ERADICATION OF ADENOCARCINOMA A549 CELLS USING 5'DFUR AND MESENCHYMAL STEM CELLS TRANSFECTED WITH THYMIDINE PHOSPHORYLASE

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by

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Authorization to Submit Thesis

This thesis of Xutu Wang, submitted for the degree of Master of Science with a major in Biological Engineering and titled "**ERADICATION OF ADENOCARINOMA A549 CELLS USING 5'DFUR AND MESENCHYMAL STEM CELLS TRANSFECTED WITH THYMIDINE PHOSPHORYLASE,**" has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Chemotherapy drugs have been widely used for many years in cancer therapy but produce horrible side effects like low blood cell count, nausea, and hair loss. Targeting cancer cells with a non-toxic prodrug, which is then converted to a chemotherapy drug in the vicinity of targeted cells, can largely reduce the side effects of chemotherapy. Gene-directed enzyme prodrug therapy (GDEPT) has been in development for more than 20 years as a cancer therapy approach to more effectively target tumor cells and reduce toxicity to healthy cells. Various GDEPT systems have advanced into clinical trials yet no GDEPT drug in the market indicating there are still barriers to overcome. GDEPT mainly consists of three parts to achieve the treatment: a nontoxic prodrug, a gene encoded for the enzyme which can convert the prodrug to toxicity drug, and a gene carrier. In this study, we used a well-known chemotherapy drug fluorouracil (5-FU) and its prodrug 5'DFUR, an enzyme called thymidine phosphorylase (TP), and human mesenchymal stem cells (human MSCs) as the gene carrier. A non-viral vector should be very safe for gene delivery when compared to a viral vector. One problem of using non-viral vectors (e.g., polyethylenimine) was the delivery efficiency of TP gene to human MSCs is extremely low. Since a viral vector has a much higher delivery efficiency than a non-viral vector, the viral vector containing TP gene was constructed for human MSCs gene delivery and resulted in very high delivery efficiency and subsequent protein expression. The TP gene was first delivered into A549 cells to test the cancer cell viability with 5'DFUR prodrug treatment. The TP enzyme-prodrug conversion rate in A549 cells was quantified by enzymatic assays. TP was overexpressed in A549 cells after gene delivery and converted 5'DFUR to 5-FU at a high rate, resulting in elimination of 90% of A549 cells in 4 days. Next, human MSCs infected by TP-encoded lentiviral vectors were co-cultured with A549 cells to test cell viability of both A549 cells and human MSCs after treated with prodrug 5'DFUR. Cell viability decreased to 10% in 5 days after the prodrug was administered. In summary, this study shows that prodrug 5'DFUR can be converted to chemotherapy drug 5-FU by TP-expressing

human MSCs at a high rate, and results in elimination of both cancer cells and carrier cells in a short time frame.

Acknowledgements

I would like to express my very great appreciation to my advisor Dr. Ching-An Peng for his patient guidance during my Master study. His willingness to give his time and knowledge so generously has been very much appreciated.

I would like to offer my special thanks to Dr. Schiele Nathan; a wonderful professor leads me into tissue and mesenchymal stem cell's world and always kindly provided helps in lab. I would also like to thank Dr. Martin who has always been an inspiring mentor for me and has served on my thesis committee. Assistance provided by Dr. Lee Fortunato on lentiviral production was greatly appreciated.
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Dedication

I would like to thank my family and friends especially my mom Xiaohua Xu who unconditional support and encouragement has gone a long way helping me fulfill my goals. I couldn't be able to complete the study without them.

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Chapter 1 Introduction

1.1 Cancer therapy

There are a variety of cancer treatments; all of which depend on the different cancers and conditions [1,2]. To date, surgery, chemotherapy, radiotherapy, and immunology therapy are still the four main cancer treatments in clinical applications [2,3]. Patients usually receive more than one kind of therapy for cancer to increase the cure possibility [3, 4, 9]. This can become problematic as one or more treatments usually cause additional side effects like nausea, hair loss, and low red blood cell count [6]. Another challenge in cancer therapy is that it is difficult to target specific cancer sites [7]. For example, chemotherapy drug treatments are delivered to cancer sites through blood vessels and travel throughout the body to cause non-specific cell damage to both healthy and diseased tissue [8]. In severe cases, these side effects can be serious and even lead to patient death [5]. In order to overcome that when administrate chemotherapy drug, many novel cancer therapies have been developed to decreased side effects.

1.2 Gene-directed enzyme prodrug therapy

Gene-directed enzyme prodrug therapy (GDEPT) system have developed over the past 20 years and have an extreme potential and opportunity in the future [13]. GDEPT is a gene therapy for cancer treatment that uses a non-toxicity prodrug instead chemotherapy drug in order to only target specific cancer sites. The inactivate prodrug in this therapy can convert to a corresponding chemotherapy drug via a specific enzyme which is only expressed around a cancer site [10]. As a result, the chemotherapy drug will only show up at the cancer site by enzyme conversion and many side effects will largely be decreased without the chemotherapy drug circulating through the whole body [12]. Usually a transfer gene encoded that specific enzyme was delivered to cancer site first to increased expression level and help the conversion activity and rate [11]. There are three essential parts of this therapy: the first is a non-toxicity prodrug capable of converting to a chemotherapy drug, secondly a transfer gene encoded to an enzyme capable of carrying out this prodrug conversion, and lastly a carrier to deliver the transfer gene [15]. GDEPT therapy makes cancer treatment easier, safer and more promising through various

choices of prodrug enzyme systems. There are many prodrug drug pairs can be use in GDEPT system such as doxifluridine (5'DFUR) and fluorouracil (5-FU). In this study, we use 5'DFUR as the prodrug and thymidine phosphorylase as the enzyme to help convert the prodrug to chemotherapy drug 5-FU. However, there are still many challenges in this therapy to overcome; problems like the delivery of a transfer gene to a cancer cell site can be modified by cancer specific promoter and cause some tumor regression [14]. The expression and activity of this transfer gene is directly related to the drug efficiency. Therefore, to optimize a gene delivery system in GDEPT it is very important to include choosing a carrier. In this study, MSCs were selected to become the gene carrier because of its specific characteristic and abilities.

1.3 Thymidine phosphorylase

Thymidine phosphorylase (TP) is nucleoside metabolism enzyme that consists of 2 identical subunits and was first described in 1953 [16]. TP was found located on chromosome 22q13 with a dimer molecular mass of 102 kDa and plays an important role in pyrimidine salvage pathway [20]. Each subunit of TP contains a thymidine binding site that can catalyze thymidine to thymine as a reverse conversion but mainly does forward direction [17]. TP has the same structure of platelet-derived endothelia-cell growth factor (PD-ECGF) which can stimulate angiogenesis [16, 17]. TP was overexpressed in many cancer cells like carcinoma [16], esophageal [18], gastric [17], breast [19] and colorectal cancer [22, 23], but the reasons for its expression remain unknown. TP has been shown to promote tumor angiogenesis, invasion, metastasis, and evasion of the immune-response and resistance to apoptosis [21]. Because of its high level in cancer cells, TP was used for cancer therapy by its crucial characteristics for activation of widely use chemotherapy agents like 5-FU. In this study, TP plays a role in the conversion of prodrug 5'FDFUR to chemotherapy drug 5-FU in GDEPT system for cancer therapy. The transfer gene of TP has been delivered to cancer site in different ways and expressed with high level activity for prodrug conversion [22].

1.4 Mesenchymal stem cells as gene carriers in GDEPT

MSCs have the capability to renew themselves through cell division and differentiate to many cell types such as chondrocytes, osteoblasts, and adipocytes [24]. MSCs have been developed for many chronic disease treatments like autoimmune, inflammatory, and degenerative disease because of their specialized characteristics [25]. In this study, mesenchymal stem cells are used as carrier encoded TP gene to cancer cells because of their tumor tropism characteristic and low immune respond [26]. MSCs have the homing ability via specific receptors on the cell surface which can interact with some cytokines (e.g., FGF-2, CCL 5, and VEGF) secreted from cancer cells [27]. When MSC's carry TP transfer gene and homing to cancer cell site, prodrug 5-DFUR will only convert to 5-FU by TP protein expressed at cancer site [28]. Compared to some other gene carriers, like viruses, MSCs cause very low immune responses, making the therapy safe [26]. The homing ability of MSCs to cancer cell site can also be affected by delivery method, passage numbers, and culture conditions. One of the challenges of utilizing MSCs during research is the loss of cell potency when sub-culturing, or a higher passage which makes expansion in vitro more difficult [29].

1.5 Gene therapy

In order to transfer new gene materials into target cells, also called gene delivery, an appropriate carrier or vector is needed [31]. The ideal vectors should deliver genes into cells with a high delivery efficiency and cause no toxicity to cells [32]. There are basically three classic gene delivery methods: chemical, physical, and viral vectors, of which each has its own characteristics, advantages and limitations [30, 33]. In this research, we focused on chemical and viral vector because they are more suitable for in vitro gene delivery [34]. Chemical vectors, including liposomes, nanoparticles, polymers, can get directly into the cells upon delivery [35]. Chemical vectors are usually safe without immunogenicity, economic, and the possibility of repeated administration [36]. Chemical vectors are a good option when you need a very safe environment, but it also has limitations such as a low efficiency of gene delivery in some cell types or conditions [37,40]. Viral vectors have a very high efficiency compared to non-viral vectors,

but always has safety concerns [38]. Viral vectors had been modified for many years to prevent further replication and to limit targeting cells to make them safer [39]. Viral vectors also risk to causing immunological reactions, and the gene size is limited when using a virus as a vector [41,42].

1.5.1 Non-viral vector: polyethylenimine

Polyethylenimine (PEI) is a cation polymer with many different types and molecular weights, an is one of the most common polymers used for non-viral vector in gene delivery [43]. 25 kDa branched PEI is the most common and effective polymer for a wide range of gene delivery because of the B-PEI/DNA polyplexes exhibit great transgene expression *in vivo* and *vitro* also used in this study [44]. PEI polymer has many advantages among many gene delivery vectors like cost-effectiveness, ease of operation, and it has a high delivery efficiency for most cell lines [45, 46]. However, PEI vector causes some toxicity to cells in certain concentrations [47]. The ratio of PEI to DNA particles is usually first test conducted in cells to find a safe amount for optimal deliver efficiency. The ratio of primary amine groups in PEI polymer of phosphate to DNA can be calculated and represents the ratio of PEI to DNA written as N/P ratio [48]. Optimal deliver efficiency with different cell line always has different N/P ratio.

1.5.2 Viral vector: lentiviral

Lentiviruses belong to the retrovirus family and can perform reverse transcription for viral genetics [49]. Retroviruses has been used as a gene vector as well, but it has many disadvantages like the inability to transduce non-dividing cells and transfection efficiency is high in vitro [52, 53]. Lentiviruses can transfect both dividing and non-dividing cells with a high delivery efficiency [51]. The inflammatory responses caused by lentiviruses transfection are also low because the surface protein on the envelope for binding target cells has been modified for safety concern [50, 53]. In this study, lentiviruses are used as a vector for delivery of TP genes into MSCs. Lentiviruses had a high delivery efficiency when compared to PEI vector and didn’t cause toxicity to cells. In this case, lentiviral vector is safe and efficient [55].

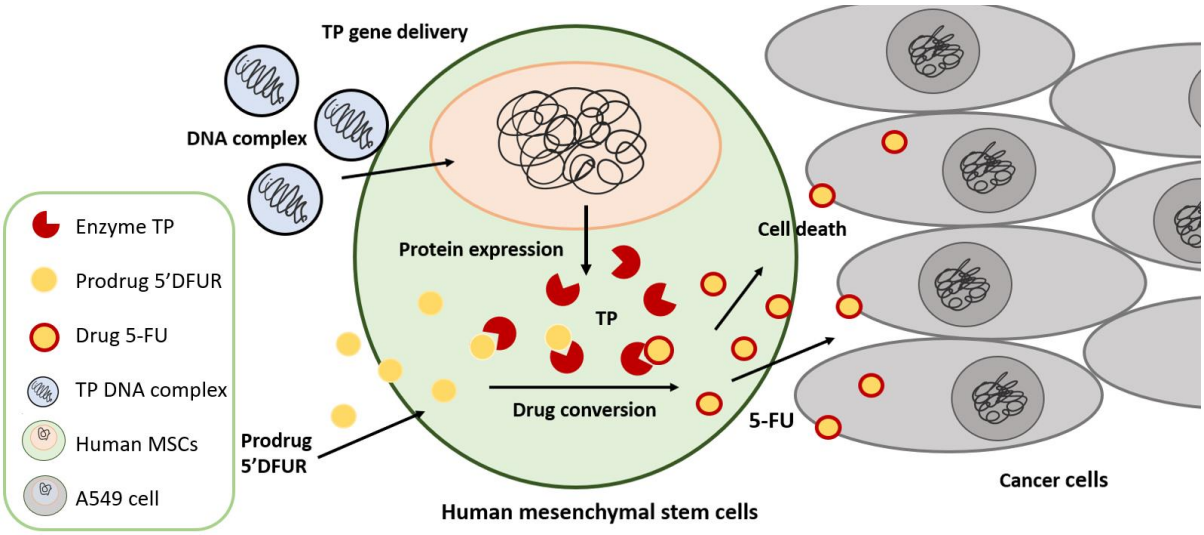


Figure 1.1 *Schema diagram of gene directed prodrug therapy when using thymidine phosphorylase (TP) as the enzyme and 5'DFUR as the prodrug for cancer therapy.*

1.6 Summary and objectives

Chemotherapy drug for cancer treatment is known for their powerful effective and convenience operation to eradicate cancer cells for a long time [1]. Chemotherapy drug can be widely used for many types of cancer but cause severe side effects at the same time include but not limited to nausea, hair loss, low red blood account [3]. Healthy cells damaged by drugs with cancer cell together when doing chemotherapy [4]. In order to decreased side effects of chemotherapy treatment, gene-directed enzyme prodrug therapy had been developed to use prodrug to target cancer site instead administrate toxicity drug all over the body [11]. Prodrug can convert to chemotherapy drug by specific enzyme only expressed around cancer site. Consequently, the chemotherapy drug seldom shows to the other place of body and damage healthy cells. In our study, a gene encoded TP enzyme first delivered into MSCs cells and co-cultured with adenocarcinoma A549 cells. And then prodrug 5'DFUR will be administrated to the co-cultured cells. Human MSCs became a perfect gene carrier for prodrug cancer therapy because its tumor tropism characteristic and cause very low immunology response. However, delivering gene into human MSCs is a challenge task [22]. The non-viral gene delivery for human MSCs extremely low. Viral vector has a high deliver efficiency but has been reported to be have some safety concern [51]. The overall goal of this study is to use non-toxicity prodrug instead of chemotherapy drugs in cancer

treatment to decrease side effects. Prodrug 5'DFUR is used to treat A549 lung cancer cells instead directly administrate chemotherapy drug 5-FU. I hypothesize the prodrug 5'DFUR will convert to chemotherapy drug 5-FU by thymidine phosphorylase expressed in human MSCs transfected by gene vectors. It is expected that A549 cells and human MSCs will both killed by chemotherapy drug 5'FU which is converted from prodrug 5'DFUR by the expressed thymidine phosphorylase in a short time.

Aims:

- Deliver TP-GFP plasmid into A549 cells by PEI vector and treat the cells with prodrug 5'DFUR. Measure the TP activity and expression level and quantify the prodrug conversion rate in A549 cells.
- Deliver TP-GFP plasmid into human MSCs by PEI or viral vectors. Co-culture the TP-GFP transfected human MSCs with A549 cells and treat cells with prodrug 5'DFUR to examine cytotoxic effects. Measure TP activity and expression level and quantify the prodrug conversion rate in co-cultured cells. Investigate the prodrug effects in different cell number ratios of human MSCs to A549 cells in the co-cultured population.

Chapter 2. Thymidine phosphorylase delivery to A549 cells by non-viral vector

2.1 Introduction

Thymidine phosphorylase (TP) has been found expressed in most all our body cells in different levels [18]. Many types of tumor cells like breast cancer cells and colon cancer cells have higher TP expression level than normal tissues [16]. 5-FU is a wide use chemotherapy drug for many years work for many tumors, and it's a good option for prodrug therapy because it has a prodrug 5'DFUR can be converted by TP catalase [17]. Prodrug 5'DFUR can be administrated to patients but remain no toxicity to cells until it meets over dose TP enzyme. Chemotherapy drug 5-FU will only appear at cancer cell sites but don't damage healthy cells. Since not all types of cancer cells has TP over expression, a lung cancer cell A549 cells with normal TP expression has been delivered by TP-GFP plasmid for prodrug treatment test in this study. Cell viability was tested by comparing TP-GFP delivered and non-delivered A549 cells with the same amount prodrug 5'DFUR treatment. A549 cells with TP-GFP plasmid delivered had a high-level protein expression and enzyme activity to convert prodrug 5'DFUR to chemotherapy drug 5-FU and finally eradicated cells in 4 days. A549 cells without TP-GFP plasmid delivered didn't show any cell death because of no prodrug conversion. Cells grew as a normal rate after prodrug treatment and grew up to 100 % confluence on day 3. Our results showed A549 cells had a TP protein over expression after non-viral gene delivery and capable to convert prodrug 5'DFUR to 5-FU with high release rate. The chemotherapy drug 5-FU converted from prodrug finally killed cells up to 90% in 4 days.

2.2 Materials and methods

2.2.1 Preparation of PEI/DNA polyplexes

10 μM 25kDa Polyethylenimine (PEI) stock solution (pH7.0) was made by dissolving 2.5 mg branch PEI (Sigma-Aldrich, St. Louis, MO) into 10 mL DI water and neutralized the pH with 0.2 M hydrochloric acid (Sigma-Aldrich). Different N/P ratios of PEI and DNA amounts were

tested to get the optimal delivery efficiency and lowest cytotoxicity to cells. 2 µg TP-GFP DNA (GenScript, Piscataway, NJ) with N/P ratio 10, 20 PEI and 4 µg TP-GFP DNA with N/P ratio 10 and 20 PEI were tested in Adenocarcinoma A549 cells (ATCC, Manassas, VA). Certain amount of PEI and DNA were mixed in 100 µL Dulbecco's Modified Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Gibco, Thermo Fisher, Waltham, MA) in a 1.5 mL sterilized centrifuge tube and incubated at room temperature for 30 minutes prior to added into cell cultures. Different amount of PEI and TP-GFP DNA were mixed to generate different N/P ratios. The map and sequence of TP-GFP are show in Appendix A.

2.2.2 TP-GFP plasmid delivery to A549 cells

A549 cells were obtained from ATCC (Manassas, VA) and saved in freezing medium in liquid nitrogen tank (Thermo Fisher). Freezing medium was consisted by 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich), 30% FBS and 60% DMEM. A549 cells were first thawed in a water bath at 37°C and cultured in DMEM with 10% FBS, 1% penicillin/streptomycin (Gibco, Thermo Fisher) in a T-75 flask at 37 °C with 5% CO_2. Cells were incubated to settle down and grow to 100% confluence in the following days. Cells were unattached by 0.25% trypsin (Gibco) and 10^4 cells were passed into three 65mm petri dishes with fresh DMEM cultured and cultured overnight for triplet experiments in each condition. The PEI/DNA polyplexes were incubated for 30 minutes in 100 µL DMEM at room temperature and added into each cell culture. Fresh culture medium was changed after 24 hours of gene delivery, TP-GFP expression was observed by the fluorescence microscope (Thermo Fisher). Cells were collected by 0.25% trypsin and pelleted by spin down at 400 x g for 5 min (Legend XTR, Thermo Fisher). TP-GFP expression and activity was tested by Western blot and enzyme activity assay after 48 hours of gene delivery.

2.2.3 Cell viability after prodrug treatment

10 mM stock solution of prodrug 5'DFUR (Sigma-Aldrich) was made by dissolved 25 mg 5'DFUR powder into 10 mL DI water and sterilized by a 0.2 µm filter. After 48 hours of PEI/TP-GFP

DNA delivered, 20 µL prodrug 5'DFUR was added into each dish and the final concentration was 200 µM. Experiments were performed in triplicate. 10 to 1000 µM 5'DFUR were tested in cells to get a LD 50 (Median Lethal Dose 50%) prodrug concentration. After 24, 48, and 72 hours of TP-GFP DNA delivered, cells were collected by 0.25% trypsin (Gibco) on each day and the cell viability was quantified by trypan blue assay (Thermo Fisher). For all samples, cells were suspended in 0.5 mL 0.25% trypsin and 0.5 mL DMEM for neutralize. 20 µL of cell suspension was mixed with 0.4% trypan blue in PBS at 1:1 ratio and use 20 µL of total mixture to count cells on a hemocytometer. The dead cells were showed blue color and live cells were showed bright under a bright field model microscope.

2.2.4 Western blot

Cells were resuspended in 100 µL RIPA buffer (Thermo Fisher) with 1µL protease inhibitor (Sigma-Aldrich) to lysis. The mixture was incubated on ice for 15 min and tip sonicated (Thermo Fisher) on ice for 10 seconds with 30 second intervals, 3 times. Proteins were collected by centrifuging the mixture at 14,000 x g for 15 min. The supernatant contained protein extraction was transferred into a new centrifuge tube and cell pellet was discarded. Laemmli 2X sample buffer (Bio-Rad, Hercules, CA) with 5% β-ME (Thermo Fisher) was added into protein extraction at 1:1 ratio and mixed well by pipette. The mixture was heat shocked at 95°C for 5 to 10 min in a water bath to let proteins stabilize. Both mixture and protein ladder (Bio-Rad) were loaded into a 12% SDS gel (Bio-Rad) and run for 1 hour at 150 V. The gel was transferred on to a nitrocellulose membrane (Azure Biosystems, Radnor, PA) immediately by Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) run for 1 hour at 20 V. The membrane was washed 3 times in tris-buffered saline buffer with 1ml/L Tween 20 (TBST) for 5 min each and blocked in 5% milk powder blocking buffer (Bio-Rad, Hercules, CA) for 1.5 hours while gently shaking at room temperature. The anti-TP primary antibody (GenScript, Piscataway, NJ) was added into the blocking buffer at 1:1000 ratio, and the membrane was kept in it while continue shaking overnight at 4 °C. Unbinding primary antibody was washed away by TBST again 3 times. The membrane was put into an IgG anti-rabbit

secondary antibody buffer (1:1500) for another hour, shaking at room temperature and finally washed in TBST 3 times. ECL A and B solutions (Thermo Fisher) were mixed and loaded on membrane for 1 min; membrane was finally detected by an image machine (Syngene, Frederick, MD).

2.2.5 Enzyme activity assay

Protein was obtained from cell lysate with the same steps described above in section 2.2.4. 40 µL of potassium phosphate buffer (pH 7.4) (Sigma-Aldrich) was added into 40 µL protein supernatant from gene delivered A549 cells. 10 µL 10mM 5'DFUR prodrug was added into mixture and placed on ice for 5 min before incubation. Initial absorbance reading of samples were measured by a spectrophotometer (Molecular Device, San Jose, CA) as a base line. Samples were incubated at 37°C for 1 hour while gentle shaking at 70 rpm. 10 µL of 2 M NaOH was added to stop the reaction after 1-hour incubation and samples were put on ice for 5 min [56]. Absorbance of all samples measured again after incubation by spectrometer. The absorbance values at 305 nm of samples before and after incubation were compared and calculated to get the enzyme activity of TP expression with a calibration curve and BAC assay of each sample. The calibration curve and BAC result is in Appendix B.

2.3 Results and discussion

2.3.1 Characterization of PEI/DNA polyplexes

Table 2.1 indicate the particle size and zeta potential of PEI/DNA polyplexes in N/P ratio 20 with 100 µg DNA. PEI/DNA polyplexes size were around 95 nm which was a propriate size for endocytosis. The charge of PEI/DNA polyplexes was around 45 mV which is also reasonable [20]. The cell surface is negative charge, and DNA is also negative charge. In order to increase the affinity of DNA to cell surface, a positive charged PEI mixed with DNA made the final charge was positive. Positive polyplexes were went to cell surface and get in easily.

Table 2.1 *Particle size and zeta potential of PEI/DNA polyplexes*

Polyplexes size (nm)	95 ± 12
Polyplexes zeta potential (mV)	45 ± 7

Figure 2.1 shows TP-GFP expression in A549 cells by PEI vector delivery in different N/P ratio and DNA amount. Cells show green color from the image indicate cells with TP-GFP expression and cells in grey color indicates the total cell number in the well. Compare the cells shows in fluorescence image and bright field image, the TP-GFP expression level in cells can be estimated. TP-GFP plasmid delivered into A549 cells with N/P ratio 10 and 2 µg DNA shows very few TP-GFP expression (A, a). TP-GFP expression level increased a little bit when increase DNA amount from 2 µg to 4 µg but fixed N/P ratio at 10 (B, b). TP-GFP expression in A549 cells also increased when increased N/P ratio from 10 to 20 but fixed DNA amount at 2 µg (C, c). The optimal TP-GFP expression level shows in A549 cells when both N/P ratio and DNA amount increased to 20 and 4 µg (D, d). However, keep increase N/P ratio can cause cytotoxicity to cells. From the results, increase DNA amount or increase N/P ratio respectively can both help TP- GFP expression in A549 cells, increase both N/P ratio and DNA amount to certain level can reach the optimal TP-GFP expression level.

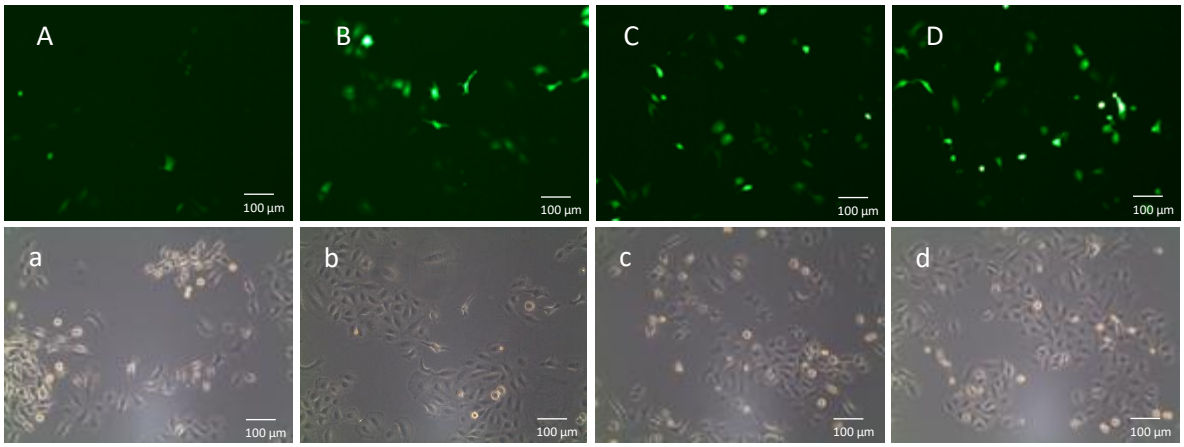


Figure 2.1 *Fluorescence and bright field images of TP-GFP expression in A549 cells by PEI gene delivered with N/P 10 and 2 µg DNA (A, a). Fluorescence and bright field images of TP-GFP expression in A549 cells by PEI gene delivered with N/P 10 and 4 µg DNA (B, b). Fluorescence and bright field images of TP-GFP expression in A549 cells by PEI gene delivered with N/P 20 and 2 µg DNA (C, c). Fluorescence and bright field images of TP-GFP expression in A549 cells by PEI gene delivered with N/P 20 and 4 µg DNA (D, d).*

Figure 2.2 indicate the TP-GFP plasmid delivery efficiency of A549 cells by PEI vector with different N/P ratios and DNA amounts. Delivery efficiency is closely related to TP-GFP expression level in A549 cells. GFP expression level in A549 cells can be visualized by fluorescence microscope. TP protein and GFP are the fusion protein which makes TP protein expression level easy to be observed. The TP delivery efficiency in each condition was quantified by cell counting assay with a hemocytometer via fluorescence microscope. The cell shows fluorescence green color count as cell with TP-GFP expression and DNA delivered succeed, the cell shows no fluorescence green color count as no GFP and TP expression and DNA delivered failed. A549 cells with N/P ratio 10 and 2 µg had only 10% delivery efficiency and protein expression which is very low. The delivery efficiency increased to 50% when DNA amount increased from 2 to 4 µg and increased to 50% when N/P ratio increased from 10 to 20. The delivery efficiency reached highest level 60% when the N/P ratio and DNA amount both increased to 20 and 4 µg. The delivery efficiency was not kept increase when approach N/P ratio more than 20 and DNA amount more than 4 µg but cause cytotoxicity. From the results, the delivery efficiency for A549 cells by PEI is highly related to N/P ratio and DNA amount, but too much PEI can cause toxicity to cells.

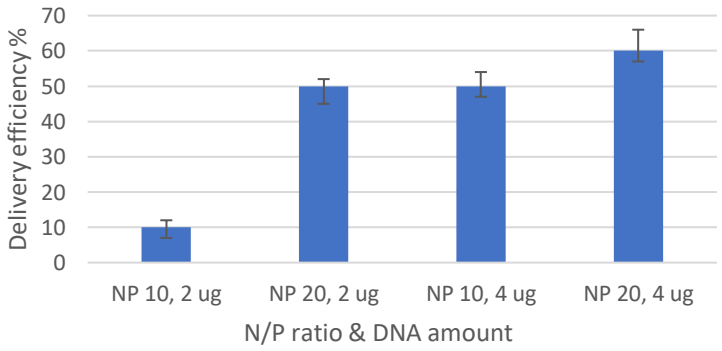


Figure 2.2 *Delivery efficiency of TP plasmid to A549 cells by PEI with different N/P ratios and DNA amount. The delivery efficiency of A549 cells with N/P 10 and 2 µg DNA is 10%. The delivery efficiency of A549 cells with N/P 10 and 4 µg DNA is 50%. The delivery efficiency of A549 cells with N/P 20 and 2 µg DNA is 50%. The delivery efficiency of A549 cells with N/P 20 and 4 µg DNA increased to 60%.*

2.3.2 Western blot

Figure 2.3 shows the western blot result of TP protein expression in A549 cells with and without TP-GFP DNA delivered. GFP expression can be easily observed under fluorescence microscope but the TP protein expression needs to be confirmed it expressed with GFP together by Western blot. The size of GFP and TP protein is 25 kDa and 50 kDa known by publications [20]. The size for GFP-TP fusion protein should be 75 k Da whether using GFP or TP primary antibody. In figure 2.3, lane 1 represent A549 cells with TP-GFP deliver showing a band at 75 kDa compare to the protein ladder. Lane 2 represent A549 cells with no TP-GFP delivered shows no band. This indicates there was no endogenous TP expression in A549 cells which indicate it is not capable to convert prodrug 5'DFUR to 5-FU. From the Western blot result, the TP protein was expressed with GFP protein in A549 cells when delivered TP-GFP plasmid by PEI.

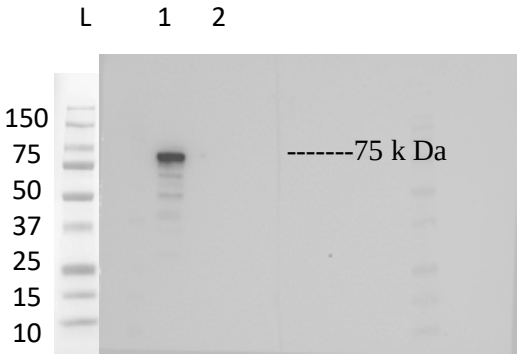


Figure 2.3 *Western blot result of comparing TP-GFP expression in A549 cells with and without gene delivered. L represent protein ladder, lane 1 shows the result of TP-GFP expression in A549 cells with TP-GFP gene delivery, and lane 2 shows the result of TP-GFP expression in A549 cells without TP-GFP gene delivery.*

2.3.3 Enzyme activity

Figure 2.4 shows the spectrometer reading of A549 cells before and after TP plasmid delivery by PEI vector. The OD reading at 300 nm of A549 cells before TP plasmid delivery is 0.12 and inreased to 0.28 after delivered TP-GFP plasmid by PEI. The increased reading 0.16 indicates the 5-FU conversion by TP protein. 5-FU release rate can be calculated by calibration curve of OD reading versus 5-FU concentration (µmol/mL) and the calibration curve of BSA protein assay (ng/mL) both shows in Appendix B. The calibration curve was obtained by measured the OD reading of series dilution of 5-FU.When calculate the 5-FU release rate of A549 cells with TP-GFP delivery, the OD reading is 0.28

which present 0.01 µmol/mL 5-FU concentration after incubation. The 5-FU release rate can be calculated with divided by 0.05 mg/mL total protein amount in cells and incubation time 1 hour, and finally get 0.2 µmol/h/mg total protein. The 5-FU release rate results shows in figure 2.5.

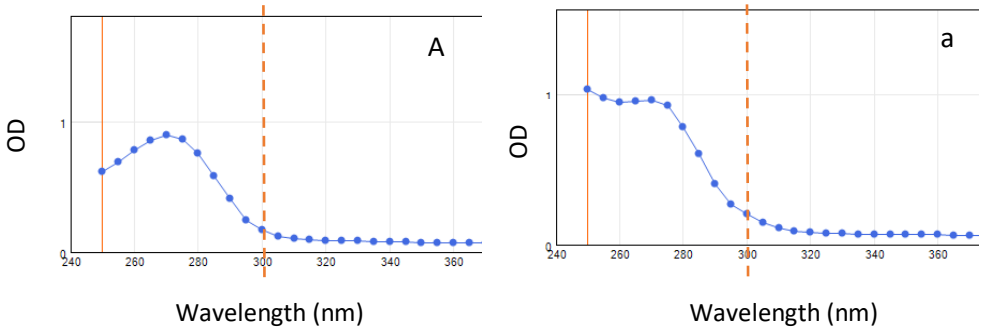


Figure 2.4 *Spectrometer reading of A549 cells with and without TP-GFP delivery is 0.12 (A) and 0.28 (B at 300 nm.*

The enzyme activity of TP protein is also very important in the whole process and closely related to prodrug-drug conversion rate. The prodrug conversion rate by thymidine phosphorylase can't reach 100% in vitro tested for many cell types [56]. It measured and quantified by 5-FU release per hour per mg total protein in cells. A549 cells without TP-GFP delivered shows only 0.04 µmol/h/mg total protein 5-FU released. The 5-FU release rate of A549 cells with TP-GFP delivered increased to 0.2 µmol/h/mg total protein 5-FU released, which is 5 times more than the one without TP-GFP delivered cells. The high 5-FU release rate represent TP protein in A549 cells have strong ability to convert prodrug 5'DFUR to chemotherapy drug 5-FU which is important to cell suicide rate and cell viability.

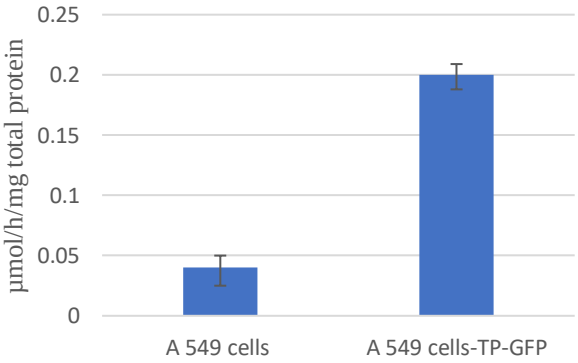


Figure 2.5 *TP activity result of A549 cells with and without TP-GFP delivered shows in 5-FU release rate. The 5-FU release rate of A549 cells with TP-GFP delivered is 0.2 µmol/h/mg total protein. The 5-FU release rate of A549 cells without TP-GFP delivered is 0.04 µmol/h/mg total protein.*

2.3.4 Cell viability of A549 cells treated with 5'DFUR

Figure 2.6 shows the result of A549 cells with TP-GFP delivered and prodrug 5'DFUR treated. Prodrug 5'DFUR was administrated to the cells after 24 hours of PEI/TP-GFP gene delivery. Cells showed normal growth on day1 after prodrug added and were stopped growing on day 2. The cell viability was not obvious decreased on day 1 (A1, a1) and day 2. However, A549 cells can be observed die a little on day 2 and stopping growing after that (A2, a2). More than half of the cells dead on day 3 (A3, a3) and most of cell dead on day 4 (A4, a4). This result shows prodrug 5'DFUR converted to chemotherapy drug within the cells by overexpressed TP. To make sure the prodrug 5'DFUR is non-toxicity to cells and A 549 cells has not enough endogenies TP expression, administrated prodrug 5'DFUR to non-TP-GFP delivered A549 cells as a control group (B1-B4, C1-C4). As shown in B1-B4, cell growth continued over 4 days of culture. Cells kept growing as A549 cells without any treatment. Growth rate of prodrug 5'DFUR treated cells is same as non-treated cells, and no cell were dead during these 4 days. Cell grew to 100% confluence on day 4. To make sure cell death was specifically caused by 5-FU converted from prodrug 5'DUFR, another control group of cells administrated 5-FU directly was observed (D1-4). Compare to the A549 cells with TP-GFP delivery and 5'DFUR treatment, cells with 5-FU directly added shows the similar morphology change and death. The cells started to die on day 2 and only a few of cells still survived on day 4. From the result of those control groups, A549 cells have not enough endogenies TP expression to convert prodrug 5'DFUR to chemotherapy drug 5-FU and the prodrug 5'DFUR has no toxicity to A549 cells.

Figure 2.6 shows the cell viability result of A549 cells whether with TP-GFP DNA delivery and prodrug 5'DFUR treatment. A549 cells without TP-GFP gene delivery treated with prodrug 5'DFUR showed no cell death in 4 days and cells continue grew to 100% confluence. The results of A549 cells without TP-GFP deliverey but with prodrug 5'DFUR treatment also shows no cell death which indicate the prodrug 5'DFUR has no toxicity to cells. Also, A549 cells have not enough TP expression to convert prodrug to chemotherapy drug 5-FU. A549 cells with TP-GFP DNA deliver but no prodrug treatment also had no toxicity and cells kept growing from 40% to 100% confluence in 4 days. This result conformed A549

cells with TP-GFP delivery cause no toxicity and no cell been killed. A549 cells with TP-GFP delivered and prodrug 5'DFUR treated show a decrease cell viability in 4 days. Cells stopped growing on day 2 when 5'DFUR added and started to die.

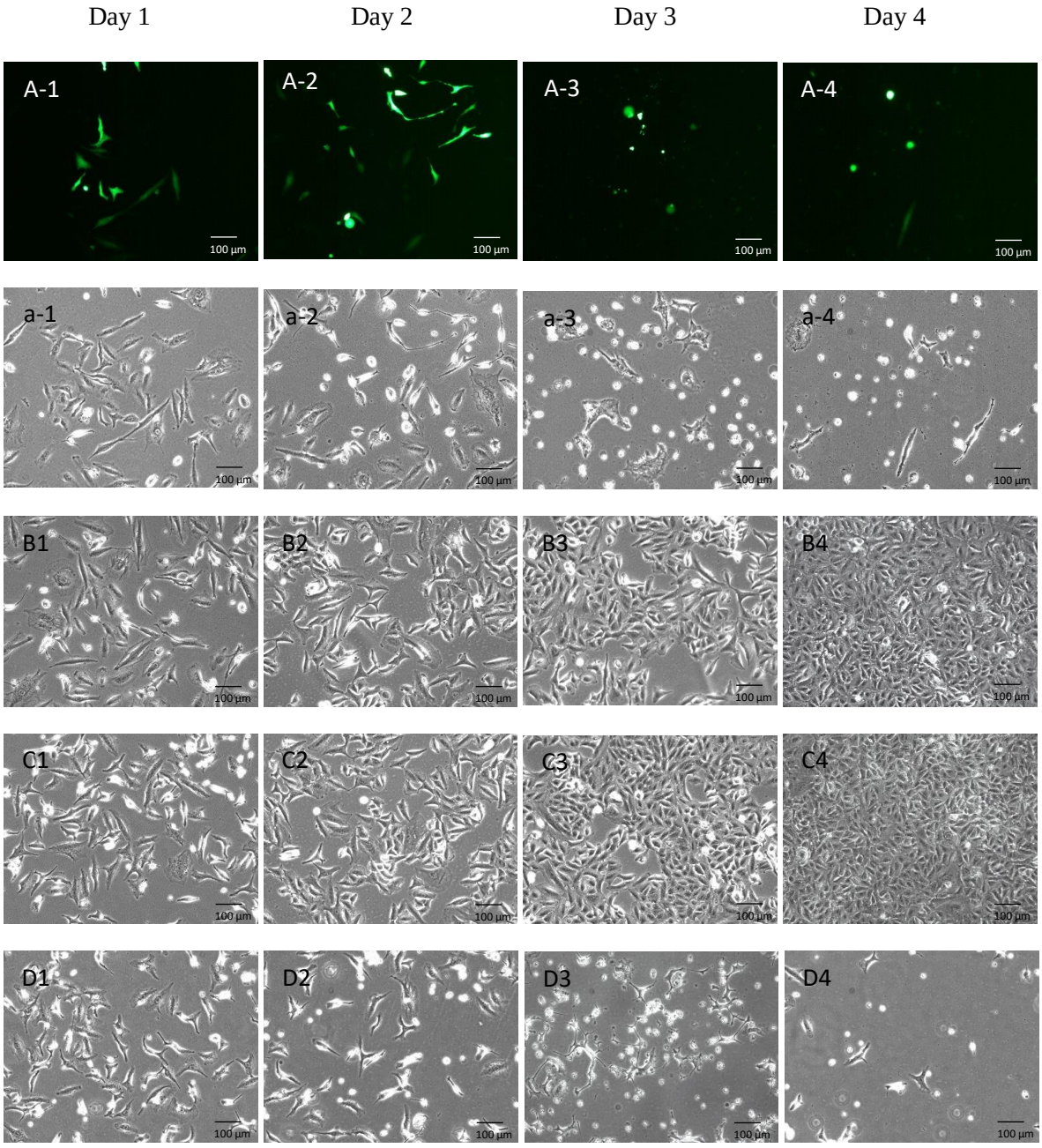


Figure 2.6 *Fluorescence and bright field images of TP-GFP delivered A549 cells by PEI vector with prodrug 5'DFUR treatment in 4 days (A1-4). Bright field images of TP-GFP delivered A549 cells by PEI vector without prodrug 5'DFUR treatment in 4 days (a1-4). Bright field images of none TP delivered A549 cells with prodrug 5'DFUR treatment in 4 days (B1-4). Bright field images of TP delivered A549 cells without prodrug 5'DFUR treatment in 4 days (C1-4). Bright field images of A549 cells with chemotherapy drug 5-FU treatment in 4 days (D1-4).*

Cell viability was decreased to 50% on day 3 and more than 90% cells were dead on day 4. Decreased cell number shows cell death caused by prodrug 5'DFUR conversion by TP overexpression TP with high level activity in A549 cells. From the result we can see, TP protein expression occurred after 24 hours of gene delivered with PEI and prodrug conversion started one day after prodrug 5'DFUR added. A549 cells with directly chemotherapy drug 5-FU treated as a comparison group of prodrug treatment shows a sharply cell viability decreased to 30% after 24 hours of 5-FU added. Only 25% cells left on day 3 and no more than 5% cells lived on day 4. The cells treated with chemotherapy drug 5-FU were die faster than cells had TP-GFP expression and treated with prodrug 5'DFUR. This result shows the prodrug conversion take times compare to directly treated chemotherapy drug. Overall, A549 cells with TP-GFP delivery and 5'DFUR treatment shows a big decrease of cell number over 4 days, it indicated the efficiency of prodrug conversion by TP enzyme expressed in A549 cells by gene delivery is high. As a result, A549 cancer cells were largely killed by this prodrug conversion.

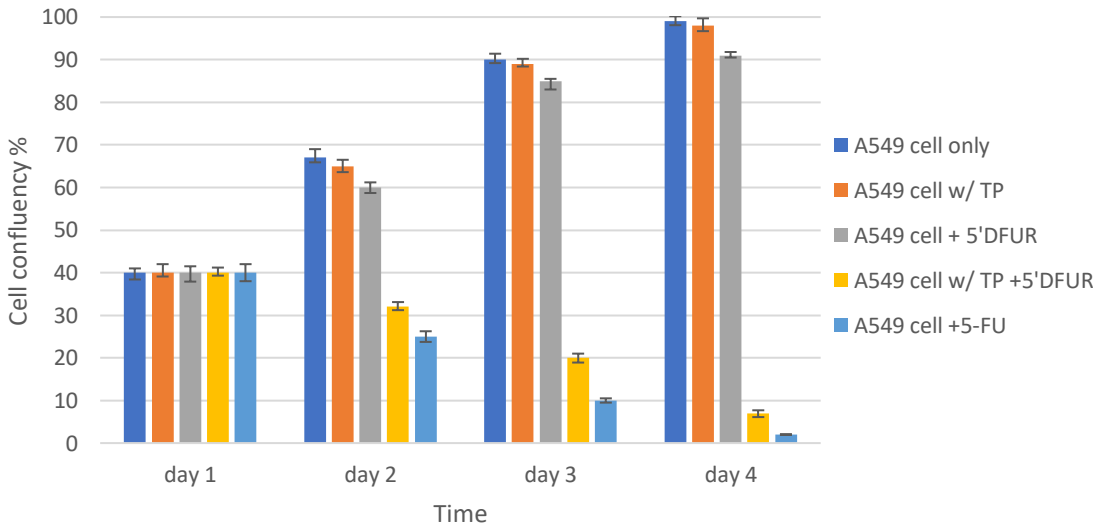


Figure 2.7 *Cell confluency of A549 cells in different conditions with prodrug 5'DFUR treatment in 4 days. The cell confluency (inoculation rate) of all sample on day 1 were about same around 40%.*

2. 4 Summary

TP-GFP delivered into A549 cells by PEI vector with different N/P ratios and DNA amounts cause different protein expression level in cells. From the results, relatively higher DNA amount and N/P ratio for same number of cells had higher delivery efficiency and protein expression. N/P ratio 20 and 4 μg DNA had the optimal protein expression among N/P ratio 10 to 20 and 2 to 4 μg DNA amount. However, too much PEI added into A549 cells can cause cytotoxicity to the cell and make cell morphology changed then dead eventually. Some cancer cells have overexpression endogenies TP and capable to convert prodrug 5'DFUR to 5-FU with no help. In this case, according to the Western blot and enzyme activity results, A549 cells had no overexpression endogenous TP to achieve the prodrug-drug conversion. A549 cells with TP-GFP plasmid delivery by PEI had an optimal deliver efficiency up to 60% and high-level protein expression which lead a 0.2 μmol/h/mg total protein 5-FU release rate. A549 cells with TP-GFP expression treated with prodrug 5'DFUR after 48 hour of gene delivery. A549 cells started to die on day 2 after prodrug 5'DFUR treatment and been eradicated on day 4. A549 cells without TP-GFP expression treated directly with chemotherapy drug 5-FU show the similar death rate and morphology changes. A549 cells without TP-GFP expression but treated with prodrug 5'DFUR show no cell death and morphology changed in 4 days of culture. Cells were kept grow to 100% confluence. In summary, our results indicate TP-GFP gene delivery to A549 cells lad to TP protein overexpression and cause more than 80% cells death in 4 days by converting prodrug 5'DFUR to chemotherapy drug 5-FU.

Chapter 3 Thymidine phosphorylase delivery to human MSCs by viral vector encoded with TP

3.1 Introduction

Using viral vectors for gene delivery is the most popular and effective type of gene therapy. This is especially true of primary cells such as human mesenchymal stem cells (human MSCs) with low delivery efficiency using non-viral vectors. In this chapter, human MSCs were delivered by the viral vector containing TP transfer gene constructed priory to reach a high gene delivery efficiency. In order to produce viral vector which contains TP gene, a virus backbone plasmid SFFV-GFP was inserted with TP sequence to obtain virus backbone SFFV-TP plasmid. Two primers amplifying the TP insertion sequence were designed to also include restriction enzyme cutting sites that allowed them to be ligated into the vector's cut-sites. The TP sequence and SFFV vector were then cut by enzyme digestion and ligated together with T4 ligase. The SFFV-TP plasmid complexed with PEI was delivered into HEK 293T cells simultaneously with two other plasmids which can produce the viral envelope and structure. This produced functional viruses containing the TP transfer gene inside. Human MSCs with a successful viral transfection had a high-level of TP protein expression when tested by Western blot and enzyme activity assay. Also, TP protein expression in human MSCs had a strong activity to convert prodrug 5'DFUR to 5-FU when tested by cell viability assay.

3.2 Materials and methods

3.2.1 SFFV-TP plasmid construction

In order to produce a viral vector containing TP sequence for gene delivery to human MSCs, a virus backbone transfer gene containing the TP sequence needed to be constructed. A pHR-SFFV virus backbone plasmid containing GFP sequence was obtained from Addgene (Cambridge, MA) to serve as a detectable marker for gene delivery. GFP expression is easily observed in viruses successfully transfect cells by fluorescence microscopy. In this study, the GFP sequence was removed by enzymatic digestion and replaced with the TP sequence as target gene. GFP sequence originally inside was considered to

keep in the vector because it functioned as a label protein which makes the virus transfection trackable and protein expression visualizable. However, it was removed for TP insertion because most of the restriction enzyme cutting sites behind GFP sequence were not usable for TP sequence. The map of plasmid HR-SFFV shows in Appendix C.

3.2.1.1 Primer design and PCR analysis

In order to insert TP sequence into pHR-SFFV-GFP vector (Addgene), two restriction enzyme sites Xho1 and EcoR1 (New England Biolabs, Ipswich, MA) were added on each side of TP sequence by a forward and a reverse primer. Both primers contain 6 random base pairs at 5' end and Xho1 or EcoR1 enzyme site next to it and finally 18 base pairs of corresponding TP sequence at the 3' end. A start codon added into the forward primer and a stop codon added into the reverse primer to control the transcription.

The forward primer is 5' TCTAGAGAATTCATGGCAGCCTTGATGACCCC 3', and the reverse primer is 5' AGAGTCCTCGAGTTACTTGTACAGCTCGTCCATGCC 3' (primers purchase from IDT, San Jose, CA). TP sequence was cloned out from plasmid pcDNA 3.1+C-eGFP (obtained from GenScript) by forward and reverse primers through a PCR machine (Bio Rad). PCR product was tested by DNA gel analysis to conform the TP sequence was cloning out correctly. DNA gel was made by dissolving 0.5 g agarose into 50 mL TAE buffer and heated up by microwave for 1-2 min. 1 µL ethyl bromine was added in agarose solution after it cooled down to 60°C. Finally, poured the mixed solution was poured into a gel cast and wait it solidified at room temperature for 15 min. 10 µL PCR product was mixed with 2 µL loading dye and added into DNA gel with a ladder. Gel was run at 200 V for 1 hour. The gel was exposed with UV light in a dark room.

3.2.1.2 Enzyme digestion and ligation

After TP sequence cloned out from its original plasmid, it was digested by 2 restriction enzymes Xho1 and EcoR1 (NEB) to created same enzyme cutting sites with pHR-SFFV vector. GFP sequence was cut out by digesting pHR-SFFV vector with same enzymes Xho1 and EcoR1. Both digested vector and

insert sequence went through heat shock at 65 °C and 80 °C respectively for 10 min to end the digest reaction. Digestion products were going through another DNA gel to analysis.

Digested TP sequence and SFFV vector were ligated together by using T4 ligation kit (Thermo Fisher). 20 µL T4 ligase and 20 µL buffer were added in a 1.5 mL centrifuge tube mixed with TP sequence and pHR-SFFV vector sequence. The mixture was kept at room temperature for 20 min to react. Heat shocked the samples at 65 °C in water bath for 10 min to end the ligase reaction.

3.2.1.3 Cell transformation, DNA isolation and amplification

Constructed plasmid pHR-SFFV-TP was isolated and amplified by cell transformation first with DH5α competent cells (NEB). DH5α bacteria cells were thawed on ice for 5 min and added 5 µL of ligation product (100ng/µL) and set the water bath to 42°C at the same time. Incubate the DNA with cells on ice for 30 min and then heat shocked the mixture at 42°C for exactly 30 second and put back on ice for another 5 min. 950 µL room temperature SOC medium was added into the mixture and incubated in a shaker with a speed at 225 rpm for 1 hour at 37°C. After 1 hour, 100uL bacteria mixture was took out and spread on a pre-warmed ampicillin resistant agar plate. The agar plate was incubated at 37°C upside down for overnight. After 18 hours incubated, colonies grown on the agar plate. Picked up 3 to 4 colonies from agar plate by a pre-sterilized hood and put cells into 5 mL LB medium with 50 µg/mL ampicillin (Sigma-Aldrich) in a 50 mL centrifuge tube. Incubated cells in a shaker with 225 rpm at 37°C. Bacteria cells were collected by spinning down next day with 6000 x g for 15 min. DNA was isolated and amplificated by mini prep with P1, P2, P3 buffers (Qiagen, Germantown, MD). Supernatant was discarded and resuspend cell pellet in 200 µL P1 buffer. P1 and P3 buffer kept on ice during the whole process. After mixing well of cells and P1 buffer, added same amount of P2 buffer with 0.1 mg/mL RNase A (Thermo Fisher) into mixture. Incubated the mixture at room temperature for 5 min then added same amount of P3 buffer to the mixture and incubated on ice for another 5 min. After incubated the cell with buffers, spin down the mixture at 13,300 x g for 20 min at 4°C.

Carefully pipetted out the supernatant into another 1.5 mL tube and added 70% volume of isopropyl (Sigma-Aldrich) into supernatant to eluted DNA. And then DNA was spun down at same speed for 30 min at 4°C. A small white pellet appeared on the bottom of the centrifuge tube, discarded the supernatant and resuspend the pellet by 70% ethanol to wash. Finally spun down the DNA again at same speed and temperature for 10 min. Discarded the ethanol and air dry the DNA pellet for 10-20 min in biosafety hood. Resuspend DNA with 100 µL DI water depending on the pellet size, the concentration and purity of amplified DNA were measured by a spectrophotometer (Thermo Fisher) was around 1000 ng/µL and 1.8, respectively.

3.2.2 Virus production and transfection

Viruses contained TP transfer gene was produced by HEK 293T cells (ATCC, Manassas, VA). A plasmid pMD2.G for virus envelope and a plasmid pCMV-dR 8.2 for virus structure (Both obtained from Addgene) with pSFFV-GFP or constructed pSFFV-TP transfer gene were first mixed with PEI in 100 µL DMEM and incubated for 30 min at room temperature. DNA/PEI mixture with N/P ratio 5 was delivered into HEK 293T cells were cultured in a T-75 flask. Experiments were performed in triplicate. 40% inoculation rate for HEK 293T cells is the best for viruses produce. Changed medium on day 1 and started to collect medium on day 2 to day 4, added fresh medium every day, and saved the medium contain viruses at -20°C. Viruses in medium can be saved for several day at 4°C. The map of plasmid pMD2.G and pCMV-dR8.2 are show in Appendix C. Mixed the medium with viruses with 10 mM polybrene and incubated for 15 min at room temperature before transfected. Filtered the mixture by 0.45 µm membrane and added into cell cultures for virus transfection. Incubated cells for 2 days and observed cells under fluorescence microscope if using pSFFV-GFP plasmid. Cells were collected by 0.25% trypsin for Western blot assay to tested TP protein expression when using SFFV-TP transfer gene.

3.2.3 Western blot

HEK 293T cells and human MSCs were collected by 0.25% trypsin and protein was extracted for Western blot assay which did in the same way in section 2.2.4.

3.2.4 Enzyme activity assay

TP activity in HEK 293T and human MSCs were measured by enzyme activity assay same as described in section 2.2.5.

3.3 Results and discussion

3.3.1 HEK 293T cells and human MSCs with viruses TP-GFP delivered

Figure 3.1 shows the results of GFP expression in HEK 293T cells and human MSCs after 48 hours of virus transfection of SFFV-GFP plasmid. HEK 293T cells shows a very high percentage of GFP expression in figure 3.1A with more than half of cells show green color when compare the fluorescence and bright field pictures. Also, the intensity of green color was very strong, and the cells were remained healthy. Figure 3.1B shows a high level of GFP expression in human MSCs by virus transfection with a strong intensity as well. About half of the cells show green color and the cell morphology remained good. In this case, the virus transfection of HEK 293T cells and human MSCs cause no toxicities but high protein expression rate. Figure 3.1 C shows the GFP expression in human MSCs by PEI vector as a compare group. GFP expression in this sample was very low and cell morphology was also changed after gene delivery. Only a few cells show protein expression and cells had some cell debris after 48 hours of gene delivery.

Since protein expression in cells is closely related to gene delivery efficiency. Usually a high protein expression level representative a high gene delivery efficiency. The delivery efficiency of HEK 293T cells by virus transfection was 60% obtained by cell count which is same when using PEI vector. The delivery efficiency of human MSCs by virus transfection was around 40% which is lower than HEK 293T cells but still high compare to human MSCs with PEI delivery which only had 10% delivery

efficiency. The result shows TP delivery efficiency of human MSCs increased from 10% to 40% when change the PEI vector to virus vector.

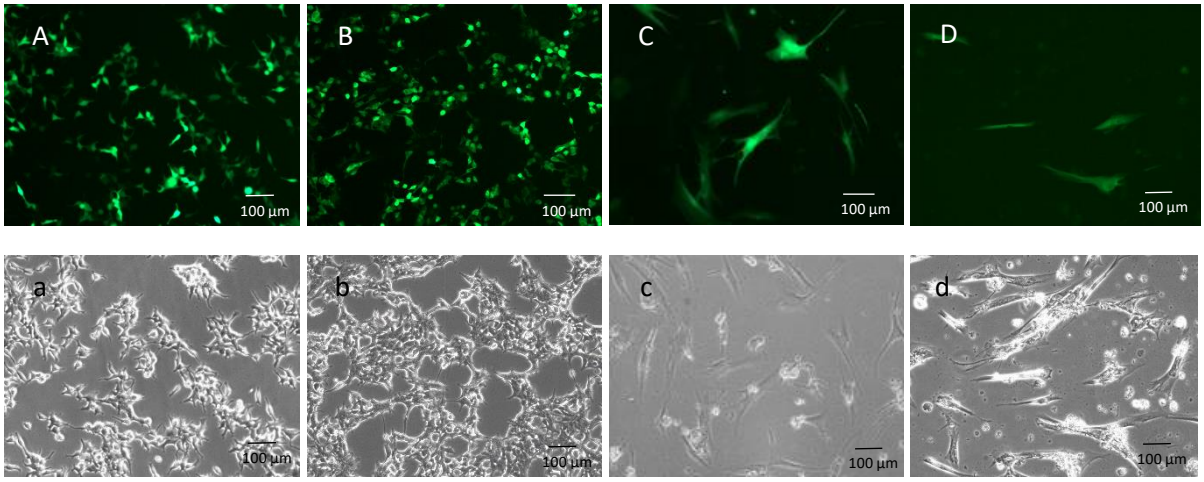


Figure 3.1 *Fluorescence and bright field images of GFP expression in HEK 293T cells by viral vector gene delivered (A, a). Fluorescence and bright field images of GFP expression in human MSCs by viral vector gene delivered (B, b). Fluorescence and bright field images of GFP expression in human MSCs by PEI gene delivered (C, c).*

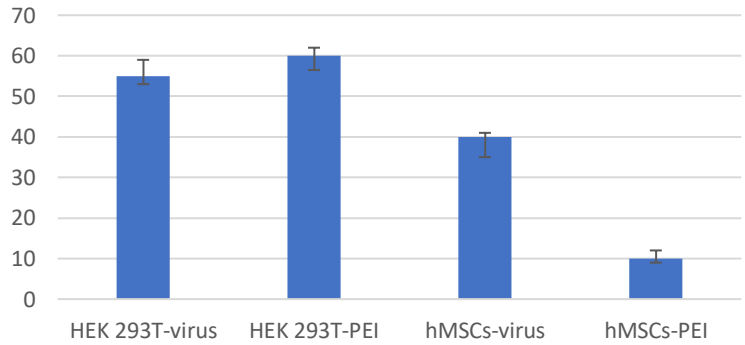


Figure 3.2 *TP delivery efficiency of HEK 293T cells and human MSCs by viral vector and PEI vector. The TP delivery efficiency of HEK 293T cells is 60% by both virus and PEI vector. The TP delivery efficiency of human MSCs is 40% by virus vector and 10% by PEI vector.*

3.3.2 DNA gel analysis of TP sequence insert

Figure 3.3 shows the PCR result of TP sequence cloning out from SFFV-TP plasmid. After plasmid SFFV-TP was constructed and amplified, a PCR analysis was tested to verify if the TP sequence inserted into SFFV vector correctly. A strong band shows on the gel lane 1 around 1.5 kb which is corresponded to the size of TP sequence. As a compared group, lane 2 shows the PCR result of SFFV-GFP which

contain no TP sequence, and had no band shows on the DNA gel with TP primers. This result shows the TP sequence was inserted into SFFV plasmid succeed and correctly

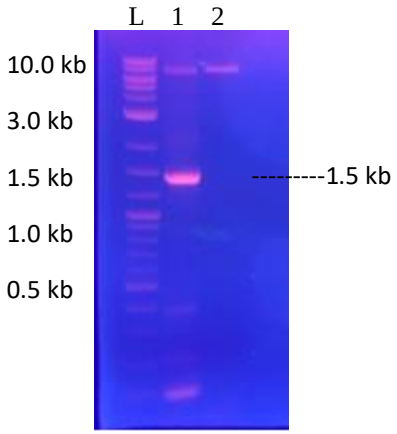


Figure 3.3 *Comparing PCR results of TP sequence cloned out from constructed SFFV-TP and commercial SFFV-GFP plasmid. L represent DNA ladder, lane 1 is the result of constructed SFFV-TP which has a band at 1.5 kb, and lane 2 is the result of commercial SFFV-GFP plasmid which has no band.*

3.3.3 Western blot

Figure 3.4 shows the Western blot result of TP protein expressed in HEK 293T cells and human MSCs. All the bands were at the correct size which located at around 55 k Da The first lane represents the protein ladder and lane 1 is the results of TP expression in HEK 293T cells by PEI delivered and lane 2 shows the results of TP protein in HEK 293T cells by virus transfected. This shows both PEI and virus vector succeed delivery plasmid into cells and get protein expression. Lane 3 and 4 represent the result of TP protein from the medium of HEK 293T cell delivered by PEI and virus vector. There are also two very light bands show at 55 kDa which means maybe a little bit TP protein came out of the cell into medium but most of the TP protein remained inside the cells. Lane 5 shows the result of TP expression in human MSCs by virus transfected with self-constructed SFFV-TP transfer gene. It indicates TP expression in human MSCs when using virus vector as the delivery carrier. Comparing to the TP expression in human MSCs by PEI transfected shows in lane 6, there is no TP expression detected. Lane 7 and 8 are the protein from the medium of human MSCs delivered with virus and PEI which barely can see. No bands show in the cell culture medium indicating TP protein didn't get out of transfected MSCs. This is very important for the GDEPT approach because TP protein has to stay inside of MSCs and

migrate to the cancer site. This result ascertain TP protein won't leak everywhere in the body and kill health cells after administrate 5'DFUR prodrug, while MSCs migrate towards the tumor site. This western blot result shows TP expression in human MSCs when using virus vector instead PEI vector, but TP expression shows in HEK 293T cells for both PEI and viral vector. This shows PEI vector have different characteristic for different cell types.

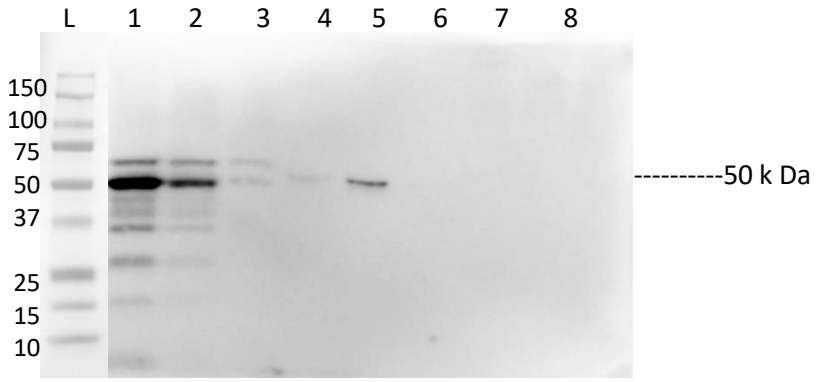


Figure 3.4 *Western blot result of TP expression in HEK 293T cells and human MSCs by viral vector and PEI vector. L represent the protein ladder. Lane 1 and 2 show the results of protein expression in HEK 293T cells by PEI and virus vector. Lane 3 and 4 show the results of protein expression in HEK 293T cells medium by PEI and virus vector. Lane 5 and 6 show the results of protein expression in human MSCs by virus and PEI vector. Lane 7 and 8 show the results of protein expression in human MSCs medium by virus and PEI vector.*

3.3.4 Enzyme activity

Figure 3.5 shows the spectrometer reading of HEK 293T cells and human MSCs before and after TP plasmid delivery by PEI and virus vector. The OD reading at 300 nm of HEK 293T cells before TP plasmid delivery is 0.11 at 300 nm and inreased to 0.28 after delivery TP plasmid by PEI (A, a-1). The OD reading at 300 nm of HEK 293T cells after delivery TP plasmid by virus is 0.29 and similar to the reading of using PEI vector (a-2). The OD reading at 300 nm of human MSCs before TP plasmid delivery is 0.1 at 300 nm and inreased a little bit to 0.14 after delivery TP plasmid by PEI (B, b-1). The OD reading at 300 nm of human MSCs after delivery TP plasmid by virus is 0.25 which increased a lot to the reading of using PEI vector (b-2). The increased reading amount indicates the 5-FU conversion. 5-FU release rate can be calculated by calibration curve and protein amount in cells described previously in section 2.3.3.

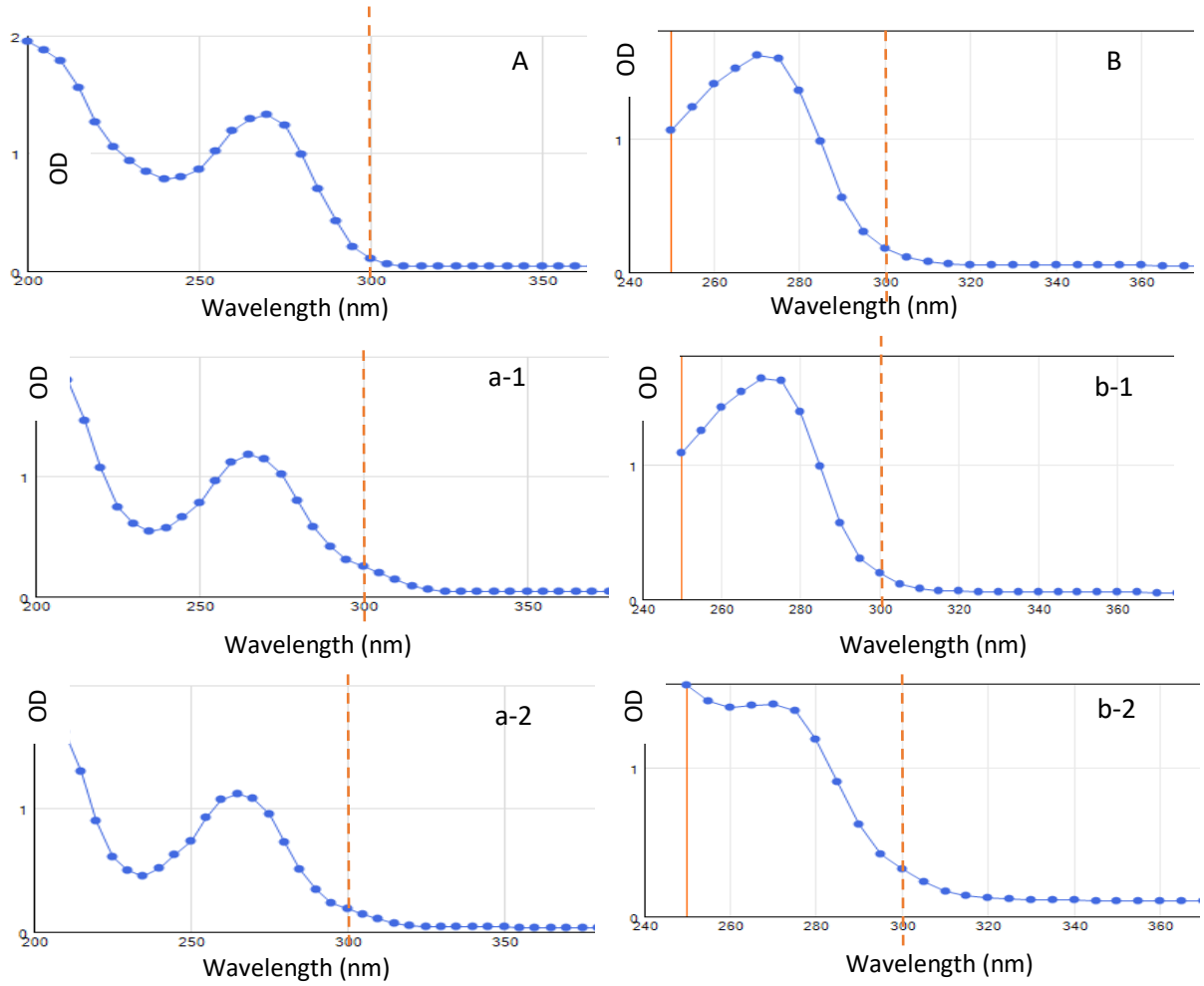


Figure 3.5 *Spectrometer reading of HEK 293T cells and human MSCs before and after TP plasmid delivery by virus and PEI vector. The OD reading is 0.11 and 0.1 at 300 nm for and HEK 293T cells and human MSCs without TP-GFP plasmid delivery (A, B). The OD reading is 0.28 and 0.29 at 300 nm for HEK 293T cells with TP-GFP plasmid delivery by PEI and virus (a-1, a-2). The OD reading is 0.14 and 0.25 at 300 nm for human MSCs with TP plasmid delivery by PEI and virus vector (b-1, b-2).*

Figure 3.6 shows the result of TP activity of the protein expression in HEK 293T cells and human MSCs by PEI and viral gene delivery. The TP protein in HEK 293T cells had a very high level of TP activity whether use viral or PEI vector. The 5-FU release rate of HEK 293T cells by two vectors are both over 0.25 µmol/h/mg total protein. The TP activity in human MSCs is much high when use viral vector compare to PEI vector. The 5-FU release rate for human MSCs by PEI vector is only 0.06 µmol/h/mg total protein. The 5-FU release rate for human MSCs by viral vector is close to 0.2 µmol/h/mg total protein which is more than 3 times of PEI vector. The 5-FU release rate represents the ability of TP convert prodrug 5'DFUR to 5-FU and closely related to the killing ability of cancer cells. The TP

expression in all four samples are capable to convert certain amount of prodrug to 5-FU in different level but is very low for human MSCs by PEI delivery. Using virus as a vector to delivery TP to human MSCs had a big increase on TP expression and activity compare to PEI vector.

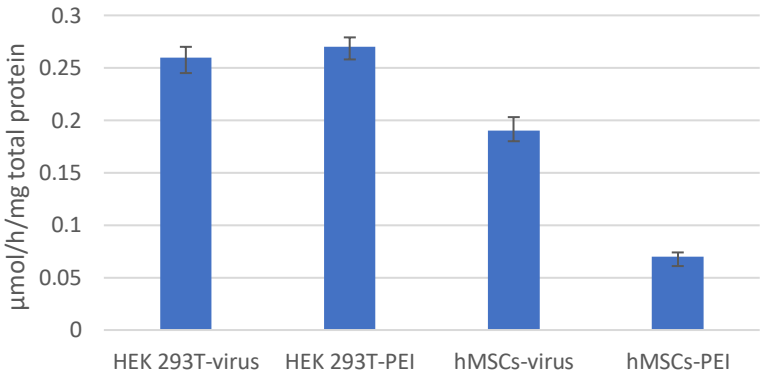


Figure 3.6 *TP activity of HEK 293T cells and human MSCs with and without TP delivered by viral vector and PEI vector shows in 5-FU release rate. The 5-FU release rates of HEK 293T cells by virus and PEI vector are 0.26 and 0.27 µmol/h/mg total protein respectively. The 5-FU release rates of human MSCs by virus and PEI vector are 0.19 and 0.06 µmol/h/mg total respectively.*

3.4 Summary

Gene delivery to human MSCs through a non-viral vector like PEI having a very low delivery efficiency and protein expression. TP sequence was delivered to human MSCs by virus vector to increase the gene delivery efficiency and protein expression level in and a higher prodrug-drug conversion rate. Virus as a gene delivery vector contain TP transfer gene were produced by HEK 293T cells. TP transfer gene was constructed by SFFV plasmid with a TP sequence insertion. TP sequence was first cloned out and amplified by two self-designed primers and digested by two restriction enzymes. SFFV plasmid has a In this case, TP expression in human MSCs was not easy to observe and the it was tested by western blot assay. Compare to the human MSCs by PEI gene delivery, human MSCs with virus gene delivery had a much higher TP protein expression level and enzyme activity. Virus production and transfection was first tested by HEK 293T cells use SFFV-GFP plasmid and the delivery efficiency of it was very high. An interested thing is that the gene delivery efficiency of HEK 293T cells with PEI vector is even a little bit higher than virus vector. The delivery efficiency of virus vector is always high for both HEK

293T cells and human MSCs but the PEI vector delivery efficiency for HEK 293T cells and human MSCs are vary. From this we can see, compare to the virus vector, the delivery ability of PEI is not stable and strongly dependents on the cell types.

Chapter 4 Thymidine phosphorylase delivery to human MSCs by viral vector encoded with TP-GFP

4.1 Introduction

Human mesenchymal stem cells (human MSCs) can be derived from bone marrow and some other tissue and present not only in fetal tissues but also many adult tissues. Human MSCs have the capability of self-renewal and can differentiate into multiple cells. human MSCs were chosen to be the gene carrier because of their cancer tropism characteristic and low immunology response [26]. There are many markers and receptors on the surface of human MSCs which can be attracted by some chemicals secreted by cancer cells. Additionally, their low immunology response helps to deliver gene-carried human MSCs into the human body. Human MSCs also have no endogenous TP expression in the human body. In this study, human MSCs were transfected with the TP gene by lentivirus and co-cultured with A549 lung cancer cells and treated with prodrug 5'DFUR. After TP protein expression in human MSCs, prodrug 5'DFUR was converted to chemotherapy drug 5-FU, and killed both A549 cells and human MSCs. Human MSCs transfected with PEI or other non-viral methods have low transfected efficiency and protein expression [29]. In this chapter, a GFP labeled TP sequence was delivered to human MSCs by lentivirus produced by HEK 293T cells. A structure plasmid, envelope plasmid and a plasmid contain TP-GFP transfer gene were delivered to HEK293T cells first by PEI at N/P ratio 5. Viruses were collected from day 2 to 4 after gene delivery and transfected to human MSCs with polybrene. Virus transfected human MSCs had very high delivery efficiency and protein expression. Cell sorting by GFP labeling showed higher percentage of protein expression. TP protein expression was confirmed by Western blot, and TP activity was tested by enzyme activity assay. The results showed TP expression and enzyme activity in human MSCs through viral transfection was very high and converted prodrug 5'DFUR to 5-FU at a reasonable rate. The optimal strategy is to delivery TP transfected human MSCs into cancer sites to express TP protein. After administering prodrug 5'DFUR, the prodrug will convert to chemotherapy drug via TP protein at the cancer site, not throughout the body.

4.2 Materials and methods

4.2.1 TP-GFP plasmid delivery to human MSCs by PEI

Human mesenchymal stem cells (human MSCs) were obtained from Lonza (Alpharetta, GA) and stored at –80 °C in 10% dimethyl sulfoxide (DMSO) and 30% FBS alpha minimum essential medium (α-MEM). Human MSCs were thawed in a 37°C water bath and cultured in 10% FBS with 1% L-glutamine and 1% penicillin/streptomycin α-MEM in a T-75 flask. Cells were incubated in an incubator with 5% CO_2 at 37°C. Human MSCs were attached to the inner surface of a T-75 flask after 5 to 6 hours and cultured for 2 more days to achieve 100% confluence. After cells grew to 100% confluence, 10^5 cells were passed to 65 mm petri dishes and cultured overnight for TP-GFP gene delivery. Experiments were performed in triplicate. 10 μM 25 kDa PEI (pH 7.0) was prepared as same way in section 2.2.1. TP-GFP DNA was mixed with a certain amount of PEI in N/P 20 in 100 μL α-MEM in a 1.5 mL sterilized centrifuge tube and incubated for 30 min at room temperature. TP-GFP DNA/PEI mixture was then added into the cell culture after incubation and mixed well with culture medium. Human MSCs were incubated at 37°C in the incubator overnight and then observed by a fluorescence microscope in following 3 days.

4.2.2 TP-GFP plasmid delivery to human MSCs by lentiviral

SFFV-TP-GFP plasmid was custom made from GenScript. 10 μg SFFV-TP-GFP plasmid was mixed with 10 μg p 8.2 and 10 μg p MDG plasmids, as well as a certain amount of PEI at N/P ratio 5 in 100 μL DMEM in a sterilized 1.5 mL centrifuge tube incubated at room temperature for 30 min. The PEI/DNA mixture in DMEM was added into a T-75 flask of HEK 293T cells with 10 mL fresh DMEM. HEK 239T cells with DNA delivered were cultured for 2 more days to produce viruses. Viruses were collected with cell culture medium at day 3 and day 4 after DNA delivery and saved in a 15 mL tube at -20°C for further use. Fresh DMEM medium was added following each collection. The virus mixture was mixed with 10 mM polybrene for 15 min at room temperature and filtered through a 0.45 μm

membrane before being added into human MSCs culture. Human MSCs were incubated for 2 days and observed the TP-GFP protein expression under a fluorescence microscope.

Experiments were conducted three times to get average results. Human MSCs were collected after 4 days of viral transfection by 0.25% trypsin for Western blot and enzyme activity assay. Cells were spun down at 400 x g for 5 min to get a cell pellet, which was saved at –80°C.

4.2.3 Western blot

Human MSCs with TP-GFP expression were collected by 0.25% trypsin and the protein was extracted from cells by the same way as described in section 2.2.4.

4.2.4 Enzyme activity assay

TP activity in human MSCs delivered with TP-GFP plasmid by virus vector was tested by enzyme activity assay and quantified by spectrometer describe previously in section 2.2.5.

4.2.5 Cell sorting assay

After 3 days of TP-GFP plasmid delivered into human MSCs, 10^6 cells were collected by 0.25% trypsin and spun down at 400 x g for 5 min. Cells were resuspended in α-MEM without FBS and went through cell sorting by fluorescent active cell sorting machine (FACS) SH 800 (Sony, New York). Human MSCs with and without TP-GFP expression were separated by FACS machine to two groups, one tube collected the cells with TP-GFP protein expression and one without. Human MSCs with TP-GFP protein expression were cultured back for further test.

4.3 Results and discussion

4.3.1 Deliver efficiency of TP-GFP in human MSCs with viral and non-viral vector delivered

Figure 4.1 shows the comparative results between non-viral PEI vector and lentiviral vector transfection to human MSCs of TP-GFP plasmid. Cells with green coloring represent cells with TP-GFP expression. As can be seen from the figures A1-4, GFP expression in human MSCs with non-viral PEI vector gene

deliver is very low and only a few cells show green color when compared with bright field and fluorescent images. TP-GFP expression in human MSC with lentiviral vector transfection had a large increase as shown in images B1-4. The total cell numbers from figure A and B are about the same, but the number of cells expressing TP-GFP protein were 3 to 4 times more in figure B. Besides the increase in the efficiency of protein expression, the cell morphology of viral vectors is better than the PEI vector. Viral vectors always have a safety concern when used for gene delivery, so the virus backbone plasmid kept improving to reduce the toxicity. As we can see in figures B 2 and 4, cell morphology is very healthy after 3 days of viral transfection. On the contrary, the cell morphology of PEI vector gene delivery was changed and was not healthy 3 days after delivery. Non-viral vectors are mostly safe, but PEI has some toxicity to cells according to some studies when used in a large amount [16]. Here, we limited the N/P ratio to under 20, which is safe for many other cells, but the human MSCs still had some toxicity and the cell debris negatively affected the health of the cells 3 days after gene delivery. Viral vector transfection of TP-GFP to human MSCs not only had higher delivery efficiency and less toxicity, but also took less time to show more intense GPF signal. TP-GFP with PEI delivery to human MSCs showed light fluorescent color on day 2 and strong fluorescence on day 3, but it only took 2 days for the viral vector transfected human MSCs to show strong fluorescent green color.

Figure 4.1 C1-4 shows the MSCs cell sorting results after 3 days of virus transfection. Cells were sorted through a sorting machine based on their GFP label. The sorting machine separated human MSCs with and without TP-GFP expression. Cells with TP-GFP expression were collected and grown back in a culture medium for 2 days. HMSCs with TP-GFP delivery after cell sorting reached an 80% protein expression efficiency.

Figure 4.2 shows the cell sorting result of human MSCs with TP-GFP plasmid delivery. The figure A shows 97% of cells distributed well in the medium. The cells resuspend in the medium may get aggregate and it cause the sorting result not very accurate. If two or more cells conjugate together and some with

GFP but some without all count for cells with protein expression. So, it shows almost all of the cells distribute well in the suspension which make the sorting result credible.

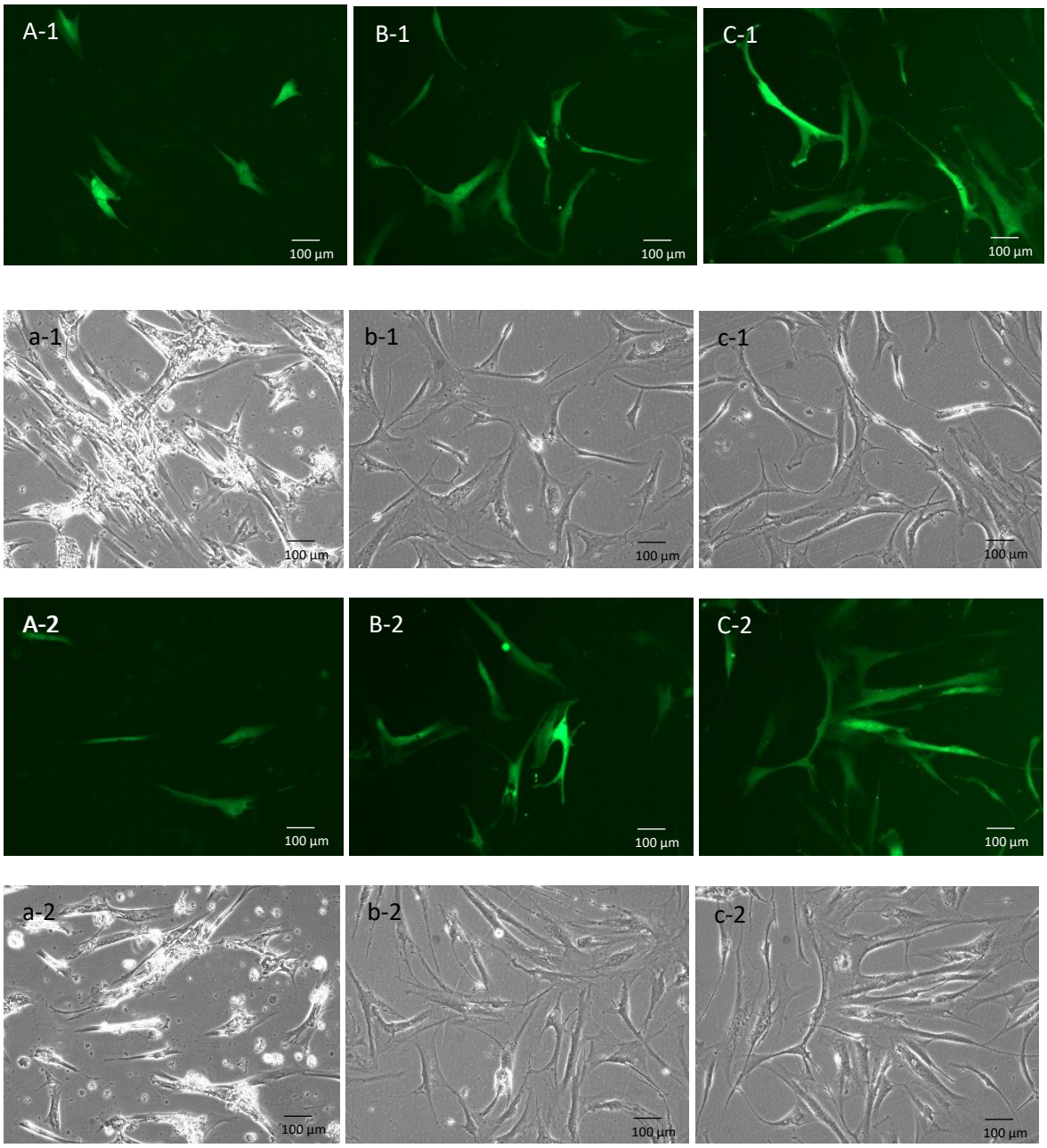


Figure 4.1 *Fluorescence and bright field images of TP-GFP expression in human MSCs by PEI vector gene delivered (A1-2, a1-2). Fluorescence and bright field images of TP-GFP expression in human MSCs by viral vector gene delivered (B1-2, b1-2), Fluorescence and bright field images of TP-GFP expression in human MSCs by viral vector gene delivered and cell sorting (C1-2, c1-2).*

In figure B1-2, it shows more than 46% of the cells in 97% of the total populations sorted as cells with GFP indicate almost 50% of the populations has TP-GFP expressed. This result corresponds to the result of plasmid delivery efficiency of human MSCs delivered with TP-GFP by virus measured from cell count. It confirmed the delivery efficiency and protein expression level of TP is high in human MSCs by virus delivery.

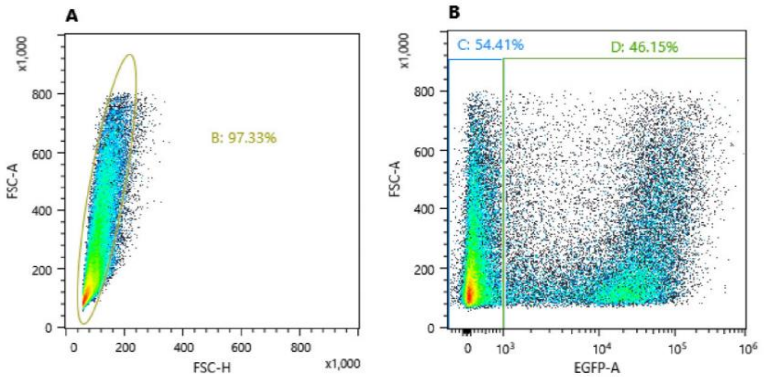


Figure 4.2 *Cell sorting of human MSCs with TP-GFP expression. (A) 97.33% of total human MSCs distributed well and went through cell sorting, (B) 46.15% of human MSCs with TP-GFP expression were sorted based on GFP level detected in cells.*

The gene delivery efficiency is closely related to protein expression percentage. Figure 4.3 represents the comparison of TP-GFP delivered efficiency of human MSCs by viral and non-viral vector. The delivery efficiency of non-viral vector PEI to human MSCs was extremely low; only 15% at optimal conditions. Using the viral vector, delivery efficiency of human MSCs sharply increased to around 50%. Therefore, the delivery efficiency of viral vector to non-viral vector in human MSCs increased 4 folds. Three days after viral transfection, human MSCs went through cell sorting to separate TP-GFP expressed cells and non-expressed cells. After cell sorting, delivery efficiency increased again to 85% of the population.

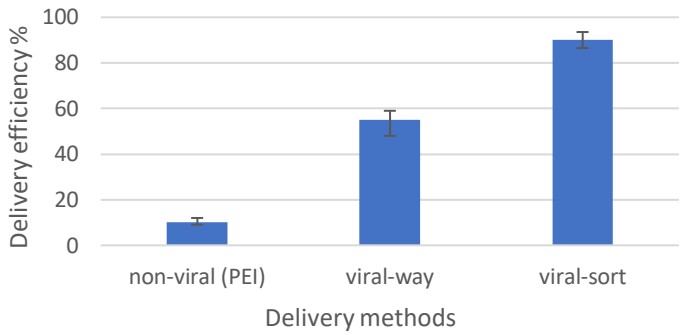


Figure 4.3 *TP delivery efficiency of human MSCs by non-viral vector (PEI), viral vector (Lentiviruses), and viral vector with cell sorting. The human MSCs delivery efficiency of PEI vector is 15%. The delivery efficiency of viral vector to human MSCs increased to 55% and keep increased to 88% after cell sorting.*

4.3.2 Western blot

Figure 4.4 shows the western blot result of TP-GFP expression in human MSCs by virus and PEI transfection. The size of fusion protein TP-GFP is 75 kDa since GFP is around 25 kDa and TP is around 50 kDa. The band shows at lane 1 at 75 kDa barely can see, meaning there were no TP-GFP expression in human MSCs by PEI vector delivery. Lane 2 shows a very strong band at 75 kDa, indicating there was a TP-GFP expression in human MSCs using lentivirus delivery. This result shows there was no TP-GFP expression in human MSCs when using the PEI vector, and TP-GFP expressed when change to viral vector.

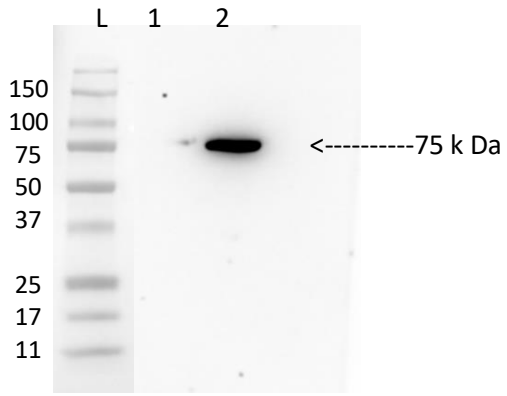


Figure 4.4 *Western blot result of human MSCs with TP-GFP delivered by virus vector. L represent the protein ladder. Lane 1 is protein expression of human MSCs without TP delivered. Lane 2 is TP-GFP expression in human MSCs by virus delivered.*

4.3.3 Enzyme activity

Figure 4.5 shows the spectrometer reading of HEK 293T cells with TP-GFP plasmid delivery by virus and human MSCs with TP-GFP plasmid delivery by virus and PEI. The OD reading at 300 nm is 0.14

for human MSCs using PEI vector which are point out by arrow (A) and increase to 0.26 (a). The OD reading at 300 nm of HEK 239T cells is 0.29 when with virus delivery of TP-GFP. The increased amount reading is indicated the 5-FU converted amount and the 5-FU release rate can be calculated by calibration curve and total protein amount in cells. The OD reading is in proportional to the 5-FU release rate, the higher of the OD reading increased, the higher 5-FU release rate of each sample. The spectrometer reading of HEK 293T cells and human MSCs without TP plasmid delivery shows in section 3.3.4 previously.

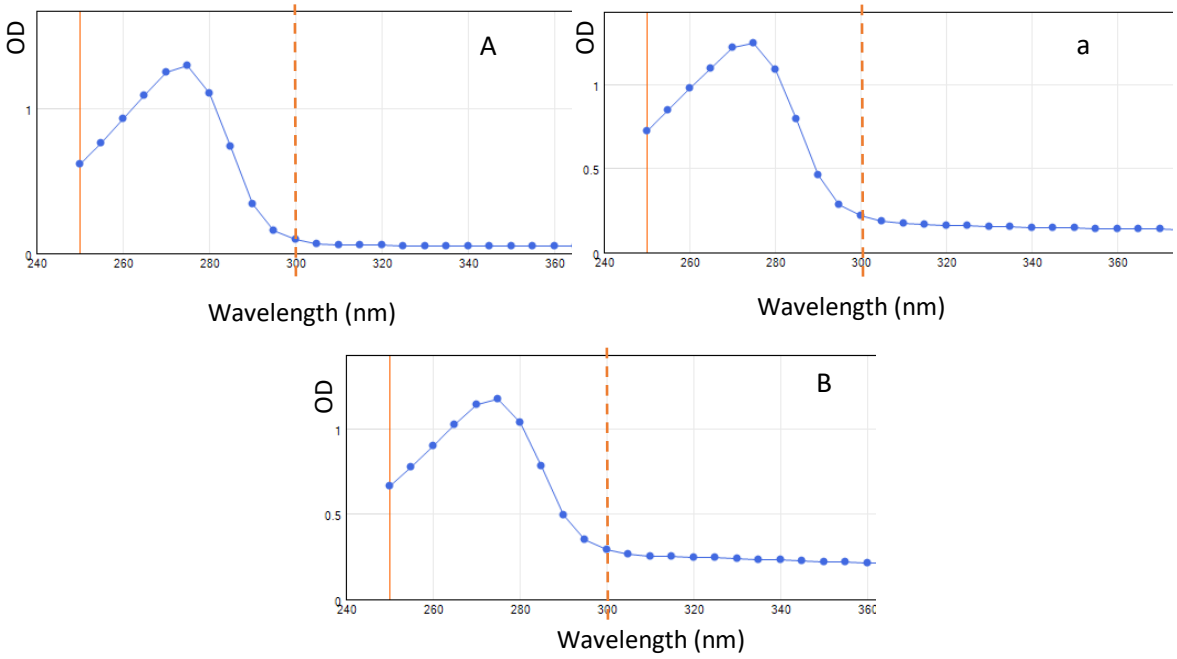


Figure 4.5 *Spectrometer reading of HEK 293T cells with TP-GFP plasmid delivery by virus and human MSCs with TP-GFP plasmid delivery by PEI vector and virus vector. The OD reading is 0.14 and 0.26 at 300 nm for and human MSCs with TP-GFP plasmid delivery by PEI vector and virus vector (A, a). The OD reading is 0.29 at 300 nm for HEK 293T cells with TP-GFP plasmid delivery by virus (B).*

Figure 4.6 indicates the 5-FU release rate in each type of cell with and without gene delivery and represents the TP activity and capability of prodrug drug conversion. The 5-FU release rate in HEK 293T cells and human MSCs without gene delivery were both only 0.01 µmol/h/g total protein which are close to 0 and can be regard as no enzyme activity to convert prodrug to drug. This confirms HEK 293T cells and human MSCs have no endogenous TP expression. The A549 cancer cells showed a 5-

FU release rate of 0.05 µmol/h/g total protein. This indicates A549 cells have a low-level of endogenous TP expression and that enzyme activity is low compare to gene delivered cells. HEK 293T cells with TP-GFP delivered by PEI vector showed the highest level of 5-FU released (0.29 µmol/h/g total protein). The gene delivery efficiency of HEK 293T cells was very high as explained previously because of the cell type. That directly caused a very high level of enzyme activity and prodrug conversion rate. The amount of 5-FU released from human MSCs by PEI vector was 0.07 µmol/h/g total protein which was higher than human MSCs with no gene delivery but still very low compared to viral vector ones. The 5-FU release rate of human MSCs with viral vector gene delivery was 0.22 µmol/h/mg total protein which was much higher than the non-viral one and represents a high enzyme activity and prodrug conversion rate.

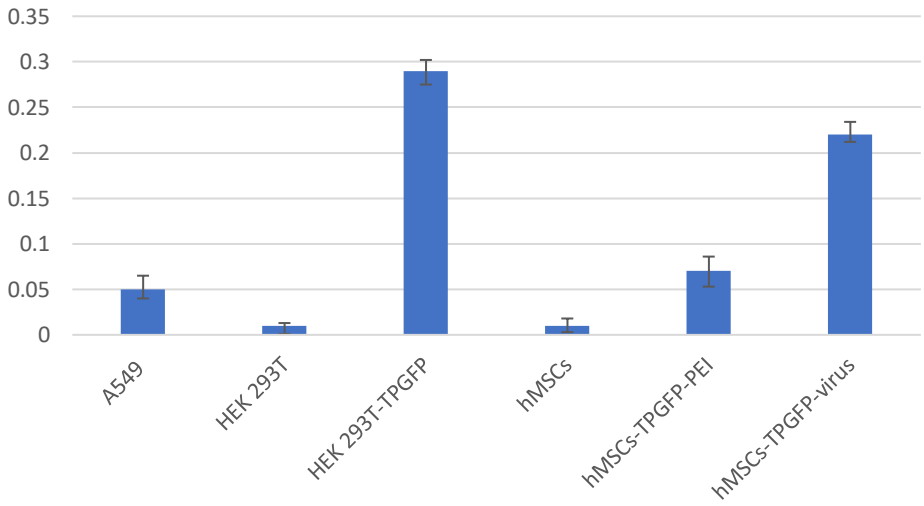


Figure 4.6 *TP activity of A549 cells, HEK 293T cells, HEK 293T cell with TP-GFP delivered by virus vector, human MSCs, and human MSCs with TP-GFP delivered by viruses and PEI vector shows in 5-FU release rate. The 5-FU release rate of A549 cells without TP-GFP DNA delivery is 0.05 µmol/h/mg total protein. The 5-FU release rate of HEK 293T cells with and without TP-GFP DNA delivery is 0.29 and 0.01 µmol/h/mg total protein. The 5-FU release rate of human MSCs with TP-GFP DNA delivery by PEI and virus vector is 0.06 and 0.22 µmol/h/mg total protein respectively. The 5-FU release rate of human MSCs without TP-GFP DNA delivery is 0.01 µmol/h/mg total protein.*

4.4 Summary

In this chapter, human bone derived mesenchymal stem cells (human MSCs) were successfully transfected with TP-GFP plasmid by both non-viral vector (PEI) and lentiviral vector. The efficiency

and safety of non-viral transfection to human MSCs using PEI as a gene delivery reagent are extreme low and slow. Using lentivirus as the gene carrier to deliver TP-GFP into human MSCs has a much higher efficiency and protein expression with no toxicity. Viral vectors always have a safety concern when used to deliver a gene *in vivo*, while non-viral vectors are usually considered to be safe. However, in this experiment, human MSCs with lentivirus gene delivery were caused no toxicity to cells at all, while the non-viral vector PEI caused some toxicity and cell debris to human MSCs even in a low amount. Another case, the gene delivery efficiency of PEI to HEK 293T cells is very high and caused absolutely no toxicity or cell morphology changes. Again, the characteristics and ability of each gene vector is strongly dependent on the type of cells. The results of TP-GFP protein expression level and enzyme activity corresponding to the gene delivery efficiency. Human MSCs with viral vector gene delivery shows a very high level of protein expression and had the strong activity required to convert prodrug to chemotherapy drug, which was sufficient for the next step of the experiment.

Chapter 5 Eradicate of A549 cells by TP-GFP transfected human MSCs

5.1 Introduction

The crucial step in gene-directed enzyme prodrug therapy is using TP carried human MSCs which migrate to cancer cell sites and administrate prodrug 5'DFUR. Ideally prodrug travels all over the body through blood vessels and is converted to chemotherapy drug at cancer site only by overexpressed TP. Chemotherapy drug kills both cancer cells and carrier cells human MSCs but no other healthy body cells. In this study, we co-cultured TP transfected human MSCs with A549 lung cancer cells *in vitro* to mimic deliver human MSCs to tumor site in body, and then added certain amounts of prodrug 5'DFUR to tested prodrug-drug conversion and cell viability. A549 cells were co-cultured with TP or TP-GFP transfected human MSCs in 2:1, 1:1, and 1:2 ratios to verify cell viability after treatment in different cell populations. The enzyme activity and 5-FU release rate were quite different in the 3 ratios used for co-culture. Higher ratio of human MSCs contained TP protein to A549 cells shows higher enzyme activity and 5-FU release rate. Cell viability of human MSCs and A549 cells co-culture were observed under microscope and quantified in the following days after prodrug treatment. Human MSCs and A549 cells both started to shrink and die on day 2 and were finally eradicated in 5 days. Human MSCs and A549 cells died in a slightly different rate because of different cell size, and diving phase. Cells without TP-GFP delivered or prodrug administrated survived and grew to 100% confluence in 4 days. These results indicate the prodrug was successfully converted to chemotherapy drug 5-FU and killed both A549 cells and human MSCs by administrated non-toxicity prodrug.

5.2 Materials and methods

5.2.1 Co-culture of TP-GFP transfected human MSCs and A549 cells

Virus transfected Human MSCs with high level TP or TP-GFP expression were co-cultured with A549 cells in a 65-mm petri dishes in 3 different ratios for triplicate experiments. Human MSCs cell number were fixed at 5×10^4 in each petri dishes and A549 cells number were adjusted to reach 2 to 1, 1 to 1,

and 1 to 2 ratios cultured in 50% DMEM and 50% a-MEM culture medium. Human MSCs were passed from a T-75 flask after 3 days of TP-GFP plasmid delivered. After human MSCs settled down in the new dishes, A549 cells were loaded into each petri dish. A549 cells usually take overnight to completely settle down.

5.2.2 Enzyme activity assay

TP activity of co-cultured human MSCs with TP or TP-GFP expression and A549 cells were tested by enzyme activity assay and quantified by spectrometer as same described in section 2.2.5.

5.2.3 Cell viability assay with prodrug 5'DFUR

After co-cultured cells completely settle down, 200 mM prodrug 5'DFUR was added into each well. Experiments were performed in triplicate. Cells were observed in following days under fluorescence microscope and cell viability was quantified by cell count via trypan blue buffer. 3 ratios of each co-cultured cells received same amount of prodrug and images were took at same days for comparison. For each sample and control, the cells were suspended in 0.5 mL 0.25% trypsin and 0.5 mL DMEM for neutralize. 20 µL of cell suspension were mixed with 0.4% trypan blue in PBS as 1:1 ratio and use 20 µL of total mixture dropped on a hemocytometer for cell count. The dead cells were blue color and live cells were show in bright under a bright field model microscope.

5.3 Results and discussion

5.3.1 Enzyme activity analysis

Figure 5.1 shows the spectrometer reading of human MSCs with and without TP-GFP delivered by virus vector and co-cultured with A549 cells in 2:1, 1:1, and 1:2 ratios. The OD reading at 300 nm of each image point out by arrow. Image A, B, and C are the results of human MSCs without TP-GFP plasmid but co-cultured with A549 cells in 2:1, 1:1, and 1:2 ratios. The OD reading at 300 nm are all around 0.1. Image a, b, and c are the results of human MSCs witt TP-GFP plasmid but co-cultured with A549 cells in 2:1, 1:1, and 1:2 ratios. The OD reading at 300 nm are 0.28, 0.23, and 0.17 respectively. Compare the

image of A to a, B to b, and C to c, the OD reading increased 0.27, 0.22, and 0.16 respectively. The increased amount indicates the 5-FU conversion. 5-FU release rate can be calculated by calibration curve and total protein amount in cells. Total protein amount in cells of each sample was measured by BAC assay.

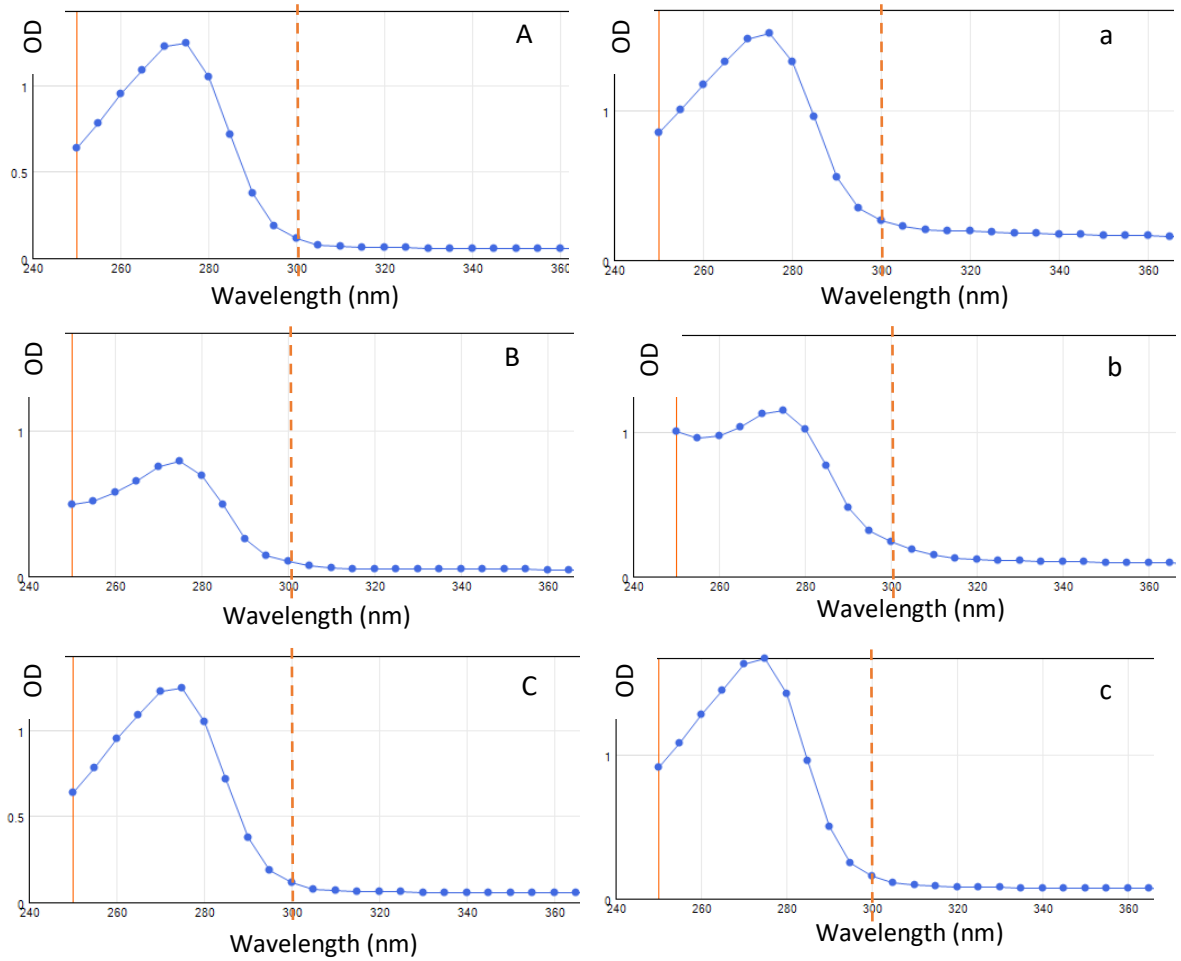


Figure 5.1 *Spectrometer reading of human MSCs with and without TP-GFP delivered by virus vector and co-cultured with A549 cells in 2:1, 1:1, and 1:2 ratios. At 2:1 cell co-cultured ratio, OD is 0.1 at 300 nm for no TP delivery sample and 0.28 for TP delivery sample (A, a). At 1:1 cell co-cultured ratio, OD is 0.1 at 300 nm for no TP delivery sample and 0.23 for TP delivery sample (B, b). At 1:2 cell co-cultured ratio, OD is 0.1 at 300 nm for no TP delivery sample and 0.17 for TP delivery sample (C, c).*

Figure. 5.2 shows the TP activity of human MSCs and A549 cells co-culture in 3 different ratios. Using self-constructed TP virus backbone transfer gene, the 5-FU release rate is 0.1 µmol/h/mg total protein when the ratio of human MSCs to A549 cells co-culture ratio equals to 0.5. 5-FU release rate increased to 0.16 µmol/h/mg total protein when the ratio of human MSCs to A549 cells is 1. The 5-FU release rate

reaches a of maximum 0.21 µmol/h/g total protein when the cell number of human MSCs to A549 cells is doubled. For the human MSCs using TP-GFP virus backbone transfer gene, the 5-FU release rate is higher for all 3 ratios. The 5-FU release rate is 0.16 µmol/h/mg total protein when the human MSCs A549 cells ratio is 0.5 and increased to 0.21 µmol/h/mg total protein when equal amount cell number for human MSCs and A549 cells. 5-FU release rate reached 0.27 µmol/h/mg total protein when human MSCs number is twice that of A549 cells. From the results, more TP protein in the cell population causes a higher 5-FU release rate meaning higher enzyme activity. More TP transfected human MSCs co-cultured with A549 cells obtained a higher 5-FU release rate with the same prodrug concentration.

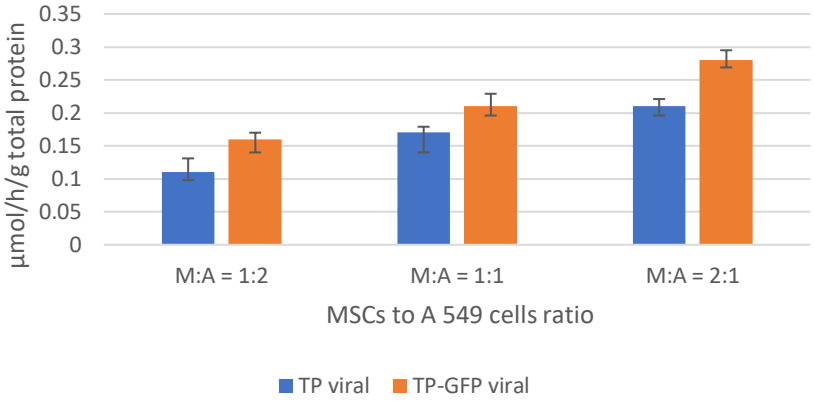


Figure 5.2 *TP activity of TP transfected human MSCs by virus vector co-cultured with A549 cells in 2:1, 1:1, and 1:2 ratios. The 5-FU release rate of human MSCs delivered by TP virus in 3 ratios of co-cultures are 0.11, 0.18, and 0.21 µmol/h/mg total protein. The 5-FU release rate of human MSCs delivered by TP-GFP virus in 3 ratios of co-cultures are 0.17, 0.21, and 0.28 µmol/h/mg total protein.*

5.3.2 Cell viability with prodrug treatment

Figure 5.3 shows the images of co-cultured A549 cells and human MSCs with TP delivery by virus in 1:1 ratio with prodrug treated. Human MSCs were transfected by TP viral vector without out GFP so the protein expression is not visible by fluorescence light. TP expression was confirmed by western blot previously. Cells were stopped growing on day 1 after 5'DFUR treatment (A1) and started to have some morphology change on day 2, and few cells unattached to the surface. Half of both A549 cells and human MSCs dead on day 3 as can be seen in the images (A2). More cells dead on day 4 and only about 20% of cells still lived and attached to surface on day 5 (A3). Compare to the co-culture cells without TP

delivery to human MSCs, both human MSCs and A549 cells were kept growing after being administered 5'DFUR. Cells grew 30% more on day 3 (B2) and to 100% confluence on day 5 (B3). Cell morphology was remained good and A549 cells were grew faster than human MSCs. That makes the final ratio of human MSCs to A549 cells changed. Different to control group, co-cultured cells with TP delivery and 5'DFUR treatment, the cells dead in a similar rate and cells remained live on day 5 are still about 1:1 ratio.

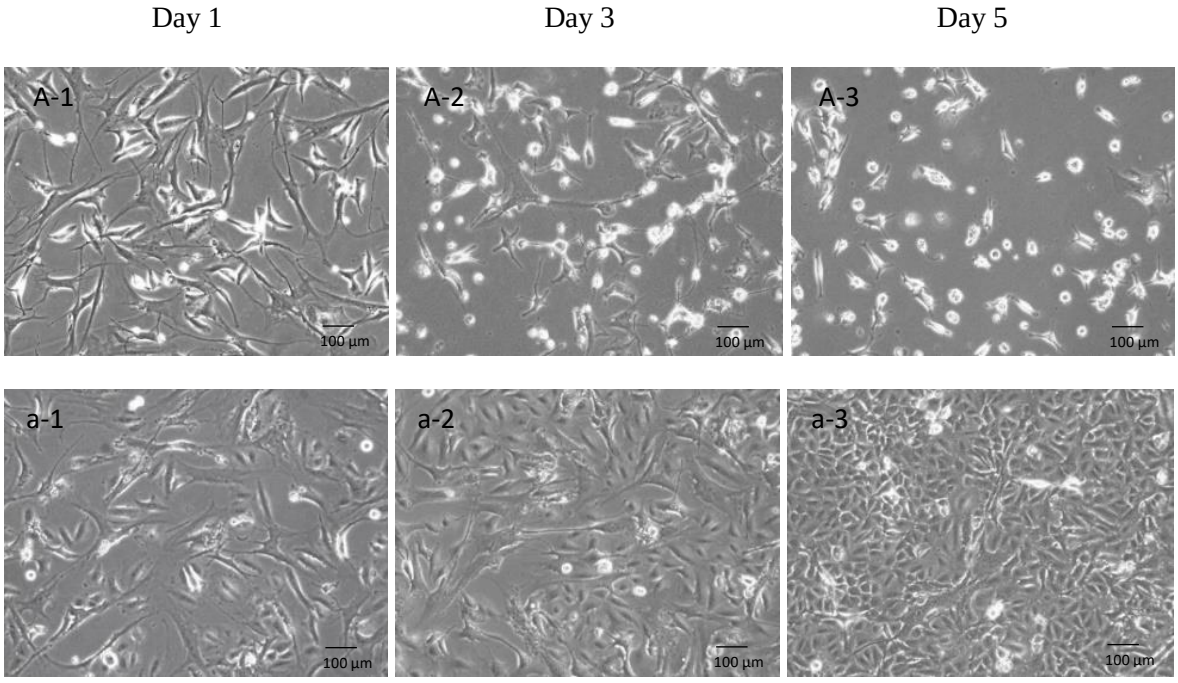


Figure 5.3 *TP transfected human MSCs co-cultured with A549 cells with prodrug 5'DFUR treatment for 5 days (A1-3). None TP transfected human MSCs co-cultured with A549 cells with prodrug 5'DFUR treatment for 5 days (a1-3).*

Figure 5.4 shows the co-culture results of TP expressed human MSCs and A549 cells with prodrug 5'DFUR added. TP transfected human MSCs were co-cultured with A549 cells in 3 different ratios which were 1:2, 1:1, and 2:1. Different ratios were tested to verify the effect on cell viability of A549 cell numbers in the co-culture population. Higher ratio of A549 cells to human MSCs indicate more cancer cells in the cell population with fixed human MSCs number. For all three ratios of co-culture, cells stopped growing on day 1 after prodrug treated and started to die on day 2. Cell viability decreased to 75%, 80%, and 85% on day 2 for 2:1, 1:1, and 1:2 ratios. Both human MSCs and A549 cells died at

a similar rate on day 2 day but the sample with higher A549 cell numbers shows a higher cell viability. On day 3, cell viability for 3 ratios decreased to 46%, 58%, and 65% which around half of the populations been killed. For the 2 to 1 ratio sample, cell viability decreased to only 25% which ¼ cells been killed in 4 days. And for 1:1 ratio and 1:2 ratio samples, cell viability also decreased to 30% and 40%. On day 5, only 10% cells survived for 2:1 sample, and cell viability decreased to 16% and 22% for 1:1 and 1:2 ratios sample. From the result, both A549 cells and human MSCs been killed after added prodrug 5'DFUR. The sample with higher human MSCs ratios has a lower cell viability over 5 days and cell viability all decreased to 10% to 20% for three samples.

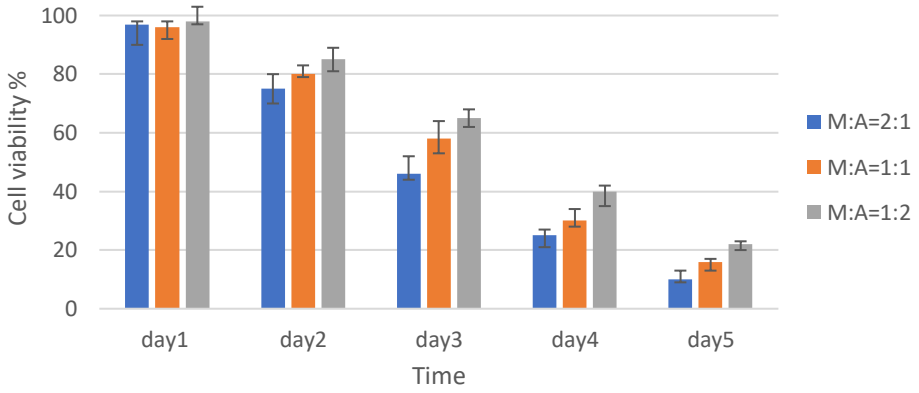
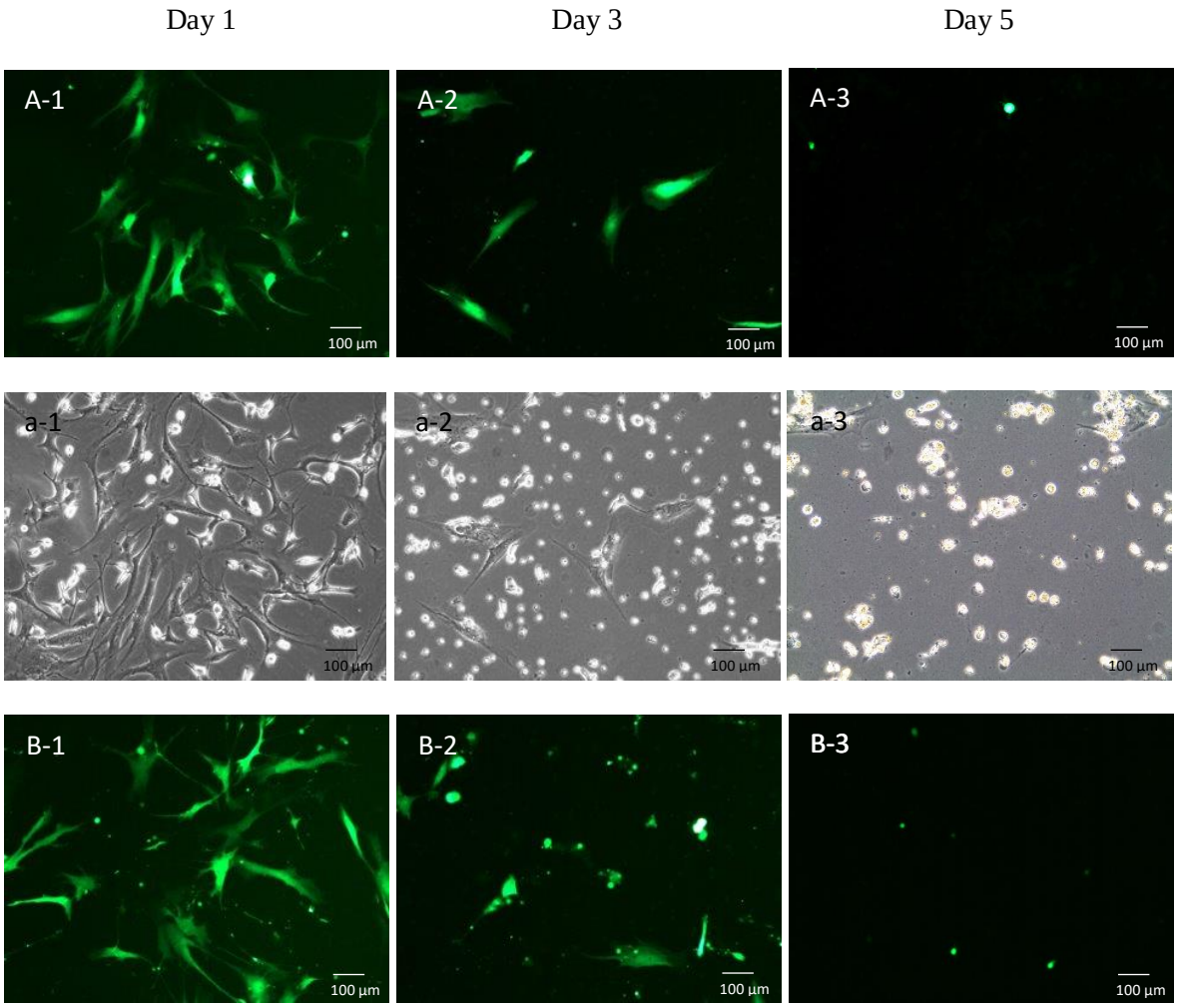


Figure 5.4 *Cell viability of co-cultured TP delivered human MSCs and A549 cells in 2:1, 1:1, and 2:1 ratio with 5'DFUR treatment in 5 days. The cell viability of three samples on day 1 is all around 97%. The cell viability of three samples on day 2 decrease to 75%, 80%, and 85%. The cell viability of three samples on day 3 decrease to 46%, 58%, and 65%. The cell viability of three samples on day 4 decrease to 25%, 30%, and 40%. The cell viability of three samples on day 5 finally decrease to 10%, 16%, and 22%.*

Figure 5.5 shows the result of co-cultured of A549 cells and human MSC with TP-GFP expression in different ratios with 5'DFUR treatment. TP-GFP expressed human MSCs were co-cultured with A549 cells after 48 hours of gene delivery in 2:1, 1:1, and 1:2 ratio with fixed human MSCs numbers. At 2:1 ratio, the cell number of human MSCs were doubled of A549 cells on day 1 with 5'DFUR treatment (A1, A4). Both human MSCs and A549 cells stopped growing on day 2 and had about half of cell dead on day 3 (A2, A5). Cell viability keep decrease on day 4 and most of cells were dead on day 5 (A3, A6). At 1:1 ratio, human MSCs numbers were same as A549 cells on day 1 after 5'DFUR treatment (B1, B4). Both human MSCs and A549 cells stopped grow on day 2 as well and dead many on day 3 (B2, B5).

Living cells keep decrease and only a few cells still alive on day 5 same to 2:1 sample (B3, B6). At 1:2 ratio, human MSCs numbers were halved of A549 cells on day 1 after 5'DFUR treatment. Both human MSCs and A549 cells stopped grow on day 2 and dead a lot on day 3 (A2, A5). As can be seen on the picture took on day 3, compare three ratios of co-cultured samples, cells dead more with the increasing human MSCs number in the population. On day 5, all three samples had about more than 80% cells dead shows on the pictures, only 10-20% of cells still attached to the surface. This result show TP protein in human MSCs convert prodrug 5'DFUR to chemotherapy drug and killed both human MSCs and A549 cells continuous in 5 days. More human MSCs in the population have higher TP activity, and results in lower cell viability.



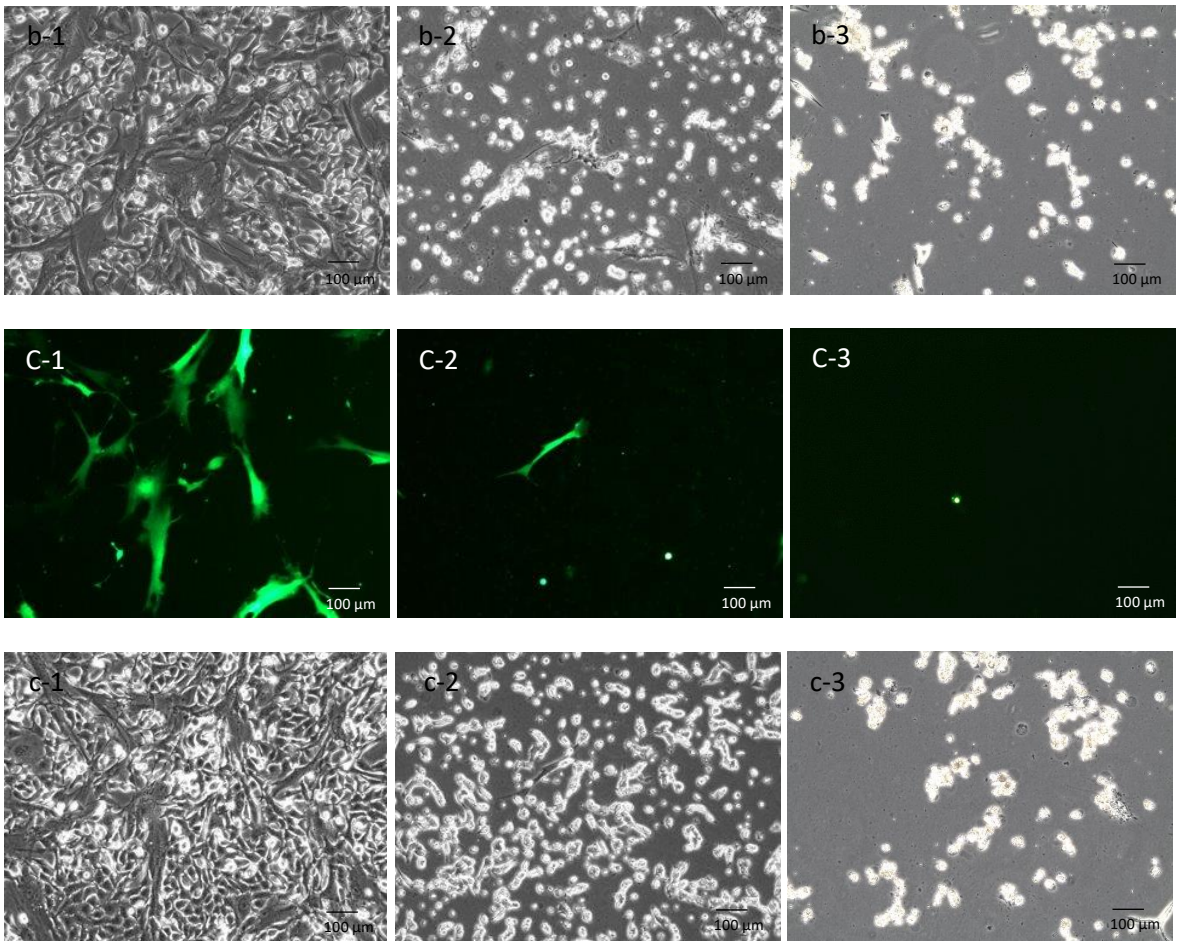


Figure 5.5 *Fluorescence and bright field images of human MSCs with TP-GFP delivered and A549 cells co-cultures in 2:1 with prodrug 5'DFUR added in following day 1, day 3, and day 5. (A1-3, a1-3), fluorescence and bright field images of human MSCs with TP-GFP delivered and A549 cells co-cultures in 1:1 with prodrug 5'DFUR added in following day 1, day 3, and day 5. (B1-3, b1-3), fluorescence and bright field images of human MSCs with TP-GFP delivered and A549 cells co-cultures in 1:2 with prodrug 5'DFUR added in following day 1, day 3, and day 5. (C1-3, c1-3).*

Figure 5.5 shows A549 cells and human MSCs co-cultured with no TP-GFP delivery but treated with 5'DFUR prodrug show no cell death for5 days, cells kept grew to 100% confluence (A1-3, B1-3). Human MSCs with no TP delivery and co-cultured with A549 cells but treated with prodrug 5'DFUR as a control group shows no cell death for 5 days and cells were kept growing to 100% confluence (Figure 5.5 C1-3). Cell morphologies were good and healthy. These result show prodrug 5'DFUR has no toxicity to cells. A549 cells and human MSCs had not enough endogenous TP expression to convert prodrug 5'DFUR to chemotherapy drug 5-FU. A549 cells with directly chemotherapy drug 5-FU treated as another comparison group of prodrug treatment (D1-3).

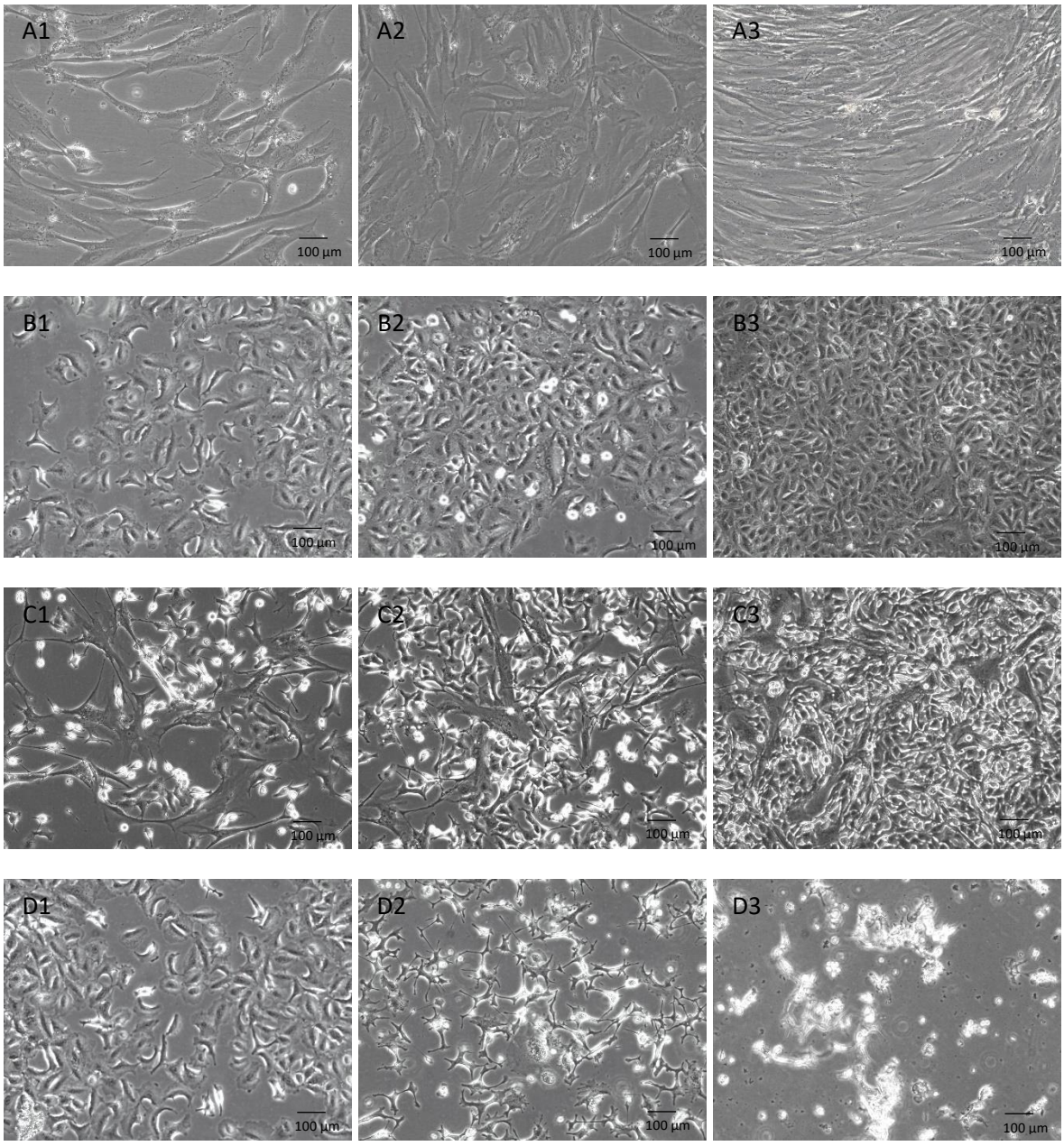


Figure 5.6 *None TP delivered human MSCs treated with prodrug 5'DFUR in day 1, day 3, and day 5 (A1-3). None TP delivered A549 cells treated with prodrug 5'DFUR in day 1, day 3, and day 5 (B1-3). Co-cultured none TP delivered human MSCs and A549 cells with prodrug 5'DFUR in day 1, day3, and day 5 (C1-3). A549 cells treated with chemotherapy drug 5-FU (D1-3).*

A549 cells were decreased to 50% on day 3 of 5-FU was added, and less than 10% cells were still alive on day 5 as shows in the picture. The cell death rate and cell morphology change of prodrug treatment with TP-GFP delivered was similar to the cells with chemotherapy drug 5-FU. This result shows the

cells treated with prodrug 5'DFUR were killed by chemotherapy drug 5-FU converted by prodrug and TP protein in another way.

Figure 5.6 indicates the cell viability of co-cultured TP-GFP delivered human MSCs and A549 cells in 2:1, 1:1, and 1:2 ratios with 5'DFUR treated for 5 days. For human MSCs and A549 cells at 2:1 ratio co-cultured, cells stopped growing on day 1. Cell viability decreased to 76% on day 2 and 40% on day 3. On day 4 of 5'DFUR treatment, cells were mostly dead, and viability was only 17%. And on day 5 only 5% cells didn't die. This result shows cells were dead very fast after prodrug treatment and were finally eradicate in 5 days when human MSC to A549 cells is 2:1. When the co-culture ratio of human MSCs to A549 cells were equal, cells also stopped growing on day one and viability decreased in the following days. The cell viability on day 2 was 79% which a little bit higher than 2:1 ratio co-culture but still confirmed cell death. On day 3, cell viability decreased to 49% meaning half of the cells were dead in 3 days and decreased to 23% on day 4. On day 5, the cell viability was only 7%. The result for 1:1 ratio co-culture was still reasonable because more than half of the cells died in 3 days and only 7% of cells were living on day 5. For the human MSCs and A549 cells co-culture ratio was 1:2, cells had been killed a little bit slower because of less human MSCs percentage in population. Cell viability had no changes on day 1 and decreased to 83% on day 2. On day 3 cell viability keep decreased to 60% and 29% on day 4. Again, on day 5only 9% cells survived.

From those results, we conclude that when human MSCs number is fixed, less A549 cells in the population cause lower cell viability for each day after addition of 5'DFUR. Compare the cell viability of co-cultures between using TP and TP-GFP plasmid, the cell viability is lower on each day for all three ratios when using TP-GFP plasmid.

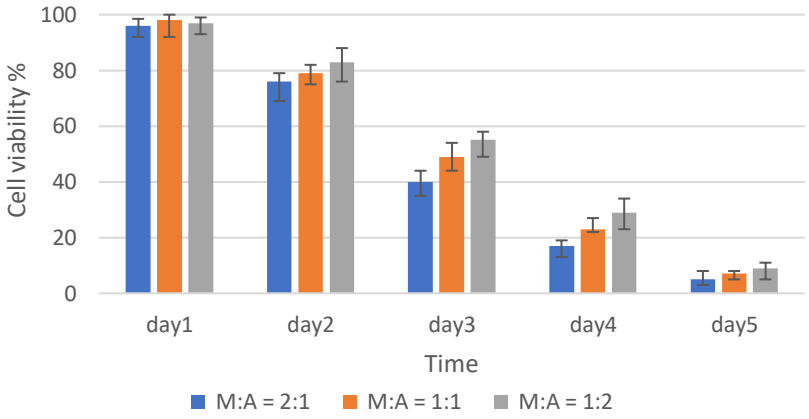


Figure 5.7 *Cell viability of co-cultured TP-GFP delivered human MSCs and A549 cells in 2:1, 1:1, and 2:1 ratio with 5'DFUR prodrug treatment in 5 days. The cell viability of three samples on day 1 is all around 97%. The cell viability of three samples on day 2 decrease to 76%, 79%, and 83%. The cell viability of three samples on day 3 decrease to 40%, 49%, and 55%. The cell viability of three samples on day 4 decrease to 17%, 23%, and 29%. The cell viability of three samples on day 5 finally decrease to 5%, 7%, and 9%.*

5.4 Summary

TP-GFP transfected human MSCs by viruses co-cultured with A549 cells had a high-level TP protein expression and enzyme activity which is similar to human MSCs without A549 cells. This indicates that co-culturing human MSCs with A549 cells will not affect TP expression and activity in cells. Comparing self-constructed TP and customer made TP-GFP plasmid, the viruses containing TP-GFP DNA showed a little bit higher transfection efficiency to human MSCs than the self-constructed one tested by western blot and enzyme activity assay. However, compare to deliver TP-GFP to human MSCs by PEI vector, TP expression were increased 30% to 50% by virus vector whether use TP or TP-GFP plasmid. Different ratios of human MSCs co-cultured with A549 cells cause different levels of prodrug conversion rate and cell viability. More human MSCs with TP expression in the populations cause higher enzyme activity, prodrug conversion rate and lower cell viability. On the contrary, more A549 cells in the populations cause lower enzyme activity and prodrug conversion rate and took longer to get the same cell viability. Overall, all samples with different ratios had high TP expression level to convert prodrug and killed 80 to 90% cells in 5 days.

Chapter 6 Conclusion and future work

More and more people are focusing on targeting therapy of cancer treatment. Gene directed enzyme prodrug therapy is an advanced cancer therapy which uses prodrug instead of toxic chemotherapy drug to reduce harmful side effects. In this study, A549 cells were delivered successfully with TP-GFP plasmid by PEI vector. The optimal condition of DNA delivery of this plasmid to A549 cells was N/P 20 with 4 μg DNA for 10^5 cells. TP-GFP expression was observed in A549 cells by fluorescence microscope and tested by the Western blot assay. A549 cells have no endogenous TP according to the Western blot result. TP activity and prodrug conversion rate in A549 cells were measured and quantified by enzyme activity assay. TP-GFP transfected A549 cells were eradicated in 4 days after prodrug 5'DFUR treatment.

TP-GFP plasmid was delivered into human MSCs by PEI vector first but the deliver efficiency was extremely low. Viruses included only TP plasmid was produced by HEK 293T cell first by self-constructed virus transfer gene. TP expression and activity were detected in human MSCs when use virus vector by the Western blot and enzyme activity assay. Viruses included TP-GFP plasmid were further constructed and delivered to human MSCs. Strong protein expression was observed by fluorescence microscope. TP and TP-GFP delivered human MSCs were co-cultured with A549 cells in 3 ratios and treated with prodrug 5'DFUR. Both human MSCs and A549 cells were gradully die in 5 days.

The most important step in GDEPT is targeting the cancer site but not allowing chemotherapy drug to go all over the body. In this study, MSCs were selected to be gene carriers for the enzyme converting prodrug to chemotherapy drug. A very significant reason we choose MSC as the gene carrier is MSCs has tumor tropism characteristic, meaning MSCs can migrate to cancer sites by some chemical released by cancer cells. After that, genes in MSCs are expressed and prodrug is converted to drug at this site. Prodrug at other site without this enzyme expression will not convert to chemotherapy drug and stay

non-toxic. At this stage, after delivered TP gene into MSCs, it co-cultured with A549 lung cancer cells but skipped “homing” step for research purpose.

- Homing function can be tested by putting TP delivered MSCs at one side of some 3D scaffold and cancer cells at the other site to see if MSCs can migrate to the cancer cells site. Another way to do it is load TP transfected MSCs in a transwell and culture cancer cells under the well of transwell. After adding some culture medium, we would merge the transwell, to see if MSCs can cross over to the other side of transwell. If the MSCs with TP delivered can succeed homing to cancer cells site, this approach is another step closer to clinical trials.
- Also, more conditions of TP delivery method could be tested for MSCs. The efficiency of genetic material delivery is also very important in GDEPT therapy and closely related to drug conversion rate. MSCs in this research mostly focused on viral vector delivery because of the high delivery efficiency. Although no toxicity had been observed when using viral vector to deliver DNA into MSCs, viral vectors always have a safety concern especially *in vivo*. So, more gene delivery methods or vectors could be tested to optimize both efficiency and safety. TP transfected human MSCs can be injected into mice with has cancer. And then prodrug can be administered to see if cancer cells can be killed and observe if side effects can be decreased comparing to mice treated with chemotherapy drug directly. In this research, TP expression in A549 cells and human MSCs was confirmed has activity and ability to convert prodrug 5’DFUR to chemotherapy drug 5-FU. The result shows cancer cells and gene carrier human MSCs both can be killed by this converted drug. This is a promising result of gene directed enzyme prodrug therapy for cancer treatment.

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Appendix A

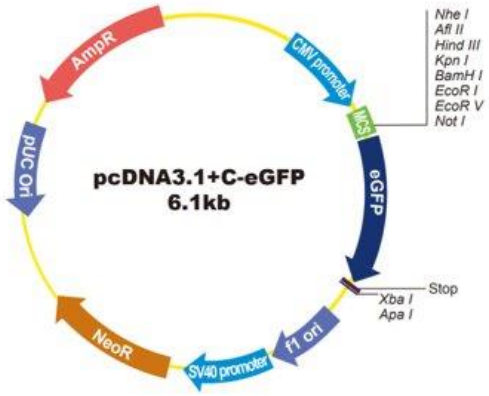

Figure A. 1 *Map of a schema of pcDNA 3.1+C-eGFP vector (from Gen script, USA) which contains a TP sequence.*

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GACTTCCCGACCCTTCGCCAGAGCCCAAGCAGCTCCCGGAGCTGATCCGCATGAAGCGAGACGGAGGCCGCCT
GAGCGAAGCGGACATCAGGGGCTTCGTGGCCGCTGTGGTGAATGGGAGCGCGCAGGGCGCACAGATCGGGGC
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Figure A.2 *Sequence of TP DNA cloned out from pcDNA 3.1+C-eGFP vector and inserted into plasmid SFFV-GFP (GenScript).*

TTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATC
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CGCCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCA
GGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCG
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Figure A.3 *Sequence of GFP DNA in pcDNA 3.1+C-eGFP vector.*

Appendix B

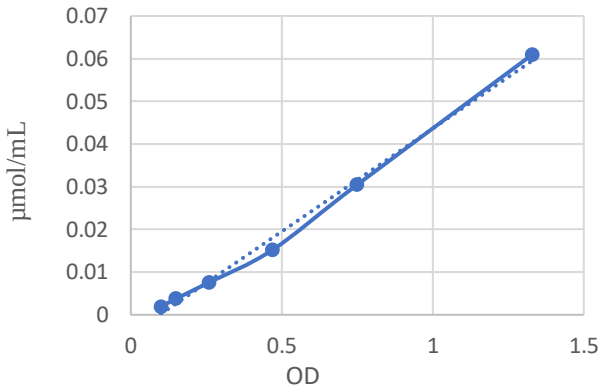

Figure B.1 *Calibration curve of OD reading at 300 nm vs. 5-FU concentration for enzyme activity calculation.*

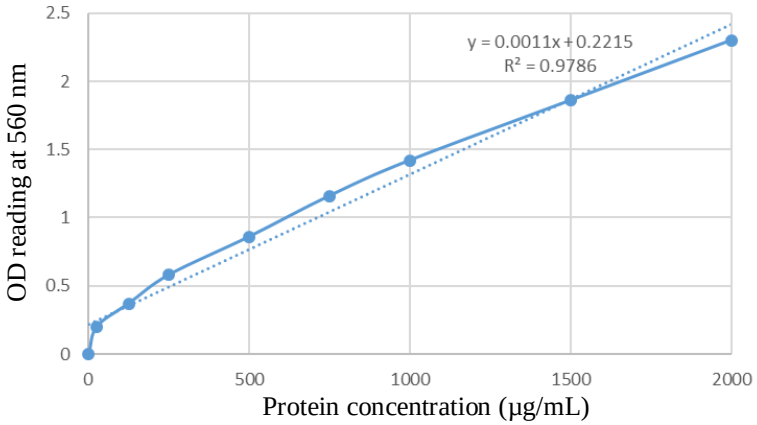

Figure B.2 *BSA protein assay calibration curve of OD reading vs. protein concentration (µg/mL).*

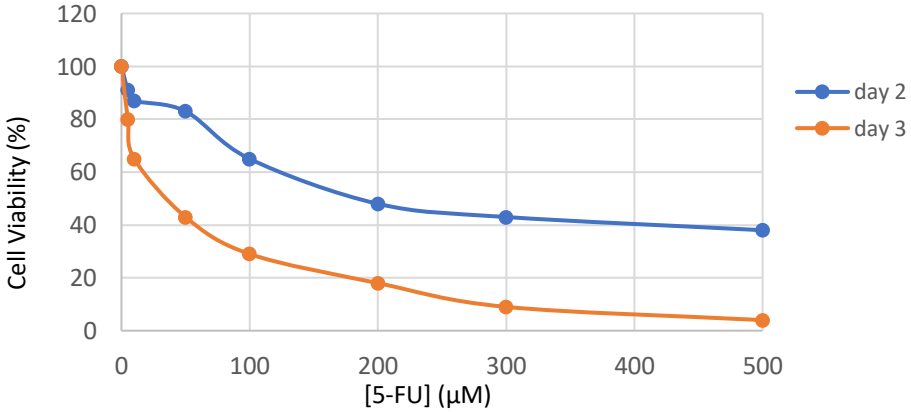

Figure B.3 *the concentration of 5-FU versus to A549 cells viability shows the LD50 on day 2 is around 200 µM.*

Appendix C

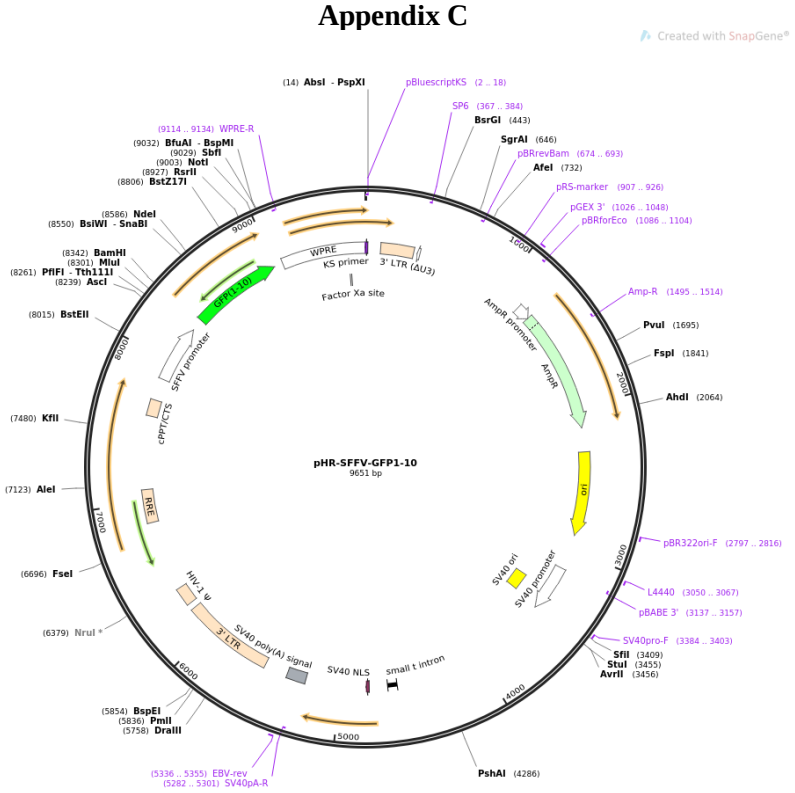

Figure C.1 *The map of plasmid HR-SFFV-GFP purchased from Addgene.*

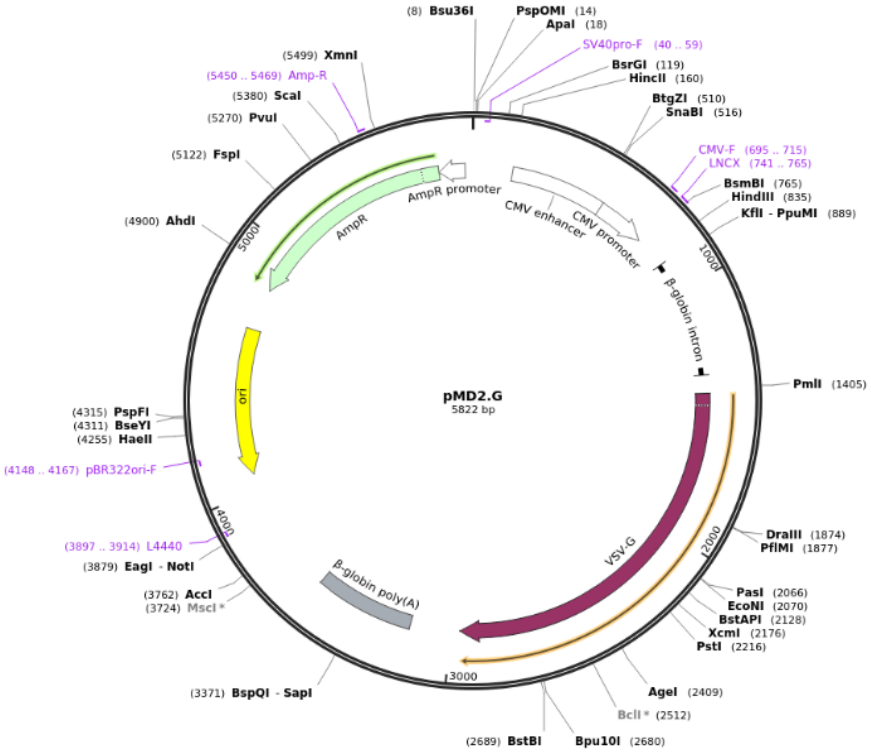

Figure C.2 *The map of plasmid MD2.G purchased from Addgene.*

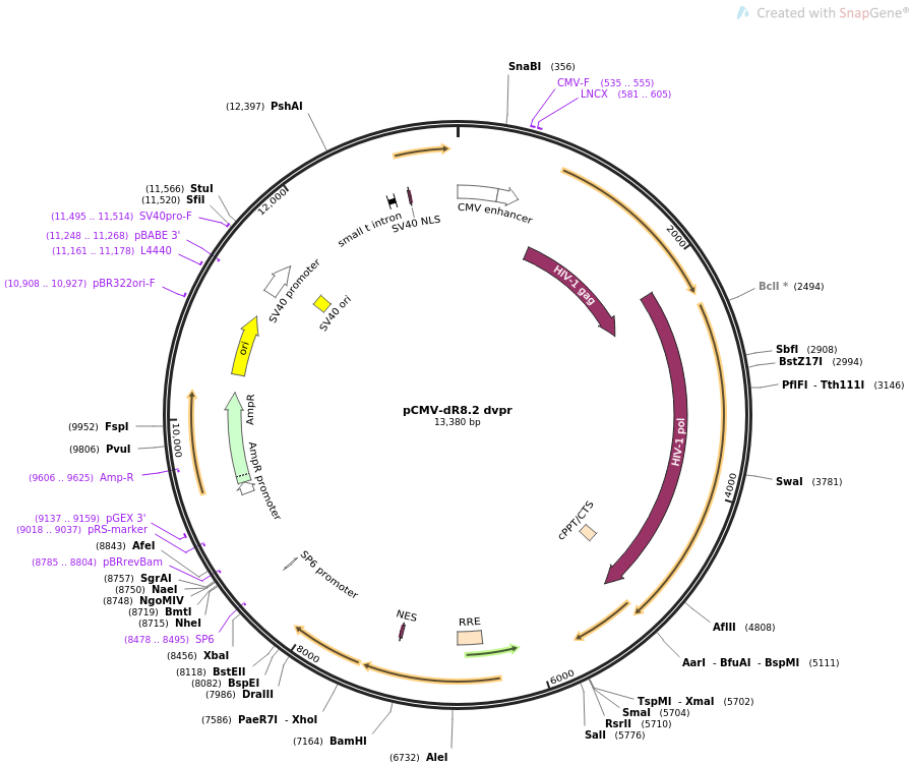

Figure C.3 *The map of plasmid CMV-dR8.2 dvpr purchased from Addgene.*