Limited expression of non-integrating CpG-free plasmid is associated with increased nucleosome enrichment

Omar Habib1,2, Rozita Mohd Sakri1,2,3, Nadiah Ghazalli1,2, De-Ming Chau1,2, King-Hwa Ling1,2, Syahril Abdullah1,2,4*

1 Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia, 2 Genetics and Regenerative Medicine Research Centre, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia, 3 Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor, Malaysia, 4 UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia

* syahril@upm.edu.my

Abstract

CpG-free pDNA was reported to facilitate sustained transgene expression with minimal inflammation in vivo as compared to CpG-containing pDNA. However, the expression potential and impact of CpG-free pDNA in in vitro model have never been described. Hence, in this study, we analyzed the transgene expression profiles of CpG-free pDNA in vitro to determine the influence of CpG depletion from the transgene. We found that in contrast to the published in vivo studies, CpG-free pDNA expressed a significantly lower level of luciferase than CpG-rich pDNA in several human cell lines. By comparing novel CpG-free pDNA carrying CpG-free GFP (pZGFP: 0 CpG) to CpG-rich GFP (pRGFP: 60 CpGs), we further showed that the discrepancy was not influenced by external factors such as gene transfer agent, cell species, cell type, and cytotoxicity. Moreover, pZGFP exhibited reduced expression despite having equal gene dosage as pRGFP. Analysis of mRNA distribution revealed that the mRNA export of pZGFP and pRGFP was similar; however, the steady state mRNA level of pZGFP was significantly lower. Upon further investigation, we found that the CpG-free transgene in non-integrating CpG-free pDNA backbone acquired increased nucleosome enrichment as compared with CpG-rich transgene, which may explain the observed reduced level of steady state mRNA. Our findings suggest that nucleosome enrichment could regulate non-integrating CpG-free pDNA expression and has implications on pDNA design.

Introduction

Plasmid DNA (pDNA) has played an essential role in advancing basic research, biotechnology and medicine. Due to its ability to facilitate transgene expression, it becomes practical to express, silence, augment the levels of a gene(s) of interest, or even introduce foreign gene(s) [1]. This allows better insight into the biology of a system and consequently offers the ability to
control and manipulate such a system for beneficial purposes such as gene therapy. As a result, pDNA has undergone various improvements to better facilitate transgene expression. Some of the improvements are the incorporation of novel promoters, introns, and chromatin insulators in the pDNA design. One of the more noteworthy improvements is the development of CpG (cytosine-guanine dinucleotides)-depleted pDNA or commonly known as CpG-free pDNA, where CpG dinucleotides are completely depleted from the whole pDNA, including the transgene.

CpGs in pDNA of bacterial origin differ from CpGs in vertebrate DNA in the frequency and methylation status. CpGs exist more frequently in bacterial pDNA (1 in 16 dinucleotides compared to 1 in 64 dinucleotides in vertebrates DNA) and are not methylated. In contrast, 60–90% of CpGs in vertebrate DNA are methylated [4]. This distinction is crucial in antigen recognition for immune defense. Toll-like Receptor 9 (TLR9) recognizes non-methylated CpG-containing DNA fragments to be foreign, and will accordingly induce an acute inflammatory response [5]. Even the presence of a single CpG in pDNA was enough to elicit profound inflammation in vivo [6]. As a result, CpG-induced inflammation leads to immune-mediated destruction of transgene-carrying cells [7, 8], repression of transgene expression due to the direct action of pro-inflammatory cytokines [9–11], and prolonged inflammation, which could result in the development of autoimmunity [12]. Moreover, studies have shown that the unmethylated CpGs in pDNA were prone to de novo DNA methylation-mediated silencing that resulted in the loss of transgene expression [13–18]. Given these points, removing CpGs from pDNA would be advantageous in an in vivo system.

The first CpG-free pDNA developed was able to significantly reduce inflammation, which includes levels of pro-inflammatory cytokines, and neutrophil infiltration indicative of minimal tissue damage, irrespective of topical lung or systemic delivery [6]. Subsequent studies also reported the use of CpG-free pDNA only induced minimal host inflammatory response and was safer than the CpG-containing pDNA [19–21]. In addition, intranasal delivery of CpG-free pDNA into mouse lungs led to a high and sustained luciferase expression up to 28 days post-administration. In comparison, a CpG-containing pDNA expression only lasted for approximately 2 weeks before declining drastically, despite the presence of the pDNA on days where the transgene was silenced [6]. Besides mouse lungs [6, 19, 22, 23], CpG-free pDNA was shown to have a better in vivo expression profile than CpG-containing pDNAs regardless of delivery methods and target tissues. These include hydrodynamic injection to mouse liver [24, 25], localized injection to rat corneas [26], and intravenous injection to mouse tumour [27]. These studies demonstrated the benefits of utilizing CpG-free pDNA to achieve higher and prolonged transgene expression in vivo. To the best of our knowledge, similar benefits have not been reported in in vitro applications.

Studies have shown that the presence of CpG often hinders transgene expression due to DNA methylation [14, 15, 28]. The elimination of CpG can potentially circumvent the issue. However, one of the major challenges is to construct the CpG-free pDNA, namely the depletion of CpG from the transgene, without changing its regulatory binding sites and coding sequence. As described earlier, even though vertebrate DNA has lower CpG frequency as compared to bacterial pDNA, the presence of CpG in the vertebrate coding gene is unavoidable [4]. Depleting CpG from vertebrate-derived transgene requires accurate and detailed gene optimization strategies, which include exclusion of AU repeat elements, UpA dinucleotides, RNA secondary structure, and cryptic splice sites. The resulting CpG-free transgene requires codon optimization to remove any rare codon that would negatively impact transgene expression [29]. Given the arduous steps involved in de novo transgene construction, the advantages of utilizing CpG-free pDNA (CpG-free backbone and transgene) as compared to CpG-free pDNA backbone with CpG-containing transgene in in vitro models are unknown. Identifying...
the pros and cons of either construct, would provide deeper understanding on the limits and potentials of the CpG-free pDNA. Here, we analyzed the in vitro transgene expression from CpG-free pDNA, specifically to determine the influence of CpG depletion from the transgene. First, transgene expression of a CpG-free pDNA was compared to CpG-rich pDNA. These two pDNAs carry luciferase reporter gene but with different pDNA backbone. Next, we constructed second generation of pDNAs with similar 0 CpG backbone but carry either zero CpG GFP (pZGFP) or CpG-rich GFP (pRGFP). The data obtained shows that the pZGFP exhibited reduced transgene expression when compared to pRGFP in several cell lines, and this was due to a reduced rate of transcription. Notably, the absence of CpG on the pZGFP transgene was associated with an increase of nucleosome density that could limit its expression.

Materials and methods
Plasmid DNA (pDNA)
In total, 4 different non-integrating pDNAs were utilized in this study. pCIKLux (kindly provided by the Gene Medicine Research Group, University of Oxford, UK) and pCpGfree-Lucia (InvivoGen, USA) each carry a luciferase reporter gene, were utilized in Section 3.1. Henceforth, these plasmids will be referred to as pCpG-rich (pCIKLux) and pCpG-free (pCpGfree-Lucia). In addition, pZGFP and pRGFP pDNAs were constructed from the pCpGfree-Lucia pDNA backbone, after excising the luciferase gene with NcoI and NheI restriction enzymes and replaced with green fluorescent protein (GFP) genes, specifically the EmGFP variants. The CpG-free GFP (ZGFP) transgene was amplified by PCR from pMOD-ZGFP::Sh (InvivoGen, USA) using the forward (5’-GAGGGCCACCATGGTTTCTAA-3’) and reverse (GCAAGCTAGCTTACTTGATACAGCTCATCCATCCCAGAG) primers designed with restriction enzyme sites to complement the pCpG-free backbone (bold: NcoI; underlined: NheI). As for CpG-rich GFP (RGFP) transgene, pLenti6.3-V5-GW-EmGFP (Thermo Fisher Scientific, USA) was used as PCR template and amplified using the forward (5’-AATCCATGGT GAGCAAGGGCGAGGAGCTGTTCAC-3’) and reverse (5’-CAAGCTAGCTTACTTGATACAGCTCGTCCATGGC-3’) primers. The resulting pZGFP and pRGFP constructs were confirmed to be mutation-free by Sanger sequencing (First BASE Laboratories Sdn. Bhd., Malaysia). Peptide and DNA sequence alignment were performed using NCBI’s Needleman-Wunsch Global Protein Sequence Alignment web service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and NCBI’s Needleman-Wunsch Global Nucleotide Sequence Alignment web service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the percentage (%) of identity.

Cell culture
HEK-293FT, H1299, MCF7, SH-SY5Y, and NIH-3T3 were cultivated in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) with L-glutamine, pyridoxine hydrochloride, and 110 mg/L sodium pyruvate, supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (10,000 U/mL), and 1.5 g/L sodium bicarbonate (Thermo Fisher Scientific, USA) at pH 7.4. The cells were incubated and maintained in an incubator with 5% CO2 humidified atmosphere at 37°C. Only cells with passage number of 30 or below were utilized to avoid any unwanted effects due to repeated cell passaging. As for the primary mouse fibroblast cells, the fibroblast isolation protocol was adapted and modified from Takahashi et al. (2007) [30]. The mouse tail fibroblast was isolated post-mortem from 4 weeks old wildtype C57BL/6J mouse strain. Briefly, the tail was excised; the superficial dermis was peeled and rinsed twice with 70% ethanol, followed by 1x Phosphate Buffered Saline (PBS) pH 7.4. The tail biopsies were minced into roughly 1cm pieces, placed on 0.1% gelatin-coated culture
dishes, and incubated in DMEM supplemented with 10% FBS, antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin), 4 mM L-glutamine, and 0.1 mM non-essential amino acids (NEAA) (Thermo Fisher Scientific, USA). The culture dishes were incubated at 37˚C with 5% CO₂. Cells that migrated out from the minced tissue pieces were transferred to new 0.1% gelatin-coated culture dishes (passage 1) and maintained in DMEM containing 10% FBS. In this study, primary tail fibroblasts were utilized within three passages to avoid replicative senescence.

**Lipid and Polymer gene transfer agent (GTA) formulation and cell transfection**

The cationic lipid-based gene transfer agent (GTA) used in this study was the Lipofectamine™ 2000 (Thermo Fisher Scientific, USA) and henceforth will be referred to as “Lipid GTA”. The lipid/pDNA complexes were prepared following the manufacturer’s protocol. The ratio of pDNA (in μg): lipid GTA (in μL) utilized was 1:3, which is the optimal ratio as reported by the manufacturer. The cationic polymer-based GTA used in this study was the branched polyethyleneimine (bPEI) with molecular weight of 25 kDa (Sigma-Aldrich, USA), and henceforth will be referred to as “Polymer GTA”. The ratio of pDNA (in μg): polymer GTA (in μg) utilized was 1:1.29, which corresponds to polymer nitrogen to DNA phosphate (N:P) molar ratio of 10:1 that is optimal for most cells [20, 22, 31]. For cell transfection, cell-seeding density used was 2.5 x 10⁴ cells/cm² and 1 μg of pDNA was utilized to transfect every 5.0 x 10⁶ cells. The amount of pDNA and cell-seeding density were scaled accordingly to accommodate tissue-culture flask area (cm²). Typically, cells were seeded and allowed to adhere for 24 hours. Next, cells were incubated in Lipid or Polymer GTA formulation for 5 hours before the formulation was discarded. Following this, the cells were cultivated in the appropriate growth medium until selected time-point(s).

**Luciferase assay**

HEK-293FT, H1299, MCF7, and SH-SY5Y cells were seeded at a density of 5.0 x 10⁴ cells/well in 24-well plate and transfected with 1 μg of pCpG-free or pCpG-rich complexed with Lipid GTA. Harvesting of cell lysates and quantification of expressed luciferase was performed using the Luciferase Assay System Kit (Promega, USA) and GloMax™ 20/20 luminometer (Promega, USA), according to manufacturer’s protocol. The amount of total protein from the cell lysates was determined based on a colorimetric method using the Bio-Rad DC Protein Assay (Bio-Rad, USA) and was utilized to normalize the luciferase expression. The expression was presented as mean relative light unit (RLU) per milligram of total protein (RLU/mg protein). Cell lysate from non-transfected cells was used as a control to exclude any background signal.

**GFP protein expression analysis by flow cytometry**

HEK-293FT, NIH-3T3, and primary mouse fibroblast cells were seeded at a density of 1.0 x 10⁵ cells/well in 12-well plate and transfected with 2 μg of pZGFP or pRGFP, complexed with Lipid or Polymer GTA. At selected time-points, adhered cells were dissociated and resuspended in PBS with 1% of FBS (Thermo Fisher Scientific, USA). The GFP expression was analyzed using BD LSRFortessa™ (BD Biosciences, USA) flow cytometer, and non-transfected cells were used for gating purposes to exclude clumped cells and as a reference for GFP⁺ cells. The minimum total event was set to 10,000. Parameters assessed were the % of GFP⁺ cells (indicates the fraction of cells expressing GFP from the gated single cell population) and mean fluorescence intensity (MFI) (indicates the average GFP intensity in a single cell from the GFP⁺ cell population).
Cell viability analysis by MTT assay
HEK-293FT cells were seeded at a density of $1.5 \times 10^4$ cells/well in a 96-well plate. For transfection, the cells in each well were transfected with 0.6 μg of pZGFP or pRGFP complexed with 1.2 μl of Lipid GTA. At Day 1 and 2 post-transfection, 20 μl of MTT (Amresco, USA) was added to the growth medium in each well. The cells were incubated at 37˚C in a humidified atmosphere for an additional 4 hours. Excess dye was then removed, and insoluble formazan crystals were dissolved in 100 μl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). Spectrophotometer readings were measured using a microplate reader (ASYS Hitech, Austria) at a wavelength of 570 nm, with 690 nm as the reference filter. Non-treated cells were used as negative control. Additional controls for this study were (i) cells treated with 1.2 μl of Lipid GTA only, and (ii) cells transfected with 0.6 μg of the circularized pCpG-free backbone (devoid of transgene) complexed with 0.9 μl of Lipid GTA. Non-treated cells were used as a control with 100% viability. The data are presented as relative cell viability (%) as compared with non-treated cells.

Gene dosage by absolute qPCR
HEK-293FT cells were seeded at a density of $5.0 \times 10^4$ cells/well in 24-well plate and transfected with 1 μg of pCpG-free or pCpG-rich, complexed with Lipid GTA. Total DNA was extracted from cells at Day 1 post-transfection using the DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the manufacturer’s protocol. Only 30 ng of extracted DNA was used as qPCR template. The pDNA copy number in samples were determined through absolute qPCR using standard curves generated with known pDNA copy numbers. The standard curves were generated using purified pZGFP and pRGFP plasmids at 1:10 serial dilution ranging between 210 ng and 2.1 pg.

Absolute qPCR was performed using the LightCycler® 480 Real-Time PCR System Instrument (Roche Diagnostics, Switzerland) [32]. Data generated were analyzed and quantified with the LightCycler® 480 Software version 1.5 (Roche Diagnostics, Switzerland), employing the second derivative maximum method [33] to calculate the Cp (Crossing Point) value. To increase specificity of the assay, Universal Probe Library (UPL) hydrolysis probes that bind specifically to the transgene were utilized. qPCR was performed in a final reaction volume of 10 μl, containing 1X LC480 Probe Master Mix (Roche Diagnostics, Switzerland), 0.1 μM of UPL Probe (Roche Diagnostics, Switzerland), 1 μM of each forward and reverse primer, DNA template, and appropriate volume of nuclease-free water. The qPCR program utilized was as follows: initial denaturation of 95˚C for 10 minutes, followed by 45 cycles of 95˚C for 10 seconds, 60˚C for 30 seconds and 72˚C for 8 seconds, and finally 40˚C for 30 seconds. Primer pairs and UPL probes are: ZGFP (forward-CTGCTCTGCTTCAAGACCTCT, reverse-AATT CCTGCTGCTGTCACAA, UPL Probe# - 79, and amplicon size- 74 bp) and RGFP (forward-TGGTCCTGCTGGAGTTCG, reverse-CTTGTACAGCTCGTCCATCG, UPL Probe# - 70, and amplicon size- 62 bp).

mRNA distribution analysis by absolute qPCR
HEK-293FT cells were seeded at a density of $1.0 \times 10^5$ cells/well in 12-well plate and transfected with 2 μg of pZGFP or pRGFP, complexed with Lipid GTA. Nuclear and cytoplasmic mRNAs were extracted from cells at Day 1 post-transfection using the Cytoplasmic and Nuclear RNA Purification Kit (Norgen Biotek Corp., Canada), according to manufacturer’s protocol. Five hundred (500) ng of extracted RNAs were reverse transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Switzerland) using both anchored-oligo (dT)₁₈ and random hexamer primers. Next, the cDNAs were diluted 10x using nuclease-free
water (Roche Diagnostics, Switzerland). Absolute qPCR was performed using 1 μl of 10x-diluted cDNAs to quantify mRNA copy number in nuclear and cytoplasmic fractions [34] as described in Section 2.7.

**Formaldehyde-Assisted Isolation of Regulatory Element (FAIRE)**

FAIRE analysis was performed based on an established protocol [35]. HEK-293FT cells were seeded at a density of 6.25 x 10^5 cells/well in T25 flask and transfected with 12.5 μg of pZGF or pRGFP complexed with Lipid GTA. At Day 1 post-transfection, cells were dissociated, fixed in suspension with 1% formaldehyde solution for 9 minutes at room temperature, and subsequently quenched with glycine (125 mM). Cells were pelleted by centrifugation at 850 x g for 5 minutes in 4˚C and washed twice with PBS. The cells were then resuspended in lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1x protease inhibitor cocktail (Roche Diagnostics, Switzerland)). Sonication was performed on the samples using Bioruptor Plus sonicator (Diagenode, Belgium) at high power for 15 cycles (15 seconds on; 15 seconds off) to obtain DNA fragments ranging in 200–500 bp. Twenty-five (25)% of the sonicated samples was reverse cross-linked by adding 0.5 μg/μl proteinase K (Nacalai Tesque, Japan) and incubated at 65˚C for 6 hours. This fraction was used as “Input” fraction because histone proteins would be eliminated during reverse cross-linking. The remaining 75% of the sample was not reverse cross-linked, and this fraction was used as “FAIRE” fraction.

Next, DNA purification was performed on both "Input" and “FAIRE” fractions by adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)(Nacalai Tesque) and vortexed for 10 seconds. The samples were centrifuged at high speed for 10 minutes at room temperature to obtain the aqueous phase. The aqueous phase was transferred, and potassium acetate (3 M, pH 5.5) was added at 1/10 of the sample volume and vortexed for 5 seconds. About 2x volumes of ice-cold 100% ethanol was added, vortexed for 10 seconds, and incubated at -80˚C for 2 hours. Samples were centrifuged at high speed for 5 minutes, and supernatants were discarded. Five hundred (500) μl of 70% ethanol was used to resuspend the DNA pellet and centrifuged at high speed for 5 minutes to discard the supernatant. The DNA pellet was air-dried for 15 minutes and resuspended in an appropriate volume of nuclease-free water to make the final DNA concentration of 35 ng/μl.

qPCR was performed in a final reaction volume of 10 μL, containing 1X LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Switzerland), 1 μM of each forward and reverse primer, 35 ng of the sample, and appropriate volume of nuclease-free water. The following qPCR program was utilized: initial denaturation of 95˚C for 10 minutes followed by 45 cycles of 95˚C for 10 seconds, 60˚C for 30 seconds and 72˚C for 8 seconds, and finally 40˚C for 30 seconds. Primers were designed to cover the hEF1α promoter region on the pDNA. Additional primers were designed to amplify the 5' region, middle region, and 3' region of the transgenes. Primers were also designed to cover the GAPDH and HSAT2 regions as internal controls.

**Primer pairs utilized were:**

- **GAPDH** (forward—CCCGTCTTTGACTCCCTAGT, reverse—GTG ATCGGTGCTGTTCC, amplicon size—64 bp),
- **HSAT2** (forward—GGTGTGCTGGGTTCC, reverse—GGTGTGCTGGGTTCC, amplicon size—74 bp),
- **hEF1α** (forward—GGAAATGTGCCTAGA, reverse—AAGGGTGGACGCAATACCAA, amplicon size—75 bp),
- **5’ RGFP** (forward—CTCTGATCTCAGCAGT, reverse—GTACGAGGAGGTGGGAG, amplicon size—70 bp),
- **Mid RGFP** (forward—GGGAGGCAACATCCTG, reverse—GGCG GTGTATGACCTTTG, amplicon size—70 bp),
- **3’ RGFP** (forward—TTGCTTGCTGTGGGAG TTG, reverse—TTGCTTGCTGTGGGAG TTG, amplicon size—62 bp),
- **5’ ZGFP** (forward—GG GTGTGCTCCCATTCTG, reverse—CACAGGAAATTGGGCCATT, amplicon size—60 bp),
- **Mid ZGFP** (forward—AGGTTATGGTCCAGAGGACAA, reverse—CTGTTAACAGTGTATC.
ACCTTCAA, amplicon size—96 bp), 3’ ZGFP (forward—CTGCTCTGTCCAAAAGACCCTA, reverse—AATTCTCTGCTGCTGTACCAA, amplicon size—74 bp). Data were analyzed using the 2^ΔΔCT method, where GAPDH was used as housekeeping control. Instead of presenting the results as nucleosome-depletion, nucleosome enrichment was determined by modifying the formula to 2^((ΔInput)−(ΔFAIRE)) and values were plotted as relative to GAPDH nucleosome enrichment.

**Statistical analysis**

Data are presented as mean ± standard error of the mean (SEM). Parametric Student’s t-test was applied to compare two unpaired groups, which are the pZGFP and pRGFP, and data were considered to be significantly different when P < 0.05. Statistical analyses were performed using GraphPad Prism software v5 (GraphPad, USA). All experiments were repeated at least twice.

**Results**

**CpG-free pDNA exhibited limited transgene expression in human cell lines**

First, we sought to recapitulate the prominent in vivo expression of CpG-free pDNA, as compared with CpG-rich [6, 22, 24–27], in in vitro model (i.e. human cell lines). This was to serve as a benchmark for the level and duration of CpG-free pDNA expression for subsequent in vitro experiments. This was done by utilizing the identical pDNAs reported previously, which are the CpG-free (0 CpG) and CpG-rich (317 CpGs) pDNAs, both expressing the luciferase reporter gene. The transgene in pCpG-rich was driven by the human immediate early cytomegalovirus (hCMV) promoter, which is a typical constitutive viral-based promoter, while the pCpG-free uses a eukaryotic-origin human Elongation Factor 1α (hEF1α) promoter. The hEF1α promoter is known to provide higher and persistent transgene expression compared to hCMV in both in vitro and in vivo systems [2]. The pCpG-rich contains 317 CpGs throughout the pDNA, where 42% of the CpGs are confined in promoter and luciferase reporter gene regions (39 and 96 CpGs respectively), whereas the pCpG-free was devoid of any CpG motif (Fig 1). Equal mass of 1μg of each plasmid was transfected: however, molar ratio of pCpG-rich: pCpG-free was 1:1.5 due to the difference in pDNA size (Fig 1).

In HEK-293FT (Fig 2A), the difference in luciferase expression level was highly significant (p<0.001) from Day 1 where pCpG-free gave ≈ 3 log₁₀ lower expression than pCpG-rich. Similarly, in the other cell lines tested, pCpG-free also showed significantly lower luciferase expression than pCpG-rich at Day 1 (Fig 2B–2D) where the differences were ≈ 2 log₁₀ in H1299 (p<0.001), ≈ 1 log₁₀ in MCF7 (p<0.001) and SH-SY5Y (p = 0.029). The expression of pCpG-free remained low in comparison to pCpG-rich even at Day 14, at ≈ 3 log₁₀ lower for HEK-293FT (p<0.001), ≈ 2 log₁₀ lower for H1299, ≈ 0.25 log₁₀ lower for SH-5YSY (p = 0.025), except for MCF7. Moreover, in HEK-293FT and SH-SY5Y, the luciferase expressions from pCpG-free were consistently and significantly lower across all time points compared to pCpG-rich (Fig 2A and 2C). As for the H1299 and MCF7, the pCpG-free luciferase expressions were observed to fluctuate (Fig 2B and 2D). In general, the luciferase expression pattern from pCpG-free was lower than pCpG-rich in all human cell lines and at most time points, despite higher copy number of pCpG-free transfected. Data indicates usage of CpG-free pDNA over CpG-rich pDNA was not advantageous in in vitro systems.

**Construction of CpG-free pDNAs with variable number of CpG dinucleotides**

The observed low expression of pCpG-free compared to pCpG-rich may be due to differences in regulatory elements and/or CpG content on transgene. Here, pDNAs with identical CpG-
free pDNA backbone (i.e. regulatory elements) with transgenes of 0 CpG (ZGFP) or 60 CpGs (RGFP) were constructed to investigate the influence of CpG content on transgene expression. The expression of the transgene (ZGFP or RGFP) was under the control of mouse cytomegalovirus (mCMV) enhancer coupled with human Elongation Factor 1α (hEF1α) promoter. A synthetic intron (SI 126) and simian virus 40 (SV40) polyadenylation signal were present upstream and downstream of the transgene, respectively, to enhance the transgene expression. In addition, mammalian and bacterial domains were separated by human IFN-β and β-globin matrix attachment regions (MARs) to avoid interference between the domains that may affect the expression (Fig 3A).

Humanized CpG-depleted (ZGFP) and CpG-rich (RGFP) GFP alleles were utilized, and the proteins encoded by both transgenes were ensured to closely resemble each other. The nucleotide sequences of ZGFP and RGFP were 77% identical, with an equal peptide length of 240 a.a. and 98% protein similarity (Fig 3B and 3C). Importantly, the fluorophore region (Leu65-Thr66-Tyr67) responsible GFP fluorescence [36] was identical between ZGFP and RGFP. This ensures that any discrepancy in fluorescence intensity is not due to differences in amino acid sequences (Fig 3B).

**pZGFP has reduced transgene expression regardless of gene transfer agent**

To determine the effects of transgene CpG depletion on gene expression, the pZGFP (0 CpG) and pRGFP (60 CpGs) were transfected into HEK-293FT. Transfection was performed using 2 gene transfer agents (GTA), Lipid and Polymer, to account for differences in pDNA delivery and release in the cells, which may influence transgene expression [37]. In both groups, the percentage of GFP-expressing cells and the mean fluorescence intensity (MFI) (Fig 4A–4D) peaked at Day 1 post-transfection and declined steadily over the course of 14 days. At single cell level, Polymer GTA resulted in higher MFI than Lipid GTA for cells transfected with either pDNA.

Transfection using Lipid GTA resulted in the highest expression of pZGFP at Day 1 with 57.9% GFP+ cells, yet it was significantly lower (p<0.001) than pRGFP by 24.6% (Fig 4A). The percentage of GFP+ cells in the pZGFP-transfected group remained significantly lower than pRGFP up to the final time-point, where pZGFP exhibited lower GFP+ cells than pRGFP by
As for the average single-cell expression, MFI of pZGFP+ cells was significantly and consistently lower compared to pRGFP from Day 1 (p < 0.001) up to Day 14 (p < 0.001) (Fig 4C). At the peak of expression on Day 1, MFI of pZGFP-transfected cells was \( \approx 3.5 \times 10^4 \) lower than pRGFP. The difference became smaller by Day 14, but still at the order of \( 10^4 \).

A similar trend was observed with Polymer GTA, where pZGFP exhibited a significantly lower percentage of GFP+ cells than pRGFP at all time points (Fig 4B). On the other hand,
Fig 3. pDNA maps, peptide, and DNA sequences alignment of pZGFP and pRGFP. (A) pZGFP and pRGFP were constructed from the identical CpG-free pDNA backbone. Antibiotic resistance gene is Zeocin™, R6Kγ origin of replication, ZGFP or RGFP is under the control of mouse cytomegalovirus (mCMV) coupled with human elongation Factor 1α (hEF1α). A synthetic intron (SI 126) and simian virus 40 (SV40) polyadenylation signal sequence flank the transgene. Mammalian and bacterial domains were confined by scaffold/matrix attachment regions, from human IFN-β (IFNβ S/MAR) and β-globin (βGlo S/MAR). The vertical black bars under RGFP represent the presence of CpGs. The maps were illustrated using SnapGene software. (B) ZGFP (Sbjct) peptide sequence was aligned to the RGFP peptide sequence (Query). Highlighted in green: % of identity; pink: the presence of gaps; blue: length of peptide sequence; yellow: GFP fluorophore. (C) DNA sequences alignment between ZGFP (Sbjct) and RGFP (Query). Highlighted in yellow are CpG motifs.

https://doi.org/10.1371/journal.pone.0244386.g003
there was no significant difference in MFI at Day 1, 2, 3 and 5 between pZGFP and pRGFP groups (Fig 4D). However, with time, the MFI of pZGFP became significantly lower than pRGFP from Day 7 ($p < 0.001$) to Day 14 ($p < 0.01$). Overall, the pZGFP (0 CpG) exhibited reduced reporter gene expression at the population and single-cell levels when compared to pRGFP (60 CpGs in transgene region) in HEK-293FT, regardless of GTA used.

Reduced expression of pZGFP was not influenced by cell species and origin

Expression of a transgene was shown to vary in different species as cell species influenced promoter activity, including the EF1α promoter [38], which was utilized in this study. Hence, to determine if the reduced pZGFP expression was species-specific, NIH-3T3 mouse cell line was transfected with pZGFP and pRGFP complexed with Lipid or Polymer GTA. Generally, with
Lipid GTA, the percentage of GFP⁺ cells was ≈ 2 folds higher (pZGFP: 60.8%; pRGFP: 86.5%) than Polymer GTA (pZGFP: 34.4%; pRGFP: 40.6%). At Day 1, the group transfected with pZGFP exhibited significantly lower GFP⁺ cells (60.8%) compared to pRGFP (86.5%) when delivered using Lipid GTA (p<0.01). pZGFP-transfected group exhibited the highest percentage of GFP⁺ cells at Day 2 (83.6%) but was still significantly lower (p<0.001) than pRGFP (97.7%). The percentage of GFP⁺ cells from pZGFP transfected group remained lower than pRGFP-transfected group at all time points, except at Day 14. A similar trend was also observed with polymer GTA (Fig 5A and 5B). Mean fluorescence intensity was also significantly lower in the pZGFP-transfected group at all time points except at Day 14, regardless of GTA used (Fig 5C and 5D).

Since primary cells are better at simulating in vivo gene expression conditions as compared to immortalized cell lines [39, 40], mouse primary fibroblast cells were utilized to assess pZGFP and pRGFP expression profiles. Due to technical challenges and early cell senescence, GFP expression was only assessed at Day 1, 2, 3, and 5 post-transfection using Lipid GTA. The % of GFP⁺ cells obtained at Day 1 with Lipid GTA was minimal, with pZGFP 2.5% lower than pRGFP (p<0.01) (Fig 6A). Despite the low % of GFP⁺ cells, the inferior expression of pZGFP compared to pRGFP was still significant. The expression of pZGFP remained at a similar level up to Day 3 before receding to a negligible value at Day 5. Conversely, increased % of GFP⁺ cells was observed from pRGFP at Day 3 (12.6%), and the expression remained higher than pZGFP at the last time-point (p<0.01) (Fig 6A). Interestingly, the low expression of pZGFP at Day 1 was also reflected at the single-cell level, as the MFI value of pZGFP was ≈ 2.8 fold lower than pRGFP (p<0.001) (Fig 6B).

Here, pZGFP expression was significantly lower than pRGFP in both NIH-3T3 mouse cell line and primary mouse fibroblasts cells. This observation, supplemented with previous experiments, demonstrates that the absence of CpG in the transgene of pZGFP resulted in lower expression in in vitro conditions.

**pZGFP expression was not compromised by cytotoxicity**

Sequence-specific toxicity was observed where non-coding viral sequences are more toxic than non-coding eukaryotic sequences [41]. Since some viruses have CpG-depleted genomes [42], complete CpG-depletion in pZGFP may appear as an infection signal to the cells, leading to the cytotoxicity of pZGFP-transfected cells. To ascertain if the low expression of pZGFP in normal HEK-293FT cell line was not due to increased cytotoxicity effect, cell viability was evaluated upon exposure to the pDNA-GTA complexes. Cells were also transfected with a circularized pCpG-free backbone (pBB) complexed with Lipid GTA as a control (Fig 7). At Day 1, the cell viability from Lipid GTA only control group was slightly reduced. When complexed with the pDNAs, cell viability was reduced even further, to as low as 55.2% in the pRGFP/Lipid GTA group. Importantly the results indicate that pZGFP/Lipid GTA (77.3%) did not exhibit increased toxicity compared to pRGFP/Lipid GTA (55.2%). To rule out the possibility if pZGFP had any latent toxic effects, the cytotoxicity effect was further analyzed at Day 2. Interestingly at Day 2, pZGFP/Lipid GTA did not exhibit increased toxicity compared to pRGFP/Lipid (68.1% versus 55.1%, Fig 7).

In addition, the effect of transgene CpG depletion on cytotoxicity was also investigated. This was analyzed by comparing the cell viability of pZGFP/Lipid and pRGFP/Lipid-transfected cells to cells transfected with pCpG-free backbone (without transgene)/Lipid GTA. At Day 1, the difference was not significant between pBB (62.3%) and pZGFP (77.3%) or pRGFP (55.2%). Similarly at Day 2, the difference was not significant between pBB (57.3%) and pZGFP (68.1%) or pRGFP (55.1%). These data indicate that the absence of CpG motif on transgene in pZGFP did not cause any evident cytotoxicity leading to the lower expression.
Comparable pZGFP and pRGFP gene dosages delivered to cells

Since the difference in expression between pZGFP and pRGFP began at Day 1, one may postulate that reduced gene dosage of pZGFP contributed to the low expression of pDNA. To determine the difference in gene dosage between pZGFP and pRGFP-transfected cells, plasmid copy number was quantified using absolute qPCR. Total DNA extracted from cells at Day 1, which were transfected with the pDNAs using Lipid or Polymer GTA, were utilized as input for qPCR. The qPCR standard curves were generated using known concentrations of purified

---

**Fig 5.** GFP expression of NIH-3T3 cells transfected with either pZGFP (0 CpG) or pRGFP (60 CpGs), complexed with Lipid or Polymer GTA. A comparison of GFP expression between pZGFP (solid line) and pRGFP (dashed line) was assessed in terms of % of GFP+ cells, (A, B), and mean fluorescent intensity (MFI), (C, D). GFP expression was measured at Day 1, 2, 3, 5, 7, 10, and 14 post-transfection. Data are presented as mean ± S.E.M. of 3 biological replicates. Asterisk (*) indicates a significant difference for a given time-point between pZGFP and pRGFP determined by Student’s t-test (*p<0.05, **p<0.01, ***p<0.001).
pZGFP and pRGFP. The PCR efficiency and $R^2$ values for pZGFP standard curve were 108.8% and 0.999, and for pRGFP standard curve were 102.3% and 0.999 (Fig 8A). These values were within acceptable range (PCR efficiency: 90–110%; $R^2 > 0.985$) and indicate the qPCR assays were efficient. Based on the copy numbers from the whole cell population, Polymer GTA (pZGFP: $7.17 \times 10^7$ copies, pRGFP: $7.88 \times 10^7$ copies) was able to deliver ≈ 1.5 fold higher pDNAs compared with Lipid GTA (pZGFP: $4.36 \times 10^7$ copies, pRGFP: $4.8 \times 10^7$ copies). However, comparing pZGFP to pRGFP within each GTA, there was no significant difference in terms of pDNA copy number (Fig 8B), indicating that the limited expression of pZGFP was not due to the difference in gene dosage when compared to pRGFP.

Expression of pZGFP was restricted at the transcriptional level, not by mRNA export

5-methylcytosine (m$^5$C) is a post-transcriptional modification that is enriched in CpG-rich regions of the mRNA to promote mRNA export for translation [43]. To elucidate if the limited expression of CpG-depleted pZGFP was due to nuclear retention of ZGFP mRNA, nuclear and cytoplasmic mRNA of transfected cells at Day 1 were quantified using absolute RT-qPCR. The ratio of cytoplasmic$_{mRNA}$/ nuclear$_{mRNA}$ (eg. ZGFP cytoplasmic$_{mRNA}$/ ZGFP nuclear$_{mRNA}$) was used to indicate the mRNA export rate from the nucleus to the cytoplasm. Ratio value > 1 indicates more mRNAs are exported and < 1 indicates increased retention of mRNAs in the nucleus. Both pZGFP and pRGFP gave ratio value > 1 (pZGFP: 1.80; pRGFP: 1.64) (Fig 9A), demonstrating that the transcribed mRNAs were exported towards the cytoplasm instead of being retained in the nucleus. There was also no significant difference in the export rate, indicating that the mRNA export rate was similar, and the limited expression of pZGFP was not due to the ZGFP mRNA retention in the nucleus.

Using the same data set, the differences of transcript levels between nuclear fraction were further explored. The total mRNA of ZGFP (nuclear$_{mRNA}$ + cytoplasmic$_{mRNA}$ $\approx 9 \times 10^6$...
mRNA copies) was observed to be much lower than total mRNA of RGFP (≈ \(1 \times 10^7\) mRNA copies). Notably, nuclear mRNA of ZGFP (≈ \(3 \times 10^6\) mRNA copies) was significantly lower (\(p<0.01\)) than nuclear mRNA of RGFP (≈ \(5 \times 10^6\) mRNA copies) (Fig 9B). This signifies that the levels of steady state mRNA of pZGFP was lower than pRGFP.

### Increased nucleosome enrichment in pZGFP as compared to pRGFP

Nucleosome enrichment in the promoter or coding sequences can result in the repression of gene expression [44]. To determine if the reduction of pZGFP mRNA levels was due to nucleosome enrichment, FAIRE-qPCR was performed on HEK-293FT cells transfected with non-integrating pZGFP or pRGFP complexed with Lipid GTA at Day 1. FAIRE involves the non-specific cross-linking of histones to the DNA, fragmentation the cross-linked DNA, isolation...
by phenol-chloroform extraction and finally quantification of selected regions by qPCR. Human Satellite 2 (HSAT2) and GAPDH were used as internal controls. HSAT2 is a satellite repeat sequence found at the centromeric regions and known to be heterochromatic region with increased nucleosome enrichment [45]. Similar fold of nucleosome enrichment of 2.34 and 2.44 in HSAT2 region was observed from pZGFP and pRGFP-transfected cells, respectively (Fig 10). This indicates that these internal control regions were highly chromatinized and the FAIRE experiment was successful. Compared to the housekeeping gene GAPDH, nucleosome density was low in overall regions of pZGFP (0 CpG) and pRGFP (60 CpGs) with

Fig 8. Gene dosage analysis of pZGFP and pRGFP at Day 1 post-transfection using Lipid and Polymer GTA. (A) qPCR efficiency for pZGFP and pRGFP were determined using serially diluted purified plasmids from 210 ng to 21 pg. (B) Plasmid copy numbers from HEK-293FT cells transfected with either pZGFP or pRGFP at Day 1 post-transfection. Data are presented as mean ± S.E.M. of 3 biological replicates. NS indicates no significant difference between pZGFP and pRGFP for each GTA, determined by Student’s t-test (’p<0.05).

https://doi.org/10.1371/journal.pone.0244386.g008
Fig 9. GFP mRNA distribution analysis of pZGFP and pRGFP at Day 1 post-transfection. mRNA copy numbers from HEK-293FT cells transfected with either pZGFP or pRGFP transfected at Day 1 post-transfection using Lipid PLOS ONE | https://doi.org/10.1371/journal.pone.0244386 December 21, 2020 17 / 25
the average of ≈ 0.4 fold and ≈ 0.15 fold, respectively. This was expected with constitutive transgene expression from abundant pDNA copies. Interestingly, transgene regions of pZGFP were consistently enriched with nucleosomes as compared to pRGFP as the average fold change difference was ≈ 2.7 fold. In addition, the hEF1α promoter in pZGFP has higher nucleosome density by 2.8 fold compared to the hEF1α promoter region in pRGFP, despite both having identical CpG-free hEF1α promoter. Overall, these results show that pZGFP (0 CpG) has higher nucleosome density than pRGFP (60 CpGs).

Discussion
CpG-free pDNA has been reported to be an effective vector for transgene expression in vivo [6, 20, 22–25, 27] and was valuable in gene therapy clinical trial [46]. Despite the wealth of in vivo studies, a detailed assessment of CpG-free pDNA in in vitro systems has not yet been reported. Understanding the in vitro expression profile of CpG-free pDNA is beneficial as sustained ectopic expression could be achieved with CpG-free pDNA, which is not susceptible to DNA methylation-mediated transgene-silencing [15, 17, 47, 48]. Hence, in this study, we first sought to compare the expression levels of pCpG-free and pCpG-rich in human cell lines. We hypothesized that pCpG-free would provide sustained transgene expression as it was devoid of CpG dinucleotides and carries the eukaryotic EF1α promoter, as compared with pCpG-rich that would exhibit poor expression due to the presence of silencing-prone CpGs and viral CMV promoter. The cell lines tested include H1299 to represent lung cells because the CpG-free pDNA was extensively validated in the lungs in vivo [6, 19, 22, 23]. Surprisingly, in contrast to in vivo studies, pCpG-free expression was consistently lower than pCpG-rich during the 14 days course in the human cell lines (Fig 2).

The difference in expression level between the in vivo and in vitro systems might be explained by the inflammatory response evoked by the pDNA. In an in vivo environment, CpG-containing pDNA interacts with Toll-like Receptor 9 (TLR9) to induce an inflammatory response [49, 50]. As a result, activation of 1) Th1-type adaptive immune response [51] leading to the destruction of transgene-carrying cells and loss of transgene expression [7, 8], and 2) pro-inflammatory cytokines, such as TNF-α and IFN-γ, repress transgene expression, regardless of viral or mammalian promoter usage [10]. Therefore, CpG-induced inflammation in vivo is a major barrier to transgene expression, unlike in in vitro system. Here, pCpG-rich that contains 317 CpGs displayed more robust transgene expression capabilities than pCpG-free in the non-immunological in vitro models that lack pro-inflammatory response. This has led us to question the relevance of CpG depletion from transgene for enhanced or sustained in vitro expression. Noteworthy, the presence of CpG in the transgene region has been shown to lead to a higher in vitro transgene expression compared to CpG-depleted transgene [52]. Unlike non-integrating pDNAs used in our study, Krinner et al. (2014) utilized an integrating system called Flp—In™, where the integrated transgene facilitates constitutive expression. Integration of transgene in the host genome has been reported to cause epigenome perturbations and unlikely to provide “actual” gene expression outcomes [53]. Moreover, the expression dynamics between non-integrating vector, such as CpG-free pDNA, and integrating vector differ
significantly [54]. Therefore, further investigation is required to understand the effects of CpG content on transgene expression in the context of non-integrating pDNA.

The pCpG-free and pCpG-rich pDNA constructs used in the earlier study had different pDNA backbones (i.e., EF1α promoter vs CMV promoter) and was not suitable to investigate the influence of transgene CpG content on expression. Therefore, we constructed pDNAs of identical CpG-free backbone with CpG-depleted transgene (pZGFP; 0 CpG) or CpG-containing transgene (pRGFP; 60 CpG). Efforts have been made to ensure that any transcriptional variability was due only to the CpG content of the transgene, and not to any other elements in the pDNA construct (Fig 3). First, these plasmids had identical regulatory elements (i.e., enhancer, promoter, introns, polyA). Next, the scaffold/ matrix attachment region (S/MAR) was included to prevent gene silencing [2] caused by transcriptional interference from the encroachment of heterochromatin from the backbone [55]. Lastly, humanized CpG-depleted allele (ZGFP; 0 CpG) and CpG-containing GFP allele (RGFP; 60 CpG) were selected to determine the influence of CpG depletion from transgene. These alleles encoded proteins that was 98% identical with GFP fluorophore (Leu65-Thr66-Tyr67) to ensure no difference in the ZGFP and RGFP ability to fluoresce that would influence protein expression analysis [36].

Next, we systematically evaluated factors that would impact transgene expression leading to the limited expression of pZGFP, as compared with pRGFP. Regardless of distinct GTAs used,
the pZGFP expression was consistently low in the human cell line, mouse cell line, and primary cells (Figs 4–6), indicating that the abated pZGFP expression was not due to the discrepancy in pDNA delivery or cell types. There was also no discernable cytotoxicity detected that might have caused the loss of pZGFP-transfected cells (Fig 7). In addition, pDNA copy number analysis revealed comparable gene dosage in both pZGFP and pRGFP-transfected cells (Fig 8). These data signify the implication of CpG-depletion on transgene expression, as the only difference between the two pDNAs was the variability in the number of CpG motifs in the transgene. This observation coincides with Krinner et al. (2014), where integrated CpG-depleted transgene in Chinese hamster ovary (CHO) cells exhibited consistently low expression compared to CpG-containing transgene up to 2 years [52]. The inferior GFP activity from pZGFP, despite having similar gene dosage with pRGFP, implies the involvement of a mechanism downstream of gene dosage. Subsequently, we discovered that the total ZGFP mRNA was significantly lower than total RGFP mRNA, clearly indicating a reduced transcription of pZGFP with CpG-depleted transgene (Fig 9). Given the identical enhancer/promoter and the backbone in pZGFP and pRGFP, the transcription activity of these pDNAs was most probably regulated by any of the epigenetic mechanisms that modulate regulatory element activity [56]. However, a limitation of this study is that epigenetic regulation may not be the sole cause for low pZGFP expression. Other possible confounding factors, such as mRNA secondary structures, RNA decay, translational efficiency, and miRNA cryptic sites [57], have not been investigated in this study.

The formation of condensed or loosed chromatin structure by nucleosome occupation affects the accessibility of transcription factors and transcription initiation complexes that dictate the levels of steady state mRNA [44]. FAIRE-qPCR analysis in our study revealed that pZGFP had higher nucleosome density at the promoter and transgene regions than the pRGFP (Fig 10). This highly indicates that the reduced levels of steady state mRNA in pZGFP is associated with the increase in nucleosome enrichment. Interestingly, earlier studies using micrococcal nuclease (MNase) digestion of nuclei and Southern hybridization have shown that transfected pDNA form nucleosomes in mammalian, despite being independent of the host genome [58–60]. As a result, pDNA expression could be improved or restricted by depleting or increasing nucleosome density on pDNA [58, 61]. In addition, studies have shown a strong correlation between CpG and nucleosome density. Global analysis of CpG-depleted genes in mouse and human revealed these regions were insensitive to DNase I and have increased chromatin inaccessibility [62]. In the context of CpG-depleted transgene, Krinner et al. (2014) reported that integrated CpG-depleted transgene exhibited lower expression levels due to increased nucleosomes within the transgene, where else integrated CpG-containing transgene was able to provide expression at higher levels due to nucleosome displacement [52]. To date, no study has shown the nucleosome enrichment as the limiting factor for CpG-free pDNA expression and its mechanism. One possible explanation is the complete CpG depletion in pZGFP may have affected the physical properties of the pDNA. The presence of CpG dinucleotides was shown to affect DNA curvature and bendability, where adding more CpGs further reduce hydrodynamic radius and limit the nucleosome occupation in the DNA [63]. The FAIRE-qPCR technique used in this study, however, only provided general nucleosome density within a DNA region, and higher resolution mapping techniques, such as MNase-Seq, would provide detailed insight into pZGFP-nucleosome interaction [64].

Our findings revealed a conundrum to CpG-free pDNA usage, as complete CpG depletion minimizes detrimental inflammation and avoids DNA methylation silencing but could also leads to limited expression due to increased nucleosome occupation. One potential solution is to include a left-handedly curved DNA sequence (TTTTT[CATGTTTTT]₃) in CpG-free pDNA, as this sequence caused nucleosome sliding and lead to significant improvement of
transgene expression in cell lines and mice [65, 66]. In summary, our data show that the CpG-free pDNA transgene expression in vitro was significantly reduced at the transcriptional level. This is most likely due to nucleosome enrichment that limits the transgene expression, and to date, this is the first report that suggests the impact of nucleosome density on non-integrating CpG-free pDNA expression.

**Author Contributions**

**Conceptualization:** Omar Habib, De-Ming Chau, King-Hwa Ling, Syahril Abdullah.

**Data curation:** Omar Habib.

**Formal analysis:** Omar Habib, King-Hwa Ling, Syahril Abdullah.

**Funding acquisition:** Syahril Abdullah.

**Investigation:** Omar Habib, Rozita Mohd Sakri, Syahril Abdullah.

**Methodology:** Omar Habib, Rozita Mohd Sakri, De-Ming Chau, King-Hwa Ling, Syahril Abdullah.

**Project administration:** Syahril Abdullah.

**Resources:** Syahril Abdullah.

**Supervision:** Syahril Abdullah.

**Validation:** Omar Habib, Rozita Mohd Sakri, De-Ming Chau, King-Hwa Ling, Syahril Abdullah.

**Visualization:** Omar Habib, Nadiah Ghazalli, Syahril Abdullah.

**Writing – original draft:** Omar Habib, Syahril Abdullah.

**Writing – review & editing:** Omar Habib, Nadiah Ghazalli, De-Ming Chau, King-Hwa Ling, Syahril Abdullah.

**References**

1. Hsu CYM, Uludağ H. Nucleic-acid based gene therapeutics: delivery challenges and modular design of nonviral gene carriers and expression cassettes to overcome intracellular barriers for sustained targeted expression. J Drug Target. 2012; 20: 301–328. https://doi.org/10.3109/1061186X.2012.655247 PMID: 22303844

2. Papadakis ED, Nicklin SA, Baker AH, White SJ. Promoters and control elements: designing expression cassettes for gene therapy. Curr Gene Ther. 2004; 4: 89–113. https://doi.org/10.2174/1566523044578077 PMID: 15032617

3. Tolmachov OE. Building mosaics of therapeutic plasmid gene vectors. Curr Gene Ther. 2011; 11: 466–78. Available: http://www.ncbi.nlm.nih.gov/pubmed/22023476 https://doi.org/10.2174/156652311798192798 PMID: 22023476

4. Bird AP. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res. 1980; 8: 1499–504. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=324012&tool=pmcentrez&rendertype=abstract https://doi.org/10.1093/nar/8.7.1499 PMID: 6253938

5. He X, Jia H, Jing Z, Liu D. Recognition of pathogen-associated nucleic acids by endosomal nucleic acid-sensing toll-like receptors. Acta Biochim Biophys Sin (Shanghai). 2013; 45: 241–258. https://doi.org/10.1093/abbs/gms122 PMID: 23369718

6. Hyde SC, Pringle IA, Abdullah S, Lawton AE, Davies LA, Varathalingam A, et al. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. Nat Biotechnol. 2008; 26: 549–51. https://doi.org/10.1038/nbt1399 PMID: 18436402

7. Ghazizadeh S, Kalish RS, Taichman LB. Immune-mediated loss of transgene expression in skin: implications for cutaneous gene therapy. Mol Ther. 2003; 7: 296–303. Available: http://www.ncbi.nlm.nih.gov/pubmed/12668125 https://doi.org/10.1016/s1525-0016(03)00013-3 PMID: 12668125
8. Mercier S, Gahéry-Segard H, Monteil M, Lengagne R, Guillette J-G, Eliot M, et al. Distinct roles of adeno-virus vector-transduced dendritic cells, myoblasts, and endothelial cells in mediating an immune response against a transgene product. J Virol. 2002; 76: 2899-911. Available: http://www.ncbi.nlm.nih.gov/pubmed/11861857

9. Lui VW, Falo LD, Huang L. Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expression in vivo. J Gene Med. 2001; 3: 384–93. https://doi.org/10.1002/jgm.201 PMID: 11529668

10. Ghazizadeh S, Carroll JM, Taichman LB. Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. J Virol. 1997; 71: 9163–9. Available: http://www.ncbi.nlm.nih.gov/entдержерقبلرقة?أرتفع=230218&أرسل=pmcentrez&رئوسة=abstract https://doi.org/10.1128/JVI.71.12.9163-9169.1997 PMID: 9371574

11. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum Gene Ther. 1997; 8: 2019–29. https://doi.org/10.1089/hum.1997.8.17-2019 PMID: 9414251

12. Celhar T, Magalhães R, Fairhurst A-M. TLR7 and TLR9 in SLE: when sensing self goes wrong. Immuno Res. 2012; 53: 58–77. https://doi.org/10.1007/s12026-012-8270-1 PMID: 22434514

13. Bestor TH. Gene silencing as a threat to the success of gene therapy. J Clin Invest. 2000; 105: 409–11.

14. Ghazizadeh S, Carroll JM, Taichman LB. Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. J Virol. 1997; 71: 9163–9. Available: http://www.ncbi.nlm.nih.gov/entдержерقبلرقة?أرتفع=230218&أرسل=pmcentrez&رئوسة=abstract https://doi.org/10.1128/JVI.71.12.9163-9169.1997 PMID: 9371574

15. Hodges BL, Taylor KM, Joseph MF, Sauberla M, Scheule RK. Long-term transgene expression from plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. Mol Ther. 2002; 5: 731–8. https://doi.org/10.1006/mthe.2002.0598 PMID: 12027557

16. Prosch S, Stein J, Staak K, Liebenthal C, Volk HD, Kruger DH. Inactivation of the very strong HCMV immediate early promoter by DNA CpG methylation in vitro. Biol Chem Hoppe Seyler. 1996; 377: 195–201. https://doi.org/10.1515/bchm3.1996.377.3.195 PMID: 8722321

17. Hong K, Sherley J, Lauffenburger D a. Methylation of episomal plasmids as a barrier to transient gene expression via a synthetic delivery vector. Biomol Eng. 2001; 18: 185–92. Available: http://www.ncbi.nlm.nih.gov/pubmed/11576873 https://doi.org/10.1016/s1389-0344(01)00100-9 PMID: 11576873

18. Yew NS, Zhao H, Przybylska M, Wu I-H, Tousignant JD, Scheule RK, et al. CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. Mol Ther. 2002; 5: 731–8. https://doi.org/10.1006/mthe.2002.0598 PMID: 12027557

19. Lesina E, Dames P, Flemmer A, Hajek K, Kirchner T, Bittmann I, et al. CpG-free plasmid DNA prevents deterioration of pulmonary function in mice. Eur J Pharm Biopharm. 2009; 74: 427–34. https://doi.org/10.1016/j.ejpb.2009.11.013 PMID: 19961934

20. Lesina E, Dames P, Rudolph C. The effect of CpG motifs on gene expression and clearance kinetics of aerosol administered polyethylenimine (PEI)-plasmid DNA complexes in the lung. J Control Release. 2010; 143: 243–50. https://doi.org/10.1016/j.jconrel.2010.01.003 PMID: 20074600

21. Alton EWFW, Baker A, Baker E, Boyd AC, Cheng SH, Coles RL, et al. The safety profile of a cationic lipid-mediated cystic fibrosis gene transfer agent following repeated aerosol administration to sheep. Biomaterials. 2013; 34: 10267–10277. https://doi.org/10.1016/j.biomaterials.2013.09.023 PMID: 24090839

22. Davies L, Hyde SC, Nunez-Alonso G, Bazzani RP, Harding-Smith R, Pringle I, et al. The use of CpG-free plasmids to mediate persistent gene expression following repeated aerosol delivery of pDNA/PEI complexes. Biomaterials. 2012; 33: 5618–27. https://doi.org/10.1016/j.biomaterials.2012.04.019 PMID: 22575838

23. Pringle I a, Hyde SC, Connolly MM Lawton AE, Xu B, Nunez-Alonso G, et al. CpG-free plasmid expression cassettes for cystic fibrosis gene therapy. Biomaterials. 2012; 33: 6833–42. https://doi.org/10.1016/j.biomaterials.2012.06.009 PMID: 22727465

24. Mitsui M, Nishikawa M, Zang L, Ando M, Hattori K, Takahashi Y, et al. Effect of the content of unmethylated CpG dinucleotides in plasmid DNA on the sustainability of transgene expression. J Gene Med. 2009; 11: 435–43. https://doi.org/10.1002/jgm.1317 PMID: 19291673

25. Cim A, Sawyer GJ, Zhang X, Su H, Collins L, Jones P, et al. In vivo studies on non-viral transdifferentiation of liver cells towards pancreatic β cells. J Endocrinol. 2012; 214: 277–88. https://doi.org/10.1530/JOE-12-0033 PMID: 22685335

26. Klausner EA, Zhang Z, Wong SP, Chapman RL, Volin M V, Harbottle RP. Corneal gene delivery: chitosan oligomer as a carrier of CpG rich, CpG free or S/MAR plasmid DNA. J Gene Med. 2012; 14: 100–8. https://doi.org/10.1002/jgm.1634 PMID: 22106057
27. Kato M, Hattori Y, Kubo M, Maitani Y. Collagenase-1 injection improved tumor distribution and gene expression of cationic lipoplex. Int J Pharm. 2012; 423: 428–34. https://doi.org/10.1016/j.ijpharm.2011.12.015 PMID: 22197775

28. Ngai SC, Rosli R, Al Abbar A, Abdullah S. DNA Methylation and Histone Modifications Are the Molecular Lock in Lentivirally Transduced Hematopoietic Progenitor Cells. Biomed Res Int. 2015; 2015: 1–11. https://doi.org/10.1155/2015/346134 PMID: 25961011

29. Graf M, Schoedl T, Wagner R. Rationales of Gene Design and De Novo Gene Construction. Systems Biology and Synthetic Biology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; pp. 411–438. https://doi.org/10.1002/9780470437988.ch12

30. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861–72. https://doi.org/10.1016/j.cell.2007.11.019 PMID: 18317886

31. de Wolf HK, Johansson N, Thong A-T, Snel CJ, Mastrobattista E, Hennink WE, et al. Plasmid CpG depletion improves degree and duration of tumor gene expression after intravenous administration of polyplexes. Pharm Res. 2008; 25: 1654–62. https://doi.org/10.1007/s11095-008-9558-7 PMID: 18317886

32. Magnusson T, Haase R, Schleef M, Wagner E, Ogris M. Sustained, high transgene expression in liver with plasmid vectors using optimized promoter-enhancer combinations. J Gene Med. 2011; 13: 382–91. https://doi.org/10.1002/jgm.1585 PMID: 21721074

33. Luu-The V, Paquet N, Calvo E, Cumps J. Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction. Biotechniques. 2005; 38: 287–93. Available: http://www.ncbi.nlm.nih.gov/pubmed/15727135

34. Bauer AP, Leikam D, Krinner S, Notka F, Ludwig C, Längst G, et al. The impact of intragenic CpG content on gene expression. Nucleic Acids Res. 2010; 38: 3891–908. https://doi.org/10.1093/nar/gkq115 PMID: 20203083

35. Nammo T, Rodríguez-Seguí SA, Ferrer J. Mapping Open Chromatin with Formaldehyde-Assisted Isolation of Regulatory Elements. 2011. pp. 287–296. https://doi.org/10.1007/978-1-61779-316-5_21 PMID: 21913087

36. Holter W, Rabson AB, Corsico CD, Howard BH. Sequence-specific toxicity of transfected retroviral DNA. Exp Cell Res. 1991; 193: 54–8. https://doi.org/10.1016/0014-4827(91)90837-5 PMID: 1847335

37. Upadhaya M, Vivekanandan P. Depletion of CpG Dinucleotides in Papillomaviruses and Polyomaviruses: A Role for Divergent Evolutionary Pressures. PLoS One. 2015; 10: e0142368. https://doi.org/10.1371/journal.pone.0142368 PMID: 26544572

38. Yang X, Yang Y, Sun B-F, Chen Y-S, Xu J-W, Lai W-Y, et al. 5-methylcytosine promotes mRNA export —NSUN2 as the methyltransferase and ALYREF as an m5C reader. Cell Res. 2017; 27: 606–625. https://doi.org/10.1038/cr.2017.55 PMID: 28418038

39. Nocetti N, Whitehouse I. Nucleosome repositioning underlies dynamic gene expression. Genes Dev. 2016; 30: 660–672. https://doi.org/10.1101/gad.274910.115 PMID: 26966245

40. Capurso D, Xiong H, Segal MR. A histone arginine methylation localizes to nucleosomes in satellite II and III DNA sequences in the human genome. BMC Genomics. 2012; 13: 630. https://doi.org/10.1186/1471-2164-13-630 PMID: 23153121
46. Alton E, Armstrong D, Ashby D, Bayfield K, Bilton D, Bloomfield E, et al. Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med. 2015; 2: 1–9. https://doi.org/10.1016/S2213-2600(15)00245-3 PMID: 26149841

47. Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J Gene Med. 2004; 6: 395–404. https://doi.org/10.1002/jgm.516 PMID: 15079614

48. Kia A, Yata T, Haji N, Hajitou A. Inhibition of histone deacetylation and DNA methylation improves gene expression mediated by the adeno-associated virus/phase in cancer cells. Viruses. 2013; 5: 2561–2572. https://doi.org/10.3390/v5102561 PMID: 24153059

49. Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J Immunol. 1999; 163: 1552–61. Available: http://www.ncbi.nlm.nih.gov/pubmed/10415059 PMID: 10415059

50. Loisel S, Le Gall C, Doucet L, Ferec C, Floch V. Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipoplexes. Hum Gene Ther. 2001; 12: 685–96. https://doi.org/10.3390/v5102561 PMID: 24153059

51. Chen D, Murphy B, Sung R, Bromberg JS. Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function. Gene Ther. 2003; 10: 991–8. https://doi.org/10.1038/sj.gt.3302031 PMID: 12765420

52. Knirrer S, Heitzer AP, Diermeier SD, Obermeier I, Langst G, Wagner R. CpG domains downstream of TSSs promote high levels of gene expression. Nucleic Acids Res. 2014; 1–14. https://doi.org/10.1093/nar/gkt1358 PMID: 24413563

53. Doerfler W, Weber S, Naumann A. Inheritable epigenetic response towards foreign DNA entry by mammalian host cells: a guardian of genomic stability. Epigenetics. 2018; 13: 1141–1153. https://doi.org/10.1080/15592294.2018.1549463 PMID: 30458693

54. Pelascini L, Altiom JM, Gonçalves MAFV. Histone deacetylase inhibition activates transgene expression from integration-defective lentiviral vectors in dividing and non-dividing cells. Hum Gene Ther. 2013; 24: 78–96. https://doi.org/10.1089/hum.2012.069 PMID: 23140481

55. Chen Z-Y, Riu E, He C-Y, Xu H, Kay M. Silencing of episomal transgene expression in liver by plasmid bacterial backbone DNA is independent of CpG methylation. Mol Ther. 2008; 16: 548–56. https://doi.org/10.1038/sj.mt.6300399 PMID: 18253155

56. Gibney ER, Nolan CM. Epigenetics and gene expression. Heredity (Edinb). 2010; 105: 4–13. https://doi.org/10.1038/hdy.2010.54 PMID: 20461105

57. Schaeble B, Sun W, Li Y-S, Fang L, Chen W. The evolution of posttranscriptional regulation. Wiley Interdiscip Rev RNA. 2018; 9: e1485. https://doi.org/10.1002/wrna.1485 PMID: 28951258

58. Reeves R, Gorman CM, Howard B. Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. Nucleic Acids Res. 1985; 13: 3599–3615. https://doi.org/10.1093/nar/13.10.3599 PMID: 3859838

59. Jeong S, Stein A. Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. Nucleic Acids Res. 1994; 22: 370–375. https://doi.org/10.1093/nar/22.3.370 PMID: 7510391

60. Riu E, Chen Z-Y, Xu H, He C-Y, Kay M. Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo. Mol Ther. 2007; 15: 1348–55. https://doi.org/10.1038/sj.mt.6300177 PMID: 17457320

61. Hebbard PB, Archer TK. Altered Histone H1 Stoichiometry and an Absence of Nucleosome Positioning on Transfected DNA. J Biol Chem. 2008; 283: 4595–4601. https://doi.org/10.1074/jbc.M709121200 PMID: 18156629

62. Beck S, Rhee C, Song J, Lee B-K, LeBlanc L, Cannon L, et al. Implications of CpG islands on chromosomal architectures and modes of global gene regulation. Nucleic Acids Res. 2018; 46: 4382–4391. https://doi.org/10.1093/nar/gky147 PMID: 29529258

63. Jimenez-Usequi I, Shim D, Yu J, Yuan C. Unmethylated and methylated CpG dinucleotides distinctively regulate the physical properties of DNA. Biopolymers. 2014; 101: 517–524. https://doi.org/10.1002/bip.22411 PMID: 24122444

64. Liedeg C, Krietenstein N, Walker M, Korber P. Nucleosome positioning in yeasts: methods, maps, and mechanisms. Chromosoma. 2015; 124: 131–51. https://doi.org/10.1007/s00412-014-0501-x PMID: 25529773

65. Kamiya H, Fukunaga S, Ohyama T, Harashima H. Effects of carriers on transgene expression from plasmids containing a DNA sequence with high histone affinity. Int J Pharm. 2009; 376: 99–103. https://doi.org/10.1016/j.ijpharm.2009.04.032 PMID: 19409470
Fukunaga S, Kanda G, Tanase J, Harashima H, Ohyama T, Kamiya H. A designed curved DNA sequence remarkably enhances transgene expression from plasmid DNA in mouse liver. Gene Ther. 2012; 19: 828–35. https://doi.org/10.1038/gt.2011.127 PMID: 21918548