CONSTRUCTION OF RECOMBINANT *OTUB1* KNOCKDOWN IN BREAST, BONE AND LIVER CANCER CELL LINES

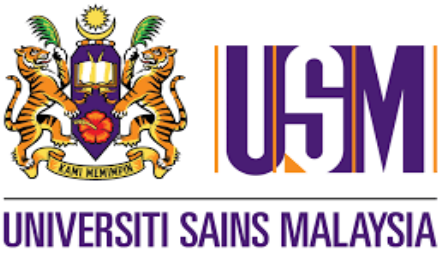
by

NOR SHILA BINTI MOHAMED NOOR

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE (MEDICAL RESEARCH)

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

2019



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DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) for the Degree of Science in Medical Research. It has not been sent to other universities. With that, this research can be used for consultation and photocopied as reference.

Sincerely,

…….

(NOR SHILA BINTI MOHAMED NOOR)

P-IPM 0016/18

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
°C/min	Degree Celsius per minute
°C/s	Degree Celsius per second
kb	Kilo base
µg/mL	Microgram per millilitre
µL	Microliter
µM	Micromolar
M	Molar
min	Minute
mL	Milliliter
mM	Millimolar
ng/µL	Nanogram per microliter
ng	Nanogram
nm	Nanometer
rpm	Revolutions per minute
V	Volt

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Cas	CRISPR associated systems
CRF	Circulating recombinant forms
CRISPR	Clustered regularly interspaced palindromic repeats
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FI	Fusion inhibitor
GFP	Green fluorescent protein
gRNA	guide RNA
HDR	Homology-directed repair
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PI	Protease inhibitor
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TALEN	Transcription activator-like effector nuclease
TAR	Trans-activation response element
TNF-α	Tumor necrosis factor α
ZFN	Zinc finger nuclease

PEMBENTUKAN REKOMBINAN OTUB1 UNTUK PENURUNAN EKSPRESI GEN DI DALAM SEL KANSER PAYUDARA, TULANG DAN HATI

ABSTRAK

Kanser adalah salah satu punca utama kematian dan morbiditi di seluruh dunia. Penyimpangan genetik penyebab kanser telah dicirikan dengan baik seperti mutasi, amplifikasi gen, translokasi, kerosakan struktur dan kehilangan pemisahan kromosom. Dengan penggunaan teknologi genomik moden, kita kini mula memahami kompleksiti kanser yang sangat besar. Menariknya pemahaman biologi sel kanser dan perkembangan tumor secara beransur-ansur membawa kepada kaedah yang lebih baik untuk merawat penyakit ini. Kemunculan alat pengeditan genom menawarkan penyelesaian menarik untuk menangani isu ini dengan mengganggu ekspresi gen kanser. Dalam kajian ini, alat pengeditan genom terkini, sistem CRISPR/Cas9, digunakan untuk menunjukkan potensinya untuk mengganggu ekspresi gen *OTUB1* dalam sel-sel kanser payudara, tulang dan hati. Gen *OTUB1* terdapat di dalam tisu dan memainkan peranan penting dalam proses fisiologi dan patologi manusia. *OTUB1* mempunyai kaitan yang sangat signifikan dengan pertumbuhan kanser. Sebelum menjalankan transfeksi, proses optimisasi prosedur dan efikasi transfeksi perlu dilakukan ke atas sel untuk mendapatkan keputusan yang optimum. Analisis mikroskop *fluorescence* dan *flow cytometry* menunjukkan *Lipofectamine 3000* merupakan metod pilihan untuk transfeksi. Hasil kajian yang memberansangkan akan membolehkan kita untuk membuat siasatan lanjut berkenaan peranan gen *OTUB1* dalam fungsi laluan isyarat dalam sel-sel kanser yang berbeza. Hasil kajian ini menunjukkan bahawa recombinan *OTUB1* berjaya dibentuk untuk proses pengurangan ekspresi *OTUB1* melalui sistem CRISPR / Cas9 yang berpotensi untuk strategi rawatan kanser di masa depan.

CONSTRUCTION OF RECOMBINANT OTIB1 KNOCKDOWN IN BREAST, BONE AND LIVER CANCER CELL LINES

ABSTRACT

Cancer is one of the leading causes of mortality and morbidity all over the world. Various types of cancer-causing genetic aberrations are well characterized such as mutations, gene amplification, translocation, structural deletion and chromosomal mis-segregation. With the use of modern genomic technologies, we are now beginning to understand the enormous complexity of cancer. The growing understanding of cancer cell biology and tumor progression is gradually leading to better methods for treating the disease. The emergence of genome editing tools offers an exciting solution to addressing this issue by disrupting expression of cancer genes. In this study, the current genome editing tool, CRISPR/Cas9 system, is used to demonstrate its potential in disrupting the expression of *OTUB1* gene in breast, bone and liver cancer cells. *OTUB1* gene is ubiquitously expressed in human tissues and play an important role in many physiological and pathological processes of human. *OTUB1* is also strongly correlated with cancer progression. Prior to transfection to knockdown *OTUB1*, optimization of transfection procedure and transfection efficacy in cell lines need to be done to get optimum results. Analysis using fluorescence microscopy and flow cytometry showed that Lipofectamine 3000 is a preferable method in transfection. These encouraging results will enable us to conduct further investigations on exploring the role of *OTUB1* gene in signalling pathways of different cancer cells. These results showed that the recombinant DNA successfully constructed to knockdown *OTUB1* using CRISPR/Cas9 system can be a promising tool for cancer treatment strategies in the future.

CHAPTER 1

INTRODUCTION

1.1 Cancer

1.1.1 General Overview of Cancer

Globally, cancer is the second leading cause of death. In 2018, an estimated 9.6 million deaths or about 1 in 6 deaths was due to cancer (WHO, 2018). Cancer can be defined as a condition in which a group of cells grow abnormally and uncontrollably by disregarding the normal process of cell division (Hejmadi, 2010).

Instead of dying, cancer cells grow continuously (Sudhakar, 2009). From the literature, there are several types of cancers according to the type of cells they start in, namely carcinoma, sarcoma, leukemia, lymphoma, myeloma, brain and spinal cord cancers (Biplop and Arun, 2018). Moreover, cancer can be categorized into two main types which are benign (cancer that does not spread to its surroundings) and malignant (cancer cells that often metastasize through blood circulation or lymph vessels) (Biplop and Arun, 2018).

Initiation and development of cancer can be influenced by external environmental factors and factors within the cell such as hereditary mutations, hormones and immune disorders (Hejmadi, 2010). All of these factors are capable of causing abnormal cell activity and proliferation. Nevertheless, it is essential to remember that it typically takes many years for these cancer-causing DNA alterations to be detected (Hejmadi, 2010). Of all the cancers, the three that causes the highest cancer cases are breast, liver and bone cancer.

1.1.2 Breast Cancer

According to Anjum, Nighat and Masood (2017), breast cancer is the most frequent cancer among women. Breast cancer is also considered as the second leading cause of death in women (WHO, 2018). Furthermore, statistics in 2018 showed that more than 600,000 women have died due to breast cancer, which accounts for about 15% of all cancer deaths among women in the world.

Statistics showed that breast cancer cases are higher in developed countries compared to other countries (WHO, 2018). However, breast cancer is also recognized as a huge public health problem in most developing countries (Balekouzou et al., 2016). Balekouzou et al., (2016) also described that the mortality rate of breast cancer varies correspondingly with age, diagnosis stages, speed of treatment management, type and severity of the tumor, and complacency.

Yip, Pathy and Teo (2014) reported that genetics caused an increased risk of breast cancer. In addition, around 15% of breast cancer patients reported family history of breast and ovarian cancer. Thus, mutations in BRCA1 and BRCA2 were identified as the most important genetic predisposed genes of breast cancer (Yip, Pathy and Teo, 2014).

1.1.3 Bone Cancer

Sarcomas of the bone are uncommon malignant tumors arising from mesenchymal or neuroectodermal tissue (Lewin et al., 2017). The most common malignancies of bone are osteosarcoma, Ewing sarcoma, and chondrosarcoma (Reed et al., 2017).

Primary malignant bone tumors comprise approximately 5% of all childhood cancers in European Countries including two major cancers among children and young adults, such as osteosarcoma and Ewing sarcoma (Gerrand et al., 2016). According to American Cancer Society (2019), over 40% of primary bone cancers in adults are chondrosarcoma.

Usually, osteosarcoma in children starts to develop at the end of the long bone and around the knee. Meanwhile tumors in adults start to develop in the axial, pelvic bones and craniofacial bones (Gerrand et al., 2016). Several risk factors that are responsible for osteosarcoma development include multiple radiation therapies treatment, Paget's disease of bone and germline abnormalities (Gerrand et al., 2016).

Chondrosarcomas are malignant cartilaginous neoplasms with various morphological features (Hans Gelderblom, Dijkstra and Carla S. Van Rijswijk, 2008). Normally, these type of tumors grow slowly and infrequently metastasize to other parts of the body. Besides that, primary chondrosarcoma arises in pre-existing normal bone (Thorkildsen et al., 2019) and subdivides into the central, periosteal and peripheral subgroups (Stevenson et al., 2018). Non-conventional chondrosarcoma variants include clear cell chondrosarcoma, mesenchymal chondrosarcoma and dedifferentiated chondrosarcoma (Stevenson et al., 2018).

1.1.4 Liver Cancer

Liver cancer is a leading cause of cancer-related deaths, accounting for 2.6 million cases worldwide in 2018 (WHO, 2018). The main types of primary liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) (Castelli, Pelosi and Testa, 2017).

HCC is the main form of liver cancer (Chuang, La and Boffetta, 2009) and has the highest incidence and mortality rate, compared to other liver cancers. According to previous studies, HCC is more common among males in Asia and Africa compared to European countries (Castelli, Pelosi and Testa, 2017), and is increasingly becoming a global problem.

The leading cause of HCC is chronic infection of Hepatitis B and C viruses (Ukawa et al., 2017). Other than that, the other predominant cause of HCC is contamination of food with aflatoxin B1 (Chuang, La and Boffetta, 2009), that damages DNA of hepatic cells and causes mutation of p53 tumour suppressor gene (Janevska, Chaloska-ivanova and Janevski, 2015). In addition, Janevska, Chaloska-ivanova and Janevski (2015) also described other mutations detected in HCC, such as c-KRAS gene and co-amplification of the cyclin D1 gene.

In populations with a low prevalence of HBV and HCV, alcohol is an essential risk factor for developing HCC (Janevska, Chaloska-ivanova and Janevski, 2015). Increased prevalence of overweight, obesity and diabetes are also associated with an increased risk of HCC (Ukawa et al., 2017).

1.1.5 Epidemiology of Cancer

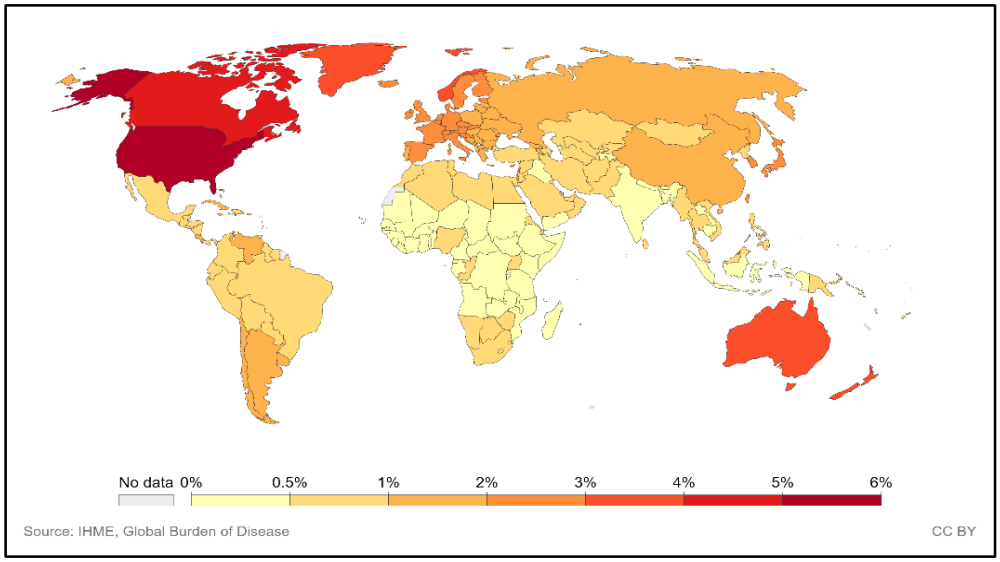


Figure 1.1 Global population with cancer in 2018, showing areas with higher prevalence of cancer.

According to WHO (2018), cancer is the second leading cause of death in the world and it is responsible for 9.6 million deaths in 2018. Furthermore, about 1 in 6 deaths is caused by cancer cases around the world.

In U.S., the number of new cases of invasive breast cancer is approximately 266,120 cases, along with approximately 63,960 cases of non-invasive breast cancer cases among women in 2018. At present, there are about 3 million women living with breast cancer. According to the statistics, about 40,920 women in the U.S. are estimated to die from breast cancer.

In Australia, there was a steep increase in the number of deaths from breast cancer, from about 1,435 in 1968 to 2,844 in 2014. Meanwhile in 2018, approximately 17,730 new cases were diagnosed in Australia. In 2012, around 94,000 new cases of breast cancer were detected among women in Africa and 48,000 died from it in sub-Saharan Africa in 2018.

1.1.6 Cancer Scenario in Malaysia

In Malaysia, cancer is becoming a serious national health problem. It is the fourth leading cause of medically certified deaths. In Malaysia in 2018, approximately 20,619 new cases in males and 23,218 new cases in females with different types of cancers were diagnosed.

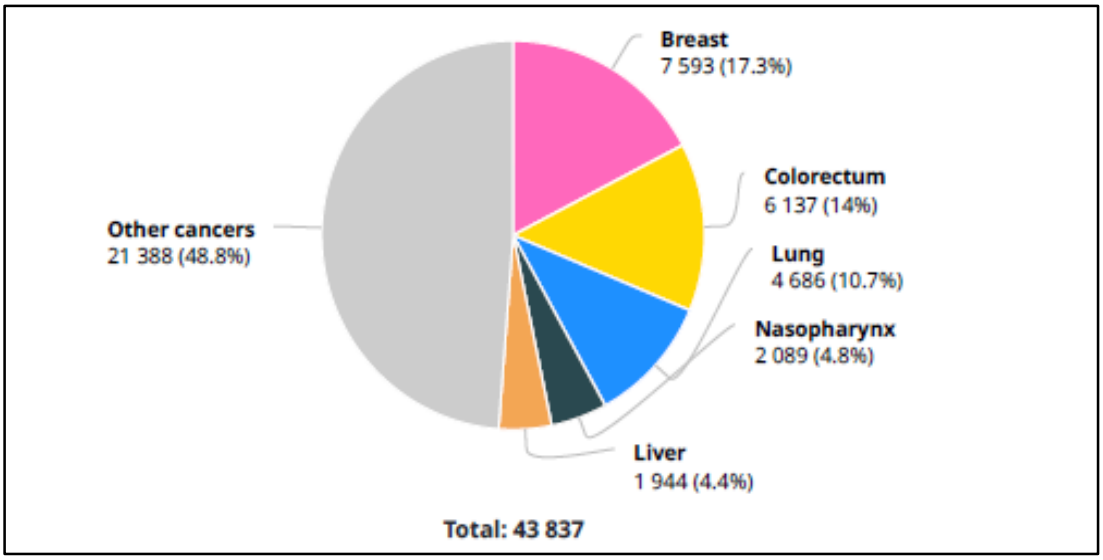
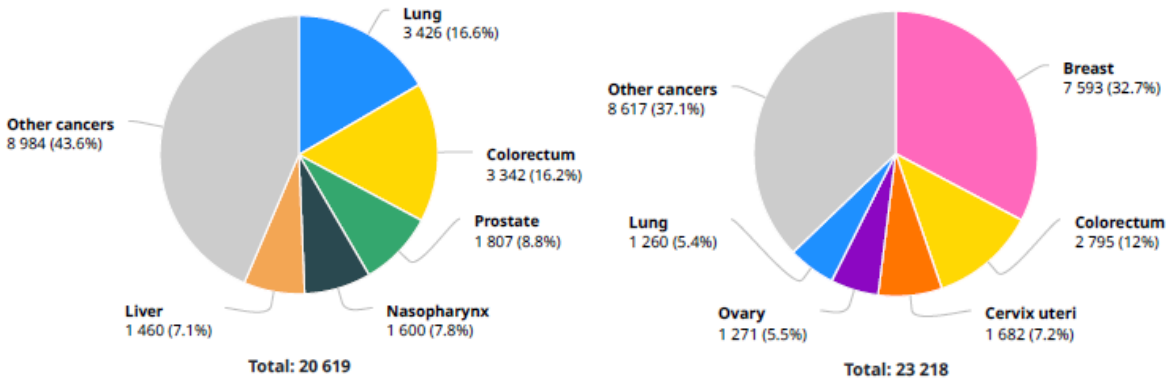


Figure 2.2 Number of overall new cancer cases in 2018 in that include both sexes and all ages. Breast cancer makes up the most number of cancer cases in Malaysia.

According to The Global Cancer Observatory (GLOBOCAN) (2018), in Malaysia, the most common type of new cancer cases in men are lung, colorectal and prostate cancer, while in women, breast, colorectal and cervix uteri cancer are the most common cases (Figure 1.3). Worldwide, similar statistics are seen with lung cancer being the most common malignancy among males (WHO, 2018). As breast cancer has the highest incidence rate among females in the world too, there is an increased awareness of wellness programmes which include screening programs, breast self-examination and awareness of the early symptoms of breast cancer (Lim, 2003).



MALE **FEMALE**

Figure 1.3 Number of new cases in males and females, including all ages in 2018. In male patients, lung cancer is most common, while in female, breast cancer is most common.

The National Strategic Plan for Cancer Control Programme (NSPCCP) 2016-2020 targets to reduce the incidence and mortality rate of cancer. This program aims to improve the quality of life of cancer patients by improving the cancer care and management from a holistic point of view (MOH, 2017). The objectives of the program are to initiate screening, detection and prevention program; diagnosis and treatment as well as rehabilitation program. In addition, these policies also focus on palliative care, and traditional and complementary medicine (TCM).

However, nowadays researchers are also focused on new approaches in cancer treatment such as targeted therapy, which can potentially target genes involved in the cancer progression pathway, such as *OTUB1*.

1.2 OTUB1

1.2.1 General Overview

OTUB1 or ubiquitin thioesterase, also known as otubain-1, is an enzyme in humans that is encoded by the *OTUB1* gene. This gene is located on locus 11q13.1 in chromosome 11. It is also a member of OTU (ovarian tumor) superfamily which is part of the deubiquitinating enzymes (DUBs) family. According to Mevissen et al., (2013), there are 16 OTU families from deubiquitinating enzymes (DUBs) family present in humans and expressed in a variety of human tissues, with high expression observed in the brain (Fagerberg et al. 2014). Most of the ovarian tumor (OTU) family function in regulating cell-signaling cascades.

OTUB1 plays an essential role in various biological processes. *OTUB1* gene is also responsible in controlling major events in cancer cell activities such as cancer initiation and progression (Saldana *et al.*, 2019).

Several studies demonstrate that *OTUB1* regulates apoptosis, therapeutic resistance, proliferation, migration and invasion in several cancer types (Saldana *et al.*, 2019). In his study, Stanišić et al., (2009) reported that *OTUB1* also interacts with ERα which is the main factor in the development of breast and endometrial cancers in cells. *OTUB1* facilitates certain types of cancer such as invasion of prostate cancer cells through RhoA activation and stimulation of tumorigenesis *in vivo* (Iglesias-Gato et al., 2015). Other than that, according to Sun et al., (2011), this gene has been identified as a novel p53 regulator. Furthermore, a previous study stated that overexpression of *OTUB1* can cause apoptosis and is capable of inhibiting cell proliferations in a p53 dependent manner.

According to Goncharov et al., (2013), *OTUB*1 stimulates cell death by modulation of stability of cellular inhibitor of apoptosis (c-IAP) protein. By silencing *OTUB1,* this mechanism affects IAP in an antagonist-induced apoptosis in many types of cancer cells and will produce a favorable effect. Thus, studies involving combined targeting of *OTUB1* in cancer cells might lead the way for novel therapeutic approaches (Goncharov et al., 2013).

Herhaus et al. (2013) showed that OTUB1 interacts specifically with phosphorylated SMAD2/3 after TGF- β stimulation. In this interaction, OTUB1 opposes p-SMAD2/3 ubiquitination and proteasomal degradation, and stimulates TGF-β signalling in human cells and *in vivo.* OTUB1 may be an essential modulator of TGF-β/ SMAD-controlled cell responses as it has a phosphorylation dependent target recognition (Herhaus et al., 2013). Thus, interfering with the OTUB1–pSMAD interaction may provide an important benefit against diseases with abnormal TGF-β signaling, such as cancer in human.

In addition to catalytic DUB activity, OTUB1 displays a catalytic-independent, non-canonical activity where it inhibits the transfer of ubiquitin onto protein substrates by sequestration of E2 ubiquitin-conjugating enzymes (Saldana *et al.*, 2019). Thus, clinical studies have associated elevated *OTUB*1 expression with invasiveness and metastasis in several tumor types including lung, breast, ovarian, glioma, colon and gastric.

Previous literature demonstrates that OTUB1 regulates many cancer-associated signalling pathways including MAPK, ERα, epithelial-mesenchymal transition (EMT), RHOa, mTORC1, FOXM1 and P53 to promote tumor cell survival, proliferation, invasiveness and therapeutic resistance (Saldana *et al.*, 2019). They suggest the importance of modulating OTUB1 as it could play a huge role in cancer control.

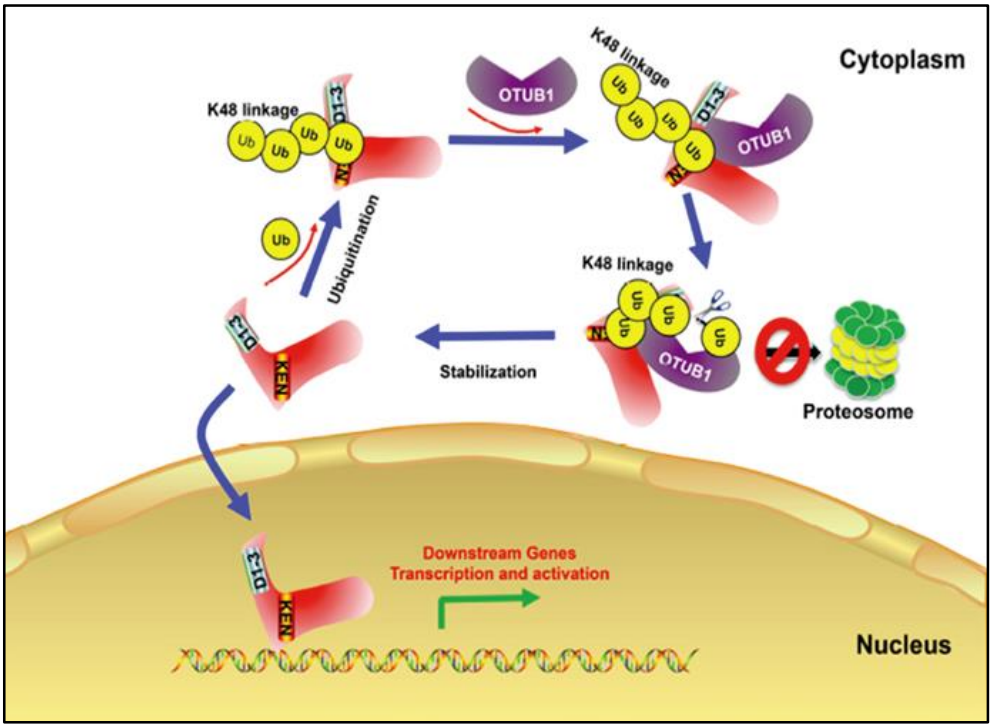


Figure 1.6 A schematic of the mechanism of OTUB1 regulates FOXM1 which promotes the role of OTUB1 in cancer progression.

Overexpression of *OTUB1* causes cancer progression and it would be important to investigate the role of *OTUB1* by constructing a recombinant knockdown of *OTUB1* to further study its role in cancer.

1.2.2 Treatment of Cancer

According to WHO (2018), established and commonly practiced cancer treatments include modalities such as surgery, chemotherapy and radiotherapy. The treatment selection is based on the existing options available at the healthcare facilities. To provide the best treatment, combining modalities of therapy is also practiced by the cancer care team to ensure an efficient treatment.

Surgery is known as the best treatment for patients whose breast cancer has not metastasized (Biplop and Arun, 2018). There are differences in the breast cancer surgeries depending on the amount of tissue containing tumor that needs to be removed (Lee et al., 2015). Meanwhile, chemotherapy can be given before the surgery as a neo-adjuvant with the purpose of shrinking the tumor size. This dual-treatment reduces the chances of cancer recurrence (Masood, 2016).

Radiotherapy is a treatment in which high-energy rays are used to kill cancer cells. According to WHO (2008), there are two broad categories of techniques used to treat patients requiring radiotherapy treatment including teletherapy and brachytherapy. Teletherapy, also known as external beam radiation therapy, is delivered in a tertiary hospital on an ambulatory basis. For certain types of cancers such as invasive cancer cases, brachytherapy is delivered with teletherapy to cure the disease. Brachytherapy may be administered in a low or high dose depending on the expertise and facilities available (WHO, 2008).

Other than that, for non-Hodgkin lymphoma, targeted radionuclide therapies such as radioimmunotherapy (RIT) is used to relieve pain in bone metastases case, and selective internal radiation therapy (SIRT) is used to treat liver cancer in selected facilities (Ministry of Health, 2017).

Recognizing the need to improve the treatment methods, in 2015, WHO updated the list of clinically proven effective treatment regimens to include 30 cytotoxic and adjuvant medicines (anti-cancer medicines). The efficacy, safety, quality and cost-effectiveness of these medications were evaluated and used in conjunction with the approved modalities as a targeted therapy against cancer (WHO, 2018).

According to Biplop and Arun, (2018), targeted therapies that target specific biological and physiological processes that are essential to tumor growth, are new modalities for cancer treatment. The purpose of targeted therapies, such as gene therapy, includes ceasing the growth and spreading of cancer cells in the body (Biplop and Arun, 2018). Targeted therapies are dynamic enough that they can include rare cancers, and accommodate different mutations.

1.3 Gene Therapy in Cancer and CRISPR

1.3.1 Overview of Gene Therapy

In the field of cancer, gene therapy can be defined as the delivery of genetic elements to the cancer cell to correct the abnormalities in the cancer tissue or to induce an immune response against the cancer cells (Akbulut, Ocal and Sonugur, 2016). Historically, in 1989, the first gene therapy was carried out and approved by the US Food and Drug Administration (FDA). The protocol utilized focused on tumor infiltrating lymphocytes collected from advanced melanoma patients that were transduced *ex vivo* with a marker gene, then expanded *in vitro* and re-infused to the patients (Wirth and Ylä-herttuala, 2014).

For a successful gene therapy in cancer, there are some prerequisites to be considered carefully, such as a suitable target to be replaced or modified, a carrier to reach the gene of interest in the cell, a successful targeting of the vector and a sufficient expression of the therapeutic genes in the target cells. In addition to that, a strong therapeutic efficacy, and safety of treatment delivered to the patients is also mandatory for the success of the treatment (Akbulut, Ocal and Sonugur, 2016).

The approaches of gene therapy are diverse including repairing, replacing or deleting the culprit gene in genetic diseases, producing incapacitating mutations in pathogen genomes to fight infectious diseases, or inducing therapeutic effects. This promising therapy can be applied to a wide range of human diseases including cancer (Zhang *et al.*, 2017).

The development of genome-editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas9) nucleases has introduced a new advancement to the field of human gene therapy (Meissner *et al.*, 2018). These programmable nucleases produce double-strand breaks (DSBs) at the targeted gene and is usually repaired by non-homologous end joining (NHEJ) which results in the disruption of the targeted gene (Zhang *et al.*, 2017). Furthermore, Cong et al., (2013) reported that CRISPR/ Cas9-mediated genome editing has been successfully employed in a variety of model organisms, human embryonic and induced pluripotent stem cells, as well as in human adult stem cells.

According to Wirth and Ylä-herttuala, (2014) gene therapy has the advantage over conventional therapies as it can be administered locally with a high therapeutic dose without risking systemic adverse effects. On top of that, since most gene therapies are single time applications, they are also cost effective.

Cancer is an accelerated and uncontrolled growth of cells that have the capacity to spread throughout the body. At the late stage, cancer is generally fatal. Therefore, it is crucial to search for a new medical advancement to help patients. Gene therapy seems to be an exciting strategy that has a promising future in clinical oncological practice (Martinez-Davila and Delgado-enciso, 2017).

1.3.2 CRISPR/Cas9 Systems

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), together with CRISPR-associated proteins (Cas) is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements (Ran *et al.*, 2013). It contains a short repeating nucleotide originally found in the genome of bacteria and archaea and functions to eliminate exogenous genetic elements (EGEs) that combine with Cas proteins (Zhang et al., 2017).

CRISPR/Cas9 can be divided into three classification systems that have been identified across a wide range of bacterial and archaeal hosts, wherein each system comprises a cluster of CRISPR-associated (Cas) genes, noncoding RNAs and a distinctive array of repetitive elements (Ran *et al.*, 2013).

The Type II CRISPR system is one of the best characterized systems consisting of three components, namely an endonuclease Cas9, a CRISPR RNA (crRNA) array that encodes the guide RNAs and a transactivating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units (Ran *et al.*, 2013; Zhang et al., 2017). The crRNA and tracrRNA molecules form a duplex structure called the guide RNA (gRNA), that can be substituted by a synthetic fused chimeric single gRNA (sgRNA), which simplifies the use of CRISPR/Cas9 in genome engineering (Oude *et al.*, 2016).

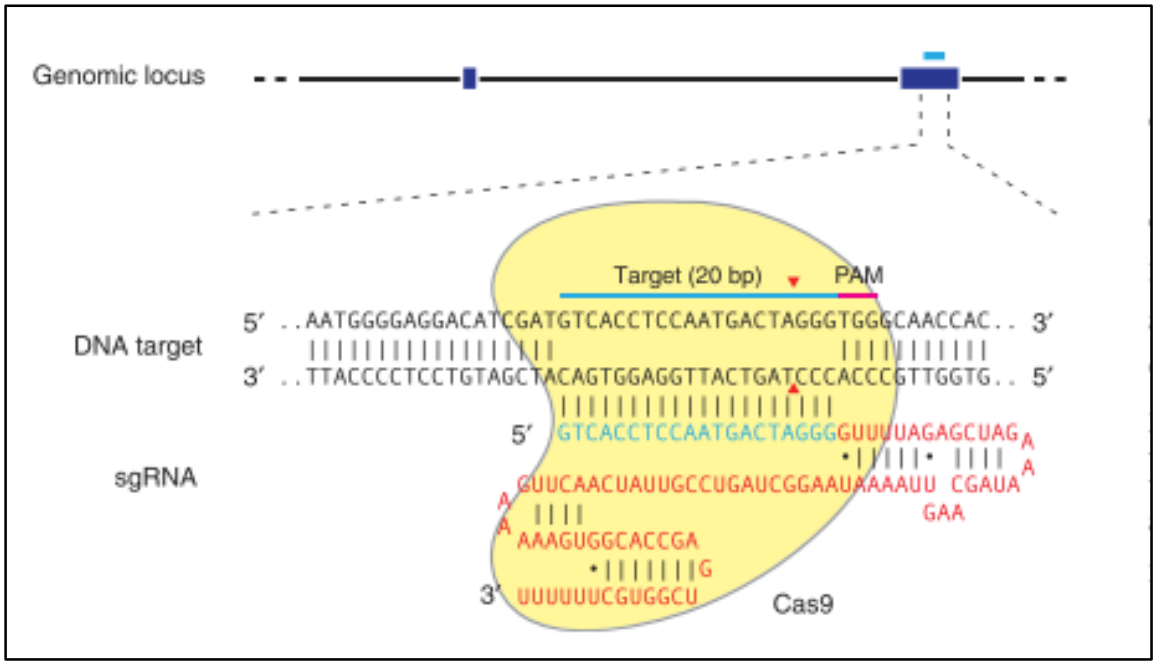


Figure 1.7 Schematic of the RNA-guided Cas9 nuclease. The Cas9 nuclease from *S. pyogenes* (in yellow) is targeted to genomic DNA by a sgRNA consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence pairs with the DNA target (blue bar on top strand), directly upstream of a requisite 5′-NGG adjacent motif (PAM; pink). Cas9 mediates a DSB ~3 bp upstream of the PAM (red triangle) (Ran *et al.*, 2013).

This simplified gRNA contains two important components namely a variable region and a basic scaffold. The earlier form of gRNA is normally composed of 18 to 20 nucleotides that can bind to the target DNA according to the base complementation pairing rule. Then, the latter form is a long scaffold-like RNA used to bind Cas9 nuclease and form a gRNA/Cas9 complex (Oude *et al.*, 2016).

Each protospacer is associated with a protospacer adjacent motif (PAM) within the DNA target which can vary depending on the specific CRISPR system. In this CRISPR-Cas system derived from Streptococcus pyogenes, the target DNA must immediately precede a 5′-NGG PAM (Ran *et al.*, 2013).

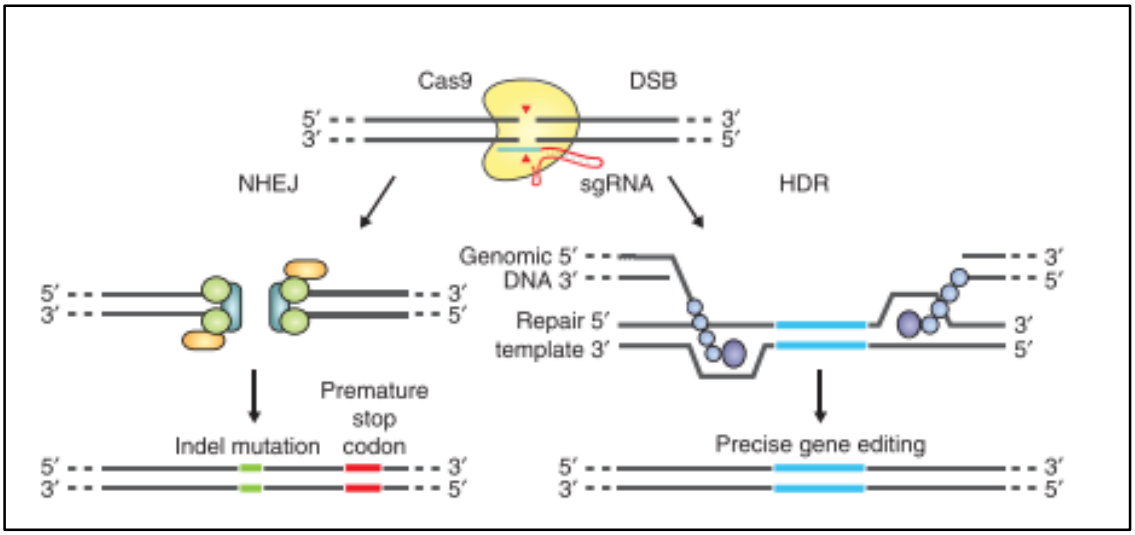


Figure 1.8 DSB repair promotes gene editing. DSBs induced by Cas9 (yellow) can be repaired in one of two ways. In the error-prone NHEJ pathway, the endogenous DNA repair machinery causes random indels at the site of junction. The indel can result in frameshifts, resulting in gene knockout. Alternatively, a repair template in the form of a plasmid or ssODN can be supplied to leverage the HDR pathway, which allows for a high fidelity and precise editing (Ran *et al.*, 2013).

According to Zhang et al., (2017), the genome-editing system requires three nucleotides, a PAM (NGG), to enable it to be recognized and bound by the gRNA/Cas9 complex to generate double-strand breaks (DSBs). The DSBs are repaired by non-homologous end joining (NHEJ) or homologous directed repair (HDR) if homologous sequences are available (Xiao-jie *et al.*, 2015).

HDR leads to precise gene correction or replacement, whereas NHEJ is error prone correction and may induce small inserts or deletions (indels). These indels eventually lead to a gene disruption or gene knockdown (Xiao-jie *et al.*, 2015). These mechanisms hold huge promise for editing any gene in any organism (Ran *et al.*, 2013).

According to Ran *et al.*, (2013), in comparison to other designer nucleases such as the zinc-finger nucleases (ZFNs) and transcription activator- like effector nucleases (TALENs), CRISPR/Cas9 offers several potential advantages over ZFNs and TALENs, including the ease of customization, higher targeting efficiency and the ability to facilitate multiplex genome editing (Zhang *et al.*, 2017).

CRISPR/Cas9 is more user-friendly than ZFN and TALEN, as primers just need to be synthesized as gRNAs as new targets to be used with CRISPR/Cas9 (Zhang et al., 2017). In contrast, retargeting of a different gene requires the construction of two new TALEN nucleases (Ran *et al.*, 2013).

CRISPR/Cas9 is more economical because there is a low cost for plasmid-mediated CRISPR/Cas9, and its genome-editing technique can be achieved in 2 weeks (Zhang et al., 2017). Cas9 is also extremely efficient at editing several cell types and organisms by targeting multiple genomic loci simultaneously by co-delivering a combination of sgRNAs to the cells of interest (Ran *et al.*, 2013).

Thus, the application of CRISPR/Cas9-based gene manipulation including gene knockout, gene knocking, gene interference or activation and other chromosome-related applications, has been widely utilized in biological and biomedical research to venture new dimension of genetic-engineering field.

Due to the versatility of CRISPR/Cas9, we will be using CRISPR/Cas9 to construct a recombinant *OTUB1* knockdown in breast, bone and liver cancer cell lines to investigate its role as a potential therapeutic target for cancer.

1.4 Problem statement

Cancer is a leading cause of death in the world with over 9.6 million deaths in 2018. Cancer can be caused by a variety of conditions including aberration of genes. Gene aberration, also known as gene mutation, can be categorized into deletion, duplication, insertion and translocation. Many gene mutations are responsible for cancer progression, including *OTUB1* gene.

OTUB1 gene regulates many cancer-associated signalling pathways such as FOXM1 and p53. According to a previous study, overexpression of OTUB1 in cancer cells promotes tumor survival, proliferation, and therapeutic resistance (Saldana *et al.*, 2019). Therefore in this study, we want to construct a recombinant *OTUB1* knockdown using CRISPR/Cas9 to evaluate its potential as a therapeutic target against cancer in the future.

1.5 Research Objectives

1.5.1 Main Objective

Constructing recombinant *OTUB1* knockdown in breast, bone and liver cancer cell lines to investigate the suitability of *OTUB1* as a potential therapeutic therapy.

1.5.2 Specific Objectives

1. Designing and constructing CRISPR/Cas9 to knockdown *OTUB1*
2. Optimizing the transfection efficiency in MCF7, HepG2 and Saos2 cell lines using Lipofectamine 3000 to ensure consistent gene expression (by using fluorescence microscopy and flow cytometry)

CHAPTER 2

METHODOLOGY

2.1 Designing and constructing of CRISPR/Cas9 to knockdown OTUB1

The full sequence of *OTUB*1 gene was retrieved from NCBI Genomic Database (NCBI reference sequence NM_017670.2). Then, sequence prediction of gRNA was done *in silico* using gRNA design software using Target Finder (Table 2.1). The selected gRNA sequence was synthesized as oligonucleotides by Integrated DNA Technologies (IDT).

Table 2.1 Online Tools Used to Design CRISPR Guide RNA

Software	URL
NCBI Sequence Database	https://www.ncbi.nlm.nih.gov/nuccore/
Target finder	https://crispr.mit.edu

2.1.1 Cloning of CRISPR nuclease into expression vector

gRNA was cloned into a Cas9 humanized expression vector, pSpCas9(BB)-2A-GFP (pX458), which was obtained from Feng Zhang lab (Addgene plasmid # 48138) (Ran *et al.*, 2013). Plasmid pX458 is a CRISPR plasmid that expresses Green Fluorescence Protein (GFP) (Figure 2.1). The GFP in this plasmid was used to monitor transfection efficiency in cells lines.

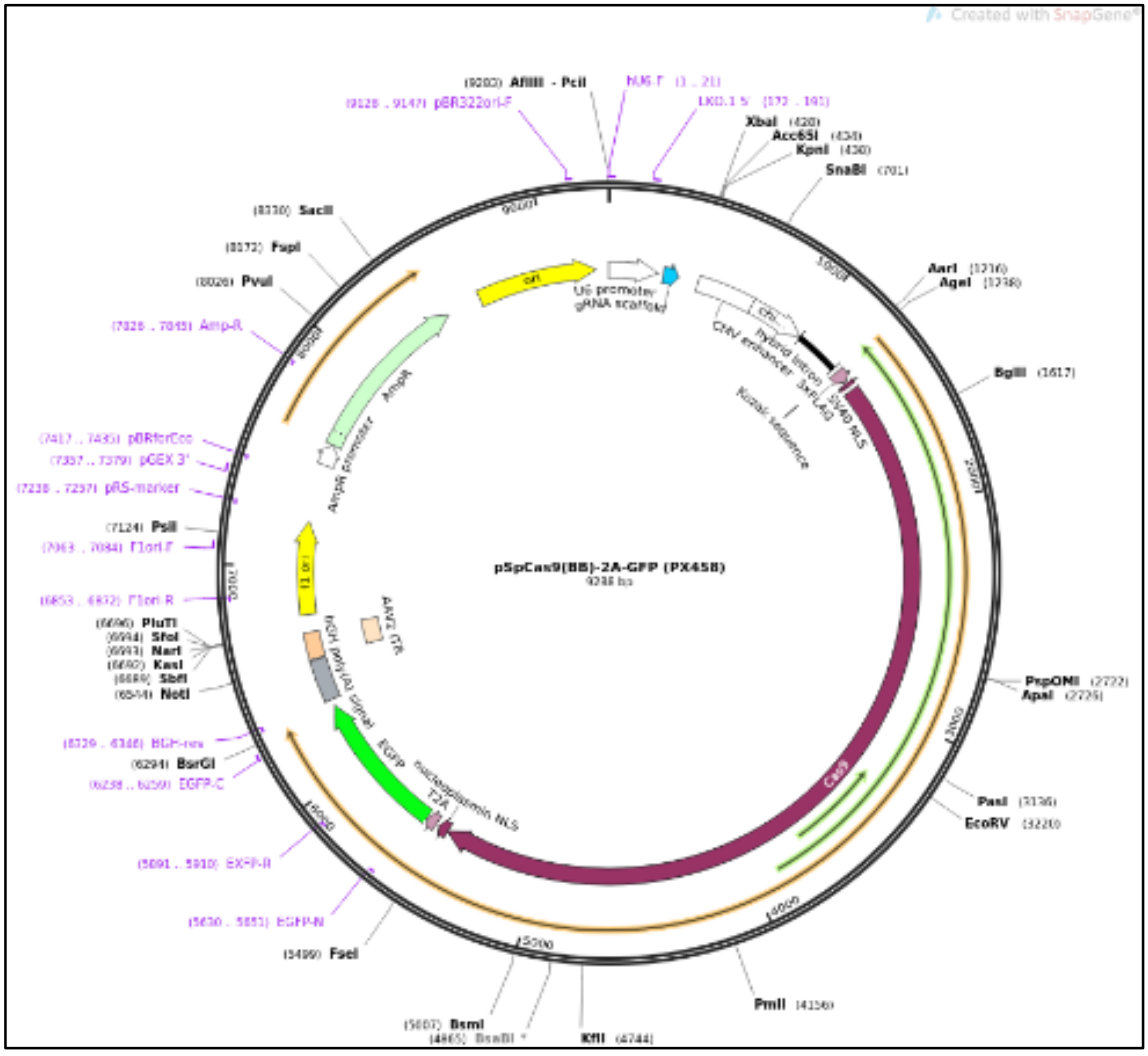


Figure 2.1 Plasmid pSpCas9(BB)-2A-GFP (pX458) that expresses Green Fluorescence Protein (GFP)

The protocol used for cloning was as described by Cong et al. 2013. Briefly, pX458 was digested with *Bbs*I. Then, the OTUB1 oligonucleotides (gRNAs) were phosphorylated and annealed in a thermocycler, similar to previous studies (Cong et al. 2013). This was followed by a ligation step as detailed below for oligo annealing and cloning into backbone vectors:

Firstly, digest 1µg of pX458 with *Bbs*I for 30 min at 37C using this mixture:

1 µg	pX458
1 µl	FastDigest *Bbs*I (Fermentas)
1 µl	FastAP (Fermentas)
2 µl	10X FastDigest Buffer
X µl	ddH2O
20 µl	Total

Secondly, gel purify digested pX458 using QIAquick Gel Extraction Kit and elute in water. After that, phosphorylate and anneal each pair of oligos using these components:

1 µl	Oligo 1 (100mM)
1 µl	Oligo 2 (100mM)
1 µl	10X T4 Ligation Buffer (NEB)
6.5 µl	ddH2O
0.5 µl	T4 PNK (NEB)
10 µl	Total

Then, anneal in a thermocycler using the following parameters:

37ºC	30 min
95ºC	5 min and then ramp down to
25ºC	5ºC/min

Lastly, set up ligation reaction and incubate at room temperature for 10 min:

X µl	*Bbs*I digested pX458 **step 2** (50 ng)
1 µl	Phosphorylated and annealed oligo duplex from **step 3** (1:200 dilution)
5 µl	2X Quickligation Buffer (NEB)
X µl	ddH2O
10 µl	Subtotal
1 µl	Quick Ligase (NEB)
11 µl	Total

The cloned vector was then transformed into DH5–alpha competent *E.coli* (NEB). For High Efficiency Transformation Protocol, thaw a tube of DH5–alpha competent *E.coli* (NEB) cells on ice for 10 minutes. Then, add 1–5 µl containing 1 pg–100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4–5 times to mix cells and DNA. Do not vortex. After that, place the mixture on ice for 30 minutes and do not mix. Heat shock at exactly 42°C for exactly 30 seconds and do not mix. Then, place on ice for 5 minutes and do not mix.

After that, pipette 950 µl of room temperature SOC into the mixture and place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate. At the same time, warm selection plates to 37°C. After 60 minutes, mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC. Lastly, spread 50–100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24–36 hours or 25°C for 48 hours.

2.1.2 Verification of CRISPR sequence

12 colonies were picked from the agar plate in 2.1.1 and colony PCR was conducted to verify that the colonies contained the insert. Colony PCR was done using the forward primer hU6 (5' – CAGCGACTCCGAAGGTGTTA – 3') and CRISPR reverse primer (5' – TGGTCTTGCGGATGTACGAG – 3') with the following parameters. Detailed steps are as listed below.

PCR Colony Screening

25 µl	Taq 2X Master Mix
2µl	Forward Primer
2µl	Reverse
21µl	H_20

95°C	5 minutes	1 cycle
95°C	0.5 minute	30 cycles
60°C	0.5 minute	
72°C	1 minute	
72°C	10 minutes	1 cycle
4°C	Hold until ready to proceed	

Amplicons were then run on a 1% agarose gel at 100 V for 30 minutes. A 650 base pair amplicon would indicate that the colony contained the insert of interest. DNA of positive colonies were purified and sent to Integrated DNA Technologies (IDT) for DNA sequencing.

2.2 Cell culture and reagents

In this study, three different cell types were used, MCF7 (breast cancer), Saos2 (bone cancer) and HepG2 (liver cancer) cell lines.

MCF7 and Saos2 cells were cultured in Gibco® Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA) and 1% penicillin and streptomycin mixture (Pen-Strep) (Invitrogen). All cells were incubated at 37°C and 5% CO_2.

HepG2 cells were cultured in Gibco® RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA) and 1% penicillin and streptomycin mixture (Pen-Strep) (Invitrogen). All cells were incubated at 37°C and 5% CO_2.

pQBI was used as the positive control, and untransfected cells were used as the negative control. pQBI was used as a positive control because of its constitutive GFP expression (Figure 2.2).

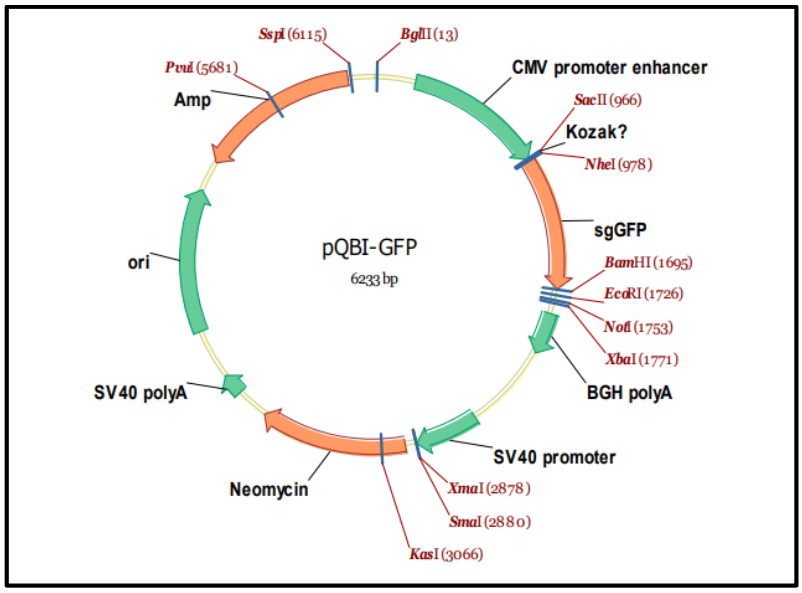


Figure 2.2 Plasmid map of pQBI. pQBI is a plasmid that constantly expresses Green Fluorescence Protein (GFP)