UNIVERSITI PUTRA MALAYSIA

GENERATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS USING POLYCISTRONIC LENTIVIRAL VECTOR IN FEEDER- AND SERUM- FREE CULTURE

AKRAM FAISAL MAHMAUD

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By

AKRAM FAISAL MAHMAUD

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GENERATION OF MOUSE INDUCED PLURIPOTENT STEM CELLS USING POLYCISTRONIC LENTIVIRAL VECTOR IN FEEDER- AND SERUM- FREE CULTURE

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AKRAM FAISAL MAHMAUD

August 2015

Chair: Syahrilnizam Abdullah, DPhil
Faculty: Medicine and Health Sciences

Somatic cells can be directly reprogrammed into embryonic stem (ES)-like cells by the introduction of several transcription factors. The generated ES-like cells, known as induced Pluripotent Stem (iPS) cells have great potential for regenerative medicine as well as for fundamental and translational research. However, there have been serious concerns regarding the safety of the use of iPS cells clinically. Firstly, the conventional viral-mediated transfer strategy can lead to multiple transgene integrations into the genome of a cell, thus increasing the risk of insertional mutagenesis. Secondly, feeder layers and serum-containing media are traditionally required for the maintenance of iPS cells. Exposure of the cells to animal products from the media may lead to the risk of xeno contamination. For this reason, the full elimination of animal-sourced ingredients and the use of serum free media are necessary. In this study, a polycistronic lentiviral vector encoding four defined transcription factor genes was used to reprogram mouse tail-tip fibroblasts into iPS cells in a feeder- and xeno-free environment. The generated iPS cells exhibited the (i) morphology and self-renewal properties of ES cells, (ii) expression of ES cell-specific pluripotent markers, and (iii) potential to differentiate into the three major distinct specialized germ layers *in vitro*. The flow cytometry, immunocytochemistry, and RT-PCR analyses revealed high expression levels of ES cell markers such as Oct4, Nanog, Sox2, Klf4, c-Myc and SEEA-1. The iPS cells were also shown to have the potential to differentiate into neural precursor and neuron cells in culture, with greater than 95% of nestin, Pax6 and βIII-tubulin expression. Although the safety profile of the cells was not analysed, this body of work describes the successful generation of iPS cells from mouse tail-tip fibroblasts without the requirement of serum and a feeder layer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN SEL INDUK TERARUH *PLURIPOTENT* TIKUS MELALUI KAEDAH VEKTOR *POLYCISTRONIC LENTIVIRUS* TANPA LAPISAN *FEEDER* DAN MEDIUM BEBAS SERUM

Oleh

AKRAM FAISAL MAHMAUD

Ogos 2015

Pengerusi: Syahrilnizam Abdullah, DPhil
Fakulti: Perubatan dan Sains Kesihatan

Pengaturcaraan semula sel dewasa kepada sel induk-serupa embrio boleh diadakan dengan menggunakan beberapa faktor transkripsi. Sel induk-serupa embrio ini dikenali sebagai *induced pluripotent stem (iPS) cells* dan mempunyai potensi yang tinggi untuk digunakan didalam bidang perubatan regeneratif dan juga di dalam kajian asas dan gunaan. Namun begitu, penggunaan sel iPS untuk tujuan perubatan adalah tidak selamat kerana beberapa faktor. Pertama, penyampaian gen secara tradisional dengan menggunakan virus boleh menyebabkan integrasi rawak ke dalam genom, yang boleh mendatangkan mutagenasi penyisipan. Selain itu, penggunaan lapisan *feeder* dan medium yang mengandungi serum untuk memelihara sel IPS boleh membawa kepada pencemaran xeno. Oleh itu, segala komponen daripada sumber haiwan perlu dielakkan. Dalam kajian ini, vektor *polycistronic* lentivirus yang mengandungi empat faktor transkripsi telah digunakan untuk pengaturcaraan semula sel *fibroblast* ekor mencit kepada sel iPS, tanpa penggunaan lapisan *feeder* dan di dalam keadaan bebas kontaminasi *xeno*. Sel iPS yang dihasilkan daripada kajian ini menunjukkan (i) morfologi dan pembaharuan kendiri yang serupa dengan sel induk embrio, (ii) ekspresi penanda *pluripotent* yang spesifik, dan (iii) keupayaan untuk berubah kepada tiga lapisan germa secara *in vitro.* Penanda ekspresi Oct4, Nanog, Sox2, Klf4, c-Myc and SEEA-1 dicatat pada paras yang tinggi yang ditentukan melalui kaedah *flow cytometry, immunocytochemistry* dan RT-PCR. Di samping itu, sel iPS yang dihasilkan berpotensi untuk berubah kepada sel pra-neuron dan sel neuron secara *in vitro* dengan paras Pax6, nestin and βIII-tubulin yang melebihi 95%. Walaupun profil keselamatan sel IPS tersebut tidak dianalisa, projek ini telah berjaya menghasilkan sel iPS yang tulen melalui ujikaji ciri-ciri sel iPS yang telah dijalankan.

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I certify that a Thesis Examination Committee has met on 17th August 2015 to conduct the final examination of Akram Faisal Mahmaud on his thesis entitled "Generation of Mouse Induced Pluripotent Stem Cells Using a Polycistronic Lentiviral Vector in a Feeder- and Serum- Free Culture" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy in Genetics.

Members of the Thesis Examination Committee were as follows:

Rajesh a/l Ramasamy, PhD
Associate Professor
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Cheah Yoke Kqueen, PhD
Associate Professor
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Abhimanyu a/l Veerakumarasivam, PhD
Senior Lecturer
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Shigeki Sugii, PhD
Assistant Professor
Duke-NUS Graduate Medical School
Singapore
(External Examiner)

_____

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy in Genetics. The members of the Supervisory Committee were as follows:

Syahrilnizam Abdullah, PhD
Associate Professor
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Norshariza Binti Nordin, PhD
Senior Lecturer
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Lai Mei I, PhD
Senior Lecturer
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature:
Name of Chairman of Supervisory Committee:
Syahrilnizam Abdullah, D.Phil.

Signature:
Name of Member of Supervisory Committee:
Norshariza Binti Nordin, PhD

Signature:
Name of Member of Supervisory Committee:
Lai Mei I, PhD

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometer
3D	three dimensional
ANOVA	analysis of variance
AP	alkaline phosphatase
ATCC	american type culture collection
CMV	cytomegalovirus
CMVenh	human cytomegalovirus enhancer/promoter
c-Myc	regulator gene that codes for transcription factor activity
DAPI	4′,6-diamidino-2-phenylindole
DMEM	dulbecco's modification of eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E2A	equine rhinitis a virus
ECM	extracellular matrix
ES	embryonic stem cells
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
hES	human embryonic stem cells
hiPS cells	human induced pluripotent stem cells
HLA	human leukocyte antigen

HSCs	hematopoietic stem cells
ICC	immunocytochemistry
ICM	inner cell mass
iPS cells	induced pluripotent stem cells
kDa	kilo-dalton
Klf4	kruppel-like factor 4
LCR	locus control region
LIF	leukemia inhibitory factor
LTRA	long-term repopulation ability
LTRs	long-term repeats
LVs	lentiviral vectors
MEFs	mouse feeder fibroblasts
mES	mouse embryonic stem cells
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
MoMLV	moloney murine leukemia virus
NANOG	homeobox protein known to regulate pluripotency
ng	nano-gram
NPC	neural progenitor cell
Oct4	octamer-4
OSKM	oct4/sox2/klf4/c-myc
P2A	porcine teschovirs-1
PBS	phosphate buffered saline

PCR	polymerase chain reaction
pDNA	plasmid dna
RA	retinoic acid
RNA	ribonucleic acid
RRE	rev-response element
RT-qPCR	real time quantitative polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SCNT	somatic cell nuclear transfer
SIN	self-inactivating
Sox2	sex-determining region y-box-2
SSEA	stage specific embryonic antigen
T2A	*thosea asigna* virus
Tuj1	neuronal class iii β-tublin
UPM	universiti putra malaysia
VSV-G	vesicular stomatitis virus glycoprotein g

CHAPTER 1

INTRODUCTION

1.1 Background

A mature cell's genome was thought to be everlastingly locked in a somatic state and unable to revert into a fully embryonic stem (ES) cell state. However, in 1962, Sir John B. Gurdon entirely altered this paradigm in producing a fully functional tadpole from an unfertilized egg containing a nucleus from a differentiated intestinal epithelium cell of a mature frog (Gurdon, 1962[a, b]). In 2006, Takahashi and Yamanaka demonstrated in an astonishingly simple strategy that the introduction of specific pluripotency genes into mature cells could change the cells into ES-like cells. These cells were named induced Pluripotent Stem (iPS) cells (Takahashi and Yamanaka, 2006). In 2007, the iPS cells findings were independently verified by Jaenisch's group (Hanna *et al*., 2007), while Hochedlinger and colleagues cloned iPS mouse (Stadtfeld *et al*., 2010). The iPS cells can subsequently be coaxed into a different range of differentiated cell types in the body, such as cardiovascular and hematopoietic lineages (Schenke-Layland *et al*., 2008), sperms (Okita, Ichisaka and Yamanaka, 2007; Hayashi *et al*., 2011), cardiomyocytes (Qian *et al*., 2012) and retinal cells (Hirami *et al*., 2009). In 2012, both John B. Gurdon and Shinya Yamanaka were awarded a shared Nobel Prize in Physiology or Medicine for their discoveries in that a somatic cell can convert back into a pluripotent stem cell state.

1.2 Problem Statements

Successful reprogramming of a unipotent state into a pluripotent state could be achieved by using different gene transfer strategies, including retroviral (Takahashi and Yamanaka, 2006; Takahashi *et al*., 2007) and lentiviral vectors (Blelloch *et al*., 2007; Hotta *et al*., 2009). However, these methods required transgene integration into the genome of a cell, which may lead to accidental activation of oncogenes (Kiskinis and Eggan, 2010). Therefore, alternative strategies such as non-integrating adenovirus expression vectors (Stadtfeld *et al*., 2008[c]), virus-free plasmids (Okita *et al*., 2008[b]; Gonzalez *et al*., 2009), synthetic messenger RNA (mRNA) modified (Warren *et al*., 2010; Mandal and Rossi, 2013), Cre/loxp system (Kaji *et al*., 2009; Soldner *et al*., 2009), piggyBac (Woltjen *et al*., 2009; Yusa *et al*., 2009) and protein reprogramming strategies (Zhou *et al*., 2009) have been developed. Unfortunately, these approaches are inferior compared to the retrovirus or lentivirus methods (Maherali and Hochedlinger, 2008).

As the lentivirus method still remains the most efficient reprogramming strategy (Winkler *et al*., 2010; Stadtfeld and Hochedlinger, 2010), more efforts have focused on generating improved and safer vectors (Maherali *et al*., 2008; Hockemeryer *et al*., 2008). One of the achievements is the development of a polycistronic lentiviral vector that encodes Yamanaka genes, which are driven by a single promoter. Reprogramming efficiency was reported to reach between 70- (Mali *et al*., 2008) to 100-fold (Zhao *et al*., 2008). This system also reduces the viral copy number integration in the transduced cells and minimizes the risk of transgene silencing (Sommer *et al*., 2009; Carey *et al*., 2009[a, b]; Shao *et al*., 2009; Xu *et al*., 2009). As yet, successful reprogramming of somatic cells into iPS cells using the polycistronic lentiviral vector (without a reporter gene or drug selection) in ES serum and feeder- free medium has not been reported.

Cellular reprogramming has been traditionally performed on animal feeder layers to maximize the production of ES-like colonies formation (Ährlund-Richter *et al*., 2009; MacArthur *et al*., 2012), along with the use of fetal bovine serum and xeno-containing products that provide an excellent environment for the maintenance and proliferation of iPS cells in an undifferentiated state. However, this approach is not ideal for clinical relevance (De Sousa, Galea and Turner, 2006; Ährlund-Richter *et al*., 2009). Hence, the full elimination of animal- sourced ingredients and the use of serum- free culture media are necessary to adhere to the Standard for Biological Ingredients (Nakagawa *et al*., 2014).

Due to these reasons, this study attempts to reprogram mouse-tail tip fibroblasts into iPS cells using a polycistronic lentiviral vector in feeder- and serum free culture conditions.

1.3 Hypothesis

It is hypothesized that tail-tip mouse fibroblasts can be reprogrammed into induced pluripotent stem cells using a polycistronic lentiviral vector that lead to ectopic expression of pluripotency-associated factors in a feeder and serum-free culture.

1.4 General Objective

The main objective of this study is to generate mouse induced pluripotent stem cells from tail-tip fibroblast using polycistron lentiviral vector in feeder- and xeno-free environment.

1.4.1 Specific Objectives

The specific objectives for this study are as to:

1. isolate and maintain mouse tail-tip fibroblasts in culture,

2. produce and assess the polycistronic lentiviral vector that encodes the "Yamanaka" reprogramming factors,

3. reprogram mouse tail-tip fibroblasts into iPS cells using the polycistronic lentiviral vector in a feeder/serum free condition,

4. characterize the derived iPS cells,

5. generate mouse neural precursor cells and neurons from the generated iPS cells.

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