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DYNAMICS OF EPIGENETIC CONTROL ON TRANSGENE EXPRESSION MEDIATED BY LENTIVIRUS IN MOUSE PLURIPOTENT STEM CELLS

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By

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DYNAMICS OF EPIGENETIC CONTROL ON TRANSGENE EXPRESSION MEDIATED BY LENTIVIRUS IN MOUSE PLURIPOTENT STEM CELLS

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June 2018

Chairman : Syahrilnizam Abdullah, D.Phl
Faculty : Medicine and Health Sciences

Autologous transplantation of patient specific iPS-derived cells for *ex vivo* gene therapy application could be the only curative option for patients that do not have compatible donor. One of the limitations of successful gene therapy is short-lived duration of transgene expression in mammalian cells cause by DNA methylation. Pluripotent stem cells have shown to possess minimal genomic methylation profile. Therefore, it is hypothesized that improve durability of transgene expression could be achieved if the transgene is introduced into a cell while it is still in a pluripotent state. The aim of this study is to assess durability of transgene expression in mouse pluripotent stem (iPS and ES) cells transduce with lentivirus carrying GFP reporter gene driven by either human elongation factor (EF1α) or cytomegalovirus (CMV) promoter and to dissect the factors that influence the duration of transgene expression in both cells. LV/EF1α/GFP and LV/CMV/GFP transduced iPS cells exhibited significant GFP expression (>80% expressing cells) with persistent mean fluorescent intensity (MFI) through out the 30 days of the study period and beyond. In ES cells, LV driven by either EF1α or CMV promoter presented lower GFP expression (>50%) but surprisingly, LV/EF1α/GFP showed significantly higher MFI when compared to LV/CMV/GFP. Analysis on the integrated copy of transgene in all transduced cells demonstrated similar copy number in the cells' genome. Significant increase in GFP intensity following 5aza-C treatment was observed in ES cells, implicating the effect of DNA methylation in transgene silencing. However, this effect was not observed in transduced iPS cells. DNA methylation study showed that the transgene promoter and the GFP region of the provirus in ES cells had higher DNA methylation when compared to that of provirus in iPS cells. The transduced iPS cells were then induced to diffeentiate into hematopoietic stem cells (HSCs). The HSCs colony derived from the LV/EFIα/GFP transduced iPS cells showed GFP^+ cells of about 68.8%, while the LV/CMV/GFP transduced iPS-derived HSCs exhibited GFP^+ cells of about 57.8%. This study demonstrated that, (1)

persistent transgene expression mediated by LV carrying GFP driven by both EF1α and CMV has been observed in iPS cells. However, a significant decrease in GFP expression in ES cell was seen, (2) GFP expression in ES cells is affected by a transgene silencing mechanism and this phenomenon was not observed in iPS cells, and (3) both LV/EF1α/GFP and LV/CMV/GFP transduced cells were able to generate HSCs upon directed differentiation and the cells retained considerable level of GFP expression. These findings could potentially be beneficial in the application of iPS-derived cells for prolonged therapeutic gene expression and could be translated in clinic for a persistent correction of genetic disorders using gene therapy technology in the future.

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

KAWALAN EPIGENETIK DINAMIK PADA EKSPRESI TRANSGEN PERANTARAAN LENTIVIRUS DALAM TIKUS SEL STEM PELBAGAI POTENSI

Oleh

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Bagi pesakit yang tidak mempunyai penderma yang serasi, pemindahan sel atau tisu *autologous* sel iPS yang telah melalui terapi genetik secara *ex vivo*, adalah merupakan satu-satunya pilihan untuk rawatan. Bagaimanapun, salah satu batasan untuk terapi genetik yang berjaya adalah jangka hayat pendek ekspresi transgen tersebut. Salah satu mekanisma gen kawalan yang lazim membawa kepada penyeyapan transgen dalam sel mamalia adalah melalui metilasi DNA. Menarik sekali sel iPS menunjukkan profil metilasi DNA yang amat rendah. Adalah dijangkakan bahawa perubahan yang bermakna kepada tempoh ekspresi transgen boleh dikecapi sekiranya transgen tersebut didedahkan ke dalam sel semasa ianya masih dalam keadaan pelbagai potensi. Oleh itu, tujuan kajian ini adalah untuk menentukan keupayaan pengekspresan transgen untuk tempoh yang panjang dalam sel stem pelbagai potensi, khususnya dalam sel stem janin (ES) dan sel iPS tikus, selain mengenalpasti faktor-faktor yang boleh mempengaruhi tempoh ekspresi transgen dalam kedua-dua sel ini. Lentivirus (LV) dengan protein pendarfluor hijau (GFP) yang didorong sama ada oleh promoter faktor pemanjangan manusia (EF1α) ataupun promoter cytomegalovirus (CMV) telah digunakan dalam kajian ini. Sel iPS yang telah dijangkiti oleh LV/EF1α/GFP dan LV/CMV/GFP menunjukkan ekspresi GFP yang signifikan (>80% sel yang nyata menunjukkan ekspresi), dengan tahap intensiti purata pendaflour (MFI) berterusan sepanjang 30 hari kajian. Dalam sel ES pula, LV yang didorong sama ada oleh faktor EF1α ataupun CMV, menunjukkan ekspresi GFP yang rendah (>50%). Namun apa yang mengejutkan adalah sel ES yang telah dijangkiti oleh LV/EF1α/GFP menunjukkan signifikasi MFI yang tinggi apabila dibandingkan dengan LV/CMV/GFP. Analisis ke atas salinan transgen yang dicantum ke dalam genom semua sel yang dijangkiti menunjukkan bilangan salinan transgen yang sama. Sel yang dijangkiti kemudiannya dirawat dengan ejen nyahmetilasi iaitu 5aza-C. Sel ES yang menunjukkan penurunan dalam ekspresi transgen, telah menunjukkan kenaikan intensiti GFP yang signifikan dengan peningkatan

konsentrasi 5aza-C. Ini menunjukkan kesan metilasi DNA ke atas penindasan transgen tersebut. Walau bagaimanapun, kesan ini tidak kelihatan pada sel iPS. Kajian metilasi DNA menunjukkan bahawa kedua-dua *promoter* dan bahagian GFP *provirus* di dalam sel ES tersebut mempunyai profil metilasi DNA yang tinggi berbanding dengan sel iPS. Sel iPS yang dijangkiti kemudiannya diaruh untuk menjadi sel stem hematopoietik (HSCs). Koloni HSCs yang terbentuk daripada sel iPS yang dijangkiti oleh LV/EF1α/GFP menunjukkan sel GFP^+ sebanyak 68.8%, manakala HSCs yang terbentuk daripada iPS sel yang dijangkiti oleh LV/CMV/GFP menunjukkan sel GFP^+ sebanyak 57.8%. Kesimpulannya, kajian ini menunjukkan bahawa, (1) ekspresi transgen yang berterusan daripada LV dengan GFP yang didorong oleh kedua-dua EF1α dan CMV telah diperhatikan dalam sel iPS. Walaubagaimanapun, terdapat penurunan ekspresi transgen yang ketara dalam sel ES, (2) ekspresi GFP dalam sel ES terjejas akibat mekanisma penyenyapan transgen dan fenomena ini tidak kelihatan dalam sel iPS, dan (3) kedua-dua sel pelbagai potensi yang dijangkiti oleh LV/EF1α/GFP dan LV/CMV/GFP berupaya menjana HSCs teraruh yang mengekalkan tahap ekspresi GFP. Penemuan ini boleh memberi manfaat untuk aplikasi sel iPS dengan ekspresi gen terapeutik yang berpanjangan dan mungkin dapat digunakan di dalam bidang perubatan untuk merawat penyakit genetik dengan berterusan melalui teknologi terapi gen.

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I certify that a Thesis Examination Committee has met on 4 June 2018 to conduct the final examination of Suleiman Yusuf Alhaji on his thesis entitled "Dynamics of Epigenetic Control on Transgene Expression Mediated by Lentivirus in Mouse Pluripotent Stem Cells" 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.

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LIST OF ABBREVIATIONS

5-AzaC	5-Azacystidine
AAV	Adeno-associated virus
Ad	Adenovirus
ANOVA	Analysis of variance
ATCC	American type culture collection
ANOVA	Analysis of variance
bp	Base pair
BSA	Bovine serum albumin
ChiP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CpG	Cytosine guanine
cPPT	Central polypurine tract
c-Myc	Regulator of gene that code for transcription factor activity
DAPI	4'6-diamidino-2-phenyndole
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimthyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxyribibonucleotides triphosphates
E2A	Equine rhinitis a virus
EB	Embryoid bodies
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraatic acid

EF1α	Human elongation factor
EGF	Epidermal growth factor
ESC	Embryonic stem cells
FACS	Fluorescence activated cells sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flt-3	FMC-like tyrosine kinase 3
HAT	Histone acetyltransferases
HCL	Hydrochloric acid
HDAC	Histone deacetylases
hES	Human embryonic stem cells
hiPS	Human induced pluripotent stem cells
HLA	Human leukocyte antigen
HP 1	Heterochromatin protein 1
HS	Hypersensitive site
HSC	Haematopoietic stem cells
HSCT	Haematopoietic stem cells transplatation
ICC	Immunocytochemistry
ICM	Inner cell mass
IFN	Interferon
IL	Interleukin
iPS cells	Induced pluripotent stem cells
IN	Integrase
kb	Kilo base
Kda	Kilo Dalton
Klf4	Kruppel-like factor 4
LB	Luria bertani

LCR	Locus control region
LIF	Leukamia inhibitory factor
LTRs	Long terminal repeats
LV	Lentivirus
MBD	Methyl-CpG-binding protein
MeCP	Methyl-CPG-protein
MEF	Mouse feeder fibroblast
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MoMLV	Moloney murine leukaemia virus
ng	Nanogram
Nanog	Homeobox protein known to regulate pluripotency
NPC	Neural precursor cells
NSC	Neural stem cells
OCT4	Octomer 4
OSKM	OCT4/SOX2/klf4/c-MYC
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
pLV	Lentiviral plasmid
RNA	Ribonucleic acid
RRE	Rev response element
RT-qPCR	Real time quantitative polymerase chain reaction
RT-PCR	Reverse polymerase chain reaction
RT	Room temperature
rpm	Revolution per minute

SCNT	Somatic cell nuclear transfer
SCID	Severe combined immunodeficiency
SIN	Self-inactivatin
SOX2	Sex determinant region y-box-2
SCID-X1	X-link severe-combined immunodeficiency
TAE	Tris acetate EDTA
TNF	Tumor necrosis factor
TSA	Tricostatin A
UPM	Universiti Putra Malaysia
VSV-G	Vesicular stomatitis glycoprotein
WBC	White blood cells
WPRE	Woodchuck post-transcriptional regulatory element

CHAPTER 1

INTRODUCTION

Delivery of genes into mammalian cells to supplement a defective gene via gene therapy may be the only curative option for many genetic diseases (Hoban, Orkin, & Bauer, 2016; O'Connor & Crystal, 2006). Various approaches using either non-viral or viral gene transfer systems have been employed for the delivery of genetic material into mammalian cells for effective gene expression in biomedical research (Hahn & Scanlan, 2010). Although non-viral gene delivery system has proven to be effective, viral mediated gene transfer is far more efficient for mammalian gene transfer. This is because some viral vectors such as Adenoa-associated virus (Marcos-Contreras *et al.* 2016), Retrovirus and Lentivirus can integrate into the host genome and provide stable transgene expression (Nayerossadat *et al.,* 2012). Unfortunately, one of the limitations of viral gene transfer vectors is the silencing of therapeutic gene or transgene expression after a certain period of adequate expression, caused by epigenetic influences in mammalian cells (Dupont *et al.*, 2015). This issue has been recognized as one of the major challenges in achieving persistent transgene expression in various gene delivery applications (Bestor, 2000; Chen, *et al.*, 2004). However, control of gene expression via epigenetics is a fundamental mechanism in mammalian cell proliferation, cellular differentiation, and genome stability (Xu. D & Juliano, 2005). It also plays a vital role in providing defense against exogenous genes serving as protective measures upon invading foreign DNA sequence (Matzke, Mette, & Matzke, 2000). Therefore, transgene being an exogenous DNA sequence may be recognized by cellular epigenetic machinery as a target for host protective defense measures, leading to inhibition of its expression (Yoder *et al.,* 1997).

Effective gene delivery strategy that provides sustained and long-term therapeutic benefits remain as one of the crucial hindrances for successful application of gene therapy in clinics (Nayerossadat *et al.*, 2012). Unfortunately, none of the existing gene delivery strategies are efficient enough to provide effective gene delivery and reliable transgene expression in target cells (Brunetti-Pierri, 2017; Wilson, 2009).

Lentivirus has been reported to remarkably transduce both dividing and non-dividing cells (Cockrell & Kafri, 2007). It is relatively safe, especially with the emergence of self-inactivating Lentivirus (Albrecht *et al.,*). The SIN LV has a deletion of few hundred nucleotides in the Long Terminal Repeat region, which abolishes the functionality of the LTR promoter activity and reduces the chances of activating oncogenes by insertional mutagenesis (Christophe Delenda, 2004). Unlike other viruses such as Adenovirus and Sendai, lentivirus is able to integrate its genome into the host, and able to carry large gene sequences. Extended duration of transgene expression via LV gene delivery in pluripotent cells was shown by Pfeifer, Bannister & Kouzarides, leading to the generation of transgenic mice that retained transgene expression for many several

generations (Bannister *et al*,. 2011). Despite successes, the main focus of the study was to produce transgenic animals, and not directed towards its use in gene therapy. Thus, no epigenetic analysis was performed in the study. Lentiviral vectors have shown promising outcome in recent gene therapy clinical trials for hematological related genetic diseases (Ferrari *et al*., 2017). In the reports, remarkable results were obtained in sickle cell anemia gene therapy clinical trials using Lentivirus carrying healthy ß-globin gene (LentiGlobin) (Ribeil *et al.,* 2017). The LentiGlobin also demonstrated extended transgene expression after transplantation and engraftment of the genetically transduced hematopoietic stem cells.

Many other studies have shown prolonged transgene expression in pluripotent cells transduced with LV (Kwok-Keung *et al.*, 2008). This efficacy of transgene expression was found to be promoter dependent in undifferentiated stem cells and varies upon directed differentiation (Hong *et al.*, 2007; Wang R, *et al.,* 2008; Herbst *et al.,* 2012a) . Different constitutive promoters in the transgene construct may modulate duration of transgene expression at varying degree (Qin *et al.*, 2010). For example, CMV promoter is known to drive inferior transgene expression compared to PGK, EF1α and CAG in pluripotent cells (Norrman *et al.,* 2010). It is speculated that CMV promoter may be sensitive to methylation compared to other promoters such as EF1α and PGK, which may be methylation resistant (Wang X. *et al.*, 2017). This may have contributed to the varying degree of transgene expression observed in pluripotent stem cells transduced with LV driven by different constitutive promoters (Hong *et al.,* 2007). Nevertheless, to date, there are limited reports underlying the mechanism of promoter dependent transgene expression variation mediated by LV in mammalian cells (Xia, *et al,.* 2007).

Application of pluripotent cells in gene therapy and regenerative medicine is at the forefront of clinical medicine (Ilic, *et al.*, 2015). Pluripotent cells have unique ability to differentiate into three primary germ layers (ectoderm, mesoderm and endoderm), which eventually generate all cell types. Pluripotent cells have been recognized as invaluable tool for understanding mechanisms in cell specific lineage differentiation, unravelling the mechanism of disease progression, drug testing, and developmental defects, as well as for the prospective use in regenerative and gene therapy of many genetic diseases. Pluripotent cells can be derived from either inner cell mass of an early developing embryo (blastocyst stage) (Bryja, *et al.,* 2006) as an embryonic stem (ES) cells, or by reprograming of somatic cells (such as skin cells) into a pluripotent state (Takahashi & Yamanaka, 2006). These cells are known as induced pluripotent stem cells. Numerous studies highlighted strong similarities between ES and iPS cells in term of functionality and molecular characteristics (Choi *et al*., 2015). Although, both ES and iPS cells are pluripotent stem cells, there is a marked variation in their global gene expression profile and imprinted genes clusters. However, they do not substantially differ from each other, especially in their differentiation potentials. It is not clear whether these variations may have a significant functional biological difference (Marks *et al.,* 2012).

DNA methylation is one of the major epigenetic effects that was reported to be associated with transgene silencing in mammalian somatic cells (Brooks *et al.*, 2004). Studies have shown that DNA methylation is minimal during embryogenesis (Smith *et al.,* 2012), and this observation was reported to be similar with that of iPS cells. As development progresses, DNA methylation dramatically increases along the developmental stages as the cells become more specialized (Smith *et al.,* 2012). Therefore, an association between pluripotency and epigenetics is suspected as DNA methylation increases exponentially after cells' specific specialization (Meshorer & Misteli, 2006a). Thus, in order to circumvent the silencing effects by epigenetic machinery in gene therapy application, the transgene could be introduced while the cells are still at pluripotent state, while their epigenetic profile is still plastic, and the cells might recognize the transgene as self.

Although, there have been many studies showing impressive duration of transgene expression in pluripotent cells, none have compared the duration of transgene expression between iPS and ES cells simultaneously, and none have compared the efficacy of the two most commonly used constitutive promoters, CMV and EF1α, in the pluripotent cells. In addition, no study has evaluated the transgene methylation profiles between the two promoters following integration in the genome of pluripotent cells. Therefore, this study may be beneficial as pluripotent cells, especially iPS cells, can be potentially used for autologous *ex vivo* gene therapy application in the future.

Therefore, **it is hypothesized that prolonged duration of transgene expression via lentiviral mediated gene delivery could be achieved if the transgene is delivered into the cells during pluripotency state.** The possible outcome of persistent duration of transgene expression in the LV transduced pluripotent cells may be due to the absence or minimal DNA methylation on the promoter and/or the reporter gene.

The general objectives of the study:-

To assess persistency of transgene expression in pluripotent cells transduced with lentivirus carrying reporter gene driven by either CMV or EF1α promoter, and to dissect the factors that contribute to the prolonged duration of transgene expression.

Specific objectives: -

1. To produce lentivirus carrying Green Fluorescent Protein reporter gene driven by CMV or EF1α promoters.
2. To assess the duration of transgene expression in different pluripotent cells lines.
3. To ascertain whether absence or limited DNA methylation on the transgene construct is a factor that contributes to extended duration of transgene expression.
4. To differentiate transduced pluripotent cells that show evidence of extended duration of transgene expression into hematopoietic progenitor cells.

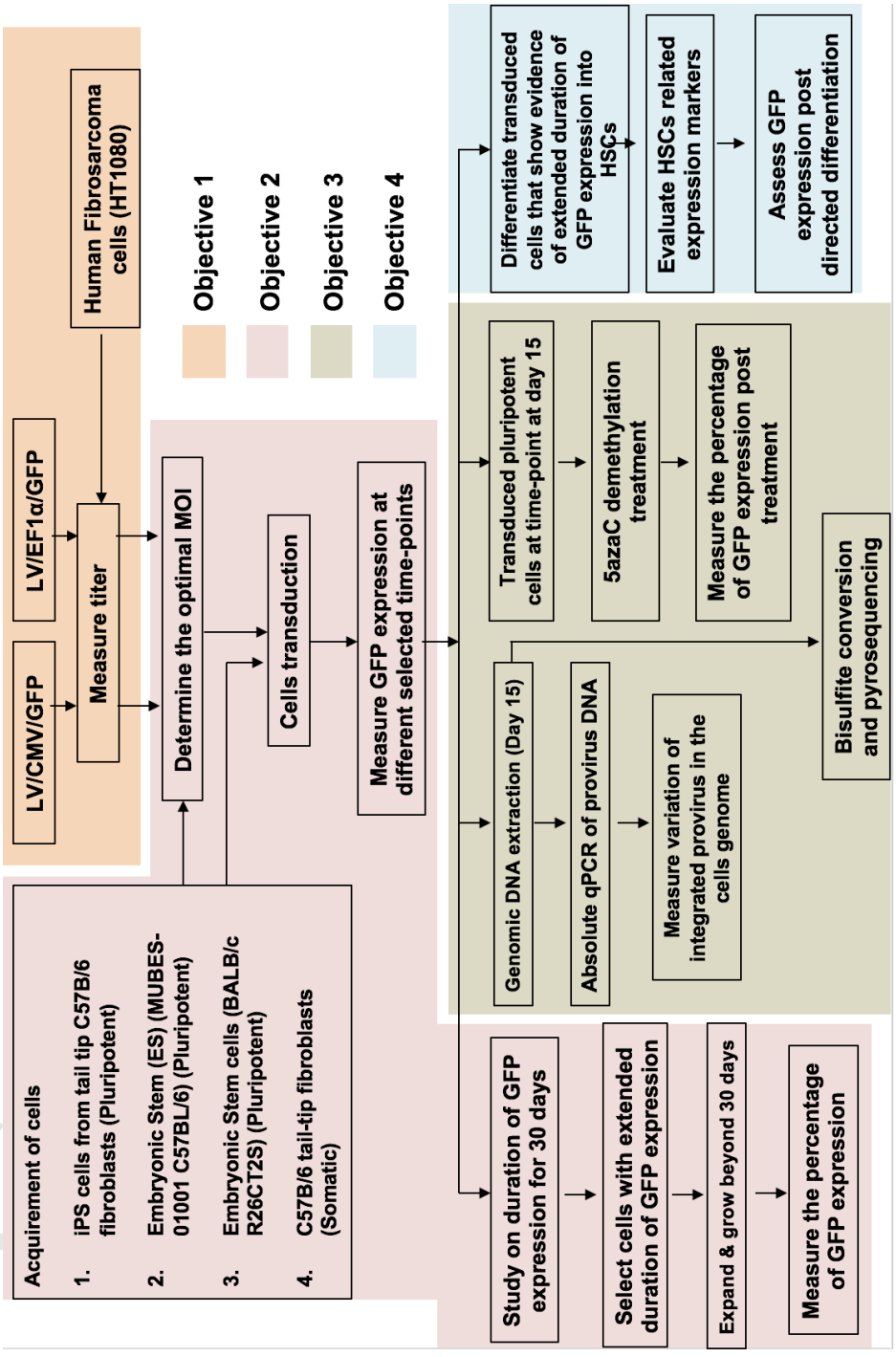

Figure 1.1: The flow chart described the general activities in each chapter of this study. It also reflected the experimental design of each objective in this project.

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