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**sp<sup>2</sup>-Iminosugar-glucosidases inhibitor  
1-C-octyl-2-oxa-3-oxocastanospermine - induced  
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Nahla Gueder

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**Thèse de Doctorat**  
*Mention Biologie Santé*  
*Spécialité Physiopathologie humaine*

présentée à *l'Ecole Doctorale en Sciences Technologie et Santé (ED 585)*

**de l'Université de Picardie Jules Verne**

par

**Nahla GUEDER**

pour obtenir le grade de Docteur de l'Université de Picardie Jules Verne

***sp<sup>2</sup>-Iminosugar -glucosidases inhibitor 1-C-octyl-2-oxa-3-oxocastanospermine - induced antiproliferative, apoptotic and necrotic effects in breast cancer cells via targeting GRP78, Stim1 and Orai1***

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## Résumé

L'altération de glycosylation est l'une des caractéristiques du cancer du sein. Ainsi le défaut de glycosylation affecte différentes protéines glycosylées responsables des différents processus cancéreux. Les canaux SOC (Store operated channels) constituent la voie majeure de l'entrée du calcium dans les cellules et sont impliqués dans la prolifération, la migration et la survie des cellules cancéreuses du sein. CO-OCS est un nouvel inhibiteur de la glycosylation avec plus de sélectivité vis-à-vis des  $\alpha$ -glucosidases, et montre des activités anticancéreuses des cellules cancéreuses du sein, sans affecter les cellules mammaires normales. L'objectif de ma thèse est d'étudier les mécanismes moléculaires par lesquels CO-OCS induit ses effets anti-tumoraux.

CO-OCS inhibe la migration des cellules cancéreuses à fort potentiel métastatique. Cet effet anti-migratoire est dû à une réduction de l'expression de la  $\beta$ 1-intégrine, de Stim1, et de l'activation des voies de signalisation FAK et ERK1/2 par CO-OCS. Dans les cellules cancéreuses peu invasives, CO-OCS diminue la prolifération et augmente la mortalité de ces cellules en affectant l'expression de 3 protéines : Stim1 et Orai1 : protéines N-glycosylées au niveau du réticulum endoplasmique (RE), et GRP78, protéine de stress du RE. Ainsi en supprimant complètement l'expression de Stim1, CO-OCS réduit la prolifération en accumulant les cellules dans les phases G1 et G2/M du cycle cellulaire. Alors que la réduction de l'expression de GRP78 et d'Orai1 par le CO-OCS augmente respectivement l'apoptose et la nécrose. Par ailleurs, l'inactivation de Stim1 atténue l'effet apoptotique induit par CO-OCS. CO-OCS réduit aussi le contenu calcique du RE. Cette réduction du calcium réticulaire est due à une fuite de calcium par le Translocon. En effet, l'Anisomycine, inhibiteur du Translocon, restaure de contenu calcique réticulaire et antagonise l'apoptose induite par le CO-OCS.

En conclusion, CO-OCS induit une accumulation de protéines mal-repliées dans le RE induisant ainsi un stress réticulaire. Trois cibles du CO-OCS ont été identifiées : l'expression de Stim1 favorise la prolifération tandis que celle d'Orai1 et de GRP78 protègent respectivement les cellules de l'apoptose et de la nécrose induites par CO-OCS. De plus, en diminuant l'expression de GRP78, CO-OCS induit une fuite du calcium du RE par le Translocon.

## Summary

Alteration in glycosylation pattern is one of the hallmarks of breast cancer. The levels and the abnormal expressions of glycan were found in breast cancer patients. Glycosylation defect can affect different glycosylated proteins which are implicated in cancerogenesis. Changes in intracellular  $Ca^{2+}$  levels can regulate different cellular processes. SOC channels are implicated in breast cancer proliferation, migration and survival. CO-OCS is a new glycosylation inhibitor with more selectivity toward the  $\alpha$ -glucosidases exhibited anti-cancer activities in breast cancer cells without affecting the normal mammary cells. The objective of my thesis is investigating the related molecular mechanisms by which CO-OCS induced its anti-tumour effects.

CO-OCS impaired breast cancer migration through decrease  $\beta$ 1-integrin expression and the activation of FAK and ERK1/2 signalling pathways. CO-OCS also induced anti-migratory effect via Stim1 protein expression down-regulation leading to inhibition of SOCE. Additionally, CO-OCS affected the expression of both Orai1 and Stim1 proteins leading to anti-proliferative effects and cell cycle arrest in G1 and G2/M phase respectively. Moreover, CO-OCS affected the expression of Stim1 at the protein level without affecting its transcript level. GRP78 implicated in CO-OCS apoptotic death. The expression of Stim1 regulated the apoptosis induced by CO-OCS via modulating GRP78 expression. Orai1 down-regulation promoted CO-OCS necrotic effect. CO-OCS induced ER- calcium depletion due to increase in ER calcium leak via the Translocon; Anisomycin (Translocon inhibitor) decreased the apoptosis induced by CO-OCS.

In conclusion, these results show that in breast cancer, by targeting Stim1, Orai1 and GRP78, CO-OCS reduced cell proliferation and induced apoptosis and necrosis cell death. Stim1 favours CO-OCS apoptotic effect while Orai1 protected from necrosis induced by CO-OCS. The inhibition of Translocon decreased CO-OCS apoptotic cell death via restoring the ER calcium homeostasis.

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## **Abbreviations**

5-FU: 5-Fluorouracil

ADP: Adenosine Diphosphate

AFP: Serum  $\alpha$ -Fetoprotein

AMPK: AMP-Activated Protein Kinase

ASK1: Apoptotic-Signaling Kinase-1

ATCC: American Type Culture Collection

ATF6: Activating Transcription Factor 6

ATP: Adenosine Triphosphate

Bcl2: B-cell lymphoma 2

BiP: Binding immunoglobulin Protein

BRCA 1&2: Breast Cancer 1 &2

BSA: Bovine Serum Albumin

C1GALT: Core-1-Synthase-Glycoprotein-N-Acetylgalactosamine-3-Beta-Galactosyltransferase-1

CA 15-3: Cancer Antigen 15-3

Ca<sup>2+</sup>: Calcium

CDKs: Cyclin-Dependent Kinases

CKI: Cyclin-Dependent Kinase Inhibitory Proteins

CREB: cAMP-Responsive Element-Binding Protein

DNA: Deoxyribonucleic Acid

ECM: Extracellular Matrix

EDTA: Thylenediaminetetraacetic Acid

EGF: Epidermal Growth Factor

EGTA: Tethylene Glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-Tetraacetic Acid

eIF2 $\alpha$ : eukaryotic Translation Initiation Factor 2

ER: Endoplasmic Reticulum

ERAD: Endoplasmic Reticulum Assisted Degradation

ERKs: Extracellular-Signal-Regulated Kinases

FAC: Focal Adhesion Complexes

FADD: Fas-Associated Death Domain

FAK: Focal Adhesion Kinase



GALNT9: Polypeptide N-Acetylgalactosaminyltransferase 9  
GCNT2: Glucosaminyl (N-Acetyl) Transferase 2  
GlcNAc: N-acetylglucosamine  
GRP78: Glucose-Regulated Proteins 78  
HER2: Human Epidermal Growth Factor Receptor 2  
HMGB1: High Mobility Group Box 1  
HSP70: 70kDa Heat Shock Proteins  
IC<sub>50</sub>: The half maximal inhibitory concentration  
IRE1 $\alpha$ : Inositol-Requiring Protein-1 $\alpha$   
JNK: Jun-N-Terminal Kinase  
MAPK: Mitogen-Activated Protein Kinase  
MMPs: Matrix Metalloproteinases  
MPTP: Mitochondrial Permeability Transition Pore  
mRNA: Messenger Ribonucleic Acid  
mTORC1: mammalian Target Of Rapamycin Complex 1  
Muc1: Mucin1  
PARP1: Poly(ADP-Ribose) Polymerase 1  
PBS: Phosphate Buffer Saline  
PERK: Protein Kinase RNA (PKR)-Like ER Kinase  
PMCA: Plasma Membrane Calcium ATPase  
RB: Retinoblastoma Protein  
ROS: Reactive oxygen species  
SICE: Store-Independent Ca<sup>2+</sup> Influx  
SOCs: Store-Operated Channels  
SRP: Signal Recognition Particle  
Stim1 or 2: Stromal interaction molecule type 1 or 2  
TBS/TBS-T: Tris-buffered saline/ Tris-buffered saline-Tween  
TCR- $\alpha$ : T-cell Antigen Receptor  $\alpha$  Chain  
TG: Thapsigargin  
TGF $\beta$ : Transforming Growth Factor Beta  
TM: Transmembrane Domain  
TNF: Tumor necrosis factor  
TRADD: TNF Receptor-Associated Death Domain

TRAF2: TNF Receptor-Associated Factor 2

TRP: Transient Receptor Potential

UGGT: (UDP)-glucose:glycoprotein glucosyltransferase

UPR: Unfolded Protein Response

# ***Introduction***

## ***Chapter I: The Mammary Gland***

### ***1.1 Mammary Gland Anatomy***

The mammary gland is an exocrine gland derived from two thickened strips of epidermal ectoderm that is totally surrounded by adipose and sub connective tissues. Its main functions are production and secretion of milk (lactation). The glandular tissue is made up of 20 sections called lobes, each lobe is composed of 20 to 40 lobules, and each lobule is composed of clusters of alveoli, which are the basic secretory part of the mammary gland.

Each acinus drains through an alveolar channel, which is lined by cuboidal, or low columnar epithelial cells (which produce and secrete milk), and surrounded by myoepithelial cells (which allow by their contraction the evacuation of milk); the two cell layers rest on a basal membrane. These alveolar channels join at the level of the lobules to drain by their turn to interlobular channels. Finally, these last channels empty into a lactiferous duct that empties onto the nipple surface (Figure 1). Milk secretion happens due to the contraction of the smooth muscle, which surrounded these channels. Both sexes develop glandular tissues in the embryonic stage, later on during puberty, females glandular tissues start to develop in response to hormones stimuli.

The arterial vascular supply of the mammary gland comes from the internal thoracic artery, the lateral artery and the intercostal arteries. In addition to this rich vascular network, the mammary gland is also characterized by a highly developed lymphatic drainage where there are the cutaneous lymphatic network and the glandular network. In fact, these rich vascular supply and lymphatic drainage play a crucial role in breast tumour progression and metastases.

### ***1.2 Mammary Gland Development***

In contrast to the other human organs, the mammary gland passes through several distinct stages from embryonic development until post-menopausal atrophy (Javed and Lteif 2013). The main development of the glandular tissues occurs at puberty in the females; in males, these tissues stay immature and do not develop after born.

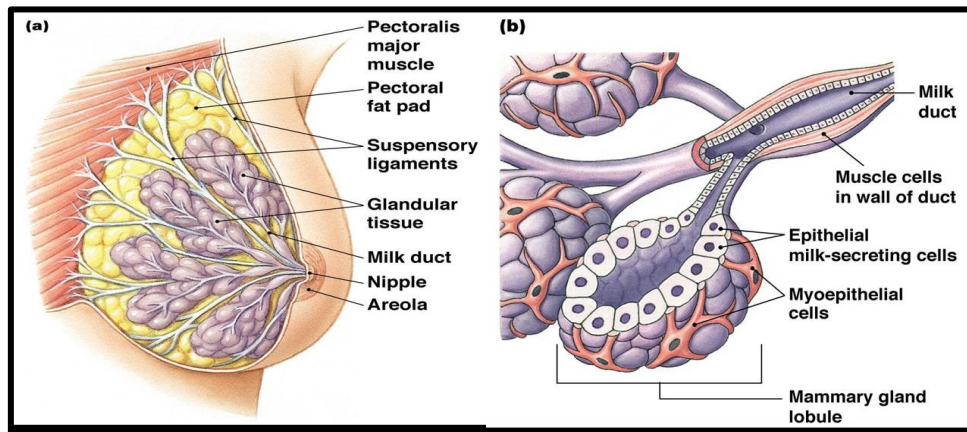


Figure 1. Histology of the mammary gland source:

<http://humanbiologylab.pbworks.com/w/page/104941359/Histology%20of%20the%20Mammary%20Gland>

### **1.2.1 Embryonic Development**

This stage starts at the fourth week of gestation; the mammary gland derived from paired ectodermal thickenings (mammary ridges or milk lines) on the embryonic ventral side from the axilla to the inguinal region. Later on, these mammary ridges begin to proliferate, raising the primary mammary buds. After 10 gestational weeks, the primary bud begins to branch, giving secondary buds; followed by the development of the mammary lobules by the 12<sup>th</sup> week. Toward the 2<sup>nd</sup> and 3<sup>rd</sup> trimesters of pregnancy, these fetal buds continue lengthening and branching to form the lactiferous ducts (Figure 2).

The glandular tissues development in this stage is induced in response to many placental hormones which are prolactin, progesterone, growth hormone, insulin-like growth factor, estrogen, adrenal corticoids, and triiodothyronine.

### **1.2.2 At Birth**

Newborn from both sexes have identical rudimentary mammary glands, each gland is composed of 15-20 mammary lobes draining into lactiferous ducts; these latter by turn drain into retro-areolar sinus (ampulla) that open on the surface of the nipple. In males, breast stays at this stage of development for the rest of their life.

### 1.2.3 At Puberty

Due to lack of hormonal stimulation, mammary gland grows slowly throughout childhood. At puberty, the growth of the glandular tissues increases inducing an increase in volume accompanied by an increase in connective and adipose tissues development.

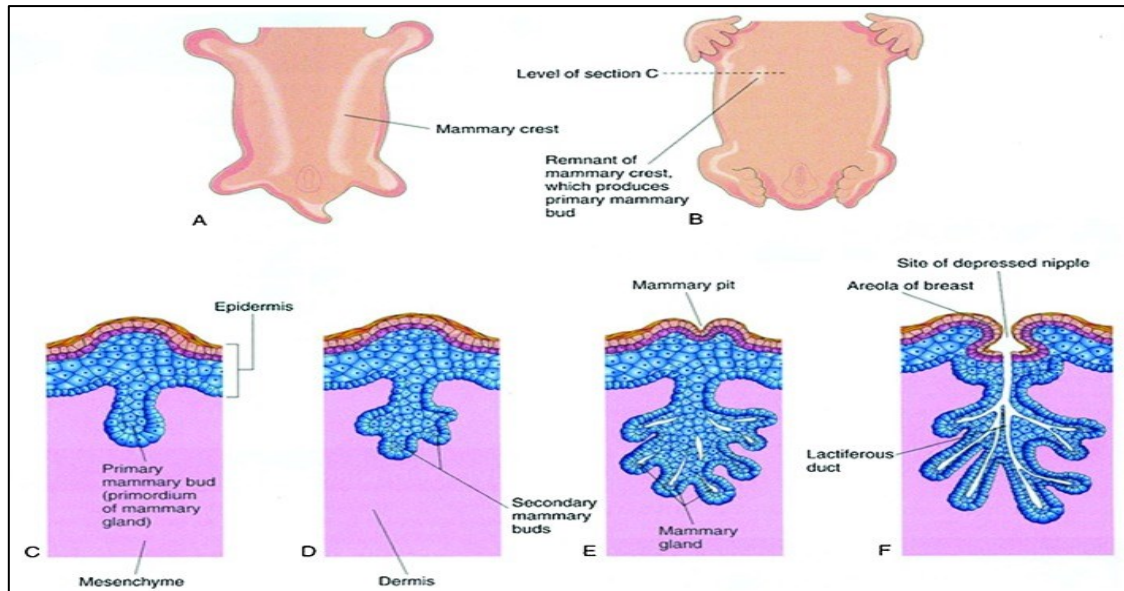


Figure 2. “Development of the mammary gland; (A) Ventral view of an embryo at 28-days gestation showing mammary crests; (B) Similar view at 6-week gestation showing the remains of the mammary crests; (C) Transverse section of a mammary crest at the site of the developing mammary gland; (D–F) Similar sections showing successive stages of breast development between the 12th week of gestation and birth”. (Javed and Lteif 2013).

### 1.2.4 During Pregnancy and Lactation

During pregnancy, the hormonal influence such as; estrogen, progesterone, prolactin and corticosteroids strongly initiates ductal, alveolar, and myoepithelial hyperplasia proliferation and differentiation. The lobular epithelial cells remain as presecretory cells until a decrease in estrogen and progesterone concentrations in the plasma after giving birth; however, these two hormones inhibit the active secretory effects of prolactin.

### 1.2.5 At Menopause

During this phase of life, women pass through variable hormonal changes. Their mammary glands undergo morphological and functional transformations due to ageing and the hormonal inhibition; however, the mammary tissue undergoes an involution characterized by a regression of the glandular mass (atrophy) and an increase in the adipose tissue.

### ***1.3 Breast Cancer***

#### ***1.3.1 Epidemiology***

According to GLOBOCAN-2012, Breast cancer (BC) is the second most commonly diagnosed cancer in the world, with about 1.67 million new cases diagnosed in 2012, representing 25% of all cancers. Anyway, it stills the most common cancer among women in developed and less developed countries; where BC is the first cause of cancer mortality in women in developing countries  $\approx 14.3\%$  and the second cause of cancer mortality in developed countries  $\approx 15.4\%$ . In France, BC is also the most diagnosed female cancer with 48,763 new cases in 2012; while BC mortality rate is about 18.8% (11 886 deaths estimated in 2012).

#### ***1.3.2 Risk Factors:***

The causes of breast cancer are not exactly known, but some risk factors for the development of this cancer have been identified. Among these factors:

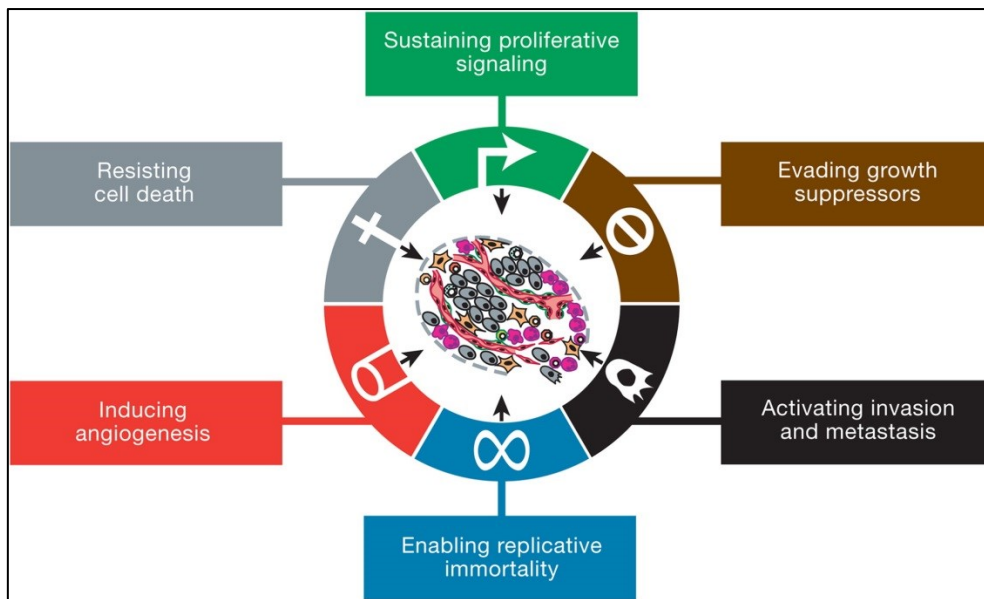
- **Age**; the risk of developing breast cancer is increased by getting older. Less than 10% of breast cancers occur before the age of 40. The incidence then increases steadily until the age of 65 years.
- **Genetic predispositions**; women with genetic mutations of BRCA1 and BRCA2 (Breast Cancer 1 and 2), which are autosomal dominant, have a risk of developing breast cancer during their lifetime in 55 to 85% of cases. These mutations represent nearly 5% of total breast cancer cases.
- **Hormonal factors**; prolonged body exposure to endogenous estrogens such as early menarche (before 11), late menopause (after 55), nulliparous women and first pregnancy after 30. Also, uses of Oral Contraceptives and post-menopausal Hormone Replacement Therapy (HRT) appear to increase the risk of developing breast cancer.
- **Lifestyle**; Cancer, in general, is promoted in particular by a high-fat diet, alcohol consumption and tobacco. Being overweight and a non-active obese person also increase the risk.

#### ***1.3.3 Breast Cancer Development***

The transformation of a normal cell into a cancer cell is a quite complicated process. It is characterized by many changes at the cellular and genetic levels; where the cells lose their

balance through having cell division out of control (proliferation signals with insensitivity to anti-proliferative signals), resistance to programmed cell death (apoptosis), angiogenesis formation and ability to spread into the adjacent tissues and distance organs. These genetic alterations are diverse such as; chromosomal deletions, translocations, gene rearrangements, duplications, amplifications and mutations. According to Hanahan and Weinberg 2011, six biological capabilities acquired during the multistep development of human tumours (Figure 3) (Hanahan and Weinberg 2011):

- Sustaining Proliferative Signaling and Self-Sufficiency in Growth Signals,
- Insensitivity to Anti-Proliferative Signals,
- Evading Apoptosis,
- Limitless Replicative Potential,
- Sustained Angiogenesis,
- and Tissue Invasion and Metastasis.



*Figure 3. Illustration of the six hallmark capabilities of cancer cells (Hanahan and Weinberg 2011)*

The complicated process of carcinogenesis can be summarized in three basic steps (Figure 4):



- **Initiation:** it is the first step in cancer development, resulting in irreversible genetic alteration as mutations, transitions, transversions and deletions in the DNA. Many factors are involved in these alterations like genotoxic agents of chemical origin, radiation, environmental or viral, as well as; it could happen spontaneously. However, these harmful genetic alterations can alter genes that regulate cell growth and other cellular mechanisms; for example; the mutation of the BRCA1 gene promotes other alterations leading to breast cancer development.

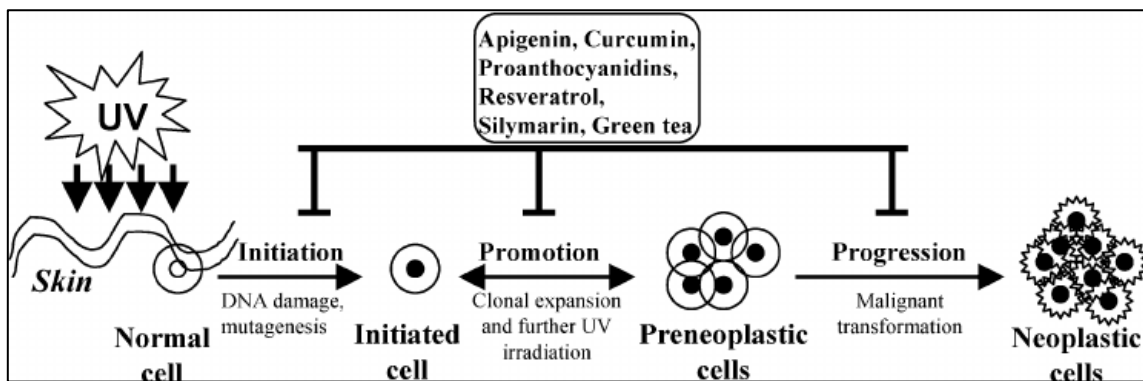


Figure 4. Schematic representation of UV radiation-induced multi-stage (initiation, promotion and progression) skin carcinogenesis (Baliga and Katiyar 2006).

- **Promotion:** it is started once the mutated DNA by an initiator escapes its cellular repair mechanisms, and the cell starts to divide carrying this new DNA defect. These mutated cells are stimulated to grow and favoured by growth factors, hormonal stimuli and the oncogenic activation as; c-Myc and Erb2. The promotion step is considered as reversible pre-cancerous cells.

- **Progression:** known as an irreversible stage where cells begin to transform into a malignant state. Other processes have accompanied this stage such as metastasis and angiogenesis. The dysregulation of several oncogenes and suppressors of breast cancer has been reported during the progression step of a tumour such as; c-myc, erbB2, CCND1, TP53, BRCA1 and BRCA2.

#### 1.3.4 Breast Cancer Types

Almost 98% of breast cancers are adenocarcinomas (cancers of epithelial nature); in general, there are two categories of breast cancer:

### 1.3.4.1 Carcinoma in Situ

In this category, the basal membrane remains integral, and the cancer cells proliferate in the lumen of the gland and are confined to the lobules. We can distinguish Ductal carcinoma in situ (DCIS) and Lobular carcinoma in situ (LCIS) (Figure 5).

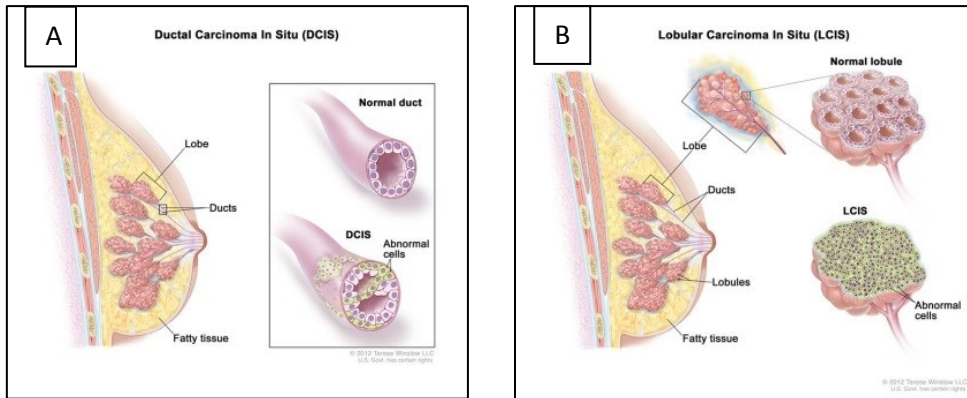


Figure 5. (A) Ductal carcinoma in situ, <https://www.cancer.gov/news-events/cancer-currents-blog/2015/dcis-low-risk>

(B) Lobular carcinoma in situ,

<https://www.cancer.gov/publications/dictionaries/cancer-terms/def/lobular-carcinoma-in-situ>

### 1.3.4.2 Invasive (Infiltrating) Cancer

In contrast to carcinoma in situ, the barrier of the basal lamina in the invasive cancer is broken, and the cells invade the underlying stroma and tissues. About 80% of diagnosed breast cancers belong to this category. It can be subdivided into invasive ductal carcinoma and invasive lobular carcinoma (Figure 6); both represent respectively 80% and 5-10% of this cancer category.

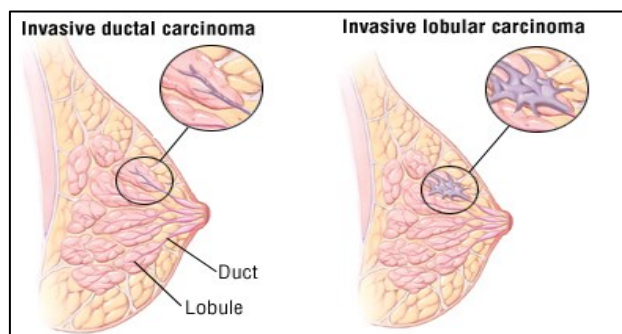


Figure 6. Invasive Carcinoma, source:

<https://www.health.harvard.edu/womens-health/breast-cancer>

### ***1.3.5 Classification of Breast Cancer***

#### ***1.3.5.1 T.N.M Classification (Staging)***

Breast cancer is classified into different stages according to the international classification of malignant tumours (T.N.M staging) (Table 1):

- T (Tumor): size and direct extent of a primary tumour.
- N (lymph Node): degree of spread to the regional lymph nodes.
- M (Metastasis): indicates whether cancer has spread to other body organs or not.

#### ***1.3.5.2 Histological Grading***

The Nottingham grading system, also known as the Scarff-Bloom-Richardson grading system) is used in grading breast tumours based on three features; Tubule formation, nuclear pleomorphism and mitotic rate. Every feature has a score between 1 and 3. The total sum of scores will determine the cancer grade;

- G1: well differentiated, where its total score between 3–5,
- G2: moderately differentiated total score between 6-7,
- G3: poorly differentiated with a total score of 8 or 9.

UPR branch	Target protein	Activation mechanism	Physiological outcomes	Role in autophagy
<b>ERN1(IRE1α)</b>	XBP1	Unfolded protein-mediated dissociation of HSPA5 leads to the homodimerization and autophosphorylation of IRE1. Activated IRE1 uses endoribonuclease (RNase) for nonconventional mRNA splicing and transmission of UPR signals from the ER to the cytosol and nucleus. The RNase function of ERN1 involves cleavage of a 26-base fragment intron from the XBP1 mRNA and subsequent religation by an uncharacterized RNA ligase and formation of a frame shift that encodes the spliced transcript isoform XBP1s	The encoded XBP1 protein subsequently translocates to the nucleus where it acts as a potent transcription factor by binding to UPR elements and activating a broad spectrum of UPR-related genes involved in ER membrane biogenesis, ER protein folding, physical expansion of the ER, ERAD, protein entry into the ER, protein quality control, protein secretion from the cell, and autophagy.	Induction and inhibition
	MAPK8	Cytoplasmic domain of activated ERN1 recruits and forms a complex with TRAF2 and MAP3K5, which in turn phosphorylates and activates MAPK8	Activated MAPK8 controls both cell survival and cell death by regulating autophagy and apoptosis accordingly. The latter is converged into NFκB signalling and CASP4-CASP12 induction.	Induction and inhibition
<b>EIF2AK3 (PERK)</b>	NFE2L2	EIF2AK3 substrate NFE2L2 is phosphorylated and translocated to the nucleus under ER stress conditions. In unstressed cells, NFE2L2 is sequestered in the cytoplasm via association with KEAP1	In the nucleus, NFE2L2 binds with the antioxidant response element and upregulates expression of genes involved in the synthesis of antioxidant proteins such as glutathione, drug transport, xenobiotic metabolism, and elimination of ROS and autophagy	Induction
	IF2S1	ER stress causes dimerization of the cytosolic kinase domain of EIF2AK3 resulting in autophosphorylation, which in turn phosphorylates EIF2S1	p-EIF2S1 blocks global protein translation, thereby lessening the ER protein burden. In addition, autophagy-mediated cell protection under various stress conditions also substantially depends on EIF2S1 activation.	Induction
	ATF4	ATF4 mRNA is selectively translated following EIF2S1 phosphorylation because it contains upstream open reading frames that allow it to escape from the p-EIF2S1-mediated translational blockage resulting in synthesis of a transcription factor	Transcription factor ATF4 translocates to the nucleus and activates a set of UPR target genes involved in amino acid metabolism, antioxidant responses, autophagy, and apoptosis.	Induction
	DDIT3	DDIT3, a potent transcription factor, is upregulated via the transcription activity of ATF4	DDIT3 potentiates autophagy and apoptosis through various molecular signals. An apoptosis signal is implicated in enhanced protein synthesis, which in turn leads to increased oxidative stress and subsequent cell death	Induction and inhibition
<b>TF6</b>	TF6	ATF6 activation includes monomer formation by reduction of ATF6 luminal intramolecular and intermolecular disulfide bonds and exposition of a Golgi-localization sequence (GLS). Those monomers then translocate to the Golgi through interactions with coat protein II (COPII). In the Golgi, ATF6 is processed into an active form of transcription factor ATF6 via the sequential activity of 2 Golgi-resident proteases, MBTPS1/S1P and MBTPS2/S2P.	In the nucleus, ATF6 binds to ER stress response elements and stimulates transcription of a subset of UPR target genes, including HSPA5 and DDIT3. ATF6 also upregulates XBP1 mRNA levels. XBP1 is then further processed by ERN1 and translocates to the nucleus to bind with the unfolded protein response elements therein. Thus, ATF6 gives positive feedback to the UPR.	Induction

Table 1. T.N.M classification, source:

<https://emedicine.medscape.com/article/2007112-overview>

### ***1.3.5.3 Molecular Classification of Breast Cancer***

In this classification, breast cancer has been divided into five molecular types (Dai, Li et al. 2015):

1. **Luminal A**; where it represents about 23% of invasive breast cancer, characterized by the expressions of estrogen and progesterone receptors without expression of HER2 with low levels of Ki-67 proteins; it does respond to hormonal therapy and has a good prognosis.

2. **Luminal B**; it counts for 42% of invasive breast cancer, characterized by the expressions of estrogen, progesterone receptors and Ki-67 proteins; the HER2 receptors can have negative or positive expressions; it has higher proliferation and histologic grade than luminal A. Its prognosis ranges from an intermediate to poor.

3. **HER2**; it is almost 11% of total breast cancers, characterized by high expressions of HER2, but negative expressions of estrogen and progesterone receptors. This type is characterized by high proliferation as well as high histological grade. In addition to that it has diffuse TP53 mutation; usually unfavourable prognosis for this sub-type.

4. **Triple-Negative Breast Cancers**; known as triple negative due to the lack of estrogen, progesterone and HER2 receptors expressions. This subtype of breast cancer is less differentiated and characterized by TP53 gene mutation in the most of the cases. These characteristics make it difficult in term of response to anti-cancer treatment; however, this aggressive sub-type does not respond to hormonal therapy that targets estrogen, progesterone receptors or Trastuzumab which is targeting HER2. Anyway, it may respond to particular chemotherapeutic agents; in general, it has a bad prognosis.

5. **Normal-like**; represents about ~8% of invasive breast cancer, where it has positive expressions of estrogen and progesterone receptors; while the expressions of HER2 and Ki-67- are negative. This sub-type has an intermediate prognosis.

### ***1.3.6 Breast Cancer Treatment***

Breast cancer management depends on many different criteria as, cancer subtype, hormonal response, stage and metastasis.

### ***1.3.6.1 Breast Surgery***

Usually, it is the first-line of breast cancer treatment; almost all patients have a breast surgery as part of their treatment. In the clinical basis, there are two types of breast surgery for breast cancer:

- **Breast-Conserving Surgery:** in this type of surgery, only a part of the breast which is containing the tumour mass will be removed this intervention also known as; lumpectomy, quadrantectomy, partial mastectomy depends on some factors as; tumour size and the anatomical site.
- **Mastectomy:** this surgery is mainly considered in advanced breast cancer, where the mammary gland is removed accompanied with or without axillary dissection and other nearby tissues removal. This surgery can also be done as a preventative procedure against breast cancer in patients who have a very high risk to develop breast cancer; especially women have BRCA1 or BRCA2 mutations in their genes as well as dense breasts images in mammography that impair surveillance in locally advanced cancer.

### ***1.3.6.2 Radiotherapy***

It is a local treatment, given as postoperative setting. Radiation dose (fractionation) and location depend on breast cancer subtype and its stage. In general, patients are receiving high-energy X-rays of 50 Gy in 25 daily fractions over 5-7 weeks. These rays are destroying breast cancer cells, through causing significant DNA lesions.

### ***1.3.6.3 Hormonal Therapy***

Breast cancer patients that have cancer cells expressing estrogen and progesterone receptors can benefit from this type of treatment; through decreasing the total amount of the estrogen hormone and blocking the action of estrogen on breast cancer cells. It can be given at different cancer stages; mainly as adjuvant therapy (after breast surgery) to help reduce the risk of recurrence, but sometimes it can be used as neoadjuvant therapy (before breast surgery) in advanced and metastatic stages. The three different types of hormonal therapy are:

- Selective estrogen receptor modulators as; Tamoxifen, Evista and Fareston.
- Aromatase inhibitors as; Arimidex, Aromasin and Femara.
- Estrogen receptor down-regulators as; Faslodex.

#### ***1.3.6.4 Chemotherapy***

Chemotherapy is an effective way of fighting cancer. Basically, they are cytotoxic molecules, which inhibit active cell proliferation and induce apoptosis through triggering different signalling pathways as; blocking DNA replication and transcription. This treatment can take place before or after breast surgery to improve patient's prognosis. Chemotherapeutic molecules used in the treatment of breast cancer can be:

- **Antimitotic:** these molecules act through targeting the microtubules and inhibiting mitosis; by their mechanism of action we can distinguish two types; Vinca Alkaloids (inhibit microtubules polymerization) or Taxanes (enhance microtubule polymerization).
- **Alkylating agents:** alkylating agents are compounds that work by covalently binding the two complementary strands of a DNA molecule and preventing the strands of the double helix from linking as should be; affecting the cancer cell ability to multiply and leading to cancer cell dies. Alkylating agents were one of the first classes of drugs has been used against cancer.
- **Antimetabolites:** (5-Fluorouracil, Gemcitabine and Methotrexate),
- **Topoisomerase inhibitors:** (Irinotecan, Etoposide, Teniposide) and Anthracyclines (Doxorubicin, Epirubicin and Idarubicin).

#### ***1.3.6.5 Targeted Therapy***

These are new cancer drugs can be used in the treatment of specific breast cancers. As chemotherapy has a general effect on health and normal and cancer cells, these targeted molecules take advantage; where can target a specific change in cancer cell either a gene or protein. Moreover, it can categorize into two main types: (1) the Monoclonal antibodies such as Trastuzumab and Pertuzumab. (2) Small-molecule drugs such as CDK4/6 inhibitors, Lapatinib and Neratinib, all of them are kinase inhibitors. For example, breast cancer patients with BRCA gene mutation can benefit of this treatment modality, where Olaparib (PARP inhibitors) which works by blocking the Poly (ADP-ribose) polymerase proteins responsible for DNA repair (Masoud and Pagès 2017).

## ***Chapter II: Tumour Development and Progression***

### ***2.1 Tumour Development***

#### ***2.1.1 Cell Cycle***

Cell cycle or cell division cycle as also known is necessary to maintain tissue proliferation and homeostasis; in which series of tightly regulated events happened in the mother cell leading to its division to produce two daughter cells. The cell cycle is divided into four major phases that go from quiescence (G<sub>0</sub> phase) to proliferation (G<sub>1</sub>, S, G<sub>2</sub>, and M phases) and back to quiescence (Norbury and Nurse 1992). Actually, the G<sub>0</sub> is static or resting phase, in another meaning, the cell does not divide in response to mitogenic factors such as growth factors and hormones (Pardee 1974). The Restriction (R) point, in which, the cell in G<sub>1</sub> phase can proliferate independently of mitogenic stimuli to enters the next phase of the cell cycle (Pardee 1974). The phase G<sub>1</sub> characterizes by an increase in the cell size and the synthesis of mRNA and proteins. During S phase the DNA synthesis and replication occur; cell goes from 2N chromosomes in G<sub>1</sub> phase to 4N chromosomes at the end of the phase S. The G<sub>2</sub> phase, in which, the cell continues to grow rapidly and prepare for division; also protein synthesis takes place during this phase. The division phase or mitosis, M phase, characterized with nuclear division and cell division that produce two daughter cells with the same genetic component as their mother cell, actually mitosis happens in short period, and it is a highly regulated phase. Mitosis can be divided into four stages: prophase, metaphase, anaphase, and telophase.

Indeed, there are five types checkpoints: at G<sub>1</sub>, S, G<sub>2</sub> and M phases; where they permit a tight control before the transition between the different phases of cell cycle (Barnum and O'Connell 2014). The cell death signalling will activate if any of these points fail to repair lesions and errors during division. (1) **The Cell Size Control Checkpoints** have been noticed in G<sub>1</sub> and G<sub>2</sub>; the appropriate amount of genetic and biosynthetic material before the division should be controlled. (2) **DNA Damage Responses Checkpoint**, which checks the different lesions in the DNA and repairs them; the Cyclin-dependent kinases (CDKs) will be activated as a result of failure to remove these lesions. (3) **Monitoring DNA Replication** is initiated at multiple origins of replication on multiple chromosomes during S phase; when replication stress is encountered, this checkpoint signalling pathway is activated. (4) **S–M Dependency Checkpoints**, the cell is able to use post-replication repair pathways to bypass



the lesion; anyway this checkpoint must ensure that mitosis is not started until replication is complete. (5) **Mitotic Spindle checkpoint**, its main role to prevent anaphase onset until all chromosomes are properly attached to the spindle; the failure to attach correctly can lead to cell death or disease (Barnum and O'Connell 2014).

### ***2.1.2 Cell Cycle Regulatory Proteins***

#### ***2.1.2.1 The Cyclin / CDK Complexes***

The cell cycle is a complex process controlled by several regulatory proteins. These proteins are ensuring the progression from one phase to another. Cyclin-Dependent Kinases (CDKs) belong to the family of protein serine/threonine kinases; have a small molecular weight between 34 to 40 kDa. Their catalytic activity is dependent on the coupling with their regulatory cyclin subunit. In human, there are 21 CDKs (Whittaker, Mallinger et al. 2017). Indeed, the role of CDKs is varying from cell growth, proliferation, and transcriptional regulation. We can distinguish cell-cycle-related subfamilies (CDKs 1-6, 11 and 14-18) and transcriptional subfamilies (CDKs 7-13, 19 and 20) (Whittaker, Mallinger et al. 2017). Their expressions rate is almost stable during the cell cycle, but their enzymatic activities undergo different phosphorylation/dephosphorylation events and modifications, anyway, they possess different domains such as phosphorylation, ATP-binding domain, cyclin-binding domain and the T-loop domains.

In G1 phase, upon to a series of activation steps start with mitogenic stimulation and end with ERK1/2 activation, which promotes finally the transcription of the D-type Cyclin; the CDK4 and CDK6 are activated after coupling with D-type Cyclin to produce active kinase complexes, that by turns enable the Retinoblastoma protein (RB) phosphorylation (Figure 7) (Shah and Schwartz 2006; Whittaker, Mallinger et al. 2017). The transcription factors HDAC1 and the E2F will liberate from the phosphorylated RB; where these two are permitting histone acetylation, and activation of many transcription genes among them cyclin E. Subsequently, the Cyclin E is transcribed and associated with CDK2. The CDK2/CyclinE activation will also phosphorylate other RB sites permitting DNA synthesis to start. At this point S phase is set off, and will be cut off once there is a blockage in the DNA-binding ability; which is achieved as a result of phosphorylation of the transcription factor E2F1 after CDK2/cyclinA activation. During the transition point between the S and G2 phase, the Cyclin A attaches to CDK1 and enables the activation FOXM1 and 2 transcription factors. In M phase, the active complex CDK1/cyclin B will trigger mitosis, regulate genes expression and

of RB dephosphorylation by the protein phosphatase 1 (PP1). The activity of CDK / Cyclin complexes is regulated by cyclin-dependent kinase inhibitory proteins (CKI).

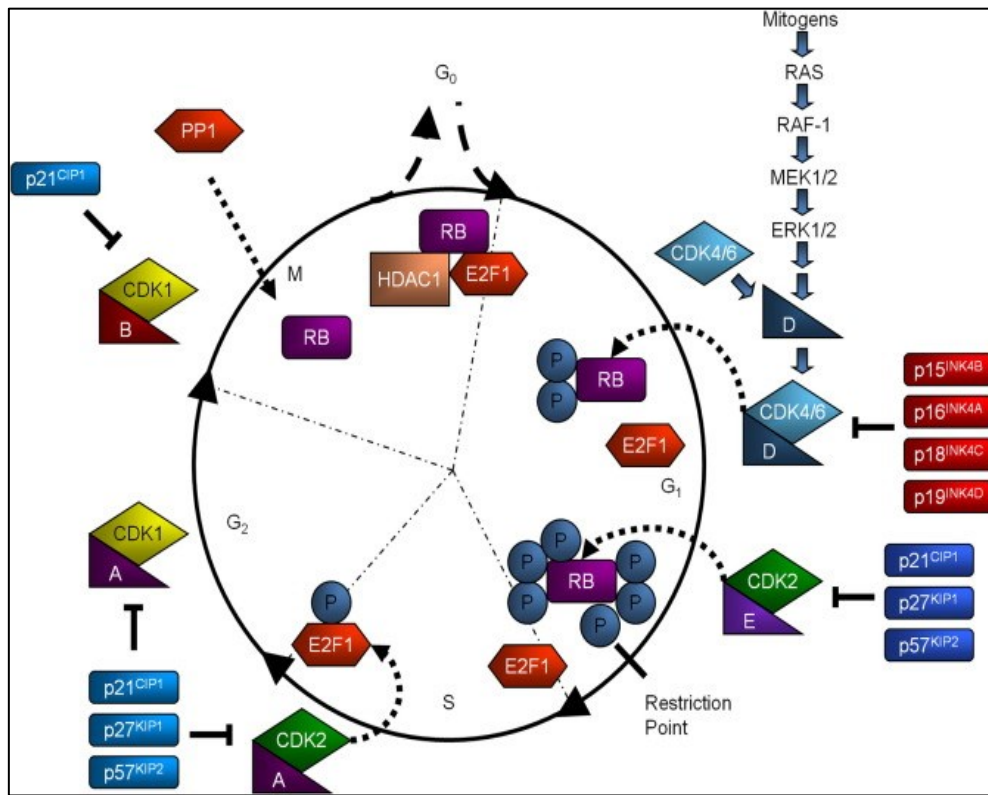


Figure 7. A simplified model of the mammalian cell cycle (Whittaker, Mallinger et al. 2017).

### 2.1.2.2 CDK / Cyclin Complexes Inhibitors

In parallel with the phosphorylation/dephosphorylation events, the CDK / Cyclin complexes can also be regulated by small polypeptides inhibitors called the CDK-inhibitors (CKIs). These inhibitors can negatively regulate the activity of CDK / Cyclin complexes. CKIs can be divided into two families in mammals. The INK4 family binds to and inhibits the CDK4/CDK6. It includes four members ( $p16^{\text{INK4a}}$ ,  $p15^{\text{INK4b}}$ ,  $p18^{\text{INK4c}}$ , and  $p19^{\text{INK4d}}$ ). The Cip/Kip family includes three members ( $p21^{\text{CIP1/WAF1}}$ ,  $p27^{\text{Kip1}}$ , and  $p57^{\text{Kip2}}$ ). The Cip/Kip family is a negative regulator of the CDK2/cyclinA, CDK2/cyclinE and CDK1/cyclinB. In contrast, this protein family can positively regulate of CDK4/6 cyclin D-complexes and permit cell progression in the early G1 phase (Canepa, Scassa et al. 2007). The role of these two families has exceeded the regulation of cell division, where they are involved in the

regulation of other cellular processes such as apoptosis, tumorigenesis and transcriptional regulation.

### ***2.1.2.3 CDK/Cyclin in Cancer***

The CDK4/6-RB pathway is a key regulator to initiate cell cycle progression from G1 to S phase, the disruption of this pathway occurs frequently in many human cancers among them breast cancer (Figure 8), (Table 2) (Ortega, Malumbres et al. 2002; Zardavas, Ponde et al. 2017). The amplification of CCND1 by 20% and the overexpression of the cyclin D1 by 70% protein have been reported in breast cancer (Zardavas, Ponde et al. 2017). Cyclin D1 expression was statistically significantly correlated with the expression of oestrogen and progesterone receptors; oestrogen can target cyclin D1 and mediates the mitogenic effects. The loss of RB expression has been reported in the basal-like phenotype breast cancer in approximately 20% of patients.

CDK1 activity was recently reported as a powerful predictor of Taxane chemosensitivity, indicating a role for CDK1 in breast tumorigenesis (Kamdje, Etet et al. 2014). CDK1 has also been implicated in the DNA damage response; BRCA1 is a direct target of CDK1; where CDK1 directly interacts with BRCA1 and phosphorylates BRCA1 at S1497 and S1189/S1191; its inhibition sensitizes cancer cells to DNA-damaging chemotherapy drugs (Johnson, Cai et al. 2009). The overexpression of the cell cycle inhibitor p16 in breast cancer linked to more aggressive phenotype (Milde-Langosch, Bamberger et al. 2001). Others CDK such as DK11 is overexpressed in triple negative breast cancers, and correlated with an advanced stage and a poor prognosis (Zhou, Han et al. 2015). By using the siRNA, *CDK11* significantly inhibited proliferation and migration as well as induced apoptosis in breast cancer cell lines (Kren, Unger et al. 2015). CDK10 gene knockdown induces resistance to Tamoxifen in breast cancer; the patients who expressed a lower level of CDK10 in oestrogen  $\alpha$ -positive tumours have a more relapsing risk (Iorns, Turner et al. 2008).

Molecular alterations of cyclin-CDK-Rb pathway across different breast cancer subtypes.				
Molecular alterations	Luminal A	Luminal B	HER2-enriched	Basal like
Cyclins	CCND1 gene amplification (29%)	CCND1 gene amplification (58%)	CCND1 gene amplification (38%)	CCNE1 gene amplification (9%)
CDKs	CDK4 gene amplification (14%)	CDK4 gene amplification (24%)	CDK4 gene amplification (24%)	-
RB	Loss of pRB Expression (6.5%)	Loss of pRB Expression (6.5%)	Loss of pRB Expression (22.5%)	RB inactivating mutations (20%) Loss of pRB Expression (65%)
INK4 Family Members	Low expression of CDKN2C (p18) gene	-	-	High Expression of CDKN2A (p16) gene

CCND1: cyclin D1; CCNE1: cyclin E1; CDK: cyclin-dependent kinase; CDKN2A: cyclin-dependent kinase inhibitor 2A; CDKN2C: cyclin-dependent kinase inhibitor 2C; pRB: protein of retinoblastoma gene.

Table 2. Molecular alterations of the CDK-RB pathway in the different subtypes of breast cancer (Zardavas, Ponde et al. 2017)

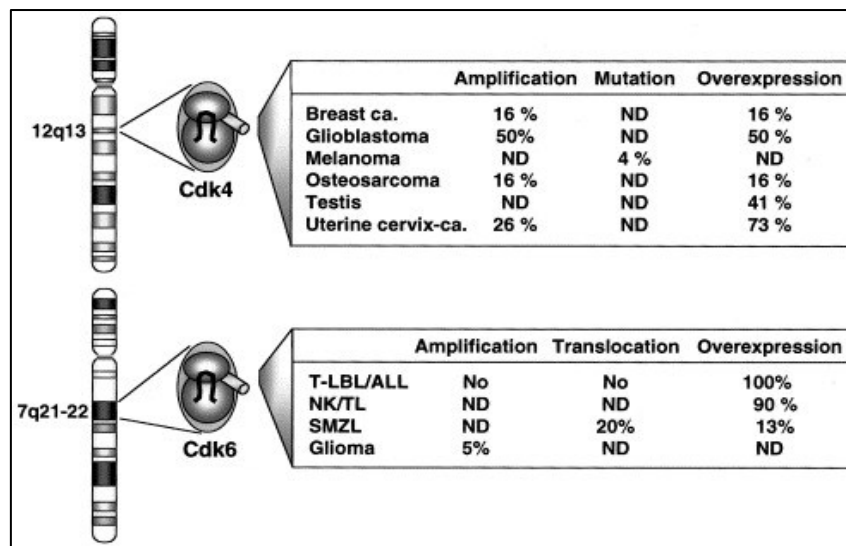


Figure 8. "Tumour-associated alterations in Cdk4 and Cdk6. Left: chromosomal location of human Cdk4 and Cdk6 genes. Right: incidence of genetic alterations in these loci found in human tumours" (Ortega, Malumbres et al. 2002)

## ***2.2 Cell Death***

This biological event happened when a cell fails to maintain the essential life functions. Different cellular criteria can be observed during this process such as; morphological, enzymological, functional or immunological criteria. During the process of death, a specific point is known as the point-of-no-return, in which, the dying cells trespass the first irreversible step in this process. The point-of-no-return could be massive caspase activation, loss of the mitochondrial transmembrane potential or complete permeabilization of the outer mitochondrial membrane, etc.

### ***2.2.1 Apoptosis***

Apoptosis is genetically programmed cell death process, essential to maintaining tissue homeostasis. Dysregulation of this mechanism leading to various pathologies, notably cancer (Cory and Adams 2002). However, Cancer cells become unable to undergo apoptosis as a result of specific apoptotic pathways impairment; such as overexpression of the anti-apoptotic regulators or inhibition of other apoptotic molecules. The activation of such mechanism will depend on the extrinsic extracellular signals (via extracellular receptors and its ligands) and/or the intrinsic intracellular signal via the mitochondrial pathway. The morphological criteria of apoptotic cells are characterized by rounding-up of the cell, retraction of pseudopods, decrease in the cellular volume, chromatin condensation, nuclear fragmentation and DNA cleavage (Wyllie, Morris et al. 1984; Kroemer, Galluzzi et al. 2009). Calcium role in both the extrinsic and intrinsic apoptosis pathways has been widely investigated; mitochondrial calcium overload is very toxic pro-apoptotic regulator, causing mitochondrial perturbation of the outer membrane, including the depolarization and the permeabilization of the outer mitochondrial membrane with the opening of the mitochondrial permeability transition pore (MPTP), and in turn the release of mitochondrial apoptotic factors into the cytosol (Giorgi, Baldassari et al. 2012).

#### ***2.2.1.1 The Extrinsic Pathway***

The extrinsic signalling pathway initiates apoptosis through specific death receptors (transmembrane receptor-mediated interactions). Transmitting the death signal from outside to inside the cell can be carried out by different death receptors and their corresponding ligands such as; FasL/Fas and TNF- $\alpha$ /TNFR1. The adaptor proteins, such as FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain) are recruited to ligand-activated and mediating the apoptotic signals. The caspases 8 and 9, which belong to

protease family, will be activated; in turn, they activate caspases 3, 6 and 7, leading to cleavage and deactivation of these targeted proteins. In the end, the activation of the enzymatic caspase cascade leads to numerous proteolytic events that mediate the apoptotic cell death process (Figure 9) (Ashkenazi 2008).

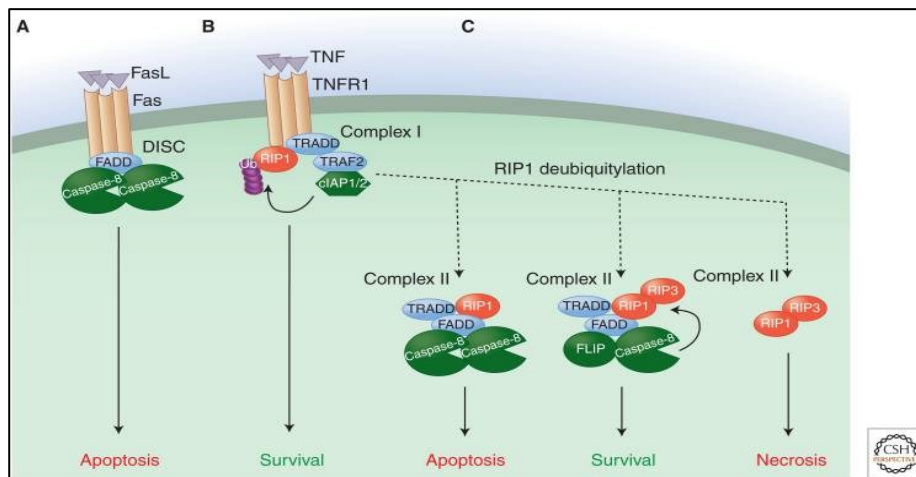


Figure 9. The extrinsic signalling pathway; FasL/ Fas and TNF- $\alpha$ /TNFR1 (Green and Llambi 2015)

### 2.2.1.2 The Mitochondrial Pathway

Apoptotic cell death can be initiated through Mitochondria (intrinsic pathway) in response to a range of different exogenous and endogenous stimuli such as DNA damage, oxidative stress, viral infection or endoplasmic reticulum (ER) stress. The mitochondria-dependent apoptosis characterized by depolarization of the mitochondrial potential; which allows the opening of the mitochondrial permeability transition pore (MPTP) permitting free passage of apoptotic factors up to 1.5 kDa in size. The MPTP is activated by  $\text{Ca}^{2+}$ , phosphate and Reactive Oxygen Species (ROS), while it is inhibition achieved by numerous factors including adenine nucleotides, low pH, and others (Kwong and Molkenin 2015). The opening of these pores releases cytochrome C from the mitochondrial intermembrane space to the cytosol; the released cytochrome C forms a complex together with Apaf-1 and pro-caspase-9 known as apoptosome (Li and Yuan 2008). This complex allows the cleavage of the pro-caspase 9 in active caspase 9. In turn, the active caspase-9 cleaves the pro-caspases 3 and 7 (Figure 10) (Suhaili, Karimian et al. 2017). Moreover, the MPTP also allows the release of the anti- and pro-apoptotic proteins of the Bcl2 family that implicated in regulating the cytochrome C/Apaf1/CASP9 signalling pathway. The Bcl2 family proteins control tightly the

apoptosis, where interact to induce or prevent apoptosis. There are three subfamilies of Bcl2 proteins; (1) the antiapoptotic Bcl2 proteins ( Bcl2, Bcl-xL, and Mcl1); (2) the proapoptotic effector proteins (Bax and Bak); and (3) the BH3-only proteins (Puma, Bid, Bim, Bad, and Noxa). Bax protein is recruited at the level of the mitochondrial membrane to allow cytochrome c release while Bcl2 inhibits the permeabilization of the mitochondrial membrane by blocking the Bax protein (Figure 11) (Shi, Chen et al. 2003; McCommis and Baines 2012).

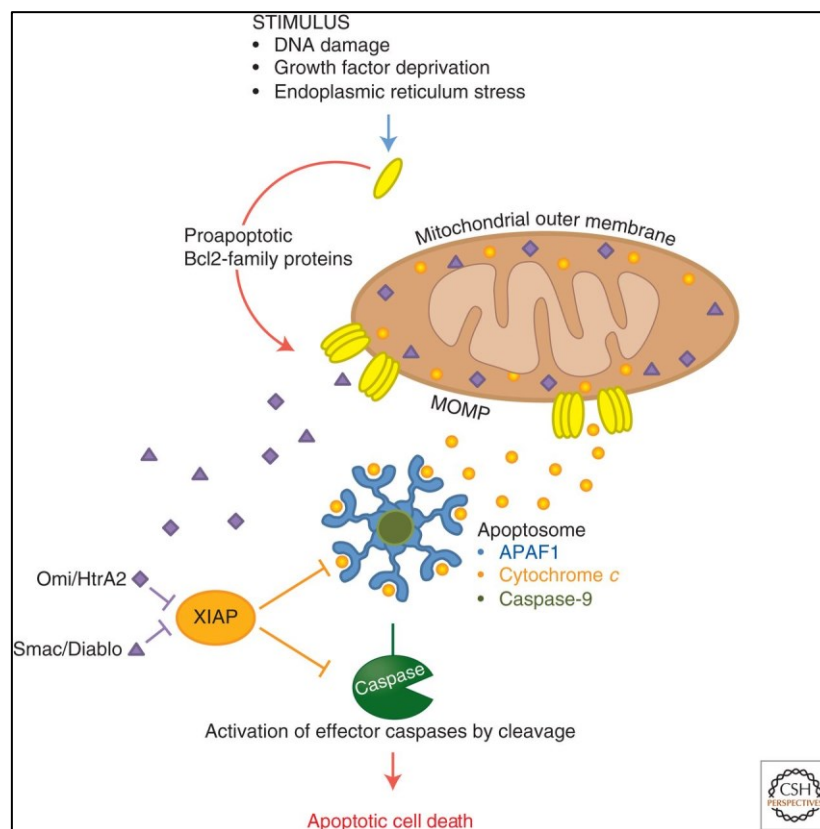


Figure 10. The Intrinsic Pathway (Green and Llambi 2015)

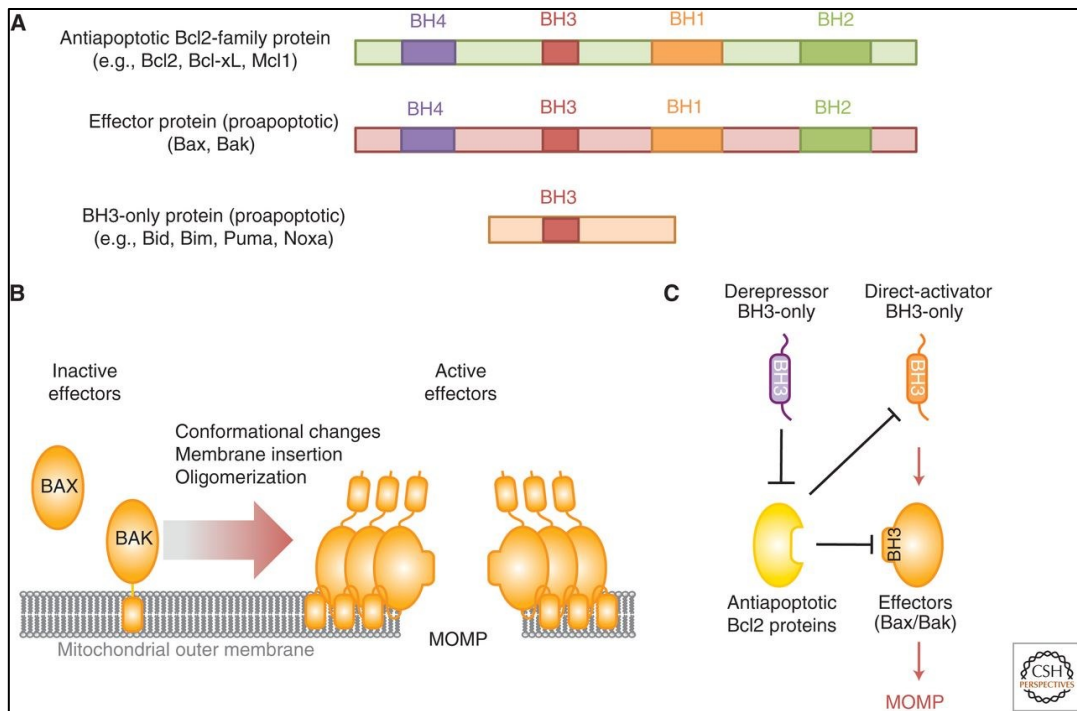


Figure 11. Regulation of mitochondrial membrane permeabilization by Bcl2 protein family (Green and Llambi 2015)

### 2.2.1.3 Apoptosis and ER

The endoplasmic reticulum (ER) is important to maintain the intracellular calcium homeostasis and proper posttranslational folding of proteins. During prolonged acute ER stress, the ER becomes unable to keep its functions; which leads at the end to the initiation of the apoptotic cell death. ER can induce apoptosis either by unfolded protein response (UPR) dependent or independent ER stress-signalling.

Upon accumulation of misfolded proteins in the ER, the UPR is activated; this activation leads to an increase in the expression of ER chaperones, an inhibition of protein translation and activation of ERAD (ER assisted degradation). The three signalling proteins; IRE1 $\alpha$  (inositol-requiring protein-1 $\alpha$ ); PERK (protein kinase RNA (PKR)-like ER kinase); and ATF6 (activating transcription factor 6) are activated as a result of ER-resident chaperone GRP78 dissociation from their luminal domains (will be discussed in chapter 4). **IRE1 $\alpha$**  oligomerization and autophosphorylation can activate the Apoptotic-Signaling Kinase-1 (ASK1); which causes activation of the Jun-N-terminal kinase (JNK) and MAPK, these, in turn, will promote apoptosis (Ron and Hubbard 2008) (Sano and Reed 2013). JNK



phosphorylation regulates the activity of Bcl2 and Bim (Lei and Davis 2003). Besides that, MAPK can induce apoptosis through the phosphorylation and activation of the transcription factor CHOP; which result in increasing of Bim expression and decreasing Bcl2 expression (Puthalakath, O'Reilly et al. 2007). Cleaved **ATF6** at Golgi activates target genes such as GRP78, GRP94 and CHOP. ATF6 can mediate apoptosis in C2C12 (an immortalized mouse myoblast cell line) via reduction of Mcl-1 Protein (Morishima, Nakanishi et al. 2011). In contrast, other work demonstrated that ATF6 has protective effects in cardiomyocytes after exposure to ischemia and reperfusion (Belmont, Chen et al. 2012). Phosphorylated **PERK** mediates the phosphorylation of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ); PERK/eIF2 $\alpha$  promotes the induction of the pro-apoptotic transcriptional factor CHOP (Sano and Reed 2013).

The induction of apoptotic calcium signalling could be achieved through sustained elevation of intracellular calcium or prolonged decrease in ER calcium. Acute calcium release from the ER can induce UPR independent ER cell death mainly by Ca<sup>2+</sup>-mediated mitochondrial cell death. Intracellular Ca<sup>2+</sup> overload is highly toxic, causing massive activation of proteases and phospholipases (Pinton, Giorgi et al. 2008). Moreover, Bcl2 family has a role in ER Ca<sup>2+</sup> homeostasis; Bcl2 overexpression prevents the decrease of ER Ca<sup>2+</sup> concentration (Baffy, Miyashita et al. 1993). Bax and Bak can initiate ER calcium-mediated apoptosis; Bax overexpression causes the release of ER Ca<sup>2+</sup> accompanied with increasing in the mitochondrial Ca<sup>2+</sup> level, which is permitting the release of the cytochrome c to the cytosol (Scorrano, Oakes et al. 2003). ER Calcium dysregulation can perturb the quality of protein folding in the ER, as well as, inducing apoptosis. Indeed, the overexpression of Calreticulin (a major calcium-binding protein in the ER) has increased the intracellular calcium concentration, inhibited the anti-apoptotic factor Bcl2, and increased the expression of Bax, p53 and caspases 8 and 3 that result in apoptotic cell death in neonatal rat cardiomyocytes (Lim, Chang et al. 2008); while Calreticulin-deficient cells are relatively resistant to apoptosis (Pinton, Ferrari et al. 2001).

#### ***2.2.1.4 Apoptosis in Cancer***

Via regulating a variety of genes, proteins and signalling cascades, cancer cells lose their ability to undergo programmed cell death that leads to uncontrolled proliferation. In cancer cells, several mechanisms allow cells to escape apoptosis; TP53 is a very important tumour suppressor, which codes the p53 protein. TP53 is mutated in ~50% of human cancers

mainly at the DNA-binding domain and can be activated in response to many stress stimuli (Aubrey, Kelly et al. 2017). The activation of p53 can directly or indirectly regulate the transcription of several genes and different cellular processes among them apoptosis; where p53 induces apoptosis through regulating the Bcl2 proteins (Maddika, Ande et al. 2007) (Marchenko and Moll 2014). The pro-apoptotic BH3-only proteins (Puma, Bim and Bid) are directly upregulated by p53; this will initiate the apoptosis due to the DNA damage (Roberts and Huang 2017). Also, the pro-apoptotic effector BAX and APAF-1 (the scaffold protein for caspase-9 activation) can be transcriptionally regulated by p53 (Aubrey, Kelly et al. 2017). Moreover, p53 is also able to regulate death receptor-induced apoptosis. The Crosstalk between the intrinsic and extrinsic pathways can be initiated by p53, which in turn, will activate the Bcl2 family protein Bid (Sax, Fei et al. 2002). It can regulate the ER-induced apoptosis via increasing the calcium level in the ER and the amount of  $Ca^{2+}$  transferred from the ER to the mitochondria (Bittremieux and Bultynck 2015). It has been shown that p53 mutation confers chemo-resistance to breast cancer patients (Olivier, Hollstein et al. 2010). The patients with a Specific mutation (missense mutation) had lower overall survival than with other mutations (Borresen, Andersen et al. 1995). Also, p53 mutations have linked to Doxorubicin resistance and the early relapse in breast cancer patients. (Aas, Børresen et al. 1996). The apoptotic effect induced by chemotherapeutic molecules such as 5-FU can depend on the expression of p53; where 5-FU induced more apoptotic mortality level in the wild-type gastric cancer cells; in contrast, no apoptosis was observed in the mutated type (Osaki, Tatebe et al. 1997).

The over-expression of Bcl2 proteins especially the anti-apoptotic proteins (Bcl-xL, Mcl-1, and Bcl-w) have been reported in many cancer types; these proteins enhance cancer cells resistant to chemotherapeutic treatment (Azmi, Wang et al. 2011; Mohammad, Muqbil et al. 2015; Radha and Raghavan 2017). In different cancer types, the upregulation of anti-apoptotic protein Mcl-1 confers resistant to apoptosis induced by chemotherapy and radiation (Warr and Shore 2008). Furthermore, apoptosis resistance in breast cancer is related to different factors that lead to dysregulation of the apoptotic signalling; among these factors the p53 gene mutations, epithelial growth factor receptor family, Bcl2 upregulation, cyclin E and p27 dysregulation. The chemo-resistance in breast cancer was correlated to the over-expression or dysregulation of Bcl2 proteins (Booy, Henson et al. 2011). Additionally, BH3-only proteins initiate apoptosis either by activation of pro-apoptotic Bax/Bak or by inhibition of anti-apoptotic Bcl-2 proteins; silencing of Bim in human leukaemia cell lines inhibited

Imatinib-induced cell death (Kuroda, Puthalakath et al. 2006). The 20S proteasome inhibitor, Velcade, is approved for the treatment of multiple myeloma (Chen et al., 14); it can enhance Bim half-life by inhibiting its proteasomal degradation. Velcade also dissociates Bim from Mcl-1, which promotes Bax/Bak mitochondrial permeabilization and apoptosis (Chen, Zhang et al. 2014; Shukla, Saxena et al. 2017).

### ***2.2.2 Autophagy***

It is a cellular catabolic process, highly conserved in eukaryotes. Autophagy is activated in response to extra- or intracellular stresses and signals (e.g. nutrient starvation and intracellular  $\text{Ca}^{2+}$  increase) or for the removal of damaged organelles (e.g. mitochondria, endoplasmic reticulum, peroxisomes and intracellular pathogens) and aggregated proteins. The defect in autophagy mechanism contributes to a variety of pathological conditions, including cancer, neurodegenerative disorders, cardiovascular diseases, and viral infection (He and Klionsky 2009).

In general, there are three forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy; all of them cause proteolytic degradation of cytosolic components at the lysosome. However, macroautophagy is the main form; in the literature, it usually called autophagy (Mizushima 2007; Glick, Barth et al. 2010). Macroautophagy is characterized by the sequestration of the cytosolic cargo within cytosolic double-membrane vesicles, known as an autophagosome, that fuses with the lysosomal membrane to form an autolysosome (Glick, Barth et al. 2010; Feng, He et al. 2013). In contrast, in microautophagy, by invagination or cellular protrusion of the lysosomal membrane, the lysosome directly engulfs the cytoplasmic materials (Glick, Barth et al. 2010; Hafner Česen, Pegan et al. 2012). In the last type, the chaperone-mediated autophagy (CMA), the soluble targeted cytosolic proteins are directly translocated across the lysosome membrane for degradation; CMA characterized by the formation of the CMA /chaperone complex (such as Hsc-70), this complex is recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A) (Glick, Barth et al. 2010; Kaushik and Cuervo 2012). Anyway, the Autophagy can be either non-selective or selective mechanisms (Smith and Wilkinson 2017). Depend on several cellular conditions, autophagy functions as stress adaptor to avoid cell death or initiator of autophagic cell death (Baehrecke 2005; Gozuacik and Kimchi 2007; Maiuri, Zalckvar et al. 2007).

Despite it was linked to non-apoptotic cell death; autophagy is generally considered as a survival mechanism. Its signalling pathways can be divided into these main steps: initiation, elongation, assembly and formation of autophagosomes, fusion with lysosomal membranes and degradation (Figure 12) (Rybstein, Bravo-San Pedro et al. 2018). Further, the electron microscopy is the gold standard and the most accurate methods for the detection of autophagosomes.

In fact, different stress conditions can promote different autophagic signalling for example in the case of metabolic stress the, preinitiation complex includes ULK1, FIP200, and ATG13 can be regulated via the activation of AMP-activated protein kinase (AMPK) and/or the inhibition of mammalian target of rapamycin complex 1 (mTORC1) (Kim, Kundu et al. 2011). In turn, the preinitiation complex promotes the activation of the initiation complex, this last composed of beclin 1, Vps34, and Vps1 that generates the lipid phosphatidylinositol 3-phosphate (PI3P). Next, a series of recruitment and activation of ATG proteins will end by the cleavage of LC3B I to LC3B II. The recruited LC3B-II associates with the autophagosomal membrane; where this autophagic marker is crucial for the targeting of autophagosomes to lysosomes (Green and Llambi 2015). Indeed, The Bcl2 family seems to have a role not only in the regulation of apoptotic cell death but also in the regulation of autophagy; where the antiapoptotic proteins Bcl2 and Bcl-xL can sequester and inhibit the Beclin 1, thus inhibiting the initiation complex (Pattingre, Tassa et al. 2005). In response to stress signals, the BH3-only proteins Bad and Bnip3 dissociate from beclin 1; which permits the initiation of the autophagic cascade (Maiuri, Criollo et al. 2007; Bellot, Garcia-Medina et al. 2009). In addition, the JNK signalling phosphorylates Bcl2, leading to its sequestration from beclin 1 (Wei, Pattingre et al. 2008).

Upon ER stress and UPR signalling, the ERAD is activated. ERAD can include two degradation mechanisms: type I which is ubiquitin-proteasome-dependent ERAD and type II which is autophagy-lysosome dependent ERAD (Rashid, Yadav et al. 2015). Type I ERAD targets only soluble misfolded proteins while autophagic pathway targets the soluble and insoluble misfolded proteins. The work of Ogata and her colleagues put the first evidence demonstrated that the UPR induced by ER stress leads to autophagosome formation and initiation of autophagy (Ogata, Hino et al. 2006). Indeed, autophagy can be induced by the three UPR Axes (table 3) (Rashid, Yadav et al. 2015).

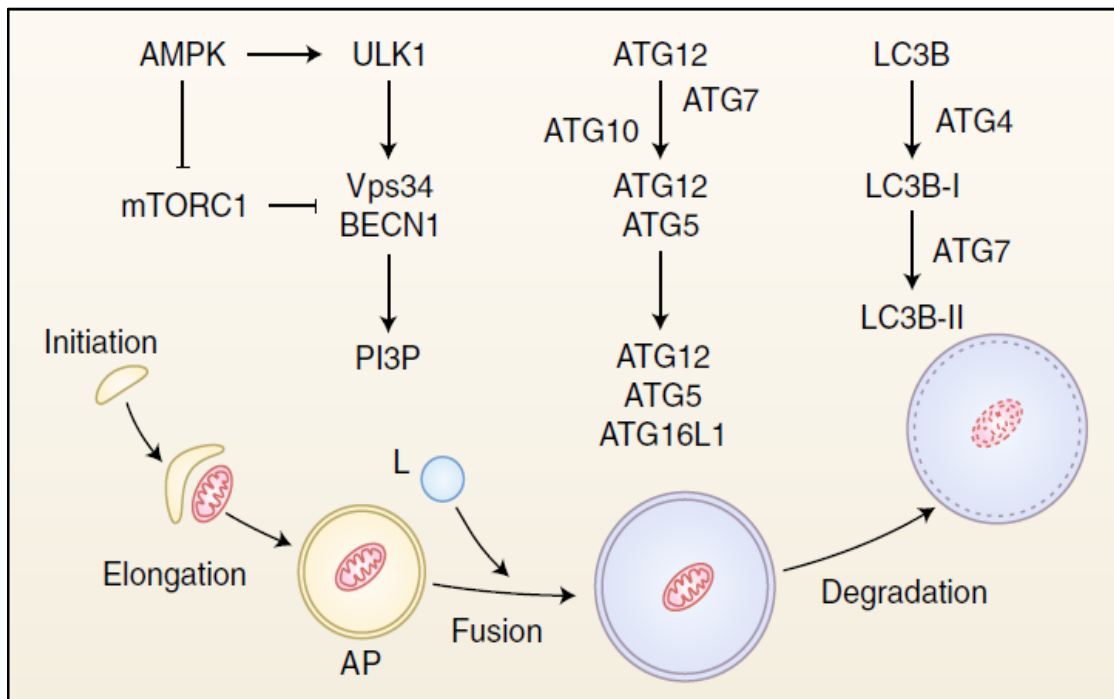


Figure 12. The main steps of autophagy (Rybstein, Bravo-San Pedro et al. 2018)

### 2.2.2.1 Autophagy and Cancer

There is a growing body of evidence linking autophagy with tumour initiation and development (Chen & Debnath, 2010; Liang & Jung, 2010). Autophagic response and activation in pre-malignant cells can inhibit oncogenic transformations. Indeed, the autophagic activity of hepatocellular carcinoma cells was less in normal hepatocytes cells (Kisen, Tessitore et al. 1993); suggesting a correlation between tumorigenesis and the down-regulation of autophagy. Maiuri, et al. have correlated many tumour suppressor proteins, which are known to be mutated in different types of cancer, such as p53, phosphatase and Tensin homolog (PTEN), Death-Associated Protein Kinase (DAPK) and Tuberous Sclerosis (TSC) to the induction of autophagy (Maiuri, Tasdemir et al. 2009).

Other oncogenes including Bcl2, AKT and mTOR, can inhibit the initiation of autophagy (Maiuri, Tasdemir et al. 2009; Morselli, Galluzzi et al. 2009; Bhutia,

Mukhopadhyay et al. 2013). In MCF-7 breast cancer cells, silencing of the antiapoptotic Bcl2 protein initiates autophagic cell death, characterized by the induction of LC-3-II, ATG5 and Beclin-1 (Akar, Chaves-Reyez et al. 2008). Moreover, the deletion of one allele of beclin1 has been reported in different cancers including breast, ovarian, and prostate cancers; the overexpression of Beclin1 in MCF-7 has initiated autophagy and inhibited tumorigenesis in vivo (Liang, Jackson et al. 1999). However, other autophagic genes have been presented as regulators involved in tumorigenesis such as UVRAG, Bif-1 and ATG 5,7 and 16 (Bhutia, Mukhopadhyay et al. 2013).

Different anticancer therapies have exhibited autophagic cell death in breast, colon and prostate cancer in vitro (Chen and Karantza 2011). Different works have demonstrated that the upregulation of autophagy in different cancer cell lines enhanced the chemo and radio-therapies resistance (Hu, Jahangiri et al. 2012; Zou, Yuan et al. 2012); while autophagy inhibition increased their sensitivity (Sotelo, Briceno et al. 2006; Carew, Nawrocki et al. 2007; Firat, Weyerbrock et al. 2012; Sui, Chen et al. 2013).

UPR branch	Target protein	Activation mechanism	Physiological outcomes	Role in autophagy
<b>ERN1(IRE1<math>\alpha</math>)</b>	XBP1	Unfolded protein-mediated dissociation of HSPA5 leads to the homodimerization and autophosphorylation of IRE1. Activated IRE1 uses endoribonuclease (RNase) for nonconventional mRNA splicing and transmission of UPR signals from the ER to the cytosol and nucleus. The RNase function of ERN1 involves cleavage of a 26-base fragment intron from the XBP1 mRNA and subsequent religation by an uncharacterized RNA ligase and formation of a frame shift that encodes the spliced transcript isoform XBP1s	The encoded XBP1 protein subsequently translocates to the nucleus where it acts as a potent transcription factor by binding to UPR elements and activating a broad spectrum of UPR-related genes involved in ER membrane biogenesis, ER protein folding, physical expansion of the ER, ERAD, protein entry into the ER, protein quality control, protein secretion from the cell, and autophagy.	Induction and inhibition
	MAPK8	Cytoplasmic domain of activated ERN1 recruits and forms a complex with TRAF2 and MAP3K5, which in turn phosphorylates and activates MAPK8	Activated MAPK8 controls both cell survival and cell death by regulating autophagy and apoptosis accordingly. The latter is converged into NF $\kappa$ B signalling and CASP4-CASP12 induction.	Induction and inhibition
<b>EIF2AK3 (PERK)</b>	NFE2L2	EIF2AK3 substrate NFE2L2 is phosphorylated and translocated to the nucleus under ER stress conditions. In unstressed cells, NFE2L2 is sequestered in the cytoplasm via association with KEAP1	In the nucleus, NFE2L2 binds with the antioxidant response element and upregulates expression of genes involved in the synthesis of antioxidant proteins such as glutathione, drug transport, xenobiotic metabolism, and elimination of ROS and autophagy	Induction
	IF2S1	ER stress causes dimerization of the cytosolic kinase domain of EIF2AK3 resulting in autophosphorylation, which in turn phosphorylates EIF2S1	p-EIF2S1 blocks global protein translation, thereby lessening the ER protein burden. In addition, autophagy-mediated cell protection under various stress conditions also substantially depends on EIF2S1 activation.	Induction
	ATF4	ATF4 mRNA is selectively translated following EIF2S1 phosphorylation because it contains upstream open reading frames that allow it to escape from the p-EIF2S1-mediated translational blockage resulting in synthesis of a transcription factor	Transcription factor ATF4 translocates to the nucleus and activates a set of UPR target genes involved in amino acid metabolism, antioxidant responses, autophagy, and apoptosis.	Induction
	DIT3	DDIT3, a potent transcription factor, is upregulated via the transcription activity of ATF4	DDIT3 potentiates autophagy and apoptosis through various molecular signals. An apoptosis signal is implicated in enhanced protein synthesis, which in turn leads to increased oxidative stress and subsequent cell death	Induction and inhibition
<b>TF6</b>	TF6	ATF6 activation includes monomer formation by reduction of ATF6 luminal intramolecular and intermolecular disulfide bonds and exposition of a Golgi-localization sequence (GLS). Those monomers then translocate to the Golgi through interactions with coat protein II (COPII). In the Golgi, ATF6 is processed into an active form of transcription factor ATF6 via the sequential activity of 2 Golgi-resident proteases, MBTPS1/S1P and MBTPS2/S2P.	In the nucleus, ATF6 binds to ER stress response elements and stimulates transcription of a subset of UPR target genes, including HSPA5 and DDIT3. ATF6 also upregulates XBP1 mRNA levels. XBP1 is then further processed by ERN1 and translocates to the nucleus to bind with the unfolded protein response elements therein. Thus, ATF6 gives positive feedback to the UPR.	Induction

Table 3. Autophagy Induced by the three UPR Axes (Rashid, Yadav et al. 2015)

### ***2.2.3 Necrosis***

In contrast to apoptosis, necrosis was defined as an unregulated accidental cell death process (passive death) as a consequence of extensive damage that disrupts cell integrity such as; high temperature, following freeze-thaw, infection, toxins, or trauma. Recently, many studies have abolished this concept; whereas necrosis can be regulated either by the chemical inhibitors of necrosis or via targeting specific proteins (Green and Llambi 2015). At the morphological level, it is characterized by cytoplasmic swelling and plasma membrane breakage which leads to a release of intracellular materials.

#### ***2.2.3.1 Necroptosis***

The molecular regulated necrotic cell death includes different forms such as necroptosis, pyroptosis, oxytosis, and mitochondrial permeability transition (MPT)-dependent necrosis (Pasparakis and Vandenabeele 2015). Pyroptosis is an inflammatory cell death induced by inflammasome activation (Lamkanfi and Dixit 2014); while necroptosis which is caspase-independent, is regulated by the kinase activity of RIP3 and MLKL proteins (Cho, Challa et al. 2009). However, necroptosis is the best characterized necrotic cell death cascade (Degterev, Huang et al. 2005) with marked depletion of cellular ATP. As the extrinsic apoptotic pathway, necroptosis is also regulated by death receptors through the TNF- $\alpha$  and FAS ligand activation, permitting its interaction with RIP1. This last will recruit cIAP1 and cIAP2, which results, in the end, deubiquitination and dissociation of RIP1. In fact, up to this, a complex of RIP1, FADD and procaspase-8 will be formed. Soon after, there are two signalling cascades depend on caspase-8 activation or inhibition. The activation of caspase-8 will induce apoptosis while the inhibition of caspase-8 will form necrosome due to RIP1 binding to RIP3. Furthermore, the auto-phosphorylated RIP3 will recruit and phosphorylate MLKL. This results in plasma membrane integrity disruption and necroptotic cell death (Figure 13) (Chen, Yu et al. 2016).

Indeed, other stimuli can be trigger necroptosis via activating RIP3 through either RIP1-dependent or -independent signalling, such as Toll-like receptors 3 and 4, T-cell receptor stimulation, interferons (IFNs) and DNA damage (He, Liang et al. 2011; Vanlangenakker, Vanden Berghe et al. 2011; Kaiser, Sridharan et al. 2013; Chen, Yu et al. 2016). Anyway, the poly (ADP-ribose) polymerase (PARP1) is able to regulate other necrotic cell death types, which are initiated in response to different stimuli like DNA alkylating agents, oxidative stress, and hypoxia. PARP1-dependent necrosis is totally independent of



RIP1 signalling cascade; where several steps are involved include the translocation of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the nucleus, the activation of calpain proteases and ATP depletion (Yu, Wang et al. 2002; Cao, Xing et al. 2007; Moubarak, Yuste et al. 2007; Berghe, Linkermann et al. 2014). The work of Vaseva et al., have shown that necrosis induced by oxidative stress was regulated via p53; where p53 triggers mitochondrial permeability transition pore and necrosis (Vaseva, Marchenko et al. 2012). Necrotic cell death study necessitates a combination of several approaches such as transmission electron microscopy (TEM), the release of high mobility group box 1 (HMGB1) from the necrotic cells and ATP depletion. Moreover, other necrotic markers were used to distinguish between the different necrotic cell deaths; for example; Necrostatin-1 inhibits RIP1 function; GSK843 and GSK872 are inhibitors for RIP3. Some of clinically used chemotherapeutics molecules such as Cisplatin, 5-FU and Etoposide induce necroptotic cell death in different cancers through targeting different proteins in the necroptotic cascade (Koo, Morgan et al. 2015).

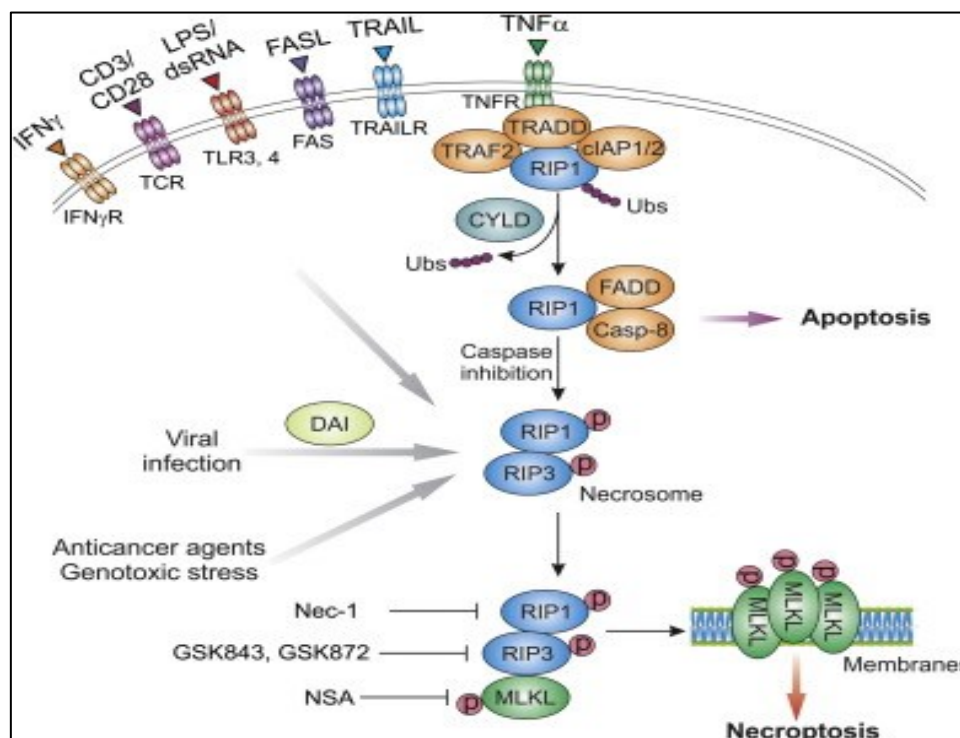


Figure 13. The Necroptotic cell death cascade (Chen, Yu et al. 2016)

## **2.3 Migration**

### **2.3.1 Introduction**

The migration process can be simply defined as the capacity of cells to translocate from location to another after their dissociation from the neighbouring cells. It is a tightly regulated mechanism in normal cells and essential for several physiological functions such as proper immune response, embryonic development, wound healing and tissue homeostasis (Wu and Thiagarajan 1996; D Luster, Alon et al. 2006; Barriga and Mayor 2015). However, aberrant cell migration intervenes in various pathologies and especially in cancer during the metastatic cascade (Yamaguchi and Wang 2004); whereas Metastasis is the dissemination of cancer cells from a primary tumour to other distant organs through blood and lymphatic vessels. The Metastatic process is started with the detachment of the cell from its surrounding and then followed by migration, invasion and adhesion. However, the metastatic capacity is achieved in response to a variety of factors including epithelial to mesenchymal transition, cell-cell adhesion, angiogenesis and modified microenvironment such as (extracellular matrix structure, growth factors, chemokines and matrix metalloproteinases) (Figure 14) (Guan 2015). The normal cells undergo Anoikis, which is the induction of apoptotic cell death through the death receptor and mitochondrial pathways caused by cell detachment from the extracellular matrix (Sakamoto and Kyprianou 2010; Guan 2015). In contrast, cancer cells are able to develop a resistance to anoikis, so they survive after their detachment (Sakamoto and Kyprianou 2010; Guan 2015). In order to migrate, cancer cells undergo cycles of four basic steps (protrusion, adhesion, translocation and retraction) to move toward other sites (Yamazaki, Kurisu et al. 2005).

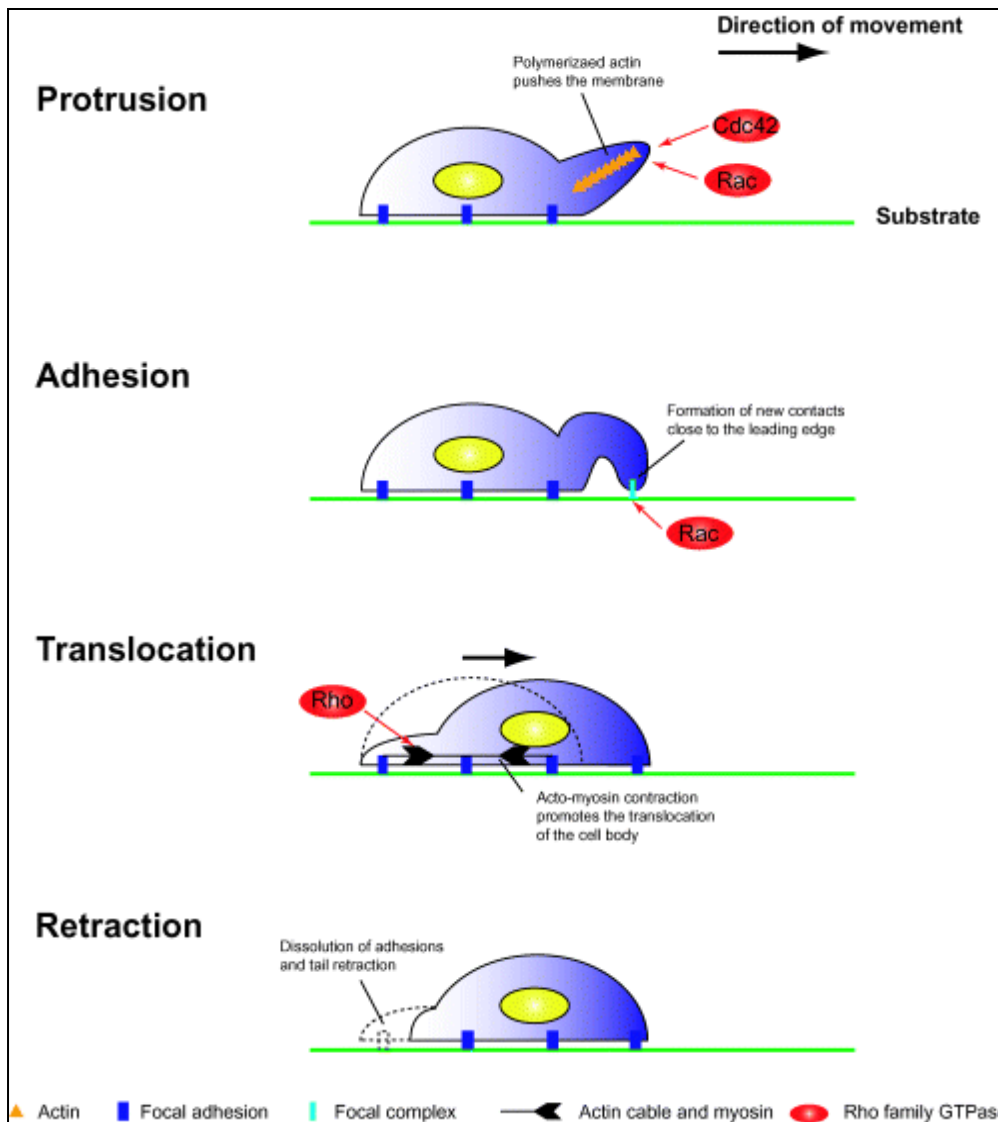


Figure 14. Cell migration on 2-D substrates. “Cell migration consists of the following four successive processes. Protrusion: when cells are polarized by extracellular stimuli, *de novo* actin polymerization occurs at the leading edge; polymerized actin filaments induce the formation of membrane protrusions such as filopodia and lamellipodia. Adhesion: protruded membranes contact the substrate and form novel integrin-dependent cell-substrate adhesions; newly formed adhesion complexes are stabilized and mature into focal adhesions. Translocation: actomyosin contractile forces translocate the nucleus and cell body. Retraction: cell-substrate adhesive structures at the trailing edge are disassembled, and the trailing edge retracts” (Yamazaki, Kurisu et al. 2005)

During the migration process, several chemoattractants bind to the extracellular membrane receptors, thus triggering cell migration via the reorganization of the actin cytoskeleton and developing cytoplasmic protrusions. The next step in this process is forming Focal Adhesion Complexes (FAC), through such complexes the cell create adhesion points with its environment (extracellular matrix and neighbouring cells) (Figure 15). Integrins proteins represent essential structure in the FAC; when integrins cluster; other cytoskeletal proteins will recruit leading to regulation in the migration of the cells capacity as well as other cellular functions.

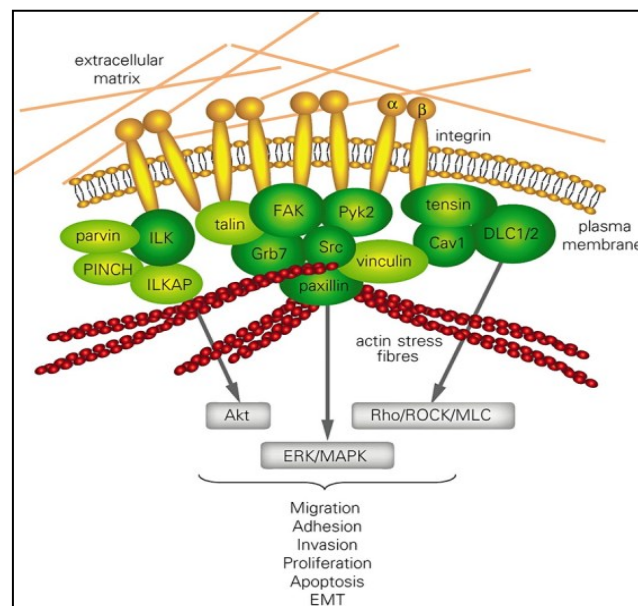


Figure 15. The focal adhesion complex. “Focal adhesions form the transmembrane linkages between the extracellular matrix (ECM) and actin cytoskeleton” (Yam, Yuk Ting Tse et al. 2009)

### 2.3.2 Integrins

Integrins are adhesion receptors at the plasma membrane, bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands (Hynes 2002). They are organized into heterodimers and compose of  $\alpha$  and  $\beta$  subunits. In human, there are almost 24  $\alpha$  and  $\beta$  combinations generating from 18  $\alpha$  and 8  $\beta$  subunits (Takada, Ye et al. 2007). Integrins have mechanical, biochemical and bidirectional properties; where they attach the cell cytoskeleton to ECM, detect the site of adhesion and transmit the signals from outside to inside and vice versa (Hynes 2002; Harburger and Calderwood 2009; Huttenlocher and Horwitz 2011). Their downstream signalling pathways that include FAK, PI3K and ERK/MAPK are involved in several cancer processes such as initiation, progression and metastasis (Hughes, Renshaw et al. 1997; Imanishi, Hu et al. 2007; Castelló-Cros, Khan et al. 2009). These proteins subunits

are distinct from each other and without homology between them (Takada, Ye et al. 2007). In this work, we are interested in  $\beta 1$  integrin, which constitutes a large subgroup of integrins. Moreover,  $\beta 1$  integrin is expressed in human breast cancer, implicated in epithelial-mesenchymal transition, metastasis, angiogenesis and tumour resistance to treatment (Schlaepfer and Hunter 1997; Aoudjit and Vuori 2001; Imanishi, Hu et al. 2007; Jahangiri, Aghi et al. 2014; Hou, Isaji et al. 2016). Integrins, in general, are composed of three domains; (1) large extracellular NH<sub>2</sub> termini compared to the other two domains; (2) transmembrane domain; and (3) Short COOH cytoplasmic domain that contains between 40 and 60 amino acids (Legate and Fässler 2009; Alexander and Bendas 2011). Additionally, they have different glycosylation sites, for example, the complex  $\alpha 5\beta 1$  contains 26 potential N-glycosylation sites (14 in the  $\alpha$  subunit and 12 in the  $\beta 1$  subunit) and located on the extracellular domain (Figure 16) Several adaptor proteins bind to specific sites on COOH  $\beta$ -integrin domain permitting integrins clustering and activation, whereas the COOH  $\beta$ -integrin domain has no enzymatic or actin-binding activity. According to Legate and Fässler, Integrin adaptor proteins can be categorized into three categories (Legate and Fässler 2009):

- 1) Adaptors have structural function including Talin, Filamin and Tensin, permitting direct coupling between the F-actin and integrins.

- 2) Adaptors have binding sites for additional focal-adhesion proteins (Scaffolding adaptors) such as Paxillin.

- 3) Adaptors have catalytic activity, among them; Focal Adhesion Kinase (FAK), Integrin-Linked Kinase (ILK), and Src (Legate and Fässler 2009).

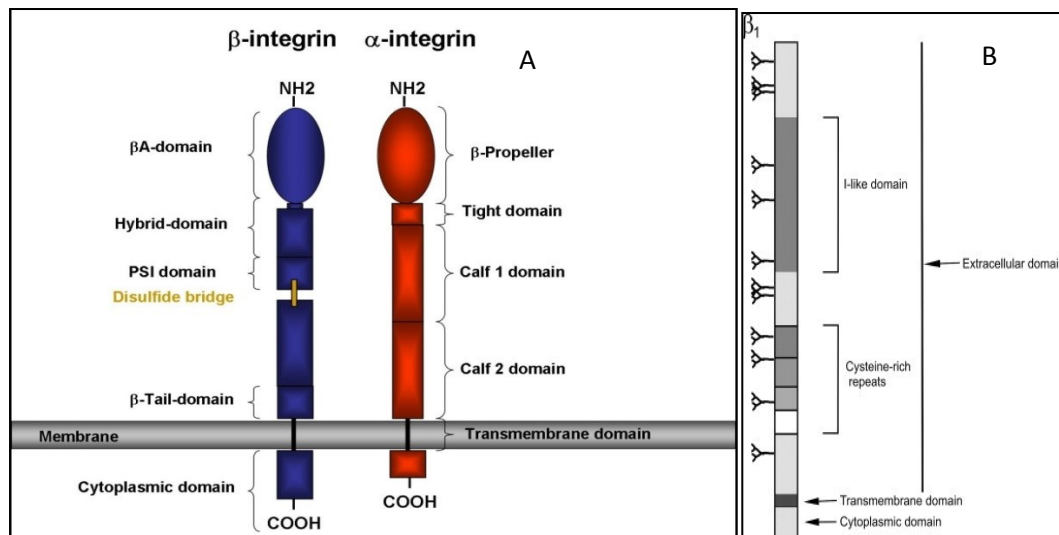


Figure 16. (A) Domain architecture of the heterodimeric integrins (Alexander and Bendas 2011). (B) The potential N-glycosylation sites of  $\beta_1$  subunit (Janik, Lityńska et al. 2010)

Integrins are glycoproteins and most of cell surface receptors are glycosylated. In fact, glycosylation can affect the function of glycoproteins; the different glycans structures cover the protein surface, thus preventing the nonspecific binding and modulate their ligand binding activity, provide protease protection and increase the stability of glycoprotein (Janik, Lityńska et al. 2010). There is much evidence demonstrate that integrin complex function depends on its glycosylation. However, the  $\alpha_5\beta_1$  is one of the best-characterized integrins; the glycosylation of  $\beta_1$  integrins has important regulatory mechanisms (Guo, Lee et al. 2002; Bellis 2004; Siddiqui, Pawelek et al. 2005; Wang, Liang et al. 2006; Zhao, Sato et al. 2008). Integrins overexpressions were reported in different cancers among them breast cancer (Bates, Bellovin et al. 2005; Diaz, Cristofanilli et al. 2005; Yu, Datta et al. 2005; dos Santos, Zanetti et al. 2012; Schaffner, Ray et al. 2013; Jahangiri, Aghi et al. 2014). Atypical expression of  $\beta_1$  integrin in cancer correlated to cell adhesion, angiogenesis, tumour progression, and metastasis (Schlaepfer and Hunter 1997; Legate and Fässler 2009; dos Santos, Zanetti et al. 2012). Moreover,  $\beta_1$  integrin expression was upregulated and correlates with lymph node metastasis and poor prognosis of breast invasive micropapillary carcinoma (Liu, Zheng et al. 2018). The work of Hou et al. demonstrated that decrease the expression of  $\beta_1$  subunit in MDA-MB-231 cells altered the expressions of other integrin subunits and impaired the cell migration (Hou, Isaji et al. 2016). The same work also exhibited that the deletion of  $\beta_1$  suppresses tumorigenesis *in vivo* (Hou, Isaji et al. 2016). Integrins can regulate the matrix metalloproteinases (MMPs), causing ECM degradation and remodelling (Borrirukwanit,

Pavasant et al. 2014; Missan, Mitchell et al. 2015).  $\alpha 5\beta 1$  can inhibit metastasis and delay the development of a primary tumour in Caveolin-1-negative head and neck squamous cell carcinoma (Jung, Ray et al. 2015). In melanoma cells, the interaction between Timp1, CD63 and  $\beta 1$ -integrin at the cell surface is involved in the acquisition of an anoikis-resistant phenotype through PI3K signalling pathway (Toricelli, Melo et al. 2013). Furthermore, integrins can confer radio-, chemo- and targeted therapy resistance in different cancers such as head and neck, breast and blood cancers (Blandin, Renner et al. 2015).

### ***2.3.3 Focal Adhesion Kinase (FAK)***

FAK is a cytoplasmic protein tyrosine kinase that acts as an intracellular mediator of extracellular changes and permitting the regulation of a variety of cellular processes like cellular adhesion, motility, proliferation and survival. The FAK gene is highly conserved and located at human chromosome 8 (Fiedorek and Kay 1995). FAK is a ubiquitous 125-kDa protein, composed of four distinct domains (Figure 17):

- An N-terminal contains the domain FERM (Band 4.1, ezrin-radixin-moesin) and consists of three distinct subdomains (F1, F2, and F3) (Ceccarelli, Song et al. 2006). FAK is autophosphorylated at tyrosine Y397, located between the end of the N-terminal and the beginning of the kinase domain (Calalb, Polte et al. 1995; Yoon, Dehart et al. 2015).
- A centrally located catalytic tyrosine kinase domain regulates FAK kinase activity and contains the tyrosine activation loop (Y576 and Y577); these sites can be phosphorylated by Src (Chan, Lai et al. 1999).
- A C-terminal domain contains two proline-rich sequences and the focal-adhesion targeting (FAT) domain; this domain has two tyrosine sites, Y861 and Y925; their phosphorylation permitting the interaction between FAK and Src SH2 domain (Calalb, Polte et al. 1995; Lindfors, Drijfhout et al. 2012). FAK and integrins are not directly connected; however, FAT domain, which contains binding sites for the adapter proteins Paxillin and Talin directs FAK to focal adhesions complex.

FAK can be activated via the activation and clustering of integrins or due to growth factors, hormones or cytokines; which are permitting its autophosphorylation on the tyrosine residue 397 and allowing FAK binding with Src protein; this interaction phosphorylates additional sites on FAK leading to full activation. Its autophosphorylation can be regulated through its FERM domain via docking into the catalytic domain leading to auto-inhibition of

FAK kinase activity, as well as via interacting with other regulators can mediate and control FAK signalling (Schaller 2010).

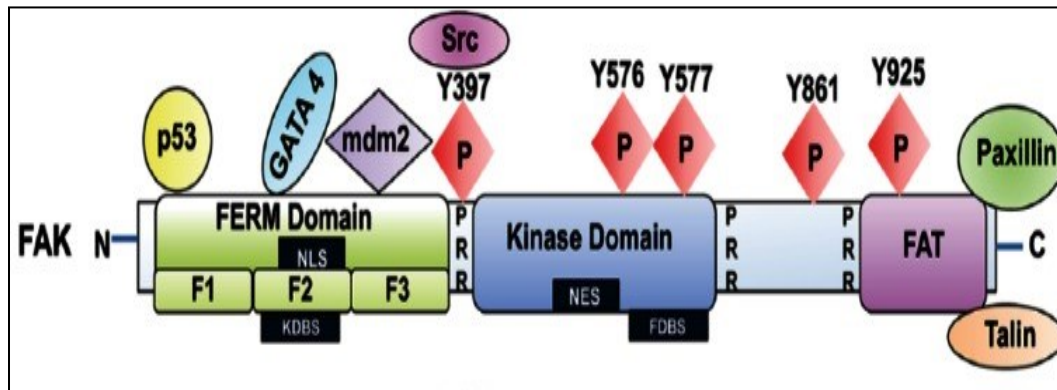


Figure 17. Schematic structure of FAK: FAK contains N- and C-terminal domains and a central kinase domain. (Yoon, Dehart et al. 2015)

In the focal adhesion complexes, FAK is mainly activated by the activation and clustering of integrins causing intracellular signalling cascades modulating cell migration and other cellular functions. Many works demonstrated that FAK able to regulate cell migration through different mechanisms; these include (Zhao and Guan 2011):

(1) FAK/Src or p130cas signalling cascade; affecting FAK association with either Src or p130cas are able to inhibit and reduce cell migration (Cary, Chang et al. 1996; Cary, Han et al. 1998).

(2) FAK interaction with PI3K leads to direct phosphorylation of Grb7; this promotes cell migration through PIK3 downstream effector Rac (Zhao and Guan 2011).

(3) FAK regulates cell migration through its effects on the assembly and disassembly of actin cytoskeleton via phosphorylating N-WASP, which is a Cdc42 downstream effector.

The expression of FAK protein is upregulated in different cancers (Table 4). Also, FAK gene amplification was correlated to FAK overexpression in cancer (Corsi, Rouer et al. 2006; Yoon, Dehart et al. 2015). Upregulated FAK expression levels were correlated to invasive and metastatic tumours in several types of cancer (Weiner, Liu et al. 1993; Agochiya, Brunton et al. 1999; Sulzmaier, Jean et al. 2014).

Different studies linked FAK proteins to many cancer processes such as cancer initiation and cell survival (1996; Golubovskaya, Zheng et al. 2009), epithelial-to-mesenchymal transition (Cicchini, Laudadio et al. 2008), invasion and metastasis (Shibue and Weinberg 2009) and tumor angiogenesis (Figure 18) (Mitra and Schlaepfer 2006). The work of Fang and his colleagues identified a new splicing mutant FAK-Del33 (exon 33 deletion) in breast cancer; indeed, they also demonstrated that the autophosphorylation of FAK at Tyr397



was strongly increased in FAK-Del33 breast tumour cells compared to wild-type (Fang, Liu et al. 2014). In addition,  $\beta$ 1-integrin /FAK signalling regulates the proliferation and invasion of metastatic cells in lung parenchyma *in vivo* (Shibue and Weinberg 2009). Furthermore, FAK promotes cell membrane expression of MT1-MMP, which leads to ECM (Wu, Gan et al. 2005).

<b>Tumour Type</b>	<b>Occurrences<sup>*</sup></b>	<b>References</b>
<b>Head and neck squamous cell carcinoma</b>	~62%	(Canel et al. 2006; He et al. 2006)
<b>Neuroblastoma</b>	~73%	(Beierle et al. 2008)
<b>Breast cancer</b>	~25-77%	(Cance et al. 2000b; Lark et al. 2005)
<b>Colorectal cancer</b>	~40-86%	(Cance et al. 2000b; Theocharis et al. 2003)
<b>Pancreatic cancer</b>	~48%	(Furuyama et al. 2006)
<b>Esophageal cancer</b>	~59%	(Miyazaki et al. 2003)
<b>Lung cancer</b>	~44%	(Wang et al. 2005)
<b>Small-cell lung cancer</b>	~59%	(Ocak et al. 2012)
<b>Ovarian cancer</b>	~68%	(Sood et al. 2004)

*Table 4: FAK Overexpression in Cancer. \* Occurrences were based on immunohistochemistry analyses (Yoon, Dehart et al. 2015)*

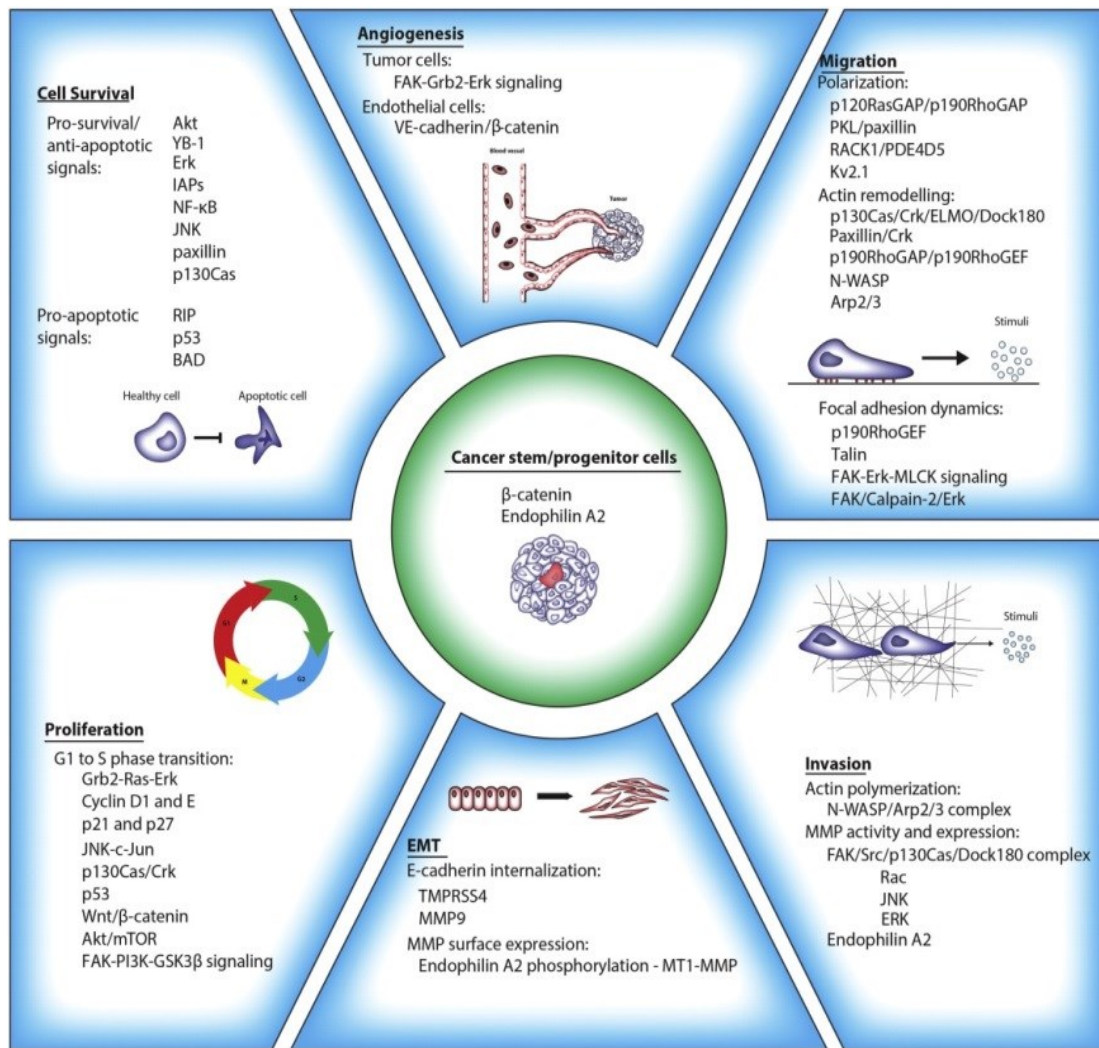


Figure 18. Summary of FAK signalling targets and pathways that regulate specific processes critical for tumorigenesis and cancer progression (Lee, Timpson et al. 2015)

### 2.3.4 Extracellular-Signal-Regulated Kinases (ERKs)

ERK1 and ERK2 are members of the family of mitogen-activated protein kinases (MAP Kinases). The MAP kinases are cytoplasmic proteins involved in the transduction of signals from the surface into inside the cell mainly via three signalling cascades; including the extracellular signal-regulated kinases (ERKs), the p38 kinase family, and the c-Jun N-terminal kinase family. Because of their homology, ERK1 and ERK2 are referred as ERK1/2; where these two MAP Kinases proteins are about 84% identical at the structural level as well as sharing different cellular functions. The MAPK cascade which involves the Ras-Raf-MEK-ERK signalling (Figure 19) modulates and required in a wide variety of cellular processes such as cell cycle progression, proliferation, differentiation, cell death, migration, cell adhesion and immune system (Ramos 2008). The defect in ERK1/2 MAP kinases signalling

has been linked to different pathologies and cancers (Ramos 2008; Roskoski 2012). The interaction between the integrins and the extracellular matrix activates FAK, which in turn targets and activates the Ras-Raf-MEK-ERK signalling (Sawai, Okada et al. 2005). Breast cancer migration and bone metastasis are highly dependent on FAK and its downstream target ERK1/2 activation (Wu, Wang et al. 2018). Rap2B is a member of the Rap family of small GTP-binding proteins, increases the intracellular calcium level leading to phosphorylation of ERK1/2 and enhancing breast cancer cell migration *in vitro* (Di, Huang et al. 2015).

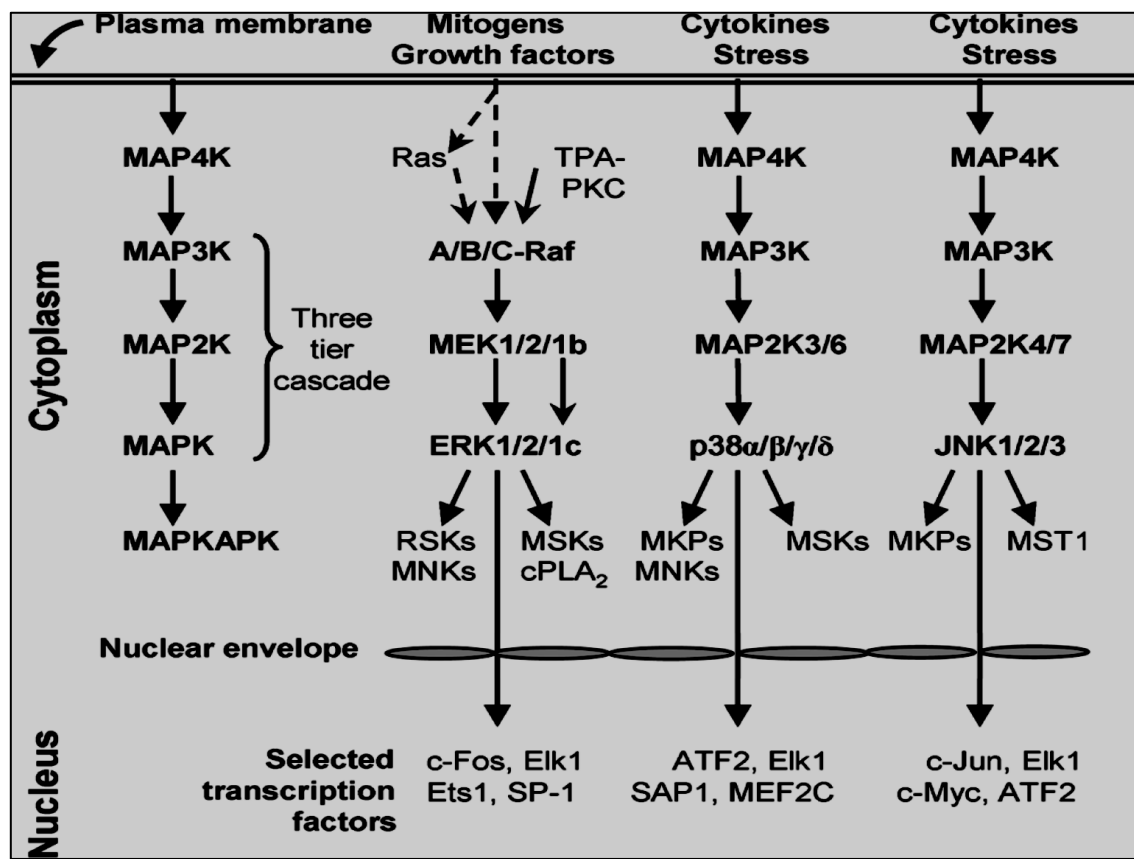


Figure 19. “The ERK, p38, and JNK MAP kinase cascades. The MAP kinases, which occur in the cytoplasm and can be translocated into the nucleus, catalyze the phosphorylation of dozens of cytosolic proteins and numerous nuclear transcription factors” (Roskoski 2012).

## 2.4 Invasion

Invasion and migration are two connected mechanisms involved in the metastatic cascade. Invasion is a direct extension and penetration by cancer cells into adjacent tissues; this process of invasion is started as a result of the penetration of the basement membrane and extracellular matrix by the mobile cancer cells. However, according to van Zijl, et al. and

Jiang,et al. , the metastatic mechanism is a multi-step process (Figure 20) that includes (van Zijl, Krupitza et al. 2011; Jiang, Sanders et al. 2015) :

- Local infiltration of cancer cells into the surrounding tissues,
- Intravasation, which is a transendothelial migration of cancer cells into blood or lymphatic vessels,
- Survival in the circulatory system,
- Extravasation,
- and Colonization of cancer cells in secondary organs.

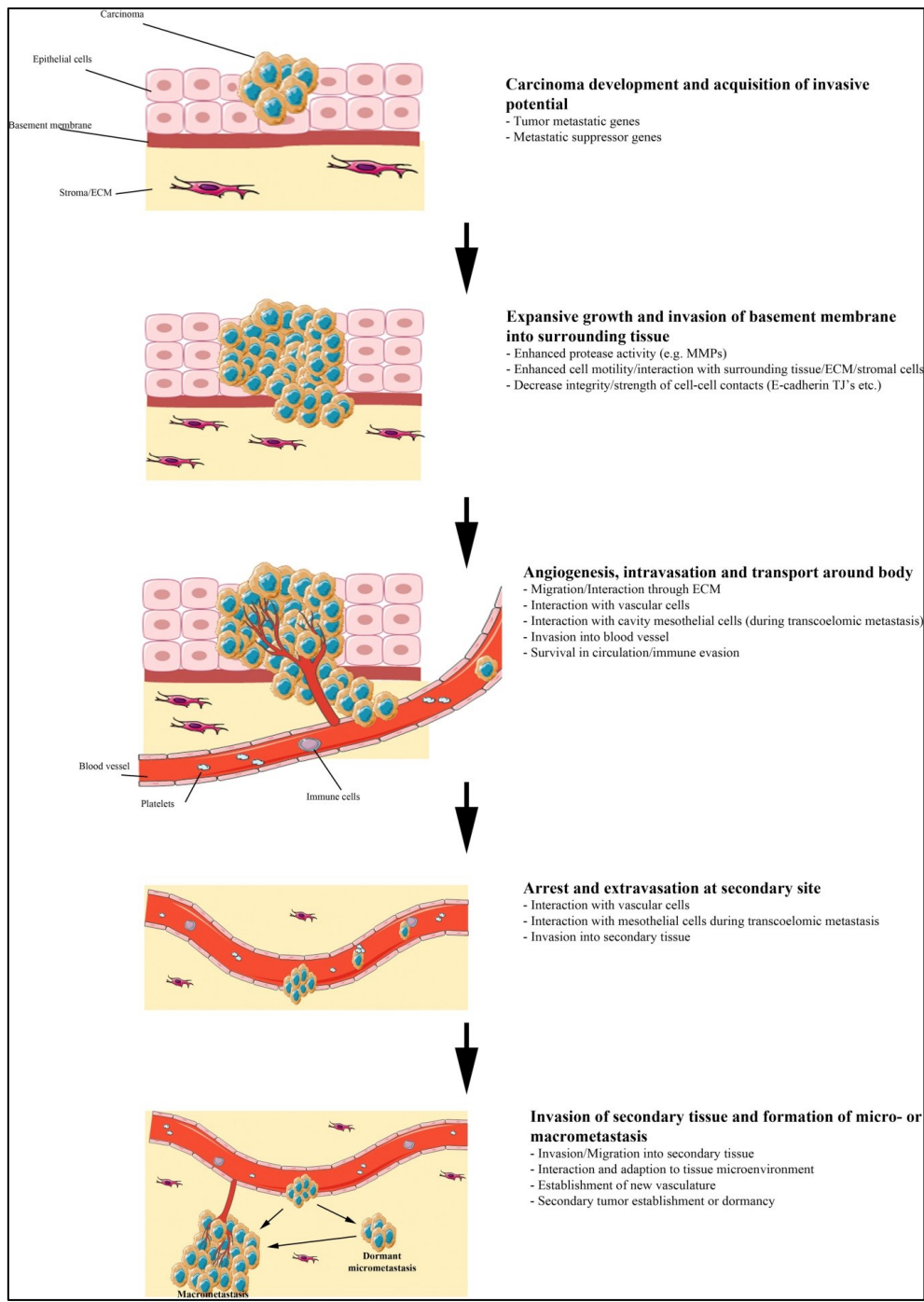


Figure 20. The metastatic cascade (Jiang, Sanders et al. 2015)

## ***Chapter III: Glycosylation***

### ***3.1 Introduction***

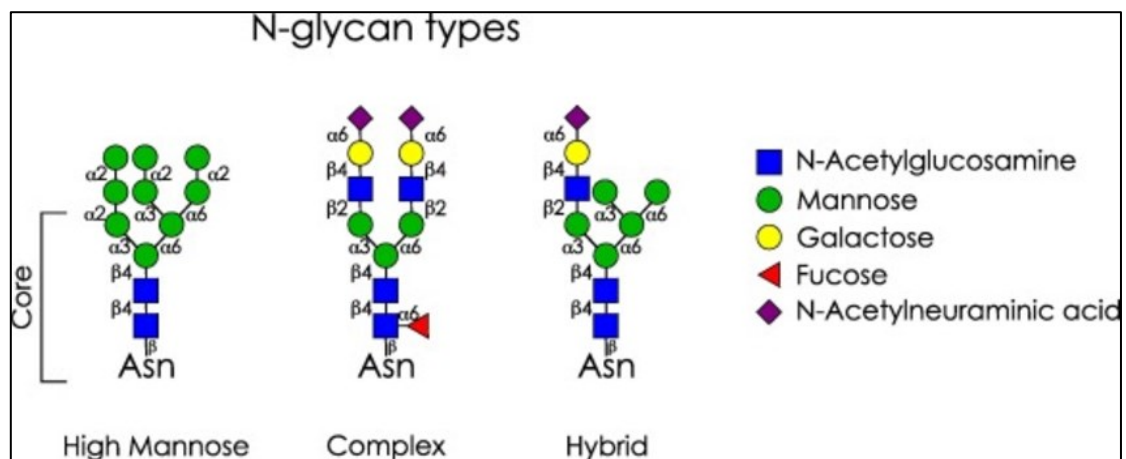
During translation and after being translated, Proteins undergo co-translational and post-translational modifications in different cellular organelles. One of the most important cellular conserved processes among eukaryotic is glycosylation. The majority of the newly secretory and membrane proteins translocate into the ER lumen for processing and maturation; this traffic is achieved by the secretory 61 translocon complex (Park and Rapoport 2012), which is located at the ER membrane. Glycosylation is vital and essential for proteins, where, alteration in Glycosylation can affect protein folding, stability, activity, localization, secretion, solubility, function and cell-cell interactions (Shental-Bechor and Levy 2008; Hanson, Culyba et al. 2009; Skropeta 2009; Culyba, Price et al. 2011). During glycosylation process, a glycan residue attached to a nascent protein or lipid by covalent bonds, which result in glycoproteins and glycolipids.

In general, glycosylation is enzymatic reactions; however, the work of Vasan et al., demonstrated another non-enzymatic form of protein glycosylation (Vasan, Zhang et al. 1996). Moreover, According to the linkage between the polypeptide and the sugars, protein glycosylation can be calcified into three main categories. These are N-linked glycosylation, O-linked glycosylation and C- linked glycosylation.

### ***3.2 N-Glycosylation***

The asparagine-linked glycosylation is a fundamental phenomenon happened in the rough endoplasmic reticulum (RER). In eukaryotic, N-glycans begin with GlcNAc $\beta$ 1-Asn; which are characterized by the attachment of the Oligosaccharide (N-acetylglucosamine abbreviated as GlcNAc) to the amino group (NH<sub>2</sub>) of an asparagine residue. This last should be located in a specific arrangement (Asn-X-Ser or Asn-X-Thr).

N-glycosylated proteins are either membrane-bound or secretory; they cannot be cytoplasmic or nuclear proteins; such as immunoglobulins, CD44, integrins, cadherins, matrix metalloproteinases, EGF receptor, HER2/neu, TGF $\beta$  receptor and fibronectin (Oliveira-Ferrer, Legler et al. 2017). Based on the core Man<sub>3</sub>GlcNAc<sub>2</sub> of N-glycan, we can distinguish three types (Figure 21) (1) Oligomannose, (2) complex, and (3) hybrid.



*Figure 21. Types of N-glycan (Higel, Seidl et al. 2016)*

The Biosynthesis of N-glycan consists of three main steps:

- **Oligosaccharide precursor synthesis (GlcNAc);** the biosynthesis of N-glycan started on a lipid molecule termed dolichol phosphate, located at ER membrane and functions as a membrane anchor for the oligosaccharide precursor via pyrophosphate linkage. The oligosaccharides assembly begin at the ER cytoplasmic side where a GlcNAc<sub>2</sub> - Man<sub>5</sub> residue is produced by the catalytic action of the glycosyltransferases. At this point, the oligosaccharide precursors translocate into ER lumen, where other 4 mannose and 3 glucose molecules will be added, giving 2 GlcNAc, 9 mannose, and 3 glucose (Figure 22).
- **Attaching glycan to a newly nascent protein;** the Oligosaccharyltransferase protein complex which is localized in the lumen of the ER regulate this step; where the 2 GlcNAc, 9 mannose and 3 glucose precursor attached to a primary asparagine residue of a nascent polypeptide. The glycan transfer to the asparagine residue needs a specific loop formation where the hydroxyl groups of Ser or Thr bind to the Asn. For that, a specific arrangement of the polypeptide Asn-X-Ser or Asn-X-Thr should be conserved. However, it is impossible for the Proline to occupy the middle residue X and forming that loop.

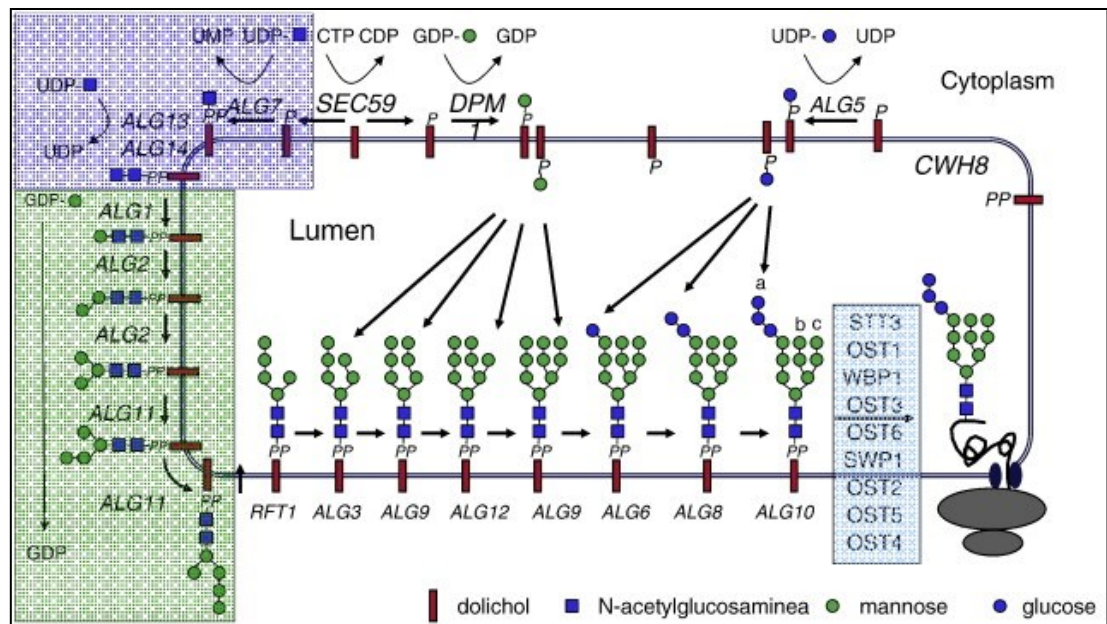


Figure 22. Scheme of the biosynthesis of the lipid-linked oligosaccharide and the transfer of protein at the membrane of the endoplasmic reticulum (Aebi 2013)

- Glycan processing;** this step takes place at ER lumen and ends in Golgi apparatus. Glucosidase I removes the terminal glucose residue from the glycan residue. Subsequently, glucosidase II cleaves the second glucose residue resulting in 2 GlcNAc, 9mannose and 1glucose. At that point, the proteins enter the Calnexin / Calreticulin (CNX/CRT) folding cycle (Figure 23), where the Calnexin is an ER membrane-bound protein and the Calreticulin is a soluble protein, retained in the ER through its C-terminal KDEL retention domain. Both of them characterized by their functions as ER chaperons. During this folding cycle, glucosidase II trims the last glucose molecule. Soon after, the properly glycosylated proteins are secreted into Golgi for further cleavage. In the other side, the misfolded proteins are recognized and reglycosylated by (UDP)-glucose:glycoprotein glucosyltransferase (UGGT), which is a soluble enzyme localized at the ER lumen. The UGGT transfers one glucose molecule to the glycoprotein precursor, permitting it to re-enter the CNX/CRT cycle, where the Calnexin and Calreticulin proteins have high affinities for the monoglucosylated proteins. In the case of unfolded Glycoproteins, the ER-associated degradation (ERAD) pathway is eventually activated.



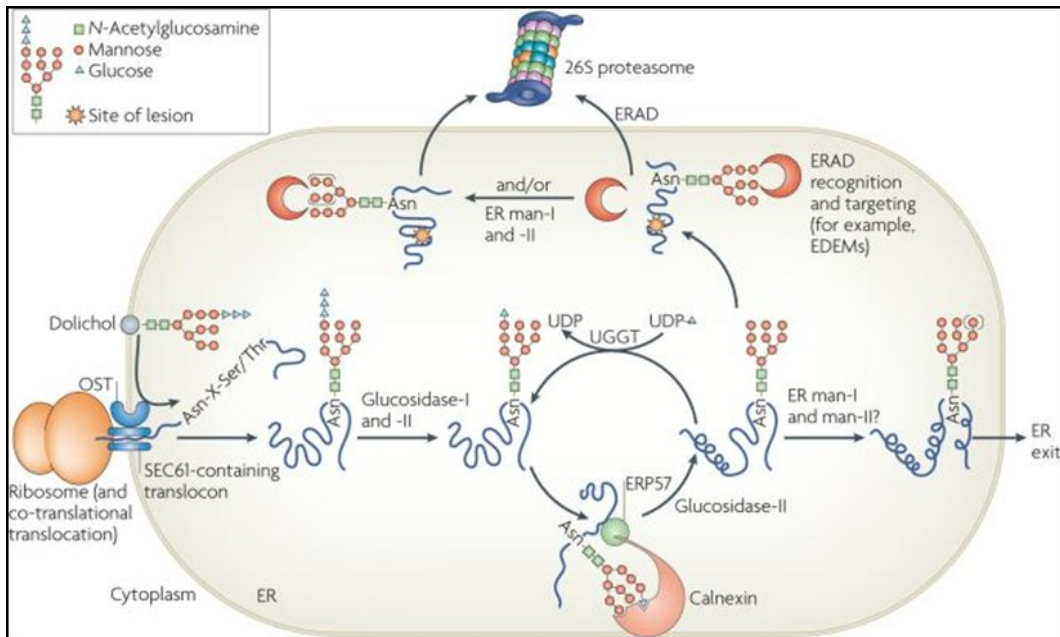


Figure 23. *N*-linked glycosylation and the calnexin/calreticulin cycle (Vembar and L Brodsky 2009)

### 3.3 O-Linked Glycosylation

This is a post-translational modification, which takes place in Golgi apparatus. After proteins have been already processed in ER lumen, the O-glycan core is synthesized by transfer sugar molecules to any amino acid has a hydroxyl group, as serine, threonine, hydroxyproline and hydroxylysine; anyway, the serine/threonine residues are more involved. In contrast to N-glycosylation where glycan precursor has transferred en bloc to the asparagine residue, the monosaccharide molecules are added one by one to the Ser/Thr residues during the O-glycan synthesis. The Golgi resident enzyme, N-acetylgalactosaminyltransferase is controlling this process. O-glycosylation can be classified on the basis of the innermost monosaccharide to (1) N-acetylgalactosamine, (2) N-acetylglucosamine, (3) fucose, (4) glucose, (5) galactose, and (6) mannose. In mammals, the most common O-linked glycan is the Mucin - N-acetylgalactosamine (GalNAc)  $\alpha$ -linked glycan. The steps involved in the synthesis of Mucin-type glycan are (Figure 24):

- 1) Catalysis of UDP-GalNAc by a ppGalNAcT (polypeptide N-acetylgalactosamine (GalNAc) transferase) forming Tn antigen.
- 2) Elongation of Mucin-type O-glycan (Tn antigen) by different glycosyltransferases; thus giving 4 different core structures of Tn antigens depending on the monosaccharide types and sequence.

3) Sialylation of Tn antigens by  $\alpha$ -2,6-sialyltransferase ST6GalNAc-I creating sialyl-Tn antigen (STn antigens) (Oliveira-Ferrer, Legler et al. 2017).

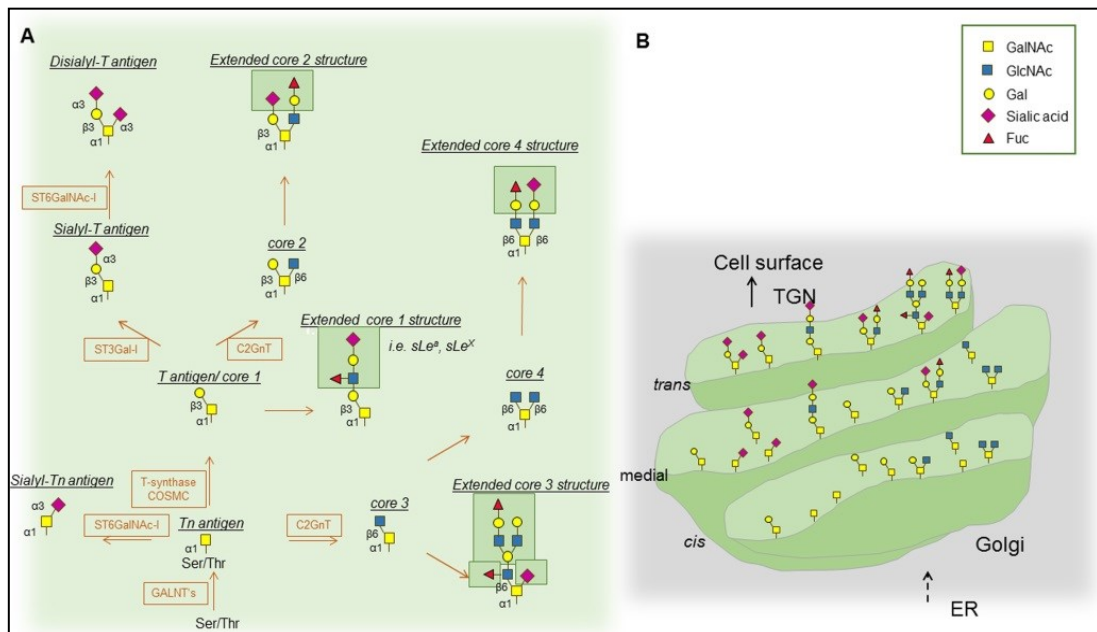


Figure 24. (A) Biosynthesis of Mucin-type O-glycans. (B) Distribution of O-glycans in the Golgi (Oliveira-Ferrer, Legler et al. 2017)

O-glycosylation is required for many secretory and membrane proteins functions; where these proteins carry Mucin-type O-glycans. Mucins proteins protect against proteolytic degradation, modulates recognition, as well as adhesion and cell-cell-communication. Specific anti-Mucin antibodies have been shown to have therapeutic potential and anti-cancer activity.

### 3.4 C-Glycosylation

Also known as C-mannosylation of tryptophan where a mannose molecule is added to the indole ring of tryptophan residue (Trp) in these specific sequences (Trp-X-X-Trp and Trp-Ser/Thr-X-Trp motifs (X indicates any amino acid)). C-linked glycosylation is a carbon-carbon bond-forming reaction by the C-mannosyltransferase. This specific linkage was observed in human RNase 2 for the first time (Hofsteenge, Mueller et al. 1994). Later on, it was observed two mannosylated types according to the tryptophan motif; where the first or first and second tryptophan residues can be mannosylated in the Trp-X-X-Trp sequence. In the other motif, in which the tryptophan linked to Ser/Thr, the mannosylation occurs only in the tryptophan residue located just before Ser/Thr (Dutta, Mandal et al. 2017). C-

mannosylation has been observed in specific proteins such as the Type I cytokine receptors, CD44 antigen, Properdin, erythropoietin receptor, Thrombospondin-1, IL-12B and others.

### ***3.5.1 Roles of Glycosylation in Different Pathologies***

Alteration in glycosylation process has been linked to many conditions such as; Genetic Disorders of Glycosylation, autoimmune diseases, and cancer progression and metastasis. These diseases vary in their biochemical and clinical presentations and usually impact multiple organs and systems. Glycosylation defects are ranging from affecting the glycan synthesis and transport to processing through a defect in the enzymes that control these steps as glycosidases and glycosyltransferases.

Over 100 known Genetic Disorders of Glycosylation (GDGs) are classified according to the location of the defects (Freeze, Eklund et al. 2015). The type I GDG results due to a failure in any steps of N-glycan precursor synthesis, while the defects in glycosidases, glycosyltransferases, and nucleotide sugar transporters cause the type II GDG. However, the impairments of other enzymes-controlled glycosylation are responsible for other variants of GDG. However, the total absence of N-glycan is a deadly condition.

Some glycosylation changes have been linked to Ulcerative Colitis and Crohn's disease (known as Inflammatory Bowel Disease). These alterations include increased sialylation of terminal precursor and reduced sulphation (Campbell, Yu et al. 2001). Glycosylated proteins are an absolute necessity for normal cell-cell interactions, especially for leukocyte trafficking (Lyons, Milner et al. 2015), whereas the absence of glycan ligand sLex leads to leukocyte adhesion deficiency type 2. Glycoproteins are used as a biomarker for the inflammatory conditions in general; the work of Takeshita et al. 2016 demonstrated that MMP-3 O-glycan changes would be used as a specific biomarker for rheumatoid arthritis activity (Takeshita, Kuno et al. 2016).

The expressions of abnormal glycoproteins precursors are a hallmark of many cancers, their implications and functional roles in cancer progression have been widely investigated. In the past years, cancer research has shown the close correlation between the development of cancer and the associated changes in glycosylation patterns; the most frequently occurring cancer-related changes include the synthesis of highly branched glycans, highly sialylated, premature termination, and reexpression of fetal-type antigens (Janik, Lityńska et al. 2010). Moreover, almost all secreted proteins are glycosylated, and the detection of glycoproteins abnormality could serve as biomarkers; Serum  $\alpha$ -fetoprotein (AFP) is a clinical biomarker for hepatocellular carcinoma and other cancers. A specific fraction of AFB (AFP-L3) has more

sensitivity and specificity for hepatocellular carcinoma (Zhao, Ju et al. 2013). The alteration of serum Mac-2-binding protein glycosylation pattern has been presented as a marker for cancer progression and metastasis (Grassadonia, Tinari et al. 2004). Alteration in N-linked GlcNAc  $\beta$ 1,6 glycan branching affects melanoma cell migration (Pochee, Janik et al. 2013). Also, it has been demonstrated that the increase in mRNA glycosyltransferases expression is implicated in several cancers glycosylation pathway (Petretti, Kemmner et al. 2000). The role of integrins in cancer metastasis is widely studied in many cancer among them breast cancer;  $\beta$ 1 integrin is O-glycosylated protein. The work of Liu, et al. exhibited that  $\beta$ 1 integrin can be modified via C1GALT1 in liver cancer (Liu, Hu et al. 2014). Moreover, it has been found that the expressions of O-glycosylation- controlled- enzymes were altered. The expression of GALNT1 was overexpressed in hepatocellular carcinoma; its high expression was associated with bad prognosis, whereas GALNT1 downregulation inhibited cell migration and invasion (Huang, Hu et al. 2015). In contrast, GALNT2 and GALNT3 were downregulated in liver cancer and pancreatic cancer respectively (Wu, Liu et al. 2011; Chugh, Meza et al. 2016).

### ***3.5.2 Glycosylation in Breast Cancer***

Many researchers are interested in studying the correlation between altered glycosylation pattern and development of breast cancer. The cell surface glycan changes in breast cancer ranging from increases in branching of N-linked glycans, sialylation, and fucosylation of glycans precursors as well as truncation of O-linked glycans. These changes are linked to cancer behaviour in several ways.

The transmembrane protein, Mucin-1 is known to impact cell migration and invasion. Muc1 is overexpressed in many cancer among them breast cancer where it is accompanied by aberrant O-glycosylation alteration (Zaretsky, Barnea et al. 2006). These changes have been linked to tumour metastasis (Horn and Schroeder 2013). It has been shown that C1GALT1 (Core-1-synthase-glycoprotein-N-acetylgalactosamine-3-beta-galactosyltransferase-1) regulates Muc-1 and leads to tumour proliferation, migration and invasion (Chou, Huang et al. 2015). Furthermore, the unglycosylated Muc1 proposed to serve as a diagnostic marker in breast cancer; in the clinical approach, Cancer Antigen 15-3 (CA 15-3) is used for monitoring breast cancer (Kabel 2017). Moreover, altered glycan sialylation leads to defect in breast cell adhesion (Ma, Dong et al. 2016). The work of Saldovala et al. demonstrated that Bi-, Tri-, and Tetraantennary-sialyl-Lewis x epitopes were increased in metastatic breast cancer and proposed them as a prognostic marker (Saldovala, Reuben et al. 2011). The levels of OGT and O-GlcNAc are increased in breast cancer cells (Caldwell, RaElla Jackson et al. 2010); in

contrast, inhibiting O-GlcNAc levels through silencing of OGT decreases cancer growth and invasion (Caldwell, RaElla Jackson et al. 2010). Alteration in glucosaminyl (N-acetyl) transferase2 (GCNT2) was linked to breast cancer metastasis through enhancing cell detachment from the extracellular matrix (Kölbl, Andergassen et al. 2015). Moreover, Pangeni et al. demonstrated that Glycosyltransferase GALNT9 (an initiator of O-glycosylation) is a possible prognostic candidate for secondary brain tumour metastases developed from breast cancer (Pangeni, Channathodiyil et al. 2015). Handerson 2005 highlighted that Beta1,6-branched Oligosaccharides are increased in lymph node metastases and predict poor outcome in breast cancer (Pangeni, Channathodiyil et al. 2015). This entire evidence increases the interest to investigate glycosylation and glycosylation inhibitors in breast cancer.

### **3.6.1 Glycosylation Inhibitors**

Treatment of cells with glycosylation inhibitors via targeting a specific glycosylation step (its relevant enzyme) produces glycoproteins with missing or altered precursors. These inhibitors exhibited effects in the treatment of storage diseases, diabetes, viral infections and cancer. Acarbose and Miglitol are clinically approved drugs for the treatment of type II diabetes. Zavesca- Miglustat is used to treat type I Gaucher disease and Niemann–Pick disease, type C. In the past two decades great effort has been made to synthesize new inhibitors of glycosylation with high selectivity and specificity to minimize their systemic side effects. Glycosylation inhibition can be achieved through various molecules such as; Metabolic inhibitors, Tunicamycin, Plant alkaloids, Substrate analogues, Glycoside primers and Tagged monosaccharides.

In this work, we are interested in Plant alkaloids known as (Iminosugars). They are mimic monosaccharides, where a nitrogen atom has replaced the oxygen atom. Iminosugars are low-molecular-weight molecules, isolated from plants, microorganisms, or synthesized. Since the 17th century, iminosugars were recommended for diabetes treatment; where they are abundant on Haarlem oil which was extracted from the leaves of *Morus alba* (Compain and Martin Olivier). The 1-Deoxynojirimycin (DNJ) was the first synthesised iminosugar in 1966 (Paulsen 1966). Next, the Nojirimycin was isolated from *Streptomyces bacteria* and exhibited antibiotic properties (Inouye, Tsuruoka et al. 1968). Glyset was the first iminosugar approved for treatment of type II diabetes in 1996. Iminosugars can be classified into five classes according to their structures (Asano, Nash et al. 2000):

- **polyhydroxylated piperidines:** they are heterocyclic amines consist of a six-membered ring containing one nitrogen atom. Deoxynojirimycin (DNJ) is the first synthesized iminosugar by Paulsen where Nojirimycin (NJ) (5-amino-5-deoxy-glucopyranose) is the first natural discovered iminosugar by Inouye. In 1968, NJ was isolated from *Streptomyces roseochromogenes*. Despite its potent inhibitory effect of  $\alpha$ - and  $\beta$ -glucosidases, NJ has an unstable hydroxyl group at C1. The synthesized DNJ is a more stable molecule. Actually, DNJ has later been isolated from natural sources. Many other molecules belong to this class such as; Nojirimycin B, Galactostatin and 1-Deoxymannojirimycin.

- **Indolizidines:** two bicyclic alkaloids belong to this family. In 1981, Castanospermine was isolated from the seeds of *Castanospermum australe*. It is a selective inhibitor of both  $\alpha$ -glucosidases and  $\beta$ -glucosidases. Castanospermine structure mimics D-glucose in the pyranose ring. Swainsonine (1,2,8-trihydroxyoctahydroindolizine) is isolated from *Swainsona canescens* in 1979. Its structure resembles D-mannose in furanose ring. Swainsonine is a potent inhibitor of both lysosomal  $\alpha$ 1-3- and  $\alpha$ 1-6-mannosidase and Golgi  $\alpha$ -mannosidase II.

- **Pyrrolidines:** these are cyclic secondary amines with a five ring (four carbon atoms and one nitrogen atom (C<sub>4</sub>H<sub>9</sub>N)). Pyrrolidines have been isolated from many natural sources as (DMDP, 2,5-dideoxy-2,5-imino-d-mannitol) or chemically synthesized.

- **Pyrrolizidines:** the structure of pyrrolizidine alkaloids composes from two cycles of pyrroles, with a carbon branch at C-1, such as dihydroxyheliotridane or a carbon branch at C-3, Such as Alexine.

- **Nortropanes:** they are polyhydroxy nortropane alkaloids; Calystegines are recently discovered as secondary metabolites of Calystegines plants, that carry 3, 4 or 5 hydroxyl groups

### ***3.6.2 Properties of Iminosugars as Potential Drug Candidates***

- These molecules are highly water soluble with good oral bioavailability; the water solubility of iminosugars is a fundamental property that plays an important role in the absorption of the drug by the digestive system after oral administration.

- Chemical stability of iminosugar molecules is another important property, which maintains its chemical and therapeutic properties during the time of storage.

- Metabolically stable molecules, where they excreted unchanged in urine with good elimination by glomerular filtration.
- They able to penetrate the blood-brain barrier because of their low molecular weight.

Tunicamycin is a nucleoside antibiotic and a potent inhibitor of N-glycosylation. It blocks the first step in the biosynthesis of N-linked glycosylation, which is the formation of dolichol-bound N-acetylglucosamine derivatives. Indeed, tunicamycin activates the UPR as a result of unfolded proteins accumulation in the endoplasmic reticulum. Many published papers reported strong anti-tumour effects of Tunicamycin in different cancer; Tunicamycin had inhibited xenografts breast tumour growth in nude mice (Banerjee, Lang et al. 2011). Furthermore, it enhanced the antitumor activity of Trastuzumab in breast cancer cells overexpressing HER2 through induction of cell cycle arrest and apoptosis *in vitro* and *in vivo* (Han, Zhang et al. 2015). On the other hand, others iminosugars attribute as potential antitumors candidates. The iminosugar Castanospermine had important anti-tumour properties *in vitro* and *in vivo*; Castanospermine inhibited The pulmonary metastasis of melanoma cancer cells (Humphries, Matsumoto et al. 1986). Also, it has been demonstrated that Castanospermine reduced tumour growth in nude mice (Pili, Chang et al. 1995). Furthermore, other work exhibited that Castanospermine prevented platelet aggregation (Spearman, Ballon et al. 1991) and tumour angiogenesis (Pili, Chang et al. 1995). Additionally, the effect of Swainsonine on different cancers was of interest and studied in many scientific papers. Indeed, Swainsonine exhibits anti-metastatic activity in melanoma cells and tumour lymphoid cells (Dennis 1986; Humphries, Matsumoto et al. 1986; Humphries, Matsumoto et al. 1990); this molecule enhances the antitumor effects of interferon. Actually, the treatment of different cancers *in vitro* and *in vivo* with Swainsonine exhibited strong anticancer effects (Olden, Breton et al. 1991; Baptista, Goss et al. 1994; Goss, Baptiste et al. 1994; Goss, Baker et al. 1995). This made it as a potent candidate for a clinical trial, however, in phase I and in phase II, however, Swainsonine exhibited toxic effects in renal cell carcinoma patients. Further, other iminosugars molecules have shown anti-tumours properties such as Deoxynojirimycin and 2-Acetamido-1,5-imino-1,2,5-trideoxy-D-glucitol.

## ***Chapter IV: UPR, GRP78, Translocon and ERAD***

### ***4.1 Unfolded Protein Response***

Secretory and membrane proteins are synthesized and modified in the lumen of ER where the ER is the main organelle for protein folding, maturation and secretion. Many proteins and proteins processing enzymes resident in the lumen of ER are involved in the proteins folding and assembly. ER stress can perturb ER function by affecting its capacity for proper proteins folding in the lumen of ER. Thus, the accumulation of unfolded proteins perturbs the ER homeostasis, leading to induction of adaptive responses called unfolded protein response (UPR) to preserve ER proper function. The UPR can be established through the activation of three diverse stress signaling pathways (Figure 25), including activating transcription factor 6 (ATF6) (Haze, Yoshida et al. 1999), double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) (Harding, Zhang et al. 1999) and inositol-requiring kinase 1 (IRE1) (Tirasophon, Welihinda et al. 1998). These three sensors are located at the ER membrane and kept as inactive forms due to their association with the ER Glucose-Regulated Proteins 78 (GRP78). The ER chaperones involved in the folding machinery include GRP78, GRP94, Calnexin, Calreticulin, and ERp57 (Gidalevitz, Stevens et al. 2013; Minakshi, Rahman et al. 2017). However, the result of this adaptive response promotes the folding capacity and efficiency via upregulations of molecular chaperones, proteins translational attenuation and an increase in ER-associated protein degradation (ERAD). Eventually, UPR is controlled by a negative feedback response as a result of restoring ER homeostasis; otherwise, the cell that suffers from severe stress will initiate ER-stress-induced cell death.

#### ***4.1.1 ATF6 Signalling***

ATF6 is a 90 kDa, ER transmembrane transcription factor, contains a 670-amino acid, and cleaved in response to ER stress. It composes of the cytosolic N-terminal domain and C-terminal domain located in the ER lumen. Upon ER stress, the C-terminal which functions as ER stress sensor (Haze, Yoshida et al. 1999), dissociates from GRP78 leading to proteolytic cleavage of ATF6 to pATF-6 at Golgi apparatus by the action of S1P and S2P proteases (Shen, Ellis et al. 2001; Minakshi, Rahman et al. 2017). This active cleaved form of ATF6 translocates to the nucleus and possesses different nuclear signals permitting the activation of



several nuclear proteins that responsible for the induction of ER stress-responsive genes (Thuerauf, Morrison et al. 2002).

#### ***4.1.2 PERK Signalling***

PERK is a single pass protein with kinase activity. Under ER stress, PERK undergoes oligomerization and auto-phosphorylation (Bertolotti, Zhang et al. 2000). It has an essential role in the regulation of proteins translation by a general inhibition of the cellular proteins translation machinery. This general inhibition achieved through direct phosphorylation of translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) (Harding, Zhang et al. 1999), which also leads to selective expression of the transcription factor ATF4. ATF is a member of cAMP-responsive element-binding protein (CREB) family (Scheuner, Song et al. 2001), has a critical role in the cellular adaptation to ER stress by regulating the genes expressions involved in protein folding, apoptosis and autophagy (Gidalevitz, Stevens et al. 2013; Chevet, Hetz et al. 2015). The work of Fawcett et al., demonstrated that ATF4 regulates the transcription factor CHOP (Fawcett, Martindale et al. 1999), which permits the regulation of the expressions of both Bax protein and Bcl2 protein and controlling the induction of cell survival cascade or the cell death signaling (McCullough, Martindale et al. 2001; Yamaguchi and Wang 2004; Han, Back et al. 2013).

#### ***4.1.3 IRE1 Signalling***

IRE1 is a type I protein located in the ER membrane and has two isoforms IRE1 $\alpha$  and IRE1 $\beta$ . IRE1 has two domains; (1) an N-terminal ER stress sensing domain and (2) a C-terminal has a cytoplasmic kinase/ ribonuclease activities. In response to ER stress, IRE1 undergoes dimerization and autophosphorylation, leading to increasing the transcription of UPR target genes through the upregulation of XBP1 expression. This last has impacts on proteins folding, ER-associated protein degradation (ERAD) and lipid synthesis (Acosta-Alvear, Zhou et al. 2007; Chevet, Hetz et al. 2015). IRE1 $\alpha$  RNase domain is involved in the degradation of RNAs (referred to a Regulated IRE1-dependent decay) such as mRNA at the ER, ribosomal RNA and microRNAs (Hollien and Weissman 2006; Hollien, Lin et al. 2009; Lerner, Upton et al. 2012; Maurel, Chevet et al. 2014; Hetz, Chevet et al. 2015). In stressed cells, this protein encodes distinct signalling ranging from pro-survival pathways through the upregulation of chaperones proteins to pro-apoptotic pathways by inhibition c-Jun N-terminal kinase (JNK) activation. Indeed, this inhibition achieved by TNF Receptor-Associated Factor 2 (TRAF2). The adaptor protein TRAF2 binds to the cytoplasmic site of IRE1, which

stimulates apoptosis signal-regulating kinase (ASK1), followed by inhibition of JNK. In turn, JNK is able to regulate and decrease the expressions of the anti-apoptotic proteins Bcl2 and Bcl-xL.

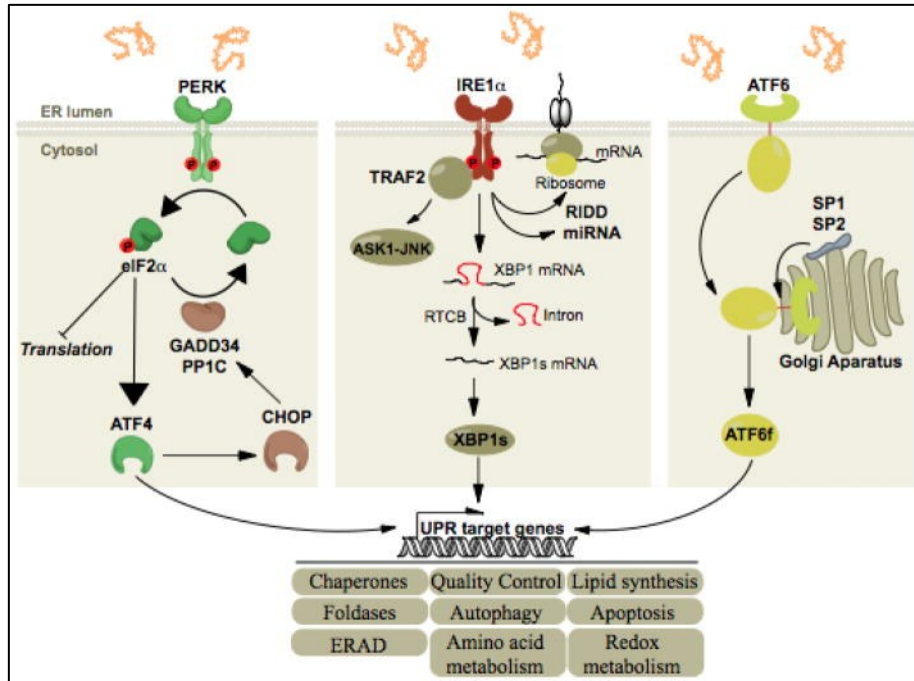


Figure 25. Stress signalling leads to selective activation of downstream cascades (Hetz, Chevet et al. 2015)

#### 4.2 70kDa Heat Shock Proteins (HSP70)

The family of HSP70 serves as ER chaperones for maintaining intracellular proteins homeostasis; it is discovered for the first time in 1962, when “A new puffing pattern induced by temperature shock and DNP in *Drosophila*” (Ritossa 1962). This work opened the door to investigate the physiological and pathological roles of HSP70 proteins. Many members belong to this family; the expressions of HSP70 proteins are upregulated by many cellular stresses; as heat, hypoxia, glucose deprivation and chemical toxins. The major member of HSP70 proteins is HSPA5, predominantly known as 78 kDa Glucose-regulated protein (GRP78) (Shiu, Pouyssegur et al. 1977) or Binding immunoglobulin protein (BiP) due to its binding to the immunoglobulin heavy chains of pre-B cells (Haas and Wabl 1983). GRP78 is the master ER chaperon through regulating the ER stresses signalling and protein quality control (folding and assembly). In spite of GRP78 belongs to the HSP70 family, the expression of GRP78 was not upregulated due to heat shock exposure. On the other hand , the

ER stress inducers such as Thapsigargin, the Calcium Ionophore A23187, and Tunicamycin upregulate the expression of HSP70 and GRP78 (Watowich and Morimoto 1988; Lee 2005).

#### 4.2.1 GRP78 Structure

GRP78 is encoded by the gene Hsp5a; located on the chromosome 9q33.3, consist of 654 amino acids and has two major functional domains (1) an N-terminal domain which characterized with ATPase domain (aa 125–280); it hydrolyzes ATP to ADP leading conformational changes in the other domains. (2) A C-terminal domain which is peptide binding domain (aa 400–500), and a KDEL motif (aa 651–654) at the end of the C-terminal tail (Figure 26) (Zhang, Liu et al. 2010). GRP78 has about 60% homology when compared with the other members of HSP70 family; where they have the same ATPase domain but they do not share the peptide binding domain. Besides its main localization in the ER lumen, GRP78 expresses in the cell surface, cytoplasm, nucleus and mitochondria. Also, a secreted form of GRP78 by tumour cells has been detected (Figure 27) (Kern, Untergasser et al. 2009; Fu, Yang et al. 2014).

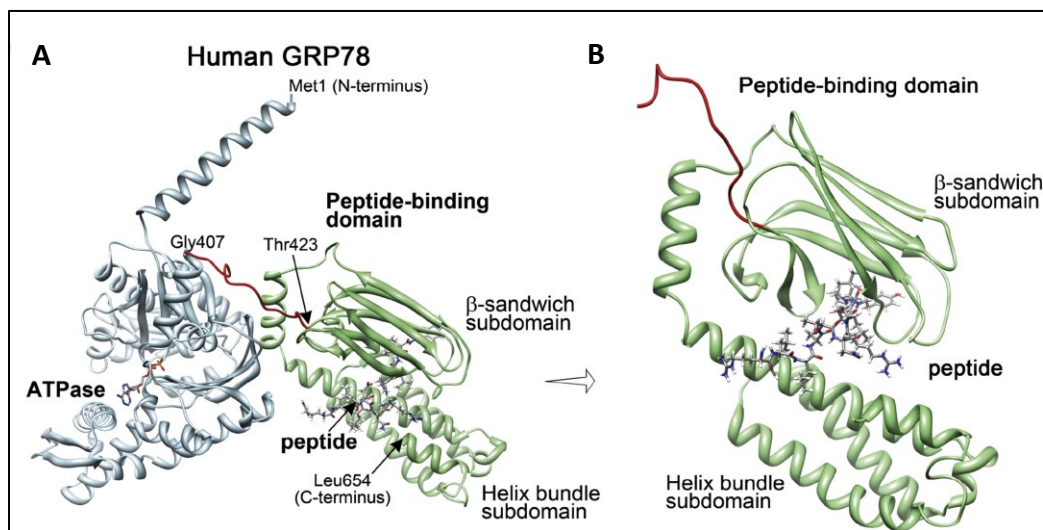


Figure 26. Modelling of the 3D structure of human GRP78

(A) “Human GRP78 is composed of a peptide-binding domain and an ATPase domain linked by a loop”. (B) “ ribbon model of the peptide-binding domain of human GRP78, which is composed of a  $\beta$ -sandwich subdomain and a helix-bundle subdomain”. “The L-peptide is “predicted to bind the peptide-binding domain of GRP78” (Wang, Lee et al. 2016)

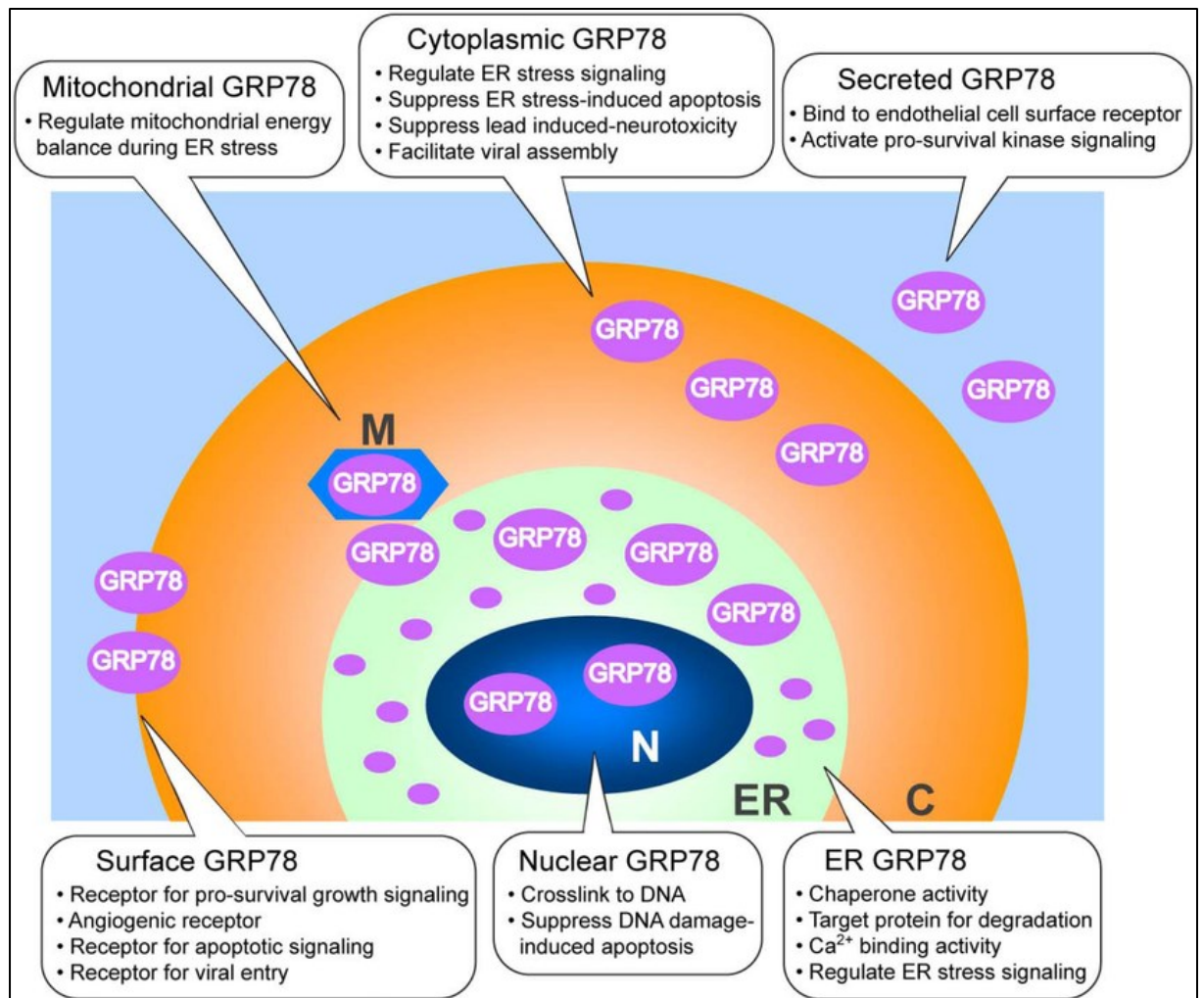


Figure 27. GRP78 localization and functions in different subcellular compartments  
(Ni, Zhang et al. 2011)

#### 4.2.2 Functions of GRP78

HSP5a gene is regulated by several transcription factors that mediate GRP78 expression (Figure 28). One of the most important roles of GRP78 is maintaining the native proteins confirmation through its binding to the hydrophilic residues on the unfolded polypeptides to prevent the aggregation of the unfolded proteins. As mentioned before, during normal physiological condition, GRP78 holds and attaches to the luminal domains of the ER stress sensors IRE1, ATF6 and PERK keeping them in inactive conformations. In stressed cells, GRP78 functions as a regulator of UPR, where it detaches away from the ER stress sensors and moves to bind the misfolded or unfolded proteins, permitting the activation of UPR via the ER stress sensors downstream signalling pathways. Indeed, as a result of UPR activation, GRP78 is a target of the UPR; where GRP78 expression is upregulated to increase the ER-folding capacity as well as it is associated with pro-survival responses by protecting

cells against ER stress-induced apoptosis. The overexpression of GRP78 leads to an inhibition in the activity of caspase-12, caspase-7, BIK and BAX, and prevents the release of cytochrome C from the mitochondria. Moreover, the GRP78 expression on the cell surface has been reported in different cancer cells; cell surface GRP78 was linked to mediate pro-survival (Papalás, Vollmer et al. 2010; Zhang, Yang et al. 2011) and pro-apoptotic signalling pathways (Raiter, Yerushalmi et al. 2014). Moreover, the work of Berger et al. reported for the first time a cell surface localization of GRP78 in malignant lymphocytes of cutaneous T-cell lymphoma (Berger, Dong et al. 1997). Next, other studies have demonstrated the localization of this protein on many tumours cell surfaces, among them breast cancer.

#### ***4.2.3 GRP78 Translocation***

The mechanism by which GRP78 can be translocated from the ER to the plasma membrane is unclear; in fact, GRP78 does not contain classical transmembrane domains. It has shown that deletion of KDEL receptor at C terminal of GRP78 leads to secretion of this protein when it is expressed in cells (Munro and Pelham 1987). However, induction of ER stress does not result in upregulation of KDEL motif expression (Llewellyn, Roderick et al. 1997). Moreover, the deletion of the C-terminal ER retention motif (KDEL sequence) in GRP78 alters its cell surface expression, anyway; the mutation of the putative O-linked glycosylation site Thr 648 of human GRP78 has no effect (Ni, Zhang et al. 2011). Tsai et al. demonstrated that GRP78 binds to the cell surface via interaction with other plasma proteins such as glycosylphosphatidylinositol-anchored proteins and its translocation needs different mechanisms in the different cancer cell lines. In HeLa but not in HCT-116 cells, blocking the ER to Golgi transport has decreased GRP78 expression on the surface. The authors also demonstrated that this process needs GRP78 substrate binding activity and is independent of its ATP binding domain (Tsai, Zhang et al. 2015). Conditions that mimic cancer environments such as ER stress and hypoxia has proposed to be initiators for GRP78 re-localization at the cell membrane. In some cancer ER stress actively promotes cell surface localization of GRP78 after using ER-stress inducers such as TG and Tunicamycin (Zhang, Liu et al. 2010; Tsai, Zhang et al. 2015), however, in other cancers, the ER-stress inducer, Tunicamycin, did not manage to express the cell surface GRP78 (Serrano-Negrón, Zhang et al. 2018).

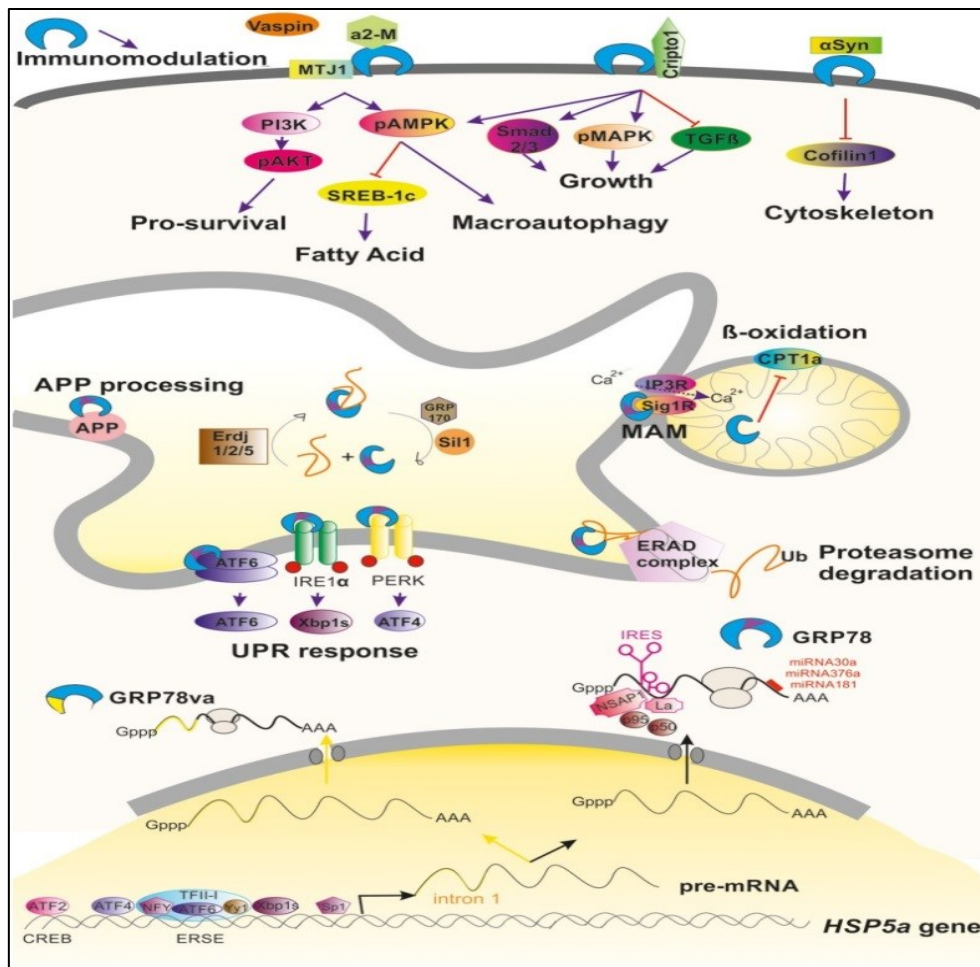


Figure 28. «“The regulation and activities promoted by GRP78; induction and regulation of the transcription of the HSP5a gene is mediated by several transcription factors that bind to ERSE or CREB motifs in the promoter of the gene; alternative processing of its pre-mRNA can occur under stressful conditions leading to retention of intron 1 (yellow line) that advance a stop codon, giving to GRP78va truncated protein that is retained in the cytosol because it lacks the ER-signaling motif (purple triangle; commonly processing GRP78 is submitted under post-transcriptional regulation either due to the action of factors on its IRES motif or by the action of different miRNAs. GRP78 is found mainly in the luminal ER where it can promote the activation of the UPR, ERAD, or MAM regulation; in some circumstances, GRP78 can be translocated to the cell surface where it can interact with multiple partners and hence modulate different pathways; it is also be secreted where it can immunomodulate”» (Casas 2017).

#### ***4.2.4 GRP78 and Calcium***

The disturbance of both endoplasmic reticulum function and/or calcium homeostasis increases GRP78 level which is generally founded in low levels. GRP78 is calcium-binding protein (Lamb, Mee et al. 2006); calcium can auto-phosphorylate GRP78 (Leustek, Toledo et al. 1991). However, it seems that calcium is essential for GRP78 association /dissociation with the T-cell antigen receptor  $\alpha$  chain (TCR- $\alpha$ ) (Suzuki, Bonifacino et al. 1991). Furthermore, chemical molecules that regulate intracellular calcium levels such as Calcium Ionophore A23187, Thapsigargin which is a non-competitive inhibitor of SERCA pumps, the calcium chelators BAPTA-AM and the translocon activator Puromycin or translocon inhibitor Anisomycin can up or down-regulate GRP78 expressions (Suzuki, Bonifacino et al. 1991; Wang, Wang et al. 2008; Hammadi, Oulidi et al. 2013; Casas 2017; Li, Yan et al. 2018) . The increase of calcium in mitochondria initiates apoptotic cell death; GRP78 overexpression has shown a protective role in primary astrocytes exposed to ischemia via decreasing the mitochondrial calcium level (Ouyang, Xu et al. 2011).

#### ***4.2.5 GRP78 in Cancer***

GRP78 has a critical survival role in cancer; GRP78 expression upregulation has been reported in a variety of cancer tissues and cancer cell lines such as breast, lung, melanoma, prostate, ovarian, gastric, hepatocellular, esophageal and renal (Daneshmand, Quek et al. ; Lin, Wang et al. ; Gazit, Lu et al. 1999; Koomagi, Mattern et al. 1999; Fernandez, Tabbara et al. 2000; Ulianich and Insabato 2014). The role of GRP78 in cancer was associated with increased aggressiveness, invasion, resistance to chemotherapy (Table 5) and poor prognosis (Reddy, Mao et al. 2003; Yeung, Kwan et al. 2008; Ren, Chen et al. 2017). Cell surface GRP78 has been reported in cancer cells but neither in benign tissues nor in immortalized cells (Liu, Li et al. 2013; Zhang, Tseng et al. 2013; Yao, Liu et al. 2015). The elevated GRP78 expression is associated with a bad survival rate in breast cancer (Zheng, Cao et al. 2014). In a retrospective cohort study of breast cancer patients, GRP78 conferred resistance to Adriamycin chemotherapy (Lee, Nichols et al. 2006). In triple-negative breast cancer, targeting of the cell surface GRP78 by anti-GRP78 antibody prevented apoptosis induced by Doxorubicin and Tunicamycin; assuming the necessity of the expression of GRP78 at the cell membrane to initiate cell death (Raiter, Yerushalmi et al. 2014). Moreover, mutant BRCA1 increased both the protein and the transcript of GRP78 expressions; siRNA against GRP78 enhanced Tunicamycin and Paclitaxel apoptotic effects in breast and ovarian cancers (Yeung, Kwan et al. 2008). In MCF-7 breast cancer, the upregulation of GRP78 expression

significantly increased cells proliferation and migration; indeed, an anti-GRP78 antibody designed against the N-terminal blocked the cell surface GRP78 receptor function, leading to an inhibition in MCF-7 proliferation and migration (Yao, Liu et al. 2015). Moreover, the authors also demonstrated that JAK2/STAT3 pathway mediates cell surface GRP78-induced action (Yao, Liu et al. 2015). In prostate cancer, the cell surface GRP78 and Cripto complex was able to enhance tumour progression via inhibiting TGF- $\beta$  signalling and activating the PI3K/AKT cascade (Shani, Fischer et al. 2008). Another work in prostate cancer has exhibited that cell surface GRP78 is involved in the formation of the  $\alpha$ 2-macroglobulin/prostate-specific antigen complex, which in turn binds to GRP78, causing activation of MEK1/2, ERK1/2, S6K, and AKT signalling pathways (Misra, Payne et al. 2011). Further, siRNA-mediated knockdown of GRP78 confirmed the implication of GRP78 in promoting cell invasion in melanoma and associated with lymph node metastasis; in the same study, GRP78 proposed as a new potential targeted therapy and/or useful biomarkers for diagnosis and prognosis. Targeting the COOH-terminus of GRP78 by polyclonal antibodies in melanoma cells and prostate cancer cells promoted apoptosis via p53 expression upregulation and inhibition of the Ras/MAPK and PI3K/AKT signalling pathways (Misra, Mowery et al. 2009). The expression of GRP78 has been linked to chemotherapy sensitivity, in lung cancer, the low expression of GRP78 was associated with the chemo-sensitivity (Wang, Wang et al. 2008).

Cancer Type	Therapy
Lung cancer	GRP78 $\uparrow$ in <b>DOX</b> and <b>TAX</b> resistant cells
Bladder cancer	GRP78 $\uparrow$ confers resistance to <b>ETOP</b>
Stomach cancer	GRP78 $\downarrow$ (versipelostatin) sensitizes xenografts to <b>Cis-Pt</b>
Breast cancer	GRP78 $\uparrow$ in <b>DOX</b> , <b>ETOP</b> , <b>TAX</b> resistant cells GRP78 $\downarrow$ (siRNA) sensitizes cells to <b>ETOP</b>
Gastric cancer	GRP78 $\uparrow$ confers resistance to <b>Celecoxib</b> GRP78 $\downarrow$ (siRNA) sensitizes cells to <b>Celecoxib</b>
Transformed fibroblasts	GRP78 $\downarrow$ (antisense) sensitizes cells to <b>UVC</b> and <b>Cis-Pt</b>
Epidermoid carcinoma	GRP78 $\downarrow$ (siRNA) sensitizes cells to <b>DOX</b>

*Table 5. GRP78 and resistance against therapeutic agents in human cancer (Li and Lee 2006)*



### 4.3 Translocon

Translocon is a heterotrimeric protein, has a three-subunit core. In human, referred as Sec61 and located at the ER membrane; while in bacteria it is termed SecYEG and located at the cytoplasmic membrane. The ER translocon complex comprises of the three subunits Sec61 $\alpha$ , Sec61 $\beta$ , and Sec61 $\gamma$  (Figure 28) which forms a bidirectional hydrophilic dynamic channel permitting the co-translational passage of newly synthesized polypeptides and the retro-translocation of unfolded proteins toward the cytosol. The dimeric Sec62/Sec63 proteins are located in the ER membrane and involved in the translocon complex. Sec translocons are expressed ubiquitously in tissues.

#### 4.3.1 Translocon Structure and Function

At the structural level, SecYE and Sec61 $\alpha$   $\gamma$  are homologues and conserved; while the Sec61 $\beta$  subunit is not homologous to SecG subunit. Sec61 $\alpha$  and Sec61 $\gamma$  are essential for protein transport; however, Sec61 $\beta$  is not implicated in the transporting traffic. Sec61 $\alpha$  is the major subunit (Figure 29), consists of 10 transmembrane  $\alpha$ -helical domains (TMs). TMs 1–5 are linked to the TMs 6–10 by a periplasmic loop. The translocon / ribosome interaction occurs via the TM helices between 6 and 7 and TM helices between 8 and 9 (Voorhees, Fernández et al. 2014). The Sec61 $\gamma$  is a single-spanning membrane protein. The Sec61 $\beta$  subunit contains one TM; this subunit does not interact with the signal recognition particle (SRP) receptor while it interacts with Rtn1p protein which is implicated in ER tubule formation (Denks, Vogt et al. 2014; Linxweiler, Schick et al. 2017).

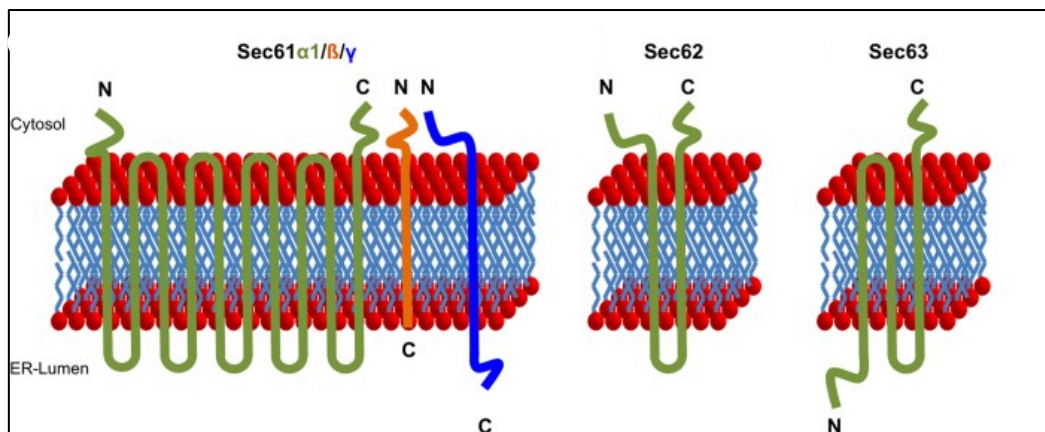


Figure 29. Structure of Sec translocons (Linxweiler, Schick et al. 2017)

Prior to the co-translational transport (Figure 30), the SRP binds to the N-terminus of the nascent polypeptide precursor; then, the SRP receptor drives the polypeptide to cross the ER membrane through the Sec61. The J1 domain of Sec63 can mediate the direct interaction

between ER chaperons and the newly nascent polypeptides. The Sec63 interacts with Sec62; in turn, the C-terminal domain of Sec62 interacts with the N-terminal domain of Sec61 $\alpha$ . The GRP78 substrate-binding domain binds to ER luminal loop 7 of Sec61; GRP78 -ATP hydrolysis is essential which is provided by Sil1 and GRP170 proteins. Also, there is a calcium-Calmodulin binding site on the N-terminal domain of Sec61 $\alpha$ . The post-translational transport is achieved by the cytosolic HSP40 and HSP70 chaperones (Figure 30).

Besides its role as protein transport channel, Sec61 functions as a passive calcium channel that permits calcium efflux from the ER lumen to the cytosol (Simon and Blobel 1991; Lomax, Camello et al. 2002; Roy and Wonderlin 2003; Schäuble, Lang et al. 2012). This passive calcium leakage can be regulated by different factors; the interaction between the Calmodulin and Sec61 $\alpha$  limits calcium leakage from the endoplasmic reticulum (Erdmann, Schäuble et al. 2011). GRP78 seals the Sec61 in results to its association to the loop 7 of Sec61 $\alpha$ , thus inhibiting ER-calcium efflux (Schauble, Lang et al. 2012). Sec62 seems to be also implicated, where the C-terminal EF-hand motif of Sec62 can mediate calcium leakage from the Sec61 (Linxweiler, Schorr et al. 2013). However, mutations and overexpression of Sec61, Sec62 and Sec63 were found in different pathological condition among them cancer (Linxweiler, Schick et al. 2017). Even during ER stress, Sec61 can inhibit calcium leakage and modulate apoptosis induced by potent ER stressors such as Thapsigargin (Hammadi, Oulidi et al. 2013); the inhibition of translocon by the translocon inhibitor, Anisomycin, can restore the ER calcium homeostasis and promote cell survival (Cassel, Ducreux et al. 2016).

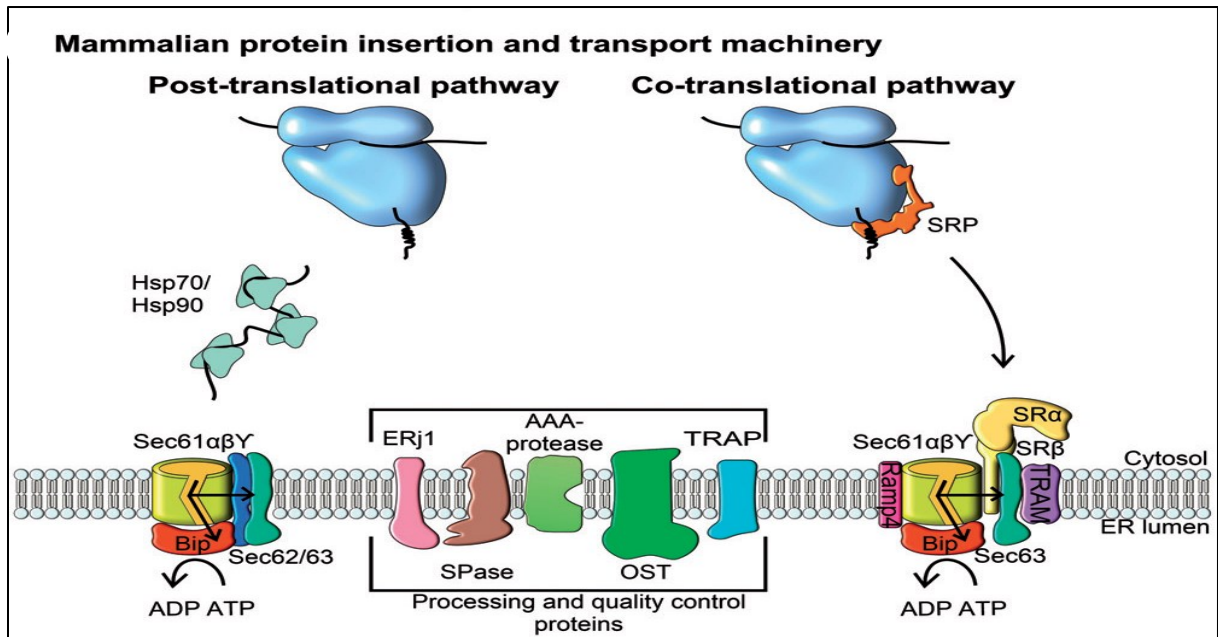


Figure 30. «“In eukaryotes, the Sec61-mediated insertion and the translocation occurs both co-translationally and post-translationally”. “During post-translational targeting, fully synthesized pre-proteins are kept in a transport-competent state by members of the Hsp90, Hsp70 and Hsp40 chaperone families”. “Translocation is mediated by the Sec61 complex in association with Sec62/Sec63 and the chaperone BiP at the luminal side of the ER membrane. BiP binds to the translocating substrates in the ER lumen and prevents their back-sliding by an ATP-dependent ratcheting mechanism”. “The eukaryotic SRP pathway delivers both membrane proteins and secretory proteins co-translationally to the Sec61 complex”. “The eukaryotic SRP receptor consists of two unrelated GTPases, SRa and SRb”. “The Sec61 translocon associates in a substrate-dependent manner with additional proteins that either bind to RNCs (Ramp4) or are suggested to assist membrane protein folding (TRAM, Sec63). BiP is also required for co-translational transport”. “Additional proteins are involved in processing and quality control (SPase; TRAP [translocon associated protein], oligosaccharyl transferase [OST], the Hsp40-homologue Erj1 or AAAproteases)”» (Denks, Vogt et al. 2014).

#### **4.4 ERAD**

ERAD, for endoplasmic reticulum-associated degradation, is an adaptive mechanism which can start almost in parallel with UPR to manage ER stress and restore ER homeostasis. ERAD can include two degradation mechanisms: type I which is ubiquitin-proteasome-dependent ERAD and type II which is autophagy-lysosome dependent ERAD (see chapter 2) (Rashid, Yadav et al. 2015). Type I ERAD targets only soluble misfolded proteins while autophagic pathway targets the soluble and insoluble misfolded proteins. ERAD activation is a result of failure to achieve proper proteins folding. It recognizes the unfolded proteins and retrotranslocates them to the cytoplasm; where these proteins will face their fate and driven to degradation by the ubiquitin-proteasome system (UPS). Four ordered steps included in this pathway; Recognition, Dislocation, Ubiquitination and Degradation (Figure 31).

##### **4.4.1 Recognition**

Glycoproteins that are unable to fold by CNX/CRT cycle will re-enter the folding cycle; properly folded proteins leave the ER to Golgi after removal of 1 to 2 mannoses residues. Trimming of at least three mannose molecules from the glycan branch triggers ERAD (Lederkremer and Glickman 2005; Shenkman, Lomonosova Iu et al. 2013). These mannose cleaving steps are tightly controlled by ER mannosidase I (ERmanI) and the ER degradation-enhancing  $\alpha$ -mannosidase-like proteins (EDEMs). The binding of non-lectin chaperone GRP78 to ERdj5 (co-chaperone-ERAD enhancer) is necessary for ERAD of both glycosylated and non-glycosylated proteins (Ushioda, Hoseki et al. 2013). The GRP78-ATP binding domain is needed for this coupling .Moreover, OS-9 and XTP3-B are lectins proteins, able of recognizing the unfolded proteins after trimming their mannoses residues, and helping their binding to retrotranslocon (Hosokawa, Kamiya et al. 2009; Bernasconi and Molinari 2011; Christianson and Ye 2014).

##### **4.4.2 Retrotranslocation**

After the recognition of unfolded proteins, retrotranslocons start to assemble for the retrotranslocation step and secrete the misfolded substrates to the cytosol. The Sec61 translocon channel serves as a retrotranslocon channel (Wiertz, Tortorella et al. 1996). GRP78 seals Sec61 pore opening which is needed for substrates to cross into the cytosol. Indeed, Derlins proteins (1.2.3) are a part of the retrotranslocon channel (Ye, Shibata et al. 2005) and the retrotranslocation- regulatory factors (Brodsky 2012). Suppressor/enhancer of Lin12-like

(SEL1L); is an important regulator seems to have an important role through connecting the recognition factors such as; OS-9, XTP3-B, EDEMs and ERdj5 to the retrotranslocon (Olzmann, Richter et al. 2013; Williams, Inoue et al. 2013).

#### ***4.4.3 Ubiquitination***

Once the unfolded substrates retrotranslocated to the cytosol, a series of enzymatic reactions starts during this third step; many ubiquitin ligases are involved, such as E1, E2, and E3 proteins in forming of the polyubiquitin chain which binds to p97 partner proteins, Ufd1 and Npl4 (Meyer, Bug et al. 2012; Lee, Park et al. 2013), then passing into the center of the p97 hexamer through the translocon. This polyubiquitinated protein is targeted by a complex capping system of the 26S proteasome.

#### ***4.4.4 Proteasomal Degradation***

Finally, these unfolded substrates reach their terminal destination. The 26S proteasome proteolytic complex is composed of a 20S proteasome and a 19S regulatory particle (Voges, Zwickl et al. 1999; Smalle and Vierstra 2004). The 19S contains ATPase subunits that undergo catalyzation for the most of ubiquitinated substrates. However, there is a selective degradation of certain substrates by the 20S proteasome (Goldberg 2005). Rpn10 and Rpn13 are able to recognize ubiquitinated chain through their Ub-binding domains (Finley 2009). The adaptor proteins such as Rad2334-36, Dsk235,36, and Ddi136 contain a ubiquitin-like domain and a ubiquitin-associated domain that connect the proteasome to the polyubiquitin chain (Schauber, Chen et al. 1998; Hiyama, Yokoi et al. 1999; Wilkinson, Seeger et al. 2001). Briefly, ubiquitin is removed from the ubiquitinated chain, and then the protein is linearized, broken into smaller polypeptides followed by degradation of amino acids by peptidases.

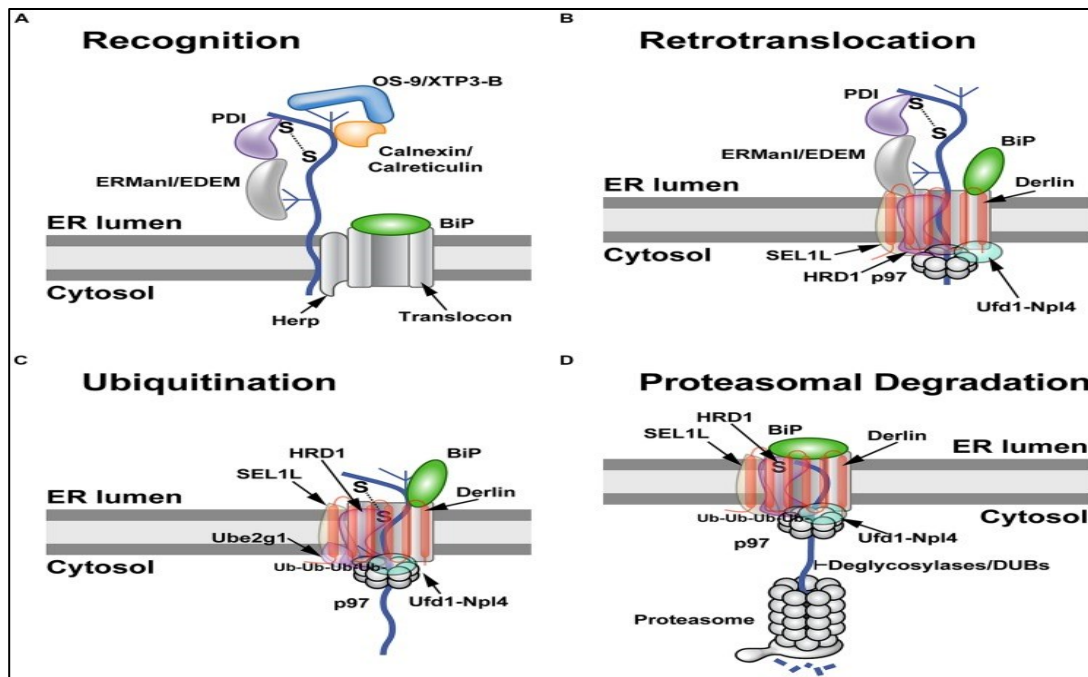


Figure 31. The ERAD process (A) Substrate recognition, (B) Retrotranslocation, (C) Ubiquitination of ERAD substrates and (D) Proteasomal degradation (Byun, Gou et al. 2014)

## ***Chapter V: Calcium***

### ***5.1 Introduction***

Calcium is a very abundant ion in the body and functions as a 2<sup>nd</sup> ubiquitous messenger, involved in the regulation of many signalling pathways modulating various physiological processes. In 1883, the British clinician Ringer demonstrated the first evidence of calcium role in physiology when a perfusion solution containing calcium has managed to maintain normal heartbeats of isolated frog heart. In non-excitable eukaryotic, the intracellular concentration of Ca<sup>2+</sup> is approximately 100 nM, while in the extracellular medium is usually between 1 and 2 mM (Petersen 2002; Berridge 2003). This gradient is controlled tightly by various plasma membrane calcium regulators and also from intracellular calcium stores organelles including the ER, Golgi apparatus and mitochondria. In excitable cell when a specific stimuli creating a calcium signal, this results in an increase in intracellular concentration of calcium to activate or inhibit a cellular process such as secretion, neuronal transmission, proliferation, migration, apoptosis, muscle contraction, cellular metabolism, and gene transcriptions (Berridge, Bootman et al. 1998; Braet 2004; Roderick and Cook 2008). Every calcium-dependent cellular process has unique calcium signature characterized by a specific amplitude, frequency, location and duration. For example, in smooth muscle cells, the type of the Ca<sup>2+</sup> signals is variable and depends on the calcium origin, which could be either extra or intracellular calcium; these signals have a variety of forms, range from sustained, temporally and localized changes (Hill-Eubanks, Werner et al. 2011). In response to vascular injury, vascular smooth muscle cells can undergo phenotypic switching associated with calcium signal changes from a depolarization-induced calcium entry by voltage-gated Ca<sup>2+</sup> channels to store-operated calcium entry (SOCE) (Matchkov, Kudryavtseva et al. 2012; Munoz, Hernandez-Morales et al. 2013).

Every type of oscillated Ca<sup>2+</sup> signalling regulates a specific cellular process; calcium plays a critical role in cell cycle progression during the different phases (G1, S and G2 / M). The study of Pande et al. by flow cytometry on fibroblasts showed that the level of free intracellular calcium level very low in cells in G0, it increases during the progression in G1, it decreases during the S phase and increases again at the G2 / M (Pande, Kumar et al. 1996). It has been shown that calcium regulates the expression of some transcription factors necessary for G1 phase (Takuwa, Zhou et al. 1993; Roderick and Cook 2008; Koenig, Linhart et al. 2010). Cellular proliferation of some cell types is highly dependent on extracellular calcium concentrations. Inhibition of cell proliferation has been measured when extracellular calcium

concentrations range from 1 to 0.1 mM; this inhibition was reversible after restoring the extracellular calcium concentration to its physiological level. Calcium overload can induce necrotic cell death (Li, Fan et al. 2010) or programmed apoptosis. Two signalling pathways are involved in apoptosis mediated by calcium; either by endoplasmic reticulum reticular stress or by mitochondrial calcium overloads (Berridge, Bootman et al. 1998).

Calcium signals were altered in cancer and linked to cancer progression; these signals can be regulated due to alteration in calcium channels and pumps expression, functionality and localization as well as via post-translational modifications. Indeed, the expressions of specific calcium regulators were observed for various cancer cell lines and in clinical samples including breast (Deliot and Constantin 2015), ovarian (Frede, Fraser et al. 2013) (Lehen'kyi, Raphael et al. 2012), brain (Ando, Kawaai et al. 2017) (Ando, Kawaai et al. 2017), prostate (Gaunt, Vasudev et al. 2016; Grolez and Gkika 2016; Tajbakhsh, Pasdar et al. 2017; Warrington, Richards et al. 2017) and oesophages (Zhu, Zhang et al. 2014; Zhang, Xie et al. 2016). Additionally, calcium phenotype signalling has changed in tumour cells when compared to non-tumour cells, such as sustained increase, faster recovery after calcium stimuli, and increase in oscillations frequency (Stewart, Yapa et al. 2015).

### ***5.2 The Principle Regulators Involved in Calcium Homeostasis***

Several types of ion pumps and proteins are involved in cellular calcium homeostasis at the level of the cytoplasm, endoplasmic reticulum, mitochondria as well as plasma membrane (Figure 32).



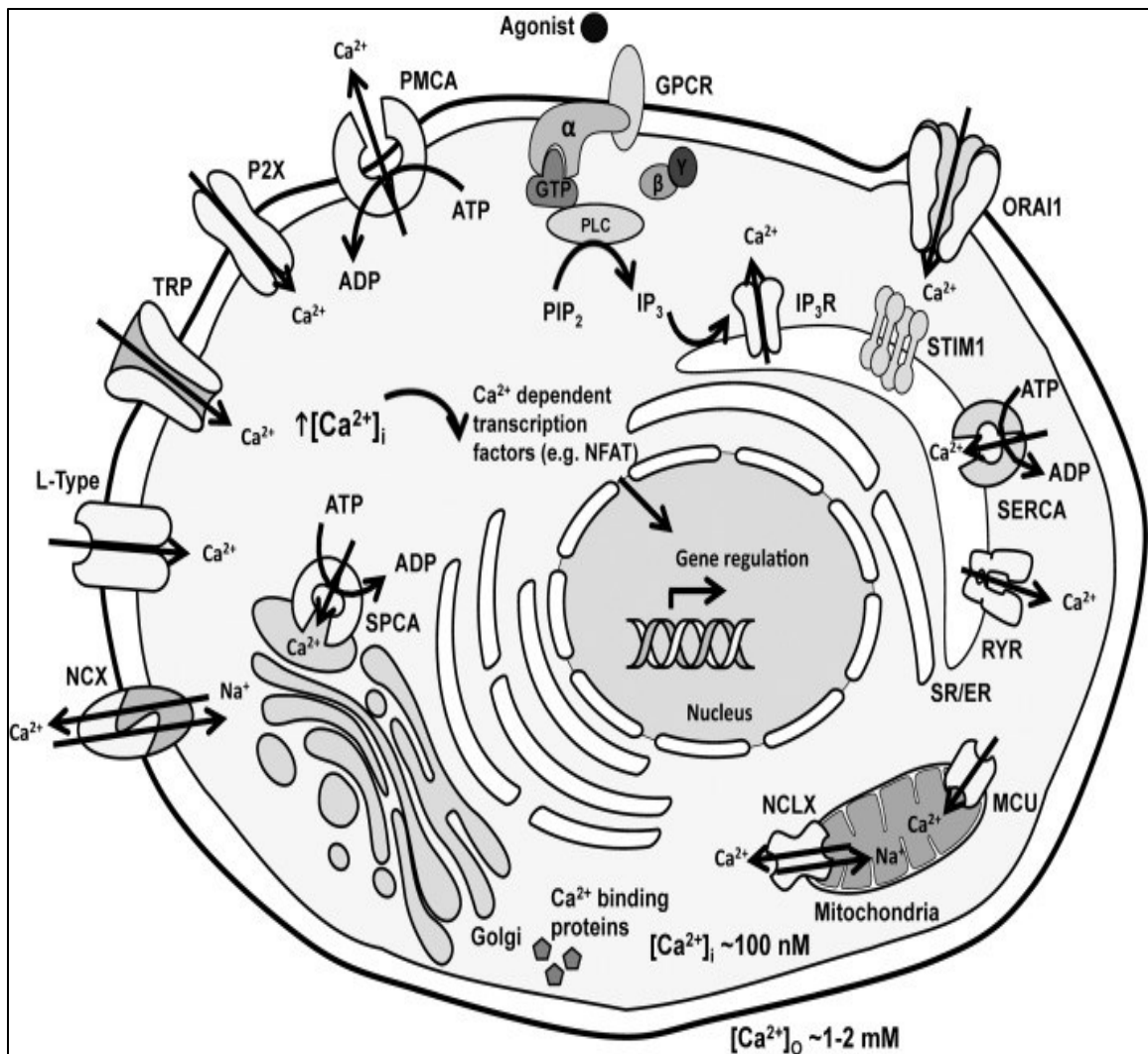


Figure 32. The ion pumps and proteins are involved in cellular calcium homeostasis (Stewart, Yapa et al. 2015)

### 5.2.1 Regulators Located at ER Membrane

#### 5.2.1.1 SERCA Pumps (Sarco-Endoplasmic Reticulum Calcium ATPase)

SERCA pumps belong to the ATPase family, located at the endoplasmic reticulum membrane. They transport the  $Ca^{2+}$  ions against their electrochemical gradient from the cytoplasm to the lumen of the endoplasmic reticulum by using the energy provided by the hydrolysis of ATP where 2  $Ca^{2+}$  ions transported by ATP hydrolyzed (Bobe, Bredoux et al. 2005); in results, filling the reticular calcium stock. There are three genes encoding SERCA pumps SERCA1, 2 and 3 (Misquitta, Mack et al. 1999; Martonosi and Pikula 2003). Each SERCA family presents different isoforms.

### ***5.2.1.2 Inositol 1,4,5-trisphosphate Receptors (IP3R)***

IP3R family has three isoforms (IP3R1, 2 and 3). IP3R are ubiquitously expressed almost in all tissues. They permit  $\text{Ca}^{2+}$  release from the ER into the cytoplasm upon stimulation by inositol 1,4,5-trisphosphate. These are a large tetrameric channel IP3R with N-terminal that contains binding and suppressor domain. The C-terminal modulates gating and channel function, which is containing multiple proteins binding sites and phosphorylation sites. Calcium has a biphasic activity, where it can activate or inhibit the activity of IP3R. In fact, at low intracellular calcium concentrations, IP3R is activated, whereas high calcium concentrations can inhibit its activity (Wright and Wojcikiewicz 2016).

### ***5.2.1.3 Ryanodine Receptors (RyRs)***

These channels are also involved in the  $\text{Ca}^{2+}$  release from the ER to the cytoplasm, by sensing low cytosolic  $\text{Ca}^{2+}$  concentration, RyRs establish positive feedback to release calcium toward the cytosol after being activated by the ryanodine even at a very low concentration (Buck, Zimanyi et al. 1992). They consist of three isoforms (RyR1, 2 and 3) and all form homotetrameric proteins (Fill and Copello 2002); the RyR1 isoform is mainly expressed in skeletal muscle (Zorzato 1989); RyR2 is founded primarily in cardiac muscle (Otsu, Willard et al. 1990), While RyR3 is expressed in neurons and other tissues (Hakamata, Nakai et al. 1992).

## ***5.2.2 Regulators at Mitochondrial Membrane***

### ***5.2.2.1 Mitochondrial Calcium Uniporter (MCU)***

MCU is a transmembrane protein located at the inner mitochondrial membrane, permits the passage of calcium from the cytosol to inside the mitochondria. Mitochondrial  $\text{Ca}^{2+}$  uptake is mediated by the mitochondrial calcium uniporter complex ~ 480 kDa; where MCU is the main pore forming unite, the MCUB and EMR (essential MCU regulator) also contribute in this complex as well as the regulatory subunits MICU1 and MICU2 (De Stefani, Patron et al. 2015; Mammucari, Raffaello et al. 2016). MICU1 enhances MCU opening while MICU2 inhibits MCU activity at high  $\text{Ca}^{2+}$  level (Patron, Checchetto et al. 2014)

## ***5.2.3 Regulators Located at Plasma Membrane***

### ***5.2.3.1 PMCA (Plasma Membrane Calcium ATPase) Pumps***

PMCA pumps belong to the ATPase family. They are very abundant, have a strong affinity for  $\text{Ca}^{2+}$  and allow the release of calcium to outside of the cell, opposite to its

electrochemical gradient (Carafoli, Genazzani et al. 1999; Carafoli, Santella et al. 2001); thereby, restoring the normal intracellular calcium concentration by using the energy of the hydrolysis of an ATP molecule (Bobe, Bredoux et al. 2005). At the structural level, PMCA pumps are very similar to SERCA pumps, composed of 10 transmembrane segments and N and C-terminal regions on the cytoplasmic side. These pumps characterized by two large cytoplasmic loops which contain ATP binding site, acidic phospholipid binding domain (PL) and Calmodulin binding domain (CaMBD) (Strehler, Filoteo et al. 2007). There are 4 PMCA isoforms coded by 4 different genes. PMCA1 and 4 are expressed in a ubiquitous way, while PMCA2 and 3 have a more specific expression (Strehler and Zacharias 2001; Strehler, Filoteo et al. 2007).

#### **5.2.3.2 $Na^+$ / $Ca^{2+}$ Exchangers**

The NCX exchangers are antiporter proteins, expressed ubiquitously, but are more abundant in the heart and nervous tissues; three isoforms of NCX have been identified (Carafoli, Genazzani et al. 1999; Philipson and Nicoll 2000). NCX allow extrusion of calcium to the outside of the cell by exchanging 3  $Na^+$  ions for 1  $Ca^{2+}$  ion. They have a much lower affinity for  $Ca^{2+}$ , but more capacity than PMCAs and their role is to rapidly decrease the intracellular calcium concentration using the energy of the  $Na^+$  gradient (Carafoli, Santella et al. 2001). In contrast, these antiporter proteins contribute to  $Ca^{2+}$  entry into the cell when the intracellular  $Na^+$  concentration increase, thus  $Na^+$  comes out while  $Ca^{2+}$  enters the cell (Blaustein and Lederer 1999; Beauge and DiPolo 2005).

#### **5.2.3.3 Voltage-Dependent Calcium Channels**

Voltage-gated calcium channels (VGCCs) open in response to membrane depolarization leading to the opening of calcium-selective pores. They permit calcium influx along the electrochemical gradient into the excitable cells such as muscles and neurons, and also implicated in the progression of different cancers (Phan, Wang et al. 2017). They characterized with their selectivity for  $Ca^{2+}$ . According to Simms and Zamponi, VGCCs may classify into two major categories; (1) high voltage-activated (HVA) channels that activate due to large membrane depolarizations; and (2) low voltage-activated (LVA) channels that need fewer voltage depolarizations (Simms and Zamponi 2014). The HVA channels are heteromultimers composed of the  $Cav\alpha 1$  subunit which assembles with other Cav unites while the LVA channels function as  $Cav\alpha 1$  subunit monomers (Simms and Zamponi 2014).

#### 5.2.3.4 SOC Channels

SOC activation permits calcium influx to the cytosol after a signal stimulates the receptor coupled to the heterotrimeric G proteins and leading to activate the phospholipase C (PLC- $\beta$ ). This last catalyzes the hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to produce two distinct second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>). IP<sub>3</sub>, in turn, activates the IP<sub>3</sub> receptor (IP<sub>3</sub>R), which is located at the ER membrane; result in calcium efflux from the ER to cytosol. To refill intracellular Ca<sup>2+</sup> stores, SOC will activate and permit calcium influx toward the cytosol after sensing calcium depletion by Stim proteins to regulate basal calcium levels (Figure 33) (Srikanth and Gwack 2012; Putney 2017).

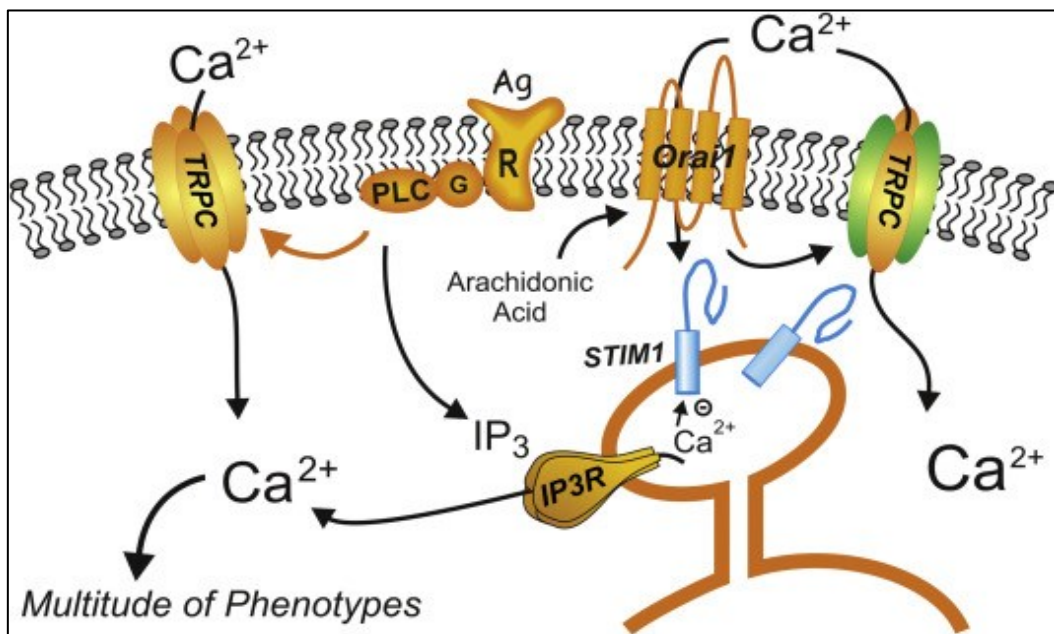


Figure 33. Signals of SOC activation (Putney 2017)

#### 5.2.3.4.1 TRP channels

TRP channels for “Transient Receptor Potential” have been identified for the first time in *Drosophila*. The TRP superfamily was grouped into 7 subfamilies: the first group TRPs includes (TRPC, TRPV, TRPM, TRPN, and TRPA) and the second group includes (TRPP and TRPML). The subfamily TRPC only has six members in humans (TRPC1, TRPC3–TRPC7). As mentioned previously, TRPC activation is initiated by a stimulant activates phospholipase C (PLC) which induces the production of DAG and IP<sub>3</sub> from phosphatidylinositol diphosphate (PIP<sub>2</sub>). The second messenger DAG activates the ROC (Receptor Operated Channel); TRPC 1, 6, 7 allow the entry of Ca<sup>2+</sup> into the cell as SOC/

TRPC after ER stores depletion. The work of Liu, et al. exhibited that TRPC1 silencing decreased SOCE; suggesting that TRPC1 contribute to SOCE (Liu, Singh et al. 2003). It also has been demonstrated that TRPC1 and Stim1 interaction happened following store depletion (Yuan, Zeng et al. 2007). Moreover, store depletion induces PLC $\beta$ 1 activity and Stim1 is essential for the activation of TRPC1-based SOCs (Shi, Miralles et al. 2017).

#### **5.2.3.4.2 Orai Channels**

Mammalian cells express 3 isoforms of Orai proteins (1.2.3) (Figure 34). They are closely related homologues with some difference in their C-terminal. All three isoforms are producing store-operated Ca<sup>2+</sup> entry (Prakriya, Feske et al. 2006; Gross, Wissenbach et al. 2007; Lis, Peinelt et al. 2007) and are widely expressed in many different tissues (Gross, Wissenbach et al. 2007; Gwack, Srikanth et al. 2007; Wissenbach, Philipp et al. 2007). However, in our study, we focused on the calcium channel Orai1, because of Orai1 remains the best-studied CRAC channel protein, seems to be the principal isoform mediating SOCE in different cells types; besides its implication in breast cancer progression; as well as Orai1 is a N-glycosylated protein.

#### **5.2.3.4.3 Orai1**

In 1989, Lewis and Cahalan were the first who recorded the CRAC currents; since that many candidates were studied as a possible CRAC pore-forming subunit (Lewis and Cahalan 1989). Orai1 canals were presented for the first time by Feske and his collaborators in 2006, where they specified Orai1 as a crucial regulator for SOCE and CRAC channel function (Feske, Gwack et al. 2006). In the same work, Orai1 was identified by using two approaches: (1) the phenotypic identification in severe combined immune deficiency patients (Feske, Prakriya et al. 2005); where this syndrome characterized by a lack of T cells activation and absence of CRAC current; the reconstitution of T cells from a SCID patient with wildtype Orai1 restored CRAC current. (2) The Linkage mapping by genome-wide SNP array screen in *Drosophila* S2 cells (Feske, Gwack et al. 2006). The same year, other publications have identified the same gene encoding Orai1 and named as CRACM1 (for CRAC Modulator 1) and introduced it as a Plasma Membrane Protein Essential for Store-Operated Ca<sup>2+</sup> Entry (Vig, Peinelt et al. 2006). Both Orai1 and CRACM1 are used in the literature to refer the same protein; anyway, the name Orai1 is the most prevalent. Since that, other reports presented that Orai1 is the main pore-forming subunit of CRAC channels; the co-overexpression of Orai1 and Stim1 induced large CRAC currents with high Ca<sup>2+</sup> selectivity (Mercer, Dehaven et al.

2006; Soboloff, Spassova et al. 2006; Peinelt, Lis et al. 2008; Yamashita, Somasundaram et al. 2011). Mutation at specific acidic residues in Orai1 impaired its  $\text{Ca}^{2+}$  selectivity. Also, Orai1 exhibits a similar pharmacological inhibitory response to 2-aminoethyldiphenyl borate (2-APB) which is already recorded in the original CRAC currents (Yeromin, Zhang et al. 2006; Yamashita, Somasundaram et al. 2011).

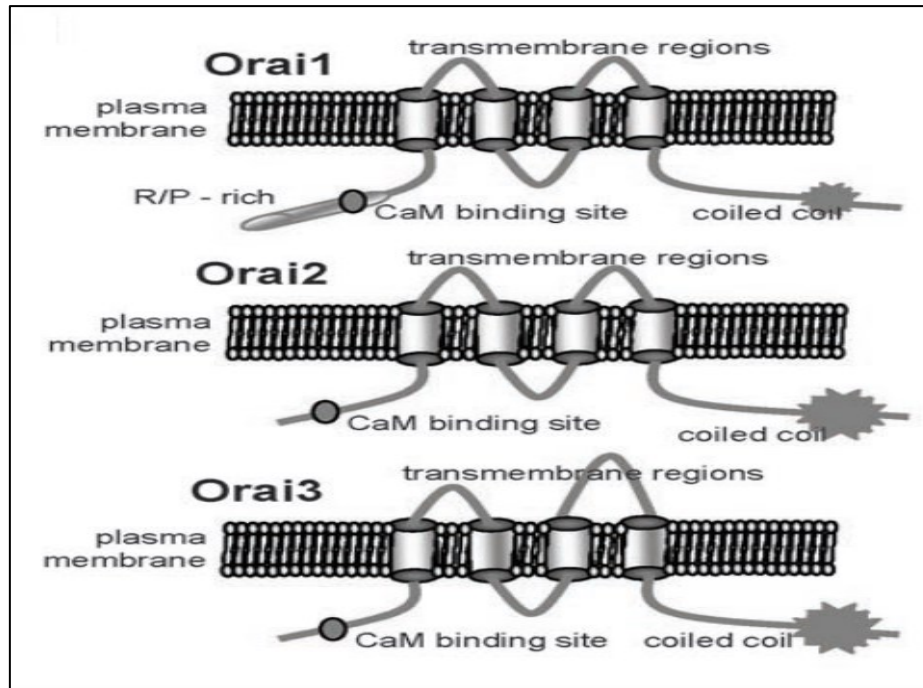
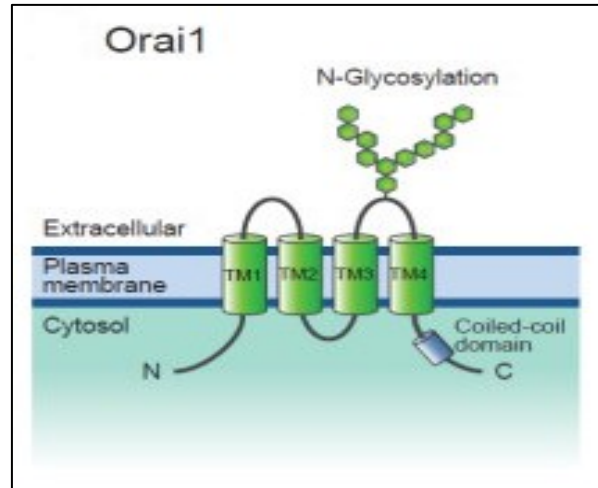


Figure 34. Orai isoforms (Frischauf et al., 2008)

At the structural level, Orai1 is a cell surface protein of mass ~34 kDa, consist of 303 amino acids located on chromosome 12q24, with 4 transmembrane segments (TM1–TM4), and two cytoplasmic N- and C- termini. Unlike Orai2 and Orai3, Orai1 has N-terminal region characterized by a domain rich in Proline and Arginine residues (Frischauf, Schindl et al. 2008) while the C-terminal regions (called coiled-coil) of Orai1 interact with the C-terminal regions of Stim (Stromal-interaction molecule) proteins permitting Stim1–Orai1 activation (mechanism of activation via Stim1 will be detailed later). In MCF-7 breast cancer cell line, independently from Stim1, Orai1 channels can be activated and regulate the basal calcium concentration via SPCA2 pumps which are initially located at the level of the Golgi apparatus (Feng, Grice et al. 2010). This type of calcium entry called store-independent  $\text{Ca}^{2+}$  influx (SICE), which is independent of SOCE and the ER stores. Moreover, SICE was not affected either by the ATPase activity of SPCA2 nor by Stim1 protein; where a mutation of the ATPase domain of SPCA2 and knockdown of the Stim1 did not modulate SICE currents (Feng and Rao 2013).

The only isoform of Orai family known to have a glycosylation site is Orai1, where it located on the Asparagine residues (Asp223) between the T3 and T4 (Figure 35) (Gwack, Srikanth et al. 2007). Also, Orai1 has phosphorylation sites at the Serine residues (Ser27-30) (Kawasaki, Ueyama et al. 2010).



*Figure 35. The glycosylation site of Orai1 located on the asparagine residues (Asp223) between the T3 and T4 (Chen, Chen et al. 2013)*

#### **5.2.3.4.4 Stim proteins**

One year before the discovery of Orai1 in 2006, a large screening by using RNA interference in *Drosophila* S2 cells permitted identifying the proteins that alter Thapsigargin - dependent  $\text{Ca}^{2+}$  and responsible for SOC activation: (Stim1 for “Stromal interaction molecule type1” and Stim2 for “Stromal interaction molecule type2”). Stim proteins presented as sensors of endoplasmic calcium concentration (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005). Mammalian cells ubiquitously express both Stim1 and Stim2 proteins (Williams, Manji et al. 2001); which are encoded by genes located respectively on chromosomes 11p15.5 and 4p15.2 (Parker, Begley et al. 1996). More precisely, Stim1 was called GOK and cloned for the first time in 1996 at an important tumour-suppressor gene region on the 11p15.5 chromosome (Parker, Begley et al. 1996). In this work, we are more interested in Stim1 than Stim2 for the following reasons:

✓ Stim1 and 2 present very similar homology (approximately 61%) (Williams, Manji et al. 2001) but different properties and functions; Stim1 is the principle SOCE activator, while Stim2 is a responsible for prolonged calcium entry; where it is a feedback regulator for basal  $\text{Ca}^{2+}$  levels in the cytosol and in the endoplasmic reticulum. Additionally, it is responsible for prolonged calcium entry (Brandman, Liou et al. 2007; Gruszczynska-Biegala, Pomorski et al. 2011).

- ✓ Both Stim proteins have glycosylated sites, but the mutagenesis of Stim1 N-glycosylation sites N 131 and N 171 profoundly alter SOCE (Kilch, Alansary et al. 2013).
- ✓ Stim1 has been studied more widely in the literature than Stim2; where several works demonstrated an important role of Stim1 in breast cancer.

#### 5.2.3.4.5 Stim1

Stim1 is a transmembrane protein and contains 685 amino acids and phosphorylated on Ser/Thr residues (Manji, et al. 2000). In contrast with Stim2 which is only found in the ER, about 75% of Stim1 is mainly localized in the ER membrane and about 25% on the plasma membrane (Roos, DiGregorio et al. 2005; Spassova, Soboloff et al. 2006; Baba 2016; Jardin and Rosado 2016). Furthermore, it has been found in the acidic stores (Zbidi, Jardin et al. 2011). A recent study demonstrated that phosphorylation of Stim1 at Y361 residue is required to trigger SOCE by enabling Stim1-Orai1 interaction and  $Ca^{2+}$  entry through Orai1 channels (Yazbeck, Tauseef et al. 2017).

The main structure of Stim1 composes of three parts (figure 36); (1) an N-terminal region, which is located in the intra-luminal compartment of the ER, contains a SAM domain (Sterile- $\alpha$ -Motif) and a calcium-binding site (conical EF-hand), this last works as a sensor for ER  $Ca^{2+}$  store depletion. (2) A C-terminal region at the cytosolic compartment, this region presents mainly the coiled-coil domain which consists of three parts (CC1, CC2 and CC3). The CC1 is the CRAC modulatory domain, which is rich in Serine/Proline and Lysine. The other two parts (CC2 and CC3) also known as SOAR/CAD for (Stim1–Orai1 Activating Region/ and CRAC Activating Domain). (3) A Transmembranous segment (TM) which connects both of N and C termini together.

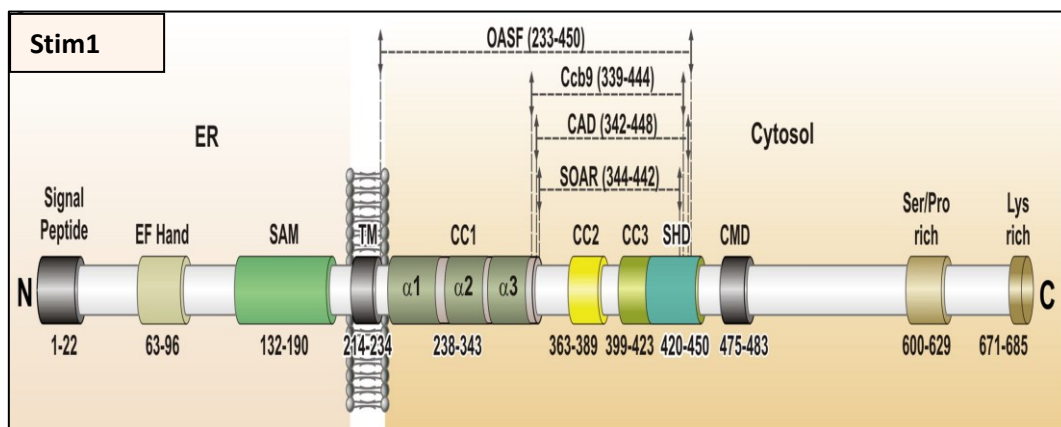


Figure 36: Schematic representation of human, full-length, Stim1 (Jardin and Rosado 2016)



The ER calcium store sensor, Stim1, is an essential component of SOCE, permits the activation of the plasma membrane pore-forming unit of SOC channels, Orai1, in response to intracellular calcium depletion. Briefly, in resting cells with full of ER  $\text{Ca}^{2+}$  stores, Stim1 is inactive dimer form. Sensing calcium stores depletion by Stim1 EF-hand in response to calcium dissociation (Marshall, Nishikawa et al. 2015) leads to Stim1 oligomerization aggregation and redistribution in the form of puncta at ER-PM junction. Next, Stim1 CRAC activating domain (CAD) binds and activates the N-terminal domain of Orai1 (Hoover and Lewis 2011). This active complex (Stim1/Orai1) permits intracellular capacitive calcium entry and refilling of calcium stores (Figure 37). The work of Le Zehang et al. demonstrated that the EF-hand of Stim2 has low calcium binding affinity similar to Stim1; Stim2 EF-SAM is more stable than Stim1 EF-SAM region, thus, Stim2 stays monomeric and does not aggregate in the absence of  $\text{Ca}^{2+}$  (Zheng, Stathopoulos et al. 2011). However, Stim1 suppression inhibited SOCE; while Stim2 suppression caused a mild to no SOCE inhibition. In contrast, Stim2 overexpression inhibited SOCE. The mutations in the Stim1 EF-hand can interfere with its property as  $\text{Ca}^{2+}$  sensor leading to constitutively SOCE even with full ER stocks (Böhm, Chevessier et al. 2013).

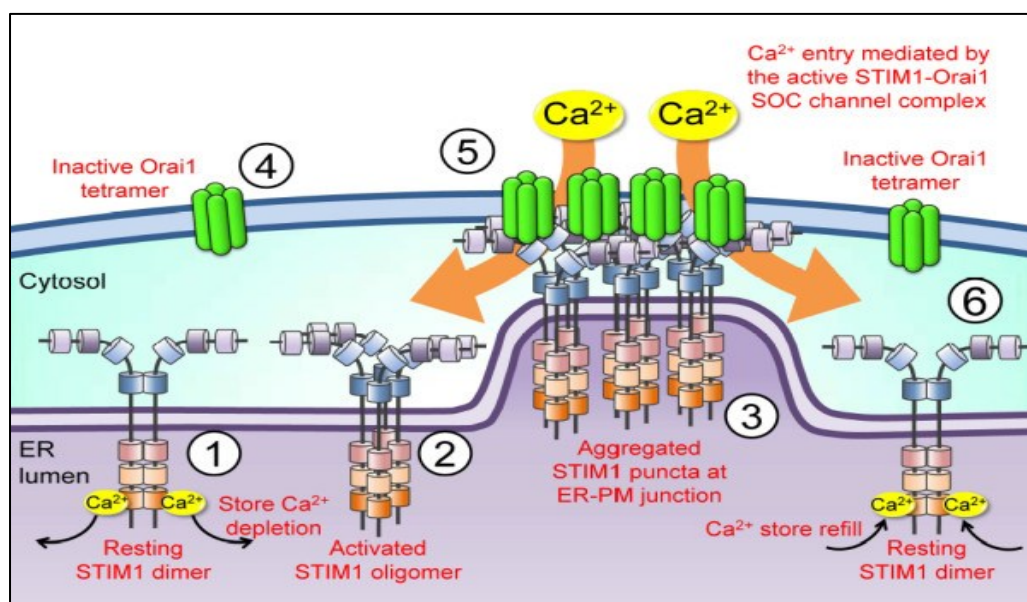


Figure. 37. Activation of SOC (Chen, Chen et al. 2013)

### 5.3.1 Orai1/ Stim1 Mutations and Pathologies

Since CRAC current has been correlated to some pathologies such as a severe combined immune deficiency (SCID), Orai1 and Stim1 found to implicate in different

conditions and diseases (Figure 38). Mutations in *Orai1* have been reported in rare immunodeficiency disorders in certain families; which characterized with the absence or severe inhibition of SOCE in T and B cells. Complete or LoF mutation in *Orai1* or *Stim1* is characterized by severe life-threatening immunodeficiency conditions; those patients are suffering from severe recurrent infections with viral, bacterial and fungal pathogens (Lacruz and Feske 2015). In the other hand, *Stim1* and *Orai1* specific mutations can lead to the constitutive  $Ca^{2+}$  influx and increase in SOCE, which also cause pathological conditions as Tubular Aggregate Myopathy and Stormorken syndrome (Lacruz and Feske 2015).

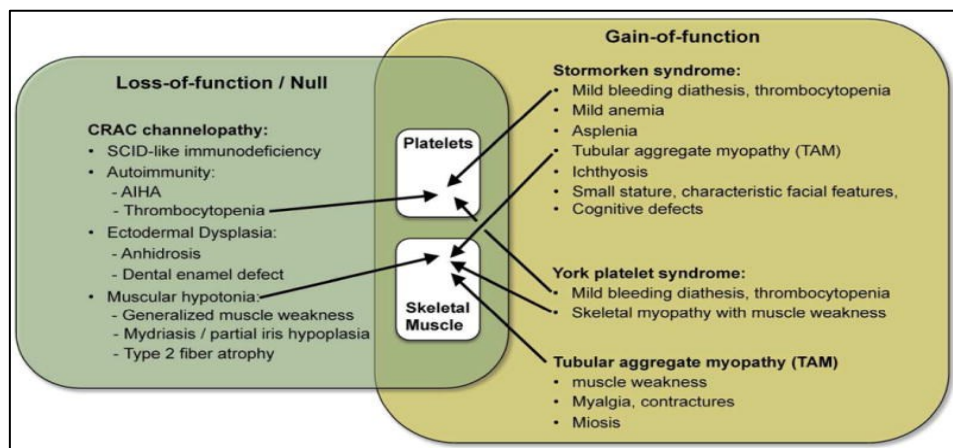


Figure. 38. Disease phenotypes associated with mutations in *Orai1* and *Stim1* (Lacruz and Feske 2015)

### 5.3.2 *Orai1* in Cancer

After the discovery of *Orai1* as the pore-forming subunit of the  $Ca^{2+}$  channel; important efforts have been made towards understanding their role in normal, pathological and cancer conditions. *Orai1* involvement in tumor progression has been demonstrated in several cancers (Table 6), including breast cancer, prostate cancer, esophageal cancer and ovarian cancer (Yang, Zhang et al. 2009; Flourakis, Lehen'kyi et al. 2010; McAndrew, Grice et al. 2011; Schmidt, Liu et al. 2014; Zhu, Zhang et al. 2014; Davis, Janoshazi et al. 2015; Stanisz, Vultur et al. 2016). *Orai1* is highly expressed in clear cell renal cell carcinoma, knockdown of *Orai1* inhibited cell migration and proliferation (Kim, Lkhagvadorj et al. 2014). The expression of *Orai1* is also increased in oesophageal squamous cell carcinoma, and correlated with patients survival (Zhu, Zhang et al. 2014). Moreover, Zhan and his collaborators have been demonstrated that *Orai1* overexpression was linked to poor prognosis in human non-small cell lung carcinoma (Zhan, Zhong et al. 2015). In contrast, there is a

down-regulation of Orai1 expression in human prostate tumour tissues (Xu, Zhang et al. 2015).

The implication of Orai1 in apoptotic cell death in cancer range from apoptotic to anti-apoptotic roles; in prostate cancer cells, down-regulation of Orai1 in LNCaP cells grown in the absence of androgens reduces apoptosis induced by Thapsigargin, TNF- $\alpha$  (Tumor Necrosis Factor  $\alpha$ ) or by chemotherapeutic agents such as Cisplatin or Oxaliplatin via mitochondrial and cytosolic mechanisms (Flourakis, Lehen'kyi et al. 2010); whereas others demonstrate that Orai1 and/or Stim1 silencing enhanced 5-Fluorouracil (5-FU) and Gemcitabine apoptotic effects (Kondratska, Kondratskyi et al. 2014).

Cancer type	Cell type	Role of SOCE	Stim1/SOC expression	Silenced protein	Effect of native expression or silencing
Lymphoma and myeloma	U937 cells	Apoptosis	↑Orai3		↑Apoptosis resistance
	8226 cells	Apoptosis	↓Orai3		↑Apoptosis resistance
Fibrosarcoma	HT 1080 cells	Migration	NA	TRPC1	↓Cell migration
Brain cancer	Glioblastoma cells	Apoptosis	NA	Orai1	↑Apoptosis
	Glioblastoma multiforme	Migration	↑Stim1; ↑Orai1	Stim1 and Orai1	↓Cell migration
	Glioblastoma multiforme	Proliferation/differentiation	↑Stim1; ↑Orai1	Stim1 and/or Orai1	↓Cell proliferation
Breast cancer	Glioblastoma	Migration	↑TRPC1	TRPC1	↓Cell migration
	MDA-MB-231	Migration	NA		
	MCF7 and T47D	Proliferation	NA	Orai3	Cycle cell arrest G1
	Breast ductal cells	Proliferation	↑TRPC1; ↑TRPC6; ↑TRPM7; ↑TRPM8; ↑TRPV6		
Cervical cancer	SiHa and CaSki cells	Migration	↑Stim1	Stim1	↓Cell migration and FA turnover
	SiHa and CaSki cells	Proliferation	↑Stim1	Stim1	↓Cell proliferation
	SiHa and CaSki cells	Angiogenesis	↑Stim1	Stim1	↓Angiogenesis
Colorectal cancer	Colorectal carcinoma cell	Migration, proliferation	↑Stim1	Stim1	↓Cell migration
	Carcinoma HT29 cells	Apoptosis	↑Stim1; ↓STIM2; ↑Orai1; ↑Orai2, ↑Orai3	STIM2	↑Apoptosis resistance
Gastric cancer	SGC7901 cells	Apoptosis, proliferation and migration	NA	Stim1	↑Apoptosis and ↓cell migration and proliferation
Liver cancer	Hepatocellular carcinoma cells	Focal adhesion	↑Stim1	Stim1	↓Cell migration
		Proliferation	NA	Stim1	↓Cell proliferation
Lung cancer	Non-small cell lung cancer cells	Apoptosis	↑Stim1	Stim1	↑Apoptosis
		Proliferation	↑TRPC1	TRPC1	↓Cellular growth
Neuroendocrine tumor	Neuroblastic N-type cells	Differentiation	↑Stim1; ↓Orai1		
Pancreatic cancer	Pancreatic adenocarcinoma Panc1 cells	Apoptosis	↑Stim1; ↑Orai1		↑Apoptosis
Pharynx cancer	Hypopharyngeal carcinoma	Apoptosis			↑Apoptosis
Prostate cancer	LNCaP	Proliferation			
		Apoptosis early state	↔ Stim1; ↔ Orai1; ↔ Orai2/↑Orai3		↑Apoptosis resistance and proliferation
	LNCaP	Androgen-independent state	↑Orai1		↑Apoptosis resistance
Renal carcinoma	Clear cell renal cell	Migration and proliferation	↑Orai1	Stim1; Orai1	↓Cell migration and proliferation
Skin cancer	Human epidermoid carcinoma	Proliferation	NA	Stim1	↓Cell proliferation
		Melanoma	Migration and proliferation	↑Stim1; ↑Orai1	Stim1; Orai1
		Proliferation	↑Stim1; ↑Orai1	Stim1; Orai1	↓Migration; ↓proliferation

Table 6. Expression of SOC channels in cancer cells (Jardin and Rosado 2016)

### 5.3.3 Orai1 in Breast Cancer

Three years after the discovery of Orai1, Yang and his colleagues had published the first evidence shown the implication of Orai1 and Stim1 in breast cancer; where both of them inhibited migration and metastasis in MDA-MB-231 breast cancer cell line by mediating SOCE (Yang, Zhang et al. 2009). Orai1 knockdown expression decreased focal adhesion complexes turnover (Figure 39), which resulted in migration inhibition (Yang, Zhang et al. 2009). MDA-MB-231 transfected with siRNA Stim1 or Orai1 have much less migration potential than control siRNA-treated cells in mouse models; therefore, Stim1 and Orai1 siRNAs significantly affected breast tumour metastasis *in vivo*. Yang, in his study, presented SOC channels as a potential therapeutic target; SKF96365 is SOC inhibitor, managed to reduce breast tumour metastasis *in vivo*. The published data of McAndrew, et al. studied the expression of Orai1 in several breast cancer cell lines, indeed, only Orai1 transcript expression was highly increased in MCF-7, MDA-MB-231, and T-47D breast cancer cell lines, neither Orai2 nor Orai3, when compared with immortalized nonmalignant cell lines (McAndrew, Grice et al. 2011). On the contrary, Faouzi et al. demonstrated the upregulation of Orai3 expression in MCF-7 and T47D breast cancer cell lines (Faouzi, Hague et al. 2011). In the triple-negative breast cancer cell line MDA-MB-231, by using siRNA, hEag1 modulated cell migration via Orai1. Additionally, it has been demonstrated that hEag1 and Orai1 are co-expressed in invasive breast cancer tissues and lymph node metastasis (Hammadi, Chopin et al. 2012).

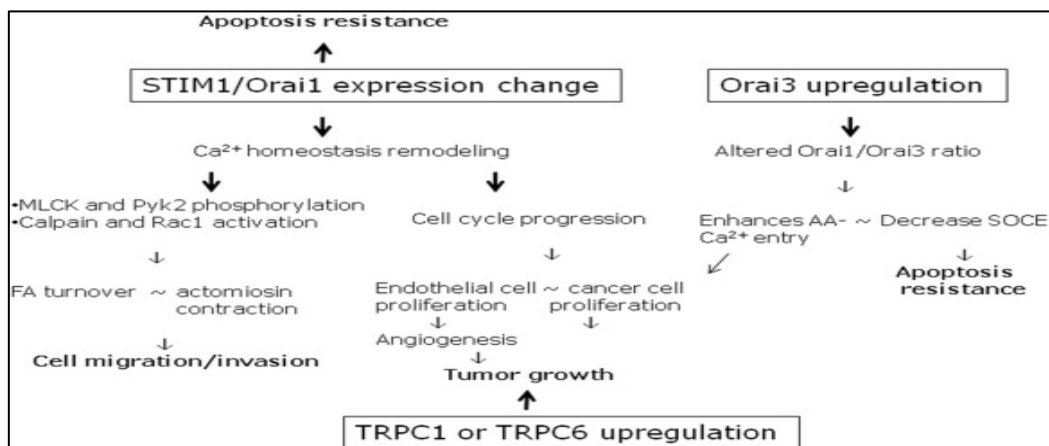


Figure 39. The Role of Stim1, Orai and TRPC channels in tumorigenesis. (Jardin and Rosado 2016)

### **5.3.4 Stim1 in Cancer**

Intracellular calcium hemostasis has an important role in maintaining proper cell function while  $\text{Ca}^{2+}$  imbalance can provoke diseases, as well as, controlling various oncogenic transformation such as apoptosis, proliferation, angiogenesis and metastasis. As one of many cellular calcium regulators, Stim1 has been studied widely in different cancers (Table 6). Many recent studies demonstrated the important role of Stim1 in cancerogenesis in a variety of human cancers, including prostate cancer, breast cancer, cervical cancer, glioblastoma, and pancreatic cancer. Inhibition of SOCE through regulating the calcium store sensor (Stim1) alters a variety of oncogenic transformation. Furthermore, several works demonstrated that Stim1 overexpressed in breast cancer (Yang, Jiang et al. 2017), colorectal cancer (Wong and Chang 2015), Prostate cancer (Perrouin Verbe, Bruyere et al. 2016), and in cervical cancer (Chen, Chen et al. 2017)

Stim1 regulates the migration ability of cancer cells by; (1) impairing FAK turnover through regulation protease Calpain and Tyrosine kinase Pyk2 (Figure 39) (Chen, Chiu et al. 2011; Yang, Tang et al. 2013). (2) Stim1 can translocate and activate Orai1 after stimulation by EGF, which is cancer cells migration stimulator. (3) Stim1 knockdown impaired prostate cancer cells migration and invasion through PI3K/AKT inactivation (Zhou, Gu et al. 2017). (4) It is capable to regulate the actomyosin reorganization (Chen, Chen et al. 2013). (5) Also, Stim1 through mediating SOCE, involved in podosome formation in cancer cells (Chen, Chen et al. 2017).

In fact, Stim1 overexpression enhances the migratory dynamics of cancer cells leading to increasing SOCE in colorectal cancer cells via an augmentation in the expression of cyclooxygenase-2 (COX-2) and the prostaglandin E2 (Wang, Sun et al. 2015), while Stim1 knockdown induces cell cycle arrest through upregulation of p21 and downregulation Cdc25C, cyclin D and cyclin E (Chen, Chiu et al. 2011; Li, Zhang et al. 2013). Moreover, Stim1 affects cell proliferation in liver cancer (El Boustany, Bidaux et al. 2008). Also, it has been linked to prostate cancer-induced apoptosis (Vanden Abeele, Roudbaraki et al. 2003).

### **5.3.5 Stim1 in Breast Cancer**

Abnormal variation of intra and extracellular calcium levels could be a pathological factor for normal mammary cells (Hu, Qin et al. 2011; Davis, Parsonage et al. 2013). In breast cancer,  $\text{Ca}^{2+}$  dysregulation has been linked to tumour progression in mammary gland through Stim1. McAndrew and his collaborators have demonstrated a significant correlation between basal-like breast cancer and Stim1<sup>high</sup>/Stim2<sub>low</sub> by using microarray data from 295 breast

cancer samples; this Stim1 ratio associated with poor prognosis and more aggressive and metastatic behaviour (McAndrew, Grice et al. 2011). Stim1 suppression inhibited cell migration and metastasis *in vitro* and in mice through regulating the focal adhesion turnover, (Yang, Zhang et al. 2009). Another work has exhibited that in MDA-MB-231 cells, silencing of Stim1 significantly inhibited migration by decrease invadopodia and podosome formation (Chen, Chen et al. 2017). TGF- $\beta$  decreased Stim1 expression, leading to SOCE inhibition, resulted in a decrease in breast cancer cell proliferation (Cheng, Wang et al. 2016). Recent work has exhibited that Stim1 is a direct target of miR-223 in breast cancer cell lines and tumour specimens in breast cancer patients; where miR-223 binds to the 3'-UTR of Stim1 and inhibiting Stim1 expression leading to decrease breast cancer cells proliferation and migration (Yang, Jiang et al. 2018). Stim1 expression in human breast cancer cases has been linked to clinicopathological factors and the prognosis in breast tumors tissues when compared to adjacent non-tumor tissues; by immunostaining method, Stim1 expression was positive in 109 of 165 breast cancer tissues, also, Stim1 expression was significantly higher in breast cancer tissues than adjacent non-tumor tissues (Yang, Jiang et al. 2017). In this last study, Stim1 overexpression was significantly associated with larger size tumours, lymph nodes metastasis and negative estrogen receptor.

# *Objectives*



## Objectives

As breast cancer is the most common cancer among women, huge efforts were done during the past decades to understand its signalling pathways and to find new treatments for this type of cancer. In fact, the early screening and some available treatment have improved survival rates dramatically. However, breast cancer is still in the focus of interest by scientists; where it is a big dilemma in the community health.

Glycosylation is a fundamental process in eukaryotic where it can affect protein folding, stability, activity, localization, secretion, solubility, function and cell-cell interactions. One of the hallmarks of cancer is aberrant glycosylation patterns. Furthermore, the abnormal glycan precursors and their implications and functional roles in cancer progression have been widely investigated and approved. The changes of glycan precursors in cancer can involve increasing in the branching of N-linked glycans, sialylation, fucosylation and truncation. These changes are linked to cancer behaviour in several ways. Specific enzymes are implicated in the N-glycosylation; indeed, the expression of glycosyltransferases and glycosidases were altered in cancers. However, the N-glycosylated proteins implicated in breast cancer development could be potential targets of the inhibitors of glycosylation.

Different glycosylation inhibitors have been shown anti-cancer activities *in vitro* and *in vivo* such as; Tunicamycin, Castanospermine and Swainsonine. However, these molecules have a generalized inhibition of glycosylation; this effect interfered and stopped the clinical trial of Swainsonine in renal cell carcinoma patients due to its toxic effects.

The synthesis of new generation of glycosylation inhibitors with more selectivity and specificity can reduce their toxic effects. Indeed, an sp<sup>2</sup>-Iminosugar  $\alpha$ -glucosidase II inhibitor 1-C-octyl-2-oxa-3-oxocastanospermine referred as “CO-OCS” with Ki 0.87  $\mu$ M for the yeast enzyme exhibited a selectivity to  $\alpha$ -glucosidases inhibition (Sánchez-Fernández Elena, Rísquez-Cuadro et al. 2012). Furthermore, CO-OCS also demonstrated antitumor effects in breast cancer cell lines MCF-7 and MDA- MB-231 without affecting normal immortalized cell line MCF-10A (Allan, Ouadid-Ahidouch et al. 2013) .The IC<sub>50</sub> value after 72 h of treatment for MCF-7 and MDA-MB-231 were 26  $\mu$ M and 44  $\mu$ M respectively. However, CO-OCS signalling pathways, as well as its principal targets, remain not studied.

The objectives of my thesis are:

- Investigating the molecular mechanisms by which CO-OCS induced its anti-tumour effects on cell migration, proliferation and survival mainly via determining the effect

of CO-OCS on the N- glycosylated proteins known to be implicated in breast cancer development.

- Studying the effect of CO-OCS on the UPR and ER stress signalling in breast cancer.

## ***Materials & Methods***

## ***1. Cell Culture***

The cell lines were used in this project, are purchased from ATCC (American Type Culture Collection). Cell culture performed within Cell Culture Hood class II, BH 2004D. For each cell line, a specific medium was used to cultivate the cells under a controlled atmosphere containing 5% CO<sub>2</sub> at 37°C. Culture medium was changed every 48 h to maintain optimal cell proliferation.

### ***1.1 Cell Lines***

Cell lines are used in this study were provided by ATCC:

- **MCF-7** (Michigan Cancer Foundation-7), adherent epithelial breast cancer cell line isolated for the first time from a pleural effusion due to metastatic adenocarcinoma, characterized by invasive behaviour and presence of estrogen receptors.
- **MDA-MB-231**, adherent epithelial breast cancer cell line with invasive phenotype, derived from a pleural effusion, characterized with Epidermal Growth Factor (EGF) and Transforming Growth Factor Alpha (TGF alpha) expression.
- **MDA-MB-435s**, adherent epithelial Melanoma cancer cell line derived from metastatic Melanoma, characterized by very invasive behaviour.
- **T-47D**, adherent epithelial breast cancer cell line, was isolated from pleural effusion, has estrogen and progesterone receptors expression.
- **MIA PaCa-2**, adherent epithelial pancreatic cancer cell line was isolated from tumour tissue of the pancreas from an old man.
- **MCF-10A**, adherent epithelial mammary immortalized but non-tumorigenic cell line.

### ***1.2 Culture Conditions***

#### ***1.2.1 Subculturing (Cell Passaging)***

Once cells growth reached 80% of confluence, cells were rinsed with PBS (Phosphate Buffered Saline from Sigma, France), then, cells were harvested by trypsin-EDTA solution (Invitrogen, Thermo Fisher, France) after 5 min of incubation at 37°C for all cell lines; except MCF-10A cell line was incubated for 20 min. To inhibit the action of trypsin, fresh culture

growth medium was added once the cells detached from the surface. Cells were counted by using hemocytometer and reseeded in a new flask (25 or 75 cm<sup>2</sup>).

### 1.2.2 Culture Medium

The next table represents the medium with different compositions for each cell line:

Cell lines	Culture medium	Provider	Freezing medium
MCF-7	<ul style="list-style-type: none"> <li>• Sterile water</li> <li>• EMEM 10X</li> <li>• Sodium bicarbonate</li> </ul>	<ul style="list-style-type: none"> <li>• Sigma</li> <li>• Lonza</li> </ul>	<ul style="list-style-type: none"> <li>• Fetal Bovine Serum</li> <li>• DMSO</li> </ul>
MDA-MB-231	<ul style="list-style-type: none"> <li>• HEPES</li> <li>• Non-essential amino acids</li> </ul>	<ul style="list-style-type: none"> <li>• 0.22% Gibco Life Technologies</li> <li>• 20 mM Gibco Life Technologies</li> </ul>	<ul style="list-style-type: none"> <li>• 10% (Dimethyl sulfoxide)</li> </ul>
MDA-MB-435s	<ul style="list-style-type: none"> <li>• L-Glutamax</li> </ul>	<ul style="list-style-type: none"> <li>• 2 mM Gibco Life Technologies</li> </ul>	
T-47D	<ul style="list-style-type: none"> <li>• Inactivated Fetal Bovine Serum (FBS) 5% (56C° for 30 min)</li> </ul>	<ul style="list-style-type: none"> <li>• Lonza</li> </ul>	
MIA PaCa-2	<ul style="list-style-type: none"> <li>• DMEM</li> <li>• inactivated Fetal Bovine Serum 5%</li> </ul>	<ul style="list-style-type: none"> <li>• Lonza</li> <li>• Lonza</li> </ul>	<ul style="list-style-type: none"> <li>• FBS</li> <li>• 10% DMSO</li> </ul>
MCF10A	<ul style="list-style-type: none"> <li>• DMEM / F12</li> <li>• EGF</li> <li>• Cholera Toxin</li> <li>• Insulin</li> <li>• Hydrocortisone</li> <li>• Fetal bovine serum 5%</li> </ul>	<ul style="list-style-type: none"> <li>• Lonza</li> <li>• 20ng/ml Sigma</li> <li>• 100mg/ml Sigma</li> <li>• 0.01mg/ml Sigma</li> <li>• 500ng/ml Sigma</li> <li>• Lonza</li> </ul>	<ul style="list-style-type: none"> <li>• Culture medium</li> <li>• 10% FBS</li> <li>• 5% DMSO</li> </ul>

Table7. Composition of different culture medium

### 1.3. Freezing and Thawing Cells

#### 1.3.1 Freeze Procedure

At 80 % of confluence, cells were detached by Trypsin/EDTA solution as mentioned before. 2 million cells were transferred to a sterile tube. Then, cells were centrifuged at 1000

RPM for 7 min. The supernatant was removed by aspiration. Pellet was quickly re-suspended by adding 1 ml of freezing media per vial. The Freezing procedure was done progressively and slowly in the freezing container CoolCell™ (Biocision, Dutscher, Thermofisher, France) overnight at -80 °C to avoid thermic shock.

### ***1.3.2 Thaw Procedure***

The vial was held in 37°C water bath until freezing medium was thawed. To inhibit the toxic effect of DMSO, 10 ml of 5% FBS culture growth medium was added. Cells were centrifuged at 1000 RBM for 7 min. The supernatant was discarded by aspiration. Next, cells were re-suspended in another 10 ml of 5% FBS culture growth medium and incubated at 37°C. Culture growth medium was discarded and replaced with fresh growth medium after 24 h, then every 48 h.

## ***2. RNA Interference and Transient Transfection***

This technique allows regulating a specific protein expression (overexpression or inhibition). Protein inhibition achieved by targeting a specific protein through specifically encoding mRNA. This is realized by using small specific nucleotide sequences (usually 21 to 25 bp) called siRNA (short interfering RNA). In this project, we used the electroporation method, this technique based on the application of electric impulses to cells, increasing their membrane permeability and permitting siRNA passage.

### ***2.1 Transfection by Electroporation***

Almost 1 Million Cells were transfected with 2 µg of Stim1, GRP78 and MCU small interfering RNAs or 4 µg of Orail small interfering RNA (Table 8). Control cells were transfected with scrambled siRNA. The transfection kit L from Lonza was used for both cell lines MCF-7 and MCF-10A, while the kit V was used for MDA-MB231 and MDA-MB435s. The efficacy of transfection was initially assessed by using real-time quantitative PCR and or Western blot. The transfected cells were treated or untreated at 24 h post-transfection and collected at 72 or 96 h post-transfection according to the experiments. The transfection was done using Amaxa® Nucleofector® Technology – two modular devices according to manufacturer's instructions and Protocol.

<b>siRNA</b>	<b>sequences (all in the 5'–3' direction)</b>
<b>Stim1</b>	<b>G.G.A.G.G.A.U.A.A.U.G.G.C.U.C.U.A.U.U</b>
<b>Orai1</b>	<b>G.C.C.A.U.A.A.G.A.C.U.G.A.C.C.G.A.C.A.dT.dT</b>
<b>GRP78</b>	<b>GGA-GCG-CAU-UGA-UAC-UAG-A55</b>
<b>MCU</b>	<b>GCC-AGA-GAC-AGA-CAA-UAC-U</b>
<b>CTL</b>	<b>siGenome non-targeting siRNA #1 – Dharmacon</b>

*Table 8. The sequences of siRNA used*

### ***3. Cell Viability and Mortality Studies***

#### ***3.1 MTT Assay Protocol***

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is referred to as MTT. Live cells have active metabolism leading to converting MTT into purple insoluble formazan crystals by mitochondrial dehydrogenase enzymes (Mosmann, 1983). These crystals participate in the mitochondria; where they are soluble in DMSO and have an absorbance at 550 nm. Died cells lose their ability to convert MTT into a formazan product. Thus, variations in colour formation in viable cells serve as a good colourimetric tool. For this experiment, cells were seeded in 6-well plates. The supernatant was discarded by aspiration. Cells were incubated with 800 µL of MTT (0.5 mg/ml in 5% FBS culture growth medium) for 60 min. After the incubation time, MTT solution was removed. In order to dissolve the formazan crystals, 800 µl of DMSO was added per sample. Then, in 96-well plate (Greiner Bio-one), 200 µl of dissolved formazan crystals was transferred in each well. The absorbance was measured by spectrophotometer at 550 nm (Infinite P200 PRO™ TECAN).

#### ***3.2 Trypan Blue Assay***

The total numbers of viable and died cells were measured by using trypan blue staining test. Trypan blue (TB) is about 960 Daltons molecule where it cannot cross the intact cell membrane; in contrast, it can enter in the cell through compromised membranes. TB assay permits for a direct distinction and counting of live (unstained) and dead (blue) cells in a population of interest. This experiment was done as follow; cells were cultured in 35 mm Petri dishes after siRNA transfection with  $8.10^4$  for MCF-7 and  $4.10^4$  for MCF-10A cells/dish. Cells were harvested at different times according to experiments by using trypsin-EDTA. Then, 1 volume of 4 % trypan blue dye was diluted to 4 volumes of suspension cell culture. Cells counting were done 6 times for each condition in at least three different

experiments using hemocytometer; the results were expressed in percentage of live and dead cells and normalized to the control condition.

1. The total number of cells is calculated by the following formula:

**Number of cells counted x 4 x (estimated volume + 400  $\mu$ L)**

2. The percentage of dead cells:

**Number of dead cells / total number of cells x 100**

3. The number of living cells:

**Number of unstained cells x 4 x (estimated volume + 400  $\mu$ L)**

#### ***4. Flow Cytometry***

##### ***4.1 Cell Cycle Study***

Flow Cytometry is a technical tool that utilizes laser-based technology to sort cells in suspension depend on several characteristics (physical and chemical) such as; number, profile, function, phenotype and size of cells. The cells pass one by one in a constant flow in front of laser beams. Sensors measure the diffracted light by the cells and transmit the information to the computer for analysis (Figure 40). However, flow cytometry allows distinguishing the distribution of cell phases (G0/G1, S, and G2M) among cell cycle. This analysis based on measuring the relative quantity of DNA within the nucleus among the different phases of cell division. Propidium Iodide (PI) is an intercalating molecule binds to DNA strands. The fluorescence emitted after PI excitation is proportional to the amount of DNA where the intensity of the fluorescence emitted by the cells in the G2 and M phases is twice larger than cells in G1 phases.



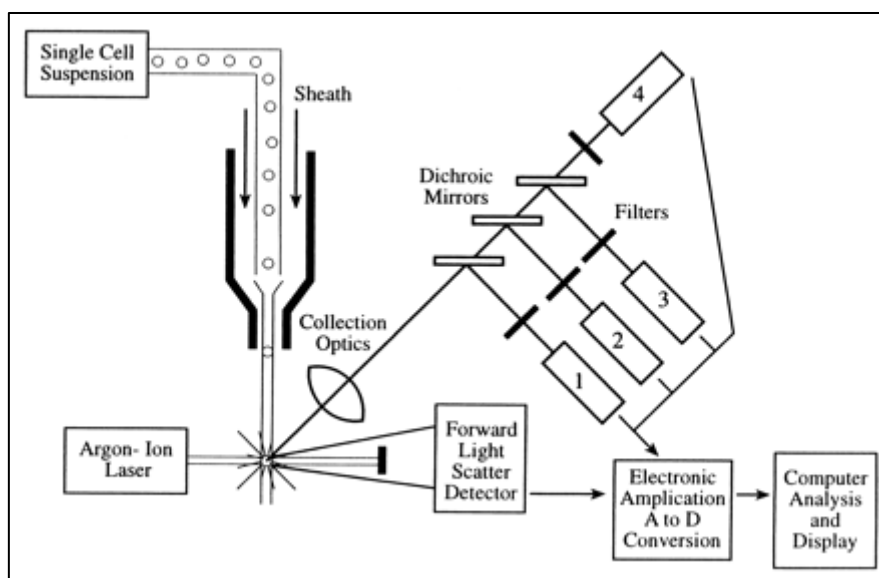


Figure 40. Schematic of a flow cytometer (Brown and Wittwer 2000)

Only viable adherent cell populations were used in this assay. Briefly, in all conditions, attached cells were rinsed twice with PBS and then detached by trypsin, followed by centrifugation for 7 min at 1000 RPM. The supernatant removed and the cell pellet re-suspended in 150  $\mu$ l of 5 mM PBS EDTA at cell concentration minimum 2000 cells / $\mu$ l. Then cells were fixed by 100% cold ethanol (350  $\mu$ l added drop by drop). The fixed cells could be stocked at 4°C for up to 2 weeks. After cells fixation for at least 6h at 4°C, cells were centrifuged for 7 min at 1000 RPM. The supernatant was removed and pallet re-suspended in (5 mM) PBS-EDTA. 2  $\mu$ L of RNase treatment (20  $\mu$ g/ml final concentrations) was applied to the cells for 30 min at room temperature to prevent the fixation of the intercalant molecule to the RNA. 100  $\mu$ l of Propidium Iodide (PI) was added to the cell at a concentration of 100  $\mu$ g/ml. Cells were passed and analyzed through BD Accuri flow cytometry.

#### 4.2 Cell Apoptosis Study

This protocol passed on the detection of phosphatidylserine phospholipids present on the surface membrane during the apoptotic process. The phospholipids are present only on the inner surface of the plasma membrane of viable cells. During apoptosis, these phospholipids translocate to the outer surface of plasma membrane. Detection of these cells is achieved by Annexin V; which has an affinity to phosphatidylserine phospholipids. Annexin V can couple to a fluorochrome (Phycoerythrin or FITC) which permits its detection (Figure 41). In parallel, another marker such as PI should be used to detect dead cells with disrupted membrane integrity due to necrotic cell death necrosis or late apoptosis.

Indeed, the cells will be classified according to their marking:

- Viable cells will be Annexin V - / Propidium Iodide -
- Early apoptosis cells will be Annexin V + / Iodide of Propidium -
- Late apoptosis or necrosis cells will be Annexin V + / Propidium Iodide +

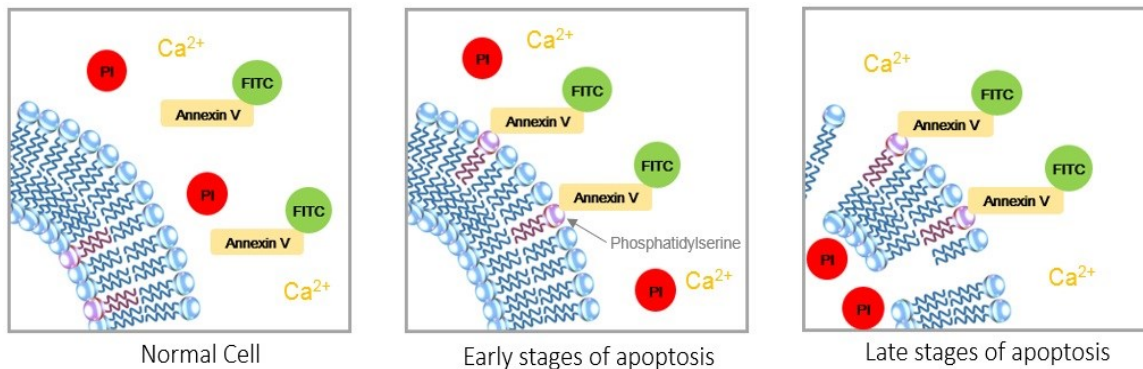


Figure 41. Apoptosis detection by Annexin V.

<https://www.nacalai.co.jp/global/reagent/research/annexin.html>

To quantify the percentage of apoptotic and necrotic cells, we used FITC Annexin V Apoptosis Detection Kit II (BD Biosciences Pharmingen, France). Growth medium that contains detached cells was collected in a 15 ml tube. The monocellular layer was washed twice with 4 ml of PBS. Cells were harvested by trypsin, re-suspended in PBS, and then added to the same 15 ml tube that contains the detached cells. Centrifugation at 1000 RPM for 7 min was done; the supernatant was discarded. Pellet was washed once by PBS, then once by the 1x binding buffer. 100  $\mu$ L of 1x binding buffer was added to re-suspend the cells, followed by 5  $\mu$ L of Annexin V and 5  $\mu$ L Propidium Iodide Staining Solutions, and then incubated for 15 min in dark place. After that, 400  $\mu$ L of 1x binding buffer was added to the mixture. Samples were analyzed by BD Accuri C6 flow cytometry.

## 5. Study of Cell Migration

### 5.1 Transwell Migration Assay:

Cell migration was studied by using cell cultures inserts (Becton Dickinson, 353097, France). These inserts characterized by a polyethylene terephthalate (PET) membrane with pores with a diameter of 8  $\mu$ m. The migratory capacity of cells is estimated by counting the

number of cells that cross these pores. The upper insert compartment contains 200  $\mu$ l of cell suspension at a rate of 40,000 cells per insert while the lower compartment contains 800  $\mu$ l of culture medium (Figure 42). After incubation time at 5% CO<sub>2</sub> and at 37°C, inserts were collected and rinsed 3 times with PBS and then the cells were fixed on the PET membrane by immersing the inserts in methanol for 10 min at 4°C. Next, inserts were immersed in the Crystal Violet to colour the cells during 20 min then rinsed by 3 washes with PBS. Before counting, the cells inside the insert were removed via a cotton swab. Only the cells that crossed the PET membrane were counted by an inverted microscope at x40 magnification. The cell number was estimated by counting of 15 fields per insert.

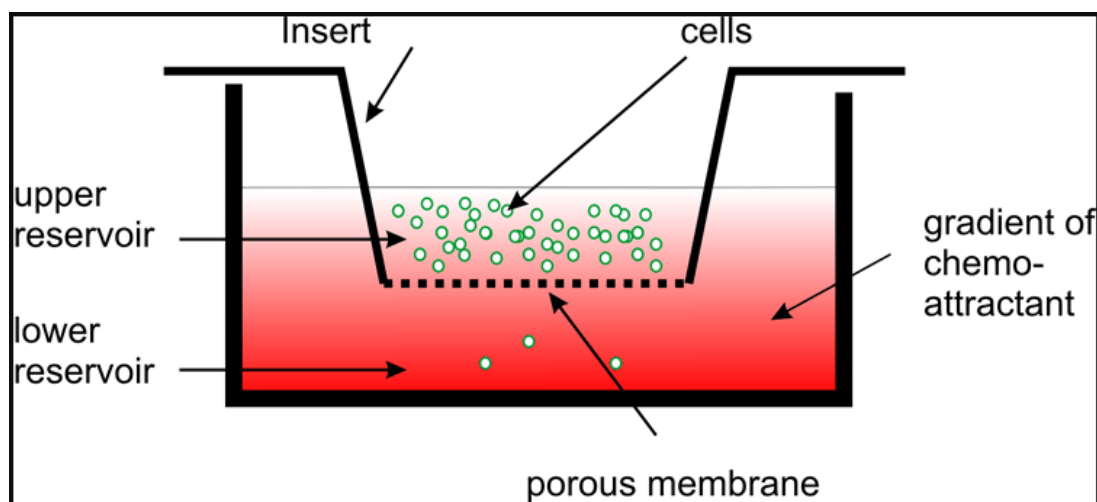


Figure 42. Transwell Migration Assay (Lautenschlaeger 2011)

### 5.2 Wound Healing Test

The migration study can also be done by the technique of "wound healing". At confluence, a wound in the cell monolayer was done by using a cone of pipette P 200. In order to follow the evolution of the closure of the wound, images were taken at the beginning and at regular intervals during cell migration. By using the Image J software, areas that do not contain cells are measured to calculate the percentage of wound closure.

## 6. Western Blot

This technique is an important tool in molecular biology which permits identification and quantification of a specific protein from a protein mixture complex. The separation of proteins based on their molecular weights through gel electrophoresis (SDS-PAGE), followed by transferring them to a nitrocellulose membrane. Then, the membrane is incubated with two

antibodies: a primary antibody which specific of the targeted protein and secondary antibody can conjugate to the primary antibody, the secondary antibody is coupled with horseradish peroxidase enzyme.

### ***6.1. Total Protein Extraction (Cell Lysis) and Concentration Measurement***

Briefly, cells for this experiment were cultured in 60 mm Petri dishes. Cultured medium was removed, then, the cells were rinsed twice with cold PBS. Proteins extraction was done over ice for 30 min by adding the 60  $\mu$ L of complete RIPA buffer (Triton1%, sodium desoxycholate, proteases inhibitor cocktail, EDTA 2 mM, 0.5 mM of sodium orthovanadate). Next, the mono-cellular layer was detached by a cell scraper; the cell suspension was centrifuged at 15,000 x g for 15 min at 4°C in order to separate the total protein (supernatant) from cellular debris. Then, the cell lysate was frozen at -20C. For accurate measurement of proteins concentration, we used the colorimetric Bio-Rad DC Protein Assay Kit. The concentration measurement assay was done with a standard curve was developed using a series of Bovine Serum Albumin (BSA) concentrations to compare the optical densities of samples to those of the BSA. The kit assay is composed of 3 reagents: A, S and B. First, 2.5  $\mu$ L of each sample was loaded in a 96-well plate. A mixture of reagents A + S was prepared (1 mL of A and 20  $\mu$ L of S), Then, 25  $\mu$ L of A+S mixture were added to each sample. Finally, 200  $\mu$ L of reagent B was added and stirred gently for 20 min. Optical densities measurements were done by spectrophotometry at 595 nm (Infinite P200 PRO <sup>TM</sup> TECAN).

### ***6.2 Gel Electrophoresis***

After calculating the total volume of protein lysates needed to load almost 50 mg of proteins on each wall; the proteins lysates were denatured by adding the reducing Laemmil buffer (Tris-base 62.5 mM, glycerol 10%, SDS 2.3%, B mercaptoethanol) and boiling the samples at 90°C for 10 min. Followed by loading them into the polyacrylamide gel (PAG). Different concentrations (10%, 12% and 15%) of PAG resolving gels were used according to the molecular weight of the targeted protein, and 5% of stacking gel was poured on top of resolving gel. Next, a running buffer (10% of 10x Tris/Glycine Buffer, 1% final concentration of SDS) was used to charge negatively the proteins permitting them to migrate through the PAG during 1 h at 200 V. The proteins were then transferred on nitrocellulose membrane through applying 60 V for 2 h. As a control step to ensure proper transfer, the membrane was stained with Ponceau S solution stain.

Next, the membrane was blocked (5% BSA or nonfat dried milk diluted in TBST) for 1 hour at room temperature. A Specific primary antibody was used to detect the targeted protein according to the provider instructions (Table 9).

Primary antibody	dilution	Provider	Incubation	Secondary antibody
<b>Stim1</b>	1:1000	Cell signaling	5% milk	Anti-rabbit
<b>GRP78</b>	1:1000	Cell signaling	5% milk	Anti-rabbit
<b>Bcl2</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b>Orai1</b>	1:250	Sigma	5% BSA	Anti-rabbit
<b><math>\beta</math>1 Integrin</b>	1:1000	Cell signaling	5% BSA	Anti-goat
<b>FAK</b>	1:500	Santa Cruz	5% BSA	Anti-rabbit
<b>p-Fak (Tyr397)</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b><math>\alpha</math>-Tubulin</b>	1:5000	Sigma	5% milk	Anti-rabbit
<b><math>\beta</math>-actin</b>	1:1000	Santa Cruze	5% milk	Anti-mouse
<b>ERK</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b>pERK</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b>AKT</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b>p-AKT (Ser473)</b>	1:500	Cell signaling	5% BSA	Anti-rabbit
<b>GAPDH</b>	1:3000	Abcam	5% milk	Anti-rabbit

*Table 9. Primary and secondary antibodies used*

In order to eliminate the non-specific binding of the primary antibody, 3 washing cycles were done for 10 min with TBST, followed by incubating the membrane with the secondary antibody anti-IgG coupled with horseradish peroxidase enzyme. The membrane was rinsed again with TBST for 3 times. Signals were detected by chemiluminescence, and captured by MF-ChemiBIS 2.0™ system (DNR BIO Imaging System) and quantified by using Quantity One software (Biorad).

## **7. Pharmacological Molecules**

The 2-oxa-3-oxocastanospermine C-octyl glycosides (CO-OCS), collaboration with Instituto de Investigaciones Químicas (IIQ), CSIC—Universidad de Sevilla, Spain, was solubilized in DMSO and stored at a concentration of 100 mM at -20°C. Cells were treated

with different concentration of CO-OCS and diluted in cell culture medium. Other molecules were used for different proposes (Table 10).

Molecules	Concentration/ duration	Provider
<b>MG-132</b>	5 $\mu$ M/ 24 h	Sigma
<b>Necrostatin-1</b>	30 $\mu$ M/ 3h	Sigma
<b>IM-54</b>	10 $\mu$ M / 3 h	Sigma
<b>Anisomycin</b>	0.2 $\mu$ M/ 48 h	Sigma

*Table 10. Chemical molecules and their used concentration*

## ***8. Molecular Biology Study***

### ***8.1 Extraction of the total RNAs***

The total RNAs are extracted according to the following protocol:

- First of all, the work plan and all the equipment used were cleaned and treated with RNase AWAY Reagent (Molecular Bioproducts). Then, the culture medium was removed, and the cells were lysed by Trizol (Sigma) for 5 min on ice (1 ml of Trizol for a Petri of 60 mm) where ice permits to decrease the activity of RNases.

- 200  $\mu$ L of 1-Bromo-3-chloropropane were added to separate the different phases. The mix was vortexed, then centrifuged for 15 min at 12,000 g at 4 ° C. Three phases were formed: an upper aqueous phase that contains the nucleic acids, an intermediate protein phase and a lower lipid phase. Only upper phase was kept, and the two other phases were discarded.

- Precipitation of the RNA was achieved by adding 500  $\mu$ l of isopropanol. The solution was then centrifuged for 15 min at 12,000 g at 4°C.

- The supernatant was discarded, and the pellet was washed with 500  $\mu$ l of 70% ethanol; then, centrifugation was performed, the mixture was discarded, and the pellets were dried.

- The dry pellets were then re-suspended in 44  $\mu$ l of sterile pure water.

This step was done in order to avoid a possible genomic DNA contamination in RNA samples. RNA samples were treated with 5  $\mu\text{L}$  of DNase buffer and 5  $\mu\text{L}$  DNase (Promega) at 37°C for 30 min.

- Next, a second phenol-chloroform RNA extraction was followed by a step of precipitation with sodium acetate and absolute ethanol; then centrifugation was done for 10 min at 12,000 g at 4°C.

- The pellets were washed with 70% ethanol and then re-suspended in 20  $\mu\text{L}$  of autoclaved pure water and stored at -80°C.

The purity and the quantity of the extracted RNAs were measured by using the NanoDrop 2000 Spectrophotometer at 260 nm. The concentration of the extracted RNA was expressed in  $\mu\text{g} / \mu\text{L}$  for each sample.

### ***8.2 Reverse Transcription-Polymerase Chain Reaction***

Retro-transcription is a step to reform complementary DNA (cDNA) from 2  $\mu\text{g}$  of RNA by using the kit High-Capacity cDNA Reverse Transcription (Applied Biosystems). For each sample, 10  $\mu\text{L}$  of Mix was prepared. The reaction mixture contains 2  $\mu\text{L}$  of RT buffer, 0.8  $\mu\text{L}$  of a mixture of four deoxynucleotides triphosphate (dNTP), 2  $\mu\text{L}$  of Random primers, 1  $\mu\text{L}$  of RNase inhibiting enzyme, 1  $\mu\text{L}$  of retro-transcriptase enzyme and 3.2  $\mu\text{L}$  of water. To this, 10  $\mu\text{L}$  of Mix, 10  $\mu\text{L}$  of the solution containing 2  $\mu\text{g}$  of RNA were added in order to obtain a final volume of 20  $\mu\text{L}$  for each sample.

The cDNAs were amplified by using a Thermocycler for three hours including the following steps: hybridization of the primers at 25°C for 10 min, the retro-transcription at 37°C for 2 h and deactivation of the enzyme at 85°C for 5 min. Then, samples are stored at 4°C.

### ***8.3 quantitative PCR / Real Time (q-PCR):***

q-PCR is a very sensitive technique and allows real-time monitoring of the number of amplicons formed from the amplification of cDNAs. q-PCR requires specific forward and reverse primers (Table 11). Detection of real-time amplification is carried out by the SYBRGreen fluorescent probe, which is an agent of intercalating double-stranded DNA. The fluorescence emitted is measured at each cycle. The cDNAs were diluted to 1/20. 10  $\mu\text{L}$  of Abgene Mix (SYBRGreen plus enzymes), 0.2  $\mu\text{L}$  of each primer (diluted to 50  $\mu\text{M}$ ) and 4.6  $\mu\text{L}$  of water was added to 5  $\mu\text{L}$  of diluted cDNA, giving a total volume of 20  $\mu\text{L}$  which was loaded in each the capillary (Roche). Next, capillaries were centrifuged and placed in the

Thermocycler (Roche, LightCycler). The values of Cp (cross point) for each targeted gene were normalized to  $\beta$ -actin after quantification by Pfaffl formula (2001).

Targets	Primers
<b>CHOP</b>	Reverse: TCCTCTTCATTTCCAGGAGGTGAA Foward : GAAGCCTGGTATGAGGACCTGCAA
<b>GRP78</b>	Reverse: CCTATGTCGCCTTCACT Foward: CACAGACGGGTCATTCC
<b>Stim1</b>	Reverse: TGT GGA GCT GCC TCA GTA TG Forward: CTT CAG CAC AGT CCC TGT CA
<b>Orail</b>	Reverse: CTGATCATGAGCGCAAACAG Forward: AGGTGATGAGCCTCAACGAG
<b><math>\beta</math>-actin</b>	Reverse: ACGTACATGGCTGGGGTG Forward: CAGAGCAAGAGAGGCATCCT

Table 11. sequences of primers used in this project.

## 9. Calcium Imaging

Fluorescence calcium imaging allows a real-time measurement for the variations of the free cytosolic calcium in many cells at the same time. This measurement can be achieved by using different fluorophores probes. The fluorescent probe Fura-2 is widely used *in vitro* calcium study. Fura-2 is a ratiometric probe, characterized by its permeability, photosensitivity and its binding capacity to the free cytosolic calcium; where it is excited at 340 nm and 380 nm, and emitted at 510 nm. Based on this, an increase in this ratio (340/380) accompanied by an important increase in the intracellular calcium. In this project, we used the Fura-2-AM probe from (Sigma) for intracellular calcium measurement.

All breast cells (treated or not with CO-OCS, transfected or not) were seeded in 35 mm Petri dishes on glass coverslips for calcium imaging experiment. At each time of treatment and transfection, cells were first loaded for 45 min with 2  $\mu$ M Fura-2/AM in culture medium (Sigma Aldrich). After Fura loading, cells were washed and kept in an extracellular saline solution (145 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose and 10 mM HEPES-NaOH, at pH 7.4). The coverslip was then transferred into a perfusion chamber of an inverted microscope (Zeiss). Fluorescence was excited at 340 then at 380 nm by using a monochromator (Polychrome IV; TILL Photonics). After passing



through a long-pass filter (510 nm), fluorescence were captured by a Cool SNAP HQ camera (Princeton Instruments). Recordings were carried out at room temperature in dark place using Metafluor software (version 7.1.7.0, Molecular Devices).

In this experiment, the cells were continuously perfused with the extracellular saline solution and chemicals were added as elucidated below. Thapsigargin was purchased from Calbiochem®. The flow rate of the whole cell chamber perfusion system was set to 1 ml/min, and the chamber volume was 500  $\mu$ L.

### ***9.1 Thapsigargin Protocol***

Both normal and cancer breast cells (transfected or not/treated or not with CO-OCS) were perfused for 1 min with the normal saline solution (2 mM  $\text{Ca}^{2+}$ ), replaced by the saline solution without  $\text{Ca}^{2+}$  for 2 min, replaced again for 10 min by the saline solution without  $\text{Ca}^{2+}$  supplemented with 1  $\mu$ M Thapsigargin (TG). Then, cells were perfused for 4 min with the saline solution containing 10 mM  $\text{Ca}^{2+}$ . Finally, cells were perfused for 4 min with a saline solution without  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  release and entry profiles were analyzed using Origin software (version.8).

### ***10. Statistical analysis***

Data are expressed as mean  $\pm$  SEM, or mean  $\pm$  S.D of at least three independent experiments. The mean values of two groups were compared by the Student's *t*-test, and the mean values of more than two groups were compared using two-way analysis of variance (ANOVA), using the Sigmastat software. The statistical significance <0.05, <0.01, and <0.001 are represented as \*, \*\*, and \*\*\* respectively.

# *Results*

## ***Part I: CO-OCS decreased breast cancer cell migration***

### **Article:**

#### **sp<sup>2</sup>-Iminosugar $\alpha$ -glucosidase inhibitor 1-C-octyl-2-oxa-3-oxocastanospermine specifically affected breast cancer cell migration through Stim1, $\beta$ 1-integrin, and FAK signaling pathways**

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### **Summary**

Breast cancer is the most common tumour in women worldwide. Cancer metastases are the first cause of all cancer-related deaths. Disruption of glycosylation process results in changes in the expression and the function of many cellular proteins, which can affect cancer development and metastasis. First, we were interested in studying the effect of CO-OCS on breast cancer cell migration capacity, through the use of two cell lines models (triple negative MDA-MB-231 and estrogen receptor-positive MCF-7), in parallel with normal breast immortalized cell line (MCF-10A). Cells were treated with CO-OCS at 40  $\mu$ M for 48 h, except MCF-7 cells, we applied a concentration of 100  $\mu$ M for 24 h. The assessment of migration was done by using transwell assay. CO-OCS significantly inhibited both breast cancer cell lines migration MDA-MB-231 by 60% and MCF-7 by 38% respectively, without significant effect on cell proliferation. In MCF-10A, CO-OCS had a slight effect (about 20%) on cell migration.

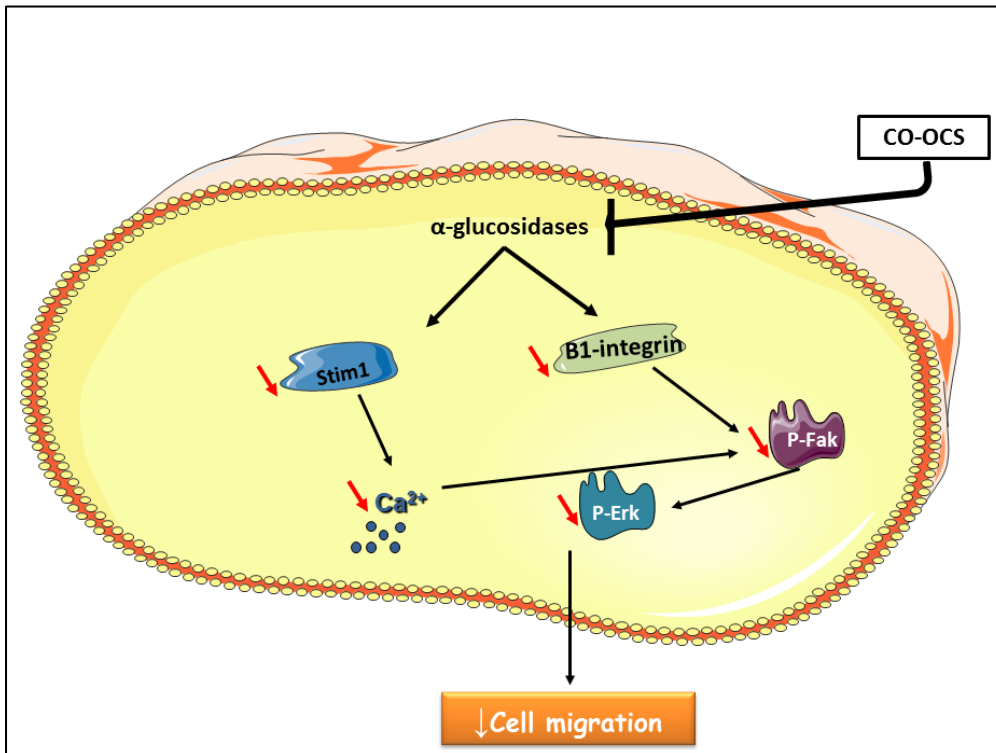
Second, to determine the underlying anti-migratory mechanism-related to CO-OCS in breast cancer cells, we investigate three N-glycosylated proteins (Orai1, Stim1 and  $\beta$ 1-integrin and its signalling cascade) that known to regulate the migration process in breast cancer. CO-OCS reduced the expression of  $\beta$ 1-integrin protein only in breast cancer cells by  $51 \pm 6\%$  and  $37 \pm 8.6\%$  in MDA-MB-231 and MCF-7 cells respectively without effecting the expression of  $\beta$ 1-integrin in normal breast cells. Additionally, CO-OCS affected the integrin downstream signalling cascades FAK and ERK1/2. The phosphorylation level of FAK in CO-OCS treated conditions was significantly decreased when compared with control conditions

after stimulation with 5% FBS for 10 min in MDA-MB-231 and MCF-7 cells. In fact, ERK1/2 was also affected, where; CO-OCS inhibited the phosphorylation of ERK1/2 at 20 min for MDA-MB-231 and at 10 min for MCF-7. We further investigated the effect of CO-OCS on the phosphorylation status of FAK and ERK1/2 in MCF-10A cells; CO-OCS had no effect on the phosphorylation levels of FAK and ERK1/2.

Stim1 and Orai1 are the two main components of SOCE channels, and both of them are essential for breast cancer cell migration. Inhibition of SOCE interferes with breast cancer cells migration; where SOCE is mediated by Stim1 and Orai1 proteins in breast cancer. We were interested in investigating the effect of CO-OCS on SOCE. By using western blot; we aimed to determine the effect of CO-OCS on the expressions of the two main regulators of SOCE (Stim1 and Orai1). CO-OCS reduced the expression of Stim1 by almost 30% in both cell lines without affecting the expression of Orai1. In contrast, CO-OCS failed to affect Stim1 transcripts level. Subsequently, we performed Fura2  $\text{Ca}^{2+}$  imaging assays in cells transfected with siRNA Stim1 in the presence or absence of CO-OCS. In MDA-MB-231 and MCF-7 cells, SOCE significantly inhibited in the siRNA Stim1 transfected cells and in CO-OCS-treated cells; no additional inhibition of SOCE was measured when the transfection and treatment were applied together. However, CO-OCS failed to affect Orai1 and Stim1 expressions in MCF-10A cells, thus, also failed to inhibit SOCE.

Finally, we demonstrated that the anti-migratory effect of CO-OCS in breast cancer cells achieved mainly through two important signalling pathways (Figure 43):

- Independent- $\text{Ca}^{2+}$  signalling pathways including; reduction of  $\beta 1$ -integrin expression, leading to inhibition of its signalling cascade FAK and ERK1/2.
- Dependent- $\text{Ca}^{2+}$  signalling pathways involving, a decrease in the expression of Stim1, which causes inhibition of SOCE, as well as impairing the activation of FAK.



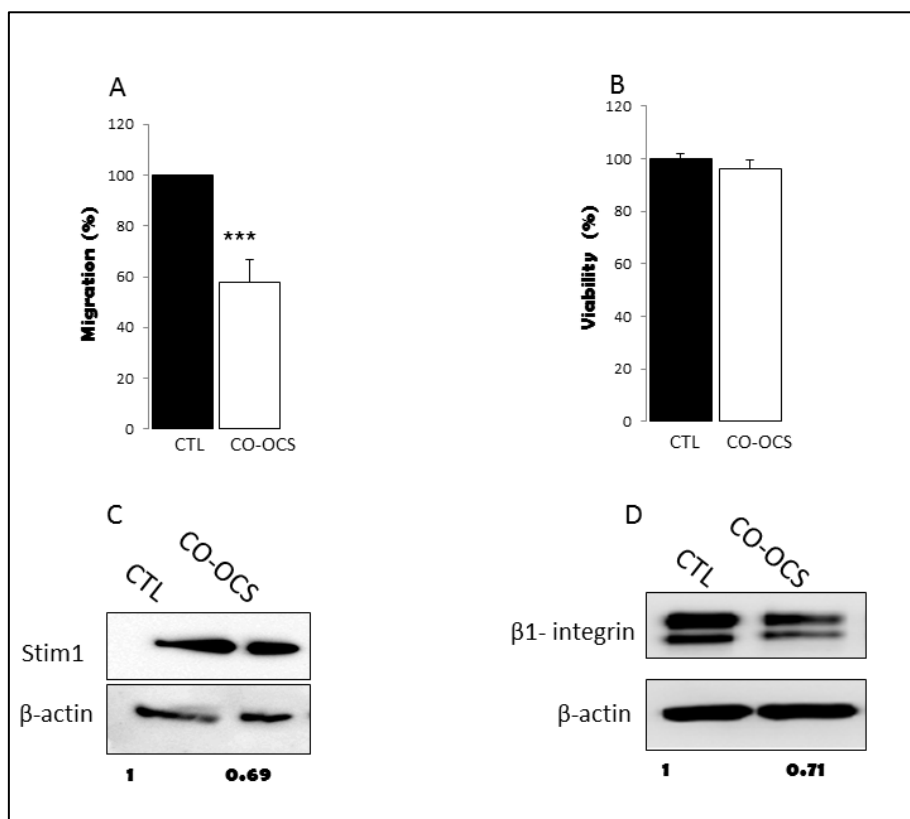
**Figure 43. Schematic representation of CO-OCS effect on breast cancer cell migration.** By inhibiting glucosidases, CO-OCS promotes Stim1 and  $\beta$ 1 integrin degradation through ERAD, which decrease breast cancer cell migration.

## ***Complementary Results***

### ***CO-OCS affected cell migration in other cancer cell types through Stim1***

- **Effect of CO-OCS on melanoma MDA-MB-435s invasive cancer cell line migration**

The origin of MDA-MB-435s cells has been largely discussed, and it appears now that they come from melanoma (Prasad and Gopalan 2015). We investigate the anti-migratory effect of CO-OCS on these cells which possess a high invasive potential, by performing transwell migration assay to assess the migration capacity after applying treatment with CO-OCS for 48 h at 40  $\mu$ M. In parallel, we performed MTT test to investigate the effect of CO-OCS on cell proliferation. CO-OCS reduced significantly cells migration by  $40 \pm 11\%$ . No effect on MDA-MB-435S cells proliferation was measured. Also, by using western blot, we checked the effect of CO-OCS on the expression of Stim1 and  $\beta$ 1-integrin. CO-OCS reduced the expressions of both Stim1 and  $\beta$ 1-integrin by 31% and 29% respectively (Figure 44).

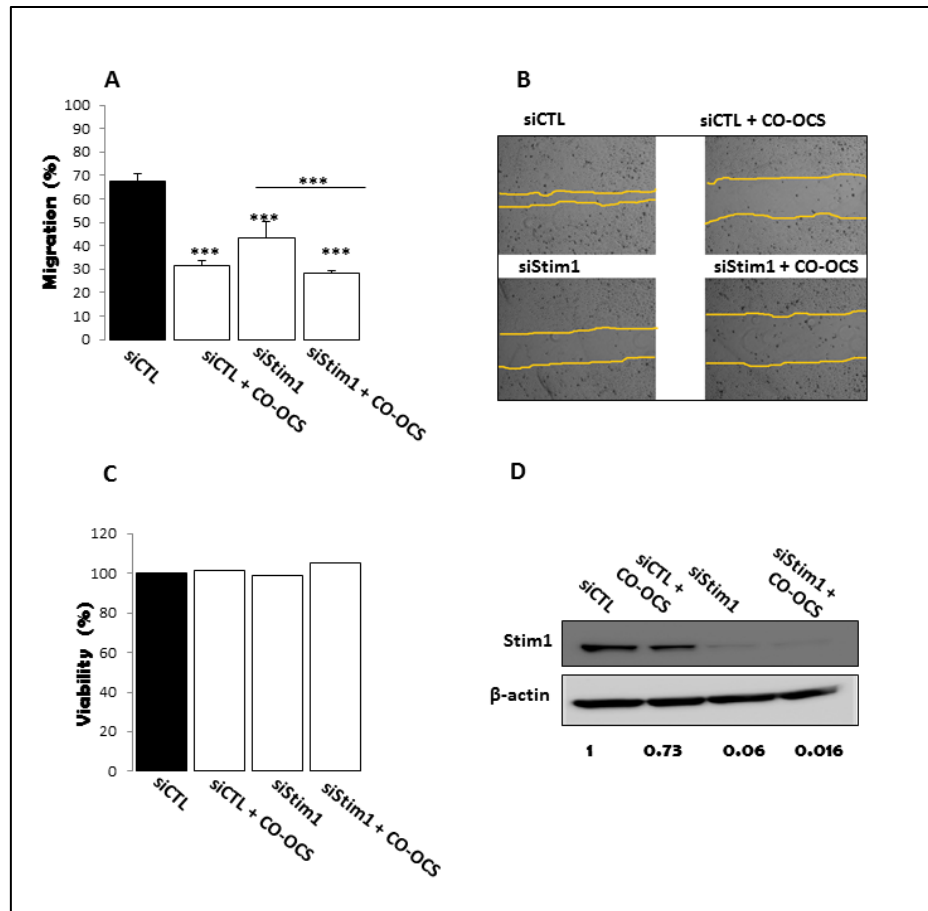


**Figure 44. CO-OCS reduced the migration in the highly invasive melanoma cell line MDA-MB-435s.** Cells were treated with 40  $\mu$ M CO-OCS for 48 h. Effect of CO-OCS on MDA-MB-435s cells migration (A) and viability (B). Effect of CO-OCS on the expression of Stim1 (C) and  $\beta$ 1-integrin (D) in MDA-MB-435S. Results are representative of three independent experiments (t-test).  $p < 0.05$  (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

- **Stim1 is implicated partially in the anti-migratory effect of CO-OCS in MDA-MB-435S cells**

In order to study the potential involvement of Stim1 in the anti-migratory effect of CO-OCS on MDA-MB-435s cells, we used a scratch wound-healing test. MDA-MB-435S cells were transfected with siStim1 or/and treated with CO-OCS. The migration was measured at 0 h and 48 h. Cells with siStim1 inhibited wound closure by  $40 \pm 9$  %. CO-OCS alone delayed wound healing after 48 h of treatment by  $69 \pm 4$  %. The inhibition of wound healing in MDA-MB-435s cells transfected with siStim1 and treated with CO-OCS was about  $72 \pm 3$  %. However, the combination of siStim1 and CO-OCS induced an additional inhibition of migration (Figure 45). It seems that CO-OCS decrease MDA-MB-435s migration partially via affecting the expression of Stim1.

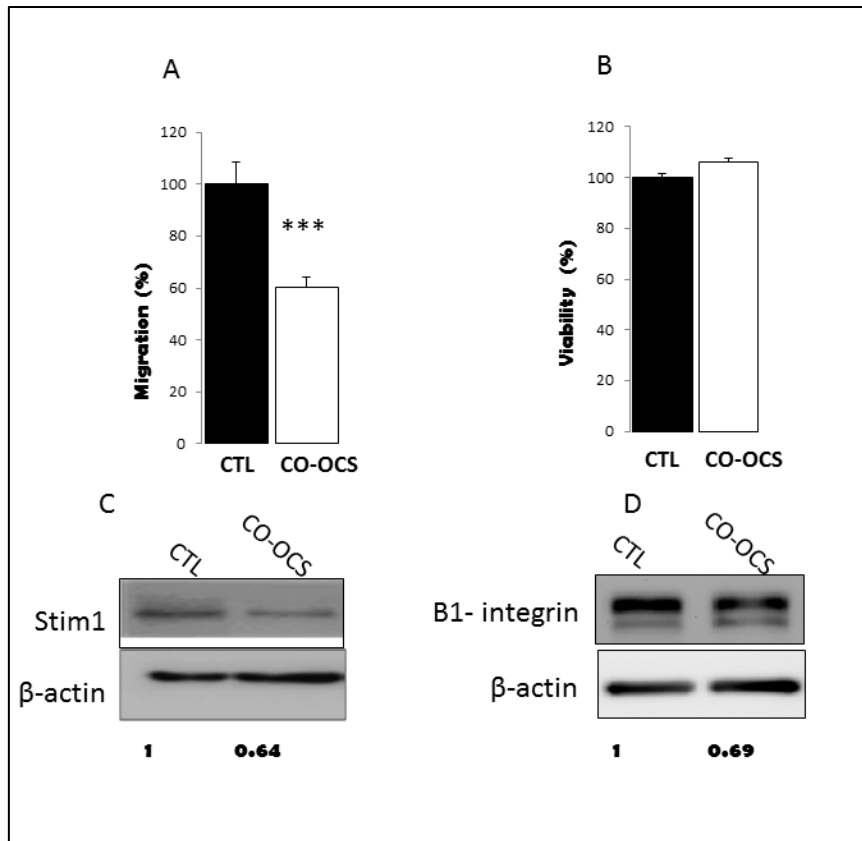




**Figure 45. CO-OCS reduced the migration of MDA-MB-435s invasive melanoma cell line via Stim1 inhibition.** Cells were weather transfected with siRNA Stim1 or/and treated with 40  $\mu$ M CO-OCS. Scratch test was done 24 h post-transfection, then, CO-OCS treatment for 48 h was applied. CO-OCS decreased MDA-MB-435s cells migration potential via Stim1 (A). Images of a scratch wound-healing test at 48 h of treatment to assess the migration capability of MDA-MB435S (B). Effect of CO-OCS on Stim1 expression at 48 h of treatment, and validation of transfection by siRNA Stim1 after 72 h in MDA-MB-435s (C). T-test,  $p < 0.05$  ( $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ ).

- **Effect of CO-OCS on T-47D invasive breast cancer cell line**

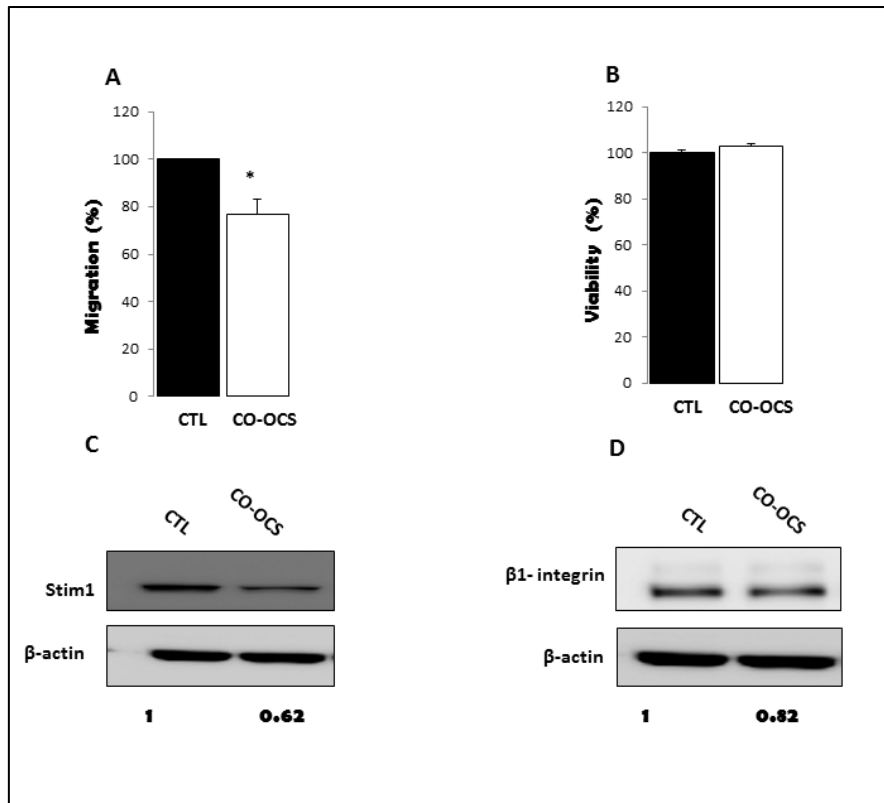
To explore the effect of CO-OCS on the migration of other breast cancer cell lines, we investigated its ability to modify cell migration in T-47D cells. Transwell chambers were used to assess the migratory potential of T-47D cells at 100  $\mu$ M of CO-OCS after 24 h of treatment, where the treatment with 40  $\mu$ M for 48 h impaired T-47D proliferation. In parallel, we performed MTT test to investigate the effect of CO-OCS on T-47D cell line proliferation. CO-OCS inhibited significantly T-47D migration by almost  $40 \pm 3\%$ ; no effect on cell proliferation was measured. Western blots were done to study the effect of CO-OCS on the expression of Stim1 and  $\beta$ 1-integrin. CO-OCS inhibited the expressions of both Stim1 and  $\beta$ 1-integrin by 63% and 31% respectively (Figure 46).



**Figure 46. CO-OCS reduced the migration in T-47D breast cancer by 40% without affecting cell viability.** Cells were treated with 100  $\mu$ M CO-OCS for 24 h. (A) Effect of CO-OCS on T-47D migration. (B) Viability measured by MTT test. (C) Effect of CO-OCS on Stim1, and (D)  $\beta$ 1-integrin expression in T-47D breast cancer cell line. T-test,  $p < 0.05$  (\* $p < 0.05$ )

- **Effect of CO-OCS on the pancreatic cancer cell line MIA PaCa-2 cells**

We also investigate the effect of CO-OCS on the migration of the invasive pancreatic cancer cell line MIA PaCa-2. The migration potential was assessed by using transwell migration assay, after 48 h of treatment with CO-OCS at 40  $\mu$ M. We checked for any potential proliferative effect induced by the treatment by using MTT on MIA PaCa-2 cells. In pancreatic cancer cells, CO-OCS inhibited significantly cell migration by  $24 \pm 4$  %. No effect on MIA PaCa-2 cells proliferation was measured. Western blot was done to study the expressions of Stim1 and  $\beta$ 1-integrin after applying CO-OCS treatment. CO-OCS reduced the expressions of Stim1 by 38% and  $\beta$ 1-integrin by 18% (Figure 47).



**Figure 47. CO-OCS reduced MIA PaCa- cell line migration.** Cells were treated with 40  $\mu$ M CO-OCS for 48 h. Effect of CO-OCS on MIA PaCa-2 cells migration (A) and viability was measured by MTT (B). Effect of CO-OCS on the expression of Stim1 (C) and  $\beta$ 1-integrin (D) in MIA PaCa-2 cells. T-test,  $p < 0.05$  (\* $p < 0.05$ )

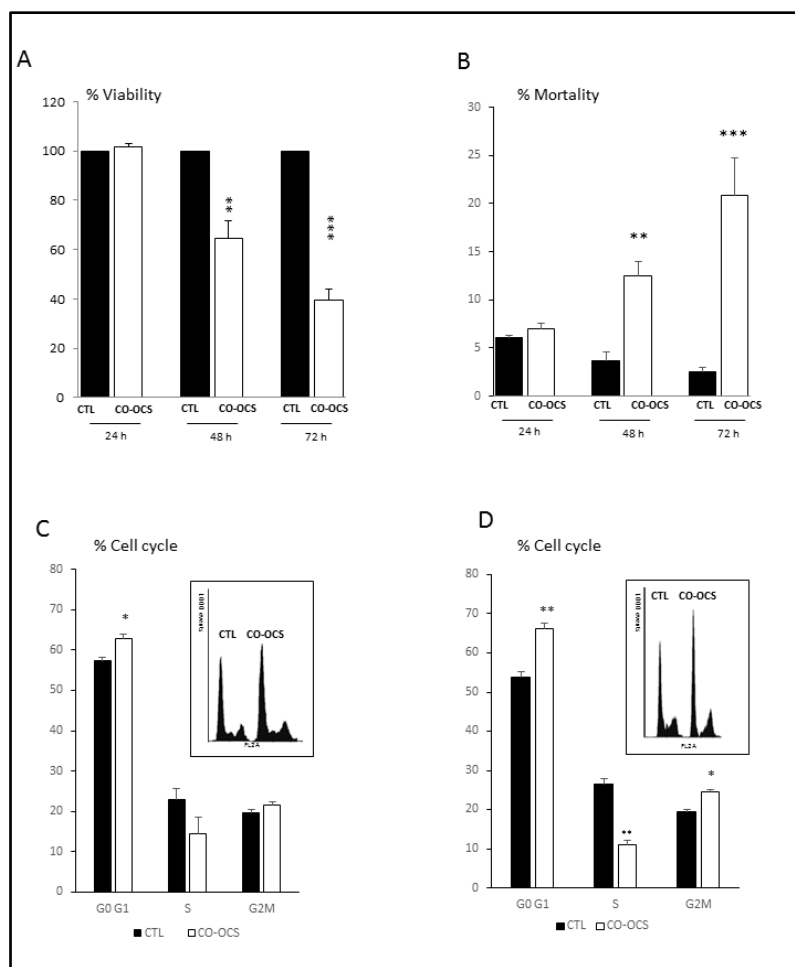
We can conclude from our performed studies on the different cancer types that CO-OCS affected cancer cell migration through Stim1 and  $\beta$ 1-integrin. However, the effect of CO-OCS on pancreatic cancer cells migration capacity appears lower when compared to that seen in melanoma and breast cancer.

**Part II: CO-OCS induced UPR and decreased Stim1, Orai1 and GRP78 expressions leading to antiproliferative, apoptotic and necrotic effects**

**A. CO-OCS induced post-translational modification on Stim1, Orai1 and GRP78 expression through its ERAD activity**

- **CO-OCS induced its antitumor effects in a time-dependent manner**

Previous work from our laboratory has demonstrated that CO-OCS induced mortality and antiproliferative effect in a dose-dependent manner with an  $IC_{50}$  of 40  $\mu$ M and 26  $\mu$ M respectively (Allan, Ouadid-Ahidouch et al. 2013). Here, we investigated the effect of CO-OCS at 40  $\mu$ M but in a time-dependent manner (24, 48 and 72 h). By using Trypan Blue method, we found that CO-OCS inhibited cell viability by  $39.4 \pm 7.6$  % and  $64.7 \pm 11.8$  % at 48 h and 72 h respectively after normalization to CTL (Figure 48.A). While it induced mortality almost by  $12 \pm 1.51$  % and  $20.8 \pm 3.84$  % at 48 and 72 h respectively (CTL for 48 h  $3.64 \pm 0.69$  % and for 72 h  $2.54 \pm 0.36$  %) (Figure 48.B). Cell cycle study was carried out by flow cytometry; CO-OCS increased statically the percentage of arrested cell in G1 phase ( $62.8 \pm 1.04$  % for CO-OCS and  $56 \pm 0.8$ % for CTL) at 48 h without important effect in S and G2M phase (Figure 48.C and Table 12). At 72 h, we measured more synchronization at G1 ( $66.27 \pm 1.28$ % for CO-OCS and  $53.87 \pm 1.2$ % for CTL), a statically significant increase in G2M ( $24.64 \pm 0.46$ % for CO-OCS and  $19.55 \pm 0.59$ % for CTL) and a decrease in the S phase (Figure 48.D and Table 12).



**Figure 48. CO-OCS reduced cell viability and induced mortality in breast cancer cells in a time-dependent manner.** MCF-7 Cells were treated with 40  $\mu$ M of CO-OCS for 24, 48 and 72 h. Trypan Blue exclusion assay, CO-OCS inhibited cell viability at 48 and 72 h (A). The effect of CO-OCS on the cell mortality at 24, 48 and 72 h estimated by trypan blue exclusion assay (B). Results of cell mortality and viability are representative of SD of at least three independent experiments. For the cell cycle study, CO-OCS arrested MCF-7 cells in the G1 phase at 48 h (C); while at 72 h, there was an elevation in G1 as well as G2/M phases (D). Results are representative of S.E.M of three independent experiments. T-test was done for Data analysis, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

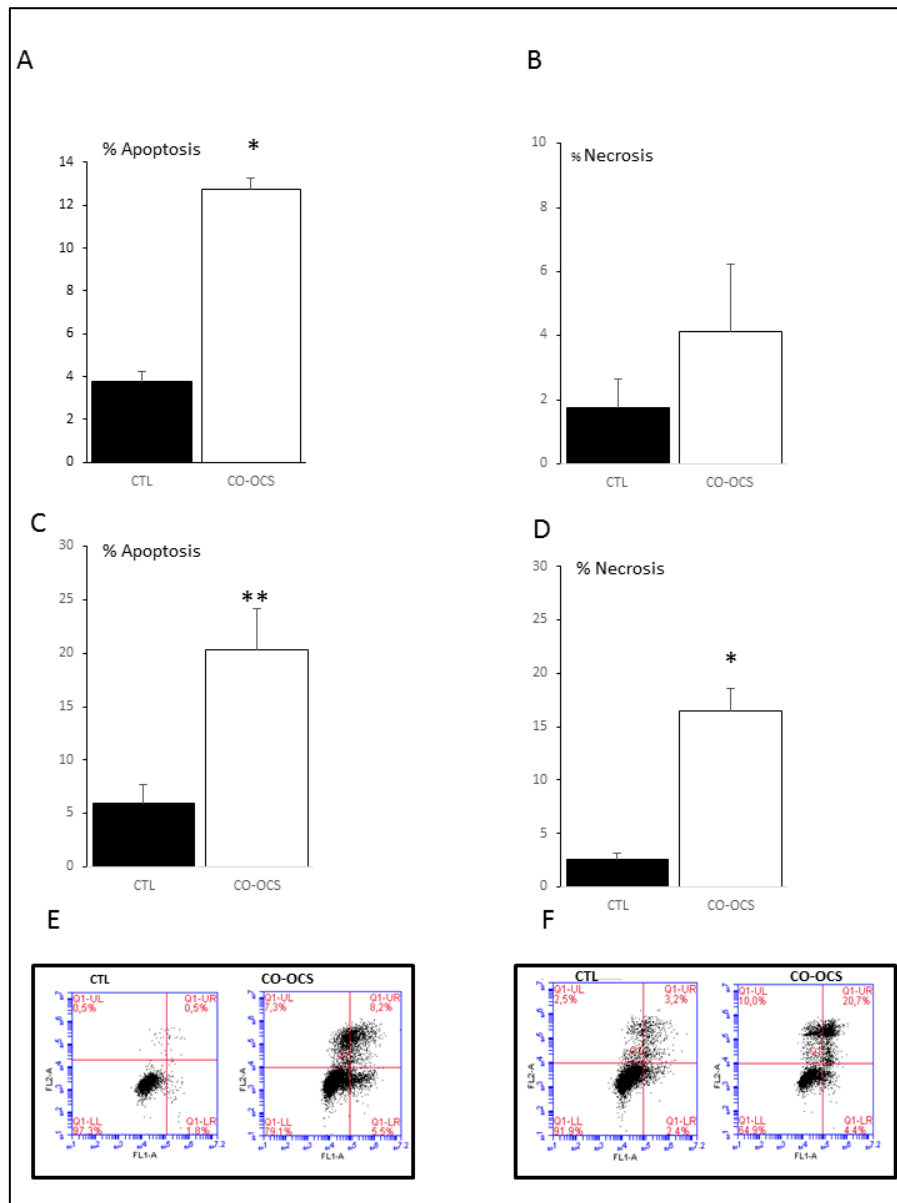
Condition	% G0/G1	% S	% G2M
CTL 48h	57.8 $\pm$ 0.8	22.9 $\pm$ 2.63	19.7 $\pm$ 0.7
CO-OCS 48h	62.8 $\pm$ 1.04	14.5 $\pm$ 4.08	21.5 $\pm$ 0.88
CTL 72h	53.87 $\pm$ 1.2	26.36 $\pm$ 1.43	19.55 $\pm$ 0.59
CO-OCS 72h	66.27 $\pm$ 1.28	11.03 $\pm$ 1.05	24.64 $\pm$ 0.46

**Table 12.** Percentage of MCF-7 cells treated with CO-OCS, at 40  $\mu$ M for 48 and 72 h, in G0/G1, S, and G2/M phases of the cell cycle. (T-test), \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **CO-OCS promoted apoptosis and necrosis at different times of treatment**

In order to determine the time by which CO-OCS induced apoptotic and necrotic cell death in MCF-7 cells, we used annexin V FITC in parallel with Propidium Iodide stain. We examined the effect of applying 40  $\mu$ M of CO-OCS on MCF-7 cells at 48 and 72 h. CO-OCS induced apoptosis at 48 h by  $12.7 \pm 0.56\%$  ( $3.7 \pm 0.49\%$  for CTL) (Figure 49.A), without increasing the necrotic cell death ( $4.1 \pm 1.3\%$  for CO-OCS and  $1.7 \pm 0.86\%$  for CTL) (Figure 49.B). Moreover, the apoptosis induced by CO-OCS was increased from  $5.9 \pm 1.6\%$  to  $20.3 \pm 3.8\%$  at 72 h (Figure 49.C) and accompanied with statically significant elevation in the necrotic cell death from  $2.5 \pm 0.65\%$  to  $16.6 \pm 2.08\%$  (Figure 49.D).

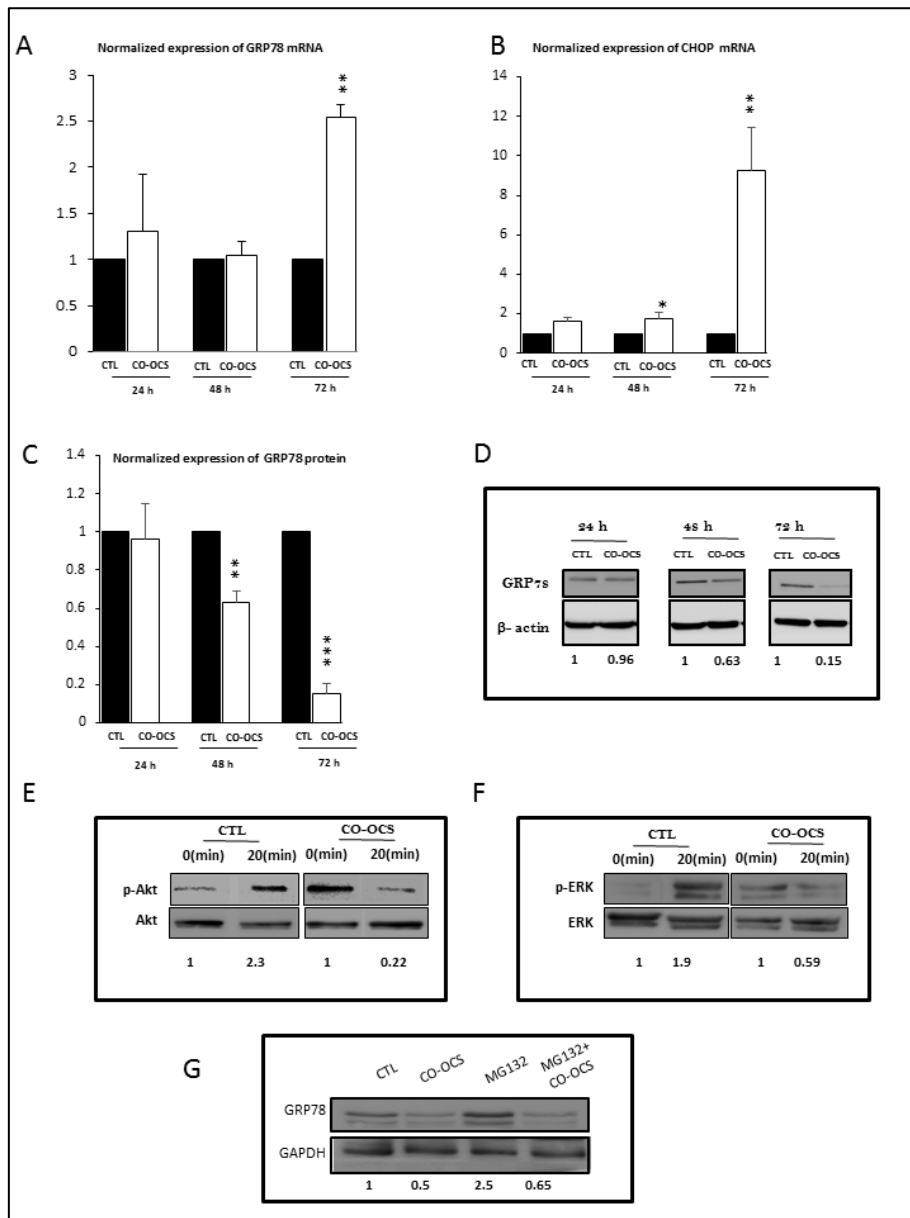




**Figure 48. CO-OCS induced apoptosis and necrosis in MCF-7 cells.** Cells were treated with CO-OCS for 48 and 72h, and the apoptosis assay was carried out by Annexin FITC and Propidium Iodide staining. Effect of CO-OCS on apoptosis (A) and necrosis (B) at 40  $\mu$ M after 48 h of incubation in MCF-7 cells. Apoptosis (C) and necrosis (D) at 40  $\mu$ M of CO-OCS after 72 h of treatment in MCF-7 cells. Representative traces of Annexin V/PI staining assay with and without CO-OCS at 48 h (E) and 72 h (F). All experiments were performed at least three times, the values indicated are mean  $\pm$  SD; (t-test) \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **ER stress and ERAD were activated in CO-OCS-treated cells**

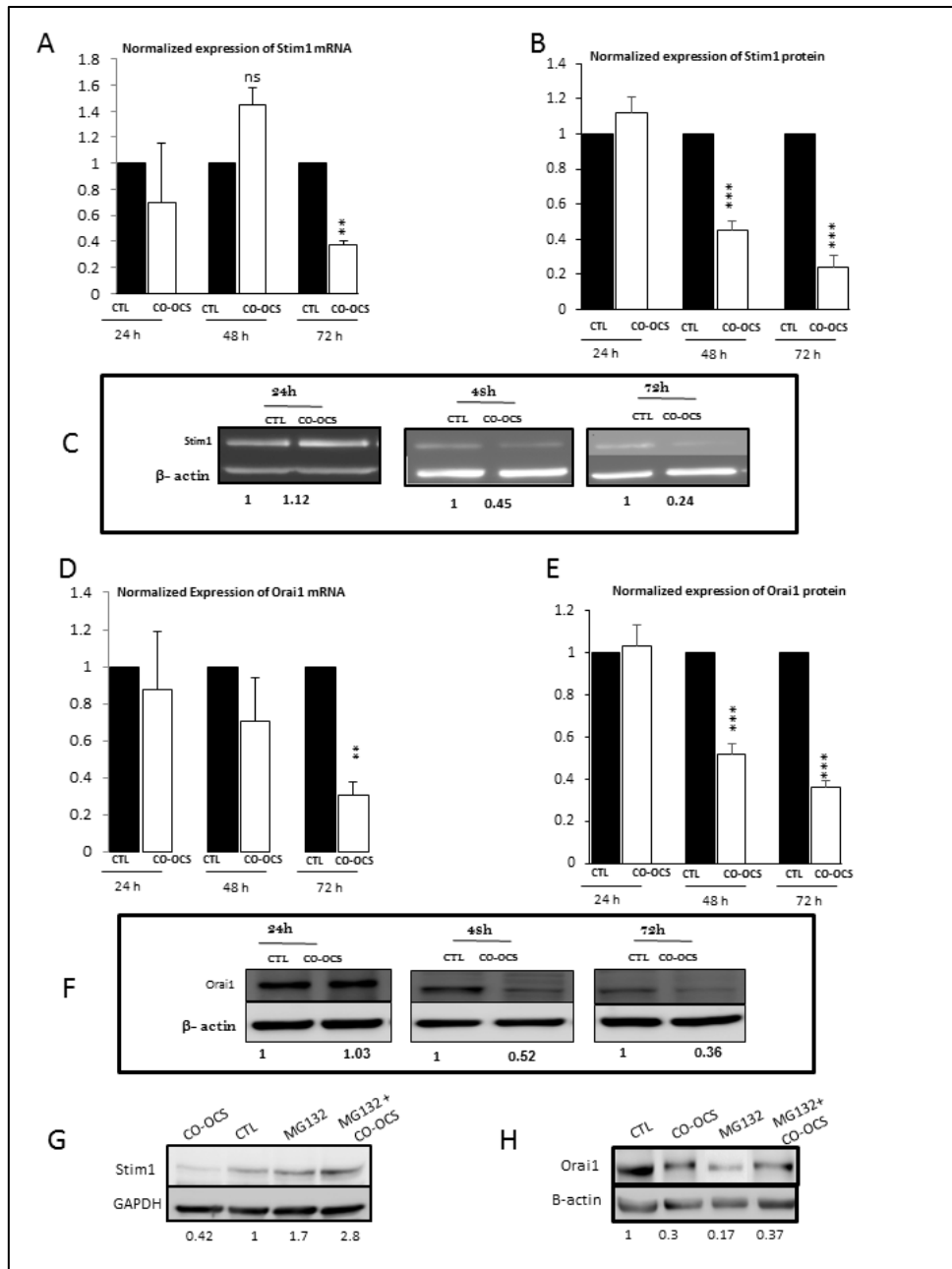
Since CO-OCS is an inhibitor of glycosylation, leads to accumulation of misfolded proteins in the ER, we explored its effect on the ER stress and ERAD. CO-OCS increased GRP78 mRNA by  $2.53 \pm 0.23$  fold at 40  $\mu$ M after 72 h of treatment (Figure 40.A). The same treatment had elevated CHOP transcript by 1.7 fold at 48 h and  $9.2 \pm 2.21$ -fold at 72 h (Figure 40.B). However, CO-OCS seems to have no effect on the expression of GRP78 at the protein level at 24 h; while GRP78 expression was decreased after 48 and 72 h of applying the treatment to  $0.63 \pm 0.15$  and  $0.15 \pm 0.05$ -fold respectively when normalized to CTL (Figure 40.C). ERAD is associated with the inhibition of Akt and ERK1/2 phosphorylation (Qin, Wang et al. 2010; Galluzzi, De Santi et al. 2012; Mulay, Wood et al. 2013). We demonstrated that CO-OCS at 72 h of treatment decreased the activation of Akt after stimulation for 20 min with medium supplemented with 10% FBS to 0.22 fold (Figure 49.E). CO-OCS also reduced the phosphorylation of ERK1/2 to 0.59 (Figure 49.F). As the effect of CO-OCS on the expression of GRP78 protein levels did not correlate with GRP78 mRNA levels, we investigated whether CO-OCS-induced GRP78 protein degradation via the proteasome. Indeed, the proteasome 26S is the principal pathway for protein degradation (Inobe and Matouschek 2014). For that reason, we treated MCF-7 cells with MG-132, an inhibitor against the proteasome 26S. Our preliminary results show that CO-OCS-induced reduction in GRP78 expression was not restored in MG-132 treated condition (Figure 49.G).



**Figure 49. CO-OCS increased the mRNA of GRP78 and CHOP and decreased the expression of GRP78 at the protein level in MCF-7 cells.** (A) Normalized Expression of GRP78 mRNA after incubation with 40  $\mu$ M of CO-OCS for 24, 48 and 72 h. (B) Expression of CHOP mRNA was normalized to CTL after applying 40  $\mu$ M CO-OCS for 24, 48 and 72 h. The effect of CO-OCS on the expression of GRP78 protein at the same concentration and time (C) and the representative Western blot (D). Representative Western blots of (E) pAkt and Akt and (F) pERK1/2 and ERK1/2 ratio with and without treatment by CO-OCS for 72 h, then stimulation or not with medium supplemented with 10% of FBS at 0 and 20 min. (G) Western blot quantification after applying CO-OCS with and without MG132; MCF-7 cells were treated with 5  $\mu$ M MG-132 for 24 h or not treated; in parallel, treatment with 40  $\mu$ M of CO-OCS for 48 h was applied. Data analysis are the mean  $\pm$  SD from three independent experiments; (t-test) \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **MCF-7 cells treatment with CO-OCS exhibited post-translational modifications and affected Stim1 and Orai1 expressions at the protein levels**

In order to investigate the effect of CO-OCS on the expression of two N-glycosylated proteins (Stim1 and Orai1) that known to have an important role in breast cancer development (Yang, Zhang et al. 2009; McAndrew, Grice et al. 2011), we explored the effect of 40  $\mu$ M of CO-OCS at 24, 48 and 72 h on the mRNA in parallel with the protein expression of Stim1 and Orai1. Our result demonstrated that Stim1 transcript was only decreased at 72 h of treatment by 0.63-fold (Figure 50.A), while the decrease of the protein expression level was measured earlier at 48 h by 0.55-fold (Figure 50.B). In fact, CO-OCS reduced much more the expression of Stim1 protein at 72 h by 0.8-fold in a time-dependent manner (Figure 50.B and C). Moreover, the effect of CO-OCS on Orai1 transcript and protein expression was almost similar to its effect on Stim1. A significant decrease of Orai1 transcript was only measured at 72 h by 0.7-fold (Figure 50.D). As shown in Figure 50.E, CO-OCS-induced a significant decrease in Orai1 protein expression at 48 h and 72 h by 0.48 and 0.64-fold respectively (Figure 50.E and F). Here also, we investigated the potential involvement of proteasome in Stim1 and Orai1 protein degradation. MCF-7 cells were treated with MG-132 alone or combined with CO-OCS. We found that Stim1 protein expression level was not affected in MCF-7 cells treated with both MG-231 and CO-OCS (Figure 50.G). MG-132 was not able to restore the expression of Orai1 protein as provided in the quantitative western blot (Figure 50.H).

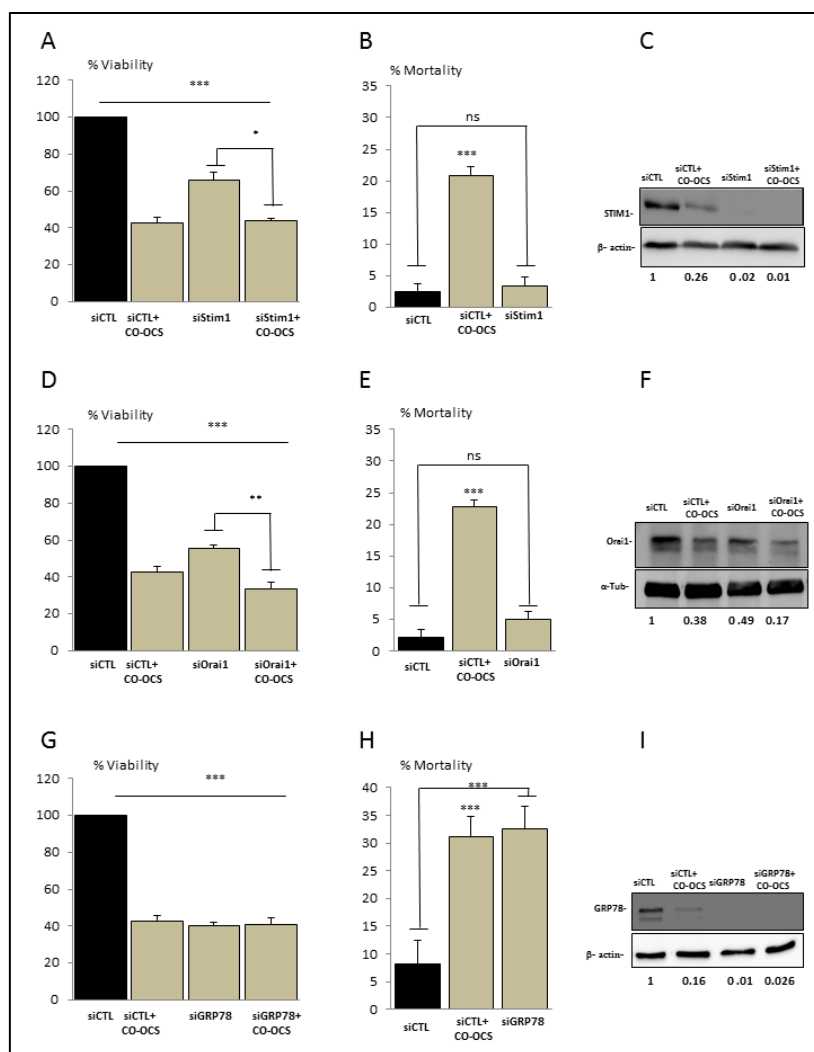


**Figure 50. CO-OCS induced the degradation of Stim1 and Orai1 proteins by the proteasome.** MCF-7 cell line was treated with 40  $\mu$ M of CO-OCS for 24, 48 and 72 h. (A) Normalized Expression of Stim1 transcript after incubation for 24, 48 and 72 h. (B) Expressions of Stim1 protein at 24, 48 and 72 h were normalized to their Controls. (C) Western blot quantification demonstrates the different effects of CO-OCS at 40  $\mu$ M on the expression of Stim1 protein. The same treatment concentration and duration were applied for Orai1 mRNA and protein studies. Normalized Expression of Orai1 mRNA (D) and Orai1 protein (E). The quantification of Orai1 protein expression by western blots at different times (F). Representative Western blots demonstrate the effect of incubating MCF-7 cells with or without 5  $\mu$ M of MG-132 for 24 h with 40  $\mu$ M of CO-OCS for 48 h on Stim1 (G) and Orai1 expression (H). Data analysis for qPCR and WB are the mean + SD from three independent experiments; (t-test) \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

**B. Stim1 and Orai 1 are involved in the anti-proliferative effect of CO-OCS, While GRP78 is more involved its apoptotic and necrotic effect.**

- **CO-OCS regulated cell viability via Stim1, Orai1 and GR78 in MCF-7 cells**

To explore whether Stim1, Orai1 and GRP78 constitute antiproliferative targets of CO-OCS in breast cancer, MCF-7 cells were transfected with siCTL, siStim1, siOrai1 and siGRP78, and treated or untreated with CO-OCS during 72h. As shown in Figure 51.A, siCTL+CO-OCS reduced cell viability by almost 60 %. About 34% of the cell viability inhibition was measured in siStim1 cells, which is the maximal effect of Stim1 on cell viability as a result of the complete reduction of Stim1 protein expression (Figure 51.A and C). However, applying CO-OCS treatment on siStim1 transfected cells induced a cumulative inhibition of cell viability by 23% when compared to siStim1 alone (Figure 51.A and Table 13). In the other hand, Orai1 reduction by siOrai1 exhibited a decrease in cell percentage by almost 45% and in Orai1 protein expression by 0.49-fold when normalized to siCTL (Figure 51.D and F). In fact, the combination of siRNA Orai1 and CO-OCS induced an additional inhibition by almost 22% in cell number as well as in Orai1 protein expression by 0.32-fold when compared to cells transfected with siOrai1 only (Figure 51.D and F and Table 13). In addition, transfecting siGRP78 also affected cell viability. We observed a reduction in cell viability by 60% in siGRP78 transfected cells (Figure 51.G); a complete inhibition of GRP78 protein expression was achieved as shown in the representative western blot (Figure 51.I). In contrast, no additive inhibition was measured in the cells transfected with siGRP78 and treated with CO-OCS (Figure 51G and Table 13). In opposition to siGRP78 which increased the mortality rate, siStim1 and siOrai1 failed to affect cell mortality (Figure 51.B, E and H). Taken together, these results clearly show that GRP78 is involved in both anti-proliferative and mortality induced by CO-OCS, while Stim1 and Orai1 seem to be only involved in the anti-proliferative CO-OCS effect.



**Figure 51. Stim1, Orail and GRP78 regulated CO-OCS-induced anti-proliferative effect.** MCF-7 cells were transfected with siCTL or one of the three siRNA siStim1, siOrail or siGRP78; 24 h post-transfection, cells were treated with 40  $\mu$ M of CO-OCS during 72 h. Trypan Blue exclusion assay was done to estimate cell viability and mortality. Effect of siStim1 alone or combined with CO-OCS on cell viability normalized to siCTL (A) and mortality (B). Normalized western blot quantification represents the efficiency of transfection (C). Effect of siOrail alone or combined with CO-OCS on cell viability normalized to siCTL (D) and mortality (E). Normalized western blot quantification represents the efficiency of transfection (F). Effect of GRP78 alone or combined with CO-OCS on cell viability normalized to siCTL (A) and mortality (G). Normalized western blot quantification represents the efficiency of transfection (H). The values indicated are mean  $\pm$  S.E.M of three independent experiments and analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

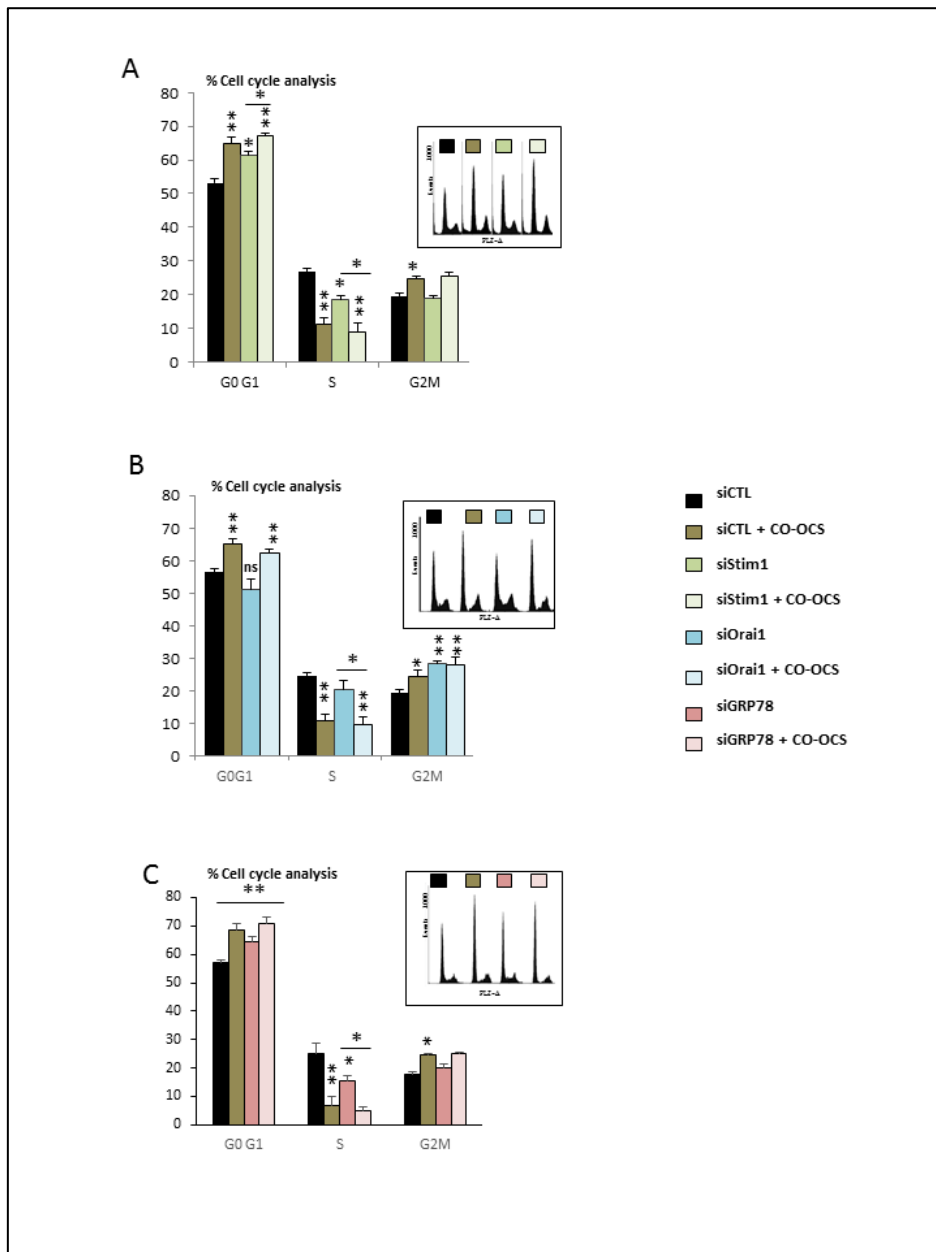
Condition	siCTL	siCTL+ CO-OCS	siStim1	siStim1+ CO-OCS	siOrail	siOrail+ CO-OCS	siGRP78	siGRP78+ CO-OCS
% Viability	100	39.42 $\pm$ 3.3	66.03 $\pm$ 4.2	43.96 $\pm$ 1.5	55.57 $\pm$ 1.98	33.62 $\pm$ 3.32	40 $\pm$ 81.6	41 $\pm$ 6.011

**Table 14.** Effect of CO-OCS at 40  $\mu$ M for 72 h with and without transfection on the viability of MCF-7. ANOVA test, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **CO-OCS arrested MCF-7 cells in G1 and G2M via targeting Stim1, Orai1 and GRP78**

CO-OCS dramatically arrested MCF-7 cells in G1 and G2M phases at 40  $\mu$ M for 72 h (Allan, Ouadid-Ahidouch et al. 2013). Our results confirmed that results (Figure 48.D). To examine the specific contribution of Stim1, Orai1 and GRP78 at the different phases of the cell cycle to the CO-OCS induced synchronization at G1 and G2M, we selectively suppressed the expression of Stim1, Orai1 and GRP78 by siRNA. Indeed, silencing of Stim1 induced G1 phase synchronization; by adding CO-OCS, we observed an additive arrest in G1 phase in the siStim1 transfected cells (Figure 52.A and Table 14). In the other hand, knockdown of Orai1 significantly enhanced MCF-7 cells synchronization in G2M phase. There no supplementary effect was measured after applying CO-OCS treatment on siOrai1 transfected cells (Figure 52 and Table 14). To confirm the involvement of GRP78 in the antiproliferative effect of CO-OCS, we suppressed its expression in MCF-7 cells. When combined, siGRP78 and CO-OCS induced the same effects as siGRP78 alone (Figure 52.C and Table 14). Collectively, these results suggested that CO-OCS induced cell synchronization in G1 via Stim1 and GRP78 while Orai1 implicated in CO-OCS-induced G2M phase.





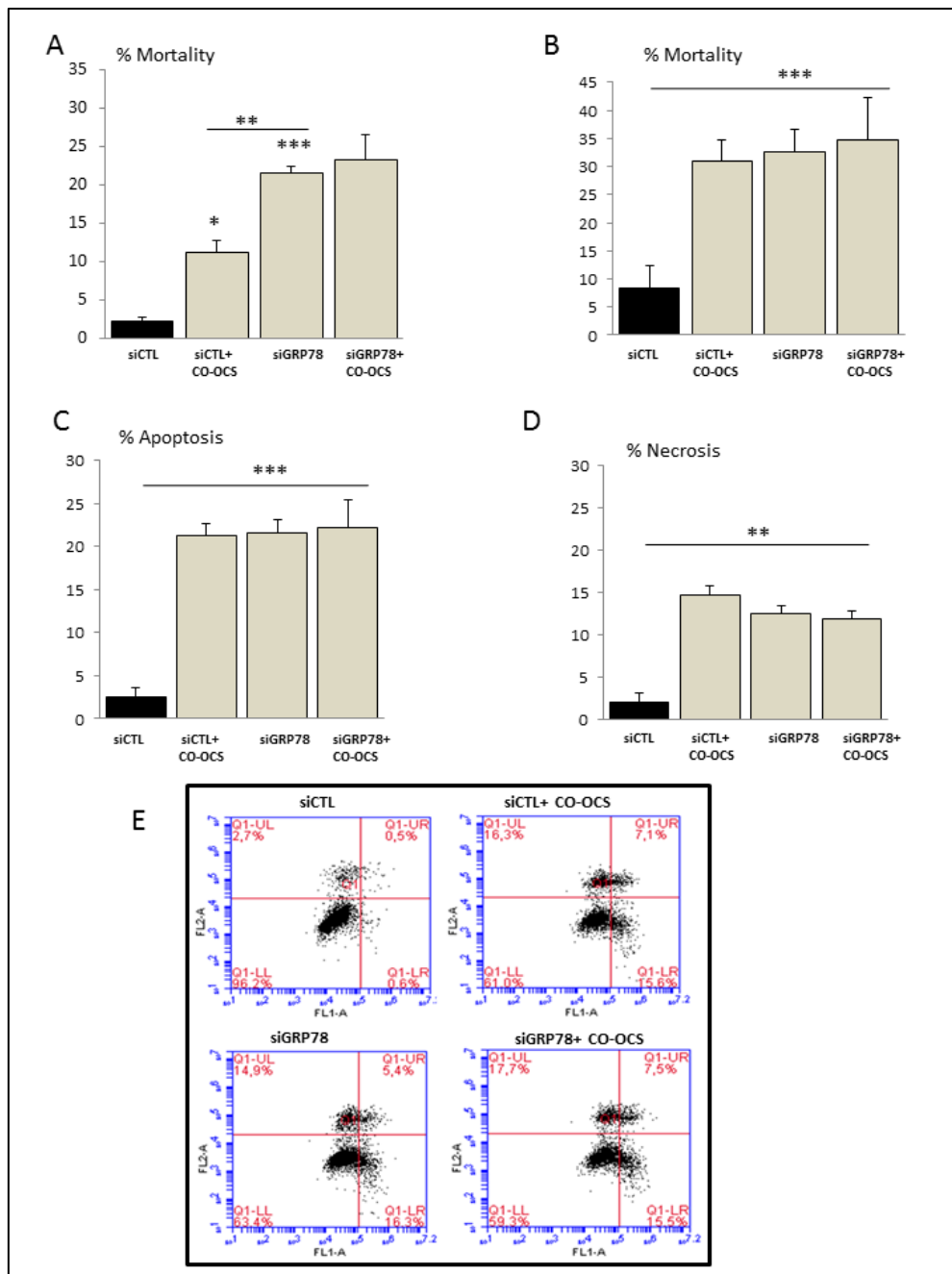
**Figure 52. Stim1 and GRP78 are implicated in CO-OCS -G1 -arrested cells while Orai1 is involved in G2M.** The same transfection protocol and treatment mentioned in Figure 51 was applied for the flow cytometry cell cycle study. The results represent the percentage of cell synchronization at the different phases G0/G1, S and G2M. (A) Effect of siStim1 combined or not with CO-OCS (72 h) at the different phases. (B) Cell cycle analysis of Orai1 silencing with and without CO-OCS incubation for 72 h on G0/G1, S and G2M phases. (C) Effect of GRP78 knockdown alone and when combined or not with CO-OCS for 72 h on the three phases of cell cycle. Data are mean  $\pm$  S.E.M of three independent experiments and analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

	%G0G1	%S	%G2M
siCTL	53.25±1.65	26.86±2.02	19.63±0.84
siCTL+ CO-OCS	64.82±1.36	11.157±1.48	24.72±0.67
siStim1	61.35±1.09	18.71±1.93	18.91±2.83
siStim1+ CO-OCS	67.05±0.74	8.77±2.09	25.44±0.81
siCTL	56.39±2.34	24.38±2.86	19.23±1.18
siCTL+ CO-OCS	65.09±1.47	10.56±2.16	24.34±1.95
siOrai1	50.89±3.55	20.42±2.73	28.24±0.90
siOrai1+ CO-OCS	62.33±1.22	9.72±2.20	27.94±2.51
siCTL	56.98±0.98	25.15±3.64	17.86±0.65
siCTL+ CO-OCS	68.41±2.36	6.96±3.22	24.61±0.64
siGRP78	64.57±1.81	15.3±1.88	20.3±0.94
siGRP78+ CO-OCS	70.82±2.54	5.16±1.07	25±0.68

**Table 14.** Effect of CO-OCS at 40  $\mu$ M for 72 h with and without transfection on the different phases of cell cycle in MCF-7. Data are mean  $\pm$  S.E.M of three independent experiments. ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **GRP78 induced CO-OCS-apoptotic and necrotic effects but not Stim1 or Orai1.**

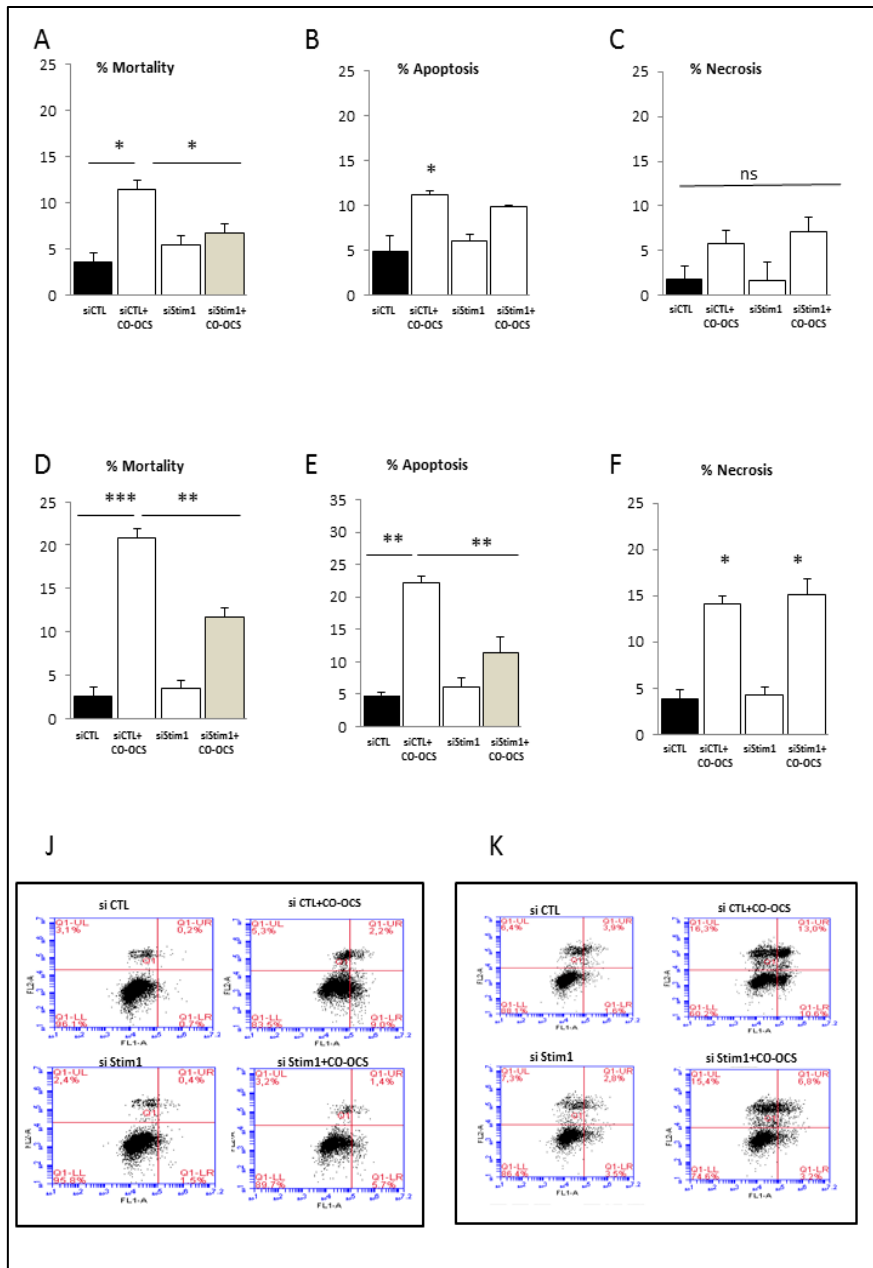
To examine whether GRP78 downregulation by CO-OCS is critical to trigger cell death, we checked the effect of siGRP78 on MCF-7 cells with and without CO-OCS. First, cell mortality was assessed by Trypan Blue dye exclusion test; siGRP78 induced  $21.59 \pm 0.89\%$  and  $32.48 \pm 4.14\%$  of mortality at 72 and 96 h post-transfection respectively ( $2.15 \pm 0.64\%$  and  $8.24 \pm 4.12\%$  for siCTL respectively at 72 h and 96 h) (Figure 53.A and B). In fact, GRP78 knockdown after 72 h induced much more mortality when compared to CO-OCS after 48 h of treatment ( $11.1 \pm 0.66\%$  for siCTL $\pm$  CO-OCS and  $21.59 \pm 1.61\%$  for siCTL  $\pm$  siGRP78) (Figure 53.A). In contrast, the combination of siGRP78 and CO-OCS treatment after 72 h of incubation failed to induce an additional effect on cell mortality ( $31.03 \pm 3.77\%$  for siCTL+ CO-OCS and  $34.71 \pm 7.47\%$  for siCT  $\pm$  siGRP78) (Figure 53.B). Second, we proceed to flow cytometry study for more detailed comprehension. As expected, siGRP78 induced apoptosis and necrosis at 96 h post-transfection up to  $21.62 \pm 1.47\%$  and  $12.36 \pm 2.37\%$  respectively ( $21.3 \pm 1.35\%$  of apoptosis and  $14.6 \pm 2.18\%$  of necrosis for siCTL $\pm$  CO-OCS). However, both apoptotic and necrotic cell death were not affected when siGRP78-transfected cells were treated with CO-OCS (Figure 53C and D).



**Figure 53. CO-OCS induced apoptotic and necrotic cell death via GRP78 regulation.** MCF-7 cell line was transfected with siCTL or siGRP78; 24 h post-transfection, cells were treated or not with 40  $\mu$ M of CO-OCS either for 48 or 72 h. Mortality was assessed by Trypan Blue exclusion assay at 72 h post-transfection and 48 h of treatment (A) and at 96 h siGRP78 and 72 h of CO-OCS incubation (A). Annexin V/PI staining assay demonstrates the percentage of apoptotic (C) and (D) necrotic cell death at 96 h post-transfection and 72 h of treatment. Representative traces of Annexin V/PI staining assay, siGRP78 at 96 h and 72 h of CO-OCS incubation. Results are representative of S.E.M of three independent experiments, analyses by ANOVA test \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

- **Downregulation of Stim1 confers apoptosis resistance to CO-OCS- treated MCF-7 cells**

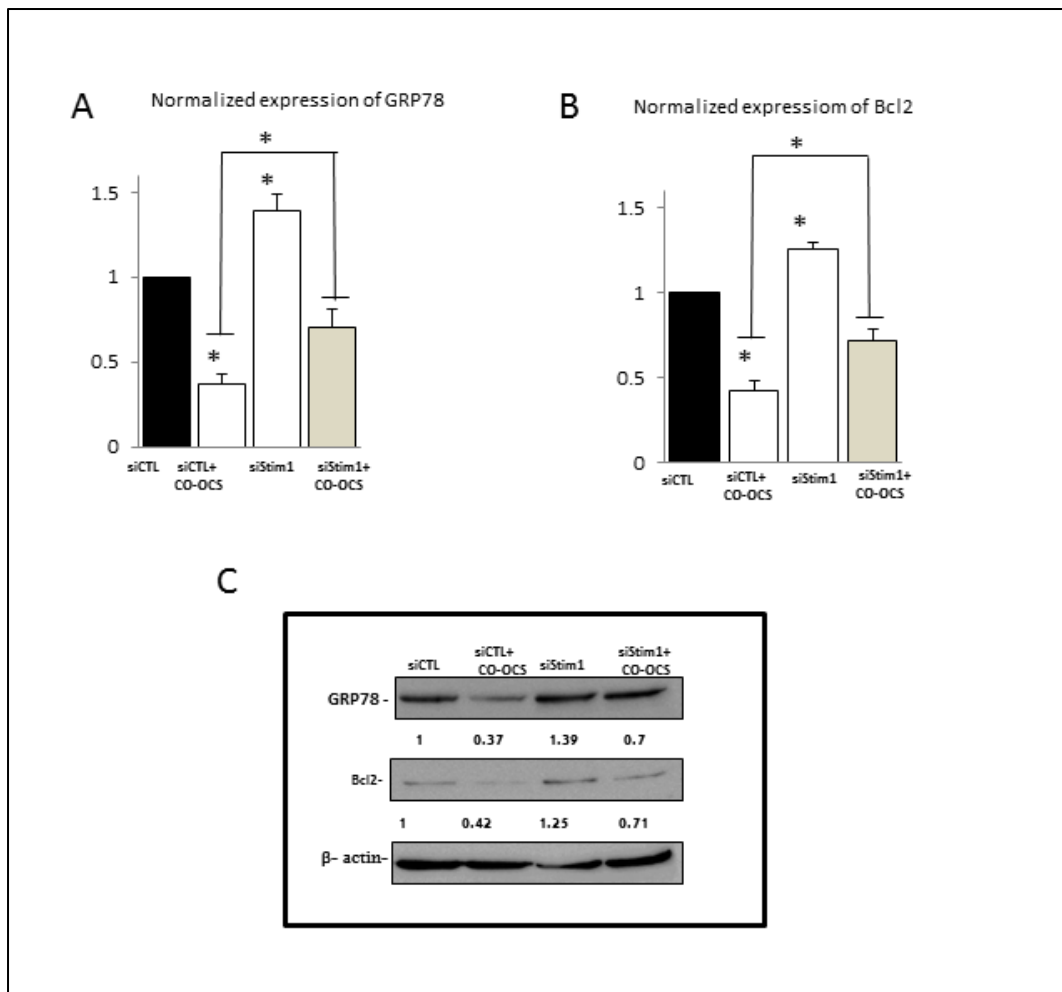
In parallel to the viability study, by using the Trypan Blue exclusion assay, we measured the percentage of mortality by direct cell counts. The decrease in Stim1 expression by siRNA exhibited no effect on cell mortality neither at 72 h nor 96 h post-transfection. Applying CO-OCS on the cells transfected with siStim1 reduced the cell mortality induced by CO-OCS at 48 h of treatment from to  $6.7 \pm 1.4\%$  ( $11.4 \pm 1.36\%$  for siCTL+ CO-OCS) (Figure 54.A). This inhibitory effect was much clearer at 72 h of treatment where the percentage of mortality was decreased from  $20.8 \pm 1.39\%$  for siCTL+ CO-OCS to  $11.68 \pm 1.4\%$  siStim1+ CO-OCS (Figure 54.B). Next, we confirmed this result by flow cytometry. At 48 h of treatment, siRNA Stim1 seems to decrease slightly CO-OCS induced apoptosis. However, Knockdown of Stim1 statically decreased the apoptosis percentage at 72 h of treatment when compared to the cells treated with CO-OCS alone ( $22.1 \pm 2.1\%$  for siCTL+ CO-OCS and  $11.3 \pm 4.1\%$  for si-STIM +CO-OCS). No necrotic effect was observed neither in siStim1 alone nor when combined with CO-OCS. In conclusion, Stim1 is a regulator of CO-OCS-induced apoptosis in MCF-7 cells.



**Figure 54. Downregulation of Stim1 expression suppressed to CO-OCS apoptotic effect with affecting its necrosis effect.** The experiments were carried out at two different durations with the same concentration of treatment. The expression of Stim1 was inhibited by transfecting siStim1; cells were kept without treatment for 24 h then treated or not with 40  $\mu$ M of CO-OCS either for 48 or 72 h; siCTL was used for all the experiments. At 72 h post-transfection and 48 h of CO-OCS treatment; effects of CO-OCS on cell mortality (A) apoptosis (B) and necrosis (C). Percentage of mortality (D) apoptotic (E) and (F) necrotic cell death at 96 h post-transfection with si Stim1 and 72 h of treatment with CO-OCS. Data are the average from three independent experiments  $\pm$  SD, analysed by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **Stim1 regulated CO-OCS-induced apoptosis via GRP78 and Bcl2**

Mitochondria have a key role in activating or protecting cells from apoptosis via Bcl2 family; the disturbance in ER homeostasis due to ER stress is also able to induce apoptotic cell death. GRP78 and Bcl-2 are involved in the control of apoptotic pathways. Moreover, knockdown of Stim1 increased GRP78 expression (Sun, Wei et al. 2017); the overexpression of GRP78 can decrease the ER stress and protects the cells from apoptosis (Jiang, Sun et al. 2016). In the other hand, Stim1 down-regulation by siRNA upregulated the expression of Bcl2 (He, Wu et al. 2017). We next sought to investigate whether GRP78 and/ or Bcl2 are involved in the anti-apoptotic effect of siStim1 in CO-OCS-treated cells. For that, we investigated the effects of Stim1 suppression with and without CO-OCS treatment by Western blot. As shown in Figure 55.A, the expression of GRP78 was significantly increased in MCF-7 cells transfected with siStim1 to 1.39-fold (Figure 55.A). In addition, the expression of GRP78 significantly increased from 0.37 fold in siCTL+ CO-OCS to 0.7 fold in siStim1 + CO-OCS (Figure 55.A). We also observed an increase in Bcl2 expression in siStim1 condition up to 1.25 fold; the expression of Bcl2 was restored from 0.42 fold in siCTL + CO-OCS to 0.71 fold in siStim1 + CO-OCS (Figure 55.B). Taken together, Stim1 was able to reduce the apoptotic effect of CO-OCS through increasing GRP78 and Bcl2 expression.

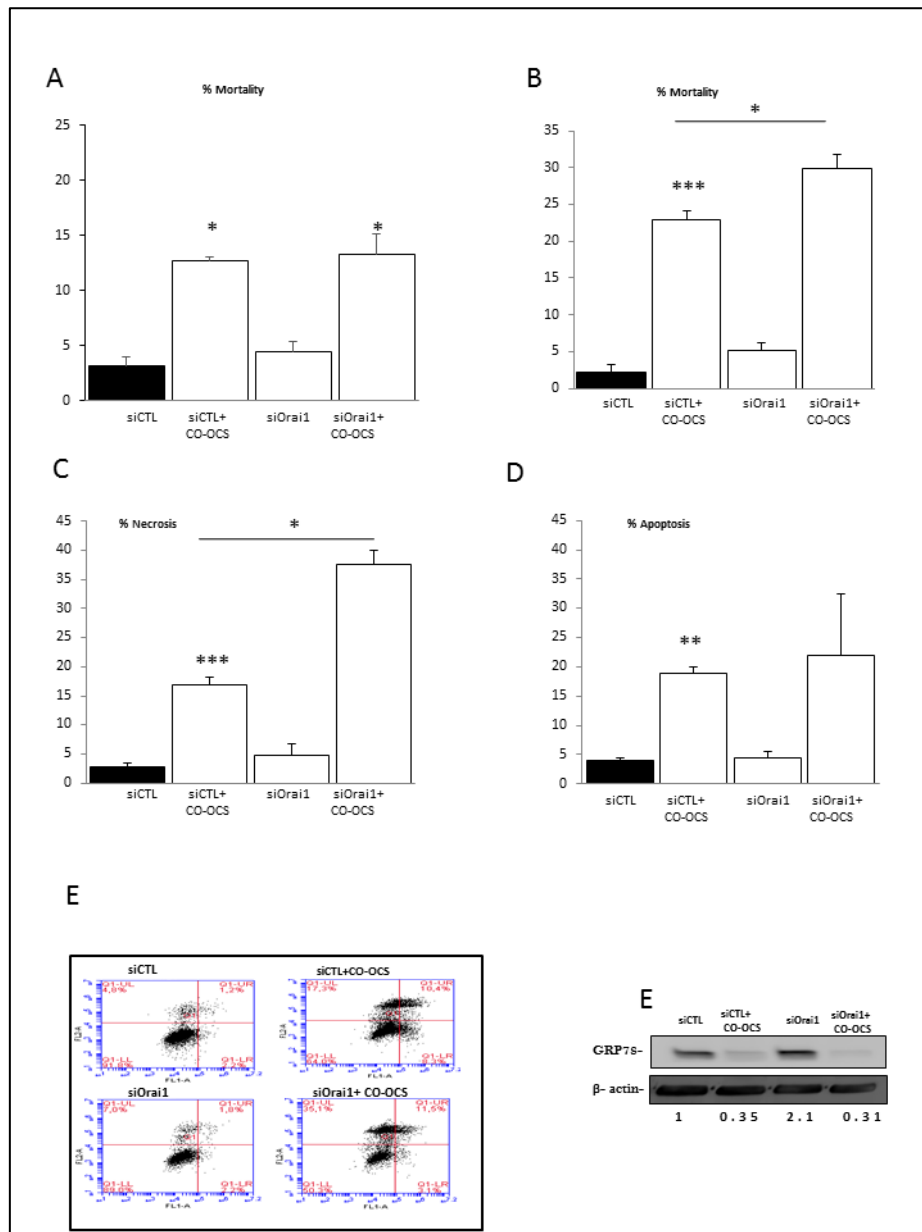


**Figure 55. Stim1 upregulated GRP78 and Bcl2 in CO-OCS treated cells.** Quantified western blots demonstrate GRP78 protein expression after siStim1 silencing in the presence of 40  $\mu$ M of CO-OCS or not during 72 h (A). Western blots quantification to evaluate the effect of siStim1 alone and combined with CO-OCS on the expression of Bcl2 (C). Representative Western-blot bands of GRP78 and Bcl2 proteins and  $\beta$ -actin served as a control (C). Data are representative of SD of three independent experiments, analyses by ANOVA test  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .



- **Orai1 sensitized breast cancer cells to CO-OCS-induced necrotic cell death**

To assess whether Orai1 under expression has a role in the effect of CO-OCS; we used Trypan Blue exclusion assay. The suppression of Orai1 expression seems to have no effect on cell mortality neither at 72 h nor 96 h post-transfection (Figure 56.A and B). In the other hand, we measured elevation in the mortality induced by CO-OCS after 72 h of treatment up to  $29.83 \pm 3.5\%$  in siOrai1 cells when compared with  $22.8 \pm 2.1\%$  mortality for siCTL + CO-OCS. Then, the impact of Orai1 knockdown on apoptosis and necrosis were evaluated by flow cytometry assay. The results showed that no statically observed changes neither in apoptosis nor necrosis percentages were measured as result of Orai1 reduction alone (Figure 56.C and D). The percentage of necrotic cell death in siOrai cells incubated with CO-OCS was significantly higher by  $37.65 \pm 2.24\%$  than  $16.68 \pm 1.29\%$  for siCTL + CO-OCS (Figure 56.C). However, no statically apoptotic effect was observed in the combined condition (Figure 56.D). Moreover, the expression of GRP78 was also investigated by western blot. Despite that, siOrai1 was able to increase the expression of GRP78, but when combined with CO-OCS, GRP78 expression in siOrai1 + CO-OCS was similar to siCTL + CO-OCS (Figure 56.F).

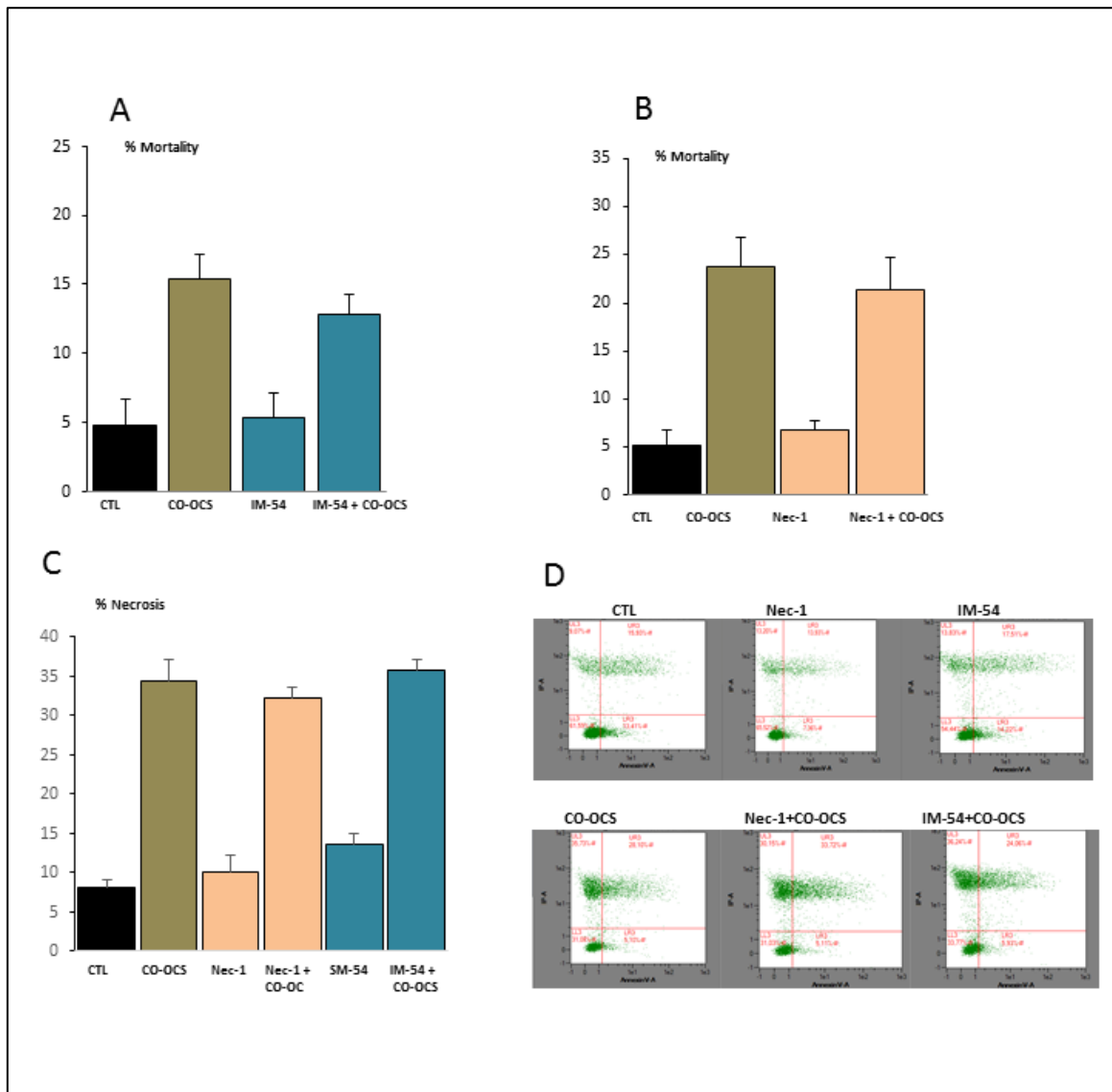


**Figure 56. Orail1 inhibition increased CO-OCS-induced necrotic cell death.** MCF-7 cells were transfected with siCTL or siOrail1; after 24 h post-transfection, cells were treated or not with 40  $\mu$ M of CO-OCS either for 48 or 72 h. percentage of mortality was estimated by Trypan Blue exclusion method at 72 h post siOrail1 knockdown and 48 h of treatment (A) and at 96 h post-siOrail1 and 72 h of CO-OCS treatment (B). Flow cytometry by using Annexin V FITC and Propidium Iodide assay exhibits the percentage of necrotic (C) and (D) apoptotic cell death at 96 h post-transfection and 72 h of treatment. Traces of Annexin V FITC and Propidium Iodide staining assay represents siStim1 at 96 h and 72 h of CO-OCS incubation. Effect of siOrail1 (96hr) on the expression of GRP78 alone and with CO-OCS (72 h) was estimated by western blot. Data are representative of SD of three independent experiments, analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.00

### **C. ER calcium stock is involved in both apoptotic and necrotic CO-OCS effect**

- **Necrostatin-1, IM-54 did not affect CO-OCS-induced mortality**

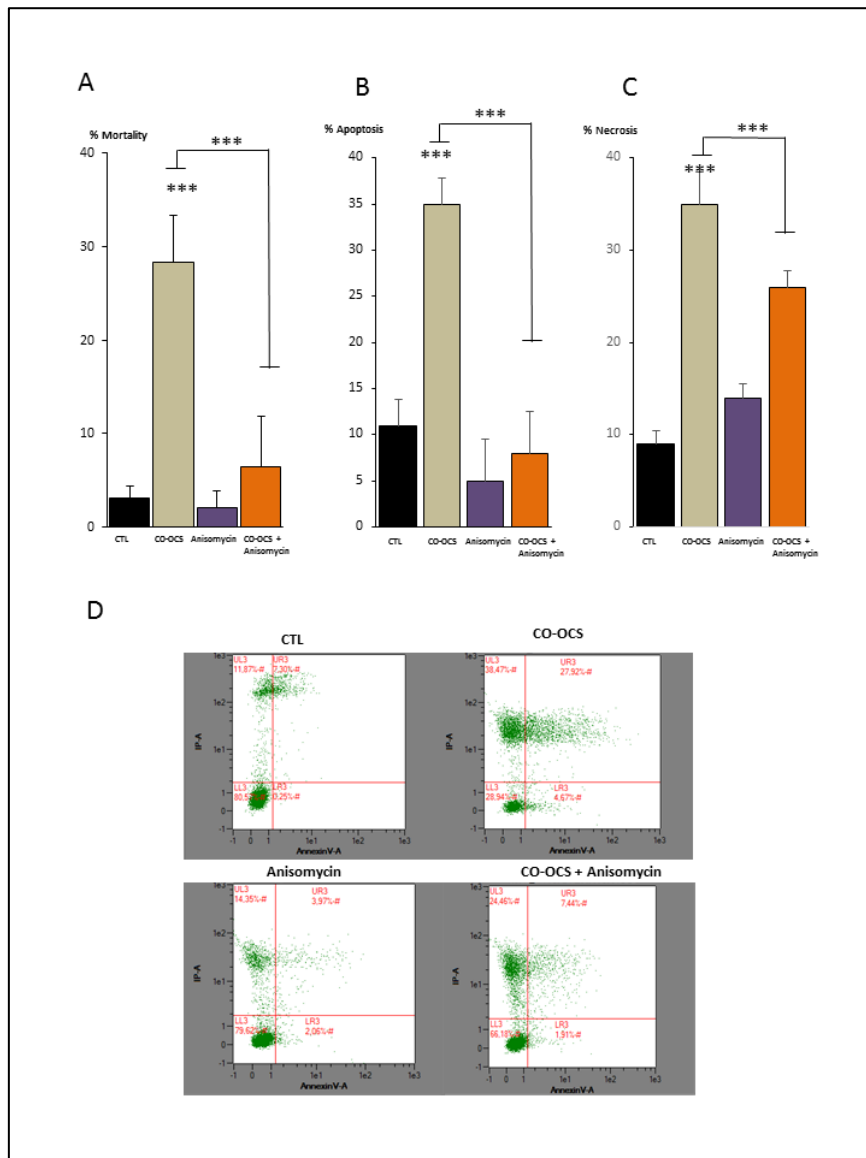
To investigate other possible death signalling pathways could be implicated in CO-OCS- induced cell necrosis; we first tested two inhibitors of necrotic cell death on CO-OCS-induced mortality by Trypan Blue then by flow cytometry. Necrostatin-1 (Nec-1) which is an inhibitor of necroptosis and IM-54 which is an inhibitor of oxidative stress induced by ROS, both of them failed to affect mortality, apoptosis or necrosis (Figure 57. A, B and C).



**Figure 57: NEC-1, IM-54 did not affect CO-OCS-induced mortality.** (A) Effect of IM-54 on CO-OCS induced Mortality; cells were pre-treated with 10  $\mu$ M of IM-54 for 3 h then treated with or without 40  $\mu$ M CO-OCS for 72 h. Effect of Nec-1 on CO-OCS induced Mortality; cells were pre-treated with 30  $\mu$ M of Nec-1 for 3 h then treated with or without 40  $\mu$ M CO-OCS for 72 h (B). Annexin V/PI staining assay demonstrates the effect of IM-54 and Nec1 on the necrosis induced by CO-OCS at 72 h (C). (D) Representative traces of Annexin V/PI staining assay after applying the IM-54 and Nec-1. Results are representative of SD of three independent experiments, analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **CO-OCS-induced cell death regulated via ER calcium store depletion**

In different cell line, ER Ca<sup>2+</sup> store depletion can trigger apoptosis (Skryma, Mariot et al. 2000). The inhibition of the ER Ca<sup>2+</sup> leak channels can modulate cell death. Translocon is a major ER Ca<sup>2+</sup> leak channel and essential for cellular Ca<sup>2+</sup> homeostasis; its inhibition can protect from apoptosis induced by UPR and ER stress. Anisomycin, a translocon inhibitor, is able to reduce the ER Ca<sup>2+</sup> leak toward the cytoplasm and inhibiting the apoptosis induced by different ER stressors such as Tunicamycin (Hammadi, Oulidi et al. 2013). Base on that, we applied Anisomycin at 0.2 μM in parallel with 40 μM of CO-OCS for 48 h. As shown in Figure 58.A, using Trypan Blue test, Anisomycin was able to decrease cell mortality induced by CO-OCS from 28.3 ± 5.1% to 6.45 ± 6.5 %. Moreover, this result was confirmed by flow cytometry; Anisomycin at 0.2 μM managed to inhibit both apoptosis from 35.1± 2.75% to 8 ± 4.48% and necrosis from 35.2 ± 3.5% to 26.12 ± 2.25 % in CO-OCS-treated cells (Figure 58.B and C).



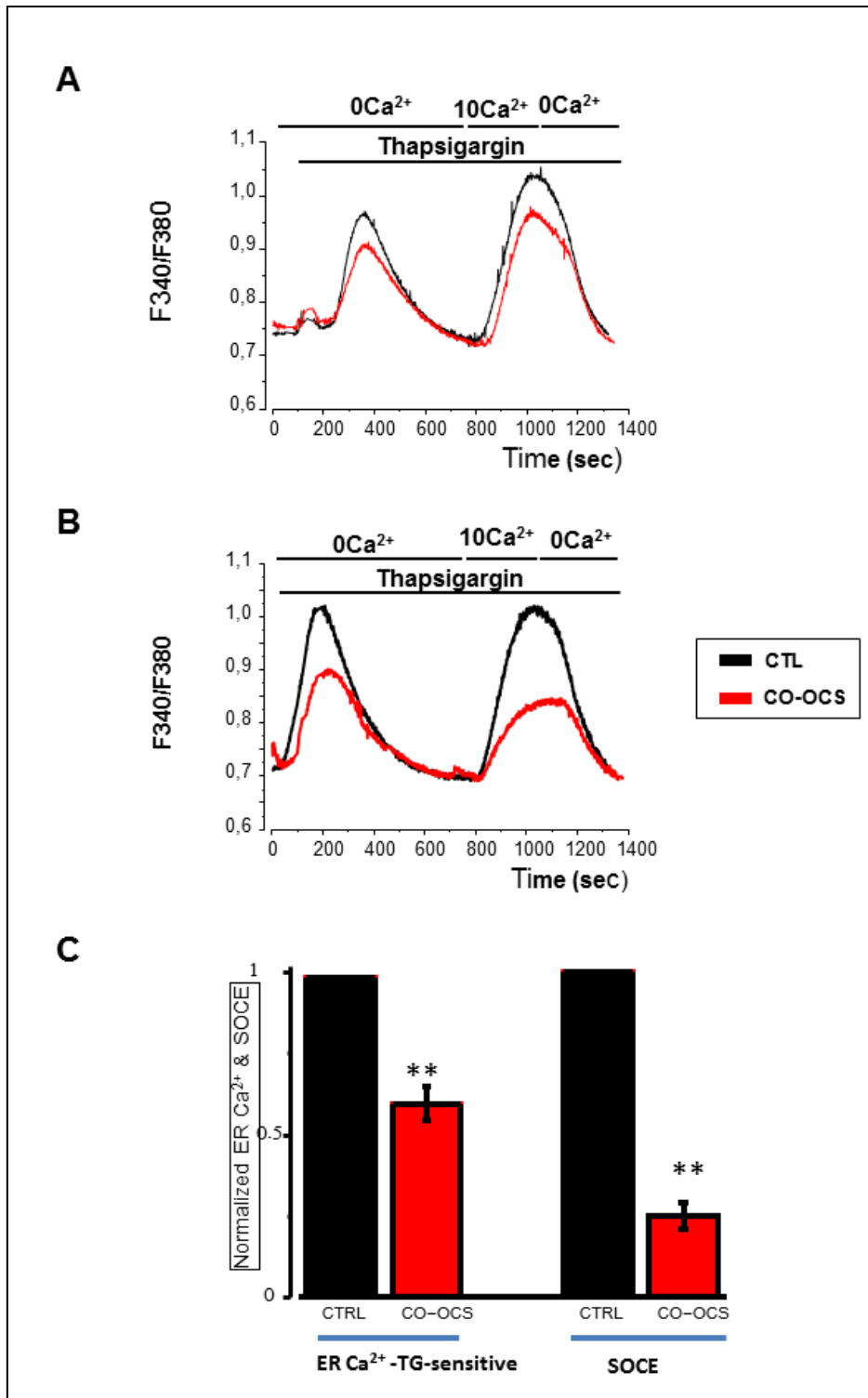
**Figure 58. Translocon inhibition regulated CO-OCS-induced cell death.** This experiment was carried out by using 0.2  $\mu$ M of Anisomycin; MCF-7 cells were treated with 40  $\mu$ M of CO-OCS and 0.2  $\mu$ M of Anisomycin in the same time and incubated for 48 h for both of them. (A) Percentage of Mortality was evaluated by Trypan Blue exclusion assay at 48 h of treatment. Annexin V/PI staining assay exhibits the percentage of apoptotic (C) and (D) necrotic cell death at 48 h of combined treatment. Representative traces of Annexin V/PI staining assay (D). Results are representative of SD of three independent experiments, analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

#### **D. CO-OCS disrupts the Reticular Calcium content as well as Calcium Entry via SOCs (SOCE)**

The three targets (GRP78, Stim1 and Orai1) of CO-OCS permit the regulation of calcium homeostasis. Indeed, GRP78 is capable of buffering 25% of the reticular calcium (Lievremont, Rizzuto et al. 1997) and its activity depends on calcium (Lamb, Mee et al. 2006). Stim1 and Orai1 are part of the SOC channels and are responsible for the SOC-type calcium influx (SOCE). The objective of this second part of our work is therefore to study the putative link existing between GRP78, the alteration of Stim1 and Orai1 expression, the capacitive entry of calcium (SOCE), the ER Ca<sup>2+</sup> Stock and the effects of CO-OCS in MCF-7 cells.

- **CO-OCS reduces the ER calcium content as well as SOCE**

First, we studied the impact of a 48 and 72 h treatment with CO-OCS on the reticular calcium-sensitive to Thapsigargin and calcium entry. Using the calcium imaging technique, we followed the variation of the F340 / 380 ratio using the classical protocol of the study of SOC entry (see Material and Methods). The perfusion of MCF-7 cells with the SERCA pump inhibitor Thapsigargin (TG at 1  $\mu$ M) in 0 mM extracellular Ca<sup>2+</sup> induces a calcium-reticular cell emptying leading to an increase in the F340 / 380 ratio. Following this Ca<sup>2+</sup> ER emptying, we perfused the cells with a calcium-rich extracellular medium (10 mM Ca<sup>2+</sup>) to better visualize SOC entry. We noticed that CO-OCS reduced the reticular calcium content as well as SOCE (Figure 59). In fact, CO-OCS decreased TG-response as well as calcium entry by  $25 \pm 8.9\%$  and  $33 \pm 7.8\%$  for 48 h and  $40.6 \pm 5.6 \%$  and  $70.17 \pm 4.06 \%$  for 72h of treatment respectively (Figure 59.A-C).



**Figure 59: CO-OCS decreased the TG-response and SOCE.** (A) showing the effect of 40  $\mu$ M of CO-OCS on MCF-7 cells at 48 h. (B) The effect of 40  $\mu$ M of CO-OCS on MCF-7 cells at 72 h. (C) The quantification of CO-OCS on ER Ca<sup>2+</sup> and SOCE at 72h. Results are representative of S.E.M of three independent experiments, analyses by T-test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

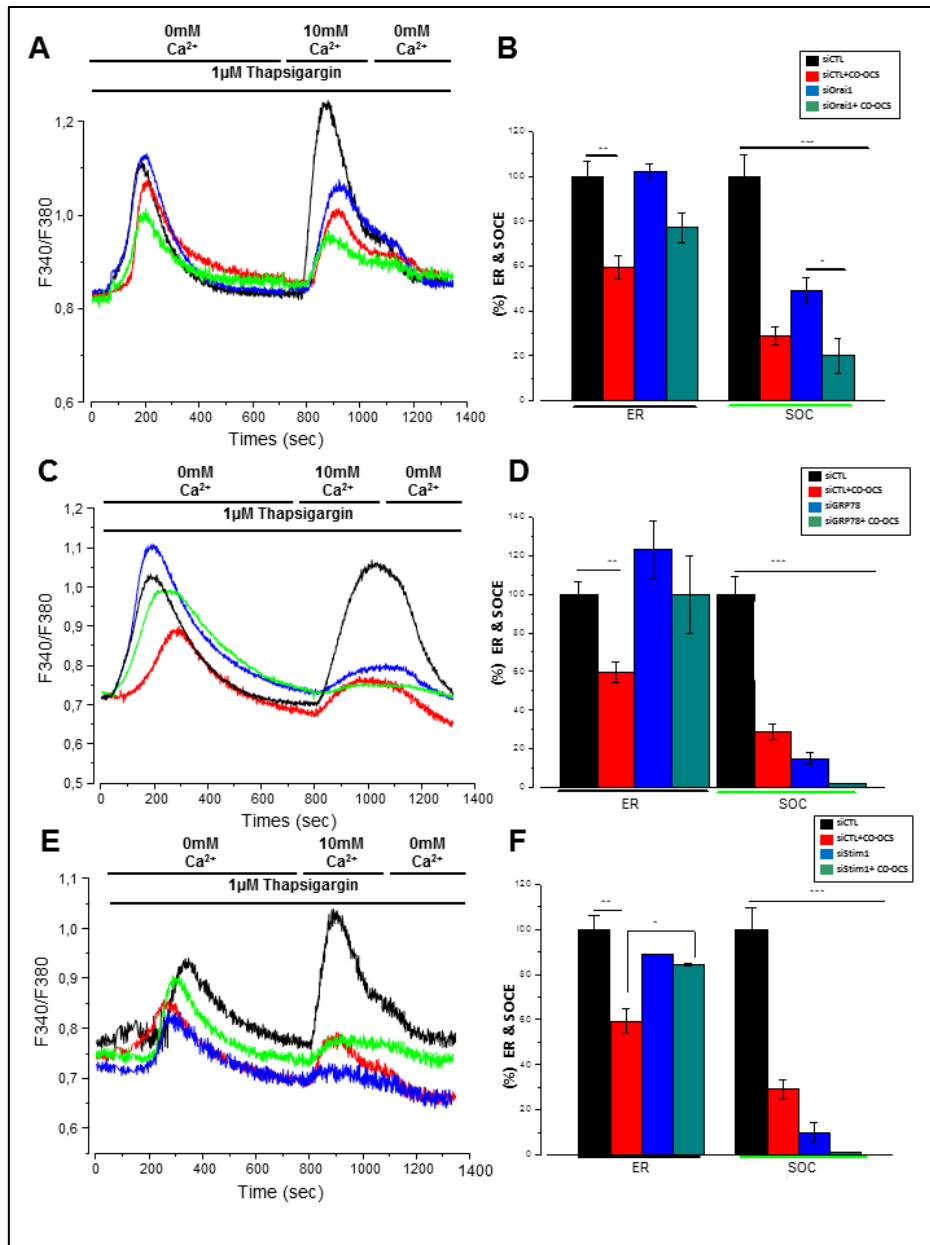


- **GRP78, Stim1, and Orai1 are involved in the alteration of the calcium signal by CO-OCS**

We have shown that treatment with CO-OCS decreases ER  $\text{Ca}^{2+}$  stock,  $\text{Ca}^{2+}$  entry and also the expression of Orai1, GRP78 and Stim1. Therefore, it seemed necessary to study the impact of CO-OCS on calcium entry (Orai1 and Stim1 are two partners involved in calcium entry) and reticular calcium (GRP78 is one of the main proteins of UPR) in cells transfected with siCTL or siStim1, siOrai1 and siGRP78 and treated for 72h. We analysed their impact on the  $\text{Ca}^{2+}$  entry and on the reticular calcium content.

- **Effect of CO-OCS on Calcium Entry and Reticular Calcium in MCF-7 cells where the expression of Stim1, GRP78 and Orai1 is invalidated by siRNAs**

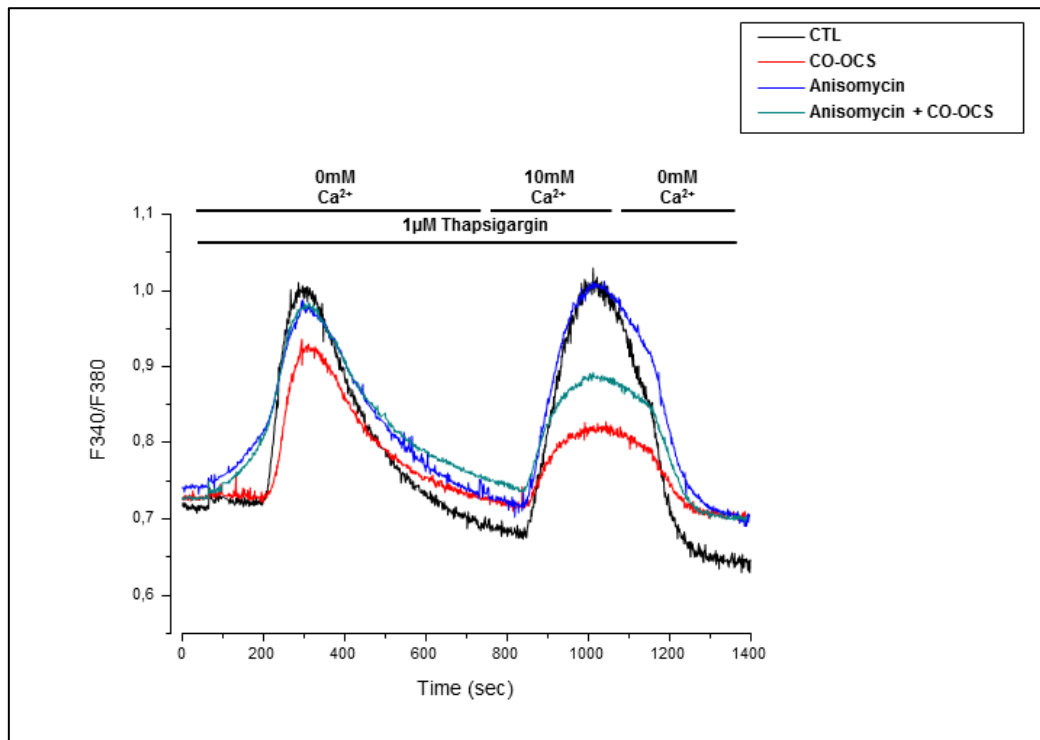
Our results show that silencing of Stim1 or GRP78 almost completely suppresses the calcium entry (Figure 60 C-F). Very interestingly, CO-OCS has no additive effect on SOCE in cells transfected with siStim1 and siGRP78 (Figure 60.C-F). In contrast, CO-OCS further reduces the calcium entry in MCF-7 cells transfected with siOrai1 (Figure 60.A and B). In parallel with the study of SOCE, we also analysed the reticular calcium content by infusing MCF-7 cells with Thapsigargin (TG at 1  $\mu\text{M}$ ) in the absence of extracellular calcium, then quantifying the F340/380 ratio. As seen previously, CO-OCS reduces the amount of reticular  $\text{Ca}^{2+}$  released from the ER following passive depletion using Thapsigargin. Interestingly, we did not observe significant differences in the amplitude of the TG response in both siStim1 and siGRP78 transfected and CO-OCS-treated cells, but not in siOrai1 cells (Figure 60.A-F).



**Figure 60: CO-OCS altered both ER Ca<sup>2+</sup> stock and store-operated Ca<sup>2+</sup> entry through alteration of the Stim1, Orai1 and GRP78 expression.** (A), (C) and (E) represent mean traces of at least 60 cells in each condition. (B), (D) and (F) the quantification of reticular Ca<sup>2+</sup>-TG-sensitive and SOCE in each condition. Results are representative of S.E.M of two independent experiments, analyses by ANOVA test\*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

- **CO-OCS did not affect the ER calcium leak or Ca<sup>2+</sup> entry when combined with the inhibitor of the Translocon**

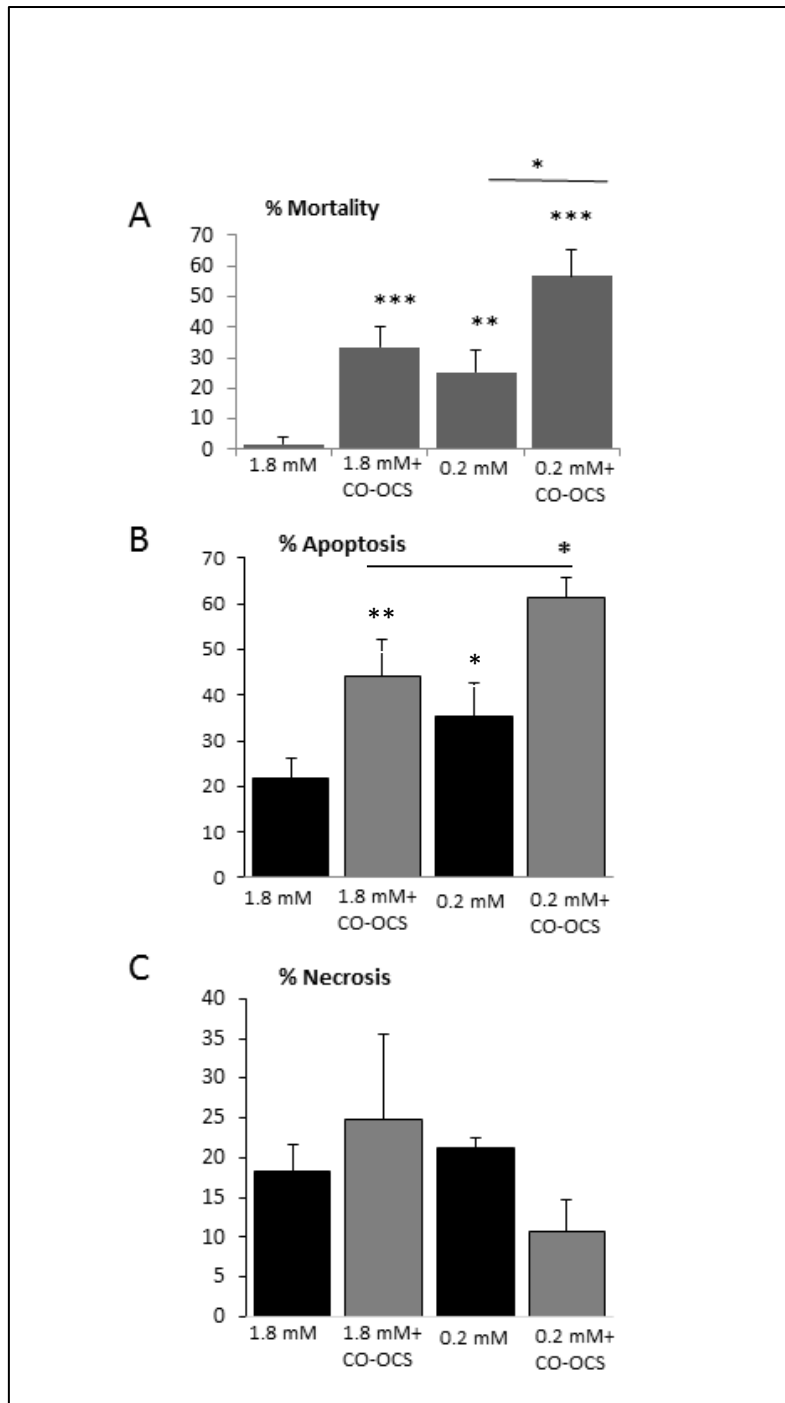
As the inhibition of Translocon decreases cell apoptosis and necrosis-induced by CO-OCS (see Figure 58), we investigated the effect of CO-OCS on the ER Ca<sup>2+</sup> leak and SOCE in presence or absence of Anisomycin. Calcium imaging was performed on cells treated with 40 μM of CO-OCS alone or combined with 0.2 μM of Anisomycin during 72h. As expected CO-OCS alone decreased ER Ca<sup>2+</sup> leak and SOCE (Figure 61). Anisomycin alone did not affect SOCE, but seems to have no effect on ER Ca<sup>2+</sup> release by TG; even we observed in two different cell batch (2 different number passages) more changes in the kinetics of ER Ca<sup>2+</sup> leak that became slower (Figure 61). Interestingly, Anisomycin suppressed completely the effect of CO-OCS on ER Ca<sup>2+</sup> and partially on SOCE (Figure 61).



**Figure 61. Anisomycin antagonised the effect of CO-OCS completely on the ER calcium leak and partially on SOCE.** The effect of 0.2 µM of Anisomycin at 72h on the calcium release from ER upon depletion by Thapsigargin and the subsequent SOCE induced by store depletion in MCF-7 cells treated or not treated with CO-OCS for 72h at a concentration of 40 µM. Results are representative of two independent experiments.

- **Effect of CO-OCS on cell mortality is independent of extracellular calcium**

We have already reported that extracellular  $\text{Ca}^{2+}$  regulates breast cancer cell survival and proliferation (Faouzi et al., 2011). Here, we first demonstrated that CO-OCS reduces calcium entry, reticular calcium content, and increases apoptosis and necrosis. Second, we demonstrated the involvement of Orai1, Stim1, and GRP78 in these calcium variations. As a result, our aim was to understand whether external  $\text{Ca}^{2+}$  could modulate CO-OCS-induced mortality, apoptosis, and necrosis. Thus, by performing Trypan Blue exclusion method and flow cytometry using an AnnexinV/IP co-labelling, we analysed the mortality, apoptosis and necrosis of CO-OCS treated MCF-7 cells both in normal culture medium (1.8 mM  $\text{Ca}^{2+}$ ) and in a medium containing 0.2 mM  $\text{Ca}^{2+}$ . The analysis of the results shows us that CO-OCS treatment for 72h in a normal medium increased the mortality ( $33.33 \pm 7.5\%$ , Figure 61.A). The reduction of the concentration of the extracellular  $\text{Ca}^{2+}$  to 0.2 mM also increased the mortality of  $25.27 \pm 7.1\%$ . However, the mortality in a low  $\text{Ca}^{2+}$  medium was much more increased in CO-OCS-treated cells ( $56.68 \pm 8.9\%$ , Figure 61.A). CO-OCS increased the percentage of apoptotic and necrotic cells to  $44.1 \pm 8.3\%$  and  $18.3 \pm 3.3\%$  respectively in the conventional culture medium (Figure 61.B and C). In a medium deficient in external  $\text{Ca}^{2+}$ , CO-OCS slightly increased the percentage of apoptotic cells to  $61.8 \pm 4.3\%$ . Concerning its necrotic effect, we didn't find a significant effect. However, in the 3 different passage number, we observed a trend of a decrease of CO-OCS necrotic effect in low  $\text{Ca}^{2+}$  medium ( $10.66 \pm 4.09\%$ ) (Figure B and C). These preliminary results indicate that effect of CO-OCS on apoptosis is independent of  $\text{Ca}^{2+}$ , unlike the necrotic effect that seems affected by extracellular  $\text{Ca}^{2+}$ .



**Figure 61. Effect of external  $\text{Ca}^{2+}$  on the cell death induced by CO-OCS in MCF-7 cells.** Cells were treated with CO-OCS for 72h in different calcium concentrations. 1.6 mM of EGTA was added to achieve 0.2 mM of calcium concentration. Effect of CO-OCS in culture medium containing 1.8 and 0.2 mM  $\text{Ca}^{2+}$  on cell mortality (A), apoptosis (B) and necrosis (C). All experiments were performed for three times, the values indicated are mean  $\pm$  S.E.M; analyses by ANOVA test \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

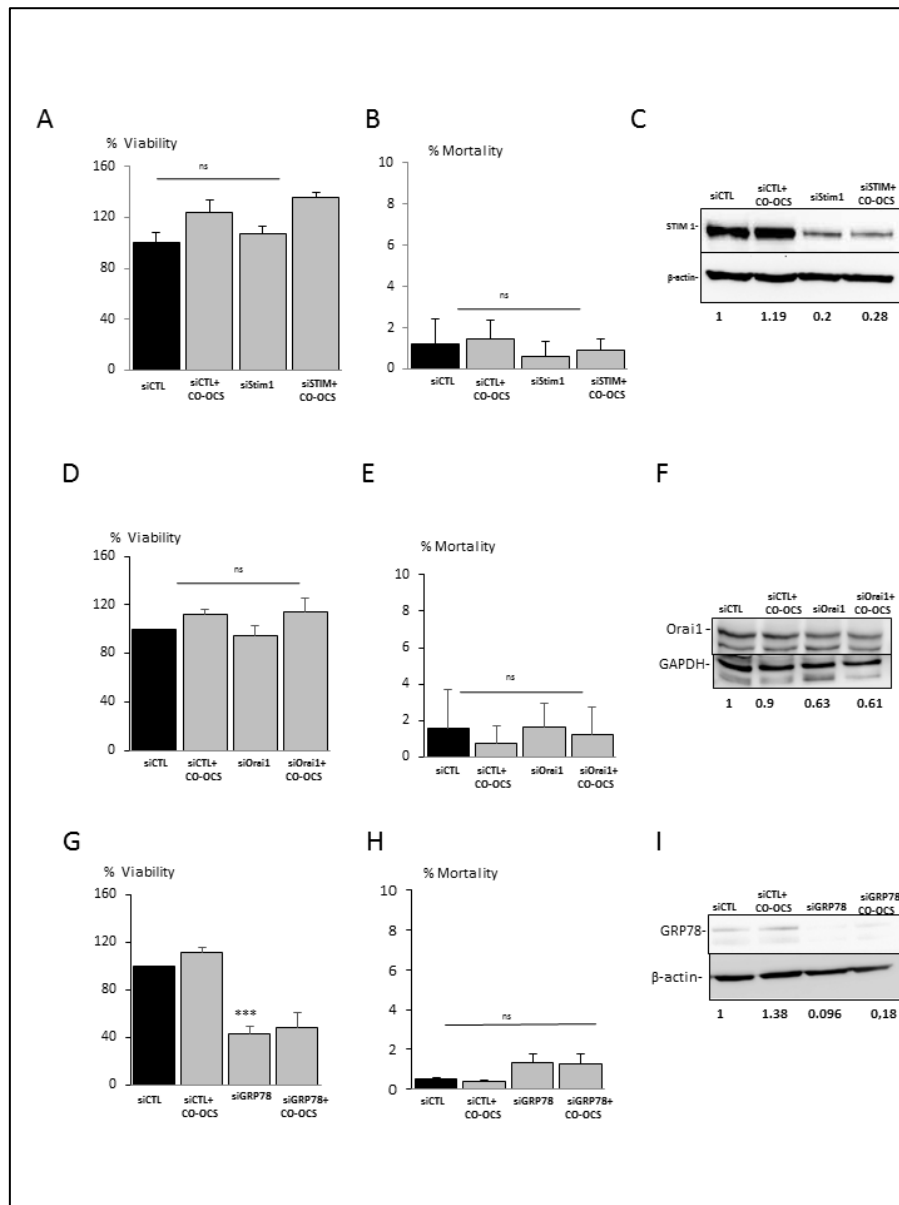
Taken together these results clearly indicate that CO-OCS, via its ability to reduce the protein expression of Stim1, Orai1 and GRP78 affected the reticular calcium homeostasis and the capacitive calcium entry. The effect of CO-OCS on apoptosis seems independent of extracellular calcium, unlike its necrotic effect.

***Part III: CO-OCS did not affect the expression of Stim1, Orai and GRP78, Calcium homeostasis and cell growth of breast normal cells***

- **CO-OCS failed to affect Stim1, Orai1 and GRP78 expression in normal breast cell line**

Researchers from our laboratory have shown that CO-OCS does not affect the viability or mortality of normal MCF-10A cells (Allan, Ouadid-Ahidouch et al. 2013). Here we investigated the impact of a 72h treatment of CO-OCS on the expression of Stim1, Orai1 and GRP78, on reticular calcium and calcium entry, then, we investigated whether the inactivation of these proteins can regulate the growth of normal cells. We found that CO-OCS failed to affect the expression of Stim1, GRP78 and Orai1 (Figure 62.C, F and I). Moreover, CO-OCS has no effect on cell mortality or cell viability. In addition, all siRNAs against Stim1, Orai1 and GRP78 failed to induce cell mortality (Figure 62.B, E and H). However, only siGRP78 was able to decrease cell viability to 43% when normalized to CTL (Figure 62.A, D and G).



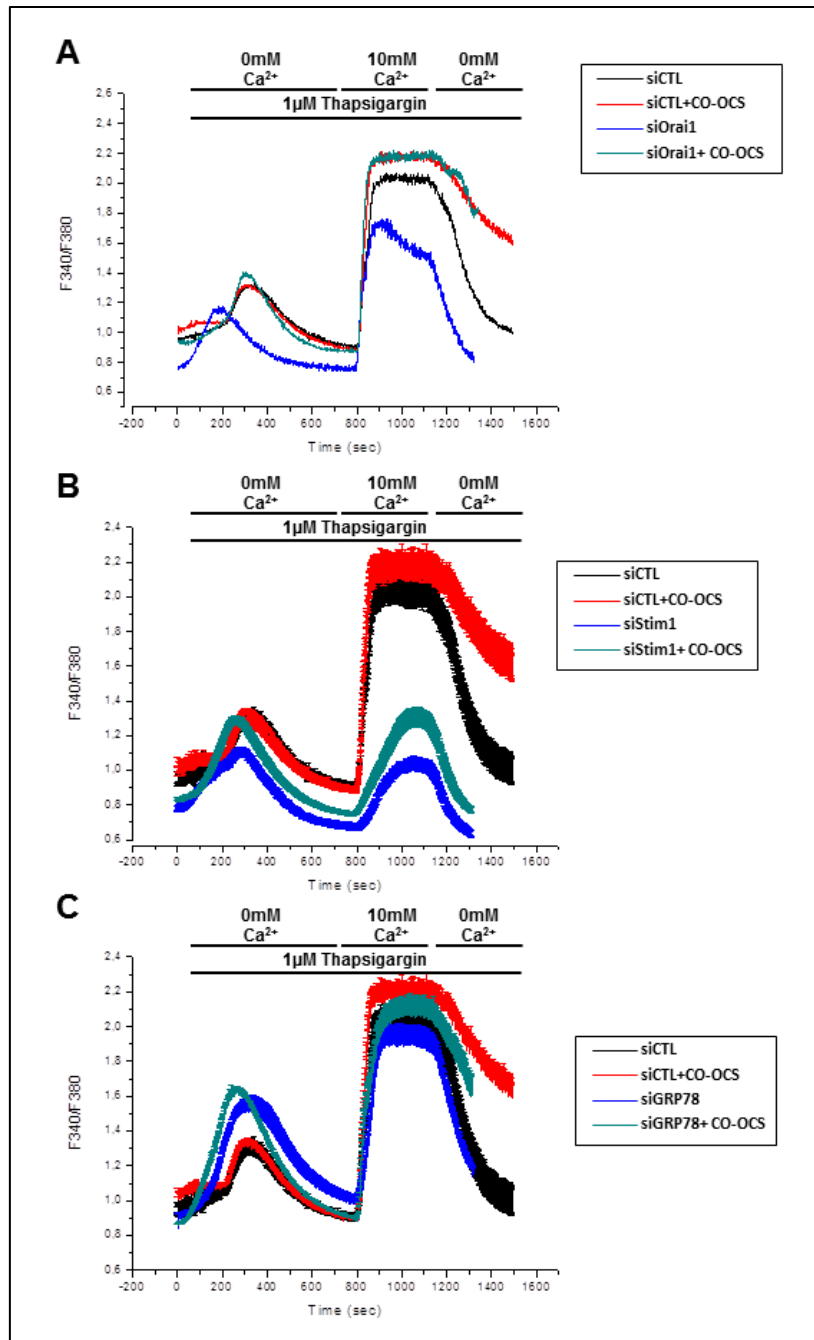


**Figure 62. CO-OCS failed to affect STIM1, Orai1 and GRP78 expression in normal breast cell line.** The same transfection protocol and treatment performed on MCF-7 cells was applied for MCF-10A cells. Effect of siStim1 after 96 h post-transfection combined or not with 40  $\mu$ M of CO-OCS (72 h) on cell viability (A) and mortality (B). Representative western blot validates siStim1 transfection (C). Effect of silencing of Orai1 for 96 h with and without CO-OCS incubation for 72 h on cell viability (D) and mortality (E). Representative western blot demonstrates transfection efficiency (F). Effect of GRP78 knockdown (96 h) alone and when combined or not with CO-OCS for 72 h on cell viability (G) and mortality (H). Representative western blot shows GRP78 inhibition by transfection method (I). Data are mean  $\pm$  S.E.M and analyses by ANOVA test \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

- **CO-OCS neither affects Ca<sup>2+</sup> ER store nor SOC Calcium Entry in normal breast cells.**

We have conducted calcium-imaging experiments to study the effect of CO-OCS on calcium entry and/or the content of reticular calcium. We applied the same protocol as that performed on MCF-7 cells. The MCF-10A cells were transfected with siCTL, siStim1, siOrai1 or siGRP78 and treated or not with CO-OCS at a concentration of 40  $\mu$ M for 72 h.

The results show that CO-OCS has no significant effect neither on the amount of calcium released from ER after the application of Thapsigargin nor SOCE (Figure 63). In addition, we observed a clear decrease in SOCE only in cells transfected with sistim1 (Figure 63.B). Moreover, the inhibition of GRP78 expression increases the release of calcium from ER after Thapsigargin perfusion; this response is not impaired by CO-OCS (Figure 63C).



**Figure 63. Effect of CO-OCS on Store-Operated Ca<sup>2+</sup> Entry and Reticular Ca<sup>2+</sup> Stock in MCF-10A cells.** Calcium recording in MCF-10A cells treated or not by CO-OCS and transfected with siOrai1 (A), siStim1 (B) and siGRP78 (C) as indicated. Results are the mean of traces of at least 30 cells in each condition

Our experiments on normal MCF-10A cells, under the same conditions as on cancer cells, show us very clearly that CO-OCS does not affect the proliferation or mortality of these cells. The absence of CO-OCS effects can be explained by the failure of this treatment to affect the expression of Stim1, Orai1 and GRP78 as well as it failed to affect neither ER Ca<sup>2+</sup> stock nor SOCE. This result reinforces the importance of these 3 targets in the specific effect of CO-OCS on cancer cells.

## ***General Discussion***

Previous research from our laboratory had demonstrated the anti-tumour effects of CO-OCS on breast cancer (Allan, Ouadid-Ahidouch et al. 2013). In this work, we investigated the related signalling pathways of CO-OCS implicated in the different cellular mechanisms in breast cancer include migration, proliferation and survival. This work was in collaboration with the Instituto de Investigaciones Químicas (IIQ), CSIC—Universidad de Sevilla, Spain. To have a more comprehensive understood of this project, we had to choose basically two breast cancer cell lines with different characteristics MCF-7 and MDA-MB-231 in parallel with the normal immortalized breast cell line MCF-10A. Different CO-OCS concentrations at 26, 40 and 100  $\mu$ M and duration of treatment for 24, 48 and 72 h were used according to the experiments.

***Part I: CO-OCS inhibited breast cancer migration mainly through calcium-dependent and independent signalling.***

The first part of the results obtained during my thesis based on the investigation of the mechanism related to the anti-migratory effect of CO-OCS in breast cancer. We demonstrated that CO-OCS impaired breast cancer migration through two distinct mechanisms; where CO-OCS inhibited  $\beta$ 1-integrin expression which in turn activated FAK and ERK1/2 proteins via signalling pathway independent of calcium. However, we also exhibited calcium-dependent mechanism which was mainly achieved via targeting Stim1 protein expression; leading to inhibition of SOCE. Interestingly, CO-OCS had no effect on the expression of both  $\beta$ 1-integrin and Stim1; also, the FAK and ERK1/2 signalling cascades were not activated in normal breast cell line MCF-10A.

Cancer metastasis to other healthy organs is a big dilemma, responsible for about 90% of cancer deaths (Seyfried and Huysentruyt 2013). Aberrant glycosylation can affect the expression and the function of many cellular proteins as well as it can affect cancer development and metastasis. Cancer cells localization in distant tissues is regulated via different proteins and cellular signalling cascades.  $\beta$ 1 integrin is one of the proteins that regulate the migration process in a number of tumour models, including breast, ovarian, pancreatic, and skin cancer (Blandin, Renner et al. 2015). It plays an essential role during the different stages of tumours metastasis including invasion, migration, intravasation, extravasation, and dissemination. Additionally,  $\beta$ 1 integrin is up-regulated in breast cancer

and correlated to lymph node metastasis and poor prognosis. It has been demonstrated that the inhibition of  $\beta 1$  integrin expression in MDA-MB-231 cells impaired cell migration (Hou, Isaji et al. 2016). Integrins can regulate the matrix metalloproteinases (MMPs), causing extracellular matrix degradation and remodelling (Borrirukwanit, Pavasant et al. 2014; Missan, Mitchell et al. 2015). Also, it is able to activate both of FAK and ERK1/2 proteins; thus, altering the migration process. Moreover, the dysregulation of glycosylation and abnormal glycoproteins have been linked to tumorigenesis.  $\beta 1$  integrin is N-glycosylated protein where it has 12 potential glycosylation sites, located on the extracellular domain. The elevation of  $\beta 1,6$  branching of N-linked oligosaccharides is common altered glycosylated form in cancer, it was linked to the inhibition of  $\alpha 5\beta 1$  integrin clustering and stimulating cell migration (Guo, Lee et al. 2002).  $\beta 1$  integrin glycosylation sites make it suitable to be affected by the inhibitors of glycosylation. Treatment of rat colon adenocarcinoma cells with the Castanospermine affected  $\beta 1$  integrin adhesion to the fibronectin (Ringear, Harb et al. 1996). Moreover, Tunicamycin inhibited hepatocarcinoma cells adhesion and migration through the down-regulation of the cell surface  $\beta 1$  integrin (She, Xu et al. 2010). We found that CO-OCS significantly affected the migration ability of breast cancer. Further, we investigated the effect of CO-OCS on the expression of  $\beta 1$  integrin at the transcript and the protein levels; both of the transcript and the protein levels were decreased.

In the focal adhesion complexes, FAK is mainly activated by the activation and clustering of integrins causing intracellular signalling cascades modulating cell migration. FAK targets and activates the Ras-Raf-MEK-ERK signalling (Sawai, Okada et al. 2005). Additionally, breast cancer migration and bone metastasis are highly dependent on FAK and its downstream target ERK1/2 activation (Wu, Wang et al. 2018). The increase in the intracellular calcium level leads to phosphorylation of ERK1/2 and enhances breast cancer cell migration *in vitro* (Di, Huang et al. 2015). Our results demonstrated that CO-OCS inhibited the phosphorylation of the integrin downstream signalling cascades FAK and ERK1/2 in breast cancer.

Both of Stim1 and Orai1 proteins have been shown to be involved in breast cancer cell migration. The silencing of Stim1 and Orai1 proteins inhibited cell migration and metastasis *in vitro* and in mice through regulating the focal adhesion turnover (Yang, Zhang et al. 2009). McAndrew and his collaborators have demonstrated a significant correlation between Stim1 expression in breast cancer and poor prognosis, aggressive and metastatic behaviour (McAndrew, Grice et al. 2011). Another work has exhibited that in MDA-MB-231

cells, silencing of Stim1 significantly inhibited migration by decreasing invadopodia and podosome formation (Chen, Chen et al. 2017). Additionally, inhibition of SOCE interferes with breast cancer cells migration; where SOCE is mediated by Stim1 and Orai1 proteins in breast cancer (Yang, Zhang et al. 2009). In MDA-MB231 cells, Stim1 and Orai1 are the principal mediators of SOCE (Motiani, Abdullaev et al. 2010). In our conditions, CO-OCS reduced the expression of Stim1 without affecting the expression of Orai1 in MDA-MB-231 cells. In contrast, CO-OCS failed to affect Stim1 transcripts level suggesting post-translational modulations. Also, CO-OCS significantly inhibited SOCE and exhibited the involvement of Stim1 in this effect. Finally, we demonstrated that in MCF-10A, CO-OCS had a slight effect on cell migration without significant effect on cell proliferation; no effects on the expression of  $\beta$ 1-integrin, the phosphorylation status of FAK and ERK1/2 and the expressions of Orai1 and Stim1 thus no effect on SOCE. We also investigated the effect of CO-OCS on cell migration and the expression of Stim1 and  $\beta$ 1 integrin of other cell lines; However, CO-OCS was able to inhibit cell migration and Stim and  $\beta$ 1 integrin expression in melanoma, breast and pancreatic cancers.

### ***Part II: CO-OCS signalling pathways through targeting GRP78, Orai1 and Stim1 induced antiproliferative and antisurvival effect in MCF-7 cells***

Almost half of the proteins undergo glycosylation in the lumen of the ER. Aberrant glycosylation is a hallmark of cancer (Janik, Lityńska et al. 2010). Moreover, targeting glycosylation can induce ER stress, disrupt ER functions and induce UPR (Wang, Wang et al. 2015). Orai1 and Stim1 are the main components of SOC channels; besides, both of them are N-glycosylated proteins (Williams, Manji et al. 2001; Gwack, Srikanth et al. 2007), implicated in the ER-calcium homeostasis. Moreover, GRP78 is one of the  $\text{Ca}^{2+}$  storage proteins in the ER (Lievremont, Rizzuto et al. 1997). GRP78 needs  $\text{Ca}^{2+}$  for its activation and it is involved in the ER stress (Lamb, Mee et al. 2006). CO-OCS is an inhibitor of glucosidases I and II (Sanchez-Fernandez, Risque-Cuadro et al. 2010) that reduces cell proliferation and increases apoptosis (Allan, Ouadid-Ahidouch et al. 2013). However, its mechanisms of action remain unclear. Here, we show that CO-OCS: (i) decreased GRP78, Stim1 and Orai1 expression at the protein level without affecting their mRNA expression, decreases both ERK1/2 and Akt phosphorylation and only the expression of Stim1 was rescued when the proteasome was inhibited; (ii) CO-OCS showed an additional effect either

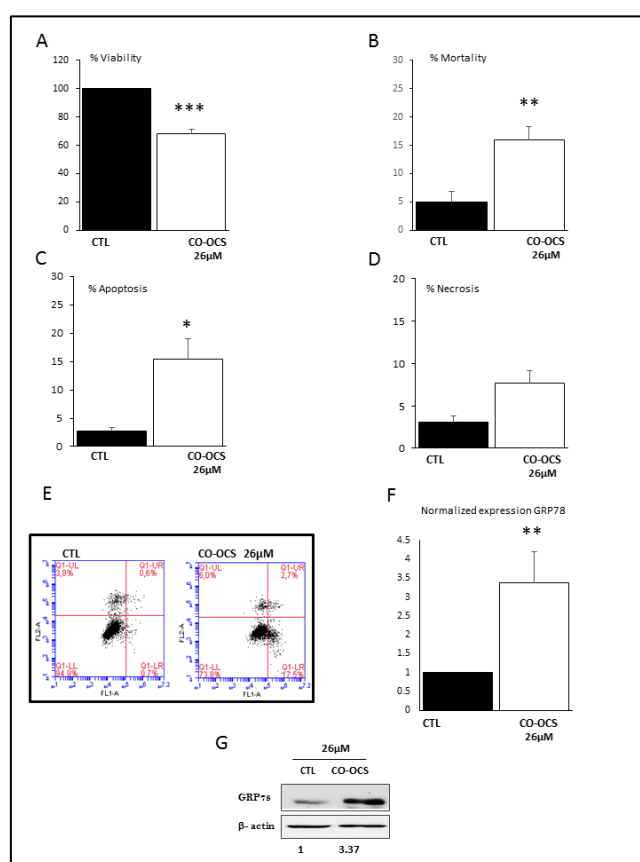


on cell proliferation or on cell cycle phases at 72h treatment in the cells transfected with siStim1 and siOrai1 but not in the cells transfected with siGRP78. (iii) The apoptotic effect of CO-OCS was completely antagonized when transfected siStim1 but not siOrai1 or siGRP78. In contrast, its necrotic effect was increased when silenced of Orai1 but not when silenced of Stim1 or GRP78. (iv) Translocon inhibitor partially reversed CO-OCS apoptotic and necrotic cell death but neither the inhibitor of necroptosis nor the inhibitor of Oxidative stress. (v) CO-OCS reduced both ER  $Ca^{2+}$  TG-sensitive and SOCE. ER  $Ca^{2+}$  TG-sensitive was restored in cells transfected with siGRP78, siStim1 or treating with Anisomycin but not in cells transfected with siOrai1. However, except for siStim1, an additional effect in cells transfected with siGRP78, siOrai1 and treated with CO-OCS was observed on SOCE. Finally, reducing extracellular  $Ca^{2+}$  concentration in the medium showed a slight increase in CO-OCS apoptosis effect with no significant effect in necrosis. Taken together, our results demonstrated that CO-OCS induced ER stress leading to UPR and ERAD activation, cell cycle arrest, apoptosis and necrosis. Stim1 and Orai1 are only involved in the anti-proliferative effect of CO-OCS probably by reducing SOCE. While GRP78 is involved in both apoptotic and necrotic effect likely via  $Ca^{2+}$  ER leak through the Translocon. Moreover, Stim1 constitute a target by which CO-OCS induced its apoptotic effect, while Orai1 protected cells from CO-OCS-induced necrosis.

- **CO-OCS induced ER stress leading to UPR and ERAD in MCF-7 cells**

Different ER stressors can upregulate CHOP and GRP78 expression at the protein and transcript levels in different cell types (Ning, Yong et al. 2015; Lei, Wang et al. 2017); Indeed, ER stress inducers such as Thapsigargin, the calcium Ionophore A23187 and Tunicamycin upregulate both the GRP78 protein and mRNA transcript expression (Watowich and Morimoto 1988; Lee 2005). Our results clearly show that CO-OCS at 40  $\mu$ M significantly increased the expression of GRP78 mRNA at 72 h while inhibited GRP78 expression at the protein level at 48 and 72 h; it seems that the effect of CO-OCS on the expression of GRP78 protein was due to protein degradation activation not due to attenuation or direct pharmacological inhibition of GRP78 mRNA. Moreover, in parallel with the inhibition of GRP78 protein; CHOP mRNA expression was increased at 48 and 72 h which indicates UPR activation. These two effects, protein degradation activation and UPR activation, were almost arrived in parallel in MCF-7-treated with 40  $\mu$ M of CO-OCS, properly as a result of acute ER stress. In agreement with our results, pharmacological inhibition of GRP78 expression at the protein and mRNA levels can activate UPR

(Kosakowska-Cholody, Lin et al. 2014). Moreover, Shi et al., have shown, in macrophages treated with deoxynivalenol, that the inhibition of GRP78 expression at the protein but not at mRNA induced UPR (Shi, Porter et al. 2009). We also incubated the cells with 26  $\mu$ M for 72 h to induce a low to a moderate effect of CO-OCS (Allan, Ouadid-Ahidouch et al. 2013). At this concentration, CO-OCS induced upregulation of the expression of GRP78 ( $3.37 \pm 0.8$  fold when normalized to control) (Figure 64.G). In both cases (moderate or high ER stress induced by CO-OCS) we observed a decrease of cell viability along with an increase of cell death ratio. However, CO-OCS at both concentrations (26 or 40  $\mu$ M) induced apoptosis, while it induced necrosis only at 40  $\mu$ M.



**Figure 64. CO-OCS increased the expression of GRP78 and induced apoptosis at a lower concentration.** MCF-7 Cells were treated with 26  $\mu$ M of CO-OCS for 72 h. Trypan Blue exclusion assay; effect of CO-OCS on cell viability (A) and cell mortality (B). Annexin V FITC and Propidium Iodide staining assay; percentage of apoptosis (C and E) and necrosis (D and E) at 26  $\mu$ M of CO-OCS after 72 h of incubation. Effect of CO-OCS on the expression of GRP78 at 26  $\mu$ M of CO-OCS for 72 h. Results are representative of SD of three independent experiments, (t-test), \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001

UPR activation promotes the folding capacity and efficiency through molecular chaperones upregulations, proteins translational attenuation and an increase in ER-associated protein degradation (ERAD). ERAD can include two degradation mechanisms: type I which is ubiquitin-proteasome-dependent ERAD and type II which is autophagy-lysosome dependent ERAD (Rashid, Yadav et al. 2015). CO-OCS-induced GRP78 degradation can be as a result of ERAD type I or II activation. It has been demonstrated that GRP78 degradation can be induced either via ubiquitin-proteasome-dependent ERAD type I (Kosakowska-Cholody, Lin et al. 2014) or autophagy-lysosome dependent ERAD type II through the cathepsin/calpain dependent degradation (Shi, Porter et al. 2009). In MCF-7 cells, the inability of MG-231 to restore CO-OCS-induced GRP78 degradation, suggests that the ubiquitin-proteasome-dependent ERAD type I is not involved; in disagreement with the results obtained by Kosakowska-Cholody et al. (2014).

CO-OCS also induced post-translational modifications at the protein level before affecting the mRNA expression of Stim1 and Orai1 at 48h treatment. While at 72h treatment, CO-OCS decreased Orai1 and Stim1 transcript expression. In contrast to GRP78 and Orai1, we demonstrated that only Stim1 protein degradation was as a result of ERAD type I activation where MG-231 was able to restore the expression of Stim1 once applied with CO-OCS.

ERAD is also characterized by a decrease of ERK1/2 and Akt phosphorylation (Qin, Wang et al. 2010; Galluzzi, De Santi et al. 2012; Mulay, Wood et al. 2013). We show that CO-OCS at 72 h of treatment decreased the activation of both Akt and ERK1/2 confirming the involvement of ERAD. Moreover, several studies have reported a link between GRP78 and Akt. Indeed, GRP78 can act as an upstream regulator of Akt activation in cancer. ER stress resistant lung cancer cells displayed GRP78 and phospho-Akt dependent chemoresistance (Lin, Wang et al. 2011). Indeed, GRP78 knockdown decreases phospho-Akt expression in these cells. Furthermore, in both ER-stressed and non-stressed PC3 prostate cancer cell line knockdown of GRP78 led to a reduction in phospho-Akt levels (Fu Y et al., 2008). All our results are in agreement that CO-OCS induced ERAD.

- **Stim1, Orai1 are involved in CO-OCS anti-proliferative effect**

Ca<sup>2+</sup> signal has a critical role in cell cycle progression during the different phases (G1, S and G2 / M) (Santella 1998); where progression in the cell cycle is mainly regulated by calcium/calmodulin pathways (Kahl and Means 2003; Choi and Husain 2006). It has been demonstrated that during each phase of cell cycle, the levels of free intracellular calcium were

changed (Pande, Kumar et al. 1996). Additionally, calcium regulates the expression of some transcription factors necessary for G1 phase (Takuwa, Zhou et al. 1993; Roderick and Cook 2008; Koenig, Linhart et al. 2010). Cellular proliferation of some cell types is highly dependent on extracellular calcium concentrations such as breast cancer cells (Journé, Dumon et al. 2004; Journé, Kheddoumi et al. 2008; Faouzi, Hague et al. 2010). In addition, it has been demonstrated that SOCE is needed for G1/S transition through Stim1-Orai1-mediated SOCE (Chen, Chen et al. 2016). The knockdown of Orai1 with siRNA attenuated glioblastoma cell growth and induced G1 phase arrest (Liu, Hughes et al. 2011); Stim1 suppression also induced G1 phase arrest in glioblastoma cell (Li, Zhang et al. 2013). In contrast, another work demonstrated that Stim1 or Orai1 inhibited proliferation and arrested cells at G2/M phase (Abdullaev, Bisailon et al. 2008). Orai1 knockdown significantly increased cell number in G2/M and decreased HEK293 cell proliferation (Borowiec, Bidaux et al. 2014). Moreover, the pharmacological inhibition or silencing of Orai1 or Stim1 in clear cell renal cell carcinoma exhibited anti-proliferative effects (Kim, Lkhagvadorj et al. 2014). Orai1 siRNA significantly inhibited cell proliferation in human non-small cell lung carcinoma and Orai1 over-expression associated with unfavourable prognosis (Zhan, Zhong et al. 2015). TGF- $\beta$  decreased Stim1 expression, leading to SOCE inhibition, resulted in a decrease in breast cancer cell proliferation (Cheng, Wang et al. 2016). Recent work has exhibited that Stim1 is a direct target of miR-223 in breast cancer cell lines and tumour specimens from breast cancer patients; where miR-223 binds to the 3'-UTR of Stim1 and inhibiting Stim1 expression leading to decrease breast cancer cells proliferation (Yang, Jiang et al. 2018). Based on that, we examined potential involvement of Orai1 and Stim1 in the antiproliferative effect of CO-OCS. Indeed, Stim1 or Orai1 knockdown or CO-OCS alone decreased MCF-7 proliferation and arrested the cell cycle progression. Silencing of Stim1 or Orai1 arrested MCF-7 in G1 and G2M respectively. CO-OCS treatment for 72h leads to cells arrest in G1 and G2M. However, the G1 phase was more significantly increased than G2M. Applying a combination of siStim1 or siOrai1 and CO-OCS for 72h increased the cell number in G1 phase in the condition concerning Stim1 while in the case of Orai1, no significant increase in G2M was measured. Interestingly neither siStim1 nor siOrai1 affected cell mortality. CO-OCS decreased cell proliferation by accumulating the cells in G1 and G2 phase. Stim1 and Orai1 are involved in different phases of the cell cycle, namely G1 and G2/M respectively. Thus, it appears from our results that CO-OCS by altering the expression of Stim1 and Orai1 accumulates cells in G1 and G2/M phase respectively.

- **GRP78 is implicated in the antiproliferative, apoptotic and necrotic effect of CO-OCS in MCF-7**

GRP78 has multi-functional roles; where it is the main ER chaperone, ER stress regulator, and initiator of UPR. Moreover, GRP78 has a key survival role in cancer; GRP78 expression upregulation has been reported in a variety of cancers tissues and cancer cell lines (Koomagi, Mattern et al. 1999; Daneshmand, Quek et al. 2007; Ulianich and Insabato 2014). The role of GRP78 in cancer has been associated with an increase in aggressiveness, invasion, resistance to chemotherapy and poor prognosis (Reddy, Mao et al. 2003; Yeung, Kwan et al. 2008; Ren, Chen et al. 2017). Moreover, the expression of GRP78 at the cell surface has been reported in cancer cells but neither in benign tissues nor in immortalized cells (Liu, Li et al. 2013; Zhang, Tseng et al. 2013; Yao, Liu et al. 2015). The elevated GRP78 expression is associated with a bad survival rate in breast cancer (Zheng, Cao et al. 2014). In MCF-7 breast cancer cell line, GRP78 overexpression significantly increased cells proliferation and migration; indeed, an anti-GRP78 antibody designed against the N-terminal blocked the cell surface GRP78 receptor function, leading to an inhibition of MCF-7 proliferation and migration (Yao, Liu et al. 2015). In addition, this last work demonstrated that JAK2/STAT3 pathway mediates cell surface GRP78-induced effect (Yao, Liu et al. 2015). Knockdown of GRP78 was able to sensitize MCF-7 colony formation to chemotherapy (Wang, Yin et al. 2009) and to reduce prostate cancer cells proliferation via maspin and COX-2 regulation (Misra and Pizzo 2004). Finally, GRP78 knockdown arrested the cells in G1 phase accompanied by a decrease in S phase in pancreatic and renal cancer cells (Niu, Wang et al. 2015, Lin, Fang et al. 2014). In agreement with these works, we found that the reduction of GRP78 protein levels in CO-OCS-treated cells affected cell viability. Silencing of GRP78 with and without treatment decreased the percentage of cell number by the same manner, and no supplementary effect was measured. Furthermore, siGRP78 induced a significant increase in G1 phase and a slight but non-significant increase in G2M phase. No additional effect in siGRP78 transfected cells was measured in G1 phase after applying the treatment. Taken together, GRP78 may constitute an antiproliferative target of CO-OCS.

The expression of GRP78 has also been linked to chemotherapy sensitivity, in different types of cancer. Thus, in lung cancer, the low expression of GRP78 associated with the chemo-sensitivity (Wang, Wang et al. 2008), in breast cancer, it conferred resistance to chemotherapy (Lee, Nichols et al. 2006; Yeung, Kwan et al. 2008; Raiter, Yerushalmi et al.

2014; Kawiak, Domachowska et al. 2017) and in colon cancer cells where GRP78 knockdown enhanced the apoptosis in 5-Fluorouracil-resistant cells (Lee, Yoon et al. 2017). Further, targeting the COOH-terminus of GRP78 by polyclonal antibodies in melanoma and prostate cancer cells promoted apoptosis via p53 expression upregulation and inhibition of the Ras/MAPK and PI3K/Akt signalling pathways (Misra, Mowery et al. 2009). GRP78 can also regulate the necrotic cell death in some cell types; the knockdown of GRP78 was able to regulate the necroptotic cell death through modulation of NF- $\kappa$ B and AP-1 pathways in airway epithelial cells (Wang, Zhou et al. 2018). In addition, the inhibition of GRP78 by siRNA induced apoptosis and necrosis in cardiac myocyte (Wang, Bi et al. 2018). Our results clearly demonstrate that both CO-OCS and siGRP78 induced similar effects on the mortality, apoptosis and necrosis. The inhibition of GRP78 failed to enhance the effect of CO-OCS neither on the apoptosis nor on the necrosis, suggesting that GRP78 is the principal target of the anti-survival effect of CO-OCS.

- **Stim1 is a regulator of apoptotic cell death induced by CO-OCS in MCF-7 cells**

ER can induce apoptosis either by UPR dependent or independent ER stress-signaling (Sano and Reed 2013). Upon ER stress and UPR activation, GRP78 is a target of the UPR; where GRP78 expression is upregulated to increase ER-folding capacity as well as it is associated with pro-survival responses by protecting cells against ER stress-induced apoptosis. GRP78 overexpression leads to an inhibition in the activity of caspase-12, caspase-7, BIK and BAX, and prevents the release of cytochrome C from the mitochondria. Moreover, the dysregulation of calcium homeostasis can increase GRP78 level (Lamb, Mee et al. 2006). Stim1 is essential for ER calcium homeostasis and implicated in breast cancer development (Yang, Jiang et al. 2018). Furthermore, knockdown of Stim1 increased GRP78 expression and UPR (Selvaraj, Sun et al. 2012; Sun, Wei et al. 2017). The calcium increase in mitochondria initiates apoptotic cell death; GRP78 overexpression has a protective role in primary astrocytes exposed to ischemia via decreasing the mitochondrial calcium level (Ouyang, Xu et al. 2011). Moreover, the Bcl2 family proteins has a role in ER Ca<sup>2+</sup> homeostasis, and controls tightly the apoptotic cell death; Bcl2 overexpression prevents the decrease of ER Ca<sup>2+</sup> concentration (Baffy, Miyashita et al. 1993); Bcl2 inhibits the permeabilization of the mitochondrial membrane by blocking the Bax protein (Shi, Chen et al. 2003; McCommis and Baines 2012). The overexpression of Bcl-2 proteins especially the anti-apoptotic proteins (Bcl-xL, Mcl-1, and Bcl-w) have been reported in many cancer types; these proteins enhance

cancer cells resistant to chemotherapeutic treatment (Azmi, Wang et al. 2011; Mohammad, Muqbil et al. 2015; Radha and Raghavan 2017). Furthermore, apoptosis resistance in breast cancer is related to different factors that lead to dysregulation of the apoptotic signalling such as Bcl2 upregulation. The chemo-resistance in breast cancer was correlated to the over-expression or dysregulation of Bcl2 proteins (Booy, Henson et al. 2011). Stim1 inhibition by siRNA can up-regulate the expression of Bcl2 (Li, Chen et al. 2013). Also, knockdown of Stim1 promoted Cisplatin-induced apoptosis and ER stress in osteosarcoma (Sun, Wei et al. 2017). In contrast, it has been demonstrated that the silencing of Stim1 decreased apoptosis in cardiomyocytes induced by hypoxia/reoxygenation through increased the Bcl-2/Bax ratio and decreased cleaved caspase-3 expression (He, Wu et al. 2017). Here, we demonstrated that in MCF-7 cells, GRP78 and Bcl2 expression can regulate CO-OCS- induced apoptosis in MCF-7 cells transfected by siStim1. Indeed, CO-OCS alone induced apoptosis and necrosis. Silencing of Stim1 in MCF-7 cells had no effect on the survival, while treating siStim1 condition with CO-OCS leads to a strong decrease in CO-OCS-induced apoptosis without affecting the necrotic cell death. In fact, CO-OCS decreased GRP78 and Bcl2 expressions. The expressions of GRP78 and Bcl2 were increased in the siStim1 cells. Also, we measured an increase in both GRP78 and Bcl2 expressions in the cells transfected with siStim1 and treated with CO-OCS more than siCTL treated with CO-OCS alone. We linked these increases of GRP78 and Bcl2 expressions to the anti-apoptotic effect of Stim1. These results strongly suggest that Stim1 favors the apoptotic effect of CO-OCS and its silencing, by re-expressing GRP78 and Bcl2, antagonized the effect of CO-OCS.

- **Orai1 confers resistance against CO-OCS-necrotic cell death in MCF-7 breast cell line**

The molecular regulated necrotic cell death includes different forms (Pasparakis and Vandenabeele 2015). Necroptosis is caspase-independent, regulated by the kinase activity of RIP3 and MLKL proteins (Cho, Challa et al. 2009). However, necroptosis is the best characterized necrotic cell death cascade (Degterev, Huang et al. 2005) with marked depletion of cellular ATP. Necroptosis is also regulated by death receptors through the TNF-  $\alpha$  (Tumor Necrosis Factor  $\alpha$ ) and FAS ligand activation, that permits the interacting with RIP1. In the end, there are two signalling cascades depend on caspase-8 activation or inhibition. The activation of caspase-8 will induce apoptosis while the inhibition of caspase-8 will form necrosome due to RIP1 binding to RIP3; eventually, necroptotic cell death (Chen, Yu et al.

2016). Indeed, other stimuli can trigger necroptosis via activating RIP3 through either RIP1-dependent or -independent signalling, (McCullough, Martindale et al. 2001; He, Liang et al. 2011; Vanlangenakker, Vanden Berghe et al. 2011; Kaiser, Sridharan et al. 2013). Anyway, the poly ADP-ribose polymerase (PARP1) is able to regulate other necrotic cell death types which are initiated in response to different stimuli. PARP1-dependent necrosis is totally independent of RIP1 signalling cascade mainly through the activation of calpain proteases and ATP depletion (Yu, Wang et al. 2002; Cao, Xing et al. 2007; Moubarak, Yuste et al. 2007; Berghe, Linkermann et al. 2014). The work of Vaseva et al., have shown that the necrosis induced by oxidative stress was regulated via p53; where p53 triggers mitochondrial permeability transition pore and necrosis (Vaseva, Marchenko et al. 2012). Some of clinically used chemotherapeutic molecules such as Cisplatin, 5-Fluorouracil and Etoposide induce necroptotic cell death in different cancers through targeting different proteins in the necroptosis cascade (Koo, Morgan et al. 2015). In breast cancer cell line MCF-7-mitoxantrone-resistant, TNF- $\alpha$  is a mediator of caspase-independent cell death; Necrostatin-1 was able to antagonize TNF- $\alpha$ -induced necrotic cell death where TNF- $\alpha$  activated JNK and RIP1 phosphorylation (Ghandadi, Behravan et al. 2017). DNA-damaging molecules induce programmed necrotic cell death in breast cancer through RIP3 and MLKL activation (Koo, Morgan et al. 2015). Indeed, ER stress induces RIP1 caspase-independent necroptosis in L929 cells; however, in the cells treated with TNF- $\alpha$ , RIP1 inhibition allowed a switch from necroptotic to apoptotic death instead of protecting the cells (Saveljeva, Mc Laughlin et al. 2015). In contrast, a shift from apoptotic to necrotic cell death was observed in cervical cancer to word the highly transformed cells (Kravchenko-Balasha, Mizrachy-Schwartz et al. 2009).

Orai1 involvement in tumour progression has been demonstrated in several cancers including breast cancer (Yang, Zhang et al. 2009; McAndrew, Grice et al. 2011). The implication of Orai1 in apoptotic cell death in cancer range from apoptotic to anti-apoptotic roles; in prostate cancer cells, down-regulation of Orai1 in LNCaP cells grown in the absence of androgens reduces apoptosis induced by Thapsigargin, TNF- $\alpha$  or by chemotherapeutic agents such as Cisplatin or Oxaliplatin via mitochondrial and cytosolic mechanisms (Flourakis, Lehen'kyi et al. 2010); whereas others demonstrate that Orai1 silencing enhanced 5-Fluorouracil and Gemcitabine apoptotic effects (Kondratska, Kondratskyi et al. 2014). The inhibition of Orai1 by siRNA enhanced 5-FU-induced cell death (Tang, Xia et al. 2017) and was able to induce apoptosis in glioblastoma cells (Liu, Hughes et al. 2011). In this work, we



demonstrated that the inhibition of Orai1 was able to promote CO-OCS necrotic effect but not apoptosis. Despite that Orai1 inhibition has no effect on MCF-7 cell survival, Orai1 silencing accompanied with CO-OCS treatment induced much more necrosis when compared to CO-OCS alone. In the other hand, the inhibition of Orai1 increased GRP78 expression. However, not like Stim1, siOrai1 failed to restore GRP78 expression in MCF-7-treated cells. We investigate the necrotic signalling cascade induced by CO-OCS in MCF-7 through using two inhibitors of necrotic cell death. Necrostatin-1 which is an inhibitor of necroptosis and IM-54 which is an inhibitor of oxidative stress induced by ROS. Both of them are failed to affect CO-OCS induced necrosis.

- **Translocon activity triggers the necrotic and apoptotic cell death induced by CO-OCS**

The homeostasis between ER calcium uptake and leak is essential for different cellular processes. Besides its roles in protein translocation after translation and retrotranslocation of misfolded proteins for their degradation; Translocon (Sec61) is a passive calcium leak channel where the ER stressors and calcium chelators deplete the ER calcium store via Translocon (Flourakis, Van Coppenolle et al. 2006). This passive calcium leak can be regulated by different factors; the interaction between the Calmodulin and Sec61 $\alpha$  limits the calcium leakage from the endoplasmic reticulum (Erdmann, Schäuble et al. 2011). Sec62 seems to be also implicated, where the C-terminal EF-hand motif of Sec62 can mediate the calcium leak from the Sec61 (Linxweiler, Schorr et al. 2013). GRP78 seals the Sec61 in results to its association to the loop 7 of Sec61 $\alpha$ , thus inhibiting ER-calcium efflux (Schäuble, Lang et al. 2012); upon ER stress, GRP78 regulates the depletion of ER calcium stores; further, the degree of calcium leak via Translocon is correlated to GRP78 expression (Hammadi, Oulidi et al. 2013). Moreover, the ER calcium release via Translocon activates SOCE (Ong, Liu et al. 2007). SOCE via targeting its regulators has been linked to cancer survival; in some cell types, SOCE inhibition confers apoptosis resistance (Flourakis, Lehen'kyi et al. 2010; Huang, Ren et al. 2017) while in other types enhances cell death (Kondratska, Kondratskyi et al. 2014; Tang, Xia et al. 2017). It has been demonstrated that in prostate cancer the pharmacological inhibition of Translocon (by Anisomycin) managed to antagonise the inhibition of ER store depletion induced by the ER stressors as well as reduced apoptosis (Hammadi, Oulidi et al. 2013). Also, in malignant glioma cells, calcium leak inhibition through Translocon reduced cell death (Johnson, White et al. 2014). The pharmacological induction of necrotic and /or late apoptotic cell death in Jurkat and peripheral blood

lymphocyte cells was decreased after inhibiting the Translocon activity (Charles, Hammadi et al. 2017). In agreement with the finding reported in the other cell lines, Anisomycin reduced CO-OCS apoptotic and necrotic cell death in MCF-7 cells. These results clearly demonstrate that Translocon is an important calcium regulator of CO-OCS apoptotic and necrotic cell death in MCF-7.

- **Are ER Ca<sup>2+</sup> store and/or SOCE involved in CO-OCS necrotic or apoptotic effect?**

To answer this question, it is interesting to remind that calcium stores are involved both in calcium signalling and protein folding. Many cellular processes and different cellular regulators are involved in ER calcium storage, e.g., SERCA activity, mitochondrial ATP production, SOCE, and reticular calcium leak. In CO-OCS-treated MCF-7 cells, the observed ER stress is presumably linked to the impairment of any of these processes. Otherwise, in many studies where the UPR is triggered in different ways, a significant decrease in the reticular stores of calcium is always reported; Such ER depletion triggers apoptosis (Hammadi, Oulidi et al. 2013; Johnson, Kono et al. 2014). Our results showed that CO-OCS-induced ER calcium depletion in a time-dependent manner. As the apoptotic and necrotic effects of CO-OCS were not affected by a low calcium medium, we suggest the involvement of calcium reticular rather than SOCE. Recently, (Sehgal, Szalai et al. 2017) have reported, in different cell lines including MCF-7, that unfolded response induced by Thapsigargin is due to ER Ca<sup>2+</sup> than SOCE.

As Anisomycin suppressed both apoptotic and necrotic effect of CO-OCS, and as CO-OCS induced Ca<sup>2+</sup> ER leak, we investigate the potential involvement of the Translocon in CO-OCS response on ER Ca<sup>2+</sup> leak. To evaluate Ca<sup>2+</sup> leak through the Translocon, we used Anisomycin to maintain the Translocon in a closed state that reduces permeability to Ca<sup>2+</sup> (Hamman, Hendershot et al. 1998). Moreover, Hammadi et al. have reported a link between ER Ca<sup>2+</sup> leak via the Translocon, ER stress and apoptosis (Hammadi, Oulidi et al. 2013). We found that Anisomycin completely inhibited CO-OCS-induced ER Ca<sup>2+</sup> leak in MCF-7 cells incubated in both CO-OCS and Anisomycin during 72h.

Moreover, Anisomycin partially antagonised the decrease in SOCE induced by CO-OCS. These findings confirm that Translocon is a major target involved in the ER Ca<sup>2+</sup> leak induced by CO-OCS. Its inhibition has a protective mechanism via restoring completely the CO-OCS ER calcium homeostasis and partially SOCE.

More and more works show the multifaceted role of GRP78. Indeed, in addition to its chaperone role, it is a reticular luminal calcium-storage protein contributing, under resting cell conditions, to around 25% of the  $\text{Ca}^{2+}$  stores (Lievremont, Rizzuto et al. 1997). Moreover, during UPR response and ER stress, GRP78 modulates calcium releasing via modulating Translocon opening (Nico, Sven et al. 2012; Hammadi, Oulidi et al. 2013). Our results show that silencing of GRP78 alone increased  $\text{Ca}^{2+}$  ER leak; they support the idea that GRP78 contributes to reducing  $\text{Ca}^{2+}$  leakage from the ER probably through Translocon (Schauble, Lang et al. 2012). Surprisingly, we found that even when restoring the ER  $\text{Ca}^{2+}$ -induced by CO-OCS, GRP78 failed to affect its apoptotic or necrotic effect. Other experiments are then needed to elucidate the link between GRP78, ER  $\text{Ca}^{2+}$ , and the apoptotic and necrotic effects of CO-OCS.

Taken into account these results, we can conclude that CO-OCS induced reticular stress and UPR reaction subsequently to a  $\text{Ca}^{2+}$  leakage from the ER and that the Translocon constitutes one of the main targets involved in this CO-OCS induced-leak.

### ***Part III: Stim1, Orai1 and GRP78 proteins constitute specific targets in breast cancer cells but not in breast benign cells***

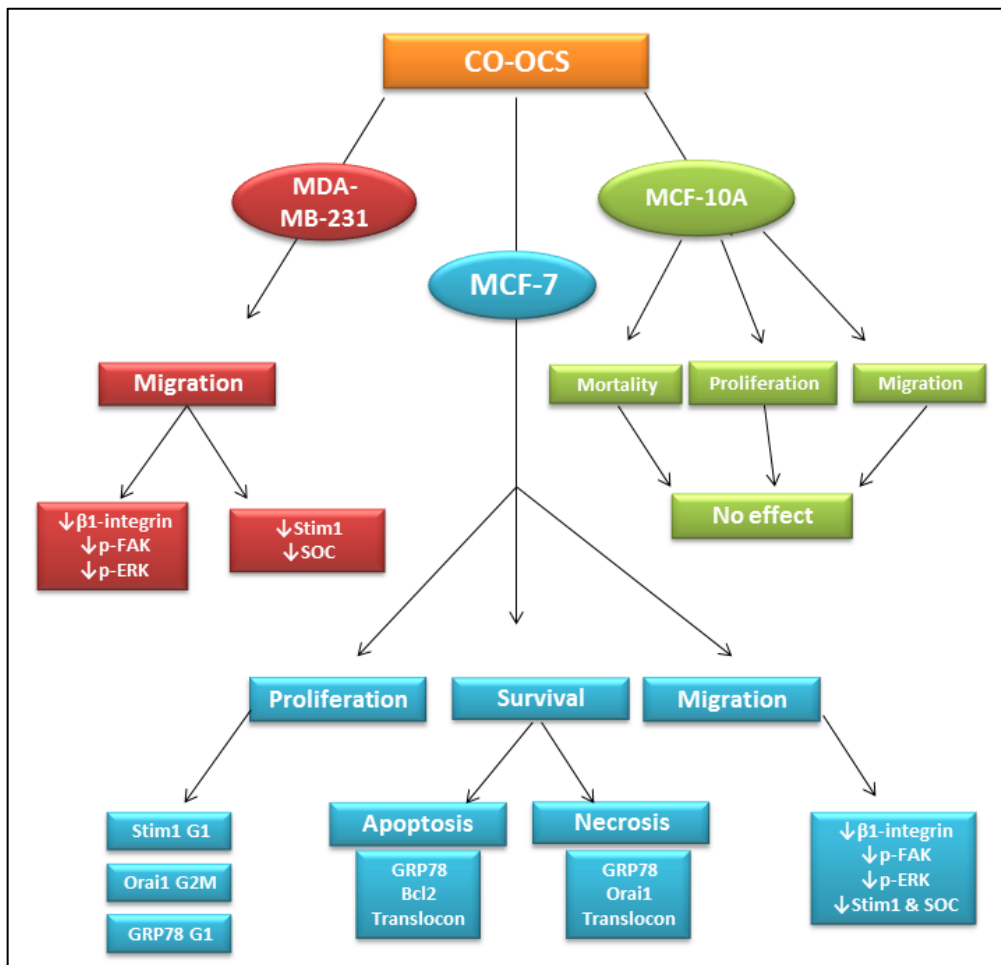
MCF-10A is an immortalized benign breast cell line derived from a benign fibrocystic mammary tissue (Soule, Maloney et al. 1990) and the most commonly used normal mammary cells in cancer studies. Previously published work from our laboratory has shown that CO-OCS does not affect the viability or mortality of normal MCF-10A cells (Allan, Ouadid-Ahidouch et al. 2013). In parallel with breast cancer cells experiments, we investigated the molecular pathways by which CO-OCS was able to induce different anti-tumours effects in breast cancer cell lines but not in the benign cell line MCF-10A. CO-OCS did not affect the expression of Stim1, Orai1 and GRP78. Indeed, Stim1, Orai1 and GRP78 are much higher expressed in breast cancer tissues than in normal tissues (Zhu, Zhang et al. 2014; Yao, Liu et al. 2015; Yang, Jiang et al. 2017). The knockdown of Stim1 and Orai1 has no effect on cell viability and mortality. Only siGRP78 was able to decrease cell viability without affecting the mortality. Moreover, CO-OCS failed to affect the ER calcium store and SOCE. These results demonstrate clearly that Stim1, Orai1 and GRP78 proteins constitutes important and specific targets in breast cancer cells but not in breast benign cells.

***General Conclusion and  
perspectives***

## General Conclusion and perspectives

During this work, we investigated the signalling pathways by which CO-OCS was able to induce antiproliferative, anti-migratory and anti-survival effects in the different breast cancer cell lines. The most important finding is that CO-OCS affect specifically breast cancer cells. CO-OCS, by altering the expression of different targets, reduced cells migration and proliferation and increased apoptosis and necrosis rate.

We can conclude our results in the illustration presented below (Figure 64):



**Figure 64.** Illustration demonstrates the effect of CO-OCS on breast cancer and normal mammary cells.

In MDA-MB-231 and MCF-7 breast cancer cell lines, CO-OCS inhibited migration through (i) the reduction of  $\beta$ 1-integrin expression and the inhibition of its signalling cascade FAK and ERK1/2; and (ii) the suppression of the expression of Stim leading to an inhibition of SOCE.

In MCF-7 breast cancer cell line, CO-OCS induced (i) antiproliferative effect via targeting Stim1, Orai1 and GRP78 causing a cell cycle arrest in G1 and G2M as well as inhibition in SOCE; (ii) apoptotic effect through decreasing the expression of GRP78 and Bcl2 and increasing the calcium leak from the ER store through the Translocon; (iii) necrotic effect through suppression the expression of GRP78 and partially by the depletion of the calcium level in the ER via the Translocon. Stim1 is an apoptotic target of CO-OCS, while Orai1 protects cells from its necrotic effect.

In the normal mammary cells MCF-10A, CO-OCS failed to induce any effect on the migration, proliferation and survival.

Our obtained results open the door to conduct more detailed experiments for more comprehensive of CO-OCS signalling pathways.

- **Is cell surface GRP78 implicated in CO-OCS-induced effects?**

GRP78 can be translocated from the ER to the plasma membrane. Conditions that mimic cancer environments could be initiators for GRP78 re-localization at the cell membrane. In some types of cancers, ER stress actively promotes cell surface localization of GRP78 after using ER-stress inducers such as TG and Tunicamycin (Zhang, Liu et al. 2010; Tsai, Zhang et al. 2015), however, in other cancers, the ER-stress inducer, Tunicamycin, did not manage to express the cell surface GRP78 (Serrano-Negrón, Zhang et al. 2018). Cell surface GRP78 has a critical survival role in cancer; where it upregulated in breast cancers tissues and cell lines (Yeung, Kwan et al. 2008; Yao, Liu et al. 2015). In fact, cell surface GRP78 expression was found in cancer cells but not in normal cells (Liu, Li et al. 2013; Zhang, Tseng et al. 2013; Yao, Liu et al. 2015). It has been demonstrated that the expression of cell surface GRP78 is essential in inducing the apoptosis by chemotherapy (Raiter, Yerushalmi et al. 2014). The upregulation of GRP78 on the cell membrane enhanced cancer cell proliferation, migration and apoptosis resistance (Misra, Mowery et al. 2009; Misra, Payne et al. 2011). Indeed, an anti-GRP78 antibody designed against the N-terminal blocked the cell surface GRP78 receptor function, leading to an inhibition of MCF-7 proliferation and migration via JAK2/STAT3 pathway (Yao, Liu et al. 2015). Also, the COOH-terminus of GRP78 seems to be involved in regulating the apoptosis in melanoma cells and prostate cancer cells (Misra, Mowery et al. 2009). Further, we will investigate the potential role of cell surface GRP78 in CO-OCS induced effect through overexpressing GRP78 on the surface and/ or an anti-GRP78 antibody against the N-terminal.

- **Evaluation the effect of CO-OCS on the UPR sensors**

Our results demonstrated the induction of ER stress and UPR by CO-OCS in MCF-7 cells through the upregulation of GRP78 and CHOP transcripts. IRE1 activation can conduct both proteasomal and lysosomal degradation pathways (Chiang, Messah et al. 2012). The UPR can be initiated through the activation of three diverse stress signaling pathways including activating transcription factor 6 (ATF6) (Haze, Yoshida et al. 1999), double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) (Harding, Zhang et al. 1999) and inositol-requiring kinase 1 (IRE1) (Tirasophon, Welihinda et al. 1998). We aim to complete these results with a more detailed study on the effect of CO-OCS on the activation of these three ER stress sensors and their downstream signalling pathways. It has been demonstrated that IRE1 activation can conduct both proteasomal and lysosomal degradation signalling pathways (Chiang, Messah et al. 2012). Thus, the role of IRE1 in CO-OC-induced protein degradation will be a subject for more investigations.

- **To investigate the role of ERAD type II in GRP78 and Orai1 degradation**

Two degradation mechanisms have been demonstrated in eukaryotic cells, ERAD type I (proteasome-dependent) and type II ( autophagy-lysosome dependent) (Rashid, Yadav et al. 2015). As our results demonstrated that the ubiquitin-proteasome-dependent ERAD type I is not implicated in GRP78 or in Orai1 degradation. Anyway, ERAD type II can undergo GRP78 degradation (Shi, Porter et al. 2009). Next, we will enlarge our study to include the autophagy-lysosome dependent ERAD type II through the cathepsin/calpain dependent degradation. We will evaluate the effect of CO-OCS on GRP78 degradation with and without an inhibitor of cathepsins. Then, we will study the involvement of ERAD type II in Orai1 degradation.

- **The potential role of PARP1 in regulating the necrotic effect of CO-OCS**

Recently, the necrotic cell death induced by different chemotherapy molecules has an emerging role in cancer research (Pasparakis and Vandenabeele 2015).The three main signalling pathways include (1) Necroptosis is caspase-independent (Cho, Challa et al. 2009), can be trigger through TNF-  $\alpha$  and FAS ligand activation which leads to RIP1, RIP3 and MLKL phosphorylation (Chen, Yu et al. 2016); (2) Necrosis induced by oxidative stress (Vaseva, Marchenko et al. 2012); (3) PARP1-dependent necrosis is initiated in response to

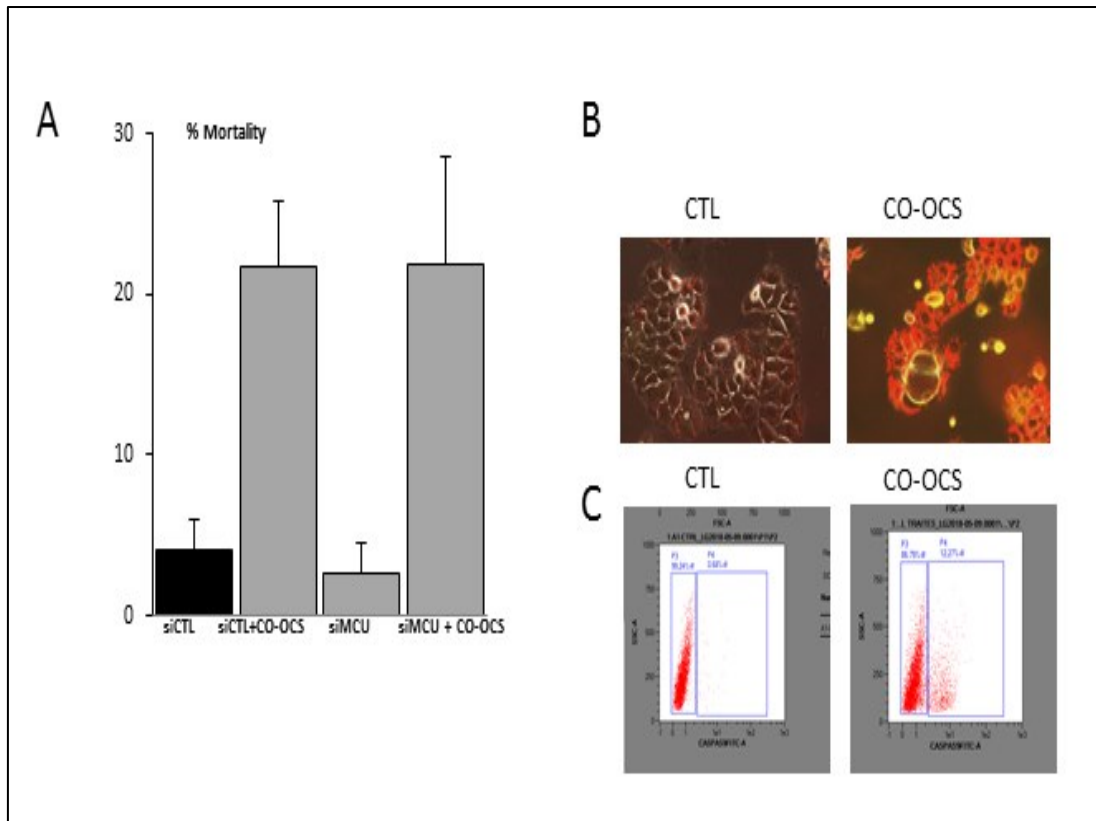
different stimuli leading to calpain activation (Yu, Wang et al. 2002; Cao, Xing et al. 2007; Moubarak, Yuste et al. 2007; Berghe, Linkermann et al. 2014). The results obtained demonstrated that CO-OCS-induced necrosis neither through RIP1 nor through ROS activation. To investigate PARP1-dependent necrosis, we will use two approaches, the effect of the PARP1 inhibitor on CO-OCS-necrotic cell death and the effect of CO-OCS on the expression of calpain.

- **Investigate the role of mitochondria and the mitochondrial calcium concentration in CO-OCS induced cell death**

Mitochondria have an essential role in initiating cell death through caspase-dependent or independent pathways through mitochondrial permeability transition pore (MPTP). ER stress leads to an increase in the number of contact sites and the length of ER-mitochondria-associated membranes (MAMs) (Marchi, Patergnani et al. 2014). Acute calcium release from the ER can induce  $\text{Ca}^{2+}$ -mediated mitochondrial cell death. Mitochondria can also initiate programmed necrosis through (1) the release of AIF (Apoptosis inducing factor) to the cytosol; (2) activation of the MPTP via BNIP3; (3) RIP kinases; and (4) the release of  $\text{Ca}^{2+}$  from the ER to the mitochondria via Nix (Baines 2010). MCU is thought to be essential in regulating mitochondria calcium uptake and breast cancer survival (Hall, Wu et al. 2014).

Our preliminary results (Figure 65) demonstrate that (A) siRNA MCU has no effect on MCF-7 survival alone and when combined with CO-OCS; (B) by using JC1 probe we tried to measure the mitochondrial membrane potential. However, we measured an increase in the red and green signals instead of a decrease; this unexpected false positive result could be explained as a result of the treatment, it seems that CO-OCS improve the coupling of the JC1 in the mitochondria. For that, another reagent selective for the mitochondria of live cells (DiOC6 (3)) will be used for further experiments. (C) The caspase 3 staining by flow cytometry demonstrated that the cell death induced by CO-OCS at least partially is caspase dependent. We are also interested in measuring the effect of CO-OCS on the concentration of calcium in the mitochondria via using a fluorescent dye-based ratiometric probe (Rhod-2) and confocal microscopy.





**Figure 65.** Preliminary results (A) effect of siMCU in MCF-7. (B) JC-1 Dye for Mitochondrial Membrane Potential. (3) Caspase-3 staining.

- **Can CO-OCS initiate other types of cell death in MCF-7 cells?**

Autophagy was linked to tumour initiation and development (Kisen, Tessitore et al. 1993). However, autophagy can initiate selective or non-selective degradation of different cellular molecules. Some anticancer molecules have exhibited autophagic cell death in breast cancer *in vitro* (Chen and Karantza 2011). In MCF-7 cells, Thapsigargin which is known to induce ER store depletion was able to initiate autophagy (Høyer-Hansen, Bastholm et al. 2007). Furthermore, in MCF-7 breast cancer cells, silencing of the anti-apoptotic Bcl2 protein initiates autophagic cell death, (Akar, Chaves-Reyez et al. 2008). In our results, CO-OCS was able to decrease the ER  $Ca^{2+}$  store depletion and the expression of Bcl2. Besides, we consider the involvement of the autophagy-lysosome-dependent ERAD in GRP78 and Orai1 degradation. Taken together, we hypothesize the induction of the autophagic cell death by CO-OCS in MCF-7 cells.

## ***Résumé de thèse***

## Introduction

Le cancer du sein étant le cancer le plus répandu chez les femmes, des efforts considérables ont été déployés au cours des dernières décennies pour comprendre ses voies de signalisation et trouver de nouveaux traitements pour ce type de cancer. En effet, le dépistage précoce ainsi que certains traitements disponibles ont considérablement amélioré les taux de survie du cancer du sein. Cependant, le cancer du sein reste un problème majeur de santé publique qui préoccupe grandement la communauté scientifique.

La glycosylation est un processus fondamental chez les eucaryotes. Elle peut affecter, d'une part, le repliement, la stabilité, l'activité, la localisation, la sécrétion et la solubilité, des protéines et, d'autre part, les interactions entre cellules. Les aberrations de la glycosylation sont l'une des caractéristiques de la cellule cancéreuse. De plus, les précurseurs anormaux des glycanes, ainsi que leurs implications et leurs rôles fonctionnels dans la progression du cancer ont été largement étudiés et leurs impacts sur le cancer approuvés. Les changements de précurseurs des glycanes sont liés au processus cancéreux de plusieurs manières. L'expression des glycosyltransférases et des glycosidases (enzymes spécifiques impliqués dans la N-glycosylation) sont, notamment, altérés dans de nombreux cancers. Cependant, les protéines N-glycosylées impliquées dans le développement du cancer du sein pourraient être des cibles potentielles des inhibiteurs de la glycosylation.

Différents inhibiteurs de la glycosylation tels que la Tunicamycine, la Castanospermine et la Swainsonine possèdent des activités anticancéreuses *in vitro* et *in vivo*. Cependant, ces molécules induisent une inhibition généralisée de la glycosylation. Leurs effets toxiques ont mis fin aux essais cliniques de la Swainsonine chez les patients atteints du carcinome rénal.

La synthèse de nouvelles générations d'inhibiteurs de la glycosylation ayant une sélectivité et une spécificité accrues est une alternative aux effets toxiques des précédents inhibiteurs. C'est le cas du 1-C-octyl-2-oxa-3-oxocastanospermine II sp<sup>2</sup>-iminosugare «**CO-OCS**», inhibiteur de l' $\alpha$ -glucosidase avec un  $K_i=0,87 \mu\text{M}$  (Sánchez-Fernández Elena, Rísquez-Cuadro et al. 2012). De plus, CO-OCS a également démontré des effets anti tumoraux dans les lignées cellulaires du cancer du sein MCF-7 et MDA-MB-231 sans affecter la lignée cellulaire mammaire normale MCF-10A (Allan, Ouadid-Ahidouch et al. 2013). La valeur de l' $IC_{50}$  après 72 h de traitement pour MCF-7 et MDA-MB-231 est respectivement de

26  $\mu\text{M}$  et 44  $\mu\text{M}$ . Cependant, les voies de signalisation du CO-OCS ainsi que ses principales cibles n'ont toujours pas fait l'objet d'études et demeurent, de ce fait, inconnues.

## **Objectifs**

Dans ce travail réalisé en collaboration avec « Instituto de Investigaciones Químicas (IIQ), CSIC - Universidad de Sevilla, Espagne » nous avons étudié :

- Les mécanismes moléculaires par lesquels CO-OCS induit ses effets anti tumoraux sur la migration, la prolifération et la survie cellulaires et déterminer son principal effet sur les protéines N-glycosylées connues pour leur implication dans le développement du cancer du sein.

- L'effet de CO-OCS sur La réponse au stress lié à l'accumulation des protéines mal repliées (UPR) dans le réticulum endoplasmique des cellules cancéreuses du sein.

Nous avons choisi de travailler sur deux lignées cellulaires du cancer du sein présentant des caractéristiques différentes (MCF-7 et MDA-MB-231). La lignée cellulaire mammaire normale immortalisée MCF-10A est choisie comme témoin. Selon les expériences, différentes concentrations de CO-OCS (26, 40 et 100  $\mu\text{M}$ ) ont été utilisées pendant plusieurs durées de traitement (24, 48 et 72 h).

## **Résultats**

**1°- CO-OCS inhibe la migration des cellules cancéreuses du sein via deux voies de signalisations, l'une dépendante et l'autre indépendante du calcium.**

La première partie des résultats obtenus est basée sur l'investigation du mécanisme lié à l'effet anti-migratoire du CO-OCS dans le cancer du sein. Nous avons démontré que CO-OCS altérerait la migration du cancer du sein par deux mécanismes distincts; un mécanisme de signalisation indépendante du calcium où CO-OCS inhibe l'expression de la  $\beta 1$ -intégrine qui à son tour active les protéines FAK et ERK1 /2. Cependant, nous avons également présenté un mécanisme dépendant du calcium, principalement obtenu via le ciblage de l'expression de la protéine Stim1, conduisant à l'inhibition de SOCE. Fait intéressant, CO-OCS n'a eu aucun effet sur l'expression à la fois de la  $\beta 1$ -intégrine et de Stim1. De plus, les cascades de

signalisation FAK et ERK1 / 2 n'ont pas été activées dans la lignée cellulaire mammaire normale MCF-10A.

Les métastases du cancer vers d'autres organes sains constituent un problème majeur de santé publique responsable d'environ 90% des décès par cancer (Seyfried and Huysentruyt 2013). La glycosylation aberrante peut affecter l'expression et la fonction de nombreuses protéines cellulaires ainsi que le développement du cancer et des métastases. La localisation des cellules cancéreuses dans les tissus distants est régulée par différentes protéines et cascades de signalisation cellulaire. L'intégrine  $\beta 1$  est l'une des protéines qui régulent le processus de migration dans un certain nombre de modèles tumoraux, notamment les cancers du sein, de l'ovaire, du pancréas et de la peau (Blandin, Renner et al. 2015). L'intégrine  $\beta 1$  joue un rôle essentiel au cours des différentes étapes de la métastase tumorale, notamment l'invasion, la migration, l'intravasation, l'extravasation et la dissémination. De plus, L'intégrine  $\beta 1$  est régulée positivement dans le cancer du sein et est corrélée aux métastases ganglionnaires et à un pronostic médiocre. Il a été démontré que l'inhibition de l'expression de l'intégrine  $\beta 1$  dans les cellules MDA-MB-231 altérait la migration cellulaire (Hou, Isaji et al. 2016). Les intégrines peuvent réguler les métalloprotéinases matricielles (MMP), provoquant la dégradation et le remodelage de la matrice extracellulaire (Borrirukwanit, Pavasant et al. 2014; Missan, Mitchell et al. 2015). En outre, elle est capable d'activer les protéines FAK et ERK1 / 2, et donc, de modifier le processus de migration. De plus, la dérégulation de la glycosylation et des glycoprotéines anormales est liée à la tumorigenèse. L'intégrine  $\beta 1$  est une protéine N-glycosylée sur laquelle il existe 12 sites de glycosylation potentiels situés sur le domaine extracellulaire. L'élévation de la ramification  $\beta 1,6$  des oligosaccharides liés à l'azote est une forme glycosylée altérée courante dans le cancer, elle est liée à l'inhibition du regroupement de l'intégrine  $\alpha 5\beta 1$  et à la stimulation de la migration cellulaire (Guo, Lee et al. 2002). Les sites  $\beta 1$  de glycosylation de l'intégrine le rendent approprié pour être affecté par les inhibiteurs de la glycosylation. Le traitement des cellules d'adénocarcinome du côlon de rat avec la Castanospermine a affecté l'adhésion de la  $\beta 1$ -intégrine à la fibronectine (Ringear, Harb et al. 1996). En outre, la tunicamycine a inhibé l'adhésion et la migration des cellules d'hépatocarcinome en régulant négativement l'expression de l'intégrine  $\beta 1$  à la surface cellulaire (She, Xu et al. 2010). Nous avons constaté que le CO-OCS affectait de manière significative la capacité de migration du cancer du sein. De plus, nous avons étudié l'effet du CO-OCS sur l'expression de l'intégrine  $\beta 1$  au niveau de la transcription et des protéines, et avons constaté une diminution de la transcription et de l'expression protéique.

Dans les complexes d'adhésion focale, la protéine FAK est principalement activée par l'activation et le regroupement d'intégrines qui provoquent des cascades de signalisation intracellulaire capables de moduler la migration cellulaire (Sawai, Okada et al. 2005). De plus, la migration du cancer du sein et les métastases osseuses dépendent fortement de la protéine FAK qui active à son tour la protéine ERK1 / 2 (Wu, Wang et al. 2018). L'augmentation du taux de calcium intracellulaire conduit à l'activation de ERK1 / 2 *via* sa phosphorylation et augmente la migration des cellules du cancer du sein *in vitro* (Di, Huang et al. 2015). Nos résultats ont démontré que CO-OCS inhibe la phosphorylation des protéines de signalisation FAK et ERK1 / 2 dans le cancer du sein.

Les protéines Stim1 et Orai1 sont impliquées dans la migration des cellules cancéreuses du sein. L'invalidation par siRNA des protéines Stim1 et Orai1 inhibe la migration cellulaire et les métastases *in vitro* sur modèle murin en régulant la protéine FAK (Yang, Zhang et al. 2009). McAndrew et ses collaborateurs ont démontré une corrélation significative entre l'expression de Stim1 dans le cancer du sein et son agressivité et potentiel métastatique (McAndrew, Grice et al. 2011). Un autre travail a montré que, dans les cellules MDA-MB-231, l'inhibition de l'expression de Stim1 inhibait de manière significative la migration en réduisant les invadopodes et la formation de podosomes (Chen, Chen et al. 2017). De plus, l'inhibition de l'entrée capacitive du  $Ca^{2+}$  (SOCE) interfère avec la migration des cellules du cancer du sein dans lequel SOCE est médiée par les protéines Stim1 et Orai1 (Yang, Zhang et al. 2009). Dans les cellules MDA-MB231, Stim1 et Orai1 sont les principaux médiateurs de SOCE (Motiani, Abdullaev et al. 2010). Dans nos conditions, CO-OCS réduit l'expression de Stim1 sans affecter l'expression d'Orai1 dans les cellules MDA-MB-231. En revanche, CO-OCS n'affecte pas le niveau de transcription de Stim1, suggérant des modulations post-traductionnelles. De plus, CO-OCS est en mesure d'inhiber de façon significative le courant SOCE via une diminution de l'expression protéique de Stim1. Enfin, nous avons démontré que dans les MCF-10A, CO-OCS a un léger effet sur la migration cellulaire sans effet significatif sur la prolifération cellulaire, aucun effet sur l'expression de l'intégrine  $\beta 1$ , le taux de phosphorylation de FAK et ERK1 / 2 et les expressions de Orai1 et Stim1, et donc aucun effet sur le courant SOCE n'a été enregistré. Nous avons également étudié l'effet du CO-OCS sur la migration cellulaire et l'expression de Stim1 et de l'intégrine  $\beta 1$  sur d'autres lignées cellulaires. Ainsi, nous avons constaté que CO-OCS induit une inhibition de la migration cellulaire ainsi que de l'expression de Stim1 et de l'intégrine  $\beta 1$  dans les cellules de cancers du mélanome, du sein et du pancréas

## **2°- CO-OCS induit l'augmentation de la mortalité cellulaire et des effets antiprolifératifs dans les cellules MCF-7 en ciblant les protéines GRP78, Orai1 et Stim1.**

Près de la moitié des protéines subissent une glycosylation dans le réticulum endoplasmique. La glycosylation aberrante est une caractéristique du cancer (Janik, Lityńska et al. 2010). De plus, le ciblage de la glycosylation peut induire un stress dans le réticulum endoplasmique (RE) et perturber ses fonctions conduisant à la réponse UPR (Wang, Wang et al. 2015). Orai1 et Stim1 sont les composants principaux des canaux SOC, de plus, elles sont des protéines N-glycosylées (Williams, Manji et al. 2001; Gwack, Srikanth et al. 2007), impliquées dans l'homéostasie calcique au niveau du réticulum endoplasmique. Par ailleurs, le GRP78 est l'une des protéines de stockage du  $Ca^{2+}$  dans le RE (Lievremont, Rizzuto et al. 1997). GRP78 a besoin de  $Ca^{2+}$  pour être activé et est impliqué dans le stress de RE (Lamb, Mee et al. 2006). CO-OCS est un inhibiteur des glucosidases I et II (Sánchez-Fernández Elena, Rísquez-Cuadro et al. 2012) qui réduit la prolifération cellulaire et augmente l'apoptose (Allan, Ouadid-Ahidouch et al. 2013). Cependant, ses mécanismes d'action restent inconnus. Dans ces travaux, nous montrons que CO-OCS: (i) diminue l'expression protéique de GRP78, Stim1 et Orai1 sans affecter l'expression de leurs ARNm, diminue la phosphorylation de ERK1 / 2 et AKT, et que seule l'expression de Stim1 revient à son niveau initial lorsque le protéasome est inhibé par un inhibiteur de protéasome; (ii) CO-OCS a également montré un effet supplémentaire sur la prolifération cellulaire et sur les phases du cycle cellulaire à 72h de traitement dans les cellules transfectées avec siStim1 ou siOrai1 mais pas dans les cellules transfectées avec siGRP78. (iii) L'effet apoptotique de CO-OCS était complètement antagonisé lors de la transfection de siStim1 mais pas de siOrai1 ou de siGRP78. En revanche, son effet nécrotique est accru lorsque l'expression de Orai1 est inhibée, mais pas lorsque Stim1 ou GRP78 sont invalidées. (iv) L'inhibiteur du translocon a partiellement inversé la mortalité cellulaire apoptotique et nécrotique induite par CO-OCS, néanmoins l'inhibiteur de la nécroptose et l'inhibiteur du stress oxydatif reste sans effet sur la mortalité cellulaire. (v) CO-OCS réduit la réponse TG au niveau du RE et la SOCE. En effet, la réponse TG est restaurée dans les cellules transfectées avec siGRP78, siStim1 ou traitée avec l'Anisomycine, mais pas dans les cellules transfectées avec siOrai1. Cependant, un effet supplémentaire dans les cellules transfectées avec siGRP78, siOrai1 et traitées avec CO-OCS a été observé sur le SOCE mais pas dans la condition impliquant siStim1. Enfin, la réduction de la concentration de  $Ca^{2+}$  extracellulaire dans le milieu a montré une légère augmentation de l'effet apoptotique du CO-OCS sans effet significatif sur la nécrose. Dans l'ensemble, nos

résultats ont démontré que le CO-OCS induisait un stress réticulaire conduisant à l'activation de l'UPR et de la dégradation de la protéine associée au réticulum (ERAD) qui a son tour induit un arrêt du cycle cellulaire et une induction de l'apoptose et la nécrose. Il semble évident que Stim1 et Orai1 ne sont impliqués que dans l'effet antiprolifératif de CO-OCS, probablement en réduisant SOCE. Bien que GRP78 soit impliqué dans les effets à la fois apoptotiques et nécrotiques probablement via une fuite de  $\text{Ca}^{2+}$  réticulaire à travers le canal Translocon. De plus, Stim1 constitue une cible par laquelle CO-OCS induit son effet apoptotique, alors qu'Orai1 protège du stress réticulaire induit par CO-OCS conduisant à la réponse UPR et à l'ERAD dans les cellules MCF-7.

Nos résultats montrent clairement que le CO-OCS à 40  $\mu\text{M}$  augmente significativement l'expression de l'ARNm de GRP78 à 72 h tout en inhibant l'expression de GRP78 au niveau de la protéine à 48 et 72 h, il semble que l'effet du CO-OCS sur l'expression de la protéine GRP78 soit dû à l'activation de la dégradation de la protéine, non à une atténuation ou à une inhibition pharmacologique directe de l'ARNm de GRP78. Par ailleurs, parallèlement à l'inhibition de la protéine GRP78, l'expression de l'ARNm de CHOP augmente à 48 et 72 h, ce qui indique l'activation de l'UPR. Ces deux effets (l'activation de la dégradation des protéines et l'activation de l'UPR), sont presque arrivés en parallèle dans les MCF-7 traitées avec 40  $\mu\text{M}$  de CO-OCS à la suite d'un stress réticulaire aigu.

L'activation de l'UPR permet un meilleur repliement des protéines en régulant positivement les protéines chaperonne, et atténue la traduction protéique ainsi que l'activation de l'ERAD. L'ERAD peut inclure deux mécanismes de dégradation: le type I, qui est un ERAD dépendant de l'ubiquitine-protéasome, et le type II, qui est un ERAD dépendant de l'autophagie et des lysosomes (Rashid, Yadav et al. 2015). La dégradation de GRP78 induite par CO-OCS peut résulter de l'activation d'ERAD de type I ou II. Il a été démontré que la dégradation du GRP78 pouvait être induite soit par un ERAD de type I dépendant de l'ubiquitine-protéasome (Kosakowska-Cholody, Lin et al. 2014), soit par un ERAD de type II dépendant de la cathepsine / calpaïne (Shibue and Weinberg 2009). Dans les cellules MCF-7, l'incapacité de la MG-231 à restaurer la dégradation de GRP78 induite par CO-OCS suggère que l'ERAD de type I dépendant de l'ubiquitine-protéasome n'est pas impliqué; en désaccord avec les résultats obtenus par (Kosakowska-Cholody, Lin et al. 2014).

CO-OCS a induit également des modifications post-transcriptionnelles au niveau de la protéine affectant l'expression des ARNm de Stim1 et Orai 1 après 48h de traitement. Au bout



de 72 h de traitement, CO-OCS diminue l'expression des transcrits d'Orai1 et Stim1. Contrairement à GRP78 et Orai1, nous avons démontré que seule l'activation de l'ERAD de type I entraînait la dégradation de la protéine Stim1, MG-231 pouvant alors restaurer l'expression de Stim1 une fois appliqué avec CO-OCS.

ERAD est également caractérisé par une diminution de la phosphorylation de ERK 1/2 et d'Akt (Qin, Wang et al. 2010; Galluzzi, De Santi et al. 2012; Mulay, Wood et al. 2013). Nous avons montré que le CO-OCS, après 72 heures de traitement, diminue l'activation d'Akt et d'ERK1 / 2, confirmant ainsi l'implication d'ERAD.

### **3°- Stim1, et Orai1 sont impliqués dans l'effet antiprolifératif de CO-OCS**

Le signal calcique joue un rôle critique dans la progression du cycle cellulaire au cours des différentes phases (G1, S et G2 / M) (Santella 1998), la progression du cycle cellulaire est principalement régulée par les voies calcium / calmoduline (Kahl and Means 2003; Choi and Husain 2006). De plus, il a été démontré que la SOCE était nécessaire pour la transition G1 / S via la SOCE médiée par Stim1-Orai1 (Chen, Chen et al. 2016). La suppression de Stim1 induit également un arrêt du cycle cellulaire en phase G1 dans une les cellules de glioblastome (Li, Zhang et al. 2013). En revanche, un autre travail a démontré que Stim1 et Orai1 inhibait la prolifération et arrêtaient les cellules en phase G2 / M (Abdullaev, Bisaillon et al. 2008). Le renversement de Orai1 a significativement augmenté le nombre de cellules dans G2 / M et a diminué la prolifération de cellules HEK293 (Borowiec, Bidaux et al. 2014). De plus, l'inhibition pharmacologique et l'inactivation d'Orai1 ou Stim1 induisent des effets anti-prolifératifs dans le carcinome à cellules claires des cellules rénales (Kim, Lkhagvadorj et al. 2014). L'utilisation de siOrai1 inhibe de manière significative la prolifération cellulaire dans les carcinomes pulmonaires (Zhan, Zhong et al. 2015). Il a été démontré que TGF- $\beta$  diminue l'expression de Stim1, entraînant une inhibition de SOCE induisant une diminution de la prolifération des cellules cancéreuses du sein (Cheng, Wang et al. 2016). Des travaux récents ont montré que Stim1 est une cible directe du miR-223 dans les lignées cellulaires du cancer du sein. Mais également que les spécimens tumoraux de patientes atteintes du cancer du sein dans lesquels miR-223 se lie au 3'-UTR de Stim1 et inhibe l'expression de Stim1, prolifèrent de façon moins importante (Yang, Jiang et al. 2018). Sur cette base, nous avons examiné l'implication potentielle d'Orai1 et Stim1 dans l'effet antiprolifératif du CO-OCS. En effet, la réduction de l'expression de Stim1 et Orai1, ou encore, l'application de CO-OCS seul a démontré que siStim1 ou siOrai1 arrête les cellules MCF-7 dans les phases G1 et G2/M du

cycle cellulaire. En revanche le traitement par CO-OCS pendant 72h conduit à l'arrêt des cellules dans les deux phases G1 et G2M. Cependant, l'accumulation cellulaire dans la phase G1 est plus importante qu'en G2/M. L'application d'une combinaison de siStim1 ou siOrai1 et de CO-OCS pendant 72h a augmenté le nombre de cellules en phase G1 dans la condition concernant Stim1, tandis que dans le cas d'Orai1, aucune augmentation significative du nombre de cellule en G2/M n'a été mesurée. Fait intéressant, ni siStim1 ni siOrai1 n'ont affecté la mortalité cellulaire. Clairement, il ressort de nos résultats que CO-OCS, en modifiant l'expression de Stim1 et Orai1, accumule les cellules respectivement dans les phases G1 et G2 / M.

#### **4°- GRP78 est impliquée dans les effets antiprolifératif, apoptotique et nécrotique de CO-OCS dans les cellules MCF-7.**

GRP78 est une protéine multifonctionnelles, elle est la principale protéine chaperonne, régule le stress et initie l'UPR. En outre, GRP78 joue un rôle essentiel dans la survie du cancer, une régulation positive de l'expression de GRP78 a été rapportée dans divers tissus et lignées de cellules cancéreuses (Koomagi, Mattern et al. 1999; Daneshmand, Quek et al. 2007; Ulianich and Insabato 2014). Le rôle du GRP78 dans le cancer a été associé à une augmentation de l'agressivité, de l'invasion, de la résistance à la chimiothérapie et à un mauvais pronostic (Reddy, Mao et al. 2003; Yeung, Kwan et al. 2008; Ren, Chen et al. 2017). De plus, l'expression de GRP78 à la surface des cellules a été rapportée dans les cellules cancéreuses mais pas dans les tissus bénins ni dans les cellules immortalisées (Liu, Li et al. 2013; Zhang, Tseng et al. 2013; Yao, Liu et al. 2015). L'expression élevée de GRP78 est associée à un mauvais taux de survie dans le cancer du sein (Zheng, Cao et al. 2014). Dans la lignée cellulaire de cancer du sein MCF-7, la surexpression de GRP78 augmente significativement la prolifération et la migration des cellules. En effet, un anticorps anti-GRP78 conçu contre l'extrémité N-terminale bloque la fonction du récepteur GRP78 à la surface des cellules entraînant une inhibition de la prolifération et de la migration de MCF-7 (Yao, Liu et al. 2015). Ce dernier travail a démontré que la voie JAK2 / STAT3 induisait l'effet produit par la GRP78 à la surface des cellules (Yao, Liu et al. 2015). Le renversement de GRP78 a permis de sensibiliser la formation de colonies de MCF-7 à la chimiothérapie (Wang, Yin et al. 2009) et de réduire la prolifération de cellules cancéreuses de la prostate via la régulation par maspin et COX-2 (Misra and Pizzo 2004). Enfin, l'inhibition de l'expression de GRP78 a arrêté les cellules en phase G1 accompagnées d'une diminution de la phase S

dans les cellules cancéreuses du pancréas et du rein (Fang, Liu et al. 2014; Niu, Wang et al. 2015). En accord avec ces travaux, nous avons constaté que la réduction des niveaux de protéine GRP78 dans les cellules traitées au CO-OCS affectait la viabilité cellulaire. La suppression de GRP78 avec et sans traitement a diminué le nombre de cellules de la même manière et aucun effet supplémentaire n'a été mesuré. En outre, siGRP78 induit une augmentation significative du nombre de cellule dans la phase G1 et une augmentation légère mais non significative du nombre de cellule en G2 /M. Aucun effet supplémentaire dans les cellules transfectées avec siGRP78 n'a été mesuré en phase G1 après l'application du traitement par CO-OCS. C'est pourquoi, GRP78 peut constituer une cible antiproliférative du CO-OCS.

L'expression de GRP78 a également été liée à la sensibilité à la chimiothérapie dans différents types de cancer (Lee, Nichols et al. 2006; Wang, Wang et al. 2008; Yeung, Kwan et al. 2008; Raiter, Yerushalmi et al. 2014; Kawiak, Domachowska et al. 2017) et dans les cellules cancéreuses du côlon où la suppression du GRP78 a renforcé l'apoptose des cellules résistantes au 5-fluorouracile (Lee, Yoon et al. 2017). En outre, cibler l'extrémité COOH de GRP78 au moyen d'anticorps polyclonaux dans des cellules de mélanome et de cancer de la prostate a favorisé l'apoptose via une régulation positive de l'expression de p53 et une inhibition des voies de signalisation Ras / MAPK et PI3K / Akt (Misra, Mowery et al. 2009). De plus, GRP78 peut également réguler la mort cellulaire nécrotique dans certains types de cellules, l'inactivation de GRP78 a permis de réguler la mort cellulaire nécroptotique par la modulation des voies de NF-KB et AP-1 dans les cellules épithéliales des voies aériennes (Wang, Zhou et al. 2018). En outre, l'inhibition de GRP78 par l'apoptose et la nécrose induites par le siARN a été observée dans les myocytes cardiaques (Wang, Bi et al. 2018). Nos résultats démontrent clairement que le CO-OCS et le siGRP78 ont induit des effets similaires sur la mortalité, l'apoptose et la nécrose. L'inhibition de GRP78 n'a pas permis d'augmenter l'effet du CO-OCS sur l'apoptose ni sur la nécrose, ce qui suggère que GRP78 est la cible principale de l'effet anti-survie induit par CO-OCS.

#### **5°- Stim1 est un régulateur de l'apoptose induite par CO-OCS dans les cellules MCF-7.**

L'inhibition de Stim1 a augmenté l'expression de GRP78 et induit l'UPR (Selvaraj, Sun et al. 2012; Sun, Wei et al. 2017) et l'augmentation du calcium dans les mitochondries initie la mort cellulaire par apoptose. La surexpression de GRP78 joue un rôle protecteur chez

les astrocytes primaires exposés à une ischémie en diminuant le taux de calcium mitochondrial (Ouyang, Xu et al. 2011). De plus, les protéines de la famille Bcl2 jouent un rôle dans l'homéostasie calcique dans le RE et contrôlent étroitement la mort cellulaire par l'apoptose. En fait, la surexpression de Bcl2 empêche la diminution de la concentration de  $Ca^{2+}$  au niveau du RE (Baffy, Miyashita et al. 1993) et Bcl2 inhibe la perméabilisation de la membrane mitochondriale en bloquant la protéine Bax (Shi, Chen et al. 2003; McCommis and Baines 2012). La surexpression des protéines Bcl-2, en particulier des protéines anti-apoptotiques (Bcl-xL, Mcl-1 et Bcl-w) a été rapportée dans de nombreux types de cancer, ces protéines renforcent les cellules cancéreuses résistantes au traitement chimiothérapeutique (Azmi, Wang et al. 2011; Mohammad, Muqbil et al. 2015; Radha and Raghavan 2017). En outre, la résistance à l'apoptose dans le cancer du sein est liée à différents facteurs conduisant à une dérégulation de la signalisation apoptotique telle que l'augmentation de l'expression de Bcl2. La chimiorésistance dans le cancer du sein est corrélée à la surexpression ou au dérèglement des protéines Bcl2 (Booy, Henson et al. 2011). L'inhibition de Stim1 par le siARN peut réguler positivement l'expression de Bcl2 (Li, Chen et al. 2013). En outre, l'inactivation de Stim1 favorise l'apoptose induite par la cisplatine et le stress réticulaire dans l'ostéosarcome (Sun, Wei et al. 2017). En revanche, il a été démontré que la suppression de Stim1 diminuait l'apoptose des cardiomyocytes induite par l'hypoxie / réoxygénation en augmentant le rapport Bcl-2 / Bax et en diminuant l'expression de la caspase-3 clivée (He, Wu et al. 2017). Ici, nous avons démontré que dans les cellules MCF-7 l'expression de GRP78 et Bcl2 peut réguler l'apoptose induite par CO-OCS dans les cellules MCF-7 transfectées par siStim1. En effet, seul CO-OCS induit l'apoptose et la nécrose. La désactivation de Stim1 dans les cellules MCF-7 n'a eu aucun effet sur la survie, tandis que le traitement de cellules MCF-7 transfectées avec siStim1 par CO-OCS entraîne une forte diminution de l'apoptose induite par CO-OCS sans affecter la mort cellulaire nécrotique. En effet, CO-OCS diminue l'expression de GRP78 et Bcl2. L'expression de GRP78 et Bcl2 sont augmentées dans les cellules transfectées avec siStim1. En outre, nous avons mesuré une augmentation de l'expression, à la fois de GRP78 et de Bcl2 dans les cellules transfectées avec siStim1 et traitées avec CO-OCS davantage qu'en condition siCTL traité avec CO-OCS seul. Nous avons lié ces augmentations d'expressions de GRP78 et Bcl2 à l'effet anti-apoptotique de Stim1. Ces résultats suggèrent fortement que Stim1 favorise l'effet apoptotique de CO-OCS et son inhibition en ré-exprimant GRP78 et Bcl2 inverse l'effet apoptotique de CO-OCS.

## **6°- Orai1 confère une résistance à la mort cellulaire par nécrose dans les cellules MCF-7 traitées par CO-OCS.**

La mort cellulaire nécrotique à régulation moléculaire comprend différentes formes (Pasparakis and Vandenabeele 2015). La nécroptose est indépendante de la caspase et régulée par l'activité kinase des protéines RIP3 et MLKL (Cho, Challa et al. 2009). Cependant, la nécroptose est la cascade de mort cellulaire nécrotique la mieux caractérisée (Degterev, Huang et al. 2005) avec un appauvrissement marqué en ATP cellulaire. La nécroptose est également régulée par les récepteurs de mort via l'activation du TNF- $\alpha$  (facteur de nécrose tumorale) et du ligand FAS, qui permet l'interaction avec RIP1. Au final, il y a deux cascades de signalisation qui dépendent de l'activation ou de l'inhibition de la caspase-8. L'activation de la caspase-8 induira l'apoptose tandis que l'inhibition de la caspase-8 formera un nécrosome en raison de la liaison de RIP1 à RIP3 et finalement la mort cellulaire nécroptotique (Chen, Yu et al. 2016). En effet, d'autres stimuli peuvent déclencher une nécroptose via l'activation de RIP3 via une signalisation dépendante ou indépendante du RIP1 (McCullough, Martindale et al. 2001; He, Liang et al. 2011; Vanlangenakker, Vanden Berghe et al. 2011; Kaiser, Sridharan et al. 2013). Quoi qu'il en soit, la poly ADP-ribose polymérase (PARP1) est capable de réguler d'autres types de mort cellulaire nécrotique qui sont initiés en réponse à différents stimuli. La nécrose dépendante de PARP1 est totalement indépendante de la cascade de signalisation RIP1, principalement par l'activation des protéases de la calpaïne et l'appauvrissement en ATP (Yu, Wang et al. 2002; Cao, Xing et al. 2007; Moubarak, Yuste et al. 2007; Berghe, Linkermann et al. 2014). Les travaux de Vaseva et al. ont montré que la nécrose induite par le stress oxydatif était régulée via p53, ainsi, p53 déclenche la nécrose et l'ouverture du pore de transition de perméabilité mitochondriale (Vaseva, Marchenko et al. 2012). Certaines molécules chimiothérapeutiques utilisées en clinique, telles que le cisplatine, le 5-fluorouracile et l'étoposide, induisent la mort cellulaire nécroptotique dans différents cancers en ciblant différentes protéines dans la cascade de nécroptose (Koo, Morgan et al. 2015). Dans la lignée cellulaire MCF-7 qui résistante à la -mitoxantrone, TNF- $\alpha$  est un médiateur de la mort cellulaire indépendante de la caspase. La nécrostatine-1 est capable d'inverser de la mort cellulaire nécrotique induite par le TNF- $\alpha$  alors que le TNF- $\alpha$  active la phosphorylation de JNK et RIP1 (Ghandadi, Behravan et al. 2017). Les molécules endommageant l'ADN induisent la mort cellulaire nécrotique programmée dans le cancer du sein par l'activation de RIP3 et de MLKL (Koo, Morgan et al. 2015). En effet, le stress réticulaire induit une nécroptose indépendante de la caspase RIP1 dans les cellules L929.

Cependant, dans les cellules traitées avec TNF- $\alpha$ , l'inhibition de RIP1 a permis de passer de la mort nécroptotique à la mort par apoptose au lieu de protéger les cellules (Saveljeva, Mc Laughlin et al. 2015). En revanche, les cellules du cancer du col de l'utérus hautement transformées passent d'une mortalité par apoptose à une mortalité cellulaire nécrotique (Kravchenko-Balasha, Mizrachy-Schwartz et al. 2009).

L'implication de Orai1 dans la progression tumorale a été démontrée dans plusieurs cancers dont le cancer du sein (Yang, Zhang et al. 2009; McAndrew, Grice et al. 2011). L'implication d'Orai1 dans la mort cellulaire dans les cancers varie de l'apoptose à l'anti-apoptose, dans les cellules cancéreuses de la prostate, la régulation négative d'Orai1 dans les cellules LNCaP cultivées en l'absence d'androgènes réduit l'apoptose induite par Thapsigargine, TNF- $\alpha$  ou par des agents chimiothérapeutiques tels que le cisplatine ou l'oxaliplatine via des mécanismes mitochondriaux et cytosoliques (Flourakis, Lehen'kyi et al. 2010); alors que d'autres démontrent que l'inhibition de l'expression d'Orai1 renforce les effets apoptotiques du 5-fluorouracile et de la Gemcitabine (Kondratska, Kondratskyi et al. 2014). L'inhibition d'Orai1 par siARN favorise la mort cellulaire induite par le 5-FU (Tang, Xia et al. 2017) et permet d'induire l'apoptose dans les cellules de glioblastome (Liu, Hughes et al. 2011). Dans ce travail, nous avons démontré que l'inhibition d'Orai1 était capable de promouvoir l'effet nécrotique du CO-OCS mais pas l'apoptose. Bien que l'inhibition d'Orai1 n'ait pas d'effet sur la survie des cellules MCF-7, l'invalidation d'Orai1 accompagné d'un traitement par CO-OCS induit un effet nécrotique plus important que le CO-OCS seul. D'autre part, l'inhibition d'Orai1 augmente l'expression de GRP78. Cependant, contrairement à Stim1, siOrai1 ne restaure pas l'expression de GRP78 dans les cellules MCF-7 traitées par CO-OCS. Nous envisageons d'étudier la cascade de signalisation nécrotique induite par le CO-OCS dans MCF-7 en utilisant deux inhibiteurs de la mort cellulaire nécrotique. La necrostatine-1 qui est un inhibiteur de la nécroptose, et l'IM-54 qui est un inhibiteur du stress oxydatif induit par ROS (espèces réactives à l'oxygène). Aucuns des deux inhibiteur n'est parvenu à affecter la nécroptose dépendante de CO-OCS.

#### **7°- L'activité du Translocon déclenche la mort cellulaire nécrotique et apoptotique induite par le CO-OCS.**

L'homéostasie calcique réticulaire est essentielle pour différents processus cellulaires. Outre ses rôles dans la translocation de protéines après la traduction et la rétrotranslocation des protéines mal repliées pour leur dégradation, le Translocon (Sec61) est un canal de fuite

passive du calcium via lequel les facteurs de stress du RE et les chélateurs de calcium appauvrissent le stock (Flourakis, Coppenolle et al. 2006). Cette fuite de calcium passive peut être régulée par différents facteurs ; l'interaction entre Calmoduline et Sec61 $\alpha$  limite les fuites de calcium du réticulum endoplasmique (Erdmann, Schäuble et al. 2011). Sec62 semble également être impliqué, où le motif C-terminal EF-hand de Sec62 peut servir de médiateur à la fuite de calcium de Sec61 (Linxweiler, Schorr et al. 2013). GRP78 bouche le canal Sec61 en raison de son association à la boucle 7 de Sec61 $\alpha$  conduisant à une inhibition de l'efflux de calcium réticulaire (Schauble, Lang et al. 2012). Lors d'un stress réticulaire, GRP78 régule la diminution des réserves de calcium du RE, de plus, le degré de fuite de calcium via le Translocon est corrélé à l'expression de GRP78 (Hammadi, Oulidi et al. 2013). Par ailleurs, la libération de calcium dans le RE via le Translocon active SOCE (Ong, Liu et al. 2007). SOCE, en ciblant ses régulateurs, est liée à la survie au cancer. Dans certains types de cellules, l'inhibition de SOCE confère une résistance à l'apoptose (Flourakis, Lehen'kyi et al. 2010; Huang, Ren et al. 2017), alors que dans d'autres types, elle favorise la mort cellulaire (Kondratska, Kondratskyi et al. 2014; Tang, Xia et al. 2017). Il a été démontré que, dans le cancer de la prostate, l'inhibition pharmacologique de Translocon (par l'anisomycine) réussissait à contrecarrer l'inhibition de l'épuisement des réserves de médicaments stockés par les facteurs de stress ainsi que la réduction de l'apoptose (Hammadi, Oulidi et al. 2013). En outre, dans les cellules de gliome malin, l'inhibition des fuites de calcium par le Translocon permet de réduire la mort cellulaire (Johnson, White et al. 2014). L'induction pharmacologique de la mort cellulaire nécrotique et / ou apoptotique tardive sur les cellules Jurkat et dans les cellules lymphocytaires du sang périphérique a diminué après l'inhibition de l'activité du Translocon (Charles, Hammadi et al. 2017). En accord avec la découverte rapportée dans les autres lignées cellulaires, l'anisomycine réduit la mort cellulaire apoptotique et nécrotique de CO-OCS dans les cellules MCF-7. Ces résultats démontrent clairement que le Translocon est un important régulateur calcique de la mort cellulaire apoptotique et nécrotique de CO-OCS dans MCF-7.

#### **8°- Est-ce que la réserve de Ca<sup>2+</sup> réticulaire et SOCE sont impliqués dans les effets nécrotiques et apoptotiques de CO-OCS ?**

Pour répondre à cette question, il est intéressant de rappeler que les réserves de calcium sont impliquées à la fois dans la signalisation du calcium et le repliement des protéines. De nombreux processus cellulaires et différents régulateurs cellulaires sont

impliqués dans le stockage du calcium dans le RE, par exemple l'activité de SERCA, la production d'ATP mitochondrial, SOCE et la fuite de calcium réticulaire. Dans les cellules MCF-7 traitées par CO-OCS, le stress réticulaire observé est probablement lié à la dégradation de l'un de ces processus. Autrement, dans de nombreuses études où l'UPR est déclenchée de différentes manières, une diminution significative des réserves réticulaires de calcium est toujours rapportée, une telle déplétion du RE déclenche l'apoptose (Hammadi, Oulidi et al. 2013; Johnson, Kono et al. 2014). Nos résultats ont montré que l'épuisement du calcium réticulaire induit par CO-OCS était fonction du temps. Comme les effets apoptotiques et nécrotiques du CO-OCS ne sont pas affectés par un milieu à faible concentration en calcium, nous suggérons l'implication du calcium réticulaire plutôt que du SOCE. Récemment, Sehgal, et ses collaborateurs ont signalé dans différentes lignées cellulaires y compris MCF-7, que l'UPR induite par la Thapsigargine est due au calcium réticulaire plutôt qu'à la SOCE (Sehgal, Szalai et al. 2017).

Comme l'anisomycine supprime les effets induits par CO-OCS (apoptotiques et nécrotiques ainsi que la fuite de  $\text{Ca}^{2+}$  réticulaire), nous avons étudié l'implication potentielle du Translocon dans la réponse de CO-OCS à la fuite de  $\text{Ca}^{2+}$  réticulaire. Pour évaluer la fuite de  $\text{Ca}^{2+}$  à travers le Translocon, nous avons utilisé l'anisomycine pour maintenir le Translocon dans un état fermé réduisant la perméabilité au  $\text{Ca}^{2+}$  (Hamman, Hendershot et al. 1998). De plus, Hammadi et al. ont signalé un lien entre la fuite de  $\text{Ca}^{2+}$  réticulaire via le Translocon, le stress réticulaire et l'apoptose (Hammadi, Oulidi et al. 2013). Nous avons constaté que l'anisomycine inhibait complètement la fuite réticulaire de  $\text{Ca}^{2+}$  induite par CO-OCS dans les cellules MCF-7 incubées à la fois en présence de CO-OCS et de l'anisomycine pendant 72h.

En outre, l'anisomycine neutralise partiellement la diminution de SOCE induite par CO-OCS. Ces résultats confirment que le Translocon est une cible majeure impliquée dans la fuite de  $\text{Ca}^{2+}$  réticulaire induite par CO-OCS. Son inhibition constitue un mécanisme de protection de l'effet de CO-OCS via la restauration complète de l'homéostasie calcique réticulaire et partielle de SOCE.

Un nombre croissant de publications montrent les différents rôles de GRP78. En effet, outre son rôle de chaperon, il s'agit d'une protéine de stockage du calcium réticulaire contribuant dans des conditions de cellules au repos, à environ 25% des réserves de  $\text{Ca}^{2+}$  (Lievremont, Rizzuto et al. 1997). De plus, lors de la réponse UPR et du stress réticulaire, GRP78 module la libération de calcium via une ouverture modulante le Translocon (Nico,



Sven et al. 2012; Hammadi, Oulidi et al. 2013). Nos résultats montrent que l'inhibition de l'expression de GRP78 seul augmente la fuite de  $\text{Ca}^{2+}$  réticulaire, ils soutiennent l'idée que GRP78 contribue à réduire les fuites de  $\text{Ca}^{2+}$  réticulaire probablement par le biais de Translocon (Nico, Sven et al. 2012).

Compte tenu de ces résultats, nous pouvons conclure que le CO-OCS induit un stress réticulaire et une réponse UPR consécutives à une fuite de  $\text{Ca}^{2+}$  réticulaire et que le Translocon constitue l'une des principales cibles impliquées dans cette fuite induite par CO-OCS.

### **9°- Les protéines Stim1, Orai1 et GRP78 constituent des cibles spécifiques dans les cellules cancéreuses mais pas dans les cellules bénignes du sein.**

MCF-10A est une lignée de cellules mammaires bénignes immortalisées dérivée d'un tissu mammaire fibrokystique bénin (Soule, Maloney et al. 1990), elles sont les cellules mammaires « normales » les plus couramment utilisées dans les recherches du cancer de sein. Des travaux publiés antérieurement par notre laboratoire ont montré que le CO-OCS n'affectait ni la viabilité ni la mortalité des cellules MCF-10A normales (Allan, Ouadid-Ahidouch et al. 2013). Parallèlement aux expériences sur les cellules cancéreuses du sein, nous avons étudié les voies moléculaires par lesquelles CO-OCS était capable d'induire différents effets antitumoraux dans les lignées cellulaires du cancer du sein mais pas dans la lignée cellulaire bénigne MCF-10A. CO-OCS n'a pas affecté l'expression de Stim1, Orai1 et GRP78. En effet, Stim1, Orai1 et GRP78 sont beaucoup plus exprimés dans les tissus du cancer du sein que dans les tissus normaux (Zhu, Zhang et al. 2014; Yao, Liu et al. 2015; Yang, Jiang et al. 2017). L'inactivation de Stim1 et Orai1 n'a aucun effet sur la viabilité cellulaire et la mortalité. Seul le siGRP78 a pu réduire la viabilité cellulaire sans affecter la mortalité. De plus, le CO-OCS n'a pas affecté les réserves de calcium des récepteurs d'urgence et le SOCE. Ces résultats démontrent clairement que les protéines Stim1, Orai1 et GRP78 constituent des cibles importantes et spécifiques dans les cellules du cancer du sein mais pas dans les cellules bénignes du sein.

## **Conclusion générale**

Au cours de ce travail, nous avons étudié les voies de signalisation par lesquelles CO-OCS était capable d'induire des effets antiprolifératifs, anti-migratoires et anti-survie dans les différentes lignées cellulaires du cancer du sein. La découverte la plus importante est que le CO-OCS affecte spécifiquement les cellules cancéreuses du sein. CO-OCS, en modifiant l'expression de différentes cibles, réduit la migration et la prolifération des cellules et augmente le taux d'apoptose et de nécrose.

Dans les lignées cellulaires de cancer du sein MDA-MB-231 et MCF-7, CO-OCS inhibe la migration (i) en réduisant l'expression de la  $\beta$ -intégrine et en inhibant ses cascades de signalisation FAK et ERK1/2 et (ii) en inhibant SOCE suite à la suppression de l'expression de Stim1.

Dans la lignée cellulaire du cancer du sein MCF-7, CO-OCS induit (i) un effet antiprolifératif via le ciblage de Stim1, Orai1 et GRP78, provoquant un arrêt du cycle cellulaire en phase G1 et G2/M, ainsi qu'une inhibition de SOCE; (ii) un effet apoptotique en diminuant l'expression de GRP78 et Bcl2 et en augmentant la fuite de calcium du stock de RE à travers le Translocon; (iii) et un effet nécrotique par suppression de l'expression du GRP78 et partiellement par la diminution du taux de calcium dans le RE via le Translocon. Stim1 est une cible apoptotique de CO-OCS, tandis qu'Orai1 protège les cellules de son effet nécrotique.

Dans les cellules mammaires non cancéreuses MCF-10A, CO-OCS n'a aucun effet sur la migration, la prolifération ou la survie.

Les résultats obtenus ouvrent un vaste champ d'expérimentation en vue de mieux comprendre les voies de signalisation de CO-OCS.

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