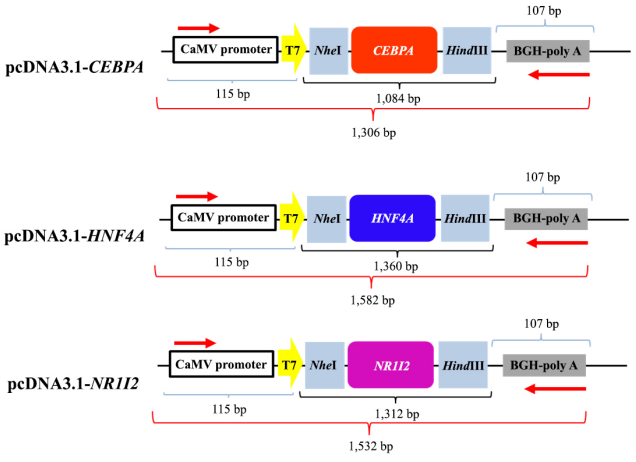

Figure 1. Construction of *CEBPA*, *HNF4A*, and *NR1I2* insertion in pcDNA3.1.

with miniprep procedure (Qiagen, Hilden, Germany), and the plasmids were restricted with *Nhe*I and *Hind*III. Plasmid were then visualized with electrophoresis on agarose gel 0.8% (b/v), 100V for 25 minutes, and compared with GeneRuler DNA Ladder (Thermo Scientific, Waltham, MA, USA). Plasmid concentration was measured using Nanodrop-2000 spectrophotometer (Thermo Scientific). One of three selected plasmids were then confirmed with sequencing analysis. Primers used for sequencing were listed in Supplementary 2.

ALMR Preparation

ALMR recombinant peptides were prepared following a patented procedure developed by Universitas Indonesia (No. IDP000069076). Design, synthesis, and functional testing of ALMR was performed as described in previous studies.[10] ALMR peptide was obtained from culture stock of pQE80L-ALMR recombinant plasmid in *E. coli* DH5α (stored at -80°C). The culture stock were cultured with LB medium overnight (16-19 hours) and the protein were expressed in an optimized condition for isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Ten mL of the culture stock that had been incubated were added into 100 mL of Terrific broth (TB) medium with 100g/mL ampicillin. The mixture was incubated at 200 rpm; 37°C for 4 hours and IPTG was added. The mixture was observed with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and compared with PageRuler Protein Ladder (Thermo Scientific). Purification of ALMR peptide was performed by nickel-nitrilotriacetic acid (Ni-NTA) matrix, and then it was dialyzed using Slide A-Lyzer Pierce (Thermo Scientific).

First dialysis was performed with distilled water containing 0.1 mM EDTA for 2 hours at 4°C. Second dialysis was performed with 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for 16 hours at 4°C. After dialysis, glycerol was added until the final concentration of peptide was 10%. The dialyzed peptide was then confirmed with 10% SDS-PAGE. Peptide concentration was determined using Lowry protocol with DCTM Protein Assay kit (Biorad, Hercules, CA, USA). Absorbances were determined with iMark Microplate Reader (Biorad) at 655 nm; 900 seconds with the shaker activated.

Human Primary Fibroblast Preparation

The use of tissue in this research was informed to the donor and the informed consents were signed afterwards. This

study has been approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia/ Cipto Mangunkusumo National Central General Hospital (No. 40B/UN2.F1/ETIK/IV/2018). Isolation of breast reduction (BRED) and cleft palate (PAL) fibroblasts were performed without enzymatic dissociation technique. The tissue was cut into pieces of about 2 mm, explanted to 24-well plates, and incubated in 37°C for about 5 minutes, until the tissue adhered to the surface. Fibroblast complete medium containing Dulbecco's modified Eagle medium (DMEM) high glucose, 1% platelet-rich plasma (PRP), 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% fungizone and 1% heparin were added to the isolated fibroblasts. Fibroblasts were incubated at 37°C with 5% CO_2. The rate of cell division of each passage was measured with population doubling time (PDT) analysis. Cell surface markers (CD73, CD90, CD105, CD45, CD34, CD19, and HLA-DR) were determined with flow cytometry.

Lipofectamine Preparation

Two µL of lipofectamine was diluted with 48 µL DMEM to obtain a final volume of 50 µL. Lipofectamine was then added to fibroblasts (100 µL/well).

ALMR Transfection

Before ALMR transfection was performed, the ALMR concentration was determined with Lowry method. The absorbances of first, second, third, and forth elution of ALMR were measured, and then compared with bovine serum albumin (BSA) serial dilution as relative concentration. After that, 4 µL of ALMR was diluted with 46 µL DMEM to obtain a final volume of 50 µL. ALMR was then added to fibroblasts (100 µL/well). The function of ALMR peptide as a delivery system was determined in CHO-K1 with eGFP marker tagging. *egfp* (720 bp) has been integrated in recombinant pcDNA3.1 (pcDNA3.1-eGFP).[9] eGFP is a 27 kDa protein consisting of 38 amino acids. Five hundred ng of pcDNA-eGFP was loaded into each well. A total of 2.8 µL pcDNA-eGFP (177.1 ng/µL) was diluted with 47.2 µL DMEM to obtain a final volume of 50 µL eGFP. eGFP was then added to the cells (100 µL/well).

Transfection of ALMR to human primary fibroblast was begun with the preparation of transcription factors transfection mix with *CEBPA*:*HNF4A*:*NR1I2* ratio as follows: 1:1:1, 1:2:1, and 1:3:1. The detailed transfection mix volume was described in Supplementary 3. DMEM was then added to the transfection mix and this mixture was added to human primary fibroblast (100 µL/well). Fibroblasts that were added with a combination of lipofectamine or ALMR and eGFP were used as experimental groups. Meanwhile, fibroblasts that were added with transfection mix without eGFP marker were used as negative control. The volume of pcDNA-eGFP was obtained by dividing the total concentration of pcDNA required (500 ng) with the initial concentration of pcDNA-eGFP (177.1 ng/µL). Therefore, 2.8 µL of pcDNA-eGFP was diluted with 47.2 µL of minimum essential medium (MEM) basal medium to obtain a final volume of 50 µL eGFP. This mixture were then administered to cells as much as 100 µL/well.

Cultivation of Transfected Fibroblasts

Hepatocyte induction medium used in this study did not contain CHIR, but contained other small molecules. Serum-free-αMEM complete medium (Gibco, Grand Island, NY, USA), Roswell Park Memorial Institute Medium (RPMI) 1640 complete medium (Gibco) with human serum albumin (HSA) (Gibco), or HepatoZYME-SFM complete medium (Gibco) with FBS was used as the induction medium. Meanwhile, human bone morphogenetic protein (BMP)-4 recombinant protein (Gibco), LY294002 (Gibco), human recombinant basic fibroblast growth factor (bFGF) (Stemcell Technologies, Vancouver, Canada), human activin A recombinant protein (Gibco), human oncostatin M (OSM) recombinant protein (Gibco), and human recombinant hepatocyte growth factor (HGF) (Stemcell Technologies) were used as small molecules. Medium replacement steps were outlined in Supplementary 4.

Analysis of Gene Expression in Transfected Fibroblasts

Fibroblasts mRNA was extracted from the transfected cells on day 3, 7, 10, 14, and 21. In addition, the mRNA of primary fibroblasts, which were not transfected and were cultivated in the same induction medium was used as a negative control. RNA obtained from rabbit liver was used as a positive control. The expression of *CEBPA*, *HNF4A*, and *NR1I2* transcription factors and hepatocyte markers glutamate-ammonia ligase (*GLUL*), albumin (*ALB*), and cytochrome P450 (*CYP*) were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control gene. The concentration of each RNA sample was equalized to 100 ng before reverse transcription with iScript cDNA (Biorad) and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) with SYBR Evagreen (Biorad) was performed. The primers

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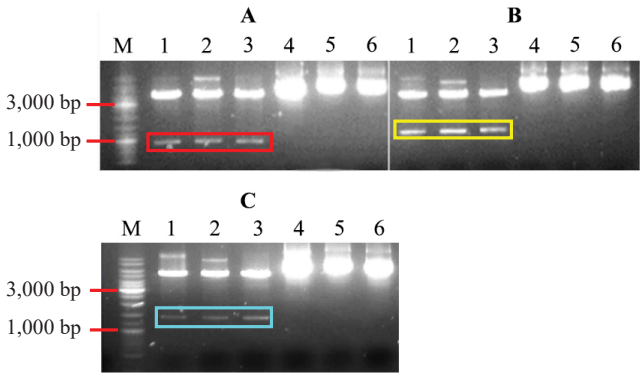

Figure 2. Visualization of recombinant plasmids before and after DNA double digestion. A: pCDNA3.1-*CEBPA*; B: pCDNA3.1-*HNF4A*; C: pCDNA3.1-*NR1I2*. Red box: *CEBPA* (1,084 bp); yellow box: *HNF4A* (1,360 bp); blue box: *NR1I2* (1,312 bp); M: GeneRuler DNA Ladder 1 kb; 1-3: after double digestion; 4-6: before double digestion.

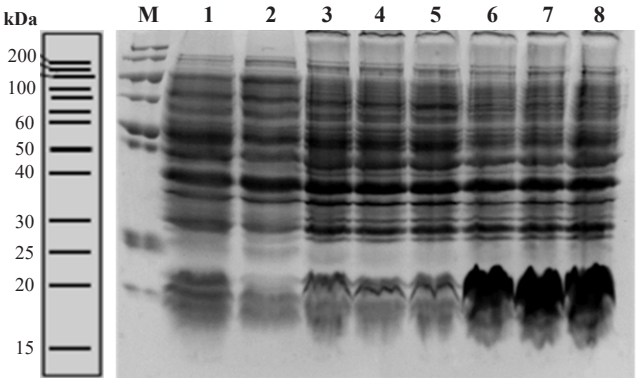

Figure 3. Expression of pQE80L-ALMR from colony 1-3 before (0 hour) and after 1 M IPTG induction (4 hours). M: PageRuler Protein Ladder; 1: Negative control DH5α; 2: Negative control plasmid pQE80wt; 3-5: Colony 1-3 before IPTG induction; 4-6= Colony 1-3 after IPTG induction.

used in qRT-PCR were listed in Supplementary 5. Measurement of gene expression was done three times. Cycle threshold (C_T) values were analyzed with Livak method.

Results

Construction of Recombinant Plasmids Containing CEBPA, HNF4A and NR1I2

Recombinant synthetic plasmids containing *CEBPA*, *HNF4A* and *NR1I2* were prepared in three seperate constructions. *CEBPA*, *HNF4A* and *NR1I2* in pcDNA3.1 (+) vectors that were transformed into competent *E. coli* cells had a length of 1,084, 1,360 and 1,312 bp, respectively. Two bands were yielded from DNA double digestion, *i.e.* one band had a similar estimated size with the insert size, and the other band was the other part of the plasmid. These results suggested that both restriction enzymes cleaved the target sequence on the appropriate position (Figure 2). The third colony of each transcription factor was chosen to be used for further analysis. The concentration and purity of each transcription factor DNA were measured by spectrophotometry (Supplementary 6). The constructions were also confirmed with sequencing. The amplified recombinant of transcription factor DNA of *CEBPA* yielded 1,306 bp using cauliflower mosaic virus (CaMV)-forward and bovine growth hormone (BGH)-reverse primers. Whereas, *HNF4A* and *NR1I2* yielded 1,582 and 1,532 bp, respectively. The complete homology and the reading frame were confirmed to reference genome sequence (Supplementary 7, Supplementary 8, Supplementary 9).

Visualization of ALMR Peptide

The size of ALMR bands obtained from the three colonies and control was about 7.1 kDa (Figure 3). ALMR from the third colony was then chosen to be expressed on a large scale and purified for further analysis. ALMR was purified in a denatured form using guanidine thiocyanate, which caused the peptide to lose its three-dimensional structure, hence the 6× histidine tag was more accessible to Ni-NTA.[18] Denatured protein returned to its native form when a physiological buffer, such as phosphate-buffered saline (PBS) was replaced through the dialysis process. In addition, the pH of the buffer also neutralized the ALMR peptides, hence avoiding cell damage and the peptide function as a delivery system can work as expected. Visualization of purified ALMR peptide was shown in Supplementary 10. Based on the BSA standard, the equation of y=3375.7x-77.164 with R^2=0.9953 was obtained and the ALMR concentration was 847.778 ng (Supplementary 11).

The Ability of ALMR as Delivery system in CHO-K1 Cells

Supplementary 12 showed that the number of fluorescent CHO-K1 cells was higher in lipofectamine transfection system compared to ALMR.

Effectiveness Validation of the Delivery System

According to population doubling time (PDT) calculation (Supplementary 13), BRED cell division was slower than PAL. BRED needed 3.198 days, while PAL needed 1.168 days to achieve twice the amount of the total cells. ALMR and lipofectamine systems were compared in both BRED

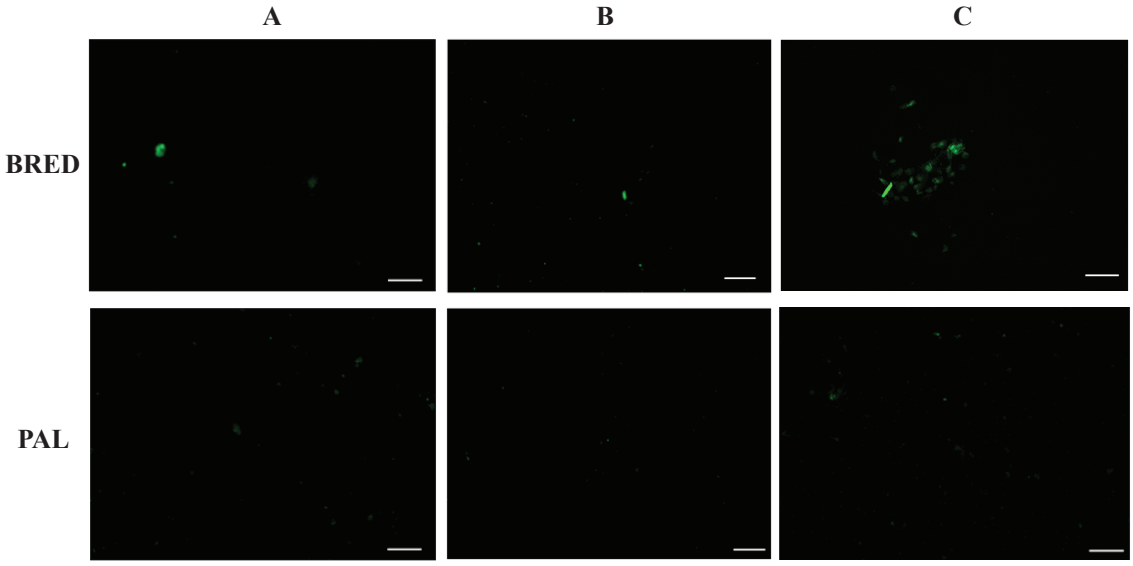

Figure 4. Observation of green fluorescence expression in BRED and PAL fibroblasts transfected using ALMR. Ratios for *CEBPA*, *HNF4A*, and *NR1I2* transcription factor DNA are as follows: A: 1:1:1; B: 1:2:1; C: 1:3:1.

and PAL fibroblasts. On the ALMR system, BRED showed more fluorescent cells than PAL. Based on fluorescence observation, ratio 1 : 3 : 1 of *CEBPA*: *HNF4A* : *NR1I2* was the best ratio for transfection in BRED fibroblasts (Figure 4). However, in the lipofectamine system, PAL showed more fluorescent cells than BRED. Ratio 1 : 1 : 1 of *CEBPA* : *HNF4A*: *NR1I2* was the best ratio for transfection in PAL fibroblasts (Figure 5).

Transcription Factors and Hepatocyte Markers Gene Expression in Transfected Fibroblasts

Both transcription factors (*CEBPA*, *HNF4A*, and *NR1I2*) and hepatocyte markers (*ALB*, *GLUL*, and *CYP*) expression

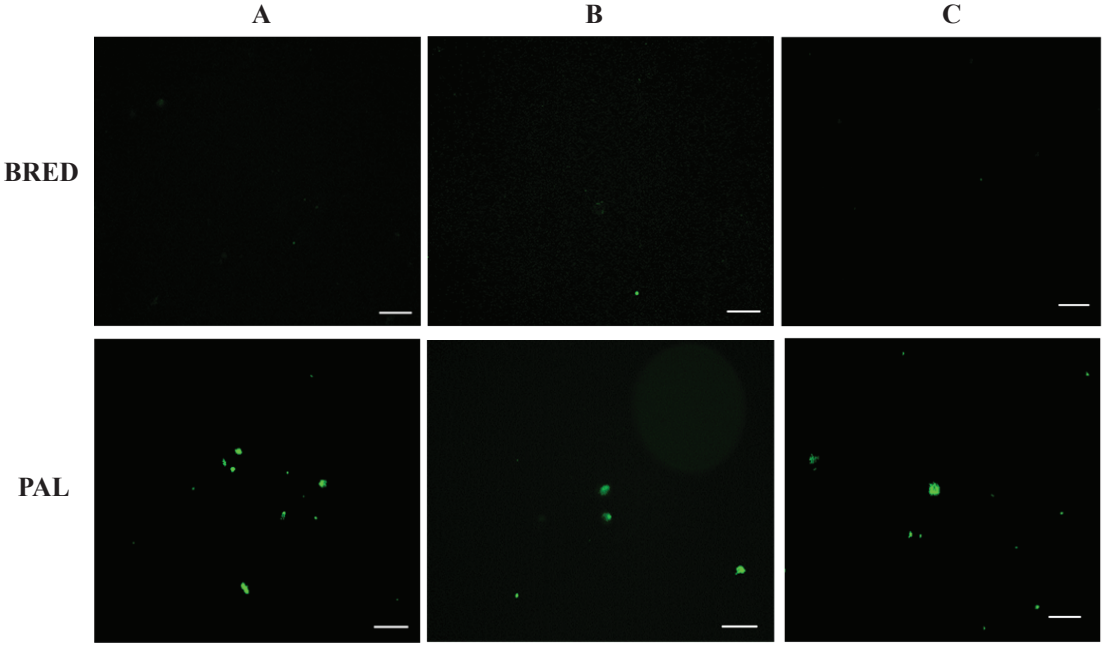

Figure 5. Observation of green fluorescence expression in BRED and PAL fibroblasts transfected using lipofectamine. Ratios for *CEBPA*, *HNF4A*, and *NR1I2* transcription factor DNA are as follows: A: 1:1:1; B: 1:2:1; C: 1:3:1..

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levels started to increase on day 3 (Supplementary 14). However, there was a slight decrease in expression level of each transcription factor gene on day 7. After day 7, the expression level of each transcription factor gene continued to increase.

Discussion

The types of fibroblasts (dividing or non-dividing cells) is important to be considered before conducting tissue biopsy for cell reprogramming. Based on our findings, the ALMR system was more effective for BRED (non-dividing cells, while lipofectamine system was more appropriate for PAL (dividing cells). This is in accordance with a previous study demonstrates that ALMR works more effectively on non-dividing cells and requires lipofectamine to increase transfection efficiency.[10] Fibroblasts are functionally and anatomically heterogeneous, determined by their function in different parts of the body. Fibroblasts from papillary cells are pro-regenerative, since they have the general characteristics of actively dividing cells. Meanwhile, fibroblasts from reticular cells are profibrotic, since they have the characteristics of non-dividing cells. BRED, breast-derived fibroblasts, slowly close the wound, with an opaque plug of dense connective tissue, whereas PAL, palate fibroblasts, close the wound with minimal scarring.[19] The age of the patient may also be associated with the characteristics of dividing fibroblast. PAL fibroblasts (obtained from a 4-year-old donor) divided faster than BRED (obtained from a 36-year-old donor). This characteristic may be affected by several factors, including the type of primary cell, sample storage duration, type of medium, cell processing technique, skills of the laboratory personnel, and contamination.[20,21]

Recombinant plasmids containing *CEBPA*, *HNF4A*, and *NR1I2* were designed to be propagated in *E. coli* and expressed in human primary fibroblast. Therefore, the transcription factor DNA can be easily cloned and is expected to be expressed when transfected into human fibroblasts. Based on our findings, the construction design was appropriate due to the increasing expression of both transcription factor genes and hepatocyte marker genes. The "up and down" in expression levels of transcription factor genes in our study is in line with results of a previous induced pluripotent stem cells (iPSc) research.[22]

Numerous combinations of transcription factors for direct reprogramming of fibroblasts into hepatocytes are interesting to be investigated with our transfection strategy.[6,23] There are similarities in the selections of transcription factors cocktail including CCAAT/enhancer binding protein (*CEBP*), hepatocyte nuclear factor (*HNF*), forkhead box (*FOX*) and GATA binding protein 4 (*GATA4*) that are related to the process of hepatocyte development from embryo to definitive endoderm, hepatoblasts, and mature hepatocytes.[23,24]

HNF4A is expressed in hepatic diverticulum development, and regulates hepatic mRNA expression during this stage, mid of hepatogenesis. Increased expression of *HNF4A* occurs in the process of cell differentiation to the hepatocyte cell line *in vitro*.[25] *CEBPA* is a transcription factor that plays a role in hepatocyte cell maturation, hepatogenesis, as well as hepatic metabolism including albumin expression and urea production.[13,26] Another core receptor used in this study, *NR1I2*, has been reported to have a modulating effect on functional liver genes, such as the *CYP* family.[13] Increased expression of *CYP3A4* in hepatocyte-like cells is important, since the production of cytochrome P450 enzymes play a role in drug metabolism.[27,28] *NR1I2* is also known to be the key of gene regulation, which has an influence on morphogenesis and differentiation.

The process of importing extranuclear material in non-dividing cells depends on the ability of extracellular material to be recognized by nuclear localization signal (NLS). Meanwhile, ALMR is a recombinant peptide that has been equipped with the NLS sequence (Rev), hence it can be recognized by cellular importers and facilitates the delivering system of extracellular DNA complexes into the nucleus via nuclear pore complex (NPC).[10] Rev is one of the HIV accessory proteins that has the ability to enter and leave the nucleus and bind to HIV mRNA (Figure 6). This is the reason why the ALMR system is more appropriate to be used in non-dividing cells (BRED) compared to PAL and CHO-K1 cells. In the lipofectamine system, the entrance of exogenous DNA relies on the cell division process when the cell membrane is more permeable.

Delivery efficiency of ALMR in CHO-K1 was lower than lipofectamine. It might be related to the efficiency of DNA release by the ALMR complexes. In the lipofectamine system, the internalization process may occur through endocytosis and is followed by endosomal escape.[15] Lipofectamine-DNA complex remains in the cytoplasm until the cell enters division phase when the nucleus disintegrates, which causes the release of DNA into the nucleus. Thus, the lipofectamine system is more appropriate for dividing cells, such as PAL fibroblasts and CHO-K1 cells.

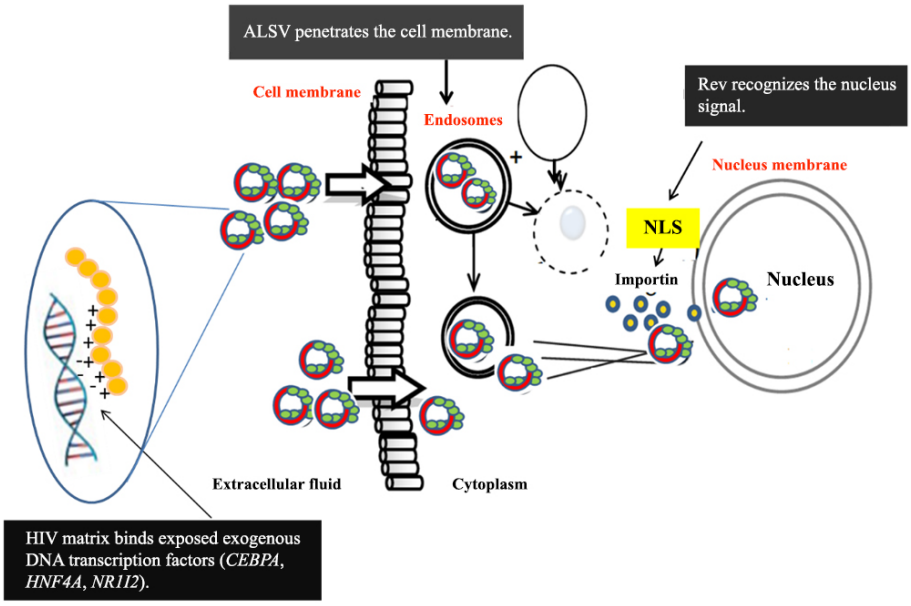

Figure 6. Delivery mechanism of transcription factor DNA to nucleus of fibroblasts by ALMR peptide. The HIV matrix in ALMR forms ionic bond with DNA of transcription factor. ALSV in ALMR helps the ALMR-DNA complex pass through the cell membrane and escapes from any endosomal bodies. NLS as part of HIV Rev in ALMR secretes importin as signal to the ALMR-DNA complex, hence the complex can be delivered to the nucleus of fibroblasts.

In the ALMR delivery system, most likely the exogenous DNA may still be in a complex with ALMR in the endosomal escape process. ALMR has an NLS amino acid sequence that can bind to importin, leading to recognition by the proteins that make up the NPC. The DNA will dissociate from the ALMR complex when it is in the nucleus area. Then, the DNA will bind to histones and form a nucleoprotein structure resembling chromatin.[10] Based on ratio optimization, *HNF4A* showed an important role in the transfection of ALMR. *HNF4A* as a nuclear receptor contains a NLS, hence it may assist the process of recognizing nuclear signals. In contrast to the lipofectamine system, the transfection efficiency seems not affected by the presence of NLS.[29]

The virus-based gene delivery systems will be possibly abandoned by the rise of the cell-penetrating peptide-based concept of cell reprogramming. The cell-penetrating peptide systems allow recombinant proteins, as well as small and large biomolecules to be delivered into the nucleus of living cells, although the alternate non-integrating systems may avoid genome integration. Nevertheless, the DNA molecule of transcription factors and short vectors might also cause insertion mutation.[30] Therefore, virus- and DNA-free systems should be concepted in further research.

The exogenous *CEBPA*, *HNF4A*, and *NR1I2* DNA are expected not to be integrated into the host genome to do the transcription process. The mammalian vector, pcDNA3.1 has its own expression system, hence the transcription process can occur. Medium replacement is required to change the cell niche that will support fibroblasts conversion to the hepatocyte cell line.[31] Continuous research to improve the quality of hepatocyte-like cells and more in-depth investigations regarding the relevant biological processes are required before clinical translation.

The results showed that ALMR was more effective to be applied on BRED than PAL fibroblasts. ALMR may have an advantage in autologous cirrhosis cell therapy which is generally needed by elderly patients. In addition, the measurement of cell viability to quantify the possible toxicity of ALMR during transfection has also not been determined. The ALMR system has the potential to convert myofibroblasts in the liver of cirrhosis patients, converting them directly into hepatocytes by delivering a set of transcription factors by the ALMR system, followed by the induction of hepatocyte differentiation. Several *in vivo* studies in animal models of cirrhosis prove the feasibility of this concept.[2,32] The construction of recombinant plasmid in this study could also be applied for other transcription factors. Cell therapy for other diseases, such as heart

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diseases, diabetes mellitus, intestinal diseases, may also be developed with this autologous cell reprogramming concept.[33]

Conclusion

Based on the results of current study, design of recombinant plasmid for transfection of DNA transcription factors, which are produced in *E. coli* system and expressed in mammalian cells has been reported to be delivered to primary human fibroblast by ALMR system. Further study is needed to investigate the effect of transcription factors transfection on the production of protein encoded by hepatocyte marker genes.

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Authors Contribution

MR, BB, RDA and JAP were involved in concepting and planning the research and drafted the manuscript. MR and STW performed the data acquisition. MR, STW and NFM performed the data analysis. MR and NFM designed the figures and tables. MR, BB, STW and NFM aided in interpreting the results. BB, RDA and JAP took parts in giving critical revision of the manuscript. All authors have read and approved the final manuscript.

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