THE EFFECTS OF PIOGLITAZONE AND MK886 ON THE mRNA EXPRESSION OF PPAR-alpha, PPAR-gamma AND ITS ASSOCIATED GENES, CELL DEATH AND MIGRATION IN MDA-MB-231 CANCER CELLS

by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

October 2015

DEDICATIONS

This thesis is dedicated to my beloved parents.

ACKNOWLEDGEMENT

First and foremost, I would like to thank God for everything. Following which I would like to express my heartfelt gratitude to my supervisor, Dr. Khoo Boon Yin for all her supports, time, patience guidance, valuable advices, financial aids and constructive encouragement throughout my study. Without her, this project would not be succeeded. I would also like to thank my co-supervisors; Dr. Chew Ai Lan and Prof. Prabha Balaram for their willingness to share the knowledge with me. Their time and advices are truly appreciated.

Not forgetting to thank the laboratory staff of INFORMM; Puan Maimunah, Puan Sabariah, Puan Noorizan, Puan Fatihah, Puan Syazwani, Puan Fatihah, Puan Izzati, Encik Nyambar and Encik Sa'aban for their valuable helps. I would also like to thank the Science Officers of INFORMM; Puan Halida, Puan Azimah and Encik Hasani for their assistance during my study in INFORMM. I would also like to extend my sincere appreciation to the administrative staffs of INFORMM, especially to Puan Nurul Jannah, Cik Ziera, Puan Siti, Encik Adli, Encik Irwan, Puan Kammini, Puan Iman and Puan Oyan for their sincere assistances.

I wish to also extend my sincere thanks to all my friends in INFORMM for their guidance and supports. Special thanks go to Kak Anizah, Thaneswary, Kak Madihah, Kak Aishah, Kak Shuhaidatul, Ms. Chan, Siang Yian, Ron, Wee Yee and Yuan Lin. I would also like to thank the members of PRN's group, DSS's group, DLNS's group,

DPKK's group, DOMT's group and DVB's group for their ideas and encouragements throughout my study.

I am grateful to the Ministry of Higher Education (MoHE) for granting me with the MyMasters scheme and Universiti Sains Malaysia for financially aiding my study with the Graduate Assistant (GA) scheme. My sincere appreciation also goes to the Fundamental Research Grant Scheme (FRGS, Grant No: 203/CIPPM/6711162) from the MoHE.

Last but not least, I would like to thank my parents for their ultimate love, encouragement and support. Without their constructive inspiration, it is not possible for me to finish my study. I would also like to thank my extended family members and friends especially the Biozone group and Kanmani for their emotional support. Finally, I would like to extend my token of appreciation to everyone else who has helped me in one way or another in completing my study.

Kalpanah

INFORMM, 2015.

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LIST OF SYMBOLS AND ABBREVIATIONS

Approximately	~
Acridine orange	AO
Base Pair	bp
Carbon Dioxide	CO_2
Complementary DNA	cDNA
Cycle threshold	Ct
Degree celcius	°C
Deoxyribo nucleic acid	DNA
Dimethyl sulfoxide	DMSO
Distilled water	dH_2O
Dulbecco's modified eagel medium	DMEM
Enzyme-linked immunosorbent assay	ELISA
Fetal bovine serum	FBS
Gram	g
Microgram	µg
Microlitre	µl
Micrometre	µm
Micromolar	µM
Milligram	mg
Millilitre	ml
Milimolar	mM
Minute	Min
Non Template Control	NTC
Percentage	%
Pioglitazone	PGZ
Phosphate Buffer Saline	PBS
Polymerase Chain Reaction	PCR
Propidium iodide	PI
Ribonucleic acid	RNA
Revolutions per minute	rpm
Standard deviation	SD
Sodium chloride	NaCl
Voltage	V

LIST OF PUBLICATIONS & PRESENTATIONS

KESAN PIOGLITAZONE DAN MK886 TERHADAP EKSPRESI mRNA PPAR-alpha, PPAR-gamma SERTA GEN-GEN YANG BERKAIT, KEMATIAN SEL DAN MIGRASI DALAM SEL-SEL KANSER MDA-MB-231

ABSTRAK

Pioglitazone (PGZ) merupakan ubat preskripsi yang digunakan untuk merawat diabetes mellitus jenis II. Ubat yang lebih dikenali sebagai perangsang sintetik untuk PPAR ini telah dikaji secara meluas oleh kumpulan penyelidikan kami sebagai ubat sasaran untuk merawat kanser payudara ER-negatif. Kanser jenis ini lebih agresif dan lebih cepat bertumbuh daripada kanser payudara ER-positif. Kanser jenis ini juga mempunyai prognosis yang lemah dan biasanya tidak bertindak-balas terhadap terapi anti-estrogen. Kajian awal kami menunjukkan bahawa rawatan MDA-MB-231 dengan PGZ mempunyai kesan yang lemah terhadap pertumbuhan sel-sel kanser dan tidak mendorong apoptosis dalam sel-sel kanser walaupun ia merangsangkan kadar ungkapan mRNA PPARα yang tinggi dalam sel-sel kanser yang berkenaan. Oleh itu, untuk mengkaji peranan ungkapan mRNA PPARα dalam MDA-MB-231 secara mendalam, kesan PGZ dan MK886 yang merangsangkan dan merencatkan ungkapan mRNA PPARα masing-masing, dan ungkapan mRNA gen-gen lain yang berkaitan dalam MDA-MB-231 akan dikenal pasti dalam kajian ini. Mekanisma biologi yang didorongkan oleh kedua-dua ubat ini turut akan dikaji dalam kajian ini. Bagi mencapai semua objektif kajian ini, ungkapan mRNA PPARα dalam MDA-MB-231 yang dirawat dengan PGZ dan MK886 akan dikenal pasti dengan menggunakan kaedah 'Real-time PCR'; kesan rawatan PGZ dan MK886 dalam pertumbuhan

MDA-MB-231 akan dikenal pasti dengan menggunakan kaedah Asai Trypan Biru dan 'Real-time PCR'; perangsangan apoptosis oleh PGZ dan MK886 dalam MDA-MB-231 akan dikenal pasti dengan menggunakan Asai Penanggaan DNA, 'Real-time PCR', Pewarnaan Sel dan Flow Sitometri, manakala pergerakan MDA-MB-231 yang dirawati oleh PGZ dan MK886 akan dikenal pasti dengan meggunakan Asai Pemulihan Luka. Kaitan ungkapan mRNA PPARα dengan gen-gen yang lain seperti PPARγ, FGF4, 5LOX, ALPI, IL6, HIF1α, BNIP3 dan VEGF dalam MDA-MB-231 yang dirawati oleh ubat-ubat juga dikenal pasti pada kajian ini menggunakan 'Real-time PCR'. Keputusan ujikaji menunjukkan bahawa rawatan MDA-MB-231 dengan PGZ meningkatkan ungkapan mRNA PPARα/γ, dan peningkatan ungkapan mRNA ini dapat direncatkan oleh rawatan MK886. Kedua-dua ubat ini dapat mengurangkan pertumbuhan MDA-MB-231 dan proses ini tidak dipengaruhi oleh ungkapan mRNA PPARα/γ, serta tidak mendorong apoptosis dalam sel-sel kanser yang dirawati. Fenomena ini telah disahkan oleh pengesanan caspase-9 dan caspase-3 dalam sel-sel kanser yang dirawati dengan menggunakan 'Real-time PCR'. Jurang yang terdapat pada luka MDA-MB-231 yang dirawati oleh PGZ tidak diperhatikan dalam sel-sel kanser yang dirawati oleh MK886 menunjukkan bahawa kesan pergerakan MDA-MB-231 yang dirawati oleh PGZ boleh dihapuskan dengan rawatan MK886. Fenomena ini turut dapat dikaitkan dengan ungkapan mRNA gen-gen lain seperti PPARα/γ, FGF4, dan 5LOX, dalam sel-sel kanser yang dirawati. Kajian ini memberi maklumat yang berguna terhadap ungkapan mRNA PPARα dan gen-gen lain yang berkait dalam MDA-MB-231. Gen-gen ini mungkin menjadi sasaran yang menarik untuk mengurangkan pergerakan kanser payudara ER-negatif manusia.

THE EFFECTS OF PIOGLITAZONE AND MK886 ON THE mRNA EXPRESSION OF PPAR-alpha, PPAR-gamma AND ITS ASSOCIATED GENES, CELL DEATH AND MIGRATION IN MDA-MB-231 CANCER CELLS

ABSTRACT

Pioglitazone (PGZ) is a prescription drug used in the treatment of diabetes mellitus type II. It is a well-known synthetic agonist for PPAR, and being studied extensively by our research group as a target drug for the treatment of ER-negative breast cancer. The ER-negative breast cancer is more aggressive and fast growing than the ER-positive breast cancer. Moreover, this type of cancer has a poor prognosis generally and is often unresponsive to anti-estrogen therapy. A preliminary research showed that treatment of MDA-MB-231 with PGZ produced little effect on cell growth and did not induce apoptosis in the cancer cells despite increasing the levels of PPARα mRNA expression in the cells. As such, to further evaluate the role of the PPARα mRNA expression in MDA-MB-231, the effects of PGZ and MK886 on the induction and inhibition of mRNA expression of PPARα, respectively, and other associated genes in MDA-MB-231 were determined in this study. The biological mechanisms induced by both drugs were also assessed in this study. To achieve all objectives of the study, the mRNA expression levels of PPARα in PGZ-treated and MK886-treated MDA-MB-231 were determined using Real-Time PCR; the growth inhibitory effects of PGZ and MK886 in MDA-MB-231 were determined using the Trypan Blue Exclusion Assay and Real-time PCR; the apoptosis induction by PGZ and MK886 in MDA-MB-231 was determined using the DNA Fragmentation Assay, Real-Time PCR, cell staining and Flow Cytometry, and the migration of PGZ-treated and MK886-treated MDA-MB-231 was determined using the Wound Healing Assay. Correlation of PPARα mRNA expression with other genes, such as PPARγ, FGF4, 5LOX, ALPI, IL-6, HIF1α, BNIP3 and VEGF, in the drug-treated MDA-MB-231 was also determined in this study using Real-time PCR. The results demonstrated that treatment of MDA-MB-231 with PGZ increased the mRNA expression of PPARα/γ, and the mRNA expression could be inhibited by MK886 treatment. Both drugs reduced the viability of MDA-MB-231 independent of the PPARα/γ mRNA expression but did not induce apoptosis in the treated cancer cells. This phenomenon was confirmed by the detection of caspase-9 and caspase-3 in the treated cells using Real-Time PCR. The wound inflicted was not healed in PGZ-treated MDA-MB-231, but it could be healed in MK886-treated cancer cells, indicating that the migration reduction in PGZ-treated MDA-MB-231 was eliminated by MK886 treatment. This phenomenon may also be associated with the mRNA expression of PPARα/γ, FGF4 and 5LOX in the treated cancer cells. This study provides useful information regarding the mRNA expression levels of PPARα and others related genes in MDA-MB-231. These genes may be attractive targets for reducing the invasiveness of ER-negative human breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Development of cancer

Cancer is fundamentally a disease that begins from a tiny cell whereby the cells turn abnormal and divide uncontrollably until a malignant tissue or a group of abnormal cells is formed (Figure 1.1.a). The cancer cells may also spread from one tissue or one organ to another through blood and lymph systems (Figure 1.1.b). The first cancer cell was discovered by Rudolf Virchow in 1880s (Mukerjee, 2010). Since then, cancers cause about 8.2 million or 14.6% of human death as reported by the World Cancer Report in 2014. There are more than 100 types of human cancers identified worldwide. As such, countless of research studies are published every year in scientific journals, to battle the major killer of humans.

The core focus of current anti-cancer research is to treat and remove cancer, as well as to prevent occurrence of cancer and chance of cancer recurrence. Scientists have found three main classes of genes that are important in controlling cell growth and playing important role in cancer cell development.

The oncogenes that cause healthy cells to grow out of control whereby promoting cancer cell growth (Becker, 2008). Meanwhile, the tumour suppressor genes that normally protect against cancer by stopping cancer cell growth and controlling cell death. Impairment of tumour suppressor genes leads to uncontrolled cell growth, cell division and cell death (apoptosis). Nearly 50% of cancers are thought to involve a damaged or loss of function of tumour suppressor gene (Cooper, 2000). On the other hand, DNA repair genes that are responsible to repair damaged genes by fixing mutations that commonly occur in DNA replication (Wood *et al.*, 2001). These genes are also useful biomarkers in establishing specific cancer diagnosis and therapeutic approaches.

Although regulating oncogenes, tumour suppressor genes and DNA repair genes are the main strategies to combat human cancers, the expression of other genes involved in cancer cell growth may also take part. Indeed, multiple genes are interacting with the surrounding to produce a phenotype for cancer development.

The mode of anti-cancer therapies may vary from inducing cell death, reducing invasiveness, or controlling occurrence and migration rate of the cancer cells. Alternative ways of cancer treatment, including traditional and natural medicines, have also been identified. However, these approaches have not reached optimum results due to the complex mechanism of this deadly disease. One of the major problems is the dynamic changes of the genome. The genetic changes include changing of gene activity and expression level of cancer genes that play important roles in proliferation, differentiation, invasiveness and metastasis that trigger the prognosis of the cancer cells (Yokota, 2000). Therefore, identifying the most prominent gene candidates and studying its effect post certain drug treatment poses high hope for cancer treatment and prevention in the near future.

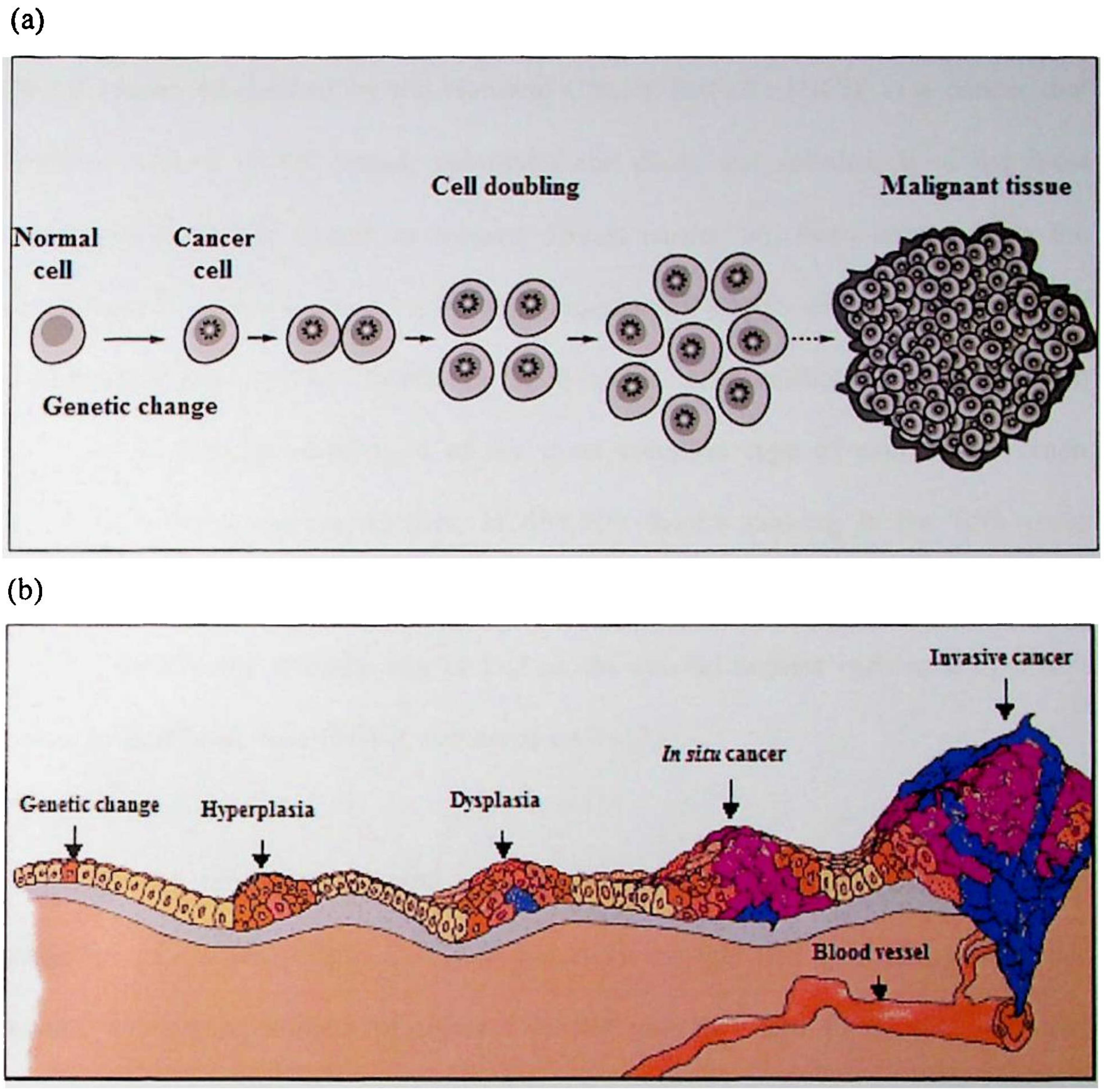

Figure 1.1: The development of cancer. **(a)** Cancer begins from a tiny cell whereby the cells turn abnormal and divide uncontrollably until a malignant tumour or a group of abnormal cells is formed (NASA, 2005). **(b)** The cells may also grow and divide into a condition called hyperplasia and further increase its tendency to dysplasia, *in situ* cancer and then spread from one tissue or organ to another through blood and lymph systems (Canadian Cancer Society, Accessed on 2015).

1.2 Breast cancer

Breast cancer, as defined by the National Cancer Institute (NCI), is a cancer that forms in tissues of the breast, habitually the ducts and lobules. It is the most commonly diagnosed cancer in women. Breast cancer has been identified as the second most common cancer with an incidence of 10.9% of cancers diagnosed worldwide (Ferlay *et al.*, 2010). In 2008 alone, 1.38 million new cases were identified in women, marking it as the most common type of cancer in women worldwide. Breast cancer resulted in 458,000 deaths ranking it the fifth most common cause of cancer deaths (Ferlay *et al.*, 2010). In Malaysia, the incidence of breast cancer in the country was ranked as the second highest incidence of breast cancer in Southeast Asia (WHO, Accessed on 2013).

In 17th century, breast cancer was related to lymph nodes based on several conducted researches (Olson, 2003). It was only in 19th century that breast cancer became a common subject of interest as the mortality age in people increased dramatically. Now, breast cancer is one of the major health issues in developed and developing countries in this globalization era. The breast cancer affects both men and women with the latter being affected at a higher risk. However, breast cancer incidence in male is less than 1% (Fentiman *et al.*, 2006). This major difference could be contributed by several factors, including menstruation, pregnancies, childbirth, breastfeeding and contraceptive pill intake, which are generally happen in female only.

Breast cancer can be divided into several types as shown in Table 1.1. The aggressiveness and responses to treatment may vary depending on the type of cancer. Among the types, triple-negative breast cancer (TNBC) is accountable for almost 15% of all breast cancers (Cleator *et al.,* 2007). TNBC lacks the main receptors namely, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth-factor receptor 2 (HER2) (Gluz *et al.,* 2009). This type of cancer is particularly more aggressive in comparison to other breast cancer types (Carey *et al.,* 2007; Dent *et al.,* 2007; Haffty *et al.,* 2006). Moreover, this type of cancer generally has a poor prognosis and is often unresponsive to the anti-estrogen therapy and do not respond to other hormone treatments. Previous reports show TNBC has a high rate of recurrence, whereby it contributes to higher death rate in breast cancer patients (Dent *et al.*, 2007; Tischkowitz *et al.,* 2007). High mortality rate contributed by TNBC could be related to its aggressiveness and limited treatment options as chemotherapy is the only systemic treatment available for TNBC to date (Reis-Filho & Tutt, 2008).

Table 1.1: Types of breast cancer and its mechanism and action.

Type	Mechanism	Site
Ductal carcinoma *in-situ*	Non-invasive	Lining of breast milk duct
Invasive ductal carcinoma	Invasive	Invade the surrounding of milk duct
Inflammatory breast cancer	Non-tumorogenic	Often affects skin
Metastatic breast cancer	Metastatic	Spread to other tissues or organs
Triple-negative breast cancer (TNBC)	Negative receptor	Tumorigenic development

1.3 MDA-MB-231 cells

MDA-MB-231 used in this study is a mesenchymal-like cell line, belonging to the TNBC (Chavez *et al.*, 2010). The lack of drug therapy approaches for TNBC leads to more researches on this type of breast cancer cell line, to determine the molecular mechanism underlying TNBC. MDA-MB-231 cell line is also a good model for ER-negative breast cancer study, and being studied extensively by our research group as a drug target for the treatment of ER-negative breast cancer. The cells are highly aggressive, invasive, poorly-differentiated characteristic and fast growing than the ER-positive breast cancer cells (Fritah *et al.*, 2008). Our preliminary studies showed that treatment of MDA-MB-231 with pioglitazone and chrysin possessed little effect on cell growth and did not induce apoptosis in the cancer cells despite inducing elevated levels of PPARα mRNA expression in the cells (Teh *et al.*, 2010).

MDA-MB-231 was derived from metastatic adenocarcinoma of the mammary gland of a 51-year-old Caucasian woman, as stated in the data sheet of American Type Culture Collection (ATCC). The morphology of MDA-MB-231 is epithelial-like with spindle shape and some of the cells remain round (Figure 1.2). Like most type of cancers, the progression of breast cancer is closely related to cell invasion, causing the main reason of death in breast cancer patients. It is well accepted now that the MDA-MB-231 cells display its proliferation by mediating the aggressive and invasive potencies of the cancer cells (Liu *et al.*, 2003). Although the invasion process of breast cancer cells is well defined, the molecular mechanism that regulates the anti-invasion, and other processes of the MDA-MB-231 cells remains unclear.

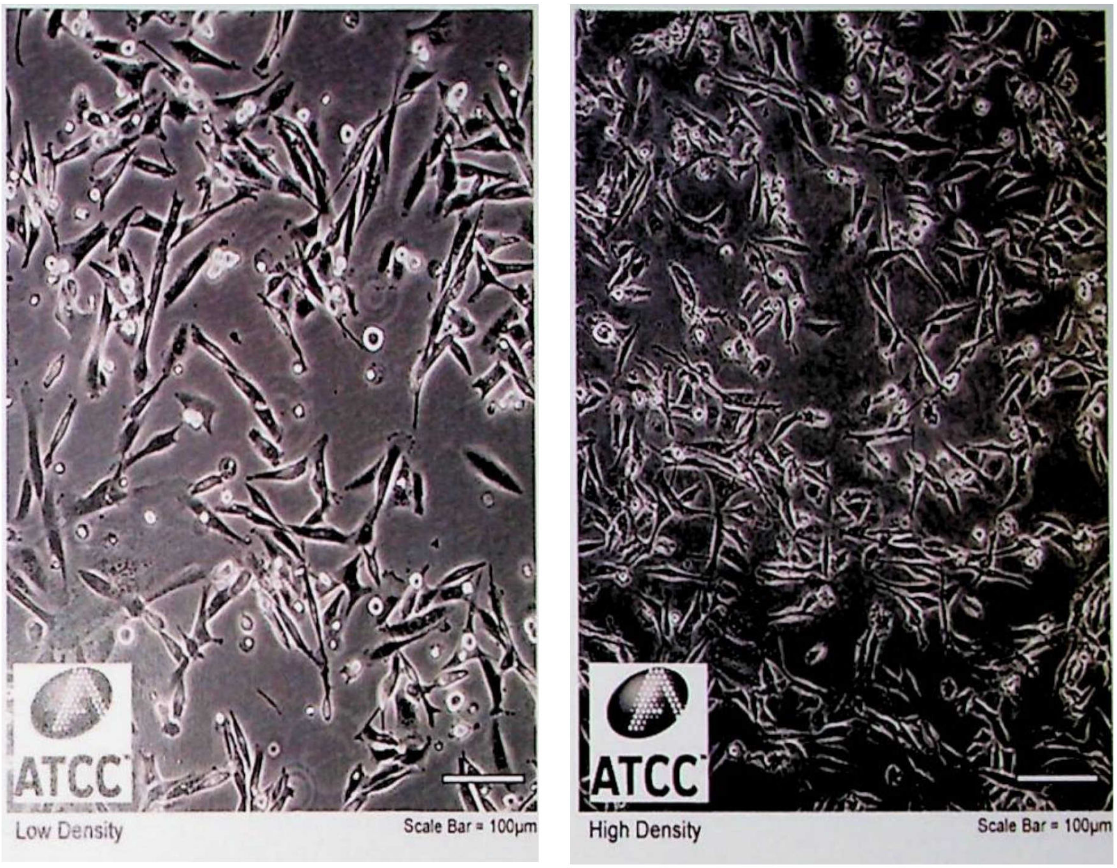

Figure 1.2: Phase contrast micrograph of MDA-MB-231 cells grown in conventional 2D culture plates. Image shows the MDA-MB-231 cells at low (left) and high (right) confluency. Scale bars: 100 µm. [ATCC, Assessed on 2014].

1.4 Gene expression in breast cancer cells

Specific gene expression can be developed as a signature to predict survival, effectiveness and sensitivity of drug treatments for breast cancer. Gene expression regulates cellular activities, such as proliferation, metastasis, apoptosis, autophagy, angiogenesis and many other biological mechanisms involved in cancer progression.

Table 1.2: The genes included in this study.

No.	Name	Abbreviation	Description
1.	Peroxisome proliferator-activated receptor-alpha	PPARα	A nuclear receptor that functions as transcription factor in gene expression regulation.
2.	Peroxisome proliferator-activated receptor-gamma	PPARγ	A nuclear receptor that regulates fatty acid storage and glucose metabolism.
3.	Cysteine-dependent aspartate-directed proteases-9	Caspase-9	An initiator caspase activates apoptosis.
4.	Cysteine-dependent aspartate-directed proteases-3	Caspase-3	An executor caspase activates apoptosis.
5.	Ki-67	Ki-67	A nuclear protein associated with cellular proliferation.
6.	Proliferating cell nuclear antigen	PCNA	A protein that is crucial for DNA synthesis and repairing.
7.	Fibroblast growth Factor-4	FGF4	A signalling molecule responsible for various biological processes.
8.	5-Lipoxygenase	5LOX	An enzyme that is essential for fatty acid transformation.
9.	Intestinal alkaline phosphatase	ALPI	A hydrolase enzyme responsible for dephosphorylation of many types of molecules.
10.	Interleukin-6	IL-6	A protein acts as both pro-inflammatory and anti-inflammatory cytokines.
11.	Hypoxia-inducible factor 1-alpha	HIFIα	A protein responsible for cellular responses to hypoxia.
12.	BCL2/adenovirus E1B kDa protein-interacting protein 3	BNIP3	A protein responsible for protection against virally induced cell death
13.	Vascular endothelial growth factor	VEGF	A signalling protein that stimulates vasculogenesis and angiogenesis.

1.4.1 PPARs

PPARs are identified as nuclear receptor that plays the role of ligand-activated transcription factors (Michalik *et al.*, 2006). The receptors regulate genes essential for cellular processes, such as differentiation, metabolism and promoting tumorigenesis (Belfiore *et al.*, 2009). PPARs were discovered by Issemann and Green in 1990 that were categorized under nuclear hormone receptor superfamily (Issemann & Green, 1990). PPARs can be divided into three subtypes namely PPARα, PPARγ and PPARδ (Berger & Moller, 2002). PPARs have been widely linked to various genetic, nutritional and environmental diseases, such as diabetes, obesity and cancers (Kersten *et al.*, 2000). Although the functions of these three subtypes can be overlapping, the focused PPARs in this study are PPARα and PPARγ, as PPARδ is often linked to colorectal cancer.

PPARα was the main focus in this study. It is expressed in both normal and tumorigenic rat mammary gland and plays a potential role in mammary gland carcinogenesis (Gimble *et al.*, 1998; Roberts-Thompson & Snyderwine, 2000). Previous studies proved that PPARα was dynamically regulated in breast cancer cells to a greater extent in MDA-MB-231 cells, in comparison to MCF-7 cells (Suchanek *et al.*, 2002). It is hypothesized that interaction of peroxisome proliferators with other compounds may exhibit carcinogenic effect (Michalik *et al.*, 2004). The study also stated that PPARα attenuates the inflammatory response that is closely linked to cancer progression and metastasis (Auguste *et al.*, 2007). The involvement of PPARα in carcinogenesis has allegedly been reported to mediate the induction of hepatocarcinogenesis in rodents (Corton *et al.*, 2000). The PPARα gene activation promotes cell proliferation in the cells. PPARα can be activated by both exogenous and endogenous ligands (Issemann & Green, 1990; Forman *et al.*, 1997; Ward *et al.*, 1998; Kliewer *et al.*, 1997; Krey *et al.*, 1997; Lin *et al.*, 1999). In addition, PPARα activation reportedly represses the apoptosis (Hays *et al.*, 2005). However, the mechanism underlying the effects of PPARα remains unclear, making it an interesting target gene for cancer study.

On the other hand, PPARγ is the most well characterized and widely studied isoform of the PPAR family. PPARγ was shown to regulate various malignancies, including breast adenocarcinomas (Mueller *et al.*,1998). In addition, its activation triggered differentiation as reported by Mueller *et al.*, 1998 and apoptosis in several breast cancer cell lines (Kilgore *et al.*, 1997; Elstner *et al.*, 1998). A previous study revealed that the ligands of PPARγ induced cell proliferation and differentiation, and PPARγ activation also leads to inhibition of invasiveness in breast cancer cells (Liu *et al.*, 2003). In the finding, Liu *et al.*, (2003) also stated that the consequence of PPARγ involvement in inflammatory was reportedly very similar to the metastasis of breast cancer and other processes, such as vascular penetration, tissue degradation and neo-angiogenesis.

Another transcriptional regulation of the transcription factors is autoactivation. Autoactivation is a process in which a substance is activated by its secretions. The members of PPAR family have been reported to have the ability to undergo this mechanism. This phenomenon was evidenced in adipose differentiation in which the expression of PPAR alone was sufficient to induce the processes of adipose conversion and differentiation (reference). Moreover, the activation was aided by binding of the transcription factor to peroxisome proliferator response elements (PPREs) and induced by its ligands, such as fatty acids. This activation is made possible through the formation of heterodimer binding with retinoid X receptors (RXR) (Magdougald & Lane, 1995). The ectopic expression of one gene is activated the others provides the ability of the genes to cross-activate the gene expression (Tonionoz *et al.* 1994). It is further elucidated that many other regulatory genes may be involved in different stages of autoactivation that involve multiple transcription factors. Thus, it is important to study the genes that shuttle early stage of the processes, such as studying the gene expression at mRNA level, in order to get a clear picture of the interconnection of the PPAR isoforms with other genes in breast cancer cells,

1.4.2 Caspases

Apoptosis is a programmed cell death that plays an important process in malignancies (Kerr *et al.*, 1994; Evan & Vousden, 2001). Apoptosis has been referred to as a standard event for validating the effectiveness of potential anti-cancer agents on given type of cancers. It is well known that the event is initiated and mediated by various crucial genes, such as caspases. Caspases belong to cysteine protease family, play crucial roles in apoptosis, necrosis and inflammation (Alnemri *et al.*, 1996). The caspases are named according to the order of discovery. The caspases that are involved in apoptosis can also be divided into two groups namely, initiator caspases (e.g. caspase-9) and downstream effector caspases (e.g. caspase-3), (O'Donovan *et al.*, 2003).

Caspase-3 is an effector caspase and is only activated during apoptosis. The caspase needs three proteins; cytochrome-c, Apaf-1 and active caspase-9 for its activation and the binding of these three proteins forms an apoptosome (Zou *et al.*, 2002; Li *et al.*, 2004). This lengthy process of apoptosis begins when the signal of induction is received whereby the mitochondrial outer membrane permeabilization occurs. Release of various proteins into the membrane space results in the release of cytochrome-c from the mitochondria. Cytochrome-c in the cytoplasm combines with Apaf-1, to form a complex termed apoptosome, in the presence of ATP, in order to activate the Caspase-9 (Debatin, 2004). The Caspase-9 subsequently activates the downstream executor Caspase-3. Activation of Caspase-3 and the subsequent degradative events trigger apoptosis. This process is named intrinsic apoptosis. On the other hand, in extrinsic apoptosis occurs when death receptors ligate with their ligands resulting in recruitment of adapter molecules that cause dimerization and involvement of Caspase-8 that activates Caspase-3 (Tait & Green, 2010). A previous study reported that the expression of Caspase-3 in breast cancer cells was very low or undetectable despite detectable range of Caspase-9 (Devarajan *et al.*, 2002). This phenomenon indicates the crucial role of Caspase-3 in the mechanism of breast cancer development. In order to reverse this situation, stimulation of cytochrome-c that aids pro-Caspase-9 activation is required (Blanc *et al.*, 2000). Therefore, it is necessary to evaluate the level of Caspase-9 and Caspase-3 in breast cancer cells post potential drug treatment.

1.4.3 Cell proliferation markers

Cell proliferation is a vital process in breast cancer initiation and progression. It is often regarded as the prognostic indicator (Tubiana & Courdi, 1989). There are two common proteins being studied for this mechanism; Ki-67 and proliferating cell nuclear antigen (PCNA). Ki-67 is a nuclear protein that is detected in the cell nucleus during the active phase of cell cycle (Scholzen & Gerdes, 2000). It is closely associated with cell proliferation. Meanwhile, PCNA is an auxiliary protein of DNA polymerase δ (Leornadi *et al.*, 1992). PCNA plays a fundamental role in DNA synthesis and is abundantly found in cell nuclei during the cell cycle (Hall *et al.*, 1990).

Ki-67 is identified as a reliable cell proliferation marker for early onset of breast cancer (Urruticoechea *et al.*, 2005). Ki-67 has been reported to express at very low level in normal breast cells (Harper-Wynne *et al.*, 2002) and only present in ER-negative breast cancer cells (Clarke *et al.*, 1997). Abundance of Ki-67 indicates a poor prognosis associated with lack of responses toward anti-cancer therapy (Urruticoechea *et al.*, 2005). On the other hand, PCNA has been reported to have prognostic value in various types of cancers (Cummings *et al.*, 1993). The research reveals that two isoforms of PCNA are found in breast cancer cells, marking it an effective marker to detect breast malignancy (Malkas *et al.*, 2006). Beforehand, it was discovered that Ki-67 and PCNA shares a weak, but positive correlation in cancers (Haersley *et al.*, 1996; Moriki *et al.*, 1996). This correlation is associated with multiple functions of PCNA in cancer cells (Prosperi, 1997).

1.4.4 Other genes associated with PPARα

(a) Fibroblast growth factor-4 (FGF4)

FGF4 is a human protein encoded by the fibroblast growth factor family. The gene is involved in many stages of cell survival mechanism, including cell growth, tissue repair, morphogenesis and invasion (Galland *et al.*, 1992). Although FGF4 has not been considered as the key gene of the family, it has been closely linked to mammary tumorigenesis in the mouse (Basilico & Moscatelli, 1992). However, contradicting statements have been linked to FGF4 until a study conducted in 1995 that showed FGF4 was expressed in 32% of tumours and breast-related cancer. The study also reported the presence of FGF4 in MDA-MB-231 (Penault-Llorca *et al.*, 1995). Another study reported that FGF4 is involved in mediating pathway to the expansion of cancer stem cells *via* estrogen receptor-based mechanism (Fillmore *et al.*, 2010). Interestingly, another study on TNBC and its signalling promoters reported that FGF4 inhibition only showed a modest effect on the growth of TNBC (Sharpe *et al.*, 2011). Hence, there is a need to study the role of FGF4 in MDA-MB-231 to obtain a clear view of the role of this gene in ER-negative breast cancer cells and if the activities of this gene is influenced by the expression of PPARα.

(b) 5-lipoxygenase (5LOX)

5LOX is an enzyme that is encoded by a human gene; ALOX5, and interacts with arachidonic acid (AA) to convert the enzyme to leukotrienes (Hoque *et al.*, 2005). AA is the major predecessor of human carcinogenesis because its metabolites enhances cell proliferation and inhibits apoptosis (Shureiqi & Lippman, 2001; Tong *et al.*, 2002; Xu, 2002). A report revealed that the products of 5LOX increase cell proliferation (Tong *et al.*, 2002). Another report showed that 5LOX sustained high growth rate of breast cancer cells (Hu *et al.*, 2011). Therefore, additional studies on the expression of 5LOX in breast cancer cells and its effect on other breast cancer-related genes post drug treatment is important, to better explain the role of 5LOX in human breast cancer.

(c) Alkaline phosphatase

ALP is the enzyme that is evaluated in serum-based rapid detection tests for cancer diagnostic (Ritzke *et al.*, 1998). The enzyme removes phosphatase from many molecules, such as nucleotides, proteins and alkaloids (Reichling & Kaplan, 1988). There are three most common isoenzymes of ALP; from intestinal (ALPI); non-specific tissue (ALPL) and placenta (ALPP). Increased level of ALP in serum is often associated with various diseases, including liver disease, hepatitis and some malignancies. Previous studies show that ALP is markedly increased in breast cancer patients (Ritzke *et al.*, 1998). Further study on the expression of ALP in non-treated and treated breast cancer cells may reveal valuable information for early diagnosis and therapeutic of ER-negative breast cancer. The isoenzyme chosen for this study is ALPI. The ALPI has been reported to be induced by the activation of PPAR isoform in human colon cancer (Kitamura *et al.*,1999). Thus, it is interesting to investigate the PPAR and ALPI correlation in breast cancer post treatment with PPAR ligand.

(d) Interleukin-6 (IL-6)

Interleukins are known as extracellular proteins that interact with hematopoietic and immune cells (Huang *et al.*,2015). Secreted by lymphocytes and macrophages, IL-6 can act as both pro-inflammatory and anti-inflammatory cytokines (Knupfer & Preiss, 2007). The cytokines are sometimes interchangeably used as growth factors. There are extended research revealed diverse functions of IL-6 and thus unable to provide a clear difference between the functions (Wordinger & Clark, 2008). Previous report demonstrated that significant defect in the immune system of breast cancer patients was related to inhibitory cytokines within the tumorigenic cells (Stewart, 1996). In addition, breast cancer aggressiveness has also been demonstrated to attribute to the pro-inflammatory characteristic of cytokines (Purohit *et al.*, 2002). Another report shows that IL-6 induces drug resistant protein, GP96 in MDA-MB-231 cells which grants survival advantage to tumor cells through drug resistance (Haverty *et al.*, 1997). Additionally, high invasiveness of ER-negative breast cancer cells has also been related to IL-6 (Bhat-Nakshatri *et al.*, 2004). Thus, it is important to study the expression of IL-6 in specific conditions of breast cancer cells; pre-treatment and post-treatment with potential anti-cancer drugs, to further elucidate the role of IL-6 in breast cancer entirely.

(e) Hypoxia-inducible factor-1-alpha (HIF1α)

Hypoxia-inducible factor (HIF) plays a key role in cell survival response to hypoxia (Bellot *et al.*, 2009). This phenomenon is mainly influenced by a major protein, HIF1α which is encoded by HIF1α gene (Semenza *et al.*,1996). The HIF1α is responsible for cellular responses to hypoxia, plays an important role for tumour environment and monitors nutrient availability, intracellular pH and anaerobic glycolysis (Dayan *et al.*, 2006). Bellot *et al.* (2009) reported that HIF1α, which is usually functioning as a tumor promoter in cancer fibroblasts, also functions as a tumor suppressor gene in breast cancer and epithelial cells. A study by Dayan *et al.* (2006) also supports the information that HIF1α is a bifunctional protein. Thus, it is essential to study the expression level of HIF1α in ER-negative breast cancer.

(f) BCL2/adenovirus E1B19 kDa protein-interacting protein 3 (BNIP3)

BCL2/adenovirus E1B19 kDa protein-interacting protein 3 or BNIP3 is often regarded as a pro-apoptotic gene in mediating apoptosis. A previous study showed that BNIP3 was a target for transcriptional repression through pRB/E2F pathway, which was also essential to the hypoxia-induced autophagy (Tracy *et al.*, 2007). This phenomenon was supported by another finding that revealed BNIP3 was required for the optimal induction of autophagy during low oxygen tension (Bellot *et al.*, 2009). The same study showed the hypoxia-induced autophagy *via* BNIP3 was indeed a survival mechanism for autophagy. Therefore, it is important to study the expression of BNIP3 in MDA-MB-231, as well as its correlation with HIF1α as the proteins may be used as the targets to evaluate the efficacy of the drug treatments in breast cancer.

(g) Vascular endothelial growth factor (VEGF)

VEGF is a signal protein that is involved in cell proliferation, migration and invasion, as well as angiogenesis (Weidner *et al.*, 1991; Ferrara, 2001). Angiogenesis is an utmost important mechanism in tumour growth progression and metastasis (Folkman, 1992). VEGF is also known as vascular permeability factor for marking it a vascular permeability agent that allows endothelial cell basement membrane disruption in breast cancer metastasis (Weiss & Cheresh, 2005). Overexpression of VEGF may lethally affect breast cancer progression, and hence provide useful information for the researchers to perform antiangiogenic therapy and treatment for advanced breast cancer by targeting VEGF (George & Sledge, 2005).

1.5 Drugs used in the study

1.5.1 Pioglitazone (PGZ)

PGZ is a prescription drug for Diabetes Mellitus Type II, belonging to the thiazolidinedione (TZD), and has a molecular formula of $C_{19}H_{20}N_2O_3S$ (Figure 1.3.a). The PGZ is a well-known synthetic ligand for peroxisome-proliferator activated receptors (PPARs) in which PPARγ is triggered to a higher extent in comparison to PPARα (Gillies & Dunn, 2000; Smith, 2001). The PGZ alters the protein synthesis of PPARs by altering the carbohydrate and lipid metabolism-related gene transcription (Smith, 2001). Previous studies have shown that diabetes and breast cancer are correlatively related through three distinct mechanisms; insulin pathway; insulin-like-growth-factor pathway and endogenous sex hormone regulation (Wolf *et al.*, 2005). Several studies proved that PPARγ ligands, including PGZ could reduce the proliferation and induced apoptosis in breast cancer cells through PPARγ activation and invasiveness reduction (Kilgore *et al.*, 1997; Elstner *et al.*, 1998; Mueller *et al.*, 1998; Liu *et al.*, 2003). The alteration of genes' transcription factor through treatment with PGZ would offer a promising approach towards breast cancer therapy.

1.5.2 MK886

MK886 is an indole derivate that is previously known as L663536. It has a molecular formula of $C_{27}H_{33}ClNO_2S.Na$ (Figure 1.3.b). The 5-lipoxygenase-activating protein (5-FLAP) is required for catalytical activation of 5LOX(Abramovitz *et al.*, 1993). As introduced in the Introduction, the 5LOX is the key enzyme in the metabolism of AA that plays a major role in breast cancer (Rioux & Castonguay, 1998). Studies have proven that inhibition of 5LOX is able to induce growth arrest and apoptosis in breast cancer cells (Avis *et al.*, 2001). Another study reveals that MK886 is an inhibitor of PPARs as well, with the ability to inhibit PPARα to a greater extent than PPARγ , and hence it has since been used widely as a PPAR antagonist (Kehrer *et al.*, 2001). However, Avis *et al.* (2001) reported contradicting results whereby 5LOX inhibitors mediate the activation of PPARγ. Therefore, studying the mechanism induced by MK886, the effect of MK886 on the expression of PPARα and its mode of action in MDA-MB-231 are warranted.

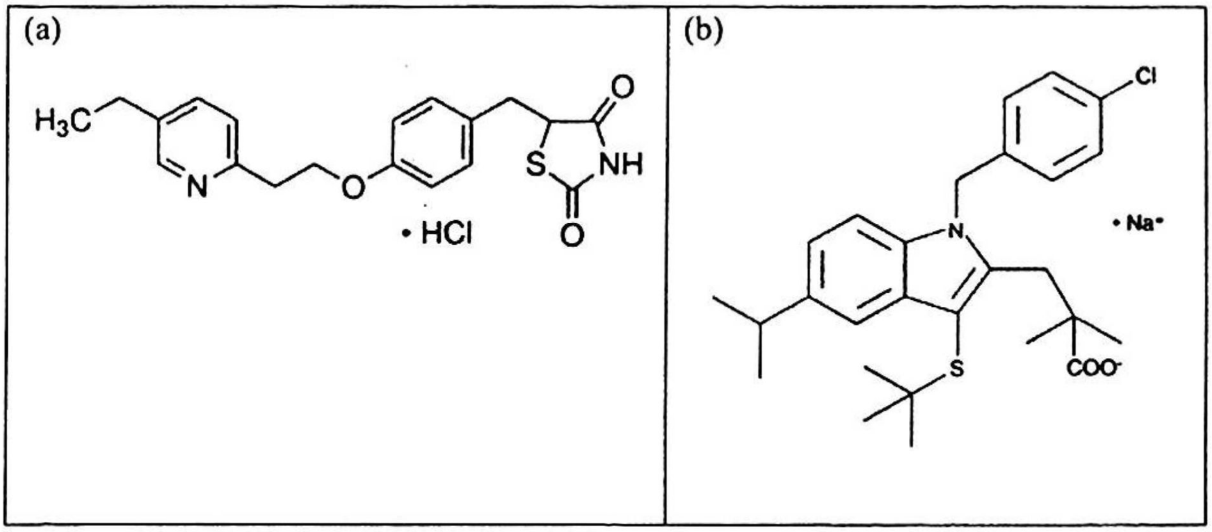

Figure 1.3: Chemical structures of (a) PGZ (Sigma Aldrich, 2014) and (b) MK886 (Cayman Chemicals, 2014). PGZ induces elevated level of PPAR whereas MK886 inhibits the expression of PPAR.

1.6 Technologies used in the study

1.6.1 Real-time PCR

Real-time polymerase chain reaction or also known as quantitative PCR method is a technique based on the principles of PCR. Similarly in both the techniques, the method is used to amplify and detect a target DNA through 5' to 3' exonuclease activity (Holland *et al.*, 1991). Like conventional PCR, qPCR is revolved with three main stages which are, denaturation, annealing and extension. However, in real-time PCR this detection is observed in quantification format in which the amplification is measured real-time and the result is exerted in numerical form unlike the conventional PCR. It is a highly sensitive, specific and reproducible method. Two general chemistries are available for qPCR. First, is double stranded DNA intercalating agents (eg: SyBr Green) which binds to dsDNA and emits fluroscences. However this method is based on the amount of dsDNA available in the reaction and may also correspond to unspecific bindings and requires a melt curve analysis to overcome this problem.

Meanwhile, with the presence of fluorogenic probes, unspecific binding amplification can be avoided altogether as a specific hybridization between the probes and template is required for fluorescent emission. The methods can be further breakdown into hydrolysis probe (eg: TaqMan), dual hybridization probes, molecular beacons and scorpions. The results are usually shown as Ct value which is generated during the exponential phase is the fractional PCR number during which the fluorescence is higher than the minimal detection limit (Arya *et al.*, 2005). qPCR has been widely used for gene expression studies (Bustin, 2000). For a more reliable result, comparative quantification (ΔΔCt) method is used. In this method, the relative expression level of the gene of interest is propotional to the difference in threshold cycles in gene of interest and the housekeeping gene (Klein, 2002).

1.6.2 Cell based assays

1.6.2.a Cell stainings

Trypan blue is a dye based assay that is commonly used to determine cell viability. The assay is based on the principle of membrane permeability (Strober, 2001). If the cells are viable, the cell membrane will be intact thus dye will not permeate. However, the dye can permeate the dead cells and bind with the intracellular proteins and the cytoplasm will be stained with blue colour. Nevertheless, since the cell viability determined by trypan blue method is purely based on cell integrity and may be compromised and the viability of the cell may be overestimated (Altman *et al.*, 2008). The results may differ based on the level of injury and the ability of the cell to repair itself (Strober, 2001). Several comparison methods were carried out and flurometric assay such as propidium iodide staining was considered as a reliable alternate method.

Meanwhile, propidium iodide is a cell stain with intercalating property. The dye which is membrane impermeant only enters compromised cells and binds to the DNA content inside a cell (Richardo & Nicoletti, 2006). The non viable cells profuse with PI will be stained in red colour. PI is commonly used for detection of cell viability as well as cell cycle analysis such as fluocytometry.