UNIVERSITI PUTRA MALAYSIA

EVALUATION OF MINICIRCLE-INDUCED PLURIPOTENT STEM CELL

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IB 2014 3

EVALUATION OF MINICIRCLE-INDUCED PLURIPOTENT STEM CELL

By

NASIBEH DANESHVAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

July 2014

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DEDICATION

Dedicated to my family, specially my parents and only brother for their love and infinite support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

EVALUATION OF MINICIRCLE-INDUCED PLURIPOTENT STEM CELL

By

NASIBEH DANESHVAR

July 2014

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Due to the risk of insertional mutagenesis, viral transduction has been increasingly replaced by non-viral methods to generate iPSCs. One technique that has not yet been explored enough is the use of "minicircle" DNA. The pMC.LGNSO plasmid is known as the parental DNA structure and is utilized to generate minicircle vectors which are episomal DNA vectors that are created as circular expression cassettes without any backbone of bacterial plasmid DNA. Their smaller molecular size gives them this ability to have more efficient transfections alongside sustained expression last for weeks in comparison to standard plasmid vectors which extremely work only for a week. Minicircle DNA also benefits from higher transfection efficiencies and longer ectopic expression. The Transfection efficiency of the minicircle vectors can be monitor during the plasmid transfections by EGFP expression under a fluorescence microscope.

This minicircle-based induction of pluripotency method is beneficial for obtaining transgene-free hiPSCs from human donors which are clinically applicable cell sources. Such techniques have the characteristic of developing patient or disease-specific cell lines to develop further translational and disease modeling researches. Here, we report the use of a single minicircle vector to generate transgene-free iPS cells from adult human mesenchymal stem cells. 50,000 cells/well was considered as the best amount of seeded cells to achieve the highest amount of transfection. Moreover, the results highlighted the utilization of antibiotics to avoid cell contamination. This experience also demonstrated that the 1:2 (3µg DNA/6 µL LTX) ratio and 150 µl of DNA/LTX complex in presence of medium at the transfection time will produce the highest level of genes expression.

The human MSCs were transfected twice using minicircle DNA/Lipofectamine LTX complex. The GFP-positive cells were observed by florescent microscopy 24 h post-transfection. Human ESC-like colonies with a tightly packed, domelike structure began

to appear 7 to 10 days after second transfection. The pluripotency of the derived iPSC lines was verified by qRT-PCR and immunochemical staining (ICC) techniques to possess pluripotent markers and cause embryoid body (EB) differentiation. Based on the results of RT-PCR, the expression of three embryonic germ layers markers showed that the EBs are successfully differentiated. Thus the iPSCs were shown to possess of the pluripotency of ESC thus has potential for use in cell-based treatment in human medicine. The developed method of iPSC producing in this study is measured as a fast and inexpensive technique, which benefits from using a non-viral vector to develop human iPSCs safely. This method also utilizes feeder-free cell culture technique to remove concerns of possible contaminations that could occur in the process of utilizing of mouse embryonic fibroblast feeder layers. This method can have a huge effect on making use of MSCs which are easily obtainable from clinical wastes discarded after child delivery.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENILAIAN MINILINGKAR-TERARUH PLURIPOTENSI STEM SEL

Oleh

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Sel dasar pluripotensi teraruh (iPSCs) manusia boleh diterbitkan daripada sel soma melalui proses pemprograman semula yang dipacu oleh penyataan suatu set faktor transkripsi tertakrif. iPSC ini juga menunjukkan sifat pembaharuan semula dan pluripotensi seperti sel dasar embrio (ESC) manusia dan dengan demikian boleh diguna untuk menjana jenis sel terbeza dengan banyak daripada ketiga-tiga lapisan germa. iPSC ini boleh dijanakan daripada hampir semua pesakit yang berlatar belakang berbeza, termasuk yang mengalami pemutatan genetik penyebab penyakit. Disebaliknya, penghasilan ESC manusia daripada sumber yang mempunyai latar belakang berbeza adalah mencabar kerana penggunaan embrio manusia adalah terhad dan diperbahasakan kerana isu etika. Teknologi iPSC telah membekal penyelidik dengan suatu alat unik untuk menerbitkan sel dasar khusus-penyakit untuk kajian dan mungkin juga untuk rawatan gangguan degenerasi mengguna sel autologus.

Oleh sebab ada risiko berlakunya mutagenesis selitan, transduksi virus telah beransur digantikan dengan kaedah bukan-virus untuk menjana iPSC. Satu teknik yang belum diteroka dengan betul ialah pengguna DNA minilingkar, suatu vektor baharu bebas DNA bakteria diguna dan berupaya untuk ternyata tinggi dalam sel. Berbanding plasmid, DNA minilingkar dimanfaatkan kerana kecekapan transjangkitan tingginya dan penyataan ektopi yang lebih lama. Justeru itu, objektif kajian ini ialah untuk mengembangkan iPSC melalui pemprograman semula sel dasar mesenkima (MSC) manusia. Plasmid MC.LGNSO ialah konstruk DNA yang mengandungi satu kaset empat faktor pemprograman semula iaitu Oct4, Sox2, Lin28, Nanog dan gen pelapor protein pendarfluor hijau (GFP). Sel dasar mesenkima manusia ini ditransjangkit dua kali dengan mengguna kompleks DNA minilingkar/Lipofectamine LTX. Sel GFP-positif telah dilihat melalui mikroskopi pendarfluor pada 24 jam pasca-tranjangkitan. Oleh sebab berlakunya pelarutan vektor DNA minilingkar semasa pemproliferatan, maka kehilangan penyataan GFP dalam iPSC berlaku secara beransur. Koloni bak hESC

dengan struktur padat, seperti kubah mula muncul 7 hingga 10 selepas transjangkitan kedua. Pada peringkat awal, koloni kekal GFP^+, tetapi dengan pengkulturan dan pempasajan secara berterusan, klon iPSC manusia bona fide ini menjadi GFP^-. Pluripotensi titisan iPSC yang diterbitkan ini telah disahkan melalui teknik qRT-PCR dan pewarnaa imunokimia (ICC) mempunyai penanda pluripotensi dan berlaku pembezaan jasad embriod (EB). Jasad embriod ini terdiri daripada kesemua tiga lapisan germa embrio. Dengan demikian, iPSCs ini mempunyai sifat pluripotensi ESC dan berpotensi untuk diguna dalam rawatan berasaskan sel dalam perubatan manusia.

ACKNOWLEDGEMENTS

In the name of Allah is the most gracious and most merciful. Praise is to Allah the cherisher and sustainer of the world. Show us the right way and O my lord! Advance us in knowledge.

Writing this thesis about the generation of induced pluripotent stem cells is very exciting and had even made me more interested in stem cell research for clinical applications.

My deepest gratitude and appreciation to my supervisor Prof. Dr. Rasedee Abdullah for his dynamic guidance and advice, and my co-supervisors Drs Rajesh Ramasamy, Syahrilnizam Abdullah and Norshariza Nordin for their help and support throughout the work.

I would also like to thank Dr. Fatemeh Shamsabadi, Dr. Parvaneh Mehrbod and Samira Eshghi my dear and close friends for keeping me company and giving me encouragement during my study and preparation of this thesis. I also thank all my colleagues and our staff of Institute of Bioscience, Universiti Putra Malaysia for their kind help and cooperation.

Last but not the least, my undying gratitude and love for my dearest Father Ali Daneshvar, my dearest mother Vajihe Ali pour and my loyal Brother Ahmad Reza Daneshvar for their inspiration, encouragement and support throughout my study.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
DMEM-F12	Dulbecco's Modified Eagle Medium-F12
DPCs	Dermal Papilla Cells
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
hESCs	human Embryonic Stem Cells
hiPSCs	human induced Pluripotent Stem Cell
ICM	Inner Cell Mass
iPSCs	induced Pluripotent Stem Cell
MEF	Mouse Embryonic Fibroblast
MSC	Mesenchymal Stem Cell
NTC	Negative Template Control
PBS	Phosphate-Buffered Saline
PI	Propidium Iodide
QRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
UC-MSC	Umbilical Cord Mesenchymal Stem Cells
UPM	Universiti Putra Malaysia

CHAPTER I

INTRODUCTION

Generation of induced pluripotent stem cells (iPSCs) can be performed using human somatic cells through a reprogramming process to express of a variety of defined transcription factors. These cells possess the same pluripotential and self-renewal properties as human embryonic stem cells (hESCs), and can be used to generate a large variety of differentiated cell types of any of the three germ layers (Ectoderm, mesoderm and endoderm). Since hESCs are from patients of various genetic and ethnical backgrounds, technical issues in production of the stem cells were challenging. The iPSCs technology has supplied researchers with a unique method to obtain patient and disease-specific stem cells for experimentation and treatment of diseases.

Using retroviruses or lentiviruses as delivery vectors to generate hiPSCs had increased the random integration of reprogramming genes into the host genome and may lead to insertional mutagenesis (Lai *et al*., 2011). On the other hand, Some of the reprogramming technologies had used oncogenes in combination with other reprogramming genes, which had the possibility of adverse effects such as risk of cancer development (Cheng *et al*., 2006; Maherali and Hochedlinger, 2008). Although using non-integrating viral (Stadtfeld *et al.*, 2008), nonviral episomal (Okita *et al.*, 2008) and excisional (Kaji *et al.*, 2009; Woltjen *et al.*, 2009) reprogramming techniques in mice have partly circumvented these concerns but translation of these methods into the production of safer hiPSCs derivatives is still difficult. This problem may be due to the low efficiency of the technique. Although hiPSCs could be produced by lentiviral transduction with consequent Cre-loxP elimination of reprogramming transcription factors (Soldner *et al.*, 2009), residual vector sequences in the genome will be a major drawback of this technique. Earlier studies had successfully derived transgene-free hiPSCs from neonatal foreskin fibroblasts using three episomal plasmids which express seven reprogramming transcription factors (Yu *et al.*, 2009). Transgene-free hiPSCs can also be derived from human foetal or neonatal cells using continual transduction of proteins with chemical treatments (Zhou *et al.*, 2009).

The aim of this study is to develop a method for generation of transgene-free hiPSCs from human umbilical cord mesenchymal stem cells (hUC-MSCs) under free-feeder layer condition and using a non-viral minicircle plasmid reprogramming system. The superiority of this method is the safe reprogramming of human adult somatic cells in the absence of viral sequences, genomic modification, feeder layer or proto-oncogenes (such as c-Myc) (Miura *et al.*, 2009). Although there is no general agreement on the reprogramming efficiency of this method, it is the purpose of the present study to assess feasibility of this technique in the reprogramming of hUC-MSCs to human iPSCs.

1.1 Hypothesis

It is postulated that the transfection of hUC-MSCs using minicircle DNA/Lipofectamine LTX complex can be an effective method in the reprogramming of the somatic cells to iPSCs based on the expression of four transcription factors, namely Oct4, Sox2, Nanog and Lin28 in a feeder-free medium.

1.2 Objective

The present study was to design with the following goals:

1. To determine the ideal ratio of transcription-cassette-harboring-minicircle DNA/ Lipofectamine LTX complex for efficient transfection of hUC-MSC
2. To induce iPSCs from hUC-MSC in feeder-free cell culture system
3. To characterize and assess the pluripotency of the reprogramming cells
4. To induce embryoid bodies (EBs) formation from iPSCs

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