UNIVERSITI PUTRA MALAYSIA

CpG-FREE PLASMID DNA EXHIBITS LIMITED In Vitro REPORTER GENE EXPRESSION DUE TO TRANSCRIPTIONAL INCONGRUITY

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By

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

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The use of efficient vector is essential in gene therapy, which has the promising potential to treat not only genetic diseases but also acquired diseases. Although plasmid DNAs (pDNA) have been shown to be a much safer alternative compared to viral vectors, limitations, like detrimental inflammatory response and transient transgene expression, hinder its clinical application. This is mainly due to the presence of unmethylated cytosine guanine (CpG) motifs on pDNA. As a solution, CpG-free pDNA (p0CpG) was developed, where CpG motifs are completely depleted from the pDNA backbone and transgene. In addition, the safety and efficacy of the p0CpG were further reinforced with significant therapeutic effect observed in a cystic fibrosis clinical trial. Given these points, extending the application of clinically relevant p0CpG towards *ex vivo* gene therapy would be a novel approach to treat many disorders. However, it was recently reported that the depletion of CpG motifs from transgene could affect the *in vitro* gene expression negatively. Similar results may also extend to p0CpG, as the p0CpG is also devoid of CpG motifs. Thus, the main objective of this study is to evaluate the performance of p0CpG *in vitro*, specifically the effect of CpG depletion from the transgene.

First, expression of luciferase reporter gene was compared between p0CpG and a CpG-containing pDNA in multiple human cell lines. Surprisingly, pCpG-free exhibited poor expression *in vitro* that contradicts published *in vivo* reports. To determine if this was due to the CpG depletion from transgene, two novel pDNAs were constructed where each has CpG-free

backbone with Green Fluorescent Protein (GFP) gene that is either CpG-free (pZGFP) or contains 60 CpGs (pRGFP). pZGFP showed significantly inferior transgene expression when compared to pRGFP, despite using different gene transfer agents (lipid or polymer-based) or cell types (human, mouse cell lines & primary mouse cells). Besides that, there was no significant difference in pDNA copy number and toxicity between the pDNAs. Therefore, the low expression of pZGFP is attributed to CpG depletion from transgene. Upon further investigation, the lower expression of pZGFP was not due to mRNA export but was due to lower transcriptional rate as observed in mRNA distribution studies. In conclusion, complete depletion of CpG motifs from transgene in p0CpG resulted in reduced transcription rate. Data obtained from this study could be used to improve and optimize p0CpG for *ex vivo* gene therapy approach in the future.

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

PLASMID DNA BEBAS-CpG MENGHASILKAN EKSPRESI GEN PELAPOR *In Vitro* YANG TERBATAS KERANA KECANGGUNGAN TRANSKRIPSI

Oleh

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Penggunaan vektor yang efisien adalah penting dalam terapi gen, yang berpotensi untuk merawat bukan sahaja penyakit genetik, malah penyakit rumit juga. Walaupun plasmid DNA (pDNA) merupakan alternatif yang lebih selamat berbanding vektor virus, aplikasi klinikal pDNA adalah terhad disebabkan oleh tindak balas keradangan yang membahayakan dan penghasilan ekspresi gen terapeutik yang terbatas. Kesan-kesan sampingan ini berpunca daripada motif cytosine-guanine (CpG) yang tidak dimetil. Sebagai penyelesaian, pDNA bebas-CpG (p0CpG) dihasilkan, di mana motif CpG dilupuskan dari tulang belakang pDNA dan transgen. Disamping itu, keselamatan dan keberkesanan p0CpG diperkukuhkan lagi apabila pengunaan p0CpG didapati berkesan untuk merawat pesakit cystic fibrosis di peringkat ujian klinikal. Oleh itu, pelanjutan penggunaan p0CpG ke arah terapi gen *ex vivo* akan menjadi satu pendekatan rawatan baru yang mampu dimanfaatkan ke atas pelbagai penyakit. Walaubagaimanapun, terbitan terkini melaporkan bahawa pelupusan motif CpG daripada transgen akan memberi kesan negatif pada ekspresi transgen *in vitro*. Kemungkinan, hasil terbitan ini boleh diperhatikan pada p0CpG kerana CpG motif juga dilupuskan dari p0CpG. Oleh itu, objektif utama kajian ini adalah untuk menilai prestasi p0CpG *in vitro*, khususnya pada kesan pelupusan CpG dari transgen.

Pertama, ekspresi gen pelapor luciferase telah dibandingkan di antara p0CpG (pCpG-free) dan pDNA yang mengandungi CpG (pCpG-rich) pada beberapa jenis sel manusia *in vitro*. Sebaliknya, pCpG-free menghasilkan

ekspresi transgen yang terhad di *in vitro* yang bercanggah dengan penemuan kajian *in vivo* yang diterbitkan. Untuk menentukan sama ada penemuan ini disebabkan oleh pelupusan CpG dari transgen, dua pDNA telah dicipta di mana masing-masing mempunyai tulang belakang bebas-CpG yang sama, tetapi mempunyai Green Fluorescent Protein (GFP) transgen yang berbeza, sama ada bebas-CPG (pZGFP) atau mengandungi 60 CpG (pRGFP). pZGFP menunjukkan kadar ekspresi jauh lebih rendah berbanding pRGFP walaupun menggunakan ejen penyampaian gen (lipid atau polimer) atau sel (sel manusia, sel mencit & sel primer mencit) yang berbeza. Selain itu, tiada perbezaan yang signifikan dari segi bilangan unit pDNA dalam sel dan ketoksikan antara pDNA. Oleh itu, ekspresi yang rendah dari pZGFP adalah disebabkan oleh pelupusan CpG daripada transgen. Dengan siasatan lanjut, ekspresi rendah pZGFP bukan disebabkan oleh pembatasan eksport mRNA tetapi disebabkan oleh kadar transkripsi yang lebih rendah. Kesimpulannya, pelupusan motif CpG daripada transgen di p0CpG menyebabkan kadar transkripsi berkurangan. Data yang diperolehi daripada kajian ini boleh digunakan untuk memperbaiki dan mengoptimumkan prestasi p0CpG untuk aplikasi terapi gen *ex vivo* pada masa hadapan.

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"Guide us to the right path"
(Quran 1: 6)

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I certify that a Thesis Examination Committee has met on 10 March 2017 to conduct the final examination of Muhammad Omar bin Habib Rahuman on his thesis entitled "CpG-Free Plasmid DNA Exhibits Limited *In Vitro* Reporter Gene Expression Due to Transcriptional Incongruity" 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 Master of Science.

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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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Name of Member
of Supervisory Committee: Dr. Chau De-Ming

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AI	autoimmunity
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AZA	5-azacytidine
bDNA	bacterial DNA
BMP-2	bone morphogenetic protein 2
bPEI	branched polyethylenimine
BSA	bovine serum albumin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CHO	Chinese Hamster Ovary
CIP	calf intestinal alkaline phophatase
CLI	Critical Limb Ischemia
CMV	cytomegalovirus
CpG	cytosine-guanine dinucleotide
CTL	cytotoxic T lymphocytes
DDW	double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
EMA	European Medicines Agency
FBS	fetal bovine serum
FDA	Food and Drug Administration
FRT	flippase recognition site
GFP	green fluorescent protein
GT	gene therapy
GTA	gene transfer agent
HDAC	histone deacetylases
hEF1α	human elongation Factor 1 α
IFN	interferons
IL	interleukin
LB	Luria-Bertani
MBP	methyl binding proteins
MIP-1α	macrophage inflammatory protein 1α
MSC	mesenchymal stem cells
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-κB
NT-3	neurotrophin 3
OEG	olfactory ensheathing glia
p0CpG	CpG-free pDNA
PAD	Peripheral Arterial Disease
PBS	phosphate buffered saline
PCR	polymerase chain reaction

pDNA	plasmid DNA
RAC	NIH Recombinant DNA Advisory Committee
RGFP	CpG-containing GFP
RLB	Reporter Lysis Buffer
RLU	relative light unit
SEM	standard error of the mean
SFDA	China's State Food and Drug Administration
TLR9	Toll-like Receptor 9
TNF-α	tumour necrosis factor-α
vDNA	vertebrate DNA
VEGF	vascular endothelial growth factor
X-SCID	X-linked severe combined immune deficiency
ZGFP	CpG-depleted GFP

CHAPTER 1

INTRODUCTION

The basic principle of gene therapy is to deliver nucleic acids into patients to treat or even cure a particular condition. The nucleic acids were delivered to ultimately modulate the expression of relevant gene(s) in target cells with the goal of treating a disease (Hsu & Uludağ, 2012). The two common clinical approaches in gene therapy are *in vivo*, where nucleic acids are delivered directly to the target cells or near the target cells in the patients, and *ex vivo*, where target cells are isolated from the patients and genetically modified *in vitro* before autologous transplantation (Naldini, 2011). Although the initial conception of gene therapy was mainly to treat incurable monogenic diseases, now gene therapy could be applied to acquired and complex diseases making gene therapy as a prospect for effective treatment strategy in medicine.

In order to deliver and facilitate the expression of nucleic acids, vectors are utilized. In fact, designing a safe and optimal vector is a major factor contributing to the success of gene therapy. Between the two most common gene therapy vectors, viral vectors are preferred over the non-viral plasmid DNA (pDNA) vectors due to their ability to efficiently transduce and deliver nucleic acids to the target cells. However, limitations of viral vectors in terms of production, cloning capacity and most importantly safety issues, such as insertional mutagenesis and immunological side effects (Chen et al., 2003; Thomas et al., 2003), which makes pDNA vectors an advantageous alternative (Tolmachov, 2011).

Recently, a pDNA known as the CpG-free pDNA (p0CpG) has emerged to be an efficient and safe vector. p0CpG was designed to be devoid of CpG (cytosine-guanine dinucleotide) motif from the whole pDNA that includes the pDNA backbone and the transgene. With depletion of the CpG motifs, the p0CpG was able to circumvent detrimental inflammatory response and provide sustained transgene expression in various pre-clinical studies by avoiding silencing by DNA methylation (Ando et al., 2012; Hyde et al., 2008). In addition, the clinical safety and efficacy of p0CpG was further reinforced with the significant treatment effect observed in Phase 2B cystic fibrosis gene therapy clinical trial with no significant adverse events (Alton et al., 2015). Interestingly, all reported studies using p0CpG were focused on *in vivo* approaches. Extending the p0CpG towards *ex vivo* approach would be a novel and highly beneficial strategy as this construct may provide sustained transgene expression by circumventing DNA methylation silencing effects.

However, to date, the evaluation of the p0CpG expression in *in vitro* model has never been reported. There is a need to validate the performance of p0CpG *in vitro* as *ex vivo* approach requires isolated cells to be genetically manipulated and validated *in vitro* before autologous transplantation. On the other hand, it was recently reported that CpG depletion from transgene resulted in limited *in vitro* transgene expression (Krinner et al., 2014). Even though this phenomenon was not studied on the p0CpG, there is a necessity to clarify this effect on p0CpG since CpG motifs are completely depleted from the whole pDNA, including the transgene, which could possibly affect the *in vitro* performance of this vector. Thus, the **problem statement** of this study is the assessment of the p0CpG performance *in vitro*, specifically the effect of CpG depletion from transgene, is necessary for future *ex vivo* gene therapy application.

Therefore, the **general objective** of this study is to evaluate the performance of p0CpG *in vitro*, specifically the effect of CpG depletion from the transgene. The **null hypothesis (H_0)** proposed is the p0CpG will exhibit a superior *in vitro* expression profile despite having CpG-depleted transgene. Conversely, the **alternate hypothesis (H_1)** is the p0CpG will exhibit limited *in vitro* expression profile due to CpG-depleted transgene.

The specific objectives of this study are:

1. To compare the *in vitro* expression of luciferase reporter gene in human cell lines between pDNAs that have been previously validated by *in vivo*, which are CpG-free pDNA and CpG-containing pDNA.

2. To construct novel pDNAs with identical CpG-free backbone carrying GFP alleles of variable CpG content as a tool to investigate the influence of pDNA backbone and transgene CpG content on transgene expression.

3. To determine external factors that could influence transgene expression of the constructed pDNAs, which include type of gene transfer agent, type and species of cells and pDNA-induced cytotoxicity.

4. To identify the levels in gene expression process that may limit pZGFP expression compared to pRGFP, specifically at the gene dosage and mRNA export levels.

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