SIMULTANEOUS KNOCKDOWN OF TELOMERASE AND SURVIVIN GENES IN HELA CELLS

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SIMULTANEOUS KNOCKDOWN OF TELOMERASE AND SURVIVIN GENES IN HELA CELLS

By

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ABSTRACT

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CHANG LOONG HUAT

Cells are tightly regulated by the cellular homeostasis to maintain the balance between cell division and cell attrition. Any occurrence of DNA damage in the cell will lead to the programmed cell death. However, normal cell that has undergone neoplastic transformation will be able to evade from apoptosis and proliferate continuously. Cancer is one of the most prominent causes of death worldwide. Annual new cases of cervical cancer are widely reported in developing countries and it is one of the common cancers among women. Several molecular markers are upregulated in cancer. Of these, survivin and human telomerase reverse transcriptase (hTERT) genes, can be used to distinguish the malignancy of the cancer cells. Survivin is an apoptosis inhibitor gene. hTERT gene is crucial in the role of maintaining the length of the telomere for effective and continuous cell division. Therefore, the expression of survivin and hTERT genes contributed to the cancer cell immortality by evading from the apoptosis and leading to continuous cell proliferation. These two genes can be targeted by using the recent discovery in molecular biology, gene silencing. The main objective in this project is to induce apoptosis of HeLa cells by silencing both

survivin and hTERT genes simultaneously. The expression vector containing the short hairpin RNAs (shRNAs) that specifically target the survivin and hTERT genes were transfected to HeLa cells. The knockdown efficacies were analysed by western blotting, Reverse Transcription Real Time Polymerase Chain Reaction (RT-qPCR), and the mitochondria-staining apoptosis assays to determine the percentage of apoptosis induction. The mRNA and protein expression of survivin and hTERT genes were found down-regulated which correlate to the increased of apoptosis induced in HeLa cells. It was discovered that the single gene silencing has induced 60% and 70% apoptosis of HeLa cells, for hTERT and survivin genes, respectively. On the other hand, the knockdown of both genes simultaneously has induced 85% of apoptosis in HeLa cells. In conclusion, cancer cell can be reintroduced to apoptosis by silencing both genes simultaneously and the effect is more prominent than silencing one gene alone.

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APPROVAL SHEET

This dissertation entitled **"SIMULTANEOUS KNOCKDOWN OF TELOMERASE AND SURVIVIN GENES IN HELA CELLS"** was prepared by CHANG LOONG HUAT and submitted as partial fulfilment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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I understand that the University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

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(CHANG LOONG HUAT)

DECLARATION

I **CHANG LOONG HUAT** hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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Date: _____

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LIST OF ABBREVIATIONS

BIR	Baculoviral IAP Repeat
cDNA	Complementary DNA
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
HPV	Human Papillomavirus
hTERT	Human Telomerase Reverse Transcriptase
hTR	Human Telomerase RNA Component
IAP	Inhibitor of Apoptosis
miRNA	Micro RNA
mRNA	Messenger RNA
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate

SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
snRNA	Small nuclear RNA
TEMED	Tetramethylethylenediamine
v/v	Volume/Volume
w/v	Weight/Volume

CHAPTER 1

INTRODUCTION

Cancer has always been one of the top leading causes of death worldwide. Cancer cell is the same cell that exists in our body but has been transformed genetically. This transformation, known as neoplastic transformation, has led the cell to gain the ability to grow uncontrollably and evaded from apoptosis. Hence, one neoplastic transformed cell will be able to undergo unlimited number of cell division to form new layer of abnormal cells instead of dying. This cluster of abnormal cells will form tumour. Aggressive division of cancer cells is known as malignant neoplasm while the slower dividing cancer cell is known as benign neoplasm. Malignant cancer cell possess higher tendency to move to other parts of the body apart from their originated organ. This movement of cancer cells through the blood or lymphatic vessels was named metastasis (Sudhakar, 2009; Mukherjee, 2011; Chang, 2018).

There are many classification of cancer, each have their own name based on the organ they are originated from and the type of cells that give rise to the cancer. The epithelial cancers were known as carcinomas, the mesenchymal cancers were known as sarcomas, blastomas were the cancers derived from embryonic tissues whereas leukemias were the ‘liquid’ cancers derived from the blood cells (Watson, 2013). Since most of the body parts can be afflicted by

cancer, there is a high tendency for human to develop cancer in their lifetime provided they are constantly exposed to the cancer-causing agents or by inheritance. Based on a statistical study of cancer in the United States of America, there will be higher new cancer cases for female in year 2018. The most common cancers among the females as reported are lung cancer, colon cancer, breast cancer, cancer of the genital system and skin cancer to name a few (Siegel et al., 2018).

By the name cancer of the genital system, it can be the cervical cancer, corpus cancer and ovarian cancer. Though the mortality rate for cervical cancer has been reduced to date, it is still a major health problem in younger women especially in developing countries (Siegel et al., 2018). Thus, it is practical for the researcher to develop a potential cure for the cervical cancer in order to further decrease the rate of mortality in younger women. Since the cervical cancer cells are common among cell culture researchers, it provides a good platform for research targeting cancer in term of the growing pattern of the cells and their weaknesses before moving on to other types of cancerous cells. The cervical cancer cell line commonly cultured in the laboratory is the HeLa cell line.

After the commencement of the ‘War on Cancer’ by President Nixon in year 1971, an abundance of scientific researchers have put on their full gears to work on the best possible cure to fight against this disease. There were many

theories on the combat against cancer but all of them converge to the 3 main methods, namely surgical resection, radiotherapy and chemotherapy. Not too long after that, a new way of cancer therapy emerges from the undying researches to cure cancer. This new method targets the basic molecule of the cell which is the ribonucleic acid (RNA) by breaking down the molecule post-transcription (Liu & Wang, 2011; Mukherjee, 2011; Watson, 2013).

RNA interference (RNAi) is the newly discovered technique to cure cancer by targeting the molecular pathway of the cancer cells. RNAi is a natural mechanism that exists in our very own body to fight against foreign particles such as viral and bacterial infection. Moreover, this mechanism is crucial as the human genome protector by degrading the repetitive sequences and wrongly transposed gene that occurred during transcriptional error. Due to its sequence-specific nature, researchers can guide this defence mechanism in the body of the cancer patient to destroy the cancer cells (Elbashir et al., 2002; Zaffaroni et al., 2007).

Cancer cells can be destroyed by a cell killing mechanism named apoptosis. Apoptosis is a programmed cell death mechanism that is tightly regulated to maintain a normal tissue homeostasis. Cell with damaged DNA will be subjected to the cell killing in case the damage is non-repairable to prevent function disruption to the body. Cancer, which is caused by the mutation of DNA, is considered as damaged cell, thus, required to be removed through apoptosis

(Lynch, 2009; Mishra et al., 2011).

One of the newly discovered molecular markers of the cancer is a protein named survivin. Classed in the inhibitor of apoptosis protein (IAP) family, survivin's main function is to inhibit apoptosis. The expression of this protein is insignificant in normal, terminally differentiated cells. However, in cancer cells, the expression of this protein increased. Thus, it is speculated that cancer cell has reactivated this protein from its dormant phase in order to provide the cell with the unlimited proliferation ability. Besides that, its function to restrict the body from performing the natural defence mechanism, the apoptosis process, from killing the cancer cells has contributed much to the immortality of cancer cells (Johnson & Howerth, 2004).

Another highly remarkable molecular marker of cancer is telomerase. Telomerase is a ribonucleoprotein that functions to maintain the telomere length of the chromosome by adding in the TTAGGG repeats. Telomere length is important for the cell to divide. Hence, the major role of telomerase is to maintain the stability of the chromosome by regulating the cellular replicative lifespan. Telomerase is notably expressed in proliferative embryonic stem cells but the telomere length maintaining ability is not to the fullest. However, in cancer cell, the telomere length is always stably maintained which led to the continuous proliferation of the cancer cell. This shows that cancer has the ability to reactivate

the normally silent telomerase activity compared to that of vast amount of human somatic cells (Stewart & Han, 2002; Mertzger et al., 2009; Shay & Wright, 2011).

The discovery of these two molecular markers of the cancer cells has open up a new road to develop a cure for cancer. By utilising the RNAi method in silencing these two genes, cancer cells will lose the ability to indefinitely proliferate. Ultimately, the cancer cell will be reintroduced to apoptosis in which they had been evaded from due to the activation of survivin and telomerase.

The main objectives in this project are listed as followed:

i) To study the knockdown effect of survivin gene, telomerase gene and both genes simultaneously in terms of mRNA level, protein level and proliferation of HeLa cells.

ii) To induce apoptosis of HeLa cells

iii) To determine the knockdown efficacies of single gene and double genes on the reduction of mRNA expression, the reduction of protein expression, the number of cell death induced and the apoptosis of HeLa cells

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

2.1.1 The Definition of Cancer

Malignant neoplasm, the medical term for cancer, is the condition whereby the cell has undergone neoplastic transformation. Therefore, the cancer cell can divide and proliferate continuously due to the evasion from the normal cellular homeostasis. A tight regulation of apoptosis and cell division is required in order to regulate the cellular homeostasis which is essential for the survival of the organisms. However, in the cases whereby the regulation mechanisms of cellular homeostasis have been disrupted, the occurrence of cancerous phenotype will usually follows (Hansen et al., 2008). The rate of proliferation of the neoplastic cells affects how the transformation will be named. The cells that are fast growing and uncontrollably dividing with limitless proliferation ability are named malignant neoplastic cells. On the other hand, normal cells that have been changed in nature and can divide uncontrollably might form the benign tumour. This contradicts with the definition of transformed cell as this type of tumour will not spread to other parts of the body and they did not proliferate continuously. They are typically just a lump of abnormal cells and are not cancerous as compared to malignant tumour. Thus, cancer can be in the form of tumour but a

tumour is not necessarily being cancerous. Tumour is a mass of cells that have abnormal growth (Hansen et al., 2008; Sudhakar, 2009; Chang, 2018).

2.1.2 History of Cancer

There are a few versions on how the word cancer originated from. Some sources stated that Hippocrates name it from the word crab in Greek which is *karkinos* due to the abnormal shape of the tumour with swollen blood vessels surrounding it and its nature in "grabbing on to the human and won't let go". Hippocrates is not the first person to discover cancer as a disease, he just named the disease and have had his Humoral Theory on cancer that was caused by the black bile resided in human body. However, the first discoverer of cancer is not known though by using the recorded history findings, cancer has been around for centuries. There were records written by Imhotep about the incurability of cancer. Furthermore, the first confirmed ancient case of cancer was diagnosed by a paleopathologist Arthur Aufderheide in year 1990 in a Peruvian mummy who has had osteosarcoma in her upper arm. Thus, cancer has appeared since long ago and persists to date (Sudhakar, 2009; Mukherjee, 2011).

2.1.3 War on Cancer

In December 1971, the president of the United States of America, Richard Nixon has declared the war on cancer. This was due to the ever increasing cases of cancer, and it has once been the top cause of death in the America. The mass-

media and an abundance of non-government organisations had been established to pressure the government to focus on the research in curing cancer. Hence, the National Cancer Act of 1971 was enacted to encourage more members of the scientific community to develop more effective cancer treatments for instance the targeted drug therapies. The government had even offered scholarship and large amounts of money to the scientist that has succeeded in developing a working drug. Most of the hospitals and cancer laboratories were developed in this time-frame.

2.1.4 Statistics of Cancer

Cancer has once been the first cause of death worldwide in the 19th-century due to the lack of medical knowledge on the cause and the treatment for this disease. However, moving on to the years after all the attention of the scientist have been focusing on cancer, the mortality of cancer is reducing gradually, decades by decades (Mukherjee, 2011). In the 2000s, cancer came as the second major cause of death worldwide which is behind cardiovascular diseases whereby the chances of developing cancer are 50% for American men and 33% for American women (Sudhakar, 2009). As more and more researches have been done on developing the more targeted therapies for cancer, the rate of death caused by cancer had been moderately decreasing though the disease remain in the top ten list as the major cause of death. This indicates the severity of cancer as a disease in consuming the lives of human being.

According to Siegel et al. (2018), there will be 1,735,350 new cases of cancer estimated to occur in the United States while the number of death caused by cancer was approximated to be 609,640 for the year 2018. The statistic research completed by the team has discovered that there was no decline in the incidence rate of cancer in American women while there was an annual 2% declination in American men from year 2005 to 2014. From the 1,735,350 estimated new cancer cases, 878,980 will occur in American women and 856,370 cases will occur in American men (Siegel et al., 2018). The higher number indicates the higher tendency of women to develop cancer in America. The cancer therapies development in the United States is considered one of the top notches, thus, the incidence rate of cancer and cancer death will definitely be higher in developing countries.

In Malaysia, the incidence rate of cancer among Malaysian males and females was 128.6 and 135.7, respectively, per 100,000 populations in the year 2006. The trend in the incidence rate is the same as compared to the Americans, whereby, the females are more prone to develop cancers. The most common cancers among Malaysian females were the cancers of the breast, lung, colorectal, cervix and nasopharynx (Omar et al. 2006). Progressing to year 2016, cancer remained in the top five position as the principle cause of death in Malaysia. Though a decade has past, more potential cancer cured has been discovered, the death rate among Malaysia females who has malignant neoplasm of the breast has increased by 0.4 percentage points summing up to 3.8% in year 2016 as compared

to that in year 2015 (Ho, 2017). This indicates the urgency in developing potential cancer cures for females in the whole world, especially in developing countries.

2.1.5 Classification of Cancer

The word cancer is a general term that symbolises all types of malignant neoplasm. A specific name is required to mention each type of cancer as there are many types of cancer available and it will be of ease for the clinician to further understand the nature of the cancer. Different types of cancer that derived from various types of cells or tissues will be named differently. There is carcinoma, sarcoma, blastoma, adenoma and glioma to name a few types of the cancer. The naming of the cancer depends on the classification of cancer and the name of the organ will be included. Carcinoma is the tumour derived from the epithelial cell. For example, uterine cervix carcinoma is the cervical cancer. Sarcoma is the tumour derived from connective tissues or those from non-epithelial tissues. For example, osteosarcoma is the bone cancer. Tumour derived from the glandular structures in the epithelial tissues will be named adenoma and this is usually a benign tumour. For instance, pituitary adenoma is a benign tumour grown on the pituitary gland. A glioma is the brain tumour which is derived from glial cells. For example, oligodendroglioma is the tumour on the oligodendrocytes of the brain. Whereas for blastoma, it is the neoplasm of the immature undifferentiated cells such as retinoblastoma, which is derived from the immature retina cells that is mostly found in young children. A targeted therapy can be developed based on the types of cancer as each type of cells would have different sets of marker. Though

there are many emerged cancer drug to target different types of cancer specifically, most of the carcinomas remain highly difficult to be cured. Carcinoma is one of the top common cancers that appear most often in human (Hamilton and Aaltonen, 2000; Idikio, 2011; Mukherjee, 2011; Watson, 2013).

2.1.6 Uterine Cervix Carcinoma

The cancer of the cervix or the medical term, uterine cervix carcinoma is the primary tumour developed on the uterine cervix lining at the lower end of the uterus which contacts with the upper vagina. Though the death rate has been declining up to date, this neoplasm has been noted to affect approximately 13,000 American women annually which led to the 4,100 number of death (Stoppler, 2017). Based on the statistical research conducted by Siegel et al. (2018), approximately 13,240 American women will develop the cancers of the cervix in year 2018. As reported by Omar (2006) and Ho (2017), uterine cervix carcinoma is one of the top 10 leading causes of cancer death in Malaysian women. Therefore, the cancers of the female need to be treated in top priority in order to reduce the mortality rate of all females worldwide. The most commonly used cervical cancer cell line in most of the cell and tissue culture laboratories is the HeLa cell line.

2.1.6.1 HeLa Cells

HeLa cells are cervical adenocarcinoma cells that is the first immortalised cell line being discovered and cultured in the laboratory. The cell line was originated from a 30 years old African American woman, Henrietta Lacks, and hence, the cell was name by the initials of the woman's name. Henrietta Lacks was diagnosed with a terminal stage of cervical cancer by the doctors at Johns Hopkins Hospital in Baltimore. Without the consent of Henrietta Lacks or her family, the doctors had extracted some of the cells from her diseased organ. The extracted cells were then cultured in the laboratory and discovered to be highly immortalised (Skloot, 2010).

This cell line has contributed abundantly to the scientific community in order to study the nature of the human cells. Other useful contribution obtained by researches conducted by using HeLa cells include the creation of polio vaccine, vaccines of influenzas and herpes, gene mapping and cloning, cell adaptability in outer space and many other useful discoveries which led to the blossoming of journal publications. Moreover, this cell line has been used by many researchers to test on the effect of drugs, hormones, steroid and almost anything infectious to make more discoveries. Thus, this is a common immortalised cell line for scientific research and the best option as the testing platform for the discoveries of cancer cure (Davis et al., 2010; Denise Watson, 2010; Claibome and Wright, 2010; Skloot, 2010).

2.1.6.2 Causes of Cervical Cancer

The current determined causes of cervical cancer are still general as the more detailed knowledge on cancer is a long pathway for the researchers and medical practitioners to go through. The most common cause of cervical cancer is the infection by Human Papillomavirus (HPV) on the epithelial of cervix by certain species of the virus. A family background of cervical cancer might be inherited to the offspring as well while the daily habit of the unhealthy lifestyle plays a major role in contributing to the progression of this disease. Other causes include the mutations in the chromosomes such as the tumour suppressor gene, oncogene and other molecular marker that have been activated as a part of the transformation of the normal cell into a cancerous cell (Sellors, 2003; Crosbie et al., 2013; Stoppler, 2017).

2.1.6.3 Molecular Changes of Cervical Cancer

The major cause of most of the cervical cancer is the Human Papillomavirus (HPV) infection. According to Gius et al. (2007), 99.7% of the invasive cervical cancer patients were discovered to exhibit the presence of HPV DNA in their cell samples but not all women that had contracted HPV infection would develop invasive cervical cancer. It was speculated that certain genetic factors play pivotal roles in addition to the HPV infection to contribute to the progression of invasive cervical cancer. Cervical lesions were initiated after the infection of HPV which leads to dysplasia and gradually progress towards invasive cervical carcinoma. The lesion was known as cervical intraepithelial

neoplasia (CIN) and was classed in different stages of neoplasias whereby each stage displayed different expression of genes (Gius et al, 2007).

The progress of cervical lesion begins with the HPV infection on normal cervical epithelial cells. This is the first stage of preinvasive lesion known as CIN1 where certain genes had changed in expression pattern causing increased cellular proliferation and suppressed body immune system which led to mild dysplasia on the basal layer of dividing cervical epithelial cells. In CIN2, the changes in certain genetic factors had provided the environment for the ease of development of new blood vessels and moderate dysplasia was noticed on the cervical epithelial cells. Progressing towards CIN3, pro-invasive genes were activated causing severe dysplasia and further develop into carcinoma in situ. Invasive carcinoma was developed beyond CIN3 whereby all the major genes that were important for the maintenance of cancer cell had been fully up-regulated (Gius et al. 2007). Figure 2.1 shows the schematic representation of the genetic changes in each stage of CIN.

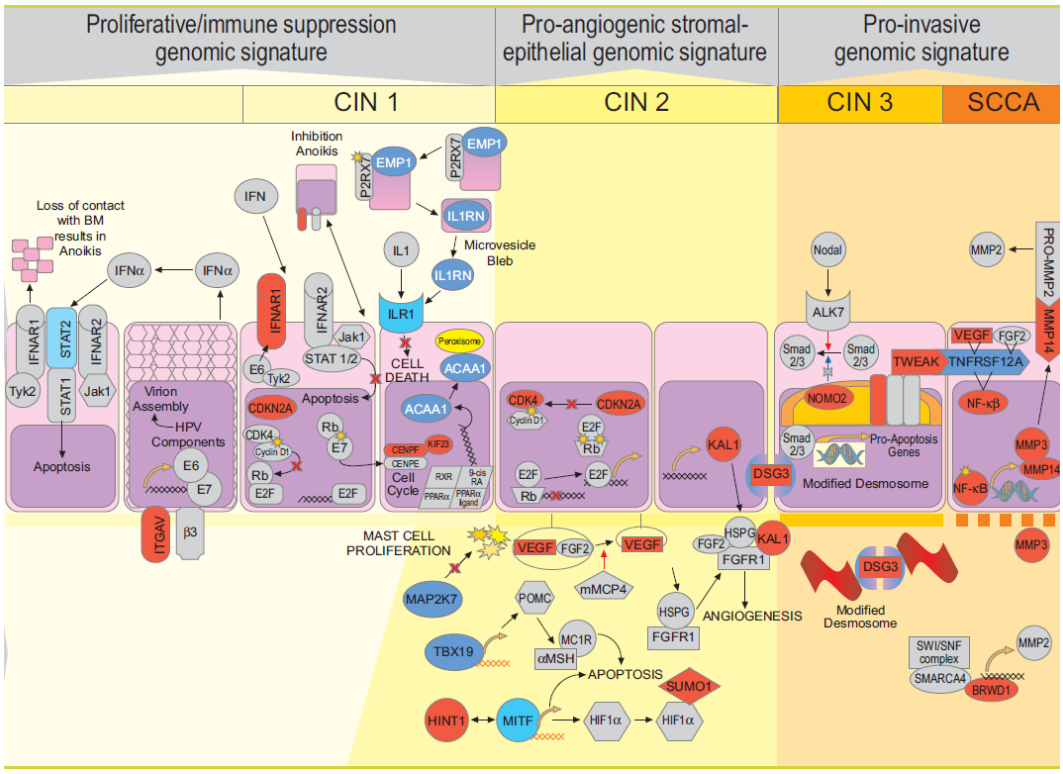

Figure 2.1: Cervical cancer carcinogenesis in schematic model (Gius et al, 2007). Genes in dark red represent increment in expression; genes in dark blue represent decrement in expression; genes in grey represent host and viral genes.

2.1.6.4 Stages of Cervical Cancer

There are five distinct stages in cervical cancer. The initial stage of this cancer is Stage 0 whereby the abnormal cells are growing on the surface of the uterine cervix lining. Stage I has small amount of minor tumour growing on the surface of the cervix which has not invade into the lymphatic nodes. In Stage II, the tumours have begun to spread to distant sites beyond the cervix and the uterus but not the lower part of the vagina or pelvic walls. The spreading of tumours to the pelvic walls and lower part of the vagina starts in Stage III and the ureters

might be blocked by the growing tumours. In the terminal stage, Stage IV, spreading of tumours have been ways beyond the uterus such as the bladder, rectum or other parts of the body and become mostly incurable (Stoppler, 2017).

2.1.7 Hallmarks of Cancer

In human body, all the cells are tightly regulated by a process called homeostasis. This includes the removal of disrupted cells or abnormal cells. Thus, in order for the normal cells to be able to successfully undergo neoplastic transformation, there are multiple steps and checkpoints the cells have to pass through (Hanahan and Weinberg, 2000). There are six discovered capabilities that the normal cells had to acquire in order to gain autonomous to transform into a neoplastic cell. These six capabilities are known as the hallmarks of cancer whereby all cancer cells had shown similarity in characteristics described by these hallmarks of cancer (Hanahan and Weinberg, 2000; Beishline and Azizkhan-Clifford, 2015).

The first of the six hallmarks of cancer is the self-sufficiency in growth signals whereby the cells had acquired the ability to grow without depending on the exogenous growth stimulation, thus, tumours can generate the growth signals on their own and enter into active cell cycle. The second capability acquired by the cells is the insensitivity to antigrowth signals. Once a normal cell has been terminally differentiated, the cell will be blocked from further growing or

proliferating by the inhibitor of growth signals by the homeostasis process. However, pre-cancerous cells are able to evade from these signals and continue to participate in active cell cycle. The maintained cellular homeostasis has always neutralised the number of cell division and cell killing. Thus, by gaining the ability to resist from cell killing, the cancer cells are able to expand in population which tallied to the third hallmark of cancer. Resisting to cell death or evading apoptosis is one important hallmark of the cancer as the cells that acquired this ability can prosper continuously which contributed much to the malignancy of cancer (Hanahan and Weinberg, 2000; Beishline and Azizkhan-Clifford, 2015).

Apart from the three hallmarks of cancer mentioned earlier, the transformed cells that gained the three capabilities had not abided to the signals released from their environment. However, the evasion from these signals does not mean that the cancer cell can continue to grow in number. There is one more mechanism that is hardwired in every cell to maintain the replicative potential of the cell. Hayflick and Moorhead (1961) had mentioned that there is a finite replicative potential in every cell and once the cells had achieved the certain amount of replication, the cells will enter into a senescence stage which is the Hayflick limit. The fourth hallmark of cancer is the unlimited replicative potential whereby this capability will enable the cancer cell to overcome the finite replication potential and proliferate uncontrollably. The fifth hallmark of cancer is the ability to sustain angiogenesis. Cell requires oxygen and nutrients to survive and maintain its functions. However, this requirement is tightly regulated by the

body as well in order to maintain homeostasis. Therefore, a neoplasm will require more blood vessels to be formed to cover up the ever-increasing need of oxygen due to the cell division and expansion. Thus, the neoplastic cell has to develop their customised source of nutrient by acquiring the angiogenic ability. Last but not least, the sixth hallmark of cancer is the capability to invade and metastasise. This is one of the major hallmarks of cancer and its appearance in terminal stage of cancer is compulsory. The cell that has acquired this ability will be able to colonise new sites of the body and starts to grow and proliferate in the new environment apart from its primary tumour form. The prognosis will be poor once the cancer cell has acquired this hallmark of cancer. Though these six hallmarks of cancer are crucial for the progressive grow of neoplasias, there might be other hidden hallmarks that are yet to be discovered by scientist due to the cunning nature of cancer cells and hence, the high difficulties in curing cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Beishline and Azizkhan-Clifford, 2015).

2.1.8 Malignancy of Cancer

The malignancy of the cancer cell is the degree of severity of the cell to human being. Malignancy of a neoplasia can be in term of its proliferation ability and speed, the invasiveness of the tumour and the final achievement of the cancerous cells, metastasis. Progressing to the stages of cancer, the malignancy increases. As mentioned earlier, there are six hallmarks of cancer. The malignant cancer cell might have achievement certain capabilities in random arrangement. It

can have only four of the hallmarks, all six hallmarks or even more than six hallmarks of cancer whereby some capabilities are repeated. Repeated capability indicates that the neoplasm might have two or more different types of mutated genes that caused the acquirement of the same hallmark of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Malignancy affects the prognosis of a cancer undoubtedly.

2.1.9 Treatment of Cancer

From the initiation of the National Cancer Act of 1971 by President Nixon onwards, the development in the discovery of cancer treatment can be summarised in three categories. Cancer will be treated by one of the following ways, the surgical resection, chemotherapy or radiotherapy. In surgical resection, it has progressed from the radical resection, which removed the cancer tumour together with all the lymph nodes connected, to using laser in the removal of tumour tissue of certain organs. Whereas in chemotherapy, the development of a single cancer drug to treat cancer was found to be not effective leading to the introduction of different combination of chemicals that can kill the cells. In earlier years of using radiotherapy to cure cancer, the researchers or doctors that perform the radiation were found to contract with cancer themselves. It was further studies that indicated the radiation itself is the cancer-causing agent besides killing the cells. Hence, the improvement in the radiotherapy areas includes the usage of several other radiation beams which have different wavelength that is safe to human, the use of safety gears while performing radiotherapy and different ways

in radiating the cancerous organ. Though all three methods worked to most of the patients, the cancer remissions are still highly unavoidable. After that, came the discoveries of hormonal therapy and immunotherapy whereby it is the admission of hormonal drugs and vaccination, respectively. Adjuvant therapy was also developed after the failure of a single treatment, whereby it is the use of chemotherapy in addition to the surgery or radiotherapy in order to kill all the remaining cancer cells in the body (Sudhakar, 2009; Liu and Wang, 2011; Mukherjee, 2011, Watson, 2013).

It was determined that the failure in those cancer treatments was not due to the inefficacy of the methods. It was the sneakiness of the cancer cell itself that made cancer hardly able to be cured besides the lack of information on the origin of cancer and the main cause of the disease genetically. For the surgery, the removal of the tumours did not necessarily remove all the cancers in the body. For chemotherapy, the drugs administered did kill all the cancer cells but together with other normal cells. For radiation, the cancers were successfully killed but the adjacent tissues were either burned to death or induced to transform into cancerous tumour (Sudhakar, 2009; Mukherjee, 2011).

After the Human Genome Project in year 1988 to year 2003, scientists around the world can get their hands of the database of the deoxyribonucleic acid (DNA) in the human body. Therefore, it will be easier for scientists to determine

the source of the mutation that give rise to cancer or specifically the precise problematic genes and even the location of the genes in the chromosome. Furthermore, the discovery of the RNA interference is a big progress towards the journey in treating cancer as the cancer can be targeted molecularly (Sudhakar, 2009; Mukherjee, 2011; Watson, 2013).

2.2 RNA Interference (RNAi)

RNA interference (RNAi) is a biological phenomenon that is first discovered in *Caenorhabditis elegans* (*C. elegans*). This phenomenon is useful in functional genomic studies whereby it enables a protein function to be discovered by degrading the target messenger ribonucleic acid (mRNA) using a double stranded ribonucleic acid (dsRNA) introduced in the cell of the organism (Hannon, 2002; Hannon, 2003).

2.2.1 History of RNAi

C. elegans, a hermaphroditic roundworm, was used as the genetic model organism due to its rapid generation time which is approximately three days, its self-fertilising ability, uncomplicated anatomy and the convenience in culturing the organisms in the laboratories (Brenner, 1974; Barr, 2003). After Fire et al. (1991) has successfully conducted the RNAi experiment in disrupting the gene expressing myofilament proteins in the muscle of *C. elegans*, he further worked with Craig Mello in more researches regarding the RNAi and later on discovered

the potential of dsRNA in initiating the RNAi as compared to that of single-stranded ribonucleic acid (RNA) strand. Their publication on this discovery in year 1998 had made them the Nobel laureate of Physiology or Medicine in year 2006 (Fire et al, 1998; Wianny et al., 2004; Mansoori et al., 2014). This phenomenon has led to the blossoming of researches in the study of loss of function of genes, bio-molecular properties of a disease and recently, a breakthrough in the study to develop the cancer cure (Paddison et al., 2002; Mansoori et al., 2014).

2.2.2 Activity of RNAi in Mammalian Cell

The RNAi phenomenon consists of some conserved genes that carried out the function as the intermediates in the pathway of RNAi. The Dicer, which is a member of the RNase III family and *Argonaute* gene are one those conserved genes that were found in the mammalian cells indicate that this phenomenon are actively involved in the cells' defense mechanism. In cell, the infection by external viral DNA or bacteriophage that give rise to dsRNA will initiate the function of RNAi to get rid of the foreign DNA that might harm the function of the cell. Other than foreign infection, the activity of transposon in the chromosomes was regulated by this mechanism as well in order to get rid of mutation due to jumping genes (Nicholson and Nicholson, 2002; Wianny et al., 2004).

2.2.3 The Pathway of RNAi

The presence of dsRNA in the nucleus of the cell will trigger the RNAi mechanism. The activation of RNAi is initiated by the cleavage of the long dsRNA into a working short interference RNAs (siRNAs) by the Dicer, a nuclease from the RNase III family. Some additional cofactors are required in stabilising the siRNAs and to recruit the dsRNA into the Dicer. These freshly produced siRNAs, which are approximately 22 to 25 nucleotides, will be incorporated into the RNA-induced silencing complex (RISC) to be unwinded into sense strand and antisense strand of the siRNAs. The antisense siRNA will be the guiding strand in the RISC to bind to the target cognate mRNA. After the binding of the antisense siRNA to the mRNA, endonuclease properties in the RISC will cleave the mRNA and hence, degrading the target mRNA leading to the cease of target protein translation (Bernstein et al., 2001; Wianny et al., 2004; Mansoori et al., 2014). The cleavage of the mRNA took place at the center region of the complementary binding between the siRNA and the target mRNA (Elbashir et al., 2001). Wianny et al. (2004) speculated that the gene expression silencing by RNAi acted coherently by promoting the degradation of RNA, inhibition of protein translation and blocking the activity of chromatin remodelers. A diagram showing the pathways involved in the silencing of the gene is shown in Figure 2.2.

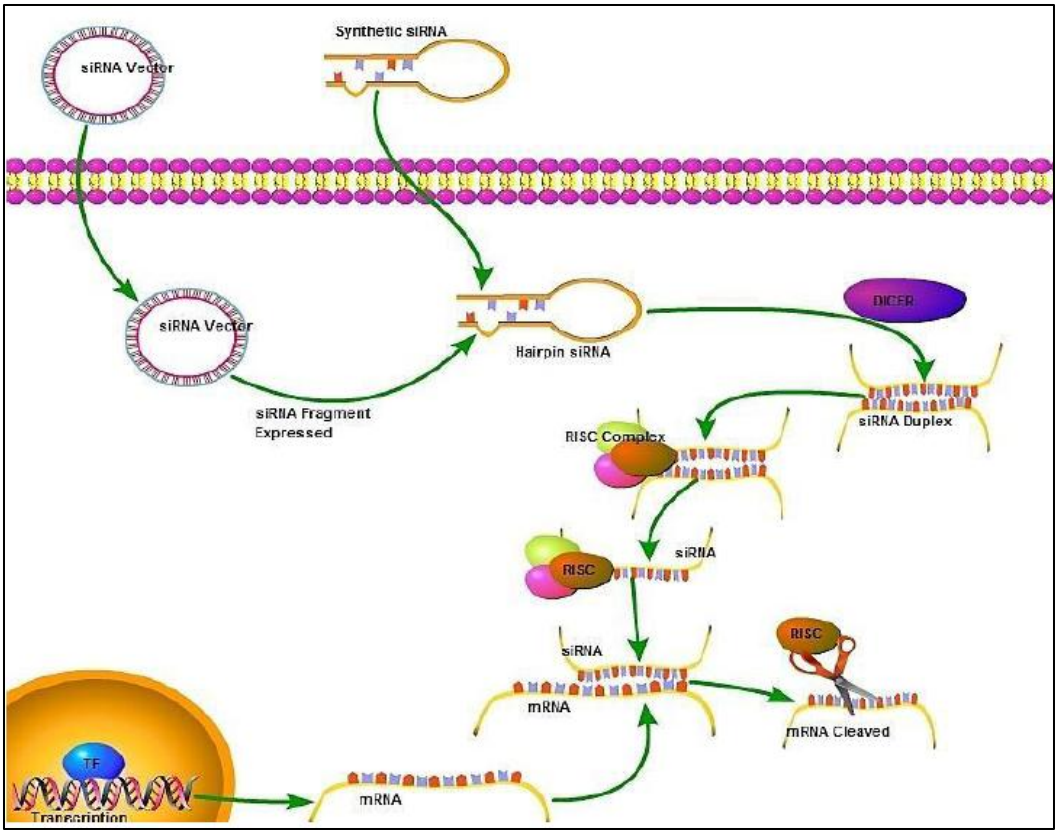

Figure 2.2: Multiple steps of mRNA degradation in the pathway of RNAi (Mansoori et al., 2014)

2.2.4 Types of Interfering RNA in RNAi

As mentioned earlier, the RNAi pathway can be initiated by the non-coding dsRNA to form a smaller version of the guiding strands which is made of less than approximately 30 nucleotides. There are many types of dsRNA that can be introduced into the cell to kick start the RNAi pathway. Previously discovered researches have focus mainly on the introduction of long dsRNA into the cell to be managed by the Dicer to form the siRNA. In plants and invertebrates, the introduced dsRNAs were of very long strand of the molecule, which may go up to

700 bp of nucleotides. This long dsRNA might cause the activation of dsRNA-responsive protein kinase as a result of interferon response to the viral infection, which directly leads the cell to apoptosis, skipping the gene silencing effect (Wianny et al., 2004 Mansoori et al., 2014). Hence, newer approaches have begun to directly use the specially designed 21 to 23 nucleotides of siRNA that mimicked the product of the Dicer to be incorporated into the RISC (Elbashir et al., 2001; Wianny et al., 2004).

There is another type of dsRNA that is similar to the siRNA, the short hairpin RNA (shRNA). ShRNA is the folded version of the siRNA with a hairpin loop in the middle. ShRNAs are expressed by using an expression vector transfected into the nucleus before transporting out into the cytoplasm to trigger the RNAi mechanism by incorporating into the RISC (Mansoori et al., 2014). Other than that, there are many more types of non-coding dsRNA that are potent triggers of RNAi which include micro RNA (miRNA), promoter-associated RNA (PAR) and small nuclear RNA (snRNA) to name a few of them (Mansoori et al,. 2014).

2.2.5 Application of RNAi

After the discovery of RNAi in year 1998, various researchers had utilised this phenomenon to study the function of genes and exploring RNAi as a therapeutic tool in many diseases. Some of its application includes the silencing

of protein kinase C isoforms in human and rat cells by Irie et al. (2002) and to determine the crucial gene involved in cell growth by Harborth et al. (2003). Wilda et al. (2002) and Park et al. (2002) had used RNAi as a therapeutic tool to silent the product of the oncogene and knocking down viral genes of human immunodeficiency virus (HIV), respectively. Recent approaches have been mostly focusing on the production of anti-cancer therapy by targeting specific genes that are essential for cancer cells. The silenced cancer gene will lead the killing of the cancer cell by a process called apoptosis.

2.3 Apoptosis

Apoptosis is known as the programmed cell death which is an alternative form of cell death that is active and inherently controlled process compared to necrosis, which is the cell death caused by the passive consequence of injurious agents. In addition to that, apoptosis can be initiated or inhibited by several physiological and pathological stimuli from the surrounding cell microenvironment (Richard, 2009).

2.3.1 Origin of Apoptosis

According to Richard (2009), apoptosis was termed after a Greek word which is used to illustrate the falling off of the flower petals. Richard (2009) stated that the works in the field of the concept of apoptosis were originated from John Kerr, an Australian pathologist. The first idea of apoptosis came from the

ischemic-induced hepatic atrophy of a rodent model. The differences between apoptosis and necrosis are the existence of individual, scattered hepatocytes containing the small, round cytoplasmic masses and fragments of pyknotic chromatin which is known as the apoptotic bodies. The location of the apoptotic bodies found was adjacent to the zones of the experimentally-induced ischemic necrosis (Richard, 2009).

2.3.2 Importance of Apoptosis

Richard (2009) stated that all the research findings had found that necrosis is conspicuous while apoptosis is subtle in a morphological perspective. The apoptosis is an important process as it can involve in the tissue homeostasis due to its roles in maintaining cells number in actively dividing cells. The homeostasis involved the continuous depletion of cells to balance the cell division with only a low level of tissue disruption. Moreover, the apoptosis are proved by the researchers to be normal, active and intrinsically controlled process that played a roles that is complementary but opposite to mitosis in the regulation of cell populations. Furthermore, the apoptosis process is cost-effective in terms of cellular components reutilization. There are two distinct stages of apoptosis whereby the first stage involved the apoptotic bodies formation while the second stage comprised of the phagocytosis and degradation of the apoptotic bodies by other cells (Richard, 2009).

Apoptosis is a crucial life process in controlling the population of cell in normal processes in the body ranging from fetal development to adult tissue homeostasis. Therefore, this highly organized physiologic event must be reactivated or induced in case of cell undergoing neoplastic transformation to prevent the malignant cell from proliferating continuously. This approach in resensitizing the cancer cells to apoptotic pathway is an important step in a cancer therapy. For instance, the approaches might include the restoration of the lost apoptosis intermediates, inactivation of the apoptosis inhibitor proteins and inducing apoptosis by targeting specific tumorigenic lesions (Carrasco et al., 2011).

2.3.3 Pathways of Apoptosis

There are two major pathways involved in the induction of the apoptosis process. All these pathways will eventually converge to the same outcome which is the execution pathway. The two major pathways of apoptosis are intrinsic pathway and extrinsic pathway. In most of the cancer cases, the disruption of the intrinsic pathway has given rise to cancer. For instance, the gene that induces apoptosis such as the p53 gene has undergone mutation leading to the disruption its function and the activation of the anti-apoptosis gene by mutation of the Bcl-2 family (Johnstone et al., 2002; Schmitt et al., 2002).

2.3.3.1 Intrinsic Pathway

In the intrinsic pathway, as mentioned by the name, it is initiated internally in the cell by the mitochondria, thus, sometimes it is called as the mitochondria pathway. Whenever a cellular stress occurred, it leads to the perturbation of mitochondria. The stresses include mutation of DNA, DNA damage, sudden heat shock of the DNA and the presence of apoptogenic agents, whereby all these might cause the disruption of cellular homeostasis. Therefore, the changes in the membrane integrity of the mitochondria cause the formation of the initiator caspases to initiate the downstream processes. The eventual release of cytochrome C from the mitochondria will directly induce the cell killing process which proceeds to the execution pathway to release the cascades of caspase (Fulda and Debatin, 2006; Elmore, 2007).

2.3.3.2 Extrinsic Pathway

On the other hand, extrinsic pathway in inducing apoptosis was initiated by factors external to the cell internal environment. This pathway depends on the action of the death receptor located on the membrane of the target cell, thus, this pathway is also known as the death receptor pathway. Once there are changes or abnormalities happening in the cell, such as DNA damage, replication error, viral infections and other disruptions on the genetic material, the adjacent cells or the immune system that have the death factors known as ligands will bind to the death receptor of the target cell. Several examples of these ligands are Fas, CD95, TNF-α, APO-1 and TRAIL. The binding of these ligands to the transmembrane death

receptor of the target cell will initiate the aggregation of several death receptors on the target cell's surface. This leads to the recruitment of an adaptor protein that functions to recruit the caspases to incorporate the death-inducing signal complex (DISC). DISC will direct the target cells to the execution pathway whereby the cell degradation will be carried out by downstream caspases (Fulda and Debatin, 2006; Elmore, 2007).

2.4 Survivin

Survivin is a protein member from the inhibitor of apoptosis (IAP) proteins family. The 16.5 kDa protein is the smallest in size as compared to other members of the IAP family (Salvesen and Duckett, 2002; Altieri, 2003a). It contains only a single Baculovirus IAP Repeat (BIR) domain at the N-terminal, a dimeric arrangement and an extended alpha-helix coiled region at the C-terminal (Ambrosini et al., 1997; Johnson and Howerth, 2004). Located on the human chromosome 17q25, survivin has three different isoform due to the availability of three alternatively spliced transcripts (Altieri, 2003a; Li, 2003; Johnson and Howerth, 2004). The expression of survivin in normal terminally differentiated cells are negligible but is highly expressed in most of the common cancer cells (Li, 2003; Shariat et al., 2003; Johnson and Howerth, 2004; Takeno et al., 2008).

2.4.1 Inhibitor of Apoptosis Protein (IAP) family

The IAP family was first discovered in year 1993 in the genomes of the baculovirus (Crook et al., 1993; Birnbaum et al., 1994). The discovery of this protein family in other organisms emerged subsequently, for instance, the nematodes, *Drosophila melanogaster*, yeast and humans. There are eight members of this family that got brought to light by the researchers, namely XIAP, NAIP, ILP-2, c-IAP1, c-IAP2, ML-IAP or livin, survivin and apollon. The similarity of the members of IAP family is the presence of the hallmark of the IAP, the baculoviral IAP repeat (BIR) domain which is an approximately 70 amino acids zinc-finger fold (Altieri, 2003b; Li, 2003; Johnson and Howerth, 2004).

2.4.2 Similarities and Differences of Survivin to Other IAPs

As mentioned earlier, all the IAPs contained the hallmark of IAP family. Each and every one of the IAPs carries at least one copy or more copies of the BIR and most importantly, the capability to inhibit apoptosis (Johnson and Howerth, 2004). However, survivin is the smallest in size compared to that of all other members in the IAP family. Survivin protein consist of only one BIR domain with the elongated alpha-helix motif at the C-terminal. The lack of RING finger motif also made survivin distinguishable from other IAPs (Li, 2003; Mobahat et al., 2014). Compared to other IAPs, survivin and livin have different level of expression in cancer cells (Li, 2003). Moreover, according to Chantalat et

al. (2000) and Verdecia et al. (2000), survivin is the only member in the family whereby it occurred in homodimerization in solution.

2.4.3 Functions of Survivin

Owing to the reason that survivin is a member of the IAP family, this protein is undoubtedly an inhibitor of apoptosis. As reported by Tamm et al. (1998), survivin has the ability to supress apoptosis induced by the extrinsic factors of apoptosis which are fas and bax other than the caspases and anticancer drugs. It was discovered that survivin has another function which is the involvement in the progress of cell cycle. Survivin is highly expressed in the G_2/M phase of mitosis. This indicates that survivin facilitates the proliferation of cancer cell by supressing the caspases activity which occur during cell cycle as a cellular check point in regulating the cell population (Li et al., 1998; Guo and Bruce, 1999; Paydas et al., 2003; Johnson and Howerth, 2004).

2.4.4 Pathways Involving Survivin in Cancer Development

As mentioned earlier, survivin is a bi-functional protein that is involved in the inhibition of apoptosis and regulates mitosis. Being one of the components of the chromosome passenger complex (CPC), survivin is an essential regulator in the segregation of chromosome and cytokinesis. In order to enhance the process of mitosis, nuclear survivin binds to two other CPC components, namely inner centromere protein antigen (INCENP) and borealin, to promote the auto-

phosphorylation of aurora kinase B. This process promotes the accurate alignment of chromosome, segregation and cytokinesis. On the other hand, mitochondrial survivin will be released upon the activation of checkpoint kinase 2 which is induced by DNA damaging stress. This release of survivin is to inhibit the process of apoptosis and enhance the survival of tumour cell. However, in normal cell, wild-type p53 will be stabilised by the DNA damage. Thus, in cancer, whenever there is mutation in p53 gene, the expression of survivin will not be regulated. Therefore, the cancer cell could be evaded from apoptosis and proliferate continuously (Mobahat et al., 2014). Figure 2.3 shows the pathways from which survivin were involved in the process of mitosis and inhibition of apoptosis.

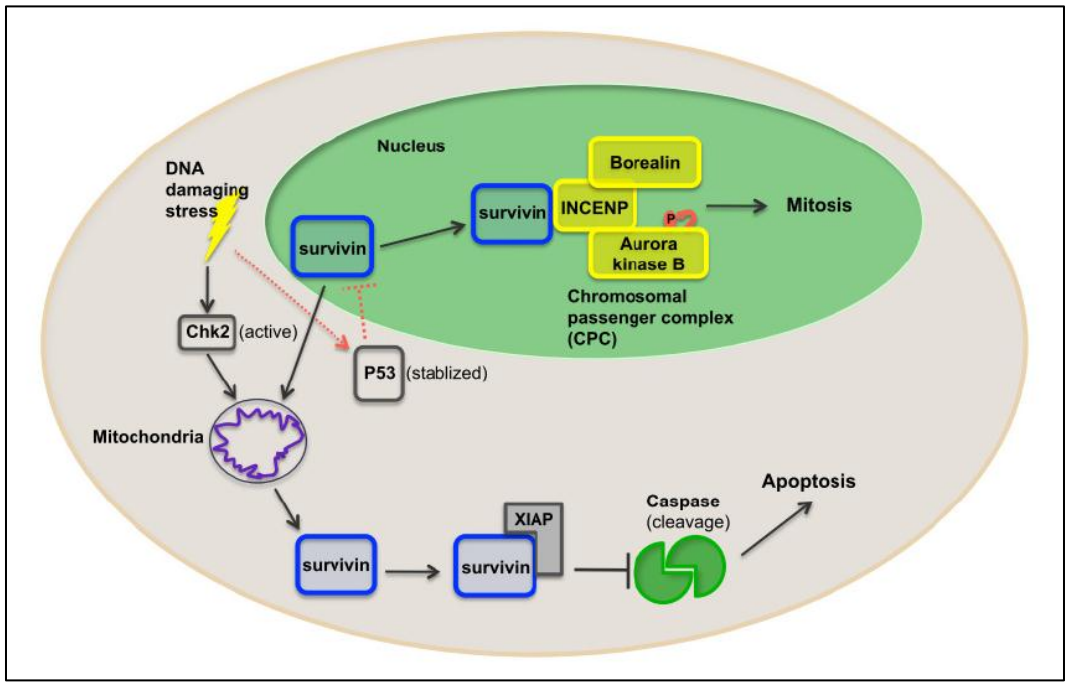

Figure 2.3: Pathways involving survivin in mitosis and the inhibition of apoptosis (Mobahat et al., 2014). Green area represents survivin expression in the cellular nucleus; grey area represents cytoplasm where mitochondria are involved in the process.

2.4.5 Applications Involving Survivin Gene

The crucial role of survivin in facilitating the prospering of cancer cells has made survivin a remarkable target in cancer therapeutic research. Many researches had target survivin expression in different types of cancer such as Azuhata et al. (2001) in neuroblastoma; Tanaka et al. (2000) in breast cancer; Karami et al. (2013) silenced survivin to treat leukemia, and Li et al. (2015) treated the adenocarcinoma, cervical cancer and hepatocarcinoma. There is an abundance of other researches that focus mainly on targeting survivin as the potent molecular marker of cancer.

As reported by Mobahat et al. (2014), all those researches that target survivin in treating various kinds of cancers had utilised different methods in suppressing the gene expression. The initial impression on targeting survivin seems to be difficult due to the nature of survivin that is neither an enzyme nor a surface protein. However, many approaches had succeeded in suppressing this protein by targeting its precursor, certain receptors linked to this protein, its promoter and even certain molecules in the activation pathways of this protein. The approaches included the transcriptional inhibition of survivin such as siRNA, ribozymes and antisense oligonucleotide; post-translational inhibition of survivin such as using the CDK inhibitors and Hsp90 inhibitors; cytotoxic vaccines that target against survivin epitopes; gene therapy by expressing the protein genes that down-regulate the function of survivin (Mobahat et al., 2014).

2.5 Telomerase

Telomerase is a ribonucleoprotein that functions to maintain the telomere length at the end of a linear chromosome. This is one of the pivotal roles of telomerase in the cell to ensure the integrity of the genome. Telomerase consists of two subunits that are important to one and another in the maintenance of telomere. These two subunits are human telomerase reverse-transcriptase (hTERT) and human telomerase RNA component (hTR or hTERC in several other publications). The expression of telomerase was found be significant in most of the malignant neoplasias while the expression was said to be absent generally in almost all normal somatic cells with several exceptions. These exceptions include those frequently dividing cells or proliferating cells such as embryonic stem cells, basal layer epithelial cells, germ line cells and activated lymphocytes (Stewart and Hahn, 2002; Zaffaroni et al., 2007; Jafri et al., 2016).

2.5.1 Functions of hTERT

The hTERT subunit in telomerase functions as the catalytic protein in the elongation of telomere length. It is a 127 kDa protein which consists of a specific telomerase domain (T-motif) that plays a crucial role in the enhancement of telomeric repeat extension (Zaffaroni et al., 2007; Drosopoulos and Prasad, 2010). Besides that, due to the structural similarity of hTERT to other reverse transcriptases, they have the similar functional properties. In overcoming the attrition of telomere during repeated DNA replications, hTERT is the rate limiting factor in the activity of telomerase. The hTR subunit is the template provider for

the catalytic counterpart, the hTERT, to carry out the pivotal function in elongation of telomere. Telomere is a tandem repeats of TTAGGG double-stranded DNA with a 3'-single-stranded overhang at its terminal. This overhang will then form a T-loop structure by folding back onto the double-stranded telomeric DNA. Therefore, telomere is the cap at the terminus of a linear chromosome to enhance the replicative lifespan of the cell. Hence, the reactivation of hTERT in cancer cells has contributed much to the limitless division of cancer cell and facilitated in the evasion from apoptosis (Zaffaroni et al., 2007; Mertzger et al., 2009; Jafri, 2016).

2.5.2 The Hayflick Limit

In year 1961, Leonard Hayflick and Paul Moorhead had discovered the limit in cell division capacity of the cultured normal cells. This phenomenon was due to the shortening of the telomere in each of the replication cycle. Thus, they termed this phenomenon as replicative senescence whereby after a certain cycle of replication, the telomeric DNA length will be exhausted and the DNA could not be replicated any further leading to the termination of cell division. This severe erosion of telomeric length would led to the chromosomal instability which in turn caused the activation of caspases activity to carry out apoptosis, hence, the process was called crisis. This phenomenon was named after the discoverer and hence the name Hayflick Limit. The activation of telomerase in cancer cells has facilitated the cells in overcoming this limit and attained the infinite replication

lifespan (Hayflick and Moorhead, 1961; Stewart and Hahn, 2002; Shay and Wright, 2011; Jafri et al., 2016).

2.5.3 Mechanistic Action Involving hTERT in Cancer

In cervical cancer, after the infection of HPV, many early (E) viral genes were expressed. Two of those early viral genes that involved in the regulation of hTERT were E6 and E7. High risk HPV infection promotes the expression of high risk E6 and E7 that worked synergistically to enhance the function and expression of hTERT protein. This enhancement was to maintain the high cellular division rate to allow the proper viral infection processes. Hence, high risk E6 and E7 play essential role in the progress of HPV infection towards the development of carcinoma in situ on the epithelial cells of the cervix and lastly, invasive cervical cancer (Katzenellenbogen, 2017). Other than that, Ram et al. (2009) has speculated that hTERT up-regulation was related to certain signal transduction pathways such as the PI3k/Akt and Rad pathways. The PI3k/Akt pathway is the key regulator for cell proliferation, cell growth and apoptosis. Thus, DNA damage-induced cancer has activated the PI3k/Akt pathway which caused the overexpression of hTERT gene as a DNA repair mechanism of the cell (Ram et al., 2009). Figure 2.4 shows the induction of telomerase post-HPV infection.

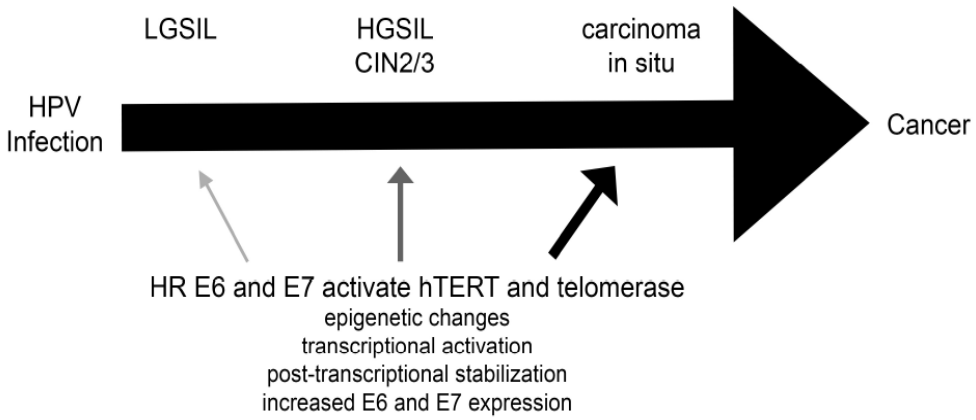

Figure 2.4: Induction of telomerase post-HPV infection (Katzenellenbogen, 2017).

2.5.4 Applications Involving hTERT Gene

The vital role of hTERT in facilitating the cancer cells to have the infinite dividing capability and evasion from apoptosis has made this gene a highly remarkable molecular marker to be targeted by cancer researchers. The applications in treating cancer by silencing this gene include George et al. (2010) in treating glioblastoma; Liu et al. (2014) in treating colon cancer; Marggisano et al. (2017) in treating thyroid cancer as well as in lung cancer by Xie et al. (2011).

Marggisano et al. (2017) has suggested that the overexpression of hTERT gene might not be co-related to the mutation of the hTERT promoter. Hence, different types of thyroid cancer samples were tested for the presence of promoter mutation and the expression of hTERT in thyroid cancer cells and normal cells. The attempts to silent the hTERT gene by using siRNA approach were then

reported to be successful in reducing thyroid cancer cell proliferation and migration even in the presence of hTERT promoter mutation (Marggisano et al., 2017). Therefore, Marggisano et al. (2017) has hypothesised that hTERT as a potential oncogene which is crucial for cancer cell proliferation and migration.

Moreover, Ram et al. (2009) has reported that hTERT overexpression has caused the resistance of cancer cells to radiation therapy and even further increased in expression. Experiments had been conducted on the Ewing sarcoma cells, breast cancer and leukemia by these researchers. They had observed the increased in telomerase activity after radiation has been carried out in attempt to kill the cancer cells. Hence, they had concluded that hTERT is one of the components in the DNA repair mechanism of body. This gene will be up-regulated when DNA damaging stress is present in the cell. Therefore, by silencing this gene directly, cancer cells will be resensitised to apoptosis and more responsive to other anticancer therapies (Ram et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 General Chemical and Biological Reagents

Several chemicals and premixed biological reagents were purchased from certain companies as followed: Dimethylsulfoxide (DMSO) from CALBIOCHEM®; Ammonium persulfate from OmniPUR®, Tetramethylethylenediamine (TEMED) from Calbiochem®, Tris-Base and Bis-Acrylamide Solution from ThermoFisher Scientific; Ethylenediaminetetraacetic acid (EDTA) from ChemAR; Glycine from 1st BASE; Tween-20, Phosphate Buffered Saline (PBS) from MP Biomedicals, Luria Betani (LB) broth and LB agar from MERCK; glacial acetic acid from Fisher Scientific; calcium chloride ($CaCl_2$) and sodium chloride (NaCl) from SYSTERM®; MTT Assay Kit from Trevigen; SYBR green qPCR kit from New England Biolabs (NEB); Chemi-Lumi One L and Blocking One from Nacalai Tesque; Anti-human hTERT antibody and Anti-human Survivin antibody from Thermo Scientific; Anti-human Actin antibody from SantaCruz Biotechnology; sodium dodecyl sulfate from Fisher Scientific; MitoLume Apoptosis Detection Kit from GeneTech; Agarose, Methanol from MERCK and Ethanol from Copens Scientific (M).

3.1.2 Reagents for Molecular Cloning

The restriction enzymes used for molecular cloning such as BamHI, HindIII, EcoRI, MfeI and XhoI were purchased from NEB; T4 DNA ligase from ThermoFisher Scientific; Ampicillin from USB®; DNA Extraction Kit, RNA Extraction kit, Gel Extraction Kit and PCR Purification Kit from PKT; RevertAid™ H Minus First Strand cDNA Synthesis Kit from (Fermentas) ThermoFisher Scientific; jetPRIME® Transfection Kit from Polyplus-transfection®.

3.1.3 Reagents for Animal Cell Culture

Eagle's Minimum Essential Medium (EMEM), Fetal Bovine Serum (FBS) and 0.25% Trypsin with 0.53 mM EDTA solution were purchased from Biowest, Penicillin-Streptomycin was from MERCK.

3.1.4 List of Prepared Solutions

Several solutions were prepared in the laboratory by mixing chemicals according to the formulations as shown in Table 3.1.

Table 3.1: List of solutions and respective formulations

Solution	Formulation
Annealing Buffer	10 mM Tris, 1 mM EDTA (pH 8.0), 50 mM NaCl
Agarose Gel Electrophoresis Buffer (TAE)	40 mM Tris, 20 mM Glacial Acetic Acid, 1 mM EDTA (pH 8.0)
Phosphate Buffered Saline (PBS)	1 PBS Tablet in 100 mL ddH_2O (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.10 mM Na_2HPO_4
Cell Lysis Buffer	10 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, 0.5% (w/v) Sodium Deoxycholate, 0.5% (v/v) Nonidet® P-40
Sample Buffer (SAB)	62.5 mM Tris, 25% (v/v) Glycerol, 2% (w/v) SDS, 0.01% (w/v) Bromophenol Blue
Tris-Glycine Electrophoresis Buffer	25 mM Tris, 190 mM Glycine, 1% (w/v) SDS, pH 8.6
Transfer Buffer	20 mM Tris, 150 mM Glycine, 20% (v/v) Methanol
Blocking Buffer	5% (v/v) Blocking One in PBST
Stripping Buffer	400 mM Glycine, 2% (v/v) Tween-20, 0.2% (w/v) SDS, pH 2.2
Washing Buffer (PBST)	0.1% (v/v) Tween-20 in PBS

3.2 Cloning of shRNA Expression Vector

3.2.1 Target Region Selection

The sequence of the specific region on the targeted survivin and human telomerase reverse transcriptase (hTERT) genes were selected by using certain

RNAi design algorithms. The algorithms are BLOCK-it RNAi from Invitrogen, GPP portal from TRC consortium and siRNA Wizard v3.1 from InvivoGen. The sequences that have no previous researches performed on and constituted the best oligonucleotide conditions were selected as the target sequence for this project. The selected survivin and hTERT target sequences were further compared against the genome database to ensure that they can target all the isoforms of the target genes. This comparison was performed by using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) website. In order to complement the RNAi experiment, a nontargeting negative control was constructed for both the survivin and hTERT genes. This negative control was constructed by scrambling the arrangement of the nucleotide sequences from the selected target sequences. Therefore, these controls, named scrambled shRNA, contained the similar nucleotide composition as the selected target sequence without significant sequence homology to the target genome. The selected target sequences and the scrambled shRNA sequences were shown in Table 3.2.

Table 3.2: List of selected target sequence and scrambled shRNA

Target name	**Selected nucleotide sequence**
Survivin	GCCAGACTTGGCCCAGTGTTT
hTERT	GGAAGAGTGTCTGGAGCAAGT
Scrambled Survivin (control)	ATGTCGACGTCTACGTTCGGC
Scrambled hTERT (control)	GAGGATATAGAGCGGTATGCG

3.2.2 shRNA Template Design

After the target sequences had been obtained, the design of the shRNA template was carried out by following the guidelines shown in the shRNA expression vector manual from ThermoFisher Scientific. The shRNA template oligonucleotide consists of several parts starting from the BamHI restriction site, sense strand of the target sequence, the loop region, the antisense strand of the target sequence, RNA polymerase III terminator and ended with HindIII restriction site. The sequence of the loop, the additional AA dinucleotide sequences and the additional of 5'–GGAA–3' downstream of the RNA polymerase III terminator were following the optimisation suggested by the shRNA expression vector manual for optimal gene silencing. The overview of the template design was shown in Figure 3.1. The list of the designed shRNA oligonucleotides for this project was shown in Table 3.3.

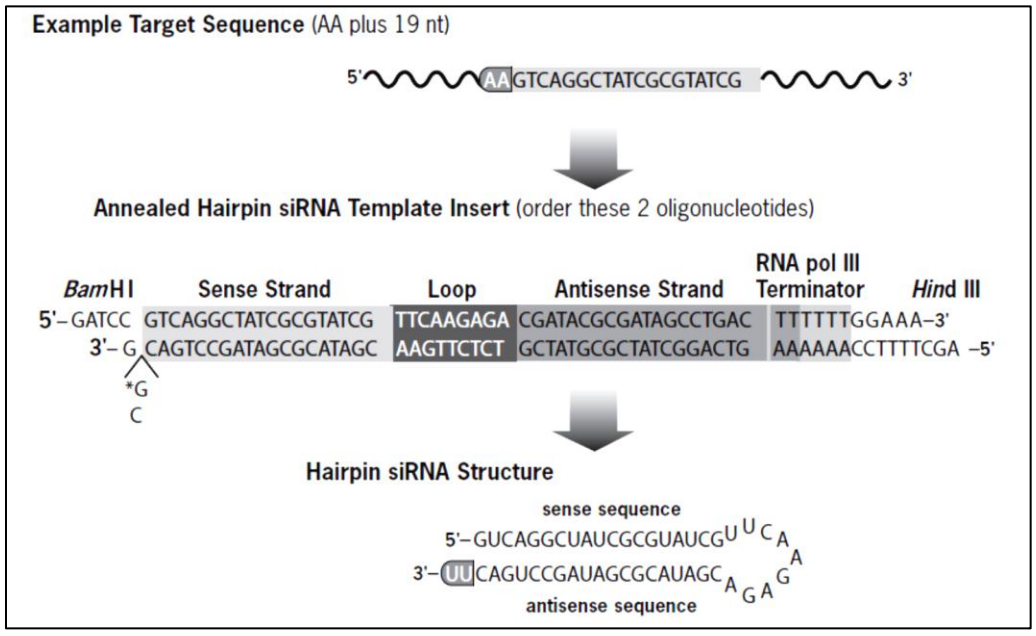

Figure 3.1: shRNA Design Template

Table 3.3: List of designed shRNA oligonucleotides

Oligo Name	Oligonucleotide Sequence
shBIRC-Up	5'–GATCCGCCAGACTTGGCCCAGTGTTTAGTTCTC AAA CACTGGGCCAAGTCTGGCTTTTTTGGAAA – 3'
shBIRC-Down	5'–AGCTTTTCCAAAAAAGCCAGACTTGGCCCAGTG TTT GAGAACTAAACACTGGGCCAAGTCTGGCG– 3'
shTERT-Up	5'–GATCCGGAAGAGTGTCTGGAGCAAGTAGTTCTC ACT TGCTCCAGACACTCTTCCTTTTTTGGAAA– 3'
shTERT-Down	5'–AGCTTTTCCAAAAAAGGAAGAGTGTCTGGAGCA AGT GAGAACTACTTGCTCCAGACACTCTTCCG– 3'
shScrBIRC-Up	5'–GATCCATGTCGACGTCTACGTTCGGCAGTTCTC GCC GAACGTAGACGTCGACATTTTTTTGGAAA– 3'
shScrBIRC-Down	5'–AGCTTTTCCAAAAAAATGTCGACGTCTACGTTC GGC GAGAACTGCCGAACGTAGACGTCGACATG– 3'
shScrTERT-Up	5'–GATCCGAGGATATAGAGCGGTATGCGAGTTCTC CGC ATACCGCTCTATATCCTCTTTTTTGGAAA– 3'
shScrTERT-Down	5'–AGCTTTTCCAAAAAAGAGGATATAGAGCGGTAT GCG GAGAACTCGCATACCGCTCTATATCCTCG– 3'

3.2.3 Oligonucleotides Annealing

The two complimentary strands of oligonucleotides were annealed by using the prepared Annealing Buffer whereby 1 µL was taken from each 10 µM oligonucleotide solution and added to 18 µL of 1X Annealing Buffer. The annealing mixture was incubated in boiling water bath (100ºC) for 5 min and gradually cooled down to room temperature (~25ºC). The annealed oligonucleotides were electrophoresed in 4% agarose gel to ensure the formation of intermolecular oligonucleotide.

3.2.4 Competent Cell

Escherichia Coli (*E.Coli*) bacteria strain DH5-α were used to prepare the competent cells. The DH5-α cells were streak on a LB agar plate and incubated at 37ºC overnight to obtain a single colony. The single colony was further cultured in LB broth overnight at 37ºC with 200 rpm agitation to obtain a healthy growing bacteria culture. From this, 1 mL of the DH5-α culture was transferred to 50 mL of LB broth in a 250 mL conical flask and incubated at 37ºC with 200 rpm agitation for 3 h. After 3 h, 1 mL of the culture in the conical flask was quantified with OD_{600} using a spectrophotometer. The best reading from the spectrophotometer would be 0.400 to ensure the bacteria had reached the exponential phase in the growth cycle.

After obtaining the 0.400 reading, the culture in the conical flask was cooled at 4ºC for 10 min. The culture was then centrifuged at 2500 rpm in 4ºC for 15 min. The pellet obtained was resuspended with 50 mL ice-cold 10% (v/v) glycerol solution. After that, the centrifugation step and the resuspension step were repeated with 25 mL and lastly, 10 mL of ice-cold 10% (v/v) glycerol solution. After the final centrifugation step, the supernatant was carefully decanted and the remnant buffer was removed by careful pipetting. The pellet was then resuspended with 1 mL of ice-cold 10% glycerol. An aliquot of 40 uL of the suspension were used as the electro-competent cells for transformation by electroporation whereas the remaining suspension were stored as the electro-competent cells stock for further transformation processes.

3.2.5 Propagation of p*Silencer*™

The shRNA expression vector used in this project was p*Silencer*™ hygro Kit purchased from previously Applied Biosystem, which is now one of the brands under ThermoFisher Scientific. The vector provided by the kit namely p*Silencer*™ 2.1-U6 hygro was further modified to enable the construction of multi-shRNA expression vector. The vector map for p*Silencer*™ 2.1-U6 hygro was shown in Figure 3.2. The modifications performed were the removal of one EcoRI site on the Hygromycin region plus the addition of MfeI and XhoI endonuclease restriction sites immediately downstream of the HindIII site. The modified expression vector was named pU6-m4 and the representative image of the modified vector was shown in Figure 3.3.

This modified vector was used to transform the prepared competent cells through electroporation. Prior to transformation, 40 µL of the competent cells were added with 1 µL of the expression vector and incubated in ice for 1 min. The electroporator settings were set as followed: 1550 V; 200 Ω resistance and 25 µF capacitance. Immediately after the electroporation, 400 µL of LB broth were added to 40 µL aliquot of transformed cells. After that, 100 µL of the transformed bacteria broth were spread with a bent glass rod on the LB agar plate containing 100 µg/µL of Ampicillin and cultured in 37ºC incubator overnight. The colonies formed on the plate were selected and further cultured in LB broth containing 100 µg/µL Ampicillin overnight at 37ºC with 200 rpm agitation to propagate the vector-containing bacteria. After that, 5 mL of the bacterial broth were

centrifuged at 14,000 rpm for 1 minute to pellet the bacteria cells. The pU6-m4 plasmids were extracted from the pelleted cells using the DNA Extraction Kit from PKT, Korea. The plasmid extraction method was according to the manufacturer's protocol. The extracted plasmid was quantified for the dsDNA content by using NanoDrop™ 2000 Spectrophotometer from Thermo Scientific™.

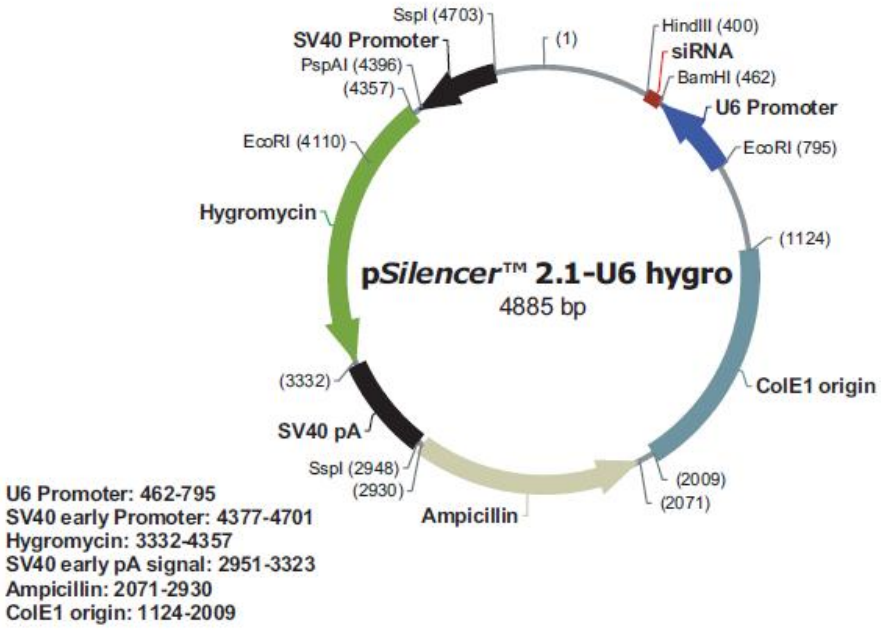

Figure 3.2: Vector Map for p*Silencer*™ 2.1-U6 hygro

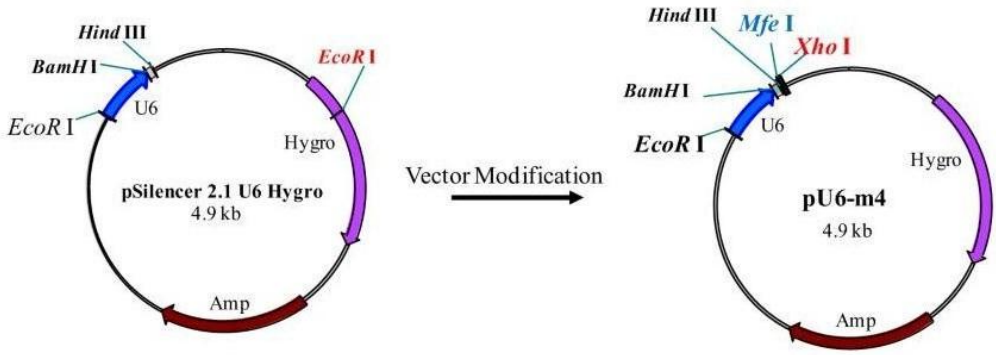

Figure 3.3: Representative Graphics of the Vector Modification

3.2.6 Cloning of Single shRNA Expression Vector

3.2.6.1 Restriction Endonuclease Digestion of pU6-m4 Vector

Following plasmid extraction, the extracted pU6-m4 vector was digested with two different restriction enzymes (RE) which are BamHI and HindIII. This double restriction digestion was to prepare the linear vector for the ligation with the annealed oligonucleotide. Approximately 1 µg of the plasmid was digested with 1 µL of (1U/µL) BamHI and 1 µL of (1U/µL) HindIII with 5 µL of 10X NEBuffer 2.1 in a total reaction volume of 50 µL by topping up with sterile dH_2O. The reaction was incubated in 37ºC for 1 h. After the digestion, the REs were heat inactivated at 80ºC for 20 min. The digestion product was electrophoresed at 100 V in 1% agarose gel to determine the completion of digestion and the presence of star activity. Provided the double restriction digestion has completed and no presence of star activity, all the remaining linearised vector was loaded in the 1% agarose gel and electrophoresed at 100 V for 40 min. The band at the expected size of ~4.9 kb was cut with a blade and gel extraction was carried out by using the Gel Extraction Kit from PhileKorea according to the manufacturer's protocol.

3.2.6.2 Ligation of Annealed Insert and pU6-m4 Vector

To ligate the annealed hTERT and Survivin oligonucleotides to the linearised pU6-m4 vector, the molar ratio of the vector and the oligonucleotides was calculated according to the following formula in order to obtain better ligation efficiency.

Formula for ratio of vector and oligonucleotides:

$$\frac{\text{Insert Length (bp)}}{\text{Vector Length (bp)}} \times \text{Vector Conc. (ng)} \times \frac{3}{1} = \text{Insert Conc. (ng)}$$

The 3:1 ratio of insert:vector was chosen in this case due to the better ligation yield obtained. Therefore, ligation reaction tube contains 20 ng of linearised vector, 0.80 ng of insert oligonucleotides, 1 µL of T4 DNA Ligase, 1 µL of T4 DNA Ligase Buffer in a total reaction volume of 10 µL by topping up with sterile dH_2O. The ligation reaction tubes of Survivin, hTERT, scrambled Survivin and scrambled hTERT were labelled pB, pT, pSB and pST respectively. The prepared ligation mixtures were incubated at 16ºC for 16 h followed by heat inactivation at 65ºC for 10 min.

3.2.6.3 Transformation of DH5-α with Single shRNA Vector

Following ligation, 1 µL of the ligation mixture from each of the reaction tubes was added into the prepared 40 µL of competent DH5-α cells and incubated in ice for 1 min prior to electroporation. The settings of the electroporator were

set exactly the same as for the transformation of the competent cells with pU6-m4. The transformed cells were added with 400 µL of LB broth immediately after electroporation and 100 µL were spread on the LB agar plate containing 100 µg/µL of Ampicillin. The plates were incubated at 37ºC overnight. The Ampicillin resistant colonies formed on the agar plate were picked with sterile toothpick onto another LB agar plate containing 100 µg/µL of Ampicillin. These colonies were propagated in preparation for colony PCR.

3.2.6.4 Colony PCR of Single shRNA Transformants

The colonies propagated were picked with sterile toothpick into 10 µL of sterile dH_2O, each labelled with their respective vectors' names and colonies' numbers. From these, 1 µL of the diluted bacteria colonies were used as the DNA template in colony PCR. The primers used for colony PCR were T7 promoter primer and 2.0 Rev primer as suggested by the p*Silencer*™ hygro kit manual. The primer sequences were shown in Table 3.4. The PCR cycle conditions were shown as followed: 94ºC initial denaturation for 3 min; followed by 30 repeats of 94ºC denaturation for 30 sec; 50ºC primer annealing for 40 sec; 72ºC elongation for 60 sec; and lastly, one cycle of final elongation at 72ºC for 5 min.

Table 3.4: Primer Sequence for Colony PCR

Primer Name	Primer Sequence	Binding Location in Expression Vector (bp)
T7 Promoter	5'–TAATACGACTCACTATAGGG– 3'	840
2.0 Rev	5'–AGGCGATTAAGTTGGGTA– 3'	333

3.2.6.5 Sequencing of Single shRNA Vectors

The products of colony PCR were electrophoresed in 2% agarose gel at 100 V for 40 min. The colonies that exhibited the expected band size of approximately 550 bp were chosen as the positive clones. Two clones were selected from each of pB, pT, pSB and pST vectors. The selected clones were cultured in LB broth containing 100 µg/µL of Ampicillin at 37ºC overnight. Plasmid extraction was performed by using the cultured clones. The extracted plasmids were prepared for PCR by using the *pfu* Tag polymerase for better amplification prior to sending out the clones for sequencing service to confirm the sequence of the clones. The sequencing results were compared against the designed sequence by using BLAST from NCBI to ensure 100% alignment were present in the extracted plasmids before downstream experiments were carried out.

3.2.7 Cloning of Multiple shRNA Expression Vector

3.2.7.1 Restriction Endonuclease Digestion of Single shRNA Vector

Double endonuclease restriction digestion was performed on the cloned single shRNA expression vector from Section 3.2.6. Only one clone was selected from the two sequenced clones for each of the single shRNA vectors. The selected clones were pB1 (survivin), pT1 (hTERT), pSB1 (scrambled survivin) and pST1 (scrambled hTERT). Three types of RE were used which are MfeI, XhoI and EcoRI. The pB1 and pSB1 were doubly digested with MfeI and XhoI while pT1 and pST1 were doubly digested with EcoRI and XhoI. The RE digestion with MfeI and XhoI will yield a linearised vector for each of the pB1 and pSB1. Whereas, the RE digestion with EcoRI and XhoI will yield the insert segment of the shRNA expression vector spanning from the U6 polymerase promoter site to the inserted shRNA oligonucleotides' terminator region.

The RE digestion for both pB1 and pSB1 plasmids were as followed: 1 µg of plasmid; 1 µL of 1U/µL MfeI and 1 µL of 1U/µL XhoI; 5 µL of NEBuffer 1.1 in a total reaction volume of 50 µL by topping up with sterile dH_2O. On the other hand, the RE digestion for both pT1 and pST1 plasmids were as followed: 1 µg of plasmid; 1 µL of 1U/µL EcoRI and 1 µL of 1U/µL XhoI; 5 µL of NEBuffer 2.1 in a total reaction volume of 50 µL by topping up with sterile dH_2O. The MfeI and XhoI RE digestion reactions were incubated at 37ºC for 1.5 h followed by heat

inactivation at 65ºC for 20 min. Whereas, the RE digestion with EcoRI and XhoI were carried out at 37ºC for 1 h and lastly heat inactivated at 65ºC for 20 min.

After double RE digestion, the digested plasmids were separated on 1% agarose gel electrophoresis at 100 V for 40 min. The expected band size of linearised pB1 and pSB1 was ~4.9 kb while the restricted segments of pT1 and pST1 have a band at approximately 450 bp in size following the indication of the 1 kb DNA ladder. The bands were excised from the gel and purified by using the Gel Extraction Kit from Phile Korea following the instructions in the manufacturer's manual.

3.2.7.2 Ligation of the Multiple shRNAs Vector

The excised single shRNA fragments were ligated to the linearised plasmid by T4 DNA Ligase following the molar ratio of insert over vector as shown previously. For every 1 µg of linearised plasmid in the reaction tube, 275 ng of single shRNA plasmid fragments were added, in addition of the 1 µL T4 DNA Ligase, 1 µL of T4 DNA Ligase Buffer and further topped up to 10 uL total reaction volume with sterile dH_2O. The ligation reaction tubes of multiple shRNA and multiple scrambled shRNA were labelled pBF, and pSS, respectively. The reactions were incubated at 16ºC for 16 h followed by heat inactivation at 65ºC for 10 min.

3.2.7.3 Transformation of DH5-α with Multiple shRNAs Vector

After ligation, 1 µL of the ligation mixture from each of the reaction tubes was added into the prepared 40 µL of competent DH5-α cells and incubated in ice for 1 min prior to electroporation. The settings of the electroporator were set exactly the same as the previous transformation processes. The transformed cells were added with 400 µL of LB broth immediately after electroporation and 100 µL were spread on the LB agar plate containing 100 µg/µL of Ampicillin. The plates were incubated at 37ºC overnight. The Ampicillin resistant colonies formed on the agar plate were picked with sterile toothpick onto another LB agar plate containing 100 µg/µL of Ampicillin. These colonies were propagated in preparation for colony PCR.

3.2.7.4 Colony PCR and Sequencing of Multiple shRNA Transformants

Downstream from the propagation of multiple shRNA transformants, amplification of the cloned plasmids were performed through colony PCR. The conditions of the amplification were the same as that performed in section 3.2.6.4, with the same set of primers. The expected band size of the amplified fragment of the multiple shRNAs plasmid was 1 kb. The sequencing procedures and the gene alignment against the database were following the same procedures as in section 3.2.6.5. The confirmed clones were pBF57 and pSS5. The confirmed plasmids were mass extracted and kept in stock before downstream processing.

3.3 Treatment of Human Cancer Cell Line

3.3.1 HeLa Cell Line (CCL-2™)

The source, from which the HeLa cell line was obtained, was from ATCC®, USA. The code used by the company to refer to this cell line was ATCC® CCL-2™. This human cervical adenocarcinoma cell line, namely HeLa cell line, was deposited by WF Scherer from a 31 years old Black female. The epithelial morphology of HeLa cell line was shown in Figure 3.4. This anchorage-dependent cell line was proven to contain the human papilloma virus (HPV), hence, it was handled with delicate care in biosafety level 2 laminar flow. HeLa cell proliferates in EMEM with 10% (v/v) FBS at 37ºC, humidified 5% CO_2 incubator.

3.3.1.1 Preparation of Complete Medium for HeLa Cell Culture

The complete medium preparation for HeLa cells was EMEM supplemented with 10% (v/v) FBS and 1% (v/v) 100X Penicillin-Streptomycin antibiotics solution. The prepared complete medium was stored in 4ºC.

Figure 3.4: Morphology of HeLa Cells

The HeLa cells were visualised under Nikon ECLIPSE T*i* Inverted Microscope at 100x magnification and acquired by using NIS Elements BR Software version 4.40.

3.3.1.2 HeLa Cell Culture and Maintenance

The cell line was maintained every 3 days by changing the medium in the $75cm^2$ T-flask from TPP®. Prior to changing the medium, the HeLa cells in the flask were rinsed gently with 3 mL of PBS solution. After that, 10 mL of complete medium were added into the flask and the HeLa cells were cultured in the mentioned appropriate conditions.

3.3.1.3 HeLa Cell Sub-culture

From the cultured and consistently maintained HeLa cells, once 70% to 80% of cell confluency was observed, the HeLa cells were sub-cultured into new T-flask. The initial step was rinsing the HeLa cells with 3 mL of PBS solution twice to remove the remnant of FBS, followed by the addition of 3 mL of Trypsin-EDTA solution. The cells in trypsin solution were incubated at 37ºC for 5 min. After that, 6 mL of EMEM containing FBS were added into the flask to terminate the trypsin reaction. The detached cells were pelleted at 600 x *g* for 5 min by using Heraeus Multifuge 1-SR (Thermo Scientific). The supernatant was decanted and the cells pellet was suspended with 1 mL of EMEM by gently pipetting technique. The suspended HeLa cells were seeded into the new flask or plate with preferred cell density according to the requirement of the downstream experiment.

3.3.1.4 Cell Storage and Thawing

After the HeLa cells had reached 80% confluency, the cells were trypsinised by following the same protocol as mentioned in section 3.3.1.3. The re-suspended cells will be aliquoted into the cryo vials with a total cell number of 5×10^5 by cell counting using haemocytometer. A total solution volume of 1 mL was made up by topping up with complete medium and 5% (v/v) DMSO were added as the cryo-preservative. This cell suspension in cryo-vial was stored in -80ºC freezer for 24 h and further moved into the liquid nitrogen storage tank.

The stored cells were thawed by immediately incubating the cryo-vial with cell suspension, obtained from the liquid nitrogen storage, in 37ºC water bath until all the cell suspension has melted. Following the thawing process, 1 part (1 mL) of the cell suspension was added with 9 parts (9 mL) of EMEM. This 1:10 dilution was to remove the residue of DMSO in the suspension that is cytotoxic. The 10 mL of cell suspension was centrifuged at 600 x *g* for 5 min. The supernatant was decanted and the cell pellet was re-suspended in 1 mL of EMEM. The re-suspended cells were then cultured in T-flask with complete medium up to a total volume of 10 mL.

3.3.1.5 Cell Counting with Haemocytometer

The Neubauer Improved 0.0025 mm^2 Haemocytometer from Blau Brand, Germany was used to perform cell counting. To prepare the HeLa cells for counting, the attached cells in the T-flask were trypsinised by following the same protocol as mentioned in Section 3.3.1.3. After that, a small amount of cell suspension was aliquoted 1 µL to be further diluted with 9 µL of EMEM. The 10 µL of diluted cell suspension was added with 50% (v/v) Trypan Blue solution. The stained cell suspension was carefully placed in between the haemocytometer slide and the coverslip by capillary action. The cells on the haemocytometer were viewed with 100X magnification under the BRAND inverted microscope. The dead cells were stained blue by the Trypan Blue dye whereas living cells will be unstained and possessed a refractile ring on the outer membrane of the cell. The cells that were located within the 16 smaller squares and any position on the left

or bottom boundary line were counted with a cell counter. The cell counting was performed by using the upper section of the haemocytometer as well the lower section in order to achieve a more consistent calculation. The cell concentration was determined by using the following formula.

$$\text{Cell Concentration} = \frac{\text{Number of Viable Cells}}{8} \times \text{Dilution Factor} \times 10^4$$

The calculated cell concentration was adjusted accordingly to the optimal number of cells required for different types of assay during cell seeding

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3.3.1.6 HeLa Cell Death Curve Analysis

In order to obtain the effective antibiotic concentration for selection of HeLa cell line and the optimal plating density for the treatment, titration on hygromycin B concentration and the cell number were performed by using the cell death curve analysis. For this, a hygromycin B stock solution was prepared at a concentration of 50 mg/mL. The hygromycin B concentration was titrated against a fixed HeLa cell density of 2×10^5 cells per well. The cells were seeded in 5 mL of complete medium for 24 h at 37ºC in a humidified 5% CO_2 incubator. The varying amounts of hygromycin B, which were 0, 50, 100, 200, 300 and 600 µg/mL, were added into the medium in each well and cultured for 96 h. The

medium was replaced every 48 h. The plate was observed every 24 h for viable cells and the lowest concentration that gave rise to massive cell death.

After the optimal hygromycin B concentration was obtained, it was tested with various cell densities to determine the optimal cell plating density. HeLa cells were seeded on a 6-well plate with the cell number of 5×10^4, 1×10^5, 2×10^5, 5×10^5, 1×10^6 and 5×10^6 in each well, respectively, for 24 h. Following the incubation, 200 µg/mL of hygromycin B solution were added into each of the cell cultures. The 6-well plate was incubated at 37ºC in a humidified 5% CO_2 incubator for 96 h. The cell culture medium was replaced every 48 h. The plate was observed every 24 h for cell viability to determine the density that enabled the cells to reach approximately 80% confluency before massive cell death begins.

3.3.1.7 HeLa Cell Transfection

For the transfection of HeLa cells with the expression vector, the transfection was performed by using the jetPRIME® Transfection Kit from Polyplus-transfection®, France. For 6-well plate, 2×10^5 HeLa cells were seeded in 2 mL complete medium in each well. In preparing the transfection mixture, 2 µg of expression vector was mixed gently with 200 µL jetPRIME®. After that, 4 µL jetPRIME® reagent was added into the previous mixture which was 1:2 DNA to jetPRIME® reagent ratio. The transfection mixture was mixed gently by pipetting and incubated for 10 min at 25ºC. During the incubation of transfection

mixture, the cells in the 6 well-plate were rinsed gently with PBS and 2 mL of EMEM with 10% (v/v) FBS were added into each well. The transfection mixture was added into each well and the plate was gently tapped from the sides to ensure well mixing of the transfection mixture with the serum containing medium. The plate was then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 h prior to the replacement of the transfection mixture with complete medium. After 24 h of incubation, 6 µL of 50 mg/mL hygromycin B solution were added into each well for selection. Following the selection, the cells were harvested every 24 h for three consecutive days. Medium replacement was carried out every 48 h. For transfection carried out in different plate size, the volume of transfection mixture and the amount of expression vector was calculated accordingly by following the instructions in the jetPRIME® Transfection Kit manual.

3.4 Gene Silencing in HeLa Cell Line

3.4.1 Knockdown Efficiency Evaluation by Cell Counting

The general knockdown efficiency of the HeLa cells transfected with all the expression vectors (pB1, pT1, pBF57, pSB1, pST1 and pSS5) were evaluated by cell counting with haemocytometer. Following the transfection of HeLa cells with respective expression vectors, the cells in three different plates, labelled Day 1, Day 2 and Day 3, were harvested separately every 24 h. The cells harvest began with the rinsing of the cells with PBS solution to remove the FBS residue, followed by the trysinisation of the cells for 5 min at 37ºC. The detached cells

were re-suspended and diluted with PBS. The diluted cells were further stained with 2X Trypan Blue solution before counting with haemocytometer for the remaining viable cells to determine the cell death number after gene silencing. The percentage of cell death was calculated by using the formula shown as followed.

$$\text{Percentage of cell death} = \frac{\text{Number of dead cells}}{\text{Number of cells in control}} \times 100\%$$

3.4.2 Knockdown Efficiency Evaluation on RNA Level

3.4.2.1 Total RNA Extraction

After the transfection of HeLa cells with all the expression vectors, the cells were harvested for the extraction of total RNA every 24 h for three consecutive days. The Tissue RNA Mini Kit from PKT was used to extract the total RNA from the HeLa cells. For every harvest, the cells in each well were first rinsed twice with PBS solution to remove the FBS residue prior to trypsinisation. After the cells have fully detached from the plate surface, two parts of FBS containing medium were added to a part of trypsin used. The cells were mixed carefully with gentle pipetting, followed by centrifugation at 600 x *g* for 5 min to pellet the cells. The whole total RNA extraction protocol was carried out in RNase contamination free condition by spraying the working bench and wiping the apparatus with RNase Quiet from Nacalai Tesque, Japan. After that, the cell pellet was re-suspended with the XPRB Buffer, mixed with β-mercaptoethanol,

provided by the Tissue RNA Mini Kit. The following procedure was all according to the protocol described in the Tissue RNA Mini Kit Handbook and hence, the final product was 50 µL of total RNA for each of the samples transfected with the respective expression vectors and control vectors. The extracted total RNAs were aliquoted in small amount for the synthesis of first strand cDNA and the remaining RNAs were kept in -80ºC ultrafreezer for long term storage.

3.4.2.2 cDNA Synthesis from the Extracted Total RNA

The extracted total RNA was measured by NanoDrop™ 2000 Spectrophotometer from Thermo Scientific™ to quantitate the amount of RNA. The amount of total RNA for each sample was standardised at 1 µg in each tube as the template for the synthesis of first strand cDNA. Assuming 100% efficiency on the enzymatic activity of the reverse transcriptase, this standardisation of the amount of RNA was to ensure the consistency in the amount of cDNA produced for each sample. RevertAid™ H Minus First Strand cDNA Synthesis Kit from Fermentas was used to reverse transcribe the total RNA into cDNA. In this protocol, 1 µg of the extracted total RNA was used as the template and random hexamer primer was chosen as the binding primer on the template to initiate the reverse transcription. The procedures for the reverse transcription were according to the manufacturer's protocol and were carried out in ThermoCycler from Bio-Rad. The synthesised cDNA were used as the template for the qPCR reaction and the remaining cDNAs were kept in -20ºC chest freezer for future experimental repeats.

3.4.2.3 Quantitation of RNA Amount Reduced by Gene Silencing

The synthesised cDNAs were used as the template for quantitative amplification with the gene specific primer. The list of primers used in qPCR reaction was shown in Table 3.5. Luna® Universal qPCR Master Mix from New England BioLabs® was used to prepare the amplification reaction mixtures. A total of 20 µL reaction volume was prepared for each sample which consists of 10 µL Luna Universal qPCR Mix; 0.5 µL of 10 µM forward primer; 0.5 µL of 10 µM reverse primer; 20 ng of cDNA template and an appropriate volume of dH_2O. The amplification of each sample was performed in triplicates for consistent results. CFX96 Touch™ Real-Time PCR Detection System from Bio-Rad Laboratories was used as the thermal cycler for the quantitative amplification. The cycling profile for the quantitative amplification was 60 s of initial denaturation at 95ºC; 40 cycles of denaturation at 95ºC for 15 s and extension at 60ºC for 15 s, plate reading was set directly after extension step, and lastly, the melt curve analysis was performed ranging from 60ºC to 95ºC whereby the temperature increment was set as 0.5ºC increase in one second.

After all the qPCR cycles had completed, the results were analysed by using the Bio-Rad CFX Manager™ Software Version 3.1 which came together with the CFX96 Touch™ Real-Time PCR Detection System. In analysing the results, ΔΔCt method was being utilised. The melt curve analysis was used as the standard to determine the specificity of the primer and the efficiency of the quantitative amplification. The obtained relative gene expression exhibited

efficiency of the targeted gene knockdown. Standard error of the mean (SEM) was used in this project to determine deviation between the readings of the triplicates. The significance level of the relative gene expression was analysed subsequently.

Table 3.5: List of Primers for Quantitative PCR

Primer Name	Primer Sequence
Sur Up	5'–AAGGACCACCGCATCTCTAC– 3'
Sur Down	5'–CAAGTCTGGCTCGTTCTCAG– 3'
hTERT Up	5'–GCATTGGAATCAGACAGCAC– 3'
hTERT Down	5'–CCACGACGTAGTCCATGTTC– 3'
β-Actin Up	5'–AGCCATGTACGTTGCTATCC– 3'
β-Actin Down	5'–TCGTAGATGGGCACAGTGTG– 3'

3.4.3 Knockdown Efficiency Evaluation on Protein Level

3.4.3.1 Total Protein Extraction

Total protein was extracted from the HeLa cells transfected with the respective expression vectors. The harvest of the cells began after 24 h of hygromycin B selection for every 24 h in three consecutive days. The transfected HeLa cells were washed with PBS solution twice prior to trypsinisation. Two volume of PBS solution was mixed with one volume of Trypsin added. The cells suspension was centrifuged at 600 x *g* for 5 min at 4 ºC. The pelleted cells in each tube were re-suspended with 100 µL of ice cold Cell Lysis buffer and protease

inhibitor cocktail in 1:100 dilution by gentle pipetting followed by incubation at 4 ºC for 60 min. After incubation, the lysis mixtures were centrifuged at 13,000 rpm for 10 min at 4 ºC. Subsequently, the supernatant will be transferred to another clean microcentrifuge tube and stored in -20ºC for further usage.

3.4.3.2 Total Protein Quantitation

The protein concentration of the extracted total protein was measured by using Pierce™ BCA Protein Assay Kit from Thermo Scientific. The bicinchoninic acid (BCA) protein quantitation assay was carried out by following the microplate procedure according to the manual provided by the kit. The provided bovine serum albumin (BSA) was used to prepare a set of protein standards to determine the concentration of protein colourimetrically. BCA working reagent was prepared by mixing 50 volume of BCA Reagent A to 1 volume of BCA Reagent B. In preparation for the protein samples for the assay, 2 µL of the extracted total protein was mixed with 8 µL of lysis buffer in the microplate. Subsequently, 200 µL of the BCA working reagent were added to each of the diluted protein samples and the mixtures were briefly shaken on a shaker for 30 s prior to 30 min incubation at 37ºC. The absorbance of the protein standards and samples were measured in the wavelength of 562 nm by using FLUOstar® Omega Microplate Reader from BMG LABTECH, Germany. A graph of absorbance against protein concentration was plotted by using the obtained protein standard data. Each protein standard and sample was prepared in triplicate, thus, the average absorbance was used to calculate the concentration of the protein.

3.4.3.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A 12% and 8% resolving gel of SDS-PAGE was prepared for the separation of Survivin protein and hTERT protein, respectively. The composition of the different percentage of the resolving gel and stacking gel was shown in Table 3.6 below. The extracted total protein was denatured by mixing with the Sample Buffer (SAB) containing 4% (v/v) β-mercaptoethanol followed by boiling in 95ºC water bath for 6 min. The denatured protein samples were then loaded into the stacking gel. For Survivin samples, 4 µg of total protein were loaded for single gene silencing whereas 8 µg of total protein were loaded for multiple genes silencing. On the other hand, for hTERT samples, 8 µg of total protein were loaded for single gene silencing whereas 15 µg of total protein were loaded for multiple genes silencing. The protein samples were electrophoresed in 1X running buffer at 135 V for approximately 70 min together with ExactPro Broad Range Prestained Protein Ladder from 1st BASE.

Table 3.6 Composition of Resolving and Stacking Gel

	Resolving Gel (12%)	Resolving Gel (8%)	Stacking Gel (4%)
40% Acrylamide/bis solution, 19:1	3 mL	2 mL	0.5 mL
0.5 M Tris, pH 6.8	-	-	1.3 mL
1.5 M Tris, pH 8.8	2.5 mL	2.5 mL	-
10% SDS	100 µL	100 µL	50 µL
ddH2O	4.35 mL	5.35 mL	3.2 mL
TEMED	50 µL	50 µL	25 µL
10% Ammonium persulfate (APS)	5 µL	5 µL	5 µL
Total	**10 mL**	**10 mL**	**5 mL**

3.4.3.4 Western Blot

Once SDS-PAGE is completed, the stacking gel was removed from the resolving gel and the unloaded lanes were cut off from the resolving gel by using a gel cutter. The gel fragment containing the samples and ladder was incubated in ice cold transfer buffer for 10 min. A PVDF membrane (DGel Sciences) was cut in appropriate size and immerse in pure methanol for 2 min. After that, the PVDF membrane was transfer into a clean container and incubated in ice cold transfer buffer for 10 min. Subsequently, both the PVDF membrane and gel fragment were sandwiched in between two western blotting filter papers and was placed on the platform of semi dry blotting machine WSE-4110 PoweredBLOT-One from ATTO Corporation, Japan. The top of the filter paper was carefully rolled with a roller to remove the air bubbles trapped in between the gel and the PVDF

membrane followed by the additional of ice cold transfer buffer to moist the sandwich. The transfer was carried out at 12 V for 30 min for Survivin and 50 min for hTERT. After the transfer process has completed, the PVDF membrane was carefully removed by clean forceps and washed in washing buffer (PBST) for 1 min prior to the blocking process.

For the blocking process, the PVDF membrane was immersed and incubated in blocking buffer at 200 rpm for 60 min. The primary antibody mixture was prepared by diluting the respective primary antibodies in blocking buffer at a ratio of 1:1000. After the blocking process, the PVDF membrane was rinsed twice with PBST for 5 min at 200 rpm shaker. Primary antibody was added on the membrane and the mixture was ensured to cover the surface of the PVDF membrane prior to shaking at 200 rpm for 60 min. The membrane was then rinsed twice with PBST for 5 min at 200 rpm shaker. The secondary antibody was prepared by diluting the antibody with blocking buffer at 1:5000 dilution factor. The membrane was then incubated in the secondary antibody solution for 2 h. Subsequently, the membrane was rinsed with PBST for 5 min at 200 rpm shaker for four times. The membrane was then viewed in Molecular Imager® ChemiDOC™ XRS+ from Bio-Rad Laboratories. The membrane was viewed under chemiluminescence option for 5 min exposure time. The image of the chemiluminescent bands on the membrane was captured and further analysed with the Image Lab™ Software Version 5.0 provided with the imager to quantitate the amount of protein reduced by gene silencing. The densitometry data obtained

from the software analysis was then calculated and the SEM of the sample triplicates was determined. Subsequently, the significance level of protein expression between three experimental repeats was determined for each sample.

3.4.4 Evaluation of the Transfection Cytotoxicity and Cell Proliferation

The HeLa cells were cultured in triplicate and transfected with the respective expression vectors in 96-well plate to perform the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Assay. After 24 h of hygromycin B selection, the cells were prepared for the MTT assay every 24 h for three consecutive days. The 96-well plate was centrifuged at 600 x *g* for 5 min. The medium in each well was carefully discarded followed by the addition of 10 µL of TACS® MTT Reagent from Trevigen® to each sample including the empty well as the reading blank. The plate was then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 h. After that, the plate was viewed under the microscope to ensure the purple crystal has fully formed in the wells prior to the addition of 100 µL DMSO solution. Subsequently, the plate was then read with FLUOstar® Omega Microplate Reader from BMG LABTECH to obtain the absorbance readings in the wavelength of 590 nm. The obtained data was compiled and the following formula was used to determine the cytotoxicity of the treatment plus the proliferation of the cells.

$$\text{Cell Viability} = \frac{\text{Corrected Value}}{\text{Dynamic Range}} \times 100\%$$

Corrected Value = Average OD of treated cells – Average OD of blank

Dynamic Range = Average OD of control cells – Average OD of blank

The obtained cell viability was subtracted from 100% to determine the percentage of inhibition. Standard error of the mean (SEM) between the triplicates was determined and the significance level of the inhibition effect of the gene knockdown between experimental repeats was studied and calculated with SPSS version 20.

3.4.5 Evaluation of the Apoptotic Induction

The HeLa cells were cultured in triplicate and transfected with the respective expression vectors in 96-well plate for the apoptosis assay. After 24 h of hygromycin B selection, the cells were prepared for the apoptosis assay every 24 h for three consecutive days. MitoLume Mitochondrial Apoptosis Detection Kit from GeneTex was used to perform the apoptosis assay. The wells containing transfected cells and the media were briefly rinsed with PBS solution. The Incubation Buffer provided by the kit was pre-warmed at 37ºC and used to dilute the MitoLume Reagent at a ratio of 1:1000. The cells were then mixed with 100 µL of the diluted MitoLume Reagent and incubated at 37ºC in a humidified 5% CO_2 incubator for 20 min. Following incubation, the 96-well plate was then centrifuged at 500 x *g* for 5 min. The supernatants were discarded and 100 µL of pre-warmed Incubation Buffer were added to each sample in the plate. The 96-

well plate was then read by the FLUOstar® Omega Microplate Reader from BMG LABTECH, Germany, using the fluorescent filter to detect the fluorescent at the excitation spectrum of 488 nm and emission spectrum of 530 nm for green monomers whereas for red aggregates, the excitation spectrum of 488 nm and emission spectrum of 590 nm were used. The fluorescence readings were recorded for further processing.

Subsequently, the same 96-well plate was used to view the apoptotic cells with Nikon ECLIPSE T*i* Inverted Microscope to qualitatively determine the induction of apoptosis. The Incubation Buffer in the sample wells were removed prior to viewing. The fluorescence filters were adjusted to view the green monomer and the red aggregates of the stained cells. The fluorescent images of the living and apoptotic cells were captured by using the NIS Elements BR Software Version 4.40 in 100X magnification separately and merged to compare the quantities of each cells. The previously obtained fluorescence readings were calculated to obtain the data of the number of apoptotic cells after gene silencing. The SEM of each sample of the triplicate and the statistical significance between all the three experimental repeats was determined.

3.4.6 Statistical Analysis

Standard error of the mean (SEM) of three independent experiments, carried out in triplicates, was determined by using the Student's t-Test provided by the Microsoft Excel® Software. The results were shown as mean ± SEM.

On top of the SEM, the statistical analysis of the collected data was performed using Statistical Package for Social Sciences (SPSS) through the IBM® SPSS® Statistics Software version 20, Chicago. The three independent experimental repeats carried out in each section were analysed to determine the statistical significance of the data. The mean values of all the results were compared by using one-way ANOVA. The experimental repeats were considered significant if $P < 0.05$ which was marked with an asterisk, * in the charts.

CHAPTER 4

RESULTS

4.1 Oligonucleotides Annealing

After the design of the shRNA of the target region has been completed, the oligonucleotides which contain the shRNA, the loop region and the multiple endonuclease restriction sites were ordered to be made. The single stranded oligonucleotides were annealed to form the double stranded DNA as the insert into the expression vector, pU6-m4. The size of the annealed oligonucleotides is approximately 65 bp. The image of the electrophoresed annealed oligonucleotides in 4% (w/v) agarose gel was shown in Figure 4.1 below.

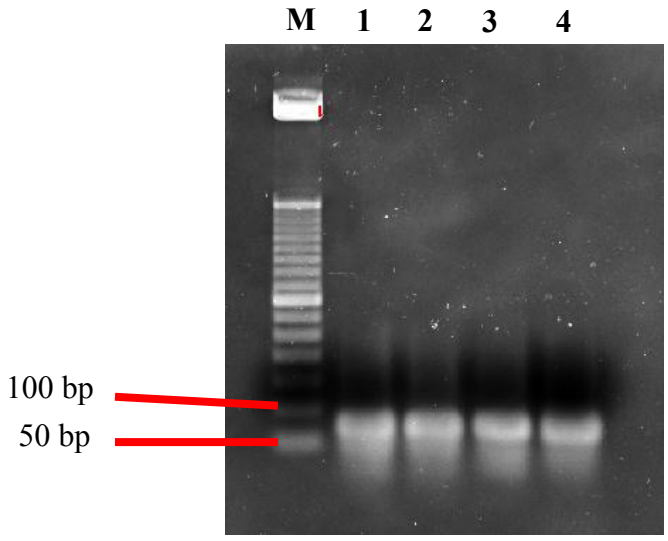

Figure 4.1: Gel electrophoresis image of annealed oligonucleotides in 4% (w/v) Agarose Gel. Lane M represents the Invitrogen 50 bp DNA-ladder whereas lane 1 to 4 represents annealed oligonucleotides of survivin, hTERT, scrambled survivin and scrambled hTERT, respectively.

4.2 Construction of Expression Vector

4.2.1 Single shRNA Expression Vector Construction

The annealed oligonucleotides were inserted into the doubly restricted pU6-m4 expression vector to form the single shRNA expression vector and it is represented in Figure 4.2 below. The insert which is approximately 65 bp was inserted into the 4.9 kb pU6-m4 vector. The expression vector for survivin, hTERT, scrambled survivin and scrambled hTERT were named pB1, pT1, pSB and pST, respectively.

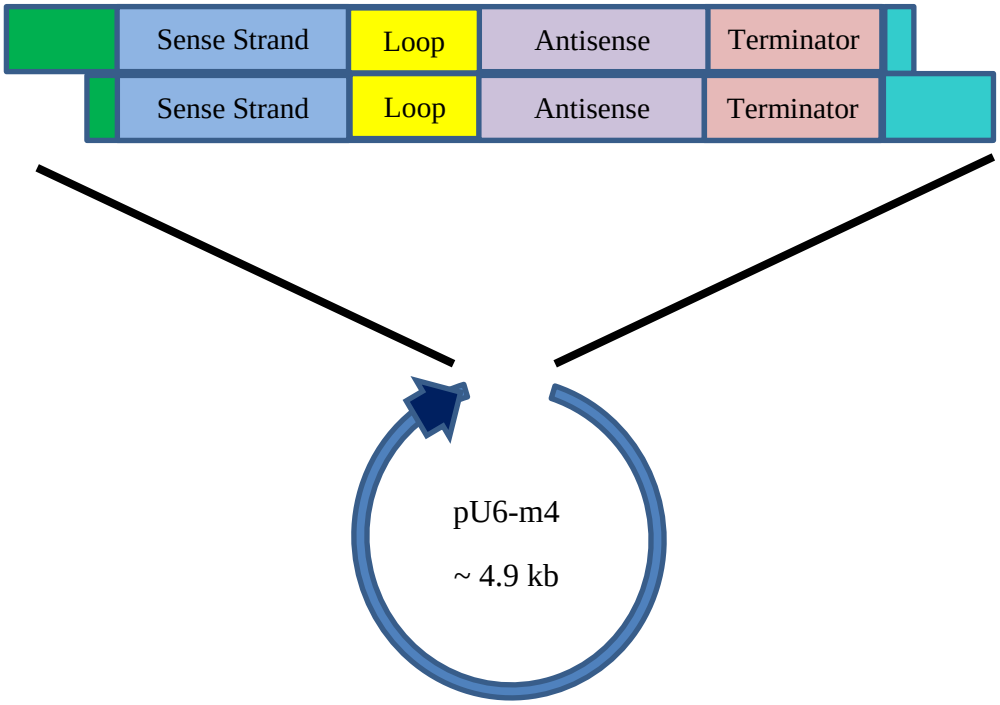

Figure 4.2: Schematic representation of the insertion of annealed oligonucleotides into the linearised pU6-m4 vector. Top: Composition of the double-stranded oligonucleotides of the shRNA; Bottom: Linearised pU6-m4 expression vector with a U6 promoter.

4.2.2 Multiple shRNAs Expression Vector Construction

The double shRNAs expression vector was named pBF57 and the double scrambled shRNAs expression vector was named pS5. These expression vectors were constructed by digesting one constructed single shRNA expression vector to form the cassette containing the U6 promoter and a single shRNA whereas another single shRNA expression vector was linearised by two different types of restriction enzyme. The construction is represented in Figure 4.3 below.

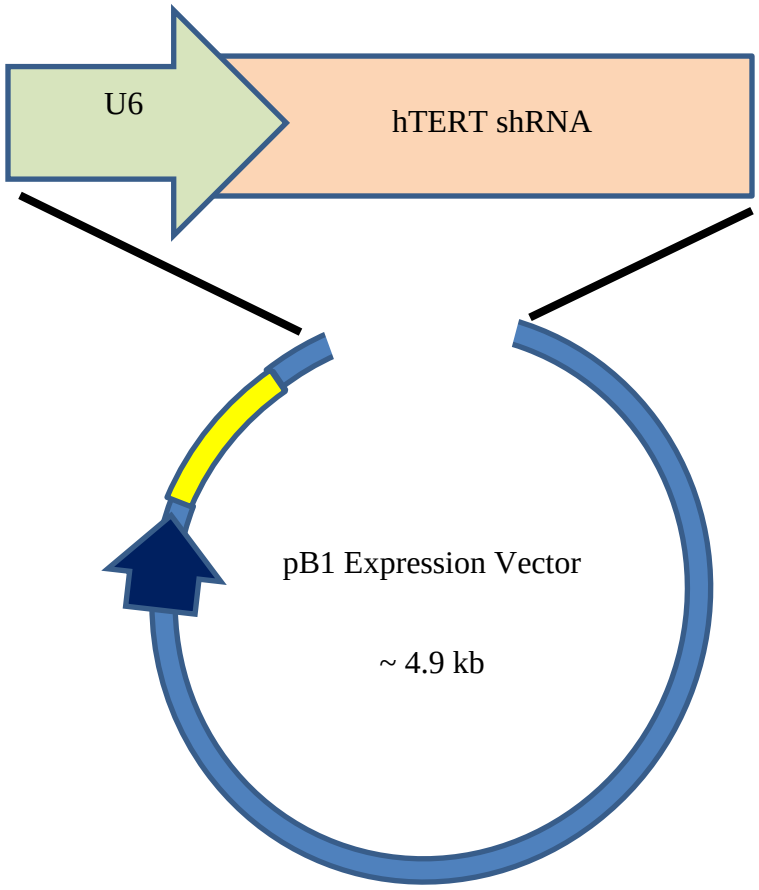

Figure 4.3: Schematic representation of the construction of the double shRNA expression vector, pBF57. Top: The cassette obtained from pT1; Bottom: The linearised pB1 vector whereby the U6 promoter and survivin shRNA was intact. The size of pBF57 was approximately 5.3 kb.

4.3 Verification of Cloned Expression Vectors

4.3.1 Agarose Gel Electrophoresis of Cloned Expression Vectors

The cloned pB1, pT1, pSB1, pST1, pBF57 and pS5 were all electrophoresed in 0.8% (w/v) agarose gel in order to check the differences in size to verify the cloning process. The electrophoresis image of the expression vectors is show in the following Figure 4.4.

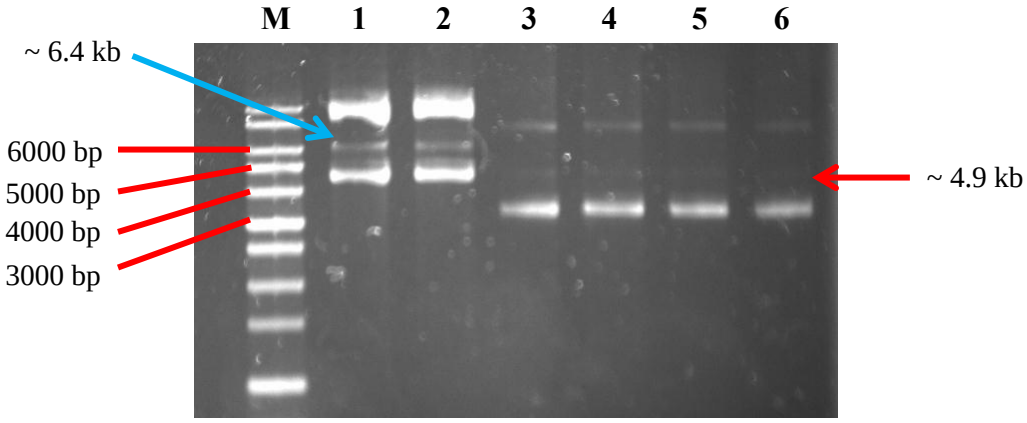

Figure 4.4: Gel electrophoresis image of cloned expression vectors. Lane M represents the GeneRuler 1 kb DNA ladder whereas lane 1, 2, 3, 4, 5, and 6 represents the purified pBF57, pS5, pB1, pT1, pSB1 and pST1, respectively. The red arrow represents the size of the linear conformation of the vector pB1, pT1, pSB1 and pT1 whereby the size of the vectors is approximately 4.9 kb. The blue arrow represents the location of the linear conformation of the vectors pBF57 and pS5 whereby the size is approximately 6.4 kb.

4.3.2 Sequencing of Cloned Expression Vectors

After the confirmation of the cloned expression vectors' size, the vectors were transformed into the competent *E. coli* cells. The transformants that showed resistance to Ampicillin were extracted for the expression vectors. The purified vectors were thereby sent for the DNA sequencing service to verify the target genes sequences were present. The partial images of the DNA sequencing electropherograms were shown in the following figures together with the alignment with the gene database by using the BLAST software. The full details of the electropherograms were attached in the Appendices section.

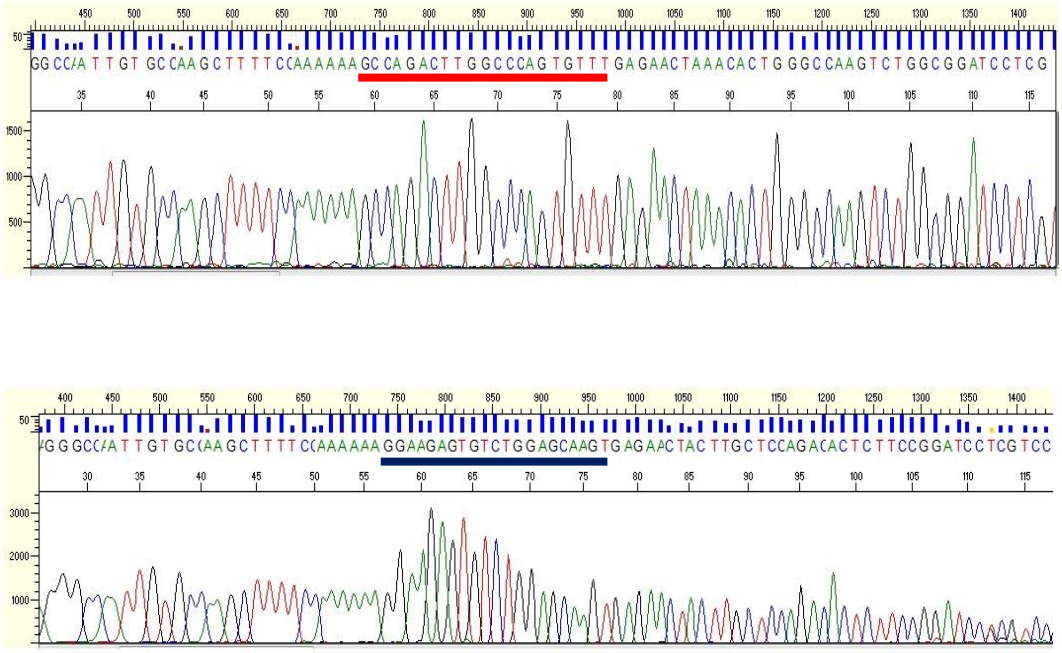

Figure 4.5: Electropherograms of the DNA sequencing of the purified expression vectors. Top: The DNA sequencing electropherogram of pB1 with the red line underlining the sequence of survivin shRNA; Bottom: The DNA sequencing electropherogram of pT1 with the blue line underlining the sequence of hTERT shRNA.

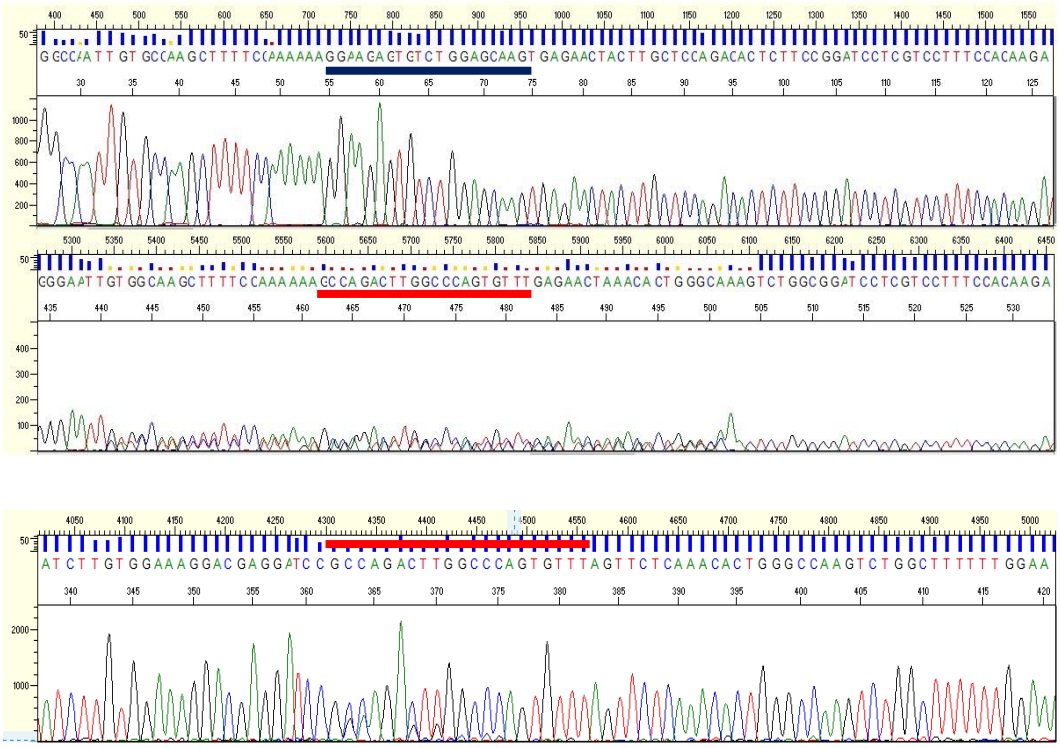

Figure 4.6: Electropherograms of the DNA sequencing of the purified pBF57 vector. The blue line shows the sequence of hTERT shRNA is present in the vector whereas the red line shows the sequence of survivin shRNA is present in the vector; the top two partial electropherograms were extracted from the same single DNA sequencing electropherogram reading by using the 2.0 Rev primer whereas the bottom partial electropherogram was extracted from DNA sequencing using T7 Promoter primer due to the low signal of survivin sequencing from the reverse primer.

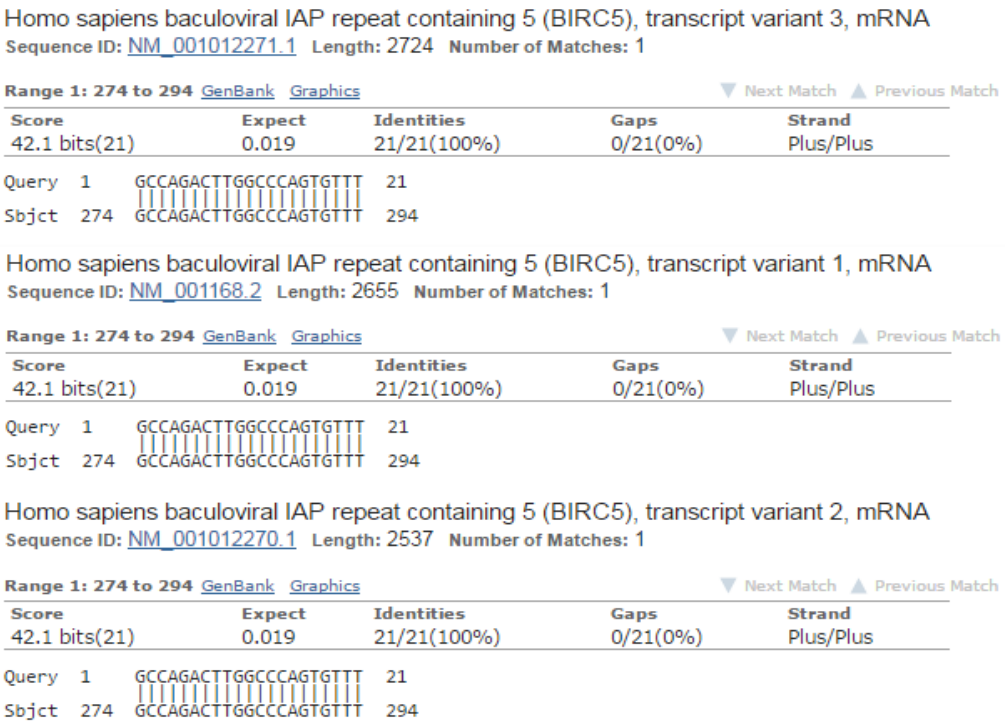

Figure 4.7: Identity and alignment of shRNA sequence of pB1 to the sequence of survivin in the Reference RNA sequence database by using BLAST.

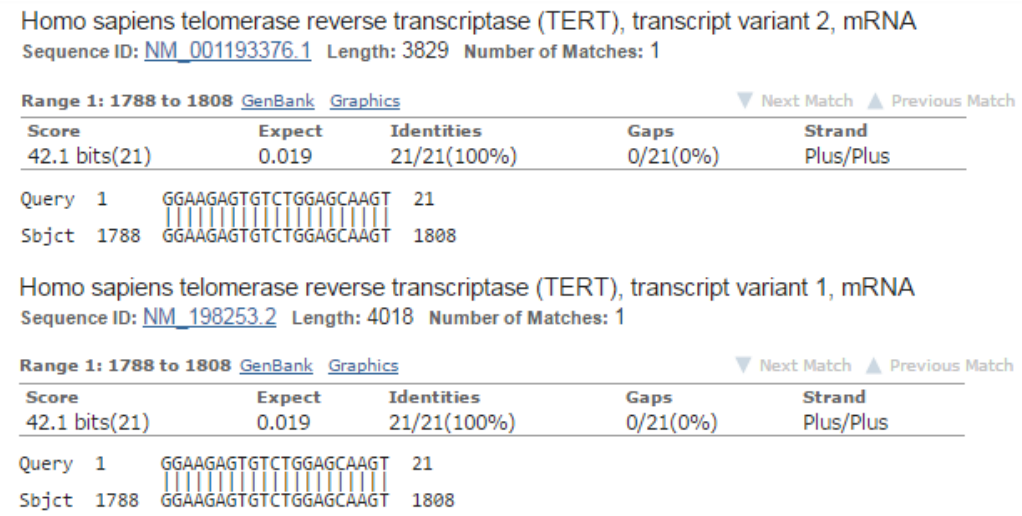

Figure 4.8: Identity and alignment of shRNA sequence of pT1 to the sequence of hTERT in the Reference RNA sequence database by using BLAST.

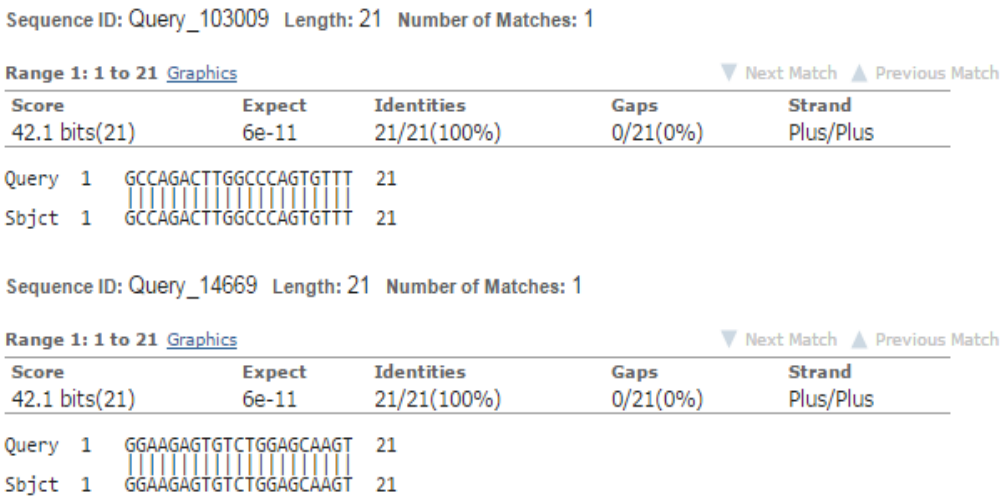

Figure 4.9: Identity of the shRNA sequences of pBF57 to the designed sequences of the survivin and hTERT shRNAs.

Since the sequenced DNA nucleotides of the cloned expression vectors showed 100% identity to the survivin and hTERT in the gene database, this indicated the successful cloning of the shRNAs into the pU6-m4 expression vector. Hence, the cloned pB1, pT1, pBF57 and all the scrambled DNA vectors were used in the downstream experiments to determine the knockdown efficiency of the targeted genes.

4.4 Cell Counting

The cloned expression vectors, pB1, pT1, pBF57, and the scrambled DNA vectors were all transfected to the HeLa cells. The number of HeLa cells seeded into each of the 6-well plate was 2 x 10^5 cells. Thereby, the cell counting was conducted each day for three days consecutively on three independent

experiments to determine the number of cell death induced upon transfection with the expression vectors. The data collected were listed in Table 4.1.

Table 4.1: Total cell count per sample and the percentage of cell inhibition.

Day and Sample Name	Experiment 1 (X 10^5 cells/ml)	Experiment 2 (X 10^5 cells/ml)	Experiment 3 (X 10^5 cells/ml)	Average Total Inhibition (%)
Control	2.500	8.250	10.000	0.000 ± 0.000
pU6m4	2.250	8.000	10.250	3.510 ± 3.616
pS5	2.000	7.500	9.500	11.364 ± 4.477
pSB	2.250	7.750	9.750	6.187 ± 2.166
pST	2.000	8.000	9.500	9.343 ± 5.359
D1 pBI	1.750	5.750	6.750	30.934 ± 0.788
D2 pBI	1.500	4.000	5.000	47.172 ± 3.612
D3 pBI	0.750	2.250	3.250	70.076 ± 1.509
D1 pTI	1.750	6.000	7.250	28.258 ± 0.874
D2 pTI	1.500	5.000	6.500	38.131 ± 1.576
D3 pTI	1.000	3.250	3.500	61.869 ± 1.576
D1 pBF57	1.500	4.500	6.250	40.985 ± 2.348
D2 pBF57	1.000	2.750	4.500	60.556 ± 3.379
D3 pBF57	0.500	1.750	1.750	80.429 ± 1.093

Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted. The differences in control cell number were due to different number of cells seeded into the well before each transfection.

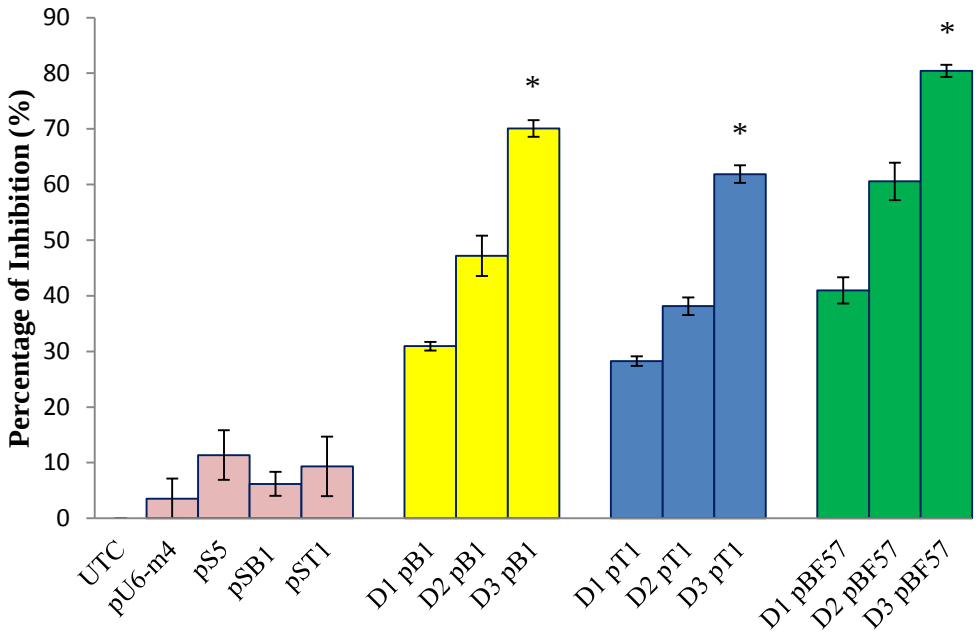

Figure 4.10: Percentage of inhibition of each sample in their particular day. Data of percentage of inhibition was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on Table 4.1 and Figure 4.10, after 72 hours of transfection, the total percentage of HeLa cells death induced after transfected with pB1, pT1 and pBF57 were 70.1%, 61.9% and 80.4%, respectively. These indicated the silencing of survivin were more effective than that of hTERT whereas silencing both genes simultaneously induced even higher number of HeLa cell death.

4.5 Quantitative RT-PCR Analysis

Since RNAi will degrade the mRNA of target gene, the mRNA expression level of the HeLa cells transfected with pB1, pT1, pBF57 and all the scrambled DNA vectors was evaluated by using the quantitative reverse transcription polymerase chain reaction (qRT-PCR). The total mRNA of each sample was extracted after 24 hours, 48 hours and 72 hours post transfection. Reverse transcription was carried out for each total mRNA extracted to obtain the double-stranded cDNA as the template for qRT-PCR analysis. The relative expression of each sample obtained was listed in Table 4.2 and Table 4.3 below.

Table 4.2: Relative expression of survivin mRNA in each sample

Day and Sample Name	Relative Expression 1	Relative Expression 2	Relative Expression 3	Average Relative Expression ± Standard Deviation
D1 pB1	0.731	0.704	0.852	0.762 ± 0.045
D2 pB1	0.560	0.585	0.658	0.601 ± 0.029
D3 pB1	0.407	0.484	0.395	0.429 ± 0.028
pSB	1.880	0.951	0.894	1.242 ± 0.319
UTC	1.000	1.000	1.000	1.000 ± 0.000
D1 pBF57	0.531	0.442	0.592	0.522 ± 0.044
D2 pBF57	0.335	0.298	0.298	0.310 ± 0.012
D3 pBF57	0.236	0.184	0.187	0.202 ± 0.017
pS5	0.854	0.833	1.170	0.953 ± 0.109
UTC	1.000	1.000	1.000	1.000 ± 0.000

Legend: pB1 is the expression vector with survivin shRNA, pSB is the expression vector with scrambled survivin shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted; UTC represents untransfected control.

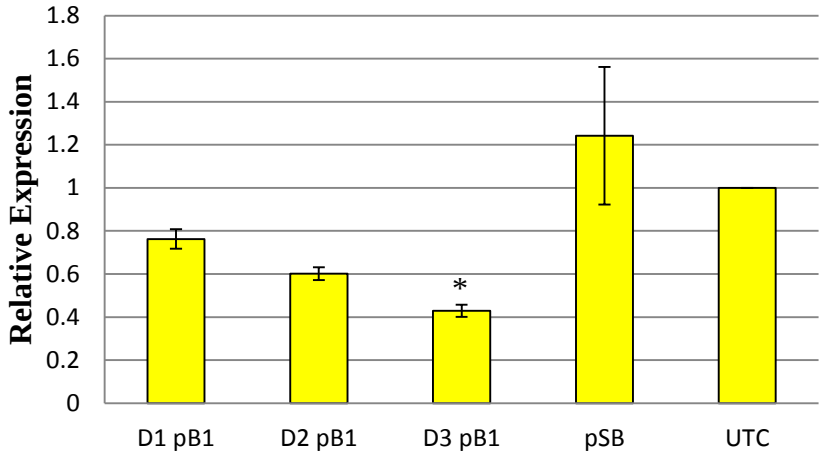

Figure 4.11: Relative expression of survivin mRNA in HeLa cells transfected with pB1 and pSB. Data of relative expression was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pB1 is the expression vector with survivin shRNA, pSB is the expression vector with scrambled survivin shRNA; D1 to D3 represent Day 1 to Day 3 of the transfection conducted; UTC represents untransfected control.

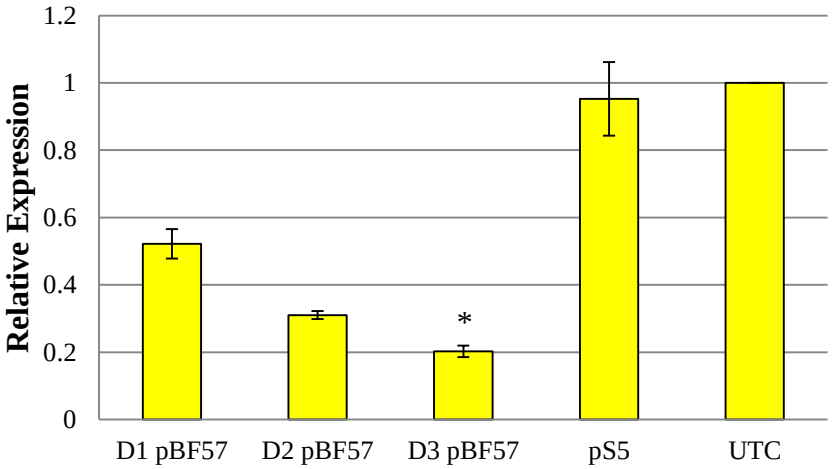

Figure 4.12: Relative expression of survivin mRNA in HeLa cells transfected with pBF57 and pS5. Data of relative expression was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted; UTC represents untransfected control.

Based on Figure 4.11 and Figure 4.12, the knockdown efficiency of pB1 and pBF57 on survivin mRNA was shown to be approximately 58 % and 80 %, respectively, after three days of transfection, showing that the mRNA expression level reduction by the knockdown of survivin singly was not as effective as knocking down both survivin and hTERT simultaneously.

Table 4.3: Relative expression of hTERT mRNA in each sample.

Day and Sample Name	Relative Expression 1	Relative Expression 2	Relative Expression 3	Average Relative Expression ± Standard Deviation
D1 pT1	1.062	0.749	0.741	0.851 ± 0.106
D2 pT1	0.782	0.571	0.647	0.666 ± 0.062
D3 pT1	0.526	0.484	0.466	0.492 ± 0.018
pST	0.954	0.877	0.935	0.922 ± 0.023
UTC	1.000	1.000	1.000	1.000 ± 0.000
D1 pBF57	1.153	0.733	0.445	0.777 ± 0.206
D2 pBF57	0.627	0.438	0.350	0.472 ± 0.082
D3 pBF57	0.336	0.138	0.216	0.230 ± 0.058
pS5	1.048	0.850	1.855	1.251 ± 0.307
UTC	1	1	1	1.000 ± 0.000

Legend: pT1 is the expression vector with hTERT shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted; UTC represents untransfected control.

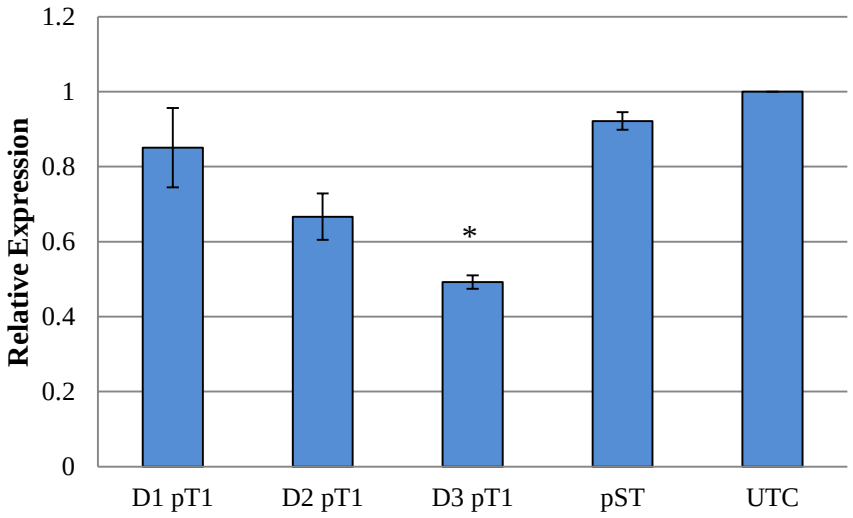

Figure 4.13: Relative expression of hTERT mRNA in HeLa cells transfected with pT1 and pST. Data of relative expression was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pT1 is the expression vector with hTERT shRNA, pST is the expression vector with scrambled hTERT shRNA, D1 to D3 represent Day 1 to Day 3 of the transfection conducted; UTC represents untransfected control.

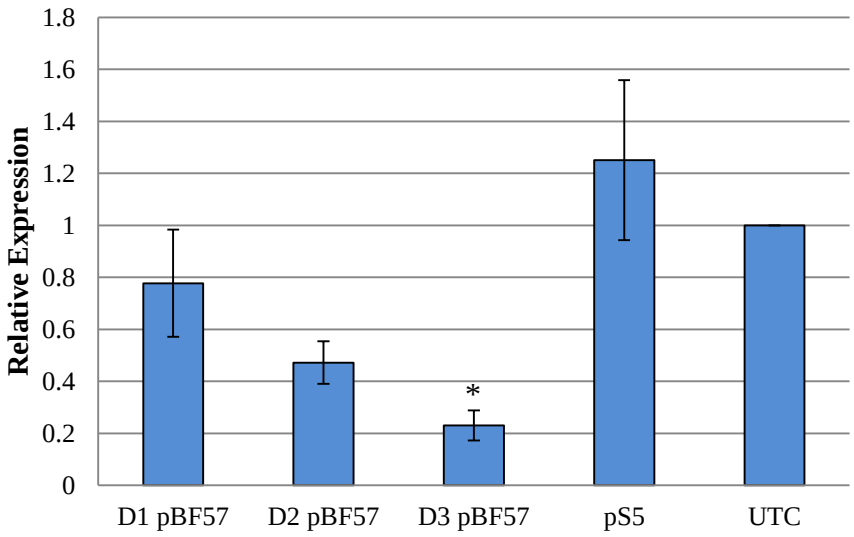

Figure 4.14: Relative expression of hTERT mRNA in HeLa cells transfected with pBF57 and pS5. Data of relative expression was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on Figure 4.13 and Figure 4.14, the knockdown efficiency of pT1 and pBF57 on hTERT mRNA was shown to be approximately 51 % and 77 %, respectively, after three days of transfection. These showed that knocking down both genes simultaneously reduced more target mRNA expression.

4.6 Western Blot Analysis

Since mRNA expression level might affect the level of protein expression, the HeLa cells transfected with each expression vector were also extracted for the total protein lysate in order to determine the level of protein inhibition by western blot analysis. The images of the western blot for each sample were show in the following figures.

4.6.1 Survivin Protein Inhibition

The following figures show the western blot of the total protein extracted from the HeLa cell line transfected with pB1, pBF57 and the scrambled DNA expression vectors, pSB and pS5.

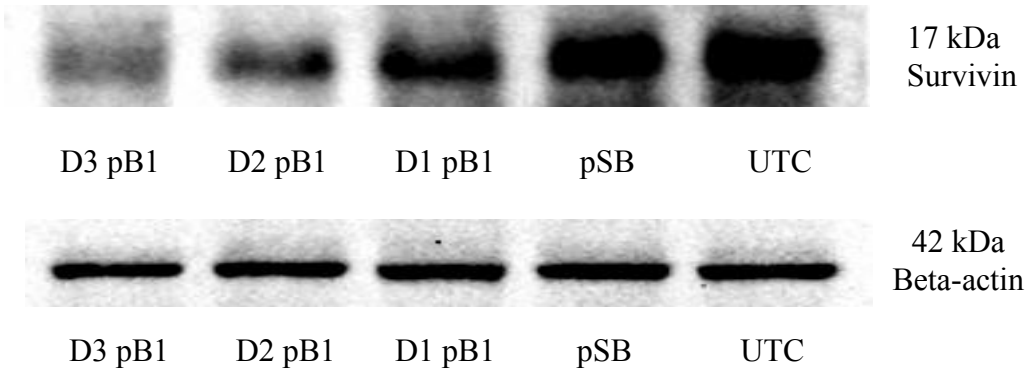

Figure 4.15: Western blot image of HeLa cells transfected with pB1 and pSB. Legend: pB1 is the expression vector with survivin shRNA, pSB is the expression vector with scrambled survivin shRNA; D1 – D3 represent Day 1 to Day 3 after transfection; UTC represents untransfected control.

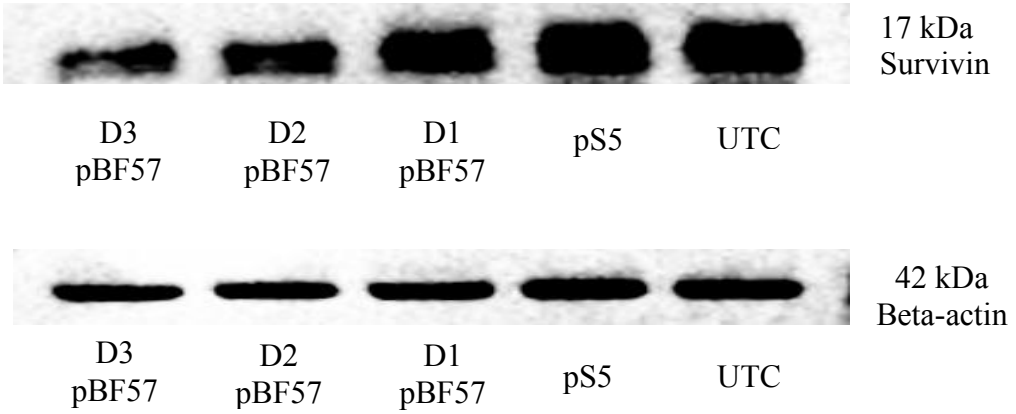

Figure 4.16: Western blot image of HeLa cells transfected with pBF57 and pS5. Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 – D3 represent Day 1 to Day 3 after transfection; UTC represents untransfected control.

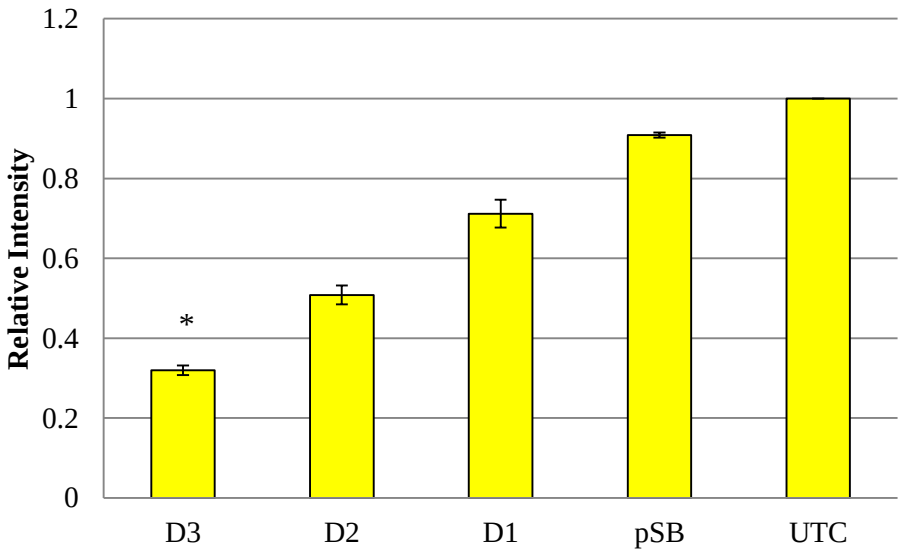

Figure 4.17: Relative intensity of western blot band from HeLa cells transfected with pB1 and pSB. Data of relative intensity was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pB1 is the expression vector with survivin shRNA, pSB is the expression vector with scrambled survivin shRNA; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

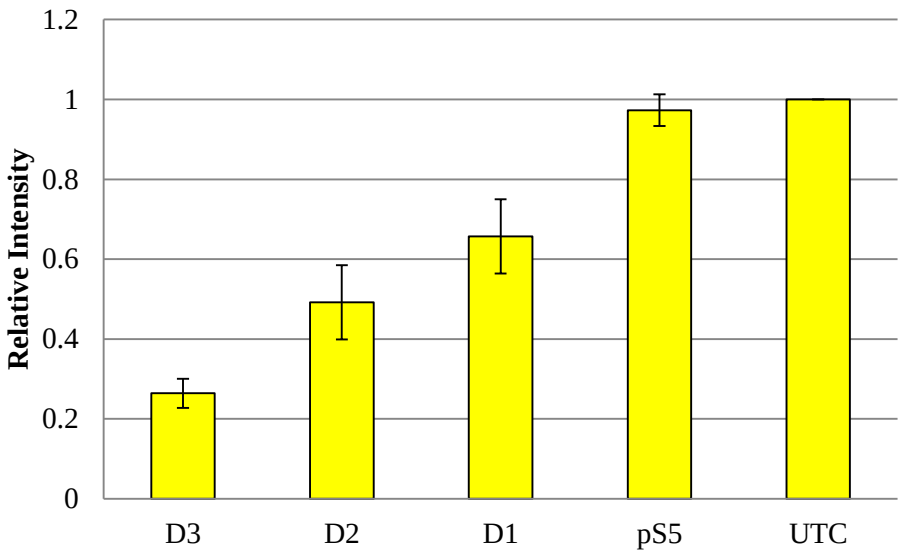

Figure 4.18: Relative intensity of western blot band from HeLa cells transfected with pBF57 and pS5. Data of relative intensity was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on Figure 4.17 and Figure 4.18, the survivin protein inhibition in HeLa cells by pB1 and PBF57 was approximately 68 % and 74 %, respectively, after three days of transfection. These showed that silencing survivin would cause a reduction in survivin protein expression and even more reduction could be observed by silencing both survivin and hTERT simultaneously.

4.6.2 HTERT Protein Inhibition

The following figures show the western blot of the total protein extracted from the HeLa cell line transfected with pT1, pBF57 and the scrambled DNA expression vectors, pST and pS5.

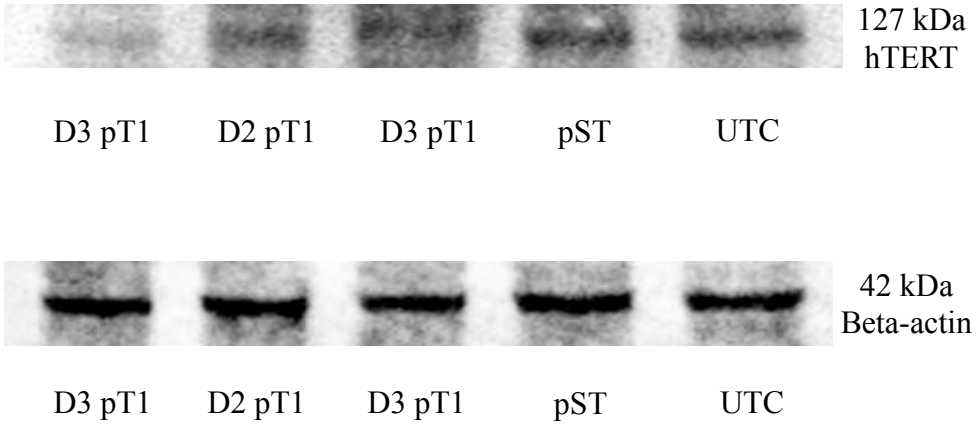

Figure 4.19: Western blot image of HeLa cells transfected with pT1 and pST. Legend: pT1 is the expression vector with hTERT shRNA, pST is the expression vector with scrambled hTERT shRNA; D1 – D3 represent Day 1 to Day 3 after transfection; UTC represent untransfected control.

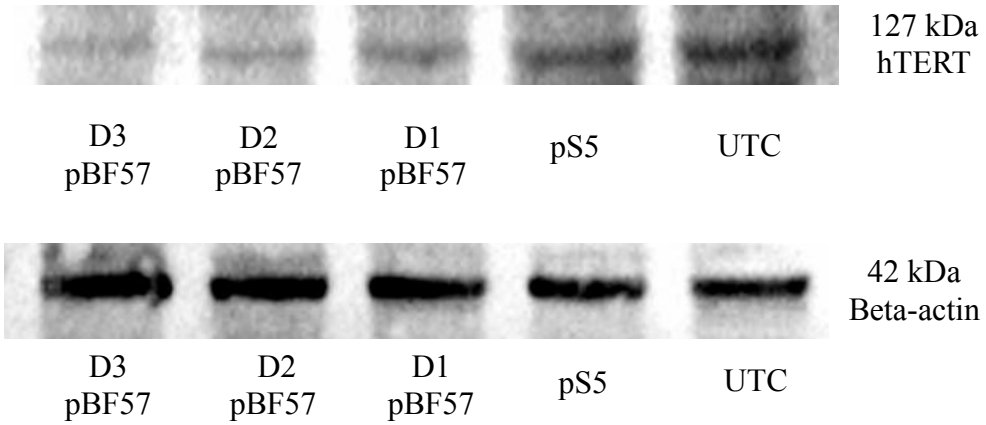

Figure 4.20: Western blot image of HeLa cells transfected with pBF57 and pS5. Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

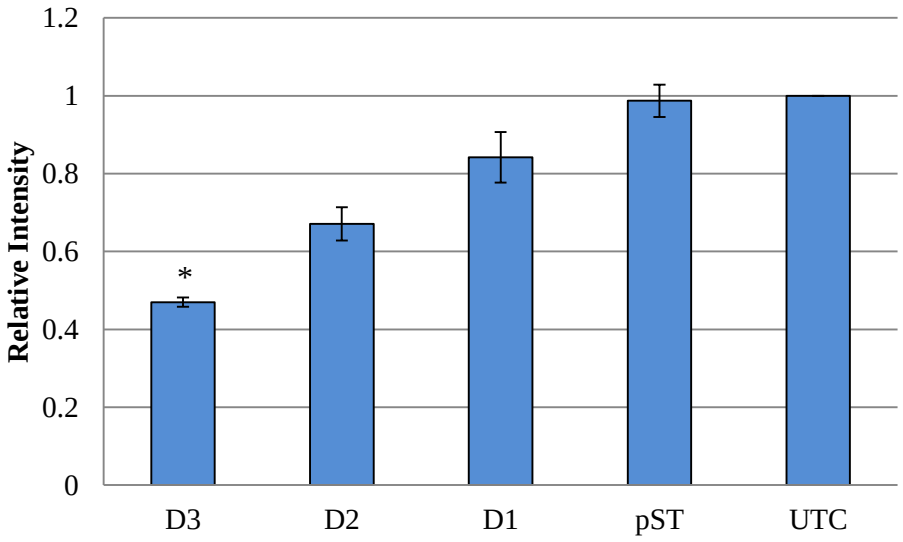

Figure 4.21: Relative intensity of western blot band from HeLa cells transfected with pT1 and pST. Data of relative intensity was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pT1 is the expression vector with hTERT shRNA, pST is the expression vector with scrambled hTERT shRNA; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

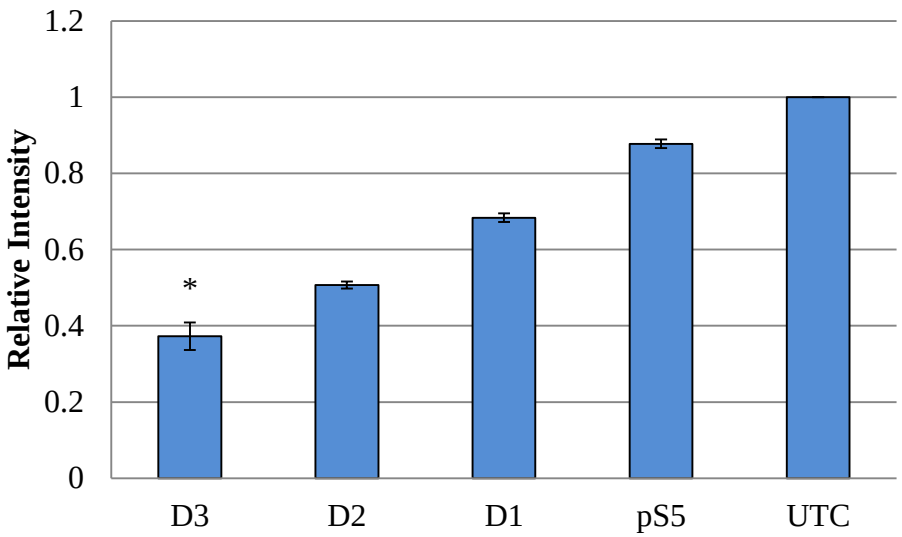

Figure 4.22: Relative intensity of western blot band from HeLa cells transfected with pBF57 and pS5. Data of relative intensity was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on Figure 4.21 and Figure 4.22, the hTERT protein inhibition in HeLa cells by pT1 and PBF57 was approximately 53 % and 63 %, respectively, after three days of transfection. These indicate that simultaneously silencing both survivin and hTERT gene would cause the higher reduction in hTERT protein expression compared to silencing hTERT gene alone.

4.7 Cytotoxicity and Proliferation Assay (MTT Assay)

The cytotoxicity effect of the expression vector to the HeLa cells was studied by using the MTT assay as well as the effect of gene knockdown on cell proliferation. Each well in the 96-well plate was seeded with 1 x 10^4 cells and transfected with pB1, pT1, pBF57 and all the scrambled DNA expression vectors. Data was collected every 24 hours for three consecutive days by using the FLUOstar® Omega Microplate Reader (BMG LABTECH). The data collected was listed in Table 4.4

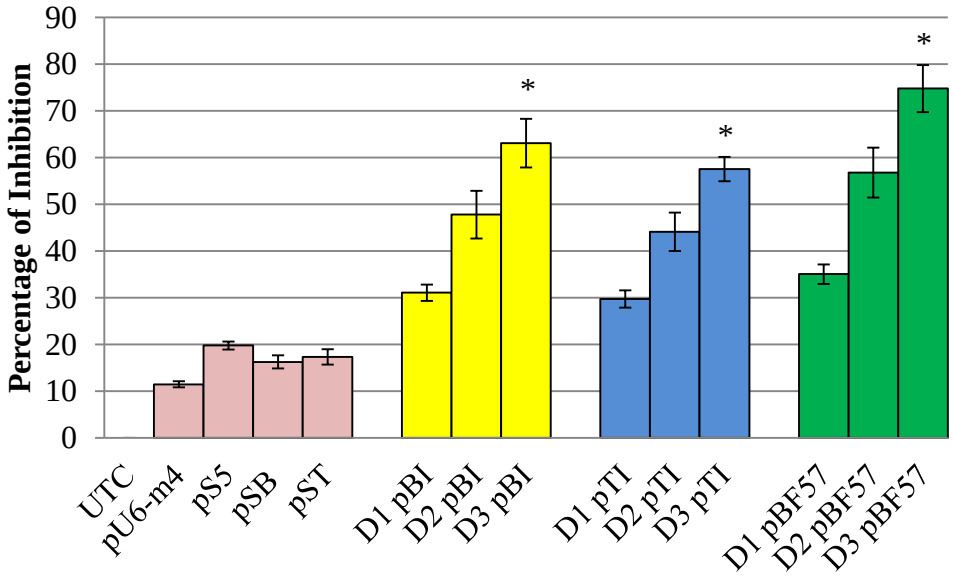

Figure 4.23: Percentage of HeLa cell inhibition in MTT assay. Data of percentage of inhibition was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Table 4.4: Percentage of cell inhibition by MTT assay

Day and Sample Name	Cell Inhibition 1 (%)	Cell Inhibition 2 (%)	Cell Inhibition 3 (%)	Average Total Cell Inhibition (%) ± Standard Deviation
Control	0.000	0.000	0.000	0.000 ± 0.000
pU6m4	12.653	10.371	11.459	11.494 ± 0.659
pS5	19.563	21.385	18.420	19.789 ± 0.863
pSB	16.935	18.303	13.635	16.291 ± 1.385
pST	15.117	20.581	16.359	17.353 ± 1.654
D1 pBI	27.588	33.173	32.488	31.083 ± 1.759
D2 pBI	40.787	57.727	44.883	47.799 ± 5.103
D3 pBI	53.975	72.027	63.217	63.0733 ± 5.211
D1 pTI	26.031	31.537	31.681	29.749 ± 1.860
D2 pTI	37.550	51.651	43.110	44.104 ± 4.101
D3 pTI	52.318	60.254	60.061	57.544 ± 2.614
D1 pBF57	31.168	35.758	38.311	35.079 ± 2.090
D2 pBF57	47.207	65.746	57.408	56.787 ± 5.361
D3 pBF57	64.747	79.433	80.138	74.773 ± 5.017

Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on Figure 4.23, the total percentage of HeLa cell death showed by MTT assays for transfection with pB1, pT1 and pBF57 were 63 %, 58 % and 75 %, respectively, after three days. The total percentage of HeLa cell death shown in this assay included apoptosis and necrosis. Hence, apoptosis assay is required to be conducted in order to determine the amount of apoptosis induced.

4.8 Apoptosis Assay

4.8.1 Apoptosis Assay by Fluorescent Microscopy

After the HeLa cells had been transfected with pB1, pT1, pBF57 and all the scrambled DNA expression vectors, the cells were treated with the reagents from MitoLume Apoptosis Detection Kit (GeneTech) and were further viewed under the Nikon ECLIPSE T*i* Inverted Microscope to determine the amount of apoptosis qualitatively. Below are the images obtained from the microscopy analysis.

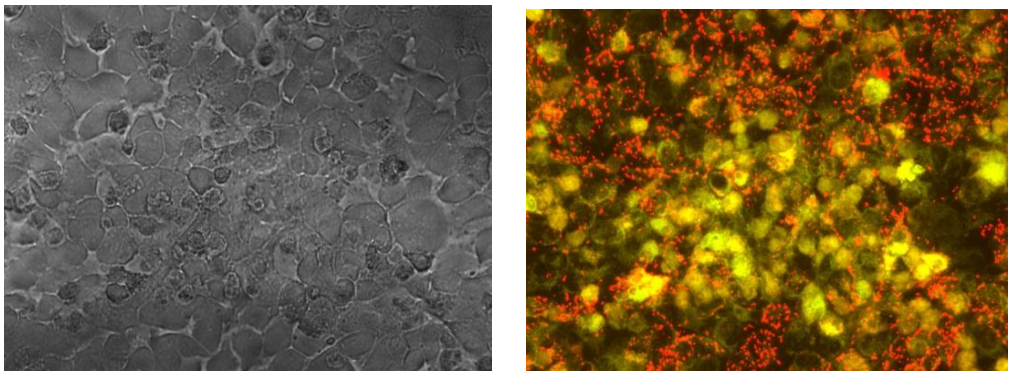

Figure 4.24: Fluorescent Microscopy Analysis of HeLa cells transfected with pB1 expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells.

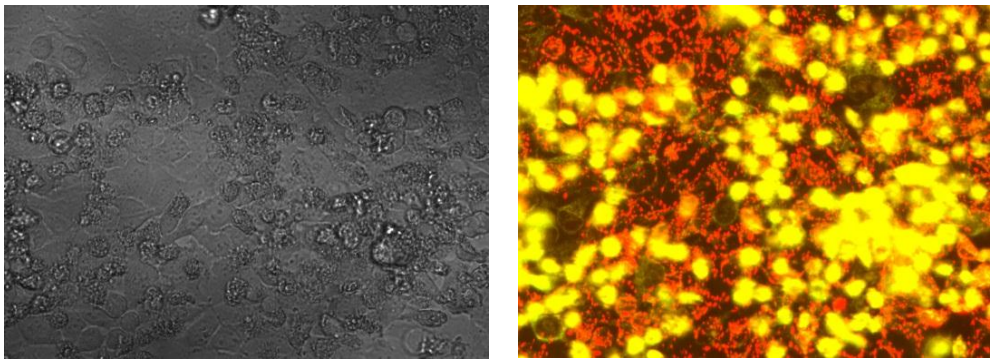

Figure 4.25: Fluorescent Microscopy Analysis of HeLa cells transfected with pT1 expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells.

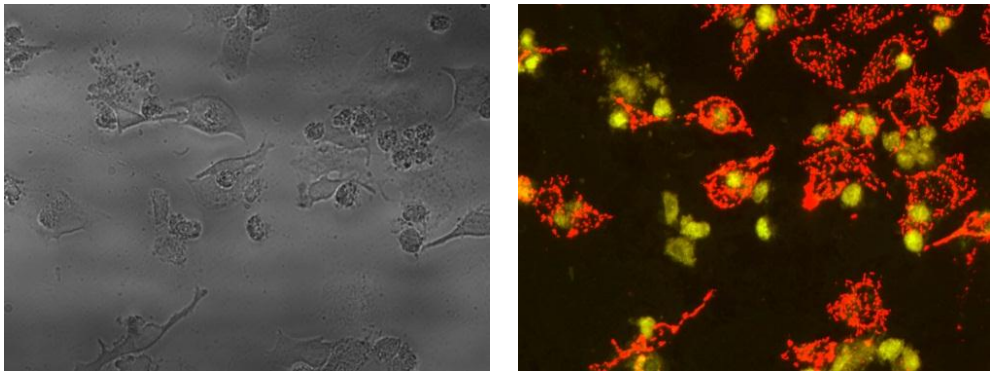

Figure 4.26: Fluorescent Microscopy Analysis of HeLa cells transfected with pBF571 expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells).

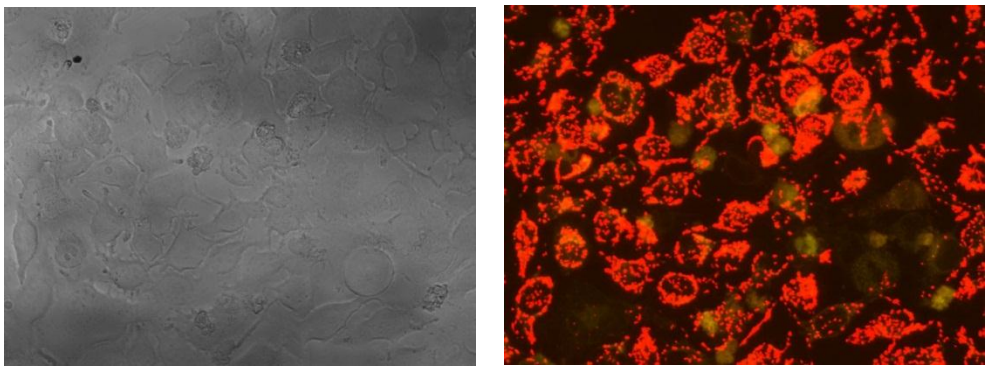

Figure 4.27: Fluorescent Microscopy Analysis of HeLa cells transfected with pSB scrambled DNA expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells).

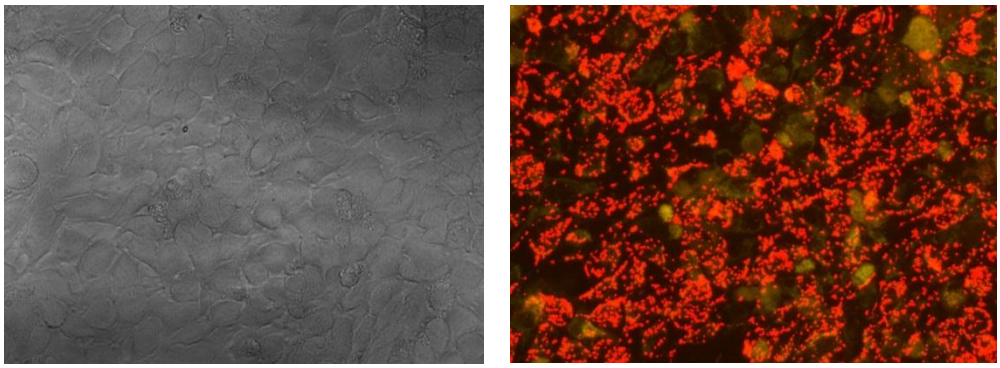

Figure 4.28: Fluorescent Microscopy Analysis of HeLa cells transfected with pST scrambled DNA expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells).

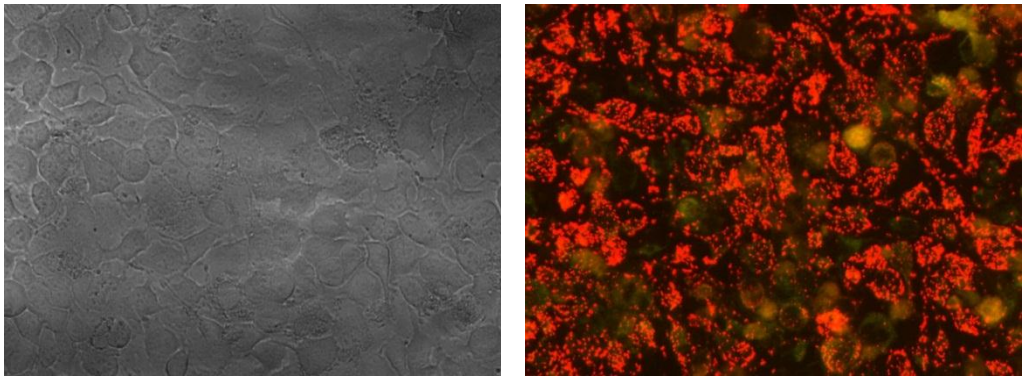

Figure 4.29: Fluorescent Microscopy Analysis of HeLa cells transfected with pS5 scrambled DNA expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells).

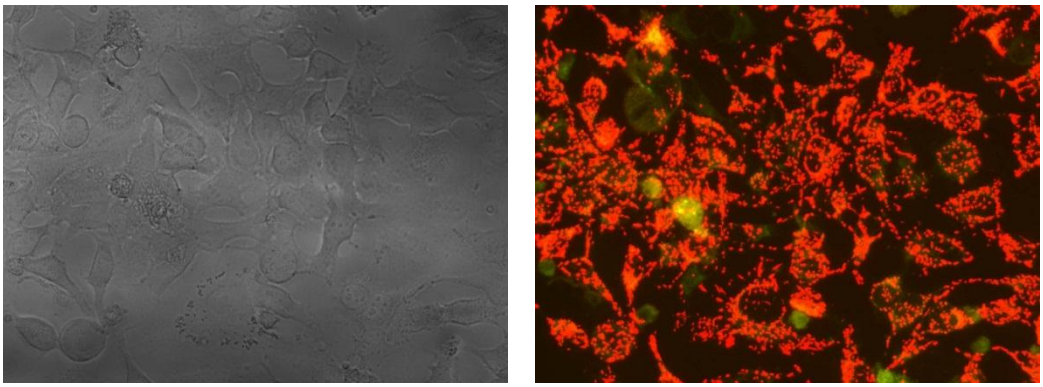

Figure 4.30: Fluorescent Microscopy Analysis of HeLa cells without transfection of expression vector after 72 hours. (Left: Bright field view of the cell culture well; Right: Fluorescent view of the cells treated with MitoLume Kit whereby green indicates apoptosis while red indicates living cells).

All the images above had shown that the transfection with pB1, pT1 and pBF57 shRNA expression vector had induced the apoptosis of HeLa cells. The amount of apoptotic cells were higher in cells transfected with pBF57 compared to that of pB1 and pT1. The amount of living cells was also greatly reduced throughout the 72 hours post-transfection.

4.8.2 Apoptosis Assay by Relative Fluorescent Unit Quantification

Other than fluorescent microscopy, the apoptosis assay analysis was further determined quantitatively by measuring the relative fluorescent unit by using FLUOstar® Omega Microplate Reader (BMG LABTECH). The readings obtained were converted to percentage of inhibition and listed in Table 4.5.

Table 4.5: Percentage inhibition of HeLa cells in apoptosis assay

Day and Sample Name	Cell Inhibition 1 (%)	Cell Inhibition 2 (%)	Cell Inhibition 3 (%)	Average Total Cell Inhibition (%) ± Standard Deviation
Control	0.000	0.000	0.000	0.000 ± 0.000
pU6m4	9.257	8.747	8.904	8.969 ± 0.151
pS5	15.785	11.516	13.917	13.740 ± 1.236
pSB	4.760	10.151	11.786	8.899 ± 2.123
pST	6.529	6.608	11.525	8.221 ± 1.652
D1 pBI	24.968	28.406	28.665	27.346 ± 1.192
D2 pBI	46.574	46.627	49.776	47.659 ± 1.059
D3 pBI	74.990	65.042	69.751	69.928 ± 2.873
D1 pTI	25.373	28.620	25.007	26.333 ± 1.148
D2 pTI	42.731	42.459	39.885	41.692 ± 0.907
D3 pTI	65.683	51.344	60.085	59.037 ± 4.172

D1 pBF57	35.071	41.536	39.339	38.649 ± 1.898
D2 pBF57	73.467	63.998	69.637	69.034 ± 2.750
D3 pBF57	97.219	76.406	82.005	85.210 ± 6.218

Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

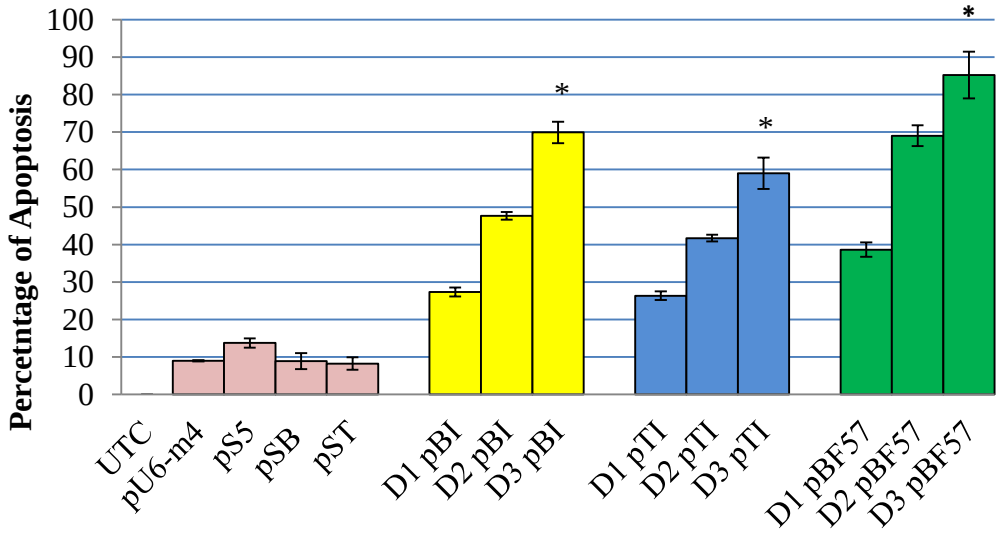

Figure 4.31: Percentage of apoptosis induced by using the relative fluorescent unit. Data of percentage of apoptosis was expressed in mean ± SEM (error bars) obtained from three independent experiments. The mean values were compared by using one-way ANOVA. * $p < 0.05$ as compared to untransfected control (UTC). Legend: pU6m4 is the expression vector without shRNA, pB1 is the expression vector with survivin shRNA, pT1 is the expression vector with hTERT shRNA, pSB is the expression vector with scrambled survivin shRNA, pST is the expression vector with scrambled hTERT shRNA, pBF57 is the expression vector with survivin and hTERT shRNAs, pS5 is the expression vector with scrambled survivin and scrambled hTERT shRNAs; D1 to D3 represent Day 1 to Day 3 of the transfection conducted.

Based on the results shown in Figure 4.31, the percentage of HeLa cells induced to apoptosis was approximately 70%, 60% and 85% for the transfection with pB1, pT1 and pBF57, respectively, for three days. These results indicate that the knockdown of survivin has induced higher percentage of apoptosis compared to that of hTERT. Moreover, silencing both survivin and hTERT genes simultaneously has induced higher percentage of apoptosis of HeLa cells compared to that of single gene.

CHAPTER 5

DISCUSSION

5.1 Genes Selection

There is an abundance of molecular markers of cancer discovered since more details of the nucleotides arrangement in human body has been made available by the Human Genome Project. To name a few of the discovered genes related to cancer, there are p53, EFGR, PARP, Brca1, Bcl-2 and many other notable genes. However, survivin and hTERT genes were selected to be the target in this project due to the pivotal roles of these genes in contributing to the immortality of cancer. Moreover, in the attempt to discover the potential cancer therapy, the specificity is of the outmost importance. These two genes were discovered to be highly expressed in cancer cells while their expression in normal terminally differentiated cells and most of the adult somatic tissues, for survivin and hTERT, respectively, are close to none or negligible (Paydas et al., 2003; Johnson and Howerth, 2004; Rudolph, 2008). Hence, by targeting these genes is equivalent to targeting cancer cells specifically omitting the unwanted side effects to other normal cells.

5.1.1 Survivin Gene in Cancer

Survivin plays an important role as the inhibitor of apoptosis in tumorigenesis or in the multistage progressive mutation of genes involved in the neoplastic transformation of a normal cell. However, other than the inhibitor of apoptosis, survivin has other potent abilities in facilitating the neoplasm of cells. According to Li (2003) and Johnson and Howerth (2004), survivin functions involved the regulation of cancer cell division as well. The high expression of survivin in G_2/M phase in the mitosis process during cell division indicates its function in blocking the cell growth regulators from sending signals that terminate cell division (Johnson and Howerth, 2004). Therefore, the silencing of survivin gene implies the removal of two major hallmarks of cancer which are of high importance in the tumorigenesis process. The capabilities granted to cancer cell by the high expression of survivin are the insensitivity to antigrowth signals and the evasion of apoptosis, based on the hallmarks of cancer as described by Hanahan and Weinberg (2000).

Furthermore, survivin has been a potent molecular target of the researchers in developing the cancer therapy. The researchers can gain two huge achievements in the cancer cure development, whereby the malignant tumours can be highly induced to apoptosis and the resensitisation of the cancer cells to apoptosis-inducing agents (Altieri, 2003b). There are due to the properties of survivin that contributed to the resistance of cancer towards other approaches of cancer therapy that utilised the induction of apoptosis. Based on the study by Li et

al. (1998), the survivin role in regulating cell division indicates its ability to bind to the mitotic spindles as well as microtubules. These bindings have given rise to the resistance of cancer towards chemotherapies that employ the microtubule-targeting agent, the taxanes, such as taxol and taxotere. Besides that, the study by Asanuma er al. (2000) had demonstrated the role of survivin in contributing the resistance of cancer cells towards radio-therapy by using a sublethal doses of radiation to trigger the upregulation of survivin mRNA expression. This can be seen in hormonal cancer therapy as well whereby the flutamide administered by Zhang et al. (2005) did not induce the apoptosis of prostate cancer cells in the presence of survivin. Therefore, by targeting survivin gene, apoptosis of cancer cells could be induced and it is possible to remove the resistance of cancer towards other anticancer therapies.

5.1.2 HTERT Gene in Cancer

The role of telomerase in maintaining the telomere length is crucial in providing the immortality to cancer cells. However, hTERT is of higher priority to be targeted since it exhibits the rate-limiting factor in the reactivation of telomerase activity. Other than its role in maintaining the integrity of the chromosome end, it has another function which is to cap the end telomere at the end of the chromosomes. This capping effect has made the cancer cell to evade from apoptosis since the DNA damage by the attrition of telomeric length has been covered by the activity of hTERT (Zaffaroni et al., 2007; Rudolph, 2008). Thus, hTERT has contributed to two major hallmarks of cancer whereby they are

the abilities to achieve unlimited replicative potential and evasion of apoptosis (Hanahan and Weinberg, 2000). Therefore, hTERT is another remarkable molecular target of anticancer therapies.

5.2 Target Region Selection in Designing shRNA

The incumbent roles of survivin and hTERT had made them the major target of researchers in the development of anticancer therapies. Thus, the two of them were chosen as the main targets in this project in order to study their silencing effects on cancer simultaneously. However, due to the availability of different splice variants of both genes, there are three isoforms of survivin protein and two isoforms of hTERT protein (Johnson and Howerth, 2004; Zaffaroni et al., 2007). In order to specifically target the mRNA and protein of these genes, the design of the shRNAs of both genes must target all the isoforms to ensure the effective knockdown of the mRNAs expression. The survivin shRNA designed in this project is able to bind with all three isoforms of survivin and hTERT shRNA will bind to all the two isoforms of hTERT.

Other than targeting all the isoforms of the genes, the design required to be specifically targeting the nucleotides within the exon region of the gene of interest to avoid the design of sequences that targets nothing after the splicing activities of the targeted genes. Moreover, the composition of the nucleotides in the shRNA must confer for the highest efficacy to provide effective knockdown. Hence, the

designed shRNA sequence that matched with all the isoforms of the target genes must be subjected to the analysis of the algorithms to determine the efficacy of the designed set of nucleotides. Furthermore, the choice of hairpin sequence in the loop of the shRNA also plays a major role in affecting the knockdown efficiency. The shRNA hairpin loop sequence in this project followed the highly recommended sequence provided by the manual of the expression vector to ensure effective knockdown of survivin and hTERT genes.

5.3 Advantages of RNAi Over Other Anticancer Therapies

There are many other methods in targeting cancer, for instance, the surgery removal of tumour tissue, chemotherapy, radiotherapy and several other hormonal therapies. Out of all of those methods, RNAi is a sequence-specific mechanism that target directly to the cancer genes. In chemotherapy, the most specific approaches include the use chemical that target the cell-growth factor of cancer cells or any drugs that initiate the generation of reactive-oxygen-species (ROS) in killing the cells. The former has been proven to be less effective in the presence of high survivin expression as this protein will block the binding of the chemical to the microtubules rendering the chemicals ineffective whereas the latter is undoubtedly effective in killing cancer cells but the killing worked to all the adjacent healthy tissues as well (Zaffaroni et al., 2007; Watson, 2013). The similar resistance to radiotherapy occurred in the presence of elevated survivin gene expression, moreover, the previous therapies utilising radiation has caused higher occurrence of new cancer cases besides killing cancer cells (Zaffaroni et al.,

2007; Mukherjee, 2011).

On the other hand, the therapies that target hormones were working on most of the cancer patients but not all. This is due to the presence of exogenous hormones from the food they consumed, for instance, the oestrogen-positive breast cancer whereby the patients' removal of the oestrogen-producing organ were suffering from cancer remissions after certain period of time (Muhkherjee, 2011). The immunotherapy of cancer had similar observation as well whereby the drug that target the gene of breast cancer worked on half of the women tested but ineffective to the other half. After a serial of researches, it was found out that there were *Her-2* positive and *Her-2* negative, an oncogene that might or might not present in breast cancer. Thus, the use of Trastuzumab as the antibody for breast cancer would not work for patients that are *Her-2* negative (Mukherjee, 2011). Therefore, RNAi is the most potent method in the development of anticancer therapies due to its sequence-specific nature in targeting the oncogene or any other crucial genes in cancer and most importantly, the mechanism has already hard-wired in the human body.

5.4 Advantages of shRNA

Most of the scientists that are dealing with RNAi will prefer to use shRNA instead of siRNA. One of the main reasons in the lack of usage of siRNA is the introduction of the siRNA into the cell cytoplasm might initiate the cell

cytotoxicity effect due to the action of dsRNA-responsive protein kinase (Wianny et al., 2004). Besides that, the initial concentration of siRNA administered into the cell will be diluted along with the progressive cell division whereas the vector-based introduction of shRNA is capable of contributing DNA integration in the cell nucleus. Thus, the shRNA is consistently expressed in the cell, even after all the progressive cell cycles (Moore et al., 2010). The durability of shRNA has also made it as the preferable choice as shRNA can be expressed in the cell for up to three years whereas the siRNA will be mostly degraded in 46 hours after the delivery into the cell. The specific off-target effects chances in shRNA are also lower than that of siRNA (Mansoori et al., 2014). Furthermore, it is more time-efficient in using shRNA as the amount of antisense oligonucleotides produced by shRNA is double of that compared to siRNA. Therefore, shRNA has more benefits to be used as the targeted therapy for cancer.

5.5 Treating HeLa Cells with shRNA

Cervical cancer was chosen to be the cancer target in this project due to its commonness among cell culture practitioners. The HeLa cell line is widely used in a majority of cell culture laboratories and it is the firstly discovered immortal cell line in the earlier years of cell and tissue culture. HeLa cell line has a shorter doubling time compared to several other cancer cell lines such as the breast cancer cell line, MDA-MB-468, and liver cancer cell, HepG2. This shorter doubling time has made many data obtainable in a short period of time and the effect of the treatment can be determined earlier.

Other than that, cervical cancer is in the top 10 list of leading cancer-caused death in many other developing countries. Even in the United States of America, there is an increasing number of new cases reported annually which may indirectly leads to higher mortality rate due to cervical cancer (Siegel et al., 2018). Therefore, there is a need to develop a potential cancer therapy for cervical cancer in order to reduce the mortality rate of cervical cancer in females worldwide. The current available HPV vaccination functions as the prevention to viral infection that might lead to cervical cancer. This vaccination does not cure cervical cancer. Hence, the development of a cancer cure specific to cervical cancer is crucial to contribute to the community of females.

5.6 Determining the Efficacy of Gene Silencing

5.6.1 Analysing the Expression of mRNA

Since RNAi is a sequence-specific post-transcriptional mechanism, the gene silencing was conducted by targeting the transcribed RNAs of survivin and hTERT. Thus, the efficacy of the expression vectors in silencing the target genes has to be evaluated from the expression of mRNA. In this project, the transfection of the expression vectors into the HeLa cells has knocked down the expression of survivin and hTERT genes in HeLa cells. The function of pB1 is to express the shRNA targeting the survivin mRNA in order to silence the expression of survivin mRNA and survivin protein. The function of pT1 is to express the shRNA targeting the hTERT mRNA to knockdown the hTERT gene and protein.

Subsequently, the function of pBF57 is to carry out the roles of pB1 and pT1 simultaneously to determine the enhancement of the silencing effects.

Therefore, in order to evaluate the expression of survivin mRNA and hTERT mRNA in HeLa cells, RT-qPCR assays were carried out. The expression of survivin mRNA was successfully down-regulated to 58% and 80% for single gene knockdown and double gene knockdown, respectively (Figure 4.11 and Figure 4.12). On the other hand, the hTERT mRNA expression was successfully reduced to 51% and 77% for single gene knockdown and double gene knockdown, respectively (Figure 4.13 and Figure 4.14).

5.6.2 Analysing the Protein Expression

Proteins will be expressed up-stream from the DNA transcriptional process by translating the transcribed mRNA. Since mRNA has been targeted in this experiment, the expression of protein needs to be evaluated to further determine the specificity of the designed shRNA in reducing the expression of survivin mRNA and hTERT mRNA. It was hypothesised that the reduction in mRNA level would lead to the reduction in protein expression. Therefore, the amount of protein expressed in HeLa cells was determined by using the Western Blot analysis. It was discovered that the silencing of mRNA indeed has led to the reduction in protein expression. The amount of survivin protein has shown to have been reduced to 74% and 68% (Figure 4.17 and Figure 4.18), while the

hTERT protein expression has been reduced to 63% and 53% (Figure 4.21 and Figure 4.22), for single gene knockdown and double gene knockdown, respectively.

5.6.3 Comparison between mRNA and Protein

The amount of mRNA and protein for both the survivin and hTERT genes were found to be different. There were differences in the knockdown efficacies between both genes and there were also differences in silencing effect on mRNA and protein level of the same gene. It was discovered that the abundance of each protein in the body was different. Certain protein are more abundant than the others and hence, the higher expression level. Survivin protein was found to be more abundant compared to that of hTERT. It was also discovered that the bigger size protein was more difficult to be silenced. Thus, the lower mRNA and protein reduction observed in hTERT gene compared to that of survivin whereby the size of survivin and hTERT protein are 17 kDa and 127 kDa, respectively. Moreover, the expression of protein level does not always co-relate to the expression of mRNA level which was probably due to the differences in half lives of the mRNA and protein (Vogel and Marcotte, 2012; Edfors et al., 2016; Liu et al., 2016).

5.6.4 Cell Proliferation Assay

As mentioned earlier, survivin and hTERT are two pivotal proteins in maintaining the survival of cancer cells. Since both genes had been silenced in the

RNA level, the amount of cancer cell death was required to be determined. It was found out that 75% of cell death was observed in double gene knockdown whereas 63% of cell death was obtained by silencing survivin alone and 58% of cell death triggered by silencing hTERT gene (Figure 4.23). Hence, there was a correlation between the reduction of mRNA and protein which were important for cancer, to cause the cancer cell death. However, cell death could include necrosis and apoptosis. In order to determine the efficacy of cancer cells induced to apoptosis by silencing survivin and hTERT genes, the apoptosis assay was carried out to measure the amount of apoptotic bodies.

5.6.5 The Trend in Gene Silencing

Gene silencing is usually conducted in the timeframe of a few days whereby the results obtained might not be optimal on the first day post-transfection of the cell with expression vector. Since this project aims to induce apoptosis of cancer cells, the transfection process has to be a transient transfection. This is because all the transfected cancer cells would eventually be dead in the culture after a few days as compared to other silencing experiment which did not target the crucial protein for cell survival. Transfection requires time in order for the cell to process the injected expression vector and to express its functions. Hence, the result on the first day might not be optimum, therefore, the experiments were carried in three days' time while most the cells were found to be dead on the fourth day which caused the extraction of sample mRNA and protein could not be accomplished. In this project, the expression of mRNA of

both survivin and hTERT were found to be decreasing from the first day post-transfection to the third day. Besides that, the amount of cell death in MTT assays and apoptosis assay were found to be increasing in percentage throughout the three days post-transfection. Thus, genes silencing has been successfully performed.

5.7 Apoptosis of Cancer Cell

The final achievement of any other cancer therapy researches has most probably the apoptosis. Similarly, the main objective of this project is to induce the apoptosis of HeLa cells. Apoptosis is the programmed cell death which is more preferable to the premature death, the necrosis. There are many advantages of apoptosis compared to that of necrosis. While the process of apoptosis is fully natural, the necrosis is triggered when there is presence of external harm factors such as toxins, infections or even trauma. The effect of necrosis is usually detrimental compared to apoptosis which is to maintain the cellular homeostasis by removing the damaged cells. The symptoms involved in apoptosis are usually insignificant or none as compared to necrosis that usually coming up with inflammation or even gangrene which is tissue death. Cell apoptosis usually does not required any medical treatment whereas necrosis usually highly requires treatment as the process might lead to death. The apoptosis process involved the blebbing of cell membrane, cell shrinkage, condensation of chromatin, fragmentation of chromosomal DNA and the formation of apoptotic bodies which will eventually be engulfed by the body immune cells. On the other hand, necrosis

involved the disruption of cell membrane, hypoxic condition due to depletion of ATP, collapsed metabolism and inflammation which is caused by swelling of the cell and cell rupture (Kate et al., 2018).

5.7.1 Induction of Apoptosis by the Expression Vectors pB1, pT1 and pBF57

From the obtained data, it was determined that pB1 has successfully induced 70% of cell apoptosis, pT1 has induced 60% of cell apoptosis whereas the combination of the two, pBF57 has induced 85% of apoptosis (Figure 4.31). These indicate the role of shRNA in targeting cancer gene is specific and the combination of gene silencing will provide an even better effect in the repression of gene functions.

For the treatment with pB1 expression vector, the 70% induced apoptosis of HeLa cells corresponded to the research done by Carter (2003) which used the antisense oligonucleotide method to supress the expression of survivin in myeloid leukemia. For the treatment with pT1 expression vector, the 60% induction of HeLa cells apoptosis coincided with the study completed by Fu et al. (2005) which has successfully silenced more than 50% of survivin expression by using the antisense oligonucleotide method in treating colon cancer.

In the combined treatment part, George et al. (2010) has combined the silencing of hTERT and interferon-gamma and obtained approximately 95% of supressed mRNA and protein expression. Ganesh et al. (2013) had used cisplatin in combination with survivin silencing to induce the apoptosis of human non-small cell lung cancer and this study had effectively knockdown close to 90% of the gene. In another research done by Fu et al. (2005), they had combined the silencing of hTERT and hTR simultaneously and achieved approximately 70% of apoptosis induction as compared to target hTERT singly which gave rise to 50% of apoptosis induction. On the other hand, the outcome of targeting survivin and hTERT genes simultaneously in this project was the induction of 85% of cell apoptosis in cervical cancer. All these discoveries showed that the treatment for cancer is better in combination as compared to singly targeting one cancer gene.

5.8 Combined Treatment in Cancer

All this while, the war on cancer has never won by only targeting one factor in cancer. Even in earlier years, the chemotherapy that had temporarily control the cancer from relapsing for more than five years was a combination of several drugs that worked together to kill of the cancer cells. For example, the VAMP treatment which is the combination of vincristine, amethopterin, mercaptopurine and prednisone has successfully control the cancer from relapsing until the cancer had gained resistance towards this combination. Then, emerged a new combination which is BVP that composed of bleomycin, vinblastine and cisplatin whereby all work together and had succeeded in treating lung cancer in

year 1974 before this regimen was rejected by a newer mutation of cancer. Other combined treatments in earlier years include the adjuvant therapy by using chemotherapy to subsequently treat cancer patient that has undergone radiotherapy or surgical removal or tumour (Sudhakar, 2009; Mukherjee, 2011).

Furthermore, Watson (2013) has mentioned the requirement of a synthetic lethal approach to perform better in curing cancer. By saying this, he meant that cancer is a combination of DNA mutation that happened in the cell before it turned malignant and ultimately, metastasise to distant sites. A synthetic lethal approach is conducted by targeting a combination of two or more genes that is crucial to the tumourigenesis and survival of cancer. This is one of the strong evidences that support the combined targeting of the survivin and hTERT genes in this project to induce apoptosis of HeLa cells. This is because by targeting these two genes, four of the major hallmarks of cancer will be removed leading to the apoptosis of cancerous cells. As mentioned earlier, survivin has granted cancer cells the abilities to ignore antigrowth signals and evasion of apoptosis whereas hTERT has given cancer cells the capabilities to has unlimited replicative potential and evasion of apoptosis. The overexpression of these two genes in cancer cells will lead to the final hallmark which is the capability to invade distant sites and metastasis. Therefore, by silencing survivin and hTERT simultaneously, all these four hallmarks of cancer are targeted and apoptosis can be highly induced.

Other than that, both of the extrinsic and intrinsic pathways of apoptosis can be triggered by targeting survivin and hTERT simultaneously. Both survivin and hTERT silencing will trigger the intrinsic pathway of apoptosis whereby the perturbation of the mitochondrial membrane integrity will cause the release of cytochrome c that eventually lead to caspases production to kill the cell. However, according to Paydas et al. (2003), survivin could bind to extrinsic factor to suppress the apoptosis process. Hence, by silencing survivin, apoptosis of the cell could be induced by the extrinsic pathway as well.

5.9 Targeting Survivin and hTERT in Normal Healthy Cells

The main reason survivin and hTERT are labelled as the potential target genes for cancer therapy is their abnormally high expression in cancer cells especially the metastatic cancer cell. Normal human cells and tissues have either no sign of expression or negligible expression of the genes (Shariat et al., 2004; Zaffaroni et al., 2007). Therefore, it would be extremely difficult to target these two genes in normal cells or there will be no observable significant effect.

However, there is exceptional case in term of survivin gene. Though it was mentioned that this gene will not be expressed in normal, terminally differentiated adult tissue, the expression of survivin in embryonic cells and stem cells are significant. These cells required high division number to be developed and undergo differentiation to form specific tissues and finally become organs. It was

reported by Uren et al. (2000) that embryonic cell that lacked survivin expression was found dead *in utero* post-gestation due to disruption in cytokinesis during cell cycle. Moreover, Xing et al. (2004) has reported that survivin plays a major role in the maturation of T lymphocytes. Though the role of survivin in inhibiting apoptosis in T cells development is not significant, its role in assisting cell division during mitosis contributed a lot to the final maturation of T lymphocytes and hence, silencing survivin in patient with defective immune system will cause the underdevelopment of required T cells (Xing et al., 2004).

On the other hand, hTERT gene is expressed in extremely low level or even not expressed in normal human somatic cells. However, hTERT gene is expressed in stem cells and the highest expression was found in pluripotent stem cells. The stem cells required hTERT gene to elongate the telomeric length at the end of the chromosomes in order for the cells to have unlimited self-renewal ability. Thus, if hTERT is silenced in a healthy human, the stem cells will be lost of the regeneration and self-renewal ability. Therefore, in certain diseases that required the use of stem cells to generate specific differentiated cells, the operation should be aborted with this hTERT deficient stem cell since none of the new pluripotent stem cells could be generated (Teichroeb et al., 2016).

5.10 Difficulties Encountered

In the cloning section at the beginning of this project, the transformation efficiency was very low which made the propagation of expression vector not effective. Hence, by following the methods suggested by Tu et al. (2005) and Li et al. (2010), whereby the competency of the *E. coli* cells had been greatly increased, had made the transformation efficiency improved. Besides that, the lipid based transfection reagent which is the DOTAP required quite an amount of time to be adjusted with the ratio of DNA over DOTAP in a single transfection to obtain acceptable transfection efficiency. Further optimisation has enhanced the transfection efficiency to approximately 80 % subsequently. Moreover, the U6 promoter used in the expression vector is a strong RNA polymerase III promoter that will pose a certain amount of cytotoxicity to the cell, and hence the presence of low percentage of cell inhibition shown in the cells transfected with scrambled DNA expression vector (Makinen et al., 2006). Thus, a milder promoter is suggested to be used together with this vector without affecting the efficiency of shRNA expression.

5.11 Future Prospects

In future, the simultaneous silencing of survivin and hTERT can be conducted in various types of cancer cell lines *in vitro*. Since survivin and hTERT are reactivated and highly expressed in almost all of the common cancer cells, this study can be performed to compare the silencing effect of the genes. Besides that, this designed expression vector has the potential to be a general cancer cure as it

targets almost all types of cancer.

In addition to that, this combination of shRNA can be expressed by using the lentiviral-based vector. The lentiviral-based vector can help in improving the efficiency and effectiveness of the shRNA expression. Moreover, the shRNA can be stably expressed in the cell post transfection without the risk of vector degradation after progressive cell-cycles.

Other than that, this expression vector can be used to treat cancer in combination with other anti-cancer drugs that target certain other crucial factors in cancer immortalities. This multiple shRNA vector can have additional shRNA targeting other hallmarks of cancer to be cloned into it because the initial modification of the vector has made this possible.

Lastly, the *in vivo* test by using this multiple shRNA vector can be performed on animal model or any living organisms. However, tough optimisation procedures are required in order to determine the best transportation system to deliver the vector into the cells without the unwanted side effects.

CHAPTER 6

CONCLUSIONS

In conclusion, the targeted cancer genes in this project, namely survivin and hTERT genes have been successfully silenced both singly and simultaneously. Survivin that act as the inhibitor of apoptosis protein in cancer must be silenced to resensitise cancer cells to apoptosis. On the other hand, hTERT that functions in the elongation of telomeric end of chromosome have to be knocked down in order to remove the limitless replicative potential of cancer cells and reintroduce the cancer cells to apoptosis. Therefore, both genes play an important role in the immortalisation of cancer cells and there is a need to knockdown both genes as one of the targeted therapy of cancer.

HeLa cells, which are the cells extracted from the uterine cervix carcinoma or also known as cervical cancer, has been successfully reintroduced to apoptosis by targeting its pivotal molecular markers, survivin and hTERT genes. HeLa cells, like most of other common cancers, have an elevated expression of both genes which contributed much to the survival and immortality of these cells. Hence, the knockdown of these molecular markers is lethal to HeLa cells. Moreover, the HeLa cells are also resensitise to other anti-cancer therapies due to the knockdown of the gene that gave rise to resistance of the cells towards anticancer drugs.

Other than that, the knockdown efficacies of the survivin and hTERT genes had been determined both singly and simultaneously. In the single gene knockdown of survivin, there was a 58% reduction of survivin mRNA expression, 68% of survivin protein expression and finally the experiment has induced 70% of cell apoptosis. For the silencing of single hTERT gene, it was discovered that 51 % of the hTERT mRNA expression had been reduced, 53% of hTERT protein had been reduced and lastly, single hTERT gene knockdown has induced 60% of cell apoptosis. By silencing both genes simultaneously, 80% and 77% of survivin and hTERT mRNA expression had been reduced, respectively, whereas 74% and 63% of survivin and hTERT protein, respectively, had been repressed. Ultimately, the simultaneous knockdown of both genes had successfully induced 85% apoptosis of HeLa cells. This signifies that the approaches in silencing cancer gene have to be conducted by targeting more than one gene to achieve higher efficacies.

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