



Production of Lentivirus Carrying Green Fluorescent Protein with Different Promoters for *in vitro* Gene Transfer

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ABSTRACT

Many diseases are potential targets for gene therapy using either non-viral or viral vectors. Unlike non-viral methods, viral vectors, such as lentiviruses, have the ability to integrate into the host chromosome, which can lead to long-term transgene expression. Lentiviruses have advantages over other types of viruses due to their capacity to transduce non-dividing cells. An optimized generation of lentiviruses carrying green fluorescent protein (GFP) reporter gene driven by either UbC (LV/UbC/GFP) or CMV (LV/CMV/GFP) promoter is described in this paper. The lentiviruses were produced by co-transfecting lentiviral expression constructs and packaging mix into 293FT lentivirus producer cell lines. Lipofectamine was highly efficient in transfecting the cells compared to Transfast and Polyethyleneimine (PEI). Following cell transfection, syncytia were clearly visible at day 2. Lentiviruses were harvested at days 1, 2 and 3 post-transfection. The highest transduction efficiency was read from LV/CMV/GFP harvested at day 2 post-transfection and LV/UbC/GFP harvested at day 3 post-transfection. Finally, the GFP expression in COS-7 cells was determined at day 2 and day 14 post-transduction for transient and stable GFP expression. It was found that the GFP expression declined overtime. However, the transduction efficiency and duration of the transgene expression in COS-7 cells transduced with LV/CMV/GFP were higher compared to LV/UbC/GFP. In conclusion, we have successfully produced lentiviruses carrying GFP with different promoters and shown that the viruses were able to infect COS-7 cells at different efficiencies. Meanwhile, the generation of the active lentiviruses will allow us to proceed to the subsequent analysis of the effect of regulatory elements in future study.

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INTRODUCTION

Gene therapy is the introduction of a therapeutic gene into target cells to replace, manipulate or supplement non-functional or malfunctioning genes to treat diseases (Yang *et al.*, 2006). Unlike non-viral methods, which are limited by their low rate of gene transfer and short duration of transgene expression, retroviral vectors have the ability to integrate into the host genome permanently, and this can further lead to a long-term transgene expression (Malik & Arumugam, 2005). Recombinant retroviruses derived from murine leukemia viruses are widely used as a gene transfer vector in research and clinical settings (Verma & Somia, 1997). However, retroviral vectors can only transduce actively dividing cell (Lewis & Emerman, 1994), and thus limit their application in gene therapy.

Lentiviruses are a genus of slow viruses of Retroviridae family, many of which, produce tumours (Kontaratos *et al.*, 2010). They resemble retroviruses in their ability to integrate the transgene into the cell genome, which is in theory, should lead to a permanent transgene expression. Hence, lentivirus vectors have appeared to be an attractive gene therapy vector due to their ability to transduce non-proliferating cells (Bukrinsky *et al.*, 1993; Gallay *et al.*, 1997; Gallay *et al.*, 1995). In 2010, lentiviruses have been applied in about 1.8% of the gene therapy clinical trials for various diseases (<http://www.wiley.co.uk/genmed/clinical/>[June2010]). Although lentiviruses are created based on human immunodeficiency virus type (HIV)-1, the lentiviruses used for the purpose of gene therapy have been modified to enhance their biosafety features (Dull *et al.*, 1998). In the first generation of HIV-derived vectors (Naldini *et al.*, 1996), the hybrid viral particles were produced from the expression of HIV-1 core proteins, enzymes, accessory factors and envelope of vesicular stomatitis virus G glycoprotein (VSVG) (Burns *et al.*, 1993). In the second version, the component plasmids of the lentivirus have been reduced to *gag*, *pol*, *tat* and *rev* genes (Zufferey *et al.*, 1997). In the latest generation of the lentivirus system (the third-generation), only a fractional set of HIV genes were used (*gag*, *pol* and *rev*). A deletion in the 3'LTR of the lentiviral expression construct resulted in a "self-inactivation" of the lentivirus after transduction into the target cells (Zufferey *et al.*, 1998), rendering the proviral components to be unable to produce more viruses. The lentivirus was generated by the co-transfection of lentiviral expression plasmid with three lentiviral component plasmids in the transformed embryonal human kidney cells (293FT). These component plasmids supply the structural and replication proteins *in trans* for the generation of a complete virion. The co-transfection process can be performed by using a myriad of transfection agents, such as FuGene, GeneJuice, Lipofectamine, Transfast, Polyethyleneimine and several others. Meanwhile, the lentivirus can be harvested a day or few days post-transfection.

The transduction efficiency of the lentivirus vectors is regulated by many regulatory elements, such as promoters, enhancers, polyadenylation signals, etc. Lentiviral vectors can be designed to carry different types of promoters, depending on the target cell type and the target gene. The commonly used human cytomegalovirus (CMV) immediate-early promoter/enhancer has been shown to generate efficient and high level of gene expression (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Meanwhile, increased transduction efficiency and transgene expression were observed with CMV promoter in the presence of central polypurine tract (cPPT) and woodchuck posttranscriptional regulatory element (WPRE) in a lentiviral gene delivery context (Barry *et al.*, 2001). Interestingly, it has been reported

that human ubiquitin C (UbC) promoter generates a higher expression level of transgene in a wider range of tissues as compared to other routinely used promoters, such as H2-K, CMV or P_{gk}-1 (Schorpp *et al.*, 1996). In a non-viral context, Gill *et al.* (2001) reported that UbC promoter directs higher and more durable transgene expression as compared to the CMV promoter in the lung of mouse.

In the present study, two types of lentivirus vectors carrying green fluorescent protein (GFP) reporter gene were produced. One was driven by UbC promoter (LV/UbC/GFP) and the other by CMV promoter (LV/CMV/GFP). Unlike LV/UbC/GFP, LV/CMV/GFP contained cPPT and WPRE. Prior to the production of lentivirus, different types of transfection reagents were tested for their transfection efficiency in order to select the most effective transfection reagent. Lentiviruses were harvested at different days of post-transduction to determine the highest titre of the lentivirus produced. Finally, the transduction efficiency of LV/UbC/GFP and LV/CMV/GFP in African green monkey kidney fibroblast (COS-7) were also checked for the transient and stable GFP expression.

MATERIALS AND METHODS

Cell Cultures

Transformed embryonal human kidney (293FT) and African green monkey kidney fibroblast (COS-7) cell lines were purchased from Invitrogen (Carlsbad, CA, USA) and American Type Culture Collection (ATCC) (Manassas, VA, USA), respectively. The 293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, MO, USA) supplemented with 6 mM L-glutamine (PAA Laboratories GmbH, Austria), 0.1 mM MEM non-essential amino acids (PAA Laboratories GmbH), 1 mM MEM sodium pyruvate (Sigma-Aldrich), 50 µg/ml Geneticin (Gibco BRL, Paisley, UK) and 10% foetal bovine serum (FBS) (Gibco). COS-7 cells were cultured in DMEM supplemented with 10% FBS. The cells were maintained at 37°C in humidified 5% CO₂ atmosphere.

Construction of the Lentiviral Expression Vectors

Plasmid pMGFP (Promega, Madison, WI, USA) was transformed into *E. coli* JM109 Competent Cells (Promega), whereas the pENTRTM4 (Invitrogen) was transformed into Library Efficiency[®] *E. Coli* DB3.1TM Competent Cells (Invitrogen). The plasmids were purified using PureLinkTM HQ Mini Plasmid Purification kit (Invitrogen). The plasmids were restriction digested and subjected to agarose gel electrophoresis to separate the GFP and pENTRTM backbone from the other digested fragments. Following gel extraction, the GFP was subcloned into the pENTRTM4. The resulting pENTRTM4/GFP was verified using a restriction enzyme analysis on agarose gel. Next, a recombination reaction was performed between pENTRTM4/GFP and pLenti6/UbC/V5-DEST (Lentivirus plasmid destination vector construct) (Invitrogen) using LR ClonaseTM II enzyme mix (Invitrogen) to produce pLenti6/UbC/GFP/V5-DEST. The product was transformed into One Shot[®] Stb13TM chemically competent *E. coli* (Invitrogen) and verified using the restriction enzyme analysis and DNA sequencing. Meanwhile, pLenti6/CMV/GFP/V5-DEST plasmid was purchased from Invitrogen and used as a control in this study.

Transfection

293FT cells were seeded in 24-well tissue culture plate at 5×10^4 cells per well. After 24 hours of culturing, the cells were transfected with 1 μ g of phMGFP, pLenti6/UbC/GFP/V5-DEST (pL/UbC/GFP) or pLenti6/CMV/GFP/V5-DEST (pL/CMV/GFP) by using Lipofectamine (Invitrogen), Polyethyleneimine (PEI) (Sigma-Aldrich) or Transfast (Promega), according to the protocol suggested by the manufacturer. The medium containing the transfection mixture was discarded and replaced with fresh DMEM complete medium at day 1 post-transfection. The transfection efficiency was evaluated as a percentage of GFP-expressing cells within the total cells acquired by flow cytometry analysis at day 2 post-transfection.

Lentivirus Production

Lentivirus vectors, pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G), were generated. Briefly, 293FT cells (1×10^6 cells per well in 6-wells tissue culture plate) were co-transfected with packaging mix (plasmids to provide structural and replication proteins *in trans* required to produce the virions) (Invitrogen) and the lentiviral expression plasmid (pL/UbC/GFP or pL/CMV/GFP) in the OptiMEM (Invitrogen) medium. The packaging mix is comprised of three expression plasmids encoding the packaging protein gag-pol (pLP1), rev-expressing construct (pLP2) and VSVG-expressing construct (pLP/VSV-G). Meanwhile, the morphology of the cells was analyzed under inverted brightfield microscopy examination for the presence of syncytia, and virus supernatant was harvested after 24, 48 and 72 hours by centrifugation at 1000 x g for 15 minutes at 4°C. The lentiviruses produced were LV/UbC/GFP (GFP expression controlled by UbC promoter) and LV/CMV/GFP (GFP expression controlled by CMV promoter).

Transduction Efficiency

COS-7 cells were seeded at 5×10^4 cells per well in 24-well tissue culture plates. On the following day, the cells were transduced with LV/UbC/GFP or LV/CMV/GFP in a complete DMEM tissue culture medium with polybrene (Sigma) at a final concentration of 6 μ g/ml. The medium containing the virus was removed and replaced with fresh complete medium at day 1 post-transduction. At day 2 post-transduction, growth medium was removed and replaced with a dissociation solution. The GFP expression of COS-7 transduced with the viruses was evaluated as a percentage of GFP-expressing cells within the total cells acquired by flow cytometry. The percentage of the GFP expression was used to calculate the titre of lentivirus. Once the titre of the viruses had been determined, the GFP expression was evaluated at day 2 and 14 post-transduction for the transient and stable expression at multiplicity of infection (MOI) of 0.8.

Flow Cytometry

The cells were dissociated and centrifuged at 250 x g for 5 minutes to remove residual media components. Next, the cell pellet was resuspended in PBS with 1% foetal bovine serum. The total events of 10,000 were set. The cells were analyzed for green fluorescence by

FACSVantage (Becton Dickinson, San Jose, CA) using CellQuest software. Dead cells were excluded and negative green fluorescence was set at around 1% for the untreated cells.

Statistical Analysis

Numerical data were expressed as mean with standard deviations. ANOVA was applied to compare the means of the experiment samples, followed by a *post-hoc* test to determine the statistical significance in the mean difference. The differences with $P \leq 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Construction of Lentiviral Expression Vector

Successful gene delivery into somatic cells relies on the ability of the gene delivery vector to deliver the gene with high efficiency. Lentivirus (LV) was selected for this study because it can transduce non-proliferating cell (Zufferey *et al.*, 1997) and integrate into the host genome (He *et al.*, 2005). In addition, Dull *et al.* (1998) reported that the LV was highly efficient for *in vivo* gene delivery and exhibited prolonged transgene expression in several tissues. In this study, the production of two types of LV vectors carrying GFP reporter gene, driven by a UbC (LV/UbC/GFP) or CMV (LV/CMV/GFP) promoter, are described. Unlike LV/UbC/GFP, LV/CMV/GFP contains two additional *cis*-acting regulatory sequences, namely, the Woodchuck Post-transcriptional Regulatory Element (WPRE) and the central Polypurine Track (cPPT).

To construct the lentiviral expression vector carrying GFP reporter gene, driven by UbC promoter, a GFP gene from pHMGFP was subcloned into a pENTR4 to generate pENTR4/GFP, which is an entry clone for the Gateway Expression System. Subsequently, the LR recombination reaction was performed between pENTR4/GFP and pLenti6/UbC/V5-DEST to generate pLenti/UbC/GFP/V5-DEST (pL/UbC/GFP). The resulting construct was verified by restriction enzyme analysis and sequencing. The lentiviral expression vector construct pLenti/CMV/GFP/V5-DEST (pL/CMV/GFP), which is driven by CMV promoter with extra

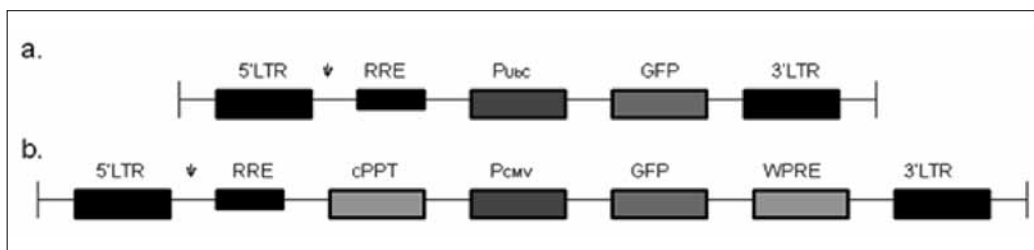


Fig.1: Genomic structure of the lentiviral expression vectors; (a) The LV construct encoding GFP under the control of the UbC promoter, (b) The LV construct encoding GFP under the control of the CMV promoter with cPPT and WPRE. Long terminal repeat (LTR), HIV-1 psi packaging signal (ψ), HIV-1 Rev-response element (RRE), Polypurine Tract from HIV (cPPT), human ubiquitin C promoter (P_{UBC}), human cytomegalovirus immediate-early promoter (P_{CMV}), Green Fluorescent Protein reporter gene (GFP), Woodchuck Posttranscriptional Regulatory Element (WPRE) are indicated

post-transcriptional regulatory element (WPRE) and transduction enhancer (cPPT), was used as a control. The genomic structure of the lentiviral expression vectors is shown in Figure 1.

Transfection Efficiency of Lipofectamine, Polyethyleneimine (PEI) and Transfast on 293FT

To produce the virus, the 293FT cells were co-transfected with lentiviral expression vector and the packaging mix. Due to the myriad of the transfection agents available, it was pertinent to select the transfection agent that could shuttle the plasmids into the cells efficiently. In this study, the transfection efficiency of Lipofectamine, Transfast and Polyethyleneimine (PEI) was compared. Plasmids pL/UbC/GFP and pL/CMV/GFP were transfected into 293FT cell lines and the cells were subjected for examination under fluorescent microscopy and flow cytometry at day 2 of post-transfection.

GFP expression was observed from the cells transfected with pDNAs by all the transfection agents tested (see Fig. 2). Transfast and Lipofectamine appeared to be more efficient at transfecting cells compared to PEI, based on the number of the cells transfected qualitatively. With Transfast, the transfected cells were evenly spread across the plate, whereas the transfection by Lipofectamine was localized to a few populations of the cells. Meanwhile, the delivery of the naked pDNAs did not show qualitatively significant GFP expression.

The flow cytometry analysis was performed to further verify the qualitative observations. The percentage of 293FT cells transfected with the pDNAs using Lipofectamine and Transfast was found to be significantly higher than PEI and naked pDNA (Figure 3). For Lipofectamine, the percentages of the cells with GFP reading were 72.15, 81.90 and 92.29 for pL/UbC/GFP, pL/CMV/GFP and phMGFP, respectively. Transfast showed GFP readings of 71.00% (pL/UbC/GFP), 86.72% (pL/CMV/GFP) and 67.60% (phMGFP). Although Lipofectamine showed a trend of higher percentage of a number of cells transfected, the values were not significantly different than Transfast. The transfection efficiency of PEI was the lowest among the transfection reagents, with GFP readings of 9.93% (pL/UbC/GFP), 13.92% (pL/CMV/GFP) and 4.30% (phMGFP). Mock transfections without transfection agent showed values that are less than 2% for all the pDNAs.

To produce the virus, the lentiviral expression plasmid construct was co-transfected with 3 other plasmids (pLP1, pLP2 and pLP/VSV-G) in packaging mix, which provide the structural, replication protein and envelope of the virus *in trans*. Prior to the production of the virus, different transfection reagents were tested for their ability to transfect the GFP expression plasmids (pL/UbC/GFP, pL/CMV/GFP and phMGFP) into the 293FT LV producer cell line efficiently. Among the transfection reagents tested, Lipofectamine exhibited the highest transfection efficiency. Therefore, Lipofectamine was used to co-transfect the packaging mix and the GFP expressing LV plasmid (either pL/UbC/GFP or pL/CMV/GFP) into the LV producer cell line to produce the virus.

Lentivirus Production and Infectivity

Lentivirus was generated by the co-transfection of 293FT cells with packaging mix (pLP1, pLP2 and pLP/VSV-G) and a lentiviral expression plasmid construct (either pL/UbC/GFP or

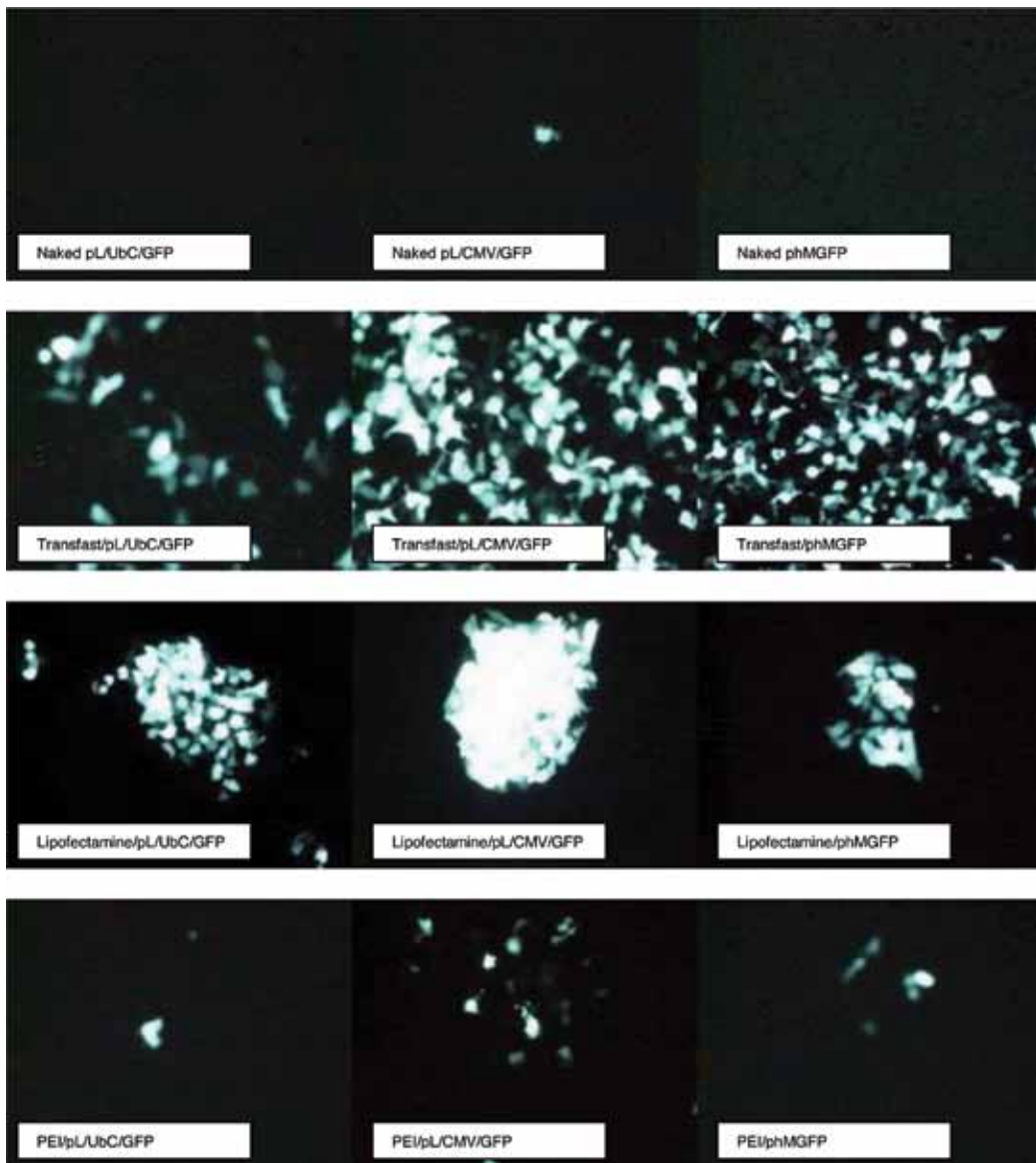


Fig.2: Fluorescent microscopy of the GFP expression in the 293FT cell lines transfected with different plasmids using different transfection reagents; Original magnification x20

pL/CMV/GFP), using Lipofectamine as the transfection reagent. As shown in Fig. 4, syncytia were clearly visible at day 2 post-transfection. The formation of syncytia is a sign for the lentivirus production. Meanwhile, the formation of the multinucleated cells implied that the packaging plasmid had been transfected and expressed successfully in the 293FT cells. The formation of syncytia was due to the expression of VSV-G glycoprotein translated from pL/VSVG, which subsequently caused the fusion of the 293FT cells into large and multinucleated cells. Lentiviruses LV/UbC/GFP and LV/CMV/GFP were harvested at days 1, 2 and 3 post-transfection. To determine the infectivity of the virus, COS-7 cells were transduced with the

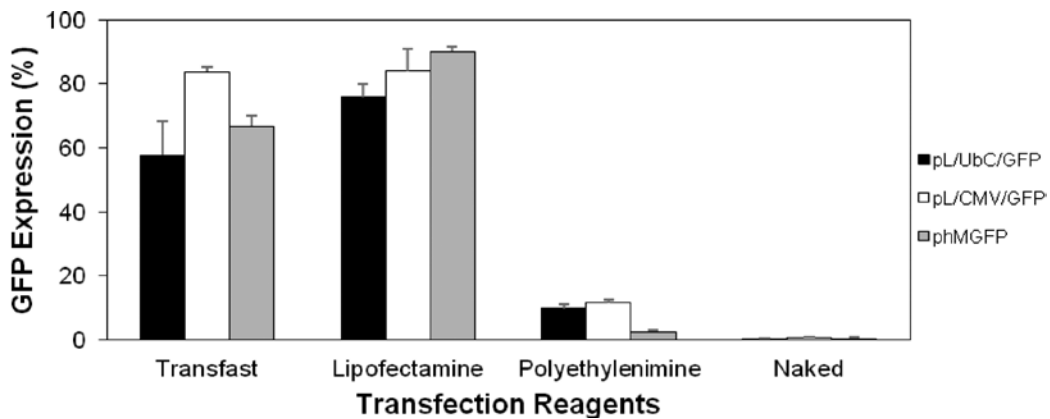


Fig.3: Transfection efficiency using Transfast, Lipofectamine and PEI in the 293FT cell line with plasmid pL/Ubc/GFP, pL/CMV/GFP and phMGFP. The GFP expression was measured at 48h post-transfection as the percentage of GFP-expressing cells within the total cells acquired. Data are presented as mean \pm SD of experiments conducted in triplicates

harvested viruses and the percentage of GFP expressions was determined by flow cytometry at day 2 of post-transduction.

Based on the information illustrated in Fig. 5, LV/Ubc/GFP harvested at day 3 post-transfection showed the highest GFP expression (32.81%) although the reading was not statistically different from the virus harvested at day 2 (19.74%). For LV/CMV/GFP, the lentivirus harvested at day 2 post-transfection showed the highest GFP reading, followed by day 3 and day 1 post-transfection, with GFP expressions of 89.41%, 83.48% and 46.86%. In general, the results also exhibit that the GFP readings from LV/CMV/GFP at all days of post-transfection were significantly higher compared to LV/Ubc/GFP. Since there were no significant differences between the values of both the viruses harvested at day 2 and day 3, it was decided that the lentiviruses should be harvested at day 2 post-transfection for the following experiments to avoid the risks of contamination.

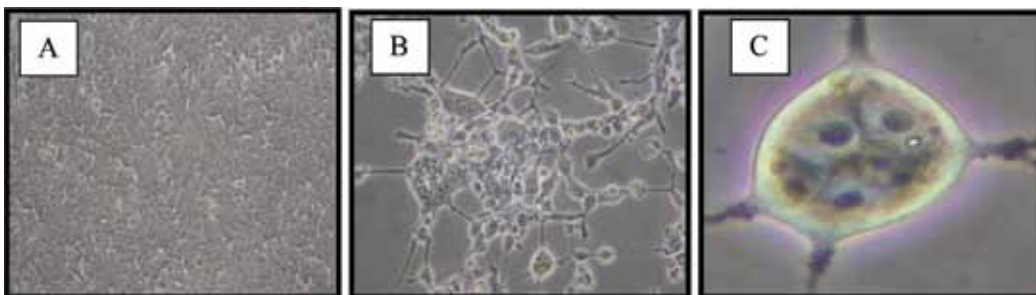


Fig.4: The morphology of the 293FT cell line transfected with lentiviral vector expression plasmid and packaging mix at day 2 of post-transfection; (A) Untreated cells, (B) Transfected cells. 293FT cells were fused together and became multinucleated (syncytia). (C) A single multinucleated cell from the original photo. 293FT cells were examined under inverted brightfield microscope at original magnification x20

The harvested lentivirus was subsequently used to transduce COS-7 cells. Higher transduction efficiency was obtained from the cells transduced with LV/CMV/GFP compared to LV/Ubc/GFP harvested at all days post-transfection even though the same parameters had been used to produce both the viruses. It was speculated that the higher GFP expression by LV/CMV/GFP was either caused by the differences in the promoter used or by the incorporation of WPRE and cPPT in LV/CMV/GFP. A previous study reported that the incorporation of WPRE and cPPT into lentiviral vector driven by CMV promoter provided increased transduction

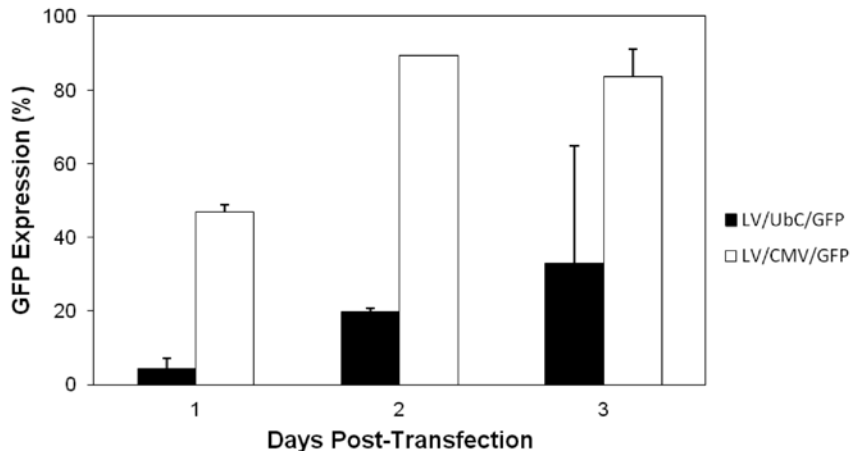


Fig.5: The GFP expression of COS-7 cells transduced with either LV/Ubc/GFP or LV/CMV/GFP harvested at days 1, 2 and 3 post-transfection. The GFP expression was measured at day 2 post-transduction as the percentage of the GFP-expressing cells within the total cells acquired. Data are presented as the mean \pm SD of experiments conducted in triplicates

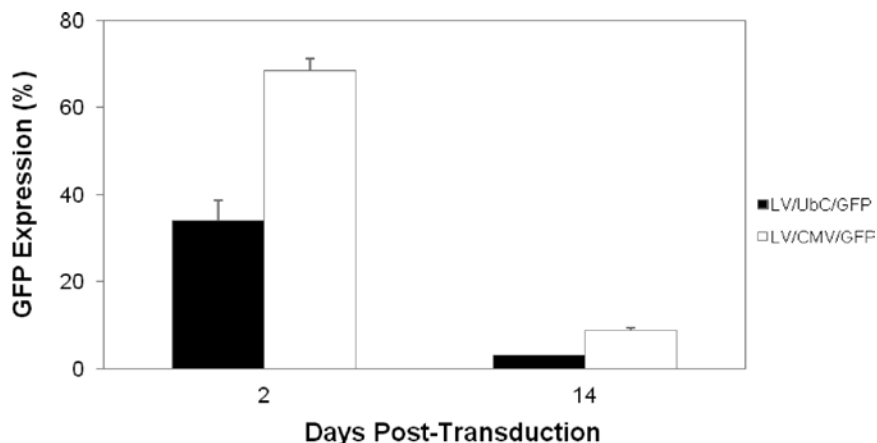


Fig.6: The GFP expression of COS-7 cell lines transduced either with LV/Ubc/GFP or LV/CMV/GFP at MOI of 0.8. GFP was measured as the percentage of the GFP-expressing cells within the total cells acquired at day 2 and day 14 post-transduction. Data are presented as the mean \pm SD of the experiments conducted in triplicates

efficiency (Barry *et al.*, 2001). However, Gill *et al.* (2001) reported that UbC promoter generated greater transgene expression as compared to CMV promoter in a non-viral gene delivery context. The main reason for the high transgene expression by the LV/CMV/GFP will only be determined when a new LV/UbC/GFP is developed with the presence of WPRE and cPPT in future investigations.

Transient and Stable Lentivirus Reporter Gene Expression

GFP activities were also evaluated for the transient and stable expression. Prior to this experiment, the Multiplicity of Infection (MOI) of 0.8 was detected as the most efficient MOI for COS-7 cells transduction (data not shown). Therefore in this study, the cells were transduced with LV/UbC/GFP and LV/CMV/GFP at MOI of 0.8 in the presence of Polybrene. The medium was replaced at day 1 post-transduction and the cells were subjected to flow cytometry analysis at day 2 and day 14 post-transduction. As illustrated in Fig. 6, the GFP expressions for COS-7 transduced with LV/UbC/GFP (33.92%) and LV/CMV/GFP (68.5%) were significantly higher at day 2 post-transduction as compared to day 14 post-transduction (LV/UbC/GFP of 3.1% and LV/CMV/GFP of 8.82%). The results also show that the GFP expression of the cells transduced with LV/CMV/GFP was approximately 2-folds higher than the cells transduced with LV/UbC/GFP at both time points of the study.

The reduction in GFP expression from the cells transduced by both viruses diminished over time, which might be due to transgene silencing or promoter attenuation. Nevertheless, the GFP readings for both LV/CMV/GFP and LV/UbC/GFP at day 14 post-transduction were still significantly high as compared to the untreated control. Similar results were also seen with the GFP expression from murine embryonic carcinoma (EC) P19 cells transduced with the LV (He *et al.*, 2005). The authors reported that the decrease in the transgene expression was due to the transcriptional silencing and not by the deletion of the transgene. Meanwhile, DNA methylation and chromatin modification have been reported as the two modes of epigenetic control of genome function (Ou *et al.*, 2007). These may lead to the GFP silencing as observed in our study. Based on the results obtained in the current study, the cells transduced by LV/UbC/GFP were probably subjected to the transgene silencing more severely as compared to the cells that were transduced by LV/CMV/GFP. Hence, it was speculated that the transgene silencing was caused by the DNA methylation at the CpGs site in the promoter region. The UbC promoter has more CpGs (88 CpGs) as compared to CMV promoter (30 CpGs), and therefore, it may be prone to the silencing effects compared to the CMV promoter. To further confirm whether methylation at the CpG site was the factor for the transgene silencing, sodium bisulfate sequencing performed to compare the percentage of methylation in the UbC and CMV regions in the upcoming study.

The reporter gene, driven by UbC promoter, has been reported to exhibit higher transgene expression *in vivo* as compared to its CMV counterpart, in a non-viral gene delivery context (Gill *et al.*, 2001). However, in the LV gene delivery context study, the LV/CMV/GFP has been shown to exhibit better transduction efficiency and a stable gene expression as compared to LV/UbC/GFP. The superior results exhibited by the LV/CMV/GFP in this study could be due to the presence of cPPT and WPRE in the viral construct. In addition, the LV vector, with

cPPT in its construct, has been shown to have high copy number of provirus integrated into the host genome (Park & Kay, 2001), and thus resulting in the higher reporter gene expression. Sirven *et al.* (2000) also reported that the inefficiency of gene transfer by lentivirus, which was due to the limitation of nuclear translocation, had been overcome by the presence of cPPT. Meanwhile, WPRE, which is purified from woodchuck hepatitis virus, has been exploited as an enhancer of the transgene expression by increasing the nuclear export of mRNA to the cytoplasm and subsequently increased protein synthesis (Mastroiannopoulos *et al.*, 2005).

CONCLUSION

The production of two types of lentiviral vectors has been described in the current study. As indicated earlier, the two types of lentiviral vectors are driven differently; one driven by a UbC promoter (LV/UbC/GFP) and the other by CMV promoter with two additional *cis*-acting regulatory sequences (LV/CMV/GFP). Among the transfection reagents tested to deliver the lentiviral construct and structural plasmids into the 293FT cells, Lipofectamine showed the highest transfection efficiency. The higher transduction efficiency and gene expression were exhibited by LV/CMV/GFP as compared to LV/UbC/GFP. The successful production of these lentiviruses will allow the researchers to further analyze the effects of the UbC promoter and the two regulatory sequences in the LV construct in the subsequent study.

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