EFFECT OF PHORBOL 12-MYRISTATE 13-ACETATE TREATMENT ON CHOLINE KINASE EXPRESSION

by

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LIST OF ABBREVIATION AND ACRONYMS

ATP	Adenosine Triphosphate
CCT	Cytidylyltransferase
CDP-choline	Cytidine-5-iphosphocholine
CEPT	Choline/Ethanolamine Phosphotransferase
Cho	Choline
CK	Choline Kinase
CPT	Cholinephosphotransferase
DAG	Diacrylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
ERK	Extracellular Signal-regulated Kinases
EthK	Ethanolamine Kinase
FBS	Fetal Bovine Serum
GTP	Guanosine Triphosphate
hCK	Human Choline Kinase
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Respossive Element
MAPK	Mitogen-activated Protein Kinase
PC	Phosphatidylcholine
PCho	Phosphocholine
PE	Phosphoethanolamine
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
TBST	Tris Buffer Saline Tween-20

LIST OF SYMBOLS

%	Percentage
rpm	Round Per Minutes
kDa	Kilo Dalton
°C	Degree Celsius
V	Voltage
α	Alpha
β	Beta
μ	Micro
n	Nano
g	Gram
m	Mili
L	Liter
w/v	Mass/volume
v/v	Volume/volume
Hrs	Hours
M	Molar
A	Ampere

KESAN RAWATAN FORBOL 12-MIRISTAT-13-ASETAT TERHADAP EKRPRESI KOLINA KINASE

ABSTRAK

Kolina kinase (CK) memainkan peranan yang penting dalam *de novo* sintesis fosfolipid. Ia mengphospholilasikan kolin kepada fosfokolin dalam kehadiran ATP dan Mg^{2+}. Banyak kajian menyatakan bahawa karsinogenesis dantumorigenesis berkait dengan peningkatan tahap CK dan ia sudah menjadi satu ciri yang penting pada sel kanser. Oleh itu, CK disasarkan sebagai potensi baru dalam rawatan kanser. Namun demikian, kajian tentang jujukan promoter CK masih kurang dijalankan. Untuk mengenal pasti tapak pengikatan faktor transkripsi yang berpotensi untuk mengawal keaktifan promoter CK, penyelidikan terhadap jujukan promoter dari -1252 sampai -1275 dijalankan dengan rawatan "phorbol 12-myristate 13-acetate" (PMA) dalam keturunan sel adenokarsinoma payu dara manusia, MCF-7.

Jujukan promoter CK -1284 telah diklonkan dalam sistem vektor pelapor berdasarkan lusiferase. Dengan cara ini, rawatan PMA didapati menurunkan keaktifan promoter CK. Tambahan pula, tahap mRNA dan protein CK yang diekspres juga menurun selepas rawatan PMA. PMA yang digelar sebagai pengaktif protein kinase C (PKC) juga mengaktifkan aktiviti kumpulan E26 transformasi jujukan spesifik, Ets. Dengan keputusan kajian ini, kita mencadangkan bahawa faktor transkripsi kumpulan cEts memainkan peranan sebagai pengawal negatif dalam promoter CK.

EFFECT OF PHORBOL 12-MYRISTATE 13-ACETATE TREATMENT ON CHOLINE KINASE EXPRESSION

ABSTRACT

Choline kinase (CK) plays main role in the *de novo* phospholipid synthesis pathway. It phosphorylates choline into phosphocholine in the presence of ATP and Mg^{2+}. Many studies have showed that the carcinogenesis and tumorigenesis are associated with the increased of CK and it has become the hallmark of cancerous cells. It is so importantly that CK has become the potential target of the anti-cancer therapy. Yet, studies on the promoter sequence of the CK remain rare. In order to identify the potential transcription factor binding sites which act on regulating the CK promoter, -1252 to -1275 of choline kinase α, (hCKα) promoter sequence was investigated for the effect of phorbol 12-myristate 13-acetate (PMA) treament in human breast adenocarcinoma cell lines, MCF-7.

A -1284 putative promoter of CKα was cloned into a luciferase (Luc) based reporter vector system. In MCF-7 cells, PMA treatment decreased the expression of Luc under the control of the CKα promoter. In addition, CKα mRNA and protein levels were decreased compare to the control in response to the PMA treatment. PMA, the protein kinase C (PKC) activator, promoted the E26 transformation specific sequencec, Ets family transcription factors activity. The results suggest that cEts family transcription factors were acting as negative regulators of CKα promoter activity.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction of Choline Kinase

In 1953, Wittenberg and Kornberg described the enzymatic action of Choline kinase (CK) in Brewer's yeast for the first time. CK is a cytosolic enzyme and the primary enzyme in the Kennedy pathway (Figure 1.1), responsible for the *de novo* synthesis of phosphatidylcholine (PC). Through this pathway, there are three reactions involved: first the free choline (Cho) is phosphorylated by CK and adenosine triphosphate (ATP) in the presence of Mg^{2+}, yielding phosphocholine (PCho), PCho with choline-phosphate cytidylyltransferase (CTP) are catalyzed to form cytidine-5-phosphocholine (CDP-choline) by phosphocholine (PCho) cytidylyl-transferase (CCT) and the final condensation reaction of CDP-choline with diacylglycerol are catalyzed by cholinephosphotransferase (CPT) producing phosphatidylcholine (Aoyama *et al.*, 2004).

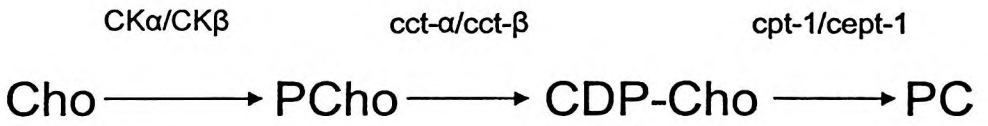

Figure 1.1 Biosynthetic pathway of phosphatidylcholine (PC) in human. CKα and CKβ for choline kinase (CK), cct-α and cct-β for P-Cho cytidylyltransferase (CCT), cpt-1 and cept-1 for cholinephosphotransferase (CPT) and choline/ethanolamine phosphotransferase (CEPT), respectively. Cho, choline; P-Cho, phosphocholine (modified from Aoyama *et al.,* 2004).

Human CK are divided into two: choline kinase alpha (CKα) and choline kinase beta (CKβ) which are encoded by two separate genes, CHKA and CHKB. For CKα, from the same CHKA gene, two isoforms, CKα1 (50kDa, 439amino acids) and CKα2 (52kDa, 457amino acids) are produced due to an alternative splicing while only CKβ (45kDa, 395amino acids) is derived from separate CHKB genes. CKα2 isoform sequence is different from CKα1 by having an additional 18 amino acids insertion (RSCNKEGSEQAQNENEFQ) in CKα1 sequence. The functional difference between CKα1 and CKα2 is still unknown. CKα1 is shorter than CKα2 and is about 60% similarity in sequences between CKα1 and CKβ (Aoyama *et al.*, 1998). The 2.7-kb gene encoding human ChKα is located on chromosome 11 and has 12 exons (NCBI). The 1.6-kb gene encoding human ChKβ is located on chromosome 22 and has 11 exons (NCBI). These three different proteins consist of choline/ethanolamine kinase (CK/EtnK) domain which allows them to catalyze the phosphorylation of Cho to PCho and also play role in the synthesis of phosphoethanolamine (PE), by catalyze the phosphorylation of ethanolamine to produce phosphoethanolamine (PE).

According to Gallego-Ortega *et al.* in 2009, CKα1 can function as both CK and EtnK while CKβ show low CK activity but high PE activity. The enzymatic activity of CKα and CKβ was suggested that in cell-free systems, choline is a better substrate for CKα1 (2.85 fold) than CKβ, whereas ethanolamine is a better substrate for CKβ (5.83 fold) than CKα1.

According to previous studies, the only active form of CK is in dimeric form protein. Since there are two types of CK, different homo- or hetero- dimer population has been proposed to be tissue-specific. Furthermore, the combination between CK isoforms results in a different level of CK activity in vitro under cell-free systems conditions. Thus, the α/α homodimer is the most active choline kinase form, the β/β homodimer is the less active, and the a/b heterodimer has an intermediate phenotype. Since CKα has two isoforms , CKα1 and CKα2, more isoform subunits dimer form combination can be developed like α1/α1, α1/α2, α2/α2, α1/β, α2/α2, α2/β and β/β. A significantly high expression of CKα was detected in both testis and liver, whereas a relatively high expression of CKβ was found in heart and liver (Aoyama *et al.*, 2002).

1.2 Role of CK in Phosphatidylcholine Biosynthesis

PC is the most abundant class of glycerolphospholipid present in mammalian cells. It is a crucial structural component in cellular membranes and serum lipoproteins and serves as a precursor for the production of lipid second messenger (Exton, 2000).

For mammalian cells, PC is biosynthesized in two pathways. One is the *de novo* pathway, from choline via Kennedy (CDP-choline) pathway and the phosphatidylethanolamine methylation pathway (PEMP) and degraded by phosphatidylcholine specific phospholipase D whereas, PEMP seems to be significant only in hepatocytes. Then, PC is hydrolyzed by phospholipase D to yield phosphatidic acid and choline. CK appears to be crucial to initiate PC

biosynthesis as well as to release the essential lipid second messengers like PCho and DAG for mitogenic signal transduction (Lacal, 2001). PCho has been proposed to promote mitogenesis in mammalian cells by acting as a mediator of growth factor-induced cell proliferation (Cuadrado *et al.,* 1993). Consistent with this finding, nuclear magnetic resonance spectroscopy techniques, have revealed higher levels of phosphomonoesthers in tumoral samples when compared to their normal counterparts (Eliyahu *et al.*, 2007). There was also a study showed that the increase level of PCho initiates the mitogenic activity in NIH3T3 and other cell types has been observed. It has been shown that phosphocholine confers mitogenic properties to mouse fibroblasts upon stimulation by platelet-derived growth factor (PDGF) or fibroblast growth factors (FGF). The increased mitogenesis rate after microinjecting the PCho into the cells produces a rather direct evidence for the role of PCho in growth factor regulated mitogenesis (Cuadrado *et al.*, 1993).

1.3 Role of CK in Tumor Development

PCho is an important metabolite that is involved in cell proliferation as well as tumor production and induction of DNA synthesis. Therefore the generation of PCho through the Kennedy pathway is considered to be one of the crucial steps in regulating growth factor for cell proliferation, tumorigenesis, invasion and metastasis (Rodriguez-Gonzalez *et al.*, 2003).

Tumor formation is associated with the alteration of phospholipid metabolism which leads to the overexpression of PCho. The overexpression of PCho is

accompanied by the higher activation of CK and studies stated that various growth factors namely, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin dependent growth factor (IDGF), and vascular endothelial growth factors (VEGF) could enchance the CK activity during the tumor production (Janardhan *et al.*, 2006).

Elevated phosphocholine level is a common characteristic feature in cell lines derived from human tumors and this parameter seems to be able to distinguish the malignant cell lines from the normal cell lines irrespective of their proliferation rates (Aboagye *et al.,* 1999). The elevated levels of phosphocholine and accumulation of intracellular phosphocholine have been investigated in a variety of different types of human tumors like breast, lung, colon, colectal and prostate as well as in diverse murine tumors (*Liu et al.,* 2002).

The implication of CKα in cell growth, proliferation, initiation and progression of cancer is well documented. Different tumoral tissues of human lung, colorectal, prostate and breast cancer and the derived epithelial and hemopoietics cell lines have displayed the overexpression of CK compared to the normal cells (Ramirez de Molina *et al.*, 2002).

CK protein levels have been found to be drastically increased in both human tumors and cell lines derived from human tumor, when compared to normal tissues or appropriate human primary cells, respectively (Janardhan *et al.* 2006).

The involvement of Rho GTPase family in cancer onset and progression has been extensively described. Previous study has showed that CK is involved in the malignant transformation along with Ras and RhoA as Ras and RhoA GTPases are upstream regulators of CKα1. (Ramirez de Molina *et al.*,2005) Ras signaling pathway through its two well-known effectors, Ral-GDS and P13K enhance the CK activity during tumour formation while third effector Raf-1 does not show any sginificant effect on CK (Ramirez de Molina *et al*, 2002). Study from Ramirez de Molina (2005) showed that overexpression of CK induces oncogenic transformation of human embryo kidney fibroblasts and canine epithelial Madin-Darby canine kidney cells.

According to Gallego-Ortega *et al.* (2009), the overexpression of CKα1 was sufficient to induce tumour growth in immunosuppressed mice in the both cell systems analyzed while cells overexpressing CKβ were not able to induce tumour growth under similar conditions. This is supported by the significant increase overexpression of CKα mRNA compared with normal cells whereas no changes for CKβ RNA levels. Through this observation, this suggested that CKβ is not an essential component in cancer formation. (Eliyahu *et al.*, 2007)

Choline phospholipid compounds of these HCT116 human colon cancer cells with (p53+/+) and without p53 (p53-/-) were observed that the loss of the p53 function resulted in increased PC and total choline, which were consistent with increased malignancy (Glunde *et al,* 2005).

1.4 Role of CKα in cell stress

Previous study on higher prokaryote and the plant *Arabidopsis* have shown that *CK* gene is induced during cell stress. An increased level of CK expression has been observed during cell stress, which is likely to initiate the cell proliferation. According to Aoyama *et al.,* 2004, CK-α is mostly involved in chemically induced cell stress such as polycyclic aromatic hydrocarbons (PAH) and carbon tetrachloride (CCl_4). Metabolism of CCl_4 by liver CYP4E, a member of P_{450} family producing trichloromethyl radical and this reactive radical causes the lipid peroxidation and leads to the cell membrane damage by producing a reactive oxygen species. During this process, the *CK* gene induction is a secondary response to the increased levels of *c-fos* and *c-jun* genes (Kitteringham *et al.,* 2000). However, the exact physiological role of *CK* gene in cell stress is not yet clarified. Besides that, CK has also been considered as a new target in the development of antimalarial drugs (Janardhan *et al.,* 2006).

1.5 CK knockout (KO)

CKβ knockout (KO) mice (*rmd* mice) are viable, but develop a rostrocaudal muscular dystrophy, while phosphatidylcholine levels are normal in most tissues analyzed except in hind limb skeletal muscle (Sher *et al.,* 2006). Therefore, CKα is sufficient to maintain normal phosphatidylcholine levels in most tissues. By contrast, the lack of CKα results in embryonic lethality, and CKα+/− heterozygous mice displays an accumulation of choline and a reduction in phosphocholine in liver and testis, suggesting that there is no CKβ compensation in CKα knockouts for phosphatidylcholine biosynthesis *in*

vivo. These results suggested different roles *in vivo* for both CKα and CKβ isoforms. Furthermore, the attenuated levels in phosphatidylethanolmine found in CKα+/− heterozygous mice suggest the involvement of CKα not only in the biosynthesis of phosphatidylcholine but also in the phosphatidylethanolmine pathway. This is also consistent with the fact that in CKβ KO mice, phosphatidylethanolmine levels are unaffected, indicating that phosphatidylethanolmine homeostasis is fully maintained with the ethanolamine kinase and CKα proteins intact (Gallego-Ortega *et al.,* 2009).

1.6 Promoter analysis of human CKα gene

Recently, Glunde *et al.,* 2008 had done a promoter analysis on isolated upstream sequence of human *CKα* gene. Similar to the promoter isolated upstream of rodent *CKα* gene, human CKα promoter had a high GC content and was TATA less. No core promoter region was recognized. Six putative hypoxia response elements (5'-CGTG-3') were identified out of which 4 closely spaced HREs (HRE 3-6) were shown to bind HIF1α. These 4 HRE sites were found to be associated with up-regulation of CKα expression in promote reporter assays under hypoxic condition. Then, two putative HRE sites were newly identified as HRE2B (-1422) and HRE7 (-222) by Bansal *et al.*, 2011 stated that hypoxia decreased choline phosphorylation, choline kinase activity and CK mRNA and protein levels. Promoter analysis studies revealed a region upstream of the *CKα* gene bearing a conserved DNA consensus binding motif, hypoxia response element-7 (HRE7) at position -222 relative to +1 translation start site, for binding the hypoxia dependent master regulator transcription factor, hypoxia-inducible factor 1α (HIF-1α).

They suggested that transcriptional control of choline phosphorylation is largely mediated via HIF-1α binding to the newly identified HRE7 as shown in Figure 1.2.

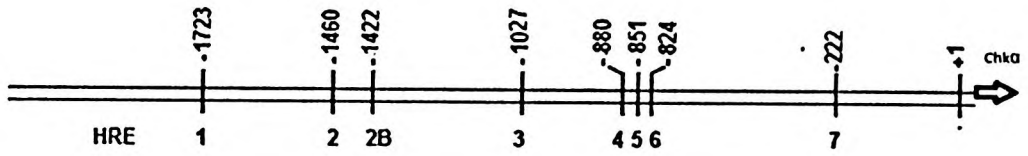

Figure 1.2 Schematic diagram of promoter region upstream of human choline kinase α gene. Figure shows putative 8 HRE sites. (Modified from Bansal *et al.*, 2011)

1.7 Choline kinase expression and activity

CK activation by mitogens and growth factors is implicated in Ras-dependent and independent carcinogenesis and tumor progression (Janardhan *et al.*, 2006).

The hypoxia-regulated expression of CK through hypoxia inducible factor 1 (HIF-1) signaling has been reported by Glunde and colleagues in a human prostate cancer cell line (Glunde *et al.*, 2008) *raf*-transformed cells have also increased CK activity and elevated PCho levels (Ratnam and Kent, 1995). Ras-signaling pathways through Ral-GDS and phosphoinositide 3-kinase (PI3K) effectors can increase the activity of CK during tumor formation and, CK activation and generation of PCho have been proposed as essential events in regulating AKT phosphorylation and activation of Raf-1 and mitogen-activated protein kinases (MAPK) (Jimenez *et al.*, 1995) Previous study has shown that CKα but not CKβ is a downstream target of oncogenic molecules such as Ras and RhoA. Thus, while Ras activates CKα through Ral-GDS and PI3K, and RhoA activates CKα through Rho and Rho-associated protein kinase (ROCK), none of these oncogenic GTPases affect CKβ activity under similar conditions (Ramirez de Molina *et al.*, 2002; Ramirez de Molina *et al.*, 2005).

Overexpression of CK will lead to the increase of downstream activity. Thus, the overexpression gives rise to carcinogenesis. CK has been proposed as a prognostic factor for the onset of breast, non-small cell lung cancer and bladder carcinomas (Ramirez de Molina *et al.*, 2007). According to Eliyahu

(2007), there are a significant 2- to 5-fold CK activation in breast cancer cells. The mRNA expression of CKα was 5- to 8-fold higher in breast cancer cell lines than in human mammary epithelial cells (HMEC), while CKβ showed a smaller (up to 2-fold) increase only in the investigated cancer cell lines. The CK-mediated reaction is suggested fast and not rate-limiting, because higher CK activity in cancer cells failed to correlate with the increased PCho level although its induction ensures increased PCho levels. The activity of CCT was found to be several folds lower than that of CK in each investigated breast cancer cell line, in agreement with the role of CCT as the rate limiting step in PtdCho synthesis and PCho accumulation (Eliyahu *et al., 2007).* There is 3 to 4-fold increase in CKα but not CKβ mRNA expression has also been reported in epithelial ovarian cancer cell lines, compared with non tumoral counterparts, with agreement to a 3-fold increased protein expression (Iorio *et al.,* 2010).

1.8 Rationale of study

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (Jemal *et al.*, 2011). Cancer has become a commonly seen in these days and many studies have been done to prevent and control cancer.

CK has been proved to play main role in carcinogenesis and tumorigenesis. This made the CK become a potential target for anti-cancer therapy. This important discovery has stimulated the scientists to figure out the way to suppress the CK expression with or without the lowest adverse effect level to

human body. HC-3 and MN58b were potent CK inhibitor, which displays a potent antiproliferative effect in several tumoral cell lines in vitro, and a strong reduction of tumor growth in nude mice xenografts (Gallego-Ortega *et al.*, 2009). Yet, lack of study has been done on the promoter sequence of CK which might consist of some promoter regions which could contribute to pharmacologically down-regulating of the CK expression.

Therefore, in this research, the CK promoter was analysed and figured out whether the -1252 to -1275 of CK promoter sequence was the predicted suppressive binding region to CK expression and check whether cEts family transcription factor as suggested by the TFSEARCH software, was the responsive binding substance to this region. Therefore, a general activator, phorbol 12-myristate 13-acetate, PMA was treated to stimulate the test to determine the pathway that was involved.

1.9 Objective

The objectives of study are:

1. To study the effect of PMA treatment on the activity of hCKα gene promoter.
2. To detect the effect of PMA treatment on hCKα mRNA expression.
3. To investigate the effect of PMA treatment on hCKα protein expression.

CHAPTER TWO

MATERIALS AND METHODOLOGY

2.1 Materials

All chemicals, reagents, commercial kits, consumables, laboratory equipments, antibodies, plasmids and primers used in this research project are listed in Table 2.1 to Table 2.6.

Table 2.1 List of chemicals and reagents

Name	Supplier
Ammonium persulfate (APS)	Biorad, Japan
BenchMark Prestained protein ladder	Invitrogen, California
Bradford reagent	Biorad, Japan
Dimethyl sulfoxide	Amresco, USA
Dulbecco□s modified Eagle□s medium (DMEM)	Invitrogen, USA
Fetal bovine serum (FBS)	Invitrogen, USA
GeneRuler DNA Ladder Mix	Fermentas, Lithuania
Lipofectamine 2000	Invitrogen, USA
Opti-MEM I Reduced Serum Medium	Invitrogen, USA
Penicillin-streptomycin	Invitrogen, USA
Phorbol ester phorbol-12-myristate-13-acetate (PMA)	Merck, Germany
Protease inhibitor	Roche, Switzerland
ProteoJet mammalian cell lysis buffer	Fermentas, Lithuania
RiboRuler High range RNA ladder	Fermentas, Lithuania
Sodium dodecyl sulfate (SDS)	Amresco, USA
Trypan Blue	Amresco, USA

Table 2.2 List of commercial kits and consumables

Name	Supplier
Dual-Glo luciferase assay system	Promega, Madison, WI
Microtiter plate (96-well, 24-well)	Nunc, Denmark
T-25 flask	Orange Scientific. E.U.
Nitrocellulose membrane	Schleicher & Schuell, Germany
96-microwell plates	Nunc, USA
24-microwell plates	TPP, Switzerland
CL-Xposure Film, 5 x 7 inches	Thermo, USA
Supersignal West Pico Chemiluminescent Substrate	Thermo, USA
Rneasy Mini Kit	QIAGEN, Germany
RevertAid H Minus First Strand cDNA Synthesis Kit	Fermentas, Lithuania

Table 2.3 List of laboratory equipments

Name	Supplier
Haematocytometer and counting chamber	Assistant, Germany
Centrifuge Mikro 22R	Hettich, USA
Centrifuge Universal 320	Hettich, USA
Luminometer Glomax 20/20	Promega, Madison, WI
Microscope Axiostar Plus	LEICA, Germany

pH 211 Microprocessor pH meter	HANNA, India
CO_2 water jacketed incubator	NUAIRE, USA
Biophotometer plus	Eppendorf, Germany
MyCycler thermal cycler	Biorad, USA
Hypercassette, 5 x 7 inches	Amresco, USA

Table 2.4 List of antibodies

Name	Supplier
Rabbit Anti-human CKα Polyclonal Antibody	In-house
Rabbit Anti-β-actin Polyclonal Antibody	In-house
Goat Anti-rabbit IgG-HRP Conjugated	Sigma, USA

Table 2.5 List of plasmids

Name	Supplier
pGL4.10-hCKα	In-house
pGL4.73 [hRluc/SV40]	Promega, USA

Table 2.6 List of primers

Name	Supplier
hCKα:	
Forward 5'-TCAGAGCAAACATCCGGAAGT-3'	1st base, Malaysia
Reverse 5'-GGCGTAGTCCATGTACCCAAAT-3'	1st base, Malaysia

2.2 Preparation of Solutions and Buffers

2.2.1 Ethanol (70%)

A 70% ethanol was prepared by mixing 30 mL of distilled water with 70 mL of absolute ethanol. The solution was stored at room temperature.

2.2.2 Sodium Dodecyl Sulphate (S.D.S)

The 10% (w/v) S.D.S was prepared by disolving 1 g of S.D.S in 10 mL of distilled water. The solution was stored at room temperature.

2.2.3 Ammonium Persulphate (APS)

The 10% (w/v) APS was prepared by dissolving 0.1 g of APS in 1 mL of distilled water. The solution was stored at -20°C.

2.2.4 Protein Electrophoresis Running Buffer

The electrophoresis running buffer was prepared by dissolving 3.02 g of Tris base, 14.26 g of Glycine, and 1.00 g of SDS in 1 L of distilled water. The mixture was stored at room temperature.

2.2.5 2x SDS PAGE Loading Buffer

The 2x loading buffer was prepared by mixing 0.60 g of Tris HCl, 1.00 g of SDS, 10 mL of glycerol, and 0.02 g of bromophenol blue. The mixture was adjusted to pH 6.8 and made up to 100 mL with distilled water. 10% (v/v) β-mercaptoethanol was added prior to use. The mixture was stored at room temperature.

2.2.6 Phosphate Buffer Solution (PBS)

The phosphate buffer solution was prepared by mixing 8.0 g of sodium chloride (NaCl), 1.1375 g of sodium dihydrogen orthophosphate (Na_2HPO_4), 0.2 g potassium dihydrogen orthophosphate (KH_2PO_4), and 0.2 g of potassium chloride (KCl). The mixture was adjusted to pH 7.2 and made up to 1 L with distilled water. The mixture was stored at 4°C.

2.2.7 Western Transfer Buffer

The Western transfer buffer was prepared by disolving 2.42 g of Tris base, 11.24 g of glycine in 1 L of distilled water. The solution was stored at 4 °C. 20% (v/v) methanol was added prior to use. The mixture was stored at room temperature.

2.2.8 Tris-buffered Saline (TBS)

The TBS was prepared by mixing 1.21 g of Tris base and 8.77 g of sodium chloride. The mixture was then adjusted to pH 7.5 and made up to 1 L with distilled water. The solution was stored at 4°C.

2.2.9 Tris-buffered Saline Tween-20 (TBST)

The TBST was prepared by adding 1 mL of Tween-20 into 1 L of TBS. The solution was stored at 4 °C.

2.2.10 Skim Milk (5% w/v) in TBST

The 5% (w/v) skim milk was prepared freshly by mixing 2.5 g of skim milk powder with 50 mL of TBS.

2.2.11 10x TAE (Tris-acetate-EDTA) buffer

The 10x TAE buffer was prepared by mixing 48.4 g of Tris base and 11.42 mL of glacial acetic acid and 0.5 M EDTA in 800 mL distilled water. The mixture was then adjusted to pH 8.5 and made up to 1 L with distilled water.

2.3 Flow Chart of Study

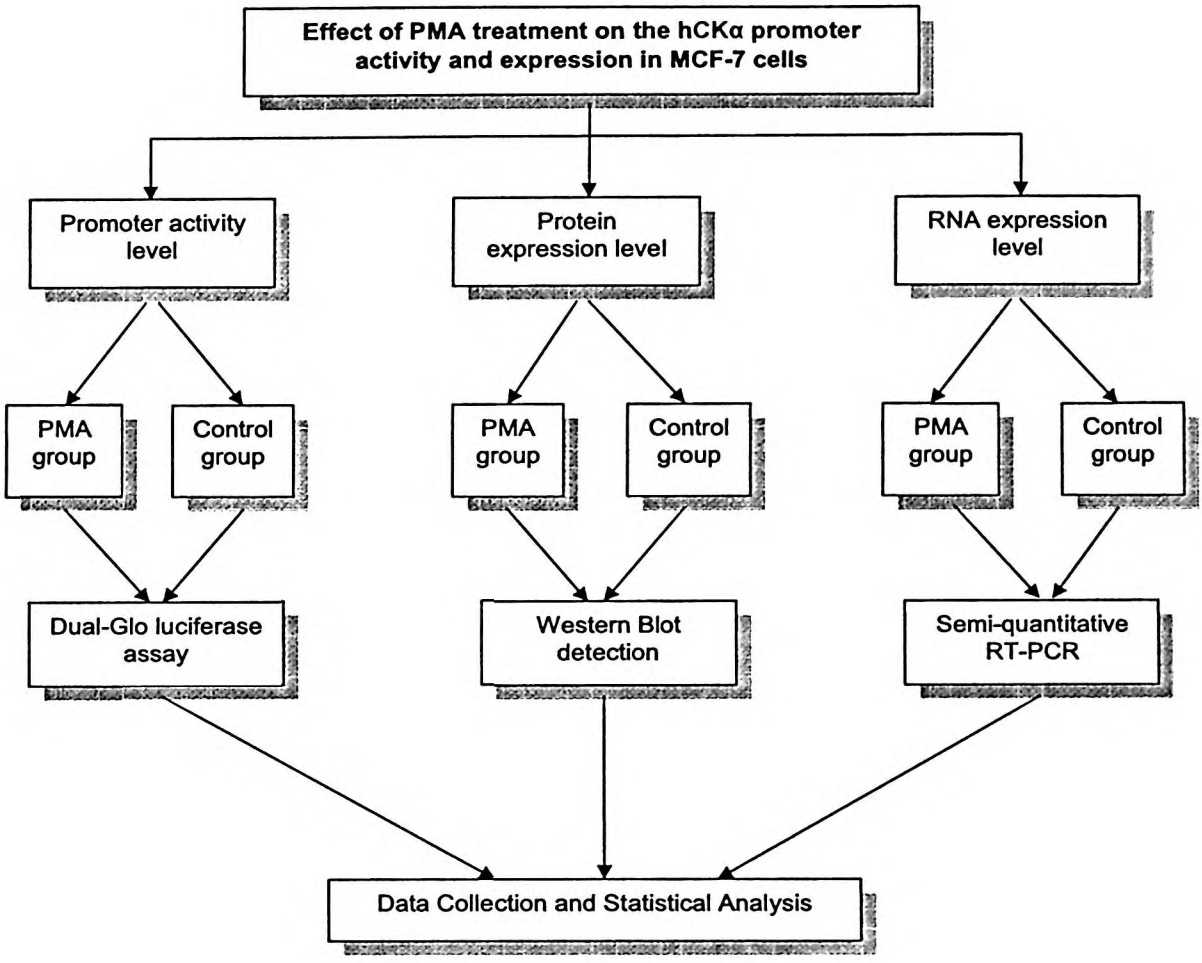

Figure 2.1 Flow chart of study.

2.4 Effect of PMA treatment on the activity of hCKα gene promoter by using Dual Glo luciferase assay system.

2.4.1 Cell lines and culture conditions

The human breast cancer cell line, MCF-7, was kindly provided by Dr. Khoo Boon Yin (INFORMM, USM) and was cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% fetal bovine serum (FBS), 10000 U/mL penicillin and 10000 µg/mL streptomycin in a 5% CO_2 humidified incubator at 37°C.

2.4.2 PMA treatment and Luciferase assay

At 70-80% cell confluence, MCF-7 cells were seeded on a 96 well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37°C. After 24 hours, MCF-7 cells were co-transfected with 200 g of pGL4.10-CKα construct kindly provided by Yee Yoke Hiang (USMKK) and 2.5 ng hRluc/SV40 as an internal control by using 0.5 µl Lipofectamine 2000 as recommended by transfection reagents protocol. After 6 hours incubation in a 5% CO_2 humidified incubator at 37 °C, cells was serum starved by changing the medium to Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum (FBS). 20 hours later, 20 ng/mL PMA was added to the PMA treated group while DMSO instead of PMA was added to the control group and incubate in 5% CO_2 humidified incubator at 37°C.

2.4.3 Dual-Glo luciferasse assay

After 6 hours PMA treatment, cells were harvested and luciferase assays were performed using the Dual-Glo luciferase assay by using Glomax 20/20 Luminometry system according to the manufacturer's manual (Promega). To perform the Dual-Glo luciferase assay, the 96-well plate containing mammalian cells was removed from the incubator. A volume of Dual-Glo Luciferase Reagent equal to the culture medium volume was added to each well and mixed. After 10 minutes, the firefly luminescence was measured by using Luminometer Glomax 20/20. To measure *Renilla* luciferase activity, a volume of Dual-Glo Stop & Glo Reagent equal to the original culture medium volume was added and mixed to each well. Firefly luciferase activity was normalized to *Renilla* luciferase activity. After 10 minutes, the *Renilla* luminescence was measured in the same plate order as the firefly luminescence was measured. This experimental protocol was shown in Figure 2.2.

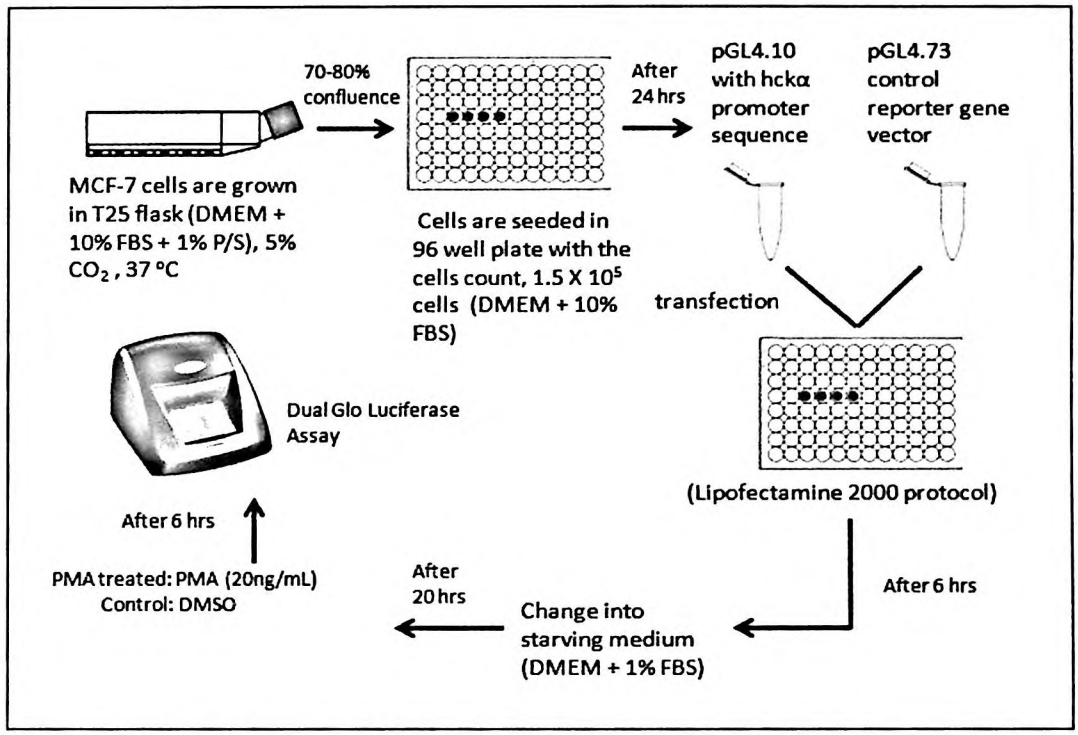

Figure 2.2 Experimental protocol for investigating the effect of PMA treatment on the activity of CK-α gene promoter.

2.5 Determination of effect of PMA treatment on the hCKα mRNA expression level by using semi-quantitative RT-PCR.

The experimental protocol of determining the effect of PMA treatment on the hCK-α RNA expression level by using semi-quantitative RT-PCR is showed in Figure 2.3.

2.5.1 PMA treatment

MCF-7 cells were cultured as mentioned in section 2.4.1. However, this time two T25 flasks were used. One was labeled as PMA treated group and another was control group. At 70-80% cell confluence, cells serum starvation was done by replacing the medium with Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal bovine serum (FBS) in the T25 flask. 20 hours later, 20 ng/mL PMA was given to the PMA treated group while DMSO instead of PMA was given to the control group and incubated in 5% CO_2 humidified incubator at 37°C.

2.5.2 Isolation of total RNA

After 6 hours PMA treatment, cells were harvested. Approximately 3 x 10^6 cells were used for RNA extraction. Total RNA was isolated by using the RNeasy Mini Kit (QIAGEN). The harvested pellet cell was loosen and lysed by adding 350 µL of the guanidine isothiocyanate (GITC)-containing buffer, buffer RLT which was added with β-Mercapthoethanol prior to use and vortexed to mix well. Then, 1 volume of 70% ethanol was added to the homogenised lysate and well mixed by pipetting. The 700 µL sample was applied to RNeasy spin column placed in a 2 mL collection tube and

centrifuged for 15 sec at 10000rpm. Then the contaminants were removed by adding 700 µL buffer RW1 to the RNeasy column and centrifuged for 15 sec at 10000rpm. After the RNeasy column transferred into a new 2 mL collection tube, 500 µL buffer RPE was added and centrifuged for 15 sec at 10000rpm. Lastly, the ready-to-use total RNA was eluted with 30 µL RNase free water. Isolated total RNA was used in the determination of the concentration of total RNA isolated and to synthesise the cDNA. The remaining was kept at -80°C.

2.5.3 Electrophoresis of total RNA

The success of total RNA isolation was determined by agarose gel electrophoresis and ethidium bromide staining. 1% agarose gel was prepared. The standard marker was RiboRuler High range RNA ladder (Fermentas). 1 µL of marker and samples were mixed with equal volume of 2x RNA loading dye. Electrophoresis was run at constant voltage of 90 volts at room temperature. RNA bands was visualised by staining the gels in 0.5 µg/mL ethidium bromide solution for 3 to 5 minutes and observed and photographed under UV light at 366 nm wavelength. The concentration of total RNA was measured at 260 nm by using biophotometer plus spectrophotometer (Eppendorf). Distilled water was used as blank while RNA sample was diluted with distilled water.

2.5.4 Reverse Transcription-Polymerase Chain Reaction

Total RNA of hCKα was reverse transcribed by using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). First, 1.0 µg of total RNA, 1.0 µL oligo $(dT)_{18}$ primer were mixed and DEPC-treated water were added to a