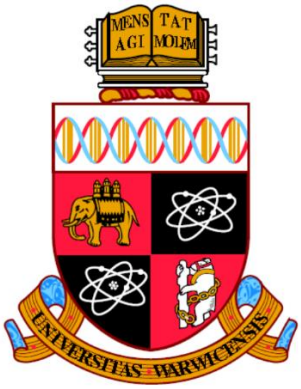

Alternative Splicing (AS) and Epithelial-to-Mesenchymal Transition (EMT) programming in cellular model of breast cancer

By

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Acknowledgements

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Last but not least, this dissertation is especially dedicated to my parents and siblings for being the reason of my existence today and for being very supportive emotionally as well as financially. I always knew that you believed in me and wanted the best for me.

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

Conference output

1. Proteome analysis in breast cancer cells reveals differential expression of signatures involved in pre-mRNA alternative splicing events in response to 17β-estradiol and CRH. Poster presentation made at the 9th International Conference on Proteomics and Molecular Medicine, Paris, France. November 13-15, 2017.

Summary

It is increasingly evident that alternative pre- mRNA splicing events (AS) play a profound role in tumor progression and metastasis, frequently via the activation of cellular processes that can trigger epithelial-to-mesenchymal transition (EMT) program in the cells. Alterations in mRNA splicing pattern can arise from altered expression of splicing factors as well as interactions with cancer-related signal transductions.

This study investigated the potential role of key hormones, estrogen and CRH in the regulation of pre-mRNA splicing mechanism, and the molecular pathways mediating CRH actions in promoting EMT in ER (+) MCF7 and ER (-) SKBR3 breast cancer cell line. Estrogen signaling has been reported to be involved in the alternative splicing of specific set of genes, and this study found that estrogen can induce the phosphorylation and nuclear translocation of a key splicing kinase, SRPK1 via Akt dependent pathway, and thus triggers the production of CD44s splice variant.

Additionally, CRH regulates numerous protein kinases including Akt, therefore the effects of CRH on SRPK1 status was also investigated. In this study, CRH was shown to modulate SRPK1 activity via similar pathway as E2 did in MCF7 cells, resulted in the accumulation of CD44v6 mRNA isoform. Apparently, while this effect of CRH on SRPK1 was not seen in SKBR3 cells, increased expression of CD44v6 mRNA was detected, suggesting that CRH exerted its effects via different pathways in SKBR3 cells. Furthermore, increased level of CD44 splice variant, particularly CD44v6 in the stimulated cells positively correlated with EMT gene expression Twist/Snail as well as the migration and invasion properties of the cells.

Additionally, EMT gene profiling analysis revealed that in addition to Twist and Snail, CRH was able to drive the transcription of several other prominent EMT markers such as VIM, TGFB1/2, NOTCH1, MMP9 and Wnt5A when either Akt or SRPK1 kinase was inhibited in the cells, thus signifying the potential convergence of CRH signaling pathway in the MCF7 cells. Given that the role and the mechanism of E2 and CRH actions in the progression of breast cancer are still not fully elucidated, the link between these two key

hormones with alternative splicing events and the activation of EMT in ER+ cells demonstrated in this project may contribute considerably to the current understanding of breast cancer development and progression.

Abbreviations

AS	Alternative Splicing
BCSC	Breast cancer stem cell
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary Deoxyribonucleic Acid
Co-IP	Co- immunoprecipitation
CRH	Corticotropin releasing hormone
CRH-BP	Corticotropin releasing hormone-binding protein
CRHR1/R2	Corticotropin releasing hormone receptor 1/ 2
CSCs	Cancer stem cells
CS-FBS	Charcoal- stripped fetal bovine serum
DAPI	4',6'-Diamino-2-phenyllindole
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagles' Medium
DTT	Dithiothreitol
E2	17β- estradiol
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ER+	Estrogen receptor positive
ER-	Estrogen receptor negative
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FASP	Filter aided sample preparation

FBS	Fetal bovine serum
FZD	Frizzled
GPCR	Glucocorticoid receptor
GR	Growth hormone receptor
GRK	G protein coupled receptor kinase
GSK-3β	Glycogen synthase kinase 3β
HER-2	Human epidermal growth factor 2
hnRNPA1	Heterogenous ribonucleoprotein A1
HPA	Hypothalamic-pituitary axis
Hr	hour
HR	Hormone receptor
IκB	Inhibitory Kappa B
ICD	Intracellular domain
IGF	Insulin-like growth factor
IL	Interleukin
JAK	Janus kinase
JNK	Jun N-terminal Kinase
kDa	Kilo Dalton
MAPK	Mitogen- activated protein kinase
MET	Mesenchymal- to- epithelial transition
Min	Minute
mg	Milligram
ml	Milliliter
mM	millimolar
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MW	Molecular weight

mTOR	Mammalian target of rapamycin
NK-κB	Nuclear factor Kappa B
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI3-K	Phosphatidylinositol-3 OH Kinase
PIP	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B/AKT
PKC	Protein Kinase C
POMC	Pro-opiomelanocortin
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RNA	ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase- Polymerase Chain Reaction
RT-qPCR	Reverse transcriptase- quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SEM	standard error of Means
SRp	Serine-Arginine Protein Kinase
SRPK1	Serine- Arginine Protein Kinase 1
STAT	Signal Transducer and Activator of Transcription
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline-1% Tween-20
TEMED	N, N, N^1, N^1-tetramethylenediamine
TN	Triple negative
TNF	Tumor necrosis factor

UCN	Urocortin
μl	microliter
μM	micromolar

CHAPTER 1

Introduction

1.1 The pathogenesis of cancer

There are more than one hundred different kinds of human cancers that have been identified and classified to date. These cancers are categorized into three main groups, namely carcinomas, sarcomas and lymphomas/leukemia and further classified according to tissue of origin such as lung, breast and colon. Approximately 80% of human cancers are shown to belong to carcinomas, a type of cancer which arise from epithelial cells (1). Unlike normal cells whose growth is strictly regulated in the organism, cancer cells replicate continuously and capable of invading surrounding tissues (malignant/invasive) as well as spreading to distant sites (metastasis) through the circulatory system.

In fact, studies of cancer cells in culture reveal some of the distinct differences in the growth requirements and behaviors of cancer cells as compared to normal cells under controlled conditions. For examples, normal cells show density-dependent of cell growth, whereas tumor cells continue growing regardless of cell densities. In addition, cancer cells display reduced dependence on serum growth factors, they can grow without attachment to surface (anchorage independence), have the capacity to grow indefinitely and to be sub-cultured continuously as well as they can display no contact inhibition of movement in a culture dish (2,3). These features suggest that cancer cells reprogram their metabolism to promote growth, survival and proliferation, such as by displaying an increased rate of glucose uptake (Warburg Effect) to maintain tumors growth and viability [reviewed in (4)]. Following this observation, it was later demonstrated that the abovementioned abnormalities appeared to be attributed to aberrations in the regulatory system which were stably inherited at the cellular level

hence suggesting the involvement of genetic changes and progressive series of alteration in the process of tumor development (2,5).

1.1.1 Biology of human cancers

Cancer cells often differ from normal cells in several biochemical properties such as secretion of growth factors, increased glycolysis, and secretion of proteases, decreased levels of cell surface proteins and disorganization of cytoskeleton. These differences might be directly associated with the abnormal growth properties of cancer cells; however, it is not clear whether these altered biochemical properties are the causes or the consequences of the transformed state of the cells. Therefore, rather than comparing the phenotypes of cancer cells and normal cells, many researches have focused on understanding the molecular basis of cancers by identifying and studying the function of specific genes that can potentially induce cell transformation and abnormal cell proliferation, which are also known as oncogenes. These oncogenes are frequently found amplified or mutated in cancer cells where in some cases oncogene amplification is closely correlated with increased malignancy in tumors (6). This is supported by tumor genome sequencing analysis in which more than 10,00 tumor genomes have been sequenced and the information generated reveals thousands of mutations and other genomic alterations identified in cancer cells (6).

Apart from increased oncogene expression due mutations or alternative splicing, loss of function of a class of genes that function as negative regulators in cancer biology can also lead to tumor development. These genes which are commonly called as tumor suppressor genes are frequently found inactivated in cancer cells and demonstrated to play critical role in the pathogenesis of various human cancers. Studies have revealed a growing list of tumor suppressor genes responsible for many inherited cancers, with one of the well-studied is *p53* gene which is found mutated in nearly 50% of human cancers. Apparently, further investigation has shown that both oncogenes and tumor suppressor genes could encode for; 1) growth factors such as platelet-derived growth factor (PDGF),

fibroblast growth factors (FGFs), epidermal growth factor (EGF) & transforming growth factors (TGF), Wnt family and Interleukin-2/-3 and 2) protein-tyrosine kinases and the related cell surface receptors (e.g. EGFR, FGFR), that are important in signal transduction pathways that regulate cell growth as well as allow cells to respond to extracellular stimuli (2). Consistently, many studies have reported the role of TGFβ, PDGF and FGF2 as key mediators of activated fibroblast recruitment in many cancers which consequently resulting in the increased proliferation of fibroblasts and positively correlates with cancer progression [reviewed in (7)]. Nevertheless, non-receptor protein-tyrosine kinases (e.g. Src, Jak) and non-tyrosine kinase receptors that can also act as oncogenes and propagate signals from growth factors by coupling with intracellular targets mediated by family of guanine nucleotide binding proteins (G proteins) hence inducing cell growth and proliferation.

Another molecular alteration found in cancer cells is changes in microRNA (miRNA) expression, a small noncoding RNA that negatively regulate protein expression. Studies on miRNA expression in tumors have demonstrated global changes in miRNA expression in various malignant cancers as compared to their normal counterparts in which some of these miRNAs have been identified as either oncogenes (e.g. miR-15, miR-17-5p) or tumor suppressor genes (e.g. miR-34, miR-15a) (8,9). miRNA inhibits gene expression at the post-transcriptional level by binding to specific messenger RNA (mRNA) at specific sequences and forms miRNA/mRNA duplex which then inhibits mRNA translation or increases the rate of mRNA degradation in the cells [reviewed in (10)]. In vitro studies have shown that upregulation of miR-296 through VEGF and EGF promotes angiogenesis and increases migration of endothelial cells, whereas another study in murine breast and lung cancer models demonstrates that VEGF triggers the expression of miR-10b and miR-196b, both of which are identified as key molecules in angiogenesis and tumor progression in these cancers (11).

1.2 Breast Cancer

Population studies show that death cases in developed countries are primarily caused by cancer and this trend is slowly followed by developing countries where cancer has now become the second leading cause of death (12,13). Globally, it is estimated that breast cancer causes more than 400,000 deaths every year and is the second leading cause of cancer-related death in women after lung cancer (14,15). Among women in the United States only, breast cancer accounts for 1 in 3 cancer cases diagnosed in patients and once the tumor has metastasized, the 5-year survival of the patient is approximately 27% (12,16). Breast cancer begins as local disease but later can acquire the ability to metastasize to the lymph nodes and distant sites. Its progression to become metastatic is often marked by genomic alterations such as acquired genomic aberrations, changes in gene expression, splicing patterns and protein functionality (17,18).

Although screening technology for diagnosing breast cancer has been improved, it can only reduce the mortality rate by offering early treatment to the patient. It is generally agreed that chemotherapy has been the most effective treatment for breast cancer. However, population studies and meta-analysis show that some tumor cells become resistant to chemotherapy and therefore survive the treatment (19). This subsequently leads to cancer recurrence and like many other cancers; the main cause of deaths in breast cancer cases is linked to metastatic spread of the tumor cells.

1.2.1 Pathogenesis of breast cancer

The breast is composed of specialized glandular tissue which includes the ducts which terminate peripherally into terminal duct lobular units and supporting connective tissue. The epithelial compartment of glandular tissue is bound by a thin basement membrane and has two-layer structure with the luminal cells as an inner layer and myoepithelial cells as an outer layer. There are several risk factors that have been associated with the development of breast cancer in women, which include age, lifestyle, family history, reproductive history, endogenous estrogen level, exogenous hormone exposure and mutations of tumor suppressor genes such as BRCA1 BRCA2, p53 and PTEN (20,21). At present, various lines of treatments are used to treat cancer such as surgery, radiation therapy, chemotherapy, targeted therapy and hormonal therapy (22).

Currently there are more than 18 subtypes of breast cancers that have been identified, in which most breast cancers are carcinoma (epithelial origin) arises from cells lining the terminal duct lobular unit of the glandular breast tissue (23). Clinical signs and symptoms of breast cancer include mastalgia (breast pain), a change in breast shape, dimpling of the skin, nipple discharge and a lump in the breast (24,25). Furthermore, there are two important phases in breast carcinoma; *in situ* phase*,* which is restricted within epithelial compartment thus can be cured by excision, and invasive phase, where cells can breach the barrier of the epithelium, infiltrate within the breast connective tissues and have the potential to spread and establish at distant sites (metastasis) (26). In addition, the ability of breast glandular tissue to increase the number of epithelial cells (hyperplasia) in response to physiological stimuli has in some cases contributed to the formation of lesions of the breast including benign breast lesion and carcinoma *in situ*. Although there is no evidence about the progression of benign hyperplasia to carcinoma *in situ*, it is crucial to distinguish between hyperplasia associated with benign lesions from the ones associated with carcinoma as the progression from *in situ* to invasive carcinoma is already widely accepted (26). In addition to this, carcinoma *in situ*

can be further divided into two types; ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), with DCIS is more commonly associated with invasive carcinoma ductal type and LCIS is associated with invasive carcinoma of lobular type (26).

1.2.2 Molecular subtypes of breast cancer

Generally, breast cancer can be categorized according to the pattern of gene expression which defines each of the breast cancer types with distinct biological and clinical characteristics. Hormones bind to the receptors on the breast cancer cells surface or in their cytoplasm and nucleus resulting in the changes in the cells. Therefore, breast cancers are often classified according to the status of human growth factor-neu receptor (HER2) and hormone receptors (HR) such as estrogen receptors (ER) and progesterone receptors (PR). There are four molecular subtypes of breast cancer; Luminal A (HR+, HER2-), Luminal B (HR+, HER2+), HER2+ and triple negative (ER-, PR-, HER2-)(27–31). Although triple negative cancers do not express any of these receptors, they often express receptors for other hormones such as androgen and prolactin [reviewed in (32)].

The status of ER and PR is usually assessed by immunohistochemistry, in which at least 1% of tumors cells needs to show positive nuclear staining to be interpreted as ER or PR positive, and if the tumor staining result shows less than 1%, it is considered as receptor negative (33). The cutoff of 1% is chosen to define receptor positivity because hormonal therapy can be benefited by the patient with even 1% of ER/PR- positive tumors (21). Meanwhile, approximately 10% to 34% of invasive breast cancer show overexpression or amplification of HER2, a proto- oncogene responsible for the production of transmembrane tyrosine kinase growth receptor and is found involved in various regulatory pathways related to proliferation, survival, motility and invasion in breast (21). HER2 overexpression is also associated with the increased translation of fatty acid synthase (FASN), which subsequently increases EGFR and HER2 signaling hence resulting in cell growth and poor prognosis in HER2-amplified breast cancer cells (34).

Meanwhile, annual reports on the status of cancer in the united states shows that HR+/HER2- breast cancer subtypes is the most common subtype, representing 72.6% of all cases, nearly six times higher than triple-negative (TN), seven times higher than HR+/HER2+ and 16 times higher than HR-/HER2+ breast cancer rate (35).

1.2.3 Triple Negative (TN) breast cancer

Triple negative breast cancer accounts for approximately 15% of all invasive breast cancers and is defined as tumors that do not express estrogen receptor (ER), progesterone receptor (PR) and HER2. As this type of breast cancer lacks the abovementioned receptors, patients with triple negative tumors often show worse prognosis as compared to luminal breast cancers. Since endocrine therapy or HER2-targeted therapy cannot be prescribed on them, therefore there is no standard form of chemotherapy for the treatment of triple negative breast cancer at present [reviewed in (36)]. It is reported that more than 75% of women carrying breast-cancer susceptibility gene mutation, *BRCA1* are showing triple-negative breast cancer phenotype (37,38). In addition, analysis of triple negative breast cancer cells profile demonstrates that these cancer cells have high expression of aldehyde dehydrogenase 1 (ALDH1A1) and the cells also display cell surface marker $CD44^{high}$ $CD24^{low}$ ($CD44^{+}CD24^{-}$), which is used to characterize breast-cancer stem cells (39). Apart from that, triple negative tumors also have high rate of tumor suppressor, *p53* gene mutations which further contributes to their enormous aggressiveness (40). Currently, one of the promising treatment to treat triple negative tumors is the poly-ADP ribosepolymerase-1 (PARP-1) inhibitors, which together with the defect of *BRCA1* DNA repair gene in these tumors would lead to cell death due to accumulation of breaks in the double- stranded DNA.

1.2.4 HER2+ breast cancer

Human epidermal growth factor receptor (HER) family is located at the cell membrane and consists of four members; HER1 (epidermal growth factor receptor, EGFR/erbB1), HER2 (neu, c-erbB2), HER and HER4 with all these receptors have intracellular tyrosine kinase domain except HER3. To date, natural ligand for HER2 is not yet known but there are several ligands identified for HER1, HER3 and HER4 such as transforming growth factor-a (TGFa) and epidermal growth factor (EGF) [reviewed in (41)]. The binding of ligand to HERs causes auto-phosphorylation of specific tyrosine residues within the catalytic kinase domain of these receptors which in turn serves as docking site for phosphotyrosine-binding domain- and src-homology 2-containing proteins, linking ErbB receptors to MAPK and PI3K pathways (42–44).

As compared to hormone-positive breast cancer, HER2+ breast cancer cells tend to be more aggressive and the treatment for HER2+ metastatic breast cancer is reported to be challenging as studies show that approximately 50% of patients develop brain metastasis [reviewed in (45)]. HER2 overexpression which is characterized by HER2 gene amplification (located in the 17q12 chromosome) and other genes associated with the HER2 pathway, accounts for 18-20% of all breast cancers is often used as a marker to select patients for anti-HER2 therapy such as trastuzumab for HER2+ metastatic breast cancer (33,46,47). In addition, more than 40% of HER2+ tumors show mutation in one of the prominent tumor suppressor gene, *p53* (48).

1.2.5 Hormone- sensitive breast cancer

Endocrine responsiveness in most of the hormone-sensitive breast cancers is predominantly dependent on the presence of a functional estrogen receptor (ER), a protein that regulates hormone-dependent growth of hormone receptor positive (HR+) breast tumors and is detected in approximately 50%-80% of all breast tumors (49). Studies in animal models have demonstrated the role of estrogen as a key hormone in breast cancer development as administration of estradiol (E2) in animal models induces breast cancer. This is parallel with other reports in which the level of estrogen has been shown to positively correlate with the increasing risk of breast cancer in women (50,51).

Mutations are more commonly reported in hormone responsive breast cancer cell. This is shown by accumulating evidences that the resistance of ER+ breast cancer cells with *PIK3CA*-mutated tumors to therapy such as aromatase therapy is associated with mutations in *ESR1,* gene that encodes for estrogen receptor alpha (ERα) (52), mutations that recently have also been reported in metastatic ER+ breast cancer tissues (53). Furthermore, apart from receptor mediated-effects that cause cell proliferation-induced mutations triggered by estrogen, estrogens can act as mutagenic agents to breast tissue via mechanisms independent of ER, by causing estrogen-induced mutations which subsequently result in the development of breast cancer (Figure 1.1).

Meanwhile in another study, it is demonstrated that the ratio between ERα/ERβ in breast cancer cells affects the pattern of pre-mRNA maturation and the splicing of biologically active estrogen-dependent genes which regulate apoptosis, cell signaling, protein ubiquitination and mRNA maturation and translation (54). Several variants of ERs which include truncated isoforms of ERs (ERα66, ERα46, ERα36) have been identified in breast cancer which are implicated in the development of endocrine therapy in ER+ tumors [Reviewed in (55)]. Studies in ER+ MCF7 breast cancer cells show that overexpression of ERα66 leads to cell proliferation and can potentially contribute to cancer development and progression thus highlighting the role of aberrant splicing which tends to occur in ER+ breast cancer [Reviewed in (56)]. Furthermore, the

complexity of estrogen receptor signaling is suggested to be attributed to the interplay of kinase networks involved in pathways associated with cancer hallmarks in ER+ breast cancers [reviewed in (57)].

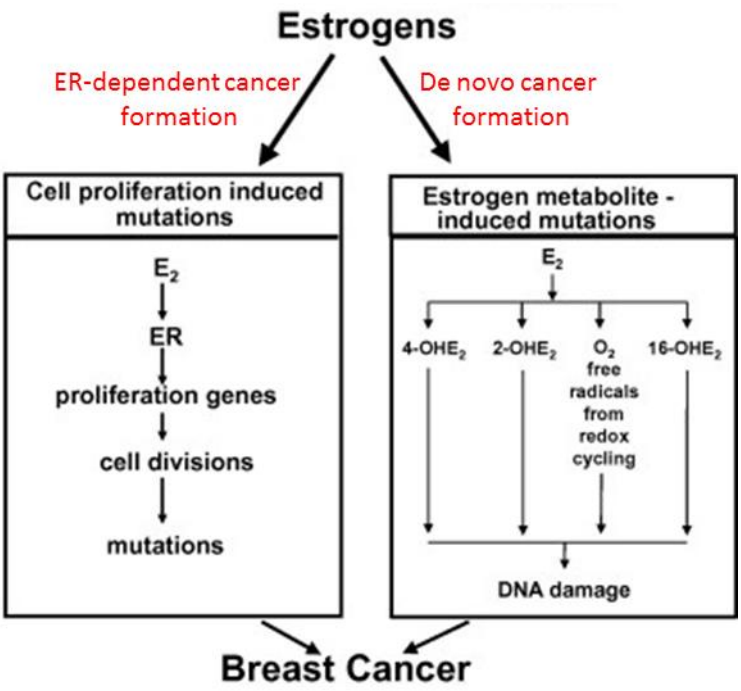


Figure 1.1 Diagrammatic representation of the effects of estrogen to breast tissue that can give rise to breast cancer via ER- dependent pathway (Left) such as through cell proliferation-induced mutations triggered by estrogen, and de novo cancer formation via non-genomic pathway that leads to estrogen-induced mutations (Right) [Adapted from Yue et al, 2013 (58)].

1.2.5.1 Steroid Hormones

Steroid hormones are derived from cholesterol and thus are structurally similar to it (Figure 1.2). Steroid hormones are produced in three endocrine organs that are specialized in steroid hormone production: adrenal cortex, ovary and testis, and during pregnancy, placenta also serves as additional major source of steroid hormones. Generally, steroid hormones are divided into five groups; mineralocorticoids, glucocorticoids, estrogens, progestins and androgens. They are distinguished by their physiological behavior and actions on specific steroid hormone receptors which are intracellular transcription factors, and subsequently regulate transcription of target genes and protein synthesis (59). In addition, second messengers such as cAMP, induced by peptide agonists have been shown to be able to modulate steroid-induced nuclear transcription by an intracellular cross-talk (60). In fact, studies have also shown that this cross-talk can take place without the presence of steroid ligand, for instance, estrogen receptor (ER) α can be activated by epidermal growth factors (EGF) via MAPK pathway (61). In addition to genomic steroid actions, rapid nongenomic effects of steroid hormones via membrane receptors involving second messenger cascades such as protein kinase C (PKC) have also been described in many studies [reviewed in (62)].

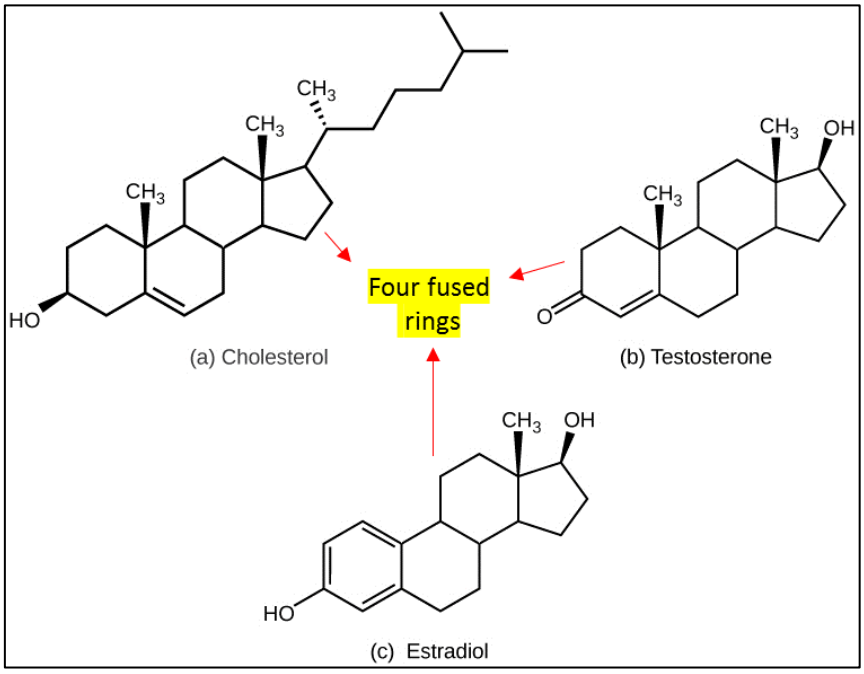


Figure 1.2 An illustration of steroid hormones structure. As cholesterol is the parent compound from which steroids are derived, their structures are similar to cholesterol with four fused rings as core structure. Adapted from (63).

1.2.5.2 Estrogen Biosynthesis

Estrogen is a female sexual hormone and a drug of choice in treating menopausal symptoms in women. This steroid hormone is a key regulator of growth and differentiation in various target tissues such as in the male and female reproductive tracts, mammary gland, skeletal and cardiovascular systems. Although estrogens are primarily produced in the ovaries, they can also be produced by other organs such as heart, skin, liver and brain (64). While E2, or 17β- estradiol is the major product from estrogen biosynthesis process, there are two other forms of estrogen; estrone (E1) which plays role mostly after menopause, and estriol (E3), the least potent estrogen that plays a larger role during pregnancy. The synthesis of estrogen in the body is regulated by aromatase, an enzyme that is responsible for the last step in the E2 synthesis and in which its expression in tissue relies on three major factors; alternative splicing

mechanisms, different transcription factors and tissue-specific promoters (65–67). In addition, the synthesis of estrogen is different in reproductive women and non-reproductive women. Estrogen synthesized in extragonadal sites such as kidney and adipose tissue mostly functions as paracrine/intracrine factor and act locally to maintain tissue specific functions (68). Whereas, ovarian-synthesized estrogen in reproductive women is released into the bloodstream where its level is dependent upon the reproductive status of the individual.

1.2.5.3 Estrogen Signaling

The physiological actions of estrogen involve complex cellular/molecular mechanisms that can be classified into two different pathways; genomic signaling (ligand-dependent, ligand-independent, DNA response elements ERE- independent) which leads to the transcription of downstream target genes and non-genomic signaling (cell-surface ER forms) which does not involve any transcriptional activity in the nucleus (69,70).

In the ligand-dependent pathway, estrogen exerts its effects predominantly through two distinct intracellular receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), that show a high degree of homology specifically at the DNA-binding domain (97%) and ligand binding domain (60%) (Figure 1.3) (69,71). As for the ligand- independent pathway, the function of ER can be modulated by extracellular stimuli such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1), in the absence of estrogen and eventually elevate the expression of ER target genes (72). Meanwhile, in the ERE-independent action of estrogen, estrogen-ER binding alters transcription of genes through the interaction with other DNA-bound transcription factors (Fos/Jun) and AP-1 binding site of the target genes (69) (Figure 1.4).

On the other hand, the non-genomic effects of estrogen can be mediated through membrane-associated binding site, possibly a form of ER that is linked to intracellular signal transduction proteins such as tyrosine-kinase protein which is

associated with MAPK signaling pathway activation that may promote proliferative and anti-apoptotic effect of estrogen in the target cells (69,73–75). In addition, estrogen is also reported to activate phosphoinositide 3-kinase (PI3K) signaling, a major pathway in the regulation of aerobic glycosylation in proliferating cells (76,77). While the tumor - promoting role of estrogen has been demonstrated to be mediated predominantly by ERα, recent studies have reported the substantial role of ERβ in promoting cell proliferation and invasion independently of estradiol in ERα-negative (MDA-MB-435 cells), in vitro. In addition to this, in vivo studies in ERβ+ MDA-MB-435 cells also showed that as compared to control cells, the growth rate of these ERβ+ cells were higher with more pulmonary metastasis detected (78). This finding indicates the importance of ERα/ERβ ratio in cells for the ERβ to exert its effect in breast cancer development.

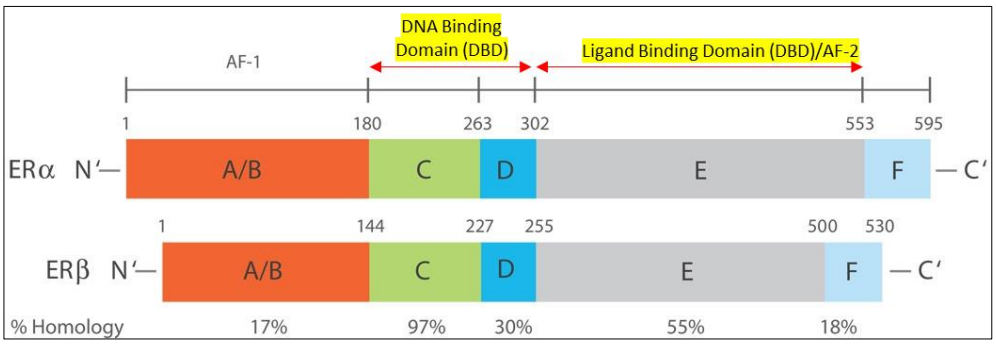


Figure 1.3 Comparison of ERα and ERβ structures and homology. There are three major modular domains of ERs; an A/B domain, a DNA binding domain (DBD) and a ligand binding domain (LBD). While ERα contains more amino acids (595) than ERβ (530), their DNA binding domains show almost perfect homology whereas their least identical domain is at the A/B and LBD which contains Activation Function, AF-1 and AF-2 binding domain respectively which are responsible for regulating gene transcription. [Adapted from Leitman et al, 2010 (71)].

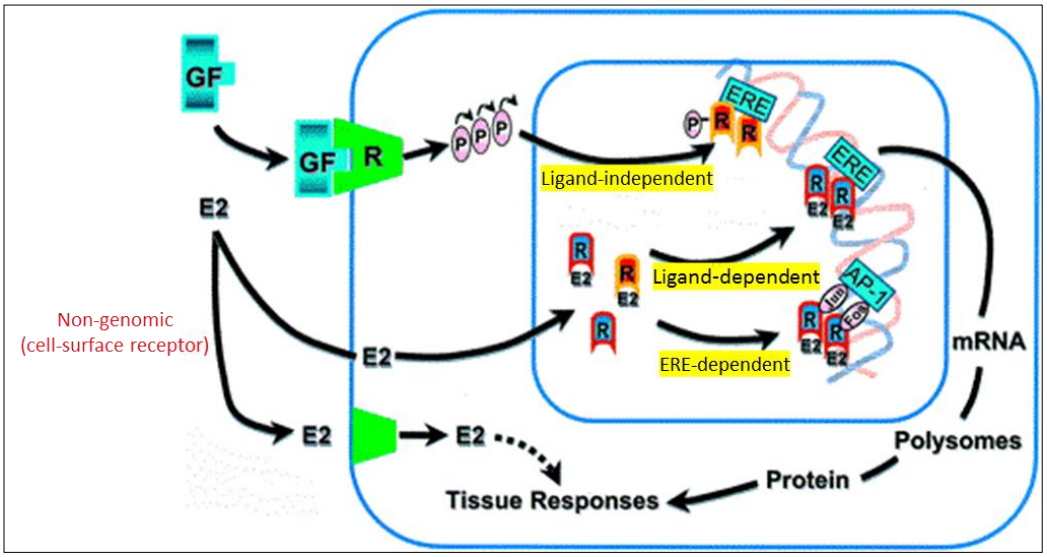


Figure 1.4 Proposed model of estrogen actions through genomic and non-genomic pathway. Genomic pathways (highlighted in yellow) lead to the transcription of target genes whereas non-genomic pathway (in red) causes rapid effects through cell surface receptor and triggers responses in target tissues [Adapted from Hall et al, 2001 (69)].

Emerging evidence also suggests the involvement of a G protein-coupled receptor, G protein-coupled estrogen receptor (GPER/GPR30) has been reported in breast cancer development as it is demonstrated to mediate both genomic and non-genomic effects of estrogen (79,80). The upregulation of GPER expression in breast cancer cells has been shown to cause the activation of yes-associated protein 1 (YAP) and transcriptional coactivator with a PDZ-binding domain (TAZ), both of which are required for GPER-induced gene transcription, cell proliferation and migration in breast cancer (81). In addition, TAZ is often found overexpressed in metastatic breast cancer and is shown to play role in the self-renewal and tumor initiation capabilities of breast cancer stem cells (82). In addition, accumulating evidence suggests that mutations in the gene encoding for ERα, *ESR1* is one of the contributing factors in the development of endocrine therapy- resistance in ERα- positive breast tumors. These mutations, which are frequently identified in metastatic breast tumors, enable tumor cells to evade

hormonal treatments by inducing activation of estrogen receptor in the absence of hormone (55).

1.2.6 Breast cancer signaling pathways

Research on signaling pathways governing the process of tumors formation has allowed better understanding on cancer biology which in turn provides valuable information for the development of new targeted therapies. The study on molecular biology of breast cancer reveals interconnection of multiple signaling pathways which include PI3K/AKT, MAP kinase signaling, p8 kinase, JNK kinase, JAK/STAT signaling pathway, transforming growth factor beta (TGFβ), Notch, Wnt and NF-κB signaling. However, a plethora of studies has shown that cellular transformation, tumorigenesis, cancer progression and drug resistance in breast cancer are frequently associated with alterations in phosphoinositide 3-kinase (PI3K/AKT) pathway [Reviewed in (83)]. In addition, alterations in this signaling pathway are found to be particularly common in ER-positive breast cancer (45% in luminal A, 30% in luminal B)(84).

1.2.6.1 PI3K/AKT

Among all the classes in PI3K families, class I_A PI3Ks is the predominant class implicated in cancer. Class I_A PI3Ks consist of a p85 regulatory subunit and a p110 catalytic subunit which are responsible in mediating signals from growth factor receptor tyrosine kinases (RTKs) such as EGFRs, HER2, IGF-IR and FGFR. The binding of these growth factors results in the recruitment of p85-p110 complex to the lipid phosphatidylinositol PIP_2 at the plasma membrane, followed by phosphorylation of phosphatidylinositol-3,4,5-trisphospate (PIP_3) by PI3K (p110) (85). PI3K signaling is negatively regulated when PIP_3 is dephosphorylated by PTEN and INPP4B, whereas the signaling pathway will lead to protein synthesis, RNA translation, cell growth and autophagy when proteins such as

AKT and PDK1 bind to PIP_3 leading to phosphorylation of AKT involving PDK1 and mTOR/Rictor complex [reviewed in (86)].

The highly proliferative property of cancer cells demonstrates that there is dysregulation in the cellular metabolism, cytoskeletal rearrangement, survival and growth control (87). One of the pathways that control these cellular processes is the PI3K/AKT pathway, one of the most frequently altered signaling pathways in human cancer (Figure 1.5). These alterations include the loss of lipid phosphatases PTEN and INPP4B and mutation/amplification of genes encoding the PI3K catalytic subunits (e.g. p110a/*PI3KCA*), the PI3K regulatory subunits (e.g. p85a/*PI3R1*) and the PI3K effectors (e.g. AKT1, AKT2, AKT3 and PDK1) [reviewed in (88)]. Mutations in PI3K pathway are also reported in 20-25% of the breast cancers studied with ER+ breast cancer shows the highest percentage of nearly 35% (89). PI3K is also demonstrated to directly and indirectly interact with estrogen receptor (ER) leading to the phosphorylation of ER and thus increasing the ligand-independent ER transcription in breast cancer cells (86). In addition, uncontrolled activation of PI3K pathway is thought to be responsible for the cells resistance to anti-estrogen treatment in ER+ breast cancer cells and the mutations in this pathway is also associated with resistance to anti-HER2 drugs (90,91).

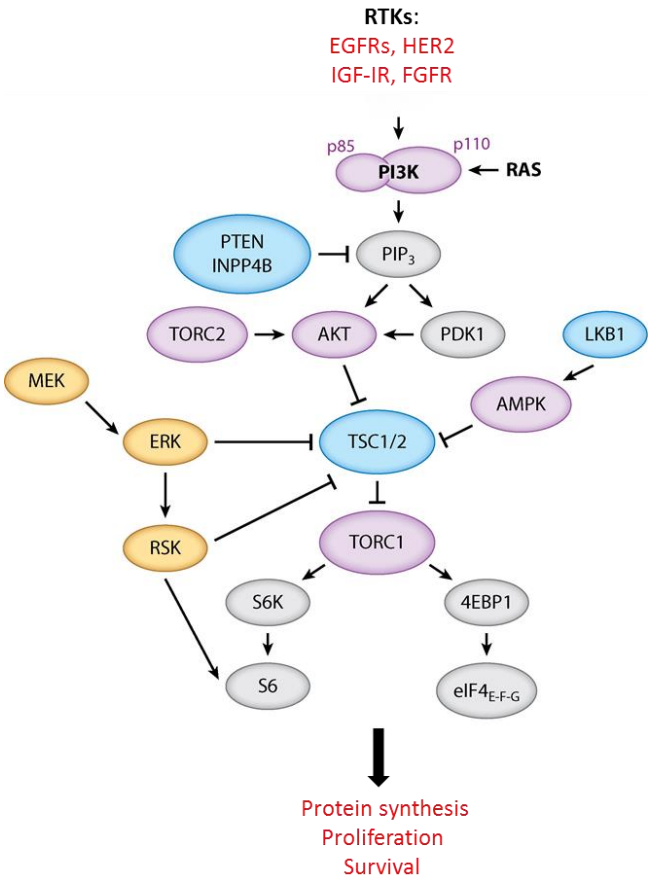


Figure 1.5 PI3K/AKT signaling pathway. Signals from receptor tyrosine kinases (RTKs) such as EGFR, HER2, IGF-IR and FGFR are mediated by p85 and p110 regulatory subunit of PI3K. Mechanisms of altered PI3K/AKT activation include the loss of PTEN and INPP4B lipid phosphatases and mutation or overexpression of p85 and p110 subunits and PI3K effectors, AKT1/2/3 which can result in increased protein synthesis, proliferation and cell survival. [Adapted from Mayer and Arteaga, 2016 (86)].

1.3 Corticotropin- Releasing Hormone (CRH) & CRH-related peptides

Corticotropin-releasing hormone (CRH) family peptides are 41-amino acid residues encoded by *CRH* gene that involve in the stress response and functions primarily in the stimulation of pituitary adrenocorticotropic hormone (ACTH) in hypothalamic-pituitary-adrenal axis (HPA) (92). CRH sequence homology between human, mouse, rat and others are approximately 76-95% with its mRNA distributed mainly in the brain and also detected in other organs such as the retina, stomach, intestine, spleen, lung and gonads (93). CRH family peptides consist of CRH, Urocortin (Ucn), Urocortin 2 (Ucn2) and Urocortin 3 (Ucn3), and their actions are mediated by specific seven transmembrane domain (7 TMD) G-protein-coupled receptor (GPCRs). While CRH is responsible for initiating stress response to stress, urocortins play substantial role in the recovery response to stress.

In the anterior pituitary, CRH stimulates the synthesis and processing of proopiomelanocortin (POMC) to generate adenocorticotropin hormone (ACTH) and its secretion into the systemic circulation which in turn stimulates the synthesis and the release of glucocorticoids (94). Apart from that, CRH also has several other physiological functions such as suppression of food intake, regulation of body temperature, growth, metabolism and reproduction. Recent findings demonstrate that peripheral CRH-related peptides can act as pro-inflammatory factors (95). In addition, studies on CRH effects in human and mice have also reported that abnormal elevation of CRH and modification to CRH system are often associated with human stress-related disorders such as anxiety, sleep disruption, depression and adverse changes in cardiovascular, metabolic and immune function (96–98). Peptides of the CRH family and their receptors have been detected in several cancer cells such as human renal carcinoma (99), tumorous adrenocortical cells (100), human endometrial, prostate, ovarian and breast cancer cells (101–105) and several lines of evidence have suggested that CRH has dual roles depending on tissue types. CRH and Ucn 1 have been demonstrated to inhibit the

human endometrial adenocarcinoma growth (101), and hepatocellular carcinoma growth and angiogenesis (106), respectively. On the other hand, dermal fibroblasts deficient of CRH has shown higher proliferation and migration rates as compared to the wild type cells (CRH+/+) (107).

1.3.1 CRH Receptors

Three types of CRH receptors have been identified; type- 1 (CRHR1) and type- 2 (CRHR2) which share 70% homology at the amino acid level (108,109) are present in mammals, chicken, *xenopus* and teleost, whilst type-3 (CRHR3) is found in catfish (93). In mammals, gene coding for CRHR1 consists of 13 exons and 12 introns whereas CRHR2 gene consists of 12 exons and 12 introns, and three splice variants (CRHR2α, 2β and 2χ) (93). Both CRH and Urocortin 1 bind to CRHR1 with higher affinity while CRHR2 exhibits low affinity to CRH but high affinity to other CRH-related peptides such as Urocortin1, Urocortin 2 & 3 (92). In addition, the actions of CRH-like peptides have been shown to be modulated by a CRH-binding protein (CRH-BP), which circulates in blood and is detected in the brain of some species (110) (Figure 1.6).

These CRH receptors belong to the class B_1 subfamily of seven transmembrane (TMD7), secretin type G protein-coupled receptors (GPCRs). GPCR is activated when an agonist binds to and promotes interaction with heterotrimeric G protein (G$\alpha\beta\gamma$) hence triggering guanine nucleotide exchange and separation of Gα subunit from G$\beta\gamma$ subunits (111), followed by initiation of downstream responses upon the interaction between G protein subunits and variety of effectors and second messenger pathways such as enzymes and ion channels (112). G protein signaling is strictly controlled by the family of regulator of G protein signaling (RGS) proteins that function as GTPase activating proteins (GAPs) on the α subunits of the Gα_i and Gα_q subfamilies of heterotrimeric G proteins to facilitate the termination of downstream signaling by the Gα and G$\beta\gamma$ subunits [reviewed in (113)]. In addition, the activation of G protein is terminated by

desensitization mechanism such as phosphorylation of receptors by GPCR kinases (GRKs) followed by recruitment of β- arrestins (βarrs) to the phosphorylated receptor (114), which engages the phosphorylated C-tail and transmembrane core of the receptor, an interaction that leads to overlapping with the G protein-binding site and thus prevent further G protein activation (115–117).

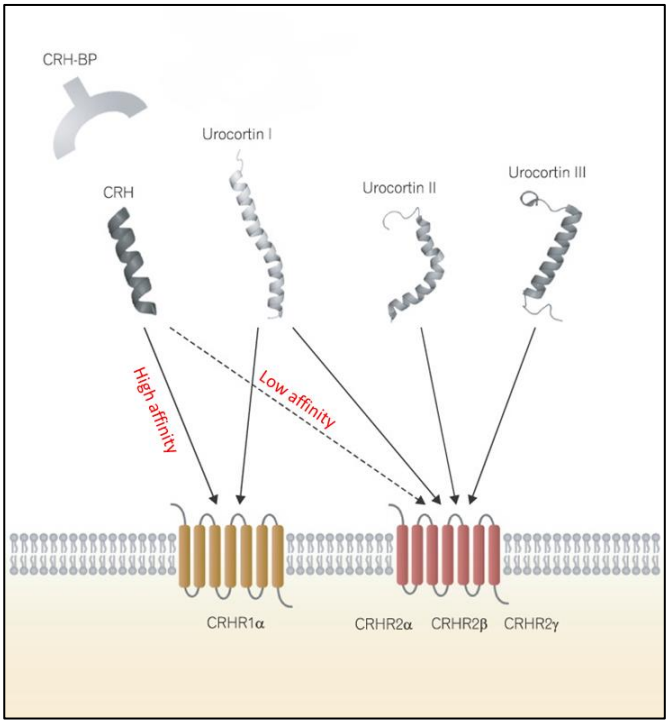


Figure 1.6 Corticotropin-releasing hormone (CRH) system showing CRHR1 and CRHR2 receptors and their splice variants. CRH binds with high affinity to CRHR1 but with low affinity to CRHR2. Urocortin 1 can bind to both receptors and Urocortin 2 & 3 bind to only CRHR2. [Adapted from Im, 2014 (118)].

Upon the binding of CRH to the CRHRs, the structural confirmation of these receptors would change and thus resulting in the signals being transduced across the cell membrane (109). The stimulation of CRHRs by CRH and CRH-related peptides induces activation of adenylyl cyclase via Gs protein-coupled with CRHRs, which increases cAMP levels (119,120). Increased production of cAMP activates protein kinase A (PKA) which in turn phosphorylates downstream effectors in cytosol and nucleus such as CREB, c-fos and Nur77, thus inducing POMC gene transcription. Even so, this activation of adenylyl cyclase signaling pathway is believed to be tissue-specific as this pathway activation is not seen in certain tissues, instead CRH is shown to activate alternative signaling cascades suggesting that CRH and CRH-like peptides can trigger different responses in target tissues (121,122). CRH has been shown to activate extracellular signal-regulated kinase (ERK1/2) via a PKA CamKII- dependent pathway that activates Nur77 transcriptional activity (123) (Figure 1.7).

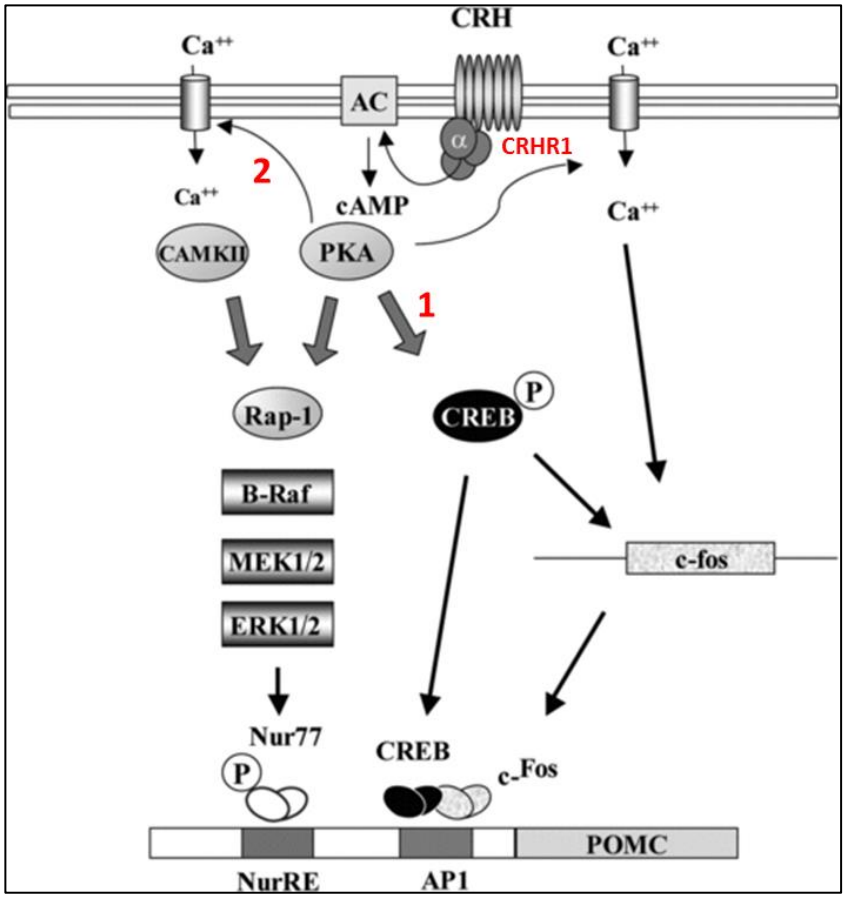


Figure 1.7 CRH binds to CRHR1 and activates PKA which subsequently triggers two main transduction pathways; (1) Calcium-independent pathway. (2) Calcium-dependent/CAMKII pathway. MAPK pathway can be activated via calcium-dependent and – independent which involves Rap-1, Raf, MEK and ERK1/2 that can lead to increased transcriptional activity of transcription factor Nur77. In addition, PKA can activate CREB thus eventually regulate the expression of POMC. [Adapted from Refojo & Holboer, 2009 (124)].

1.3.2 CRH and Cancer

Variations of the CRHR1 gene have been reported as one of the risk factors for depression after childhood maltreatments (125,125,126) and *CRHR1* polymorphism is associated with recurrence in major depressive disorder (MDD) and is found to mediate irritable bowel syndrome (IBS) (127,128). Whereas, polymorphism and gene variation in *CRHR2* can attenuate stress response thus reducing symptoms of PTSD disorder in women (129). In addition to this, several studies have demonstrated the potential of CRH receptors in modulating various intracellular protein kinases such as protein kinase A (PKA), protein kinase B (PKB/Akt) and p42/p44 and p38 mitogen activated protein kinases (MAPKs) [reviewed in (109)]. As one hypothalamic factors mediating behavioral and neuroendocrine responses to stresses, CRH has been detected in a stress environment such as in tumor microenvironment, thus influencing cancer progression.

The presence of CRH family peptides and their two corresponding CRHR1 and CRHR2 in various human cancers, for examples malignant melanomas and small-cell lung cancers as well as in endocrine-related cancers such as breast cancer and prostate cancer has also been previously reported (130–135). Besides, genomic studies have reported approximately 20% of human cancers show mutations in GPCRs suggesting the important role of GPCR in cancer biology (136). At present, CRH has been shown to play both promoting and inhibitory roles in tumorigenesis. CRH receptors have been shown to modulate inflammation and tumor growth, for example in mouse model of colitis-associated cancer (CAC), CRHR1 has been demonstrated to induce tumorigenesis and regulate colon cells survival and proliferation through NFκB and STAT3 signaling pathways (137). In addition, CRH and Ucn2 has been shown to induce migration of RM-1 prostate cancer cells through CRHR1 and CRHR2 (138). The role of CRH and Fas ligand (FasL), a protein that plays significant role in promoting tumor cells' counter attack towards immune cells thus favoring tumor survival and progression, has been shown in ovarian carcinoma (105). Furthermore, studies also showed that CRH upregulated MMP2 and MMP9 levels in Ishikawa cells hence enhancing migration and invasion

properties of the cells (139). In addition, its tumor-promoting effects has been reported in hormone-responsive MCF7 breast cancer cell line where it was shown to promote cell motility and invasiveness (104).

On the other hand, inhibitory roles of CRH have also been reported in various tumors. The anti-proliferative effect of CRH has been previously reported in human endometrial adenocarcinoma and human mammary cancer cells (101,140). In addition, it has also been demonstrated that CRH, via CRHR1 inhibits human breast cancer cell growth and invasion in a concentration-dependent manner (103), and that the biological activity of CRH may be regulated by estrogen in which estrogen was shown to increase the abundance of CRHR2 and CRHR1 splice variant, *CRHR1 (Δ12)* mRNA which subsequently prevents the inhibitory effect of CRHR1 on cell invasion in ER+ breast cancer cells (141). Furthermore, CRH was also shown to promote apoptosis in ER+ breast cancer cell line, MCF7 (134), and inhibit TGFβ1 – mediated MCF-7 cell migration via CRHR1/R2, and the inhibition in MDA-MB-2331 was demonstrated via CRHR2 (142).

1.4 Alternative Splicing (AS)

In the mature mRNAs, introns are excised from the pre-mRNAs transcript by the process of alternative splicing (AS). This splicing event regulates gene expression mechanism to generate greater protein diversity from a single gene and is tightly regulated by splicing regulatory proteins in a cell- or tissue- specific manner, and at different developmental stages (143–145). Splicing regulatory proteins which are also known as spliceosomes comprise of five types of small nuclear ribonucleoprotein (snRNP; U1, U2, U4, U5 and U6) and at least 150 of additional proteins that control the process of intron excision and exon joining in pre-mRNA splicing process (146). AS commonly consists of either included or skipped of single cassette exon in the spliced mRNAs which can potentially produce protein isoforms with diverse functions such as in protein-protein interactions and post-translational modifications [reviewed in (147)]. However, there are other types of AS which include alternative 5’ or 3’ splice sites that produces short or long forms of an exon, mutually exclusive exons and intron retention (Figure 1.8).

The mechanism of actions of two main regions in pre-mRNA transcript; exonic splicing silencer (ESE) and intronic splicing enhancer (ISE) involve the binding of regulatory proteins such as SR proteins (serine/arginine-rich proteins) or heterogeneous nuclear ribonucleoproteins (hnRNPs). In addition, certain splicing silencers form a pre-mRNA secondary structure that blocks the activity of SR proteins in recognizing the splicing enhancer sequence on pre-mRNA transcript (148).

Recently, mutations have been revealed to play roles in the disruption of canonical splice sites or abolishment the splicing enhancers and silencers function leading to the production of aberrantly spliced mRNA or failure to a functional protein resulting in the pathogenesis of diseases such in tumor development and progression. Mutations in splicing elements of susceptible genes such as *LKB1, KIT, CDH17, KLF6, BRCA1* and alterations in splicing regulators that affect *Ron, RAC1* and *CD44* gene expression have been detected in cancers (148).

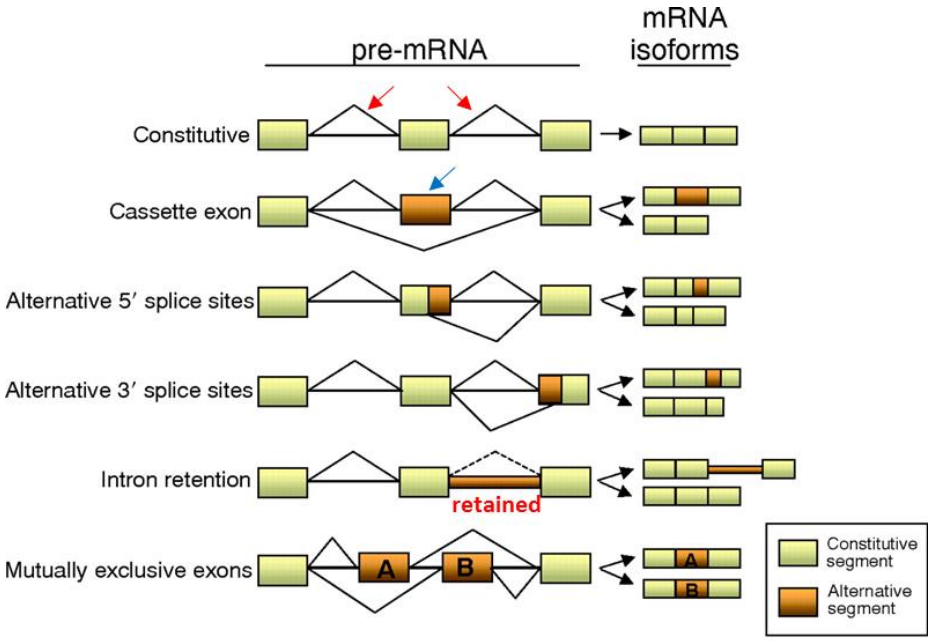


Figure 1.8 Different types of alternative splicing generate several combinations of exons in mature mRNA transcripts. Green boxes indicate constitutive exon sequences, blue arrow indicates alternative regions and red arrows indicate alternative splice patterns that can take place. [Adapted from Srebrow & Kornblihtt, 2006 (148)].

1.4.1 Alternative splicing in cancer

Although there are several other regulatory checkpoints in the gene expression pathway such as polyadenylation of mRNA precursor and translation, alteration in alternative splicing (AS) can cause the most profound impacts of all. Given that approximately 90% of human genes undergo AS, its deregulation can influence the downstream profile of various genes important in cell developmental programs and consequently causes diseases including cancer.

Alternative splicing in cancer usually can contribute to tumor survival, proliferation, invasion and metastasis due to aberrant expression of mRNA transcripts. An example of this is the alternative splicing of 5' splice sites of exon 2 in the BCL-X gene that produces two variants that oppositely regulate cell death (149). In addition, studies in human breast, colon carcinoma and colorectal adenocarcinoma cells have shown that accumulation and overexpression of alternatively spliced protein isoforms such as ΔRon and Rac1b are sufficient to transform cells in culture (150,151). In fact, ΔRon which skipped the exon 11 of the original pre-mRNA transcript has also been identified to induce invasive phenotype of human gastric carcinoma (152).

Furthermore, dysregulation of cancer-related signal transductions such as the Ras-PI3K-AKT pathway can also contribute to alterations of splicing patterns in cells (Figure 1.9). This pathway has been reported to trigger changes in the activity of SR proteins, particularly SF2/ASF and 9G8 as AKT has been demonstrated to phosphorylate these SR proteins in vitro (153,154). In addition, the activity of another SR protein, SRp40 can be affected by the induction of this pathway by insulin, resulting in the inclusion of alternative exon in protein kinase C (PKC) βII pre-mRNA (155,156). Besides, Ras- dependent pathway is also implicated in the regulation of CD44 (157), one of the most studied alternatively spliced genes in cancer, whose splice variants correlate with tumor development and metastasis (158,159). The regulation of exon 5 alternative splicing in CD44 is shown to be dependent on the activity of nuclear RNA-binding protein, Sam68 which is phosphorylated by ERK after the induction by phorbol-ester in T-lymphoma cells. Furthermore, another Ras-dependent co- activator SRm160, a factor

that promotes cell invasiveness has also been identified to trigger inclusion of exon 5 in CD44 in HeLa and 293T cells (160).

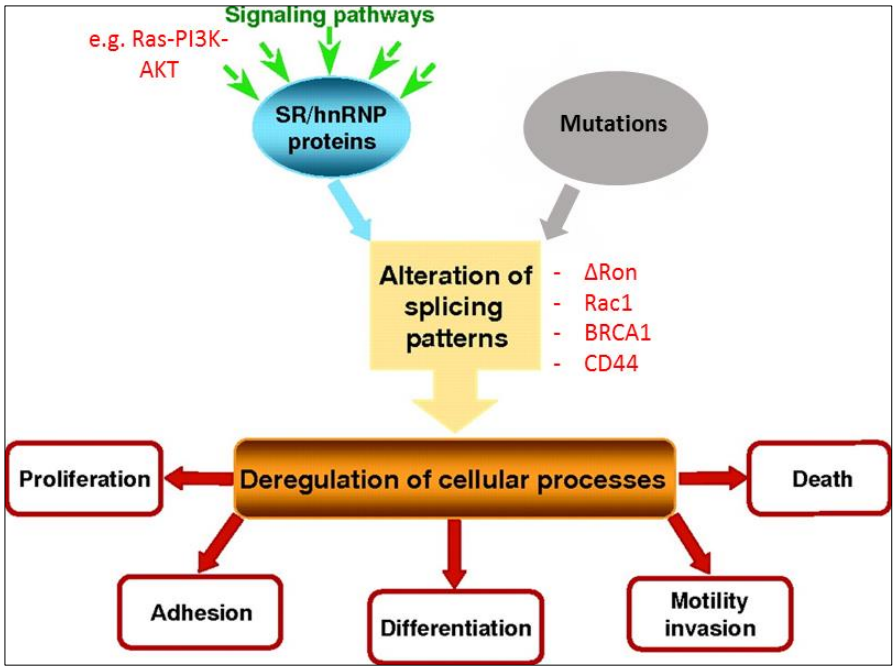


Figure 1.9 Mutations and Activation/Deregulation of signaling pathways such as Ras-PI3K-AKT can lead to changes in the activity of splicing factors thus causing alterations in the splicing of oncogenes such as Ron, Rac1, BRCA1 and CD44 hence promoting tumorigenesis and cancer metastasis. [Adapted from Srebrow and Kornblihtt, 2006 (148)].

A number splicing alteration that promote cell growth and survival has been identified in ER+ and ER- breast cancers, which among the well- studied genes are *ER*, *HER2* and *CD44*. Aberrant splicing of *ER1* locus leads to the generation of a splice variant, *ERα36* that regulates non-genomic estrogen signaling pathways and subsequently resulting in 4-hydroxytamoxifen resistance in breast cancer cells (161). In addition, Δ16HER2 which lacks exon 20 of *HER2* has been identified in 9% of HER2-amplified breast cancer and shown to confer endocrine therapy- resistance in HER2 positive breast cancer (162,163). Whereas, alternative splicing of CD44 gene can produce several

isoforms such as CD44s and CD44v, in which although both have been implicated in the development of breast cancer, CD44v is found to be more associated towards better prognosis in hormone-responsive breast cancer, while CD44s is more commonly found in HER2+ or metastatic breast cancer cells (164).

1.4.2 Serine-Arginine Protein Kinase 1 (SRPK1)

There are three classes of splicing kinases in human which include the serine-arginine protein kinases (SRPK1/2/3), the CDC-like kinases (CLK1/2/3/4) and the pre-mRNA processing factor 4 kinases (PRP4K) with each has distinct cellular localization based on their different roles in splicing regulation (165). The PRP4K is a lesser-known splicing kinase and shown to regulate spliceosome assembly through the phosphorylation of splicing factors PRP6 and PRP31, whilst CLK1 and Serine-Arginine protein kinases (SRPKs) family are responsible for the phosphorylation of serine residues in SR protein.

SRPKs family comprises of more than 50 members that have been detected in the genomes of mammals, fungi, insects, nematode and plants (166). Most of the functional studies on mammalian SRPKs were based on SRPK1, which is one of the first protein kinases to be studied in literature, followed by SRPK2 and SRPK3 which were identified based on sequence homology with SRPK1 (167). While the functions of SRPK1 and SRPK2 are in the regulation of SR proteins distribution through phosphorylation, SRPK3 has been identified to be involved in normal muscle growth and homeostasis (167,168).

SRPK1 is a 92kDa SR protein kinase found in the cytoplasm of most cell types and tissues that act as downstream AKT target for transducing growth signal from cell surface to the nucleus (169). It is anchored in the cytoplasm by networking with chaperones and is thought to be the key splicing regulator in the alternative splicing mechanism (170). Zhong et al demonstrated that SRPK1 binds to the co-chaperones Hsp40/DNAjc8 and Aha before interacts with major molecular chaperones Hsp70 and

Hsp90 in the cytoplasm (171). Following this, SRPK1 dissociates from these chaperone complexes which can be triggered by many factors such as stress signal, and translocates to the nucleus and initiate SR proteins phosphorylation and alter the splice site of target mRNA (171).

Cytoplasmic SRPK1 phosphorylates RS domain of serine arginine-rich (SR) proteins that induces them to relocate back to the nucleus where they can influence splice site usage for alternative splicing (172–174). Studies show that additional phosphorylation is required to recruit the already phosphorylated SR proteins in the nucleus to nascent pre-mRNA transcript and this second phosphorylation is mediated by CLK1 (175). These sequential phosphorylation events are proposed to be regulated by two factors; distinct cellular localization of the kinases (SRPK1 can be found in both cytoplasm and nucleus, CLK1 in nucleus only) and substrate specificity of the splicing kinases. For example, cytoplasmic SRPK1 phosphorylates the Arg-Ser repeats (RS1) at N-terminal of splicing protein SRSF1, whereas CLK1 phosphorylates Ser-Pro repeats (RS2) at C- terminal of SRSF1 (Figure 1.10) (173,176). Recently, studies have suggested that after binding to SR proteins to phosphorylate them, CLKs may require a release factor that can unleash them from the phosphorylated SR proteins to make it fully functional and SRPK1 is shown to function as release factor for CLK1 (Figure 1.11) (177,178). This therefore indicates that SRPK1 and CLK1 interact and work synergistically, rather that competitively, in phosphorylating RS domain of SR proteins and thus promoting spliceosome assembly in the nucleus (179).

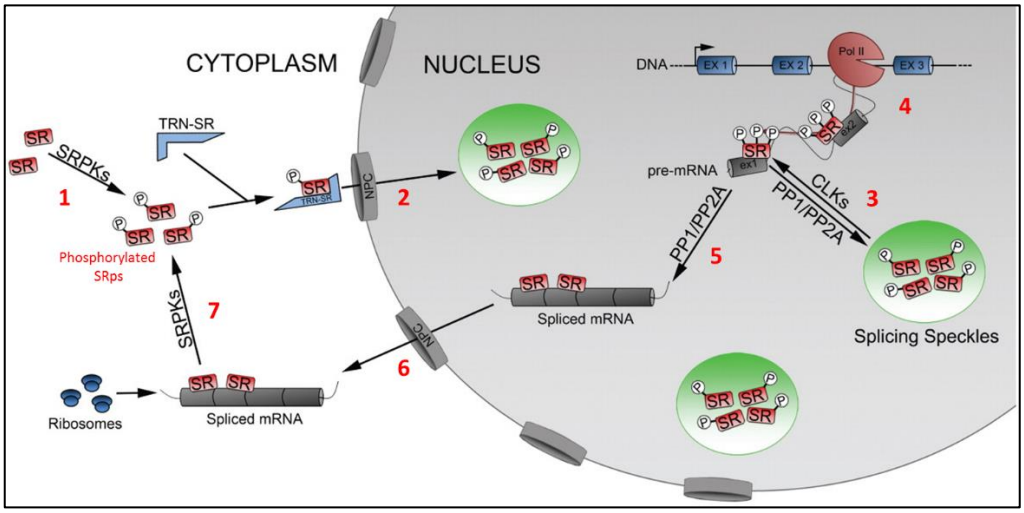


Figure 1.10 SRPK1 phosphorylates SR proteins in the cytoplasm hence induces SR proteins shuttling into the nucleus. CLK1 mediates additional SR proteins phosphorylation and promotes theirs release from splicing speckles to nascent pre-mRNA transcript. Once splicing completed, SR proteins bound to mRNA are dephosphorylated by nuclear phosphatases (PP1/PP2A), resulting in the recycling of the SR to the cytoplasm for the translation regulation or re-phosphorylated by SRPKs for the next round of splicing. [Adapted from Corkery et al, 2015 (165)].

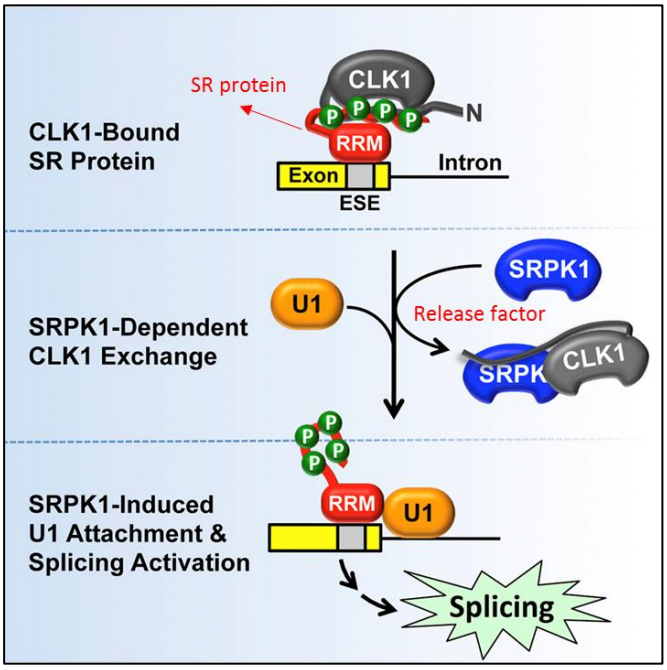


Figure 1.11 SRPK1 and CLK1 works in a cooperative manner in the nucleus of cells, where in addition to phosphorylating SR proteins, SRPK1 acts as release factor that remove CLK1 from tightly bound SR proteins and promotes U1 binding to pre-mRNA transcript thus stimulating mRNA splicing. [Adapted from Aubol et al, 2016 (179)].

Furthermore, in mammalian central nervous system, SRPK1, through the phosphorylation of SR proteins, has been shown to regulate alternative splicing events leading to the production of neuron-specific protein isoforms important in neuronal development, learning, memory and cell communication (180,181). For example, SRPK1 was demonstrated to phosphorylate SRp38 that led to the alternative splicing of glutamate receptor subunit 2 (GluR2) by promoting inclusion of flip exon in GluR2 pre-mRNA transcript (182). In addition, SRPK1 also plays role in the splicing of exon 10 in Tau protein which is implicated in the pathogenesis of Frontotemporal Dementia as well as Alzheimer disease (183). Studies also show that by decreasing the SRPK1 expression in cell will result in the decrease of phosphorylated SR proteins such as SRp20, SRp30c, 9G8, SRp40, SRp55 SRp75, Tra2β1 and ASF/SF2, in a dose-dependent manner (165,170,184,185). This data suggests that since phosphorylation of SR proteins by

SRPK1 would determine the pattern of splicing, the level of SRPK1 in cells is crucial to ensure balanced level of phosphorylated and dephosphorylated SR proteins.

Although SRPK1 activity is important for regulation of various cellular functions as shown by studies in which SRPK1 knockout mice demonstrated severe aberrations of cells functioning (186), its overexpression at both protein and mRNA has been shown to promote breast and colonic tumorigenesis (172,187,188). In fact, recent data from The Cancer Genome Atlas (TCGA) reveals that the expression of SRPKs are frequently altered in cancers as shown in Table 1.1, in which high expression of SRPK1 positively correlates with tumor progression (189).

In cancer cells, SRPK1-SR proteins activity has been demonstrated to contribute to the splicing regulation of various target genes splicing such as MAP2K2 and Rac1b. Depletion of SRPK1 kinase in breast, colon and pancreatic cancer cells resulting in reduced phosphorylation of SR proteins including SRSF3, SRSF4 and SRSF6 leading to altered splicing of MAP2K2 mRNA causing it unable to phosphorylate its targets, MAPK1 and MAPK3 which eventually leading to the induction of apoptosis (170,172). It has also been shown that nuclear translocation of SRPK1 in cancer cells can be induced by upstream signals such as growth factors, that can lead to activation of signaling pathways involved in various cellular events. This is demonstrated in studies by which EGF was shown to induce cytoplasmic SRPKs translocation into the nucleus via the activation of AKT, a protein kinase that plays substantial role in cell survival, and thus triggers splicing events mediated by AKT-SRPK-SR network (190).

In addition, SRPK1 has been shown to target RBM4 in myeloid leukemia cells, thus inhibiting RBM4 binding to *MCL1*, a gene member of *BCL2* family that regulates apoptosis in cells. This subsequently leads to exon 2 exclusion from the final mRNA transcript hence producing anti-apoptotic gene isoform in cells (165). Furthermore, increased level of MCL1 gene isoform, $MCL1_L$ has been shown to positively correlate with SRPK1-RBM4 network, where elevated level of SRPK1 causes increased accumulation of RBM4 in the cytoplasm and thus interfere with MCL1 alternative splicing, leading to the production of anti-apoptotic protein isoform in MCF7 and MDA-

MB-231 breast cancer cells (191,192). Consistently, SRPK1 has also been identified as one of the determinants in metastatic breast cancer as it was shown to facilitate tumor cell migration in breast cancer (193).

As studies have repeatedly identified alterations that affect splicing in diverse cancer types such as mutations in splice-site sequences and mutations in genes encoding splicing factors, manipulation of splicing might provide therapeutic benefits in cancer. For examples, splicing might be modulated by using spectrum of compounds that modulate spliceosome assembly for examples by inhibiting SF3B1 which plays essential role in initiating assembly of spliceosome components or by inhibiting the phosphorylation of SR proteins through the inhibition of CLKs and SRPKs (194).

Table 1.1 The roles of splicing kinases in cancers (195).

Kinase	Expression	Types of cancers	Role in cancer	Ref.
SRPK1	↑	breast, esophagus, lung, pancreas, prostate	Oncogene (glioma, cancers of prostate, liver, breast, colon, pancreas, lung) Tumor suppressor (inhibits tumoral transformation of mouse embryonic fibroblasts)	Amin et al. (2011), Bullock et al. (2016), Gong et al. (2016), Hayes et al. (2007), Liao et al. (2017), Ren et al. (2015), Sigala et al. (2016), Wu et al. (2013)
	↓	retinoblastoma, treatment-resistant		Krishnakumar et al. (2008), Schenk et al.
	⇅	colon cancer		Hayes et al. (2007), Wang et al. (2014)
SRPK2	↑	cancers of lung and colon, subset of acute	Oncogene (leukemia, colon cancer)	Gout et al. (2012), Jang et al. (2008), Wang et al.
SRPK3	↓	rhabdomyosarcoma	Tumor suppressor (rhabdomyosarcoma)	Zhang et al. (2015)
CLK2	↑	most cancers	Oncogene (breast cancer)	Yoshida et al. (2015)
PRP4	↓	thymoma, kidney chromophobe subtype	Tumor suppressor (hepatocellular carcinoma)	Liu et al. (2013), Gao et al. (2013), Giroux et al.

1.5 Epithelial- to- Mesenchymal Transition (EMT)

There are three different subtypes of EMT; type 1 is associated with implantation, embryo formation and organ development which does not contribute to fibrosis or invasive phenotype. For example, EMT is important during embryogenesis to facilitate proper anchoring of the placenta and allowing it to function in nutrient and gas exchange (196,197). The formation of primitive streak where epithelial cells in this tissue express E-cadherin, is considered the first sign of gastrulation which subsequently leads to the formation of three germ layers that generate all tissue types of the body (198). At the molecular level, canonical Wnt signaling has been shown to orchestrate the EMT process during gastrulation and this process cannot be initiated in the embryos deficient in Wnt3 (199).

Meanwhile, type 2 is related with wound healing, tissue regeneration and organ fibrosis following trauma and inflammatory injury. Inflammatory cells and fibroblasts mediate organ fibrosis by releasing various inflammatory signals and components such as collagens, laminins and elastin. This fibrosis- associated EMTs can be found in kidney, liver, lung and intestine (198). In addition, studies in 133 kidney fibrosis patients using double labelling of the tubular epithelial cells with cytokeratin, vimentin, α-SMA or zona occludens 1 (ZO-1) showed that EMT was evident in a number of samples, suggesting that organ fibrosis can be reversed if novel therapeutic interventions can be developed to suppress EMTs (200).

Whereas, type 3 EMTs has been described in cancer cells that have undergone genetic changes affecting oncogenes and tumor suppressor genes which favor the development of localized tumors. Type 3 EMTs may induce invasive properties in carcinoma cells thus enabling the cells to metastasize and facilitating life-threatening cancer progression (198). The underlying biochemical mechanisms in the acquisition of invasiveness by primary epithelial cancers is proposed by many studies to be due to activation of EMT program (201). EMT is often associated with the loss of cell-cell contact and disruption of intracellular tight junction which eventually causes cells to

acquire motile mesenchymal characteristic (202). Moreover, carcinoma cells that have acquired mesenchymal phenotype usually express mesenchymal markers such as vimentin, and are considered to be the cells that enter into subsequent steps of the invasion- metastasis cascade, namely intravasation, transport through the circulation, extravasation, formation of micrometastases, and eventually colonization of new sites (Figure 1.12) (203,204) .

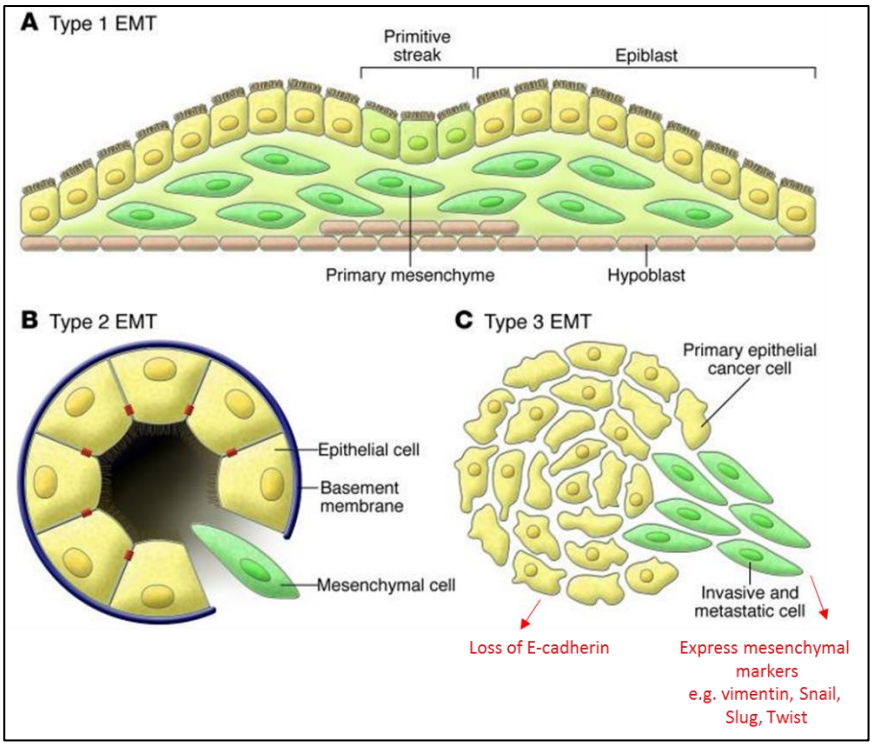


Figure 1.12 Three types of EMTs in human. [Adapted from Kalluri and Weinberg, 2009 (198)].

It has been reported that the activation of EMT- inducing transcription factors, notably Snail, Slug, Twist and FOXC2 in cancer cells are emanated from EMT- associated signals such as EGF, PDGF and TGF-β, which is also depending on series of intracellular signaling networks including ERK, MAPK, PI3K, Akt, Smads, RhoB, β-catenin and Ras (198,205). Overexpression of these transcription factors can lead to the down-regulation of E-cadherin expression in epithelial cells causing the cells to detach from their surroundings, and eventually gaining access to the lymphatic or blood vessels (Figure 1.13) (206,207). In fact, cells that have undergone EMT often re-program their metabolic and oncogenic pathways, which is also a hallmark of cancer, in order to survive the new target site (208). Some other signatures of EMT are the increased presence of N-cadherin and vimentin, nuclear localization of β-catenin and increased production of mesenchymal-specific transcription factors such as ZEB1/2, apart from Snail, Slug, Twist (144,209).

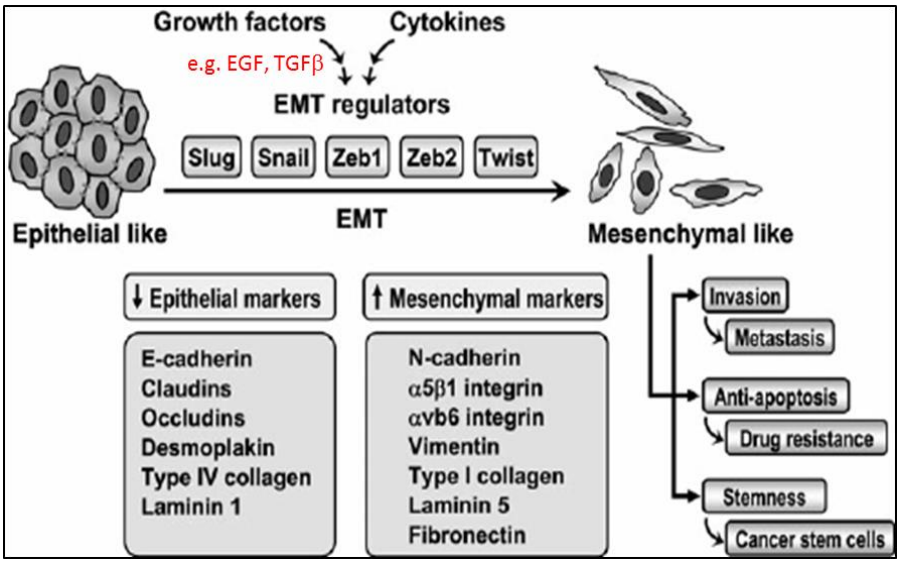


Figure 1.13 Cancer cells transform from epithelial-like to mesenchymal-like due to epithelial markers suppression and mesenchymal markers upregulation, which eventually lead to cancer metastasis. [Adapted from Shih and Yang, 2001 (210)].

Recent evidence suggests that EMT is also partly responsible for the emergence of cancer stem cells (CSCs), a subset of cell population among heterogeneous tumor cells that exhibit stem cell-like features. For examples, the induction of EMT by TGF-β treatment or forced suppression of E-cadherin expression in mammary epithelial cells gives rise to $CD44^{high}$ $CD24^{low}$ cells, one of the known markers of breast CSCs (211). In addition, the emergence of EMT- associated CSCs in breast cancers is also reported to take place after an immune response, as $CD8^{+}$ T-cells can trigger dedifferentiation of breast cancer cells, leading to the formation of $CD44^{high}$ $CD24^{low}$ stem cell-like cells (212). In a clinical setting, it is possible to trace EMT process by examining Circulating Cancer Cells (CTCs). Studies on these CTCs from patients with metastatic breast tumors revealed that mesenchymal markers were enriched in clusters CTCs rather than single migratory cell which might propose that either EMT causes single cell to form cluster cells or mesenchymal transformation happens in pre-existing cluster of CTCs in the blood stream (213,214).

These findings provide evidence that EMT involved in the breast cancer metastatic process and is a critical phenomenon that is believed to contribute to chemotherapy-resistance of various cancer cells.

1.5.1 Molecular mechanisms of EMT in cancer cells

Understanding the regulation of EMT is of utmost important to find effective and specific therapies for the treatment of metastatic cancer. The complex process of EMT involving crosstalks between multiple pathways and the activation distinct EMT-associated transcription factors, miRNAs and lncRNAs makes the onset of EMT in cancer cells does not rely on additional genetic alterations (213,215).

Studies in breast cancers found that association between lung metastasis relapse and TGFβ expression can only be seen in ER-negative primary breast tumors but not in ER+ breast tumors (216). In addition, overexpression of HER2 using heregulin (HRG) in breast cancer cells has resulted in increased expression of EMT signature, Slug but not Snail or Twist, with concurrent phosphorylation of Akt and heat shock factor 1 (HSF-1) (217). Meanwhile, ER-positive breast cancer model, MCF7 has been demonstrated to undergo EMT changes after the treatment with estrogen and the cell adhesion molecules L1 (218,219). Furthermore, another subtype of breast cancer, Triple negative (TN) cells have been shown to express high levels stem cells and EMT markers, $CD44^{high}$ $CD24^{low}$ and Twist, respectively, as compared to hormone-sensitive or HER2-positive breast cancers (220). However, although some EMT signatures can be detected or induced in breast cancer subtypes, co-expression of epithelial and mesenchymal markers can still be predominantly observed in all cell subtypes, hence suggesting that 'partial' EMT, rather than complete EMT, might represent a state with higher cell plasticity between breast cancer groups (215).

Generally, it is known that the activation and expression of the EMT- associated transcription factors take place as a response to various signaling pathways. Previous studies have revealed that EMT is controlled by family of transcription factors which include Twist, Snail, Slug, ZEB1 and ZEB2 that act by binding to the promoter region of genes responsible for cell-cell adhesion in epithelial cells such as E-cadherin (*CDH1*) and repressing their expression (145,221). Some of the well-recognized signaling pathways in

inducing EMT are TGF-B (Transforming Growth Factor B), Wnt /β- catenin and Notch (family of transmembrane proteins) (145,222,223).

1.5.1.1 Transforming growth factor-B

Amongst all the pathways involving in tumor invasion and metastasis such as NF-kB, MAPK/ERK and PI3K/AKT, TGF-β signaling pathway has been known as the major inducer of EMT in vitro and acts through various intracellular messengers. Typically, there are three isoforms of TGFβ (TGF-β1, 2, and 3) and the EMT event observed in cancer is commonly regulated by TGF-β1 (224). The TGF-β effect is enough to initiate the formation of motile cancer stem cells (CSCs) by upregulating the oct4, Nanog, N-cadherin, Vimentin, Slug, Snail and suppressing E-cadherin (significant signature of EMT) and Ck18 (213,225). It has been reported that TGF-β induces breast cancer cell invasion via Smad3- and Smad4- dependent manner (226). In addition, analysis of circulating tumor cells (CTC) of metastatic breast cancer reveals that these CTC express known EMT regulators, including TGF-β pathway components which support the role of this pathway in EMT-associated blood-borne dissemination of human breast cancer (227).

The Smad pathway involves activation of the TGF Type II receptor (TGFR2) by TGFβ, followed by phosphorylation of the Type I receptor (TGFR1) which in turn causes the activation of Smad complex. This complex then translocates to the nucleus and triggers EMT by binding to the SNAIL1/2 and ZEB1/2 promoters, inducing their transcription and suppressing expression of E-cadherin and occludin (Figure 1.14) (213,228,229). Ji et al. reported that high abundance of Snail1 in the nucleus correlates with the reduction of E-cadherin in breast cancer cell (230). Consequently, this whole cascade leads to tumor development, invasion and metastasis of cancer cells (231,232) . Additionally in non-Smad pathway, TGF-β induces EMT and cancer cell invasion by activating mTOR signaling through the activation of phosphatidylinositol 3-kinase (PI3K) as well as through the activation of mTOR complex 2 (mTOR2) which promotes

cytoskeleton changes and elevates the EMT-associated protein expression in cancer cells (233).

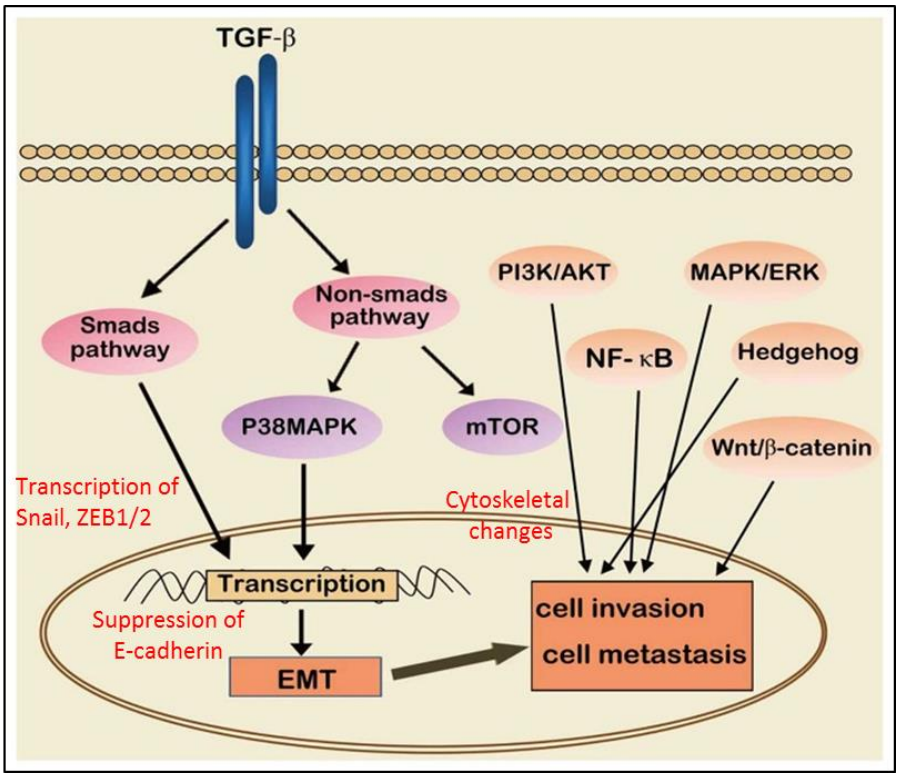


Figure 1.14. TGF-β signaling pathway in cancer invasion and metastasis. TGF-β induces EMT, invasion and metastasis of cancer cells via Smad/non-Smad pathways that leads to the transcription of EMT regulators such as Snail and ZEB1/2, and subsequently resulting in the suppression of E-cadherin expression. Other pathways that also involve in tumor invasion and metastasis are PI3K/AKT, NF-kB, Hedgehog, MAPK/ERK, p38MAPK, Wnt/β-catenin signaling pathway. [Adapted from Li et al, 2015 (234)].

1.5.1.2 Wnt Signaling

A plethora of studies confirmed that Wnt signaling is inappropriately active in several cancers such as in human liver cancer, colon cancer, ovaria cancer, and its signal transduction is associated with the loss of *BRCA1*, which is a hallmark for aggressive basal-like breast cancer (235). Even more, high expression of the EMT-related transcription factor Twist in mammary epithelial cell is also linked with Wnt signaling activation (236). The role of Wnt signaling in promoting EMT has also been demonstrated in which knocking down of Wnt3 reversed the nuclear accumulation of B-catenin as well as reduced the expression of EMT markers (213).

The Wnt pathway is activated once Wnt ligand binds to the transmembrane receptors of the Frizzled family and the signals then transduces across plasma membrane by low-density lipoprotein receptor-related protein (LRP) (Figure 1.15) (213,221,237). This binding causes β-catenin to release from APC complex followed by β-catenin accumulation in the nucleus. In the nucleus, β-catenin will then form β-catenin/TCF/LEF transcriptional complex to target genes associated with EMT (221,237). In addition, it has also been shown that the action of β-catenin/TCF complex in activating EMT program in the breast cancer is mediated by Axin2, which in turn regulates GSK3β, a nuclear kinase that is responsible for controlling the stability of transcription factor, Snail in the nucleus (230). This eventually leads to the activation of target genes such as OCT4, Nanog, Sox-2 which are crucial for cancer metastasis as well as for maintaining self- renewal ability of cancer stem cells.

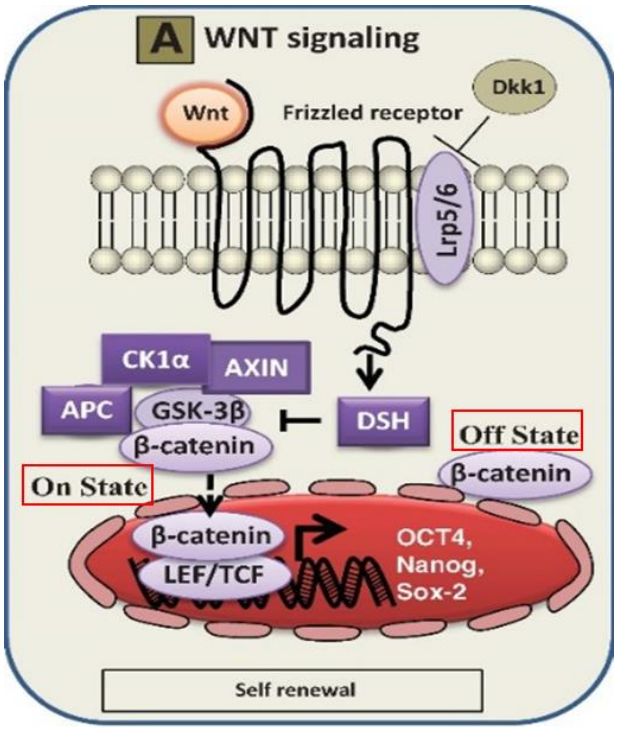


Figure 1.15 Wnt protein is a ligand that transmit extracellular signal to intracellular signaling cascade by binding through frizzled receptor. In the presence of WNT ligand (on state), the binding to frizzled receptors results in b-catenin accumulation in the nucleus. In the absence of the Wnt ligand (off state), b-catenin is sequestered by complex molecules such as Axin1 and APC (destruction complex). Ubiquitination and degradation process will take place because of b-catenin phosphorylation within this complex. [Adapted from Vaz et al, 2014 (238)].

1.5.1.3 Notch

It has been reported that Notch signaling pathway is involved in the developmental process such as cell proliferation, survival, apoptosis and differentiation. It is also shown to be involved in the acquisition of EMT in cancer cells. Elevated expression of Notch-1 and its ligand Jagged-1 was observed to be linked with poor prognosis of various cancers for example breast cancer, bladder cancer, leukaemia and prostate cancer (239–244). Figure 1.16 shows the mechanism of action of Notch signaling as well as the cross talks between Notch signaling and other signaling such as TGFβ, FGF and PDGF that can lead to the activation of intracellular notch and thus increasing the transcription of EMT regulators including Twist, Snail, Slug, and ZEB1/2 (245).

In general, interaction of Notch receptor (NICD) which contains elements for nuclear localization, with the neighboring Notch receptor causes the NICD to be cleaved by enzymes and thus translocate to the nucleus (221,246,247). In the nucleus, the NICD activate the expression of genes that promote tumor development such as NF-kB, Akt and p21 by binding to transcription repressor complexes (247–249). Furthermore, Snail can be upregulated directly by Notch by recruiting the intracellular domain of the Notch (ICN) to the Snail-1 promoter. This will cause over-expression of Snail which will in turn down regulate the expression of E-cadherin and consequently trigger the EMT process in the cells (250).

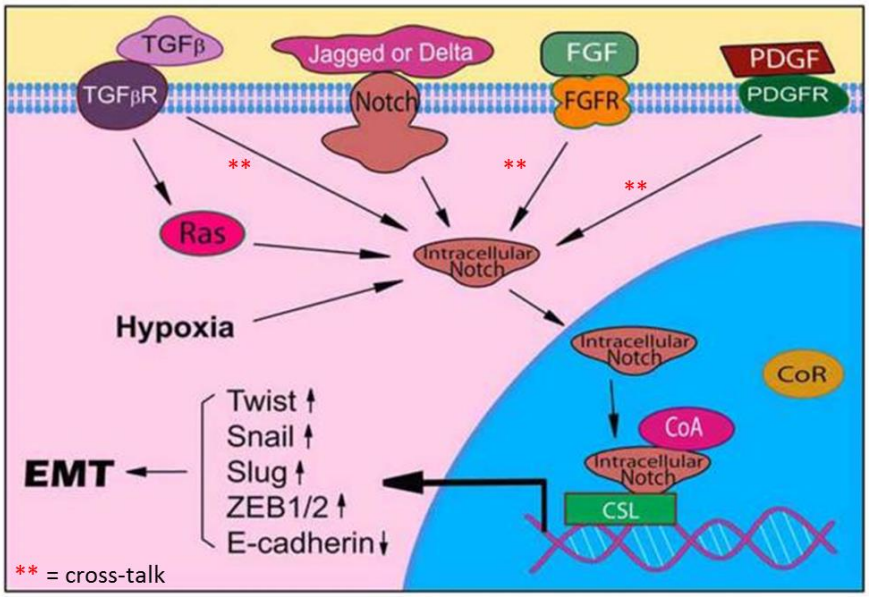


Figure 1.16 Diagram showing mechanism of Notch signaling and the cross-talks with other signaling pathways, in promoting EMT. [Adapted from Wang et.al, 2010 ((245)].

1.5.2 Link between Alternative Splicing (AS) and Epithelial-mesenchymal Transition (EMT)

There are also several ways alternative splicing can play a role in EMT particularly through the regulation of EMT-associated signaling pathways. Shapiro et al suggested that the occurrence of alternative splicing in EMT is controlled by one or more members of splicing factors such as RBFOX, MBNL, CELF, hnRNP and ESRP (251). The connection between AS and EMT was first established when specific CD44 splice variants were detected in metastatic pancreatic cancer cells that was not present in the primary tumor (252).

CD44 is a transmembrane glycoprotein where its pre-mRNA transcript is subject to intricate alternative splicing (AS) including ten adjacent exons (Figure 1.17) that can be included in combination or single and its altered splicing has been reported to be linked with the signaling pathways associated with cell growth (253,254). Some of the pathways that can be influenced by CD44 in cancer cells are Wnt/β-catenin pathway, TGFβ, Notch and PI3K pathway (Figure 1.18) (255). Previously, it was demonstrated that the alternatively spliced CD44 gene (CD44v4-7 and CD44 v6-7) were highly expressed particularly in metastatic pancreatic carcinoma as opposed to the parental tumor (256). In addition, studies on CD44v6 also demonstrate that this variant is crucial for the formation of a complex with the receptor tyrosine kinase Met and hepatocyte growth factor (HGF), an crucial step for the acquisition of metastatic features by cancer cells (257,258).

In 2011, Brown et al revealed that a switch or shift in CD44 expression from variant isoforms (CD44v) to standard isoform (CD44s) was required to accelerate EMT and breast cancer progression and was demonstrated to be negatively controlled by epithelial specific splicing factor, ERSP1 (145). In this study, it was found that CD44v was predominantly expressed in epithelial cells, and the induction of EMT by TGFβ in the breast cancer cells has resulted in the exclusive expression of CD44s, increased ratio of CD44s/CD44v mRNA, and this increased CD44s expression was also positively correlated with the expression of mesenchymal cell marker, N-cadherin, thus indicating that CD44

isoform switching occurs during EMT and CD44s is essential for EMT (145). In addition, studies on primary breast tumors revealed that except CD44s, mRNA and proteins expression of all analyzed CD44 isoform correlated with cancer stem cells (CSC) phenotype $CD44^{+}/CD24^{-}$(164). Following this, further analysis showed that CD44s isoform was positively correlated with ALDH1, another biomarker of malignant stem cells (164,259). Consistently, CD44s has also been shown to activate EMT signature, ZEB1 and maintain mesenchymal phenotype of breast cancer cells via TGFβ signaling pathway (260). Recently, ERK/Ras signaling which is substantial for cell cycle progression has been shown to induce CD44v6 splicing (261). Furthermore, collaboration between Wnt signaling, specifically Wnt- target gene, Met and CD44v6 was also observed in colorectal cancer and is required in colorectal cancer progression (262).

Besides CD44, studies have also identified other gene targets with alternative isoforms affecting EMT and correlating with invasive phenotypes in cancer cells. For example, mis-regulated splicing of tyrosine kinase receptor, Ron in breast and colorectal tumors is the most widely known example of the link between alternative splicing (AS) and the activation of EMT. The skipping of exon 11 results in the production of Ron spliced variant, ΔRon and its over-expression stimulates EMT and confers increased cell motility and matrix invasion (151). It has been observed that the expression level of splicing factor, ASF/SF2 critically determines the generation of ΔRon which is found to play significant roles in tumor progression (263).

Notably, the role of estrogen signaling in AS and EMT have been reported in several studies. For example, the induction of EMT by TGFβ in prostate cancer cell line that endogenously expresses both ERα and ERβ shows that EMT was negatively correlated with the presence of ERβ while ERa was not affected, suggesting that loss of ERβ promotes EMT in prostate cancer (264). In addition, studies in breast epithelial cells induced with estrogen (E2) reveals that estrogen can cause genomic aberrations such as changes in gene expression resulting in disrupted integrin signaling and apoptosis pathway, and EMT by reducing the expression of epithelial marker, E-cadherin and increasing mesenchymal markers such as n-cadherin and vimentin (265). Estrogen signaling has been previously studied to be implicated in the alterative splicing of several

target genes such as CRHR1 in breast cancer (141) and Fibulin-1 in ovarian cancer that produces Fibulin-1C isoform which may be involved in ovarian carcinogenesis (266). In addition, estrogen receptor-α gene (*ESR1*) has been shown to cooperate with homeobox transcription factor (BARX2) in MCF7 breast cancer cells, where both proteins bind to different ESR1 gene promoters and regulate the expression of ESR1 protein isoforms (66kDa and 46kDa). The upregulated expression of ESR1 promotes the survival, cell growth and invasion property in breast cancer by promoting increased expression of active matrix metalloproteinase-9 (MMP9) and the expression of tissue inhibitor of metalloproteinase (TIMP 1 & 2) genes (267) .

This finding as well as several other studies provides evidence that modification of constitutive splicing events can induce EMT-related phenotype change as a result of gene isoform produced which promotes and supports transformation of cells from epithelial to mesenchymal state (144).

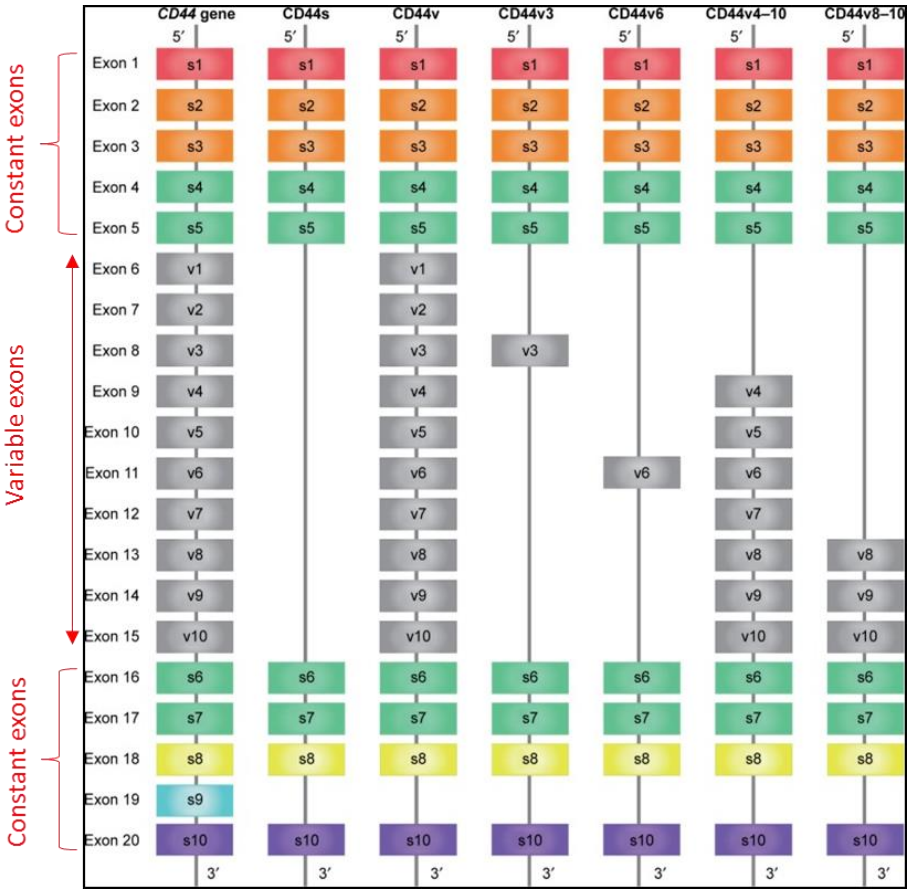


Figure 1.17 CD44 gene contains 20 exons and alternative splicing typically produces two main type of isoforms; CD44s (short/standard) and CD44v (variant). [Adapted from Chanmee et al, 2015 (268)].

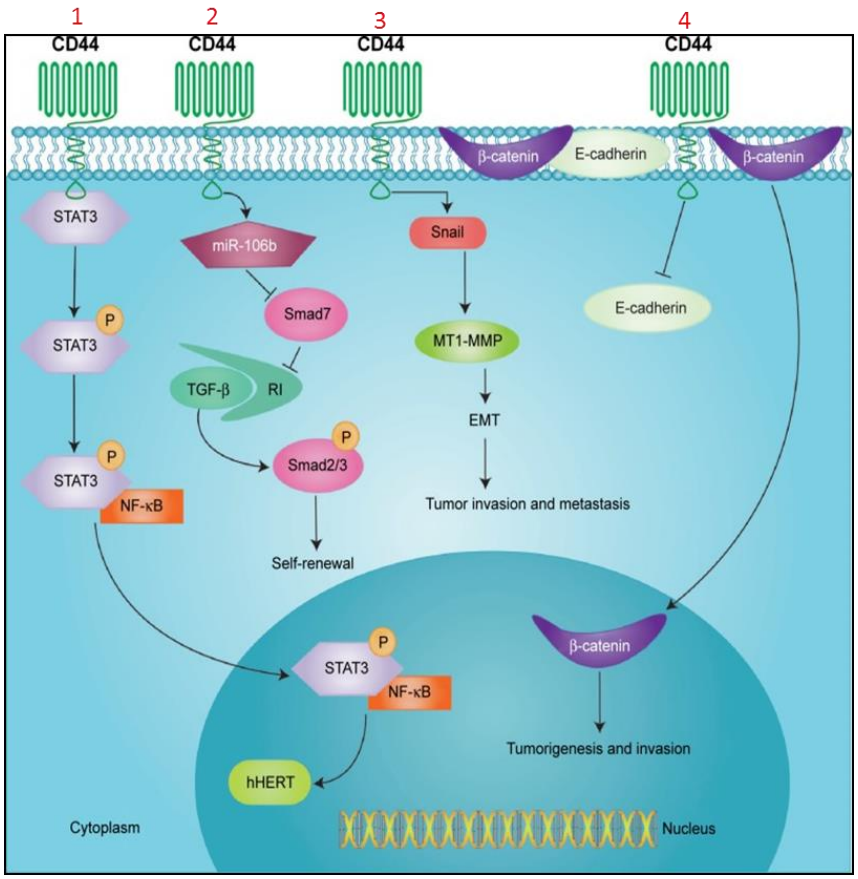


Figure 1.18 Illustration of signaling pathways stimulated by CD44. [Adapted from Xu et al, 2015 (269)].

1.6 Cancer Stem Cells (CSCs)

Cellular heterogeneity and plasticity within tumors has been one of the greatest challenges in cancer therapeutics. Many solid tumors contain a small population of highly tumorigenic CSCs, which contribute significantly to tumor initiation and metastasis. Generally, cancer initiation and development is proposed to follow two models; through accumulation of mutations which promote the loss or gain of specific proteins, where each cancer cell has similar potential to grow a tumor, and/or the existence of cancer stem cells (CSCs), a small population of stem-like cells that are responsible for the generation of highly proliferative, therapy resistant progeny cells as well as the development of the disease (270). Notably, accurate distinction between CSCs and normal stem cells is needed as they share numerous properties apart from their ability to renew themselves, such as both are demonstrated to express surface markers including CD44 or the enzyme aldehyde dehydrogenase (ALDH), and the activation of EMT- associated signaling pathways such as Wnt and Notch (271). In addition to these markers, studies in tumor-initiating population from hepatocellular carcinoma show another cell surface marker, epithelial cell adhesion molecule (EpCAM), which is expressed in normal epithelial progenitor cells (272).

Furthermore, several studies in various tumor types such as osteosarcoma, glioblastoma, breast cancer, lung cancer, ovarian cancer and colon cancer demonstrate that glucose uptake, glycolytic enzyme expression, lactate production and ATP content in CSCs are markedly increased as compared to other differentiated cancer cells in vitro and in vivo (270). However, growing evidence reveals that CSCs are less glycolytic and have preference for mitochondrial oxidative metabolism, in which CSCs show an increased oxygen consumption rates and higher mitochondrial ROS than differentiated cancer cells (273,274). These discrepancies in CSCs metabolism are suggested to be possibly due to metabolic adaptability of CSCs which might allow CSCs to survive in unfavorable circumstances during tumor progression, such as at metastatic sites (275,276). For example, in-vitro CSCs studies that have non-physiological high glucose

and oxygen concentration may favor a glycolytic phenotype of CSCs as opposed to oxidative phosphorylation (OXPHOS) phenotype (276).

Heterogeneity within tumors such as breast cancer has been well-demonstrated, in which it comprises of cells that can be subdivided into clinical subtypes based on cellular marker expression, and with molecular alterations across the subtypes of breast cancer, such as mutational activation of PI3K signaling in luminal breast cancers, *TP53* and *BRCA1* mutations in triple negative breast cancer (TN) and *PTEN* deletions in HER2-positive breast cancers, that have been shown to increase CSCs frequency in patient samples and pre-clinical models (277). Recently, breast cancer stem cells have been shown to maintain their plasticity while transitioning between two different phenotype states; a more proliferative epithelial-like state identified by the expression of CSC marker ALDH, and a more invasive, mesenchymal-like state characterized by the expression of $CD44^{+}/CD24^{-}$. These transitions are mediated by epigenetic alterations regulated by cytokine and chemokine signaling and/or transcriptional regulation, a concept that supports the link between CSCs and the process of EMT as proposed in previous studies (211,278,279). Differences in the CSC types have also been reported across these breast cancer subtypes. Notably, TN breast cancer contains high proportion of $CD44^{+}/CD24^{-}$ and ALDH1- expressing CSCs, HER2-positive breast cancer contains high proportion of ALDH1-expressing CSCs, and hormone-positive (HR+) breast cancer is characterized by a low proportion of cells expressing CSC markers (277,280,281).

1.6.1 Relationship between CSC, EMT and gene splicing in cancer

The EMT process which is often found to promote tumor metastasis and cancer cells dissemination has also been proposed to impart a self-renewal capability to these disseminating cancer cells. This is supported by studies in which cells that have undergone EMT show similar behavior to stem cells isolated from normal or neoplastic cell population (211). Furthermore, studies in head and neck squamous cell carcinoma (HNSCC) show that Snail- induced EMT promotes the maintenance of CSC-like phenotype of the cells as well as enhances their sphere-forming capability, chemo-resistance and invasive ability (282). The molecular links between EMTs and the acquisition of stem-cell traits have also been elucidate, which among them is through the regulation of microRNAs that function as stemness suppressors such as miR-200 family, whose expression are negatively regulated ZEB1 transcription factor (283). In addition, the role of TGF-β through Type 1 TGF-β receptor (TβR1) in modulating EMT and 'stemness' of cells has been revealed, in which this cytokine activates effector Smads 2/3/Smad4 complexes and associate with transcription factors such as ZEB1 and Zeb2/SIP1, thus resulting in the suppression of E-cadherin expression during EMT (284). Of note, SIP1 is important in the activin-mediated maintenance of human embryonic stem cells, which further suggests the connection between TGF-β signaling, EMT and stemness of cells (285).

Furthermore, the process of EMT and the reverse process, mesenchymal-epithelial transition (MET) have been implicated in the plasticity between CSCs types and their connection with the molecular subtypes of breast cancer with previous studies have revealed that overexpression of EMT-associated transcription factors such as Snail and Twist can lead to increased CSCs population in tumors, whereas MET ensures that metastatic cells can return to their highly proliferative state and causes tumor relapse at new sites (286). Experimental evidence have also suggested that differentiated populations of normal mammary epithelial cells can be transformed to mammary epithelial stem-like cells through the induction of EMT (211). In line with this, it has been

shown that cells derived from human mammary epithelial cells can acquire both stem and tumorigenic characteristics of CSCs through EMT-induced Ras-MAPK pathway activation (287). Consistently, gene expression profiling in MCF7 cell line demonstrated high expression of EMT-associated genes such as Vimentin, ZEB1, ZEB2, β-catenin and MMP1 in the cells enriched with $CD44^+/CD24^-$ marker (279). In addition, the significant role of specific genes and proteins in the maintenance of breast cancer cells differentiation and proliferation was further evaluated. Studies have found that the loss of WISP2 expression in breast cancer cells in vitro has caused the induction of EMT and increased the stemness of the cells (288). In addition, EMT gene Wnt1 was found to be differentially expressed in cancer cells grown in sphere culture where those cells with suppressed Wnt1 expression showed lack of stem cell- like properties such as reduced sphere forming ability and decreased ALDH activity (289).

The role of alternative splicing has also been observed in the generation of CSCs, as the analysis in $CD44^+/CD24^-$ population of breast cancer cells show that the fate of breast CSCs is determined by regulated splicing of α6 Integrin. It was observed that these CSCs contain distinct epithelial and mesenchymal population and with the expression of α6A and α6B integrin subunits was especially detected on epithelial and mesenchymal population, respectively (290). In fact, Olsson et al. has demonstrated that tumors with high expression of ALDH1 (CSC marker) also has marked level of CD44 splice variant, CD44s expression, which is also a widely established marker for EMT (164). Furthermore, analysis of $CD44^+$ population in HNSCC cells shows that cells with a high level of CD44 splice isoform, CD44v and low level of EGFR expression display both EMT and CSC- like phenotypes and exhibit resistance to radiotherapy treatment (282).

Aims & Objectives

In mammary cancer, growing evidence show that estrogen signaling plays critical roles in the regulation of target genes that may affect various cellular processes that lead to tumorigeneses. Notably, alterations in signaling pathways though mutations or post-translational modifications such as alternative pre-mRNA splicing are more frequently found in hormone responsive breast cancer. More importantly, alternative splicing (AS) was suggested to be responsible for the widespread changes in the mRNA isoform during the epithelial-to-mesenchymal transition (EMT) event, a process that marks the early event of cancer metastasis. Studies in ER+ MCF7 cells have revealed the involvement of estrogen receptors (ERs) in many oncogenic signaling pathways, whilst corticotropin-releasing hormone, (CRH) a hypothalamic hormone involved in adaption to stress has also been demonstrated to play roles in the development of various human cancers (291). However, the molecular mechanism through which both hormones can potentially promote oncogenesis needs to be further elucidated. Therefore, using MCF7 and SKBR breast cancer cell lines as ER+ and ER- cellular model respectively, this study explored the potential role of estrogen and corticotropin-releasing hormone (CRH) in influencing cancer progression through the regulation of alternative pre-mRNA splicing mechanism. The link between alternative splicing and EMT was assessed by studying the effects of altered splicing factors activity on the transcription of various EMT-associated genes. It was hypothesized that estrogen (E2) as well as CRH can cause changes in the global protein expression in hormone-responsive breast cancer cells leading to upregulation of oncogenic proteins and activation of signaling networks that can potentially trigger biological processes associated with cancer metastasis. In detail, the aims of this project were:

1. Proteomic analysis of MCF7 breast cancer cell line (ER+ cells) exposed to estrogen (17β-estradiol).

2. Isolation of MCF7 breast cancer stem cell subpopulation.

3. Identification of potential role of Estrogen and CRH in promoting cancer through the regulation of alternative splicing (AS) mechanism.

4. To study the relationship between increased production of CD44 splice isoforms with epithelial-to-mesenchymal transition (EMT) event.

5. Profiling of CRH-induced transcriptional activation of EMT- associated gene expression in MCF7 cells and their effects on cellular behaviours.

CHAPTER 2

Material and Methods

2.1 Cell culture

Human breast cancer cell lines (MCF-7 and SKBR3) were purchased from American Type Culture collection (ATCC, USA). MCF-7 cells were maintained in Dulbecco's Modified Eagle Media, DMEM (Lonza) supplemented with 10% FBS (v/v) and 1% Penicillin Streptomycin (v/v).

SKBR3 cells were cultured in RPMI 1640 media (Sigma Aldrich, UK) supplemented with 10% (v/v) fetal bovine serum (FBS),1% sodium bicarbonate (v/v) and 1% Penicillin Streptomycin (v/v) and incubated at 37^{o}C with 5% CO_2.

2.1.1 MCF-7 cell culture

MCF-7 cells vial was removed from liquid nitrogen and warmed in 37^{o}C water bath for 1-2 min. The thawed cells were then transferred into 15mL tube, diluted with fresh complete DMEM and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and cells were resuspended in DMEM and transferred into tissue culture flask. The flask was kept in 37^{o}C, 5% CO_2 incubator for 3-4 days before cell passage.

For MCF-7 cell passage, medium from cell culture flask was aspirated and the cells were washed once with PBS followed by 1-2 min incubation with Trypsin-EDTA (Sigma Aldrich, UK) in the incubator. Warmed complete DMEM was added into the flask, and the cell suspension was mixed several times. Then, 1/5 of the cell suspension was transferred to a new sterile cell culture flask incubated for 3-4 days. The media was changed every 4 days and experiments were performed on cells that were 70-80% confluent and with cells less than 30 passages.

For cell freezing, confluent cells were trypsinized as described above and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and cells were resuspended with 5ml freezing medium (4.5ml complete DMEM + 0.5ml DMSO). Cell suspension was then aliquoted into 1ml cryovial and placed in a cryofreezing container in -80^{o}C freezer overnight, before transferring them into liquid nitrogen.

2.1.2 SKBR-3 cell culture

Vial containing SKBR-3 cells (ATCC ^{R} HTB-30^{TM}) was thawed in a 37^{o}C water bath for 1-2 min. The thawed cells were then transferred into 15mL tube, diluted with fresh complete RPMI 1640 media and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and cells were resuspended in RPMI 1640 media and transferred into tissue culture flask. The flask was kept in 37^{o}C, 5% CO_2 incubator for 6-7 days and the media was changed every 3 days before cell passage.

For SKBR-3 cell passage, the old medium from cell culture flask was aspirated and the cells washed once with PBS followed by 1-2 min incubation with Trypsin-EDTA (Sigma Aldrich, UK) in the incubator. Warmed complete RPMI 1640 media was added into the flask, and the cell suspension was mixed several times. Then, 1/3 of the cell suspension was transferred to a new sterile cell culture flask incubated for 3-4 days. The media was changed every 3 days and experiments were performed on cells that were 70-80% confluent and with cells less than 30 passages.

For cell freezing, confluent cells were trypsinized as described above and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and cells were resuspended with 5ml freezing medium (4.5ml complete RPMI 1640 + 0.5ml DMSO). Cells suspension was then aliquoted into 1ml cryovial and placed in a cryofreezing container in -80^{o}C freezer overnight, before transferring them into liquid nitrogen.

2.2 Breast Cancer Stem Cells (CSCs), $CD44^+CD24^-$ isolation

2.2.1 Removal of $CD24^+$ cells

MCF7 cells were grown in T25 flask and trypsinized once the cells reached 80% confluent. Cells were resuspended in cold 1x MagCellect Plus buffer provided in the MagCellect Human $CD24^-CD44^+$Breast Cancer Stem Cells Isolation Kit (R&D Biosystems). Then, 0.5ml of these cells containing 5x 10^6 cells were added into the round bottom tube, followed by 25 μl Biotinylated CD24 antibody (biotinylated mouse anti-human CD24 antibody), and incubated at 2-8°C for 15 min. The samples were then washed and resuspended in cold 1x MagCellect Plus Buffer, before incubated with 50 μl of MagCellect Streptavidin Ferrofluid (a solution containing BSA and preservative) at 4°C for 15 min. Following this, 3 ml of the cold buffer was added to the tube, centrifuged at 300 x g for 8 min, resuspended in cold buffer and finally the tubes were placed in the MagCellect magnet for 6 min at room temperature. The $CD24^-$ cells in the supernatant were collected and placed into a new tube.

2.2.2 Isolation of $CD44^+$ cells

The collected $CD24^-$ cells from previous step was centrifuged at 300x g for 8 min, resuspended in the cold MagCellect plus buffer, and incubated with 10 μl of Biotinylated CD44 antibody (biotinylated mouse anti-human CD44 antibody) for 15 min at 4°C. Then, cells were washed with 3 ml cold buffer followed by centrifugation at 300 x g for 8 min and the cell pellet was resuspended again with cold buffer. 50 μl of MagCellect Streptavidin Ferrofluid was added in the tube and incubated at 4°C for 15 min, before placed in the MagCellect magnet for 6 min. The unwanted cells ($CD44^-$) were removed by aspirating the supernatant, and the ones left at the bottom of the tubes are cells with $CD44^+CD24^-$ properties. These cells were then transferred into sterile ultra-low attachment plate for at least 7 days to allow the formation of clusters/mammosphere to

take place. Additionally, in different tubes the collected cells were mixed again with 100 μl cold buffer and incubated with 10 μl of PE- and APC- conjugated Human CD24 (APC-conjugated mouse anti-human CD24 antibody) and CD44 Detection Antibodies (PE-conjugated mouse anti-human CD44 antibody). The tagged cells were then analysed using standard flow cytometry procedures to confirm the presence of the breast cancer stem cells (CSC) marker.

2.3 Cell Treatments

Cells were serum-starved overnight prior to treatments with various agonists/antagonists/inhibitors. There were no vehicle controls used in each experiment, however, untreated cells were used as controls and referred as 'untreated' in all experiments.

17β-Estradiol (Estrogen, Sigma Aldrich UK) Treatment

For all experiments, Estrogen dissolved in 100% Ethanol was used at a final concentration of 10nM. MCF-7 cells were treated with 10nM of estrogen for various time points depending on the type of experiments. This concentration was used following series of preliminary experiments by other members of the group.

CRH Treatment

CRH (Abcam) in distilled sterile water was used at a final concentration of 100nM. MCF-7 and SKBR-3 cells were treated with 100nM CRH for various time points according to experimental protocol and these time points and concentration were used following series of preliminary experiments by other members of the group.

Kinase Inhibitor Treatment

SRPIN340 (SRPK1 inhibitor, Tocris Bioscience UK) dissolved in 100% ethanol was used at a final concentration of $10\mu M$, whereas AKT inhibitor, MK2206 (Seleckchem) dissolved in DMSO was used at a final concentration of 5μM. MCF-7 and SKBR-3 cells were treated for 2hr with 10μM of SRPK1 inhibitor and with 5μM of AKT inhibitor. These concentrations were chosen based on recommendation by supplier and by previous studies done by (292).

2.4 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Real- time PCR is a technique that allows amplification of specific target sequences in a sample, and at the same time permits the analysis of the products while reaction is still in progress. This is achieved by using fluorescent dyes which react with the amplified product and emit fluorescent signals that can be measured by an RT-PCR instrument thus allowing kinetic measurements of product accumulation.

For all my RT-qPCR experiments, sample preparation started from cell growing, isolation of RNA, quantification of RNA content, reverse transcription of RNAs (cDNA synthesis) and finally quantitative PCR reactions were performed using SYBR Green-based fluorescent dye.

2.4.1 RNA Isolation

Total RNA was isolated according to the manufacturer's instruction (Sigma GenElute Mammalian Total RNA Kit, Sigma Aldrich UK). For the RNA extraction from cultured cells, the cells were grown until 60-70% confluent in 6-well plates. In the case of hormones or inhibitor treatment, cells were treated in serum-free media for 24h prior to RNA isolation. The cells were then washed with ice-cold phosphate buffered saline (PBS) and lysed by adding 250μl or 500μl QIAzol lysis buffer containing 1% of 2-mercaptoethanol

(v/v), depending on the density of cells in each well. The cell lysate was then transferred into filtration column to separate the RNA from cell debris, followed by mixing with 70% ethanol for the precipitation stage. This mixture was loaded into binding column and washed several times with Wash Buffer 1 and 2. Finally, 50-80μl of elution buffer was added to elute the isolated RNA to be used in the next step.

2.4.2 RNA Quantification (Nanodrop)

The integrity of the isolated RNA was quantified by measuring the absorbance value at 260nm using NanoDrop 1000 UV-Vis spectrophotometer (Nanodrop Technologies). The ratio obtained from A260/A280 and A260/A230 were used to select samples with best RNA quality.

2.4.3 cDNA Synthesis/Reverse Transcription PCR

Prior to the reverse transcription step, RNA was treated with RNase-free DNase 1 (Invitrogen) to remove any genomic DNA contamination. Following the DNase treatment, cDNA synthesis was performed on 1μg RNA samples using High Capacity RNA-to- cDNA kit (Applied Biosystems). Briefly, template RNA was mixed with reaction mixture containing 20X RT Enzyme Mix (MuLV and RNase inhibitor protein), 2X RT Buffer (includes dNTPs, random octamers and oligo dT-16) and nuclease-free water with the final volume of 20μL in 0.5ml PCR tubes. The reverse transcription was started by incubating the tubes at 37^{o}C for 60 min followed by 95^{o}C for 5 min and finally was hold at 4^{o}C before the cDNA tubes were stored at 2-8^{o}C for short term storage and -15 to -25^{o}C for long term storage.

2.4.4 SYBR® Green-based quantitative RT-qPCR

RNA expression of target genes was quantitatively examined using SYBR® Green-based method. 1μl/well of cDNA was used as a template and 19μl of PCR mixture reactions was added to each well of ABI 96-well plate. The mixture components contained 2x SYBR Green PCR Master Mix SensiFast Lo-Rox (Bioline) (10μl/well), Reverse Primer (1μl/well, 10μM), Forward Primer (1μl/well, 10μM) and 7μl nuclease-free water (Invitrogen). The plate was then centrifuged at 300xg for 3 min before loaded into ABI 7500 fast real-time PCR system. The program settings used to perform the PCR reaction was as followed:

Pre-PCR stage: 60°C, 1 min.
Holding stage: 95°C, 20s
Cycling stage: 95°c, 3 s (denature) & 60°C, 30 s (extend)
Melt curve stage: 95°C, 15 s & 60°C 1 min
Post-PCR stage: 60°C, 1 min

Each of the gene expression level was determined using comparative C_T method and normalized to the expression level of GAPDH which served as endogenous control.

2.5 Human Epithelial to Mesenchymal Transition (EMT) RT^2 Profiler PCR Array

The human EMT RT^2 profiler array (Qiagen) allows the analysis of expression profiles of 84 key genes that change their expression during EMT process. As EMT and the reciprocal mesenchymal to epithelial transition (MET) are key processes implicated in both tumour metastasis and stem cell development, this array includes cell surface receptor, extracellular matrix, and cytoskeletal genes mediating cell adhesion, migration, motility and morphogenesis. Genes controlling cell differentiation, development, growth, proliferation and signal transduction and transcription factor genes that involved in EMT are also included.

Similar to quantitative RT-PCR, sample preparation for this experiment involves cDNA synthesis using the provided kit, RT^2 First Strand cDNA Synthesis followed by the quantitative gene expression analysis using SYBR Green-based dye, performed on Applied Biosystem 7000 Real-Time PCR System. The relative expression of each gene was determined using the ΔΔCT method.

2.5.1 RT^2 First Strand cDNA Synthesis

Genomic DNA Elimination

The same amount of RNA isolated from method described in 2.4 were used to prepare the cDNA for EMT array experiment. Briefly, the reagents of the RT^2 First Strand kit (Qiagen) were thawed and genomic DNA elimination mix for each RNA sample was prepared as described in Table 2.1.

Table 2.1 Genomic DNA elimination mix.

Component	Amount
RNA	25ng-5μg
Buffer GE	2μl
RNase-free water	Variable
Total volume	**10μl**

The mixture was then incubated for 5 min at 42°C then immediately kept on ice for 1 min before proceeded to the next step.

Reverse Transcription

Reverse transcription mix was prepared as in Table 2.2. 10μl of reverse- transcription mix was added into the tube containing 10μl of genomic DNA elimination mixture from the previous step. The mixture was mixed well by pipetting up and down followed by incubation at 42°C for exactly 15 min before the reaction was immediately stopped by heating at 95°C for 5 min. In the final step, 91 μl of RNase-free water was added to the tube, mixed well and placed on ice before proceeding with the real-time PCR protocol.

Table 2.2. Reverse-transcription mix

Component	Volume for 1 reaction
5x Buffer BC3	4 μl
Control P2	1 μl
RE3 Reverse Transcriptase Mix	2 μl
RNase-free water	3 μl
Total Volume	**10 μl**

2.5.2 RT^2 Profiler Real-Time PCR Array

The EMT RT^2 Profiler PCR Array plate format C (PAHS-090) was used for this experiment.

The plate and gene layout are as shown in Figure 2.1 and Table 2.3, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	RF	RF	RF	RF	RF	GDC	RTC	RTC	RTC	PPC	PPC	PPC

Reference genes
Genomic DNA control
Reverse transcription control
Positive PCR control

Figure 2.1 RT^2 EMT Profiler Array plate layout.

Table 2.3. Each well in this plate measures the expression of a transcript related to a pathway in epithelial to mesenchymal transition.

AHNAK A01	AKT1 A02	BMP1 A03	BMP7 A04	CALD1 A05	CAMK2N1 A06	CAV2 A07	CDH1 A08	CDH2 A09	COL1A2 A10	COL3A1 A11	COL5A2 A12
CTNNB1 B01	DSC2 B02	DSP B03	EGFR B04	ERBB3 B05	ESR1 B06	F11R B07	FGFBP1 B08	FN1 B09	FOXC2 B10	FZD7 B11	GNG11 B12
GSC C01	GSK3B C02	IGFBP4 C03	IL1RN C04	ILK C05	ITGA5 C06	ITGAV C07	ITGB1 C08	JAG1 C09	KRT14 C10	KRT19 C11	KRT7 C12
MAP1B D01	MITF D02	MMP2 D03	MMP3 D04	MMP9 D05	MSN D06	MST1R D07	NODAL D08	NOTCH1 D09	NUDT13 D10	OCLN D11	PDGFRB D12
PLEK2 E01	DESI1 E02	PTK2 E03	PTP4A1 E04	RAC1 E05	RGS2 E06	SERPINE1 E07	GEMIN2 E08	SMAD2 E09	SNAI1 E10	SNAI2 E11	SNAI3 E12
SOX10 F01	SPARC F02	SPP1 F03	STAT3 F04	STEAP1 F05	TCF3 F06	TCF4 F07	TFPI2 F08	TGFB1 F09	TGFB2 F10	TGFB3 F11	TIMP1 F12
TMEFF1 G01	TMEM132A G02	TSPAN13 G03	TWIST1 G04	VCAN G05	VIM G06	VPS13A G07	WNT11 G08	WNT5A G09	WNT5B G10	ZEB1 G11	ZEB2 G12
B2M H01	HPRT1 H02	RPL13A H03	GAPDH H04	ACTB H05	HGDC H06	RTC H07	RTC H08	RTC H09	PPC H10	PPC H11	PPC H12

The PCR component mix (Table 2.4) was prepared in a 5ml tube and subsequently dispensed into the RT^2 Profiler PCR Array plate.

Table 2.4. PCR component mix.

Component	96-well Array Format C
2x RT^2 SRBYR Green Master mix	1350 μl
cDNA synthesis reaction	102 μl
RNase-free water	1248 μl
Total volume	2700 μl

The plate was then centrifuged to precipitate the mixture and remove bubbles at 1000 g at room temperature for 1 min. the plate was then placed in real-time cycler (ABI 7000) which was programmed as below:

Table 2.5. Cycling conditions for ABI cycler.

Cycles	Duration	Temperature
1	10 min	95°C
40	15s	95°C
	1 min	60°C

2.6 Immunofluorescence

Preparation of coverslips

Circular No.1 microscope coverslips (VWR UK) were sterilized by immersing in absolute ethanol for 30 min and allowed to air dry in the cell culture hood before being placed in a sterile 8-well plate, one in each well. Then, cells were seeded on the glass coverslips at a density $1x10^5$ cells/well. 2ml of growth media containing fetal bovine serum (FBS) and Penicillin/Streptomycin was added into each well and the plate was kept in the incubator at 37^{o}C until it reached 80% confluence. For treatments, cells were treated at 60-70% confluent and was left growing up to 80% confluent before proceeding to subsequent steps.

Fixation

Coverslips were removed, transferred to a new sterile well and washed three times with ice-cold Phosphate Buffer Saline (PBS). Then, cells were fixed with 4% paraformaldehyde (v/v) (VWR Chemical) (in PBS) for 10 min at room temperature followed by washing three times with ice-cold PBS. Cells were incubated in Wheat Germ Agglutinin (Alexa Fluor 488) for 10 min at room temperature for plasma membrane staining (optional).

Permeabilization

Cells were incubated in ice-cold PBS containing 0.2% Triton X-100 (v/v) (Fisher Scientific) and washed in PBS three times.

Blocking & Immunostaining

To minimize non- specific binding signal, cells were incubated with 5% BSA (w/v) (VWR UK) in PBS for 1 hour at room temperature prior to incubation with primary antibody diluted in 1% BSA (w/v), for 1 hour (room temperature) or overnight (4^{o}C). Unattached

antibodies were removed three times with PBS followed by incubation with secondary antibody (in 1% BSA, 1:400) for 1 hour in the dark at room temperature.

Counter staining

Nuclear staining was done by adding 1μg/ml DAPI for 5 min and fixed again with 4% paraformaldehyde (w/v) for 10 min. Fixation solution was removed by rinsing with PBS three times and air dried.

Mounting

A drop of mounting medium, DPX Mounting Medium (Fisher Scientific) was added directly onto the cells and coverslips were pressed gently onto glass slides and stored in the dark (wrapped with aluminium foil) at 2-8^{o}C overnight before viewing them under the fluorescence microscope (ZEISS LSM 750)

2.7 Western Blot

2.7.1 Sample Preparation

Whole Cell Protein extraction

Cell culture plate was placed on ice and cells were washed with ice-cold PBS. Then cells were lysed directly from the plate by adding 100μl ice-cold RIPA lysis buffer (Insight Biotechnology Ltd) per 10^7 cells containing protease inhibitor and phosphatase inhibitor following 1:100 dilution. Adherent cells were scraped with plastic scraper and the cell lysate were transferred into a pre-cooled microcentrifuge tube followed by centrifugation at 12,000 x g for 15 min at 4^{o}C. The supernatant was aspirated and placed in a fresh tube for protein quantification.

Cytoplasmic & Nuclear Protein Extraction

Nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) according to manufacturer's instruction. Briefly, Cells were washed with ice-cold PBS and harvested with Trypsin-EDTA followed by centrifugation at 500x g for 5 min. Supernatant was removed and ice-cold CER 1 (with added protease & phosphatase inhibitor, Thermo Scientific) to the cell pellet according to table below:

Table 2.6. Reagent volumes for different packed cell volumes. For HeLa cells, $2x10^6$ cells is equivalent to 20 μL packed cell volume.

Packed cell volume (μL)	CER I (μL)	CER II (μL)	NER (μL)
10	100	5.5	50
20	200	11	100
50	500	27.5	250
100	1000	55	500

The tubes were then vortex at maximum speed for 5s followed by incubation on ice for 10 min. Ice- cold CER II was then added to the tube, vortexed on the highest setting for 5s and incubated on ice for 1 min. Next, the tube was vortexed again for 5s on highest setting and centrifuged for 5 min in a microfuge tube at 16,000x g (11570 rpm). Immediately the supernatant (cytoplasmic extract) was transferred to a clean pre-chilled tube and stored in -80^{o}C until use.

For nuclear protein extraction, pellets produced from previous step was resuspended with ice-cold NER (containing protease & phosphatase inhibitor), vortexed for 15s and placed on ice for 10 min. This step was repeated for every 10 min, for a total of 40 min. Finally, the tube was centrifuged at maximum speed (16,000xg) for 10min and the supernatant was transferred to a clean pre-chilled tube. All samples were kept on ice for protein quantification step or stored in -80^{o}C until use.

2.7.2 Protein Quantification

The protein content from each sample was measured in duplicates using BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions. Briefly, 10 µl of BSA standard and protein samples was added into each well in the 96-well plate followed by the addition of 200 µl of working reagent. The plate was then incubated in 37°C incubator for 30 min before protein samples were measured using a plate reader (GENioS, Tecan) at the absorbance of 570nm. The content of protein in each sample was calculated using linear equation from BSA standard curve. A representative of BSA standard curve is shown in Figure 2.2.

Once the amount of protein to be loaded was determined, samples were mixed with equal volume of 2x Laemmli buffer (Sigma Aldrich UK) and denatured at 95°C for 7 min before processed for gel electrophoresis or stored at -20°C for future use.

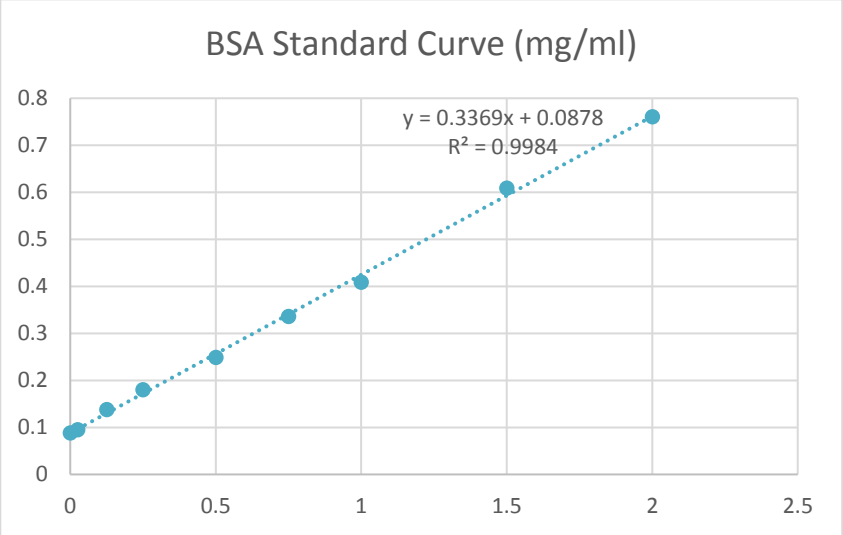


Figure 2.2 Serial dilution of BSA standard was prepared according to manufacturer's protocol and concentration ranged between 0-2mg/ml.

2.7.3 SDS-PAGE Gel Electrophoresis

Gel Preparation

10% resolving and 10% stacking gel with 0.75mm thickness for electrophoresis was casted according to Table 2.7.

Table 2.7. Reagents and chemical for resolving and stacking gels.

10% Resolving Gel (v/v)	
Distilled H_2O	4.48 ml
1.5M Tris pH 8.8	3.0 ml
ProtoGel 30% (v/v), GENEFLOW Ltd UK.	4.0 ml
10% SDS (w/v)	120 μl
Ammonium Persulphate (APS), Sigma Aldrich UK	40 μl
TEMED, Sigma Aldrich UK	10 μl
10% Stacking Gel (v/v)	
Distilled H_2O	5.8 ml
0.5M Tris pH 6.8	2.5 ml
ProtoGel 30%, (v/v)	1.5 ml
10% SDS, (w/v)	100μl
Ammonium Persulphate (APS)	100μl
TEMED	10μl

Electrophoresis

Equal amounts of protein samples in Laemmli buffer (10-20μg) were loaded into each well alongside with the PageRuler Pre-stained protein ladder (Thermo Scientific). The gel was run in 1x SDS Running Buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS (w/v), for 30 min at 20 Amp (A) and the power was increased to 40 Amp for another 30 min.

2.7.4 Protein Transfer/Blotting

Proteins from the gel were transferred to nitrocellulose membranes (Amersham UK) for one hour at 100 V. The transfer buffer was prepared by mixing ice-cold 10% of 10x Tris-glycine, (v/v) (Fisher Scientific), 10% of methanol, (v/v) and 80% of filtered water.

2.7.5 Blocking and Antibody Staining

Nitrocellulose membrane was incubated with Odyssey Blocking Buffer (LICOR Biosciences) at room temperature with consistent shaking for one hour. The membrane was then incubated with primary antibody diluted at 1:1000 in the 5% BSA (w/v) (VWR Chemical) overnight at 4^{o}C, followed by three washes with TBST, 20 min each. Next, the membranes were incubated with secondary antibody (700CW conjugated goat anti-mouse or 800CW conjugated goat anti-rabbit, LICOR Biosciences), at the dilution of 1:5000 at room temperature for one hour with consistent shaking before washing with two times TBST and final wash with TBS for 15 min.

2.7.6 Visualization of Proteins

Protein bands were visualized with Odyssey Infrared Imaging System and bands were quantified based on OD value with the Odyssey software. For the detection of endogenous control protein, membranes were stripped using the LICOR Stripping Buffer (LICOR Biosciences) for 20 min, washed with TBST, followed by incubation with Odyssey

Blocking Buffer for 1 hr before proceeding to the primary and secondary antibodies incubation steps.

2.8 Co-Immunoprecipitation (Co-IP)

Experiments were performed following manufacturer's instructions. Briefly, 200μg of protein sample was mixed with 1-10 μl of primary antibody and incubated on rotating shaker at 4°C overnight. Then, the samples were incubated on rotating shaker at 4°C overnight in PBS containing 20 μl of Protein A/G Plus Beads (Santa Cruz), a genetically-engineered protein that combines binding profiles of Protein A and Protein G, that provides binding for all species of antibody and IgG subclasses recognized by either Protein A or Protein G. Immunoprecipitants were collected by centrifugation at 4000 x g for 5min at 4°C and washed three times by resuspension and centrifugation (5min at 4000 x g) in PBS. The samples were eluted into 40 μl of Laemmli buffer, boiled for 2-3 min at 95°C, and analysed by SDS-PAGE/Western Blot (Method 2.7). Unused samples were stored at -20°C until use. A schematic diagram of the protocol is as depicted in Figure 2.3.

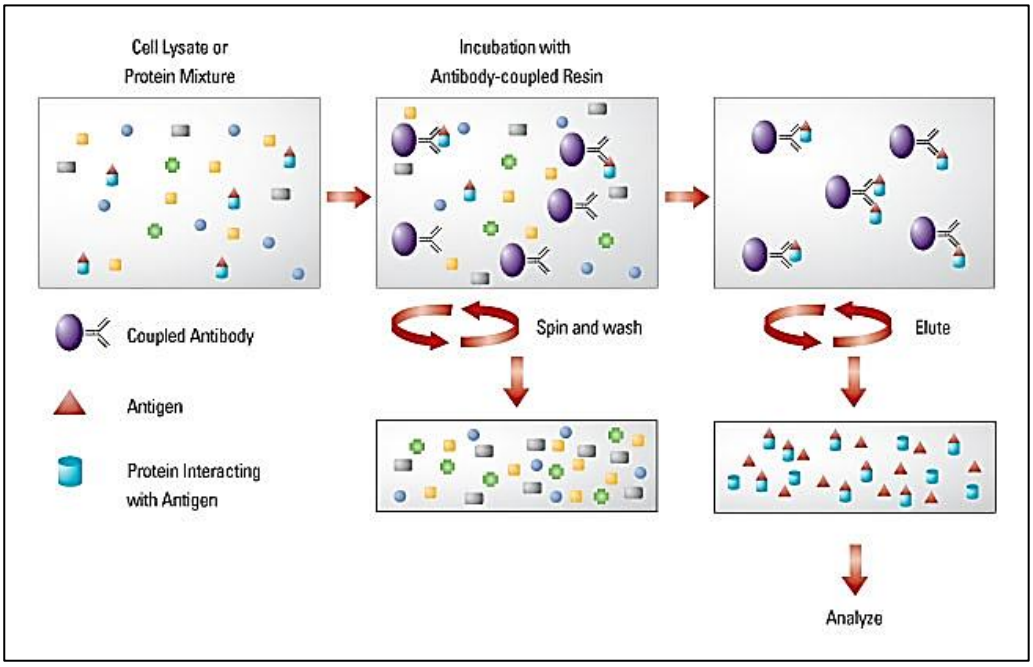


Figure 2.3. Schematic diagram of standard co-immunoprecipitation procedures (www.thermofisher.com).

2.9 Filter-Aided Sample Preparation (FASP)/ Proteomics Analysis

Filter-aided sample preparation (FASP) method is a commonly used for proteomics analysis as it allows processing of essentially any class of protein, irrespective of their subcellular location. In general, proteins are extracted using sodium dodecyl sulfate-DTT buffer (SDS-DTT), followed by detergent removal with 8M urea on standard filtration device. As the 10,000k filter has been shown to efficiently retain small proteins (5-10kDa) and efficiently elute peptides up to 5,000 Da, this filter was used for the entire experiments. Pure peptides are eluted after trypsin digestion, making them suitable to be used for single-run proteome analysis.

Sample preparation for mass spectrometry

2.9.1 Protein extraction

Attached cells were washed with ice-cold PBS and lysed directly from the plate by adding 100µl of 0.1-1 % SDS-DTT (SDT)-lysis buffer (w/v), followed by incubation for 5 min at 95^{o}C. The proteins were solubilized by a quick sonication to reduce the viscosity of the lysates before centrifugation at 13,000 rpm for 15 min.

2.9.2 Detergent removal and trypsin digestion

Aliquot of lysates corresponding to 100µg of proteins was mixed with 400 µl of 8M urea in 0.1M Tris/HCI pH 8.8 (Sigma, U5128) in an Eppendorf tube, vortexed and transferred to filter units of the 10k NMWCO spin columns. The filter unit was then centrifuged at 14,000 x g for up to 30 min at room temperature and the collected flow-through was discarded. This whole step was repeated twice. Then, 400 µl of 0.05 M iodoacetamide in 8M urea in 0.1 M Tris/HCI pH 8.8 were added to the concentrate followed by centrifugation at 600 rpm for 1 min and incubation for 5 min (without mixing) possibly in the dark, and finally centrifuged at 14,000 x g for 30 min.

Next, 400 μl of 8M urea in 0.1 M Tris/HCl pH 8.0 (Sigma, U5128) was added to the filter unit and centrifuged at 14,000 x g for 20 min. This step was repeated twice with 400 μl of 100mM ammonium bicarbonate (NH_4HCO_3). Following this, 120 μl of trypsin solubilized in NH_4HCO_3 was added, mixed at 600 rpm for 1 min and incubated in water bath overnight at 37°C. The next day, the filter units were transferred to fresh collection tubes and centrifuged at 14,000 x g for 40 min before adding 50 μl of 0.5M NaCl for further centrifugation at 14,000 x g for 20 min. Finally, the sample was acidified and purified with 1 μl of 5% Trifluoroacetic acid, TFA (v/v) to promote peptide binding to the resin of a C18 column.

2.9.3 Desalination of peptides samples

The ZipTip was rinsed ten times with 10 μl 100% Acetonitrile, ACN (v/v) and acidified peptides were loaded onto the tip by pipetting through 10 μl of the sample for at least ten times. For salt removal, the tip was immersed in 10 μl 0.1% TFA (v/v) by pipetting for at least ten times followed by rinsing with 10 μl of 50% ACN/0.1% TFA (v/v) for ten times to elute the peptides. The peptides samples were then centrifuged for 3 min to remove organic solvents and samples were subsequently applied to the high-performance liquid chromatography column and were run for 2hrs for each sample. Raw MS files were processed with a freely available software, MaxQuant and peak list files produced were searched by the MASCOT search engine against human database.

2.10 xCELLigence® Real-Time Cell Analysis (RTCA)

Continuous monitoring of cell viability, which is mediated by internal and external factors, provides better understanding of molecular and biochemical pathways mediating cell viability. RTCA instruments utilize gold microelectrodes embedded in the bottom of microtiter well, to non-invasively monitor cell status in real-time without generating potential artefacts. This label-free impedance measurement given as cell index (CI), allows detection of changes in cell number, adherence, morphology and viability, with enhanced sensitivity without the need for over-expression of reporter and target proteins.

2.10.1 Xcelligence proliferation assay protocol

The experiments were performed following manufacturer's instructions. Briefly, 100μl of media was added into each well of E-Plate 16 (ACEA Biosciences, USA), a specially designed 16-well plate with micro-electrode configuration that covers 80% of each well bottom's surface area. The real-time measurement of impedance across the electrodes provides a sensitive immediate detection of the cellular condition and response from low cell numbers to confluency. Prior to adding the cells, the plates were placed in the RTCA analyser and baseline measurement was taken every 1 min for 5 mins. Then, 100μl of cells (approx. 3000 cells) to each well and the plates were left at room temperature for 30 min, before loading into the analyser for real-time measurement for 24hrs. After 24hrs, 5 μl of stimulating compound/ treatment was added into the respective wells.

2.10.2 Invasion/Migration Assay

Invasion Assay

The experiments were performed according to manufacturer's instructions. For the invasion and migration experiment, CIM-plate was used instead of E-plate 16. CIM-plate (ACEA Biosciences, USA) is a modified- Boyden chamber designed with a removable top and bottom chamber, featuring the same micro-electrode configuration for the bottom chamber of a microporous polyethylene terephthalate (PET) membrane with the median pore size of this membrane is 8μm (Figure 2.4).

Prior to experiment, Matrigel (Corning) was diluted to 1:10 or 1:20 with cold serum free media and 30μl of this was carefully added to the middle of the upper chamber well without introducing any bubbles. Then, the plates were left in the hood for 4hrs to ensure that the Matrigel was completely solidified. Following this, 160ml of pre- warmed media was added to the lower chamber well before upper chamber was locked on top of the lower chamber. Next, 30μl of serum free media was then added to the top chamber wells and the plate was loaded into the analyser for 1 hour for the plate to equilibrate inside the chamber. Afterward, baseline measurement was taken every 1 min for 5 mins, before 100 μl of cells was added to the upper chamber. The plate was then left for 30 mins in the 37^{o}C incubator to allow cells adherence, before measurement was taken at every 15 mins for at least 10 hrs. Only data from a 2-10 hour were analysed for migration and invasion studies the cells will begin to proliferate after 10hours.

Migration Assay

The experiments for migration assay were the same as the invasion assay described above, except that the upper chamber of the plates was not coated with Matrigel prior to experiment.

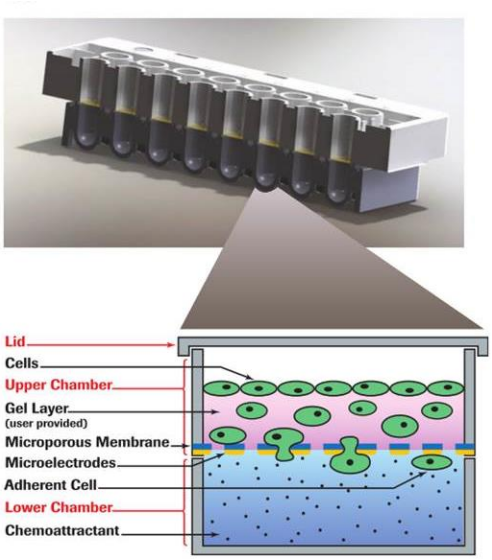


Figure 2.4 The diagram illustrates the view of upper and lower chambers for a single well. The bottom surface of the upper chamber chamber was composed of mircoporous memrane that cells can migrate through. Gold electrodes on the underside of this membrane detect the presence of the adherent cells.

Statistical analysis

Statistical analysis (Student t-test and 1-way ANOVA) for all experiments were performed using GraphPad software. All the results shown were the mean of standard error of means (SEM) of at least three independent experiments, where significance was assumed when p-value < 0.05.

For the qRT-PCR assays, relative target gene expression was normalized against endogenous control, GAPDH using the ΔΔCt method.

As for the RT^2 EMT profiler array, gene expression was analyzed using the web-based Qiagen data integration and visualization tool (https://www.qiagen.com/gb/shop/genes-and-pathways/data-analysis-center).

CHAPTER 3

Analysis of the effects of estrogen (E2) on the proteome profile of MCF7 breast cancer cell line

Introduction

Estrogen (E2) has been shown to induce various physiological effects, in which, their actions are not only required for normal development growth of reproductive tissues in female but are also implicated in tumorigenesis arise from these tissues. Studies show that E2 can exert proliferative effects in breast cancer and act as a survival agent such as by promoting the expression of anti-apoptotic protein Bcl-2 (293). Although E2 has been demonstrated to play critical role in breast cancer progression, primarily in hormone-responsive breast cancer, the molecular basis of this association is not yet fully studied.

Previous studies have used various methods to identify protein targets of E2 actions, for example by using 2-D-PAGE, however, this method provides limited coverage of the protein profile. Therefore, in this experiment, mass spectrometry was used to identify changes in global proteome profile by estrogen in hormone-responsive breast cancer cells MCF7. Through this method, greater protein coverage (generally >2000 proteins) can be obtained and highly quantitative proteome analysis can be done to elucidate sets of proteins regulated by E2 and their association with signaling networks or biological events leading to breast cancer progression and metastasis. Additionally, numerous studies have suggested that the presence of subpopulation of cells known as cancer stem cells (CSCs) within breast cancer cells is one of the factor that contribute to cancer metastasis. Therefore, this chapter was also aimed to investigate the presence of CSCs within MCF7 cells and to elucidate protein markers that connect CSCs with cancer progression.

Results

3.1 Estrogen (17β-estradiol, E2) regulates the expression of a core splicing kinase, serine-arginine protein kinase 1 (SRPK1) protein in MCF7 breast cancer cells

Approximately 50%-80% of all breast tumors have been found to express estrogen receptors (ERs), and studies have reported that breast cancer progression and hormonal therapy resistance in hormone- responsive breast cancer was frequently found to be associated with estrogen signaling in the cells (57). To investigate the effect of steroid hormone, 17β-estradiol (E2) on the global protein expression in ER+ breast cancer, proteome analysis was performed in cellular model of ER+ breast cancer cell, MCF7 cell line. Following the concentration and treatment period used in other studies, cells were treated with 10nM for 24hr, and analyzed using LC-MS/MS in triplicates.

It was demonstrated that of all 3000 proteins identified from the experiment, fold-change analysis measured against control samples (untreated) showed a total of 157 proteins were found to have statistically significant changes ($p<0.1$) in expression, with 93 proteins were upregulated and 64 proteins were downregulated in response to E2 treatment (Figure 3.1A). Comparison in gene ontology analysis (Figure 3.1B) of both the upregulated and downregulated proteins showed that E2 signaling seemed to target proteins predominantly expressed in the cytosol, nucleus, mitochondrion and cytoskeleton of the cell.

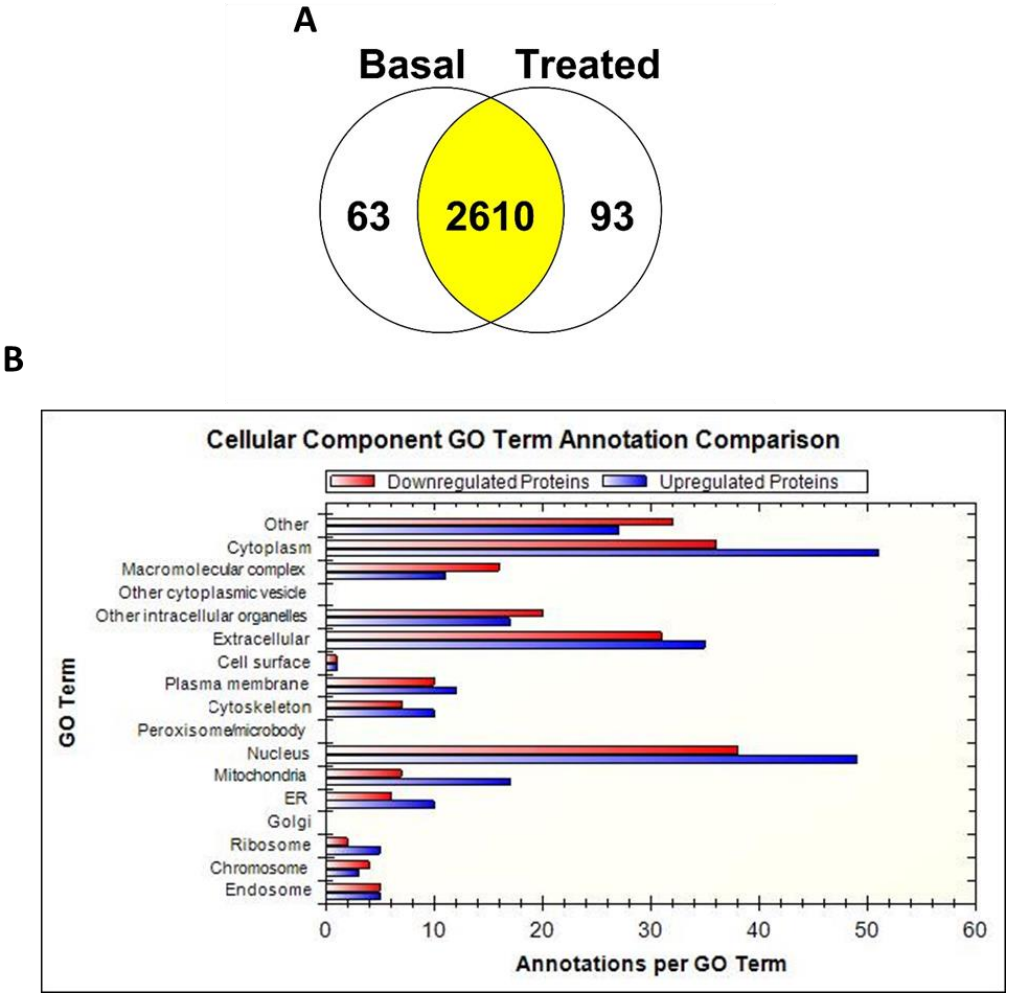


Figure 3.1 **(A)** Venn diagram shows proteins identified from quantitative profile of control vs E2-treated samples. A total of 2610 protein were identified as having identical values (statistically insignificant) in both samples, whereas 63 proteins were found upregulated (downregulated by E2) and 93 proteins were upregulated in the stimulated cells. **(B)** Comparison of Gene Ontology (GO) analysis for cellular component between upregulated and downregulated proteins in response to E2. Changes are considered significant when *p-value* < 0.1 and the fold change is two and above.

At present, there are only a few studies on proteomic analysis of breast cancer, specifically on hormone- responsive MCF7 cells. One of the studies in which MCF7 cells were exposed to mitogenic concentration of estrogen has identified statistically significant changes in the expression of twelve proteins which include those that are already implicated in the progression of breast cancer such as stathmin (STMN1), calreticulin (CRTCC), heat shock 71kDa (HS7C) and alpha- enolase (ENOA) (294). In addition, other studies have also analyzed the pathways that participate in the differential response to estrogen and estrogen- induced proliferation and apoptosis in MCF7 cells and has identified 26 proteins associated with GPCRs, PI3K/AKT, Wnt and Notch signaling pathways such as PRPF6 (proteins involved in pre-mRNA splicing/co-activator of androgen receptor), FAK1, Rap1GAP (GPCR signaling), BCL3 (Wnt signaling), TLE3 (Wnt/ Notch) (295).

Notably, some of the identified proteins above such as STMN1, ENOA, and PRPF6 were also found upregulated in this study (Table 3.1). In addition to these three proteins, my study has unexpectedly identified serine-arginine protein kinase 1 (SRPK1), which is one of the well-established key kinases in regulating alternative splicing mechanism in cells, among the ER-signaling targets and found to be upregulated with as high as 7.7-fold as compared to that in control cells.

Table 3.1 List of 93 upregulated proteins in response to estrogen treatment in MCF7 cells, as compared to control. Some of these are known proteins involved in the regulation of mRNA splicing mechanism and in the activation of oncogenic signaling pathways in cells (red font).

	Identified Proteins (93/2794)	Alternate ID	T-Test (p-value): (p < 0.1)	Fold Change by Category
1	Protein S100-A7	S100A7	0.051	INF
2	DnaJ homolog subfamily C member 21	DNAJC21	0.0018	INF
3	Calcium-binding and coiled-coil domain-containing protein 2	CALCOCO2	0.00064	INF
4	CAP-Gly domain-containing linker protein 1	CLIP1	0.035	87
5	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase MESH1	HDDC3	0.011	14
6	Membrane-spanning 4-domains subfamily A member 14	MS4A14	0.034	8.5
7	Zinc finger protein ZPR1 (Fragment)	ZPR1	0.022	8.3
8	Proteasome assembly chaperone 2	PSMG2	0.006	7.8
9	SRSF protein kinase 1 (Fragment)	SRPK1	0.041	7.7
10	Endophilin-A2	SH3GL1	0.03	7.4
11	Proteasomal ubiquitin receptor ADRM1	ADRM1	0.0084	6.2
12	Acid ceramidase	ASAH1	0.019	6.2
13	H/ACA ribonucleoprotein complex subunit 4	DKC1	0.075	5.8
14	UPF0489 protein C5orf22	C5orf22	0.016	5.7
15	Aflatoxin B1 aldehyde reductase member 2	AKR7A2	0.035	5.6
16	Signal recognition particle 19 kDa protein	SRP19	0.026	5.5
17	Pancreatic progenitor cell differentiation and proliferation factor	PPDPF	0.074	5.5
18	Mitochondrial import inner membrane translocase subunit Tim8 A	TIMM8A	0.035	5.3
19	ATP synthase subunit epsilon-like protein, mitochondrial	ATP5EP2	0.049	5.2
20	Putative E3 ubiquitin-protein ligase UBR7	UBR7	0.011	5
21	Trans-Golgi network integral membrane protein 2	TGOLN2	0.027	4.5
22	Coronin OS=Homo sapiens	CORO7-PAM16	0.062	4.4
23	Zinc finger protein 428 (Fragment)	ZNF428	0.035	4.3
24	Quinone oxidoreductase	CRYZ	0.036	4.2
25	ATP synthase subunit e, mitochondrial	ATP5I	0.014	4
26	Prolyl endopeptidase	PREP	0.084	3.7
27	Profilin	PFN2	0.05	3.7
28	Poly(ADP-ribose) glycohydrolase ARH3	ADPRHL2	0.08	3.6
29	Periodic tryptophan protein 1	PWP1	0.035	3.6
30	Splicing factor 3B subunit 5	SF3B5	0.012	3.5
31	Methionine aminopeptidase 1	METAP1	0.018	3.4
32	UPF0160 protein MYG1, mitochondrial	C12orf10	0.066	3.3
33	UPF0488 protein C8orf33	C8orf33	0.035	3.3
34	39S ribosomal protein L49, mitochondrial	MRPL49	0.04	3.3
35	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A	0.0046	3.2
36	Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL	0.097	3.2
37	Histone-lysine N-methyltransferase SETD7	SETD7	0.043	3
38	Bleomycin hydrolase	BLMH	0.044	2.9
39	Cytochrome c oxidase subunit 6C	COX6C	0.033	2.9
40	Cytochrome c oxidase copper chaperone	COX17	0.031	2.9
41	Prefoldin subunit 1	PFDN1	0.0068	2.8
42	Non-histone chromosomal protein HMG-17	HMGN2	0.074	2.8
43	OTU domain-containing protein 6B	OTUD6B	0.048	2.7
44	N-terminal Xaa-Pro-Lys N-methyltransferase 1	NTMT1	0.013	2.7
45	Histidine triad nucleotide-binding protein 2, mitochondrial	HINT2	0.059	2.7
46	Glutaredoxin-related protein 5, mitochondrial	GLRX5	0.014	2.7
47	Peptidyl-prolyl cis-trans isomerase FKBP10	FKBP10	0.0069	2.6
48	Fumarylacetoacetase	FAH	0.0091	2.6
49	CD2 antigen cytoplasmic tail-binding protein 2	CD2BP2	0.035	2.6
50	Histidine triad nucleotide-binding protein 1	HINT1	0.052	2.6

	Identified Proteins (93/2794)	Alternate ID	T-Test (p-value): (p < 0.1)	Fold Change by Category
51	Eukaryotic translation initiation factor 6	EIF6	0.049	2.6
52	40S ribosomal protein S6	RPS6	0.07	2.6
53	Pseudouridine-5'-phosphatase	PUDP	0.084	2.6
54	Coronin-1A	CORO1A	0.062	2.6
55	Protein canopy homolog 2	CNPY2	0.029	2.6
56	Lysosomal alpha-glucosidase	GAA	0.033	2.6
57	3-hydroxyisobutyrate dehydrogenase, mitochondrial	HIBADH	0.072	2.5
58	Delta-aminolevulinic acid dehydratase	ALAD	0.017	2.5
59	Probable aminopeptidase NPEPL1	NPEPL1	0.097	2.5
60	ATPase ASNA1	ASNA1	0.067	2.5
61	Myristoylated alanine-rich C-kinase substrate	MARCKS	0.019	2.4
62	Calcineurin subunit B type 1	PPP3R1	0.08	2.4
63	Coiled-coil domain-containing protein 58	CCDC58	0.042	2.4
64	Catechol O-methyltransferase	COMT	0.061	2.2
65	U6 snRNA-associated Sm-like protein LSm2	LSM2	0.049	2.2
66	Ribonucleoside-diphosphate reductase large subunit	RRM1	0.094	2.2
67	Nascent polypeptide-associated complex subunit alpha	NACA	0.048	2.1
68	NADH dehydrogenase (Ubiquinone) flavoprotein 1, 51kDa, isoform CRA_c	NDUFV1	0.064	2
69	Transmembrane protein 109	TMEM109	0.012	2
70	Proteasome subunit alpha type-1	PSMA1	0.077	2
71	Phosphatidylethanolamine-binding protein 1	PEBP1	0.049	2
72	3'(2'),5'-bisphosphate nucleotidase 1	BPNT1	0.098	1.9
73	SPRY domain-containing protein 4	SPRYD4	0.0084	1.9
74	Glutathione S-transferase omega-1	GSTO1	0.06	1.9
75	Actin-related protein 2	ACTR2	0.056	1.8
76	RNA-binding protein 8A	RBM8A	0.073	1.6
77	Triokinase/FMN cyclase	TKFC	0.068	1.6
78	Cytosolic non-specific dipeptidase	CNDP2	0.04	1.6
79	UV excision repair protein RAD23 homolog A	RAD23A	0.0058	1.6
80	Ras-related protein Rab-2A	RAB2A	0.032	1.6
81	Thioredoxin domain-containing protein 5	TXNDC5	0.037	1.5
82	40S ribosomal protein S14	RPS14	0.049	1.5
83	Growth factor receptor-bound protein 2	GRB2	0.021	1.5
84	PEST proteolytic signal-containing nuclear protein	PCNP	0.046	1.5
85	Plastin-2	LCP1	0.067	1.5
86	Alpha-enolase	ENO1	0.058	1.5
87	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	0.018	1.4
88	Tumor protein D52	TPD52	0.08	1.3
90	Ubiquitin-conjugating enzyme E2 K	UBE2K	0.04	1.2
91	Cluster of Tubulin beta chain	TUBB	0.018	0.03
92	Charged multivesicular body protein 4a	CHMP4A	0.018	1.9
93	Cluster of Stathmin	STMN1	0.051	0.00000001

Furthermore, validation experiments by western blot and immunostaining experiment confirmed that SRPK1 protein expression was detected to be upregulated following estrogen treatment at 10nM for 24hr, as shown in Figure 3.2 and Figure 3.3, respectively. Previous studies have demonstrated that elevated level SRPK1 protein expression has been observed various cancers such as breast, pancreatic and colorectal (172). In addition, it has also been reported that the production of oncogenic protein isoforms is one of the factors contributing to the tumorigenesis , predominantly in ER+ breast cancer, in which this regulation of mRNA splicing mechanism involves SRPK1 (17,296–298).

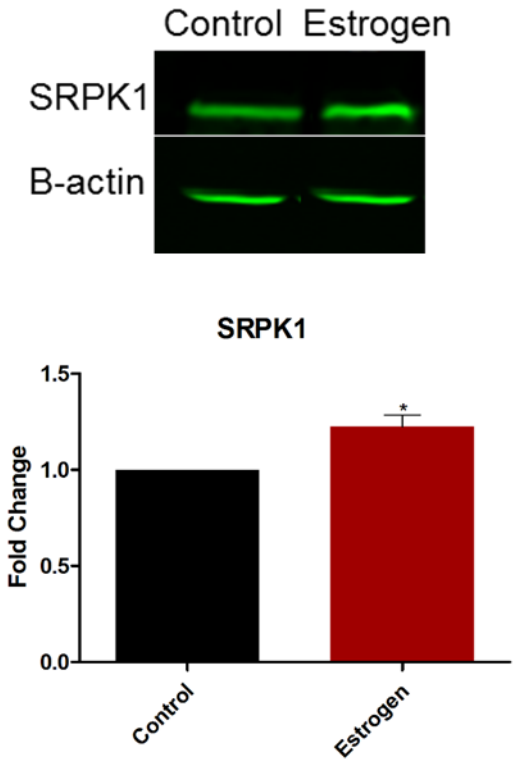
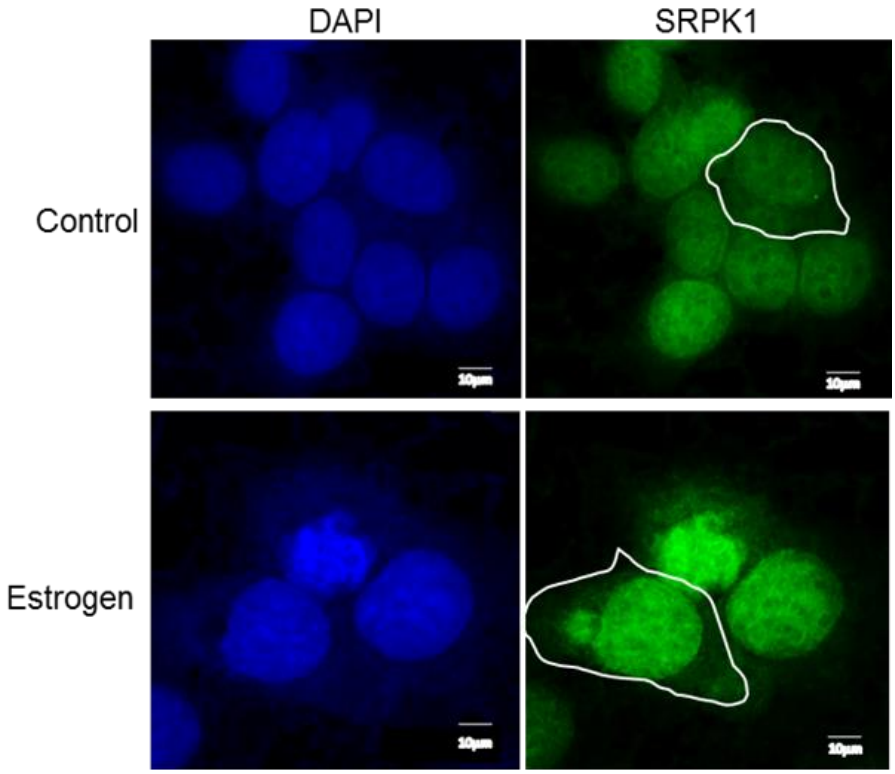


Figure 3.2 Western blot results confirmed the upregulation of SRPK1 protein expression following estrogen treatment. *p <0.05 (n=4).



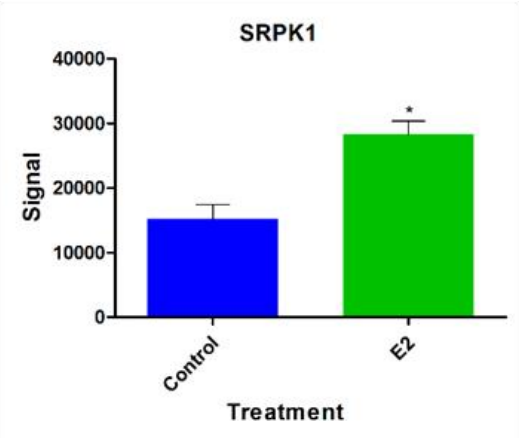


Figure 3.3 MCF7 cells were treated with 10nM of estrogen (17β-estradiol) for 24 hr before detection using anti- SRPK1 antibody. Secondary antibody used was FITC, shown in green. DAPI nuclear staining was shown in blue. Protein signal was measured by drawing shape around the cell as shown above, and intensity was measured using ImageJ. Representative images are shown. n=6, scale bar =10um.

Further analysis using STRING, a biological database and web resource of known and predicted protein-protein interactions, shows the interaction between SRPK1 and SR proteins such as SRSF1, SRSF2, SRSF3, SRSF4, SRSF5, SRSF6, SRSF7 and SRSF9 in human (Figure 3.4A). The role of SRPK1 kinase in the phosphorylation of serine-arginine (SR) splicing proteins has been well documented and studies show that differential level of phosphorylated SR proteins can affect splice site selection of pre-mRNA transcript during alternative splicing of target genes, which can subsequently lead to the production of oncogenic protein isoforms. For example, increased SRPK1 kinase activity in the nucleus has been demonstrated to induce differential phosphorylation of SRp30, SRp40, SRp55 and SRp75 which in turn altered the splice site choice of EIA gene thus producing E1A-9S, E1A-13S and E1A-12S isoforms in cells (174). Therefore, upregulation of SRPK1 in the stimulated cells may suggest the potential role of estrogen in modulating splicing mechanism, which might subsequently trigger the activation of various oncogenic signaling networks in the cells.

In addition to SRPK1, other targets of E2 actions such as DNAjc21, which belongs to Hsp40 heat-shock proteins family important in the formation of chaperone complex with Hsp70 and SRPK1 in the cytoplasm was also detected in the treated cells. Some of Hsp40 proteins family functions include protein translation, folding, unfolding, translocation and degradation through the interaction with Hsp70 proteins (299). Previous studies have demonstrated that SRPK1 binds to the co-chaperones Hsp40/DNAjc8 and Aha before interacts with major molecular chaperones Hsp70 and Hsp90 in the cytoplasm (171), and it dissociates from these chaperone complexes upon stress signals which triggers its translocation to the nucleus, and initiate SR proteins phosphorylation and alter the splice site of target mRNA (171). Furthermore, mutations in DNAjc21 has also been shown promote cancer-prone bone marrow failure (BMF) syndrome and the loss of its expression inhibits cell growth(300). In cytoplasm, DNAjc21 interacts with 60S Ribosome maturation factors and recruits heat shock 70 kDA protein 8 (HSPA8) to stimulate ATPase activity.

Another protein that showed increased expression following E2 treatment was the splicing factor SF35B, a non-snRNP multiprotein complex essential for spliceosome formation in which it ensures accurate excision of introns from pre-mRNA transcript by recognizing the branch site of pre-mRNA within the major and minor spliceosomes (301). In addition, HNRNPDL (JKTBP), a family of splicing repressor protein hnRNPs, was also found amplified under estrogen treatment. It acts as transcriptional regulator and functions primarily in mRNA biogenesis and mRNA metabolism. In epithelial cells, hnRNPDL expression was found highly elevated in response to pro-inflammatory cytokines such as interleukin-6 (IL-6), and was shown to regulate the expression level of NF-κB-repressing factor (NRF) by increasing the translation initiation of the NRF (302). NF- κB pathway is one of the signaling pathways implicated in oncogenesis, where its constitutive activation can lead to the dysregulation of genes involved in many cellular processes such as proliferation, migration and apoptosis. Even more, hnRNPDL was also demonstrated to promote cell proliferation and stimulate the expression of EGFR in prostate cancer cells (303). Apart from that, consistent with STRING analysis (Figure 3.4B), my finding was in line with the established interaction between hnRNPDL and Polypyrimidine tract-binding protein 1 (PTBP1), in which their expression was found to be negatively correlated in this study.

Other than the abovementioned splicing- related proteins, there were other functional proteins whose expression were found to be amplified in response to estrogen, such as Ras-related protein Rab-2A (RAB2A), Growth factor receptor-bound protein 2 (GRB2) and Tumor protein D52 (TPD52). RAB2A is a member of Ras-oncogene family that activates ERK ½- MAPK (Figure 3.4C), and is required for the cell-surface targeting and GPCRs signaling (304). Consistently, as one of RAB2A targets, the expression of MAPK was found upregulated by 0.5-fold although not statistically significant, with borderline p-value of 0.14. It has also been shown to promote breast cancer stem cells (BCSCs) expansion and tumorigenesis by upregulating the activity of transcription factors Zeb1 and β-catenin nuclear translocation resulting in the activation

of Wnt signaling, a prominent signaling pathway implicated in the epithelial-to-mesenchymal transition (EMT) event in the cells (305).

Meanwhile, GRB2 is a proto-oncogene and an adapter protein frequently found overexpressed in breast cancers and was involved in EGF-induced ERK and AKT activation and cell proliferation (Figure 3.4 D) (306). In addition to its role in positive signaling via the Ras/Erk pathway, GRB2 was also found to act as adaptor in Wnt signaling activation by FAK, through Grb2-rac-jnk-c-jun pathway (307). Whereas in lung cancer, GRB2 was demonstrated to be involved in pathway activation that leads to lung cancer invasion and metastasis (308). Furthermore, while tumor protein TPD52 overexpression has been shown to promote lung squamous cell carcinoma aggressiveness (309), high level of TPD52 expression was found to be positively correlated with patient's survival in ERBB2-amplified breast cancer cells (310).

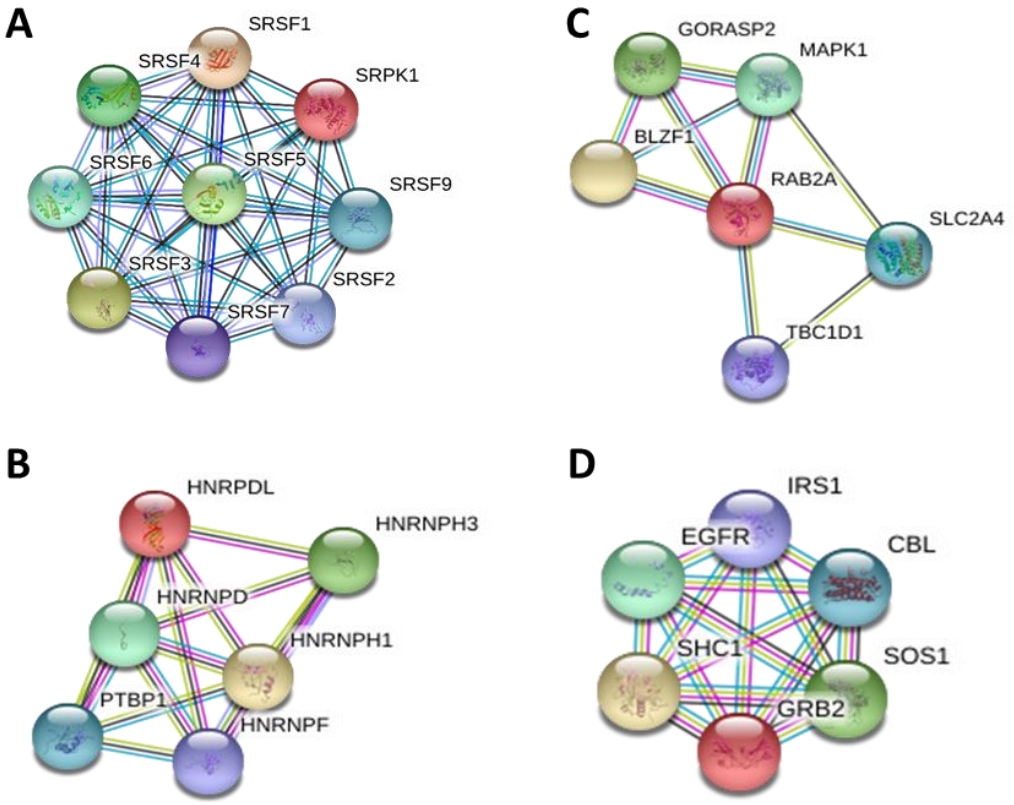


Figure 3.4 STRING analysis showing the protein-protein interaction network of selected proteins found upregulated in response to estrogen. (A) SRPK1, (B) HNRPDL, (C) RAB2A, and (D) GRB2.

On the other hand, there were a number of downregulated proteins that are related to splicing mechanism in cells such as DHX15 which is a functional partner of SRPK2 and splicing repressor,hnRNPA1, RUXG; a core component on spliceosomal, PTPB1; family of splicing repressor hnRNPs, and RAB21 which is required in the promotion of cancer cell invasion and migration as reported in previous studies (311,312).

The differential expression of splicing molecules, as well as cancer-associated proteins following estrogen treatment suggests the complexity of E2 signaling in the cells which might involve interactions between various signaling pathways in the cells, as

well as it establishes the potential of E2 signaling in regulating SRPK1-driven molecular pathway in the mRNA splicing event in breast cancer cells.

3.2 Analysis of breast cancer stem cells (CSCs) isolated from parent MCF7 cells.

Following the finding that estrogen (E2) can modulate the activity of a core splicing kinase, SRPK1, it was hypothesized CSCs subpopulation in cancer cells could potentially promote cancer progression via alternative splicing event. This is supported by the fact that one of the hallmarks of CSCs is high CD44 expression, a cell surface protein whose splice variants have been demonstrated to accelerate cancer metastasis in various human cancers.

The existence of cancer stem cells (CSCs) or circulating-tumor cells (CTCs) which has the characteristics of stem cells (e.g. to self-renew)) has been successfully demonstrated in breast cancer patients (313). Since CSCs was thought to play role in cancer metastasis and hormone therapy resistance, a magnetic separation method was used to test whether these cells could be isolated from MCF7 cell line. These CSCs are known to exhibit positivity for few markers such as $CD44^{high}/CD24^{low}$. This CSCs population was successfully isolated using various other methods in breast cancer cell lines such as SUM149,HCC194 and MCF7 (269).

In fact, high counts of CTCs/CSCs have been used as indicator of bad prognosis in patients with metastatic breast cancer (314). However, since the percentage of CSCs is relatively low in MCF7 breast cancer cells, only a small fraction of them could be collected in this experiment and mammosphere-forming ability of the collected cells was observed for several days. This mammospheres-forming ability of is often used to isolate and identify CSCs, as unlike other cells, CSCs are not anchorage-dependent and are able to survive, proliferate and form multicellular spheroids (termed as ‘mammospheres’), in the absence of attachment (in suspension) (315). The ability of CSCs to form

mammospheres or clusters is also one of the factors that is shown to be associated with increased metastatic ability, with at most 50-fold of metastatic potential as opposed to single CSCs (316).

Figure 3.5 shows the morphology of parent MCF7 cells grown in complete culture media, before CSCs was isolated from $5x10^6$ of MCF7 cells suspension. Following the isolation of CSCs subpopulation, these single cell suspension of CSCs were seeded in ultra-low attachment plates, in the presence of 1% charcoal-stripped fetal bovine serum (CS-FBS) in the media and the plates were kept in the 37^oC incubator for at least 7 days as CSCs have been known to have a slow population- doubling time (317), without changing the media throughout the incubation period. It was found that, single cells started to form small multicellular clusters after day 2, and the clusters appeared larger at day 6. The cells were kept growing in the plate up until day 15, before the cells started to die. The successful isolation of CSCs subpopulation from MCF7 cell line suggests that these cells indeed contain CSCs, and that the magnetic separation method can serve as another technique to separate the stem cells from their parent cells. In addition, this also shows that MCF7 cell line can be a suitable model to further study the biology of breast cancer stem cells. However, considering that these CSCs exist only in a very small number within breast cancer cell lines (approximately 1%), it can be an obstacle to obtain enough samples to conduct the study, without further enrichment method.

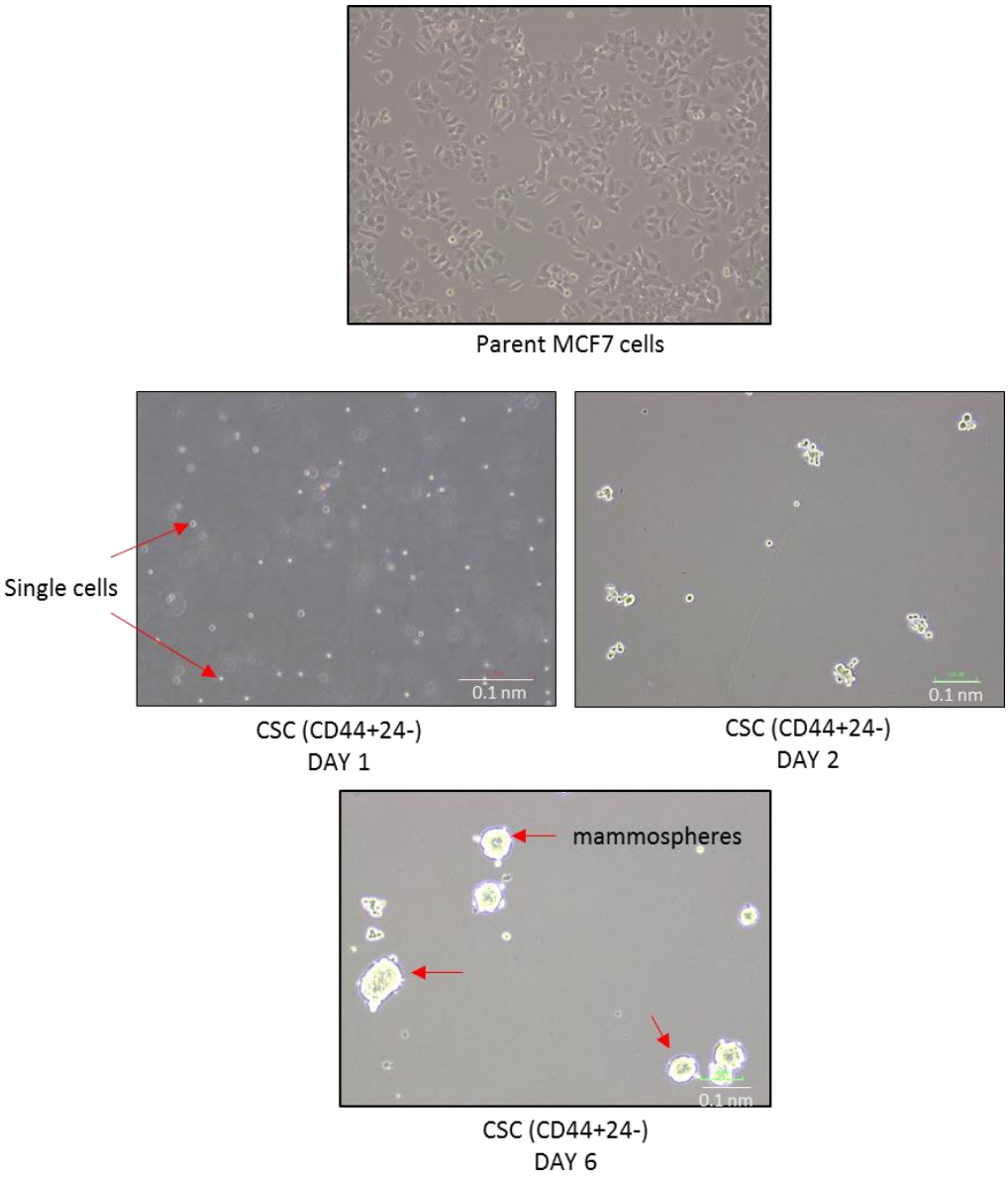
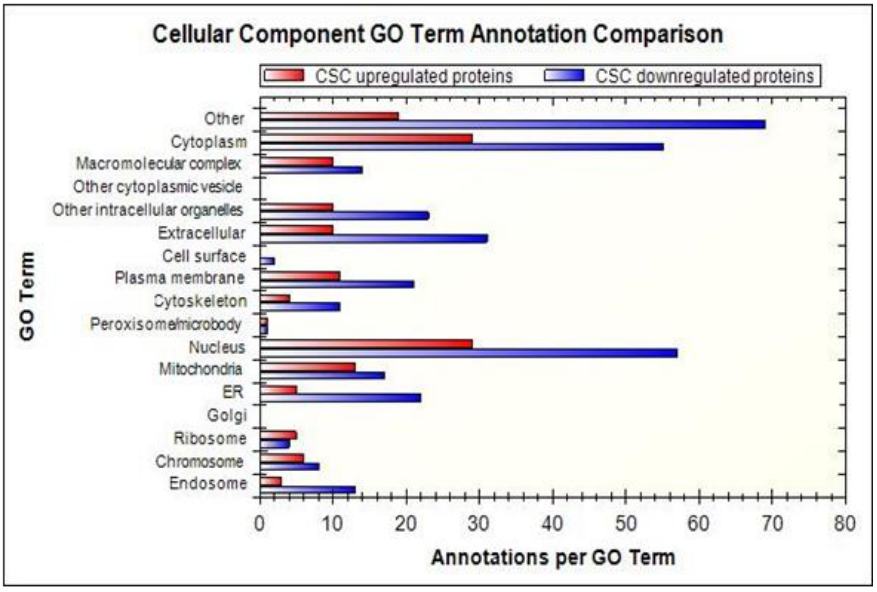


Figure 3.5 Mammosphere-forming experiment. The CSCs subpopulation were isolated from parent MCF7 cells using MagCellect CD44+CD44- breast CSC isolation kit following the manufacturer's protocols, and single cells (Day 1) were seeded in ultra-low attachment plate. Single cells started to form multicellular clusters on Day 2, and the spheres gradually expanding, as shown in Day 6. Pictures were taken using Nikon phase-contrast microscope at 10x objective.

To investigate the effects of estrogen on protein expression profile in CSCs, especially proteins involved in splicing mechanism and metastatic process as demonstrated in parent MCF7 cells, these CSCs were treated with 10nM E2 for 24 hr and quantitative proteomics analysis was carried out as described in Chapter Method 2.9. Results from the mass spectrometry analysis demonstrated that out of 2479 total proteins detected in the samples, 64 of them were highly expressed in stimulated cells as compared to control, and 136 were suppressed after the exposure with estrogen.

Further analysis on gene ontology annotation of these differentially expressed proteins indicated that the downregulated proteins are mainly in cytoplasm and nucleus (Figure 3.6A). In addition, STAT6, a member of STAT transcription factor which play a key role in mediating interleukin 4 (IL4) biological response via JAK-STAT signaling pathway was found among the significantly reduced proteins (Figure 3.6B). The JAK-STAT pathway has been known to be involved in normal stem cell development, and recently it was demonstrated that this pathway was also found deregulated in breast cancer stem cells (318). On the other hand, E2 significantly stimulated the expression of proteins that reside mainly in the cytoplasm and nucleus, and one of the protein identified among the upregulated protein was Cyclin D1 (CCND1), which is a known target of ER signaling that promotes hormonal therapy resistance breast tumors (319,320).

A



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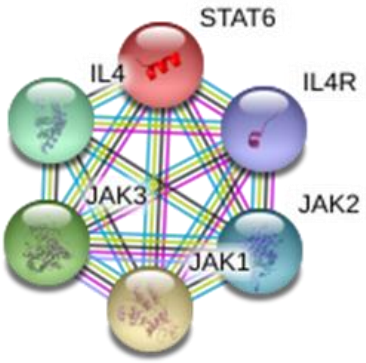


Figure 3.6 **(A)** GO annotation of proteins affected by estrogen treatment in cancer stem cells (CSCs) shows that most of the targets are the proteins found in cytoplasm, nucleus, ER and mitochondria. **(B)** One of the downregulated protein was STAT6, which is one of the key players in JAK-STAT pathway, a pathway that has been found deregulated in breast cancer stem cells.

Discussion

The role of estrogen receptor signaling in the progression of one of breast cancer subtypes, ER– positive tumours, has long been recognized where more than 80% of lymph node metastasis and approximately 65-70% of distant metastases retain ERα expression [reviewed in (321)]. In addition, estrogen (E2) has been shown to facilitate metastatic process through rapid cytoskeletal remodelling resulting in the increased migration and invasion ability of the cells through $G\alpha_{13}$/RhoA/ROCK/moesin cascade (322,323). Previous studies have shown that estrogen receptors (ERs) signaling can interact with multiple cytosolic kinases such as Src kinase, Akt, MAPK and PI3K in ER+ breast cancer (57,321).

Changes in MCF7 protein expression profile by estrogen

Proteome analysis conducted in this study demonstrated that E2 can positively modulate the expression of another protein kinase, Serine-Arginine Protein Kinase 1 (SRPK1), a key regulator in pre- mRNA splicing event. Although proteomics studies on E2-stimulated ER+ MCF7 breast cancer cells have been reported before (294,295,324), as well as estrogen has been demonstrated to regulate the transcription of hundreds of genes implicated in breast cancer proliferation and survival, the potential effects of estrogen in modulating splicing related proteins are not yet fully explored. Validation experiments through the analysis of total SRPK1 protein expression and mRNA expression further confirmed that E2 can stimulate SRPK1 expression both at protein and mRNA level in MCF7 cells. Therefore, finding from this study reveals another target of E2 action in ER+ breast cancer cells, which may potentially trigger signaling networks that can influence various downstream molecular events such as alternative splicing, hence promoting cancer development and progression. This is because, elevation of SRPK1 activity has been extensively reported in various cancers undergoing EMT, which include prostate, ER (+) breast and non-small cell lung carcinoma (171,174,190,193).

Alongside SRPK1, the expression of other splicing related proteins was also detected to be differentially expressed, such as DNAjc21 (upregulated), which is important in the formation of chaperones complexes with SRPK1 and Hsp70 in the cytoplasm. Interestingly, the expression of splicing repressor PTPB1 (family of hnRNPs) and component of spliceosomal protein, RUXG were found downregulated in response to estrogen. Given the significant altered pre-mRNA splicing event in cancer progression, these results reveal that estrogen is most likely to influence breast cancer progression, one of the ways via the modulation of splicing proteins activity in the cells.

Effects of estrogen on splicing-related protein expression in cancer stem cells (CSCs)

The existence of CSCs which have the characteristics of stem cells such as self-renewal ability, has been previously demonstrated in breast cancer patients (313). These cells, which exhibit markers such as high CD44/low CD24 ($CD44^{+}CD24^{-}$) marker on its cell surface, is suggested to be one of the factors that contribute to hormone therapy resistance and cancer recurrence and metastasis in patients. Furthermore, as splice variants of CD44 gene has also been shown to accelerate metastasis process in various cancers, the differential expression of splicing related proteins in response to estrogen was also investigated.

Of note, while presence of cancer stem cells (CSCs) sub-population in MCF7 cell line was successfully demonstrated in this study, detailed analysis of the results showed that none of splicing related proteins were found affected by the treatment. However, estrogen was shown to downregulate STAT6, a member in JAK-STAT pathway, a pathway involves in inflammatory response and in normal stem cell pathway development. Recently, JAK-STAT pathway was also found to be deregulated in breast cancer stem cells (318). In addition, one of the E2 signaling target genes, Cyclin D1 (CCND1) that play roles in the processes promoting hormonal therapy resistance in breast cancer was found upregulated by the treatment.

Furthermore, it has been reported in several studies that CSCs notably has different epithelial and mesenchymal characteristics among them, and this characteristic

is not necessarily homogenic as parent cells, for example, CSCs found in ER-positive metastatic breast cancer frequently lack estrogen receptor expression (325). Thorough search on markers specific for metastatic CSCs, such as the expression of tyrosine kinase receptor (c-MET)(326) was carried out, and it was not found among the upregulated proteins in the CSCs isolated from this study. Therefore, although the isolated CSCs were shown to have the properties associated with increased metastatic ability such as ability to form mammospheres, the criteria may not be enough to be considered as CSCs of mesenchymal/ metastatic type, thus may suggest that these CSCs might be from the epithelial type. In addition, since the detection method used was not a targeted proteomic study, in which protein abundance is a key determinant of detection, this could be a limiting factor and sample enrichment is needed before deeper analysis could be done. Therefore, further studies on CSCs were not continued in this project.

CHAPTER 4

Role of estrogen (E2) and corticotrophin-releasing hormone (CRH) in pre-mRNA alternative splicing (AS) in ER+ and ER- breast cancer cells

Introduction

Alternative splicing (AS) takes place in nearly all the mammalian genes and is regulated by protein complex called spliceosome. It is an essential regulatory mechanism that regulates proper production and function of proteins. Numerous studies have demonstrated that aberrant splicing can lead to various human diseases through the production of protein isoforms that can trigger disease development or progression in human, or by modifying protein functions important in maintaining human health. The role of estrogen in the alternative splicing of several target genes has been previously demonstrated, such as PKM2, CRHR1, FAS/CD95 and FGFR2 in MCF7 cells (327), which eventually contribute to breast carcinogenesis and development.

In line with this finding, results from my previous chapter showed that estrogen (E2) can potentially regulate the expression of splicing kinase, SRPK1 in ER+ cells. This Serine-Arginine protein kinase acts downstream of protein kinase B (AKT), and can be found predominantly in the cytoplasm of most cell types (169). In the cytoplasm, SRPK1 forms complex with major molecular chaperones HSP70 and HSP90 before leaving the complexes upon stress signal, and moves to the nucleus to phosphorylate serine-arginine (SR) proteins and initiate pre-mRNA splicing in the nucleus (170,171).

Furthermore, studies have shown that alterations in cellular immunity such as in cytokine production induced by stress signal may promote cancer progression (328–331). Studies show that stress- induced alterations through the action of such as CRH can potentially increase disease susceptibility. This is supported by previous findings in which the nucleotide sequences in the POMC promoter, a CRH target gene, has been found in the genome of human diseases such as HIV-s, human MAT-1 breast cancer oncogenes and in proinflammatory molecules, for example interleukin-1b converting enzyme (332). This has led to the hypothesis that CRH may influence progression of certain endocrine-related diseases such as breast cancer. In addition, dual roles of CRH in promoting and inhibiting breast cancer progression have also been previously

reported (103,104), in which both effects were shown to be time- and cell types-dependent.

Even more, physiological stress has been found to likely cause poor outcome in breast cancer patients, one of it by inducing poor response to therapy (333). The presence of stress molecules, corticotrophin-releasing hormone (CRH) and its related peptides in various cancers such as breast and prostate cancer further supports the notion that this hormone plays role in tumorigenesis (134,135). However, whilst reviews and meta-analysis have reported the association between stress and cancer, little is known about the underlying mechanism of actions of stress molecules in tumorigenesis.

Therefore, in this chapter, various molecular biology techniques were used to investigate the downstream effects of estrogen- driven upregulation of SRPK1 activity on the pre-mRNA alternative splicing mechanism in ER+, as studies showed that altered splicing event occurred more predominantly in hormone-responsive breast cancer. Additionally, since CRH has also been demonstrated to regulate protein kinase B (AKT), a protein kinase known to regulate SRPK1 phosphorylation, CRH was also used as extracellular stimulus to examine its potential effect on SRPK1 as well as the role of CRH signaling in promoting cancer progression in ER+ and ER- cells through the regulation of alternative splicing mechanism. This was done by analysing the activity of serine-arginine (SR) splicing proteins after the treatment with both hormones. In addition, CD44 gene, whose splice variants, especially CD44s and CD44v have been implicated in tumorigenesis was selected to measure the consequence of altered activity of splicing proteins on the production of CD44 splice isoforms in both MCF7 (ER+) and SKBR3 (ER-) cell lines.

Results

4.1 Detection of SRPK1 protein in co-chaperone complexes

Estrogen- induced upregulation of SRPK1 protein expression found in the proteomic experiment has led to further investigations on the regulation of this splicing kinase activity and the alternative splicing (AS) mechanism mediated by this protein in the cells. Previous studies have shown that cellular distribution of SRPK1 is essential for its function in the regulation of serine-arginine proteins (SRps) phosphorylation, and SRPK1 was demonstrated to interact with molecular cochaperone, Hsp70/Hsp90 complex in the cytoplasm, that facilitates folding of the kinase into active conformation for the kinase-mediated signaling (171,334). The active form of SRPK1 proteins are then released from these cochaperone complexes, and it was also demonstrated that these active SRPK1 kinases were capable to translocate to the nucleus for SR proteins phosphorylation upon dissociation from cochaperone complexes in the cytoplasm (171).

The association between SRPK1 and the Hsp70/Hsp90 containing complexes was interrogated using co-immunoprecipitation (co-IP) method followed by western blot against SRPK1 protein, and indeed, SRPK1 protein was detected in the Hsp70/Hsp90 cochaperone as shown in Figure 4.1. This finding is therefore consistent with current understanding that SRPK1 interacted directly and formed complexes with major heat-shock protein Hsp70 as well as Hsp90 (190).

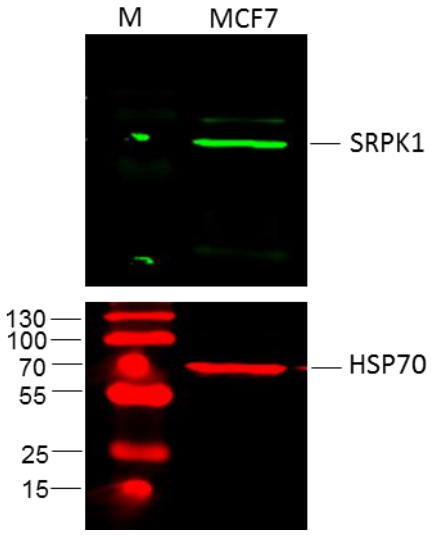


Figure 4.1 Detection of SRPK1 in Hsp70/Hsp90 co-chaperone complex confirms the association between SRPK1 with the major heat-shock complexes in the cells. The co-chaperone complexes were immunoprecipitated by using anti- Hsp70, followed by western blot against SRPK1 protein.

4.2 Analysis of estrogen (E2)- induced SRPK1 phosphorylation and nuclear translocation

4.2.1 Time-course determination of SRPK1 activity by E2

As the protein discovery analysis showed that E2 can stimulate the expression of SRPK1 in MCF7 cells, further investigation was performed to confirm this finding and to examine how E2 signaling regulates SRPK1 activity in the cells. The fundamental role of SRPK1 kinase in regulating alternative splicing mechanism in breast cancer has been reported in several studies, for example, it was reported that depletion of SRPK1 kinase in breast cancer cells resulted in reduced phosphorylation level of SR proteins, including SRSF3, SRSF4 and SRSF6, leading to altered splicing of MAP2K2 gene and inhibition of apoptosis in the cells (170,172). In addition, it is also known that catalytically active SRPK1 translocates to the nucleus upon phosphorylation by upstream kinases such as AKT, and dissociation form Hsp70/Hsp90 co-chaperone complexes and regulates the phosphorylation of SR proteins in the nucleus (171,335). In fact, the detection of SRPK1 protein signal in stimulated cells by immunofluorescence in previous section (Figure 3.3) also displayed generally higher detection of SRPK1 protein in the nuclear compartment as compared to cytoplasm.

Therefore, to determine whether E2 regulation of SRPK1 activity can also lead to its nuclear translocation, time-course experiment followed by western blot was performed in cytoplasmic and nuclear compartments of MCF7 cells, before and after E2 stimulation. As E2 was shown to induce SRPK1 protein expression, it was hypothesized that it can also trigger SRPK1 nuclear translocation in the cells. Therefore, MCF7 cells were treated with 10nM estrogen at various time points; 0, 0.5, 1, 2, 24 hr, and cytoplasmic and nuclear proteins were extracted. Since dissociation of SRPK1 from co-chaperone complexes releases active SRPK1 kinases, immunohistochemistry was performed against phopho-SRPK1 (Thr 601) in both cell compartments, and signal detection was normalized against total SRPK1 level. It was observed that in line with the

hypothesis, E2 indeed can stimulate SRPK1 nuclear translocation and both the cyto- nuc level of SRPK1 was found to be the highest at 24 hr after stimulation (Figure 4.2).

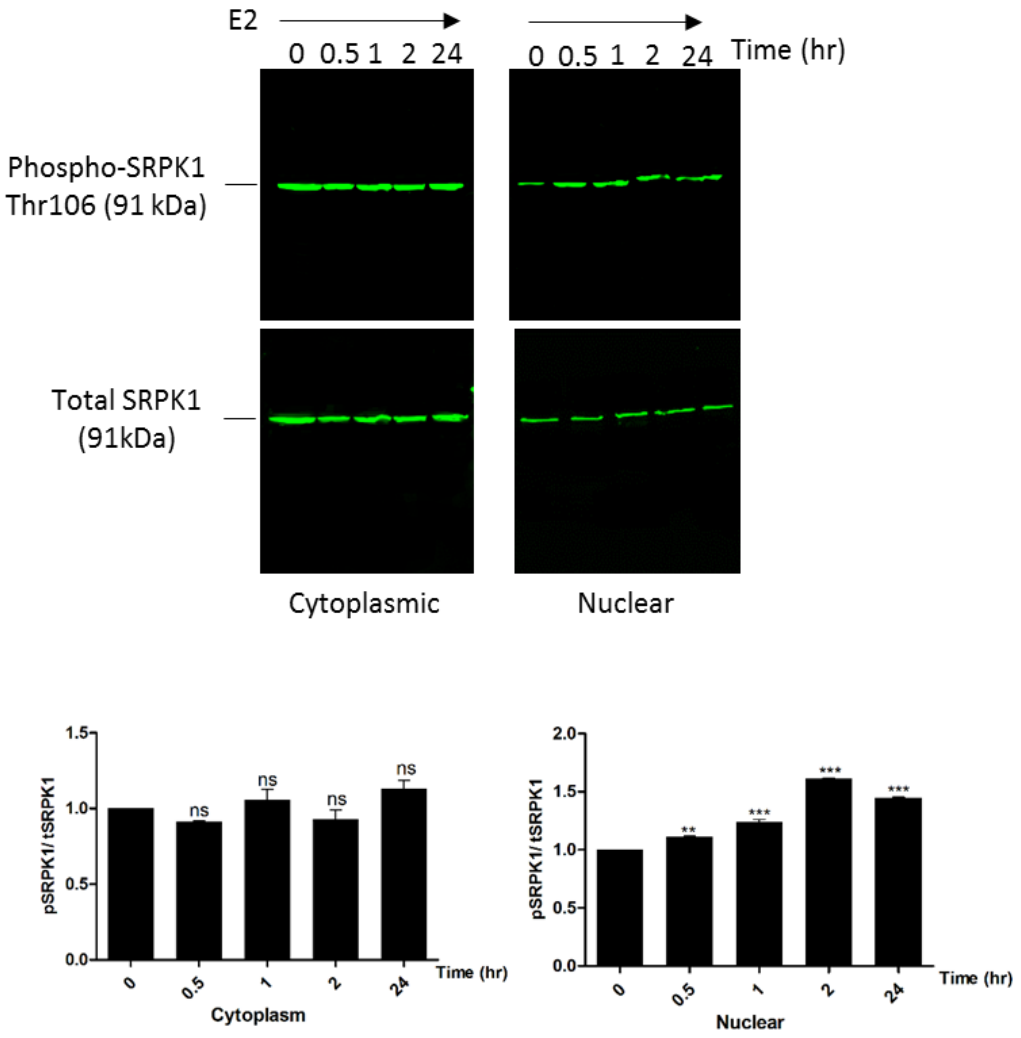


Figure 4.2 MCF7 cells were treated with 10nM E2 at 0, 0.5, 1, 2, 24hr. Cytoplasmic and nuclear proteins were extracted and phospho-SRPK1 (Thr601) signals were detected in both cell compartments using anti-pSRPK1 antibody and results were normalized against total SRPK1 level. Phosphorylation level was detected increased in both compartments after 24hrs of estrogen treatment. Representative blots are shown.

4.2.2 Analysis of phosphorylation and nuclear translocation of SRPK1 protein by estrogen (E2)

Following the time-course experiment, E2-driven SRPK1 phosphorylation and nuclear translocation were further examined, and SRPK1 mRNA expression was also investigated. Cells were treated with 10nM E2 at 24hr (this concentration and treatment period were used in all subsequent experiments) before proteins and RNA were extracted. The cyto-nuc level of phospho-SRPK1 (pSRPK1) were detected by western blot, whilst the expression of SRPK1 mRNA after estrogen stimulation was determined by real-time quantitative PCR (RT-qPCR). Figure 4.3A confirms that stimulated cells demonstrated statistically significant increase in the level of pSRPK1 in the cytoplasm and nucleus, as compared to unstimulated cells. Consistently, it was also found that the expression of SRPK1 mRNA was upregulated in the stimulated cells (Figure 4.3B).

4.2.3 Effects of Akt kinase inhibition on E2- induced SRPK1 phosphorylation

Previous findings revealed that Akt regulates the phosphorylation of SRPK1 in the cells (190), therefore the role of Akt in this E2- driven SRPK1 phosphorylation was assessed by inhibiting Akt kinase activity in the stimulated cells. Stimulated MCF7 cells were pre-treated with Akt kinase inhibitor (MK2206), at 5mM for 2hr before proteins and RNA were extracted. It was demonstrated that reduced Akt kinase did not affect much of basal phosphorylation of SRPK1, this kinase inhibition in stimulated cells showed markedly reduced pSRPK1 level (Figure 4.3A). This finding was also consistent with SRPK1 mRNA expression, in which, Akt kinase inhibition blocked E2- driven stimulation of SRPK1 mRNA expression in the cells (Figure 4.3B), suggesting that Akt plays role in E2 signaling in the regulation of SRPK1 kinase in MCF7 cells.

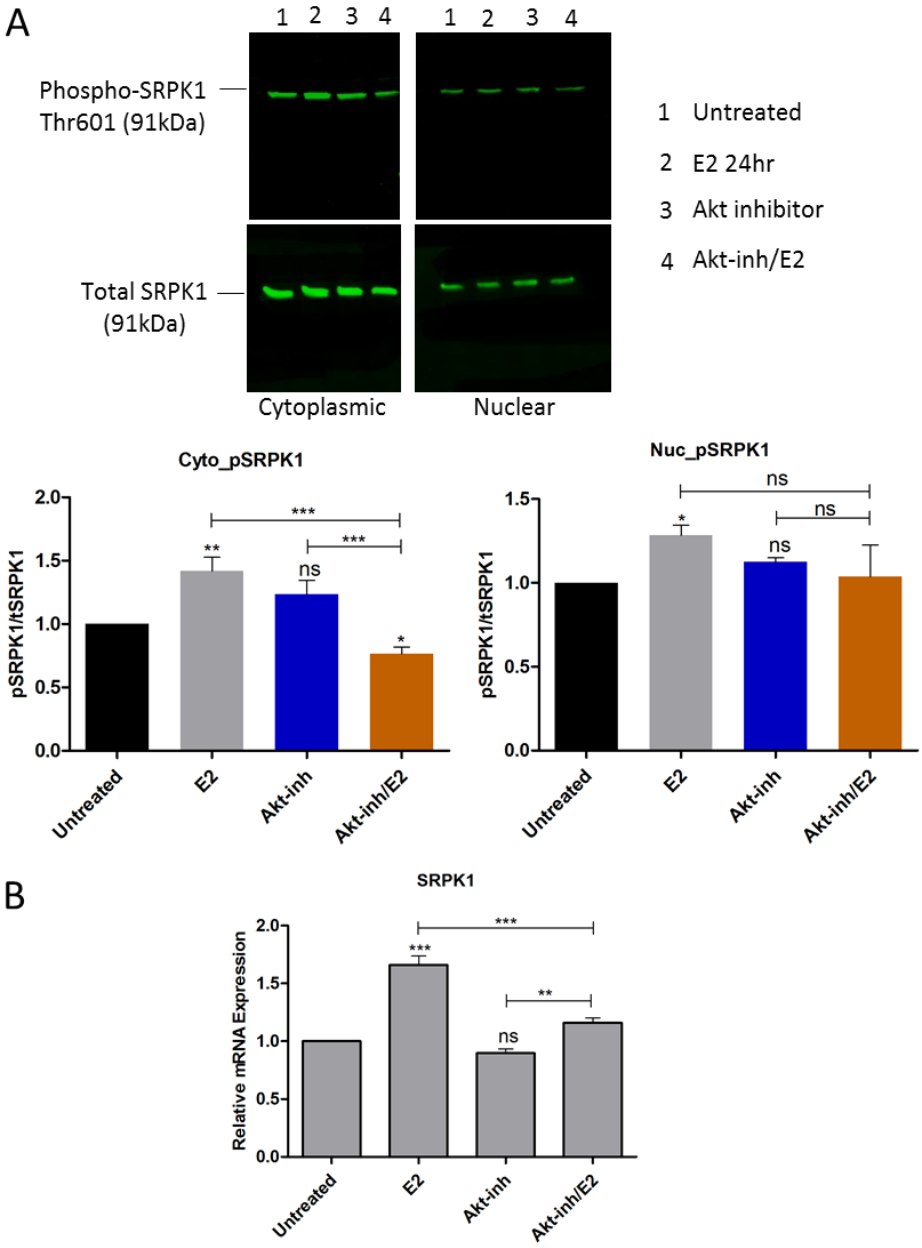


Figure 4.3 (A) Western blot analysis of phospho-SRPK1 showed that estrogen (E2) stimulates basal phosphorylation of SRPK1 (pSRPK1) and its nuclear translocation. In addition, pre-treatment of the stimulated cells with Akt inhibitor (MK2206) has led to reduced pSRPK1 signal detection, although reduced level of Akt activity did not affect basal pSRPK1 level. As compared to Akt kinase inhibition alone, inhibition of Akt kinase in stimulated cells also showed significantly reduced signal of pSRPK1 in cytoplasm. (B) Similarly, this finding was also evident at mRNA level, in which E2 was found to upregulate SRPK1 mRNA expression. However, reduced Akt kinase inhibited this E2-driven induction of SRPK1 mRNA expression.

4.3 Analysis of Corticotrophin- releasing hormone (CRH)-induced SRPK1 phosphorylation and nuclear translocation

There is growing evidence that stress may affect cancer progression, one of the ways through alterations in cellular immunity such as in cellular activation, cytokine production and cell trafficking (328–331). Previous studies suggest that physiological stress factors may also contribute to poor outcome in breast cancer patients which include poor response to therapy (333). In addition, one of the stress molecules, corticotrophin-releasing hormone (CRH) was thought to be implicated in tumorigenesis after studies reported the presence of CRH receptors and CRH family peptides in endocrine-related cancers such as breast and prostate cancer (134,135). Although reviews and meta-analysis have reported the association between stress and cancer, little is known about the underlying mechanism of actions of stress molecules in tumorigenesis.

Therefore, this study was also aimed to examine the mechanism of stress-induced changes in cancer, specifically by studying the effects of CRH and the functional consequences of CRH signaling in influencing cancer progression. CRH was used as extracellular stimulus to investigate its effects on SRPK1 status, as previous studies have reported that Akt is one of protein kinases regulated by CRH, and Akt has also been shown to regulate SRPK1 phosphorylation in the cells. Furthermore, the suitability of cellular model, estrogen receptor positive (ER+), MCF7 breast cancer cell line for studying the effects of CRH in the regulation of splicing molecules is described in this section.

4.3.1 Time-course determination of SRPK1 activity by CRH

CRH through activation of G protein-coupled receptor (GPCR) exerts acute effects affecting the activity of intracellular pathways leading to transcriptional effects of its target genes. Therefore, following the final concentration of CRH used by previous student in this group, the effects of CRH on SRPK1 status were investigated. MCF7 cells were treated with 100nM CRH at various time points; 0, 0.5, 1, 2, 4, 24hr, and cytoplasmic and nuclear proteins were extracted, followed by western blot analysis against phospho-SRPK1 (Thr601). Interestingly, it was observed that CRH can significantly induce SRPK1 phosphorylation and nuclear translocation in MCF7 cells 24hrs after stimulation (Figure 4.4), suggesting that CRH may influence tumorigenesis via modulation of key protein kinase in the regulation of splicing mechanism, SRPK1 in ER+ MCF7 cells.

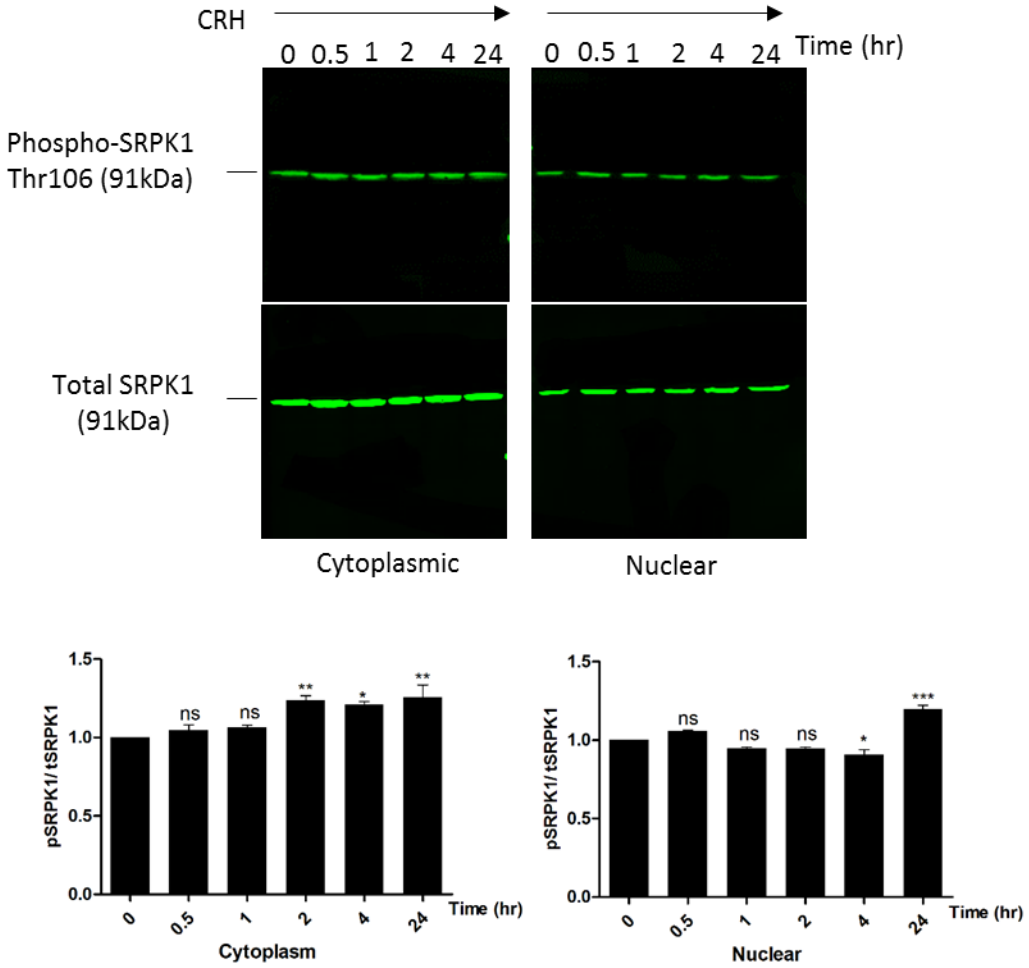


Figure 4.4 MCF7 cells were treated with 100nM CRH at 0, 0.5, 1, 2, 4, and 24hr. Cytoplasmic and nuclear proteins were extracted and phospho-SRPK1 (Thr601) signals were detected in both cell compartments by using anti-pSRPK1 antibody and results were normalized against total SRPK1. CRH was shown to significantly stimulate SRPK1 phosphorylation and nuclear translocation after 24hrs of treatment. Representative blots are shown.

4.3.2 Measurement of SRPK1 protein phosphorylation, nuclear translocation and mRNA expression in CRH-stimulated MCF7 cells

Following time-course experiment, MCF7 cells were treated with 100nM CRH for 24hrs before cytoplasmic and nuclear protein and RNA extraction, followed by western blot and RT-qPCR analysis of phospho-SRPK1 (pSRPK1) protein level and SRPK1 mRNA expression, respectively. As expected, Figure 4.5A shows that raised signal of pSRPK1 was detected in stimulated cells, and cellular fractions analysis revealed that pSRPK1 signal increased nearly two-fold as compared to untreated cells. Furthermore, CRH was also demonstrated to have transcriptional effects on SRPK1, as SRPK1 mRNA expression was shown upregulated by 50% as compared to untreated cells (Figure 4.5B). In addition, cellular distribution of pSRPK1 in stimulated cells was also observed by immunostaining, and consistent with western blot results, the overall pSRPK1 signal was detected higher in stimulated cells, and pSRPK1 signal in the nucleus of stimulated cells was slightly increased than that in unstimulated cells (Figure 4.6).

4.3.3 Effects of Akt kinase inhibition on CRH-induced SRPK1 phosphorylation

As previous studies have demonstrated that CRH signaling through GPCR was shown to regulate Akt kinase, the effects of reduced Akt kinase on the regulation of CRH-driven SRPK1 phosphorylation was further assessed. Stimulated cells were pre-treated with Akt kinase inhibitor (MK2206) for 2hr, followed by western blot detection of pSRPK1 in cellular fractions. Results showed that basal level of pSRPK1 was increased in cytoplasm, but it did not seem to affect much of nuclear pSRPK1 level when Akt kinase was inhibited (Figure 4.5A). However, inhibition of Akt activity in stimulated cells has led to reduced SRPK1 phosphorylation and nuclear translocation, as shown by markedly decreased signal of pSRPK1 in cytoplasm and nuclear fractions.

Furthermore, mRNA expression analysis by RT-qPCR also revealed that while reduced Akt kinase in the cells did not lead to statistically significant change in basal SRPK1 mRNA expression, inhibition of this protein kinase activity in stimulated cells has abolished the transcriptional effects of CRH on SRPK1 (Figure 4.5B). Overall, these findings have so far demonstrated that CRH has two effects via Akt; increased mRNA transcription of SRPK1, and increased phosphorylation and nuclear translocation of the SRPK1, suggesting that CRH may influence cancer progression through the regulation of SRPK1-mediated splicing signaling pathway in the cells.

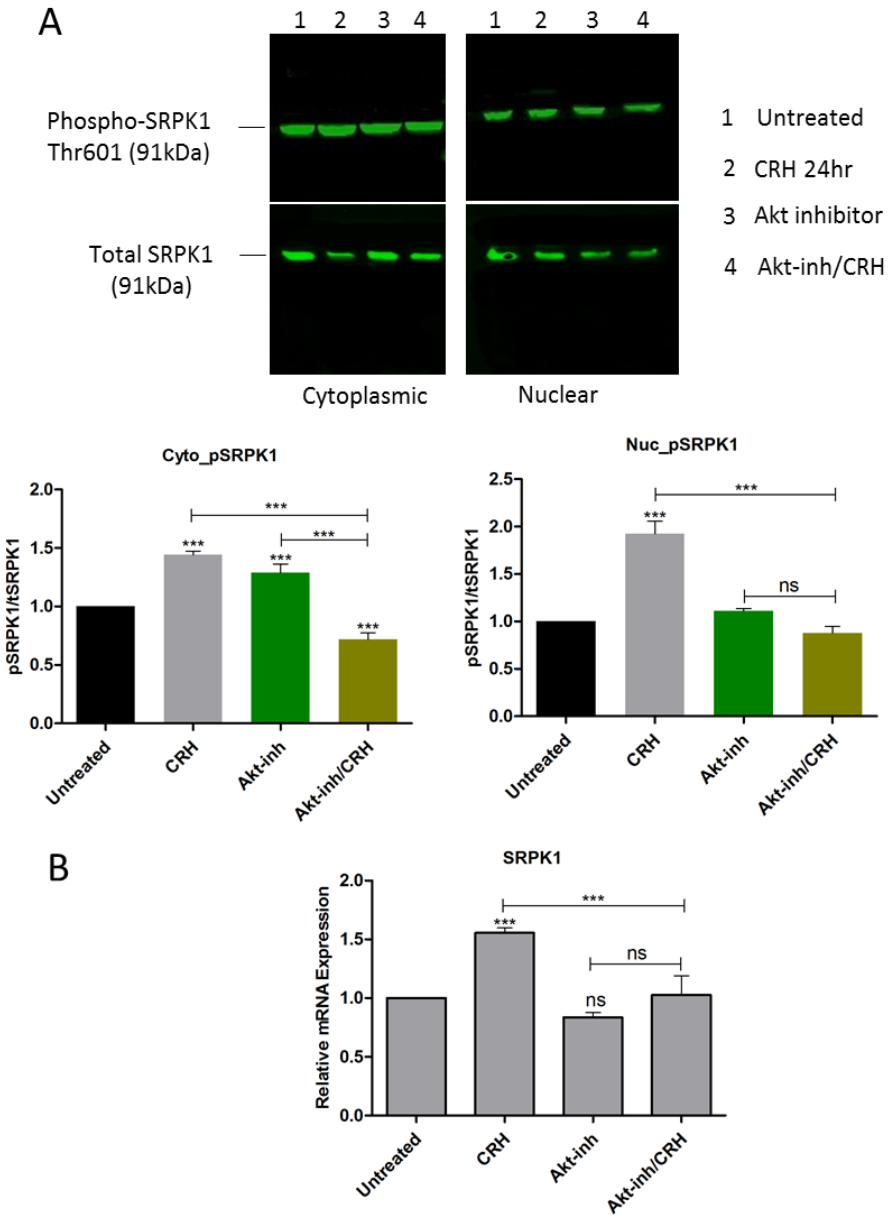


Figure 4.5 (A) Western blot analysis of phospho-SRPK1 in MCF7 showed that CRH can induce basal phosphorylation of SRPK1 (pSRPK1) in both cytoplasm and nuclear cells compartments. This CRH-driven stimulation was shown to be mediated by Akt kinase, as pre-treatment of the stimulated cells with Akt inhibitor (MK2206) has led to reduced pSRPK1 signal detection. Meanwhile, reduced level of Akt activity slightly affected basal pSRPK1 level in the cytoplasm only. In addition, as compared with Akt kinase inhibition alone, the inhibition of Akt kinase in stimulated cells also showed significantly reduced signal of pSRPK1 in cytoplasm. (B) Similar finding was also evident at mRNA level, in which, while CRH can induce SRPK1 mRNA expression, reduced Akt kinase was shown to inhibit this effect of CRH on SRPK1 mRNA expression.

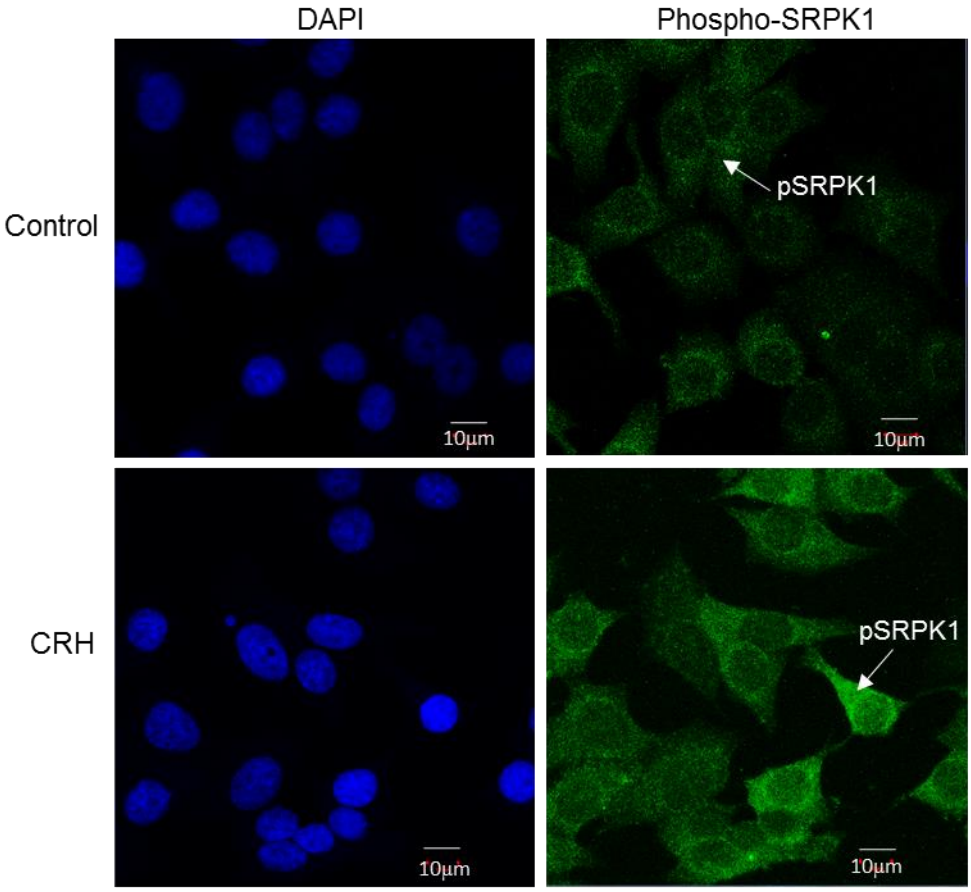


Figure 4.6 MCF7 cells were treated with 100nM of CRH for 24hrs before phospho-SRPK1 (Thr601) was detected using anti-pSRPK1 antibody. Green fluorophore shows the signal for pSRPK1, whereas blue fluorophore (DAPI) was showing the cells' nucleus. Overall, higher signal detection for pSRPK1 was observed in stimulated cells (white arrow), as compared to unstimulated cells. Representative images are shown as above, n=6, scale bar =10um.

4.3.4 Effects of CRH on SRPK1 phosphorylation in ER- SKBR3 cells

Following the demonstration that CRH can regulate SRPK1 phosphorylation, nuclear translocation and mRNA transcription in estrogen receptor positive (ER+) MCF7 breast cancer cell line, similar studies was also carried out in ER- breast cancer cell line, SKBR3 which is identified by overexpression of HER2 receptor at the cell membrane. Furthermore, these cells do not possess hormone receptors (estrogen or progesterone receptors), therefore, is also called estrogen receptor negative (ER-) breast cancer cell line. As compared to ER+ breast cancer, the ER- breast cancer cells tend to be more aggressive and accounts for approximately 18-20% of all breast cancers. Therefore, this cellular model of breast cancer can be a good model to study whether CRH can exert similar effects as it did in MCF7 cells.

SKBR3 cells were stimulated with 100nM CRH for 24hrs, before protein and RNA extraction followed by western blot and RT-qPCR, respectively. It was found that cytoplasmic level of phospho-SRPK1 protein was significantly high in the stimulated cells, but was low in the nucleus, as compared to control (Figure 4.7A). In addition, RT-qPCR analysis also revealed that CRH can upregulate SRPK1 mRNA expression in this cell line, similar to MCF7 (Figure 4.7B). Furthermore, when the cells were treated with Akt inhibitor, basal level of pSRPK1 phosphorylation was detected increased, whilst the nuclear level was decreased. Even more, inhibition of Akt kinase in the stimulated cells seemed to affect the phosphorylation level of SRPK1 protein in the cytoplasm but not in the nucleus (Figure 4.7A). In addition, gene expression analysis demonstrated that inhibition of Akt kinase did not affect the expression of basal SRPK1 mRNA. However, pre-treatment of stimulated cells with Akt inhibitor resulted in the decreased of SRPK1 expression (Figure 4.7B). These findings suggest that CRH can stimulate SRPK1 phosphorylation in cytoplasm and upregulate the transcription of SRPK1 mRNA in SKBR3 cells, both of which are mediated by Akt kinase. However, unlike in MCF7, CRH does not stimulate nuclear translocation in these cells.

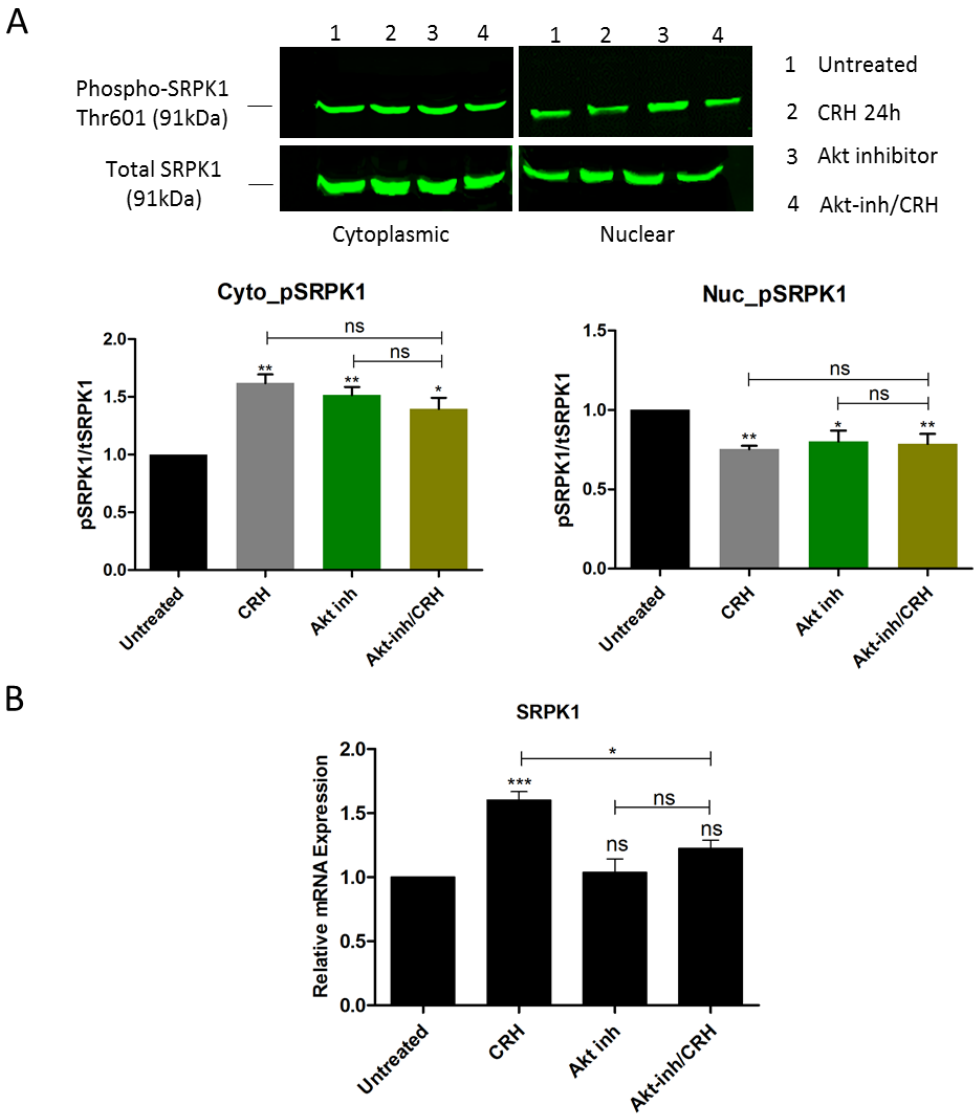


Figure 4.7 (A) Western blot analysis of phospho-SRPK1 in SKBR3 showed that CRH stimulated basal phosphorylation of SRPK1 (pSRPK1) in the cytoplasm, but not SRPK1 nuclear translocation, as nuclear pSRPK1 showed reduced signal. In addition, this effect of CRH in the cytoplasm was mediated by Akt kinase, as pre-treatment of the stimulated cells with Akt inhibitor (MK2206) has led to reduced pSRPK1 level. Reduced Akt activity was shown to increase basal pSRPK1 in the cytoplasm but decreased the level of pSRPK1 in the nucleus. (B) Similar finding was also evident at mRNA level, in which while CRH upregulated SRPK1 mRNA expression, reduced Akt kinase blocked the CRH-driven transcription of SRPK1 mRNA in the cells. Furthermore, inhibition of Akt kinase activity did not affect the transcription level of SRPK1 mRNA in the untreated cells.

4.4 Measurement of E2- and CRH-induced of Serine-Arginine (SR) proteins phosphorylation in ER+ and ER- cells

Serine- arginine proteins (SRp) have broad roles in gene expression, however their most essential function is in constitutive and alternative pre-mRNA splicing and mRNA translation. In general, the RS domain of SR proteins is extensively phosphorylated by protein kinases, from SRPKs and CLKs family, they are predominantly phosphorylated by SRPK1 on serine residues, which subsequently influences SR proteins activation state and localization in the cells. The SRPK1 kinase role in regulating serine-arginine (SR) protein phosphorylation has been well characterized in several studies. Therefore, following the demonstration of E2 and CRH effects in modulating SRPK1 protein phosphorylation, nuclear translocation and mRNA expression, the effects of SRPK1 differential phosphorylation in the cells were further interrogated, by examining activation state of SR proteins through quantification of their phosphorylation signals in cellular fractions.

Analysis of SR protein phosphorylation in ER+ MCF7 cells by estrogen

MCF7 cells were treated with either E2 (10nM) or CRH (100nM) for 24hrs, before cyto- and nuclear protein extraction, followed by immunoblotting against anti-phosphoepitope SR proteins. It was found that, increased level SRPK1 phosphorylation and nuclear translocation in treated cells resulted in differential phosphorylation of SR proteins (SRp75, SRp55, SRp40 and SRp30) in the cells.

In the E2- stimulated cells, it was demonstrated that only signal for phospho-SRp30 was detected increased as compared to unstimulated cells, whereas no statistically significant change was observed on the phosphorylation level of SRp75, SRp55 and SRp40 (Figure 4.8). Furthermore, basal level of phospho- SRp55 and – SRp30 in the nucleus was detected increased when Akt kinase was inhibited, and pre-treatment of stimulated cells with Akt kinase inhibitor has led to reduced phosphorylation signal of SRp40 and SRp30.

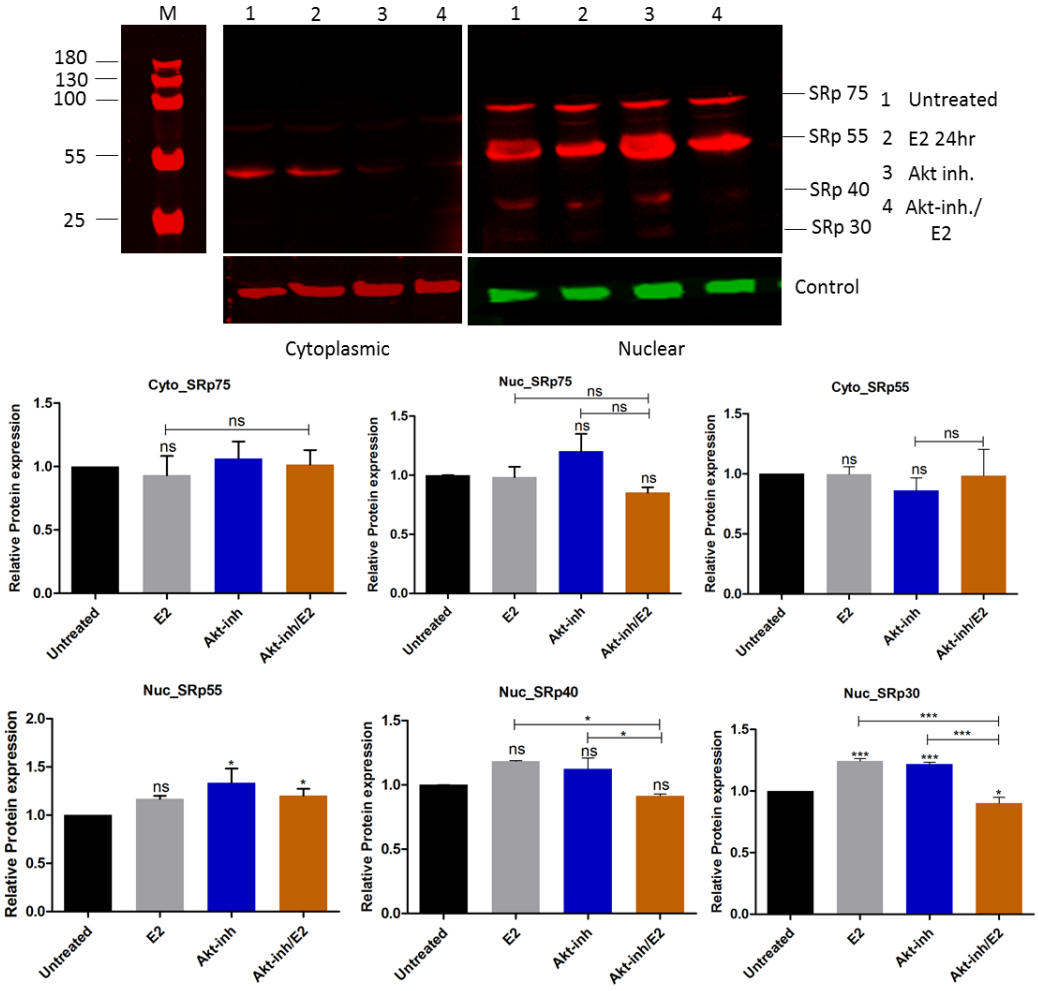


Figure 4.8 Immunoblotting results showed increased signal of phospho-SRp30 in the stimulated cells, but no significant change was observed in phospho-SRp75, -SRp55 and -SRp40 level, as compared to unstimulated cells. Inhibition of Akt kinase also resulted in increased signal detection of basal phospho- SRp55 and – SRp30 level in the nucleus, but reduced Akt kinase in stimulated cells significantly decreased the phosphorylation level of SRp40 and SRp30 in the nucleus. Furthermore, E2 was shown to induce SRp40 and SRp30 phosphorylation in cells where Akt activity was inhibited. Data was normalized to a-tubulin (cytoplasmic control) and Lamin B1 (nuclear control). n=7, *=p<0.05, **=p<0.01, ***=p<0.001, ns= not significant. Representative blots are shown.

Analysis of SR protein phosphorylation in ER+ MCF7 cells by CRH

Furthermore, detection of the subcellular distribution of SR proteins in individual cells in response to CRH was monitored by immunofluorescence against anti-phosphoepitope SR proteins prior to the detection of phosphorylation level in protein lysate. MCF7 cells were stimulated with CRH for 24hrs and the subcellular localization of SR proteins was quantified. It was found that stimulated cells showed significantly stronger phospho-SR proteins signal in both cytoplasm and nucleus, as compared to untreated cells (Figure 4.9).

Following this, the phosphorylation level of SR proteins was measured quantitatively in whole cell protein lysate via western blot by using the same anti-phosphoepitope-SR proteins antibody. Consistent with finding from immunofluorescent experiment, Figure 4.10 that quantification of SR proteins phospho-epitope in CRH-stimulated cells demonstrated that phospho-SRp55 and- SRp30 level in the nucleus was increased, whilst the phosphorylation of SRp75, cytoplasmic SRp55 and SRp40 seemed unaffected by the treatment.

In addition, Akt inhibitor treatment did not change basal phosphorylation status of all SR proteins in the cells, but its inhibition in the stimulated cells has resulted in significant attenuation of CRH- driven activation of SRp55 and SRp30 in the nucleus, suggesting CRH signal transduction was mediated by Akt in this mechanism. In addition, although CRH did not induce SRp40 phosphorylation, the phosphorylation signal was found reduced when Akt activity was inhibited the CRH-stimulated cells. These results further confirm the essential role of Akt in SRPK1-mediated regulation of SR protein phosphorylation demonstrated in previous studies (190). Interestingly, it was also observed that SRp55 seemed to be hyperphosphorylated in basal state, suggesting that its phosphorylation may involve other kinases such as SRPK2.

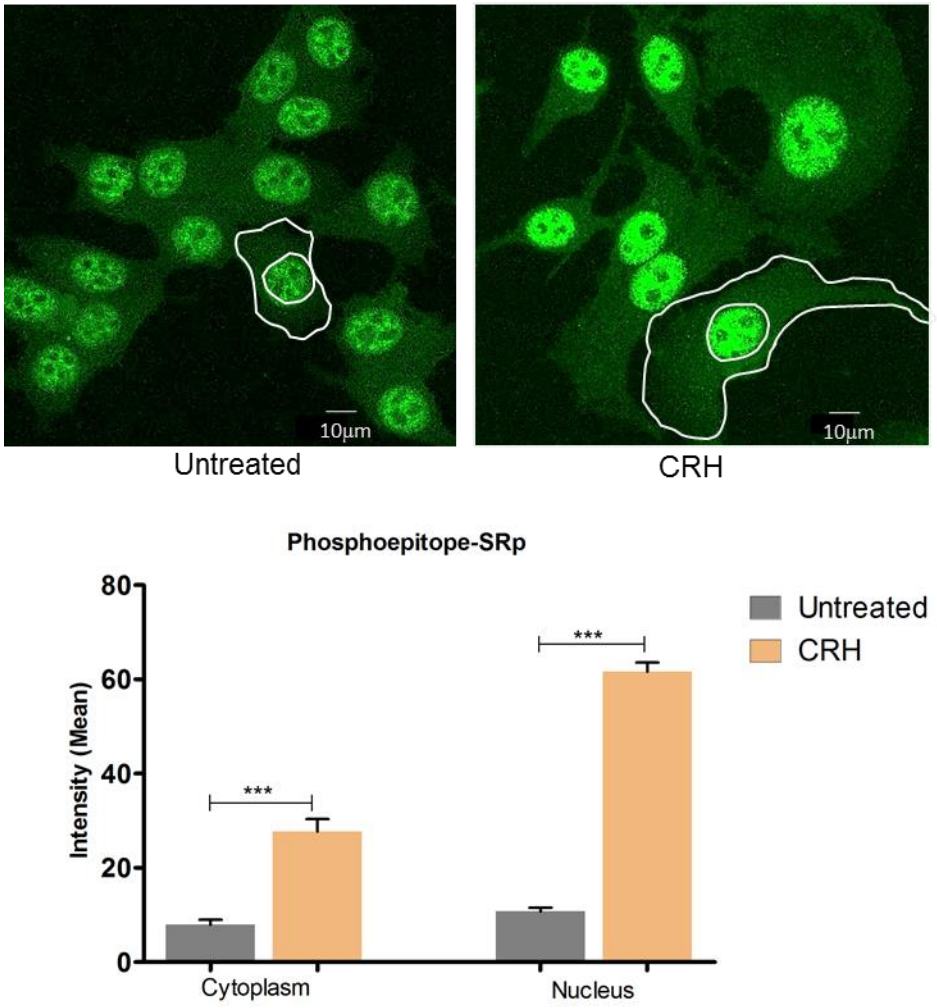


Figure 4.9 Immunostaining results against phospho- SR protein in individual MCF7 cells stimulated with CRH for 24hrs. Signal quantification using ImageJ (free-form shape was drawn around the cyto and nuc region) shows that higher signal of phospho-SR proteins was detected in both cytoplasm and nucleus of the stimulated cells, as compared to untreated cells. Green = phospho-SR protein, n=5. Scale bar = 10µm.

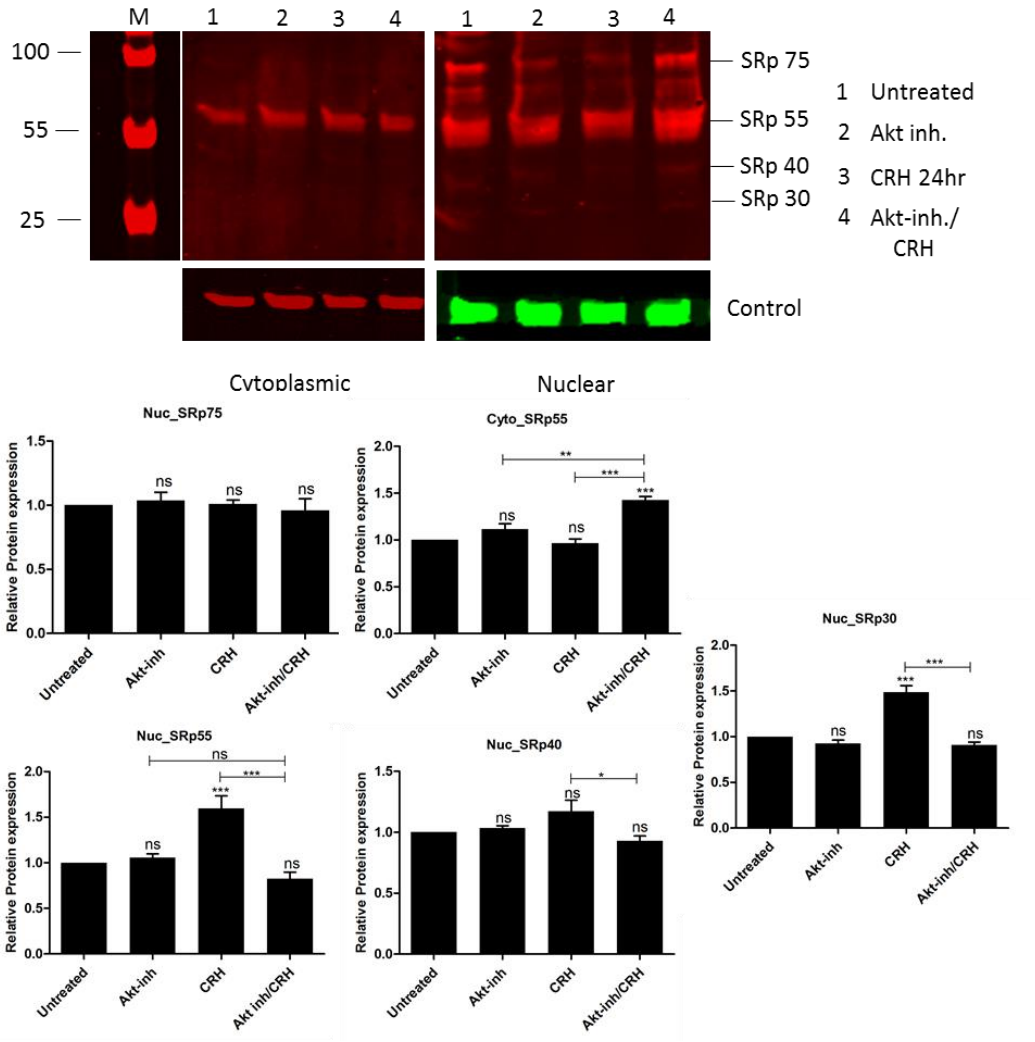


Figure 4.10 Results showed increased signal of phospho- SRp55 and - SRp30 in the nucleus of stimulated cells, but no significant change was observed for phospho-SRp75, cytoplasmic SRp55 and SRp40, as compared to unstimulated cells. Inhibition of Akt kinase resulted in no significant change in the basal phosphorylation signal of all SR proteins. However, reduced Akt kinase in stimulated cells significantly attenuated CRH effects on the phosphorylation level of SRp55 and SRp30 in the nucleus. Although CRH did not elevate SR40 phosphorylation, the inhibition of Akt in stimulated cells has reduced the phosphorylation signal of this protein. Data was normalized to a-tubulin (cytoplasmic control) and Lamin B1 (nuclear control). n=7, *=p<0.05, **=p<0.01, ***=p<0.001, ns= not significant. Representative blots are shown.

Following these differential phosphorylation status of SR proteins, further investigation was done to determine whether these changes were due to increased expression of SRPK1 in the cells. Therefore, MCF7 cells were pre-treated with 10μM SRPK1 inhibitor (SRPIN340) for 2hr, followed by stimulation with 100nM CRH for 24hr. It was demonstrated that basal phosphorylation signal of all nuclear SR proteins was reduced when SRPK1 kinase was inhibited (Figure 4.11). Moreover, when SRPK1 kinase was inhibited in the stimulated cells, CRH was shown to no longer able to elevate SRp55 and SRp30 phosphorylation in the nucleus, suggesting that SRPK1 is indeed involved in the CRH-induced phosphorylation of SR proteins in cellular model of ER+ cells. Although reduced SRPK1 kinase affected basal SRp75 and SRp55 phosphorylation in the nucleus, the signal for phospho-SRp75 and SRp55 was observed increased when SRPK1 activity was reduced in stimulated cells.

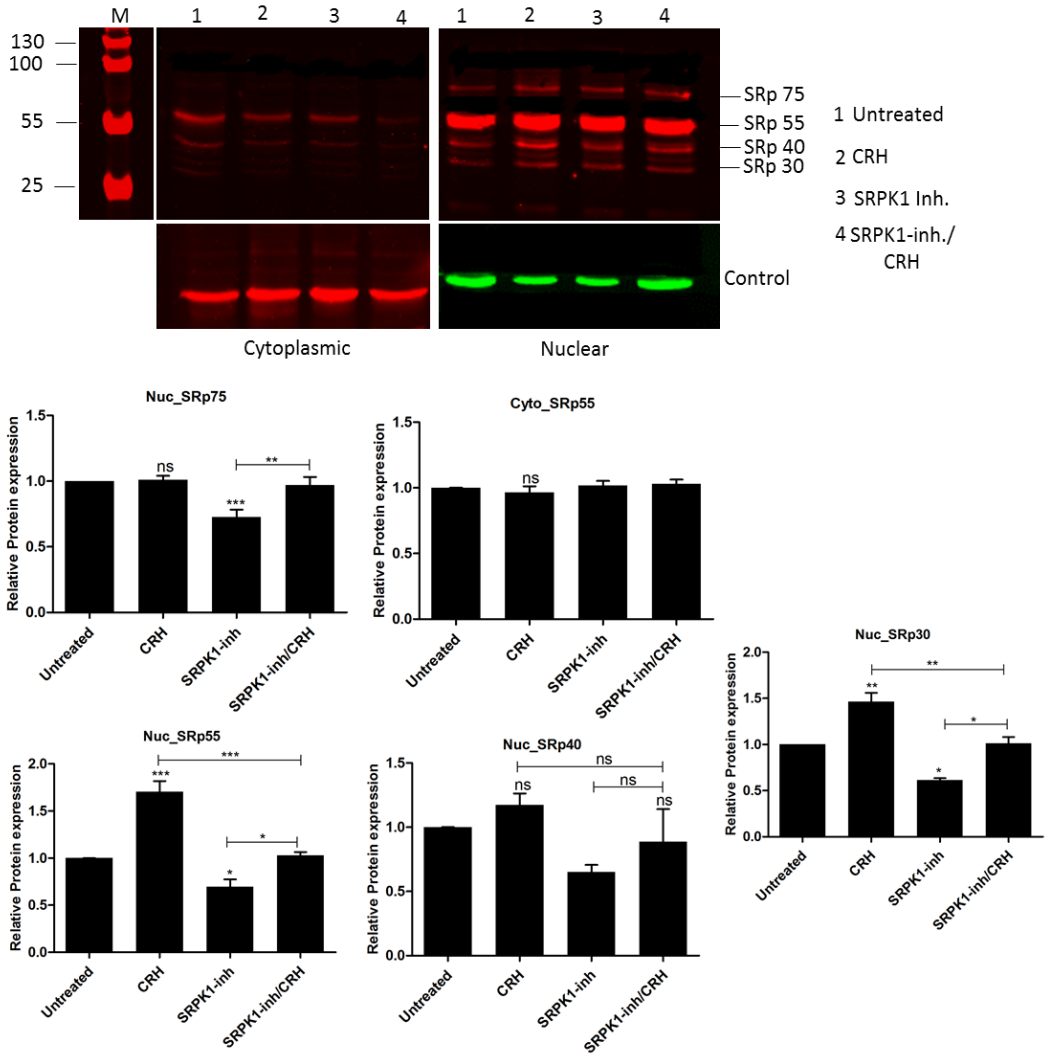


Figure 4.11 Basal phosphorylation of all nuclear SR proteins was found reduced when SRPK1 kinase was inhibited, and the effects of CRH on the phosphorylation of SRp55 and SRp30 was no longer seen when SRPK1 kinase was inhibited in the stimulated cells. Although reduced SRPK1 kinase affected basal SRp75 and SRp55 phosphorylation in the nucleus, the signal for phospho-SRp75 and SRp55 was observed increased when SRPK1 activity was inhibited in the stimulated cells. In addition, CRH was shown to induce phosphorylation of SRp75, nuc-SRp55 and SRp30 in cells where SRPK1 activity was inhibited. Data was normalized to a-tubulin (cytoplasmic control) and Lamin B1 (nuclear control). n=7, *=p<0.05, **=p<0.01, ***=p<0.001, ns= not significant. Representative blots are shown.

Analysis of SR protein phosphorylation in ER- SKBR3 cells by CRH

In the analysis of SRPK1 phosphorylation and nuclear translocation in SKBR3 cell line (Chapter 4.4.4), it was demonstrated that CRH did induce SRPK1 phosphorylation in the cytoplasm, but not nuclear translocation. To investigate the effect of this response to phosphorylation status of SR proteins in stimulated cells. Given that SRPK1 is one of the key kinases responsible in the regulation of SR protein phosphorylation for the activation of splicing program in the nucleus, it was hypothesized that decreased level of phospho-SR proteins would be detected in the cells, primarily in the nuclear fraction. Therefore, SKBR3 cells were stimulated with CRH at 100nM for 24hrs, followed by cyto- nuclear protein extraction and western blot.

The level of phospho-SR proteins was quantified and compared with untreated cells, and results showed that CRH significantly reduced basal phosphorylation level of SRp55, both in cytoplasm and nucleus, as well as nuclear SRp40 (Figure 4.12). In addition, although SRp75 and SRp30 phosphorylation signal seemed decreased as compared to untreated cells, the change was not statistically significant. Apparently, when Akt kinase was inhibited, none of the basal SR proteins phosphorylation level was affected, except the cytoplasmic phospho-SRp55 protein which was reduced as compared to unstimulated cells. Similarly, comparing with stimulated cells, inhibition of Akt activity in the stimulated cells only significantly affected cyto-SRp55 which was shown increased, but the phosphorylation level of the rest of SR proteins was not changed. Notably, the phospho- signal for SRp75, SRp55, SRp40 and SRp30 was shown decreased in these cells as compared to the cells treated with Akt kinase inhibitor alone, suggesting that while CRH signaling in modulating SRPK1-mediated SR protein phosphorylation in SKBR3 cell line may not involve Akt, CRH may have inhibitory role on SR protein phosphorylation when Akt activity is inhibited.

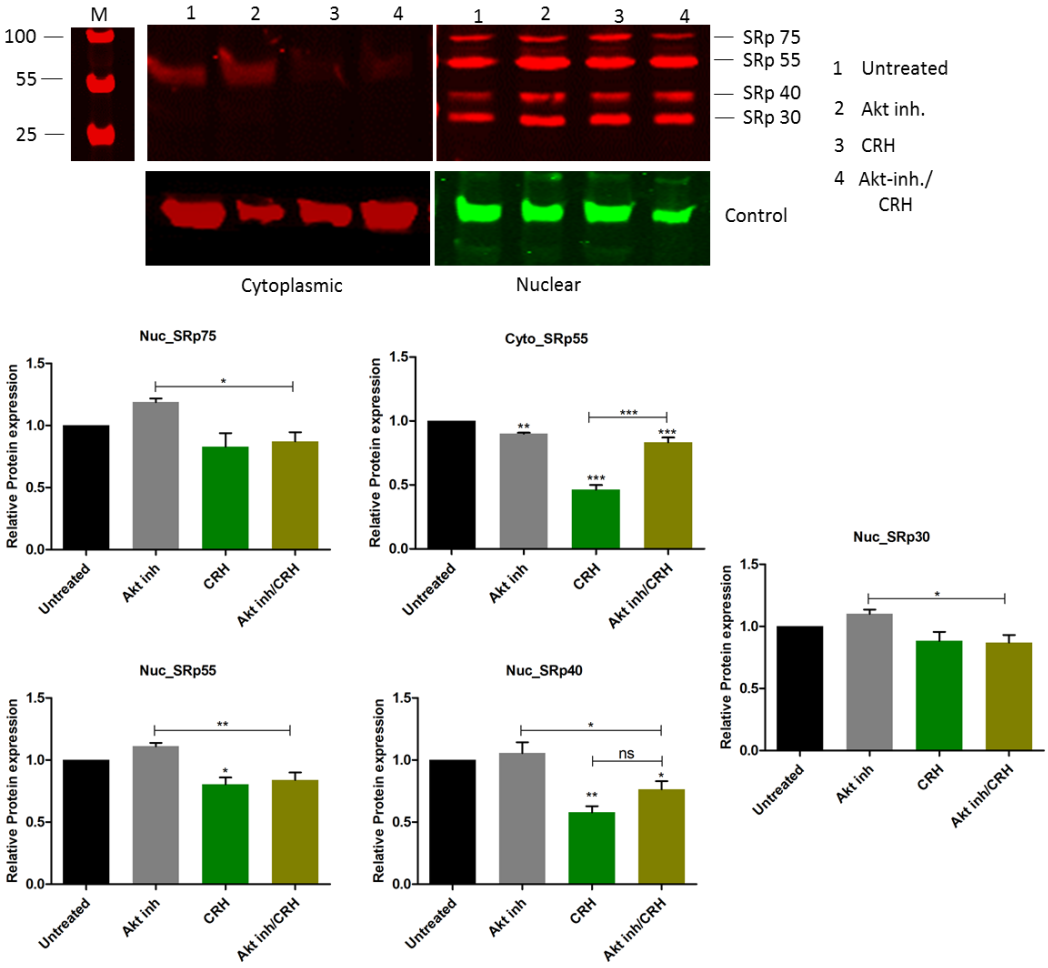


Figure 4.12 Western blot results showed that basal phosphorylation of cyto-SRp55, Nuc-SRp55 and SRp40 decreased in the CRH-stimulated cells. In addition, inhibition of Akt activity did not change much of SR protein basal phosphorylation, except for cyto-SRp55 that was shown reduced. Inhibition of Akt kinase in the stimulated cells also has led to similar response on the SRp phosphorylation level, except for cyto-SRp55 and nuc-SRp40 which was shown increased as compared to CRH-stimulated cells. Furthermore, in cells where Akt was inhibited, CRH significantly reduced SRp75, nuc- SRp55, SRp40 and SRp30 phosphorylation. Data was normalized to a-tubulin (cytoplasmic control) and Lamin B1 (nuclear control). n=7, *=p<0.05, **=p<0.01, ***=p<0.001, ns= not significant. Representative blots are shown.

Next, to examine whether SRPK1 was responsible for the reduced phosphorylation signal of SR proteins, SKBR3 cells were pre-treated with the specific SRPK1 kinase inhibitor (10μM) for 2 hr, before stimulation with CRH at 100nM for 24hrs. Results showed that inhibition of SPRK1 kinase has led to significant decrease of basal phosphorylation level of cyto-SRp55, SRp40 and SRp30, confirming the key role of SRPK1 in regulating basal SR protein as previously shown in several studies (171,185). In addition, when SRPK1 was inhibited in the stimulated cells, further reduction of phospho- SRp55 and SRp40 was detected in the nucleus, suggesting the involvement of SRPK1 in the regulation of Sr protein phosphorylation in the CRH- treated cells (Figure 4.13). Furthermore, phospho-SRp75, cytoplasmic phospho-SRp55 and phospho-SRp30 level showed no significant change in response to the reduced SRPK1 activity in the stimulated cells.

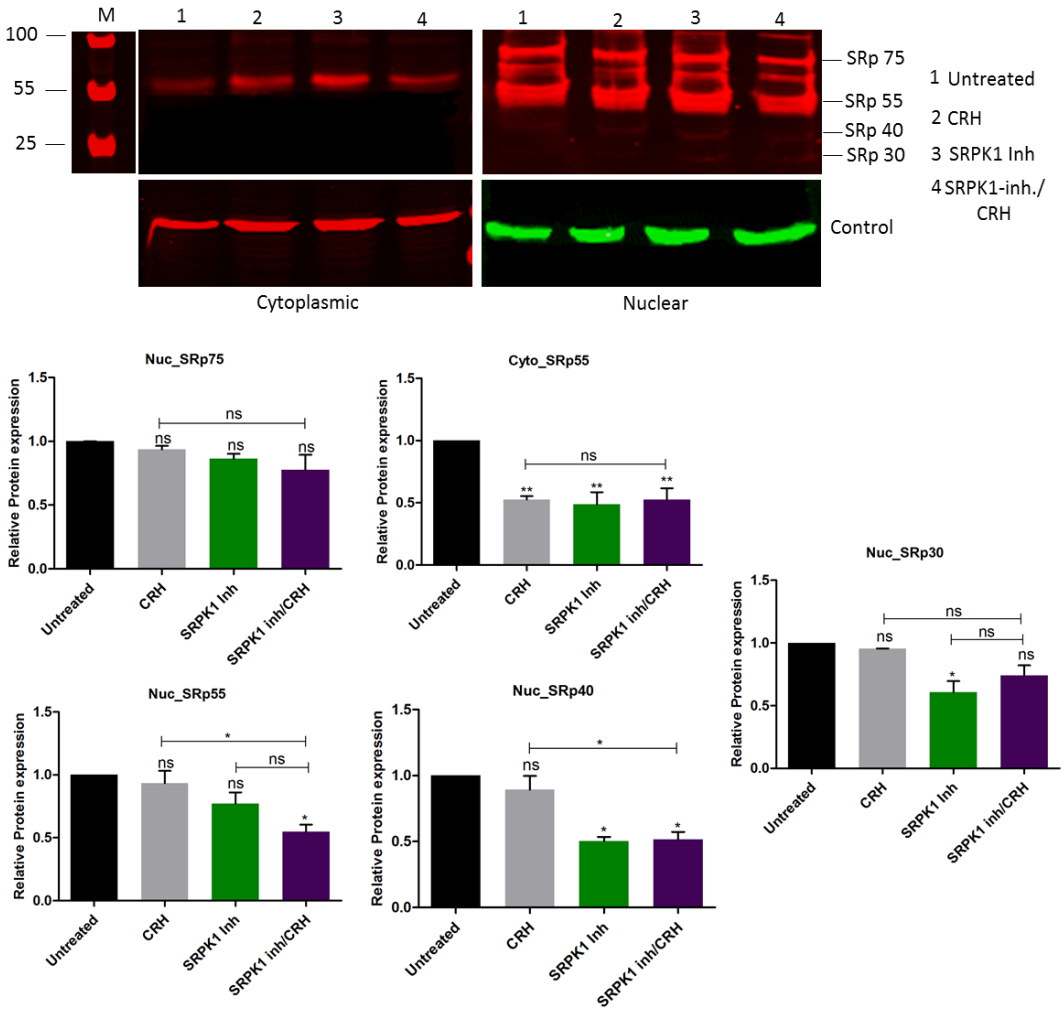


Figure 4.13 Basal phosphorylation level of cyto-SRp55, nuc-SRp40 and nuc-SRp30 was shown reduced when SRPK1 kinase was inhibited, and the inhibition of SRPK1 activity in stimulated cells has led to further reduction of nuc-SRp55 and nuc-SRp40. While other SRps showed reduced signals in this cell, however the change was not statistically significant. In addition, basal phosphorylation level of SRp75 and nuc-SRp55 was not affected by the decreased activity of SRPK1 in the cells. Data was normalized to a-tubulin (cytoplasmic control) and Lamin B1 (nuclear control). n=6, *=p<0.05, **=p<0.01, ***=p<0.001, ns= not significant. Representative blots are shown.

4.5 Detection of splicing repressor, hnRNPA1 protein and mRNA expression in MCF7 and SKBR3 cell lines

Several studies have demonstrated that splicing repressor protein, hnRNPA1 was found to be highly upregulated in cancer cells as compared to its normal counterpart, and its role in the regulation of splicing mechanism has also been well-studied (253,336). Therefore, to investigate the expression of hnRNPA1 at both protein and mRNA level in response to external stimuli, estrogen (E2) and CRH, MCF7 cells were stimulated with E2 (10nM) and CRH (100nM) for 24hrs, followed by protein and RNA extraction.

It was demonstrated that the expression of hnRNPA1 protein and mRNA expression were significantly increased by approximately 2-3 folds when MCF7 cells were stimulated with either E2 or CRH [Figure 4.14A (i)(ii) and Figure 4.14B (i)(ii)]. Furthermore, basal expression of hnRNPA1 protein was shown upregulated when Akt kinase was inhibited in the cells. Interestingly, inhibition of Akt kinase activity in E2- or CRH- stimulated cells has significantly abolished the effects of E2 and CRH on both hnRNPA1 protein and mRNA expression, suggesting that upregulation of hnRNPA1 protein expression and mRNA transcription by both stimuli were Akt dependent. Interestingly, the pattern of hnRNPA1 expression in response to E2 and CRH seemed similar with that of SRPK1 expression, as shown in Chapter 4.3.2 and Chapter 4.4.2.

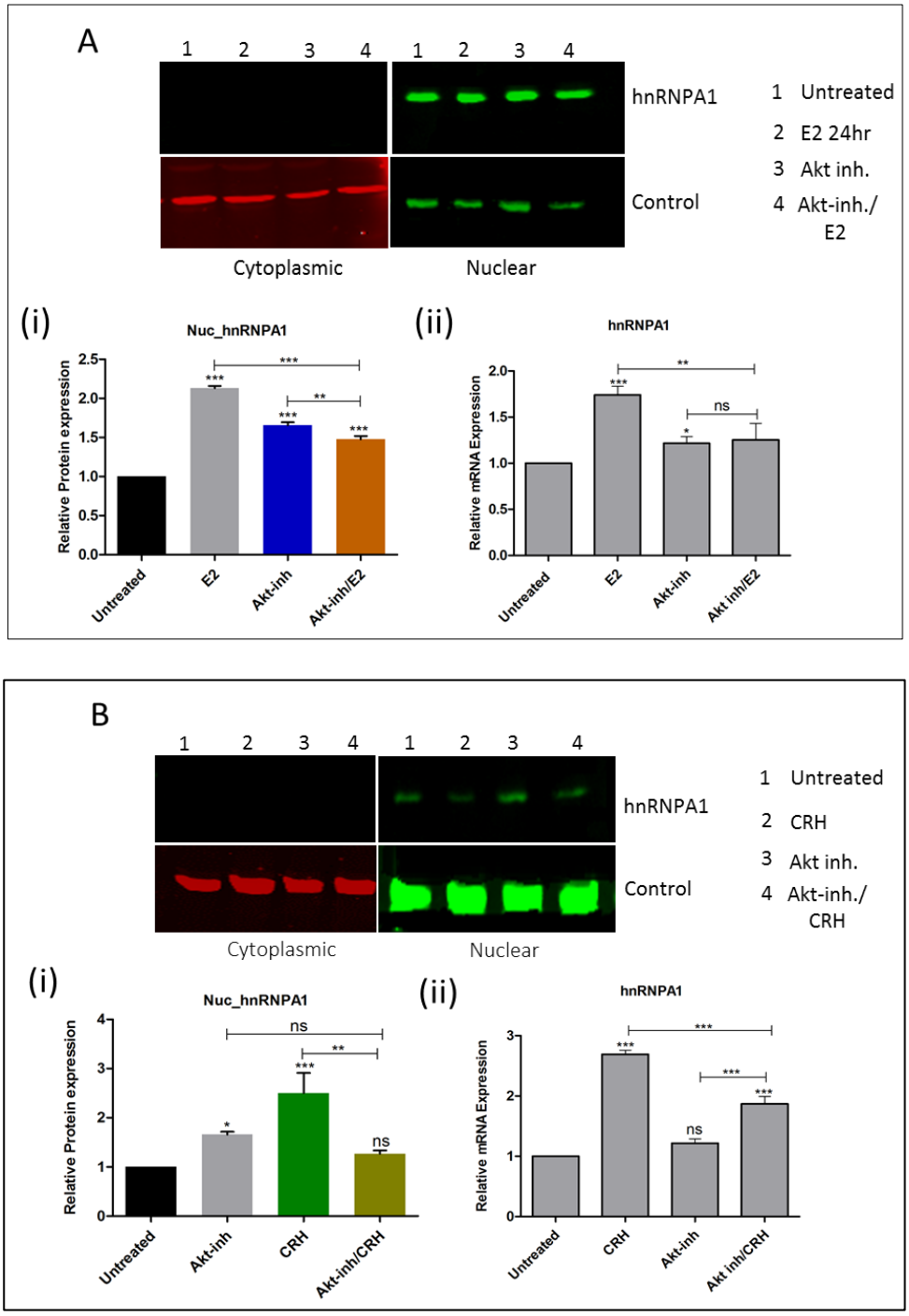


Figure 4.14 MCF7 cells were stimulated with **(A)** 10nM estrogen (E2), and **(B)** 100nM CRH, both for 24hrs. Both **A(i) & B(i)** hnRNPA1 protein expression and **A(ii) & B(ii)** mRNA expression increased when cells were stimulated with either E2 or CRH. Inhibition of Akt kinase resulted in increased basal expression of hnRNPA1, however, reduced Akt kinase in the stimulated cells has attenuated E2 and CRH effects on hnRNPA1 protein and mRNA expression.

To investigate whether SRPK1 plays role in this signaling pathway that led to upregulation of hnRNPA1 expression in MCF7 cell line, cells were pre-treated with SRPK1 kinase inhibitor at 10μM for 2hr, before stimulation with CRH at 100nM for 24hrs. Figure 4.15 (i)(ii) shows that while reduced SRPK1 kinase did not significantly affect hnRNPA1 protein and mRNA expression, the inhibition of SRPK1 activity in CRH stimulated cells resulted in significant downregulation of nearly 2-fold of hnRNPA1 protein, and ~1.5-fold of its mRNA expression, suggesting that SRPK1 may not involve in basal regulation of hnRNPA1 expression, but it plays essential role in the regulation of hnRNPA1 expression in stimulated cells.

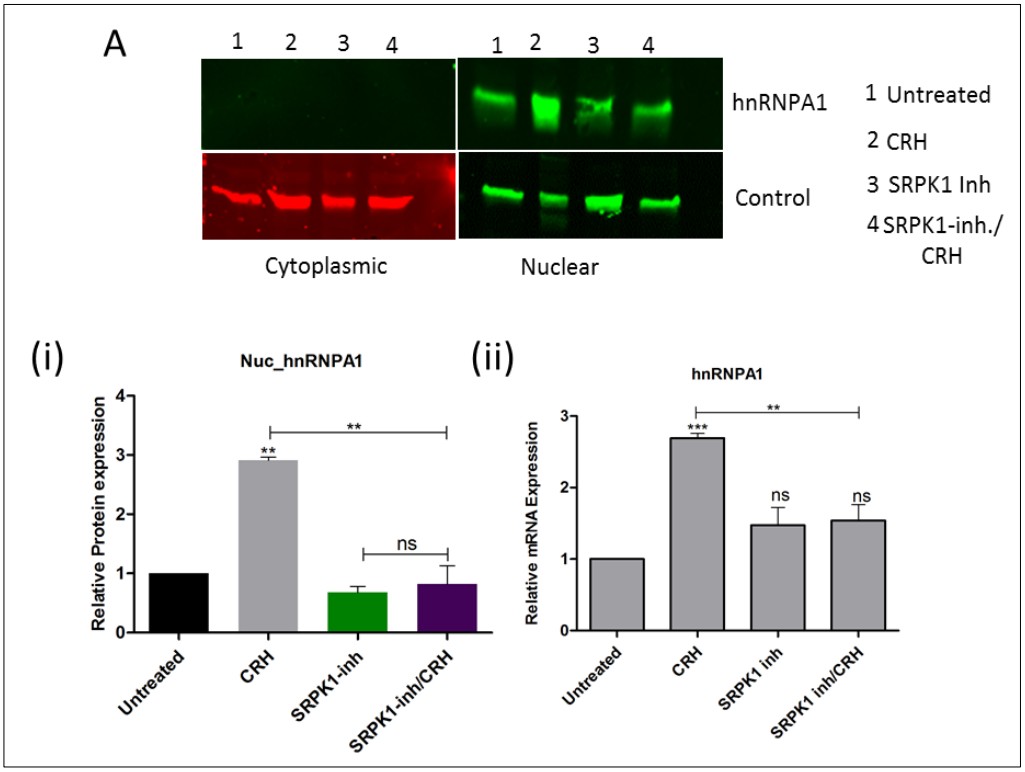


Figure 4.15 MCF7 cells were pre-treated with the specific SRPK1 inhibitor (SRPIN340) for 2hr, before stimulation with 100nM CRH for 24hrs. Results showed that inhibition of SRPK1 activity has no significant effect on hnRNPA1 **(i)** protein, and **(ii)** mRNA expression in the cells. However, reduced SRPK1 kinase in the stimulated cells has significantly abolished CRH effects on hnRNPA1 expression both at protein and mRNA level.

Furthermore, the effects of CRH on the regulation of hnRNPA1 expression was also investigated in SKBR3 cell line. Cells were treated with CRH at 100nM for 24hrs, before protein and mRNA extraction, followed by western blot and RT-qPCR for the analysis of hnRNPA1 protein and mRNA transcript, respectively. Results showed that CRH has no effect on basal hnRNPA1 expression at both protein and mRNA level, in SKBR3 cells [Figure 4.16 (i)(ii)]. Interestingly, in cells where Akt was inhibited, CRH was shown to have no effect on hnRNPA1 protein and mRNA expression.

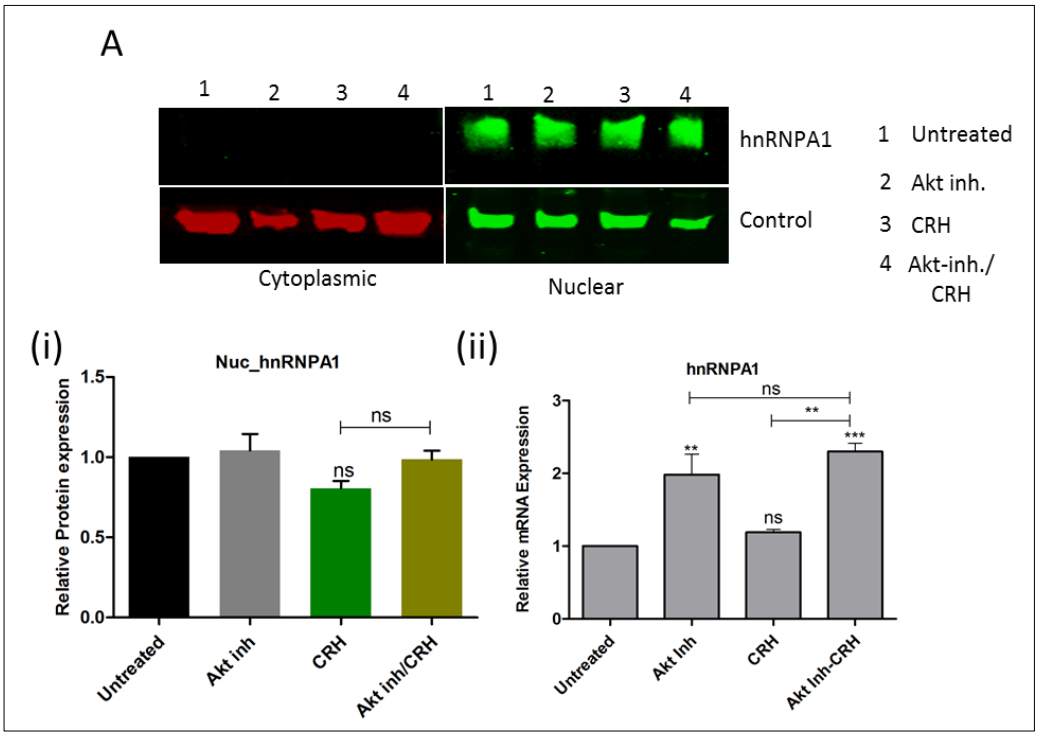


Figure 4.16 SKBR3 cells were stimulated with 100nM CRH for 24hrs. Results showed that CRH did not affect basal expression of hnRNPA1 (i) protein, and (ii)mRNA. In addition, inhibition of Akt kinase upregulated mRNA expression of hnRNPA1 in untreated cells, and reduced Akt kinase in stimulated cells also led to increased hnRNPA1 mRNA transcription. Furthermore, CRH has shown no effect on hnRNPA1 in cells where Akt is inhibited.

4.6 Effects of differential splicing factors expression on CD44 mRNA splicing

Following the activation of SRPK1 by E2 and CRH, which directly or indirectly led to differential expression of splicing factors, Serine- Arginine proteins (SRp) and hnRNPA1, the effects of these SRPK1-mediated changes on the choice of mRNA transcript splice site was examined in both cell lines, MCF7 and SKBR3. Previous studies reported that apart from nuclear SRPK1 translocation, differential phosphorylation and distribution of SR proteins and hnRNPA1 can trigger alternative splicing event and contribute to the re-programing of pre-mRNA splicing resulting in the accumulation of abnormal splice isoform in cells (171). In addition, hnRNPA1 has been shown to be involved in the splicing regulation of CD44, a cell adhesion molecule important in cell-to-cell junction (337). CD44 splice isoform, CD44v6 and CD44s were observed to be overexpressed in invasive breast tumours and have been associated with the cancer metastasis process (145,260,338).

Therefore, using CD44 as a splicing reporter of altered splicing event in response to the activation of SRPK1 splicing kinase and splicing factors in the stimulated cells, MCF7 cell lines were stimulated with E2 (10nM) and CRH (100nM) for 24hrs before RNA extraction followed by RT-qPCR to analyze the splicing pattern of CD44 mRNA and the production of its splice variants, CD44s and CD44v6. Primers specific for total CD44, CD44s and CD44v6 sequences were used to quantify the level of these transcripts in the cells (Figure 4.17).

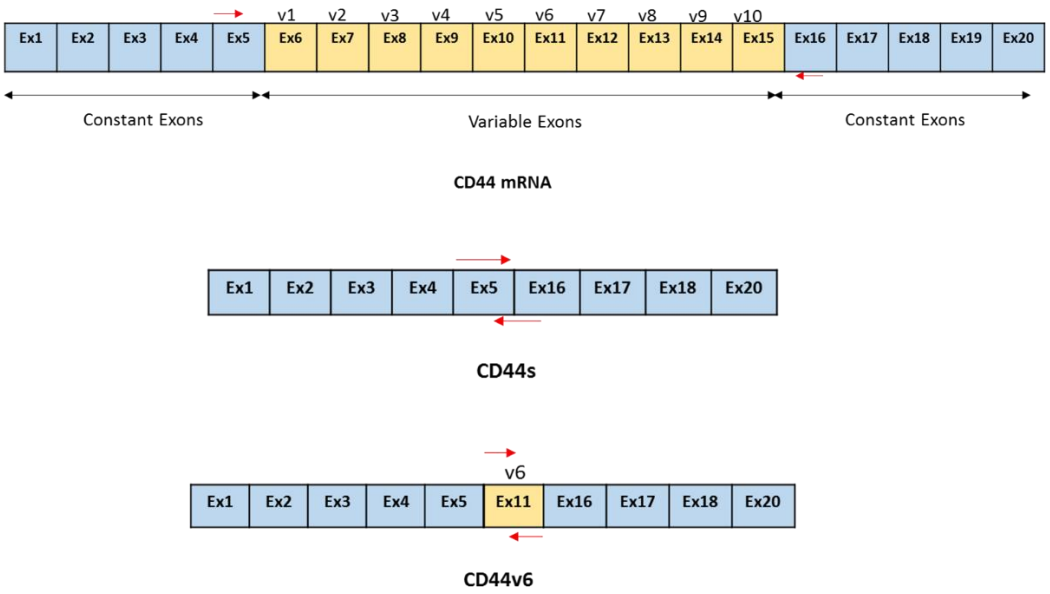


Figure 4.17 Map of CD44, CD44s and CD44v6 mRNA transcript showing the primer pairs (red arrow) used in the RT-qPCR analysis of mRNA level in this study. CD44 total primer sequence covered the regions in constant exon 5 and exon 16. CD44s was based on the exon 5 sequence (forward) and 14 nucleotides of 5' of exon 16 + 7 nucleotides of 3' of exon 5 sequence (Reverse), while CD44v6 primers were based on the sequence specific to exon 11.

Results from MCF7 cells stimulated with E2 showed that basal level of CD44s was increased by 50% as compared to unstimulated cells, but there was no significant change in CD44v6 (Figure 4.18A). Furthermore, when the stimulated cells were pre-treated with Akt inhibitor, the level of CD44s and CD44v6 was further increased, suggesting that Akt attenuates E2 effects in inducing CD44 splice variants production. In addition, although reduced Akt activity has led to decreased basal CD44s level only, however Akt kinase inhibition in stimulated cells has caused nearly 3-fold increase in CD44s, and 2-fold increase of CD44v6 mRNA transcript, suggesting that while the production of CD44s is dependent on Akt, E2 effects on both CD44s and CD44v6 is enhanced when Akt activity was inhibited in cells.

In CRH- stimulated cells, it was demonstrated that inhibition of Akt kinase did not affect the production of both CD44s and CD44v6 as compared to untreated cell (Figure 4.18B). Furthermore, results from stimulated cells showed that CRH showed no effect on CD44s but upregulated the expression of CD44v6. However, inhibiting Akt activity in the stimulated cells resulted in marked increase of CD44s, by nearly 10 times, and CD44v6 by 4 times, suggesting that CRH effect in inducing aberrant CD44 pre-mRNA splicing is amplified when Akt kinase is inhibited in the cells. Overall, this abnormal accumulation of alternatively spliced CD44 mRNA transcript was observed to occur in a manner that correlated with increased SRPK1 nuclear translocation in E2- and CRH-stimulated cells, and with reduced hnRNPA1 expression in stimulated cells when Akt was inhibited, suggesting that splicing repressor effect of hnRNPA1 is Akt dependent, and can only be seen when it is activated by either E2 or CRH in the MCF7 cells. These findings also suggest that decision for splice site selection during pre-mRNA splicing involves interactions of multiple molecular factors such as splicing kinase and both splicing promoters (e.g. SR proteins) and splicing repressor (hnRNPA1).

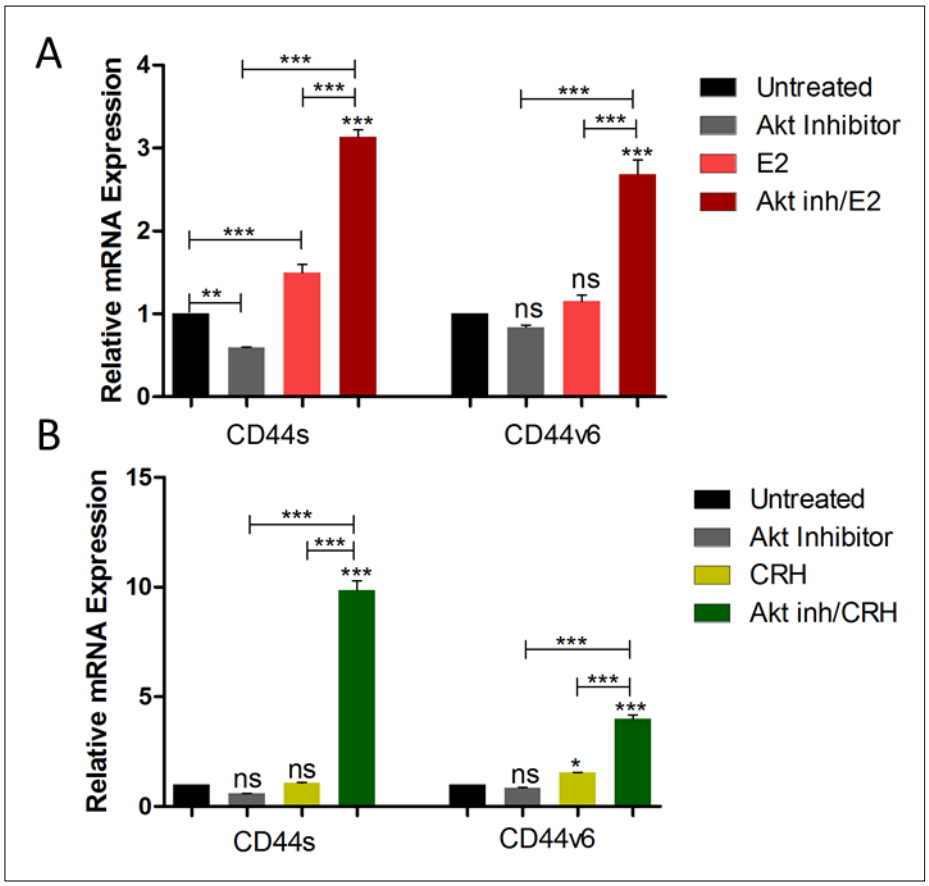


Figure 4.18 CD44 splice variants analysis in MCF7 cell line. (A) Cells were stimulated with E2 (10nM) for 24hrs. Results showed that E2 induced CD44s production, but not CD44v6. Meanwhile, inhibition of Akt kinase reduced basal level CD44s, and inhibition of Akt in the stimulated cells resulted in significant increase of both CD44s and CD44v6 in the cells, suggesting the E2 effect on CD44s and CD44v6 was enhanced in the presence of Akt inhibitor. (B) Stimulation of MCF7 cells with CRH (100nM) for 24hrs did not affect basal level of CD44s but has slightly increased the level of CD44v6. In addition, while inhibition of Akt showed no effect on basal level of both CD44 isoforms, its inhibition in the stimulated cells has resulted in significant increase of CD44s and CD44v6 mRNA transcript.

The effect of reduced SRPK1 activity in the production of CD44 splice isoforms was also investigated. MCF7 cells were pre-treated with the specific SRPK1 inhibitor (10μM) for 2hr, before stimulation with CRH (100nM) for 24hrs, followed by RNA extraction for RT-qPCR analysis of CD44 splice variants level. Results showed that reduced SRPK1 kinase has significantly reduced basal level of CD44v6, but not CD44s (Figure 4.19). Interestingly, reduced SRPK1 in the stimulated cells resulted in the increased of CD44s mRNA transcript, but significantly decreased the level of CD44v6. In addition, CRH was shown to induce CD44s production in cells where SRPK1 was inhibited, suggesting that CD44s production can only be induced by CRH when SRPK1 is reduced. Overall, these results suggest that SRPK1 may involve in the signaling mechanism leading to the production of CD44v6, and that the accumulation of CD44s might require the action of other factors as well. Similar findings was shown in other studies (171), in which depleting SRPK1 alone in cell has little effect but knocking down both SRPK1 and SRPK2 kinases resulted in reduced accumulation of stress-induced mRNA isoform in cells.

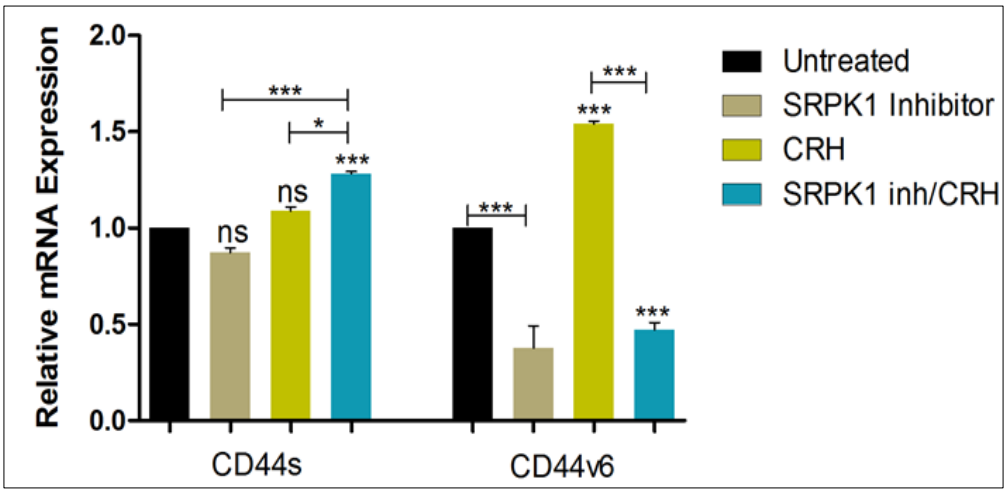


Figure 4.19 CD44 splice variants analysis in MCF7 cell line. Inhibition of SRPK1 reduced basal CD44v6 level, but inhibition of this kinase in CRH-stimulated cells resulted in significant increase of CD44s, but significant decrease in CD44v6 level in the cells.

Following the demonstration of CD44 splice isoforms accumulation in MCF7 cell line, similar study was investigated in SKBR3 cell line. Cells were treated with CRH (100nM) for 24hrs, followed by RNA extraction and RT-qPCR analysis of CD44 isoforms mRNA transcript. Results showed that reduced Akt kinase did not affect much of the CD44s and CD44v6 level in untreated cells (Figure 4.20A). In addition, CRH was found to reduce basal level of CD44s mRNA by 50%, but significantly induced the production of CD44v6 by 4-fold in the cells. Interestingly, inhibiting Akt in these stimulated cells resulted in increased CD44s level, but 4-fold decreased of CD44v6, suggesting that CRH effect on the CD44v6 production was via Akt. In addition, this finding also suggests that Akt inhibitor reversed CRH effect on the CD44s mRNA transcript production in SKBR3 cell line.

Furthermore, basal level of both CD44s and CD44v6 was shown decreased when SKBR3 cells were treated with SRPK1 kinase inhibitor (10μM) for 2hr (Figure 4.20B). It was also found that inhibiting the SRPK1 kinase in the stimulated cells resulted in increased CD44s production, but marked decrease of CD44v6, suggesting that CRH effect on CD44v6 production was mediated by SRPK1, and similar to Akt, SRPK1 inhibitor was also shown to reverse CRH effect on CD44s. Altogether, these results showed that CRH affect SKBR3 cells, a model for ER- cells, differently, in which CD44v6 expression showed positive correlation with hnRNPA1 expression, while CD44s expression was regulated in an opposite way, where it was negatively correlated with hnRNPA1 level in cells. The summary of CD44s and CD44v6 production in both MCF7 and SKBR3 cell line is shown in Table 4.1.

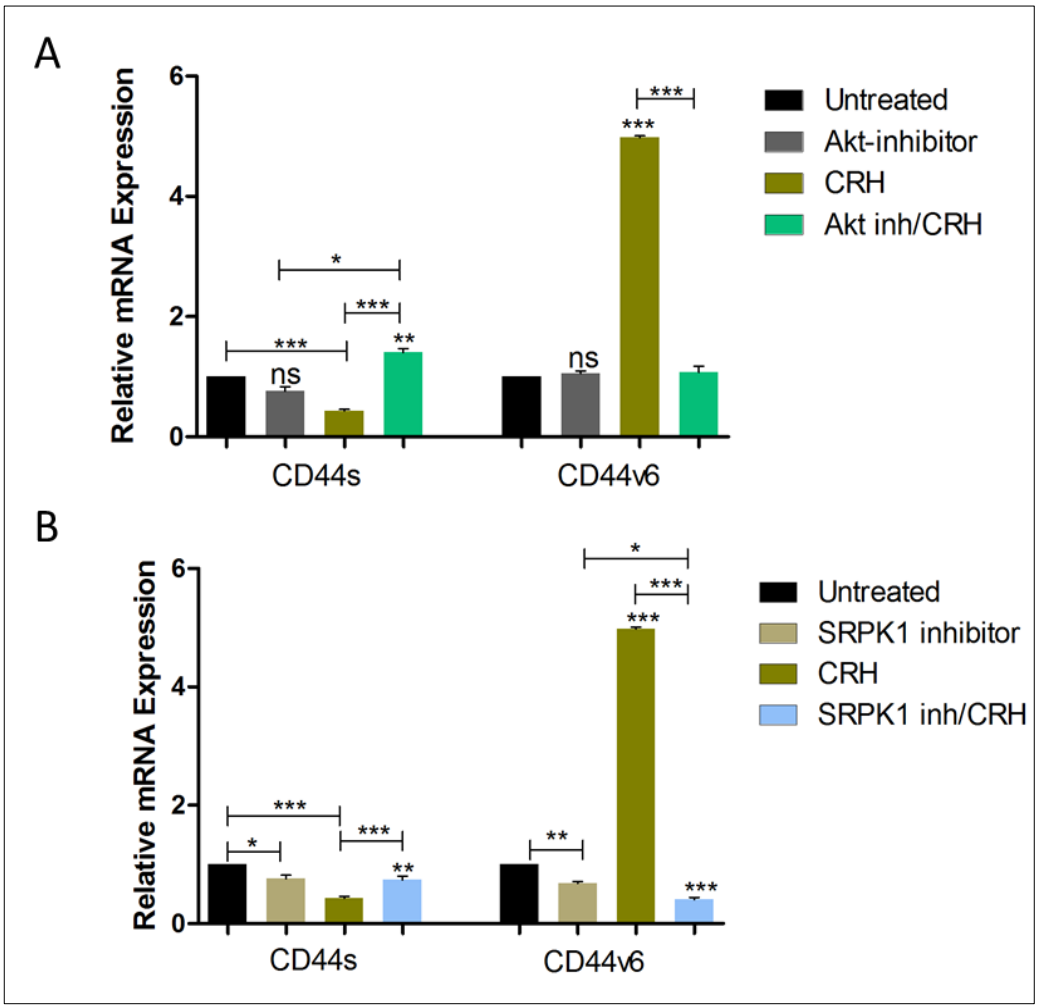


Figure 4.20 CD44 splice variants analysis in SKBR3 cell line. (A) Stimulation of SKBR3 cells with CRH (100nM) for 24hrs reduced basal CD44s mRNA level, but significantly induced CD44v6. While inhibition of Akt did not affect basal CD44s and CD44v6 level, its inhibition in the stimulated cells has resulted in increased CD44s level, but reduced CD44v6 mRNA transcript in the cells. (B) Inhibition of SRPK1 reduced basal level of both CD44 isoforms, however inhibition of this kinase in CRH-stimulated cells resulted in significant increase in CD44s and significant decrease in CD44v6 level in the cells.

Table 4.1 Summary of differential level of CD44s and CD44v6 in MCF7 cell line (ER+ cells) and SKBR3 cell line (ER- cells) in response to various treatments.

Cellular models	ER+		ER-	
Treatments /Targets	CD44s	CD44v6	CD44s	CD44v6
Akt Inh	↓	–	n.a	n.a
E2	↑	–	n.a	n.a
Akt Inh/E2	↑↑	–	n.a	n.a
Akt Inh	–	–	–	–
CRH	–	↑	↓	↑↑↑
Akt Inh/CRH	↑↑↑	↑↑	↑	↓↓↓
SRPK1 Inh	–	↓	↓	↓
CRH	–	↑	↓	↑↑↑
SRPK1 Inh/CRH	↑	↓↓	↑	↓↓↓

Discussion

In the present study, the potential role of hormones (estrogen and CRH) in regulating alternative splicing event in ER+ and ER- breast cancer cells was investigated, following the preliminary finding that estrogen could trigger overexpression of splicing kinase, SRPK1 in the ER+ cells. Interrogation on the hormone-driven upregulation of SRPK1 on the targets downstream of SRPK1 was carried out. This was done by analysing the phosphorylation level of serine-arginine (SR) proteins in cytoplasmic and nuclear compartments, the expression of splicing repressor, hnRNPA1 and the mRNA expression of CD44 splice variants as reporter genes for alternative pre-mRNA splicing process induced by estrogen and corticotrophin-releasing hormone, CRH in the cells.

Estrogen-driven alternative splicing (AS) in ER+ breast cancer cells

The analysis of phospho-SRPK1 in cytoplasmic and nuclear compartments demonstrated that most of SRPK1 proteins were predominantly found in the nuclear compartment of the cells, suggesting that E2 does not only induce SRPK1 expression but also its translocation from cytoplasm to the nucleus. This finding fit with current understanding of SRPK1 action, in which it phosphorylates SR proteins in the nucleus and triggers the assembly of spliceosome units for the initiation of mRNA splicing events. In addition, studies have shown that nuclear level of SRPK1 protein induces accumulation of SR proteins and changes in the level of splicing factors are frequently found to correlate with alternative splicing events in malignant tumor cells (339–341).

Notably, uninterrupted activity of SRPK1 kinase is important to ensure precise regulation of SR proteins phosphorylation for an accurate mRNA splicing to take place in the cells. Previous studies have shown that altered cellular distribution of SRPK1 in response to osmotic stress resulted in differential phosphorylation of SR proteins, thus leading to altered splicing of E1A gene (171). The analysis of phosphorylation level of

several SR proteins (SRp75, 55, 40 and 30) in this study demonstrated that E2-induced SRPK1 protein nuclear translocation led to increased level of phospho- SRp 30, whilst the phosphorylation level of SRp75, 55 and 40 remained unaffected. Of note, apart from SR proteins, which are also called splicing inducers, the mechanism of alternative splicing is also depends on various other factors, such as the activity of splicing repressors. One of the best studied is hnRNPA1, whose expression is found to be differentially regulated in several cancers (337,342–345). Although proteome analysis showed that E2 treatment led to the downregulation of one of the hnRNPs family proteins (i.e. PTPB1), protein and gene expression analysis demonstrated that estrogen significantly increased both hnRNPA1 protein and mRNA expression which was also positively correlated with SRPK1 pattern of expression, indicating that hnRNPA1 is one of E2 signaling targets and E2 can stimulate hnRNPA1 transcription in breast cancer cells. In parallel with this, a recent study showed that estrogen can elevate the expression of hnRNPA1 in skin cancer, resulting in the reduction of oncogene, MDM2 expression and thus inhibiting carcinogenesis in melanoma (346).

Following the upregulated activity of splicing kinase and the above-mentioned splicing factors, the effects of altered splicing molecules on the production of CD44 splice variants, CD44s and CD44v6, which are generated as a result of aberrant alternative splicing of CD44 in cells were investigated. This study found that, CD44s level was increased in response to E2 treatment suggesting positive correlation between increased SRPK1 expression and CD44s production as shown in Figure 4.21. In addition, increased level of hnRNPA1 did not seem to repress this alteration in CD44 splicing, suggesting that its action in the cells may not directly involved in CD44 splicing, but it may activate or block other molecules during the event that causes an increase level of CD44s and CD44v6.

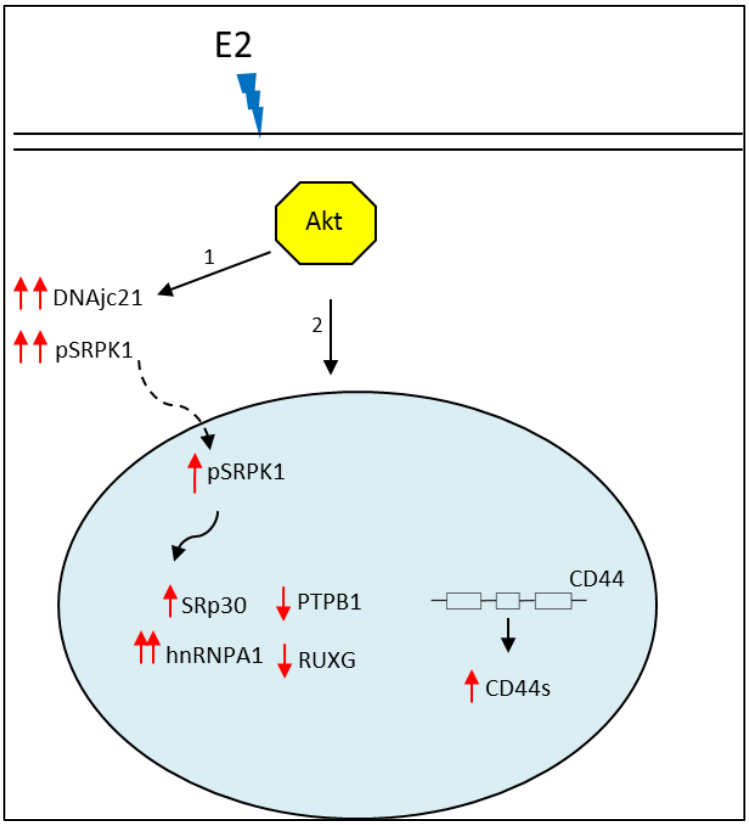


Figure 4.21 Illustration of E2 effects on splicing factors and CD44 pre-mRNA splicing in ER+ cells.

Previous studies have suggested that ER signaling in cells is also controlled by post- translational modifications such as phosphorylation. Changes in phosphorylation status of ERα and ERβ by kinases such as MAPK and AKT/PKB can lead to ERα dysfunction in the pathogenesis of breast cancer (327). Furthermore, Akt has also been demonstrated to regulate SRPK1 activity in the cells, and this connection between activated AKT and SRPKs (AKT-SRPKs-SR pathway) is demonstrated to play a central role in most of the induced splicing events in EGF-treated cells (169,170,190). In addition, other studies also showed AKT act as one of SRPKs effectors. For example, SRPK1 has been demonstrated to mediate TGFβ-induced proliferation by regulating Akt and JNK phosphorylation in oesophageal carcinomas (347). Moreover, overexpression of SRPK1 in hepatocellular carcinoma was demonstrated to induce concurrent phosphorylation of PI3K (p110a subunit), indicating functional link between SRPK1 and PI3K/AKT signaling (172,348). In addition to this, studies also suggested that abnormal expression of SRPK1 will interfere with PHLPP-mediated dephosphorylation of AKT, thus leading to constitutive activation of AKT (186). These bidirectional relationships between SRPKs and AKT, and the ability of estrogen to modulate SRPK1 activity suggests that E2 signaling can cross-talk with SRPK1 signaling pathway, hence triggering activation of molecules important for cellular functions.

Therefore, to determine the role and position of AKT in this E2- mediated SRPK1 signaling activation, stimulated cells were pre-treated with Akt inhibitor, MK2206. Notably, it was found that reduced AKT kinase activity significantly dampened the effects of E2 on SRPK1 activation (measured by phospho-SRPK1 protein antibody), which subsequently resulted in reduced detection of phospho-SR proteins signals in the cells, especially SRp40 and SRp30, as illustrated in Figure 4.22. Interestingly, both hnRNPA1 protein and mRNA expression were also found significantly decreased, which may suggest AKT-SRPK1-hnRNPA1 network in this E2- induced splicing signaling event in ER+ breast cancer cells. Previous studies showed that E2 can upregulate hnRNPA1 expression resulting in the prevention of carcinogenesis in melanoma (346). Furthermore, studies in endometrial cells demonstrated that E2 may indirectly upregulated hnRNPA1 by

inducing the expression of Pyruvate Kinase M splice variant, PKM2 via c-Myc-hnRNPA1 axis (349), as c-Myc has been shown to induce the transcription of hnRNPA1 in lung, pancreatic and breast cancer (350–353). In other studies, down-regulation of hnRNPA1 has also been found to promote breast cancer progression from non- malignant to the malignant type (345). Furthermore, degradation of hnRNPA1 together with activation of SRPK1 has been to affect pre-mRNA splicing in HeLa cells, and subsequently contribute to enhanced cell migration (354). Therefore, results from this study regarding the pattern of hnRNPA1 expression suggests that E2 may induce hnRNPA1 expression through signaling mechanism involving Akt.

Interestingly, reduced level of key splicing event regulator SRPK1 in these cells did not lead to reduced accumulation of CD44 splice isoform as hypothesized, instead, the expression of both CD44s and CD44v6 was found further increased, although Akt was initially shown to play a role only in the production of basal CD44s. This may probably be due to the decreased level of hnRNPA1, which acts by antagonizing the function of serine-arginine (SR) proteins. Therefore, more SRps can bind to splicing enhancer motif and trigger the assembly of spliceosome for the splicing event to initiate. In addition, the inhibition of Akt may affect hnRNPA1 activity, thus affect its splicing repressor function in the cells, as Akt has also previously been shown to indirectly involve in modulating hnRNPA1 activation (355). This finding somehow suggests that any shifting in the E2-induced AKT-SRPK1-hnRNPA1 network may cause more detrimental effects to the cells, which in this case, higher level of CD44 splice isoforms was generated in the cells.

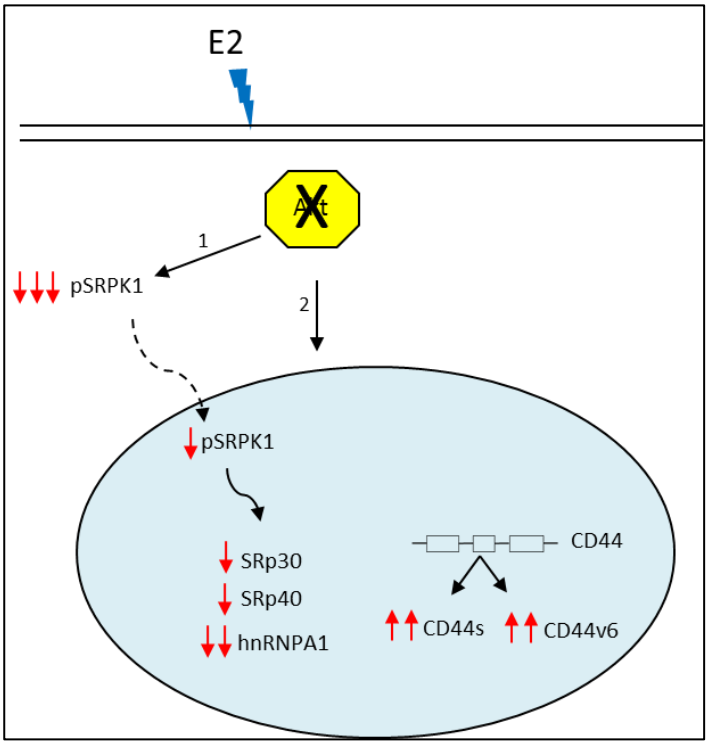


Figure 4.22 Illustration of differential expression of splicing factors and CD44s and CD44v6 splice variants when E2- stimulated cells were pre-treated with Akt inhibitor

CRH-driven alternative splicing (AS) in ER+ and ER- breast cancer cells

Following the finding that estrogen (E2) can regulate the SRPK1- mediated alternative splicing mechanism in ER+ MCF7 breast cancer cells, further investigations were carried out to assess the impact of one of stress-molecules, Corticotrophin- releasing hormone (CRH) in the pathogenesis of cancer via AS mechanism in the cells. In addition, Akt, which was shown to regulate SRPK1 phosphorylation is also regulated by CRH. Therefore, CRH was used as extracellular stimulus to investigate its effect on SRPK1 status in ER+ and ER- cells. The actions of CRH have been shown to be involved in tumorigenesis since the presence of CRH family peptides and their receptors were detected in several cancer cells, including breast cancer (101,103,137,356). To date, studies have reported the role of CRH receptors (137,356) and the alternatively spliced CRHR1 receptors (141) in cancer, but the mechanism by which CRH can potentially facilitate cancer progression by regulating the activity of splicing molecules is not yet explored. In addition, as it was shown that AKT can regulate SRPK1 phosphorylation, and AKT is one of the kinases regulated by CRH, the effects of CRH on SRPK1 status in MCF7 cell line, a cellular model for ER+ cell were investigated. Furthermore, as aberrant splicing is frequently found in estrogen-responsive breast cancers, and for comparison, this study was also conducted in estrogen receptor- negative (ER-) breast cancer cell line SKBR3.

As illustrated in Figure 4.23, this study found that CRH can upregulate SRPK1 phosphorylation in a similar fashion as estrogen did in ER+ MCF7 cell line. The analysis of phospho- SRPK1 proteins in cytoplasm and nuclear compartments of the cells showed that signals for SRPK1 proteins were mostly detected in the nucleus, suggesting that CRH may also stimulate nuclear translocation of SRPK1 protein. This also suggests that CRH and E2 uses similar pathway in modulating SRPK1 activity in ER+ cells. In addition, immunostaining of phospho- SRPK1 also demonstrated that phospho-SRPK1 were distributed more in the nucleus of the cell treated with CRH, as compared to untreated cells.

However, illustration in Figure 4.23 shows that although CRH was found to upregulate SRPK1 activity in ER- SKBR3 cells, cyto-nuclear protein determination showed that most of the phosho-SRPK1 signals were detected in the cytoplasm, rather than the nucleus. As cytoplasmic SRPK1 functions mainly in re-phosphorylation of cytoplasmic SR proteins back into the nucleus for the next round of splicing, nuclear level of SRPK1 is one of the factors that activates splicing event by facilitating the release of fully functioning SR proteins from CLKs through additional phosphorylation (177,179), this finding demonstrates that CRH actions may impact molecular subtypes of breast cancers differently, and that this result suggests that it can potentially regulate the mechanism of SRPK1-mediated alternative splicing in ER+, but not in ER- cells.

Following this, the analysis of splicing proteins activity in ER+ cells via immunoblotting and immunostaining showed that in agreement with elevated level of nuclear SRPK1 proteins, the phosphorylation signals of SRp55, 40 and 30 were detected increased and the expression of splicing repressor, hnRNPA1 was also upregulated in the nucleus. In contrast, phospho-epitope signals of these SR proteins and hnRNPA1 expression in ER- cells were found lower than basal level, which further highlight the potential of CRH to differentially regulate the activity of splicing factors in different types of cells.

Furthermore, when mRNA splicing activity was assessed by the level of CD44s and CD44v6 splice isoforms through mRNA expression analysis, it was found that both CD44 splice variants were found slightly increased in ER+ cells. This result suggests that CRH can induce alternative splicing of CD44 mRNA via SRPK1-mediated regulation of splicing proteins in the ER+ cells. In ER- cells, CD44s level was shown significantly decreased and CD44v6 was particularly elevated nearly 5-fold in the stimulated cells. Although this result in ER- was not anticipated as CRH initially was not shown to induce SRPK1 activity in the nucleus, this finding suggests that CRH may modulate splicing mechanism in ER- breast cancer cells, but via other pathways that is may probably not mediated by SRPK1 kinase, and it requires the involvement other factors, unlike that in

ER+ cells. In fact, in addition to SRPK1, there are other protein kinases known to be involved in the regulation of splicing mechanism and in splicing proteins phosphorylation such as SRPK2, cdc2- like kinase (CLK/STY) and DNA topoisomerase 1 (335,357). Furthermore, studies also demonstrated that Akt1 and Akt2 can directly target the RS domain of SR proteins with distinct specificity than SRPK1 (358), and cyclic AMP (cAMP)-dependent PKA can phosphorylate splicing protein ASF/SF2 at Serine 119; which this phosphorylation could potentially be involved in the regulation of RNA binding (359).

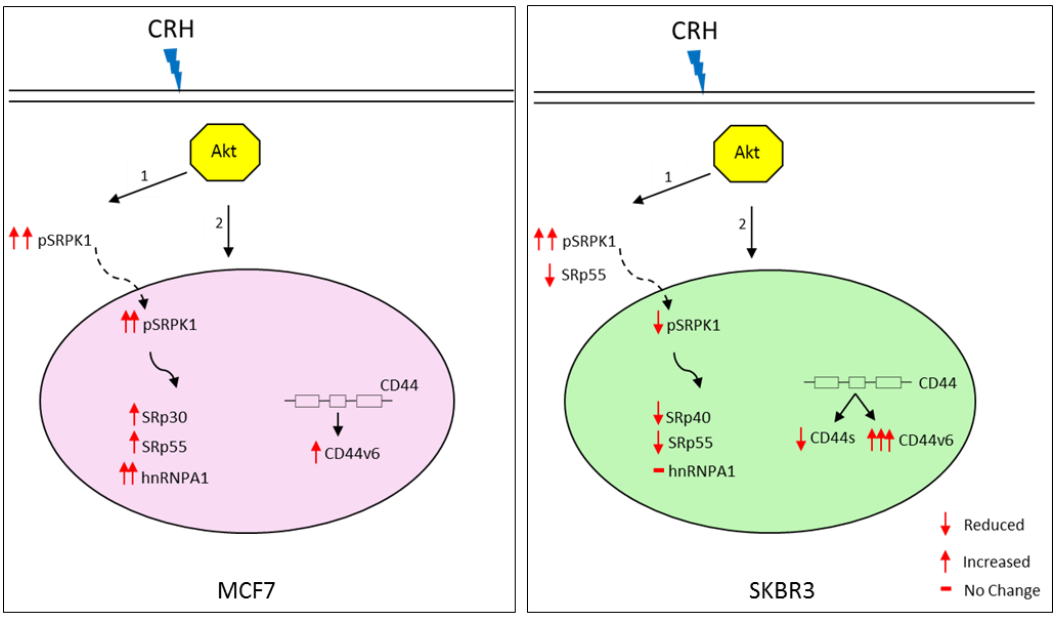


Figure 4.23 Illustration of differential expression of splicing factors and CD44s and CD44v6 splice variants when cells were treated with CRH in MCF7 and SKBR3.

Previous studies have demonstrated the potential of CRH receptors in modulating various intracellular protein kinases such as protein kinase A (PKA), protein kinase B (PKB/AKT) and p42/p44 and p38 mitogen activated protein kinases in various cells such as in rat Leydig cells, endothelial cells and in human epidermoid carcinoma cell line (MAPKs) (reviewed in (109)). Therefore, to investigate the involvement of AKT in CRH- induced regulation of SRPK1 activity, CRH- stimulated cells were treated with AKT kinase inhibitor (MK2206). The results in ER+ cells demonstrated although the reduced activity of Akt did not affect basal nuclear SRPK1 protein and mRNA level, both protein and mRNA level were significantly decreased when the AKT was inhibited in the stimulated cells (Figure 4.24). As predicted, signal detection for SR proteins, primarily SRp40 and SRp30, and hnRNPA1 protein and mRNA expression level were decreased in the cells. This finding shows that CRH induction in ER+ breast cancer cells seems to follow the proposed AKT-SRPK1-hnRNPA1 network above. In addition, findings from splicing assay show that inhibition of Akt in the stimulated cells resulted in 9-fold increase of CD44s mRNA, and 3-fold increase in CD44v6 mRNA. This finding is consistent with the finding previously found in E2-stimulated cells where Akt was inhibited, suggesting that Akt inhibitor enhances the effects of E2 and CRH in inducing CD44s and CD44v6 mRNA transcription in ER+ cells.

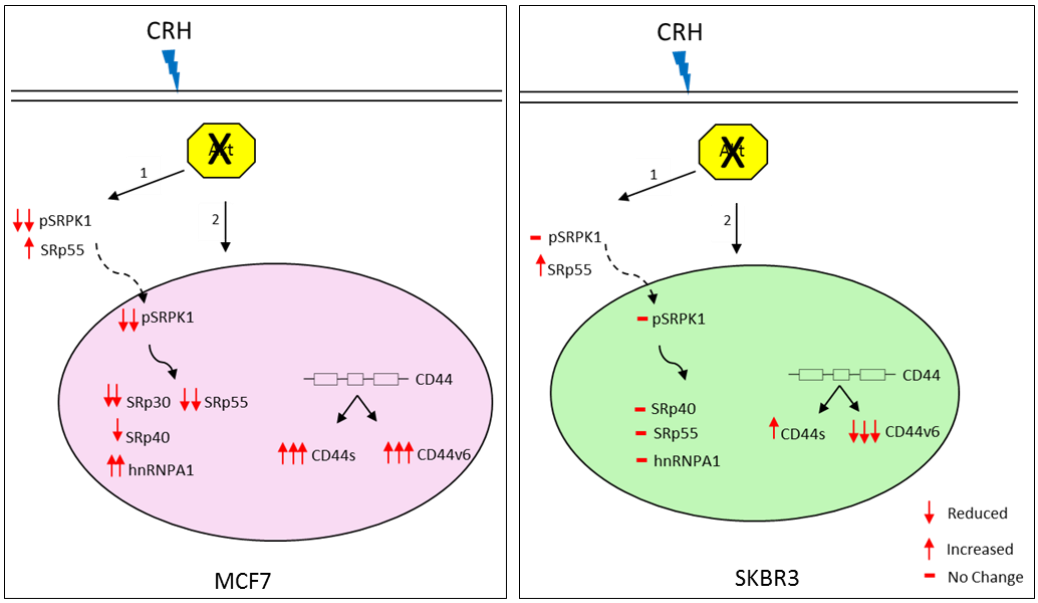


Figure 4.24 Illustration of differential expression of splicing factors and CD44s and CD44v6 splice variants in stimulated cells in the presence of Akt Inhibitor, MK-2206.

Furthermore, Figure 4.24 also illustrates that reduced AKT kinase in ER- cells has no effect on the basal level of SRPK1 mRNA level but led to slightly lower level of phopsho-SRPK1 protein in the nucleus. Interestingly, although pre-treatment of CRH-stimulated cells with Akt inhibitor downregulated SRPK1 mRNA expression, no change was detected in the level of nuclear phospho-SRPK1, suggesting that Akt mediates the effect of CRH on SRPK1 at mRNA level only. Likewise, generally no changes were demonstrated in the basal level of nuclear SR proteins phosphorylation and hnRNPA1 protein expression when Akt was reduced, as well as in CRH-stimulated cells where Akt was inhibited. However, as opposed to ER+ cells, hnRNPA1 mRNA expression remained upregulated in these cells. Previous studies have shown that other than Akt, hnRNPs can be regulated by cellular signaling pathways involving kinases such as Protein kinase A which phosphorylates hnRNPA1 at Ser-16, and Mitogen- activated protein kinase (MAPK) Interacting Kinases 1 and 2 (MNK1 and MNK2) via the activation of p38 MAPK signaling cascades (360,361). Therefore, the mRNA expression results in ER- SKBR3 cells

reflects that, Akt may play inhibitory role the transcriptional activity of CRH on hnRNPA1, in which this transcriptional effect of CRH may potentially be mediated by either of the kinases mentioned above. Furthermore, although Akt was not shown to play role in the transcription of basal CD44s and CD44v6 mRNA level, its inhibition in the stimulated cells resulted in increased production of CD44s, but reduced production of CD44v6, suggesting that the transcription of CD44v6 by CRH is Akt dependent in ER- cells.

Further investigations on the role of SRPK1 in CRH- induced activation of splicing factors, ER+ and ER- cells were examined by simultaneously treating the cells with SRPK1-kinase inhibitor, SRPIN340. The finding demonstrated that the inhibition of SRPK1 kinase in MCF7 cells resulted in decreased signal detection of phospho- SRp55, 40 and 30 in the nucleus, and it was also shown to have inhibitory effect on hnRNPA1 transcription in the cells. This subsequently led to reduced accumulation of only CD44v6 splicing isoform, but not CD44s mRNA (Figure 4.25). In contrast, although CRH did not have a major effect on the regulation of SRPK1-mediated splicing proteins in ER- SKBR3 cell line, decreased SRPK1 kinase activity was demonstrated to further reduce the phosphorylation level of SR proteins, suggesting the important role of SRPK1 kinase in regulating SR protein phosphorylation in cells. This is in line with findings from previous studies in which knocking down of SRPKs resulted in the failure to elevate SR protein phosphorylation in HeLa cells (171). Furthermore, the inhibition of SRPK1 kinase in CRH-stimulated ER- cells resulted in a slight increase of CD44s mRNA level, but CD44v6 mRNA was shown significantly reduced, a finding similar to that in ER+ MCF7 cell line. Overall, the results from splicing assay in both cell types suggest that the production of CD44s splice variants may not be entirely dependent on SRPK1 kinase only, whereas, CD44v6 isoform production is shown to be highly dependent on intact activity of SRPK1 kinase, especially in ER- cells (Figure 4.25).

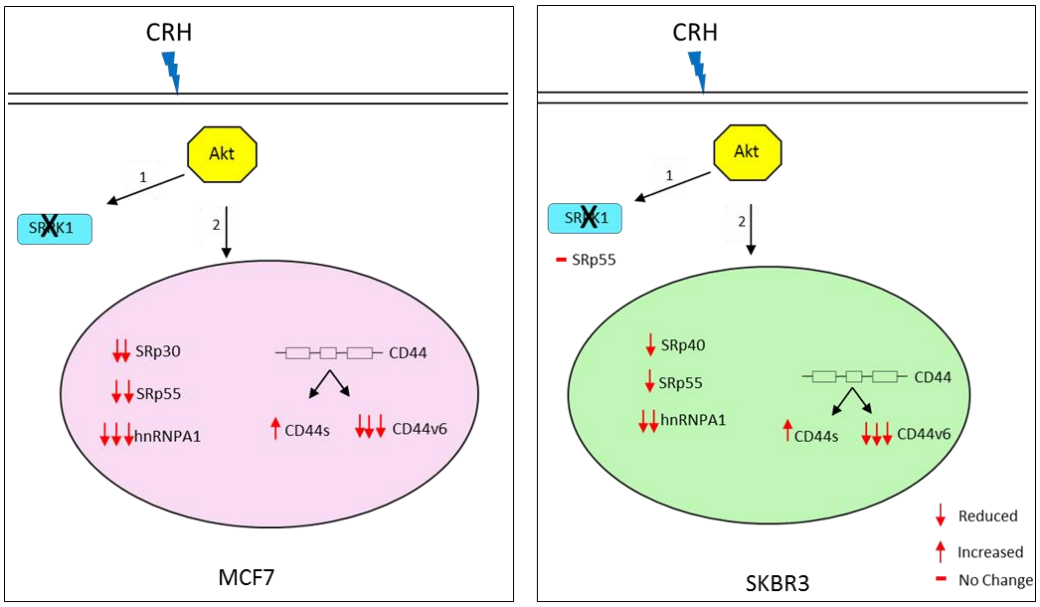


Figure 4.25 Illustration of differential expression of splicing factors and CD44s and CD44v6 splice variants when cells were treated with CRH either in the presence or absence of SRPK1 inhibitor, in both cell lines, MCF7 and SKBR3.

In summary, the results show that E2 and CRH can regulate SRPK1-mediated splicing factors activity through similar signaling mechanism via Akt in ER+ MCF7 cell line, and splicing results suggest that Akt plays inhibitory role in E2- and CRH- induction of CD44s and CD44v6 mRNA transcription. Meanwhile in ER- SKBR3 cell line, CRH did not exert similar effects on SRPK1 activity and splicing factors activation, and these effects of CRH were independent of Akt activity in the cells, and the production of CRH- induced CD44 splice isoforms was differentially dependent on Akt, in which Akt inhibitor reverses inhibitory effect of CRH on CD44s mRNA transcription but it plays role in the processes leading to accumulation of CD44v6 in ER- cells.

CHAPTER 5

Effects of altered pre-mRNA alternative splicing event in triggering epithelial-to-mesenchymal transition (EMT) in breast cancer cell lines

Introduction

Alterations in splicing activity is one of the common signatures in cancer cells, in which the resulting splice isoforms can encode proteins that promote cell proliferation, inhibit apoptosis and stimulate uncontrollable cell growth by triggering cellular processes such as epithelial-to-mesenchymal transition (EMT) event in the cells. The alterations in alternative pre-mRNA splicing (AS) was first connected with EMT was when splice variants of CD44, a cell surface protein were identified in metastatic pancreatic cancer cells that was not present in the primary tumor (252). In addition, apart from changes in cells morphology, the occurrence of EMT is evident at the molecular level, through the activation of signaling pathways leading to the change in the expression of such as transcription factors, microRNA and various genes that play role in cellular functions such as cytoskeletal re-organisation, cell motility, immune response cell growth (362).

Therefore, this chapter was aimed to analyse the impact of hormone- driven aberrant alternative splicing process on the metastatic potential of ER+ and ER- cells by measuring the expression level of EMT-associated gene markers and the cell invasion and migration properties by using real-time PCR and Xcelligence real-time cell monitoring assay. In addition, a study using EMT profiler PCR array was also carried out to elucidate the transcriptional effects of Akt and SRPK1 kinase on EMT genes, as well as the potential diversion of CRH signaling mechanism when either of these two kinases were inhibited in the cells.

5.1 Correlation between altered pre-mRNA alternative splicing and activation of Epithelial-to-Mesenchymal Transition (EMT) in cells

Previous studies have demonstrated that CD44, CD44s and CD44v6 were linked to EMT process by regulating downstream targets, transcription factor Snail1 (SNAI1) and membrane bound metalloproteinase (MMP), both of which are known regulators of EMT program (363). Therefore, the expression of EMT markers, Twist, Snail and Vimentin were examined to investigate whether differential expression of CD44s and CD44v6 lead to changes in EMT associated genes in both cell lines.

MCF7 cells were treated with E2 (10nM) or CRH (100nM) for 24hrs, followed by RNA extraction and RT-qPCR analysis of Twist, Snail, and Vimentin mRNA expression. Results showed that E2 did not significant upregulate Twist and Snail expression, but the expression of these EMT genes were increased in CRH-stimulated cells. Furthermore, basal expression of Twist and Snail was shown decreased in cells where Akt was inhibited. Interestingly, when Akt activity was inhibited using Akt inhibitor (5μM) for 2hr in either E2- or CRH- stimulated cells, the Twist and Snail mRNA level was found significantly increased, similar to the pattern of CD44s and CD44v6 expression previously found in these cells, suggesting that while Akt involves in the transcriptional activity of Twist and Snail, it however attenuates the potential effects of E2- or CRH- in modulating Twist and Snail mRNA expression in the cells (Figure 5.1A and Figure 5.1B).

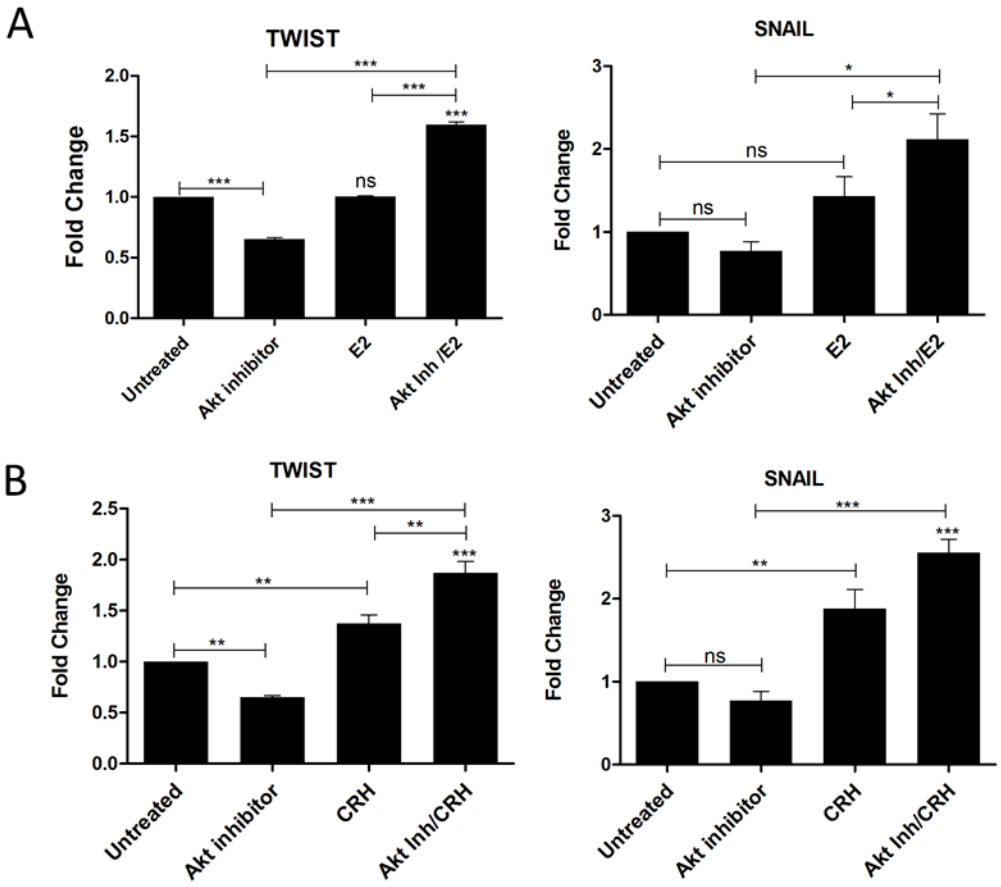


Figure 5.1 The analysis of EMT- associated mRNA expression, Twist and Snail in MCF7 cell line. (A) The treatment of MCF7 cells with E2 (10nM,24hrs) showed no effect on basal Twist and Snail mRNA expression, but inhibition of Akt in the stimulated cells led to upregulation of both mRNA. In addition, reduced Akt activity significantly reduced the transcription of Twist but has no effect on Snail. (B) Stimulation with 100nM CRH for 24hrs resulted in increased Twist and Snail mRNA expression but inhibiting Akt activity in the stimulated cells further enhanced the CRH effect on both mRNA transcripts. Meanwhile, treatment with Akt inhibitor alone (5μM) for 2hr has no effect on basal Snail expression but reduced basal Twist mRNA expression.

In addition, the effect of modulating SRPK1 activity by using the specific SRPK1 inhibitor was also investigated. Basal expression of Twist mRNA was shown decreased when cells were treated with SRPK1 inhibitor at 10μM for 2hr, but Snail mRNA expression remained unaffected suggesting that SRPK1 involves in the transcription of Twist (Figure 5.2). However, inhibiting SRPK1 activity has no effect on CRH-induced Twist and Snail mRNA expression, which further suggests that transcriptional effect of CRH on Twist and Snail does not dependent on SRPK1 activity. In addition, in cells where SRPK1 was inhibited, CRH was shown to upregulate Twist and Snail mRNA expression, suggesting the potential involvements of other kinases in mediating CRH effect on the induction of EMT gene expression in MCF7 cells.

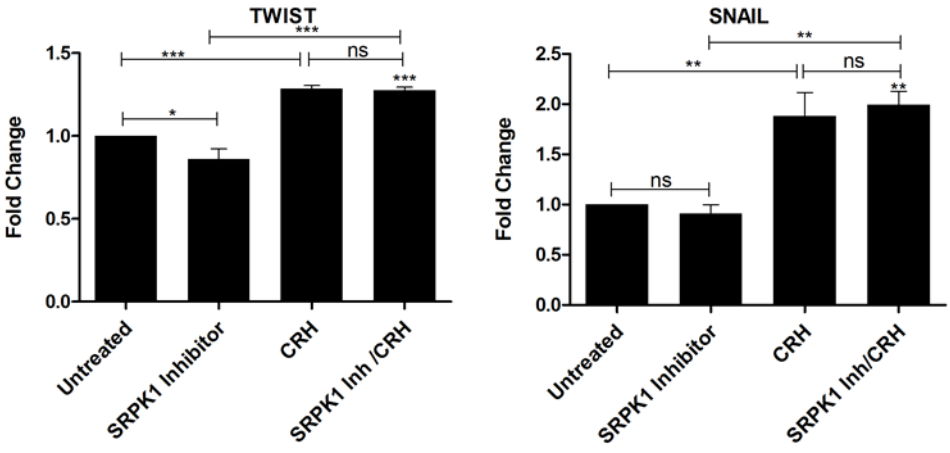


Figure 5.2 The treatment with SRPK1 inhibitor (10μM) for 2hr reduced basal Twist mRNA level but has no effect on Snail mRNA, and its inhibition in CRH- stimulated cells did not affect both mRNA expression. In cells where SRPK1 was inhibited, CRH was shown to induce Twist and Snail mRNA expression.

Furthermore, the expression of Twist and Snail was also investigated in SKBR3 cell line. Cells were treated with CRH (100nM) for 24hrs, followed by RNA extraction and RT-qPCR analysis of Twist and Snail mRNA expression. Results showed that although CRH did not affect basal expression of both Twist and Snail mRNA, pre-treatment of the stimulated cells with Akt inhibitor (5μM) for 2hr has led to nearly 3-fold increase of Twist and 1-fold increase of Snail mRNA expression in the cells suggesting that although Akt was not shown to be involved in the regulation of basal Twist expression, its activity restrains CRH effect on the transcriptional activity of Twist. In addition, this finding also suggests that Akt not only plays inhibitory role in the regulation of basal Snail mRNA expression, but also in the CRH-stimulated cells (Figure 5.3).

Overall, these results demonstrated that while CRH has no effect on the basal expression of Twist and Snail in SKBR3 cell line, as compared to that in MCF7 cell line, the transcriptional activity of both Twist and Snail was upregulated when Akt was inhibited in stimulated cells of both cell lines, suggesting that Akt masks the role of either E2 or CRH in the regulation of EMT gene expression, Twist and Snail in MCF7 and SKBR3 cell lines.

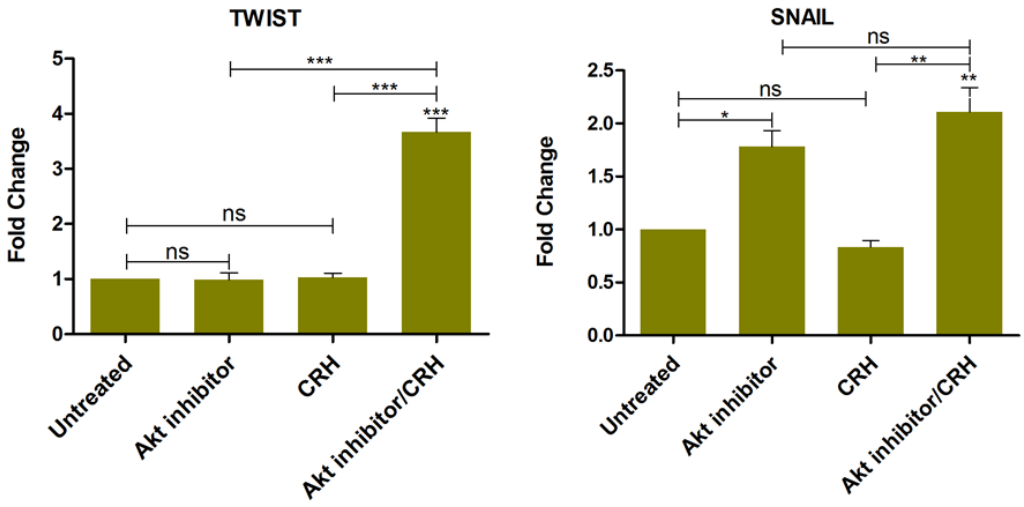


Figure 5.3 The expression of EMT- associated gene, Twist and Snail mRNA in SKBR3 cell line. Cells were stimulated with CRH (100nM) for 24hrs, followed by RNA extraction and RT-qPCR. Results showed that CRH has no effect on basal transcriptional activity of Twist and Snail, however when the stimulated cells pre-treated with Akt inhibitor (5μM, 2hr), the expression of Twist and Snail increased significantly. In addition, inhibition of Akt activity has no effect on basal Twist expression but has increased Snail mRNA expression by 1-fold.

5.2 Transcriptional regulation of Epithelial-Mesenchymal Transition (EMT) genes by CRH using EMT RT^2 Profiler PCR Array

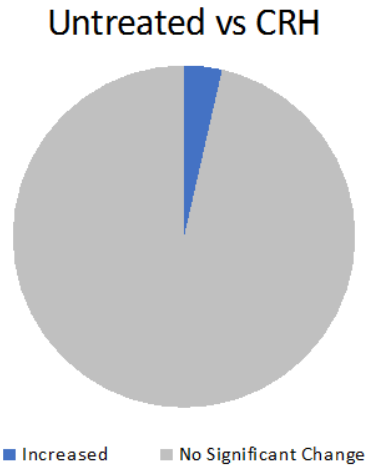
Results so far have demonstrated that CRH and E2 can potential alter the expression of splicing factors; SR proteins and hnRNPA1, by modulating SRPK1 activity via Akt. This subsequently induces the production of CD44 splice variants, CD44s and CD44v6 and upregulates the expression of EMT genes, Twist and Snail, both of which signaling pathways are differentially dependent on Akt or SRPK1 kinase in MCF7 cell line (ER+ cells). Whereas in SKBR3 cell line (ER- cells), CRH was not shown to exert similar effect on the modulation of SRPK1 and splicing factors; SR proteins and hnRNPA1. However, the accumulation of both CD44 mRNA isoforms and differential expression of Twist and Snail in response to CRH were still observed. This suggests that CRH probably exerts it effect via different signaling route in this cell line, which eventually affecting CD44 splicing and promoting EMT gene expression, similar to that in MCF7 cell line.

Therefore, having established the potential of CRH and E2 in inducing altered splicing event leading to elevated expression of known EMT-associated genes in the MCF7 cell line, the expression of other genes known to be involved in processes promoting EMT such as in cell adhesion, cytoskeletal remodeling and cell motility were further investigated in stimulated cells. In addition, as the transcription of Twist and Snail was demonstrated to be either unaffected or enhanced when either Akt or SRPK1 was inhibited in CRH- stimulated cells, the impact of specific kinase inhibition on CRH signal transduction was also investigated. Therefore, MCF7 cells were treated with CRH (100nM) for 24hrs, with or without the presence of Akt (5μM) or SRPK1 inhibitor (10μM) before 500μg of RNA was extracted, followed by qRT-PCR using the RT^2 Epithelial- to-Mesenchymal Transition (EMT) Profiler PCR Array plate, consisting of 84 EMT-associated target genes. Changes were considered significant when p-value <0.05, and fold-change was greater than two.

Table 5.1 shows that out of 84 genes, three of them (MAP1B, OCLN, RGS2) were upregulated by two-fold following the treatment with CRH, as compared to untreated cells. These genes, especially MAP1B (Microtubule Associated Protein 1B) and RGS2 (Regulator of G protein-coupled receptor signaling) have been demonstrated to be part of MAPK signaling pathway and were found overexpressed in the majority of breast cancers (364,365).

Table 5.1 Untreated vs CRH. List of upregulated genes in the qRT-PCR analysis 84 EMT-associated genes in ER+ breast cancer cells.

SYMBOL	FOLD REGULATION
MAP1B	2.0019
OCLN	2.007
RGS2	2.0019

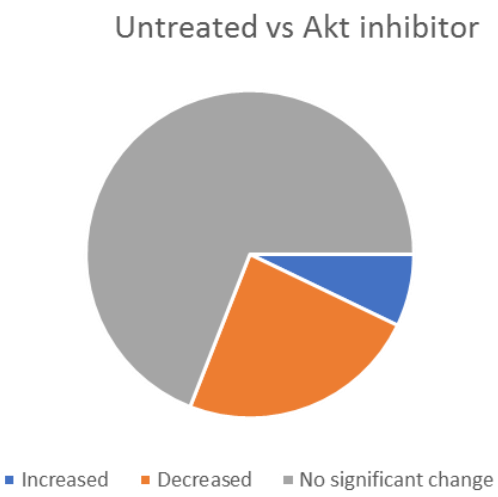


Additional experiment showed that inhibition of Akt activity in control cells led to significant increase of six (7%) of EMT-associated genes expression (VCAN, MMP3, MMP9, TFPI2, PTP4A1, ITGA5), while 20 genes (24%) were found significantly downregulated (Table 5.2), indicating a key role of Akt in numerous cellular signaling networks in the cells.

Table 5.2 Untreated vs Akt Inhibitor. List of upregulated genes (Blue) and downregulated genes (Orange) in the qRT-PCR analysis 84 EMT-associated genes in ER+ breast cancer cells.

SYMBOL	FOLD REGULATION
VCAN	33.0937
MMP3	16.616
MMP9	4.0368
TFPI2	2.0147
PTP4A1	2.011
ITGA5	2.0083

SYMBOL	FOLD REGULATION
TIMP1	-23.1738
COL3A1	-5.6585
CAV2	-5.6155
IGFBP4	-4.025
TGFB2	-4.0158
CDH1	-3.9723
GSC	-3.9681
MST1R	-3.9653
NOTCH1	-3.9385
NUDT13	-2.8383
TCF3	-2.8368
CAMK2N1	-2.8249
KRT19	-2.8221
DSC2	-2.8214
SNAI3	-2.8195
ITGB1	-2.802
ERBB3	-2.7974
TMEFF1	-2.7974
CTNNB1	-2.7916
WNT5A	-2.0013



These results led to investigation on the actions of CRH in cells in the presence of Akt inhibitor. Table 5.3 shows the significant changes in EMT gene expression in response to the treatment, and the pie-chart displays percentages of genes affected with as much as 44% of them (37 out of 84) were found significantly high which mostly were the most representative markers known to be involved in the processes leading to EMT in cancer cells such as TGFB1, SNAI3, CTNBB1, NOTCH1 and WNT5A. Studies have shown that while TGFB plays role in growth inhibition and apoptosis, it can also contribute to tumor progression and metastasis through the induction of EMT once cells overcome the TGFB-induced anti-oncogenic responses (366). Notably, TGFB signaling can regulate and crosstalk with other signaling pathways such as Wnt/b-catenin and NOTCH pathway that leads to TGFB target genes such as *Snail* and *MMP9,* causing increased cell invasion and trans- endothelial migration (245,367). In addition, the role of Notch signaling has been known in inducing EMT, where elevated expression of Notch and its ligand Jagged-1 have been demonstrated in various cancers including breast cancer and the Notch receptor, NICD can activate the expression of genes such as Twist, Snail and ZEB1/2 that promote tumor development via NF-kB and Akt signaling (250). Furthermore, Twist expression has been reported to play role in the activation of WNT5A gene expression and subsequently leads to WNT5A-mediated EMT process in breast cancer cells (368).

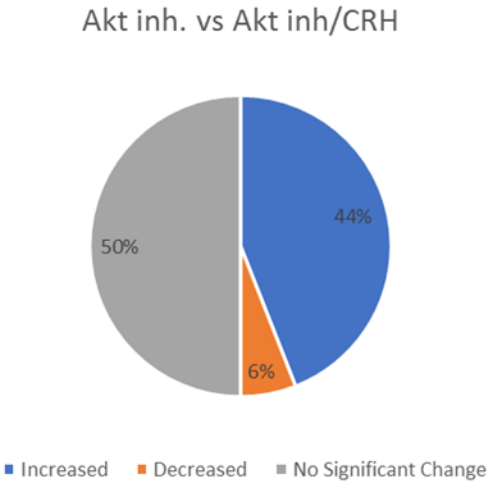
Furthermore, the results also showed that only 6% (5 out of 84) namely ITGA5, MMP3, MMP9, PTP4A1, VCAN was shown markedly suppressed under the treatment and apparently, these pattern of EMT gene expression were opposite with the pattern of expression when cells were treated with Akt inhibitor alone. This also indicates the change of direction in CRH signal transduction which probably involves other kinases, as studies on kinome proposed the existence of kinases network and their interplay that contributed to poor clinical outcome in ER+ breast cancer (57). Based on this data, we investigated potential interactions among the significantly altered genes by introducing these 42 up- and down-regulated genes into a molecular tool, STRING. The STRING analysis revealed that 34 out of 42 genes are closely connected in a single network,

demonstrating the tight relationship among the molecules (Figure 5.4). The interaction between each factor during the multiple signaling crosstalk can create feedback loops in the cells that either supports the EMT states or inhibit the process. For example, overexpression of Tissue Inhibitor of Metalloproteinases (TIMP1) and E-cadherin (CDH1) when Akt was inhibited in the stimulated cells might contribute to decreased expression of MMP-3 and MMP-9, which play role in the degradation of both matrix and non-matrix proteins in the extracellular matrix (ECM) during EMT and contribute to tumor cell migration (369). However, increased expression of TGFB can lead to the formation of heterodimeric complex via SMAD2, which then binds to DNA-sequence-specific transcription factors and activate the transcription of target gene such as SNAIL3 (370), which has been shown to repress E-cadherin (CDH1) expression in cells.

Table 5.3 Akt Inh. vs Akt Inh./CRH. List of upregulated genes (Blue) and downregulated genes (Orange) in the qRT-PCR analysis 84 EMT-associated genes in ER+ breast cancer cells.

SYMBOL	FOLD REGULATION
TIMP1	25.103
TCF3	6.0764
NUDT13	4.346
RGS2	4.345
TMEFF1	4.3359
TGFB2	4.3148
COL5A2	4.3144
CAV2	4.3104
GSC	4.3027
SNAI3	4.2841
IL1RN	4.2754
COL3A1	3.0612
OCLN	3.0531
KRT19	3.047
CAMK2N1	3.0424
ITGB1	3.0352
IGFBP4	3.0339
DSC2	3.032
CDH1	3.0297
MST1R	3.0127
NOTCH1	3.0107
CTNNB1	3.0046
ERBB3	3.0013
STEAP1	2.1732
ILK	2.1593
WNT5A	2.1573
TGFB1	2.1548
MAP1B	2.1534
PTK2	2.1533
GSK3B	2.1482
EGFR	2.1468
VIM	2.144
ITGAV	2.1432
SMAD2	2.1429
RAC1	2.1405
F11R	2.1364
DSP	2.1328

SYMBOL	FOLD REGULATION
VCAN	-30.6642
MMP3	-15.3962
MMP9	-3.7363
PTP4A1	-2.6383
ITGA5	-2.6225



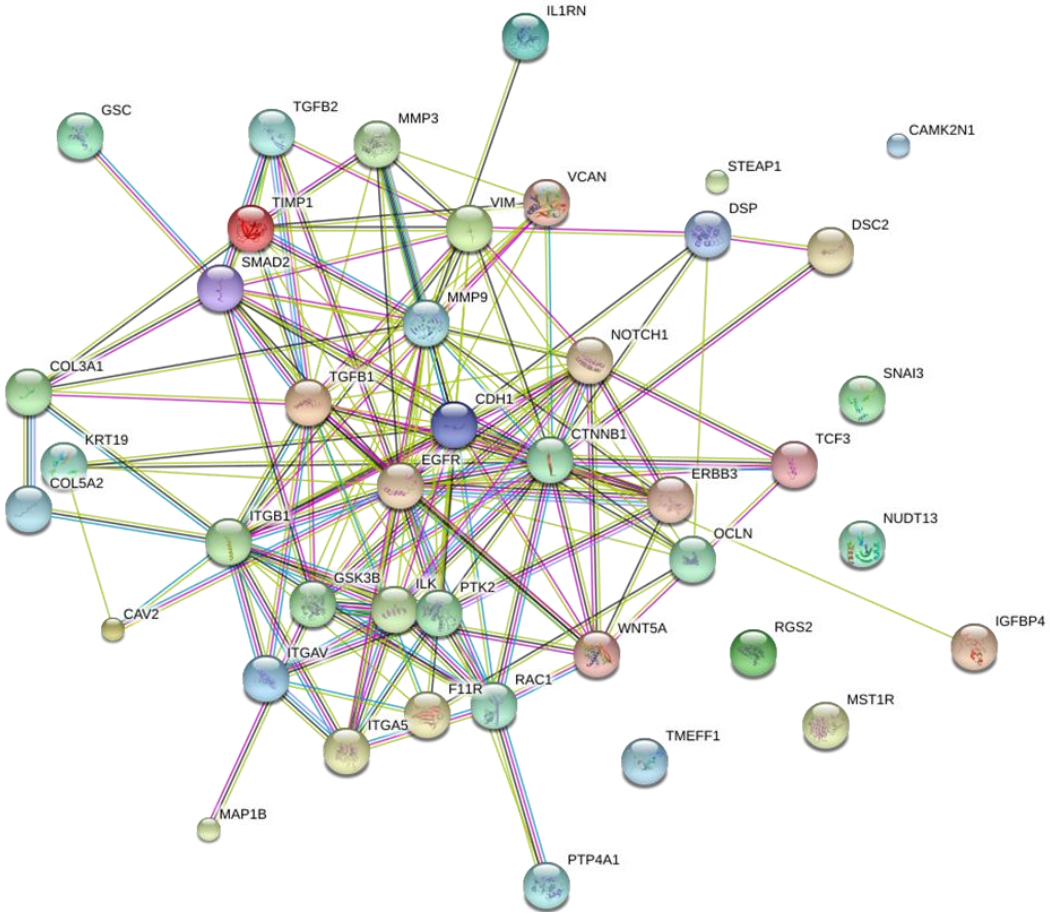


Figure 5.4 STRING analysis of known and predicted protein interactions existing among the 42 significantly altered gene expression in cells simultaneously treated with Akt inhibitor and CRH as compared against cells treated with Akt Inhibitor only. The analysis shows that 34 out of 42 altered EMT gene signatures expression are closely connected in a single network.

Table 5.4 summarises and compares the results from EMT gene profiler array experiment between untreated cells, Akt inhibited cells and CRH-stimulated cells. Overall, these findings suggest that basal expression of most EMT-associated genes was not much affected by CRH. However, the inhibition of Akt kinase led to up- and down-regulation of numerous EMT gene transcription, suggesting its key role in EMT-related intracellular signaling pathways such as Wnt/β-catenin and TGFβ pathways. This finding suggests that Akt blocked the CRH-driven upregulation of EMT genes such as GSK33b, SNAI3 and SMAD2. Interestingly, CRH was demonstrated to reverse the effects of Akt inhibition on basal expression of VCAN, MMP3, PTP4A1 and ITGA5.

Table 5.4 Summary of the EMT gene expression pattern in response to various treatments in MCF7 cells. Blue represents Increased expression, while Red represents Decreased expression.

	Untreated			Akt Inhibitor			
Akt Inh	VCAN	CAMK2N1	NOTCH1				
	MMP3	CAV2	NUDT13				
	MMP9	CDH1	SNAI3				
	TFPI2	COL3A1	TCF3				
	PTP4A1	CTNNB1	TGFB2				
	ITGA5	DSC2	TIMP1				
		ERBB3	TMEFF1				
		GSC	WNT5A				
		IGFBP4					
		ITGB1					
		KRT19					
		MST1R					
CRH	MAP1B						
	OCLN						
	RGS2						
Akt inh/CRH				CAMK2N1	GSK3B	PTK2	VCAN
				CAV2	IGFBP4	RAC1	MMP3
				CDH1	IL1RN	RGS2	MMP9
				COL3A1	ILK	SMAD2	PTP4A1
				COL5A2	ITGAV	SNAI3	ITGA5
				CTNNB1	ITGB1	STEAP1	
				DSC2	KRT19	TCF3	
				DSP	MAP1B	TGFB1	
				EGFR	MST1R	TGFB2	
				ERBB3	NOTCH1	TIMP1	
				F11R	NUDT13	TMEFF1	
				GSC	OCLN	VIM	
						WNT5A	

Additionally, the impact of inhibiting SRPK1 on the transcriptional activity of EMT-associated genes was also investigated. Table 5.5 shows that reduced SRPK1 activity resulted mainly in reduced level of nine EMT genes (11%); BMP7, CAV2, COL3A1, EGFR, KRT19, NOTCH1, TGFB2, VPS13A and WNT5A. Previous studies have shown that some of these genes such as BMP7, EGFR, NOTCH1 and WNT5A are targets of TGFβ signaling pathway, or they are part of signaling pathways that is directly or indirectly associated with TGFβ pathway. BMP7 is a member of bone morphogenic proteins (BMPs) family that can function as TGFβ ligand, and activate TGFβ signaling pathway by binding to TGFβ receptors (371). Furthermore, genes such as NOTCH1, TGFB and WNT5A represent the most well-established signaling pathways involved in processes that promote EMT, suggesting that SRPK1 kinase play role in the induction of these pathways in ER+ cells.

Comparison between the impact of inhibiting either Akt or SRPK1 in the cells demonstrated that Akt regulates 26 of 84 EMT, whereas SRPK1 regulates 9 of them (Table 5.6). Interestingly, some of the genes from both group are overlapping which includes CAV2, COL3A1, KRT19, NOTCH1, TGFB2, and WNT5A, suggesting that the transcriptional regulation of these EMT genes involves both kinases.

Table 5.5 Untreated vs SRPK1 Inhibitor. List of downregulated genes in the qRT-PCR analysis 84 EMT-associated genes in ER+ breast cancer cells.

SYMBOL	FOLD REGULATION
COL3A1	-10.2601
TGFB2	-7.2598
CAV2	-3.5866
BMP7	-2.5686
KRT19	-2.5547
WNT5A	-2.5512
NOTCH1	-2.5497
EGFR	-2.5492
VPS13A	-2.528

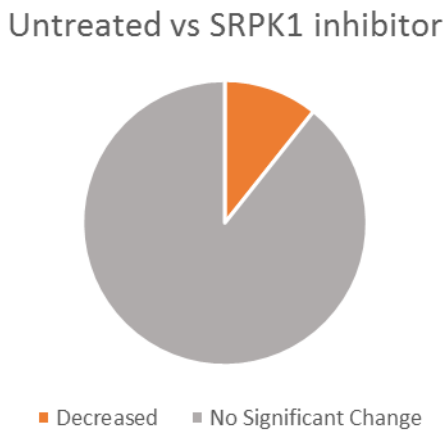


Table 5.6 Comparison between the pattern of expression when cells were treated with either Akt or SRPK1 inhibitor vs untreated cells. Blue represents Increased expression, while Red represents Decreased expression.

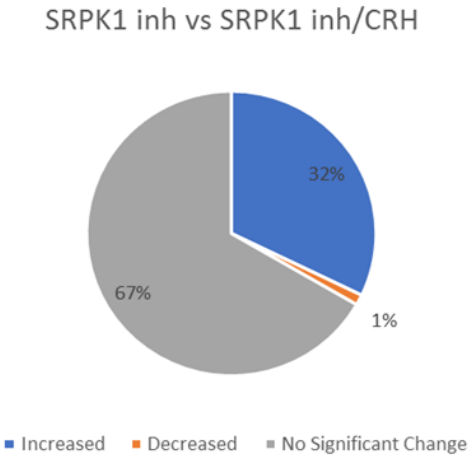
	Akt Inibitor			SRPK1 Inhibitor
Untreated	VCAN	CAMK2N1	NOTCH1	BMP7
	MMP3	CAV2	NUDT13	CAV2
	MMP9	CDH1	SNAI3	COL3A1
	TFPI2	COL3A1	TCF3	EGFR
	PTP4A1	CTNNB1	TGFB2	KRT19
	ITGA5	DSC2	TIMP1	NOTCH1
		ERBB3	TMEFF1	TGFB2
		GSC	WNT5A	VPS13A
		IGFBP4		WNT5A
		ITGB1		
		KRT19		
		MST1R		

Moreover, when SRPK1 was inhibited in CRH- stimulated cells, 27 EMT genes were upregulated, including the nine genes that were downregulated in cells treated with SRPK1 inhibitor alone. This result suggests that CRH can reverse the effect of SRPK1 inhibitor, and CRH can potentially divert its signal through other kinase and thus modulating transcriptional activity of various other EMT genes, as lister in Table 5.7.

Table 5.7 SRPK1 Inh. vs SRPK1 Inh/CRH. List of upregulated genes (Blue) and downregulated genes (Orange) in the qRT-PCR analysis 84 EMT-associated genes in ER+ breast cancer cells.

SYMBOL	FOLD REGULATION
COL3A1	11.4201
TGFB2	11.3713
IL1RN	4.0517
KRT19	4.0045
STEAP1	2.9764
OCLN	2.8637
COL5A2	2.8471
EGFR	2.8465
CAV2	2.8418
RGS2	2.8396
VPS13A	2.8392
NOTCH1	2.8357
NUDT13	2.8356
TCF3	2.835
BMP7	2.8345
MSN	2.1427
ZEB2	2.0407
TMEFF1	2.0284
VIM	2.0258
ITGB1	2.0228
SNAI2	2.0224
STAT3	2.0163
CALD1	2.0124
PLEK2	2.0103
WNT5A	2.0084
MMP9	2.0073
CAMK2N1	2.0065

SYMBOL	FOLD REGULATION
TMEM132A	-2.782



5.3 Analysis of cell invasion and migration properties in stimulated cells

In addition to altered expression pattern of EMT signatures, we determined the change in cell motility, another functional characteristic of EMT to determine whether the changes in gene expression were translated into altered biological responses. Cell proliferation and invasion were determined by using Xcelligence system, as described in Chapter Method 2.10. This is a label-free and real-time impedance-based assay systems with the plates designed to contain inter-digited gold microelectrodes to monitor the viability of cultured cells (372). The electrical impedance of the cell population was measured by electrodes in each well, and this measurement provides quantitative information about the status of the cells. Seeding concentration was determined prior to experiment and we determined that the optimum cell seeding density to monitor cell behavior of both MCF7 and SKBR3 cells is 40,000 cells/well.

For cell proliferation study, cells were seeded in E-plate 16 and the cells were automatically monitored every 15min over 24hr before stimulants were added and the cell behavior in response to the treatments was monitored continuously using RTCA for another 24hr and expressed as CI (Cell Index) value. Likewise, for the invasion study, cells were treated with stimulant in serum-free media (SFM) 24h prior to experiment and $4x10^4$ cells were seeded in a 10% (v/v) Matrigel-coated (to simulate extracellular matrix in tissues and to provide layer which the cells would have to invade) CIM-plated 16. Epidermal Growth Factor (EGF) in SFM was used as chemoattractant in the lower chamber and the cells were monitored from 0-8 hr with untreated cells served as control (in all experiments).

In line with previous findings on the role of E2 in promoting cancer cell proliferation (373–376), our MCF7 results in Figure 5.5A shows a slight increase in proliferation when treated with E2 at 10nM. In addition, the treatment with Akt inhibitor alone led to the reduction of cell proliferation, and inhibition of Akt kinase was also shown to reduce the cell proliferation in E2- and CRH- stimulated MCF7 cells (Figure 5.5B and 5.5C). In addition, CRH was shown to promote cell proliferation properties via

Akt in SKBR3 cells (Figure 5.5D). Overall, these findings suggest that E2 promote MCF7 cell proliferation properties, and the presence of Akt inhibitor blocks the effect of E2 on cell proliferation, whilst CRH via Akt, promotes cell proliferation in ER- cells but not ER+ cells.

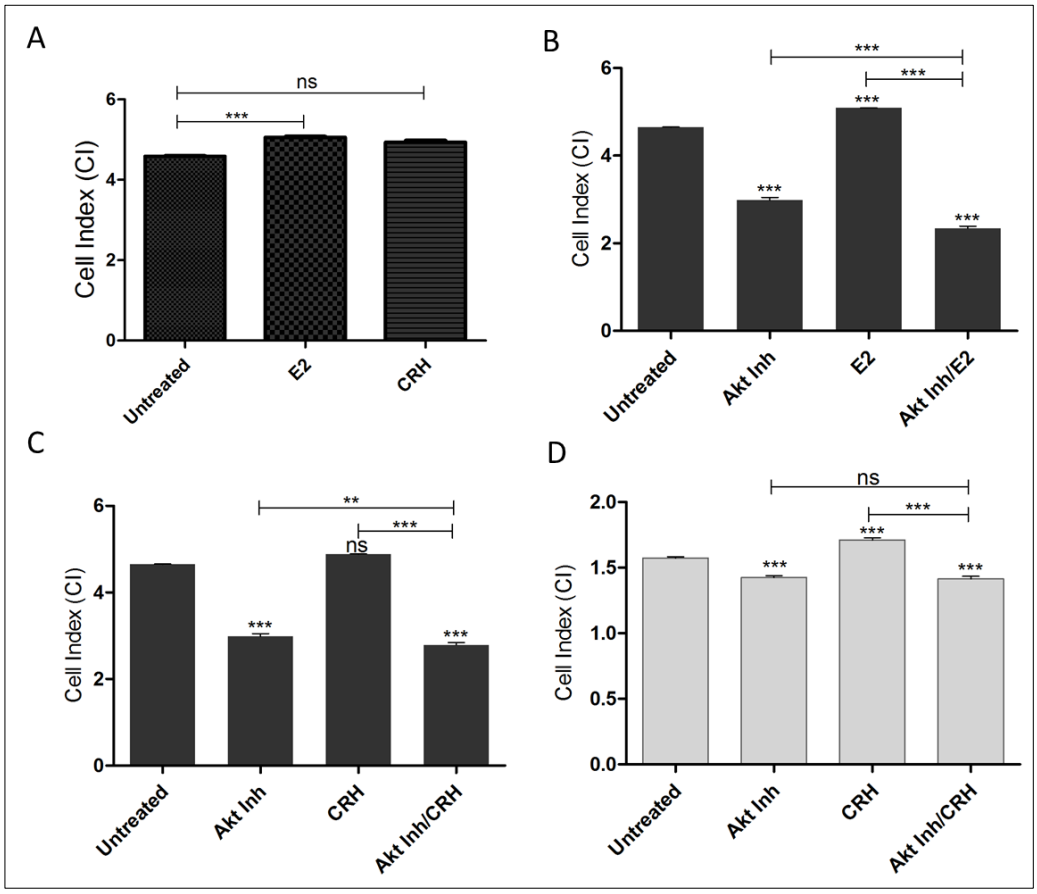


Figure 5.5 (A) MCF7 cells were stimulated with E2 or CRH alone at 10nm and 100nm for 24hr respectively and cell proliferation was determined by using Xcelligence system. (B) & (C) Stimulated MCF7 cells were co-treated with Akt-inhibitor, MK-2206 at 5μM for 2hrs (D) SKBR3 were treated with CRH, 100nM in the presence or absence of Akt Inhibitor and cells index were measured.

Next, we investigated the potential of CRH in promoting breast cancer invasion following their potent effect in regulating the transcriptional activity of various EMT-associated genes. Figure 5.6 shows that, consistent with non-invasive property of MCF7 cells, the untreated MCF7 cells shows limited invasive potential which gradually decreasing over time. Consistent with previous report (104), our data shows that CRH promotes the invasion of MCF7 cells in vitro, possibly by inducing the expression of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9)(139). This effect is somewhat different from earlier finding by Lal et.al (141) which could be explained by different experimental conditions used and variability in the cell passage number. In addition, as reduced Akt kinase in CRH-stimulated triggered the transcription of several EMT genes, our data shows that it resulted in increased cell migration properties but showed no effect on the invasion properties of the stimulated cells (Figure 5.7). Meanwhile, reduced SRPK1 kinase dampened CRH effect on cell migration and invasion (Figure 5.6 and Figure 5.7), an observation that agrees with reports regarding the role of SRPK1 as one of cancer metastasis determinants (193,377).

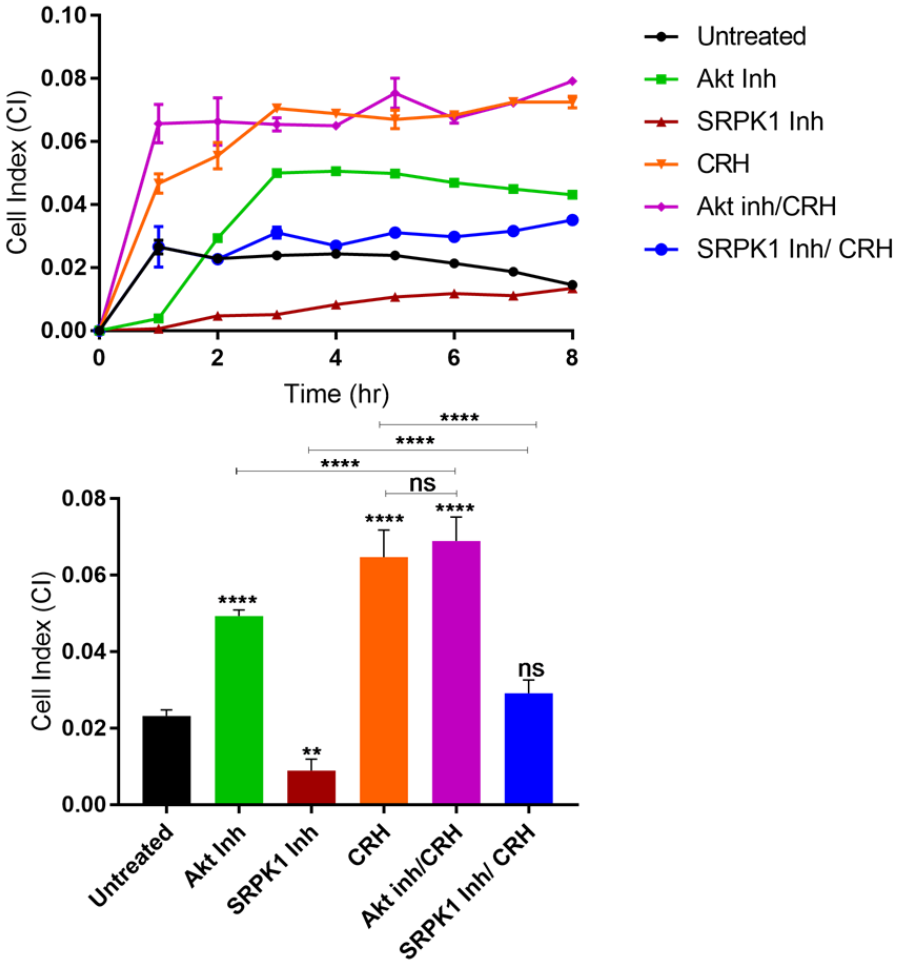


Figure 5.6 Cell Invasion assay measured with xCELLigence system. MCF7 cells were treated with 100nM CRH for 24hr, in the presence or absence of either Akt inhibitor (5μM) or SRPK1 inhibitor (10μM). Data represent mean ±SEM of at least two independent biological replicates measured in triplicates.

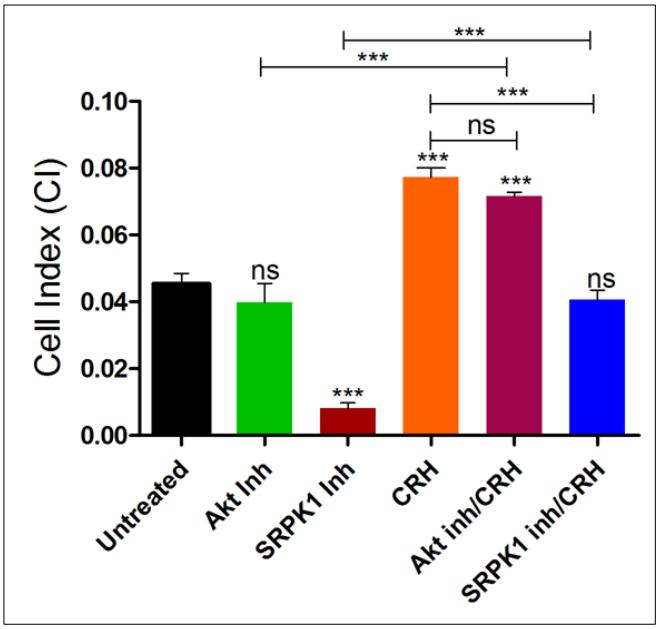


Figure 5.7 Cell migration assay of MCF7 treated with 100nM CRH for 24hr, in the presence or absence of either Akt inhibitor (5μM) or SRPK1 inhibitor (10μM).

Additionally, slightly different pattern of cell invasion property was observed in ER- cells SKBR3, in which CRH did not potentiate the invasive properties of cells, and inhibition of Akt kinase in the stimulated cells seemed showed no effect on the invasive properties of the cells (Figure 5.8). Notably, inhibition of SRPK1 significantly decreased the cell invasion in CRH-induced cells, consistent with the finding from Roosmalen etc al which demonstrated that depleting the SRPK1 activity in ER- mammary carcinoma (mouse) completely abolished the metastatic potential of the cells (193).

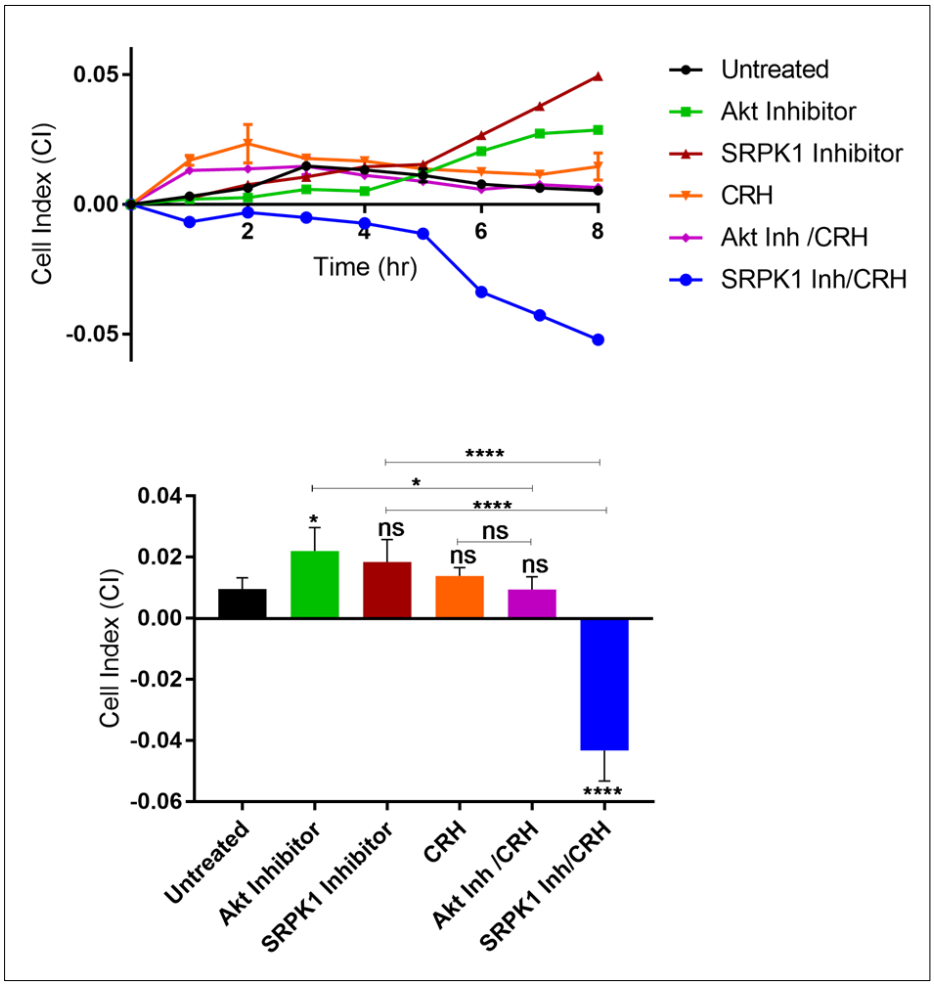


Figure 5.8 Cell Invasion assay measured with xCELLigence system. SKBR3 cells were treated with 100nM CRH for 24hr, in the presence or absence of either Akt inhibitor (5μM) or SRPK1 inhibitor (10μM). Data represent mean ±SEM of at least two independent biological replicates measured in triplicates

In addition, the action of CRH was further investigated by pre-treatment with either CRH-R1 antagonist (NBI 27914) or CRH-R2 antagonist (Asstressin-2B) in ER+ MCF7 cell line. Results showed that blocking the CRH-R1 resulted in significantly reduced cell invasion as compared to the invasion properties when MCF7 cells were pre-treated with CRH-R2 inhibitor (Figure 5.9), suggesting that CRH may promote invasive properties of ER+ cells via CRHR1.

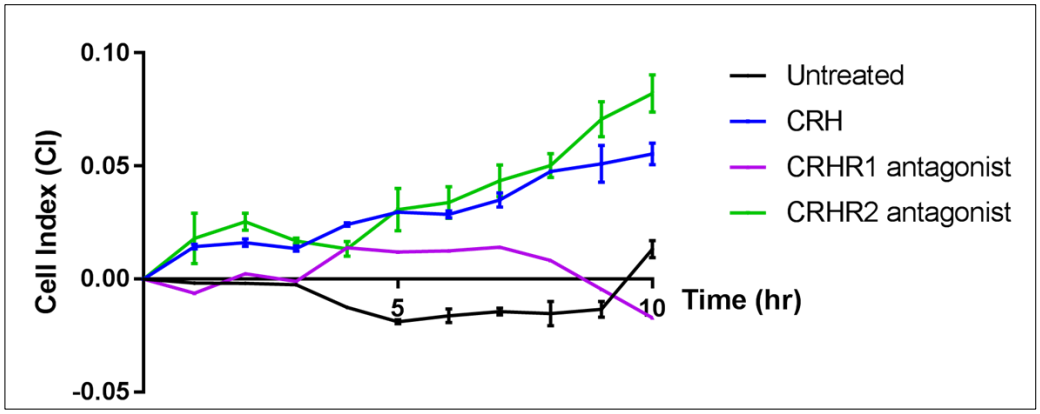


Figure 5.9 Cell Invasion assay measured with xCELLigence system. MCF7 cells were pre-treated with either CRH-R1 inhibitor (NBI 27914) or CRH-R2 inhibitor (Asstressin-2B) before stimulation with 100nM CRH for 24hr. Data represents mean ±SEM of at least two independent biological replicates measured in triplicates.

Discussion

The association between increased CD44 splice variants and EMT induction in breast cancer cells

One of the types of Epithelial-to- mesenchymal transition (EMT) in organism involves the conversion of epithelial cells to migratory and invasive cells. Growing evidence also reveal that EMT event can be supported by three regulatory events; small non-coding RNAs, differential splicing and translational and post-translational control which influence protein stabilisation and localisation (378). Studies have shown evidence on how splicing can trigger EMT through post- transcriptional mechanism such as alternative pre-mRNA splicing. Examples of the target genes that have been identified to be affected by altered splicing hence producing oncogenic variants that can trigger EMT are *CD44, FGFR2*, *RON*, and *CTNND1*.

Therefore, this study investigated the possibility of hormone- induced alteration of splicing factors activity leading to accumulation of CD44s and CD44v6 splice variants and activation of processes leading to EMT by examining the expression of selected known EMT markers i.e. Twist and Snail in cellular models of ER+ and ER- breast cancer cells. Previously, it was shown that while both splice isoforms contribute to cancer progression, CD44s expression was found to be more strongly linked with breast cancer metastasis, as this isoform was found highly expressed in TGFβ-induced EMT and showed positive correlation with EMT marker, ZEB1, and ALDH1, one of the malignant stem cells biomarkers (164,260). Meanwhile, high level CD44v6 has been demonstrated to play crucial role in the acquisition of metastatic features in human carcinoma cells such as colorectal and pancreatic carcinoma (256–258,262), and in the promotion of cell motility in breast cancer cells (379).

This thesis found that the expression of Twist and Snail were positively correlated with the level of CD44s and CD44v6 in ER+ cells, in which the expression of these EMT markers were increased in stimulated cells, especially in cells treated with CRH, and Twist and Snail mRNA expression was enhanced when the stimulated cells were pre-treated with AKT kinase inhibitor. Akt kinase is a downstream target of the PI3K/AKT pathway, and plays important role in cancer cell survival, cell cycle entry and glucose metabolism, and alterations in this signaling route are frequently found in cancer such as breast cancer (83,85,380). The PI3K/AKT pathway is activated often by signals from extracellular stimuli that activate receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) (381). The regulation of signaling downstream this pathway is highly complex and multifaceted, in which Akt regulates its downstream effectors by two general mechanisms; acute regulation through post-translational modification by phosphorylating its substrates, thus controlling their activity and localization, and through long-term changes in cellular behaviour by phosphorylation, hence affecting the activity of various transcription factors (381). Therefore, while Akt was shown to play a role in the transcription of basal Twist expression, this result from this study shows that it may not involve in the E2- and CRH- induced signaling mechanism that leads to increased transcriptional activity of Twist and Snail mRNA. This reflects that there probably be a convergence of signaling machinery in stimulated cells where Akt is inhibited in ER+ cells.

This finding supports the hypothesis made from earlier finding in CD44 splicing assay that, whilst Akt was demonstrated to play major part in this signaling pathway leading to cancer progression and activation of EMT- associated genes, this study somehow shows that blockade of its signaling cascade may lead to similar or a more unfavourable effect due to potential signal diversion or the presence of compensatory loops that may trigger activation of various other protein effectors or signaling networks in the cells. The difference in Twist and Snail mRNA expression in response to each treatment in MCF7 cells was as illustrated in Figure 5.10.

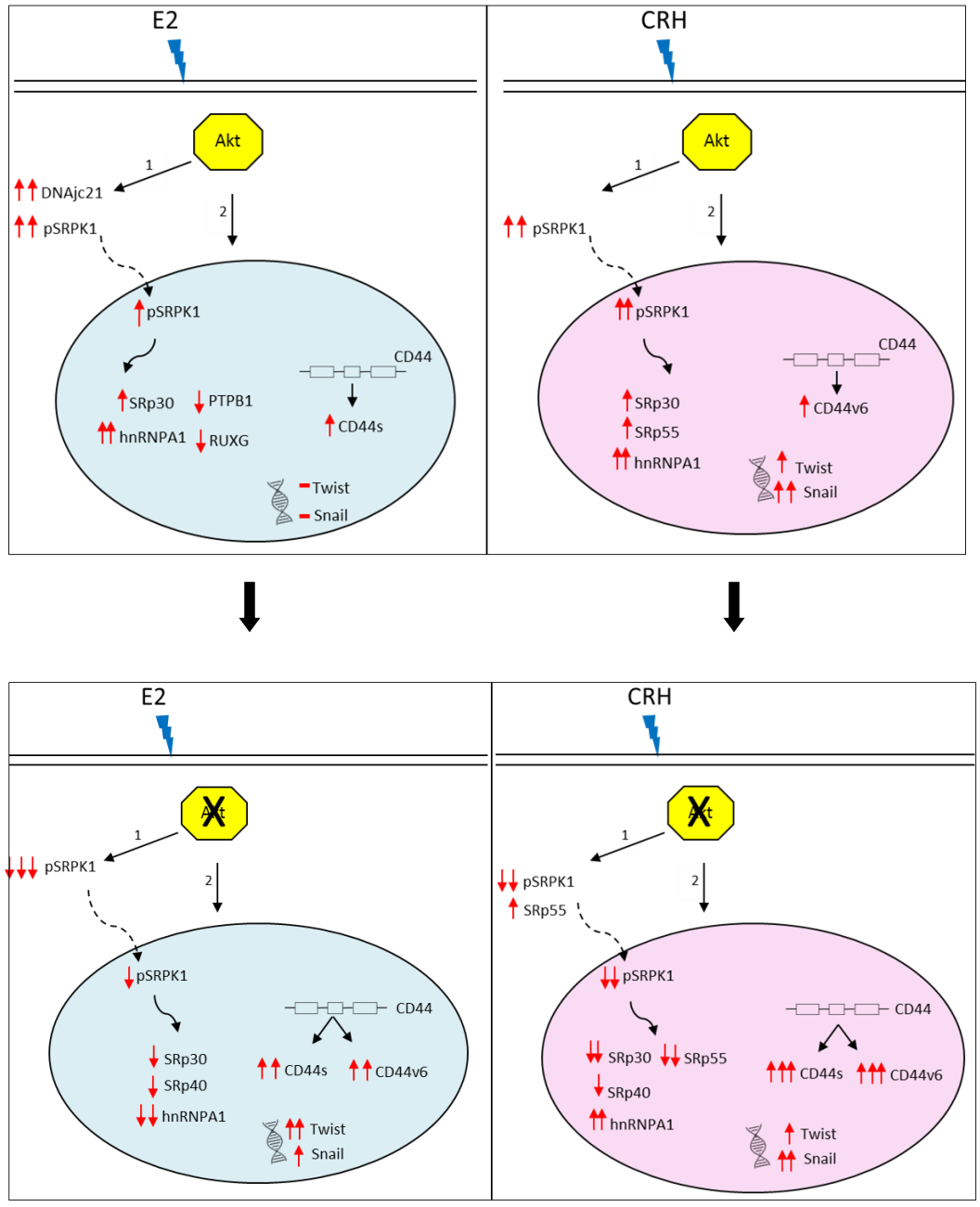


Figure 5.10 Illustration of differential expression of splicing factors, CD44s and CD44v6 splice variants, and Twist/Snail mRNA expression when cells were treated with either E2 or CRH, in the presence or absence of Akt inhibitor in MCF7 cells.

Interestingly, although reduced SRPK1 activity in the stimulated cells resulted in significantly reduced level of CD44v6 variant, this did not affect the expression of Twist and Snail as illustrated in Figure 5.11, which may further suggest that the upregulation of Twist and Snail previously found in the stimulated cells was due to the increased amount of CD44s in the cells, but not CD44v6. This finding is therefore consistent with growing evidence that CD44s plays more significant role in the cancer metastasis, as it was revealed that isoform switching from CD44v to CD44s and increased ratio of CD44s/CD44v mRNA is required to accelerate EMT in breast cancer cells (145). The demonstrated higher level of CD44s than CD44v6 mRNA in stimulated cells with reduced AKT or SRPK1 kinase, may also indicate that isoform switching was taking place in the cells, and that cells were stimulated towards becoming more mesenchymal type than the non-stimulated counterparts in the ER+ breast cancer cells. In addition, the comparison between estrogen and CRH mechanism of action in regulating splicing proteins in MCF7 is as shown in Table 5.8.

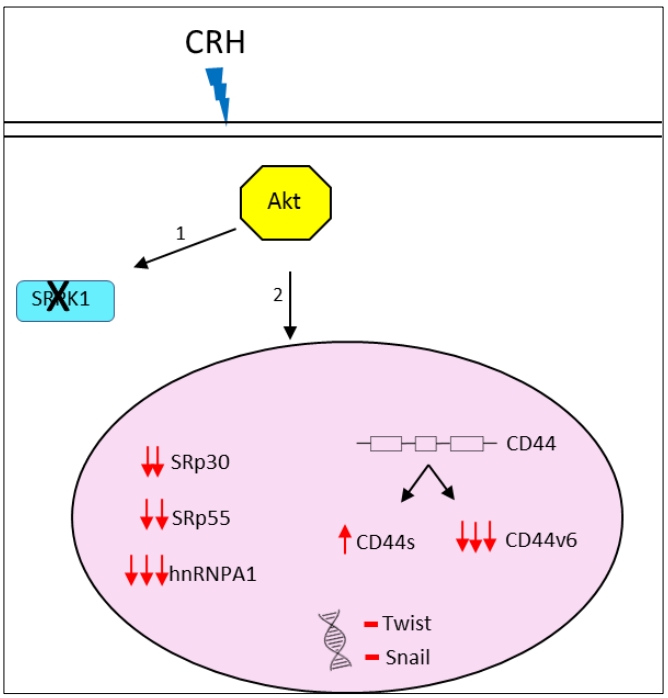


Figure 5.11 Illustration of differential expression of splicing factors, CD44s and CD44v6 splice variants, and Twist/Snail mRNA expression when cells were treated with CRH, in the presence of SRPK1 inhibitor in MCF7 cells.

Table 5.8 Comparison between E2 and CRH action in MCF7 cells.

Protein Expression		MCF7	
		E2	CRH
CYTOPLASM	pSRPK1	↑↑	↑↑
	SRp75	–	
	SRp55	–	
	SRp40		
	SRp30		
	hnRNPA1		
NUCLEUS	pSRPK1	↑	↑↑
	SRp75	–	
	SRp55	–	↑
	SRp40	–	
	SRp30	↑↑	↑
	hnRNPA1	↑↑	↑↑
mRNA expression	CD44s	↑	–
	CD44v6	–	↑
	Twist	–	↑
	Snail	–	↑↑

Furthermore, in the CRH- stimulated ER- SKBR3 cells, similar finding was also observed regarding the correlation between CD44s level and the induction of EMT gene expression, Twist and Snail, in which, the expression of these EMT markers was positively correlated with CD44s level, but not CD44v6, regardless of AKT kinase activity (Figure 5.12). The correlation between CD44s and Snail expression has also been described in previous studies, in which Snail was shown as one of CD44s targets in cancer cells (363). This result may also suggest that CD44v6 does not influence the transcriptional activity of Twist and Snail in ER- cells. Additionally, the difference in CRH actions in MCF7 and SKBR3 is compared in Table 5.9.

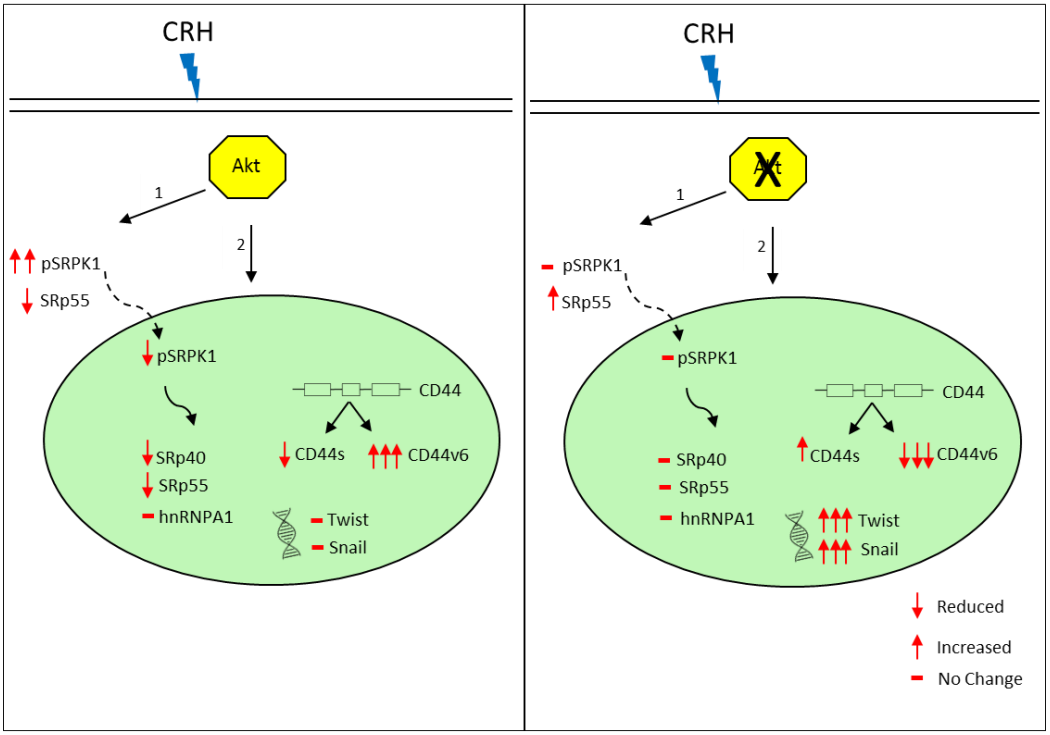


Figure 5.12 Illustration of differential expression of splicing factors, CD44s and CD44v6 splice variants, and Twist/Snail mRNA expression when cells were treated with CRH, in the presence or absence of Akt inhibitor in SKBR3 cells.

Table 5.9 Comparison of CRH action in MCF7 cells and SKBR3 cells.

Protein expression		MCF7 (ER+)	SKBR3 (ER-)
		CRH	CRH
CYTOPLASM	pSRPK1	↑↑	↑↑
	SRp75		
	SRp55		↓
	SRp40		
	SRp30		
	hnRNPA1		
NUCLEUS	pSRPK1	↑↑	↓
	SRp75		
	SRp55	↑	↓
	SRp40		↓
	SRp30	↑	
	hnRNPA1	↑↑	–
mRNA expression	CD44s	–	↓
	CD44v6	↑	↑↑↑
	Twist	↑	–
	Snail	↑↑	–

Profiling of 84 EMT gene transcription regulated by CRH in MCF7 cells

As CRH has been demonstrated to potentially induce EMT genes expression in breast cancer cells, the effects of CRH as well as the impact of inhibiting either Akt or SRPK1 kinase in the stimulated cells on the transcriptional regulation of 84 selected EMT signature genes were further interrogated using human EMT genes profiling PCR array. This can provide information about the expression of groups of EMT genes that may be co-ordinately regulated in ER+ cells. This assay can also reveal the correlation between differential expression of these genes and the change in cells behaviour undergoing EMT such as increased proliferation, migration or invasion.

In this study, using fold change of >2.0 and p-value <0.05 to determine significant differences in mRNA expression between two groups, it was found that untreated cells showed increased transcription of VCAN, MMP3, MMP9, TFPI2, PTP4A1 and ITGA5 when AKT was inhibited in the cells. These genes such as VCAN and MMPs involve in extracellular matrix (ECM) for example in the regulation of intercellular signaling and cells communication with ECM, as well as in the proteolysis of ECM, respectively. Increased expression of these genes has been found to correlate with increased cancer cells growth, differentiation, and migration. As expected, many more EMT genes were found downregulated, some of which were the most representative markers of EMT such as TIMP1, TGFB2, NOTCH1, KRT19, ERBB3, CTNNB1, and WNT5A suggesting the important role of Akt in signaling pathways leading to activation of these genes in cells. In addition, while NOTCH1 and WNT5A overexpression is well known driver in metastasis, decreased expression of KRT19 has been found to be strongly associated with the promotion of metastasis, whereas the mesenchymal phenotype of some cancer cells were shown to have loss ERBB3 leading to increased tumour survival (365). This finding indicates the major role of AKT in various cell signaling pathways leading to cancer progression and a potential presence of feedback loops that leads to the activation of alternative signaling pathways, when AKT kinase was inhibited.

In addition, growing evidence has demonstrated the central role of SRPK1 in the regulation of splicing mechanism in cells. Alterations in its expression can lead to pathogenesis of many diseases such as cancer and cancer metastasis, for examples, its overexpression has been shown to inhibit apoptosis in breast cancer cells through aberrant splicing of RBM4 mRNA (192), and increased SRPK1 expression is also correlated with angiogenesis, through aberrant splicing of VEGF (187). In addition, other than cancer, upregulation of SRPK1 activity has also been shown to promote alternative splicing of Tau gene, resulting in the pathogenesis of Alzheimer disease (183). The profiling of EMT- associated gene expression from this study demonstrated that while none of the EMT gene expression were found upregulated, basal expression of COL3A1, TGFB2, CAV2, BMP7, KRT19, WNT5A, NOTCH1, EGFR and VPS13A were reduced when SRPK1 kinase was inhibited, suggesting that SRPK1 directly or indirectly involves in the transcription of these genes in the cells. Interestingly, some of these downregulated genes namely CAV2, COL3A1, KRT19, NOTCH1, TGFB2 and WNT5A were found commonly downregulated in cells where Akt was inhibited (Figure 5.13), suggesting that the activation of these gene expression probably follows the Akt-SRPK1 axis, a network proposed in previous studies (190). In addition, this finding also suggests that transcriptional effect of SRPK1 on BMP7, EGFR and VPS13A is independent of Akt in ER+ cells.

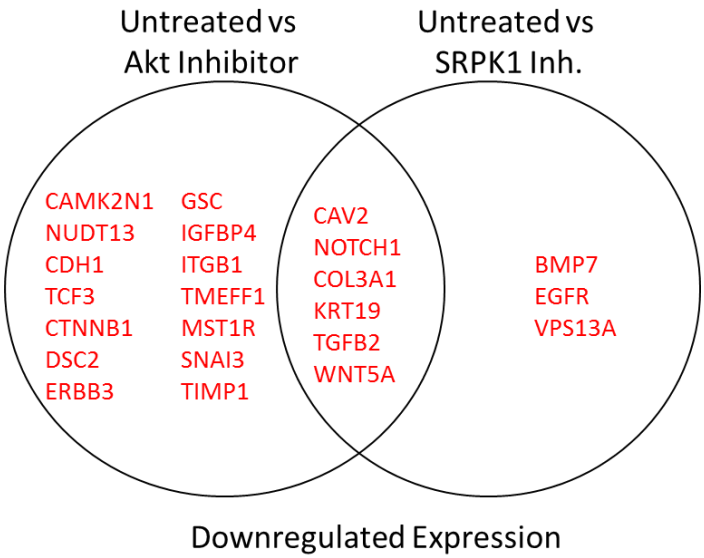


Figure 5.13 Diagram shows the list of downregulated EMT genes in response to the treatment with Akt inhibitor or SRPK1 inhibitor in MCF7 cells, each was compared to untreated cells. Commonly downregulated genes are shown in the overlapped region.

Furthermore, the expression of Occludin, OCLN (2.007-fold), RGS2 (2.0019-fold), and MAP1B (2.0019-fold) was found upregulated in response to CRH treatment. Occludin is a tight junction transmembrane protein and plays a role in the formation and regulation of the tight junction permeability barrier. In addition, gene encodes for the regulator of G protein- coupled receptor, RGS2 and gene important in the regulation of cytoskeletal re-organisation in the cells, MAP1B were also increased. Notably, when Akt was inhibited in these stimulated cells, the expression of VCAN (30.7-fold), MMP3 (15.4-fold), MMP9 (3.74-fold), PTP4A1 (2.64-fold) and ITGA5 (2.62-fold) was found downregulated. Interestingly, the fact that these genes were found upregulated when Akt was inhibited in control cells suggests that CRH can reverse the effects of Akt inhibitor on the activation of these gene expression. In addition, NOTCH1, KRT19, ERBB3, WNT5A and 33 other EMT markers such as ILK, TGFB1, IL1RN, CTNNB1 (β-catenin) and GSK3B were found upregulated in these cells. This finding suggests that while CRH alone was shown not able to modulate GSK3B and β-catenin gene expression, inhibiting the activity of AKT kinase in the cells resulted in significant increased expression of both genes, suggesting that Akt masks the transcriptional effect of CRH, through CRH-receptors (CRH-R), on the above-mentioned mRNA transcripts.

This gene expression result is in line with findings from previous student in this group, which showed that at protein level, AKT was demonstrated to restrain the inhibitory effects of CRH, through CRHR1, on β-catenin though a potential converging mechanism involving AKT, GSKB and the Wnt signaling pathway in ER+ breast cancer cells (141). In addition, as the expression of these upregulated genes were not found increased in CRH- stimulated cells, it also suggests that CRH can drive the transcriptional regulation of these genes, but its effect was blocked by Akt in the cells. As illustrated in Figure 5.14, among other upregulated genes found when Akt was inhibited in stimulated cells were the MAP1B, OCLN, and RGS2, which suggests that the transcription of these three genes by CRH is independent of Akt activity in the cells.

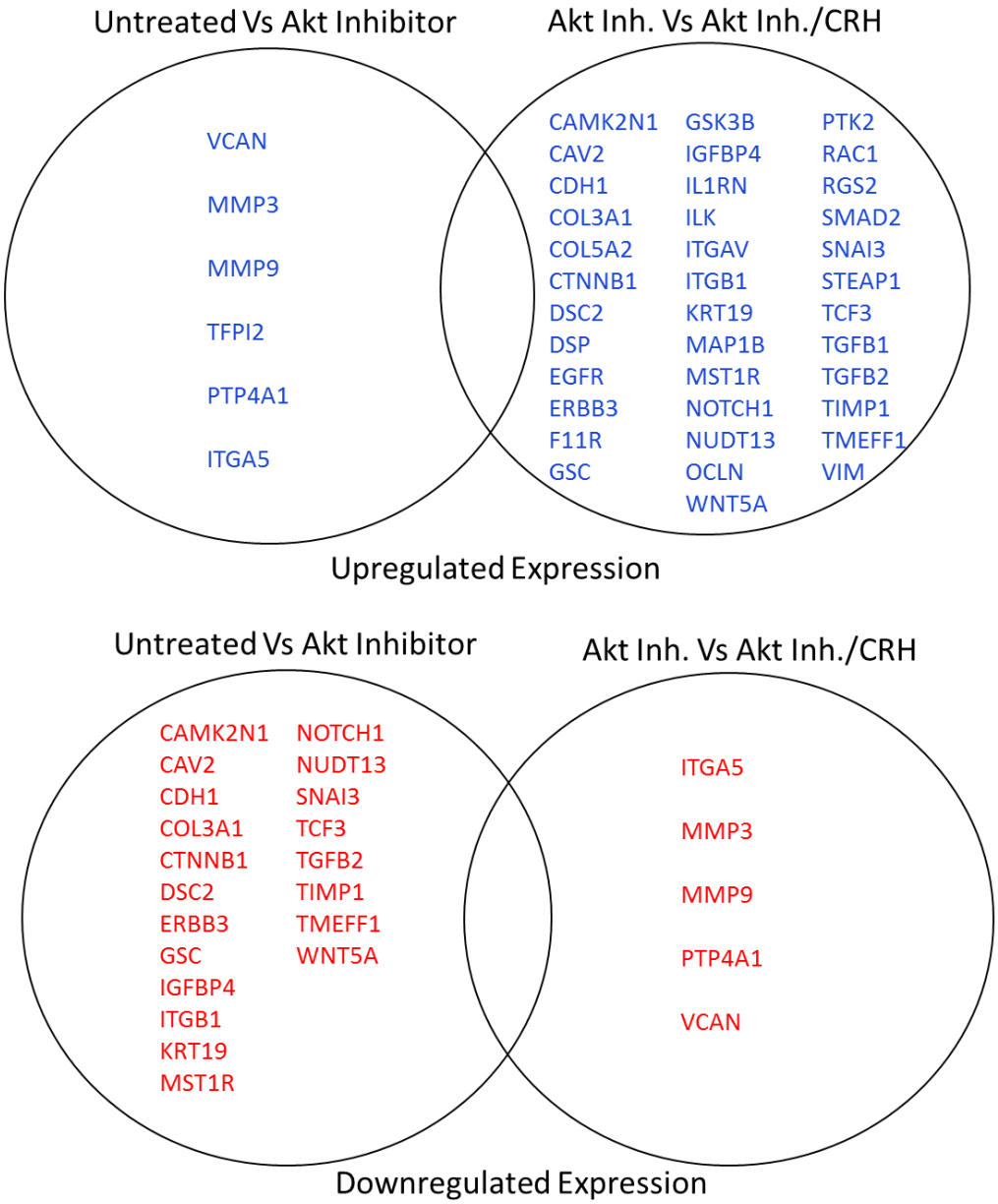


Figure 5.14 Comparison between upregulated and downregulated EMT genes between cells treated with Akt inhibitor alone and in CRH-stimulated cells when Akt was inhibited.

Moreover, when SRPK1 was inhibited in the stimulated cells, 27 genes such as VIM, STAT3, and MMP9 were found upregulated. Vimentin (VIM) is a widely used marker for cells undergoing EMTs that take place during embryogenesis and metastasis (382), whilst STAT3 activation has been shown to contribute to resistance to apoptosis in breast cancer cells though the NF-kB pathway activation (383). Apparently, these upregulated genes also included all the genes that were found downregulated when SRPK1 was inhibited in the control cells. This result suggests that CRH can reverse the inhibitory effects of SRPK1 inhibitor on the transcription of those genes in the ER+ cells. Notably, results from this experiment showed that the expression of most of the EMT genes were significantly affected when Akt or SRPK1 was inhibited in the cells, as shown in Table 5.10, suggesting the direct or indirect roles of these kinases in regulating cellular processes leading to the transcription of various EMT genes in the cells.

Table 5.10 Comparison in EMT gene expression profile when stimulated cells were treated with either Akt inhibitor or SRPK1 inhibitor.

	Akt Inh-CRH				SRPK1-inh/ CRH				
Akt-Inh	CAMK2N1	GSK3B	PTK2	VCAN					
	CAV2	IGFBP4	RAC1	MMP3					
	CDH1	IL1RN	RGS2	MMP9					
	COL3A1	ILK	SMAD2	PTP4A1					
	COL5A2	ITGAV	SNAI3	ITGA5					
	CTNNB1	ITGB1	STEAP1						
	DSC2	KRT19	TCF3						
	DSP	MAP1B	TGFB1						
	EGFR	MST1R	TGFB2						
	ERBB3	NOTCH1	TIMP1						
	F11R	NUDT13	TMEFF1						
	GSC	OCLN	VIM						
			WNT5A						
SRPK1 Inh					BMP7	IL1RN	OCLN	TGFB2	TMEM132A
					CALD1	ITGB1	PLEK2	TMEFF1	
					CAMK2N1	KRT19	RGS2	VIM	
					CAV2	MMP9	SNAI2	VPS13A	
					COL3A1	MSN	STAT3	WNT5A	
					COL5A2	NOTCH1	STEAP1	ZEB2	
					EGFR	NUDT13	TCF3		

Impacts of differential expression of EMT genes on cell migration and invasion properties

In addition to EMT profiling, functional assays to monitor the association of cancer cells behaviour with the expression of various EMT genes studied were performed. In line with previous findings, this study demonstrated that E2 promoted MCF7 cell proliferation, but CRH has no proliferative effect in MCF7 cells (384). In addition, consistent with the downregulation of the majority of EMT genes in basal cells when Akt was inhibited, which some of them play roles in cell growth and proliferation such as ERBB3 and KRT19 (365), reduced cell proliferation ability was displayed in these cells, as well as in the stimulated cells with reduced Akt activity.

Previous studies have demonstrated discrepancies in CRH role in tumor progression, which some have shown that CRH induces colon cell survival and proliferation via NF-kB and STAT3 pathway (137), and it was also revealed to promote cell migration and invasion in breast cancer and prostate cancer via CRHR1 (103,104,138). In other studies, CRH was found to inhibit invasion in breast cancer (291), and cell growth and proliferation in human endometrial carcinoma (101), hepatocellular carcinoma (106) and in dermal fibroblast (107). This thesis has demonstrated that in agreement with findings from Androulidaki et.al (104), that while CRH via Akt, did not promote breast cancer cells proliferation, it was shown to induce cell migration and invasion in ER+ MCF7 cells. Notably, the increased in cell migration and invasion observed in the stimulated cells might be explained by the elevated level of Snail which was shown to contribute to tumor cell migration and invasion via NF-kB pathway in cancer cells (385).

Furthermore, a slightly different effect of CRH was observed in ER- SKBR3 cells, in which CRH via Akt was shown to promote cell proliferation. However, it showed no effect on SKBR3 cells invasion, which again correlated with unaffected expression of Snail in the cells.

Previous studies have revealed that SRPK1 is one of the key determinants in cancer metastasis, as it was shown to promote cell proliferation, migration and invasion in ovarian cancer, prostate cancer and breast cancer (193,386,387). Follow up experiment on cellular behaviour in cells where SRPK1 was inhibited showed that the cell migration and invasion properties was decreased in MCF7 cells. This observation might be associated with downregulation of EMT genes, such as KRT19, BMP7, NOTCH1, and TGFB2 which were shown to be positively correlated with growth and invasive ability of cancer cells (365,388,389). This finding may suggest that SRPK1 may enable cancer cells to acquire invasive ability through the activation of EMT-associated signaling pathways such as TGFβ, NOTCH and Wnt signaling whose role in cell motility has been widely demonstrated in various cancers. In addition, in cells where SRPK1 was inhibited, CRH was shown to reverse the effect of SRPK1 inhibitor, leading to the upregulation of KRT19, BMP7, and MMP3 and subsequently resulted in a slight increase of cell invasion index. Notably, reduced invasion property was also demonstrated in SKBR3 cells when SRPK1 was inhibited in stimulated cells suggesting that SRPK1 can promote cell invasion in both ER+ and ER- cells.

Additionally, when the action of CRH was further investigated by pre-treating stimulated cells with CRH receptors inhibitors, CRH-R1 inhibitor (NBI 27914) or CRH-R2 inhibitor (Asstressin-2B), it was found that cell invasion was reduced when CRHR1 was blocked, as compared to cells where CRHR2 was inhibited, in both breast cancer subtypes. Interestingly, proteome analysis using Nano-flow UltraHPLC in cells depleted with SRPK1 kinase showed that GNAS2, a protein important in the activation of adenylate cyclase activity by G protein signaling pathway was found downregulated (data not shown). Furthermore, phosphosite analysis in these cells also demonstrated reduced detection of signal specific for phosphorylation site of corticotropin- releasing hormone receptor (CRH-R1/R2) at Tyrosine 208 position (data not shown). These findings may possibly suggest that SRPK1 kinase might potentially regulate the activity of CRH receptors, in which reduced phosphorylation of these receptors might lead attenuation of the receptors signaling activity, and thus leading to reduced pro-

migration and invasion effects of CRH in breast cancer cells. However, further studies are needed to verify this finding.

CHAPTER 6

Discussion

Recent studies have found that pre-mRNA splicing patterns are frequently altered in cancer, and in addition to mutations that alter mRNA splicing, altered expression of splicing-regulating proteins can also promote oncogenesis. In addition, alterations in alternative splicing are also evident in cells undergoing epithelial- to-mesenchymal transition (EMT) program. Notably in breast cancer, the role of hormones such as estrogen in the pathogenesis of the disease has been well- established. Additionally, hormonal stress response is also associated with cancer biology and studies on the role of stress hormones, such as corticotropin-releasing hormone (CRH) in breast cancer show the complexity of its actions in cancer development. Therefore, this study was set out to assess the potential role of key hormones (Estrogen and CRH) and their key molecular pathways in promoting cancer progression through the mechanism of pre-mRNA alternative splicing and epithelial-to-mesenchymal transition EMT) event in cellular models of breast cancer.

This study has demonstrated that estrogen (E2) and CRH can potentially alter proteome profiles in ER+ MCF7 cell line, through the modulation of a key kinase in mRNA splicing mechanism, Serine- Arginine Protein Kinase 1(SRPK1). The effects of both hormones on SRPK1 activity and nuclear translocation resulted in increased phosphorylation level of serine- arginine (SR) proteins and hnRNPA1 expression, leading to altered CD44 mRNA splicing hence increased production of CD44 splice isoforms, CD44s and CD44v6 in ER+ cells. Furthermore, increased level of CD44 splice variants, particularly CD44v6 in CRH-stimulated cells correlated with upregulation of epithelial-to-mesenchymal transition (EMT)-associated gene expression Twist/Snail and increased cell migration and invasion properties in the stimulated cells.

Additionally, EMT gene profiling analysis revealed that in addition to Twist and Snail, CRH was able to enhance the transcriptional activity of several other prominent EMT markers such as VIM, TGFB1/2, NOTCH1, MMP9 and Wnt5A when either Akt or SRPK1 kinase was inhibited in the cells, potentially due to convergence in signaling mechanism in the cells. In the ER- SKBR3, CRH has no effect on SRPK1 nuclear translocation thus leading to unchanged level of SR proteins phosphorylation and hnRNPA1 expression. Notably, differential expression of CD44 splice isoforms were still demonstrated in the cells, and increased cell invasion was also observed, indicating that CRH exerted its effect in these cells via different signaling mechanism that enabled the cells to acquire invasive properties, one of the hallmarks of EMT.

Conclusions and future works

Accumulating evidence suggests that aberrant alternative splicing is linked to various diseases including cancer. Mutations or alterations in the activity of splicing-related proteins can influence the splicing pattern of susceptible target genes, hence producing oncogenic gene isoforms. It has been reported that altered pre-mRNA splicing accounts for at least 15% of human diseases such as cystic fibrosis, spinal muscular atrophy, as well as it can induce proliferative and invasive properties in cancer cells (390,391).

This thesis explored the possibility of a female sex hormone, estrogen and stress-associated hormone, CRH to trigger aberrant splicing event and EMT program in breast cancer cells. One of the main contributions of this work is to discover that both hormones can upregulate splicing kinase, SRPK1, which is one of the central switches in alternative pre-mRNA splicing program. This finding has therefore led to deeper investigation on the processes and the key molecular factors involved that lead to alteration in pre-mRNA splicing by using CD44 as reporter gene. The relationship between hormone-induced alternative splicing and activation of epithelial-to-mesenchymal transition (EMT) program was also addressed, in which, accumulation of CD44 splice variants positively correlated with EMT gene expression. Another contribution of this study is the fact that CRH can potentially divert its signaling pathway during the transcriptional regulation of EMT gene whenever Akt or SRPK1 kinase activity was inhibited. This emphasizes the complexity of CRH signaling in cancer cells, which may probably involve cross-talks with several other molecular networks and eventually trigger the transcription of various other EMT signature genes in breast cancer cells.

Future works

Findings from this study were obtained from experiments using breast cancer cell lines as models to investigate the potential role of hormones (estrogen and CRH) in promoting cancer progression via alternative pre-mRNA splicing event and EMT program in the cells. Therefore, for the future works, animal models (in vivo) could be used to strengthen and complement these findings. In addition, a recently-developed and improved molecular biology tool such as CRISPR/Cas9 system can be utilized for targeted genome editing to ensure more efficient downregulation/silencing (time-wise, reversible, more than one targets at a time) of genes of interest.

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