The Use of Enhanced Green Fluorescent Protein-Expressing Cell-Based Platform for Cancer Drug Screening

Research Thesis

Presented in partial fulfillment of the requirements for graduation
with research distinction in the undergraduate colleges of The Ohio State University

by

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ABSTRACT

Green Fluorescent Protein from the jellyfish Aequorea victoria is a well-recognized marker for gene expression. During the course of this study, plasmid containing the Enhanced Green Fluorescent Protein (EGFP) gene controlled by human cytomegalovirus (CMV) was amplified using Escherichia coli (E. coli) bacteria. The transfected bacteria were then plated onto a LB agar plate containing antibiotic and allowed to form colonies. Then a single colony of E.coli was picked, transferred to LB liquid medium and incubated overnight on a air shaker. After extraction, the plasmid was used to engineer human Lung cancer A549 cells that express EGFP controlled by human cytomegalovirus promoter. A total of three attempts were made to transfect A549 cells using a lipid vector, Lipofectamine 2000.Each time, the fluorescence of transfected cells was detected using a fluorescence microscope. The results of the study helped establish several optimal parameters concerning successful transfection of A549 were established including the initial cell seeding density before addition of DNA and transfection vector, the ratio of DNA to Lipofectamine 2000, Geneticin concentration for selection of transfected cells, and the transfection efficiency when using linearized vs circular plasmids.

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CHAPTER 1: BACKGROUND

1.1 Background

Green Fluorescent Protein (GFP) and it's variants such as EGFP have been reported to be a powerful tools for high-throughput screening technologies [1]. The gene, once transfected into living cells, can lead to real-time analysis of molecular events [1]. Furthermore, fluorescence in stable EGFP expressing cells lines can be easily monitored using the fluorescent microscope. This makes it a promising technology for cell-based drug screening platforms as a decrease in EGFP fluorescence is shown to be directly quantifiable to cytotoxicity in drug screening assays [2]. Therefore, the use of EGFP expressing cell-based platforms can provide promising results for cancer research, especially concerning drug discovery and screening.

Cancer is one of the leading causes of death around the globe [3], and so a topic of great interest in modern medicine and research. Therefore, it is crucial to explore new, more reliable platforms for testing anti-tumor drugs, and their efficacy on a particular kind of cancer. Additionally, these drug testing platforms can also be used to test new antitumor drugs. Thus, an EGFP expressing cell-based drug testing platform would be a promising approach towards testing cytotoxicity of antitumor drugs in an *in vitro* environment. Furthermore, even though with

growing emphasis on cancer research in the past ten years, the number of potential anticancer agents advanced for development increased [4]. But, the success of these new agents at clinical trial level is approximately 10% [4]. This poses as a huge hurdle for the development of new antitumor drugs. However, EGFP cell-based drug screening technology can help decrease failure rates during clinical trials, especially when used in three-dimension (3D) platforms.

In contrast to two-dimensional (2D) or monolayer cell cultures, 3D platforms mimic *in vivo* environment [4]. Hence, drug cytotoxicity results obtained using 3D drug screening assays will be a lot more similar to results seen *in vivo*. Mainly because, in 3D platforms, tumor cells are co cultures with other cells and components normally present in the tumor microenvironment [4]. That includes endothelial cells, resident fibroblasts, leukocytes, pericytes, and the Extracellular Matrix (ECM). The ongoing interactions among the tumor cells, other 'normal' cells and components of the microenvironment have shown to increase tumor resistance and reduce antitumor drug cytotoxicity [5]. Furthermore, the tumor microenvironment has also been identified as a key contributor for cancer initiation, growth, and proliferation [6]. Hence, for *in vivo* like drug cytotoxicity results it is imperative to recreate the tumor microenvironment in *in vitro* settings.

Previously in our lab, a stable line of EGFP expressing Human Breast Cancer cells (MCF-7) were engineered with CMV and Survivin promoters. These cells were then used in 3D high throughput drug screening platform to test the cytotoxicity to various antitumor drugs.

1.2 Objectives

The goal of this study was to engineer a stable line of EGFP expressing Human Lung Cancer cells (A549). This cell line can then be used in the construction of a 3D drug screening assay to test cytotoxicity of drugs on A549 cells. To achieve this goal the following objectives were pursued in this study.

1. To amplify the EGFP plasmid controlled by the CMV promoter through transformation in the Escherichia coli (E.coli) bacterium.
2. To culture A549 cells in order to observe cellular morphology and cell growth rate.
3. To transfect A549 cell with EGFP plasmid, and to establish parameters including:
    a. The initial seeding density of A549 cells 24 h before transfection.
    b. The appropriate DNA plasmid to transfection vector ratio.
    c. The effects of high and low serum concentrations in the cell culture medium before and during transfection.

d. The effects of using linearized vs circular plasmid on the transfection efficiency.

CHAPTER 2: INTRODUCTION

2.1 A549 - Cancer Cell Line

A549 cell line was originally developed in 1972 by Giard et al. from a 58-year-old Caucasian male with lung adenocarcinoma[7]. The tumor was surgically removed from the patient and the cancerous lung tissue was then cultured in an *in vitro* environment to generate a stable cell line[7]. Research shows that like all other cancer types, A549 lung adenocarcinoma develops as a result of the uncontrollable proliferation of normal body cells, alveolar basal epithelial cells in this case[7,8]. Under normal conditions, the main function of these squamous cells is the transportation of polar substances such as water and electrolytes across the alveolar wall [7]. However, once these cells turn cancerous they differ from normal cells in many ways [8]. Including that they lose some if not all of their functions, become resistant to apoptosis signals, and are able to make use of surrounding body cells and blood vessels to form what is known as a 'tumour microenvironment' to fulfill their nutrient requirements [8]. However, it is also true that A549 cells resemble type II pneumocytes that are one of the two types of cells that line the alveoli in a number of characteristics, and so are often used in research as models of these cells [9].

In terms of morphology, these cells appear to have an elongated, irregular shape. They also seem to grow within close proximity of one another [10], which is especially visible in cultures with high confluency or high initial seeding densities. Furthermore, these cells proliferate at a very fast rate, with a doubling time of around 20 h [11].

2.2 EGFP as a Marker for Gene Expression

GFP, extracted from the Jellyfish *Aequorea victoria* is a well-recognized indicator for gene expression and product in experimental procedures [12]. GFP absorbs blue light (maximum 395 nm) and emits green light (maximum 509 nm) [13] which causes it to appear green when observed under the fluorescence microscope. During the course of this study an improved version of GFP, EGFP was used which is constructed by changes in the GFP coding sequences [12]. These changes make EGFP 35 times brighter than wild-type GFP [12]. Hence, the improved reporter protein allows for increased sensitivity when studying the toxicity of anti-tumor drugs on cells transfected with EGFP.

2.3 CMV as Promoter for Cancer Drug Screening

The human cytomegalovirus (CMV) is a member of the herpesvirus family. The state of the viral genome during persistence in the human body is still unknown, but it known that herpes viruses do not integrate into the host genome and rather

persist as free genomic DNA [14]. However that being said, CMV is commonly used as a constitutive promoter for gene expression in human cells as it is mostly unaffected by environmental pressures [15,16].

2.4 Amplification of Plasmid via Transformation

Transformation is one of the processes of Horizontal Gene Transfer (HGT) in bacteria, where a bacteria uptakes extracellular DNA [17,18]. In nature HGT between bacterial cells helps the bacteria to adapt to gain new characteristics. These characteristics prove beneficial for as they can aid in adaptation to a new environment, resistance to antibiotics, and spread of pathogenicity in pathogenic strains [18]. Furthermore, in the long term HGT can also contribute to evolution of bacterial species [18]. Hence, making use of the same bacterial characteristic, the plasmid containing the EGFP gene and CMV promoter was amplified within Escherichia coli (E.coli) bacteria.

2.5 Generation of Stable EGFP Expressing A549 Cell Line

Transfection can be described as entry of foreign nucleic acids (DNA or RNA) into the cell [19]. Hence, this technology can be used to study gene expression and monitor gene products [19]. However, transfection can be stable or transient. In transient transfection, the gene gains entry into the cell but is not incorporated into its genome [19]. Therefore, it is expressed for a short period of time. On the other

hand, stably transfected genes are integrated into the cells genome and so the gene product can be observed for long periods of time. The technology is also well known for its therapeutic and scientific effects in gene therapy such as knocking down a particular gene or delivering a gene of interest to cure a disease to reduce symptoms [19]. Similarly, during the course of our study, we aimed to stably transfect A549 with the EGFP plasmid to generate a cell line with long lasting EGFP expression to be later used for drug toxicity studies.

CHAPTER 3: MATERIALS AND METHODS

3.1 Culture of A549

A cryopreserved vial of A549 was thawed, by keeping the vial in a water bath at 37℃ for about 2 minutes. The cells were then resuspended in the cryopreservation medium and were centrifuged for 5 minutes at 1000 rpm. The supernatant was removed, and the cells were resuspended in cell culture medium containing DMEM (Gibco), 10% FBS (Atlanta biologicals), 1% NEAA (Gibco), and 1% PennStrep (Gibco) to seed into a T-25 flask. The cells were allowed to proliferate and the morphology was observed. Furthermore, these cells were passaged every three days using 0.25% Trypsin (Thermofisher Scientific) to suspend the seeded cells. The trypsin was then neutralized by cell culture medium and the mixture was centrifuged for 5 minutes at 1000 rpm.

3.2 A549 Cell Growth Rate

A cell growth curve experiment was carried out to study the doubling time and growth rate of A549 cells. On Day 1, twelve wells of two six-well plates were seeded with 3×10^5 cells in each well. After 24 hours' incubation, 1 ml of 0.25% Trypsin-EDTA (Gibco) was added to two wells to digest cells, and the plate was kept in the incubator for 5 minutes. Then 2 ml of cell culture medium was added to

these wells to neutralize the trypsin. The contents of both the wells were mixed by gently pipetting the mixture up and down several times and then were centrifuged separately at 1000 rpm for 5 minutes. The supernatant was removed, and cell culture media (500ml - 1000ml) was added to resuspend the cells in both centrifuge tubes. 10 µl of cells suspended in cell culture medium was added to 10 µl of Trypan Blue (Gibco). The cell number was then counted using the hemacytometer. Cells in four of the 1 mm^2 boxes were counted (Figure 1), and the results were averaged by adding the number of cells in all four boxes and then dividing the total by 4[20]. The cell number within each well was then calculated using the following formula [20]:

$$\frac{mean\ number\ of\ cells\ in\ four\ 1\ mm^2\ boxes \times 10^4}{ml\ of\ suspension}$$

The same calculations were repeated for the second well. The cell numbers obtained from both the wells were averaged and the resulting value was noted down as the cell count for Day 1. The same processes were repeated with a 24-hour interval every day for six days, and a cell growth curve was plotted.

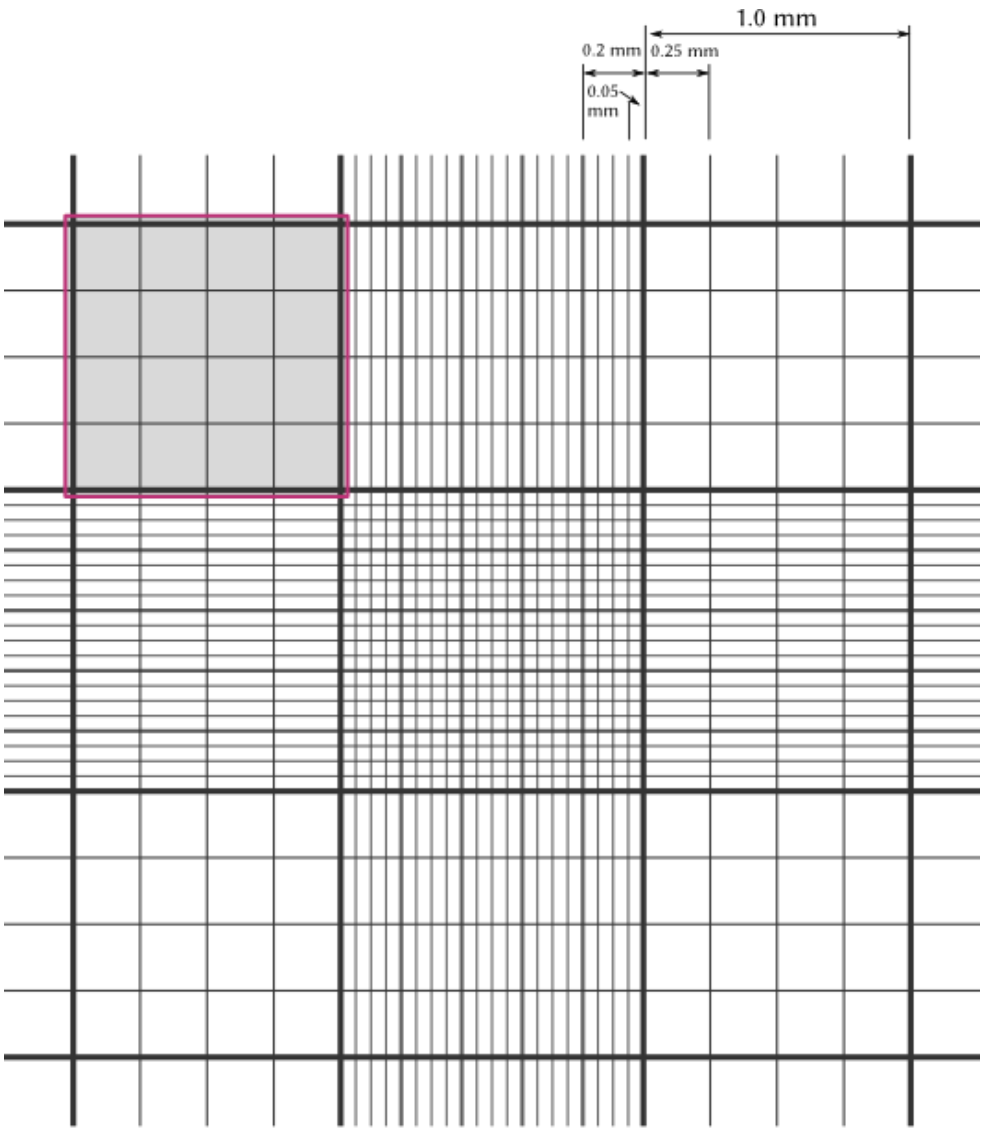

Figure 1. Pictorial representation of a hemacytometer grid [20].

3.3 Cryopreservation

Along the course of the study, some cells were cryopreserved for future use in the lab. The cells were detached from the surface of the T-75 flask using 5 ml of 0.25% Trypsin-EDTA (Gibco) and then neutralized with 10 ml cell culture medium. After which they were then centrifuged at 1000 rpm for five minutes, and the supernatant was removed. The cell number was then counted using a hemacytometer, following the same calculation procedure as explained earlier. Roughly around 1,000,000 cells suspended in 1 ml of cryopreservation media containing 90% FBS (Atlanta biologicals) and 10% DMSO (Thermofischer Scientific) were added to each cryopreservation vial. These vials were then stored at -80℃ for future use.

3.4 Plasmid Amplification

The plasmid containing EGFP sequence with CMV promoter was expanded within the bacterium, Escherichia coli. First E. coli was placed in ice, and Kanamycin solution was prepared to contain 0.03g of antibiotic per 1 ml of water. 1g of Lysogeny broth (LB) medium was melting by heating. 80µl of kanamycin solution was added to the LB medium, and the medium was equally divided and poured into three plates. These plates were then kept under UV light for 1 hour, so the medium can solidify and coat the plates. 2µl of the plasmid was transferred to the

E. coli and the vial was kept back on the ice for 45 minutes. After which E.coli with the plasmid was heated at 42℃ for 30 seconds, and immediately put back on the ice for another 3-5 minutes. Then 900µl of Super Optimal Broth (SOB medium) was added to the vial with the bacteria and the resulting mixture was put in a shaker incubator at 37℃ for 1 hour. Afterward, the mixture was centrifuged at 9,400 rpm for 3 minutes, and 800µl of SOB medium was removed, leaving behind 100µl to resuspend the bacterial cells. Then, the plates coated with LB medium earlier , were drawn and incubated overnight at 37℃.

The next day, 10 ml of liquid LB medium was added to a 50 mL centrifuge tube, along with 8µl of kanamycin solution. A single colony of E.coli was selected using a pipette tip and added to the solution containing LB medium and kanamycin. This tube was then put into a shaker incubator overnight at 37℃.

The next morning, the plasmid was isolated from the bacterial cells using the DNA/RNA extraction kit (Viogene). Firstly, 1.5 ml of the solution containing bacterial cells, LB medium, and kanamycin solution was transferred to another tube and was centrifuged at 8000 rpm for 5 minutes. The supernatant was removed, and 250µl of buffer A containing resuspension solution and RNase A (Viogene) was used to resuspend the pelleted cells [21]. Then 250µl of buffer B (lysis solution) (Viogene) was added and mixed thoroughly by inverting the tube up and

down several times until the lysate became clear [21]. The mixture was then incubated for 2- 3 minutes before 350µl of buffer C (Viogene) was added to neutralize it [21]. The tube was inverted up and down until precipitation was seen. The solution was then centrifuged at 8000 rpm for 10 minutes, and the supernatant was filtered using the Mini *plus* column placed onto a collecting tube[21]. The solution was centrifuged again at 8000 rpm for 1 minute and the flow through was discarded [21](the DNA is in the membrane, and impurities are in the flow through). The filtration column was washed with 0.5ml of WN buffer (Viogene) by centrifuging at 8000 rpm for 1 minute and the flow through was discarded [21]. The second time, the column was washed with 0.7ml of WN buffer by centrifuging at 8000 rpm for 1 minute and the flow through was discarded [21]. The column was then again centrifuged a third time at 8000 rpm for 1 minute and was then left without a lid so that the ethanol can evaporate. Then the collecting tube was replaced with a new 1.5 ml centrifuge tube and 30 µl of Elution buffer (Viogene) was added to the center of the membrane. The column was left to stand for 2-3 minutes, after which it was centrifuged at 8000 rpm for 3 minutes [21].

Lastly, the DNA concentration was determined using the Nanodrop spectrophotometer, and the plasmid was stored at -20℃ to be later used for cell transfection.

3.5 A549 Transfection

On Day 1, four wells of a 24-well plate were seeded with 1×10^5, 1.2×10^5, 1.6×10^5, and 2×10^5 cells in 500 µl of cell culture medium (without antibiotic) containing DMEM (Gibco), 10% FBS (Atlanta), and 1% NEAA (Gibco). After 24 hours had passed, wells seeded with 1.6×10^5 and 2×10^5 cells on Day 1 were selected for transfection. Since the cells in these two wells were 85-90% confluent [22].

0.8 µg of DNA was diluted in 50 µl of Opti-MEM I Reduced Serum Medium (Gibco) and was gently mixed by pipetting up and down several times [22]. Then 2.0 µl of Lipofectamine 2000 (Invitrogen, ThermoFischer Scientific) reagent was added to 48 µl of Opti-MEM I Reduced Serum Medium and the resulting mixture was incubated for 5 minutes at room temperature [22]. Afterward, the DNA solution and the Lipofectamine solution were combined to make up a final volume of 100 µl [22]. This mixture of the two solutions was then incubated at room temperature for 20 minutes [22]. 100 µl of the solution containing DNA-lipo complexes was added to each of the two wells, along with 400 µl of cell culture medium (without antibiotic) [22]. The cells were then incubated at 37 ℃ for 24 hours, after which each well was passaged to 10 wells with 500 µl of fresh cell culture medium (without antibiotic) in each well.

The next day, selective medium with two concentrations of geneticin (G-418,Gibco); 500 µg/ml [23,24] or 800 µg/ml [23,25], DMEM, 10%FBS, and 1% NEAA was prepared. 500 µl of selective medium with 500 µg/ml of Geneticin was added to five wells with the initial seeding density of 1.6×10^5 cells and five wells with the initial seeding density of 2×10^5 cells. Similarly, medium containing 800 µg/ml geneticin was added to the remaining 10 wells. The cells were passaged five times using the cell culture medium containing two concentrations of geneticin before they were checked for gene expression under the fluorescence microscope.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 A549 Morphology

The morphology of A549 cells was observed under a light microscope at 40× and 100× magnification (Figure 2). The cells seemed to have a polygonal [26,9]. spindle-like shape [27], with some being more elongated and stretched out than the rest. Furthermore, most if not all of the viable cells were observed to be tightly attached to the surface of the 6-well plate [27] and only dead cells were seen as suspended in the cell culture medium.

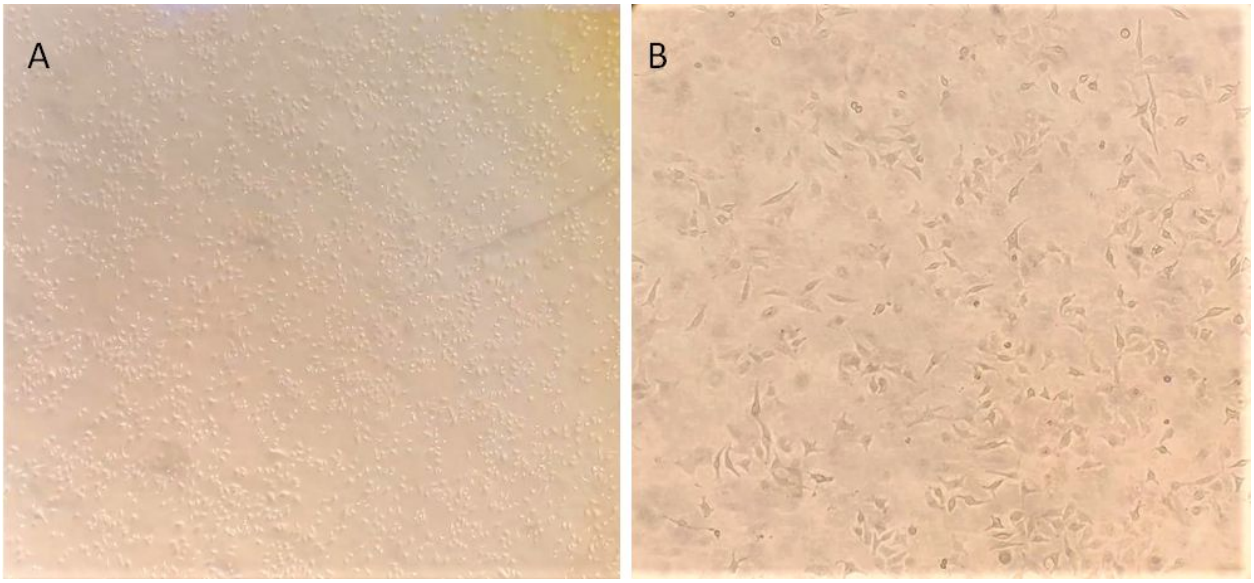

Figure 2. Images of A549 cell viewed under the light microscope at two different magnifications. A: At 40× magnification. B: At 100× magnification.

4.2 A549 Cell Growth Curve

Typically a cell growth curve can be divided into four distinct phases. Namely the initial latent or lag phase, followed by a logarithmic (log) growth phase, which is then followed by a plateau or stationary phase, and finally the decline phase (Figure 3) [28]. The lag phase is characterized by little or no division as in this phase the cells usually familiarize themselves with their surroundings [28]. Once, the cells adapt to the culture condition they enter the log phase, where they proliferate exponentially [28,29,30]. Cells are usually subcultured or passaged in this phase in order to allow more space and nutrients for proliferation [29], until they reach the stationary phase. In this phase cell division halts, and no significant increase in the cell number is observed as the cells participating in active mitotic cycle drops to less than 10% [28]. This is the followed by the decline phase, where a reduction in the number of viable cells is seen with passing time [28].

In the course of this study a cell growth curve was plotted (Figure 5) for A549 cells using the results from the cell growth rate experiment (27,9). Following the initial seeding of 300,000 cells per well on Day 1, the cells entered the lag phase of growth and showed slight increase in number on Day 2 (Figure 4) and Day 3. However, the cells entered the log phase on Day 4 (Figure 4) and the cells were

observed to double in number. Afterwhich on Days 5 and 6 the cells showed characteristics of the stationary period as the observed cell number was approximately around 500,000 cells on both days. Finally on Day 7, a decrease in cell number was seen, and it can be concluded that the cells had entered the decline phase by the end of the experiment.

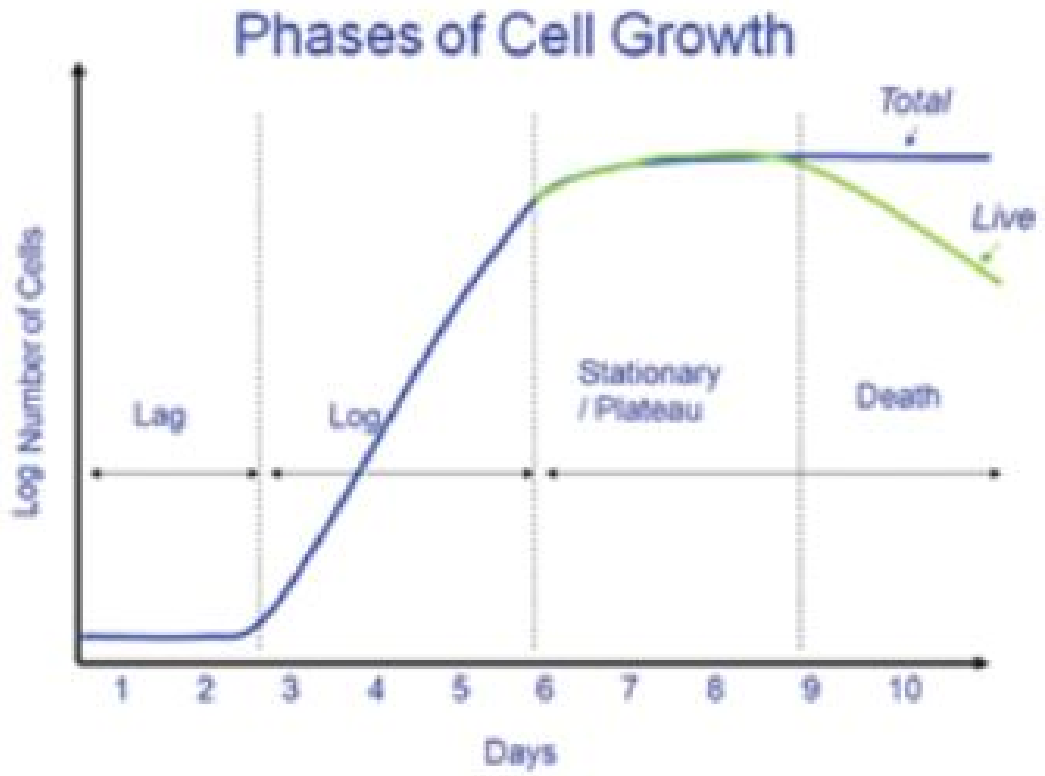

Figure 3. Graphical representation of the phases of cell growth [28].

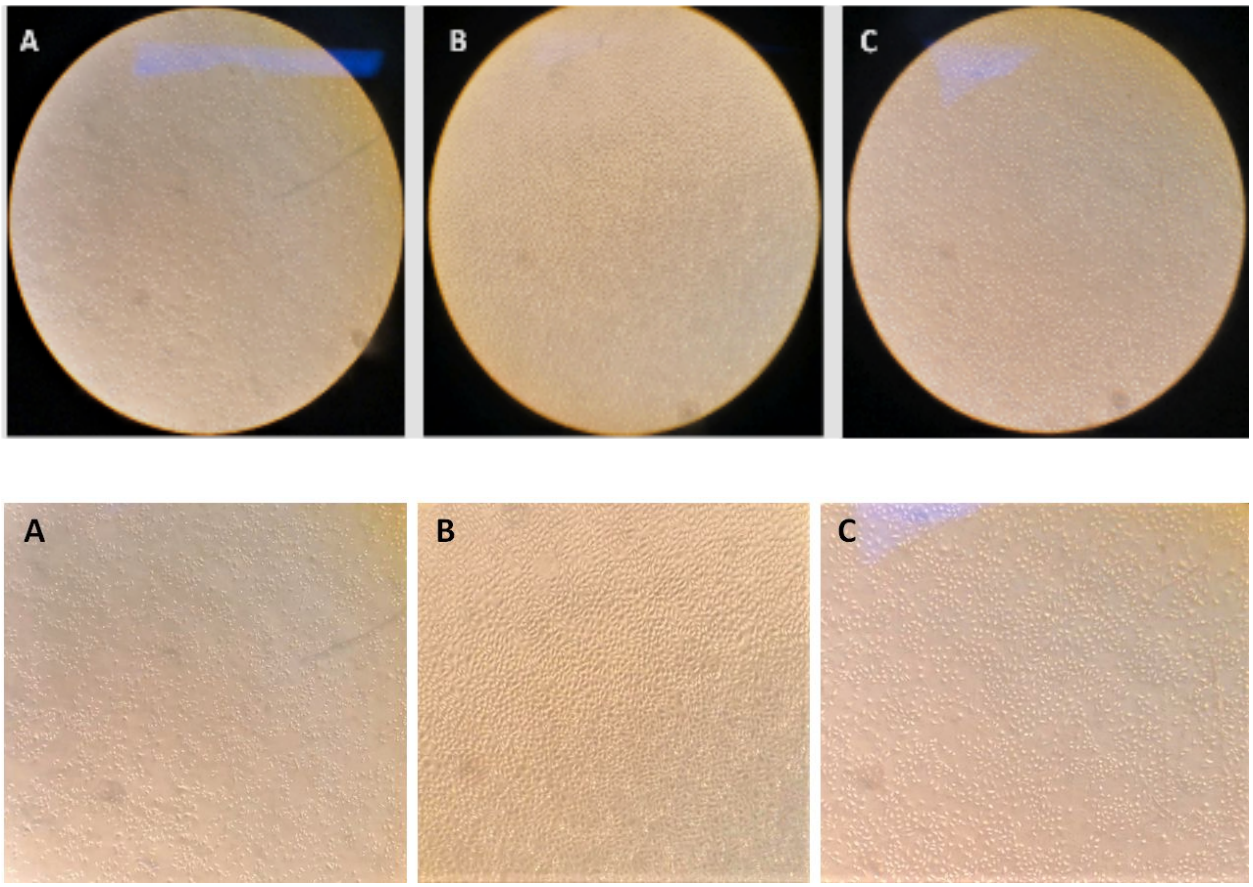

Figure 4. A549 cell culture images at Day 2, Day 4, and Day 6. A: Confluency of cells at Day 2 (24 h after seeding). B: Confluency of cells at Day 4. C: Confluency of cells at Day 6.

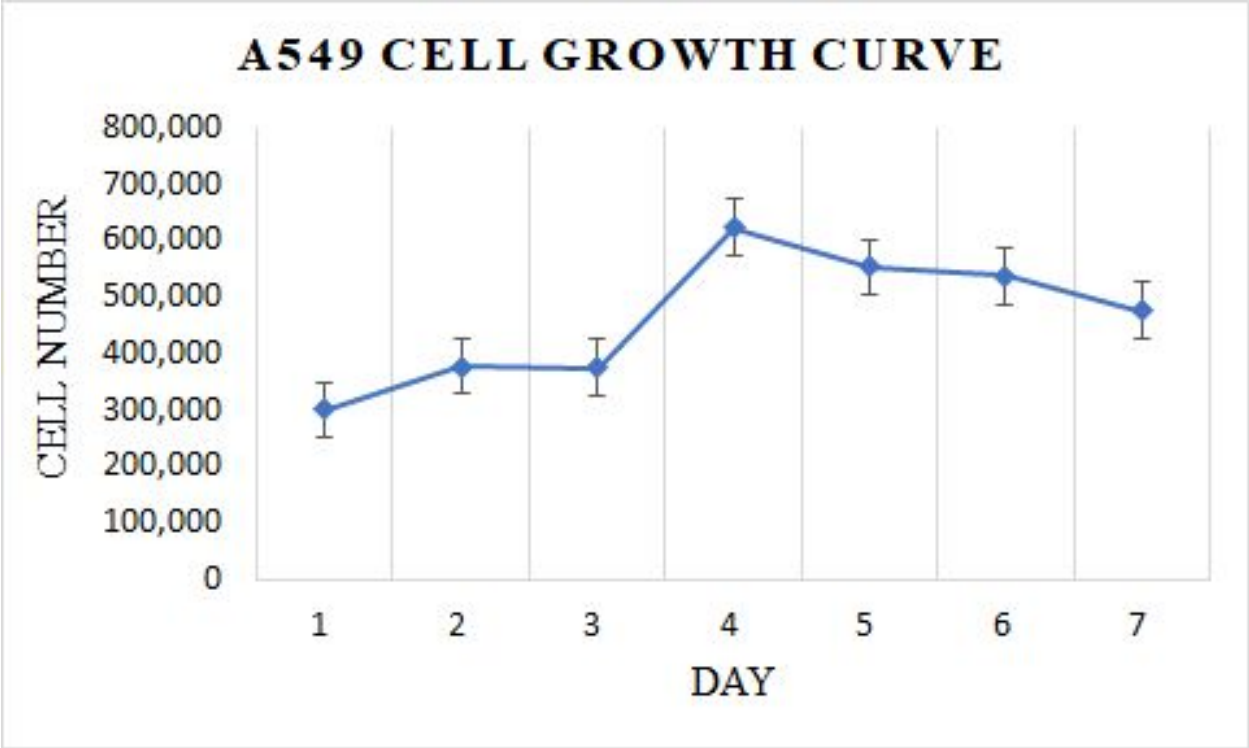

Figure 5. Cell Growth Curve for A549 cells. Day 1 shows the initial seeding density. Each subsequent data point shows the mean of the number of cells present in two wells on the corresponding day.

4.3 Transfection of A549 Cells

A total of three attempts were made to transfect the cells with the plasmid containing the EGFP gene controlled by CMV promoter. Each time, the protocol was varied from the last attempt to generate a stable EGFP expressing cell line.

In the first attempt, the exact protocol by the manufacturer was followed (27,28) without any alterations. However, a few cells showed fluorescence in the wells. To select for the successfully transfected cells the two wells which relatively showed the highest number of transfected cells were subcultured with 800 ug/ml of G148. However, after 4 passages, all of the cells started dying, meaning that the transfected cells were not stable enough to replicate and increase in number. In other words, these cells were transiently transfected. But, after this attempt, the initial seeding density of A549 cells 24 h before transfection was established to be 1.6×10^5 and/or 2.0×10^5 for future transfection experiments. Because only the cells in wells with these two seeding densities reached 70 - 90% confluency [31] required for transfection. Whereas cells in the other two wells with initial seeding densities of 1×10^5 and 1.2×10^5 were less than 50% confluent at the time of transfection (after 24h).

In the second attempt, the DNA to lipofectamine 2000 (DNA: Lipo 2000) ratio was increased from 1:2.5 to 1:3 [31,32,33] to rule out if a low DNA to lipo ratio is the

cause of low transfection efficiency seen in the first attempt. But, 24 h after transfection a lot of cells were seen dead floating in the media. The wells were subcultured in order to promote the growth of healthy cells. However, these cells also did not seem healthy and mostly all of the cells were dead after 48 h. It was hypothesized that a possible explanation for the rapid cell death observed after transfection could be due to reported cytotoxic effects of lipofectamine on mammalian cells [34,35]. When transfecting Human Embryonic Kidney (HEK293) cells, Mansouri et. al reported a less than 30% survival rate for cells after DNA transfection using Lipo 2000.

Therefore, In the final attempt, the DNA: Lipo 2000 was reduced to 1:2 [31] to reduce the cytotoxic effects of lipo 2000. This time, the quantity of serum (FBS) in the cell culture medium (without antibiotic) was reduced from 10% to 3% [36] prior to as well as during cell transfection. Primarily because FBS is known to reduce transfection efficiency due to its heterogeneous makeup [36,37]. Thus, some proteins present in FBS can interfere with lipid-mediated transfection [38]. In a study by Wallenstein et al. it was observed transfection of Embryonic Stem (ES) cells carried out after 3 days of serum starvation resulted in an approximately 33% increase in plasmid expression. Therefore, confirming the role of serum proteins or FBS in reducing transfection efficiency. Furthermore, linearized DNA was also

used for transfection along with the previously used circular DNA to study the effect of linear and circular DNA topology on transfection efficiency.

The alterations mentioned above, did show a much higher transfection efficiency than the two attempts done before (Figure 7). However, due to reduced serum in the cell culture medium, the cells showed a slower growth rate than previously observed[36]. Furthermore, lower transfection efficiency was observed in transfection with linearized DNA in contrast to transfection with circular DNA (Figure 7).

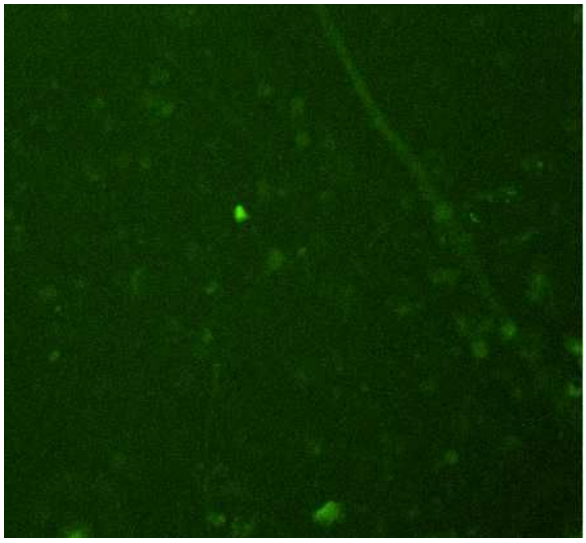

Figure 6. Image of A549 cells under fluorescent microscope after Attempt 1.

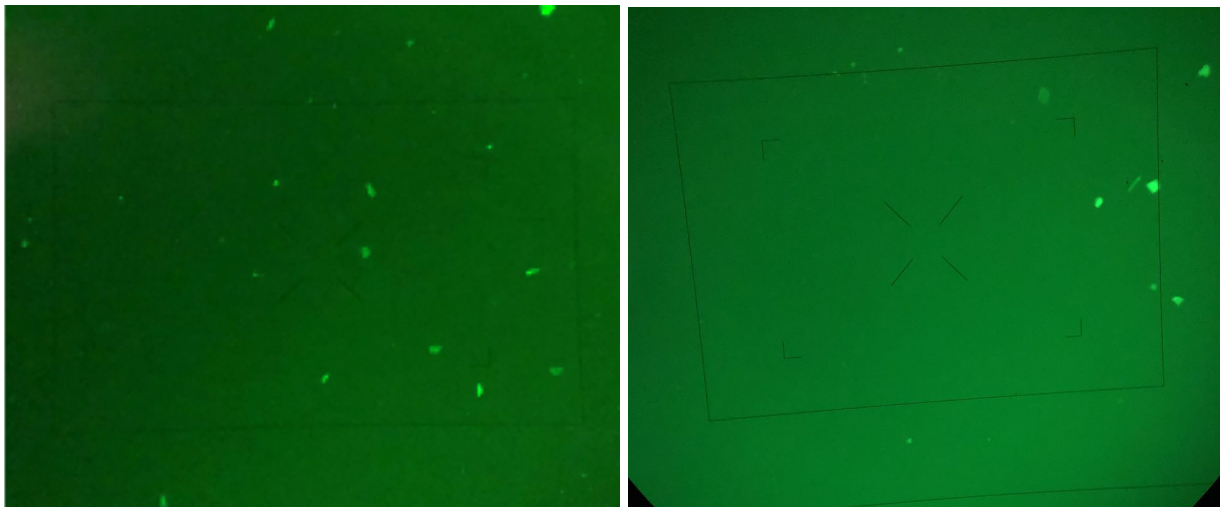

Figure 7. Image of A549 cells under fluorescent microscope after Attempt 3. The image on the left shows a well with cells transfected with circular DNA whereas the image on the right shows a well with cells transfected with linearized DNA.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

For the goal of engineering of a stable EGFP expressing A549 cell line, several different observations were made including the initial seeding density of A549, suitable DNA:Lipo 2000 ratio, concentration of serum in culture media before transfection, and the efficiency of transfection using circular vs linear DNA. All of which provide essential data and knowledge for the following transfection experiments performed with A549 cells. However, in future to increase the transfection efficiency and success other transfection methods or transfection vectors can be used.

The main governing principle underlying the process of lipofection is that the cationic (positively-charged) lipid interacts with the anionic (negatively-charged) phosphate backbone of the DNA or RNA[39,40]. This leads to the formation of the lipid-DNA complex or lipid-RNA complex respectively which too have an overall positive charge [39,40]. Hence, these complexes are attracted towards the negatively charged cell membrane [39,40]. However, the exact mechanism of the uptake of these complexes into the cell is still unknown, but it is widely believed to occur through endocytosis [39,40,41] and phagocytosis [39]. Hence, one of the main challenges of the lipid-mediated DNA transfection or lipofection in cells is hypothesized to be the entrapment of the cationic lipid-DNA complex into an

endocytic vesicle to gain entry into the cell [41]. Furthermore, the DNA from these complexes then has to gain entry inside the nucleus to be incorporated into the genome, the exact mechanism for which is also still unknown [39,42]. Hence, the use electroporation instead of chemical transfection vectors such Lipofectamine 2000 used in this study would bypass the complications which arise in the transport of the plasmid from the extracellular environment to the intracellular environment. A study by Maurisse et al. aimed at comparing the transfection efficiency and cell viability of different transfection methods and vectors on different cell lineages. The results showed that electroporation/nucleofection showed superior results when compared to other methods of transfection including Lipofectamine 2000 and Lipofectamine plus, and Polyethylenimine (PEI). The several cell lines transfected using nucleofection all showed high transfection efficiency (Figure 8) ranging from 40% - 80% with minimal toxicity. Furthermore, when compared to Lipofectamine 2000 and other methods, nucleofection showed a lot more promising results (Figure 9). Similarly, Bradburne et al. studied optimal conditions for nucleofection of pmaxGFP plasmid in A549 cells. The results showed that the observed fluorescence based on different transfection conditions ranged from completely absent to highly fluorescent, and the cell viability ranged from 72 - 100% [43].

Once a stable EGFP expressing cell line is established, the intensity of fluorescent signals can be correlated to drug toxicity in drug screening experiments. Hence, a three-dimensional (3D) cell culture model can be engineered for high throughput drug screening assays. The development of this 3D drug testing assay can lead to the formation of an extracellular matrix (ECM), cell-cell interactions, and cell-ECM interactions [44], all of which helps to mimic the *in vivo* tumor micro environment in an *in vitro* (laboratory) setting. Therefore, the proposed A549 drug screening platform can not only help test efficacy of different anti tumor drugs on A549 cells, but can also help in discovery of new drugs to treat lung cancer. Investment in 3D drug testing is especially important since Cancer is one of the leading causes of death worldwide [45]. Therefore since the 3D drug screening assays have been reported to show results similar to those seen *in vivo,* they can help accelerate the process of antitumor drug discovery and testing.

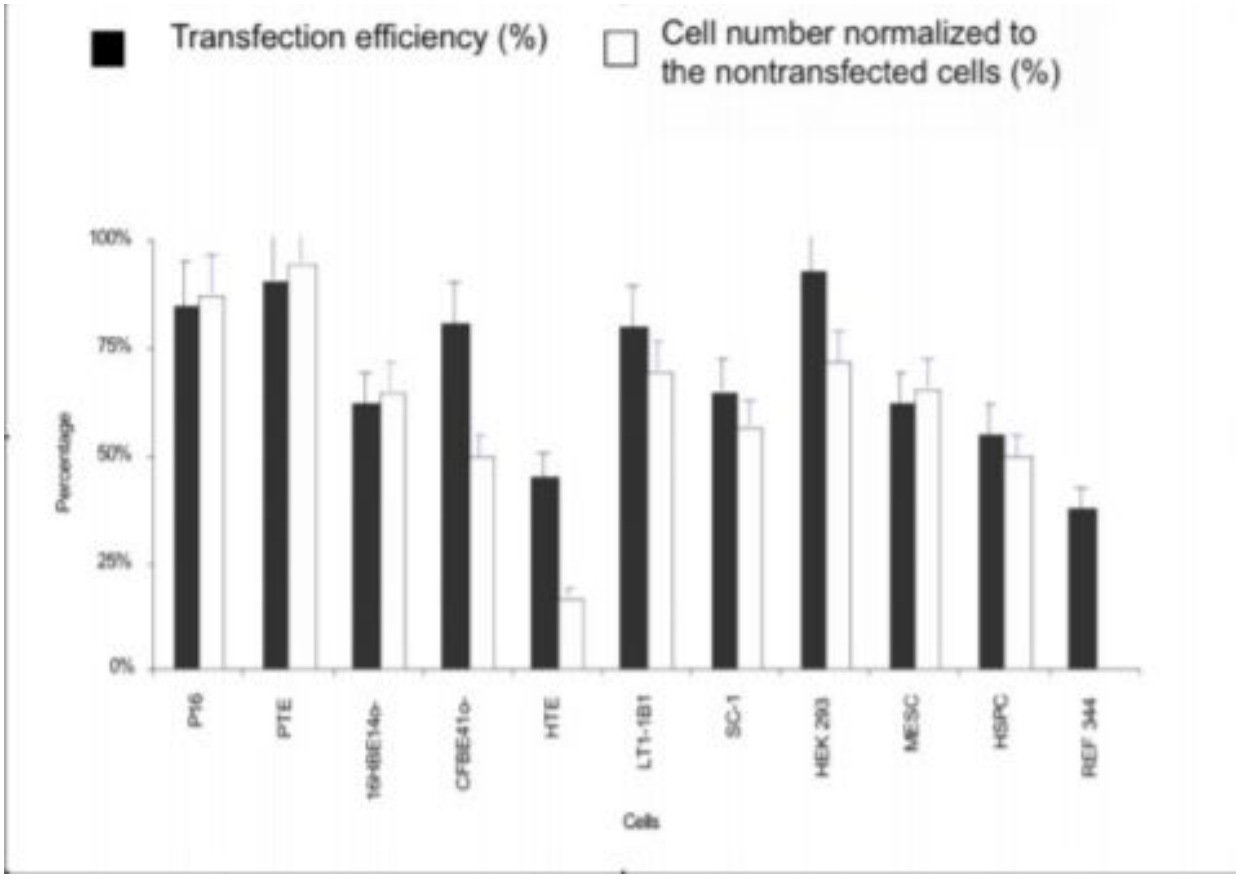

Figure 8.The transfection efficiency obtained 48 hours after nucleofection of different cell lines [42].

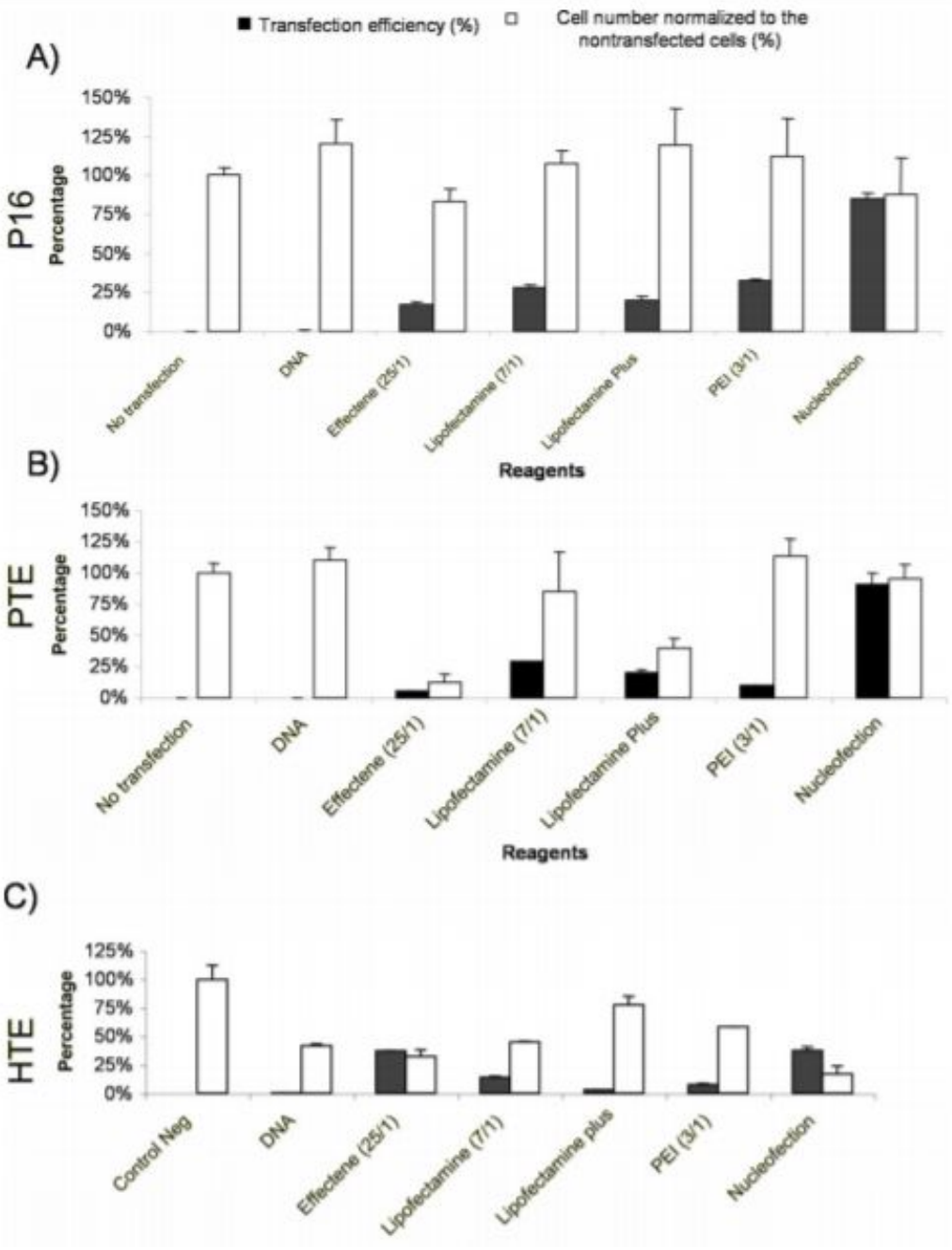

Figure 9. Comparison of the transfection efficiency obtained by using different transfection methods [42].

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